STUDIES ON SOME CRUSTACEANS OF TROPICAL WATERS WITH SPECIAL REFERENCE TO PIGMENTS

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DOCTOR OF PHILOSOPHY

in

FOOD SCIENCE

By

***SACHINDRA NM*, M. F. Sc**

Department of Meat, Fish and Poultry Technology

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSORE – 570 013, INDIA

SEPTEMBER 2003

To My wife
and
Children

01 September 2003

Dr NS Mahendrakar
Scientist
Meat, Fish and Poultry Technology

Fax: 91- 821- 2516308/ 2517233
E - mail: nsmahendra@yahoo.com
Web: http://www.geocities.com/nsmahendra
Phone: 0821 - 2514840 (O)
0821 - 2545822 (R)

CERTIFICATE

I hereby certify that the thesis entitled ***"Studies on some crustaceans of tropical waters with special reference to pigments"*** *submitted by* ***Mr Sachindra NM*** *for the degree of* ***Doctor of Philosophy*** *in* ***Food Science*** *in* ***University of Mysore****, Mysore, is the result of the research work carried out by him in the Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute, Mysore, under my guidance during the period 2000 – 2003.*

(NS Mahendrakar)
Guide

DECLARATION

I hereby declare that this thesis on ***"Studies on some crustaceans of tropical waters with special reference to pigments"*** *which is submitted for the degree of* ***Doctor of Philosophy*** *in* ***Food Science*** *to* ***University of Mysore****, Mysore, is the result of the research work carried out by me in the Department of Meat, Fish and Poultry Technology, Central Food Technological Research Institute, Mysore, under the guidance of* ***Dr NS Mahendrakar*** *during the period 2000 – 2003.*

I further declare that the result of this work has not been previously submitted for any degree or fellowship.

(NM SACHINDRA)

C O N T E N T S

SYNOPSIS

SYNOPSIS

Seafood processing industry is one of the major food industries in India. Nearly 190,000 tonnes of crustaceans particularly shrimps are processed annually in these export oriented industries. Export of frozen shrimps during the period 2000 – 01 was 110,000 tonnes valued at Rs 44,820 million. These shrimp processing industries generate large quantities of shrimp waste in the form of head and body carapace. These byproducts are valuable source of proteins (35 – 40% DWB), chitin (10 –15% DWB), minerals and natural carotenoids. At present they are being used in small quantities as shrimp meal for aquaculture and poultry diets and for production of chitin/chitosan. However a considerable quantity of this valuable byproduct is being wasted, resulting in not only the loss of valuable components but also environmental pollution.

Studies on efficient utilization of shrimp industry byproducts have been concentrated on recovery of protein and chitin from the waste. Not much attention has been given towards recovery of other valuable marketable products like carotenoids. There is a great demand for natural carotenoids as a replacement for currently used synthetic carotenoids in foods and feeds. The studies on characterization of carotenoids in crustaceans are restricted to species from temperate waters. The scientific data on quantitative and qualitative distribution of carotenoids in crustaceans from Indian waters is lacking. There is a need for development of suitable methods for recovery of carotenoids from the byproducts of shrimps form Indian waters and evaluating their suitability as coloring ingredients in food and feed.

In view of the above, studies were carried out to determine the yield and chemical composition of body components from 4 species of shallow water shrimps namely *Penaeus monodon, P indicus, Metapenaeus dobsoni, Parapenaeopsis stylifera*, two

species of deep sea shrimps namely *Solonocera indica* and *Aristeus alcocki*, one species of fresh water prawn *Macrobrachium rosenbergii,* one species of crab each from marine water (*Charybdis cruciata*) and fresh water (*Potamon potamon).* Total carotenoid content in different body components was determined. The qualitative distribution of carotenoids was determined by identifying the major carotenoids by thin layer chromatography (TLC), absorption spectra and by high performance liquid chromatography (HPLC). Carotenoid esters from the extracts of different body components were analyzed for fatty acid profile by gas chromatography (GC).

In order to recover the carotenoids from the shrimp waste, extractability of carotenoids in different organic solvents and solvent mixtures was evaluated and the conditions for solvent extraction were optimized by a statistically designed experiment. Studies were also carried out on extractability of carotenoids in different vegetable oils. The optimized conditions for oil extraction of carotenoids were established. The effect of hydrolysis of waste with different proteases prior to extraction in oil on the yield was studied and the hydrolysis and extraction conditions were optimized.

The effect of antioxidants and storage in different packaging conditions on the stability of recovered carotenoids was evaluated. The suitability of recovered carotenoids as colorants in fish products was assessed by incorporation of carotenoids in fish sausages. The pigmentation efficiency of carotenoids in ornamental fishes was evaluated by fish feeding experiments.

The whole write up is divided into three parts:

Part I includes introduction, review of literature, structure of carotenoids, scope and objectives of investigation. The introduction includes a brief account of fish production in India, processing and export of seafoods, waste generation in Indian shrimp

industries, utilization of waste and the need for the study. The literature review covers published reports on classification, function and distribution of carotenoids, occurrence of carotenoids in various aquatic animals, role of carotenoids in aquaculture, effect of processing on carotenoids in aquatic food products and recovery of carotenoids from crustacean waste. Scope and objectives covers, the need for the study, major objectives and program of work.

Part II deals with the actual investigation work and is divided into 6 chapters, each containing a brief introduction, design of experiments, results and discussion. Results of each chapter are supported by suitable statistical analysis.

Chapter 1 covers the details on yield and chemical composition of different body components from different species of shrimps, prawn and crabs.

Chapter 2 deals with qualitative and quantitative distribution of carotenoids in different body components of crustaceans studied.

Chapter 3 includes studies on recovery of carotenoids from shrimp waste by solvent extraction. The extractability of shrimp waste carotenoids in different organic solvents and solvent mixtures and optimization of solvent extraction condition are included in this chapter.

Chapter 4 presents oil extraction process for carotenoids, which includes selection of suitable vegetable oil for extraction, optimization of conditions for oil extraction and effect of enzymatic hydrolysis of shrimp waste on yield of oil recoverable carotenoids.

Chapter 5 covers studies on effect of different antioxidants and packaging systems on stability of solvent extracted and oil extracted carotenoids.

Chapter 6 includes the details of study on use of recovered carotenoids as colorants in fish sausages and as pigment source in ornamental fish diets.

Part III covers summary and conclusion of the investigation and bibliography.

The salient findings of the investigation are

- Yield of waste (head and carapace) was higher in deep-sea shrimps (62 – 66%) than in shallow water shrimps (48 – 56%). The yield of waste in fresh water prawn was 60%. Content of crude protein (8.2 – 10.2%), true protein (6.3 – 9.7%), fat (1.1 – 8.1%) was higher in head than in carapace (7.8 – 9.5% crude protein, 5.2 – 8.2% true protein, 0.75 – 2.0% fat), while ash (4.0 – 6.5%) and chitin content (3.3 – 4.4%) were lower in head than in carapace (4.9 – 9.0% ash, 4.4 – 6.3% chitin).
- The yield of meat in crabs was 28.8 – 29.7% and that of shell was 34.4 – 35.7%. Chitin content was higher in marine crab shell (8.2%) than in fresh water crab shell (4.4%).
- Total carotenoid content varied between species and body components. Highest carotenoid content was observed in head of deep-sea shrimp *A alcocki* (185.3 μg/g) and marine shrimp *P stylifera* (153.1 μg/g). High levels of carotenoids were also observed in carapace of *A alcocki* (117.4 μg/g), *S indica* (116.0 μg/g) and *P stylifera* (104.7 μg/g). Low levels of carotenoids were observed in shrimp *P indicus* and fresh water prawn *M rosenbergii* and crabs.
- The major carotenoids in shrimps, fresh water prawn and marine crab was astaxanthin and its esters. β-Carotene and zeaxanthin was at low levels in these species. Zeaxanthin was the major carotenoid in fresh water crab.

- The carotenoid esters from the crustaceans studied contained palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:0), stearic (C18:0) and oleic (C18:1) as major fatty acids.
- A 50 : 50 mixture of isopropyl alcohol and hexane was found to give higher carotenoid yield from shrimp waste compared to individual solvents, namely acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, hexane or 50 : 50 mixture of acetone and hexane .
- The optimized conditions for solvent extraction of carotenoids were 60% hexane in solvent mixture, solvent mixture to waste ratio of 5 : 1 in each extraction and 3 numbers of extractions. A regression equation for predicting the carotenoid yield as a function of three processing variable (hexane % in solvent mixture, solvent level to waste and number of extractions) was derived by statistical analysis.
- Extractability of shrimp waste carotenoids was higher in refined sunflower oil compared to groundnut oil, gingelly oil, mustard oil, soybean oil, coconut oil and rice bran oil and the carotenoid content in oil could be increased by repeated use of pigmented oil for extraction of carotenoids from fresh waste for 3 times.
- The pigments in waste can be recovered in oil by mixing the sunflower oil with waste in a ratio of 2 : 1 (oil : waste), heating the mixture at 70°C for 150 min, centrifuging the treated waste and recovering the pigmented oil by phase separation. A regression equation was arrived at to predict the carotenoid yield as a function of oil level to waste, temperature and time of heating waste in oil.

- The oil extraction yield of carotenoids can be increased by hydrolysis of waste with protease prior to oil extraction and bacterial protease alcalase was found to be better than plant protease papain or animal protease trypsin for hydrolysis.
- Optimum oil extraction yield can be obtained by hydrolysis of waste with 0.75% (of waste) of alcalase at 37°C for 150 min, adding sunflower oil to the hydrolysed waste in a ratio of 2 : 1 (oil : waste), heating at 70°C for 90 min and recovering the pigmented oil. A regression equation was derived to predict the carotenoid yield at different levels of processing variables namely, enzyme concentration, incubation time and heating time in oil. By using the hydrolysed waste for carotenoid recovery, heating time can be reduced from 150 min to 90 min to get optimum yield.
- Solvent extracted carotenoids can be stored by mixing with carriers such as sodium alginate or cornstarch. Addition of antioxidants and storing the pigmented carrier in light barrier packaging materials such as metallised polyester were found to reduce the degradation of the pigment. Tertiarybutyl hydroxyquinone (TBHQ) at a level of 200 ppm was found to be more effective antioxidant than α-tocopherol (200 ppm) for stabilization of pigments against oxidative degradation.
- In order to reduce the degradation of oil extracted carotenoids during storage, antioxidants, preferably TBHQ (200 ppm) should be added to the pigmented oil and stored in amber colored bottles.
- The addition of recovered carotenoids in fish sausage formulation at a level of 5 – 10 ppm improved the color and flavor of the product. The added carotenoids were stable during thermal processing of sausage.

- The addition of carotenoids in diets for ornamental fish koi carp (*Cyprinus carpio koi*) enhanced the skin coloration and total carotenoid content in the body.

The studies indicated that the waste (head and carapace) yield from the shrimps and prawn was in the range of 48 – 66%. The waste contains high levels of carotenoid and could be used as a source of natural carotenoids. Carotenoids in the waste can be better recovered by extracting with a mixture of isopropyl alcohol and hexane than the use of a polar solvent alone. Carotenoids can also be extracted using sunflower oil after hydrolyzing the waste with protease. To stabilize the carotenoids against degradation during storage, the addition of antioxidants and storing in light barrier materials can be adopted. The recovered carotenoids can be used as colorants in fish products and as pigment source in diets for ornamental fishes.

PART I

INTRODUCTION

INTRODUCTION

India, with its vast fishery resources, is one of the major contributors to the world fish production. The majority of fishery resources in India lie in the 2 million sq. km of exclusive economic zone (EEZ) along the 8129 km of coastal line. In addition to the marine waters, the other water bodies, 64000 km of perennial rivers, 1097 million ha of reservoirs, 1.3 million ha of lakes, 1.4 million ha of brackish water, 2.4 million ha water area of ponds and tanks contribute significantly to the fishery resources of the country (Dixitulu and Paparao 1994).

India ranks 8th in the world in the marine fish production and 2nd in inland fish production. The country contributes nearly 4.6% to the total world fish production of 130.21 million tonnes (year 2001). The trends in fish production in India (Table I) indicate that the catches increased 4.23 to 5.96 million tonnes during the period 1992 - 2001.

Table I. Fish production trend in the world and in India (1992 –2001)

Year	World	India	
	(in `000 MT)	(in `000 MT)	(% of world production)
1992	100847	4233	4.2
1993	104425	4606	4.4
1994	112351	4785	4.3
1995	116412	4952	4.3
1996	120198	5231	4.4
1997	122542	5386	4.4
1998	117790	5276	4.5
1999	126651	5593	4.4
2000	130434	5690	4.4
2001	130207	5965	4.6

Source: www.fao.org

The increase in total fish production during the years is attributed to diversification of fishing grounds and spurt in the fish production through aquaculture. The production trend of crustaceans in India during 1992 – 2001 (Table II) indicate that the production increased in the early nineties, but reduced in mid nineties. The reduction in total crustacean production was mainly attributed to the slump in shrimp production from aquaculture due to the disease outbreak. The total production is increasing at present as efforts are being made to control the disease outbreaks in shrimp farms.

Table II Crustacean production trend in world and in India (1992 – 2001)

Year	World	India	
	(in `000 MT)	(in `000 MT)	(% of world production)
1992	5243	324	6.2
1993	5309	404	7.6
1994	5840	514	8.8
1995	6254	447	7.1
1996	6589	446	6.8
1997	7024	395	5.6
1998	7639	448	5.9
1999	7840	455	5.8
2000	8148	439	5.4
2001	8436	Not available	-

Source: www.fao.org

The fishing efforts are largely confined to the inshore waters and 90% of the production from marine sector comes from within a depth range of 50 – 70 m. Efforts are

being made to tap the resources in the deep-sea. There is a good potential for harvesting the fishes and crustaceans from beyond 50 m depth (Table III).

Table III Estimated fishery potential of the Indian EEZ (in '000 tonnes)

Group	Upto 50 m	Beyond 50 m	Total
Fishes	1724	1493	3217
Crustaceans	232	8	240
Others	254	189	443
Total	2210	1690	3900

Source: www.mpeda.com

Efforts are also being made to increase the fish production by aquaculture. There is a vast potential to improve the fish production by mariculture, brackish water aquaculture and fresh water fish culture. More importance is given towards development of shrimp culture to augment the shrimp production from natural waters. However, out of 1.2 million ha of potential area for shrimp farming only 0.15 million ha is being utilized (Swamy 2001). With more water area being utilized for shrimp culture, and the incentives given by the government and the agencies like marine product export development authority (MPEDA) for shrimp culture, the shrimp production from aquaculture is increasing considerably in recent years.

Major quantity of fish produced in India is consumed fresh (3.9 million tonnes) and 24 % of fish harvested is processed, mainly for export. There are 400 seafood freezing plants along the Indian coast, with a built-in-capacity of 7284 tonnes per day (www.mpeda.com). Nearly 190,000 tonnes of crustaceans are processed annually in these export oriented seafood processing industries. The export of seafood from India during 2000 - 01 was 440,000 tonnes valued at Rs 64438.9 million (Sathiadasan and Hassan

2002). Shrimps, valued at Rs. 44820 million, contributed 25.4 % of the total quantity of seafood exported.

Shrimp processing for freezing normally involves removal of head and body carapace. It is estimated that the generation of byproducts in the form of head and body carapace from the Indian seafood industry is around 100, 000 tonnes (Gopakumar 1993). These byproducts are good source of protein (35 – 40% DWB), chitin (10 – 15% DWB), minerals and natural carotenoids. At present, these byproducts are being used in small quantities as shrimp meal for use aquaculture and poultry feed, and for production of chitin/chitosan. A considerable quantity of these byproducts is being wasted, resulting not only into the loss of valuable components but also environment pollution.

Research efforts on effective utilization of shrimp waste were mainly focused on recovery of chitin. Not much attention has been paid towards extraction of other valuable components such as carotenoids. There is a great demand for natural carotenoids for use as colorants in fish products and as pigment source in aquaculture diets. The synthetic pigments like carophyll red (canthaxanthin) and carophyll pink (astaxanthin) presently used in fish culture are very expensive. Thus natural carotenoids recovered from shrimp waste will have profound utility value in fish culture as well as in fish products industries.

The research efforts on characterization of carotenoids in crustaceans are mostly restricted to species from temperate waters. The information on carotenoids in crustaceans from tropical waters, especially from Indian waters is lacking. Further, the recovery of carotenoids from byproducts of Indian shrimps of commercial value and their utilization has not been attempted so far.

In view of the above, the investigation work was conducted to generate information on quantitative and qualitative distribution of carotenoids in some

crustaceans of commercial importance from Indian waters. The recovery of carotenoids from shrimp waste by different methods, factors affecting their recovery and utilization of the recovered carotenoids in food and feed has been investigated.

REVIEW OF LITERATURE

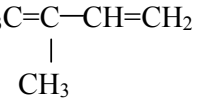
REVIEW OF LITERATURE

CAROTENOIDS

Carotenoids are a class of fat-soluble pigments found principally in plants, algae, photosynthetic bacteria and animals. They are responsible for the colors in fruits, vegetables, fish, crustaceans, egg and other plant and animals. Although animals are incapable of synthesizing carotenoids, they incorporate carotenoids from their diets and provide bright coloration, serve as antioxidants and can be a source of vitamin A (Britton et al 1985). There are more than 600 known naturally occurring carotenoids (Ong and Tee 1992) and more carotenoids are continued to be identified (Mercadante 1999). It was in the beginning of the 20th century the studies on carotenoids started when Tswett (1911) discovered the diversity of carotenes and xanthophylls and suggested the term carotenoids as a generic name for them Since then numerous studies have been carried out on structure and chemistry of carotenoids. The progress in carotenoid research is documented by Karnankhov (1990).

CLASSIFICATION OF CAROTENOIDS

Carotenoids are isoprenoid polyenes formed by joining of eight C5 isoprene units in a regular head to tail manner except in the center of the molecule, where the order is tail to tail and molecule is symmetrical (Gross 1991).



Isoprene

The carotenoids can be divided into two major classes depending on the degree of substitution (Gross 1991). The first class is the highly unsaturated carotene hydrocarbons,

which contain no oxygen. The example being lycopene and β-carotene. The second class is the oxygenated derivatives of carotenes called xanthophylls. Xanthophylls contain one or more oxygenated group substituants on the terminal rings (Haard 1992). The examples being astaxanthin, lutein, zeaxanthin .

In addition to structural differences, carotenes and xanthophylls also differ in their diversity and distribution (Latsch 1990). Generally carotenes have greater distribution in plants than in animals, while xanthophylls are more widely distributed both in plants and animals (Shahidi et al 1998).

It is generally accepted that animals are unable to synthesize carotenoids *de novo*, but are able to modify dietary plant carotenoids (Buchecker 1982). Thus the distribution of carotenoids in animal sources is primarily the result of specific dietary habits, absorption and metabolic transformation (Torrison 2000). In animals the astaxanthin is the most widely distributed xanthophylls, followed by lutein and zeaxanthin (Haard 1992).

FUNCTIONS OF CAROTENOIDS

The well understood nutritional role of carotenoids is their provitamin activity. Vitamin A is an essential micronutrient for animals. It is essential for vision, growth, reproduction and normal development of skin and mucosa (Shimizu et al 1981). This vitamin can be produced within the body of animals from certain carotenoids particularly β-carotene (Britton et al 1995). Other carotenoids, which act as provitamin A, are α-carotene, cryptoxanthin, carotenic acid ethyl esters (Gross 1991), astaxanthin, canthaxanthin and echinenone (Latscha 1990). Although animals are incapable of synthesizing carotenoids, they incorporate carotenoids from their diet and can be source of vitamin A activity (Britton et al 1995). Plant carotenoids ingested by the animals are

enzymatically converted to vitamin A (Gross 1991). Fishes obtain their vitamin A by conversion of some provitamin A carotenoids (Guillou et al 1989).

The provitamin activity of xanthophylls has been demonstrated by feeding experiments. The conversion of ingested astaxanthin and canthaxanthin to vitamin A_1 and A_2 has been demonstrated in fish guppies and platies (Gross and Budowski 1966). Astaxanthin was found to be converted into β-carotene in the intestinal wall of the fish *Heteropneustis fossilis* (Goswami 1984). Radioisotope studies have confirmed that in rainbow trout astaxanthin gets converted to vitamin A_1 and A_2 as it crosses the intestinal wall (Schiedt et al 1985). The studies on bioconversion of astaxanthin in rainbow trout indicated that the xanthophylls are first converted to echinenone then to β-carotene and finally vitamin A_1 and A_2 (Guillou et al 1989). Some xanthophylls having no provitamin activity in mammals are found to exhibit their activity in lower animals which can metabolize xanthophylls to β-carotene and to vitamin A (Goodwin 1986).

The various colors noticed in flowers, seeds, fruits, microorganisms and higher animals are attributed to carotenoids. The color of flowers has important role in reproduction as coloration attracts animals that disperse pollen, seeds or spores (Delgado-Vargus et al 2000). It has been reported that in Phycomyces the mating recognition system is disturbed by excess accumulation of carotenoids intracellularly and at later stages carotenoids are involved in mating by inhibiting cell-to-cell recognition system (Oosaki et al 1996)

Carotenoids have been found to be involved in the process of reproduction in animals both directly and indirectly (Torrison et al 1989). The characteristic coloration in some fishes attracts females at spawning time (Goodwin 1984). In Salmons the mobilization of carotenoids from muscle to the integuments and ovaries occurs at the time

of sexual maturation (Kitahara 1983). Carotenoids were found to have beneficial effects on the endocrine system with respect to gonadal development and maturation, fertilization and hatching in fish (Tucon 1981) and on the reproduction process in various animals (Latscha 1990). In Salmons, the increased content of carotenoids in their egg was found to improve their viability (Craik 1985).

In photosynthetic organisms, carotenoids are known to function as accessory pigments in light harvesting and as photo protector against oxidative damage (Delgado-Vargus et al 2000). Carotenoids absorb visible light and transfer the energy efficiently to chlorophylls (Delgado-Vargus et al 2000). The photosynthetic functions of carotenoids are determined by their associated proteins. The carotenoids are found to interact with amino acids near the cell membrane surface making contacts with hydrophobic side groups of protein in the middle layer (Barber et al 1997). It has been demonstrated (Durnford et al 1996) that in algae the energy harvesting complexes are bound to chlorophyll and carotenoids independently, increasing their absorption spectra and consequently having more efficient energy utilization.

Carotenoids have been found to protect against photosensitization in photosynthetic organisms (Goodwin 1980b) and non-photosynthetic bacteria (Mathews-Roth and Sistron 1960). The therapeutic value of carotenoids as photoprotectants in human has been established (Mathews-Roth 1982). The photoprotection of carotenoids against autooxidation was demonstrated in the yeast *Phaffia rhodozyma,* where as a protective mechanism, the astaxanthin content was found to increase when the organisms are exposed to singlet oxygen (Schroeder et al 1996).

In higher plants, carotenoids serve as photoprotector against light damage (Delgado-Vargus et al 2000). The excess light absorbed by plants is dissipated by

xanthophyll cycle, thus avoiding the cellular damage and protecting the photosynthetic mechanism (Armstrong and Hearst 1996). It has been stated that in stressed cells higher levels of xanthophylls are maintained to work as an adaptative function to protect the photosynthetic apparatus (Phillips et al 1995).

One of the important characteristics of carotenoids is their ability to act as antioxidants, thus protecting cells and tissues from damaging effects of free radicals and singlet oxygen. The free radicals and singlet oxygen produced in the body by the normal aerobic metabolism are highly reactive (Darley-Usmar and Halliwell 1996). These oxidants can react with various components of living cells such as proteins, DNA or lipids and cause structural changes leading to diseases such as ageing (Ames and Shigenga 1992), atherogenesis (Esterbauer et al 1992), ischemia (Takayama et al 1992), infant retinopathy (Phelps 1987) and carcinogenesis (Breimer 1990). Carotenoids have been found to be important in protecting against diseases and age related phenomena caused by oxidants (Halliwell 1996).

The antioxidant mechanism of carotenoids is attributed to their ability to quench singlet oxygen and scavenge free radicals (Hirayama et al 1994). The singlet oxygen quenching ability of several carotenoids has been studied. Lycopene was found to be more effective carotenoid with respect to quenching of singlet oxygen (Tinkler et al 1994). Astaxanthin was found to be twice as effective as β-carotene and 80 times more effective than the antioxidant tocopherol (Di Mascio et al 1991). Studies have shown that astaxanthin is a better agent to destroy free radicals than other carotenoids (Nielsen et al 1996).

Mathews-Roth (1993) attributed the higher antioxidant activity of canthaxanthin and astaxanthin than β-carotene and zeaxanthin to the structural difference. It is reported

that antioxidant activity of carotenoids depends on the number of double bonds, ketogroups and presence of cyclopentane rings, which enhance their activity (Chen et al 1996). The higher antioxidant activity of astaxanthin was attributed to its 13 conjugated double bonds, thus quenching more singlet oxygen (Lee and Min 1990). Miller et al (1996) stated that antioxidant activities of carotenoids are influenced by polarities that are increased with the presence of functional group in terminal rings. Studies have indicated the isomer specific activity of carotenoid, cis-isomer showing better antioxidant activity (Stahl and Sies 1993). It has been reported that luteine, lycopene and β-carotene acts as prooxidants, but acts as antioxidants in presence of γ-tocopherol (Haila et al 1998) and tocopherols protect the carotenoids against radical autooxidation (Heinonen et al 1997).

The antioxidant activity of carotenoids in muscle foods has been reviewed by Mortensen and Skibsted (2000). Most studies on carotenoids as antioxidants in muscle foods have been performed on fish and poultry. In frozen rainbow trout, the presence of astaxanthin was found to delay the development of thiobarbituric acid reactive substances (TBARS), a measure of lipid oxidation (Bjerkeng and Johnsen 1995). Lipid oxidation was found to be less after frozen storage of rainbow trout that had been fed with high amounts of astaxanthin (Jensen et al 1998). A significant improvement was found in the quality of minced meat from rainbow trout supplemented with dietary canthaxanthin compared to a product made form unsupplemented trout (Clark et al 1996).

β-Carotene showed a slight antioxidant effect in chicken muscle (Andersen et al 1993). β-Carotene and zeaxanthin in the liver of supplemented chicks decreased lipid oxidation (Woodall et al 1996). In chilled chicken muscle β-carotene was found to act as an antioxidant at high content of tissue vitamin E (Ruiz et al 1998).

The antioxidant effects of carotenoids have been studied by meat model systems (Mortensen and Skibsted 2000). The combination of vitamin E, selenium and β-carotene in the diet was found to induce oxidative stability in rat liver slices and homogenates (Chen et al 1993). Synergism between β-carotene and α-tocophereol has been observed in membrane model system (Palozza and Krinsky 1992). Canthaxanthin was found to reduce TBARS and metmyoglobin formation in oxymyoglobin-phosphotidyl choline liposome model system (Clark et al 1995).

The pharmacological effects of carotenoids are well documented. Health benefits of carotenoids related to their antioxidative potential include enhancement of immune system function (Benedich 1989), protection from sunburn (Mathews-Roth 1990) and inhibition of development of certain types of cancer (Nishino 1998). Xanthophylls found in green leaves are believed to function as protective antioxidants in the macular region of human retina (Snodderly 1995).

It has been suggested that carotenoids influence the strength and fluidity of cell membranes thus affecting its permeability to oxygen and other molecules (Delgado-Vargus et al 2000). Carotenoids are found to have a significant effect on immune response and in intracellular communications (Hong and Sporn 1997). The mixtures of canthaxanthin with low-density lipoproteins were found to inhibit macrophage formation in human monocytes (Carpenter et al 1997). The effectiveness of β-carotene in treatment of certain kinds of cancers has been demonstrated (Taylor-Mayne 1996). The consumption of marine algae rich in carotenoids was found to diminish the risk of being affected by certain types of cancers (Murakami et al 1996). The antimutagen activity of carotenoids from green pepper was presumed to be due to blocking of entrance of toxic compounds into cell (Quintannr-Hernadez et al 1996). It was suggested that the

antimutagenecity of marigold extract is due to formation of extracellular complex between lutein and the mutagen 1-nitropyren, thus limiting the bioavailability of mutagens and consequently its mutagenecity (Gonzalez-de-Mejia et al 1997).

DISTRIBUTION OF CAROTENOIDS

Carotenoids are widely distributed throughout the living world. However, they are synthesized *denovo* only by higher plants, algae and microorganisms. The carotenoids isolated from animal cells are the result of metabolic changes in the ingested carotenoids (Goodwin 1980a), and the plant carotenoids are the source of animal carotenoids (Gross 1991). In animals carotenoids are found to be responsible for color of birds, fish, insects and some invertebrates.

The distribution and diversity of carotenoids was found to be dependent on the capacity of organisms to perform a *denovo* biosynthesis and the ability of the animals to absorb and metabolize pigments (Latscha 1990). The biosynthetic pathways of carotenoids are depicted in figure I (Britton 1976) and figure II (Margalith 1992). It is generally observed that carotenoids have greater qualitative and quantitative distribution in plants or carotenogenic organisms than in animals (Latscha 1990). In plants, the most common carotenoid found is β-carotene, while in animals xanthophylls are more widely distributed.

Figure I. Summary of carotenoid biosynthesis pathways

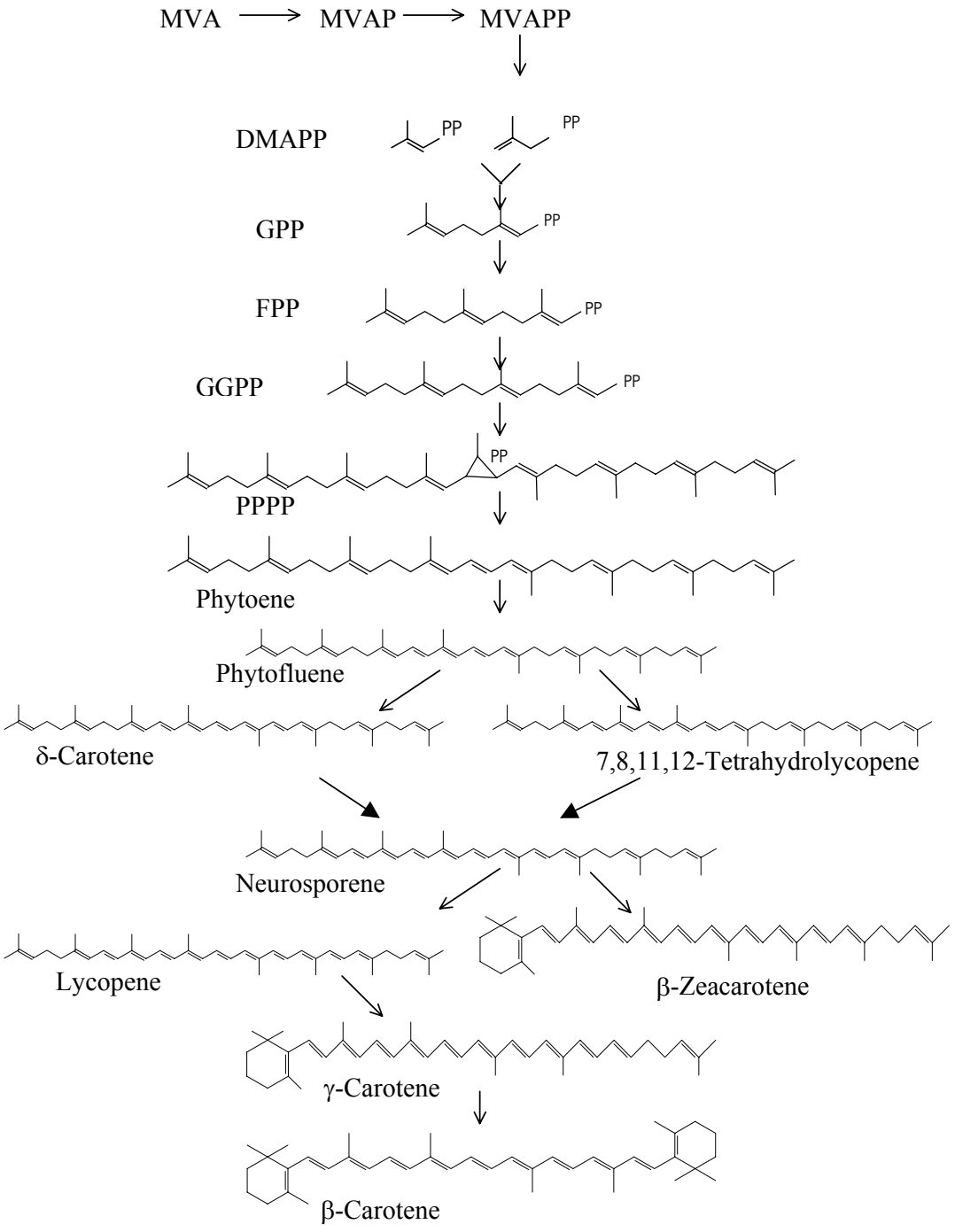


Figure II. Biosynthetic pathway of β-carotene

(MVA –Mevalonic acid, MVAP – MVA-5-phosphate, MVAPP – MVA pyrophosphate, IPP – Isopentenylpyrophosphate, DMAPP – Dimethylallylpyrophosphate, GPP – Geranylpyrophosphate, FPP – Farnesylpyrophosphate, GGPP – Geranylgeranylpyrophosphate, PPPP- Perphytoenepyrophosphate)

Carotenoids in algae

In green and red algae, the more commonly occurring carotenoids are β-carotene, lutein, violoxanthin, neoxanthin and zeaxanthin, while in brown algae fucoxanthin was found to be more abundant (Latscha 1990). Palermo et al (1991) reported the presence of β-carotene, zeaxanthin, fucoxanthin and fucoxanthinol in the red algae. Oxygenated carotenoid derivatives such as echinenone, canthaxanthin and astaxanthin are also found in algae (Goodwin and Britton 1988).

Several studies have been conducted on the carotenoids in the green algae *Haematococcus pluvialis.* Astaxanthin is the major pigment group in this species of green algae. *Haematococcus* is commercially cultured to produce astaxanthin. Studies have been carried out on effect of various factors such as, effect of light intensity and illumination cycle on astaxanthin production in *Haematococcus* (Kobayashi et al 1997), elevated temperature (Tjahjono et al 1994a), oxidative stress (Kobayashi et al 1993), carbon source (Barbera et al 1993) and nutrient limitation (Harker et al 1996a). Attempts have been made to increase the astaxanthin formation in *Haematococcus* by hybrid formation by protoplast fusion (Tjahjono et al 1994b), culturing algae in airlift photo bioreactor (Harker et al 1996b), addition of nutrients (Fabregas et al 2000), mixotrophic cultivation (Orosa et al 2001), mutation and CO_2 enriched growth environment (Usha et al 2001a, 2001b).

Carotenoids in higher plants

In photosynthetic tissues of higher plants carotenoids are present in chloroplasts as a mixture of α- and β-carotene, cryptoxanthin, lutein, zeaxanthin, violaxanthin and neoxanthin (Delgado-Vargus et al 2000). Plants also contain colorless intermediate products such as phytoene and phytofluene (Shahidi et al 1998). The xanthophylls

normally occur in free forms, but during autumn senescence, as chloroplasts disintegrate, xanthophylls are released into cytoplasm and are esterified before getting destroyed oxidatively (Goodwin 1980b).

In non-photosynthetic tissues, the carotenoids are sporadically distributed with many structural variations (Goodwin 1980b). In fruits, the chloroplasts of green unripe fruit gradually change to chromoplasts on ripening with stimulation of carotenoid synthesis (Goodwin 1980b). The red colour of tomato fruit is provided by lycopene, and its concentration increases significantly during ripening (Ronen et al 1999). Shi and Maguer-Mle (2000) reviewed the properties of lycopene in tomatoes with respect to bioavailablity, health aspects and the effects of food processing techniques. Ronen et al (1999) demonstrated that the mechanism of lycopene accumulation in tomato is based on differential regulation of expression of carotenoid biosynthesis genes. Cloning studies by Isaacson et al (2002) on tomato revealed the function of carotene isomerase in carotenoid biosynthesis in plant in the dark and non-photosynthetic tissues. Fraser et al (2001) studied the elevation of carotenoids in tomato by genetic manipulation.

Carotenoid content in vegetables and fruits in different geographical zone such as West Africa (Smith et al 1996), Egypt (Farag et al 1998), Tanzania (Mosha et al 1997) and India (Rajyalakshmi et al 2001) has been investigated. Breithauft and Bamedi (2001) screened vegetables and fruits of tropical and subtropical regions and reported that they are a rich source of cryptoxanthin esters. Cano et al (1996) studied carotenoid profiles of papaya during ripening and reported that trans-zeaxanthin and cryptoxanthin are the major xanthophylls, lycopene was the major hydrocarbon carotenoid and fatty acid esters of xanthophylls are the major carotenoid esters, and the lycopene content increased

during ripening. Wine grapes were found to contain neoxanthin, violoxanthin, lutein and β-carotene as major carotenoids (Bureau et al 1999).

The classic example of carotenogenic root is carrot, which contains β-carotene as main pigment, with xanthophylls contributing only 5% (Goodwin 1976). In red carrot, lycopene accumulates in place of β-carotene due to suppression of cyclising enzyme (Goodwin 1976). Some sweet potatoes also contain significant amounts of β-carotene (Goodwin 1976).

Carotenoids have also been identified in woods. Lutein and β-carotene have been identified in oak woods, and was suggested that lutein could be used as a marker to distinguish between wood samples (Masson et al 1997).

Carotenoids in microorganisms

Photosynthetic bacteria often produce new and specific pigments and accumulate acyclic pigments characterized by methoxy group at position 2, additional double bonds at C-3, 4 and ketogroups conjugated to double bond system, eg. Spheroidenone (Goodwin 1980a). Some green photosynthetic bacteria such as *Chlorobium* spp uniquely produce carotenoids with aromatic rings such as chlorobactene (Goodwin 1980a). In non-photosynthetic bacteria, carotenoids appear sporadically and when present have unique characteristics, examples being *Staphylococcus* accumulating C_{30} carotenoids, *Flavobacterium* C_{45} and C_{50}, some mycobacteria accumulating C_{40} carotenoid glycosides (Goodwin 1992).

Investigations have been carried out to use *Brevibacterium* sp as a commercial source of canthaxanthin (Nelis and Leenheer 1989). Orange and dark pigmented *Bradyrhyzobium* strains were found to produce canthaxanthin as major pigment (Lorquin

et al 1997). Extremely halophilic bacteria isolated from salt were found to produce canthaxanthin (Asker and Ohta 1999). Jong et al (2001) characterized the physiological properties of carotenoid production by halophilic *Erythrobacter* spp. Calo et al (1995) suggested that *Halobacter salinarium* may be a valuable source of astaxanthin and related ketocarotenoids for the food industry.

Several studies have been carried out on factors affecting carotenoid production by bacteria. Decrease in growth temperature was found to increase the carotenoid production in *Rhodococcus rhodochrous* (Takaichi and Ishidu 1993). Zheng et al (1999) used reducing sugars of rice for elevation of carotenoid production by *Rhodococcus* spp. Xiao and You (2000) achieved maximum pigment yield from *Rhodococcus* using starch-sucrose or hydrolysed sugar media. Studies on influence of carbon and nitrogen sources on zeaxanthin production in *Flavobacterium* indicated that aspergine acts as primary nitrogen source for production of the pigment (Alcantara and Sanchez 1999). Fong et al (2001) studied the carotenoid accumulation in psychrotrophic *Arthrobacter agilis* in response to thermal and salt stress.

Studies have been carried out to use genetic engineering as a tool for carotenoid production by bacteria. Misawa et al (1994) produced β-carotene in *Zymomonas mobilis* and *Agrobacterium tumefaciens* by introduction of biosynthetic genes from *Erwinia uredovora*. Zeaxanthin was produced in non-carotenogenic *Escherichia coli* by transforming with different carotogenic plasmids (Ruther et al 1997). Increased production of zeaxanthin in *Synechocystis* sp was achieved by genetic engineering techniques (Lagarde et al 2000). Misawa and Shimada (1998) reviewed the aspects of genetic engineering for production of carotenoids by bacteria.

Among numerous yeast strains only few, such as *Sporodiobolas, Sporobolomyces, Cryptococcus, Rhodosporidium, Rhodotorula* and *Phaffia* are known to produce pigments (Andrews et al 1976, Johnson and Lewis 1979, Miller et al 1976). The main carotenoids in *Rhodotorula* are β and γ-carotene, torulene and torularhodin (Liu et al 1973). Sakaki et al (1999) reported that *Rhodotorula glutinis* sensitizes its system of carotenoid biosynthesis according to the extra cellular compounds. The conditions for carotenoid production by *Rhodotorula glutinis* have been optimized (Buzzini 2000). Wang et al (2001) evaluated the effect of various additives on the carotenoid content of *Rhodotorula.* Vijayalakshmi et al (2001) optimized the growth parameters for the production of carotenoids by *Rhodotorula gracilis*.

Phaffia rhodozyma contains astaxanthin as its principal carotenoid (Andrews et al 1976). Several studies have been carried out to enhance the astaxanthin production by *P rhodozyma*. An et al (1989) attempted increased astaxanthin production by *P rhodozyma* by mutation. Effect of mutation on astaxanthin production by *Phaffia* has been studied by several workers (Fang and Chiou 1996, Bon et al 1997, Gill et al 1996). The effect of various nutrients on astaxanthin production by *Phaffia* has been studied (Gill et al 2001, An 2001, Fang and Jon 2002).

Studies have been carried out on carotenoid production by other yeasts also. Metabolic engineering was evaluated for production of β-carotene and lycopene in *Sachharomyces cerevisiae* (Yamano et al 1994). *Rhodosporidium* has been evaluated as a potential source of β-carotene (Miguel et al 1997). The food grade *yeast Candida utilis* was engineered to confer a novel biosynthetic pathway for production of carotenoids (Shimada et al 1998).

Carotenoids in aquatic animals

Carotenoids are responsible for the color of many important fish and shellfish products. It is remarked that grading or pricing of several fish and crustaceans is related to the intensity of redness (Sacton 1986). As carotenoids in animal tissues are solely derived from their dietary intake, aquatic animals are not exception. However, they differ in the requirements and assimilation of carotenoids (Shahidi et al 1998). Based on the mechanism of carotenoid metabolism aquatic animals are grouped in three categories (Tanaka 1978).

1. Red carp type, those that can convert β – carotene or lutein or zeaxanthin or their intermediate to astaxanthin (Figure III), examples being gold fish, red carp.
2. Sea bream type, those that cannot convert β-carotene and normally can only transfer the pigments from diet to tissue, examples being sea bream, trout, and salmon.
3. Prawn type, those that can convert β - carotene to astaxanthin, but not lutein (Figure IV), examples being crustaceans.

The primary source of carotenoids for aquatic animals is phytoplankton. The ingested carotenoids may be assimilated as such or may be converted to other form or may be completely catabolized, or may be passed out via feces (Haard 1992). Torrisen (2000) reviewed the dietary delivery of carotenoids and outlined the mechanism of digestion and absorption of carotenoids (Figure V).

In crustaceans, astaxanthin is formed from β - carotene or zeaxanthin through oxidative transformation (Katayama et al 1971). In sea bream, *Chrysophrys major*, zeaxanthin is converted to astaxanthin by oxidative metabolic pathway (Tanaka et al 1976). Matsuno et al (1981) studied the oxidative transformation of zeaxanthin and lutein

to astaxanthin, doradoxanthin and fritschiellaxanthin in gold fish. The oxidative metabolism of carotenoids in gold fish involves oxidation of 4,4`, 3,3` position of β - end group and epimerization of 3` position (Ookubo et al 1999).

The carotenoids also follow reductive metabolic pathway in some aquatic organisms. The reductive metabolic pathway of carotenoids in aquatic animals involves removal of keto group at C – 4 and C – 4` and conversion of β - ring to ε – ring (Matsuno et al 1985a). In rainbow trout astaxanthin is reductively metabolized to deepoxyneoxanthin via 4 – ketozeaxanthin and zeaxanthin (Schiedt et al 1985). Miki et al (1985) proposed the possible reductive metabolism of astaxanthin to tunaxanthin in yellow tail, *Seriola quinqueradiata* (Figure VI).

In red sea bream also astaxanthin is converted to tunaxanthin (Fugita et al 1983). In chum salmon dietary astaxanthin is converted to zeaxanthin via 4 – ketozeaxanthin (Kitahara 1983). In brook trout conversion of astaxanthin differs in different organs, as in muscle it is converted to zeaxanthin and lutein, while in ovary β - carotenol tetranol is produced in addition to these two pigments (Ando et al 1990).

Carotenoids in finfish

The distribution of carotenoids in finfishes varies with species, habitat and their food habits. Commonly found carotenoids are tunaxanthin in yellow fish, astaxanthin in red fish, zeaxanthin in anchovies, flatfish and shark, tunaxanthin, lutein and zeaxanthin in brackish water fish and lutein and zeaxanthin in fresh water fish (Matsuno and Hirao 1989). Several other carotenoids have been isolated and characterized from fishes and new carotenoids are continuously being identified.

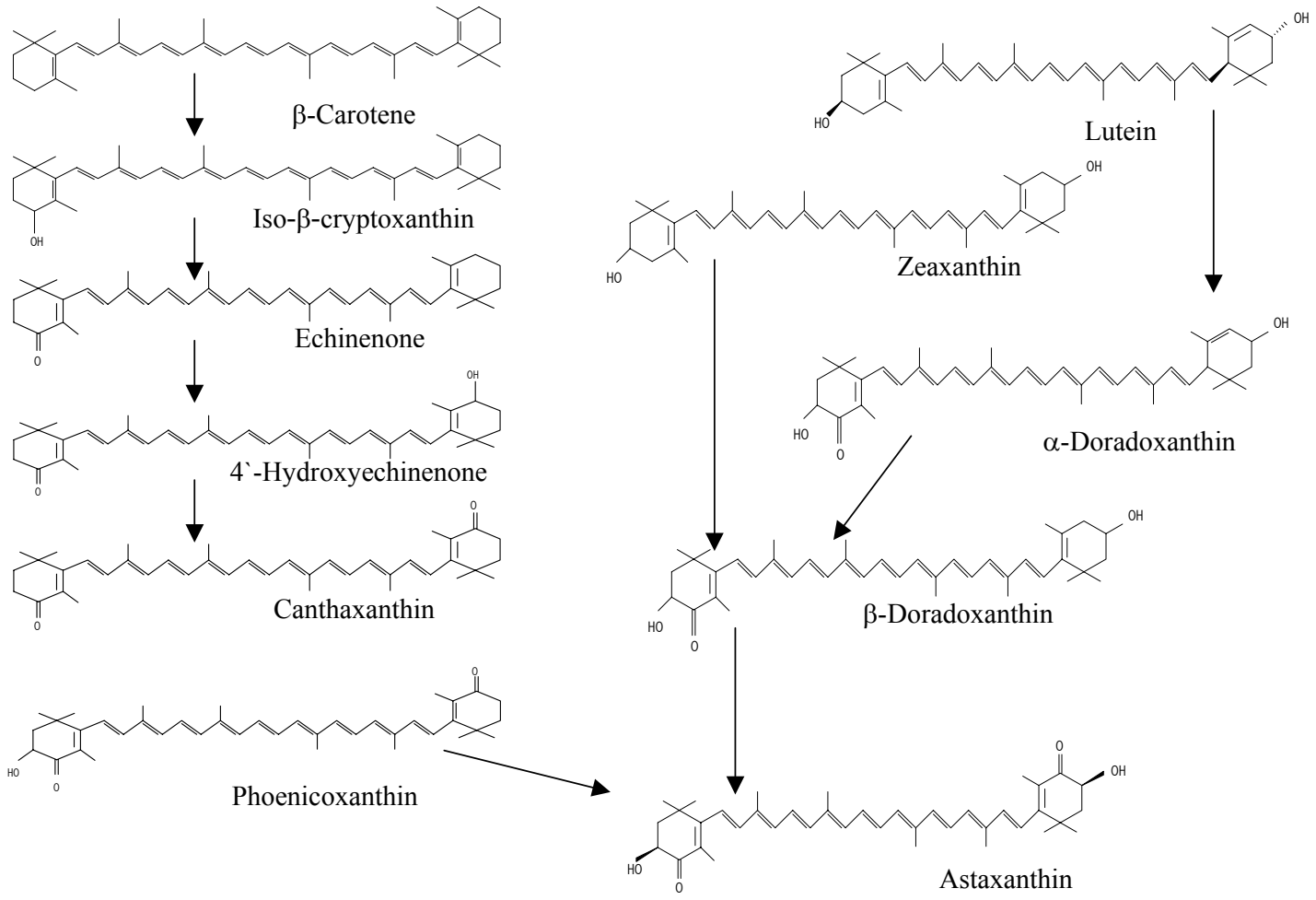


Figure III. Astaxanthin formation in red carp type fish

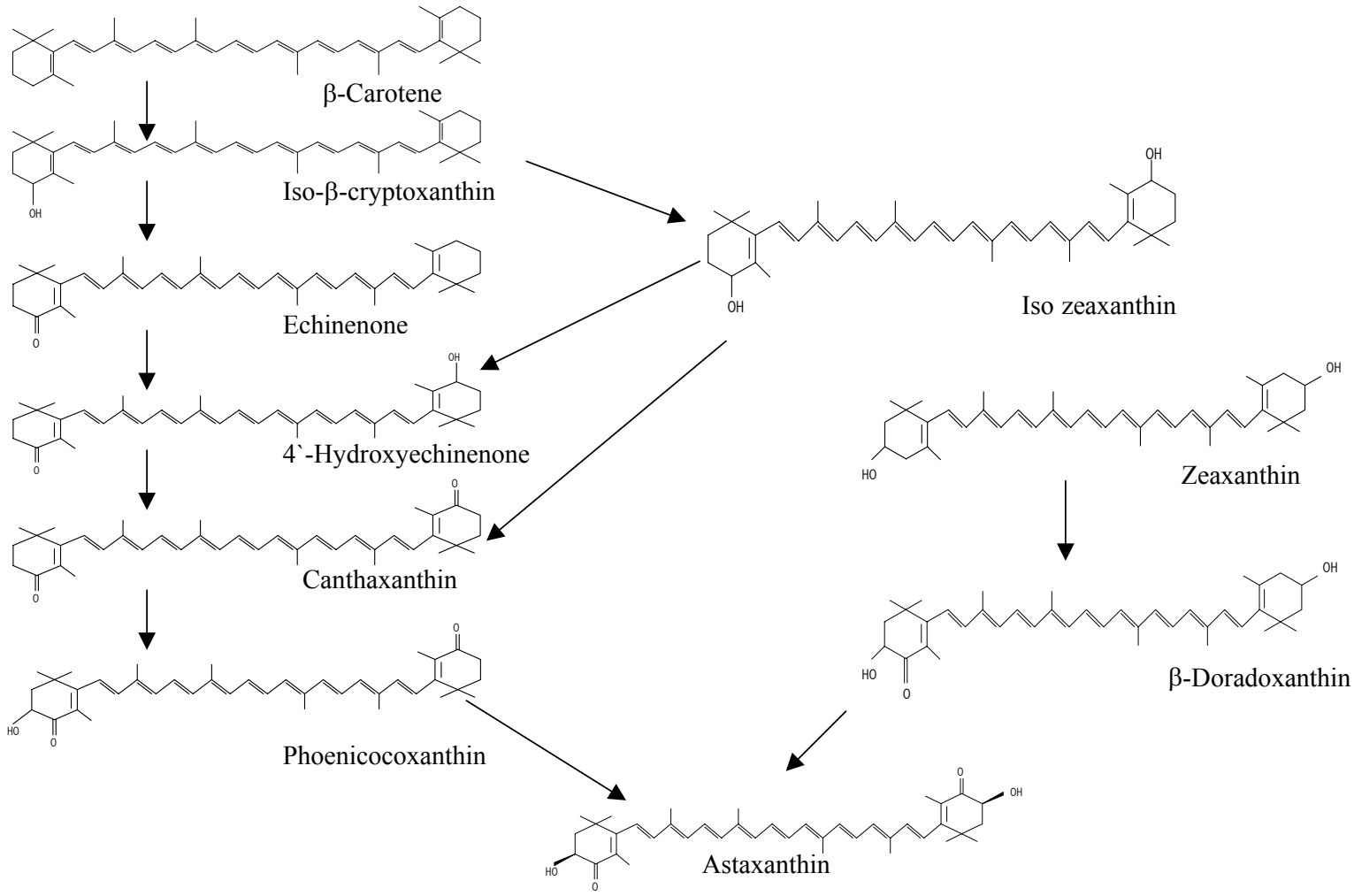


Figure IV. Astaxanthin formation in prawn

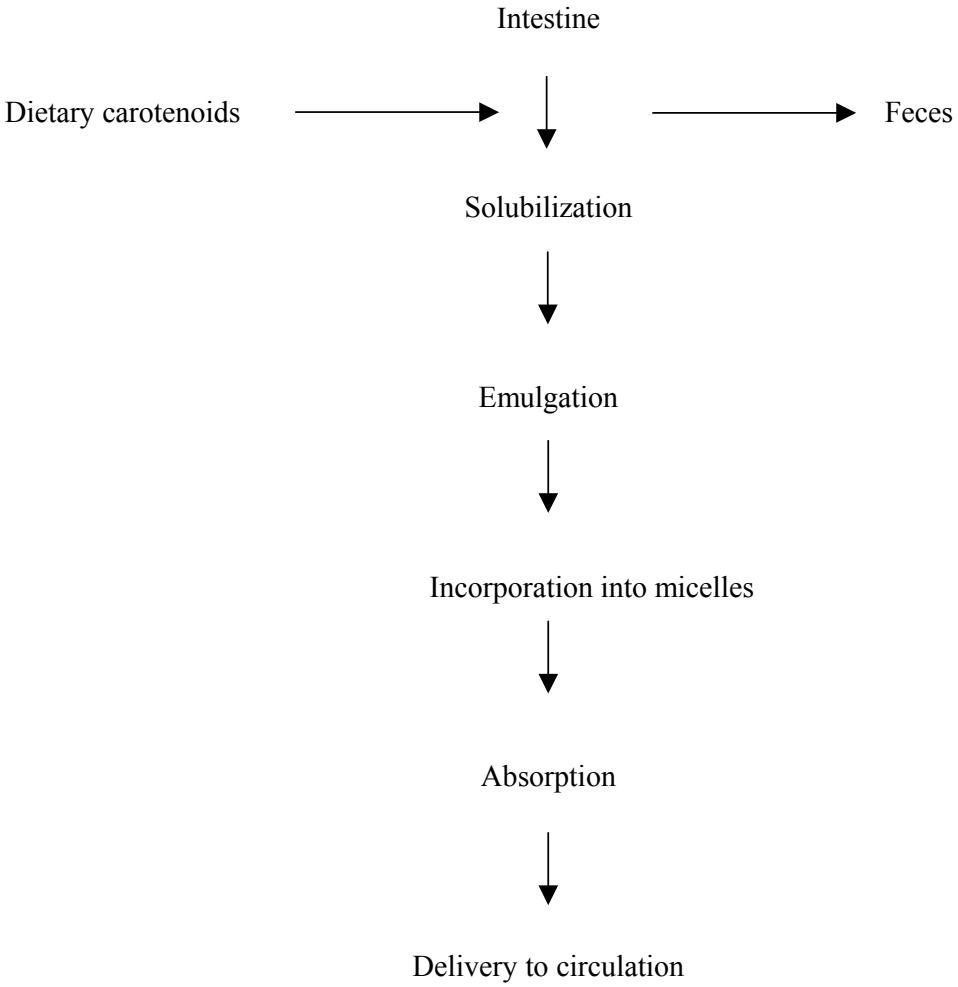


Figure V. Digestion and adsorption of carotenoids

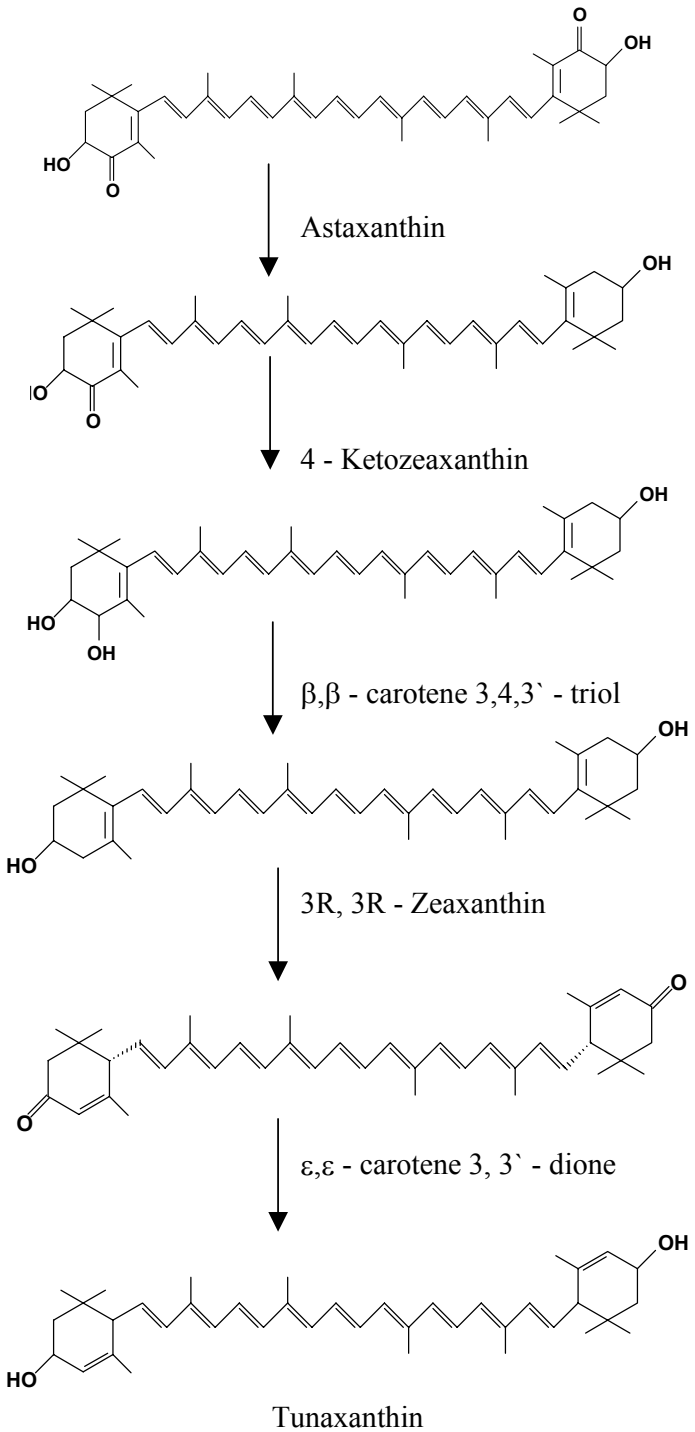


Figure VI. Reductive metabolic pathway of carotenoids in yellowtail, *Seriola quinqueradiata*

The main pigment found in sweetfish of Japan was zeaxanthin with minor quantities of cryptoxanthin, cynthiaxanthin, astacene, lutein and β-carotene (Matsuno et al 1974a). Tunaxanthin was found to be the major pigment in horse mackerel, puffers and porcupine fish, while zeaxanthin was present in considerable quantities in stripped mullets (Matsuno et al 1974c). In Chinese snakehead (*Channa argus*), tunaxanthin was found to be major pigment followed by lutein, zeaxanthin, cynthiaxanthin and β-carotene (Matsuno et al 1974b).

Matsuno et al (1976c) isolated carotenoids such as cryptoxanthin, diatoxanthin, cynthiaxanthin in addition to zeaxanthin from sea smelt. All the 19 species of fish in the family Percichtyes had similar carotenoid pattern with tunaxanthin as the major pigment (Matsuno and Katsuyama 1976a). The fishes in the family clupidae were found to contain tunaxanthin along with zeaxanthin, astaxanthin and doradoxanthin (Matsuno and Katsuyama 1976b). Czeczuga (1979) studied the carotenoids in the fishes from gadidae family from Polish waters and reported the presence of β-carotene and 4-hydroxy-4-keto-β-carotene in burbot (*Lotu lotu*) and isozeaxanthin in cod (*Gadus cacarius*). In the Arctic char, *Salvelinus alpinus* from Norwegian waters, astaxanthin and its esters were found to be the major pigment (Scalia et al 1989). Wild and cultured yellowtail had similar carotenoid pattern, tunaxanthin being the major pigment (Ha et al 1992). Lee et al (1996) observed that β-carotene, zeaxanthin and diatoxanthin are the major carotenoids in mandarin fish and Korean perch. Canthaxanthin and astaxanthin were the major pigments in spiny dogfish shark (Czeczuga and Czeczuga 1999a).

The red to pink coloration of fish belonging to salmonids group are generally due to astaxanthin (Matsuno et al 1980) and the color is the major factor affecting the acceptance of salmons by the consumers (Ostrander et al 1976). Highest astaxanthin

content (310 – 465 μg/100g) was observed in salmons than in trouts (Elmadfa and Majchrzuk 1998). Henmi et al (1987) studied the distribution and nature of carotenoids in salmon muscle and reported that astaxanthin is the major pigment, which is bound to muscle actomyosin by weak hydrophobic bonds. It is observed that salmon actomyosin forms complex with free astaxanthin and its monoesters, but not with its diester (Henmi et al 1989). By resonance Raman spectral and circular dichroism studies Henmi et al (1990) indicated that carotenoid protein interaction in salmon muscle is weak and astaxanthin has trans configuration *in vivo*.

Matsuno et al (1975a) studied the carotenoids in six species of fish of Gobineou family from fresh water and reported that cynthiaxanthin is the major pigment followed by zeaxanthin. Lutein and zeaxanthin was found to be the major carotenoid in fresh water mullets, while zeaxanthin and diatoxanthin were the major pigment in mullets from marine waters (Matsuno et al 1975b). Even though, tunaxanthin is the characteristic pigment of marine fish, Matsuno et al (1976a) isolated this pigment from fresh water perch, *Coreoperca kawameberi*. In two species of fish from Korean fresh water, *Odontobutis platylphala* and *O odontobutis,* Kim et al (1998) isolated cynthiaxanthin, diatoxanthin and tunaxanthin, which were rarely found in fresh water fish. Baek et al (1999) observed that zeaxanthin content decreases with a concomitant increase of cryptoxanthin and cynthiaxanthin after spawning in Korean fresh water fish Korean bittering and bride bittering.

Several new carotenoids are regularly being isolated from fishes. The presence of rhodoxanthin, a retro carotenoid was first reported by Matsuno and Katsuyama (1979) in *Tilapia*. Matsuno et al (1976b) isolated two new carotenoids parsiloxanthin and dihydroparsiloxanthin from Japanese catfish and postulated the metabolic pathway for

their production (Matsuno and Nagata, 1980). Other hydrocarotenoids that are isolated (Matsuno 1989) from Japanese catfish are 7`,8`- dihydro-β-β-carotene, 7,8,7,8`-tetrahydro-β-β-carotene, 7`,8` - dihydro-β-cryptoxanthin, 7,8 – dihydrolutein and 7`,8` - dihydro-diatoxanthin .

Yamashita et al (1996) reported the presence of a new apocarotenoid micropteroxanthin in black bass *Micropterus salmonides.* Two new carotenoids, 4-ketolutein D and 4-ketolutein F, were isolated from integuments of red filefish, *Banchiostegus japonicus* (Tsaushima and Matsuno 1998). An apocarotenoid galoxanthin was isolated first time by Yamashita et al (1998) from cultured ayu, and they postulated that the pigment was produced by eccentric cleavage of zeaxanthin. A new acetylenic carotenoid gobiusaxanthin has been isolated from fresh water goby, *Rhinogobius brunneus* (Tsushima et al 2000). Salmoxanthin and deepoxysalmoxanthin has been isolated from fishes belonging to salmonidae (Matsuno et al 2001).

Carotenoids in crustaceans

Crustaceans such as shrimp, prawn, lobster, krill and crab contain astaxanthin as their main pigment (Latscha 1990). Crustaceans absorb the pigments from the diet (Davies 1985) and deposit them as such or transfer them metabolically to keto or hydroxy derivatives (Castillo et al 1982). The pigments may be present in free forms, esterified or as bound form to macromolecules such as protein or chitin (Goodwin 1984). The complex forms of carotenoids in crustacean were identified to be carotenolipoproteins, chitinocarotenoids and carotenoproteins (Ghidalia 1985). Carotenolipoproteins are the complex of carotenoids, lipids and proteins found mainly in ovaries and eggs, while chitinocarotenoids are found in exoskeleton, where they are formed by a Schiff base bonds between terminal basic nitrogen bonds of chitin and keto group of carotenoids

(Ghidalia 1985). Fox (1973) investigated the properties of chitinocarotenoids in the red kelp crab, *Taliepus muttalli* and reported that, carotenoids from chitinocarotenoids can be isolated only after decalcification of the shell.

Carotenoproteins are the carotenoid protein complexes, which are extensively studied (Zagalsky et al 1970, Shahidi et al 1998). Association of the carotenoids with protein results in display of various colors in crustaceans (Zagalsky 1985) and cleavage of this complex results in color change due to the liberation of free carotenoid (Nelis et al 1989). The carotenoproteins are soluble in aqueous media and are stable (Zagalsky et al 1990).

Crustacyanin, the blue carotenoprotein from lobster, is studied by several workers. Zagalsky and Cheesman (1963) purified and characterized crustacyanin from lobster carapace. They reported that the complex has an absorbance maxima (λ_{max}) of 632 nm. Jenkens and Button (1964) reported that denatured crustacyanin shows a λ_{max} of 479 nm in ethanol, indicating the release of carotenoid, and the color of the pigment complex changes from blue to purple, yellow or red on denaturation. The appearance of red color in cooked lobster has been attributed to the release of astaxanthin from the carotenoprotein upon heating (Fox 1979). Ovoverdin is the green carotenoprotein isolated from lobster eggs and shows similar characteristics to crustacyanin when denatured (Miki et al 1982).

Quarmby et al (1977) studied the quaternary structure of the crustacyanin and characterized the apoprotien subunits. Electron microscopic study of crustacyanin revealed that the structure is formed from a linear array of eight crustacyanin molecule coiled in a helical manner into a compact configuration (Zagalsky and Jones 1982). The characteristics of carotenoids in crustacyanin have been studied by Renstrom et al (1982)

by protein carotenoid recombination technique. In crustacyanin, the carotenoid astaxanthin has been found to be bound to apoprotein within an internal hydrophobic calyx (Clarke et al 1990). Keen et al (1991) studied the complete sequence of protein subunits of crustacyanin and reported that in crustacyanin, the carotenoid environments are characterized by a preponderance of aromatic and polar residues and the absence of charged side-chains. Krawczyk and Britton (2001) isolated three spectral forms of blue crustacyanin having absorbance maxima of 632 nm, 660 nm and 780 nm respectively. A yellow carotenoprotein complex of astaxanthin and protein has also been isolated from lobster carapace (Zagalsky 1982).

Carotenoproteins from other crustaceans has also been isolated and characterized. Carotenoprotein from the crayfish, *Procambarus clarkii* was found to be of two types with red and blue color (Milicua et al 1985, 1990). The blue carotenoprotein is similar to crustacyanin in its characteristics (Garate et al 1986a). Ando and Tanaka (1996) reported that the blue carotenoprotein contains free astaxanthin alone, while the red carotenoprotein comprises of both free astaxanthin and astaxanthin esters. The chemical properties and the effect of different denaturing agents on blue carotenoprotein of crayfish have been reported (Garate et al 1986b).

Muriana et al (1993) characterized the blue carotenoprotein from shrimp *Peaneus japonicus* and reported that all-trans-astaxanthin is the main carotenoid. Nur-E-Borhan et al (1995) purified two blue carotenoproteins from *Penaeus monodon* each differing in molecular weight and absorbance maxima, both consisting of six subunits with astaxanthin as prosthetic group. Okada et al (1995) studied the carotenoproteins in cultured black tiger prawn and reported that the different color of the cultured prawn is due to the varied composition of the blue carotenoprotein and the red carotenoid fractions

in the muscular epithelium. The blue carotenoprotein from crab *Carcinas marinus* was found to be in octameric form (8 subunits) each subunit containing two astaxanthin molecules as prosthetic group (Garate et al 1984).

Studies have been carried out to identify the prosthetic carotenoid groups in carotenoproteins and astaxanthin and canthaxanthin were the carotenoids, which are usually isolated (Zagalsky 1983). Other carotenoids isolated were derivatives of astaxanthin such as 7,8-didehydroastaxanthin and 7,8,7`,8`-tetrahydroastaxanthin from purple carotenoprotein and asteriarubin from blue carotenoprotein (Zagalsky et al 1990).

Astaxanthin, in addition to being present in complex with proteins is also present in free form as the major carotenoid in crustaceans. Several other carotenoids have also been isolated from crustaceans. Balachandran (1976) reported the presence of lutein, astaxanthin and astacene in Indian prawn *Parapaeneopsis stylifera.* Astaxanthin was found to be present in both enatiomeric and meso forms in shrimp *Pandalus borealis* (Renstrom et al 1981). Fernandez and Burgos (1981) isolated phoenicoptenone and celaxanthin for the first time from crustaceans from Indian Ocean. Ha et al (1985) reported that both cultured and wild prawns contain astaxanthin, phoenicoxanthin and β-carotene as major carotenoids. In shrimp (*Penaeus monodon*) heads astaxanthin was found to be present in 3 optical isomers viz.,3R,3`R; 3R,3`S and 3S,3`S (Wu and Sun 1993). Astaxanthin and β-carotene were the major pigments in the shrimp *Penaeus japonicus* (Negre-Sadargues et al 1993). The carotenoids in the prawn were found to be affected by the molting stage (Hung et al 1999) and the carotenoid concentration reflects the molting physiology (Hung and Hu 2000).

Astaxanthin and its esters were isolated as major carotenoids from the shrimp *Pandalus borealis* (Shahidi 1995). Okada et al (1994) observed that cultured black tiger

prawn *Penaeus monodon* preferentially accumulates astaxanthin monoester in exoskeleton when the total carotenoid content exceeds 8 mg%. The astaxanthin esters from brown shrimp *Crangon vulgaris* were found to be composed of myristic, palmitic, palmitoleic, steric and oleic acids (Snauweart et al 1973a). However, Renstrom and Liaaen-Jensen (1981) noted no preferential selection of fatty acids in the astaxanthin esters of *Pandalus borealis*.

From the fresh water prawn *Macrobrachium rosenbergii*, Maugle et al (1980) isolated astaxanthin and its esters as primary pigments and by feeding experiments indicated that that this aquatic organism is capable of converting β-carotene to astaxanthin via isocryptoxanthin, echinenone and canthaxanthin. Free and esterified carotenoids were found to be the main pigments in deep-sea shrimps also (Neger-Sadragues et al 2000).

In crayfish along with astaxanthin other pigments such as idonirubin, idoxanthin and canthaxanthin have been isolated (Milicua et al 1990). Czeczuga and Czeczuga (1999b) compared the carotenoids in four species of crayfish and reported that canthaxanthin, adonixanthin and astaxanthin are predominant. Studies on metabolism of astaxanthin during embryonic development of crayfish revealed that free astaxanthin and lutein represent the main pigment occurring in the yolk at the end of embryonic period (Oliver et al 2000).

Matsuno et al (1974d) isolated astaxanthin, β-carotene, echinenone, canthaxanthin, phoenicoxanthin, lutein, zeaxanthin and 4-ketozeaxanthin from crab *Scyllarides squamosus* and *Parribacus ursus*. From the carapace of crab *Sesarama*, Matsuno and Watanabe (1974) isolated doradoxanthin as the principle carotenoid. Freschielloxanthin has been isolated from the crab *Sesarama* (Matsuno and Ookubo

1982). In the carapace of crab *Paralithodes brevipes*, astaxanthin and 7,8-didehydroastaxanthin were identified as major carotenoids (Matsuno and Maoka 1988). Lutein was isolated along with astacene and canthaxanthin from the snow crab *Chinocetes opilio* (Shahidi and Synowiecki 1991).

Studies have been carried out to isolate and characterize the carotenoids in Antarctic krill, *Euphausia superba*. Among the marine animals krill was found to contain highest carotenoid content (Czeczuga 1981). Yamaguchi et al (1983) reported that astaxanthin diester is the major pigment in krill. The astaxanthin content in krill eye lipids were found to be ten times higher than in the whole krill lipids (Rzhavskaya and Menyaeva 1981). Shibata (1983) observed no changes in the carotenoid content of krill during different fishing seasons.

Carotenoids in other aquatic animals

Occurrence of carotenoids has been observed in various other aquatic animals such as mollusks, echinoderma, tunicates, sea anemone, marine sponges etc (Matsuno 2001). Presence of mytiloxanthin and isomytiloxanthin is reported from the mussel *Mytilus edulis* (Khare et al 1973). Hertzberg et al (1988) isolated 19 different carotenoids from the mussel *Mytilus edulis*. Maoka and Matsuno (1988) isolated pectanol and 4-hydroxyaloxanthin from Japanese sea mussel *Mytilus corscus*. Occurrence of mactraxanthin (Matsuno and Sakaguchi 1983), amarouciaxanthin (Matsuno et al 1985c) and fucoxanthinol (Matsuno et al 1986) is reported from clams. Fujiwara et al (1992) isolated crassostreoxanthin from the oyster *Crassostrea gigas*.

Maoka et al (1989b) screened 9 species of cephalopods for presence of carotenoids and reported that the three stereoisomers of astaxanthin are the major pigments. Katagiri et al (1986) characterized some of the unique carotenoids of

gastropods. Echinenone, fristchiellaxanthin, phoenicoxanthin and their metabolites have been isolated from the gastropod, spindle shell (Matsuno and Tsushima 1989). Presence of two new trihydroxy carotenoids β, ε - carotene – 3,4,3`-triol and β,β - carotene –3,4,3`-triol in chitons is reported (Tsushima et al 1989). Triophaxanthin and hopkinsioxanthin have been isolated from the animals of the group nudibranchs (McBeth 1972). Two new apocarotenoids, α-citraurin and β-citraurinol, have been isolated from sea hare (Yamashita and Matsuno 1990).

In sea urchins β-echinenone was observed to be the major carotenoid (Tsushima and Matsuno 1997). Cucumarioxanthin, a novel carotenoid has been identified in sea cucumber (Tsushima et al 1996). Several acetylenic carotenoids have been isolated from starfish (Maoka et al 1989a). In tunicates alloxanthin is the major carotenoid (Ookubo and Matsuno 1985). The metabolic product of fucoxanthin, namely amarouciaxanthin, was isolated from the tunicate *Amaroucium pliciferum* (Matsuno et al 1985b). Hetrzberg et al (1969) isolated a unique carotenoid actinoerythrin along with perdinin, 2,2`-dinor-astaxanthin from sea anemone.

The coloration of marine sponges has been attributed to the presence of carotenoids. The carotenoids in marine sponges are investigated to be aryl carotenoids (Yamaguchi 1982). The aryl carotenoids isolated from sponges include isoagelaxanthin (Tanaka et al 1982), isoclathriaxanthin (Tanaka and Yamamoto 1982) and tethyanine (Tanaka and Yamamoto 1984). Carotenoid sulfates such as bastaxanthin have also been isolated from sponges (Liaaen-Jensen et al 1982). Presence of methoxylated carotenoids such as aaptopurpurin has been observed in marine sponges (Ramadahl et al 1981).

CAROTENOIDS IN AQUACULTURE

Aquaculture has become one of the major practices for continuous supply of aquatic animals. Aquatic animals grown in wild depend on the diets for their carotenoid requirements. Aquatic animals, which are cultured, do not show the same coloration as that of their wild counter part (Spinelli and Mahnken 1978). Pigmentation of cultured species like salmonids and crustacean is done through dietary manipulation (Shahidi et al 1998). Consumers have preference for red colored products of salmonid fishes (Skoneberg et al 1998). Feeding pigment-enriched diet is regarded as one of the most important management practice for marketing farmed salmon (Moe 1990). Sylvia et al (1996) indicated that redness has a significant role as an indicator of product quality of salmonids.

Carotenoid pigmentation of fish is affected by dietary pigment source, dosage level, duration of feeding and dietary composition (Bjerkeng 2000). Both synthetic carotenoids and natural pigment sources have been used for pigmentation of cultured fishes. Synthetic astaxanthin and canthaxanthin either alone or in combination is most commonly used for pigmentation of salmonids (Storebakken and Choubert 1991, Storebakken and No 1992). However it has been noted that synthetic canthaxanthin produces yellow-orange color, not a natural color of wild grown salmons (Torrison et al 1989).

Shahidi et al (1993) noted that feeding of Arctic char for 15 weeks with feed containing 75 ppm of astaxanthin or canthaxanthin is sufficient to impart color to the fillets. Dietary canthaxanthin was not only deposited as such in cultured Arctic char, but also reductively metabolized to echinenone, 4`-hydroxyechinenone and β-carotene (Metusalach et al 1997). Idoxanthin, a metabolite of astaxanthin was found to be the

major carotenoid in Arctic char fed with astaxanthin containing diet (Hansen et al 1997). Astaxanthin was found to be more efficiently utilized than canthaxanthin for flesh pigmentation in salmons (Bjerkeng et al 1992). Kinetic study of astaxanthin and canthaxanthin in the diet of rainbow trout indicated that the retention time of astaxanthin in serum is higher than that of canthaxanthin (Gobantes et al 1997). Kim et al (1999) observed that astaxanthin supplemented fish had the greatest change in body pigmentation than those supplemented with lutein or cynthiaxanthin. In sea bream, synthetic carotenoid supplementation increased the total carotenoid content in the skin, but had no effect on muscle pigment content (Gomes et al 2002).

Bjerkeng et al (1997) compared the pigmentation efficiency of astaxanthin isomers in salmonids and observed that muscle carotenoid concentration tends to be higher in trouts fed with all E-astaxanthin than those fed with mixture of E and Z-isomers. Z-astaxanthin was found to be deposited in low concentration in muscle of cultured Atlantic salmon (Bjerkeng and Berge 2000) and Arctic char (Bjerkeng et al 2000).

The use of synthetic pigments in aquaculture is not favored in many countries. In EC countries the presence of canthaxanthin in smoked fish fillets is prohibited (Tantillo et al 2000). However, Baker (2002) analysed the risks involved in canthaxanthin in aquafeed applications and concluded that use of canthaxanthin in salmon feeds presents no health risk to consumers.

The best alternative to synthetic carotenoids would be use of natural carotenoids for fish pigmentation. Several natural sources such as crustacean waste, algae and yeasts have been used for the pigmentation of cultured aquatic animals.

Crustacean waste is one of the important sources of natural carotenoids. Lambertson and Braekkan (1971) analysed several marine products for occurrence of

astaxanthin and reported that krill contains high level of astaxanthin. Pigmentation of cultured salmonids has been achieved with inclusion of crustacean waste in their diets (Saito and Reiger 1971). However, direct use of crustacean waste as a pigment source in feed results in variable pigment level, susceptibility to deterioration, bulkiness, high transportation cost and high chitin content (Haard 1992). The use of crustacean meals as pigment source in feed is not desired because of low carotenoid content and high calcium and chitin level (Simpson et al 1981). Thus attempts have been made to use concentrated carotenoid extracts from crustacean waste.

Spinelli and Mahnken (1978) used oil extracts of red crab for pigmentation of coho salmon and observed that feed containing 6 – 9 mg% carotenoid imparted a good coloration after 120 days of feeding. Feeding of silver salmon with a diet containing krill extracts resulted in similar coloration and carotenoid content of flesh as in naturally grown fish (Yamazaki et al 1983). Mori et al (1990) made similar observation in cultured coho salmon fed with diet containing extracts from krill and mysid shrimp. Ya et al (1991) suggested that carotenoproteins extracted from lobster waste contains high level of astaxanthin and low level of chitin, thus can be used as an inexpensive source of pigment in cultured salmonid species.

Choubert (1979) observed yellow brown pigmentation of cultured rainbow trout fed with β-carotene rich spirulina algae. Use of green algae *Hematococcus pluvialis* spores for pigmentation of rainbow trout indicated that pigmentation efficiency of these algae is lower than synthetic astaxanthin due to high level of esterified astaxanthin in algae and poor digestibility of ingested spores (Sommer et al 1992). The retention of algal carotenoids in the trout muscle was found to be lower than that of synthetic carotenoid. Feeding experiments with diet containing microalgae *Chlorella vulgaris* indicated that

this algae was an acceptable feed additive for enhancement of color of rainbow trout muscle (Gouveia et al 1997). Inclusion of pigment extract from the flower *Adonis aestivalis* in diet was found to impart bright pink coloration in rainbow trout (Kamata et al 1990).

The yeast *Phaffia rhodozyma* has been suggested as a best alternative to synthetic astaxanthin for salmonid pigmentation (Tangeras and Slinde 1994). Higgs et al (1995) demonstrated that fish receiving the *P rhodozyma* pigment had high astaxanthin concentration and more intense color in the muscle than those receiving synthetic astaxanthin.

Carotenoids have been used for pigmentation of cultured crustaceans also. Yamada et al (1990) observed that dietary astaxanthin was incorporated to the body tissue of *Penaeus japonicus* at higher rate than β - carotene or canthaxanthin. Negre-Sadargues et al (1993) reported that feeding of shrimps with a diet containing mixture of astaxanthin and canthaxanthin results in better pigmentation than feeding the pigments individually. In lobsters feeding with pure carotenoids such as β-carotene, echinenone and canthaxanthin resulted in accumulation of astaxanthin in exoskeleton (D`Abramo et al 1983).

Liao et al (1993) noted a marked increase in carotenoid content in carapace of prawns fed with zeaxanthin rich spirulina-supplemented diet, with transformation of zeaxanthin to astaxanthin. Sheenan et al (1998) used diets supplemented with pigmented microalgae *Dunaliella salina* for pigmentation of cultured crayfish *Cherax quadricarinatus*. Boonyaratpalin et al (2001) suggested that use of β-carotene rich micro algae *Dunaliella salina* in the diet of *Penaeus monodon* results in similar pigmentation as

feeding with astaxanthin rich diet due to metabolic conversion of β - carotene to astaxanthin.

Dietary astaxanthin has been found to improve the production yield in shrimp farming by its influence on survival, growth and resistance to disease (Gabaudan 1996). Astaxanthin supplemented diet was found to shorten the molting cycle of in *Penaeus japonicus* (Petit et al 1997). Pangantihon et al (1998) reported the involvement of astaxanthin in the reproduction in *P monodon* and recommended its inclusion in the diet of brood stock. Hung et al (2001) suggested that it is essential to maintain a certain level of astaxanthin in the diet for post larvae of *P monodon* for better survival.

EFFECT OF PROCESSING ON CAROTENOIDS IN AQUATIC PRODUCTS

Several studies have been carried out to assess the effect of various processing and storage methods on quantitative and qualitative characteristics of carotenoids in aquatic products. The changes, which are brought about by processing, include slight shift in color, cis/trans isomerization and complete loss of color as depicted in figure VII (Simpson 1982).

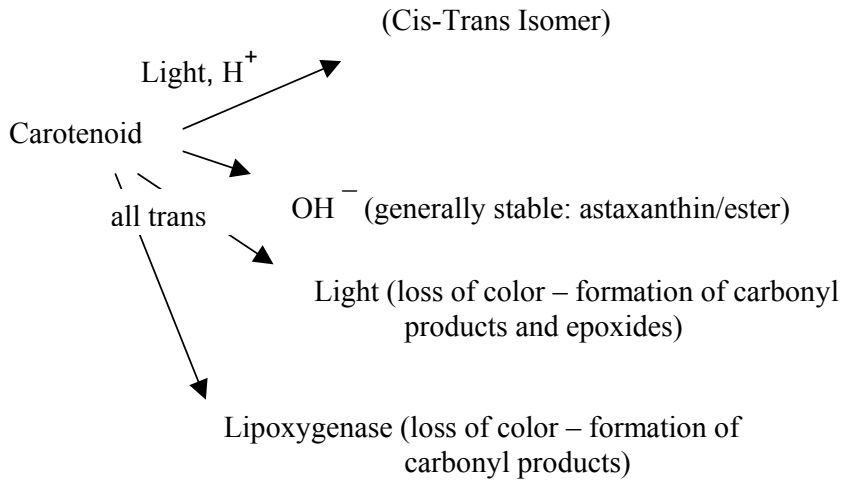


Figure VII. Degradation of carotenoids

Lipoxygenase was found to be responsible for discoloration of red fish during storage at refrigeration temperatures (Tsukuda 1972). Yakovleva and Tseluiko (1970) observed a transition of carotenoids from the skin of fish into the subcutaneous fat during chilling and cold storage as freezing resulted in the drop of carotenoid level in skin with a simultaneous increase in carotenoid content in subcutaneous fat of Black sea gray mullet. Pigment migration and subcutaneous yellowing during frozen storage of Caspian sprat (Pavel'eva and Vlasova 1973), and herring and mackerel (Lyubavina et al 1972) have also been reported. Addition of CO_2 to refrigerated seawater (RSW) was found to improve the color retention of ocean perch during storage at –1°C (Longard and Regier 1974). Song et al (1977) reported that storage of yellow sea bream at –5°C for 3 months results in complete fading. However No and Storebakken (1991) observed no changes in carotenoids of rainbow trout fillets during frozen storage at –20°C for 6 months. Chistophersen et al (1992) reported that carotenoids in frozen salmonids are sensitive to light and less sensitive to O_2 transmission rate of the packaging material. Scott et al (1995) did not observe any changes in pigment content in cultured rainbow trout fillets during frozen storage.

Choudhry (1977) studied the effect of thermal processing and storage on the carotenoid content of channel catfish and reported that the Hunter a* (redness) values were significantly reduced by cooking. The comparative study on effect of heat processing of chum salmon and sockeye salmon indicated that the chum salmon tends to exhibit an apparent fading than sockeye salmon (Masuda et al 1976). Heating of krill homogenate at 100°C for different period indicated that the total carotenoid content is reduced and the effect being more on free astaxanthin and its monoester than on diester form (Miki et al 1983). Color intensity in canned shrimps-in-brine increased, as

percentage of impurities such as calcium and magnesium in common salt increased (Godavary Bai 1987).

Tray drying of shrimp meal results in drastic reduction of carotenoid content in the meal (Simpson et al 1976). Carotenoid content in Antarctic krill meal was found to decrease during storage at room temperature (Tanaka et al 1981). Ghosh and Nerkar (1991) reported that a dip in 10% NaCl solution for 30 mins before drying considerably reduces the pigment loss during drying and storage of shrimps. Drying of recovered carotenoproteins at temperatures above 45°C was found to lower the levels of carotenoids (Ramaswamy et al 1991). Addition of antioxidants was found to prevent pigment degradation during storage of crawfish meal (Meyers and Bligh 1981, Chen and Meyers 1982).

Savagon et al (1972) reported that astaxanthin undergoes oxidative degradation in irradiated shrimp during storage, which could be prevented by vacuum packaging. Snauwaert et al (1973b) observed no immediate effect of irradiation on pigment content in shrimps, but significant losses in pigment content during further storage. The radiation stability of carotenoids was attributed to the effect of proteins on orientation of carotenoids (Snauwaert et al 1974)

Torrison et al (1981) reported that acid ensilaging of shrimp waste results in gradual conversion of astaxanthin diester to monoesters during storage of silage. However, Guillou et al (1995) observed no such changes during storage of shrimp waste silage.

RECOVERY OF CAROTENOIDS FROM CRUSTACEAN WASTE

Crustacean exoskeleton is one of the important natural sources of carotenoids, particularly astaxanthin. Several studies have been carried out to recover the pigment

from crustacean processing discards. Methods such as extraction of carotenoids using organic solvents and edible oils and recovery of carotenoids as carotenoprotein have been attempted.

Solvent extraction process for recovery of carotenoids from crustacean waste has been limited to analytical purposes. Meyers and Bligh (1981) extracted pigments from heat processed crawfish waste using a ternary system of ether, acetone and water. Britton (1985) outlined the protocols for solvent extraction of carotenoids as analytical tool. Carotenoids in shrimp waste can be extracted using cold acetone and subsequently partitioned using petroleum ether (Mandeville et al 1991). Kozo (1997) used 80% alcohol for extraction of astaxanthin from crustacean waste that is acidified to remove calcium and neutralized with alkali. Masatoshi and Junji (1999) used acetone for extraction of carotenoids from acidified shrimp waste. Supercritical CO_2 method with ethanol as cosolvent has also been attempted for astaxanthin extraction from crawfish shells (Charest et al 2001).

As carotenoids in crustacean wastes are fat soluble, vegetable oils have been used to extract pigments from waste. Anderson (1975) patented a process for extraction of carotenoids from shrimp processing waste wherein soybean oil is added to the waste, mixed, heated and the oil fraction recovered by centrifugation. Spinelli and Mahnken (1978) developed a 3-stage counter current extraction process for recovery of astaxanthin containing oil from red crab waste. Chen and Meyers (1982) used enzymatic hydrolysis of homogenised crawfish waste with a protease and subsequent extraction with soy oil for recovery of carotenoids. In the patented process for utilization of crustacean shell waste (Meyers and Chen 1985), the crawfish waste is homogenised, acidified and heated with soybean oil to recover pigment-enriched oil. The extraction of carotenoids using different

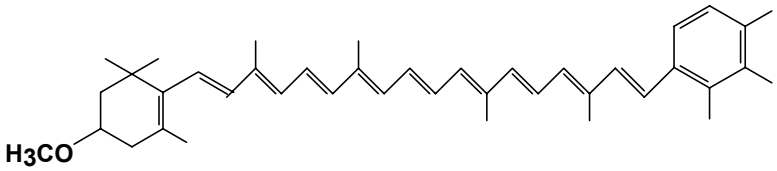
oils such as soybean, cottonseed, herring, menhaden and salmon oil was attempted by Chen and Meyers (1984), and power model was developed for the estimation of pigment in different oil, based on absorbance maxima and extinction coefficient of astaxanthin in different oils. No and Meyers (1992) demonstrated that the process of oil extraction of carotenoids from crawfish waste can be integrated with production of chitin and chitosan. Cod liver oil also has been used to extract pigments from processing discards of snow crab and shrimp waste (Shahidi and Synowiecki 1991). Yamaguchi et al (1986) adopted supercritical CO_2 extraction for separation of oil from krill, which contained astaxanthin as main pigment. A method has been developed based on silica gel column chromatography for concentration of carotenoids in krill oil (Hara et al 2001). Conditions for supercritical CO_2 extraction of astaxanthin from crab shell waste using ethanol as cosolvent has been standardized by Felix-Valenzuela et al (2001). Charest et al (2001) developed a quadratic model relating to the yield of astaxanthin from crawfish waste by supercritical CO_2 extraction.

Torrison et al (1981) attempted acid ensilaging as a method for stabilization of astaxanthin in shrimp waste during storage prior to oil extraction. Acid ensilaging of crawfish waste was found to stabilize the astaxanthin in the waste and also increase the recovery of astaxanthin in soy oil (Chen and Meyers 1983). The crude oil extract from shrimp waste silage was found to be more concentrated in astaxanthin than the oil obtained from raw shrimp waste (Inoue et al 1988). Omara-Alwala et al (1985) reported that the use of propionic acid enhances the recovery of astaxanthin from crawfish waste by 35% when extracted using vegetable oil. Guillou et al (1995) observed that silaging treatment of shrimp waste was effective in stabilizing astaxanthin in the waste and also increasing the yield of carotenoid recovery by solvent extraction.

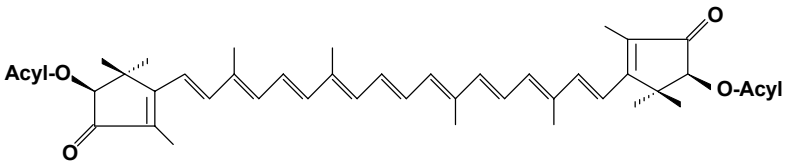
As carotenoids are more stable as complex with proteins, studies have been carried out on recovery of carotenoids as carotenoproteins. Simpson and Haard (1985a, 1985b) developed an enzymatic technique for extraction of carotenoprotein from shrimp waste using chelating agents like EDTA and the proteolytic enzyme trypsin. Zagalsky (1985) indicated that decalcfication of finely ground crustacean carapace using agents like EDTA is necessary for extraction of carotenoproteins. Cano-Lopez et al (1987) used trypsin from Atlantic cod instead of bovine trypsin for increased recovery of carotenoprotein from shrimp waste. Trypsin hydrolysis of snow crab waste followed by ammonium sulphate precipitation yielded carotenoprotein with increased carotenoid content (Manu-Tawiah and Haard 1987). Lobster waste has also been used to recover carotenoprotein with the aid of bovine trypsin and cod trypsin (Simpson et al 1992, Ya et al 1991). Drying characteristics of carotenoprotein recovered from lobster waste has been evaluated at different temperatures and relative humidity levels (Ramaswamy et al 1991). Carotenoprotein from crawfish waste has also been extracted by a fermentation process (Cremades et al 2001).

STRUCTURE OF SELECTED CAROTENOIDS

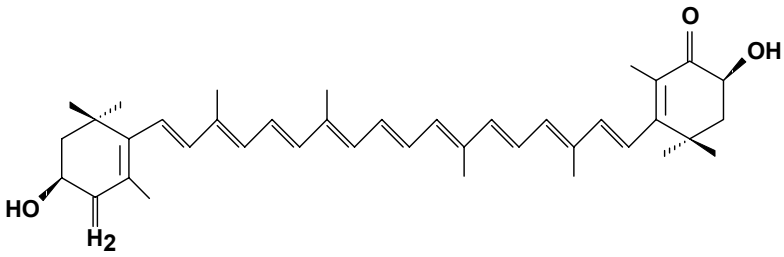
STRUCTURE OF SELECTED CAROTENOIDS



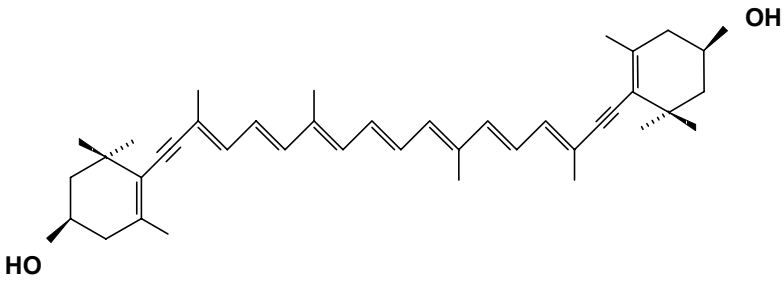
Aaptopupurin



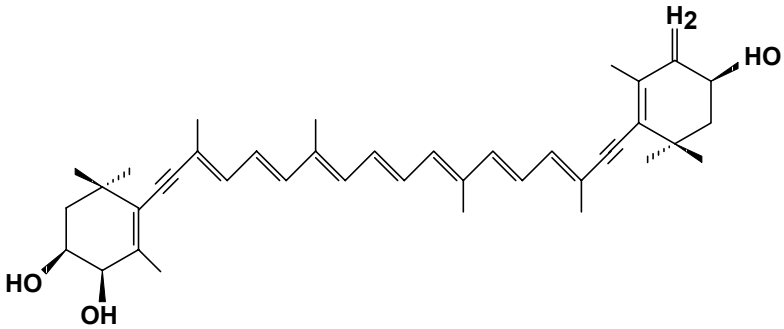
Actinoerythrine



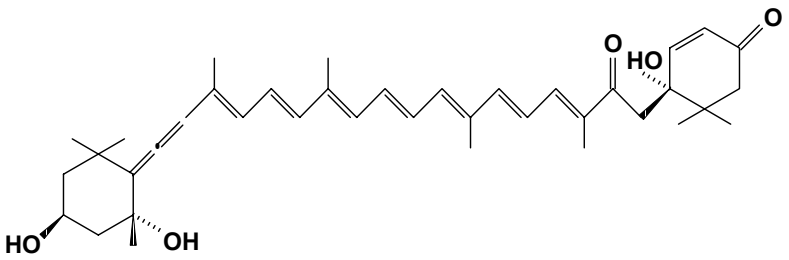
Adonixanthin



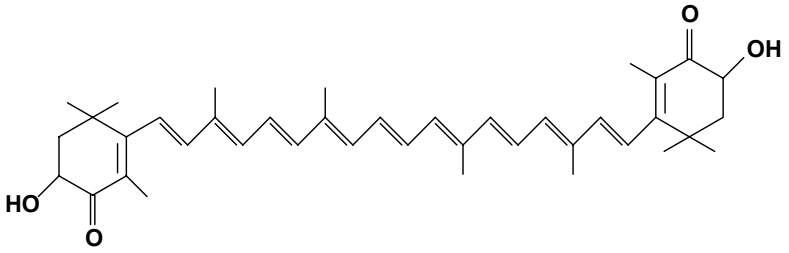
Alloxanthin



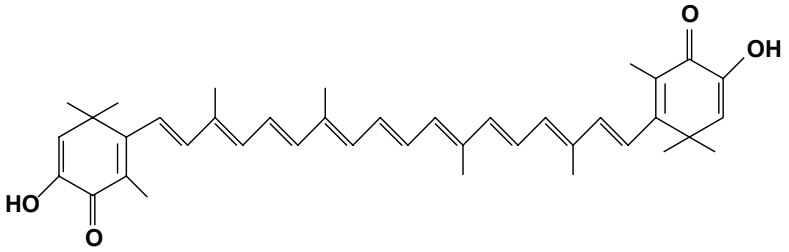
4-Hydroxyalloxanthin



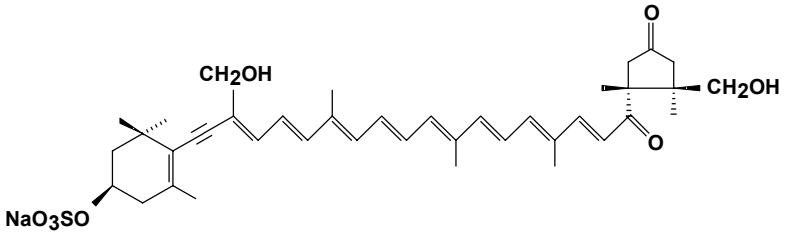
Amarouciaxanthin



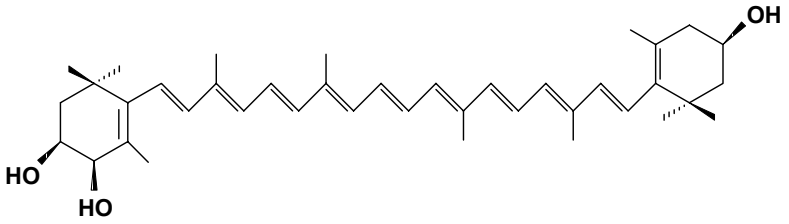
Astaxanthin



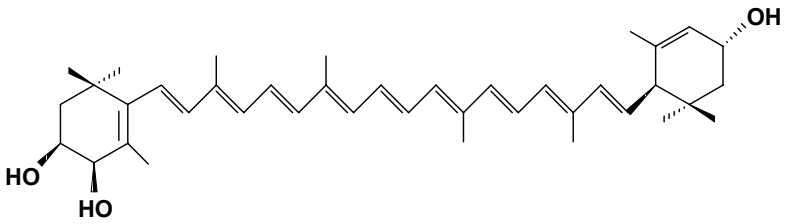
Astacene



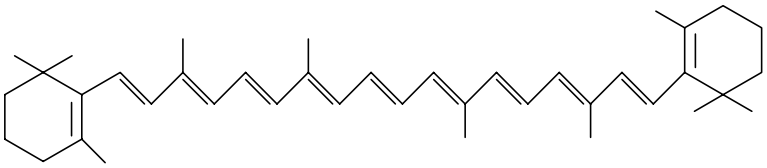
Bastaxanthin



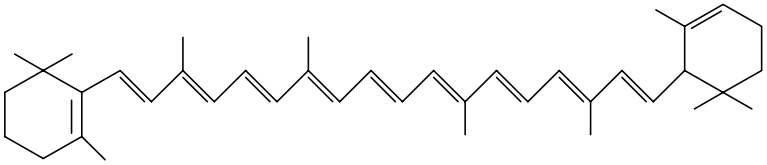
β,β-Carotene-3,4,3-triol



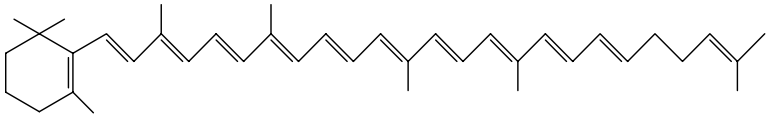
β,ε-Carotene-3,4,3-triol



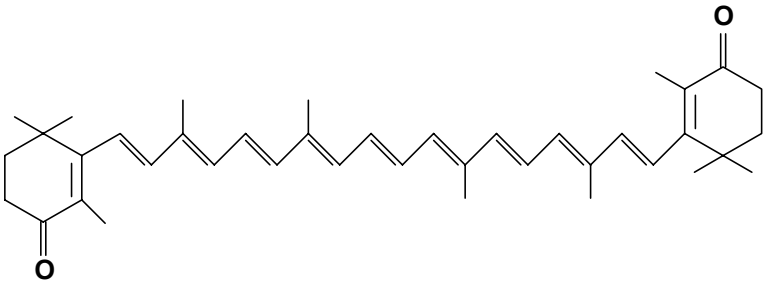
β-Carotene



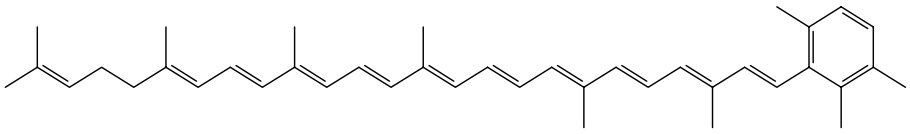
α-Carotene



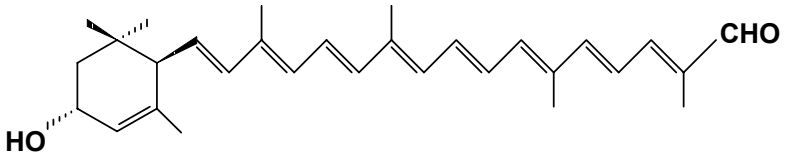
γ-Carotene



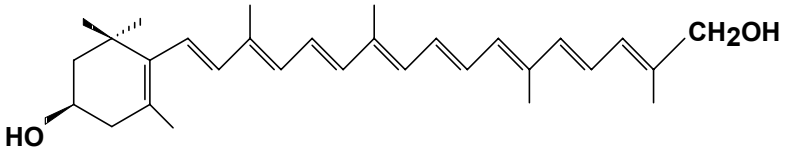
Canthaxanthin



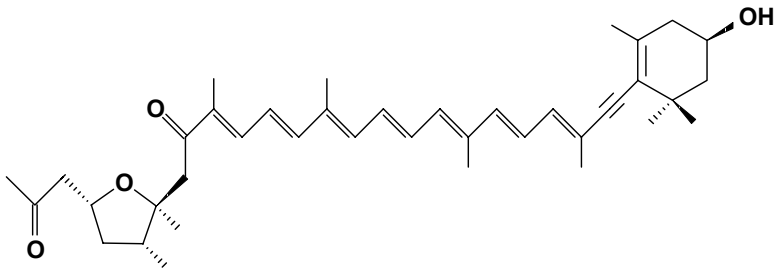
Chlorobactane



α-Citraurin



β-Citraurinol



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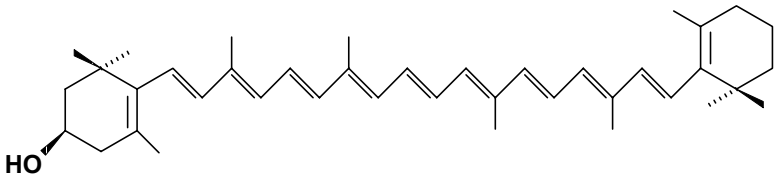
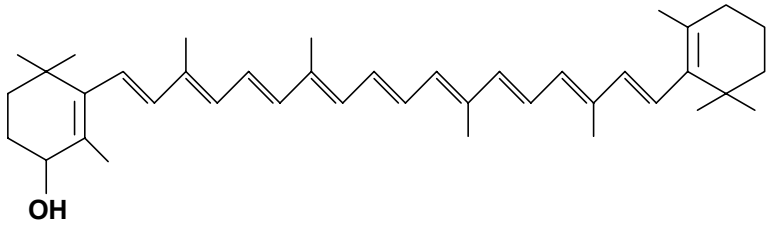


Cryptoxanthin



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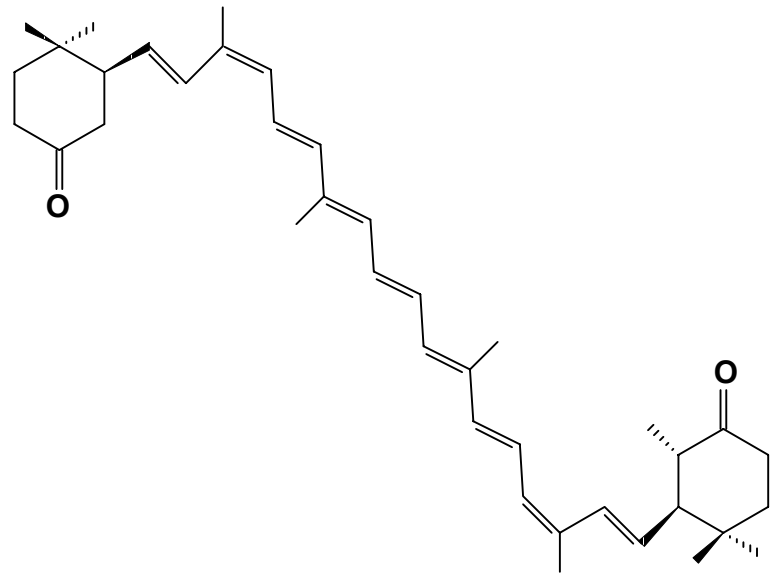
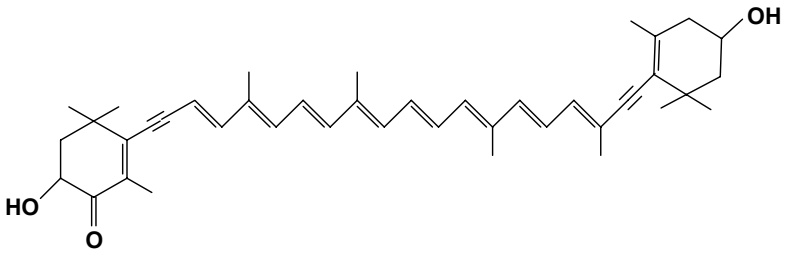
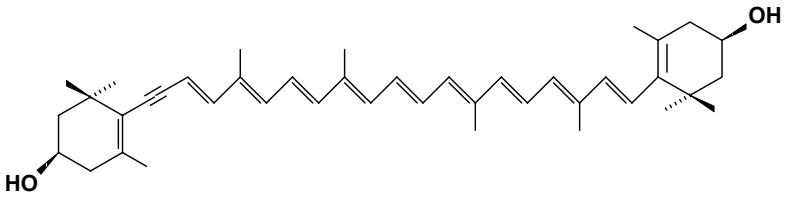


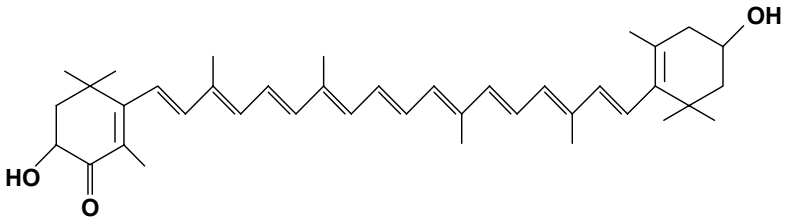
Cucumariaxanthin



Cynthiaxanthin



Diatoxanthin



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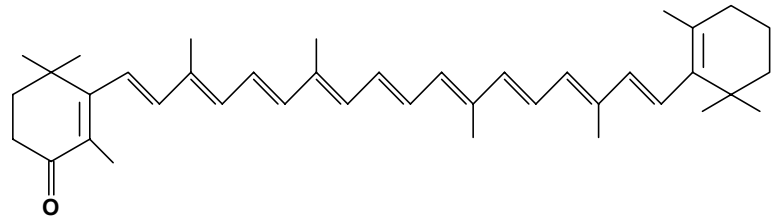
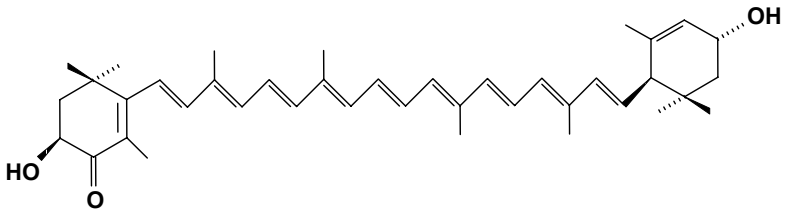


Echinenone



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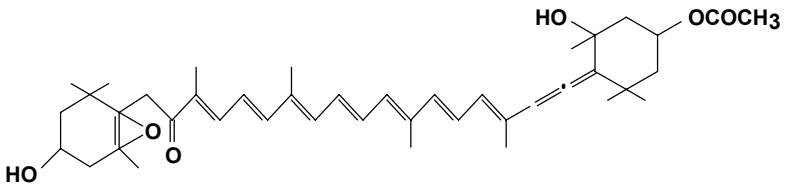
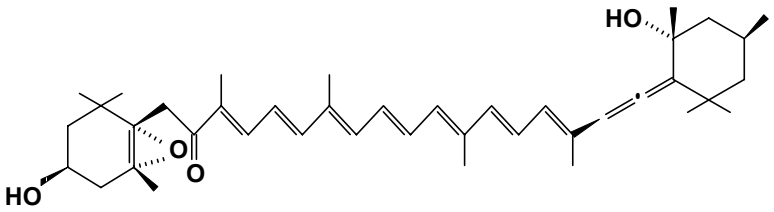
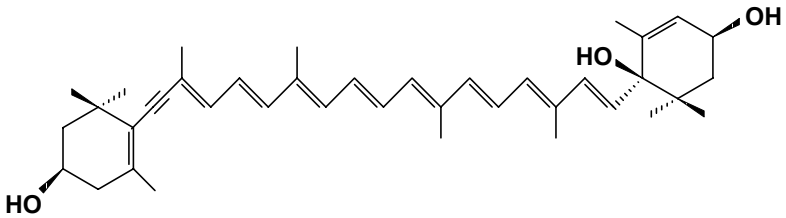


Fucoxanthin



Fucoxanthinol



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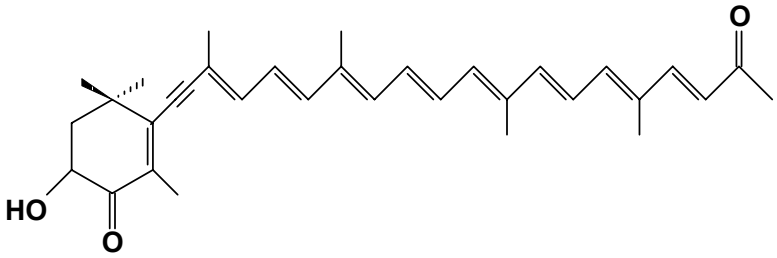

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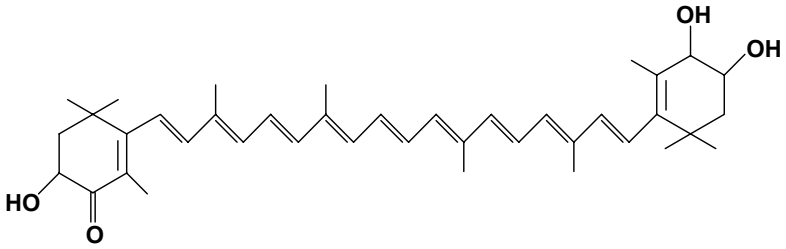

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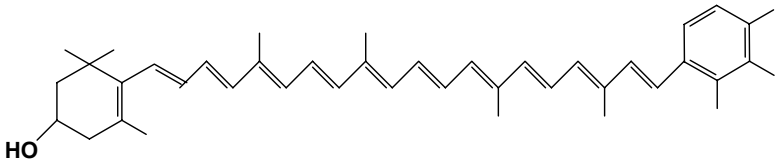
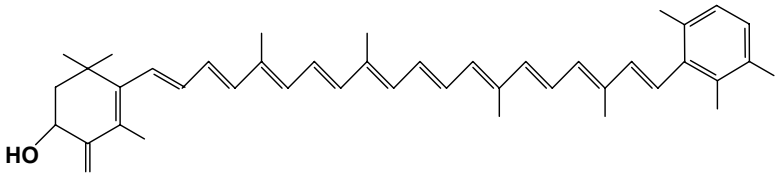


Isoagelaxanthin



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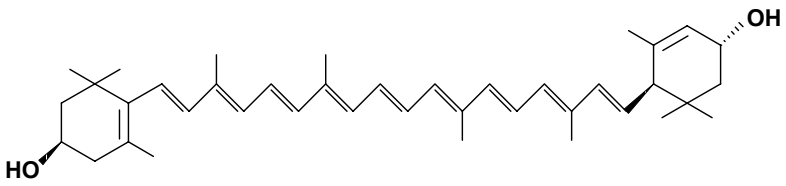
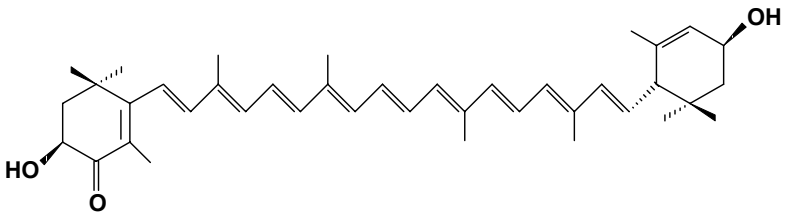
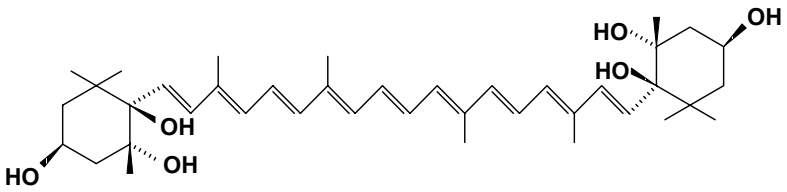


Lutein



Ketolutein

Lycopene



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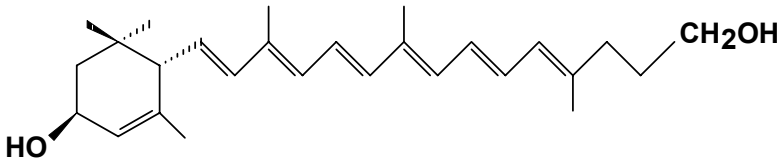
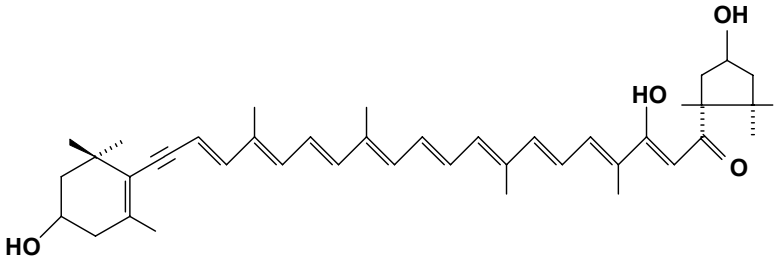


Micropteroxanthin



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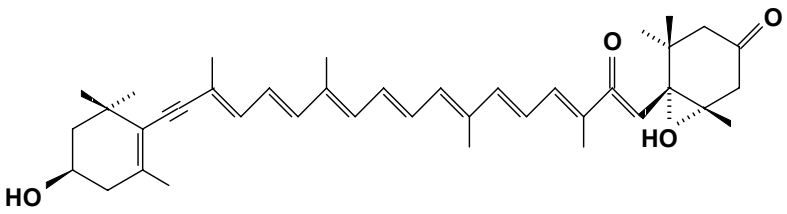

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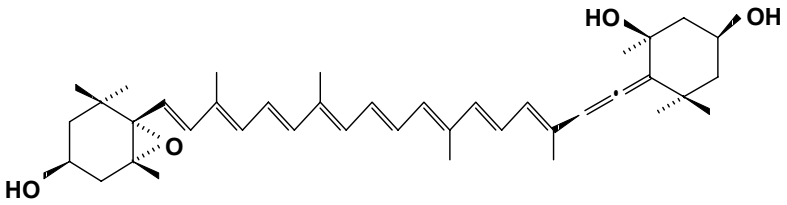

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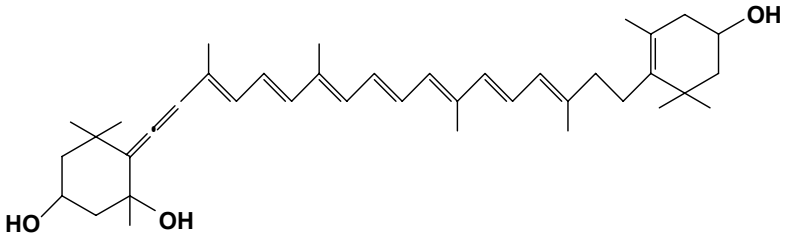

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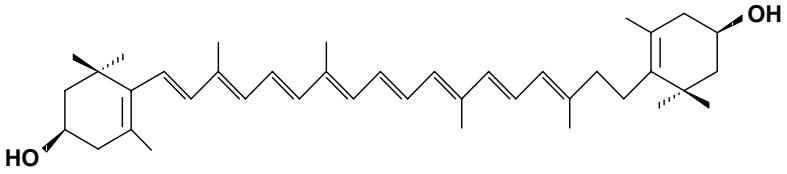
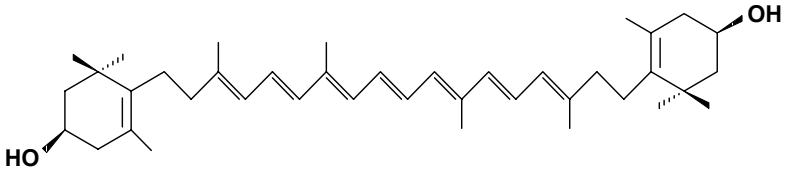


Parsiloxanthin



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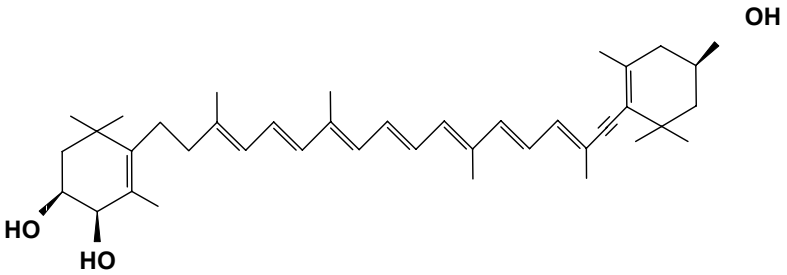

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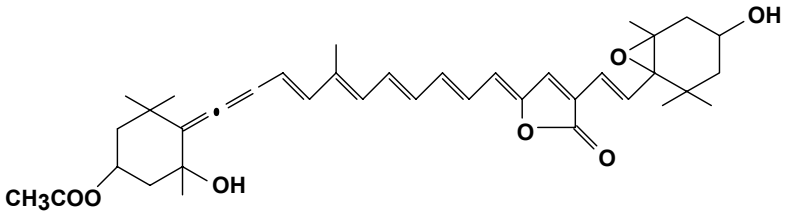

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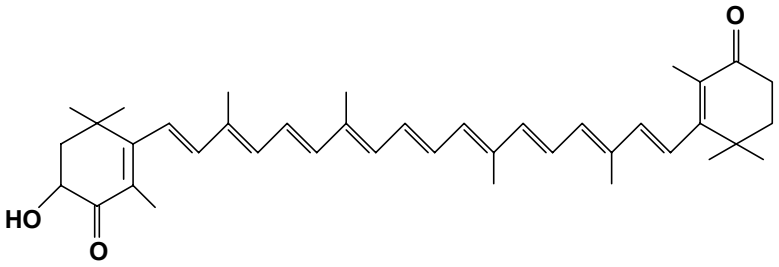

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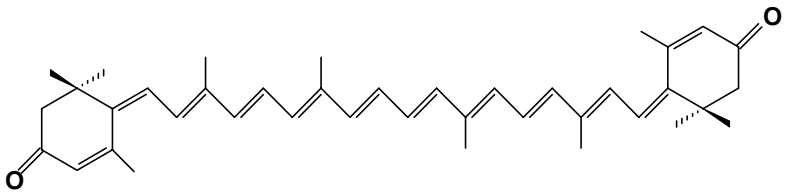
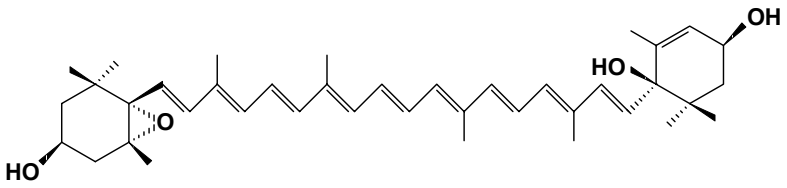


Rhodoxanthin



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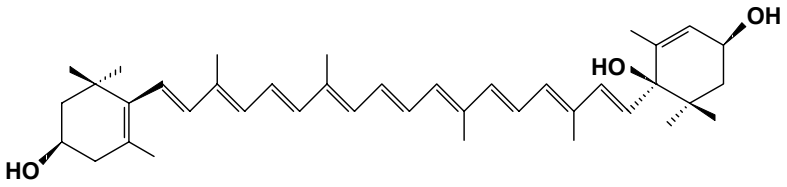

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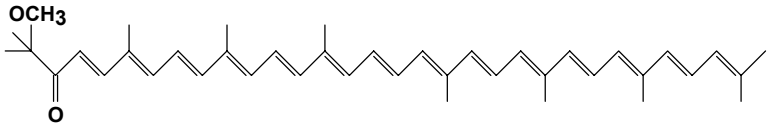

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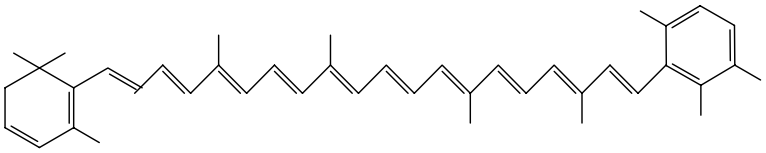

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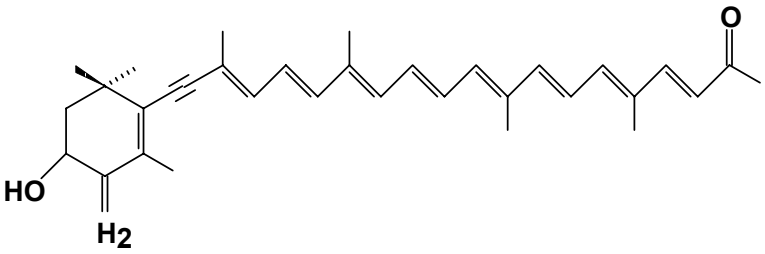

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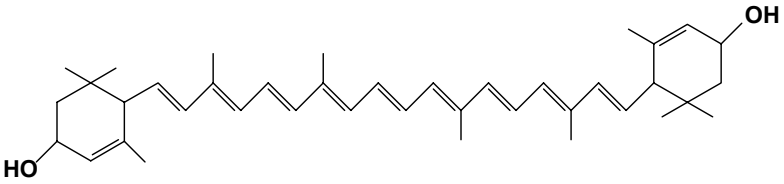

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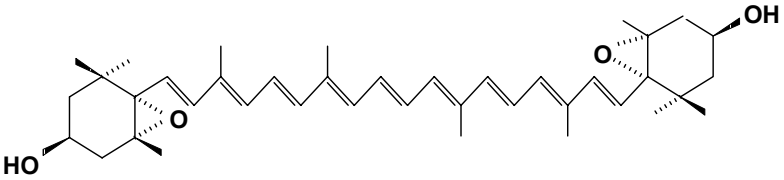

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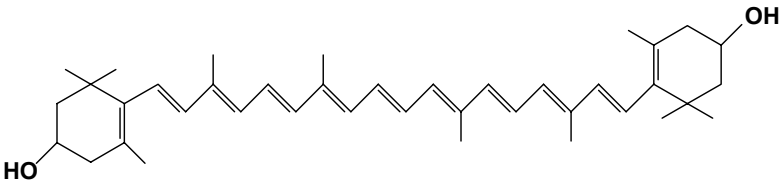

Zeaxanthin

SCOPE AND OBJECTIVES

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The review of literature indicates that information on carotenoids in aquatic animals is restricted to species from temperate waters. Scientific data on carotenoids in crustaceans of tropical waters is lacking. Further the recovery of carotenoids from byproducts of Indian shrimps of commercial value and their utilization has not been attempted so far. In view of this the investigation was carried out with following objectives,

- ❖ To assess the yield and chemical composition of different body components of crustaceans (shrimp, prawn and crab) from Indian marine and fresh waters.
- ❖ To evaluate quantitative and qualitative distribution of carotenoids in different body components of crustaceans from Indian waters.
- ❖ To study the factors affecting extractability of carotenoids in organic solvents and in vegetable oils, and optimization of extraction conditions.
- ❖ To stabilize the recovered carotenoids during storage.
- ❖ To utilize the recovered carotenoids as colorants in fish products and as pigment source in ornamental fish diets.

Program of work

1. Yield and chemical composition

a. Yield of meat, head and body carapace from shallow water shrimps *Penaeus monodon, P indicus, Metapenaeus dobsoni and Parapenaenopsis stylifera*, deep sea shrimps *Solonocera indica and Aristeus alcocki,* and fresh water prawn *Macrobrachium rosenbergii.*

b. Yield of meat and shell from marine crab *Charybdis cruciata* and fresh water crab *Potamon potamon.*

c. Chemical composition (moisture, crude protein, true protein, fat, ash and chitin) of different body components of shrimps, prawn and crab.

2. Quantitative and qualitative distribution of carotenoids

a. Total carotenoid content in different body components of shrimp, prawn and crab.

b. Identification of major carotenoids in carotenoid extracts from different body components by thin layer chromatography (TLC), absorption spectra and high performance liquid chromatography (HPLC).

c. Determination of fatty acid profile of carotenoid esters from carotenoid extracts.

3. Extractability of shrimp waste carotenoids in organic solvents

a. Extraction yield of carotenoids in different organic solvents and solvent mixtures.

b. Optimization of conditions namely, level of non-polar solvent in solvent mixture, solvent level to waste and number extraction for solvent extraction of carotenoids, by a statistically designed experiments

4. Extractability of carotenoids in vegetable oils

a. Extraction yield of carotenoids in different vegetable oils

b. Optimization of conditions namely, level of oil to waste, time and temperature of heating for recovery of carotenoids in vegetable oil.

c. Effect of hydrolysis of shrimp waste with proteases on oil extraction yield of carotenoids.

5. Stability of carotenoids recovered from shrimp waste

a. Influence of pigment carriers, antioxidants and type of packaging materials on stability of solvent extracted carotenoid during storage.

b. Effect of antioxidants and storage methods on stability of oil extracted carotenoids.

6. Utilization of recovered carotenoids in food and feed

a. Incorporation of recovered carotenoid in fish sausage formulation at different level and quality evaluation of fish sausage.

b. Preparation of fish feeds containing recovered carotenoids and evaluation of pigmentation efficiency of diets containing carotenoids on ornamental fish by feeding experiments.

PART II

CHAPTER 1

SHRIMPS, PRAWN AND CRABS:

BODY COMPONANTS

AND CHEMICAL COMPOSITION

CHAPTER 1

SHRIMPS, PRAWN AND CRABS: BODY COMPONENTS AND CHEMICAL COMPOSITION

Crustaceans comprise nearly 20% of Indian aquatic food production. Among the crustaceans, shrimps are the major commodities towards which fishing efforts are directed. About 85 species of shrimps are known to exist in Indian waters. The major species of shrimps, which are commercially important, are the ones belonging to the penaeid group, namely *Penaeus monodon* (Tiger shrimp), *Penaeus indicus* (White shrimp), *Metapenaeus dobsoni* (Brown shrimp) and *Parapenaeopsis stylifera* (Flower shrimp). *P monodon* is also one of the major species, which is cultured in brackish waters. It is estimated that the fishery potential of penaeid shrimps in the Indian Exclusive Economic Zone (EEZ) is around 178,000 tonnes (www.mpeda.com). With the emphasis is being given on diversification of traditional marine resources, fishing for deep-sea (beyond 50 m depth) shrimps is also considered important. Deep sea shrimps mainly *Solonocera indica* and *Aristeus alcocki* is being harvested from the deep waters off the Indian coast. The estimated potential of deep-sea shrimps is around 3000 tonnes. Several species of marine crabs are harvested from Indian waters, the commercially important species being *Scylla serrata, Portunes pelagicus, P sanguinolentus* and *Charybdis cruciata*. In fresh waters the prawn *Macrobrachium rosenbergii* is the main species not only harvested from reservoirs and rivers, but also cultured in large quantities. Fresh water crabs, particularly *Potamon* spp, is captured by traditional fishermen from rivers and reservoirs. The processing of these crustaceans involves removal of the head and exoskeleton to obtain the meat. The reports on yield and chemical composition of body components from some species of shrimps and crabs are available (George and

Gopakumar 1987, Gopakumar 1993). This study was carried out to compare the yield and chemical composition in different species of crustaceans from Indian waters.

1.1. Material and Methods

1.1.1. Materials

Different species of shrimps, prawn and crab were used for the study. The species of shallow marine water shrimps used for the study are *Penaeus monodon* (Photoplate 1.1), *Penaeus indicus* (Photoplate 1.2), *Metapenaeus dobsoni* (Photoplate 1.3) and *Parapenaeopsis stylifera* (Photoplate 1.4). *Solonocera indica* (Photoplate 1.5) and *Aristeus alcocki* (Photoplate 1.6) represented deep-sea shrimps, while *Macrobrachium rosenbergii* (Photoplate 1.7) represented fresh water prawn. Species of crabs used for the study were marine crab *Charybdis cruciata* (Photoplate 1.8) and fresh water crab *Potamon potamon* (Photoplate 1.9).

Samples of shrimp and crab from marine waters available in the local market, which have been transported from the landing centers (10 - 15 hours delay), were collected and transported to the laboratory under iced condition. *M rosenbergii* was collected from the local prawn farm and transported to the laboratory under iced condition. Fresh water crab was collected from the local market. All the reagents and chemicals used for the study were of AR grade.

1.1.2. Methods

1.1.2.1. Yield of body components

Shrimps and prawn were processed by removing the head and body shell (carapace) and the yield of meat, head and carapace was determined by weighing. Crabs

were processed by separating the meat from body and the claws. The gills, viscera, etc. were discarded and the yield of meat and shell was determined by weighing.

1.1.2.2. Chemical composition

Proximate composition namely moisture, crude protein, fat and ash in the different body components of shrimp, prawn and crab was determined by standard methods (AOAC 1990). Moisture content was determined by oven drying the homogenized sample at 102±1°C to a constant weight and calculating the loss in weight. Total nitrogen was determined by using the Kjeltec autoanalyser and protein content calculated by multiplying the total nitrogen by 6.25. Fat content was determined by Soxtec apparatus. The moisture free sample was incinerated oven at 550°C to a constant weight and the residue was weighed as ash. Chitin content was determined by the method of Spinelli et al (1974). One gram of moisture free sample was digested with 100 ml of 2% NaOH at 100°C for 1 hour. The digested material was filtered through a coarse sintered glass and residue was digested again with alkali and filtered. The residue was treated with 100 ml of 5% HCl at room temperature (28±2° C) for 15 hours, filtered and washed with hot distilled water. The washed residue was dried at 100° C for 6 hours and weighed and chitin content determined. Nitrogen content in the chitin obtained was determined by using the Kjeltec autoanalyser. True protein content was calculated by the formula, True protein = (Total nitrogen – Chitin nitrogen) X 6.25.

1.1.3. Statistical analysis

The determination was carried out in 6 replicates except for samples from *Aristeus alcocki and Macrobrachium rosenbergii,* for which 4 replicates were used. The data was analyzed for significant difference by Analysis of Variance (ANOVA) technique and

mean separation was accomplished by Duncan's multiple range test using the software STATISTICA (Statsoft Inc 1999).

1.2. Results and discussion

1.2.1. Shrimps and Prawn

1.2.1.1. Yield

Yield of meat ranged from 34.4 to 51.5%, head from 33.5 to 53.4% and carapace from 7.4 to 15.1% in different species (Table 1.1). Highly significant ($p \leq 0.001$) difference was observed in yield of different body components in individual species (ANOVA Table 1.1a). Lowest yield of meat (34.4% and 37.4%) with a corresponding higher yield of head (53.4% and 47.5%) was obtained in two species of deep-sea shrimps. The yield of head (52.5%) was also higher in fresh water prawn, with lowest yield (7.4%) of carapace. Yield of different body components between species also showed a significant ($p \leq 0.001$) difference (ANOVA Table 1.1b).

Results indicated that processing of deep sea shrimps yield higher (62 – 66%) quantity of waste (head and carapace) compared to the waste (48 – 56%) in processing of shallow water shrimps. The waste in prawn was also higher (60%) compared to marine shrimps. The waste from processing of Indian shrimps was quoted to be in the range of 40 – 50% (Gopakumar 1993). Barratt and Montano (1986) reported that in tropical shrimps the head generally constitutes 34 – 45% and the body shell constitutes 10 – 15%, which is in agreement with the present results. Ariyani and Buckle (1991) reported that the amount of waste generated in shrimp processing varies from 40 – 80% depending on species and type of processing.

1.2.1.2. Chemical composition

Moisture content (Table 1.2) in meat varied from 79.3 to 83.6%, in head from 71.1 to 81.1% and in carapace from 66.9 to 79.8%. A significant ($p \leq 0.001$) difference was observed in moisture content in different body parts in individual species (ANOVA Table 1.2a). In case of *P monodon, P indicus* and *M dobsoni* there was no significant ($p \geq 0.05$) difference in moisture content of meat and head. Significant difference ($p \leq 0.001$) was observed in moisture content of different body components between different species (ANOVA Table 1.2b).

Crude protein content ranged from 7.8 to 15.4% in different body components, highest being (13.6 – 15.4%) in meat (Table 1.3). There was significant difference in crude protein content between body components in individual species (ANOVA Table 1.3a) and between species (ANOVA Table 1.3b). True protein content showed similar pattern to that of crude protein (Table 1.4 and ANOVA Table 1.4a & 1.4b). Fat content was highest (8.1%) in the head of deep-sea shrimp *Aristeus alcocki* and was lowest (0.35%) in the meat of prawn *M rosenbergii* (Table 1.5). In general the fat content was higher in head compared to meat and carapace of all species, and significantly ($p \leq 0.001$) differed due to body components and species (ANOVA Table 1.5a & 1.5b). Ash content was highest in carapace (9.0%) and head (6.5%) of *S indica* (Table 1.6) and a significant difference ($p \leq 0.001$) was observed in ash content between different body components and also between different species (ANOVA Table 1.6a & 1.6b).

King et al (1990) observed a variable moisture and fat content in shrimps depending on species and size. The proximate composition in shrimp (*Metapenaeus endeavor*) was reported to be 75.1 – 77.6% moisture, 14.2 – 15.2% protein, 0.7 – 1.5% fat and 4.5 – 6.9% ash (Ariyani and Buckle 1991). Protein content in four species of

crustaceans from Korean waters was found to range between 12.74 – 20.80% (Jeong et al 1999). Balogun and Akegbejo (1992) also reported a wide variation in proximate composition of *Penaeus notiatis, Parapenaeopsis atlantica* and *Macrobrachium rosenbergii* from Nigerian waters. The results of the present study and the earlier reports suggest that the proximate composition of various body components of shrimp and prawn varies according to species and size.

The chitin content in meat ranged from 0.01 to 0.13%, in head 3.3 – 4.4% and in carapace 4.4 – 6.3% (Table 1.7) with a significant ($p \leq 0.001$) difference due to body components and species (ANOVA Table 1.7a & 1.7b). Chitin is one of the important constituents of exoskeleton of crustaceans. The chitin content in dried crustacean ranges from 20 – 50% (Ornum 1992). Chitin content (% wwb) in the offal of shrimp *Metapenaeus* spp ranged from 2.6 – 3.6% (Ariyani and Buckle 1991).

1.2.2. Crab

1.2.2.1. Yield

Crabs are normally processed by removing the meat from body and claw. The shell, gills and viscera are discarded. The total meat (body and claw meat) yield was 29.7% in marine crab and 28.8% in fresh water crab (Table 1.8). Corresponding shell yield was 34.4% for marine crab and 35.7% in fresh water crab. Even though significant difference was observed in yield of meat and shell in fresh water crab ($p \leq 0.001$) (ANOVA Table 1.8a) and marine crab ($p \leq 0.05$) (ANOVA Table 1.8b), no significant difference ($p \geq 0.05$) was observed in comparative meat yield (ANOVA Table 1.8c) and shell yield (ANOVA Table 1.8d) between two species of crabs. George and Gopakumar (1987) reported that the yield of meat in greater in claw (42 – 47.3%) than in the crab

body (23.6 – 36.0%). Jamieson (1981) observed that in commercial crab processing the waste represents 75 – 80%.

1.2.2.2. Chemical composition

Chemical composition in meat and shell was significantly ($p \leq 0.001$) different in both marine and fresh water crabs (ANOVA Table 1.8a & 1.8b). The moisture content in meat of crab was 81.7 – 81.9% and that of shell was 48.3 – 55.5% (Table 1.8), which is in agreement with the report of George and Gopakumar (1987). Moisture content was significantly different ($p \leq 0.01$) in shells of two species of crab (ANOVA Table 1.8d), but not in meat ($p \geq 0.05$) (ANOVA Table 1.8c). No significant difference ($p \geq 0.05$) was observed in crude protein content and true protein content of meat and shell between two species (ANOVA Table 1.8c & 1.8d). The crude protein content in the marine crabmeat was lower (15.5%) than the reported value of 19.16 – 20.92% (George and Gopakumar 1987), which may be due to the species variation. Fat content, differed ($p \leq 0.001$) in meat of two species (ANOVA Table 1.8c), but not in shell ($p \geq 0.05$) (ANOVA Table 1.8d). Ash content showed a significant difference in meat ($p \leq 0.001$) and shell ($p \leq 0.001$) between crabs from different waters (ANOVA Table 3.8c & 3.8d).

The exoskeleton of crabs also has been recognized as one of the important source of chitin. Chitin content was significantly ($p \leq 0.001$) (ANOVA Table 1.8d) higher in marine crab shell (8.2%) compared to fresh water crab shell (4.4%) (Table 1.8). The chitin content in the exoskeleton of snow crab *Chinocetes opilio,* was 9.2% (Manu-Tawaiah and Haard 1987).

1.3. Conclusion

Deep-sea shrimps yielded higher quantity (62 – 66%) of waste (head + carapace) than shallow water shrimps (48 - 56%), while prawns produced about 60% waste. Head

contained more crude protein (8.2 – 10.2%), true protein (6.3 – 9.3%), fat (1.1 – 81.%), less ash (4.0 – 6.5%) and chitin (3.3 – 4.4%) compared to corresponding values of 7.8 – 9.1%, 5.2 – 8.2%, 0.35 – 2.0%, 4.9 – 9.0% and 4.4 – 6.3% for carapace. Crabs yielded 28.8 – 29.7% meat and 34.4 – 35.7% shell. Marine crab shell had more chitin (8.2%) than fresh water crab (4.4%).

Photoplate 1.1

Marine shrimp *Penaeus monodon*

Photoplate 1.2.

Marine shrimp *Penaeus indicus*

Photoplate 1.3

Marine shrimp *Metapenaeus dobsoni*

Photoplate 1.4

Marine shrimp *Parapenaeopsis stylifera*

Photoplate 1.5

Deep-sea shrimp *Solonocera indica*

Photoplate 1.6

Deep-sea shrimp *Aristeus alcocki*

Photoplate 1.7

Fresh water prawn *Macrobrachium rosenbergii*

Photoplate 1.8

Marine Crab *Charybdis cruciata*

Photoplate 1.9

Fresh water Crab *Potamon potamon*

Table 1.1. Yield (%) of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***Penaeus monodon*[1] (n=6)**	51.3 ± 3.51^{xa}	34.4 ± 1.56^{ya}	14.3 ± 2.59^{za}
***Penaeus indicus*[1] (n=6)**	51.5 ± 1.83^{xa}	33.9 ± 2.03^{ya}	14.6 ± 0.68^{za}
***Metapenaeus dobsoni*[1] (n=6)**	51.4 ± 1.75^{xa}	33.5 ± 1.83^{ya}	15.1 ± 1.35^{za}
***Parapenaeopsis stylifera*[1] (n=6)**	44.0 ± 1.06^{xb}	45.0 ± 1.16^{xb}	11.0 ± 1.14^{yb}
***Solonocera indica*[1] (n=6)**	34.4 ± 0.77^{xc}	53.4 ± 1.45^{yc}	12.2 ± 0.90^{zb}
***Aristeus alcocki*[1] (n=4)**	37.4 ± 0.84^{xd}	47.5 ± 0.49^{yd}	15.1 ± 0.54^{za}
***Macrobrachium rosenbergii*[2] (n=4)**	40.1 ± 1.83^{xe}	52.5 ± 2.18^{yc}	7.4 ± 0.62^{zc}

Different superscripts on values in individual columns (a,b,c,d,e) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.1. Yield

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	4124.01	2	2062.01	107.55	15	7.17	287.59***
P indicus	4080.01	2	2040.01	39.82	15	2.66	768.33***
M dobsoni	3931.57	2	1965.79	41.33	15	2.76	713.44***
P stylifera	4478.61	2	2239.31	19.01	15	1.27	1766.94***
S indica	5090.53	2	2545.27	17.68	15	1.18	2160.06***
A alcocki	2200.77	2	1100.39	3.76	9	0.42	2637.41***
M rosenbergii	4345.23	2	2172.62	25.52	9	2.84	766.36***

b. between species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	1741.26	6	290.21	114.51	31	3.69	78.57***
Head	2575.50	6	429.25	82.26	31	2.65	161.77***
Carapace	223.70	6	37.28	57.90	31	1.87	19.96***

*** $p \leq 0.001$

Table 1.2. Moisture content of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	82.2 ± 0.62^{xa}	81.0 ± 1.96^{xa}	77.1 ± 3.10^{ya}
***P indicus*[1] (n=6)**	81.5 ± 0.81^{xab}	79.5 ± 1.25^{xa}	77.0 ± 2.52^{yab}
***M dobsoni*[1] (n=6)**	82.7 ± 0.83^{xac}	80.6 ± 1.29^{xa}	76.3 ± 3.04^{yb}
***P stylifera*[1] (n=6)**	83.3 ± 0.66^{xc}	81.1 ± 0.57^{yb}	79.7 ± 0.22^{za}
***S indica*[1] (n=6)**	83.6 ± 0.94^{xc}	80.4 ± 0.57^{ya}	76.9 ± 0.90^{zab}
***A alcocki*[1] (n=4)**	81.0 ± 0.18^{xb}	77.2 ± 0.69^{yc}	79.8 ± 0.90^{za}
***M rosenbergii*[2] (n=4)**	79.3 ± 1.61^{xd}	71.1 ± 1.15^{yd}	66.9 ± 1.49^{zc}

Different superscripts on values in individual columns (a,b,c,d) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.2. Moisture content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	85.09	2	42.55	69.22	15	4.61	9.22**
P indicus	59.56	2	29.78	42.83	15	2.86	10.43**
M dobsoni	129.73	2	64.87	58.01	15	3.87	16.77***
P stylifera	39.95	2	19.98	4.05	15	0.27	74.04***
S indica	138.17	2	69.09	10.05	15	0.67	103.08***
A alcocki	31.15	2	15.57	3.98	9	0.44	35.24***
M rosenbergii	319.35	2	159.68	18.39	9	2.04	78.12***

b. between species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	63.58	6	10.60	23.02	31	0.74	14.27***
Head	338.61	6	56.44	44.02	31	1.42	39.74***
Carapace	479.57	6	79.92	139.49	31	4.50	17.76***

** $p \leq 0.01$; *** $p \leq 0.001$

Table 1.3. Crude protein content (% wwb) of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	14.4 ± 0.37^{xa}	9.9 ± 0.59^{ya}	9.1 ± 0.48^{za}
***P indicus*[1] (n=6)**	15.4 ± 1.09^{xb}	9.7 ± 0.55^{ya}	9.0 ± 0.53^{za}
***M dobsoni*[1] (n=6)**	14.3 ± 0.35^{xa}	10.2 ± 0.31^{ya}	9.8 ± 0.54^{yb}
***P stylifera*[1] (n=6)**	13.3 ± 0.77^{xc}	9.0 ± 0.70^{yb}	7.9 ± 0.25^{xc}
***S indica*[1] (n=6)**	13.7 ± 0.51^{xac}	8.2 ± 0.14^{yc}	7.8 ± 0.21^{zc}
***A alcocki*[1] (n=4)**	15.1 ± 0.11^{xab}	8.6 ± 0.36^{ybc}	7.9 ± 0.19^{zc}
***M rosenbergii*[2] (n=4)**	13.6 ± 0.53^{xc}	8.2 ± 0.63^{yc}	7.8 ± 0.62^{yc}

Different superscripts on values in individual columns (a,b,c) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.3. Crude protein content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	151.67	2	75.84	3.68	15	0.25	309.11***
P indicus	149.86	2	74.93	8.89	15	.59	126.39***
M dobsoni	76.32	2	38.16	2.56	15	.17	22.77***
P stylifera	98.74	2	49.37	5.69	15	0.38	130.11***
S indica	127.67	2	63.83	1.59	15	0.11	600.54***
A alcocki	124.21	2	62.10	0.54	9	0.06	1042.68***
M rosenbergii	83.84	2	41.92	3.20	9	0.36	118.04***

b between species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	20.16	6	3.36	12.38	31	0.40	8.45***
Head	21.03	6	3.51	7.90	31	0.25	13.76***
Carapace	22.42	6	3.74	5.82	31	0.19	19.92***

*** $p \leq 0.001$

Table 1.4. True protein content (% wwb) of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	14.4 ± 0.37^{xa}	8.9 ± 0.60^{ya}	7.7 ± 0.49^{zab}
***P indicus*[1] (n=6)**	15.4 ± 1.09^{xb}	8.7 ± 0.54^{yab}	7.5 ± 0.56^{za}
***M dobsoni*[1] (n=6)**	14.3 ± 0.35^{xacd}	9.3 ± 0.44^{ya}	8.3 ± 0.52^{zb}
***P stylifera*[1] (n=6)**	13.3 ± 0.77^{xd}	8.1 ± 0.75^{yb}	6.4 ± 0.22^{zc}
***S indica*[1] (n=6)**	13.5 ± 0.51^{xcd}	6.3 ± 0.11^{yc}	5.2 ± 0.28^{zd}
***A alcocki*[1] (n=4)**	15.1 ± 0.11^{xab}	6.6 ± 0.39^{yc}	5.4 ± 0.19^{zd}
***M rosenbergii*[2] (n=4)**	13.6 ± 0.53^{xad}	7.3 ± 0.77^{yd}	6.3 ± 0.67^{yc}

Different superscripts on values in individual columns (a,b,c,d) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.4. True protein content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	151.67	2	75.84	3.68	15	0.25	309.12***
P indicus	216.59	2	108.30	8.97	15	0.60	181.04***
M dobsoni	126.22	2	63.11	2.96	15	0.20	320.05***
P stylifera	153.09	2	76.55	5.95	15	0.40	192.84***
S indica	244.39	2	122.20	1.73	15	0.12	1060.17***
A alcocki	219.82	2	109.91	0.58	9	0.07	1695.99***
M rosenbergii	123.55	2	61.78	3.97	9	0.44	140.19***

b. between species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	21.26	6	3.54	12.33	31	0.40	8.90***
Head	44.87	6	7.48	9.33	31	0.30	24.86***
Carapace	46.03	6	7.67	6.18	31	0.20	38.48***

*** $p \leq 0.001$

Table 1.5. Fat content (% wwb) of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	1.2 ± 0.12^{xa}	3.6 ± 0.22^{ya}	0.66 ± 0.247^{za}
***P indicus*[1] (n=6)**	1.4 ± 0.23^{xa}	4.0 ± 0.40^{yac}	0.96 ± 0.102^{zac}
***M dobsoni*[1] (n=6)**	2.1 ± 0.23^{xb}	3.5 ± 0.23^{ya}	1.1 ± 0.15^{zc}
***P stylifera*[1] (n=6)**	1.8 ± 0.36^{xc}	4.9 ± 0.12^{yb}	2.0 ± 0.12^{xd}
***S indica*[1] (n=6)**	0.94 ± 0.075^{xd}	1.1 ± 0.15^{yd}	0.35 ± 0.055^{zb}
***A alcocki*[1] (n=4)**	2.7 ± 0.42^{xe}	8.1 ± 1.28^{ye}	3.0 ± 0.90^{xe}
***M rosenbergii*[2] (n=4)**	0.35 ± 0.065^{xf}	4.4 ± 0.39^{ybc}	0.55 ± 0.069^{xab}

Different superscripts on values in individual columns (a,b,c,d,e,f) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.5. Fat content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	151.67	2	75.84	3.68	15	0.25	309.12***
P indicus	216.59	2	108.30	8.97	15	0.60	181.04***
M dobsoni	126.22	2	63.11	2.96	15	0.20	320.05***
P stylifera	153.09	2	76.55	5.95	15	0.40	192.84***
S indica	244.39	2	122.20	1.73	15	0.12	1060.17***
A alcocki	219.82	2	109.91	0.58	9	0.07	1695.99***
M rosenbergii	123.55	2	61.78	3.97	9	0.44	140.19***

b. between different species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	16.46	6	2.74	1.81	31	0.06	48.87***
Head	124.34	6	20.73	6.83	31	0.22	94.04***
Carapace	25.20	6	4.30	3.02	31	0.10	43.06***

*** $p \leq 0.001$

Table 1.6. Ash content (% wwb) of body components in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	0.94 ± 0.143^{xa}	4.2 ± 0.50^{ya}	6.9 ± 0.43^{za}
***P indicus*[1] (n=6)**	1.3 ± 0.22^{xb}	4.2 ± 0.31^{ya}	5.2 ± 0.61^{zbd}
***M dobsoni*[1] (n=6)**	1.0 ± 0.16^{xa}	4.0 ± 0.47^{ya}	5.7 ± 1.00^{zb}
***P stylifera*[1] (n=6)**	0.81 ± 0.099^{xac}	4.6 ± 0.48^{yab}	5.6 ± 0.35^{zb}
***S indica*[1] (n=6)**	0.68 ± 0.044^{xcd}	6.5 ± 0.25^{yc}	9.0 ± 0.15^{zc}
***A alcocki*[1] (n=4)**	0.65 ± 0.156^{xcd}	4.0 ± 0.21^{ya}	4.9 ± 0.27^{zd}
***M rosenbergii*[2] (n=4)**	0.60 ± 0.117^{xd}	5.0 ± 0.50^{yb}	6.2 ± 0.19^{zb}

Different superscripts on values in individual columns (a,b,c,d) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.6. Ash content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	107.74	2	53.87	2.28	15	0.15	354.83***
P indicus	57.27	2	28.64	2.58	15	0.17	166.71***
M dobsoni	67.74	2	33.87	6.29	15	0.42	80.73***
P stylifera	75.45	2	37.73	1.79	15	0.12	315.80***
S indica	217.32	2	108.66	0.45	15	0.03	3639.94***
A alcocki	39.90	2	19.95	0.41	9	0.05	436.73***
M rosenbergii	69.59	2	34.79	0.90	9	0.10	34.79***

b. between different species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	1.73	6	0.29	0.64	31	0.02	14.10***
Head	29.27	6	4.88	5.22	31	0.17	28.98***
Carapace	61.36	6	10.23	8.84	31	0.29	35.85***

*** $p \leq 0.001$

Table 1.7. Chitin content (% wwb) of body components in different species of shrimp[1] and prawn[2]

Species	Body component		
	Meat	**Head**	**Carapace**
***P monodon*[1] (n=6)**	0.13 ± 0.023^{xa}	3.6 ± 0.40^{ya}	5.0 ± 0.41^{za}
***P indicus*[1] (n=6)**	0.10 ± 0.038^{xb}	4.1 ± 0.36^{yb}	5.0 ± 0.38^{za}
***M dobsoni*[1] (n=6)**	0.07 ± 0.018^{xc}	3.5 ± 0.19^{ya}	4.9 ± 0.51^{za}
***P stylifera*[1] (n=6)**	0.04 ± 0.012^{xc}	4.2 ± 0.06^{yb}	5.2 ± 0.28^{za}
***S indica*[1] (n=6)**	0.05 ± 0.012^{xc}	4.2 ± 0.08^{yb}	6.3 ± 0.12^{zb}
***A alcocki*[1] (n=4)**	0.01 ± 0.001^{xd}	3.3 ± 0.15^{ya}	4.4 ± 0.14^{zc}
***M rosenbergii*[2] (n=4)**	0.04 ± 0.009^{xc}	4.4 ± 0.36^{yb}	5.8 ± 0.30^{zd}

Different superscripts on values in individual columns (a,b,c,d) and rows (x,y,z) indicate significant difference ($p \leq 0.05$)

ANOVA Table 1.7. Chitin content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	75.69	2	37.84	1.67	15	0.11	339.85***
P indicus	80.46	2	40.23	1.34	15	0.09	448.96***
M dobsoni	73.58	2	36.79	1.50	15	0.10	36.79***
P stylifera	90.25	2	45.12	0.40	15	0.03	1687.03***
S indica	120.51	2	60.26	0.17	15	0.01	6026.00***
A alcocki	41.67	2	20.84	0.12	9	0.02	1532.66***
M rosenbergii	72.05	2	36.03	0.66	9	0.07	489.50***

b. between different species

Body component	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	0.05	6	0.009	0.01	31	0.0004	20.14***
Head	5.13	6	0.86	2.15	31	0.07	12.35***
Carapace	12.23	6	2.04	3.70	31	0.12	17.08***

*** $p \leq 0.001$

Table 1.8. Yield (%) of meat and shell & chemical composition (%) of marine and fresh water crab (n=6)

Parameter	Meat		Shell	
	Marine crab (*Charybdis cruciata*)	**Freshwater crab (*Potamon potamon*)**	**Marine crab (*Charybdis cruciata*)**	**Freshwater crab (*Potamon potamon*)**
Yield	29.7 ± 3.90^{ax}	28.8 ± 1.47^{ax}	34.4 ± 1.17^{bx}	35.7 ± 0.98^{bx}
Moisture	81.7 ± 1.04^{ax}	81.9 ± 1.25^{ax}	48.3 ± 2.98^{bx}	55.5 ± 4.31^{by}
Crude protein	15.5 ± 0.38^{ax}	15.1 ± 0.30^{ax}	11.5 ± 0.66^{bx}	11.1 ± 0.34^{bx}
True protein	15.4 ± 0.41^{ax}	15.0 ± 0.30^{ax}	8.1 ± 0.59^{bx}	7.7 ± 0.31^{bx}
Fat	2.3 ± 0.19^{ax}	1.2 ± 0.08^{ay}	0.39 ± 0.058^{bx}	0.34 ± 0.059^{bx}
Ash	1.5 ± 0.12^{ax}	2.0 ± 0.17^{ay}	28.4 ± 1.10^{bx}	25.4 ± 1.40^{by}
Chitin	0.07 ± 0.002^{ax}	0.04 ± 0.002^{ay}	8.2 ± 0.14^{bx}	4.4 ± 0.19^{by}

Different superscripts (a & b: body components within species, x & y: same body component between species) indicates significant difference ($p \leq 0.05$)

ANOVA Table 1.8. Yield and proximate composition of marine and fresh water crab

a. Fresh water crab: between meat and shell

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Yield	168.75	1	168.75	15.61	10	1.56	108.13***
Moisture	2085.60	1	2085.60	100.72	10	10.07	207.06***
Crude protein	47.52	1	47.52	1.03	10	0.10	461.21***
True protein	158.78	1	158.78	0.94	10	0.09	1690.52***
Fat	2.45	1	2.45	0.05	10	0.005	484.44***
Ash	1641.74	1	1641.74	9.93	10	0.99	1653.93***
Chitin	57.39	1	57.39	0.19	10	0.02	3034.35***

b. Marine crab: between meat and shell

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Yield	65.33	1	65.33	83.03	10	8.30	7.87*
Moisture	3349.02	1	3349.02	49.73	10	4.97	673.46***
Crude protein	46.02	1	46.02	2.89	10	0.29	159.16***
True protein	157.47	1	157.47	2.61	10	0.26	602.36***
Fat	11.41	1	11.41	0.19	10	0.019	601.38***
Ash	2171.91	1	2171.91	6.13	10	0.61	3541.38***
Chitin	198.75	1	198.75	0.10	10	0.01	19557.79***

c. Meat: between species

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Yield	6.75	1	6.75	86.92	10	8.69	0.78^{NS}
Moisture	0.08	1	0.08	13.29	10	1.33	0.06^{NS}
Crude protein	0.45	1	0.45	1.17	10	0.12	3.86^{NS}
True protein	0.41	1	0.41	1.31	10	0.13	3.11^{NS}
Fat	3.60	1	3.60	0.21	10	0.021	175.17***
Ash	0.83	1	0.83	0.21	10	0.021	38.82***
Chitin	0.002	1	0.002	0.00008	10	0.000008	246.02***

d. Shell: between species

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Yield	5.33	1	5.33	11.71	10	1.17	4.55^{NS}
Moisture	155.81	1	155.81	137.16	10	13.72	11.36**
Crude protein	0.61	1	0.61	2.75	10	0.28	2.23^{NS}
True protein	0.48	1	0.48	2.25	10	0.22	2.12^{NS}
Fat	0.007	1	0.007	0.035	10	0.003	2.02^{NS}
Ash	26.76	1	26.76	15.85	10	1.58	16.89**
Chitin	43.12	1	43.12	0.29	10	0.03	1483.50***

^{NS} $p \geq 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

CHAPTER 2

CAROTENOID DISTRIBUTION IN SHRIMPS, PRAWN AND CRABS

CHAPTER 2

CAROTENOID DISTRIBUTION IN SHRIMPS, PRAWN AND CRABS

Carotenoids are widely distributed in crustaceans. They are responsible for the color of many crustaceans. The distribution of carotenoids in crustacean is dependent upon species and the their habitat. Carotenoids in the crustaceans from temperate waters are extensively investigated (Shahidi et al 1998). However, the reports on carotenoids in the crustaceans from tropical waters are scanty. This investigation was carried out to determine the quantitative and qualitative distribution of carotenoids in some of the crustaceans from Indian waters.

2.1. Material and methods

Different species of crustaceans procured as explained in section 1.1.1 were used for the study. All the solvents and chemicals used for extraction of carotenoids were of AR grade. HPLC grade methanol and triple distilled water was used for HPLC analysis of carotenoid extracts. Synthetic astaxanthin (Sigma, USA), β-carotene (Sigma, USA) and zeaxanthin (Extrasynthese, France) were used as standard carotenoids. Astaxanthin monoester and diester were purified by subjecting the carotenoid extract from shrimp waste to thin layer chromatography (TLC) and scrapping off the corresponding bands for monoester and diester from the developed plates, suspending in acetone, filtering and concentrating the filtrate. The filtrate is dissolved in petroleum ether and subjected to TLC and purification twice. Fatty Acid Methyl Ester (FAME) standards for Gas Chromatography (GC) were from Sigma, USA.

2.1.1. Total carotenoid content

Total carotenoid content as astaxanthin in different body components was determined by the modified method of Saito and Reiger (1971), as explained by Simpson and Haard (1985a). Ten gram of homogenized sample was blended with 25 ml of cold acetone using Polytron PT3100 homogeniser at 5000 rpm for 2 min. The homogenate was filtered through Whatman No. 1 filter paper. The residue was extracted three more times with 25 ml portions of cold acetone and the resulting filtrates were pooled into a separating funnel and partitioned with 50 ml of petroleum ether. The lower layer was drawn off into a second separating funnel and treated with 50 ml portion of petroleum ether as before and the process was repeated till the petroleum ether extract was visibly colorless. The petroleum ether layer was combined and washed 4 – 5 times with 100 ml of 0.1 % saline. Then the petroleum ether layer was dried by shaking with 25 g anhydrous sodium sulphate for 30 mins. The dried petroleum ether extract was filtered through Whatman No. 1 filter paper and the residue containing sodium sulphate was washed several times with petroleum ether. The washings were pooled with filtrate, flushed with nitrogen, and then evaporated under vacuum at 40°C using a rotary flash evaporator. The resulting carotenoid concentrate is taken up in petroleum ether and made upto a known volume. The absorbance of the extract, which was appropriately diluted, was measured at 468 nm using Spectronic 21 spectrophotometer. The concentration of the carotenoids as astaxanthin in the extract was calculated using the equation,

$$\text{Carotenoid content (}\mu\text{g astaxanthin/g sample)} = \frac{A_{468nm} \times V_{extract} \times \text{Dilution factor}}{0.2 \times W_{sample}}$$

Where, A is absorbance, V is volume of extract and 0.2 is the A_{468} of 1μg/ml of standard astaxanthin.

2.1.2. Qualitative distribution of carotenoids

2.1.2.1. Thin Layer Chromatography (TLC) of carotenoid extract

Concentrated carotenoid extract in petroleum ether was subjected to TLC using Silicagel G plates. Forty-five grams of silicagel G was mixed with 90 ml distilled water to make slurry and coated on glass plates (20 x 20 cm) using the applicator to a thickness of 0.5mm. The coated plates were air-dried for 60 min and then dried in an oven at 102°C for 2 h. Twenty five to fifty microlitre of carotenoid extract was potted on TLC plates along with standard astaxanthin, β-carotene and zeaxanthin and eluted with a mobile phase of acetone : hexane (25 : 75) (Naturose Tech Bull 1998). The Rf value of standards and the separated bands of the sample extracts were noted.

2.1.2.2. Absorbance maxima (λ_{max}) in different organic solvents

The dried carotenoid extract was dissolved in different organic solvents namely, petroleum ether, hexane, ethanol, acetone and benzene. The absorbance spectra of the carotenoid extract in each solvent was determined between 400 to 600 nm using Shimadzu UV-1610 spectrophotometer, and the wavelength(s) of maximum absorbance (λ_{max}) was noted.

2.1.2.3. High Performance Liquid Chromatography (HPLC) of carotenoid extract

Carotenoid extract from 3 experiments for each sample was subjected to HPLC analysis by the method of Taylor and Ikawa (1980). The conditions used for HPLC were, μ Bondapack C18 column (3.9 mm I.D x 30 cm) (Waters), 15 min concave mobile phase gradient of 80 – 100% methanol in water, 2.0 ml /min flow rate, injection volume 20 μl carotenoid extract in acetone, and measurement of eluent absorbance at 440 nm. The details of gradient program were as follows, where B is methanol.

0.01 min	B. Concentration	80%
0.01 min	B. Curve	5
15 min	B. Concentration	100%
35 min	B. Concentration	100%
35.01 min	STOP	

To identify the peaks, the Retention Time (RT) of sample peaks was compared with the RT of standard astaxanthin, β-carotene, zeaxanthin and that of prepared astaxanthin monoester and diester.

2.1.3. Fatty acid profile of carotenoid esters

Fatty acids were isolated from carotenoid extracts and fatty acid methyl esters were prepared by the method explained by Renstrom and Liaaen-Jensen (1981) and fatty acid methyl esters were determined by gas chromatography (GC). The carotenoid extracts from all the extractions were polled together for analysis. The carotenoid extract in ether (5 ml) were saponified with 10% KOH in methanol (5 ml) overnight. After saponification, the ether layer containing carotenoids was removed and the aqueous hypophase was acidified to pH 4.0 and the fatty acids were isolated by ether extraction. The ether was evaporated from the fatty acid isolate and the fatty acids were dissolved in 0.5 ml benzene. To the fatty acids in benzene, 0.5 ml of 0.5N methanol-HCl was added and the mixture boiled for 3 min. Water (1 ml) was added to the boiled mixture and the organic phase was separated and dried to get methyl esters of fatty acids. The methyl esters of fatty acids in chloroform were analyzed by GC using Shimadzu GC15A fitted with FID detector. The conditions for GC were, DEGS 15% Shimadzu column (3 m), column temperature of 180°C, injection temperature of 220°C, detection temperature of

230°C, N_2 flow rate of 40 ml/min, and injection volume of 1 µl. Peaks were identified by co-chromatography with authentic FAME standards.

2.1.4. Statistical analysis

The total carotenoid content determination was carried out in 6 replicates except for samples from *Aristeus alcocki and Macrobrachium rosenbergii,* for which 4 replicates were used. The data was analyzed for significant difference by Analysis of Variance (ANOVA) technique and mean separation was accomplished by Duncan's multiple range test using the software STATISTICA (Statsoft Inc 1999).

2.2. Results and Discussion

2.2.1. Total carotenoid content

Total carotenoid content (µg/g) in shrimps ranged from 10.4 to 21.4 in meat, 35.8 – 185.3 in head and 59.8 – 117.4 in carapace (Table 2.1). A significant difference ($p \leq 0.001$) was observed in carotenoid content between different body components of individual species (ANOVA Table 2.1a). In case of *Penaeus monodon, P indicus* and *Solonocera indica,* the carotenoid content was higher in carapace than in head, while in *Parapenaeopsis stylifera* and *Aristeus alcocki* the carotenoid content was higher in head than in carapace. Highest carotenoid content was observed in head of *A alcocki* (185.3 µg/g) followed by head of *P stylifera* (153.1 µg/g)). The carotenoid content of body components between species differed significantly ($p \leq 0.001$) (ANOVA Table 2.1b). Comparatively prawn *Macrobrachium rosenbergii* had lower carotenoid content in meat (2.7 µg/g), head (34.4 µg/g) and carapace (40.7 µg/g). In general the carotenoid content was highest in all the body components of the deep-sea shrimp *A alcocki*.

Carotenoid content in the crab was low, highest being 11.0 μg/g in the shell of marine crab (Table 2.2). Significant difference was observed in carotenoid content between body components of both the crabs ($p \leq 0.001$), meat between two crabs ($p \leq 0.05$) and shell between two crabs ($p \leq 0.001$) (ANOVA Table 2.2).

The total carotenoid content in crustaceans was found to vary depending on species (Lambertson and Brakken 1971). The reports on carotenoid content in crustaceans from tropical waters are limited. Okada et al (1994) analysed tiger prawn (*P monodon*) from waters of Indo-Pacific region and reported that the total carotenoid content varies from 23 – 331 μg/g in the exoskeleton, with a lower level in prawns having a pale blue body color and highest being in prawn having dark gray body color. In the waste from the shrimp, *Pandalus borealis* from Canadian waters, the total carotenoid content ranged from 30.9 to 35.8 μg/g (Guillou et al 1995). The total carotenoid content in the Norwegian shrimp (*Phasiphaea* sp) offal was 19.9 μg/g (Lambertsen and Braekkan 1971).

The carotenoid content in crabs has been reported to be low. Shahidi and Synowiecki (1991) reported that the carotenoid content in the shells of snow crab *Chinocetes opilio,* was 14 μg/g. The carotenoid content in blue crab *Callinectes sapidus* was 4.63 μg/g (Felix-Valenzuela et al 2001). In the present investigation also the carotenoid content in the crab was low compared to shrimps and prawn.

The results indicated that the commercially important shrimp species harvested from the Indian waters contain variable level of carotenoids. The waste from the shallow water shrimp *P stylifera,* and the deep-sea shrimps contain highest carotenoid level. Fresh water prawn and crabs showed comparatively lower level of carotenoids.

2.2.2. Qualitative distribution of carotenoids

Thin layer chromatographic separation of carotenoid extracts from body components of shrimp, prawn and marine crab yielded 4 distinct bands. The separated bands were with Rf 0.34 (orange), 0.50 (orange), 0.76 (orange) and 0.96 (yellow), while the fresh water crab extract yielded an additional yellow band at Rf 0.30. The orange band at Rf 0.34 corresponds to astaxanthin, while yellow bands at Rf 0.30 and at 0.96 correspond to zeaxanthin and β-carotene respectively as indicated by the TLC of standards (Figure 2.1). The orange bands at Rf 0.50 and at 0.76 correspond to astaxanthin monoester and astaxanthin diester respectively as quoted in the literature (Naturose Tech Bull 1998). The results indicated that astaxanthin, astaxanthin monoester and diester, and β-carotene are the major pigments in the different body components of shrimp, prawn and marine crab, while zeaxanthin also could be separated from the fresh water crab extract using TLC.

TLC is still used as an effective method for purifying and preliminary identification of carotenoids (Delgado-Vargus et al 2000), but to be supported with other method of identification. Absorption maxima (λ_{max}) of carotenoids in different organic solvents are also used as tools in their identification (Britton 1985). Absorption spectra of carotenoid extracts from shrimp, prawn and marine crab showed single peak of absorption maxima (λ_{max}, nm) (Table 2.3 and Figure 2.2) at 469 in petroleum ether, 470 in hexane, 475 in ethanol, 478 in acetone and 485 in benzene. While the extracts from fresh water crab showed two peaks in each solvent, 447 & 475 in petroleum ether, 450 & 476 in hexane, 451 & 478 in ethanol, 452 & 478 in acetone and 462 & 487 in benzene. The λ_{max} of carotenoid extracts from shrimp, prawn and marine crab correspond to that of astaxanthin while that from fresh water crab to that of zeaxanthin as quoted in the

literature (Britton 1985). The absorption maxima of carotenoid extracts in different solvents confirm the findings of TLC separation.

High performance liquid chromatography (HPLC) is the preferred column chromatography to carry out the quantitative and qualitative analysis of carotenoids (Britton 1991). The HPLC profile (Chromatogram 1 to chromatogram 9) of carotenoid extracts indicate that astaxanthin and its esters were the major carotenoids in the extract from shrimp, prawn and marine crab, while zeaxanthin was the major carotenoid fraction in fresh water crab. In shrimps astaxanthin content (% of total carotenoids) ranged from a low of 14.9 in the carapace of *S indica* to a high of 42.2 in the meat of *P stylifera* (Table 2.4). Astaxanthin monoester content (% of total carotenoids) ranged from 20.5 in meat of *P indicus* to 49.8 in the meat of *A alcocki*. Composition of β-carotene and zeaxanthin was low in carotenoid extracts in shrimp highest being 10.3% in the meat of *S indica* and 12.2% in the meat of *P monodon* respectively. In general zeaxanthin content was higher in body components of *P monodon* compared to other species of shrimp. The results indicate that astaxanthin and its esters contribute 63.5 – 92.2% to the total carotenoid content in shrimps analyzed.

In freshwater prawn *M rosenbergii* along with astaxanthin and its esters β-carotene was also found to be a major pigment (Table 2.4). β-Carotene content ranged from 5.5% in carapace to 29.6% in head. Astaxanthin content was higher than its esters and the total astaxanthin and esters content ranged from 51.9 to 64.3%. Zeaxanthin content was low in prawns.

Astaxanthin and its esters were found to be major pigments in marine crab *Charybdis cruciata*, with a total content of 67.6 in meat and 65.5% in shell (Table 2.5). β-Carotene content was 3.6% in meat and 5.1% in shell. In freshwater crab, *Potamon*

potamon, zeaxanthin was the major pigment both in meat (42.0% and shell (74.8%). The total content of astaxanthin and its esters in freshwater crab was 36.5% in meat and 14.8% in shell and β-carotene content was 7.4% in meat and 3.6% in shell.

Astaxanthin and its esters have been found to be the major carotenoids in crustaceans (Shahidi et al 1998). In the Indian shrimp, *P stylifera*, Balachandran (1976) reported the presence of astaxanthin as the major pigment. Okada et al (1994) reported that astaxanthin in free, mono and diester forms constitutes 86 – 98% of total pigments in *P monodon*. They also reported the presence of small amounts of β-carotene (3.6%) and zeaxanthin (1.5%) in the exoskeleton of *P monodon*. Astaxanthin and its esters have also been isolated as major carotenoid from the shrimp *P borealis* (Shahidi et al 1992) and *Penaeus japonicus* (Negre-Sadargues et al 1993) and in deep-sea shrimp from Atlantic waters (Negre-Sadragues 2000).

It is reported that tiger prawn preferentially accumulates astaxanthin monoester in exoskeleton when the total carotenoid content exceeds 8 mg% (Okada et al 1994). In the present study a carotenoid content (mg%) of more than 8 was observed in carapace of *P monodon* (8.7), *M dobsoni* (8.3), *P stylifera* (10.5), *S indica* (11.6), *A alcocki* (11.7), and head of *P stylifera* (115.3) and *A alcocki* (18.5) (Table 2.1). Correspondingly higher content of astaxanthin monoester was observed in all the above except the carapace of *M dobsoni*.

Fresh water prawn *M rosenbergii* can convert dietary β-carotene to astaxanthin. In the present study, β-carotene was also found to be a major pigment along with astaxanthin and its esters. The presence of β-carotene in large quantities may be due to composition of feed for the cultured prawns used in the study.

There are no reports on the composition of carotenoids in the freshwater crab *Potamon sp*. In the marine crab accumulation of astaxanthin, β-carotene and zeaxanthin has been reported (Matsuno et al 1974d). The present study indicates that the fresh water crab used in the study preferentially accumulates zeaxanthin as major carotenoid.

2.2.3. Fatty acid profile of carotenoid esters

The fatty acid profile of carotenoid esters from carotenoid extract of different shrimps and prawn (Table 2.6) indicates that C16:0, C17:0 and C18:0 are the major saturated fatty acids and C16:1, and C18:1 are the major unsaturated fatty acids, with which carotenoids are esterified in majority of samples analysed. Short chain fatty acids like C8:0 and C10:0 were present in considerable quantities in the carotenoid esters from carapace of *P monodon* and C10:0 in meat of deep-sea shrimp *A alcocki*. Saturated fatty acids predominated (51.1 – 83.2%) in carotenoid esters from all the body components of *P monodon, P indicus* and *P stylifera*, meat and head of *M dobsoni*, in meat and carapace of *S indica* and meat of *A alcocki* and *M rosenbergii*.

In carotenoid esters from crabs (Table 2.7) unsaturated fatty acids were higher than the saturated fatty acids. C16:0 was the major saturated fatty acid in the carotenoid esters from marine crab meat (20.0%) and shell (14.7%), while C17:0 (21.3%) was the major saturated fatty acid in the shell of fresh water crab. Among unsaturated fatty acids C16:1 predominated in carotenoid esters from marine crab shell (36.2%), C18:1 in marine crab meat (34.0%), C18:3 in fresh water crab shell (42.0%) and C20:1 in fresh water crab meat (57.7%).

The results indicate that the major fatty acids associated with the carotenoid esters in the crustaceans analyzed are palmitic acid (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), palmitoleic acid (C16:1) and oleic acid (C18:1). Even though Snauweart et

al (1973a) reported the dominance of these fatty acids in carotenoid esters from brown shrimp *Cragnon vulgaris*, Renstrom and Liaaen-Jensen (1981) observed no such preferential selection of fatty acids in the carotenoid esters of shrimp *P borealis*. Renstrom and Liaaen-Jensen (1981) observed that astaxanthin esters of shrimp, *P borealis* contain only even number fatty acids. No such observations were made in the present study. They also reported the high composition of unsaturated fatty acids in carotenoid esters and concluded that the marine animals living in cold waters contain more of unsaturated fatty acids than those living in warm waters.

Gopakumar and Nair (1975) reported that the composition of fatty acids in the lipid extract of the meat from three species of Indian shrimps is almost equally distributed between saturated and unsaturated fatty acids, with predominance of C16:0, C18:0 and C18:1 fatty acids. Palmitic acid, palmitoleic acid, stearic acid and oleic acid were also found to be the major fatty acids in the lipids of fresh water prawn *M rosenbergii* (Nair and Gopakumar 1984) . The reported fatty acid profiles of meat from the Indian shrimps and prawn and the fatty acid profile of carotenoid esters observed in the present study indicate that the carotenoids are esterified with the predominant fatty acid present in the body of crustaceans.

2.3. Conclusions

Highest level of carotenoids (μg/g) was noted in the head of deep-sea shrimp *Aristeus alcocki* (185.3) followed by the head of marine shrimp *Parapenaeopsis stylifera* (153.1). Carapace of *A alcocki* (117.4 μg/g), *Solonocera indica* (116.0 μg/g) and *P stylifera* (104.7 μg/g) also contained high level of carotenoids. Among shrimps *Penaeus indicus* showed low level of carotenoids. Fresh water prawn also showed low level of carotenoids in all the body components. The carotenoid content was very low in both

fresh water and marine crabs. Astaxanthin and its esters were the major carotenoids in the carotenoid extracts from shrimp, prawn and marine crab. Presence of β-carotene and zeaxanthin at low levels was also observed in these species. Zeaxanthin was the major pigment fraction in the carotenoid extracts from fresh water crab. The major fatty acids in the carotenoid esters from the crustaceans studied were found to be palmitic (C16:0), heptadecanoic (C17:0), palmitoleic (C16:1), stearic (C18:0) and oleic (C18:1) acids.

Table 2.1. Total carotenoid content (μg/g) in different species of shrimp[1] and prawn[2]

Species	Body Component		
	Meat	**Head**	**Carapace**
***Penaeus monodon*[1] (n=6)**	17.4 ± 5.99^{xa}	58.4 ± 7.73^{yab}	86.6 ± 13.88^{za}
***Penaeus indicus*[1] (n=6)**	10.4 ± 0.92^{xb}	35.8 ± 6.83^{yb}	59.8 ± 11.02^{zb}
***Metapenaeus dobsoni*[1] (n=6)**	11.1 ± 1.61^{xb}	51.3 ± 4.09^{yb}	83.3 ± 13.87^{za}
***Parapenaeopsis stylifera*[1] (n=6)**	16.0 ± 2.21^{xa}	153.1 ± 40.06^{yc}	104.7 ± 11.39^{zc}
***Solonocera indica*[1] (n=6)**	15.9 ± 2.09^{xa}	67.7 ± 6.30^{ya}	116.0 ± 11.84^{zc}
***Aristeus alcocki*[1] (n=4)**	21.4 ± 1.73^{xc}	185.3 ± 17.02^{yd}	117.4 ± 6.70^{zc}
***Macrobrachium rosenbergii*[2] (n=4)**	2.7 ± 0.84^{xd}	34.4 ± 5.88^{yb}	40.7 ± 4.36^{zd}

Different superscripts on values in individual columns (a,b,c,d) and rows (x,y,z) indicates significant difference ($p \leq 0.05$)

ANOVA Table 2.1. Total carotenoid content

a. between body components

Species	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
P monodon	14509.76	2	7254.88	1441.40	15	96.09	75.50***
P indicus	7304.91	2	3652.46	844.97	15	56.33	64.84***
M dobsoni	15723.23	2	7861.62	1058.69	15	70.58	111.39***
P stylifera	57991.50	2	28995.75	8697.35	15	579.82	50.01***
S indica	300051.80	2	15025.90	821.50	15	61.43	244.59***
A alcocki	54256.85	2	27128.43	1012.30	9	112.48	241.19***
M rosenbergii	3340.81	2	1670.40	162.85	9	18.10	92.31***

b. between species

Body component	SS Effect	Df Effect	MS Effect	SS Error	Df Error	MS Error	F Value
Meat	971.53	6	161.92	253.97	31	8.19	19.76***
Head	104902.9	6	17483.81	9811.54	31	316.50	55.24***
Carapace	23711.30	6	3951.88	4073.56	31	131.41	30.07***

*** $p < 0.001$

Table 2.2. Total carotenoid content (μg/g) in marine and fresh water crab

	Meat	Shell
Marine crab (*Charybdis cruciata*)	3.4±0.61	11.0±0.45
Fresh water crab (*Potamon potamon)*	4.1±0.37	6.9±0.64

ANOVA Table 2.2. Total carotenoid content in marine and fresh water crab

a. between meat and shell

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Fresh water crab	23.27	1	23.27	2.72	10	0.27	85.59***
Marine crab	174.80	1	174.80	2.88	10	0.29	605.83***

b. between species

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Meat	1.37	1	1.37	2.56	10	0.26	5.36*
Shell	52.21	1	52.21	3.04	10	0.30	171.74***

* $p < 0.05$, *** $p < 0.001$

Table 2.3. Absorbance maxima (λmax) of carotenoid extracts from different body components of shrimp, prawn and crab in different organic solvents

Carotenoid extract	Solvent				
	Petroleum ether	Hexane	Ethanol	Acetone	Benzene
Shrimp, Prawn and marine crab (all body components)	469	470	475	478	485
Fresh water crab meat and shell (two peaks)	448 475	450 476	451 478	452 479	462 487

Table 2.4. Composition (% of total carotenoids) of major carotenoids in the carotenoid extract from different species of shrimp and prawn (by HPLC) (n=3)

Species	Body Component	Astaxanthin	Astaxanthin monoester	Astaxanthin diester	β-Carotene	Zeaxanthin	Unidentified
P monodon	**Meat**	22.2±1.68	43.1±1.74	15.2±1.19	1.1±0.09	12.2±1.57	6.2±0.92
	Head	24.3±1.05	22.6±1.01	20.3±1.48	4.9±1.05	5.7±0.90	22.2±3.07
	Carapace	28.8±1.45	44.0±1.73	13.9±1.61	1.7±0.49	5.5±0.89	6.2±1.79
P indicus	**Meat**	32.9±2.43	20.5±2.25	17.9±2.17	5.5±0.89	1.7±0.52	21.4±1.69
	Head	25.5±1.25	27.3±1.35	19.3±1.66	5.5±0.67	1.4±0.40	17.7±4.05
	Carapace	24.3±1.05	26.8±1.71	25.1±2.07	3.8±0.95	1.1±0.35	18.8±3.86
M dobsoni	**Meat**	26.7±1.65	21.1±1.15	20.8±1.57	7.3±1.00	0.5±0.21	23.6±2.10
	Head	24.2±1.00	22.4±0.95	21.3±1.15	6.5±0.85	0.9±0.25	18.3±1.77
	Carapace	33.2±1.63	22.4±0.95	21.2±1.00	4.4±1.19	0.6±0.21	18.32.39
P stylifera	**Meat**	42.2±2.00	26.0±1.41	10.3±1.90	7.3±1.25	1.3±0.32	12.7±0.70
	Head	22.6±1.58	29.1±4.14	29.6±1.87	4.4±1.88	1.7±0.55	14.6±5.39
	Carapace	18.8±1.57	32.1±2.07	20.3±0.90	1.6±0.57	1.0±0.36	26.2±2.77

Table 2.4 (Contd). Composition (% of total carotenoids) of major carotenoids in the carotenoid extract from different species of shrimp and prawn (by HPLC) (n=3)

Species	Body Component	Astaxanthin	Astaxanthin monoester	Astaxanthin diester	β-Carotene	Zeaxanthin	Unidentified
S indica	**Meat**	23.2 ±1.02	24.3±1.37	19.5±0.70	10.3±0.90	1.8±0.35	20.8±4.06
	Head	19.4±1.51	23.9±4.20	20.2±1.25	5.5±0.51	2.8±0.31	24.7±1.23
	Carapace	14.9±3.19	39.0±1.55	19.4±1.30	1.1±0.20	1.5±0.25	25.1±2.31
A alcocki	**Meat**	15.1±1.46	49.8±2.38	24.0±1.21	0.8±0.25	0.6±0.20	9.8±2.04
	Head	25.4±0.98	46.3±1.06	20.5±0.93	1.0±0.58	1.2±0.50	5.8±0.91
	Carapace	26.5±1.11	40.7±1.41	21.0±2.61	1.6±0.56	4.3±1.09	5.8±2.66
M rosenbergii	**Meat**	29.7±1.56	12.3±1.38	12.9±1.80	21.8±3.57	0.3±0.15	24.4±1.91
	Head	24.6±1.69	12.8±1.31	14.5±1.36	29.6±3.56	1.3±0.23	16.2±0.81
	Carapace	29.8±1.25	18.2±1.90	16.3±1.55	5.5±1.59	0.8±0.38	29.4±6.35

Table 2.5. Composition (% of total carotenoids) of major carotenoids in the carotenoid extract from marine and fresh water crab (by HPLC) (n=3)

Species	Body Component	Astaxanthin	Astaxanthin monoester	Astaxanthin diester	β-Carotene	Zeaxanthin	Unidentified
Marine crab ***(C cruciata)***	**Meat**	17.3±1.12	26.4±2.04	23.9±2.08	3.6±1.33	0.49±0.35	27.6±3.90
	Shell	23.6±1.43	15.2±1.30	26.7±2.79	5.1±1.44	5.0±1.69	24.4±2.80
Fresh water crab ***(P potamon)***	**Meat**	9.3±0.61	11.2±0.98	16.0±1.14	7.4±1.56	42.0±2.61	14.1±1.05
	Shell	7.2±1.04	3.7±1.21	3.8±1.14	3.6±1.16	74.8±4.16	6.9±2.12

Table 2.6. Fatty acid profile (%) of carotenoid esters from shrimp and prawn

Fatty acid		*P monodon*			*P indicus*		
		Meat	**Head**	**Carapace**	**Meat**	**Head**	**Carapace**
Saturated	**C 6:0**	2.8	1.6	3.5	0.35	0.21	0.60
	C 8:0	1.5	4.7	23.5	1.2	0.59	1.0
	C 10:0	2.6	3.3	15.9	2.2	0.94	2.0
	C 12:0	0.65	0.24	0.23	0.31	0.37	0.50
	C 13:0	1.1	1.9	0.34	0.85	0.16	0.92
	C14:0	0.43	0.53	0.13	1.9	2.0	1.2
	C 15:0	12.5	20.1	10.2	1.9	0.80	9.1
	C 16:0	18.5	5.6	9.8	24.4	23.1	19.6
	C 17:0	14.9	13.6	8.9	11.9	7.5	17.7
	C 18:0	11.4	16.2	8.0	13.4	15.4	11.5
	C 20:0	3.9	-	2.7	0.13	-	6.6
Total		**70.3**	**67.8**	**83.2**	**56.6**	**51.1**	**62.5**
Unsaturated	**C 14:1**	5.2	2.3	0.55	1.3	0.97	0.75
	C 15:1	2.6	7.7	3.0	-	-	-
	C 16:1	9.2	4.0	1.1	11.7	11.5	11.0
	C 18:1	4.8	1.7	1.2	19.1	26.2	13.6
	C 18:2	-	1.1	0.15	2.1	6.4	2.3
	C 18:3	1.6	11.9	6.9	0.41	-	-
	C 20:1	3.5	1.5	2.0	1.2	-	-
	C 20:4	-	1.5	0.32	-	-	-
Total		**26.9**	**31.7**	**15.2**	**35.8**	**45.1**	**27.7**
Others (unidentified)		2.8	0.50	1.6	7.6	3.8	9.8

Table 2.6(contd). Fatty acid profile (%) of carotenoid esters from shrimp, and prawn

Fatty acid		*M dobsoni*			*P stylifera*		
		Meat	**Head**	**Carapace**	**Meat**	**Head**	**Carapace**
Saturated	**C 6:0**	0.77	1.1	-	0.91	1.4	2.7
	C 8:0	0.68	2.3	-	2.7	10.6	8.9
	C 10:0	0.71	0.70	1.2	3.1	9.4	13.4
	C 12:0	0.47	1.1	3.9	0.13	3.9	0.96
	C 13:0	9.1	1.3	-	2.5	-	0.18
	C14:0	4.3	0.78	2.2	1.5	2.5	0.39
	C 15:0	-	4.2	1.5	26.7	7.6	8.0
	C 16:0	27.8	10.2	16.7	12.3	13.9	26.6
	C 17:0	7.5	14.2	5.3	11.5	5.2	10.2
	C 18:0	8.9	19.6	1.6	18.4	10.5	5.0
	C 20:0	-	-	0.34	-	2.9	-
Total		**60.2**	**55.5**	**32.7**	**77.3**	**67.9**	**76.3**
Unsaturated	**C 14:1**	0.84	0.53	0.48	0.75	0.35	2.0
	C 15:1	-	-	-	-	4.1	7.6
	C 16:1	9.1	3.1	12.7	7.3	0.23	11.6
	C 18:1	10.1	14.2	7.0	2.1	22.9	-
	C 18:2	12.8	9.4	34.1	1.2	-	1.4
	C 18:3	-	11.4	10.9	3.4	0.92	-
	C 20:1	-	-	-	0.82	0.23	-
	C 20:4	-	-	-	-	-	-
Total		**32.8**	**38.6**	**65.2**	**15.6**	**28.7**	**22.6**
Others (unidentified)		7.0	5.9	2.1	7.1	3.4	1.1

Table 2.6(contd). Fatty acid profile (%) of carotenoid esters from shrimp, and prawn

Fatty acid		*S indica*			*A alcocki*		
		Meat	**Head**	**Carapace**	**Meat**	**Head**	**Carapace**
Saturated	**C 6:0**	-	-	3.5	0.43	0.18	0.22
	C 8:0	1.3	1.2	6.2	0.29	1.2	0.98
	C 10:0	1.9	2.0	5.5	10.6	0.31	1.6
	C 12:0	0.91	1.0	3.1	4.7	0.16	4.4
	C 13:0	-	3.8	-	0.24	-	-
	C14:0	1.4	1.3	-	1.2	3.0	2.1
	C 15:0	1.2	-	-	0.71	0.44	1.4
	C 16:0	16.9	23.2	14.3	20.5	19.4	16.3
	C 17:0	11.7	8.5	37.6	2.7	4.8	5.1
	C 18:0	15.5	3.1	12.9	8.4	6.7	6.9
	C 20:0	-	0.85	-	0.60	-	-
Total		**50.8**	**45.0**	**83.1**	**50.4**	**36.0**	**39.0**
Unsaturated	**C 14:1**	0.90	1.2	-	0.21	0.89	0.65
	C 15:1	-	2.7	-	0.56	-	-
	C 16:1	9.6	13.5	4.6	6.3	14.2	12.4
	C 18:1	12.8	14.3	-	25.5	41.5	33.2
	C 18:2	17.2	21.0	10.0	3.2	1.8	10.6
	C 18:3	3.2	1.1	-	0.85	2.1	0.33
	C 20:1	5.5	-	-	3.7	-	-
Total		**49.2**	**53.8**	**14.6**	**40.3**	**60.5**	**57.2**
Others (unidentified)		-	1.2	2.3	9.3	3.5	3.8

Table 2.6(contd). Fatty acid profile (%) of carotenoid esters from shrimp, and prawn

Fatty acid		*M rosenbergii*		
		Meat	**Head**	**Carapace**
Saturated	**C 6:0**	-	-	-
	C 8:0	1.6	-	-
	C 10:0	2.5	-	1.0
	C 12:0	0.49	0.20	-
	C 13:0	9.5	0.11	-
	C14:0	4.5	2.5	0.77
	C 15:0	-	0.67	-
	C 16:0	29.1	17.9	22.8
	C 17:0	7.9	2.0	4.2
	C 18:0	9.3	12.6	14.8
	C 20:0	-	-	0.58
Total		**64.9**	**36.0**	**44.2**
Unsaturated	**C 14:1**	0.88	0.66	-
	C 15:1	-	0.88	0.87
	C 16:1	9.5	9.4	11.6
	C 18:1	10.5	26.0	29.0
	C 18:2	13.4	8.6	9.8
	C 18:3	-	4.1	0.81
	C 20:1	-	10.6	0.75
Total		**34.3**	**60.2**	**52.8**
Others (unidentified)		0.8	3.8	3.0

Table 2.7. Fatty acid profile (%) of carotenoid esters from crab

Fatty acid		Marine crab (*C cruciata*)		Fresh water crab (*P potamon*)	
		Meat	**Shell**	**Meat**	**Shell**
Saturated	**C 6:0**	-	-	-	-
	C 8:0	-	-	-	1.8
	C 10:0	1.8	9.1	1.4	1.8
	C 12:0	-	7.8	-	0.66
	C 13:0	0.88	-	-	-
	C14:0	-	-	-	-
	C 15:0	-	-	-	-
	C 16:0	20.0	14.7	6.0	4.5
	C 17:0	5.6	-	5.1	21.3
	C 18:0	13.0	5.6	3.2	5.2
	C 20:0	-	-	-	-
Total		**41.4**	**37.2**	**15.7**	**35.3**
Unsaturated	**C 14:1**	-	-	-	-
	C 15:1	1.9	7.9	-	-
	C 16:1	6.5	36.2	1.2	1.7
	C 18:1	34.0	6.5	16.5	10.1
	C 18:2	11.9	5.2	-	11.0
	C 18:3	-	7.1	9	42.0
	C 20:1	-	-	57.7	-
Total		**54.3**	**62.9**	**84.3**	**64.7**
Others (unidentified)		4.3	-	-	-

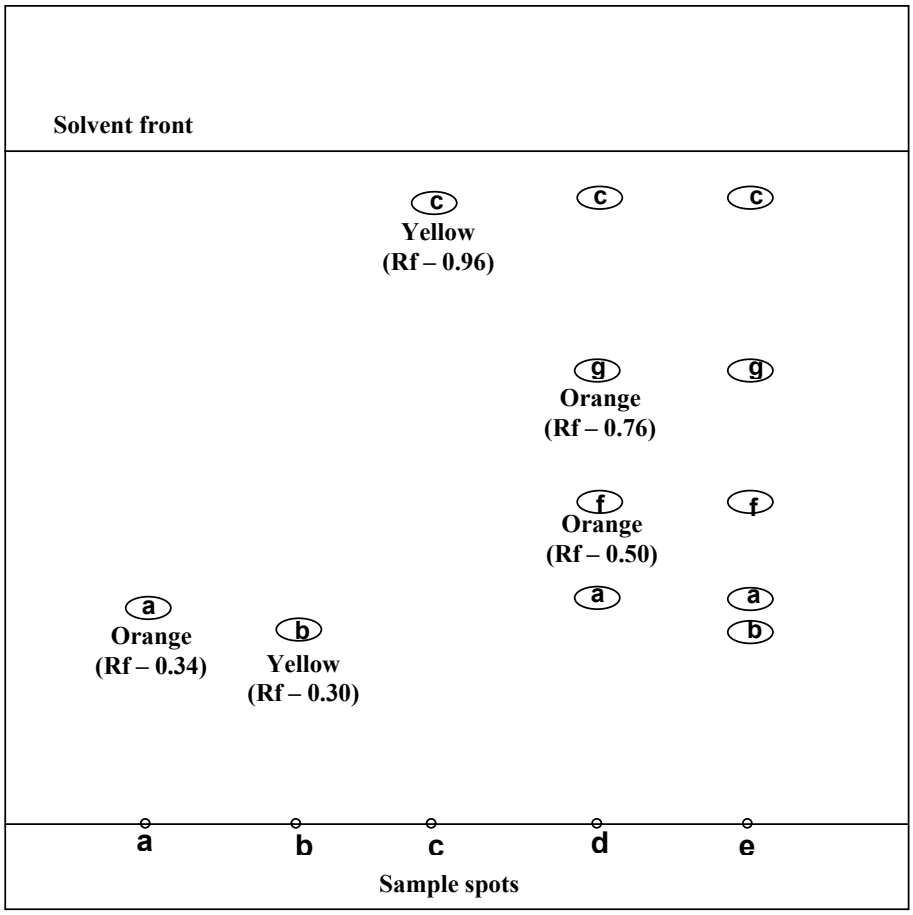


Figure 2.1. Typical thin layer chromatogram of the carotenoid standards (a,b,c) and carotenoid extract from shrimp, prawn and crab. Mobile phase – Acetone : Hexane (25 : 75).

a: Astaxanthin

b: Zeaxanthin

c: β-Carotene

d: Carotenoid extract from different body components of shrimp, prawn and marine crab

e: Carotenoid extract from meat and shell of fresh water crab

f: Astaxanthin monoester

g: Astaxanthin diester

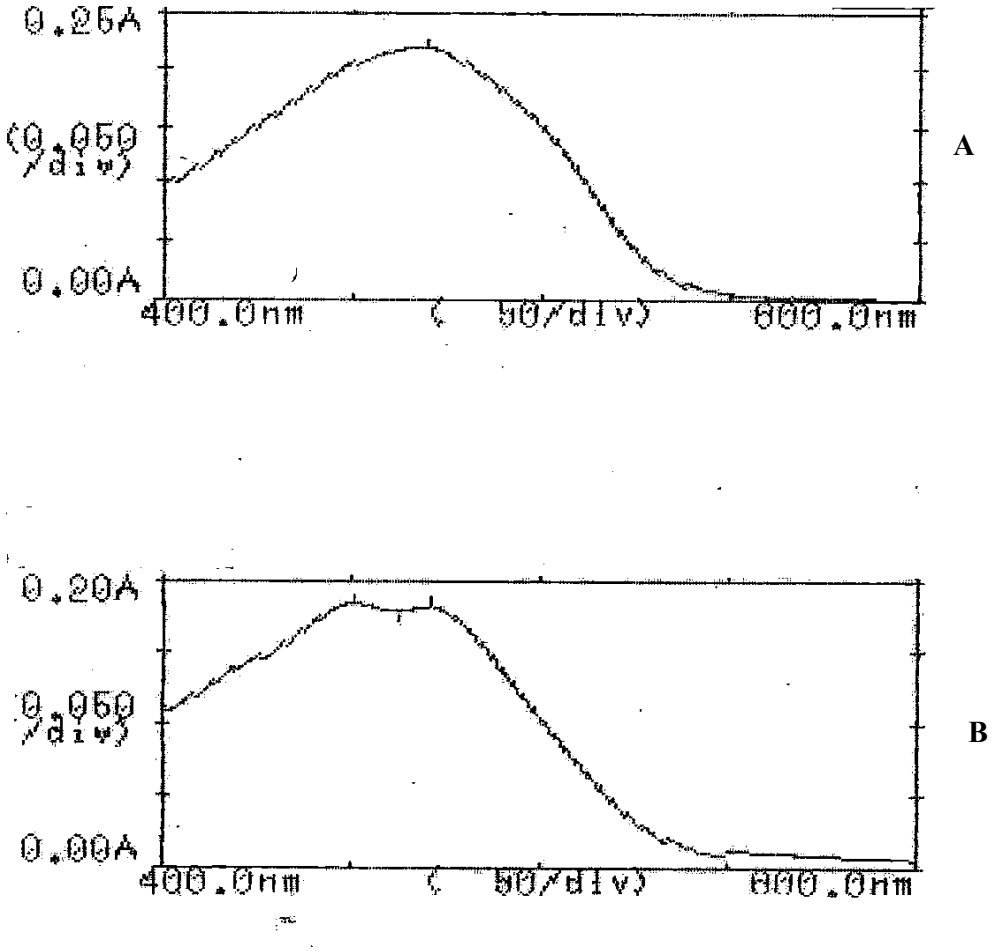
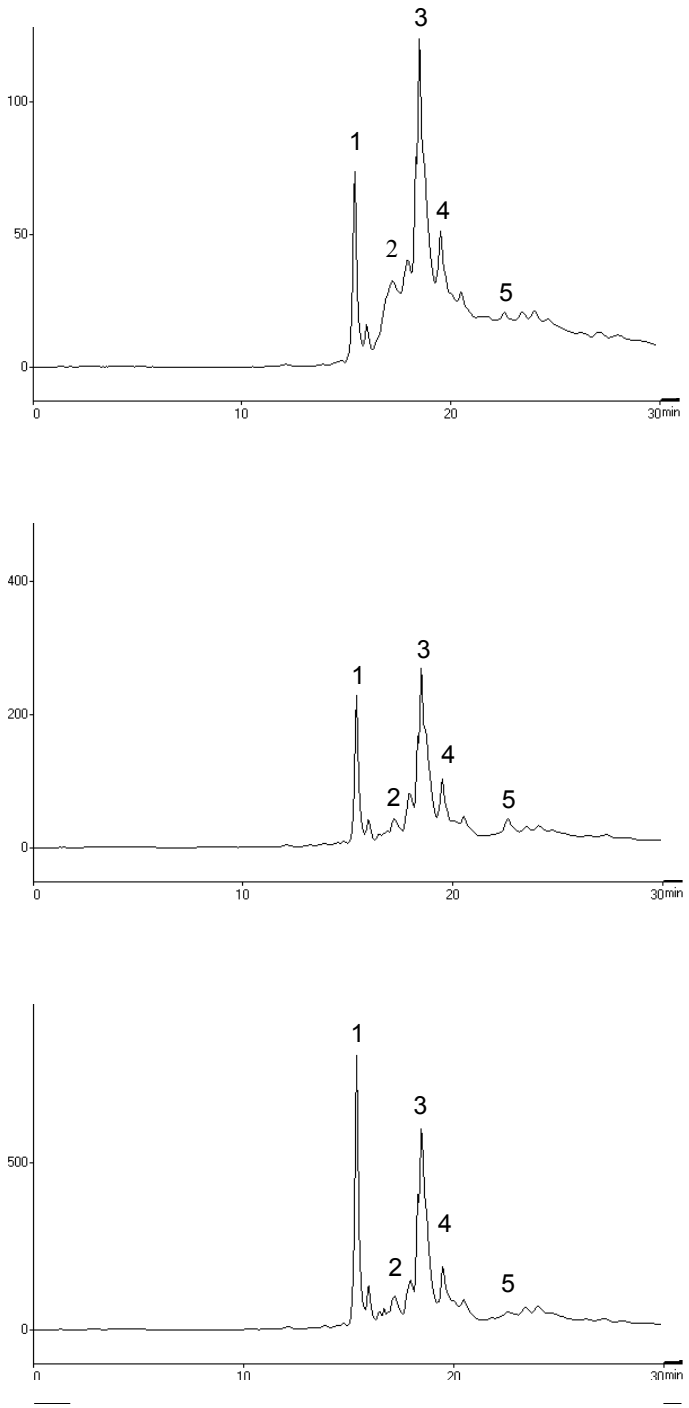
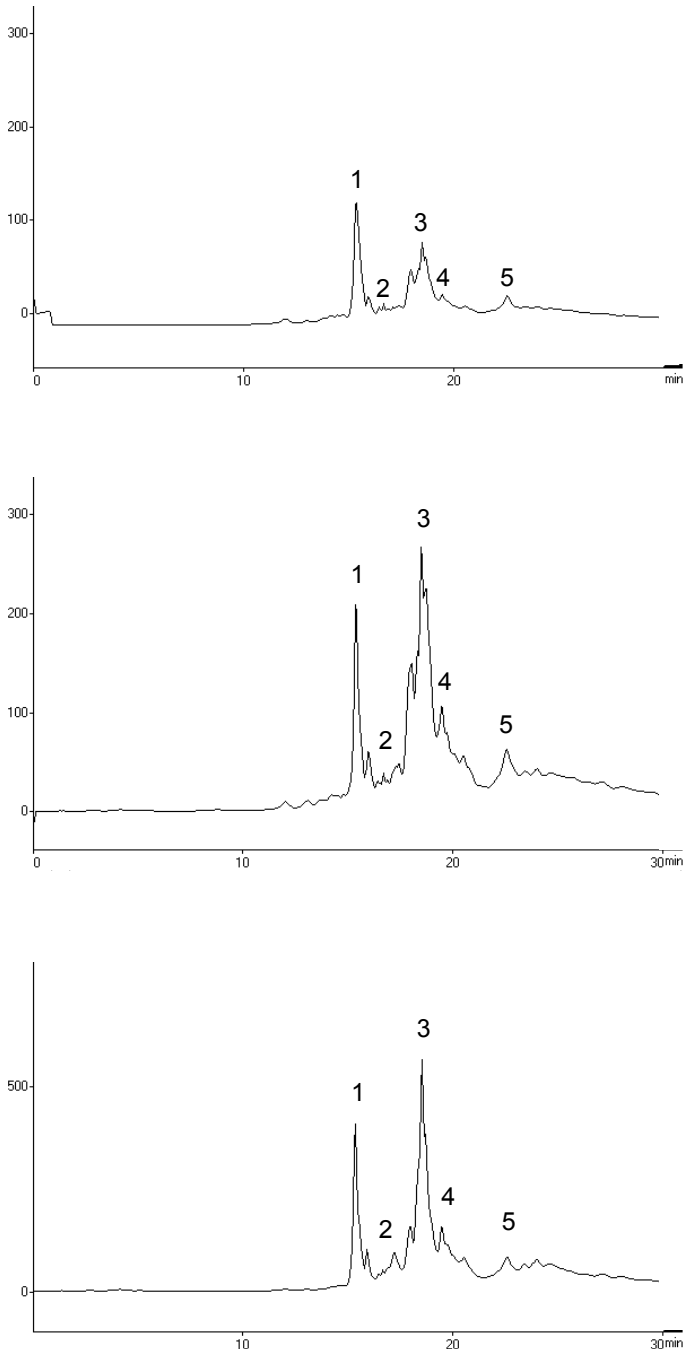


Figure 2.2. Typical absorption spectra of carotenoid extract (in hexane) from different body components of shrimp, prawn, marine crab (A) and fresh water crab (B)



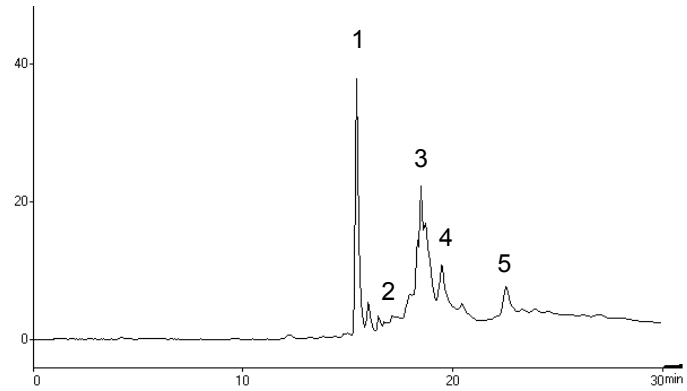
Chromatogram 1. HPLC profile of carotenoid extracts from *Penaeus monodon*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)

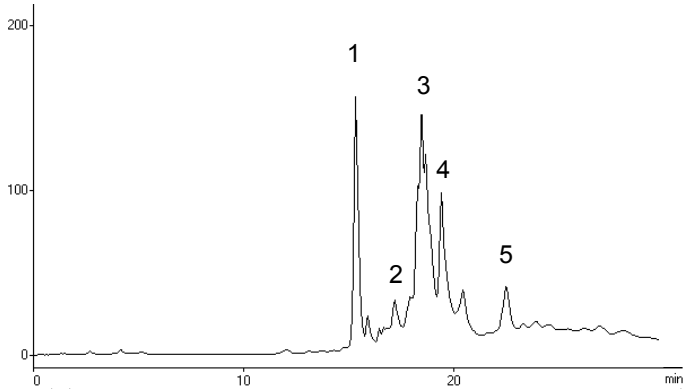


Chromatogram 2. HPLC profile of carotenoid extracts from *Penaeus indicus*

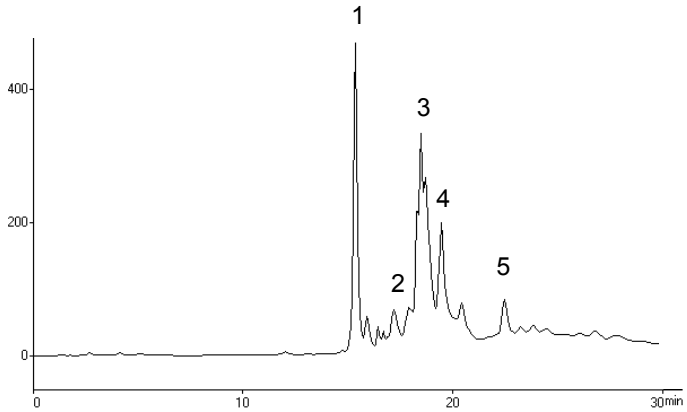
(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



Meat



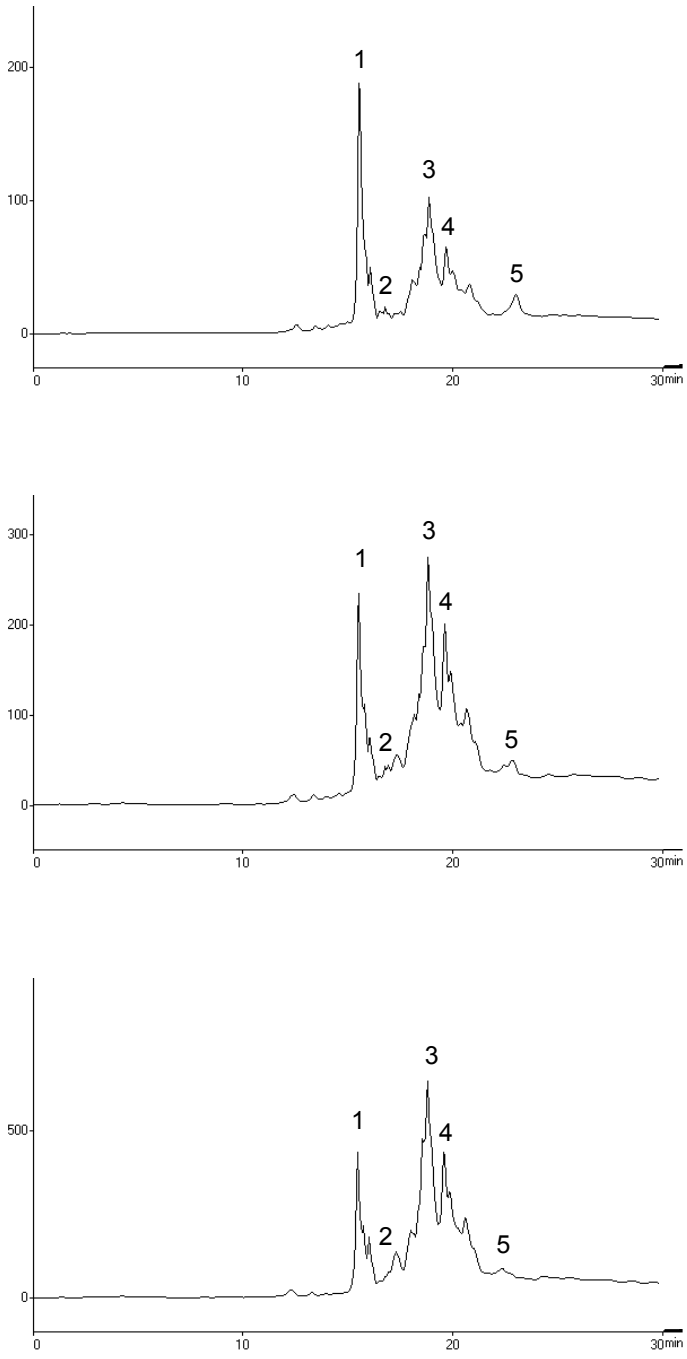
Head



Carapace

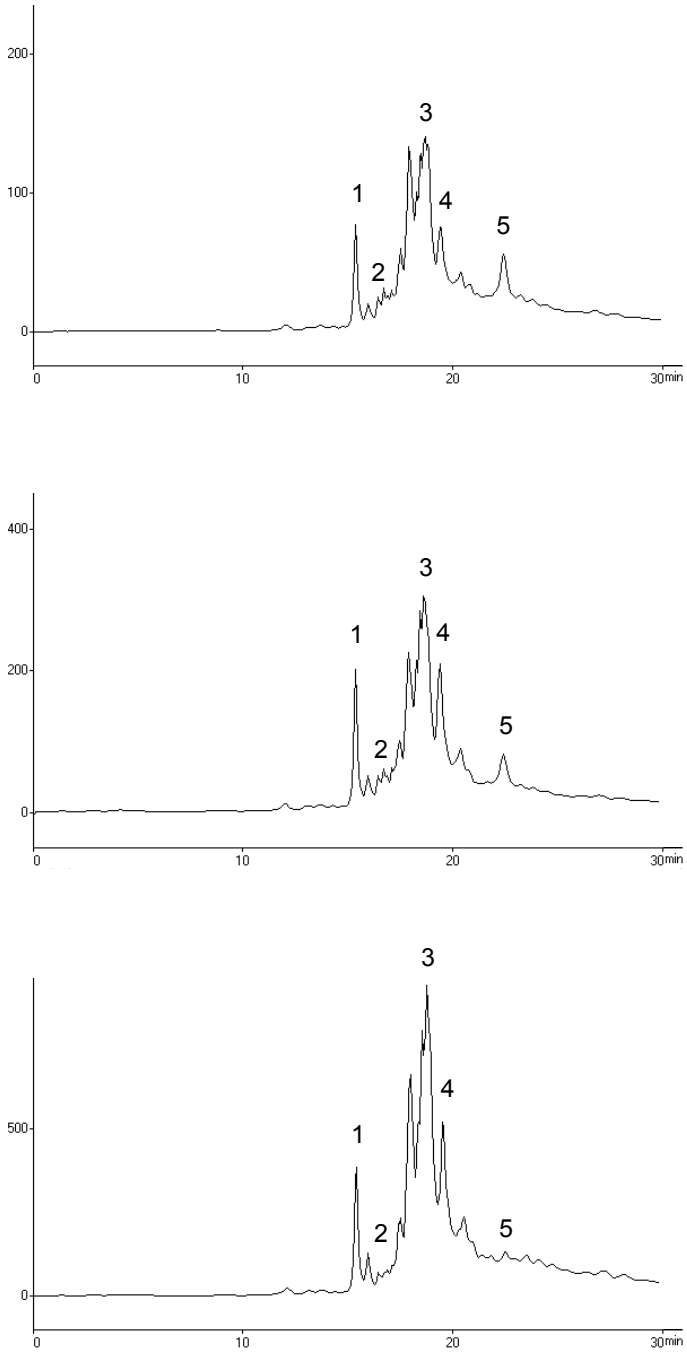
Chromatogram 3. HPLC profile of carotenoid extracts from *Metapenaeus dobsoni*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



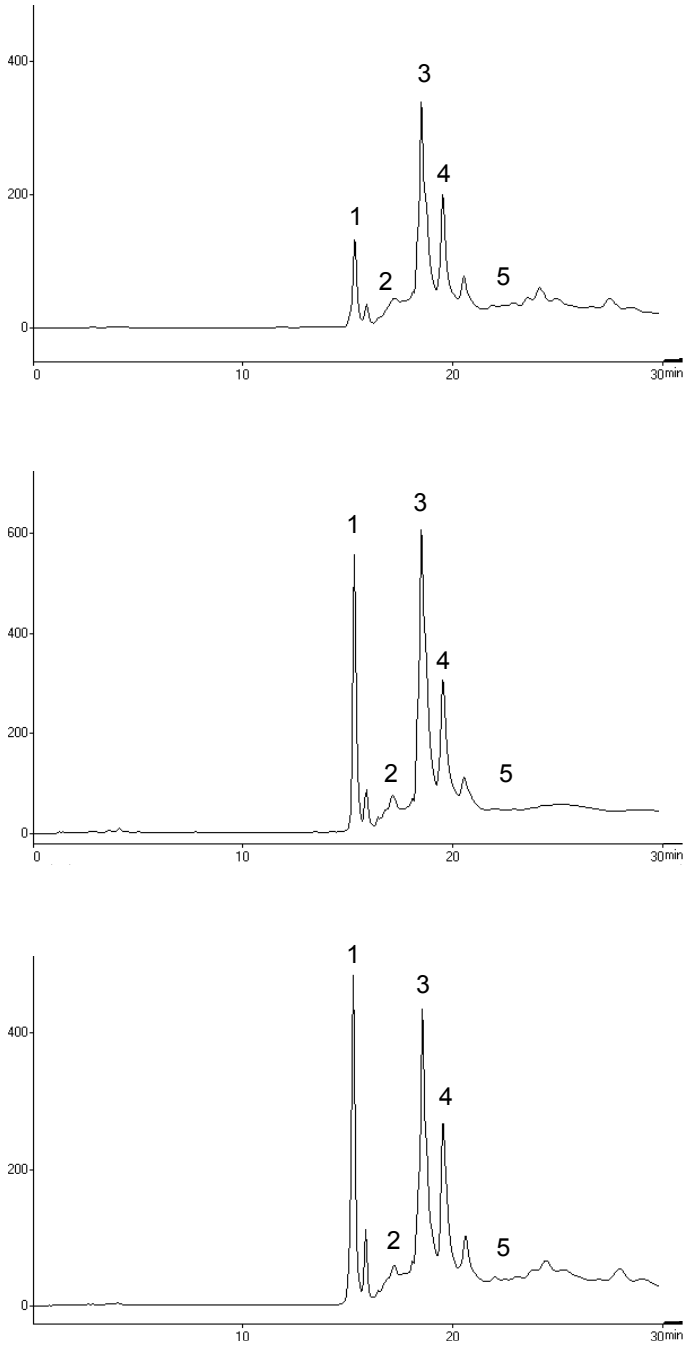
Chromatogram 4. HPLC profile of carotenoid extracts from *Parapenaeopsis stylifera*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



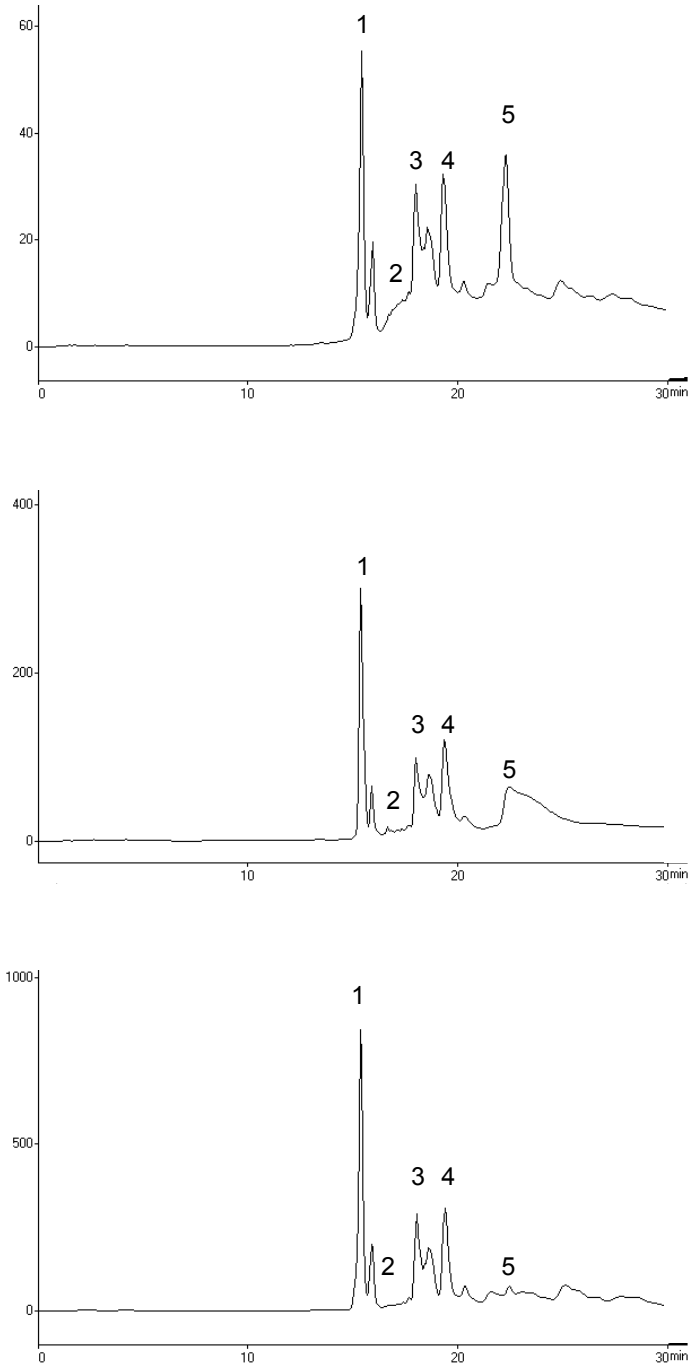
Chromatogram 5. HPLC profile of carotenoid extracts from *Solonocera indica*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



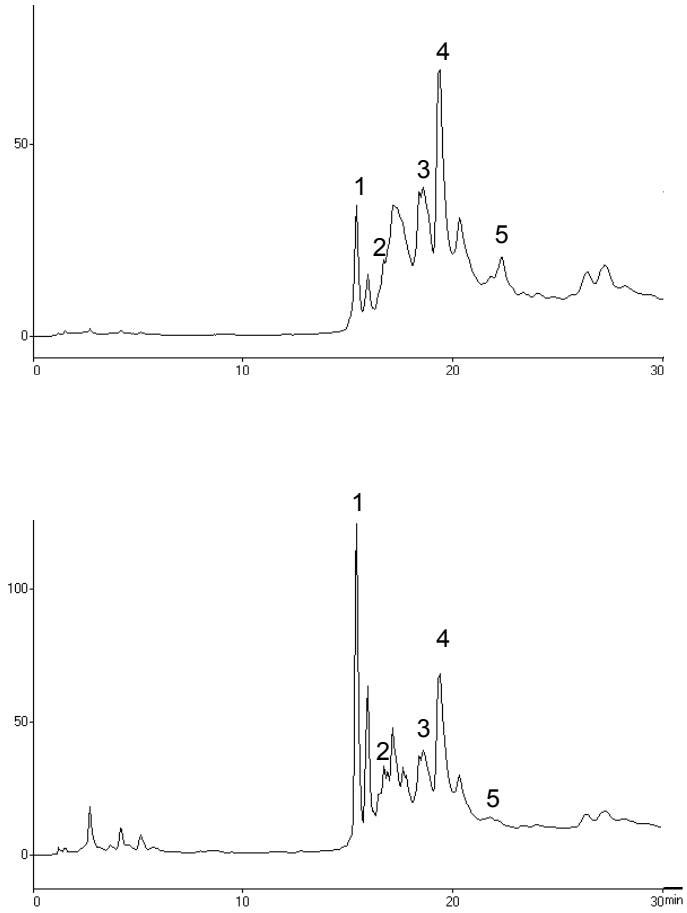
Chromatogram 6. HPLC profile of carotenoid extracts from *Aristeus alcocki*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



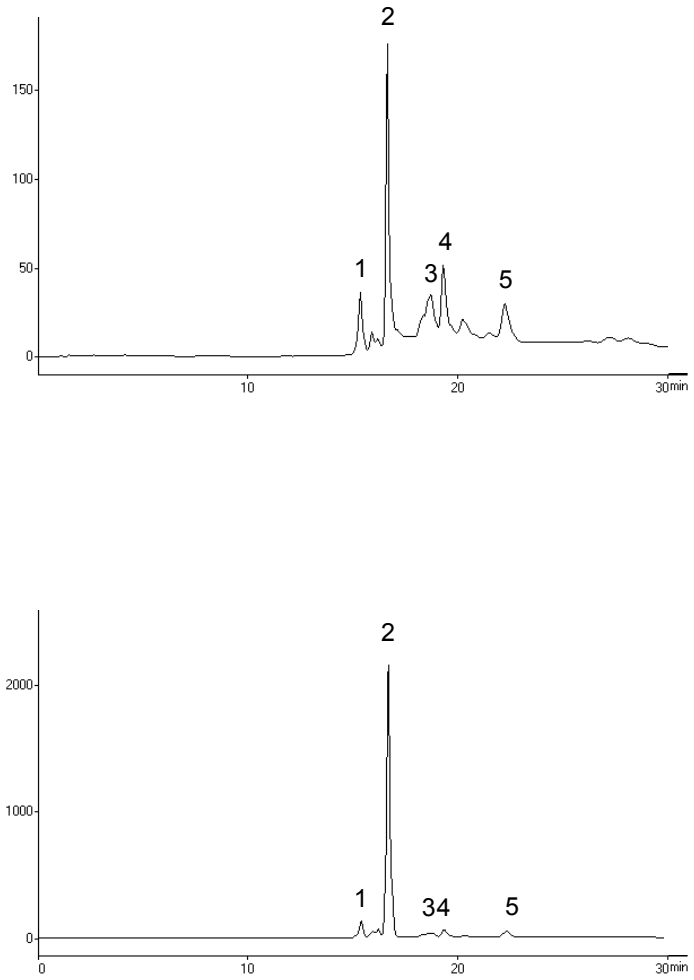
Chromatogram 7. HPLC profile of carotenoid extracts from *Macrobrachium rosenbergii*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



Chromatogram 8. HPLC profile of carotenoid extracts from marine crab *Charybdis cruciata*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)



Chromatogram 9. HPLC profile of carotenoid extracts from fresh water crab *Potamon potamon*

(1: Astaxanthin, 2: Zeaxanthin, 3: Astaxanthin monoester, 4: Astaxanthin diester, 5: β-Carotene)

CHAPTER 3

RECOVERY OF CAROTENOIDS FROM SHRIMP WASTE BY SOLVENT EXTRACTION

CHAPTER 3

RECOVERY OF CAROTENOIDS FROM SHRIMP WASTE BY SOLVENT EXTRACTION

Shrimp waste, head and carapace, comprise 45 – 55% of the whole shrimp. Large quantities of shrimp waste are being produced in the shrimp processing industries. The shrimp waste is one of the important natural sources of carotenoid. The recovery of these valuable components from the waste would not only improve the economy of the shrimp processing plant, but also would minimize the pollution potential of the shrimp waste. Shrimp waste being one of the cheapest raw materials for carotenoid recovery, the extracted carotenoid would be a good alternative for synthetic carotenoid. Use of organic solvent for recovery of carotenoid from shrimp waste is limited to the analytical purposes only (Britton 1985, Masatoshi and Junji 1999, Meyers and Bligh 1981). Hence studies were conducted to determine the yield of carotenoids from shrimp waste in different organic solvents and their mixtures, and optimization of conditions for solvent extraction of carotenoids by a statistically designed experiment.

3.1. Experimental design and methodology

Shrimp waste from *Penaeus indicus* comprising of head and carapace was collected from the shrimp processing plants situated at Mangalore and transported to the laboratory under frozen condition. The material was thawed in running water before use and homogenized in a laboratory mixer.

3.1.1. Yield of carotenoids in different organic solvents / solvent mixture

Carotenoids in the homogenized shrimp waste were extracted using different organic solvents and solvent mixtures as explained in section 2.1.1. The solvents (AR

grade) used were acetone, methanol, ethyl methyl ketone, isopropyl alcohol (IPA), ethyl acetate, ethanol, petroleum ether and hexane. The solvent mixture was prepared my mixing equal quantities of a polar and non-polar solvent. The solvent mixtures used were acetone and hexane, and IPA and hexane. In case of the carotenoid extract in petroleum ether, hexane and solvent mixture, the addition of petroleum ether for phase separation was avoided and are directly washed with saline and dried and concentrated and the carotenoid content in the concentrate was measured spectrophotometrically as explained in section 2.1.1.

3.1.2. Carotenoid yield at each stage of extraction

Carotenoids from homogenized shrimp waste were extracted using 50 : 50 mixtures of IPA and hexane as explained in section 3.1.1. After 1st extraction, the carotenoids in the filtrate was brought into hexane by washing the filtrate with 0.1% saline and the hexane phase was dried, concentrated and the carotenoid content in the concentrate was measured. The residue after 1st extraction was reextracted with solvent mixture 4 more times and the carotenoid yield in every extraction was determined as above.

3.1.3. Optimization of conditions for solvent extraction of carotenoids

As the experiment on recovery of carotenoids in different solvents showed that a mixture of IPA and hexane gives highest yield, this solvent mixture was used for optimization studies. The conditions for extraction were optimized with respect to hexane % in the solvent mixture, solvent level to waste and number of extractions using Box-Behnkan experimental design (Box and Behnkan 1960). The experiment was designed using the software STATISTICA (Statsoft Inc 1999). The experimental design used determines the effect of combination of process variables (factors) and their interactions

on the response variable. The experimental design involved 3 factors namely hexane % in the solvent mixture (X1), solvent level to waste (X2) and number of extractions (X3), each at 3 equidistant levels (-1, 0, +1) and the response variable was the carotenoid yield (Y). In total, 15 combinations of factors were used. The combination of factors at the center of level was run in triplicate. The factors, their levels and codes for the level were as follows.

Factors	**Codes**	**Level**		
		-1	**0**	**+1**
Hexane % in the solvent	X1	10	45	80
Solvent level to waste	X2	2	5	8
Number of extraction	X3	1	3	5

The combination of factors for 15 runs was as follows.

Run no.	**X1**	**X2**	**X3**
1	-1 (10)	-1 (2)	0 (3)
2	+1 (80)	-1 (2)	0 (3)
3	-1 (10)	+1 (8)	0 (3)
4	+1 (80)	+1 (8)	0 (3)
5	-1 (10)	0 (5)	-1 (1)
6	+1 (80)	0 (5)	-1 (1)
7	-1 (10)	0 (5)	+1 (5)
8	+1 (80)	0 (5)	+1 (5)
9	0 (45)	-1 (2)	-1 (1)
10	0 (45)	+1 (8)	-1 (1)
11	0 (45)	-1 (2)	+1 (5)
12	0 (45)	+1 (8)	+1 (5)
13	0 (45)	0 (5)	0 (3)
14	0 (45)	0 (5)	0 (3)
15	0 (45)	0 (5)	0 (3)

The extraction of carotenoids and determination of their concentration was carried out as explained earlier (section 3.1.1).

3.1.4. Statistical analysis

All the statistical analyses were carried out using the software STATISTICA (Statsoft Inc 1999). Analysis of variance technique and Duncan's multiple range tests were used to determine the significant difference in yield between different solvents and for mean separation respectively. Optimization data was analyzed for effect of each factor and their interactions on the carotenoid yield by ANOVA technique. The optimization data analyzed for determination of regression coefficients to arrive at the regression equation. Regression model containing 10 coefficients including linear and quadratic effect of factors and linear effect of interactions was assumed to describe relationships between response (Y) and the experimental factors (X1, X2, X3) as follows,

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i . X_j$$

Where β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the second order interaction coefficient. 3D response graph, and profile for predicted values and desirability level for factors were plotted using the software (Statsoft Inc 1999).

3.2. Results and discussion

3.2.1. Yield of carotenoids

The solvent extracted carotenoid was in the paste form with an orange red color (Photoplate 3.1). Highest carotenoid yield (43.9 μg/g waste) from waste of *P indicus* was obtained when the carotenoids were extracted with a mixture of IPA and hexane, followed by IPA (40.8 μg/g) and acetone alone (40.6 μg/g) (Table 3.1). The lowest carotenoid yield was obtained with two non-polar solvents, petroleum ether (12.1 μg/g) and hexane (13.1 μg/g). The extraction yield differed significantly ($p \leq 0.01$) between

solvents (ANOVA Table 3.1). Even though 50 : 50 mixtures of IPA and hexane gave significantly ($p \leq 0.05$) higher yield than IPA alone, no significant ($p \geq 0.05$) difference was observed in carotenoid yield between acetone and 50 : 50 mixture of acetone and hexane. Eventhough, the observations made in the experiments was with respect to the wastes from *P indicus,* it would apply to the waste from any other species of shrimps

Maximum quantity (77.8 % of total carotenoids) of carotenoids was extracted in the first extraction itself, when extracted with a 50 : 50 mixture of IPA and hexane (Table 3.2). The 2^{nd} extraction yielded 15.6 % of total carotenoid. There was a significant difference ($p < 0.001$) in the carotenoid yield at different stages of extraction (ANOVA Table 3.2).

Britton (1985) recommended the use of water miscible polar organic solvents, usually acetone, methanol or ethanol for extraction of carotenoids from tissues containing water. Delgado-Vargus et al (2000) discussed the advantages and disadvantages of various organic solvents for extraction of carotenoids and suggested that polar solvents are generally good extraction media for xanthophylls but not for carotenes. For wet tissues use of non-polar solvents is not recommended as their penetration through the hydrophobic mass that surrounds the pigment is limited (Delgado-Vargus et al 2000). De Ritter and Purcell (1981) postulated that complete extraction of carotenoids from plant tissues could be achieved with samples of low moisture content by use of slightly polar plus non-polar solvents. In the present study, the increased extraction yield of carotenoids by the mixture of IPA and hexane may be due to the reason that along with xanthophylls, increased amount of carotenes are also extracted due to the inclusion of a non-polar solvent in the extraction medium.

Even though acetone is used as a common extraction medium for carotenoids, the present study indicated that IPA is a better polar solvent for extraction of carotenoids from shrimp waste. Further it is stated that, when IPA or mixture of IPA and hexane was used for oil extraction, more antioxidants were extracted and oils with extended stability were obtained (Procter and Bowen 1996). Shrimp waste is known to contain antioxidants (Li et al 1998), thus use of IPA and hexane for extraction of carotenoids may improve their stability during storage.

It is stated that when tissue contains a large amount of water, the first extraction with polar solvents may remove little pigment, but as it dries the tissues, the carotenoid yield increases in the subsequent extractions (Britton 1985). However, in the present study nearly 93.4% of carotenoids were extracted in the first two extractions itself.

3.2.2 Optimization of conditions for carotenoid extraction

The extraction with 50: 50 mixtures of IPA and hexane at solvent to waste level of 2.5 gave higher carotenoid yield than other solvents as observed above. In order to determine the combined effect of different level of hexane in the solvent mixture (X1), solvent level to waste (X2) and number of extraction (X3) on carotenoid yield (Y), optimization experiments were conducted. All the three factors namely, hexane % in solvent mixture ($p \leq 0.01$), solvent level to waste ($p \leq 0.01$), number of extraction ($p \leq 0.001$) and the interaction between X1 and X2 ($p \leq 0.05$), X1 and X3 ($p \leq 0.01$), X2 and X3 ($p \leq 0.01$) had significant effect on the carotenoid yield (ANOVA Table 3.3). A significant ($p \leq 0.01$) lack of fit indicates that there is still some statistically significant variability left that cannot be accounted for by the factors and their interactions.

The main effects indicated in ANOVA Table 3.3 are the combination of both linear (L) and quadratic (Q) effects. The estimate for linear effect is interpreted as the

difference between the average response at the low and high setting for the respective factors, while the estimate for quadratic effect is interpreted as the difference between the average response at the center of setting and combined high and low setting for the respective factors. (Statsoft Inc 1999). The interaction effects are presented as linear-by-linear effect, which can be interpreted as half the difference between the linear main effect of one factor at the low and high setting of another.

The regression coefficients for main effects and their interactions (Table 3.3) are obtained by the regression analysis of the data to fit suitable regression equation for carotenoid yield as a function of linear and quadratic effects of main factors and the linear-by-linear interaction effects. The regression equation for the carotenoid yield was derived to be,

$$Y = - 0.44366 + (0.21985\ X1) + (2.11016\ X2) + (13.65674\ X3) + (- 0.00135\ X1^2) + (- 0.07938\ X2^2) + (- 1.25022\ X3^2) + (0.00659\ X1 * X2) + (- 0.02276\ X1 * X3) + (- 0.29520\ X2 * X3)$$

The regression equation was used to arrive at the predicted value of carotenoid yield at each combination of processing variables (factors). The closeness (correlation coefficient r = +0.9882) of observed and the predicted carotenoid yield (Table 3.4) indicates that the regression equation arrived at can be used to determine the carotenoid yield at different levels of the 3 factors, which are influencing the carotenoid yield. The frequency distribution of residuals (observed – predicted response) (Figure 3.1) indicates that the difference between observed and predicted carotenoid yield follows a normal distribution with maximum number of values (11 out of 15) falling between a narrow ranges of – 1.0 to + 1.0.

The response surface graph of the effect of hexane % in the solvent mixture and solvent level to waste when number of extraction was kept at the center of the setting (3)

shows that the rate of increase in carotenoid yield was lower above 60 % hexane in the solvent mixture and solvent to waste level of 5 (Figure 3.2). The response surface graph (Figure 3.3) of the effect of hexane % in combination with number of extraction at constant solvent level to waste (5) indicates that the carotenoid yield was highly influenced by the change in number of extractions. The response surface graph (Figure 3.4) of the effect of solvent level to waste and number of extraction when the hexane % in the solvent mixture was kept constant (45%) confirms that the influence of number of extractions was higher than the solvent level to waste on the carotenoid yield.

The profiles for predicted response and the desirability level for factors (Figure 3.5) indicates that 60% hexane in the solvent mixture, solvent to waste level of 5, and 3 extractions gives optimum carotenoid yield at an optimum desirability score of 0.90294 (in a scale of 0 to 1). The desirability profiles show which levels of predictor (X1, X2 and X3) variables produce the most desirable predicted responses on the dependent variable (Y) and is determined as the geometric mean of desirability score at different level of one independent variable holding the levels of other independent variables constant at specified values by a desirability function (Statsoft Inc 1999). These profiles indicate that increase in the hexane level above 60% in the solvent mixture, solvent to waste level above 5 and number of extraction above 3 would not increase the yield significantly.

3.3. Conclusion

Use of a mixture of polar and non-polar solvents, namely IPA and hexane for extraction of was gave highest carotenoid yield from shrimp waste. The optimized conditions for the solvent extraction of carotenoid from shrimp waste was found to be 60 % hexane in the solvent mixture of IPA and hexane, solvent to waste level of 5 in each extraction and 3 number of extractions. The use of IPA and hexane instead of normally

used acetone is beneficial in the large-scale extraction of carotenoids from shrimp waste, as cost of IPA and hexane is lower than that of acetone and the yield of carotenoid is higher. The present experiment was conducted using the waste from the shrimp *P indicus,* which has shown lowest level of carotenoids among the marine shrimps analyzed (Chapter 2). The results obtained would be applicable to waste from other species of shrimps and prawns also.

Photoplate 3.1

Solvent extracted carotenoids

Table 3.1. Carotenoid yield in different solvents and solvent mixtures (n = 6)

Solvent/Solvent mixture	Carotenoid yield (μg/g waste)
Acetone	40.6±1.55[a]
Methanol	29.0±3.39[b]
Ethyl methyl ketone	36.8±1.93[c]
Isopropyl alcohol (IPA)	40.8±3.01[a]
Ethyl acetate	36.9±2.93[c]
Ethanol	31.9±2.23[d]
Petroleum ether	12.1±1.76[e]
Hexane	13.1±0.91[e]
Acetone : Hexane (50 : 50)	38.5±1.00[ac]
IPA : Hexane (50 : 50)	43.9±0.73[f]

Values with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 3.1. Carotenoid yield indifferent solvents

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid yield	6871.42	9	763.49	228.83	50	4.57	166.82**

** $p \leq 0.01$

Table 3.2. Carotenoid yield in isopropyl alcohol : hexane (50:50) at different stage of extraction (n = 6)

Stage of Extraction	Carotenoid yield (% of total yield)
1st Extraction	77.8 ± 3.14^{a}
2nd Extraction	15.6 ± 3.35^{b}
3rd Extraction	4.5 ± 0.65^{c}
4th Extraction	1.2 ± 0.40^{d}
5th Extraction	0.87 ± 0.434^{d}

Values with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 3.2. Carotenoid yield at different stages of extraction

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid yield	25953.97	4	6488.49	109.56	25	4.38	1480.54***

*** $p \leq 0.001$

ANOVA Table 3.3. Carotenoid yield as function of hexane % in the solvent mixture, solvent level to waste and number of extraction

Factor	SS	df	MS	F value
1. Hexane % (L+Q)	49.20	2	24.60	722.60**
2. Solvent level to waste (L+Q)	39.96	2	19.98	586.95**
3. Number of extraction (L+Q)	519.92	2	259.96	7636.37***
Interaction				
1 x 2	1.91	1	1.91	56.23*
1 x 3	10.15	1	10.15	298.16**
2 x 3	12.55	1	12.55	368.62**
Lack of fit	14.99	3	4.99	146.74**
Pure error	0.068	2	0.034	

L – Linear, Q - Quadratic

* - $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Table 3.3. Regression coefficients for main factors and their interactions

	Factor/Interaction	Regression Coefficient
	Mean/Interaction (β_0)	-0.44366
1 (X1)	Hexane % in the solvent mixture (L) (β_i)	0.21985
	Hexane % in the solvent mixture (Q) (β_{ii})	-0.00135
2 (X2)	Solvent Level to waste (L) (β_i)	2.11016
	Solvent Level to waste (Q) (β_{ii})	-0.07938
3 (X3)	Number of extraction (L) (β_i)	13.65674
	Number of extraction (Q) (β_{ii})	-1.25022
	1L x 2L (β_{ij})	0.00659
	1L x 3L (β_{ij})	-0.02276
	2L x 3L (β_{ij})	-0.29520

Regression Equation

$$Y = - 0.44366 + (0.21985\ X1) + (2.11016\ X2) + (13.65674\ X3) + (- 0.00135\ X1^2) + (- 0.07938\ X2^2) + (- 1.25022\ X3^2) + (0.00659\ X1 * X2) + (- 0.02276\ X1 * X3) + (- 0.29520\ X2 * X3)$$

Table 3.4. Observed and predicted values of carotenoid yield

Run no	X1	X2	X3	Y - Observed	Y- Predicted
1	10	2	3	34.61	32.92
2	80	2	3	36.63	35.96
3	10	8	3	35.22	35.90
4	80	8	3	40.01	41.71
5	10	5	1	20.25	21.22
6	80	5	1	28.88	28.83
7	10	5	5	38.98	39.02
8	80	5	5	41.23	40.26
9	45	2	1	21.29	22.01
10	45	8	1	31.56	29.91
11	45	2	5	38.53	40.17
12	45	8	5	41.72	40.99
13	45	5	3	39.17	38.99
14	45	5	3	39.00	38.99
15	45	5	3	38.80	38.99

X1: Hexane % in the solvent mixture

X2: Solvent level to waste

X3: Number of extraction

Y: Carotenoid yield

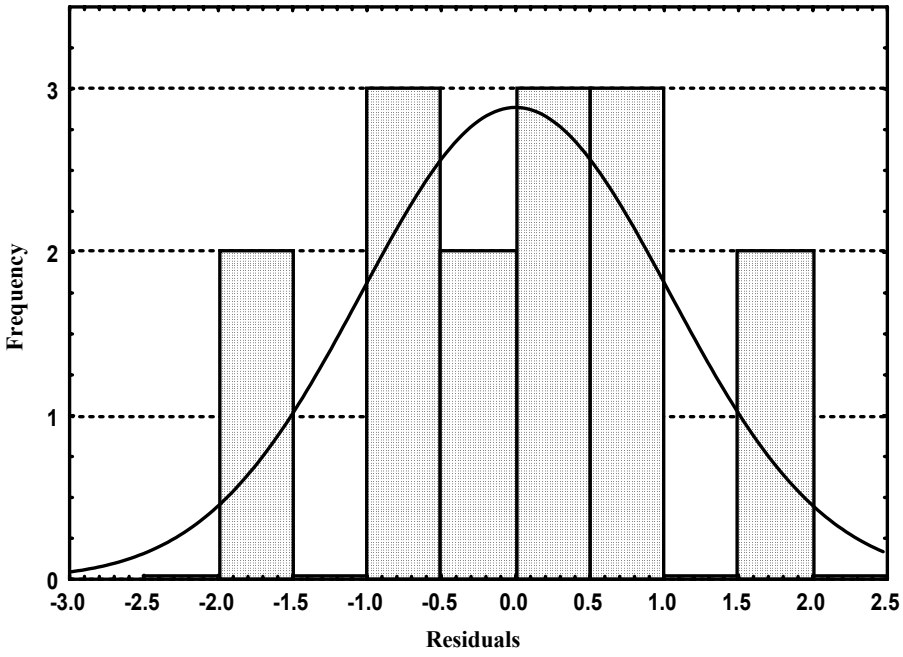


Figure 3.1. Frequency distribution of residuals between observed and predicted carotenoid yield

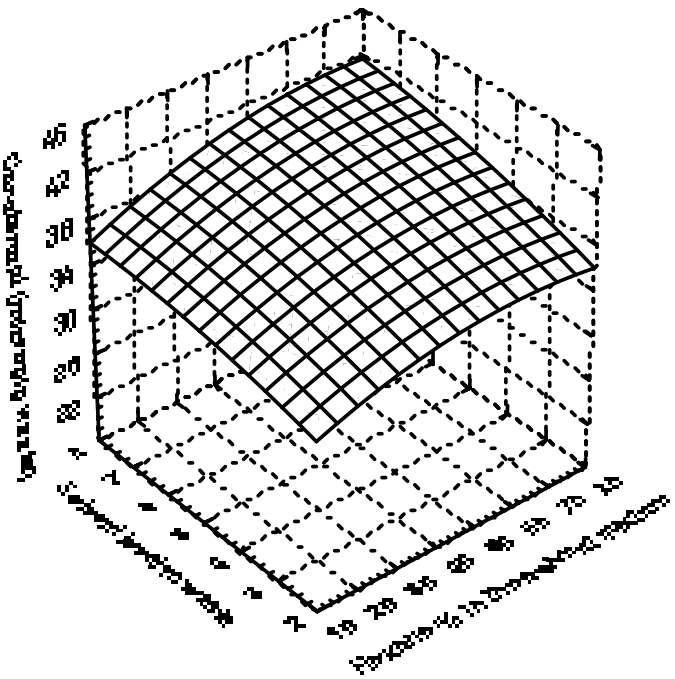


Figure 3.2 Response graph for carotenoid yield from shrimp waste as a function of hexane% in the solvent mixture and solvent level to waste (Number of extractions = 3)

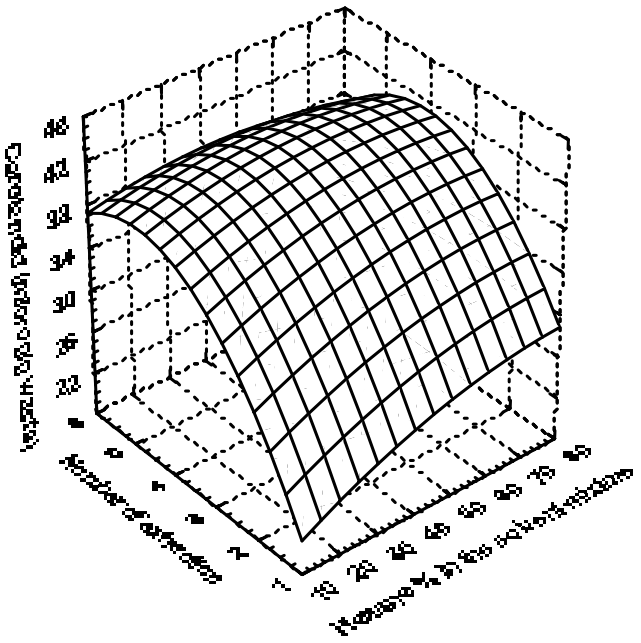


Figure 3.3 Response graph for carotenoid yield from shrimp waste as a function of hexane% in the solvent mixture and number of extraction (solvent level to waste = 5)

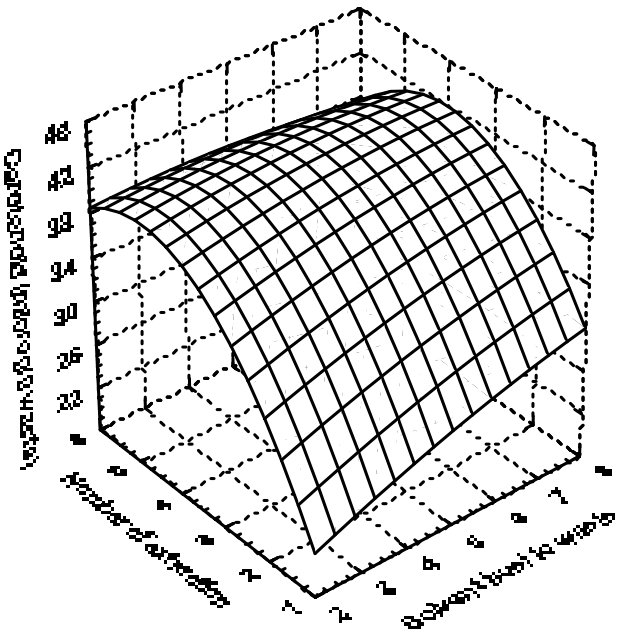


Figure 3.4 Response graph for carotenoid yield from shrimp waste as a function of solvent level to waste and number of extraction (hexane% in the solvent mixture = 45)

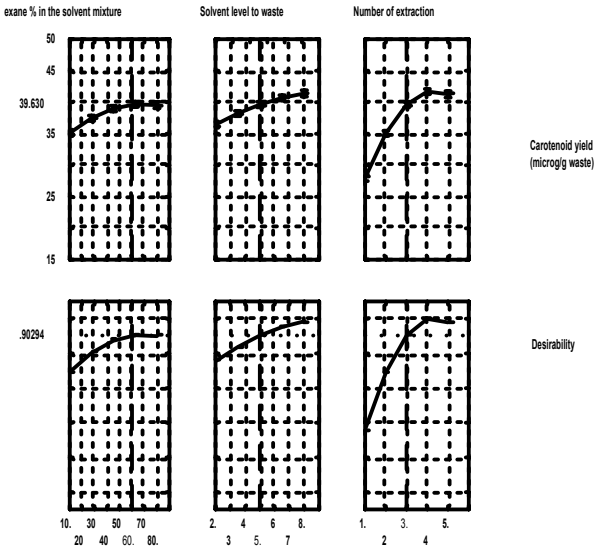


Figure 3.5 Profiles for predicted values of carotenoid yield and the desirability level for different factors for optimum carotenoid yield

CHAPTER 4

EXTRACTABILITY OF SHRIMP WASTE CAROTENOIDS IN VEGETABLE OIL

CHAPTER 4

EXTRACTABILITY OF SHRIMP WASTE CAROTENOIDS IN VEGETABLE OIL

Carotenoids are a group of oil soluble pigments. The oil solubilization characteristics of carotenoids have led to studies on recovery of these pigments in oils. The use of soy oil for extraction for carotenoids from crustacean waste has been reported (Anderson 1975, Chen and Meyers 1982, Meyers and Chen 1985). The present study was carried out to investigate the extractability of shrimp waste carotenoids in different vegetable oils, and to optimize the conditions for oil extraction. Further as carotenoids occur in crustaceans as complex with proteins, the effect of enzymatic breakdown of the complex with the aid of proteases has also been studied.

4.1. Experimental design and methodology

Shrimp waste from *Penaeus indicus* collected and processed as explained in section 3.1 was used for the study. Refined sunflower oil, groundnut oil, gingelly oil, mustard oil, soybean oil, coconut oil and rice bran oil were the vegetable oils used in the study. The proteases used for the study were the bacterial protease alcalase (activity > 0.6 Anson units/g, Merck, Germany), plant protease papain (0.6 Anson units, LOBA Chemie, India) and animal protease trypsin (0.2 Anson units, LOBA Chemie, India).

4.1.1. Absorption maxima (λ_{max}) and extinction coefficient ($E^{1\%}_{1cm}$) of standard astaxanthin in vegetable oils

Absorption maxima and extinction coefficient of standard astaxanthin in different vegetable oils was determined by the method of Chen and Meyers (1984). Twenty-five micrograms of standard astaxanthin (Sigma, USA) was dissolved in 5 ml of vegetable oil and its absorption spectra were determined between 400 to 600 nm using Shimadzu UV-

1619 spectrophotometer. The wavelength of maximum absorption (λ_{max}) and the absorption at the λ_{max} was noted. The extinction coefficient was calculated using the equation,

$$E^{1\%}_{1cm} = \frac{(A \times Y) \times 10^6}{100 \times X}$$

Where, A = absorbance at λmax, Y = dilution factor, X = weight of standard astaxanthin in μg.

4.1.2. Yield of carotenoids in different vegetable oils

Carotenoid in the homogenized waste was extracted using different vegetable oils using the modified method of Chen and Meyers (1982). Ten grams of homogenized waste was mixed with 20 ml of oil (oil/waste = 2) and heated in a water bath at 70°C for 2 h, filtered using a muslin cloth and the filtrate centrifuged at 5000 rpm for 10 min. The pigmented oil layer from the supernatant was separated using a separating funnel. The volume of the pigmented oil recovered is noted and the carotenoid content in the suitably diluted pigmented oil was measured spectrophotometrically at wavelength of λ_{max} of astaxanthin in particular oil. The carotenoid yield is calculated using the equation,

$$\text{Carotenoid (}\mu\text{g/g waste)} = \frac{A \times V \times D \times 10^6}{100 \times W \times E}$$

Where, A= absorbance at λ_{max}, V = volume of pigmented oil recovered, D = dilution factor, W = weight of waste in grams and E = extinction coefficient.

4.1.3. Concentration of carotenoid in oil

Since sunflower oil gave higher extraction yield, further experiments were carried out using sunflower oil. The carotenoid in the shrimp waste was extracted using sunflower oil as in section 4.1.2. The oil recovered was repeatedly used 3 times for

extraction of carotenoids from fresh waste keeping oil to waste level at 2 for each extraction. The carotenoid content in the oil at every extraction is calculated as mg carotenoid per 100 g oil using the equation (Chen and Meyers 1982),

$$\text{mg carotenoid/100 g pigmented oil} = \frac{A \times D \times 10^5}{100 \times S \times E}$$

where, A = absorbance at 487 nm (λ_{max}), D = dilution factor, S = specific gravity of sunflower oil (0.91), E = extinction coefficient of astaxanthin in sunflower oil (2290).

4.1.4. Optimization of conditions for extraction of carotenoids in oil

The conditions for optimized extraction yield of carotenoids from shrimp waste using sunflower oil was determined with respect to temperature of heating the homogenized waste with oil (X1), time of heating (X2) and oil level to waste (X3), using the Box-Benhkan design (Box and Benhkan 1960), with the aid of the software STATISTICA (Statsoft Inc 1999). The design used determines the influence of three main factors (X1, X2, X3) and their interactions on the carotenoid yield (Y). The factors, their levels and codes for the levels were as follows,

Factors	**Codes**	**Level**		
		-1	**0**	**+1**
Temperature of heating (°C)	X1	40	70	100
Time of heating (min)	X2	60	120	180
Oil level to waste (oil/waste, v/w)	X3	0.5	2	3.5

The combination of factors for 15 runs was as follows.

Run no.	X1	X2	X3
1	-1 (40)	-1 (60)	0 (2)
2	+1 (100)	-1 (60)	0 (2)
3	-1 (40)	+1 (180)	0 (2)
4	+1 (100)	+1 (180)	0 (2)
5	-1 (40)	0 (120)	-1 (0.5)
6	+1 (100)	0 (120)	-1 (0.5)
7	-1 (40)	0 (120)	+1 (3.5)
8	+1 (100)	0 (120)	+1 (3.5)
9	0 (70)	-1 (60)	-1 (0.5)
10	0 (70)	+1 (180)	-1 (0.5)
11	0 (70)	-1 (60)	+1 (3.5)
12	0 (70)	+1 (180)	+1 (3.5)
13	0 (70)	0 (120)	0 (2)
14	0 (70)	0 (120)	0 (2)
15	0 (70)	0 (120)	0 (2)

The extraction of carotenoids and determination of the yield was carried out as explained in section 4.1.2.

4.1.5. Carotenoid yield from enzyme hydrolyzed shrimp waste

Ten gram of homogenized shrimp waste was mixed with enzyme (0.25% and 0.5% of each enzyme, w/w of waste) dissolved in 10 ml of phosphate buffer (0.2 M, pH 7.0) and incubated at 37°C for 2 h. After incubation 20 ml of refined sunflower oil was added to the hydrolyzed waste and heated in a water bath at 70°C for 150 min, the pigmented oil was recovered, and the yield of carotenoids determined as explained in section 4.1.2. Recovery of pigment from waste without addition of enzyme under same conditions served as control.

The enzyme used for hydrolysis which gave the higher carotenoid yield (Y) by subsequent oil extraction was chosen for further experiments on optimization. The three

process variables namely enzyme concentration to waste (X1), incubation time (X2) and time of heating hydrolyzed waste in oil (X3) were optimized. The homogenized shrimp waste was mixed with three different levels of enzyme (dissolved in buffer) and incubated for 3 different periods. To the hydrolyzed waste refined sunflower oil was added at a level of 2 (oil/waste) and heated in a water bath at 70°C for three different periods. The 15 combinations of the independent variables (X1, X2, X3) arrived as explained earlier (Section 4.1.4) was as follows,

Factors	**Codes**	**Level**		
		-1	**0**	**+1**
Enzyme concentration (% of wet waste)	X1	0.25	0.75	1.25
Incubation time (min)	X2	30	150	270
Heating time in oil (min)	X3	30	90	150

The combination of factors for 15 runs was as follows.

Run no.	**X1**	**X2**	**X3**
1	-1 (0.25)	-1 (30)	0 (90)
2	+1 (1.25)	-1 (30)	0 (90)
3	-1 (0.25)	+1 (270)	0 (90)
4	+1 (1.25)	+1 (270)	0 (90)
5	-1 (0.25)	0 (150)	-1 (30)
6	+1 (1.25)	0 (150)	-1 (30)
7	-1 (0.25)	0 (150)	+1 (150)
8	+1 (1.25)	0 (150)	+1 (150)
9	0 (0.75)	-1 (30)	-1 (30)
10	0 (0.75)	+1 (270)	-1 (30)
11	0 (0.75)	-1 (30)	+1 (150)
12	0 (0.75)	+1 (270)	+1 (150)
13	0 (0.75)	0 (150)	0 (90)
14	0 (0.75)	0 (150)	0 (90)
15	0 (0.75)	0 (150)	0 (90)

The extraction of carotenoids and determination of the yield was carried out as explained earlier (section 4.1.2).

4.1.6. Statistical analysis

Analysis of variance, Duncan's multiple range tests, regression analysis of data, prediction of carotenoid yield, plotting of histogram of residuals, 3D response graphs and profiles for predicted yield and desirability value were carried out using the software STATISTICA (Statsoft Inc 1999). The regression model was assumed as indicated in section 3.1.4.

4.2. Results and discussion

4.2.1. Absorbance maxima and extinction coefficient of standard astaxanthin in different vegetable oils

The absorbance maxima (λ_{max}) of standard astaxanthin ranged from 486 to 504 nm and the extinction coefficient from 2145 to 2333 depending on the vegetable oil used (Table 4.1). Chen and Meyers (1984) reported that astaxanthin has absorbance maxima of 485 nm and an extinction coefficient of 2155 in refined soy oil, and indicated that these values depend on the purity and extent of refining of the oil. In the present observation, astaxanthin had a λ_{max} of 487 nm and extinction coefficient of 2145 in soy oil. The deviation in the present study from the reported value may be due to variation in degree of purity of oil used. The λ_{max} and extinction coefficients determined were used for further experiments on determination of carotenoid yield in different oil and optimization studies.

4.2.2. Yield of carotenoids in different oils

The pigmented oil recovered oil was orange red in color (Photoplate 4.1). Highest carotenoid yield of 26.3 μg/g waste was obtained by extraction with sunflower oil and lowest (16.1 μg/g waste) in mustard oil (Table 4.2), with a significant difference

($p \leq 0.001$) in extraction yield between oils (ANOVA Table 4.2). However the extraction yield of carotenoid in soy oil, coconut oil and rice bran oil was similar ($p \geq 0.05$) to that in sunflower oil.

The experiments on concentration of carotenoids in oil indicated that the carotenoid content (mg/100 g oil) in the oil increased significantly ($p \leq 0.001$) from an initial level of 1.6 after 1st extraction to 4.2 after 4th extraction (Table 4.3 and ANOVA Table 4.3). However the increase in carotenoid content was significant ($p \leq 0.05$) upto 3rd extraction and no significant difference ($p \geq 0.05$) was observed in carotenoid content of pigmented oil between 3rd and 4th extraction.

The use of vegetable oil for recovery of carotenoids from the waste from crustaceans of temperate waters has been reported. In the patented process Anderson (1975) used soybean oil to recover carotenoid from shrimp waste. Soybean oil has also been used for extraction of carotenoid from red crab (Spinelli and Mahnken 1978). Meyers and Chen (1985) used soybean oil to recover pigments from acidified crawfish waste. Evaluation of soybean, cotton seed, herring, menhaden and salmon oil for recovery of carotenoids from crawfish waste indicated that soybean oil gives higher carotenoid yield (Chen and Meyers 1984). In the present study it is observed that refined sunflower oil gives higher carotenoid yield than the other vegetable oils used.

Chen and Meyers (1982) reported that the carotenoid content in the pigmented soy oil could be increased 3 times by repeated use of pigmented oil at oil to waste level of 1 for extraction of carotenoids from fresh crawfish waste. In the present study it is observed that the pigment level in the oil can be increased by 2.6 times by reusing the pigmented oil 3 times for extraction of carotenoids from fresh shrimp waste. The lower rate of concentration of carotenoids in the pigmented oil observed in the present study may be

due to the higher level of oil to waste (2) employed in the study. Chen and Meyers (1982) have also made similar observations at higher level of oil to waste

The carotenoid yield by oil extraction was found to be lower than that obtained by solvent extraction (43.9 μg/g in 50 : 50 isopropylalcohol and hexane, Table 3.1). However the advantage of oil extraction process is that the pigmented oil finds use as carotenoid source in aquaculture feeds, as oil serves as pigment carrier as well a source of lipid energy (Spinelli and Mahnken 1978). The use of oils as an ingredient in feed preparation is mainly as source of energy. Thus concentration of carotenoids in the oil would be advantageous, as required carotenoid concentration in the feed and can be achieved by minimum addition of pigmented oil without affecting the energy balance.

4.2.3. Optimization of conditions for oil extraction of carotenoids

As earlier experiment has shown that sunflower oil gives higher carotenoid yield, optimization experiments with respect to influence of temperature of heating waste with oil (X1), time of heating (X2) and oil level to waste (X3) on carotenoid yield (Y) was carried out using sunflower oil. All the three processing variables namely temperature ($p \leq 0.001$), time of heating ($p \leq 0.01$) and oil level to waste ($p \leq 0.01$) and the interaction between X1 and X2 ($p \leq 0.01$), X1 and X3 ($p \leq 0.05$), and X2 and X3 ($p \leq 0.05$) had significant effect on carotenoid yield (ANOVA Table 4.4). The influence of some unaccountable factors other than main factor and their interactions is indicated by the significant ($p \leq 0.01$) lack of fit.

The regression equation for the carotenoid yield (Y) as a function of three processing variables (X1, X2, X3) and their interactions, using the constant, linear and quadratic regression coefficients of main factors and linear-by-linear regression coefficients of interactions (Table 4.4) was derived to be,

$$Y = -27.0392 + (0.8354\ X1) + (0.2444\ X2) + (7.6249\ X3) + (-0.0051\ X1^2) + (-0.0006\ X2^2) + (-1.0935\ X3^2) + (-0.0007\ X1 * X2) + (-0.0165\ X1 * X3) + (-0.0094\ X2 * X3)$$

The predicted carotenoid yield arrived at using the above regression equation are close (correlation coefficient r = + 0.9616) to the observed carotenoid yield (Table 4.5) and indicate the usefulness of the equation for prediction of carotenoid yield at different combinations of the three processing variables, which are affecting the oil extraction yield of carotenoids. The frequency distribution of residuals (Figure 4.1) indicate that the difference between observed and predicted yield falls between –1.5 and +1.5, with 7 out of 15 values between – 0.5 and + 0.5.

The response surface graph (Figure 4.2) for carotenoid yield in sunflower oil as a function of temperature and time of heating waste with oil at oil to waste level of 2 indicates that carotenoid yield increases as the temperature increases upto 70°C and then the yield decreases, while the rate of increase in carotenoid yield is marginal above a heating time of 150 min. Similarly carotenoid yield increases with increase in oil to waste level of 2 and then decreases slightly (Figure 4.3 and 4.4).

The desirability profile for optimum carotenoid yield indicates that the maximum desirability level of 1.0 (in a scale of 0 to 1) can be achieved with a temperature of 70°C, heating time of 150 min and oil to waste level of 2. The carotenoid yield and desirability level reduced considerably at a temperature above 70°C, while there was a marginal decrease in carotenoid yield above 150 min of heating. The carotenoid yield remained constant between oil to waste level of 2 and 2.5, and reduced marginally with further increase in oil level.

Chen and Meyers (1982) obtained maximum pigment yield from crawfish waste using a soy oil process involving 1 : 1 ratio of oil to waste, heating the waste with oil at a temperature of 80 – 90°C for 30 min. However in the present study it is observed that increase in the extraction temperature above 70°C results in decrease in carotenoid yield. As carotenoids are degraded at higher temperature, it is advisable to use lower temperature for longer time for optimum extraction yield of carotenoids from shrimp waste.

4.2.4. Recovery of carotenoids from enzyme hydrolyzed shrimp waste

The effect of hydrolysis of shrimp waste prior to oil extraction varied significantly ($p \leq 0.001$) with respect to type and level of enzyme used (ANOVA Table 4.6). Hydrolysis of waste for 2 h at 37°C with bacterial protease alcalase at 0.5 % level (w/w of waste) gave the maximum carotenoid yield (28.6 μg/g waste) by oil extraction (Table 4.6). Hydrolysis of waste using papain and trypsin also gave higher oil extraction yield (24.4 – 25.3 μg/g waste) than that from the unhydrolysed waste (23.7 μg/g waste). With respect to effectiveness, the order of enzyme preference for hydrolysis was alcalase > trypsin > papain.

Since hydrolysis of waste with alcalase gave higher oil extraction yield of carotenoids, the optimization studies were carried out using alcalase. The study included optimization of enzyme concentration (X1) and incubation time (X2) for hydrolysis and time of heating (X3) hydrolyzed waste with sunflower oil (1: 2 = waste: oil) at 70°C. It is observed that enzyme concentration ($p \leq 0.01$) and incubation time ($p \leq 0.05$) had significant effect on carotenoid yield in oil (ANOVA Table 4.7). Time of heating in oil had no significant ($p \geq 0.05$) effect on yield, as also the interaction between 3 variables. A

non-significant ($p \geq 0.05$) lack of fit indicated that the carotenoid yield is purely influenced by the enzyme concentration and incubation time.

The regression equation for oil extraction yield (Y) of carotenoids from hydrolyzed waste as a function of linear and quadratic effect of main factors and linear-by-linear effect of their interaction, containing the regression coefficients (Table 4.7) was found out to be,

$$Y = 19.49951 + (15.31125\ X1) + (0.01610\ X2) + (0.02142\ X3) + (-6.67833\ X1^2) + (-0.00004\ X2^2) + (-0.00004\ X3^2) + (-0.00204\ X1 * X2) + (-0.01533\ X1 * X3) + (-0.00003\ X2 * X3)$$

The closeness (correlation coefficient r = +0.9888) of observed and predicted carotenoid yield (Table 4.8) and the frequency distribution of residuals (Figure 4.6) indicate that the regression equation fits well to the model. It is observed that all the 15 residual values falls in a very narrow range of – 0.6 to + 0.6 and follows a normal distribution.

The response surface graphs (Figure 4.7 and 4.8) indicate that the carotenoid yield is highly influenced by the enzyme concentration. The rate of increase in yields increases considerably with an increase in enzyme concentration upto 0.75% of waste and reaches maximum at an enzyme concentration of 1%. The incubation time and heating time in oil has a marginal effect with a gradual increase in carotenoid yield upto 210 min of incubation time and with an increase in heating time (Figure 4.9).

The desired level of process variables for optimum hydrolysis of waste and subsequent oil extraction of carotenoids with a desirability level of 0.95452 was found to be 0.75% (of wet waste) of enzyme concentration, 150 min of incubation time and 90 min

of heating of hydrolyzed waste with oil (Figure 4.10). Above these levels of process variables, the increase in carotenoid yield is marginal.

As carotenoids occur as carotenoprotein complexes, it is necessary to cleave the bond between carotenoid and protein to liberate the carotenoids (Nelis et al 1989). In oil extraction process for recovery of carotenoids the bond is cleaved by thermal treatment. However, still some carotenoids may be firmly bound in the complex. Enzymatic hydrolysis of crawfish waste with proteolytic enzyme for cleavage of carotenoprotein complex and enhanced recovery of carotenoids was attempted by Chen and Meyers (1982). They observed that hydrolysis of crawfish waste with 0.6% (of waste) of Milizyme 8X, a bacterial protease, at 45°C for 1 h resulted in considerable increase in the amount of carotenoids extracted in soy oil. Chen and Meyers (1983) reported that acid ensilaging of crawfish waste enhanced the oil extractability of pigments due to stimulation effect of acid on the *insitu* protease activity. Guillou et al (1995) also observed the enhanced recovery of pigments from ensiled shrimp waste. In the present study also enhanced recovery of carotenoids in oil was observed when the shrimp waste was hydrolyzed with a bacterial protease, alcalase.

4.3. Conclusion

Extraction of carotenoids from shrimp waste using refined sunflower oil gave higher carotenoid yield. The carotenoid content in the pigmented oil can be increased by reusing the pigmented oil for extraction of carotenoids from fresh waste. The optimized conditions for the oil extraction of carotenoids from shrimp waste were found to be adding oil to the waste in a ratio of 2 : 1 and heating the mixture at 70°C for 150 min. The pigmented oil can be recovered by centrifuging the treated waste and phase separation. Enzyme hydrolysis of shrimp waste using proteases prior to extraction can enhance the

extractability of carotenoids in oil. Bacterial protease, alcalase was found to be more suitable for hydrolysis than plant protease papain and the animal protease trypsin, with respect to carotenoid yield in oil. Optimum yield can be obtained by hydrolysis of shrimp waste with 0.75% of enzyme at 37°C for 150 min, adding sunflower oil to hydrolyzed waste in a ratio of 2 : 1 and heating at 70°C for 90 min. The pigmented oil recovered would find use as carotenoid source in aquaculture feeds.

Photoplate 4.1

Pigmented sunflower oil containing extracted carotenoids

Table 4.1. Absorption maxima and extinction coefficient of standard astaxanthin in vegetable oils

Oil	Absorption Maxima (λ_{max})	Extinction coefficient
Sunflower oil	487	2290
Groundnut oil	487	2440
Gigelly oil	486	2266
Mustard oil	504	2255
Soya oil	487	2145
Coconut oil	486	2311
Rice bran oil	487	2333

Table 4.2. Carotenoid yield in different vegetable oils (n = 6)

Oil	Carotenoid yield (μg/g waste)
Sunflower oil	26.3 ± 2.31^{a}
Groundnut oil	23.1 ± 1.56^{b}
Gigelly oil	23.9 ± 1.32^{b}
Mustard oil	16.1 ± 1.85^{c}
Soya oil	24.8 ± 1.51^{ab}
Coconut oil	24.7 ± 2.42^{ab}
Rice bran oil	24.3 ± 1.59^{ab}

Values with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 4.2. Carotenoid yield in different vegetable oils

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid yield	400.97	6	66.83	118.27	35	3.38	19.78***

*** $p \leq 0.001$

Table 4.3. Carotenoid content in the concentrated pigmented oil (n = 6)

Number of extraction	Carotenoid content (mg/100g oil)
1	1.6 ± 0.05^{a}
2	3.0 ± 0.20^{b}
3	3.9 ± 0.28^{c}
4	4.2 ± 0.19^{c}

Values with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 4.3. Carotenoid content in concentrated pigmented oil

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid yield	24.52	3	8.17	0.761	20	0.038	214.56***

*** $p \leq 0.001$

ANOVA Table 4.4. Carotenoid yields as a function of temperature of heating, time of heating and oil level to waste

Factor	SS	df	MS	F value
1. Temperature (L+Q)	77.38	2	38.69	1328.54***
2. Time (L+Q)	38.79	2	19.40	666.00**
3. Oil level to waste (L+Q)	39.30	2	19.65	674.79**
Interaction				
1 x 2	6.28	1	6.28	215.89**
1 x 3	2.20	1	2.20	75.61*
2 x 3	2.85	1	2.85	98.00*
Lack of fit	14.47	3	4.16	142.76**
Pure error	0.058	2	0.029	

L – Linear, Q - Quadratic

* - $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Table 4.4. Regression coefficients for main factors and their interactions

	Factor/Interaction	Regression Coefficient
	Mean/Interaction (β_0)	-27.0392
1 (X1)	Temperature (L) (β_i)	0.8354
	Temperature (Q) (β_{ii})	-0.0051
2 (X2)	Time (L) (β_i)	0.2444
	Time (Q) (β_{ii})	-0.0006
3 (X3)	Oil level to waste (L) (β_i)	7.6249
	Oil level to waste (Q) (β_{ii})	-1.0935
	1L x 2L (β_{ij})	-0.0007
	1L x 3L (β_{ij})	-0.0165
	2L x 3L (β_{ij})	-0.0094

Regression equation

$$Y = -27.0392 + (0.8354\ X1) + (0.2444\ X2) + (7.6249\ X3) + (-0.0051\ X1^2) + (-0.0006\ X2^2) + (-1.0935\ X3^2) + (-0.0007\ X1 * X2) + (-0.0165\ X1 * X3) + (-0.0094\ X2 * X3)$$

Table 4.5. Observed and predicted values of carotenoid yield in sunflower oil

Run no	X1	X2	X3	Y - Observed	Y- Predicted
1	40	60	2	16.00	17.43
2	100	60	2	19.52	20.55
3	40	180	2	24.13	23.11
4	100	180	2	22.63	21.20
5	40	120	0.5	17.98	17.87
6	100	120	0.5	19.66	19.96
7	40	120	3.5	22.57	22.27
8	100	120	3.5	21.28	21.38
9	70	60	0.5	20.11	18.79
10	70	180	0.5	22.51	23.64
11	70	60	3.5	24.52	23.39
12	70	180	3.5	23.53	24.86
13	70	120	2	27.37	27.39
14	70	120	2	27.56	27.39
15	70	120	2	27.22	27.39

X1: Temperature of heating

X2: Time of heating

X3: Oil level to waste

Y: Carotenoid yield

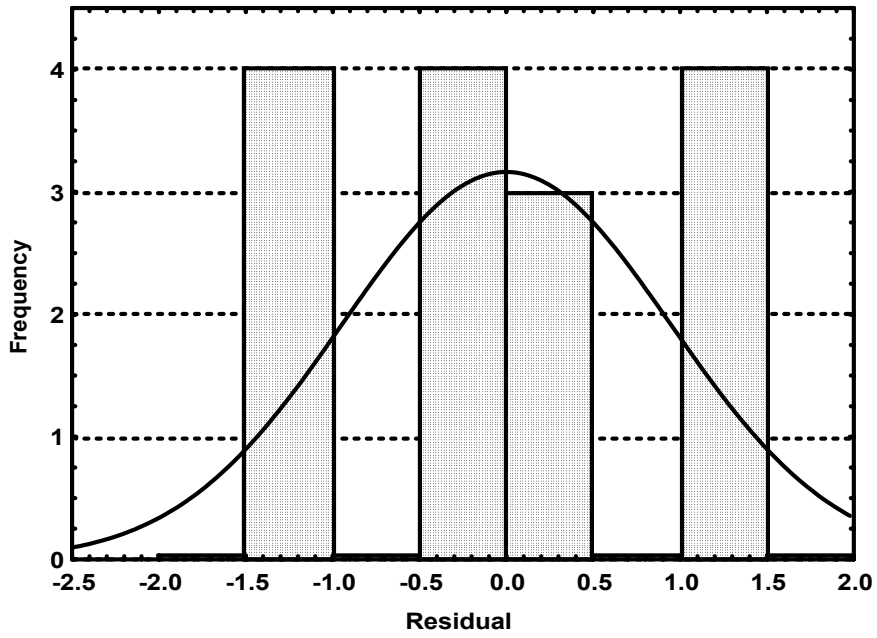


Figure 4.1. Frequency distribution of residuals between observed and predicted carotenoid yield in sunflower oil

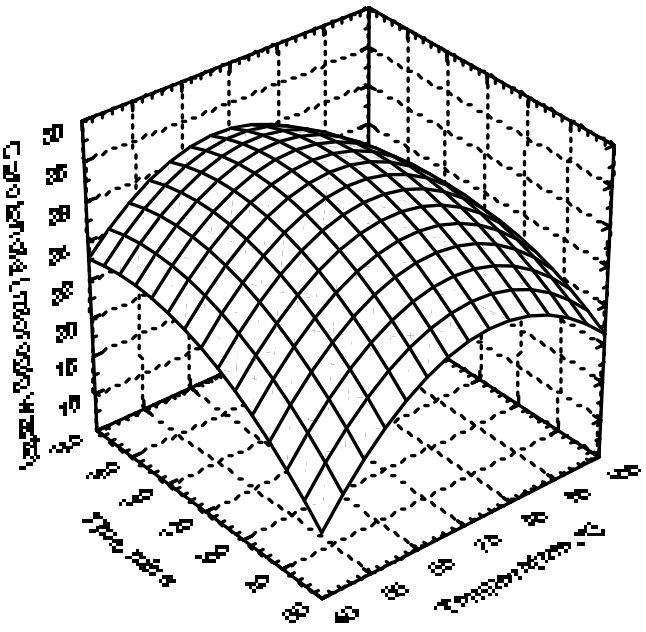


Figure 4.2 Response surface graph for carotenoid yield from shrimp waste in oil as a function of temperature and time of heating waste with oil (oil/waste = 2)

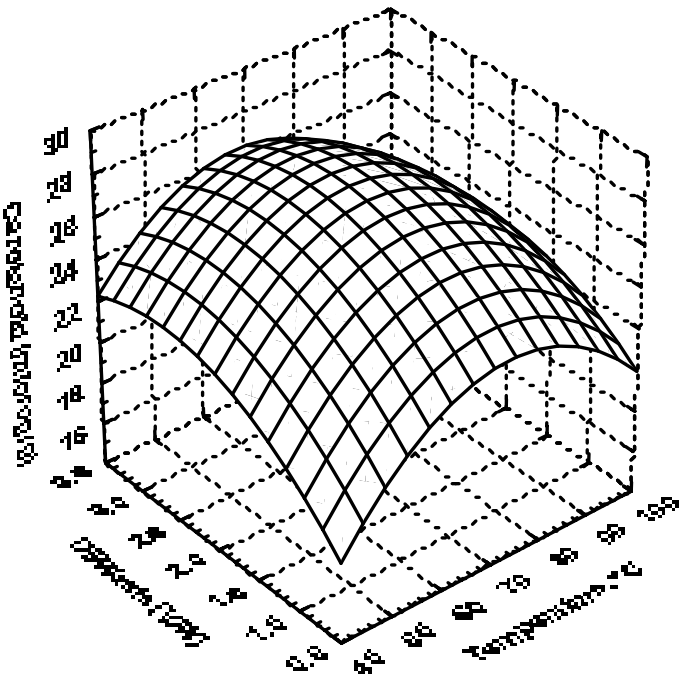


Figure 4.3. Response surface graph for carotenoid yield from shrimp waste in oil as a function of temperature of heating and oil to waste ratio (time of heating waste with oil = 120 min)

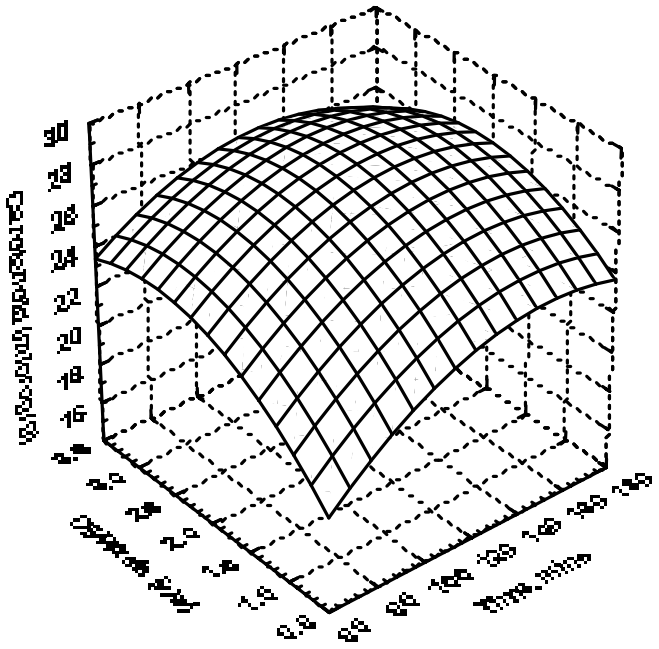


Figure 4.4. Response surface graph for carotenoid yield from shrimp waste in oil as a function of time of heating and oil to waste ratio (temperature of heating = 70°C)

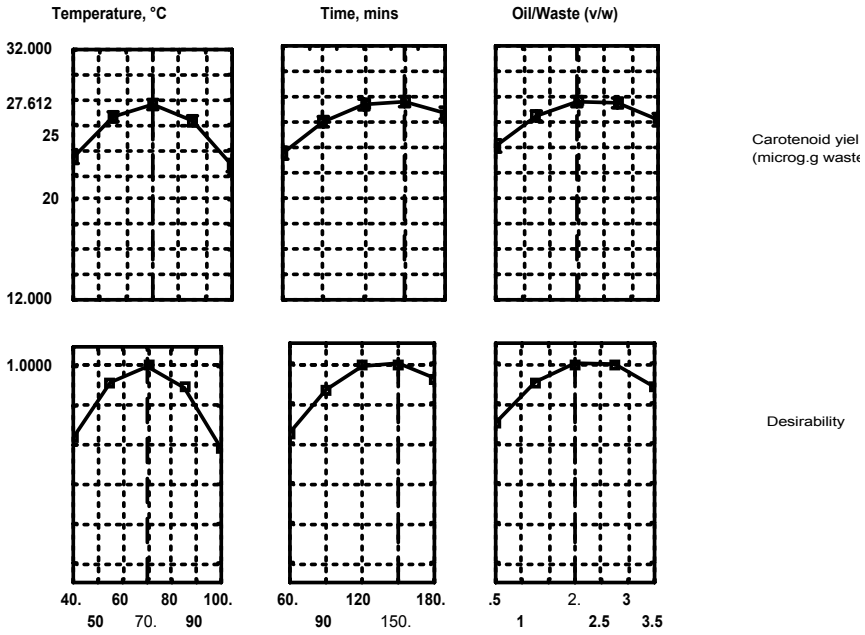


Figure 4.5 Profiles for predicted carotenoid yield and the desirability level for different factors for optimum carotenoid extraction yield in sunflower oil

Table 4.6. Carotenoid yield from shrimp waste in sunflower oil after hydrolysis with different proteases (n = 6)

Enzyme	Concentration (% of wet waste)	Carotenoid yield
Control	-	23.7±0.25[a]
Alcalase	0.25	27.3±0.20[b]
Alcalase	0.50	28.6±0.32[c]
Papain	0.25	24.4±0.30[d]
Papain	0.50	24.8±0.33[e]
Trypsin	0.25	24.5±0.40[de]
Trypsin	0.50	25.3±0.25[f]

Values with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 4.6 Carotenoid yield from shrimp waste in sunflower oil after hydrolysis with different proteases

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid yield	113.33	6	18.89	3.09	35	0.088	214.20***

*** $p \leq 0.001$

ANOVA Table 4.7. Carotenoid yield as a function of enzyme concentration, incubation time and time of heating in oil

Factor	SS	df	MS	F value
1. Enzyme concentration (L+Q)	36.32	2	18.16	286.74**
2. Incubation time (L+Q)	4.43	2	2.22	34.99*
3. Time of heating in oil (L+Q)	1.41	2	0.707	11.16^{NS}
Interaction				
1 x 2	0.060	1	0.060	0.948^{NS}
1 x 3	0.846	1	0.846	13.36^{NS}
2 x 3	0.185	1	0.185	2.92^{NS}
Lack of fit	0.843	3	0.181	4.44^{NS}
Pure error	0.127	2	0.063	

L – Linear, Q - Quadratic

^{NS} – $p \geq 0.05$, * - $p \leq 0.05$, ** $p \leq 0.01$

Table 4.7. Regression coefficients for main factors and their interactions

	Factor/Interaction	Regression Coefficient
	Mean/Interaction (β_0)	19.49951
1 (X1)	Enzyme concentration (L) (β_i)	15.31125
	Enzyme concentration (Q) (β_{ii})	-6.67833
2 (X2)	Incubation time (L) (β_i)	0.01610
	Incubation time (Q) (β_{ii})	-0.00004
3 (X3)	Heating time (L) (β_i)	0.02142
	Heating time (Q) (β_{ii})	-0.00004
	1L x 2L (β_{ij})	-0.00204
	1L x 3L (β_{ij})	-0.01533
	2L x 3L (β_{ij})	-0.00003

Regression equation

$$Y = 19.49951 + (15.31125\ X1) + (0.01610\ X2) + (0.02142\ X3) + (-6.67833\ X1^2) + (-0.00004\ X2^2) + (-0.00004\ X3^2) + (-0.00204\ X1 * X2) + (-0.01533\ X1 * X3) + (-0.00003\ X2 * X3)$$

Table 4.8. Observed and predicted values of carotenoid yield in sunflower oil from enzyme-hydrolyzed waste

Run no	X1	X2	X3	Y - Observed	Y- Predicted
1	0.25	30	90	24.49	24.58
2	1.25	30	90	28.02	28.44
3	0.25	270	90	26.51	26.10
4	1.25	270	90	29.55	29.46
5	0.25	150	30	24.61	24.89
6	1.25	150	30	29.46	29.42
7	0.25	150	150	26.59	26.63
8	1.25	150	150	29.60	29.32
9	0.75	30	30	28.21	27.83
10	0.75	270	30	28.54	28.67
11	0.75	30	150	28.35	28.22
12	0.75	270	150	29.54	29.92
13	0.75	150	90	29.42	29.39
14	0.75	150	90	29.12	29.39
15	0.75	150	90	29.62	29.39

X1: Enzyme concentration

X2: Incubation time

X3: Heating time

Y: Carotenoid yield

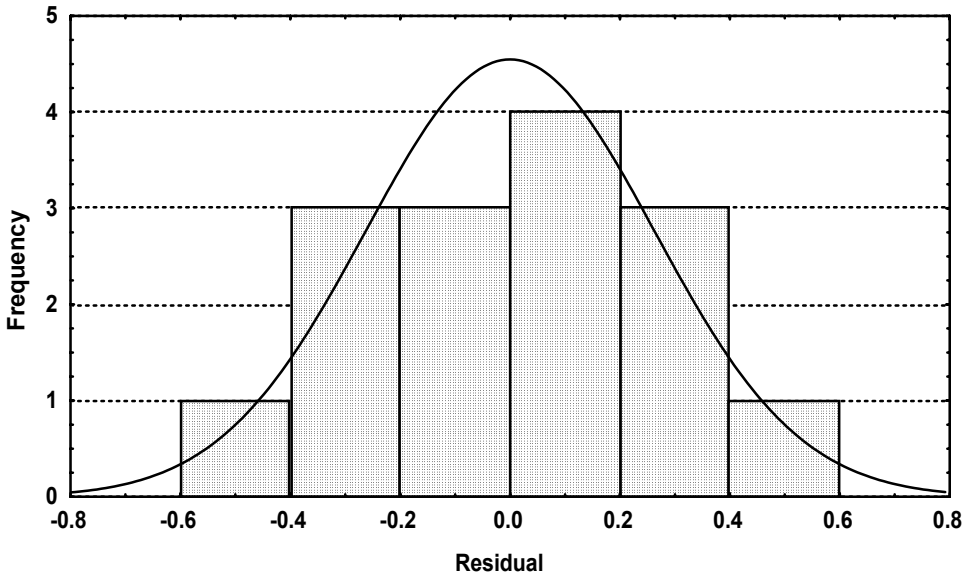


Figure 4.6. Frequency distribution of residuals between observed and predicted yield of carotenoids in sunflower oil from enzyme hydrolyzed shrimp waste

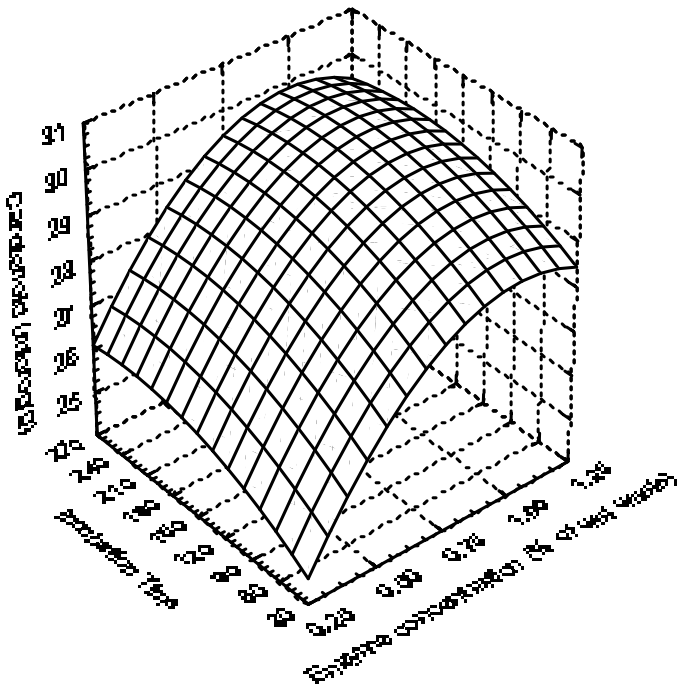


Figure 4.7. Response surface graph for carotenoid yield in sunflower oil from hydrolyzed shrimp waste as a function of enzyme concentration and incubation time (heating time in oil = 90 min)

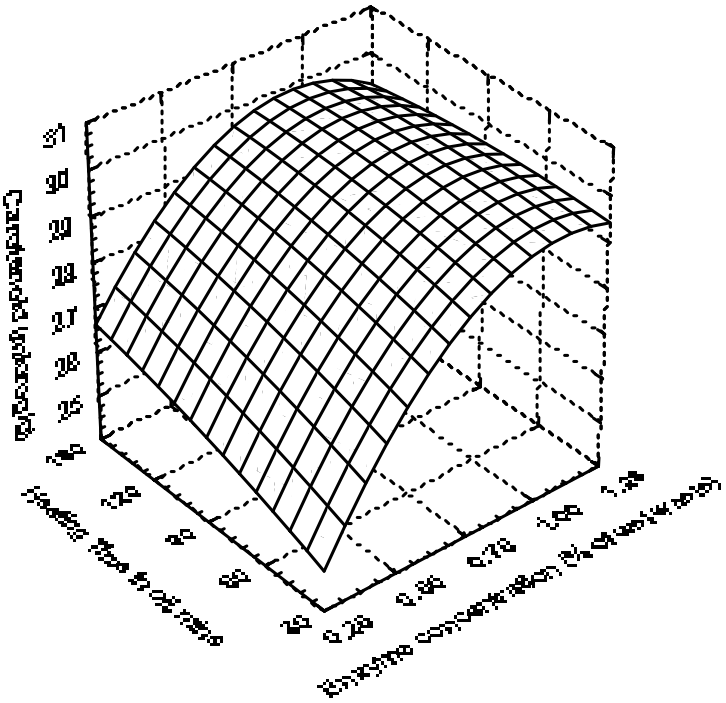


Figure 4.8. Response surface graph for carotenoid yield in sunflower oil from hydrolyzed shrimp waste as a function of enzyme concentration and heating time in oil (Incubation time = 150 min)

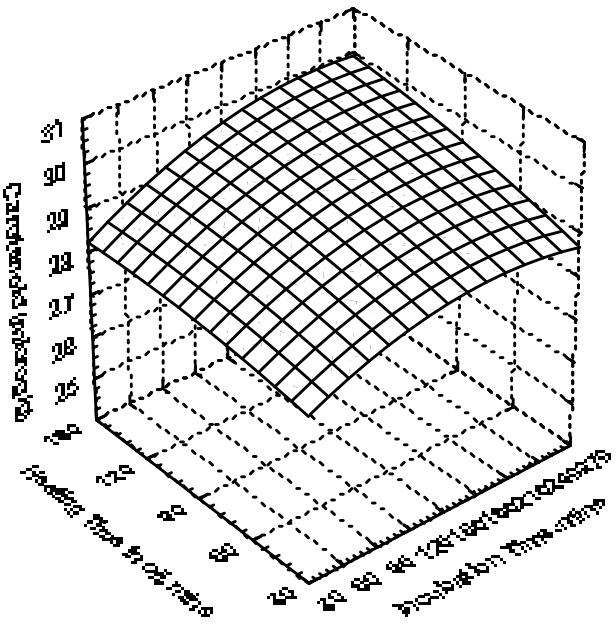


Figure 4.9. Response surface graph for carotenoid yield in sunflower oil from hydrolyzed shrimp waste as a function of incubation time and heating time in oil (enzyme concentration = 0.75% of wet waste)

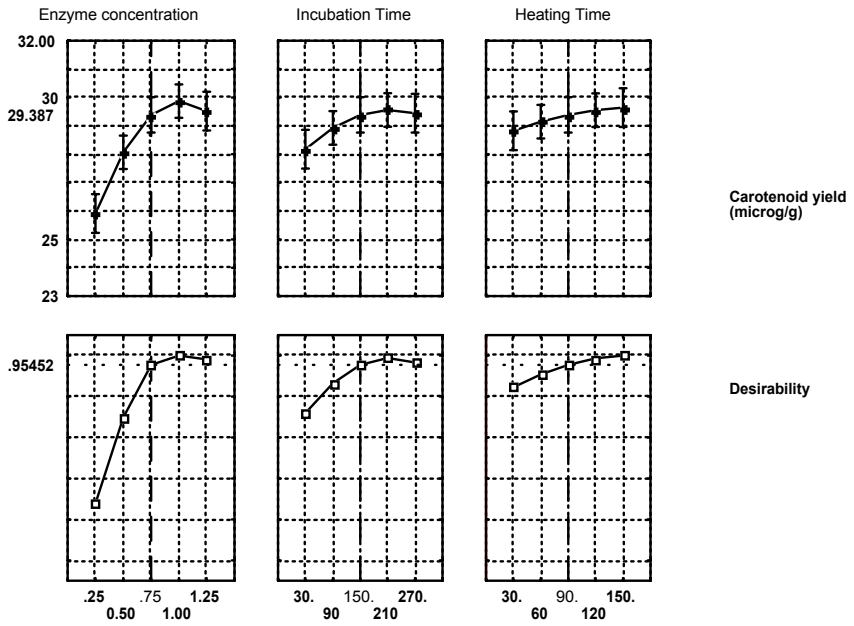


Figure 4.10. Profiles for predicted carotenoid yield and the desirability level for different factors for optimum carotenoid extraction in sunflower oil from enzyme hydrolyzed shrimp waste

CHAPTER 5

STABILITY OF CAROTENOIDS RECOVERED FROM SHRIMP WASTE

CHAPTER 5

STABILITY OF RECOVERED CAROTENOIDS

Carotenoids are highly unstable compounds and need to be protected from excessive heat, exposure to light and oxygen in order to prevent their breakdown. The processing and storage affect the carotenoids. For prevention of breakdown of pigments they are normally protected from exposure to light and oxygen by suitable storage conditions. Antioxidants have been used to prevent the oxidative breakdown of carotenoids in food materials. Chen and Meyers (1982) have reported the stabilization of carotenoids in soy oil by addition of ethoxyquin to crawfish waste before oil extraction of pigments. This study was carried out to investigate the effect of antioxidants, pigment carriers and different storage conditions on the stability of carotenoids recovered from shrimp waste.

5.1. Methodology

5.1.1. Solvent extracted carotenoids

Carotenoids in the waste from the shrimp *Penaeus indicus* was extracted using a mixture of Isopropyl alcohol and hexane as explained in section 3.1. Hexane extract containing carotenoids was concentrated to 50 ml by evaporating the solvent using flash evaporator. Fat content in the hexane concentrate was determined by evaporating an aliquot of the extract. Antioxidant, Tertiarybutyl hydroxyquinone (TBHQ) or α-tocopherol was added to the hexane extract at a level of 200 ppm (of fat content). The extract without antioxidant served as control. Carrier was added to the concentrated hexane extract at a rate of 15% of waste and the solvent evaporated completely to obtain the pigmented carrier. Cornstarch and sodium alginate were used as pigment carriers. The

pigmented carrier was packed in metallised polyester or polypropylene pouches and stored at ambient temperature (28±2°C) for 6 m.

Carotenoid content in the pigmented carrier during storage was determined at monthly intervals by extracting the pigments in hexane and measuring the carotenoid content spectrophotometrically as explained in section 2.1.1. Hunter L, a*, b* values were measured using Hunter LabScan XE (port size: 1.20", Area View: 1.00", 2°, C illuminant)

5.1.2. Oil extracted carotenoids

Carotenoid in the waste from shrimp *P indicus* was extracted using sunflower oil by adopting the optimized conditions as explained in chapter 4. To the pigmented oil, antioxidant TBHQ or α-tocopherol was added at a level of 200 ppm. The pigmented oil without antioxidant served as control. The pigmented oil was then stored in transparent and amber colored bottles at ambient temperature (28±2°C) for 6 m. The pigmented oil during storage was sampled at monthly intervals for analysis. The absorbance of the pigmented oil was read at 487 nm and the Hunter L, a*, b* values were measured.

5.1.3. Statistical analysis

All the statistical analysis was carried out using the software STATISTICA (Statsoft Inc 1999). The experiments were carried out in 4 replicates. The data was subjected to analysis of variance (ANOVA) and Duncan's multiple range tests. The relationship between carotenoid content / absorbance at 487 and Hunter L, a* b* was determined by correlation analysis.

5.2. Results and discussion

Solvent extraction of carotenoids from shrimp waste yields a product in thick paste form. It is difficult to use the paste in food applications, as uniform mixing of paste with food ingredients is a problem. Thus it is necessary to prepare and store the product in an easy to use form. Starch or alginates is normally used as an ingredient in many of the comminuted meat and fish products. Thus starch or alginate was assessed as pigment carrier for solvent extracted carotenoids.

The results indicated that the carotenoid content in the pigmented carriers decreased during storage (Figure 5.1). Presence of antioxidants, packaging material and storage period had a significant effect ($p \leq 0.001$) on the total carotenoid content, while the total carotenoid content was not affected ($p \geq 0.05$) by the carrier used (ANOVA Table 5.1a). Highest reduction (from the initial carotenoid content) was observed in the absence of antioxidant and storing in polypropylene pouches, in pigmented alginate (60.3%) and starch (62.8%) at the end of 6 m storage (Table 5.1). Lowest reduction at the end of 6-month storage was observed in pigmented alginate (22.1%) and starch (22.7%) containing 200 ppm TBHQ and packed in metallised polyester pouches. Similar to total carotenoid content, the percentage reduction in the carotenoid content was also significantly ($p \leq 0.001$) affected by antioxidants, packaging material and storage period (ANOVA Table 5.1b).

At the end of the 6 m storage period, the difference in % reduction of carotenoid content between polypropylene packed and metallised polyester pouch packed concentrate (with same carrier and antioxidant or control) ranged from 3.6 (starch & TBHQ) to 8.4 (starch & α-tocopherol). While the differences between % reduction carotenoid content in control and antioxidant containing samples in polypropylene

pouches ranged from 9.9 (starch & α-tocopherol) to 36.5 (starch and TBHQ) and in metallised polyester pouches from 10.6 (starch & α-tocopherol) to 32.3 (alginate and TBHQ). This indicates that the antioxidants have more influence on prevention of carotenoid degradation than the packaging materials used.

With decrease in carotenoid content, there was an increase in Hunter L value (Figure 5.2), decrease in a* (Figure 5.3) and b* values (Figure 5.4) of pigmented carriers. The changes in Hunter L, a*, b* values were significantly ($p \leq 0.001$) affected by antioxidants, carrier used, packaging material and storage period (ANOVA Table 5.1c, 5.1d, 5.1e). Hunter L value indicates lightness, a* redness and b* yellowness. The significant difference in lightness in two carriers was mainly due to the fact that alginate is light brown in color, while starch is white. As the whiteness increases the L value increases. Thus the pigmented starch is lighter (higher L value) than the pigmented alginate. The correlation coefficients (Table 5.2) indicate that the reduction in carotenoid content results in reduction of color intensity of pigmented carrier and thus the reduction in a* value ($r_{alginate} = 0.98$; $r_{starch} = 0.94$) and b* value ($r_{alginate} = 0.94$; $r_{starch} = 0.91$), and increase in lightness ($r_{alginate} = - 0.85$; $r_{starch} = - 0.92$).

The reductions in absorbance (at 487 nm) of pigmented oil during storage (Figure 5.5) indicate the degradation of carotenoids, which was significantly affected by antioxidants ($p \leq 0.001$), packaging material ($p \leq 0.05$) and storage period ($p \leq 0.001$) (ANOVA Table 5.2a). Highest reduction was observed in pigmented oil without antioxidant stored in transparent bottle and lowest reduction in pigmented oil containing TBHQ and stored in amber colored bottle. With reduction in absorbance, which is indicative of carotenoid loss, the lightness (L value) increased (Figure 5.6), redness (a*) (Figure 5.7) and yellowness (b*) (Figure 5.8) decreased. Hunter L, a*, b* values were

significantly ($p \leq 0.001$) affected by the presence of antioxidants, packaging material and period of storage (ANOVA Table 5.2b, 5.2c, 5.2d). Correlation coefficients (Table 5.3) between absorbance and Hunter L ($r = - 0.95$), a* ($r = 0.98$) and b* ($r = 0.98$) values are indicative of the positive relationship between absorbance and a*, b* value and negative relationship between absorbance and L value.

Carotenoids are highly unstable compounds and their degradation in foods is mainly due to oxidation, dependent upon contact with oxygen, light, heat and presence of pro- and antioxidants (Haard 1988). The stability of carotenoids has been studied in model systems. It is hypothesized that mechanism of carotenoid degradation is similar to lipid oxidation, and the antioxidants, which inhibit lipid oxidation, also decrease the degradation of carotenoids (Frankel 1985). Scita (1992) observed that in a model system β-carotene shows faster degradation with effect of light in the presence of oxygen, the degradation rate increasing with increment in oxygen turnover, and β-carotene is stabilized with antioxidants, thus concluding that the degradation is by the effect of free radicals. Synergism between carotenoid and antioxidant has also been observed in membrane model system (Haila et al 1998). The protection of β-carotene from being degraded has been attributed to the recycling of one electron oxidized β-carotene by the antioxidant α-tocopherol (Palozza and Krinsky 1992).

Antioxidants and suitable packaging conditions are commonly used to protect color degradation in many of the food items. Antioxidants are commonly used to prevent oxidation. Carotenoids are also known to have antioxidant property (Burton 1989). Mortenssen and Skibsted (2000) indicated that carotenoids, like other antioxidants are degraded by radicals when functioning as antioxidants and the presence of other antioxidants is thus important for the preservation of color as they scavenge the free

radicals before they react with carotenoids. Tocopherol is commonly used antioxidant to prevent oxidative degradation of color during storage of fish and shellfish (Ingemansson et al 1993). Li et al (1998) used sodium erythorbate to prevent astaxanthin degradation in frozen rockfish.

It is stated that oxidation of fat in crawfish waste with formation of peroxides probably would oxidize the associated astaxanthin simultaneously and develop discoloration (Budowski and Bondi 1960). The addition of antioxidant ethoxyquin to crawfish meal was thus found to stabilize the astaxanthin against degradation (Chen and Meyers 1982). Chen and Meyers (1982) also observed 99.0% pigment retention in pigmented soy oil containing 0.04% ethoxyquin as antioxidant, and storing in opaque bottles for 7 months. Solvent extraction adopted for recovery of carotenoids in the present study also extract lipids. Thus addition of antioxidants is beneficial to prevent lipid oxidation and subsequent carotenoid degradation. The addition of antioxidants to the oil extracted carotenoids and storing them in amber colored bottles showed improved stability of carotenoids during storage. TBHQ was found to be better antioxidant than α-tocopherol for stabilization of extracted carotenoids. The relative activity of antioxidants is based on combination of factors like solubility, oxygen partial pressure, reactive species with which it reacts, etc (Di Mascio et al 1991).

The lower rate of carotenoid reduction during storage of pigmented carriers in metallised polyester pouch is due to the fact that the metallised polyester films have good oxygen barrier and light barrier properties due to the presence of aluminum laminate in the film. The improved stability of oil-extracted carotenoids in the amber colored bottle is mainly due to the prevention of photo oxidation of carotenoids.

5.3. Conclusion

As carotenoids degrade on exposure to light and oxygen, they need to be protected against oxidation during storage. Solvent extracted carotenoids can be stored by mixing the extract with carriers such as sodium alginate or starch. Addition of antioxidants such as TBHQ or α-tocopherol and storing them in metallised polyester pouches reduces the carotenoid degradation during storage. The oil-extracted carotenoid can be protected from degradation by addition of antioxidants and storage in amber colored bottles.

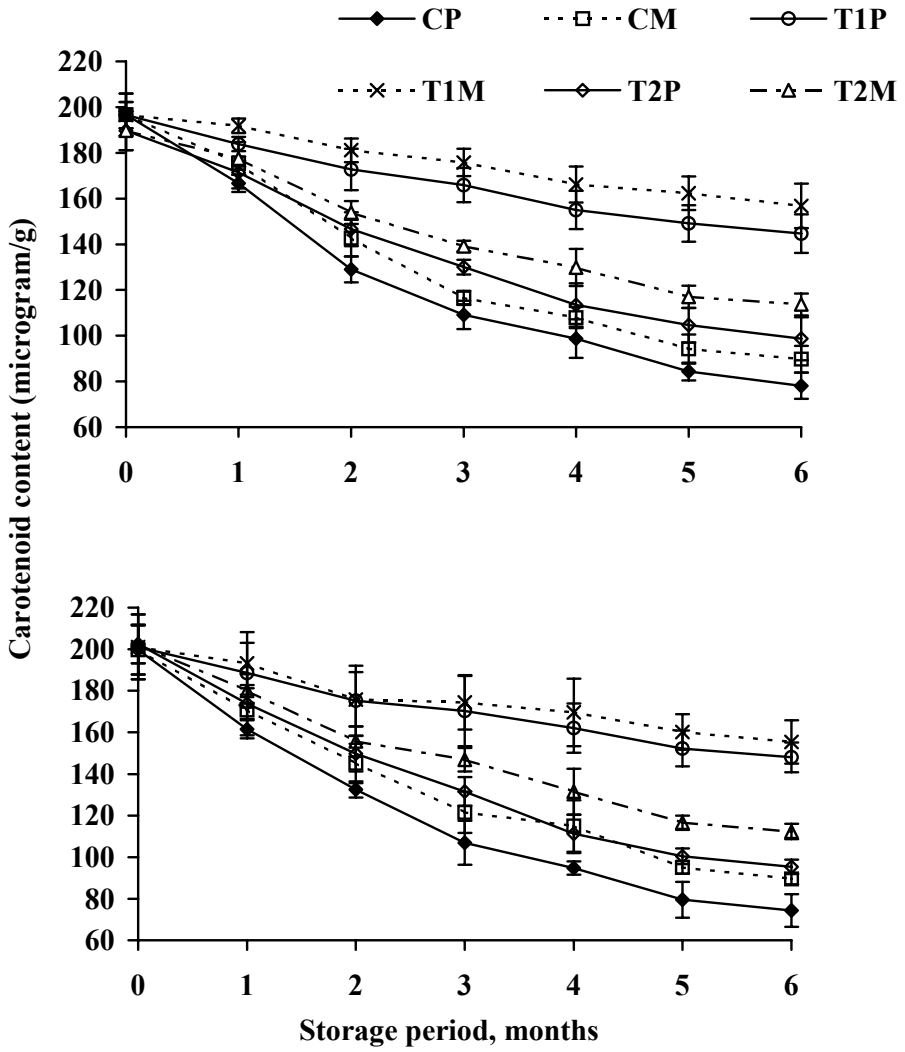


Figure 5.1. Carotenoid content during storage of solvent extracted carotenoid in alginate or starch as carrier with or without antioxidant, packed in polypropylene (P) or metallised polyester (M) pouches (n = 4)

(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

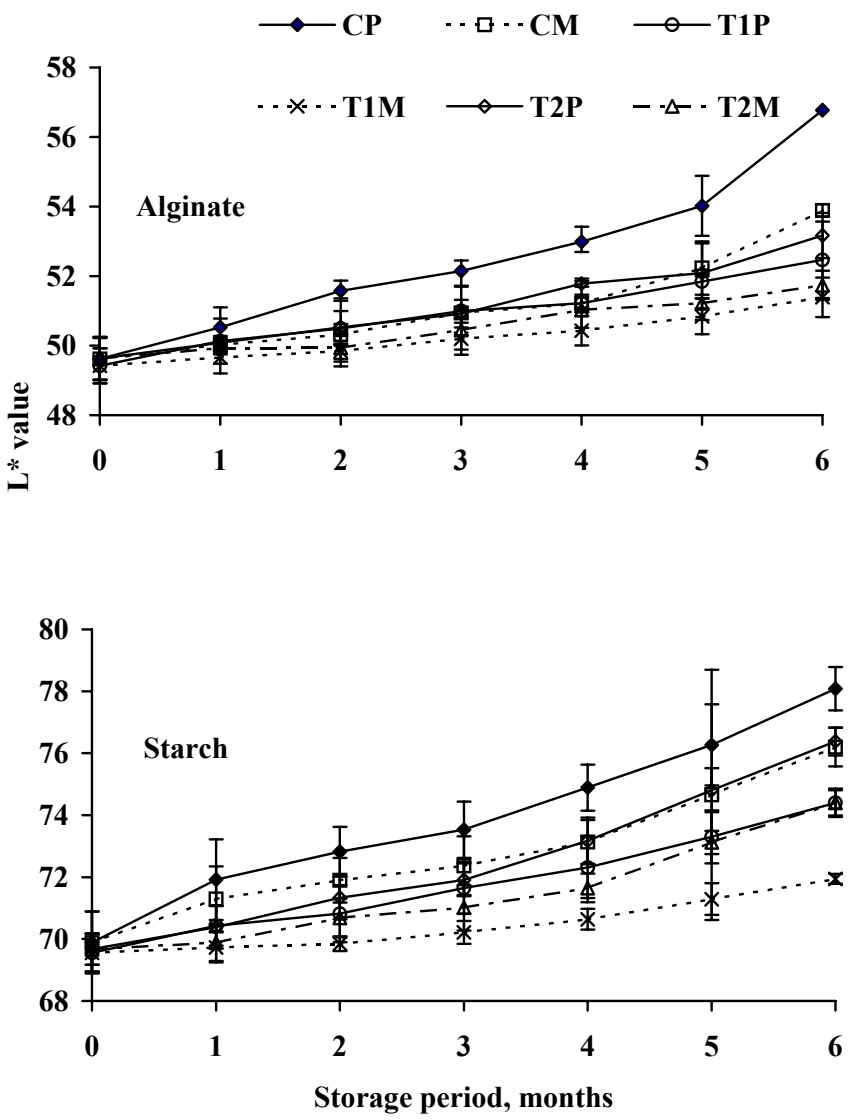


Figure 5.2. Hunter L value during storage of solvent extracted carotenoid in alginate or starch as carrier with or without antioxidant, packed in polypropylene (P) or metallised polyester (M) pouches (n = 4)

(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

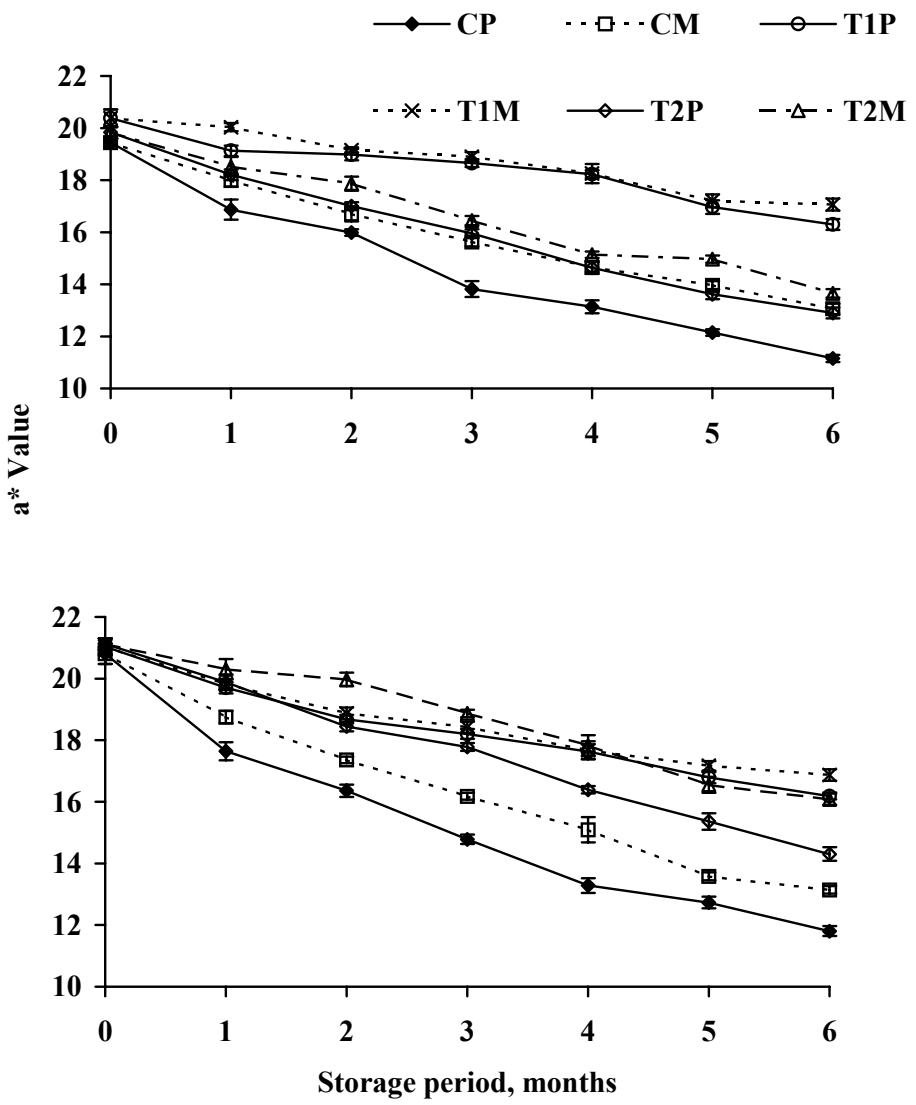


Figure 5.3. Hunter a* value during storage of solvent extracted carotenoid in alginate or starch as carrier with or without antioxidant, packed in polypropylene (P) or metallised polyester (M) pouches (n = 4)

(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

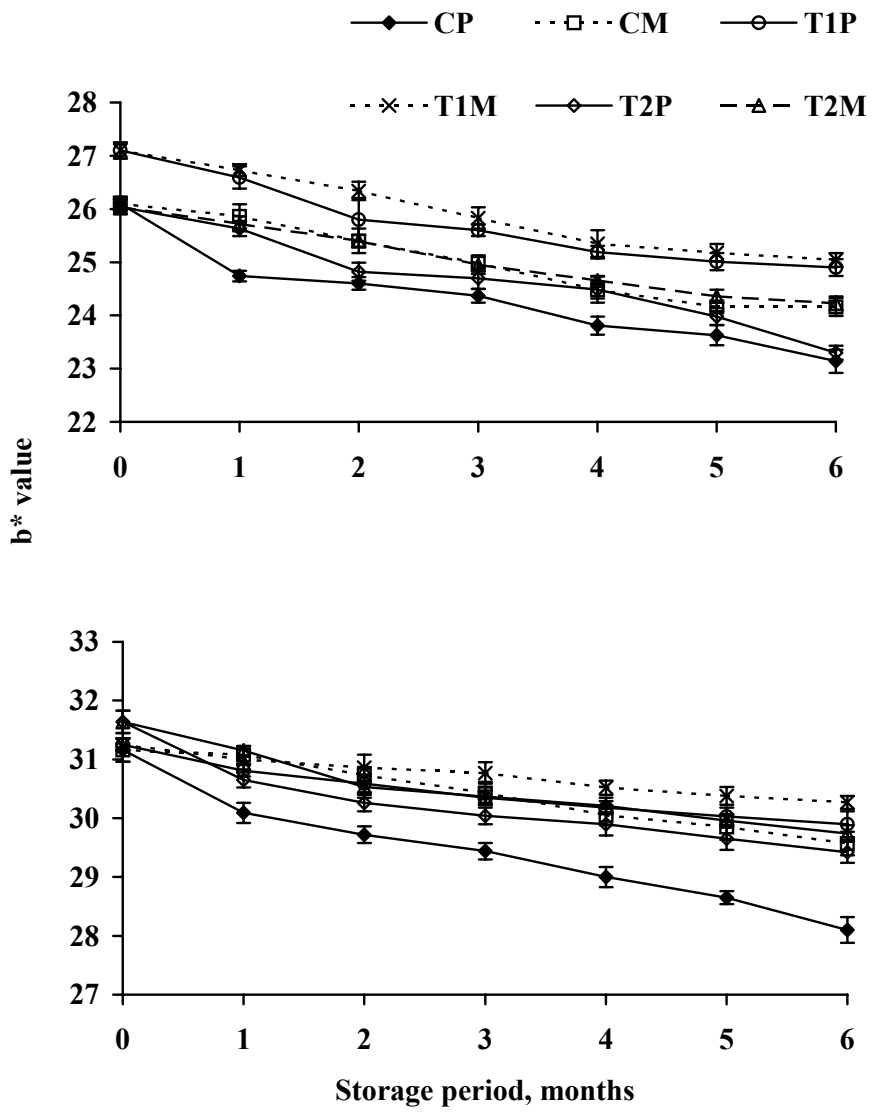


Figure 5.4. Hunter b* value during storage of solvent extracted carotenoid in alginate or starch as carrier with or without antioxidant, packed in polypropylene (P) or metallised polyester (M) pouches (n = 4)

(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

Table 5.1 Percentage reduction (from initial) in carotenoid content during storage of solvent extracted carotenoids in alginate or starch as a carrier with or without antioxidant, packed in polypropylene (P) or metallised polyester (M) pouches (n=4)

Carrier	Antioxidant	Packaging	Storage period, Months					
			1	2	3	4	5	6
Alginate	**Control**	**P**	15.2±4.20	34.4±1.07	44.6±1.13	49.9±2.57	57.1±1.97	60.3±2.35
		M	10.7±3.67	27.5±1.66	40.7±2.17	45.1±1.53	52.1±3.38	54.4±3.04
	TBHQ (200 ppm)	**P**	8.6±1.07	14.2±2.12	17.6±1.86	23.0±2.73	25.9±2.30	28.1±2.32
		M	4.6±2.81	10.0±0.73	12.6±1.43	17.4±1.70	19.3±1.44	22.1±2.57
	α-Tocopherol (200 ppm)	**P**	11.2±1.07	24.2±0.70	32.6±2.69	41.3±2.72	45.9±2.26	49.0±2.93
		M	8.1±0.50	20.3±0.95	27.9±2.38	32.8±2.31	39.4±1.42	41.1±2.25
Starch	**Control**	**P**	18.9±3.33	33.6±2.44	46.5±2.30	52.5±2.76	60.2±2.51	62.8±2.46
		M	14.6±1.01	27.3±0.73	39.1±1.37	42.5±3.53	52.3±2.47	55.1±2.06
	TBHQ (200 ppm)	**P**	6.2±0.76	12.9±1.58	15.4±1.94	19.3±1.25	24.1±2.10	26.3±2.50
		M	3.9±0.21	12.5±1.36	13.3±0.88	15.7±2.10	20.2±2.19	22.7±1.33
	α-Tocopherol (200 ppm)	**P**	14.2±0.79	26.1±1.12	35.0±3.51	45.1±4.03	50.4±2.13	52.9±1.47
		M	11.1±0.74	23.1±1.56	27.4±2.88	35.1±2.69	42.3±3.55	44.5±3.27

ANOVA Table 5.1. Effect of antioxidants, carriers and packaging materials on the stability of solvent extracted carotenoid during storage

a. Carotenoid content

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidant	118335.66	2	59167.83	54331.65	325	167.17	353.93***
Carrier	108.75	1	108.75	54331.65	325	167.17	0.65^{NS}
Packaging	7250.75	1	7250.75	54331.65	325	167.17	43.37***
Storage period	291006.66	6	48501.11	54331.65	325	167.17	290.12***

b. % Reduction in carotenoid content

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidant	30932.98	2	15466.49	5598.92	278	20.14	768.00***
Carrier	71.29	1	71.29	5598.92	278	20.14	3.54^{NS}
Packaging	2144.44	1	2144.44	5598.92	278	20.14	106.49***
Storage period	362072.20	5	7241.44	5598.92	278	20.14	359.58***

c. Hunter L Value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidant	175.86	2	87.93	262.28	325	0.807	108.86***
Carrier	37227.14	1	37227.14	262.28	325	0.807	46088.88***
Packaging	87.79	1	87.79	262.28	325	0.807	108.69***
Storage period	725.52	6	120.92	262.28	325	0.807	149.71***

ANOVA Table 5.1 (Contd.)

d. Hunter a* Value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidant	475.92	2	237.96	203.78	325	0.627	379.56***
Carrier	49.08	1	49.08	203.78	325	0.627	78.28***
Packaging	48.69	1	48.69	203.78	325	0.627	77.67***
Storage period	1334.46	6	222.41	203.78	325	0.627	354.76***

e. Hunter b* Value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidant	47.18	2	23.59	36.08	325	0.111	212.72***
Carrier	2237.79	1	2237.79	36.08	325	0.111	20176.45***
Packaging	17.56	1	17.56	36.08	325	0.111	158.36***
Storage period	146.52	6	24.42	36.08	325	0.111	119.23***

^{NS} – $p \geq 0.05$, *** $p \leq 0.001$

Table 5.2. Correlation coefficient between carotenoid content, Hunter L, a*, b* values of solvent extracted carotenoid

a. Alginate as carrier

Variable	Carotenoid content	L	a*	b*
Carotenoid content	1.00	-0.85	0.98	0.94
L	-0.85	1.00	-0.88	-0.85
a*	0.98	-0.88	1.00	0.96
b*	0.94	-0.85	0.96	1.00

b. Starch as carrier

Variable	Carotenoid content	L	a*	b*
Carotenoid content	1.00	-0.92	0.94	0.91
L	-0.92	1.00	-0.95	-0.92
a*	0.94	-0.95	1.00	0.93
b*	0.91	-0.92	0.93	1.00

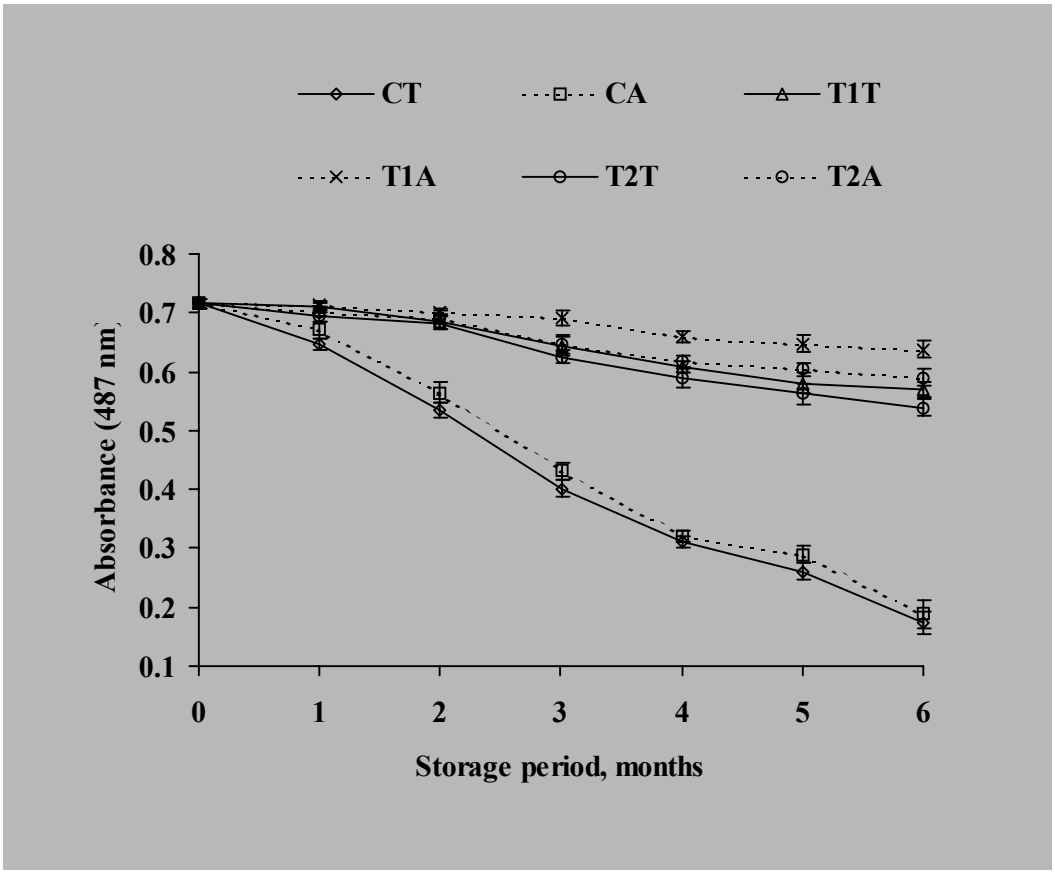


Figure 5.5. Absorbance (at 487 nm) of pigmented oil, with or without antioxidants during storage in transparent (T) and amber colored (A) bottles (n=4) (C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

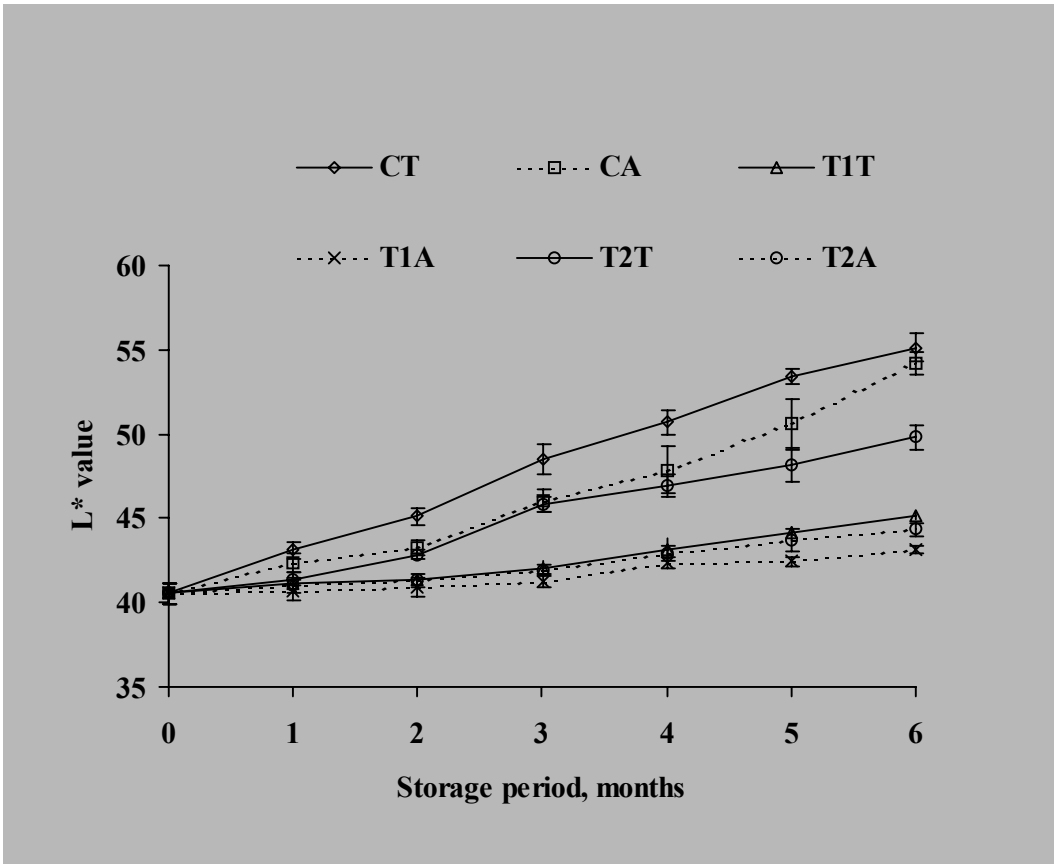


Figure 5.6. Hunter L value of pigmented oil, with or without antioxidants during storage in transparent (T) and amber colored (A) bottles (n=4)
(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-Tocopherol)

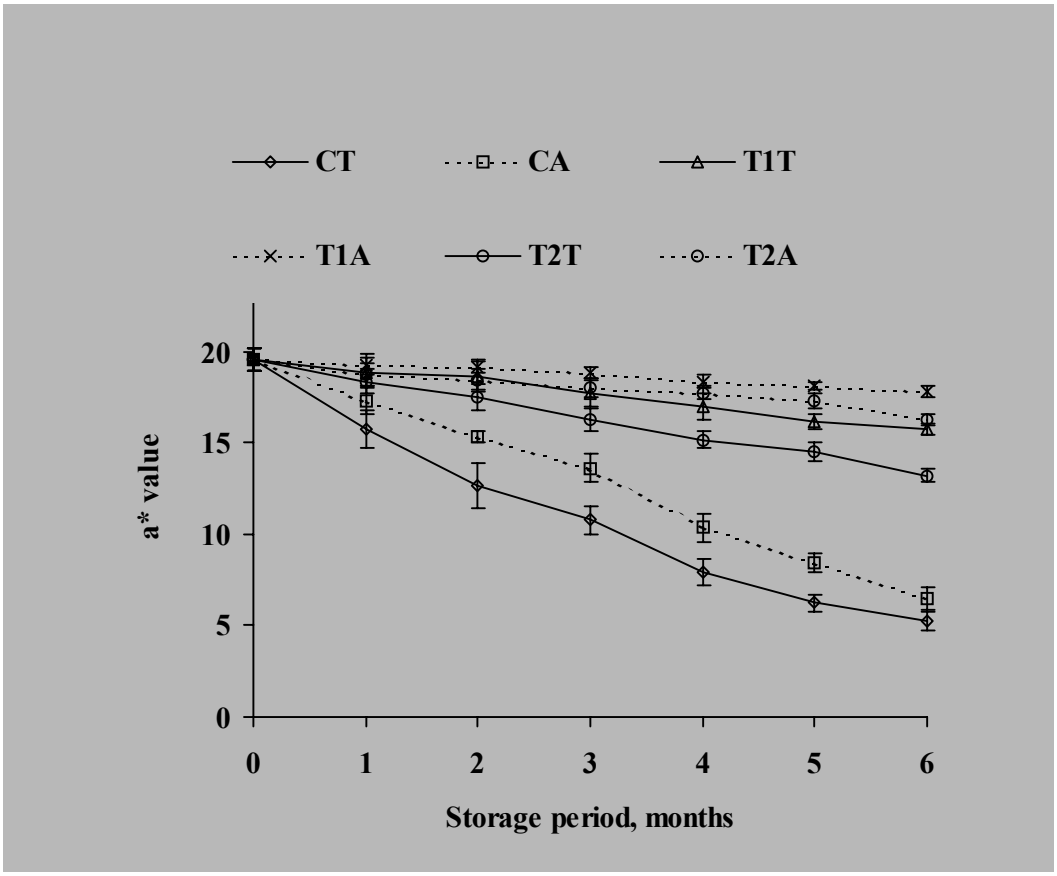


Figure 5.7. Hunter a* value of pigmented oil, with or without antioxidants during storage in transparent (T) and amber colored (A) bottles (n=4)
(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm α-tocopherol)

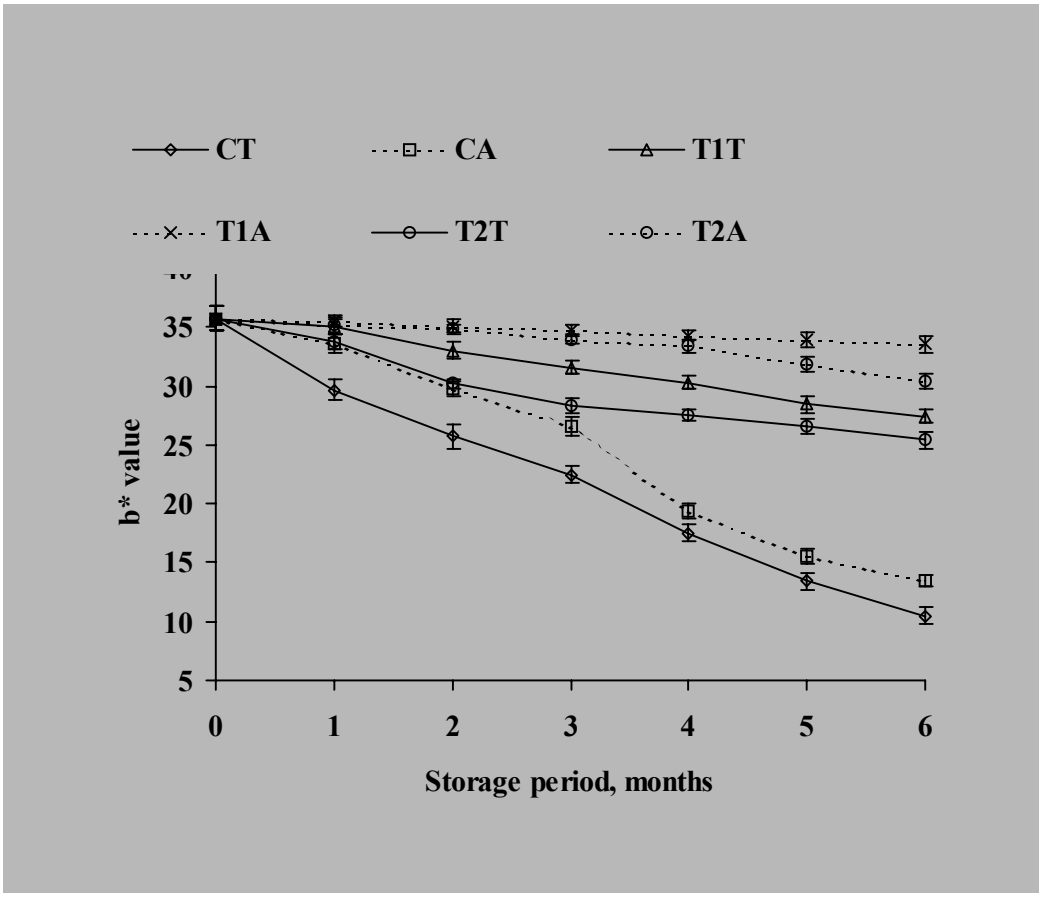


Figure 5.8. Hunter b* value of pigmented oil, with or without antioxidants during storage in transparent (T) and amber colored (A) bottles (n=4)
(C: without antioxidant; T1: 200 ppm TBHQ; T2: 200 ppm Tocopherol)

ANOVA Table 5.2. Effect of antioxidants, packaging on the stability of oil extracted carotenoid during storage

a. Absorbance at 487 nm

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidants	1.62	2	0.809	0.77	158	0.0049	166.60***
Packaging	0.029	1	0.029	0.77	158	0.0049	5.94*
Storage period	1.518	6	0.253	0.77	158	0.0049	52.10***

b. Hunter L value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidants	786.18	2	393.09	515.08	158	3.26	120.55***
Packaging	135.25	1	135.25	515.08	158	3.26	41.48***
Storage period	1266.06	6	211.01	515.08	158	3.26	64.71***

ANOVA Table 5.2 (contd)

c. Hunter a* value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidants	1204.14	2	602.07	526.14	158	3.33	180.80***
Packaging	96.76	1	96.76	526.14	158	3.33	29.06***
Storage period	937.98	6	156.33	526.14	158	3.33	46.94***

d. Hunter b* value

Source of variation	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Antioxidants	3021.40	2	1510.70	1619.50	158	10.25	147.43***
Packaging	440.67	1	440.67	1619.50	158	10.25	43.01***
Storage period	2975.04	6	495.84	1619.50	158	10.25	48.39***

* - $p \leq 0.05$; *** $p \leq 0.001$

Table 5.3. Correlation coefficient between absorbance at 487 nm, Hunter L, a*, b* values of oil extracted carotenoids

Variable	Absorbance at 487 nm	L	a*	b*
Absorbance at 487 nm	1.00	-0.95	0.98	0.98
L	-0.95	1.00	-0.97	-0.97
a*	0.98	-0.97	1.00	0.99
b*	0.98	-0.97	0.99	1.00

CHAPTER 6

APPLICATION OF SHRIMP WASTE CAROTENOIDS IN FOOD AND FEED

CHAPTER 6

APPLICATION OF SHRIMP WASTE CAROTERNOIDS IN FOOD AND FEED

Various fish mice products such as sausage, kamaboko are very popular in urban cities as ready to eat products. Color is one of the important sensory attributes, which determines the consumer acceptability of these products. Synthetic coloring agents or color developers are included in the formulation of fish products to improve the color. The main commercial colorants being used in seafood products include carmine, carmosine, caramel, paprika, and annatto dye (Lee et al 1992). Koizumi and Nonaka (1980) used ferihaemochrome forming nitrogenous bases such as imidazole and amino acid derivatives to develop pink color in fish sausage. Use of L-xylose, 2-ketohexonic acid, along with potassium bromate, pH adjustor and surfactants for coloring the surface of fish meat products is reported (Akiji 1985, 1986). There is a need for alternate natural coloring ingredients to reduce the health risks associated with synthetic food additives. The reports on use of shrimp carotenoids as colorants in fish products are scanty.

Color also plays an important role in marketability of cultured fishes like salmons and crustaceans. In order to obtain the color of the flesh of cultured species similar to those obtained from natural waters, addition of pigments in their diet is practised. The color of ornamental fishes is also an important factor, which determines the demand for such fishes. A variety of carotenoids, with more emphasis on synthetic carotenoids have been tested for effective coloration of cultured fishes (Bjerkeng 2000, Shahidi et al 1998). The utilization of shrimp carotenoids as pigment source is restricted to the direct addition of shrimp offal or shrimp meal in the aquaculture diet.

This study was carried out to assess the suitability of carotenoids recovered from shrimp waste as coloring agent in fish sausages as an alternative to the synthetic coloring agents, and to evaluate the pigmentation efficiency of shrimp waste carotenoids in the ornamental fish, koi carp.

6.1. Material and methods

6.1.1. Fish sausage with added carotenoids

Pigmented starch was prepared from the carotenoid extract of shrimp waste as explained in section 5.1.1, and used as coloring agent. The carotenoid content in the pigmented starch ranged from 397.0 to 439.9 μg/g. Fish sausage was prepared using the minced meat from pink perch, *Nemipterus japonicus*. The formulation of fish sausage included, 500 g fish meat, 14.3 g salt, 10.7 g sugar, 1.4 g sodium tripolyphosphate, 60 mg chilly oleoresin, 0.8 g pepper powder, 0.8 g garlic powder, 65 g cornstarch, 35 ml refined vegetable oil and 70 ml chilled water. Sausage mix (700 g) was prepared by mixing the ingredients in sequence in a bowl chopper. The mix was stuffed into synthetic casings and cooked at 90°C for 45 min to obtain cooked sausage. For control (A) batch no carotenoids were added. To prepare fish sausage with 5-ppm carotenoid (B), the preparation was carried out as above by replacing 8.0 – 8.8 g (depending on carotenoid content in the pigmented starch) of cornstarch with pigmented starch. Similarly, to prepare sausage with 10-ppm carotenoid (C), 16.0 – 17.6 g of cornstarch was replaced with pigmented starch in the formulation. The preparation of sausage in three formulations (C, T1 and T2) was carried out 4 times.

6.1.1.1. Determination carotenoid content, Hunter L, a*,b* values and sensory color and flavor

Carotenoid content in the fish meat, sausages mix and cooked sausage was determined as explained in section 2.1.1. Hunter L, a*, b* values were determined by using Hunter LabScan XE (section 5.1.1). Sensory analysis of cooked sausage for color and flavor was carried out on a 9-point Hedonic scale (1: dislike extremely; 9: like extremely) employing 10 trained panelists.

6.1.2. Feeding experiments with diet containing shrimp waste carotenoids

6.1.2.1. Diet formulation

Carotenoid concentrate obtained by solvent extraction as explained in section 3.1 was used as pigment source in diets. The carotenoid content in the concentrate was 5.2 mg/g. Isonitrogenous (35 - 36% protein) and isocaloric (ME = 3500 kcal/kg) diets were prepared using groundnut oil cake, rice bran, vegetable oil and carotenoid concentrate. The composition of ingredients in the 3 diets, C (control), T1 (5 ppm carotenoid) and T2 (25 ppm carotenoid) was as follows,

Ingredient	Diet		
	C	T1	T2
Groundnut oil cake	645 g	645 g	645 g
Deoiled rice bran	350 g	350 g	350 g
Vegetable oil	5 g	4 g	-
Carotenoid extract	-	1 g	5 g

Groundnut oil cake, rice bran and vegetable oil were mixed in the proportion and the mix was made into dough by addition of water. The dough was pressure cooked (0.7 kg/cm^2, 15 min), dried (50°C, 4 h) by spreading on trays and powdered. For treatment diets (T1 and T2) carotenoid concentrate was thoroughly mixed with the powder. Proximate composition of the feeds was determined by standard methods (AOAC 1999) as explained in section 1.1.2.2. Carbohydrate content was determined by difference and the energy value (ME, Kcal/kg) was calculated by taking into account of energy value of protein (4 Kcal/g), fat (9 Kcal/g) and carbohydrate (4 Kcal/g). Carotenoid content in the diets was determined by method explained in section 2.1.1.

6.1.2.2. Fish feeding

Feeding experiments were conducted in glass aquariums of size 61 cm x 30 cm x 30 cm. For each diet, duplicate tanks were used. In each tank 10 numbers of koi carp (*Cyprinus carpio koi*) juveniles (2.0 – 2.5 cm length) was stocked. Feeding was done at a rate of 2% of body, spread in two feedings per day. Feeding was continued for 9 wks and weight of fishes was taken every 3rd week. At the end of 9 weeks, the fishes were starved for a day and sacrificed.

6.1.2.3. Hunter color values and carotenoid content of experimental fish

Hunter L, a*, b* values of the surface of fish was measured in triplicates for each tank (6 replicates per diet) using Hunter LabScan XE (section 5.1.1). Carotenoid in the whole fish was determined in duplicates for each tank (4 replicates per diet) by extracting the carotenoid from the homogenized fish and measuring the content spectrophotometrically (section 2.1.1).

6.1.3. Statistical analysis

The data was subjected to analysis of variance (ANOVA) and Duncan's multiple range tests using the software STATISTICA (Statsoft Inc 1999).

6.2. Results and discussion

6.2.1. Carotenoids as colorants in fish sausage

The carotenoid content in the fish meat used for preparation of sausage was 0.34 μg/g (Table 6.1). Carotenoid content in the sausage mix and cooked sausage prepared without added carotenoid was 0.41 and 0.36 μg/g respectively. Cooking of sausage resulted in a marginal reduction ($p > 0.05$) in the carotenoid content from 4.98 μg/g to 4.86 μg/g and 9.82 to 9.45 μg/g in sausages added with 5 ppm and 10 ppm carotenoid respectively. There was a significant difference ($p \leq 0.001$) in carotenoid content between 3 formulations of sausage mix and cooked sausage (ANOVA Table 6.1), but not between ($p \geq 0.05$) sausage mix and cooked sausage of same formulation.

Hunter L values decreased, a* and b* values increased with increase in carotenoid content (Table 6.1) and showed a significant difference ($p \leq 0.001$) between 3 formulations (ANOVA Table 6.1). However, a* values were not significantly different between sausage mix and cooked sausage of same formulation.

The addition of carotenoid in the sausage formulation enhanced the visual coloration of the cooked sausage (Photoplate 6.1). The sensory analysis of cooked sausage (Figure 6.1) indicated that, the color and flavor score for sausage with added carotenoid was higher than that without added carotenoid. A significant difference was observed in color ($p \leq 0.001$) and

flavor ($p \leq 0.05$) scores between sausages of 3 formulations (ANOVA Table 6.1). However, there was no significant difference ($p \geq 0.05$) in color and flavor scores for sausages containing two different levels of added carotenoids.

Color is one of the important quality criteria, which determines the acceptability and marketability of many of fish mince products. Attempts have been made to impart color to fish paste products by using various coloring substances. Hideo (1988) used immersion in onion skin pigment extract as a technique to color the surface of fish paste product, and reported that it is difficult to achieve uniform coloration with this technique. Takahito (1993) used hydrolyzed pigments from tissue cultured cells of common madder to color fish paste products, but suggested the use of alum, organic acids and carbonates for stabilization of color during processing. Osterlie et al (2001) evaluated the use of synthetic astaxanthin as coloring agent in fish pastes and reported that synthetic astaxanthin may be added during processing of pastes without negatively affecting the product flavor.

The present study indicated that the carotenoids extracted from shrimp waste could be effectively used as coloring agent in fish sausage overcoming the disadvantages reported for other coloring agents. The advantage of the extracted carotenoids is that, it not only enhances the color, but also improves the flavor of the product. Further, the study revealed that the carotenoids added in the sausage preparation are stable during processing and do not require any stabilizers. Synthetic coloring agents are not advised for use in food products due to safety aspects. Thus the shrimp waste carotenoids would be a beneficial alternative to synthetic coloring agents hitherto used as coloring agents in fish products.

6.2.2. Carotenoids for pigmentation of ornamental fish

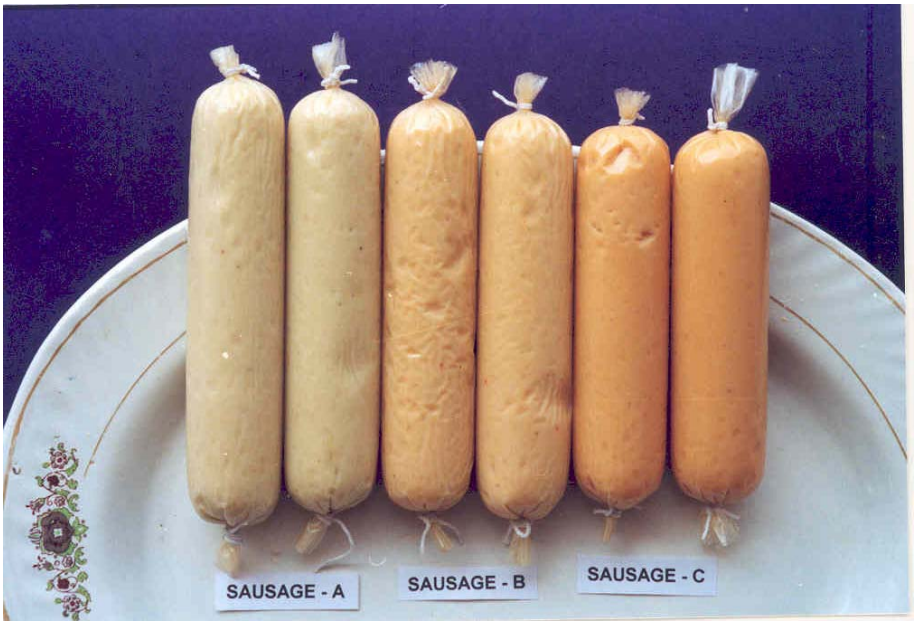
The three diets used (C, T1 and T2) had a protein content of 34.6%, 36.4% and 34.8% and carotenoid content (μg/g) of 0.82, 5.11 and 24.15 respectively (Table 6.2). The weight of fishes fed with diets containing carotenoids was slightly higher that those fed with control diet (Figure 6.2). The inclusion of shrimp waste carotenoids enhanced the skin coloration of the ornamental fish, koi carp (Photoplate 6.2). The carotenoid content in fishes fed with carotenoid containing diet was 3.3 μg/g (T1: 5 ppm diet) and 4.3 μg/g (T2: 25 ppm diet) (Table 6.3) and showed a significant difference ($p \leq 0.001$) between fishes fed with 3 diets (ANOVA Table 6.3). Hunter L ($p \leq 0.01$), a* ($p \leq 0.001$) and b* values ($p \leq 0.05$) were significantly different between 3 groups of fishes fed with different diets (ANOVA Table 6.3). Even though carotenoid content and L values differed between fishes fed with two diets containing 5 ppm and 25 ppm carotenoid, the difference in a* and b* values between two were marginal ($p \geq 0.05$) (Table 6.3).

Studies have been carried out on use of crustacean processing waste for pigmentation of salmons (Saito and Reiger 1971; Haard 1992)). But the disadvantages with direct feeding of offals include, variable pigment levels and high chitin content (Torrison et al 1981). Synthetic astaxanthin or canthaxanthin have been used in salmon diets for pigmentation (Simpson et al 1981). Shahidi et al (1993) noted that by feeding Arctic char with diets containing 75 ppm of synthetic carotenoids for 15 weeks, a carotenoid level of 5.56 ppm in the tissue could be achieved. However, Bjerkeng et al (1990) reported that carotenoid concentration of flesh of trout does not increase when dietary pigment concentration is increased above 50 ppm. The reports on use of carotenoids for pigmentation of ornamental

fish are scanty. In the present study the improved skin coloration was also reflected in increased total carotenoid content and Hunter L, a*, b* values, indicating that carotenoid extracts from shrimp waste can be successfully used as a source of pigments in ornamental fish diets.

6.3. Conclusion

Use of carotenoids recovered from shrimp waste in fish sausage formulations enhances the color of the product. The addition of carotenoid extracts also improves the flavor of the product. The added carotenoids were stable during thermal processing of the sausage. Inclusion of shrimp waste carotenoids in the diets for koi carps enhanced the skin coloration and carotenoid content. Thus the carotenoids recovered from shrimp waste can be effectively used as a natural source of pigments for coloring of fish mince products and pigmentation of ornamental fishes.



Photoplate 6.1

Fish sausage prepared with and without added carotenoid

A: without carotenoid

B: with 5 ppm carotenoid

C: with 10 ppm carotenoid

Table 6.1. Carotenoid content, and Hunter L, a*, b* values of fish meat, sausage mix and cooked fish sausage (n =4)

Sample		Carotenoid content	Hunter colour values		
			L	a*	b*
Fish meat		0.34±0.061[a]	51.1±1.21[a]	-1.2±0.31[a]	8.5±0.42[a]
Sausage mix	**A**	0.41±0.110[a]	68.4±0.55[b]	-0.72±0.201[ab]	12.9±0.50[b]
	B	5.0±0.03[b]	65.1±1.40[c]	5.3±0.31[c]	20.6±0.40[c]
	C	9.8±0.13[c]	61.8±0.46[de]	8.9±0.42[d]	24.1±0.26[d]
Cooked sausage	**A**	0.36±0.082[a]	66.3±1.63[c]	-0.17±0.332[b]	16.3±0.84[e]
	B	4.9±0.07[b]	62.9±1.65[e]	5.5±0.90[c]	21.0±0.87[c]
	C	9.5±0.19[c]	60.1±1.67[d]	9.2±0.99[d]	23.7±0.70[d]

A: Control; B: 5 ppm carotenoid; C: 10 ppm carotenoid

Values in columns with different superscripts differ significantly ($p \leq 0.05$)

ANOVA Table 6.1. Carotenoid content, and Hunter L, a*, b* values of fish meat, sausage mix and cooked fish sausage

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid content	416.74	6	69.46	0.242	21	0.012	6028.43***
Hunter L value	771.49	6	128.58	36.23	21	1.73	74.53***
Hunter a* value	481.03	6	80.17	6.93	21	0.330	243.12***
Hunter b* value	820.23	6	136.71	7.81	21	0.372	367.42***

*** - $p \leq 0.001$

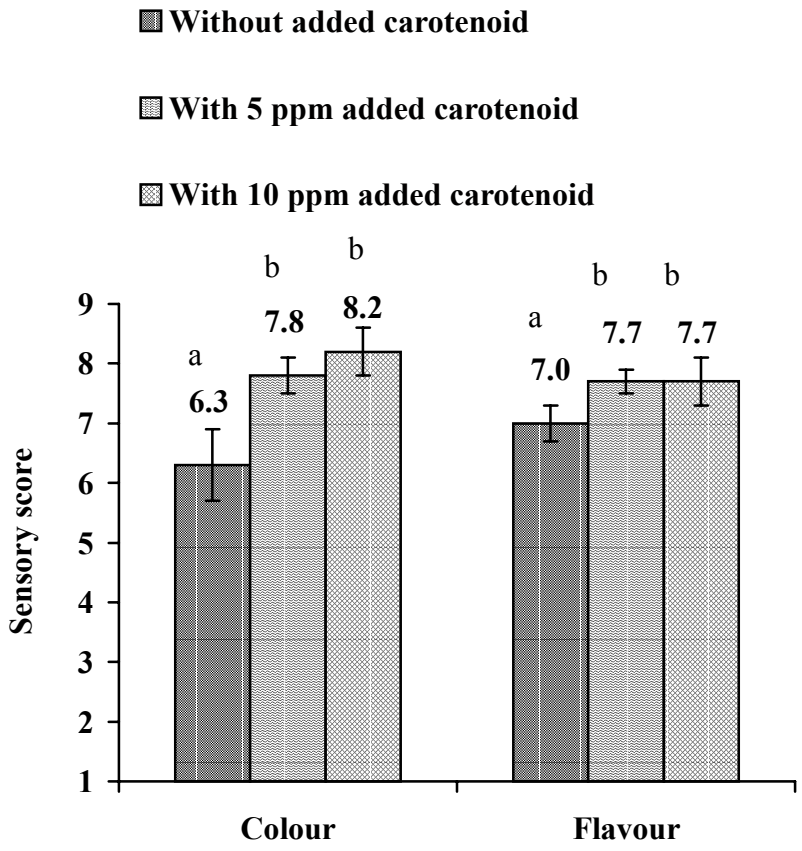


Figure 6.1. Sensor scores for color and flavor of fish sausage prepared with or without added carotenoid (n = 4)

(Values with different letters differ significantly, $p \leq 0.05$)

ANOVA Table 6.2. Sensory scores for cooked fish sausage

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Color	7.67	2	3.83	1.80	9	0.200	19.19***
Flavor	1.26	2	0.631	0.805	9	0.089	7.05*

*- $p < 0.05$, *** - $p < 0.001$

C: Control

T1: 5 ppm carotenoids

T2: 25 ppm carotenoids

Photoplate 6.2

Fish fed with three experimental diets

Table 6.2. Proximate composition, carotenoid content and calorific values of different experimental diets

	Diet		
	C	**T1**	**T2**
Moisture (%)	5.9	5.2	5.2
Protein (%)	34.6	36.4	34.8
Fat (%)	3.9	3.3	3.6
Ash (%)	11.2	11.4	11.1
Carbohydrate (%) (By difference)	44.4	43.7	45.3
ME (Kcal/kg) (Calculated)	3511	3501	3528
Carotenoid content (μg/g)	0.82	5.11	24.15

C: Control diet

T1: Diet with 5 ppm carotenoid

T2: Diet with 25 ppm carotenoid

Table 6.3. Carotenoid content and Hunter L, a*, b* values of fishes fed with diet containing shrimp waste carotenoids (n=4)

Diet	Carotenoid content[1]	Hunter Color Values[2]		
		L	a*	b*
C	1.8±0.13[a]	62.4±3.08[a]	-1.3±0.24[a]	8.0±2.58[a]
T1	3.3±0.10[b]	59.2±1.78[a]	-0.85±0.065[b]	9.7±2.33[ab]
T2	4.3±0.15[c]	55.2±3.14[b]	-0.68±0.147[b]	11.5±1.34[b]

[1] Values are mean of 4 determinations from duplicate tanks

[2] Values are mean of 6 determinations from duplicate tanks

Values in columns with different superscripts differ significantly ($p \leq 0.05$)

C: Control diet

T1: Diet with 5 ppm carotenoid

T2: Diet with 25 ppm carotenoid

ANOVA Table 6.3. Carotenoid content and Hunter L, a*, b* values of fishes fed with diet containing shrimp waste carotenoids

Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F Value
Carotenoid content	12.58	2	6.29	0.15	9	0.016	386.96***
Hunter L value	155.35	2	77.68	112.49	15	7.50	10.36**
Hunter a* value	1.38	2	0.69	0.43	15	0.028	24.35***
Hunter b* value	38.56	2	19.28	69.59	15	4.64	4.16*

* - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$

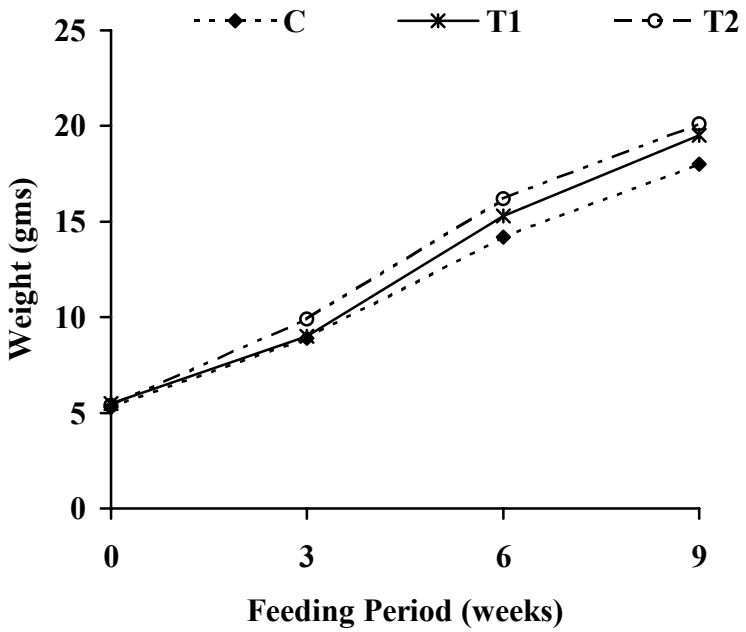


Figure 6.2. Weight of fishes (10 numbers) fed with different diets during 9 weeks of feeding period

C: Control diet

T1: Diet with 5 ppm carotenoid

T2: Diet with 25 ppm carotenoid

PART III

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Shrimp processing is one of the major seafood industry in India. Large quantities (~ 100,000 tonnes) of shrimp waste in the form of head and body carapace is produced annually from these processing plants. Shrimp waste is one of the important natural sources of carotenoids. The recovery of these valuable components from the waste would not only improve the economy of the plant but also reduces the pollution potential of the waste. The information on carotenoids in crustaceans from tropical waters, especially from Indian waters, is scanty. Further, recovery of carotenoids from byproducts of Indian shrimps of commercial value and their utilization has not been studied so far.

Studies were carried out to determine the yield and chemical composition of body components from 4 species of shallow water shrimps namely *Penaeus monodon, P indicus, Metapenaeus dobsoni, Parapenaeopsis stylifera*, two species of deep sea shrimps namely *Solonocera indica* and *Aristeus alcocki*, one species of fresh water prawn *Macrobrachium rosenbergii,* one species of crab each from marine water (*Charybdis cruciata*) and from fresh water (*Potamon potamon).* Total carotenoid content in different body components was determined. The qualitative distribution of carotenoids was determined by identifying the major carotenoids by thin layer chromatography (TLC), absorption spectra and by high performance liquid chromatography (HPLC). Carotenoid esters from the extracts of different body components were analyzed for fatty acid profile by gas chromatography (GC).

In order to recover the carotenoids from the shrimp waste, extractability of carotenoids in different organic solvents and solvent mixtures was evaluated and the conditions for solvent extraction were optimized by a statistically designed experiment. Studies were also carried out on extractability of carotenoids in different vegetable oils.

The optimized conditions for oil extraction of carotenoids were established. The oil extraction yield of carotenoids was increased by enzymatic hydrolysis of waste using different proteases and the hydrolysis and extraction conditions were optimized.

The effect of antioxidants and storage in different packaging conditions on the stability of recovered carotenoids was evaluated. The suitability of recovered carotenoids as colorants in fish products was assessed by incorporation of carotenoids in fish sausages. The pigmentation efficiency of carotenoids in ornamental fishes was assessed by fish feeding experiments.

The salient findings of the investigation are

- Yield of waste (head and carapace) was higher in deep-sea shrimps (62 – 66%) than in shallow water shrimps (48 – 56%). The yield of waste in fresh water prawn was 60%. Content of crude protein (8.2 – 10.2%), true protein (6.3 – 9.7%), fat (1.1 – 8.1%) was higher in head than in carapace (7.8 – 9.5% crude protein, 5.2 – 8.2% true protein, 0.75 – 2.0% fat), while ash (4.0 – 6.5%) and chitin content (3.3 – 4.4%) were lower in head than in carapace (4.9 – 9.0% ash, 4.4 – 6.3% chitin).
- The yield of meat in crabs was 28.8 – 29.7% and that of shell was 34.4 – 35.7%. Chitin content was higher in marine crab shell (8.2%) than in fresh water crab shell (4.4%).
- Total carotenoid content varied between species and body components. Highest carotenoid content was observed in head of deep-sea shrimp *A alcocki* (185.3 μg/g) and marine shrimp *P stylifera* (153.1 μg/g). High levels of carotenoids were also observed in carapace of *A alcocki* (117.4 μg/g), *S indica* (116.0 μg/g)

and *P stylifera* (104.7 μg/g). Low levels of carotenoids were observed in shrimp *P indicus* and fresh water prawn *M rosenbergii* and crabs.

- The major carotenoids in shrimps, fresh water prawn and marine crab was astaxanthin and its esters. β-Carotene and zeaxanthin was at low levels in these species. Zeaxanthin was the major carotenoid in fresh water crab.
- The carotenoid esters from the crustaceans studied contained palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:0), stearic (C18:0) and oleic (C18:1) as major fatty acids.
- A 50 : 50 mixture of isopropyl alcohol and hexane was found to give higher carotenoid yield from shrimp waste compared to individual solvents, namely acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, hexane or 50 : 50 mixture of acetone and hexane .
- The optimized conditions for solvent extraction of carotenoids were 60% hexane in solvent mixture, solvent mixture to waste ratio of 5 : 1 in each extraction and 3 numbers of extractions. A regression equation for predicting the carotenoid yield as a function of three processing variable (hexane % in solvent mixture, solvent level to waste and number of extractions) was derived by statistical analysis.
- Extractability of shrimp waste carotenoids was higher in refined sunflower oil compared to groundnut oil, gingelly oil, mustard oil, soybean oil, coconut oil and rice bran oil and the carotenoid content in oil could be increased by repeated use of pigmented oil for extraction of carotenoids from fresh waste for 3 times.

- The pigments in waste can be recovered in oil by mixing the sunflower oil with waste in a ratio of 2 : 1 (oil : waste), heating the mixture at 70°C for 150 min, centrifuging the treated waste and recovering the pigmented oil by phase separation. A regression equation was arrived at to predict the carotenoid yield as a function of oil level to waste, temperature and time of heating waste in oil.
- The oil extraction yield of carotenoids can be increased by hydrolysis of waste with protease prior to oil extraction and bacterial protease alcalase was found to be better than plant protease papain or animal protease trypsin for hydrolysis.
- Optimum oil extraction yield can be obtained by hydrolysis of waste with 0.75% (of waste) of alcalase at 37°C for 150 min, adding sunflower oil to the hydrolysed waste in a ratio of 2 : 1(oil : waste), heating at 70°C for 90 min and recovering the pigmented oil. A regression equation was derived to predict the carotenoid yield at different levels of processing variables namely, enzyme concentration, incubation time and heating time in oil. By using the hydrolysed waste for carotenoid recovery, heating time can be reduced from 150 min to 90 min to get optimum yield.
- Solvent extracted carotenoids can be stored by mixing with carriers such as sodium alginate or cornstarch. Addition of antioxidants and storing the pigmented carrier in light barrier packaging materials such as metallised polyester were found to reduce the degradation of the pigment. Tertiarybutyl hydroxyquinone (TBHQ) at a level of 200 ppm was found to be more effective antioxidant than α-tocopherol (200 ppm) for stabilization of pigments against oxidative degradation.

- In order to reduce the degradation of oil extracted carotenoids during storage, antioxidants, preferably TBHQ (200 ppm) should be added to the pigmented oil and stored in amber colored bottles.
- The addition of recovered carotenoids in fish sausage formulation at a level of 5 –10 ppm improved the color and flavor of the product. The added carotenoids were stable during thermal processing of sausage.
- The addition of carotenoids in diets for ornamental fish koi carp (*Cyprinus carpio koi*) enhanced the skin coloration and total carotenoid content in the body.

The studies indicated that the waste (head and carapace) yield from the shrimps and prawn was in the range of 48 – 66%. The waste contains high levels of carotenoid and could be used as a source of natural carotenoids. Carotenoids in the waste can be better recovered by extracting with a mixture of isopropylalcohol and hexane than the use of a polar solvent alone. Carotenoids can also be extracted using sunflower oil after hydrolyzing the waste with protease. To stabilize the carotenoids against degradation during storage, the addition of antioxidants and storing in light barrier materials can be adopted. The recovered carotenoids can be used as colorants in fish products and as pigment source in diets for ornamental fishes.

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