ผลของการเสริมวิตามินอีและแอสทาแซนทินที่มีต่อการเจริญพันธุ์ของกุ้งขาว *Penaeus vannmei* เพศผู้

นางสาวสรวงสุดา สุภาสัย

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EFFECTS OF VITAMIN E AND ASTAXANTHIN SUPPLEMENTATION ON MATURATION OF MALE WHITE SHRIMP *Penaeus vannamei*

Ms. Suangsuda Supasai

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By	Ms. Suangsuda Supasai
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Thesis Advisor	Associate Professor Somkiat Piyatiratitivorakul, Ph.D.
Thesis Co-advisor	Assistant Professor Thaithaworn Lirdwitayaprasit, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for Master's Degree

……...Dean of the Faculty of Science

(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

……..Chairman

(Assistant Professor Charoen Nitithamyong, Ph.D.)

……..Thesis Advisor

(Associate Professor Somkiat Piyatiratitivorakul, Ph.D.)

……..Thesis Co-advisor

(Assistant Professor Thaithaworn Lirdwitayaprasit, Ph.D.)

……..Member

(Professor Piamsak Menasveta, Ph.D.)

……..Member

(Voranop Viyakarn, Ph.D.)

สรวงสุดา สุภาสัย : ผลของการเสริมวิตามินอีและแอสทาแซนทินที่มีต่อการเจริญพันธุ์ของกุ้งขาว *Penaeus vannamei* เพศผู้. (EFFECTS OF VITAMIN E AND ASTAXANTHIN SUPPLEMENTATION ON MATURATION OF MALE WHITE SHRIMP *Penaeus vannamei*) อ.ที่ปรึกษา : รศ.ดร. สมเกียรติ ปียะธีรธิติวรกุล, อ.ที่ปรึกษาร่วม : ผศ.ดร. ไทยถาวร เลิศวิทยาประสิทธิ, 98 หน้า. ISBN : 974-17-6205-4

ศึกษาผลของวิตามินอีและแอสทาแซนทินต่อการเจริญพันธุ์ของกุ้งขาว *Penaeus vannamei* เพศผู้ โดยใช้อาหารอัดเม็ดที่มีวิตามินอี 3 ระดับ (200, 400 และ 600 ppm) และแอสทาแซนทิน 3 ระดับ (100, 300 และ 500 ppm) ทดลองในกุ้งขาวเพศผู้อายุ 5 เดือน ระยะเวลาในการทดลอง 40 วัน ก่อนเริ่มการทดลองกุ้งทุกตัวยังไม่สร้างถุงน้ำเชื้อ ผลการศึกษาไม่พบความสัมพันธ์ร่วมระหว่างวิตามินอีและแอสทาแซนทินต่อการเติบโต การรอดชีวิต และการเจริญพันธุ์ของกุ้งขาวเพศผู้ วิตามินอีและแอสทาแซนทินไม่มีผลต่อการเติบโตของกุ้งขาว กุ้งที่ได้รับวิตามินอีที่ระดับสูง (600 ppm) มีอัตรารอดสูงกว่ากุ้งที่ได้รับวิตามินอีที่ระดับต่ำ (200 ppm) อย่างมีนัยสำคัญ (P<0.05) ในขณะที่ระดับของแอสทาแซนทินไม่มีผลต่ออัตรารอดชีวิต ทั้งวิตามินอีและแอสทาแซนทินมีผลต่อน้ำหนักถุงน้ำเชื้อ โดยกุ้งที่ได้รับวิตามินอี 400 ppm มีน้ำหนักถุงน้ำเชื้อมากที่สุด ในส่วนของแอสทาแซนทินพบว่า กุ้งที่ได้รับแอสทาแซนทิน 500 ppm มีน้ำหนักถุงน้ำเชื้อมากที่สุด วิตามินอีมีผลต่อปริมาณเซลล์อสุจิและเปอร์เซ็นต์เซลล์อสุจิที่มีชีวิตอย่างมีนัยสำคัญ โดยกุ้งที่ได้รับวิตามินอีที่ระดับกลาง (400 ppm) มีปริมาณเซลล์อสุจิสูงกว่ากุ้งที่ได้รับวิตามินอีที่ระดับต่ำ (200 ppm) และ ระดับสูง (600 ppm) กุ้งที่ได้รับวิตามินอีที่ระดับกลางและสูง มีเปอร์เซ็นต์เซลล์อสุจิที่มีชีวิตสูงกว่ากุ้งที่ได้รับวิตามินอีที่ระดับต่ำอย่างมีนัยสำคัญ อย่างไรก็ตาม วิตามินอีไม่มีผลต่อความผิดปกติของเซลล์อสุจิ ในขณะที่แอสทาแซนทินมีผลต่อความผิดปกติของเซลล์อสุจิอย่างมีนัยสำคัญ โดยกุ้งที่ได้รับแอสทาแซนทินที่ระดับกลางและสูง (300 และ 500 ppm) มีความผิดปกติของเซลล์อสุจิต่ำกว่ากุ้งที่ได้รับแอสทาแซนทินที่ระดับต่ำ (100 ppm) อย่างมีนัยสำคัญ วิตามินอีและแอสทาแซนทินไม่มีความสัมพันธ์ร่วมกันต่อการสะสมของวิตามินอีและแอสทาแซนทินในเนื้อเยื่อกุ้ง ทั้งวิตามินอีและแอสทาแซนทินไม่มีผลต่อการสะสมของวิตามินอีทั้งในกล้ามเนื้อกุ้งและเฮปาโทเพนเครส แต่พบว่าที่ระดับเดียวกันของวิตามินอีมีการสะสมของวิตามินอีในเฮปาโทเพนเครสมากกว่าในกล้ามเนื้ออย่างมีนัยสำคัญ การเสริมวิตามินอีไม่มีผลต่อการสะสมของแอสทาแซนทินในเนื้อเยื่อกุ้งทั้ง 2 ชนิด ในขณะที่การเสริมแอสทาแซนทินมีผลต่อการสะสมของแอสทาแซนทินในกล้ามเนื้ออย่างมีนัยสำคัญ โดยพบว่ากุ้งที่ได้รับแอสทาแซนทินที่ระดับสูง (500 ppm) มีการสะสมของแอสทาแซนทินสูงกว่ากุ้งที่ได้รับแอสทาแซนทินที่ระดับต่ำ(100 ppm) อย่างมีนัยสำคัญ แต่แอสทาแซนทินทุกระดับไม่มีผลต่อการสะสมของแอสทาแซนทินในเฮปาโทเพนเครส ที่ระดับเดียวกันของวิตามินอีพบว่ามีการสะสมแอสทาแซนทินในเฮปาโทเพนเครสมากกว่าในกล้ามเนื้ออย่างมีนัยสำคัญ ในขณะที่แอสทาแซนทินที่ระดับกลางและสูง (300 และ 500 ppm ตามลำดับ) มีการสะสมแอสทาแซนทินในเฮปาโทเพนเครสมากกว่าในกล้ามเนื้ออย่างมีนัยสำคัญ แต่แอสทาแซนทินที่ระดับ 100 ppm ไม่มีความแตกต่างของการสะสมแอสทาแซนทินระหว่างกล้ามเนื้อกับเฮปาโทเพนเครสอย่างมีนัยสำคัญ จากการศึกษาแสดงให้เห็นว่าการเสริมวิตามินอีและ/หรือแอสทาแซนทินในอาหารสามารถช่วยพัฒนาการเจริญพันธุ์ของกุ้งขาวเพศผู้

ภาควิชา…….-……. ลายมือชื่อนิสิต……

สาขาวิชา…..เทคโนโลยีชีวภาพ…… ลายมือชื่ออาจารย์ที่ปรึกษา…….

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SUANGSUDA SUPASAI : EFFECTS OF VITAMIN E AND ASTAXANTHIN SUPPLEMENTATION ON MATURATION OF MALE WHITE SHRIMP *Penaeus vannamei* THESIS ADVISOR : ASSOC. PROF. SOMKIAT PIYATIRATITIVORAKUL, Ph.D. THESIS CO-ADVISOR : ASSIT. PROF. THAITHAWORN LIRDWITAYAPRASIT, Ph.D. ; 98 PP. ISBN: 974-17-6205-4

This study was to determine effects of dietary vitamin E and astaxanthin on maturation of male white shrimp *Penaeus vannamei*. The experiment was conducted using 3x3 factorials involved completely randomized design. The Combinations of 3 levels of vitamin E (200, 400 and 600 ppm) and 3 levels of astaxanthin (100, 300 and 500 ppm) were prepared from the same basal diet. Five month-old *P. vannamei* males were selected from an intensive rearing pond. At the beginning, only shrimp with no appearance of spermatophores were used for the experiment. The feeding experiment was operated 40 days in 8 replications. The results showed no interactions between dietary vitamin E and astaxanthin on growth, survival, reproductive performances (spermatophore weight, amounts of sperm, percentage of live and abnormal sperm), and vitamin E and astaxanthin content in whole body. Both vitamin E and astaxanthin had no significant effect on growth. Astaxanthin had also no significant effect on survival rate of the shrimp. For vitamin E, shrimp fed diets supplemented with 600 ppm vitamin E had significantly higher survival rate than those fed 200 ppm (P<0.05). Effects on reproductive performances: significantly higher mean of spermatophore weight was observed in shrimp fed diets with vitamin E 400 ppm, compared to those fed diets with vitamin E 200 and 600 ppm (P<0.05), while astaxanthin at 500 ppm provided significantly higher spermatophore weight than those shrimp fed diet containing 300 ppm astaxanthin (P<0.05). For sperm counts, all levels of astaxanthin showed no significant effect on number of sperms per spermatophore but vitamin E at 400 ppm had significantly higher sperm number than those fed with vitamin E 200 and 600 ppm (P<0.05). Shrimp fed with vitamin E 400 and 600 ppm had significantly greater live sperms than those fed 200 ppm (P<0.05) while astaxanthin showed no effect on live sperms. Percentage of abnormal sperms investigated as percentage of abnormal body sperm indicated no significant effect of both vitamin E and astaxanthin, when evaluated in term of percentage of abnormal spike, vitamin E enriched diets did not show any significant difference but shrimp fed diets containing astaxanthin 300 and 500 ppm had significantly lower abnormal spike than those fed diet containing 100 ppm of astaxanthin (P<0.05). Vitamin E content in all tissues was no significant effect among levels of both vitamin E and astaxanthin. However, vitamin E content in hepatopancreas was higher than in muscle at the same level of both vitamin E and astaxanthin (P<0.05). For astaxanthin accumulation, both vitamin E and astaxanthin had no effect on astaxanthin accumulation in hepatopancreas (P<0.05). In muscle, vitamin E had no significant effect among levels on astaxanthin content, while shrimp fed with astaxanthin 500 ppm had significantly higher astaxanthin content than those fed 100 ppm. At the same level of astaxanthin, shrimp fed with astaxanthin 300 and 500 ppm had higher astaxanthin content in hepatopancreas than in muscle, but shrimp fed with astaxanthin 100 ppm had no significant effect between muscle and hepatopancreas. The present study suggested that supplementation of vitamin E and/or astaxanthin in practical shrimp diets as appropriate amount can enhance reproductive performance in male white shrimp *P. vannamei*.

Department……-……..….. Student' signature ……

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CHAPTER I

INTRODUCTION

Penaeus vannamei (new name: *Litopenaeus vannamei*) or Pacific white shrimp has been the second non-indigenous shrimp species with potential for aquaculture in Thailand because it can grow well in low salinity area and require low protein content. Therefore, its dietary cost also has been cheaper than that of *Penaeus monodon*. Pacific white shrimp can also be stocked at small size, breed in captivity and has uniform growth rate when cultured in intensive rearing pond.

Production of nauplii depends on adequate broodstock maturation. However, high quality of shrimp larval productions depends on broodstock which imported from abroad such as Maxico, Panama and Peru. As a result, the culture for this species also has high cost. Moreover, broodstocks from above mentioned countries may carries viral diseases that has caused mass mortality of pond-reared shrimp Thus, pond-reared broodstocks are becoming widespread in Thailand. However, pond-reared broodstocks are not popular, due to poor reproductive performance (Menaseta *et al.*, 1993; Ramos *et al.*, 1995). Most researches concerned with improvement for the maturation and reproduction of penaeid species has focused primarily on female. Production of nauplii depends, among other variables, on adequate female maturation, and thus ovarian development in wild and pond-reared females has been compared histologically. In some studies, a higher quantity and quality of nauplii from wild stocks have been reported (Menasveta *et al.*, 1993; Cavalli *et al.*, 1997). In other studies, there were practically no differences between wild and captive stocks (Menasveta *et al.*, 1994a; Palacios *et al.*, 1999).

However, problems with the captive reproductive penaeids are attributable, not only to females, but also to males. When wild males were compared to pond-reared males, the wild males had better reproductive performance in terms of fertilization rate, hatching rate and number of nauplii (Ramos *et al.*, 1995) and in term of spermatophore weight (Pratoomchart *et al.*, 1993). Conversely, there are indications that male reproductive problems may have been responsible for the limited success experienced in the captive of reproduction of several penaeids species

(Leung-Trujillo and Lawrence, 1987). Nutrition deficiency may be one of the reasons for such problems in captive male (Cahu and Fakhfakh, 1990).

An optimal diet is one of the crucial factors for sexual maturation and reproduction of shrimp. Shrimp maturation diet is similar to other shrimp diets as it compose of basal ingredients e.g. carbohydrate, protein, lipid, vitamins and minerals but it has additional these fundamental aspects for improvement in maturation. Vitamin E and astaxanthin are interestingly nutritional factors for improvement to maturation in broodstocks.

Vitamin E is likely to be that of antioxidant protection for membrane-bound polyunsaturated fatty acids (PUFA) (Conklin *et al.*, 1997). It affects phagocytosis and humoral and cellular immune responses, and enhances proliferation and bacteria activity of phagocytes. Vitamin E stimulates cell proliferation in immunopoietic organs. It also modulates prostaglandin biosynthesis (Blazer and Wolke 1992). Vitamin E is an essential vitamin for *P. vannamei* (He *et.al.*, 1992) and can improve the percentage of normal sperm and the rate of ovarian maturation in *L. setiferus* (Chamberlain, 1988). It also plays a major role in reproductive performance. For example, high vitamin levels play a positive role when provided in combination with high levels of PUFA and cholesterol for *P. vannamei* broodstocks (Wouters *et.al.*, 1999)

In addition, several recent studies have suggested that carotenoids, including astaxanthin are potent antioxidants in *in vitro* memebrane models and that the operate synergistically with vitamin E (Nishigaki *et al.*,1994). The major pigment found in shrimp, astaxanthin, cannot be synthesized by most animals (Goodwin, 1984). The proposed functions for astaxanthin in aquaculture have been those of; provitamin A activity, antioxidant properties, positive effect on embryonic or larval development, cellular protection from photodynamic damage, enhancement of growth, maturation and reproduction, fecundity, formation of in-chain epoxides that act as oxygen reserves under anoxic condition (Goodwin, 1960).

The concentration and distribution of vitamin and pigments storage in penaeid shrimp varies with species, life history stages, tissues or organs of the animals and rearing conditions, mainly the food sources, whereas, it is still unclear about effective levels of vitamin E and astaxanthin on reproductive performance of male *P. vannamei*. Therefore, this study is interesting because their levels are important for

effective shrimp maturation. The result should be use as a criterion in the maturation diets.

The purposes of the present study were to determine effective levels of vitamin E and astaxanthin supplementation in practical diets on maturation of male white shrimp *P. vannamei*, using spermatophore weight, sperm count, percentage of live and abnormal sperm and their tissues accumulation as indicators.

CHAPTER II

LITERATURE REVIEW

2.1 Pacific white shrimp *Penaeus vannamei*

2.1.1 Natural history of Pacific white shrimp *Penaeus vannamei*

P. vannamei (Pacific white shrimp or Western white shrimp) prefers to live on muddy areas at depth from the shoreline down to about 72 meters [235 feet] (Dore and Frimodt, 1987). The distribution of this marine shrimp is mostly in the eastern pacific region from Sorona, Maxico to the northern Peru (Perez Farfante and Kensley, 1997). This is the leading form-raised species in the western hemisphere, representing more than 95% of its production. These shrimp can grow in low salinity areas. Moreover, they have an ability to resist to environmental changes, such as water quality and temperature, better than other cultured species. Throughout Latin America, hatcheries maintain of broodstock, which some of them are pathogen-free, some of them pathogen-resistant and some of them in captivity for almost 30 years. Farmers throughout Asia are switching to *P. vannamei*, and it has a potential to become the dominant species in Asia.

The coloration of *P. vannamei* is translucent white, thus it is most commonly known as the white shrimp. The body of this species often has a bluish hue that is due to a predominance of blue chromophores, which are concentrated near the margins of the telson and uropods (Eldred and Hutton, 1960).

Its carapace is translucent, permitting the color of ovaries to be seen. In females, the gonad which is first whitish, turns golden brown or greenish brown on the day of spawning (Brown and Patlan, 1974). The males deposit the spermatophore only on hard-shell females, which will spawn in a few hours later. The courtship and mating behavior begins in the afternoon in relation to light intensity. Regression of developing ovaries is very rare and development of the ovaries leads almost every time to spawning. The spawning process begins by sudden jumps and active swimming of the female and the whole process lasts about one minute. The cortical reaction is very rapid and first segmentation occurs in a few minutes (Ogle, 1992).

The number of eggs varies according to the size of the animal. (*P. vannamei* of 30 to 45 g size; 100,000 to 250,000). Cleavage to the first nauplius stage occurs approximately 14 hours after spawning (AQUACOP, 1979). This species has six nuaplii stages, three protozeal stages, and three mysis stages in its life history (Kitani, 1986).

2.1.2 Male reproductive system

The male reproductive system is composed of a pair of testes and vas deferentia terminating in ampoules containing spermatophores, or sperm packets, at gonopores in the base of the fifth pair of pereiopods. The testes of *P. vannamei* consist of multiple, independent testicular lobes, each of which made connection with anterior proximal vas deferens by means of collecting tubes. The multi-lobed, transparent testes are located above the heart, which is situated dorsally over the digestive gland. Talbot *et al*.,(1989) reported that one testicular lobe of *P. setiferus* contained several stages of spermatogenic cells. Chow *et al*.,(1991a) described five stages in the cycle of changes in the seminiferous tubule accompanying development of spermatogenic cells: 1) primary spermatocyte production, 2) spermatogenesis to late spermatids, 3) lumen formation by retreat and lining of sustentacular cells, 4) emptying of sperm to proximal vas deferens, and 5) disappearance of the lumen and resumption of stage 1.

The vasa deferentia consist of four distinct regions: proximal, medial (ascending and descending), distal and terminal ampoule, and they are in the ampoule were the process of spermatophore formation takes place (Talbot *et al*., 1989; Ro *et al*., 1990; Chow *et al*.,1991b). The milky-colored vasa deferentia extend posteriorly from the testes, and ventrally connect to the gonopores. In mature males, spermatophores are clearly visible through the exoskeleton from ventral or lateral views at the fifth pair of pereiopods. The two spermatophores, one from each terminal ampoule, are fused longitudinally at the time of extrusion (mating with a female) and then are referred as the compound spermatophore or simply the spermatophore in open thelycum species (Bray and Lawrence, 1992).

Males have a specialized structure called the petasma which is presumed to be used in spermatophore transfer. The petasma is roughly triangular, membranous flap

connected to the first pair of pleopods. The petasma in young animals is unjoined, but becomes zippered in subadulthood (Bray and Lawrence, 1992).

In mature *P. vannamei* males, the petasma is symmetrical, semiopen, not hooded, lacking distomedian project ions, and has short ventral costae, not nearly reaching distal margin and distinctly gaping (Perz Farfante and Kensley, 1997).

Motoh (1981) reported that specimens at average size of 11 mm CL possess a small rudimentary petasma in the form of a knife-shaped projection situates on the subapical portion of the protopod. Until at the size of 31 mm CL, the modified endopod closely resembles the petasma of the adult, the two halves are now large enough so that their inner margins meet at the median line and are thus united or fused together with the aid of numerous minute hooks. However, The two components could be easily separated by physical force. The spermaphores are extremely complex, consisting of a sperm mass encapsulated by a sheath and bearing various attachment structures (anterior wing, leteral flap, caudal flange, dorsal plate), as well as adhesive and glutinous materials (Chow *et al*., 1991).

Spermatozoa are contained within the spermatophore in a viscous, slightly grayish or milky medium. These sperms are non-motile with characteristic sperical portion and cap, with spike extending outward from the sperical portion (King, 1948). Leung-Trujillo (1990) has shown that number of sperm is positively correlated with male total weight in *P. setiferus*. An adult male *P. setiferus* of 35 g may carry some 70 million sperms per spermatophore.

For *P. vannamei*, Leung-Trujillo and Lawrence (1985) shown that an adult male produced 32 million sperms per a compound spermatophore. In addition, Alfaro (1996) reported that a 20-g male *P. vannamei* synthesized 11 million sperms per compound spermatophore at the first assessment. After ejaculation, the second assessment gave 4 million sperms per a compound spermatophore.

2.1.3 The development of male reproductive system

Castille and Lawrence (1989) reported that the maturation was visually evaluated on the basis of spermatophores in the terminal ampullae and devided in to 3 stages namely:

***Immature stage*:** The spermatophores were not visible through the exoskeleton. No immature stage males were sampled.

***Developing stage*:** The spermatophores were partially visible through the exoskeleton.
***Mature stage*:** The spermatophores were clearly visible through the exoskeleton and could be manually ejected with gentle pressure.

Leung-Trujillo and Lawrence (1991) observed developmental stages of the spermatophore at the terminal ampoule level into four stages as the followings:
***Stage I (undeveloped)*:** Small amount of an opaque, milky white substance with a very fluid consistency removed from terminal ampoules. No organization or presence of sperm detected.
***Stage II (early developing)*:** Fluid of higher viscosity than at Stage I. Small, thin pieces of hardened material of variable form interspersed in the fluid were also observed (approximately 1-3 mm.). No sperm or sperm mass detected.
***Stage III (late developing)*:** First evidence of a recognizable spermatophore: semicylindrical sperm sac containing sperm, wing (in *P.setiferus* and *P.stylirostris* only), and the presence of attachment structures. Spermatophore is unhardened, extremely thin, soft, papery, and white in color.
***Stage IV (mature)*:** Spermatophore in final form. Spermatophore completely hardened with characteristic yellow to golden tint, ready for transfer to female.

Heizmann *et al*.,(1993) reported that spermatophore regeneration in *P. vannamei* was shown to be closely related to the intermolt cycle, the spermatophore and sperm mass was arrived in the terminal ampulla during the night of molting.

2.1.4 Practical aspects of reproduction in maturation system

2.1.4.1. Unilateral eyestalk ablation

Ablation presumably reduced the inhibitor control over reproduction resulting from the synthesis and release of Gonad Inhibitory Hormone from the eyestalk neurosecretory complex.

In male, the reproductive performance of eyestalk ablation has been explored for several species. Chamberlain and Lawrence (1981) reported that male eyestalk ablation increasing gonad size, development synchrony and mating frequency in subadult *P. vannamei*. Leung-Trujillo and Lawrence (1985) demonstrated increase gonad weight, gonad weight, gonad index, spermatophore weight and sperm counts

for ablated males *P. vannamei* with no adverse effect on sperm quality. In unilateral eyestalk ablated male, *P. setiferus* accelerated spermatophore production time by approximately 2 days, and significantly increased spermatophore weight and sperm count without affecting sperm quality (Leung-Trujillo and Lawrence, 1991). Ablation of male *P. stylirostris* showed no significant increase in fertilization rates or numbers of natural matings (Ottogalli *et al*., 1989). No effects on reproduction were discerned in ablated male *P. canaliculatus* but the intermolt period was shortened (Choy 1987). Browdy and Samocha (1985) reported no significant differences in mating frequency, spawn size, fertilization or hatch for females mated with ablated versus nonablated *P. setiferus*. Eyestalk ablation of males is not usually practiced in commercial maturation units, as spermatophore development rates are not considered to be a limiting factor for nauplii production.

Moreover, the exogenous steroid hormones are reported to improve in spermatophore quality. Alfaro (1996) reported that 17 α–methyltestosterone induced an important in spermatophore quality of *P. vannamei* by injecting a single dose of 0.1 and 0.01 μg/g body weight, sperm counts increased 3 fold in 20-g males. Additionally, a low percentage of sperm abnormalities were obtained.

Negabhushanam and Kulkarni (1981) reported the positive effect of testosterone propionate and testosterone acetate on androgenic gland activity and spermatogenesis/spermatophore synthesis in *Parapenaeopsis hardwickii*. These results could be the consequence of a pharmacological effect, activating spermatogenesis related molecules or it could be that these molecules were complementary to an androgenic gland hormone (AGH) for the intensification of spermatogenesis/ spermatophore synthesis.

2.1.4.2. Enviromental factors

The level and rate of change of a number of enviromental factors have been correlated with reproduction of penaeid shrimp in their natural habitat. These include temperature, salinity, photoperiod, light intensity and spectrum, and substrate. Each of these factors could play a role in the initiation or deferral of reproductive process for given species.

The optimization of the environmental factors for maturation in captivity includes duplication, and whenever possible amplification, of those elements which

may be natural stimulatory cues while concurrently eliminating or reducing those system elements which may depress or block reproductive performance. Thus monitoring stability of environmental factors is an important component of maturation system design (Browdy, 1992). Handling of animals along with careful cleaning regimes can all be very significant in reducing stress in the system (Wyban and Sweeney, 1991).

2.1.4.3. Nutrition

Nutritional factor plays critical roles in the promotion sexual maturation and mating, the enhancement of fertility and fecundity and the establishment of viability and quality of seed. In maturation systems, natural food including crustaceans, fish and polychaetes are commonly used only in combination with or without supplementation of dried feeds. Combination diets of fresh frozen marine organisms have outperformed diets composed of any of the components fed single (Chamberlain and Lawrence 1981, Bray *et al*., 1990)

Marine polychaetes (bloodworms: *Glycera dibranchiata*) are considered by many maturation system managers to be essential for successful nauplii production of *P. vannamei*. Bloodworm supplementation has been increased reproductive performance (Gomez and Arellano, 1987) and it has been suggested that certain polyunsaturated fatty acids in bloodworms may help trigger maturation (Lytle *et al*., 1990). Some evidence pointed potential stimulation of reproductive performance by feeding frozen reproductive adult Artemia (Browdy *et al*., 1989). Moreover, Some reports emphasized feeding of mussels or trochus for maturation (AQUACOP, 1977). Macroalgae have also been used as a supplement to maturation diets. Fresh frozen squid is commonly fed in maturation systems, with other components added according to ability and price with the goal of providing rich diverse. Feeding of frozen shrimp should, however, be avoided since there is a possibility of disease transmission.

Several recent studies focused on improvement of dried feed supplements. Freshness of pelleted feeds is critical. Galgani *et al*.(1989) reported improved reproductive performance for *P vannamei*, *P. stylirostris* and *P.setiferus* when part of the diet was replaced with adequately formulated pellets including broken squid or shrimp. Adding soy lecithin supplements to the dry feed component of maturation

diets (40% squid 60% pellets, dry weight) significantly increased nauplii production, hatch and sperm counts compared with unsupplemented controls (Browdy *et al*., 1992)

Nutrition is profoundly important to reproduction of penaeus, and the success of reproduction is closely related to nutrient ingestion accompanying spermatogenesis/spermatophore synthesis. Nutritional value of fresh diets can vary in nutritional quality with species, lifestage, season of collection, nutritional condition and storage methods. Therefore, pelleted diets are additional choice for stimulation of broodstock. The improvements of current fed diets for captive broodstock prior and during maturation have been examined and may yet prove effective as means of improving reproductive performance (Primavera *et al*., 1979).

There has long been and interested artificial diet for penaeid broodstock. Artificial diets offer many advantages over the use of fresh-frozen diets including a reliable supply, minimal preparation time, known nutrient contents and reduced tank fouling. Artificial diets also offer the opportunity to orally administrative drugs such as hormones or supplementary vitamins.

Shrimp maturation diet is similar to other shrimp diets as it compose of basal ingredients e.g. carbohydrate, protein, lipid, vitamins and minerals but it has the addition of these fundamental aspects. Vitamin E and carotenoids, such as astaxanthin, are interestingly nutritional factors for improvement to the maturation in broodstocks.

2.2 Carotenoids

2.2.1 Nature and chemical structure of carotenoids

Carotenoids are natural, lipid soluble pigments known as lipocromes. In nature, they are produced via an isoprenoid pathway shared with such diverse chemical compound as essential fatty acid, steroids, and vitamin A, D, E and K. Within the various classes of natural pigments, the carotenoids are among the most widespread and structurally diverse pigmenting agents. They are almost universally distributed, occurring in the most primitive bacteria and algae up to the highly developed flowering plants and mammals. At least 600 different natural carotenoids have been identified. They are responsible for many of brilliant yellow to red colors

in plants and animals, as well as the variety of bluish, greenish, purplish, brownish and blackish colors seen in many fishes and crustaceans (Latscha, 1990, 1991).

Carotenoids are structurally related to vitamin A and β-carotene, the main source of vitamin A for animals. Both in nature and through chemical synthesis, β-carotene can be considered as the basic compound for many chemical reactions. It consists of 40 carbon atoms arranged in two β-ionone rings connected by a chain of conjugated double bonds representing the chromophore which is responsible for typical color of carotenoids **(Figure 2.1)**.

Despite their distribution in almost all living matters and their widespread abundance in a variety of aquatic animals, carotenoids are synthesized *de novo* only by plants and some microorganisms. Thus, animals are dependent on an exogenous dietary supply of carotenoids to meet metabolic nutritional requirements. Provision of a natural source of these pigments is impractical and limited. Efficiency is primarily related to the proximity of the dietary carotenoids to astaxanthin in the metabolic pathway of synthesis **(Figure 2.2)**.

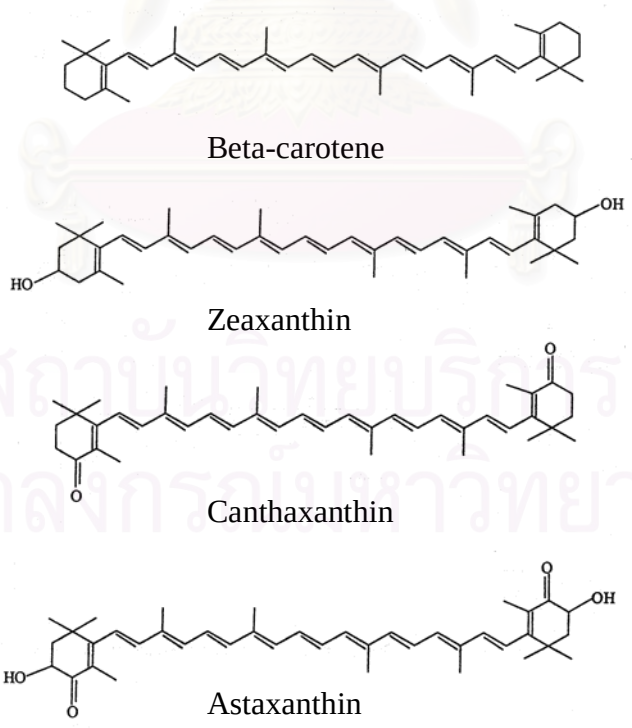

Figure 2.1. The chemical structure of some selected carotenoids

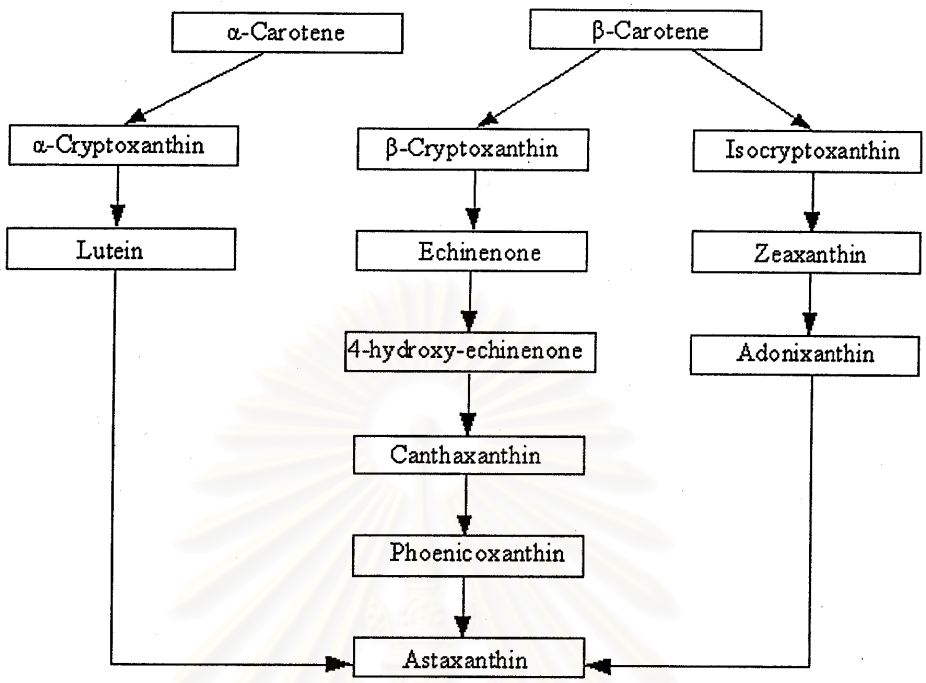

Figure 2.2. Metabolic path ways of carotenoid synthesis in crustaceans (Latscha, 1990)

Bicyclic carotenoids, β-carotene or xanthophylls (zeaxanthin) are ingested from plants and may be converted to astaxanthin (Simpson *et al.*, 1981; Torrissen *et al.*, 1989; Latscha, 1990). Organism feeding on lower forms may be able to absorb pigments that are the intermediate between β-carotene and astaxanthin. These carotenoids may be deposited unaltered or metabolically transformed. Animals often demonstrate a marked degree of selectivity in absorbing specific carotenoids or metabolically transforming them. The type of pigments absorbed and the specific rate of absorption can vary considerably between families or species of animals. Thus, while some carotenoids may be used as astaxanthin precursors by certain species, they may not be used by others.

The ability of marine organisms to convert dietary carotenoids into astaxanthin falls into three general categories (Meyers and Chen, 1982).

1. Those which cannot oxidize the β-ionone rings and can only utilize the specific oxygenated derivatives like astaxanthin for deposition are called the salmonid or sea bream type.

2. Those which can oxidize the 4and 4’ positions of the β-ionone rings and can covert dietary zeaxanthin to astaxanthin depositing the majority of dietary carotenoids unchanged are called the carp type.

3. Those which can oxidize the 3 and 3’ positions and 4 and 4’ positions of the β-ionone and may convert β-carotene, zeaxanthin or intermediates to astaxanthin are called the crustacean type.

2.2.2 Carotenoids in crustaceans

Most crustaceans contain carotenoids pigments in some combination in carapace as well as in eyes, blood, eggs, midgut gland, and ovary. The usual pigments isolated from various classes of crustacean are astaxanthin, β-carotene, echinenone and canthaxanthin. Such carotenoids can be obtained directly from dietary origin or derived from metabolic transformation of another dietary carotenoids.

Astaxanthin has been described as the most frequent end-product of carotenoid metabolism in crustacean (Katayama *et al.*, 1972) and identified as the main pigment in the adult prawn, *Penaeus japonicus* (Katayama *et al.*, 1971, 1972; Katagiri *et al.*, 1987). Tanaka *et al*.(1976) indicated that astaxanthin was the most prominent in both carapaces and internal organs of seven crustacean species and other carotenoids that isolated from them included β-carotene, echinenone, canthaxanthin, lutein, zeaxanthin, 3-hydroxy-canthaxanthin and 3,3’-dihydroxy-carotene. There are various factors affected the amount and distribution of carotenoids in crustaceans, including embryogenesis, sexual cycle, molting, background colors and hormonal control (Goodwin, 1960).

Yamada *et al*. (1990) examined the effect of diatary carotenoids, *i.e*., β-carotene, astaxanthin and canthaxanthin on pigmentation of *P. japonicus*. All three carotenoid sources were deposited in tissue of prawn as astaxanthin esters. After 8 weeks, total carotenoid and astaxanthin ester concentrations in prawns fed with astaxanthhin-supplemented feed were significantly higher than those of prawns supplied β-carotene or canthaxanthin- supplemented feed. Total carotenoid and astaxanthin concentrations increased as dietary levels of pigment increased up to 200 ppm, deposition also increases to a maximum of 29.1mg kg^{-1}body weight.

Negre-Sadargues *et al*.(1993) examined the utilization of synthetic carotenoids in *P. japonicus* and found the accumulation of dietary astaxanthin in the integument (carapaces and epidermis) and hepatopancreas. The investigation of carotenoid composition in the exoskeleton of *P. monodon* demonstrated astaxanthin being the major carotenoids, accounting for 86-98 % of total carotenoids (Okada *et al*., 1994). Captured prawn mainly accumulated astaxanthin monoester in their exoskeleton, converting all precursors to astaxanthin until a certain level (8 mg 100 g^{-1}) of carotenoid was reached. It was postulated that *P. monodon* may need to store carotenoids, mainly free astaxanthin, in order to build carotenoprotein. When the prawn receives sufficient free astaxanthin, excess dietary caroenoids are accumulated as astaxanthin esters or other carotenoids like β-carotene.

Astaxanthin and astaxanthin esters are the primary pigments of the fresh water prawn, *Macrobrachium rosenbergii* (Maugle *et al*., 1980). Evidence suggested that *M. rosenbergii* can convert β-carotene into astaxanthin via isocrytaxanthin, echinenone, and canthaxanthin. Eyestalk ablation affected deposition and metabolism of carotenoids, in all likelihood through acceleration of the molting cycle (Maugle *et al*., 1980; Castillo and Negre-Sadargues, 1991). D′Abramo *et al*. (1983) reported the effectiveness of dietary β-carotene, echinenone and canthaxanthin in the production of the primary tissue carotenoid astaxanthin of juvenile American lobsters, *Homarus americanu*s. Level of pigmentation produced by these astaxanthin precursors was related to its proximity to the astaxanthin and end products. Free astaxanthin represented the bulk of carotenoids in the unhatched embryo whereas larval, postlarval and juvenile stages exhibited the typical carotenoid pattern in which esterified forms of astaxanthin predominated.

Carotenoid astaxanthin, is the main pigment of crustaceans. Due to the presence of two identical asymmetric C-atoms at position 3, astaxanthin exists in the form of four different optical isomers [(3S, 3′S), (3S, S′R), (3R, 3′S) and (3R, 3′R)], of which two [(3S, 3S′R), (3R, 3′S)] are identical **(Figure 2.3)**. Various studies have focused on these isomers relative to pigmentation (Foss *et al*., 1987; Katsuyama *et al*., 1987). Of the geometric isomers known, the *trans*-form is the most stable and abundant.

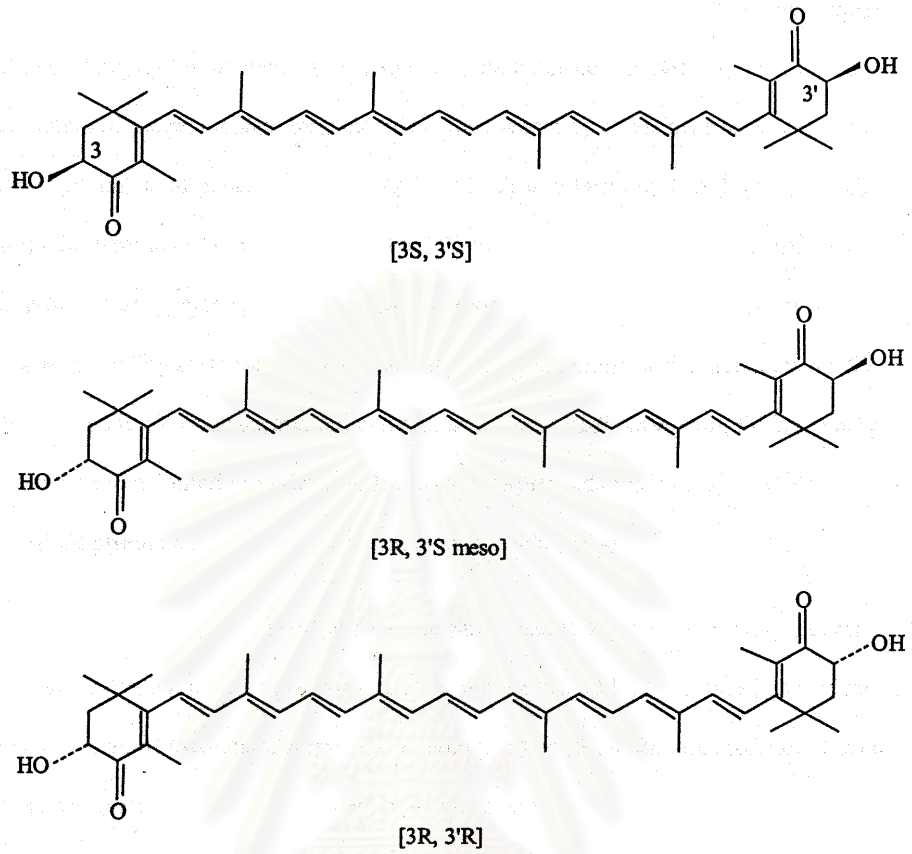

Figure 2.3. Optical isomers of astaxanthin

Astaxanthin is represent in nature ether in the free form, esterified to long chain fatty acids, or associated with proteins forming carotenoproteins. All forms are found in crustaceans. The bulk of pigments in the crustacean epidermal tissue is in the ester form with dominate in the exoskeleton. A keto group is generally essential for formation of carotenoid protein complexes and two keto groups in astaxanthin, are necessary for optimal bonding.

The total carotenoid concentration found in caridae and penaeidae range from about 60 mg kg^{-1} to as much as 499 mg kg^{-1}. The latter have been observed in some members of the penaeidae. The interspecific differences of the total carotenoid concentration may be as large as 300%. Most wild specimens of penaeid species involved in shrimp farming have levels of total body carotenoids ranging between 80 to 200 mg kg^{-1}. Qualitative composition varies in group-and species-specific. The vast majority of decapod crustaceans are characterized by a predominant accumulation of astaxanthin which accounts for 65-98% of the total tissue carotenoid

content. In the case of *P. monodon*, the remaining 2% may be attributed to various yellow to red carotenoids which may represent transient products of carotenoid metabolism (Latscha, 1991).

The carotenoid content of crustaceans is essentially based on body-weight. However, content is more precisely related to the animal's body surface because the vast majority of carotenoid pigments are concentrated in the thin exoskeleton. Given a specific carotenoid content, pigment concentration will decrease with growth suggesting an apparent pigment loss over time. This apparent loss of carotenoid was a decline. In the average concentration of carotenoid was the loss of pigments contained within the exoskeleton during molting. While the latter loss may be as high as 85% of the total carotenoid content in some lobster species, only a relatively small proportion attributed to the impact of body mass or surface increase (Meyers and Latscha, 1997). The larger proportion (approximately 70%) is attributed to the impact of body mass or surface increase. Total average losses in *P. monodon* during various pigmentation trials resulted in a mean loss of 33.6% after 30 days of culture (Meyers and Latscha, 1997).

2.2.3 Function of dietary carotenoids

2.2.3.1 Aquatic ecosystems

Astaxanthin is the most common carotenoid of the marine invertebrate zooplankton (Fisher *et al*., 1964). Astaxanthin in selected species of crustaceans found in free and esterified forms (Foss *et al*., 1987). Studies of copepods, genus *Calanus* and *Euphausids*, have repeatedly demonstrated the presence of astaxanthin (in the free and esterified 3R, 3′R form) as the predominant. In the investigation of 11 species of Euphausiacea, including *Euphausia superba*, astaxanthin or its esters were the only carotenoid found (Fisher *et al*., 1955). Such crustaceans form a vital link in the transfer of nutrients from phytoplankton to zooplankton to higher marine invertebrates, with conversion of astaxanthin by euphausids to vitamin A.

The tissue carotenoid, astaxanthin, of marine zooplankton cannot be synthesized *de novo*, but requires assimilation of a precursor which serves as a template for production (Kleppel *et al*., 1985). In crustaceans, the chief precursor is

β-carotene. The change of carotenoid levels in body of natural marine zooplankton was found to be correlated with *in situ* feeding activity (Kleppel *et al*., 1985).

2.2.3.2 Biological function of astaxanthin

Torrisen (1990) indicated that among the proposed functions for astaxanthin in aquaculture have been those of; provitamin A activity, antioxidant properties, positive effect on embryonic or larval development, cellular protection from photodynamic damage, enhancement of growth, maturation and reproduction rate and fecundity, formation of in-chain epoxides that act as oxygen reserves under anoxic conditions.

Carotenoids could be devided into two groups according to their physiological functions. Phenological functions are intraspecific and interspecific interactions and behavioral pattern influenced by color, such as luring prey, warning, camouflaging or reproduction. In confine of α-tocopherol aquaculture, animals in all likelihood are deprived of their natural food sources. Thus, if respective carotenoids normally present in the natural diet are not include in the feed , the color of integuments and eggs of fish and crustaceans will fade or disappear completely. In natural environment, functions related to predation and defense provided by carotenoids, along with sex attraction based on color, are ecological and biological significance.

2.2.3.3 Metabolic function

Torrisen (1990) reported that the vital role of carotenoids in the physiology and overall health of plants and animals, and concluded that carotenoids are essential and should be included in all aquatic animal diets. Similarity in function of astaxanthin and canthaxanthin to alpha-tocopherol (vitamin E) and retinol (vitamin A) has led to the suggestion that these two carotenoids be listed among the fat soluble vitamins.

Apart from its obvious role in pigmentation, increasing attention is being directed toward defining the biological activities of astaxanthin in aquatic animal, with the majority of the research focusing on the Atlantic salmon and rainbow trout. The review articles by Torrisen *et al*. (1989) and Torrisen. (1990) concluded that carotenoids were essential nutrients for salmonids and noted similarity in action of

astaxanthin comparable to that of vitamin A and E. Other studies using ^{14}C-labeled compounds in salmonid feeding trials have demonstrated the function of astaxanthin in various metabolic processes as well as its association with vitamin A metabolism.

Among the diversity of physiological functions, provitamin A activity of carotenoids contain β-ionone rings like astaxanthin is centrally important. Vitamin A is not available from plants and is derived from the conversion of provitamin A active carotenoids.

2.2.3.4 Antioxidant properties

Carotenoids can be characterized by their capacity to interact with a chemically reactive species of oxygen called "singlet oxygen" (Ranby and Rabek, 1978). The latter participates in oxidative reactions which can impair or destroy important cellular parts, including membranes, enzymes, and DNA nucleic acids. Singlet oxygen reactions can lead to formation of "free radicals" another species of reactive molecules capable of causing damage to cellular components. Certain carotenoids, notably β-carotene, due to their particular chemical structure, can neutralize the reactivity of singlet oxygen, thus serving as an effective antioxidant protector. β-carotene is recognized as a lipid antioxidant which causes lipid peroxidation and photosensitivity. β-carotene's lipid protecting affect complements that of vitamin E, depending on the oxygen content of the tissue. Increasing investigations of antioxidant are demonstrating the beneficial metabolic role of β-carotene as an antioxidant vitamin in protection against chemically induced toxicity (Kornhauser *et al*., 1994).

Antioxidant activity of astaxanthin is higher than β-carotene 10 times, and vitamin E approximately 100 times (Miki, 1991). Palozza and Krinsky (1992) studies further document the role of astaxanthin as an extremely effective antioxidant. Carotenoids are also active in cross membrane calcium transfer, serving as oxygen reservoirs in the neuronal calcium transfer, serving as oxygen reservoirs in the neuronal respiratory chain and protecting sensitive tissues reactive compounds from damage due to oxidation. Thus, carotenoids are now thought to function as antioxidants during hemolymph transport and within eggs, protecting nutrients and embryonic tissues from oxidative damage. They also confer structural stability to

lipoproteins by forming nonstochiometric complexes, thus protecting the nutrients until they are required and used. Canthaxanthin and astaxanthin are more effective antioxidants than β-carotene in stabilization of free radicals (Terao, 1989).

2.2.3.5 Membrane stabilization

Carotenoids in conjunction with protein moieties, *i.e*., carotenoproteins of the crustaceans, appear to improve the stabilization of proteins and their tertiary structure (Chessman *et al*., 1967). These interactions are believed to be involved in changes in membrane permeability and composition and consequently may contribute indirectly to the maintenance of water balance.

In nauplius to postlarval stages, shrimp initially obtains precusors of astaxanthin in vitellin reserves, then in algae and *Artemia* (Petit *et al*., 1991). These investigators also studied the ontogeny of carotenoid metabolism in *P. japonicus* and hypothesized a storage of astaxanthin in their eggs. This condition suggested a possible role of carotenoids in reproduction and during embryonic and post-embryonic development. While astaxanthin is deposited as the free form in larvae, it occurs mainly as esterified forms in postlarvae with the percentages of these forms increasing until stage P20. The ability to metabolize precursor pigments appeared in the postlarval stages and was linked to the development of oxidation and esterification pathways of carotenoids.

Desy *et al.* (1995) studied carotenoid metabolism in early developmental stages of the European lobster *Homarus gamarus* and observed a rapid decrease of pigment concentration immediately after hatching. Free astaxanthin represented the massive of carotenoids in the unhatched embryo whereas larval, postlarval, and juvenile stages exhibited the typical carotenoid pattern in which esterified forms of astaxanthin predominated. During certain physiological processes, such as molting or maturation, significant quantities of pigments may be relocated to other tissues like hemolymph, midgut gland, gonads, and eggs. Menasveta *et al*.(1994b) showed that prawns fed astaxanthin added diet has significantly greater egg diameter but no increase in maturation or spawning frequency of non-ablated prawns fed with the astaxanthin added diet.

2.3 Vitamin E

Vitamin E was initially discovered as an antisterility factor in rats, but it has now been generally recognized as essential for all animals. It is a naturally occurring antioxidant, which specifically inhibits the oxidation of unsaturated fatty acids.

The term vitamin E refers to a group of four naturally occurring tocols and four tocotrienols having a common molecular structure made up of a chromanol and an isoprenoid side chain, each one exhibiting different stereochemistry and biological activity **(Figure 2.4)** (IUPAC-IUB commission on Bichemical Nomenclature, 1974). Vitamin E activity in plant foods derives from α-, β-, γ-, and δ-tocopherols and corresponding to tocotrienols. In animal tissue α-tocopherol is the predominant form.

The nutritionally important isomers of α-tocopherol are the naturally occurring *d*-α-tocopherol and chemically synthesized *dl*-α-tocopherol and their acetates or succinate esters, which are used as food additives and in pharmaceutical preparations. Tocopherols are stable to visual light but are destroyed by ultraviolet light and darken gradually on continued exposure to light. On the contrary, tocopherol acetates are less readily soluble in ethanol and practically unaffected by the oxidizing influences of air, visual light, and ultraviolet light.

Vitamin E deficiency is manifested by a variety of symptoms that are highly variable from species to species. Some of these are: testicular degeneration, fetus resorption, deposition of carotenoid pigments in the masculature of small intestine and uterus, increased fragility of erythrocytes, increased urinary excretion of creatine, etc. Current information on certain biochemical, hematological, and clinical aspects is available (Lubin and Machlin, 1982). Compared with vitamins A and D, vitamin E is relatively nontoxic.

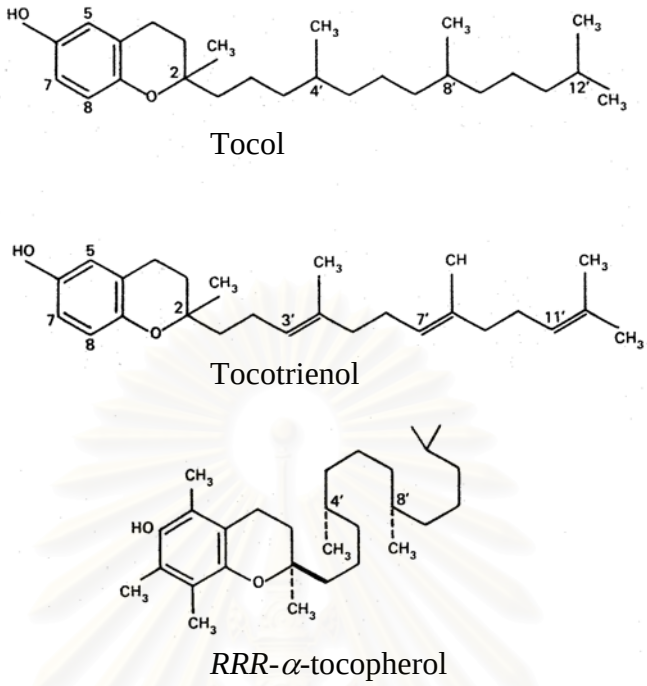

Figure 2.4. Basic structure of tocol and tocotrienol compounds

In aquatic animal, the primary role of vitamin E is likely to be that of antioxidant protection for membrane-bound polyunsaturated fatty acids. These fatty acids are particularly susceptible to autoxidation cascade (lipid peroxidation) and vitamin E is the only major antioxidant located in the membranes of cells (Burton and Trabor, 1990). The same role can be expected for crustaceans; indeed, studies with filter-feeding microcrustaceans (Conklin and Provasoli, 1977) suggested the essentiality of α-tocopherol (vitamin E) could be extended from vertebrates to crustaceans. As of α-tocopherol is synthesized only by plants, algae, either directly or indirectly, are the ultimate source of this compound for crustaceans in nature.

Vitamin E requirements in fish increase when levels of dietary polyunsaturated fatty acids (Schwarz *et al*., 1988; Roem *et al*., 1990) and their degree of unsaturation increase. Requirements of vitamin E also increase with factors that exacerbate the potential for lipid peroxidation, such as oxidation of feed oils (Hung *et al*., 1981). However, within the feedstuff oils themselves there are some endogenous tocopherols, including α-tocopherol, that serve as feed antioxidants. Hung *et al*.(1981) found the endogenous tocopherols in good quality fish oil, by themselves,

can provide adequate antioxidant protection. The most common added form of vitamin E used in the formulations of commercial diets is α-tocopherol acetate. The acetate portion of this form of vitamin E is removed by hydrolysis during digestion and the active form of vitamin, α-tocopherol, is incorporated into membrane lipids and serve as antioxidant (Hung *et al*., 1982; Bjorneboe *et al*., 1990).

The *P. vannamei* larvae fed with diet without vitamin E supplement had significantly the lowest survival and exhibited a darkening of the hepatopancreas. This result suggested that vitamin E is an essential nutrient in shrimp diets for *P. vannamei* (He *et al*., 1992).

To study vitamin E dietary requirements of juvenile *P. vannamei*, He and Lawrence (1993) used vitamin E stripped corn oil in combination with fatty acids extracted from menhaden oil to eliminate endogenous vitamin E in the diet. The growth of *P. vannamei* juveniles reared on this diet was comparatively slow compared to that of those fed a control containing vitamin E. However, with addition of synthetic antioxidant, butylated hydroxytoluene (BHT), to the diet, the shrimp grew well, even in the absence of supplemental vitamin E (He and Lawrence.,1993). Based on their studies, the requirement for vitamin E is approximately 100 mg/kg of diet.

In adult shrimp, the use of α-tocopherol by the broodstock of *P. indicus* indicated that hepatopancreas appeared to be the main storage organ for α-tocopherol. In males, its level was ten times higher than in the muscle. In females, the stocks of hepatopancreas and muscle were used during ovarian maturation and α-tocopherol was transferred into eggs, the α-tocopherol level of egg decreases during successive spawning (Alverz *et al*., 1989). Cahu *et al*.(1993) studied that the highly unsaturated fatty acids (HUFA) supplementation in penaeid broodstock diet induced a decrease of α-tocopherol concentration in both broodstock organs and eggs, proving that a large quantity of α-tocopherol is required as biological antioxidant to sustain egg hatchability. Thus, a HUFA supplementation in broodstock diet must be accompanied by an α-tocopherol supplementation. In addition, the other report found that final maturatin and spawning were also induced by diets containing the eicosapentaenoic acid (EPA) and vitamin E in *P. monodon* (Yano, 1995).

Wouter *et al*. (1999) studied the effect of feeding enriched Artemia biomass on *P. vannamei* broodstock with reference to reproductive performance and larval

quality; the result indicated that high vitamin levels (vitamin C, vitamin E, and astaxanthin) played a positive role only when these vitamins provided in combination with high levels of PUFA and cholesterol. Moreover, vitamin E levels were low in immature ovaries, increased substantially during ovarian maturation, and then decreased again upon spawning. High vitamin E levels were retained in the nuaplii (Wouter *et al*., 2001). The finding of this study, combined with those reported in related studies, suggested the importance of n-3 HUFA for larval development, of vitamin C for egg development and hatching, and of vitamin E for ovarian maturation and larval development (Wouter *et al*., 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental design

A completely randomized design (CRD) involving 3x3 factorials was used in the study. Three concentrations of vitamin E (200, 400 and 600 ppm) and three concentrations of astaxanthin (100, 300 and 500 ppm) were utilized. Eight replications were done in each treatment combination. The experiment was carried out for 40 days.

3.2 Diet and dietary preparation

Vitamin E (200, 400 and 600 mg kg feed^{-1}) and astaxanthin (100, 300 and 500 mg kg feed^{-1}) were added into basal diet to formulate the experimental diets. Carophyll pink, a synthetic astaxanthin provided by Hoffman-La Roche Switzerland, and vitamin E, the (DL) α-tocopherol acetate actived form 50 %, were used as sources of astaxanthin and vitamin E in the experiment. The basal diet ingredients are shown in **Table 3.1** and concentrations of vitamin E and astaxanthin in basal diet are demonstrated in **Table 3.2.** All ingredients were grounded into 200 mesh-size powder and mixed by a twin blade-rolling mixer for 30 minute. Then, vitamin E and astaxanthin were gently added as designed concentration. After the mixtures become homogenized, each mash was pelleted using a California Pelleting Machine (CPM), steamed at 95 ^{o}C for 5 min and dried by hot air oven at 60 ^{o}C for 2 hours. The size of the finishing pellet was 2-mm in diameter and 4 mm in length. The pellets were kept in dark container, flushed with nitrogen gas and storaged at –20 ^{o}C until used. The experimental diets were analyzed for crude protein, lipid, ash, fiber and moisture contents with methods described by AOAC(1995).

Table 3.1. Feed ingredients of basal diet

Ingredients	Dry weight (g 100^{-1} g of diet)
Fish meal	55
Wheat flour	10
Soybean meal	8
Shrimp head meal	8
Refined tuna fish oil	6
Mineral mixture[a]	2
Vitamin mixture[b]	2
Cholesterol[c]	1
Lecithin[d]	1
Binder[e]	5
Selenium[f]	0.03
Vitamin C[g]	0.5
Vitamin E[h]	200-600
Astaxanthin[i]	100-500
Cellulose	to make up volume to 100

[a]Mineral mixture 100 g contains: K_2HPO_4 2.0 g, $Ca_3(PO_4)_2$ 2.720 g, $MgSO_47H_2O$ 3.041 g, $NaH_2PO_42H_2O$ 0.790 g.

[b]Vitamin mixture 100 g contains: ρ-aminobenzoic acid 10.0 mg, biotin 0.40 mg, inositol 400.0 mg, nicotinic acid 40.0 mg, Ca-pantothenate 60.0 mg, pyridoxine-HCl 12.0 mg, riboflavin 8.0 mg, thiamin-HCl 4.0 mg, menadione 4.0 mg, cyanocobalamine 0.08 mg, calciferol 1.20 mg, folic acid 0.80 mg, choline chloride 120.0 mg.

[c]Ninety five percent cholesterol, laboratory grade, Sigma.

[d]Soy lecithin, feed grade.

[e]Wheat gluten, Lab Inter.

[f]Three percent selenium, Altech®.

[g]Stay C 35 %, Roch®.

[h]Fifty percent alpha-tocopherol acetate, feed grade, Roche®

[i]Chlorophyll pink 8%, Roche®.

Table 3.2. Vitamin E and astaxanthin concentrations in experimental diet

Treatments	Vitamin E (ppm)	Astaxanthin (ppm)
1	200	100
2	200	300
3	200	500
4	400	100
5	400	300
6	400	500
7	600	100
8	600	300
9	600	500

3.3 Experimental animals

Shrimp used in the experiment were male white shrimp *P. vannamei*, with average weight of 20.71 g, collected from Rungsit farm reared about the age of 5 months. The male white shrimp were acclimated under laboratory condition for at least 15 days before the experiment. At the beginning, only shrimp with no appearance of spermatophores were used for the experiment. Individual shrimp were reared in net partition in experimental tank. Initially, healthy adolescent animals were randomly selected, and transferred into each experimental unit. The four animals in each experimental tank were acclimated with chopped squid for a few days. If no sign of unhealthy shrimp was observed, the feeding regime of the designed experiment was then begun.

3.4 Experimental tank and rearing condition

The rearing system in this experiment was an indoor closed recirculating water system covered with shading to reduce light intensity for about 70 %. The system consisted of reared plastic tanks, size 500 L with 1.2 m diameter and then 350 liters of sea water were added to the tanks. Ponds with 2 and 8 ton capacity were used as sedimentary and biological system, respectively. The salinity of seawater used in this study was 30 ppt .

3.5 Feed strategy

The experimental diets regime; pelleted diet and fresh diet were fed 3 times per day, 0700, 1200 and 1700. At acclimation period (15 days), the fresh diet was chopped squid (*Loligo* sp.) given 5% of shrimp body weight at 0700 and the basal diet fed at 1200 and 1700 at 2.5% of shrimp weight per day. Then, the experimental diets fed at 0700 1200 and 1700 at 2.5% of shrimp weight per day for the experimental period (40 days). Uneaten diets, fecal matters and particular detritus were removed before the first feeding each day.

3.6 Data collections

At the end of the experiment, spermatophores were obtained by cutting from muscle of the fifth pereiopod of shrimp. The amount of spermatozoa was determined in term of total sperm count, whereas the quality of sperm was determined percentage of live and abnormal sperm following Leung-Trujillo and Lawrence(1987) protocol. The growth rate was determined at the end of feeding experiment. The molting and survival rate were collected daily. Finally, The remainder shrimp were sacrificed and then muscle and hepatopancreas were collected for vitamin E and astaxanthin analysis using high performance liquid chromatography as a method described by Manz and Philipp (1981) and Weber (1988), respectively.

3.7 Water quality

Ammonia, pH , dissolved oxygen and nitrite in water were determined weekly by test kids developed by Faculty of Veterinarian Science, Chulalongkorn University, Thailand). Temperature, salinity and alkalinity were monitored everyday during the experiment.

3.8 Statistical analysis

Effects of vitamin E and astaxanthin on weight gain, survival rate, spermatophore weight, sperm counts, percentage of live and abnormal sperms, and tissue vitamin E and astaxanthin contents were analyzed using analysis of variance and Duncan’s New Multiple Range Test.

CHAPTER IV

RESULTS

4.1 Water quality and dietary proximate analysis

Water quality of the experiment is described in **Table 4.1**. The proximate analysis of experimental diets, vitamin E and astaxanthin contents are shown in **Table 4.2**. Average protein in those diets was 45.99 $\pm$ 0.55 %. Vitamin E concentrations in diet supplemented with vitamin E 200, 400 and 600 ppm were 182.32±33.96, 301.84±13.47, and 528±14.42 ppm, respectively. Astaxanthin concentrations in diet supplemented with astaxanthin E 100, 300 and 600 ppm were 93.92±5.12, 285.91±6.05, 448.26±20.46 ppm, respectively.

Table 4.1. Water quality during the experiment

Parameters	Range
Salinity (ppt)	29-30
Temperature (^{o}C)	24-26
Dissolved oxygen (mg l^{-1})	6.5-7.8
Alkalinity (mg l^{-1})	110-160
pH	7.8-8.1
Ammonia (mg l^{-1})	0-0.5
Nitrite (mg l^{-1})	0-0.25

4.2 Interactive effect of vitamin E and astaxanthin on growth, survival and reproductive performance

The results showed no interactive effect between vitamin E and astaxanthin on growth, survival rate and reproductive performance such as spermatophore weight, sperm counts, and percentage of live sperms and abnormal sperms **(Appendix A)**. Therefore, the effects of vitamin E and astaxanthin on these parameters were separately discussed.

Table 4.2. Proximate analysis of experimental diets as fed basis (MEANS ± S.D.)

Proximate	Treatments*									
	Basal diet	1	2	3	4	5	6	7	8	9
Protein (%)	45.33± 0.45	46.37± 0.30	45.53± 0.75	46.18± 0.50	46.18± 0.53	45.54± 0.36	46.49± 0.55	45.24± 0.33	45.57± 0.41	46.86±0.14
Lipid (%)	13.08± 0.67	11.08± 0.59	12.52± 0.75	12.64± 0.55	13.20± 0.36	13.53± 0.68	13.49± 0.69	13.27± 0.26	12.91± 0.79	13.07± 0.20
Fiber (%)	3.02± 0.85	3.25± 1.02	2.56± 0.97	3.56± 1.28	2.89± 0.77	3.45± 1.67	3.25± 1.87	2.69± 0.56	2.78± 1.22	3.19± 1.75
Ash (%)	14.37± 0.09	14.09± 0.14	13.93± 0.08	14.39± 0.09	14.58± 0.03	14.26± 0.16	14.21± 0.03	14.00± 0.01	13.75± 0.11	14.60± 0.11
Moisture (%)	10.59± 0.33	11.38± 0.10	11.11± 0.54	9.71± 0.44	10.55± 0.51	11.89± 0.45	11.01± 0.36	10.62± 0.74	12.08± 0.02	11.45± 0.45
Vitamin E(ppm)	56.44 ±0.92	184.34 ±55.43	208.43 ±64.65	154.19 ±1.77	289.34 ±25.58	376.49 ±8.98	239.68 ±35.66	514.53 ±30.54	443.22 ±37.60	628.11 ±9.86
Astaxanthin (ppm)	1.43 ±0.31	98.89 ±9.23	290.59 ±31.15	426.45 ±32.37	94.21 ±6.99	279.18 ±51.60	451.31 ±18.95	88.66 ±13.69	287.67 ±40.03	467.03 ±12.32

* Reference to Table 3.2 CHAPTER 3

4.2.1 The effect of dietary vitamin E and astaxanthin on growth

Growth parameters were evaluated using weight and length gains. Both vitamin E and astaxanthin had no significant effect on both parameters of male *P. vannamei*. The effect of dietary of vitamin E and astaxanthin on growth is shown **Table 4-3** and **4-4**, respectively.

Table 4.3. Effects of dietary vitamin E on growth, survival rate and reproductive performance (MEANS ± S.D.) in male white shrimp *P. vannamei*. Means with the same superscripts in each row indicate no significant difference (P<0.05).

Parameters	Dietary vitamin E concentrations (ppm)		
	200	400	600
Weight gain (g)	2.508±1.438	3.331±1.760	2.605±1.696
Length gain (mm)	5.300±2.716	6.826±4.668	5.375±3.321
Survival rate (%)	$95.833±9.517^{b}$	$98.958±5.103^{ab}$	$100±0^{a}$
Spermatophore weight (g)	$0.0344±0.0091^{b}$	$0.0469±0.0162^{a}$	$0.0418±0.0118^{ab}$
Sperm counts (x 10^6 cells · one spermatophore^{-1})	$0.774±0.406^{b}$	$1.267±0.519^{a}$	$1.001±0.385^{b}$
Live sperms (%)	$90.508±7.442^{b}$	$93.417±5.054^{a}$	$95.479±2.657^{a}$
Abnormal body of sperm (%)	1.281±1.570	0.961±2.027	0.713±0.563
Abnormal spike of sperm (%)	30.819±10.450	31.107±12.685	31.128±11.435

Table 4.4. Effects of dietary astaxanthin on growth, survival rate and reproductive performance (MEANS ± S.D.) in male white shrimp *P. vannamei*. Means with the same superscripts within each row indicate no significant difference (P<0.05).

Parameters	Dietary astaxanthin concentrations (ppm)		
	100	300	500
Weight gain (g)	2.348±1.650	3.211±1.718	2.925±1.589
Length gain (mm)	6.409±3.875	6.048±3.981	5.167±3.332
Survival rate (%)	97.917±7.0582	96.875±8.446	100±0
Spermatophore weight (g)	0.0408 ± 0.0154^{ab}	0.0369 ± 0.0117^{b}	0.0457 ± 0.0126^{a}
Sperm counts (x 10^6 cells · one spermatophore^{-1})	0.985±0.471	1.031±0.473	1.056±0.508
Live sperms (%)	93.383±4.533	92.971±6.242	93.475±6.018
Abnormal body of sperm (%)	1.030±1.337	1.057±1.871	0.832±1.317
Abnormal spike of sperm (%)	38.013 ± 11.683^{b}	29.302 ± 9.663^{a}	26.137 ± 9.735^{a}

4.2.2 The effect of dietary vitamin E and astaxanthin on survival rate

No interactive effect of vitamin E and astaxanthin was found on survival rate of shrimp. Shrimp fed diets supplemented with dietary vitamin E 600 ppm (100%) had significantly higher survival rate (P<0.05) than those fed 200 ppm (95.83 $\pm$ 9.52 %). While shrimp fed with dietary vitamin E 400 ppm had no significant effect among groups on survival rate (98.96 $\pm$ 5.10 %) **(Figure 4.1 A)**. Dietary astaxanthin had no significant effect on survival rate. **(Figure 4.1 B)**.

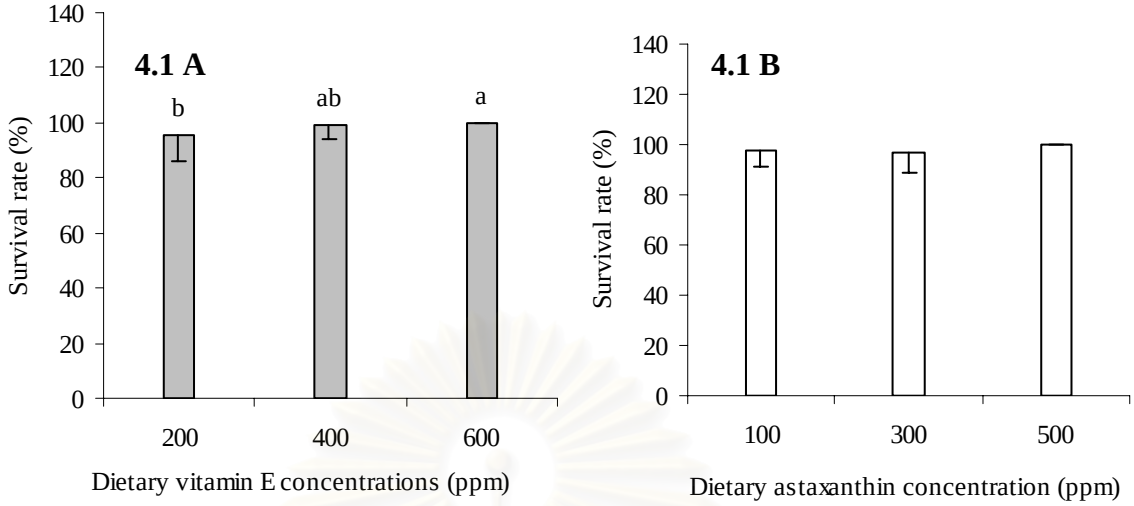

Figure 4.1. The effect of vitamin E (4.1 A) and astaxanthin (4.1 B) on survival rate of male white shrimp *P.vannamei*

Note: The same superscripts on the histogram indicate no significant difference of mean values of treatments (P<0.05).

4.2.3 The effect of dietary vitamin E and astaxanthin on reproductive performance

4.2.3.1 Spermatophore weight

The effect of dietary vitamin E and astaxanthin on spermatophore weight is shown in **Figure 4.2**. Shrimp fed diets supplemented with 400 ppm vitamin E had significantly higher mean spermatophore weight (P<0.05) than those fed 200 ppm (0.0469 $\pm$ 0.0162 and 0.0344 $\pm$ 0.0091 g, respectively) while shrimp fed 600 ppm vitamin E was no significant difference among levels **(Figure 4.2 A)**. Shrimp fed supplemented 500 ppm astaxanthin had significantly higher means of spermatophore weight (P<0.05) than those fed with 300 ppm astaxanthin (0.0457 $\pm$ 0.0126 and 0.0369 $\pm$ 0.0117 g, respectively) while shrimp fed 100 ppm astaxanthin was no significant difference among levels **(Figure 4.2 B)**.

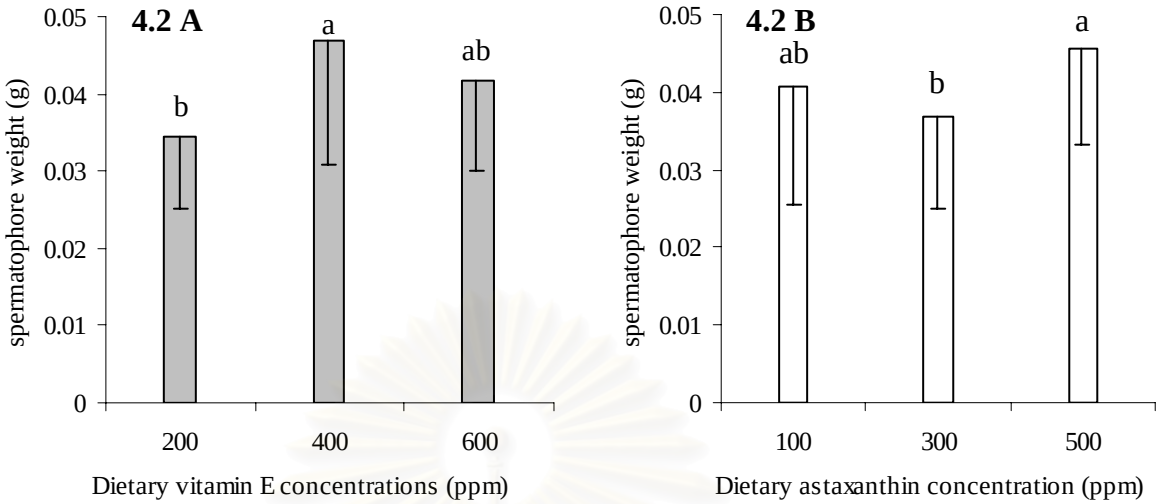

Figure 4.2. The effect of vitamin E (4.2 A) and astaxanthin (4.2 B) on spermatophore weight of male white shrimp *P.vannamei*

Note: The same superscripts on the histogram indicate no significant difference of mean values of treatments (P<0.05).

4.2.3.2 Sperm counts (cells / spermatophore)

The effect of dietary vitamin E and astaxanthin on sperm counts was discussed separately because there was no significant interaction between vitamin E and astaxanthin.

The result of sperm counts on male white shrimp *P. vannamei* was only significant difference (P<0.05) for shrimp fed with vitamin E **(Figure 4.3)**. Shrimp fed diets supplemented with vitamin E 400 ppm, the amount of spermatozoa (1.267 x 10^6 ± 0.519 x 10^6 cells / spermatophore), had significantly higher spermatozoa (P<0.05) than those fed 200 ppm and 600 ppm (0.774 $\pm$ 0.0406 and 1.001 $\pm$ 0.385 x 10^6 cells / spermatophore , respectively) **(Figure 4.3 A)**. Astaxanthin had no significant effect among groups for sperm counts **(Figure 4.3 B)**.

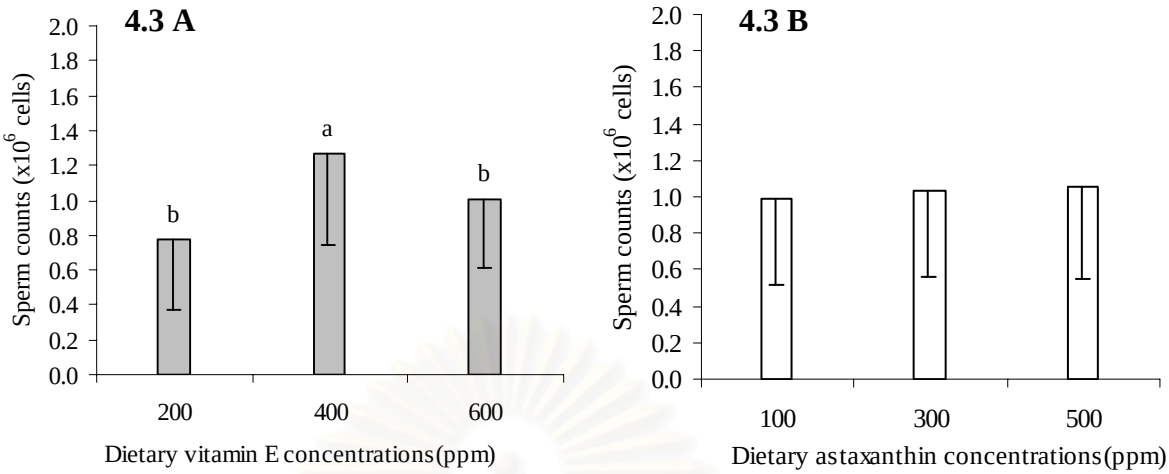

Figure 4.3. The effect of vitamin E (4.3 A) and astaxanthin (4.3 B) on sperm counts white shrimp *P.vannamei.*

Note: The same superscripts on the histogram indicate no significant difference of mean values of treatments (P<0.05).

4.2.3.3 Percentage of live sperm

No interactive effect of vitamin E and astaxanthin was found on percentage of live sperm of white shrimp *P. vannamei*. Therefore, the effect of vitamin E and astaxanthin on was discussed separately **(Figure 4.4)**.

There was no significant effect of astaxanthin on percentage of live sperm **(Figure 4.4 B)**, but shrimp fed diets supplemented with vitamin E 400 and 600 ppm, percentage of live sperm (93.42 ± 5.05 and 95.48 ± 2.66 % respectively), were significantly higher (P<0.05) than shrimp fed diets supplemented with vitamin E 200 ppm (90.51± 7.44 %) **(Figure 4.4 A)**.

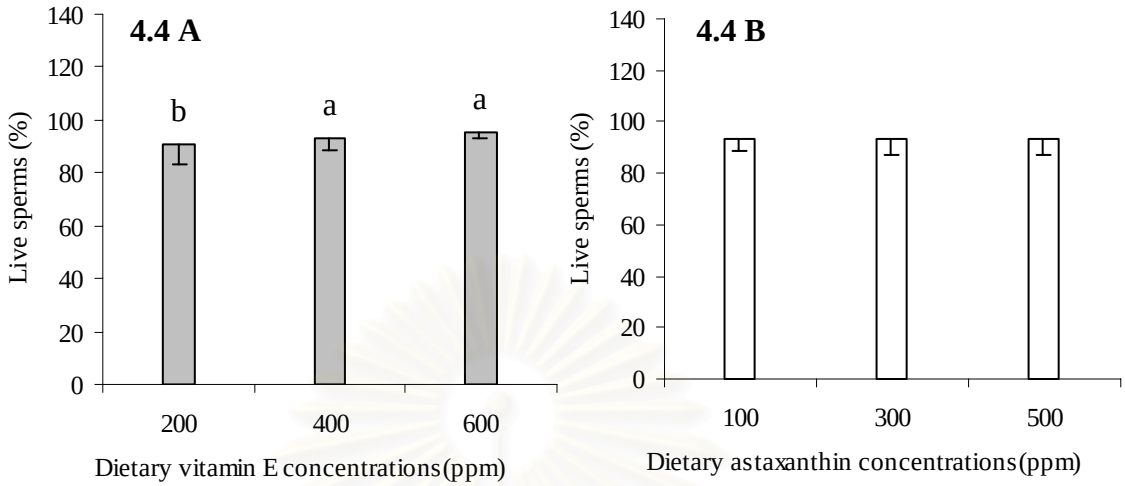

Figure 4.4. The effect of vitamin E (4.4 A) and astaxanthin (4.4 B) on percentage of live sperm

Note: The same superscripts on the histogram indicate no significant difference of mean values of treatments (P<0.05).

4.2.3.4 Percentage of abnormal sperm

Percentage of abnormal sperm was divided into two parts; percentage of abnormal body sperm and percentage of abnormal spike **(Figure 4.5)**. No interactive effect of vitamin E and astaxanthin was found on both percentage of abnormal body sperm and percentage of abnormal spike of white shrimp *P. vannamei*, therefore, the effect of vitamin E and astaxanthin on was discussed separately **(Figure 4.6)**.

In part of percentage of abnormal body, both vitamin E and astaxanthin had no significant effect **(Table 4.3 and 4.4)**. While percentage of abnormal spike, vitamin E had no significant difference **(Figure 4.6 A)**. Shrimp fed diets supplemented with astaxanthin 300 and 500 ppm, percentage of abnormal spike (29.30 ± 9.66 and 26.14 ± 9.74 respectively), were significantly lower than those fed diets containing astaxanthin 100 ppm (38.01 ± 11.68) (P<0.05) **(Figure 4.6 B)**.

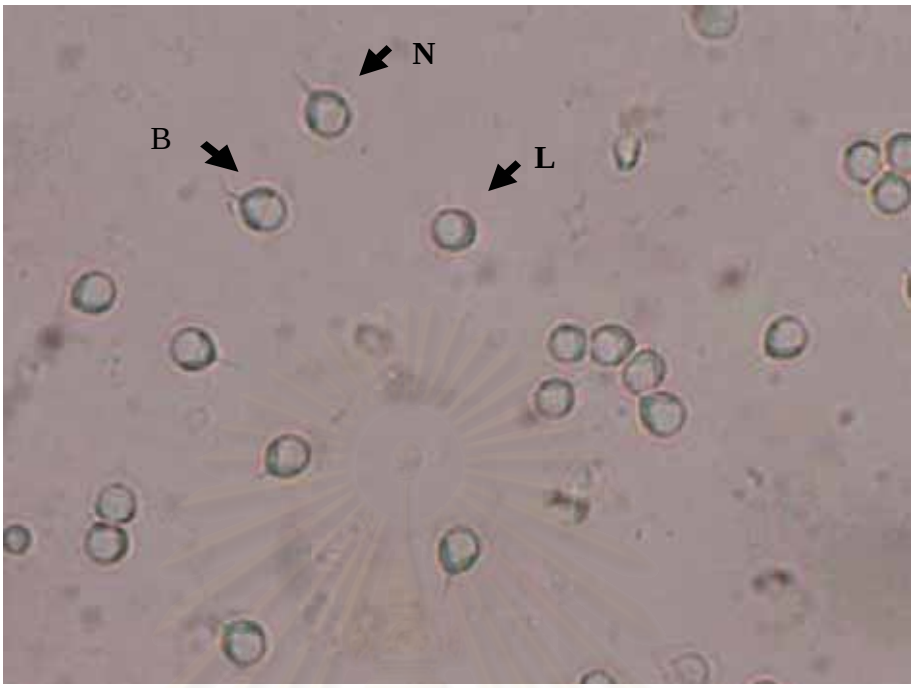

Figure 4.5. Characteristics of normal and abnormal sperms

N = Normal sperm found both body and straight spike.

L = Abnormal sperm had spikeless.

B = Abnormal sperm had bended spike.

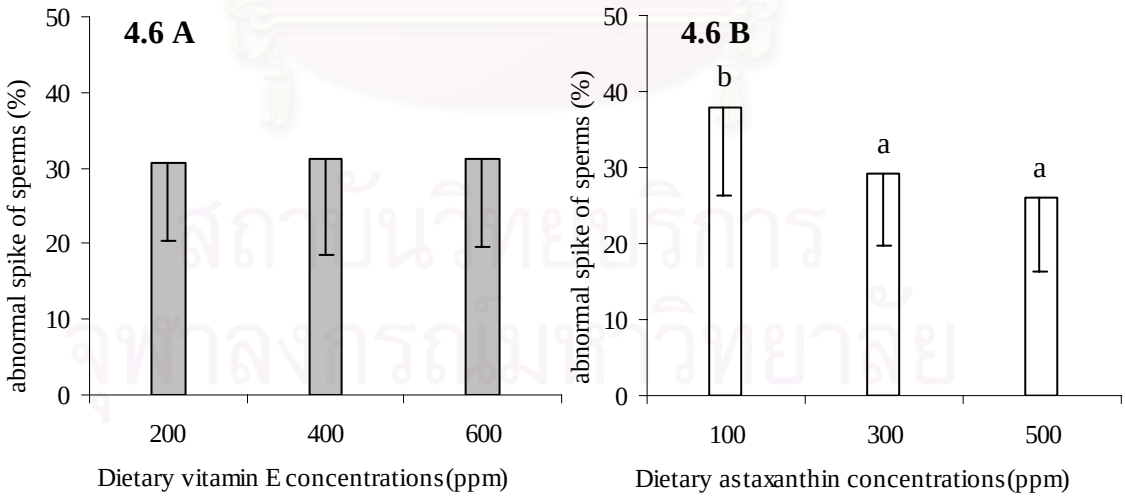

Figure 4.6. The effect of vitamin E (4.6 A) and astaxanthin (4.6 B) on percentage of abnormal spike of sperm

Note: Means with the same superscripts on the histograme indicate no significant difference (P<0.05).

4.5 The effect of dietary vitamin E and astaxanthin on α-tocopherol acetate accumulation in shrimp tissue

There was no significant interaction between vitamin E and astaxanthin. Therefore, effects of vitamin E and astaxanthin on α-tocopherol acetate accumulation in muscle and hepatopancreas were discussed separately.

Both vitamin E and astaxanthin had no significant effect on α-tocopherol acetate content in both muscle and hepatopancreas. However at the same level of dietary vitamin E or astaxanthin, α-tocopherol acetate accumulation in hepatopancreas had significantly higher content than in muscle ($P<0.05$) for all diets **(Figure 4.7)**.

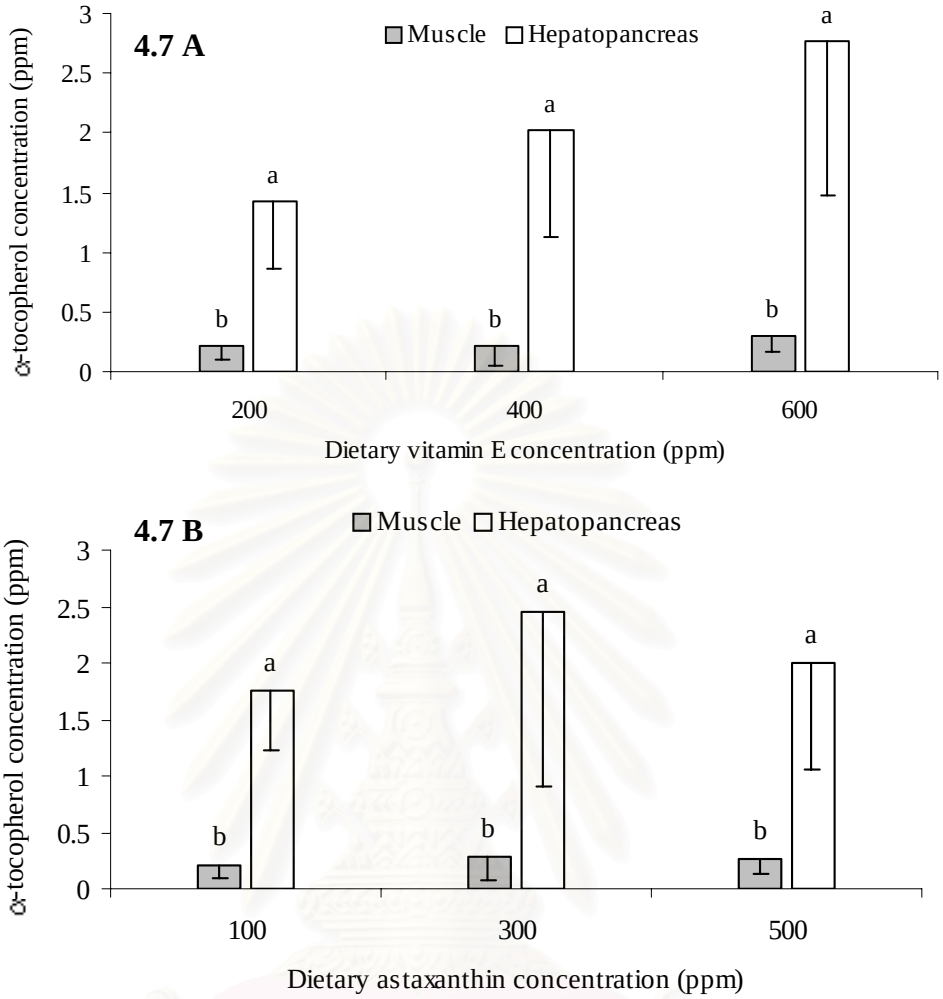

Figure 4.7. Comparison between muscle and hepatopancreas at the same level of vitamin E (4.7 A) or astaxanthin (4.7 B) on α-tocopherol acetate accumulation

Note: Means with the same superscripts on the histograme indicate no significant difference (P<0.05).

4.6 The effect of dietary vitamin E and astaxanthin on astaxanthin accumulation in shrimp tissue

Effects of dietary vitamin E and astaxanthin on astaxanthin content in muscle and hepatopancreas were discussed separately, due to no interaction between vitamin E and astaxanthin.

The effect of vitamin E was no significant difference in both muscle and hepatopancreas, while astaxanthin was significant difference (P<0.05) on astaxanthin accumulation in muscle **(Figure 4.8)**. Shrimp fed diet supplemented with astaxanthin 500 ppm had significantly higher astaxanthin content (P<0.05) in muscle than those fed with astaxanthin 100 ppm (6.927 $\pm$ 2.910 and 4.749 $\pm$ 1.339 ppm, respectively),

but shrimp fed diets supplemented with astaxanthin 300 ppm had no significant effect muscle among levels of astaxanthin on astaxanthin content in muscle (5.224 $\pm$ 1.549 ppm). For astaxanthin accumulation in hepatopancreas, astaxanthin was no significant difference among levels in this tissue.

However, the same level of diets supplemented with astaxanthin 300 and 500 ppm had significantly higher astaxanthin content in hepatopancreas than in muscle (P<0.05), while the level of dietary astaxanthin 100 ppm had no significant difference of astaxanthin content between muscle and hepatopancreas. Shrimp fed dietary vitamin E had significantly higher astaxanthin accumulation in hepatopancreas than in muscle (P<0.05) for all diets **(Figure 4.9)**.

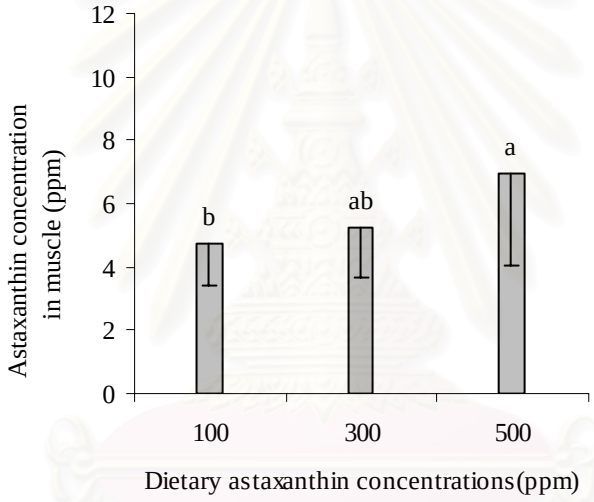

Figure 4.8. The effect of dietary astaxanthin on astaxanthin accumulation in muscle

Note: Means with the same superscripts on the histograme indicate no significant difference (P<0.05).

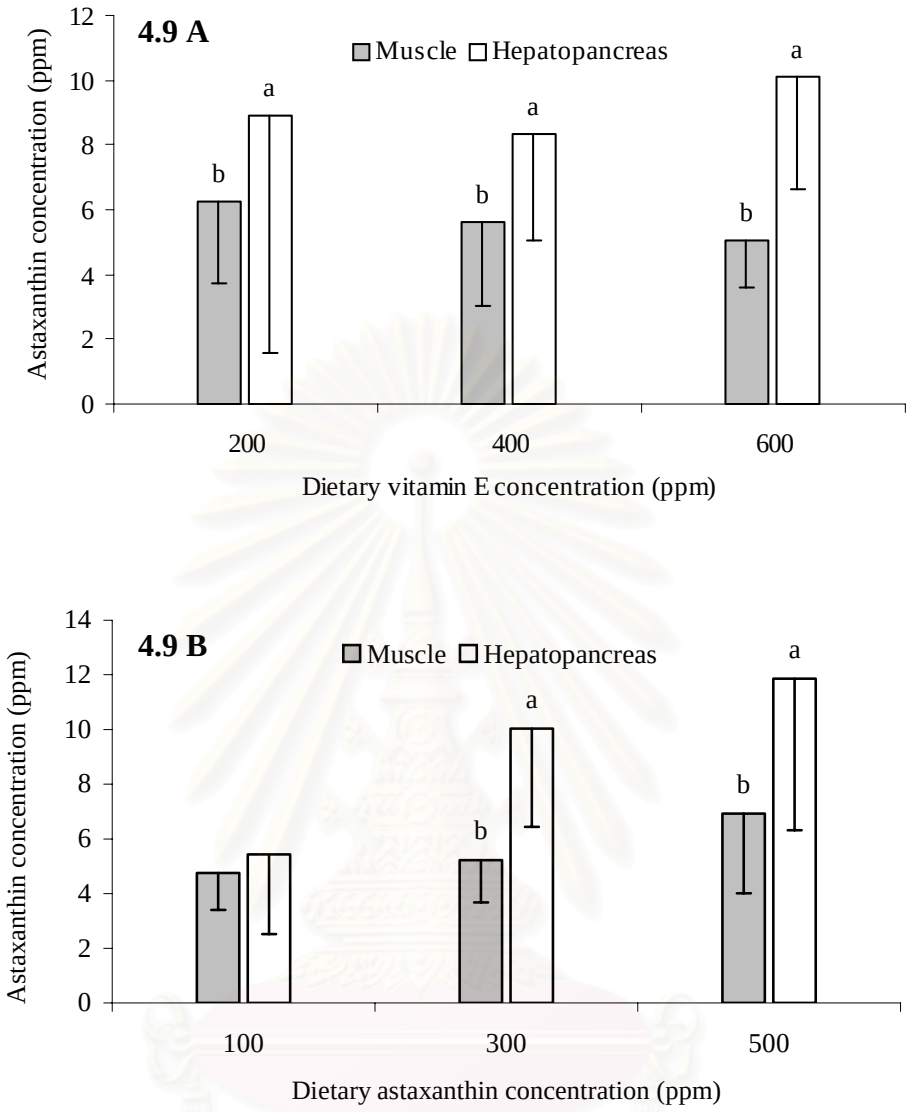

Figure 4.9. Comparison between muscle and hepatopancreas at the same level of vitamin E (4.9 A) or astaxanthin (4.9 B) on astaxanthin accumulation

Note: Means with the same superscripts on the histograme indicate no significant difference ($P<0.05$).

CHAPTER V

DISCUSSION

The result of this study illustrated that vitamin E and astaxanthin could enhance reproductive performance in male white shrimp *P. vannamei*. The study indicates no interactive effects of astaxanthin on reproductive of male white shrimp because they may perform in different functions and metabolisms of both vitamin E and astaxanthin.

Vitamin E affects on reproductive performance in this experiment namely spermatophore weight **(Figure 4.2)**, sperm counts **(Figure 4.3)**, and percentage of live sperm **(Figure 4.4)**.

An optimal diet is a crucial factor for the sexual maturation and reproduction of shrimp. Despite the importance of broodstock nutrition, publications on this topic are limited. However, supplements of vitamins to support maturation and/or reproduction are essential factors. Vitamin E is an essential vitamin in shrimp, including *P. vannamei* (He *et al*., 1992). In broodstodk, vitamin E improves the rate of ovarian maturation in *L. setiferus* (Chamberlain, 1988), fed diet supplementation with 500 mg kg^{-1} tocopheryl acetate. In the trial with *F. indicus* spawner, Cahu *et al*. (1991) demonstrated an increase in hatching percentage when raising dietary α-tocopherol levels from 40 to 350 mg kg^{-1}. The α-tocopherol egg levels reported that by Cahu *et al.* (1995) are very similar to those found in wild *P. vannamei* and Wouters *et al.* (1999) found 325-393 $\mu g\ g^{-1}$ dry matter α-tocopherol in mature ovaries and nuaplii.

Nevertheless, vitamin E supplementation in broodstock nutrition is mainly focus on female, while there was few reports for male crustacean. However, vitamin E have also crucial vitamin in males. Evans and Bishop (1992) reported that vitamin E prevented loss of spermatogenesis in male rats. Dietary deficiencies of vitamin E in males causes degenerative spermatogonium in rat (Bridgelius-Flohe and Traber, 1994), resulting in a lower sperm concentration (Cooper *et al*.1987). Dietary vitamin E supplementation was hasten early spermatogenesis in young boar (Marin-Guzman et al., 2000).

Vitamin E deficiency causes testicular degeneration in different species. It is therefore possible that testicular degeneration occurs in groups fed low levels of vitamin E (Marin-Guzman *et al*., 2000). Although other factors can be exacerbate the dietary vitamin E requirement of animals; e.g. dietary levels of lipids. Fatty acids, particurlarly n-3 HUFA, phospholipid and sterols play a crucial role in shrimp reproduction. The importance of n-3 HUFA has also been deduced from their presence in natural organisms that are successful mature (Meejing, 2003; Lytle *et al*., 1990). Several studies have evaluated the effect of dietary fatty acid levels on broodstock performance (Paibulkichakul, 2002; Cahu *et al*., 1995).

However, these lipids can be oxidized from oxidative stress. Rimm et al.(1993) suggested that vitamin E played specific roles beyond that of its antioxidant function. Decreases in lipid peroxidation in reproductive system have been occurred from mechanism of vitamin E (Brigelius-Flohe *et al*., 2000). Vitamin E is known to readily reduce alkyl peroxy radicals of unsaturated lipids (Burton *et al*., 1983), alter cellular membrane structure (Lucy, 1972) and change memebrane-bound enzyme system (Korsan-Bangtsen *et al*., 1974) which may result in spermatogenesis.

The experiment found that diet supplemented 600 ppm vitamin E had lower amount of spermatozoa than those fed 400 ppm vitamin E. Moreover, at 600 ppm vitamin E supplementation had no significant spermatophore weight compare with 200 and 400 ppm vitamin E. These results thus support the concept that vitamin E, perhaps at dietary levels substantially above the animal's requirement, reduce prostaglandin biosynthesis through its antioxidant function (Marin-Guzman *et al*., 2000). The prostaglandin biosynthesis is important because it increases the number of spermatozoa and revealed sexual activity (Cornwell *et al*., 1974; Marshall and Hafs, 1976).

In this study, no interaction of vitamin E and astaxanthin was found on vitamin E accumulation in both muscle and hepatopancreas. Vitamin E accumulation had no significant concentration at the same tissue in both muscle and hepatopancreas However, vitamin E accumulation in hepatopancrreas found increasing vitamin E concentration when dietary vitamin E levels were increased, whereas muscle had no this event. At the same levels of vitamin E supplementation found vitamin E concentrations were higher in hepatopancreas than in muscle **(Figure 4.7 A)**.

This result is supported by Cahu *et al*.(1995) who found vitamin E concentration was higher in hepatopancreas than in muscle.

This study found that the optimum concentration of vitamin E is 400 ppm in practical male white shrimp *P. vannamei* diets. This result is correlated with several authors **(Table 4.5)**.

Table 4.5. Vitamin E concentration in artificial shrimp broodstock diets as reported by several authors.

Species	Dietary vitamin E requirements* (mg. kg^{-1})	References
M. japonicus	482	Alava *et al*. (1993)
L. stylisrostris	440	Bray *et al*. (1990)
F. indicus	300	Cahu *et al*. (1995)
P. monodon	500	Marsden *et al*. (1997)
P. vannamei	500	Mendoza *et al*. (1997)

*One mg equals 1 I.U. vitamin E (acetate) all concentrations are calculated as $mg.kg^{-1}$diet on dry weight basis.

For supplemented astaxanthin effect, its effects on reproductive performance were spermatophore weight and percentage of abnormal spike of sperm cell. To begin with the effect of astaxanthin supplementation on spermatophore weight (**Figure 4.2 B)**, males fed 500 ppm astaxanthin had higher spermatophore weight than those fed 300 ppm astaxanthin. There was no report describing spermatophore weight for astaxanthin supplementation in pacific white shrimp diets. However, nutrition factors are of prime importance in ensuring the quality of the semen (Adiyodi, 1985). Thus, it may indicate that shrimp fed high level (500 ppm) of dietary astaxanthin accumulated astaxanthin for spermatophore synthesis. Conversely, a group fed 100 ppm dietary astaxanthin had no different spermatophore weight among dietary astaxanthin levels.

Ceballos-Va′zguez *et al*. (2003) reported that larger spermatophore produced larger quantities of sperm than smaller spermatophore. When amounts of sperm were compared among levels of astaxanthin supplementation in the present study, no

different in amounts of sperm were found. In accordance, Paibulkichakul (2002) observed male shrimp fed diets supplemented with astaxanthin 300 and 500 mg.kg^{-1} had no effect on amounts of spermatozoa, whereas, males fed 100 ppm astaxanthin supplementation had lower amounts of spermatozoa than males fed 500 astaxanthin supplementation. However, spermatophore weight and amount of sperm which are not a good criterion for selecting males for reproductive purposes. A better criterion for selecting male broodstock is sperm quality.

The types of morphologically abnormal sperm have been reported in white shrimp: malformed body; spike bent and spike lost (Wang *et al*., 1995). Percentage of abnormal sperm is related to the maturing process of the vas deferens, which is not mature by the time, the testes begin to produce sperm (Alfaro, 1996). The vas deferens is the organ for final sperm maturation by completing the formation of subacrosome and spike sections (Shigekawa and Clark, 1986). The present study observed that males fed 300 and 500 ppm dietary astaxanthin were significantly lower abnomal spikes of spermatozoa than those fed low dietary astaxanthin (100 ppm) **(Figure 4.6 B)**. Astaxanthin may promote spermatozoa in males shrimp via the provitamin A activity. Retinoid act in testicular development, especially on germ cells, via retinoic acid receptor and/or retinoid X receptors (Boullogne *et al*., 1999). Alkmal *et al*. (1997) reported that retinoic acid receptor alpha played an essential role in the spermatogenesis. Characterization of retinoic acid receptor alpha expression revealed time and location of the vitamin A requirement during spermatogenesis.

Astaxanthin components in muscle and hepatopancreas of male shrimp mainly were all-trans-astaxanthin. Similar with report by Muriana *et al*.(1993) as the main compound of astaxanthin in hepatopancreas and muscle of shrimp *P. japonicus* was all-trans-astaxanthin. According to Jeckel *et al*.(1989), the form of carotenoids that accumulated depends largely on the diet.

In this study, there was no interactive effect of vitamin E and astaxanthin accumulation in both muscle and hepatopancreas. In muscle, groups fed 500 astaxanthin supplementation had higher astaxanthin content than those fed 100 astaxanthin supplementation, while groups fed dietary 300 ppm astaxanthin had no different astaxanthin content among levels of astaxanthin **(Figure 4.8)**. This result is supported by Paibulkichakul (2002), shrimp fed with 500 mg kg^{-1} dietary astaxanthin had higher astaxanthin content than 100 and 300 mg kg^{-1} in

muscle. In hepatopancreas, this study had no different astaxanthin content among levels of astaxanthin. In study I of Paibulkichakul (2002) found at 500 mg kg^{-1} astaxanthin supplementation had higher astaxanthin content than 300 mg kg^{-1} astaxanthin in diets. While in his study II, he found that there was no significant difference among levels (100 and 500 mg kg^{-1}) on astaxanthin content in hepatopancreas.

At same levels of dietary astaxanthin 300 and 500 ppm had significantly higher astaxanthin content in hepatopancreas than in muscle, while at 100 ppm astaxanthin had no significant difference of astaxanthin content among organs in this study **(Figure 4.9 B)**. Therefore, astaxanthin contents accumulate in hepatopancrease higher than in muscle. However, at 100 ppm astaxanthin may be not enough for spermatogenesis process.

The main tissue of astaxanthin deposits in integument (82-94 %), but this study had not evaluated, whereas at maturation stage found that free astaxanthin and esterified carotenoid accumulate in midgut gland and then they are mobilized from there to reproductive organ (Harrison, 1990). Dall *et al*. (1995) conducted biochemical studies on wild *P. esculentus* determining astaxanthin and its esters as principal carotenoids. Free astaxanthin predominant in maturing ovaries, which are reproductive organ, increasing from 2 to 34 ppm. In the digestive gland, free astaxanthin and esters increased from 20 to 120 ppm. In the integument, carotenoid levels remained relatively constant (90 ppm) throughout the maturation cycle. Dall *et al.* (1995) also emphasize that carotenoids play a crucial role as vitamin A precursor. Therefore, the effect of astaxanthin on reproductive performance can probably be attributed via provitamin A activity. Carotenoids, particularly astaxanthin, are strong scavangers of free radicals and may be protect sperm from oxidative deterioration.

For growth and survival rate of male *P. vannamei*, there was no interactive effect between dietary vitamin E and astaxanthin. In general, vitamin E and astaxanthin have a potential effect on shrimp growth and/or survival rate (He *et al*., 1992; He and Lawrence, 1993; Paibulkichakul, 2002; Petit *et al*, 1997). The present study had no significant growth in both dietary vitamin E and astaxanthin. In some study, Chein and Jeng (1992) reported that the different pigment sources and their various levels had not effect on growth rate but positive correlation between pigment concentration in shrimp tissue and survival rates was observe in *P. japonicus*. In

accord of Negre-Sadagues *et al.* (1993), shrimp fed diet supplemented with astaxanthin were not significantly different on growth rate.

In the present study, astaxanthin supplemented diets did no promote survival rate of the experimental shrimp. The similar results also reported by Negre-Sadagues *et al.* (1993) and Boonyaratpalin *et al.* (2001). Shrimp may be accumulate high content of astaxanthin for spermatophore synthesis and/or spermatogenesis.

The result of this study found that vitamin E had effect on survival rate **(Figure 4-1 A)**. There was supported by He *et al*., 1992 and He and Lawrence, 1993. This effect of vitamin E on survival rate can probable be attributed to the antioxidant properties. Free radicals, initiated by various factors including active oxygen, attached lipid and protein in biomembranes.

In summary, vitamin E and astaxanthin supplementation in diet can promote reproductive performance in male white shrimp (*P. vannamei*). Therefore, the practical male maturation diet could supplemented with 400 ppm vitamin E and/or 300 ppm astaxanthin.

CHAPTER VI

SUMMARY

1. The present study found no interaction between dietary vitamin E and astaxanthin on growth, survival rate and reproductive performance of male white shrimp *P. vannamei*. Both vitamin E and astaxanthin supplementation had no effect on growth of male white shrimp. Astaxanthin had no effect on survival rate, while vitamin E had effect on survival rate. The high concentration of vitamin E had high survival rate.

2. Vitamin E and astaxanthin supplementation could enhance reproductive performance in male white shrimp. Both dietary vitamin E and astaxanthin had effect on spermatophore weight. Only dietary vitamin E had effect on sperm count and live sperms by its antioxidant properties while dietary astaxanthin had only effect on abnormal sperm via the provitamin A activity.

3. There were no interactive effects between vitamin E and astaxanthin on α-tocopherol acetate and astaxanthin accumulation in muscle and hepatopancreas of male white shrimp *P. vannamei*. Both vitamin E and astaxanthin had no effect on α-tocopherol acetate content in both muscle and hepatopancreas of male white shrimp but same concentrations of both vitamin E and astaxanthin had higher α-tocopherol acetate content in hepatopancreas than in muscle. For astaxanthin accumulation in shrimp tissue, Dietary vitamin E had no effect on astaxanthin content in all tissues of male white shrimp, while astaxanthin had only effect on astaxanthin content in muscle. At same levels of dietary astaxanthin 300 and 500 ppm had higher astaxanthin contents in hepatopancreas than in muscle.

4. This study suggested that the optimum concentration of vitamin E 400 ppm and/or astaxanthin 300 ppm added in practical diet could enhance maturation of male white shrimp *P. vannamei.*

This study demonstrated the potential dietary vitamin E and astaxanthin to improve maturation of male white shrimp *P. vannamei* and can be directly applied to shrimp culture and to further studies.

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APPENDICES

APPENDIX A

STATISTICAL ANALYSIS

Table 1A. Effects of vitamin E and astaxanthin supplementation on growth of male white shrimp *P. vannamei* during the experiment

General Linear Models Procedure

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	36.81861603	4.60232700	1.83	0.0896
Error	58	145.83744964	2.51443879		
Corrected Total	66	182.65606567			

R-Square	C.V.	Root MSE	WEIGHT Mean
0.201573	56.12350	1.585698	2.825373

General Linear Models Procedure

Dependent Variable: WEIGHT

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	9.05276723	4.52638361	1.80	0.1744
AX	2	7.52388341	3.76194170	1.50	0.2325
VITE*AX	4	20.24196539	5.06049135	2.01	0.1046

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	8.30478291	4.15239146	1.65	0.2007
AX	2	7.55965046	3.77982523	1.50	0.2309
VITE*AX	4	20.24196539	5.06049135	2.01	0.1046

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	3.3309	23	400
A			
A	2.6054	24	600
A			
A	2.5080	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	3.2110	21	300
A			
A	2.9254	24	500
A			
A	2.3482	22	100

Table 1A. (Continued)

General Linear Models Procedure

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.99325320	0.12415665	0.89	0.5290
Error	58	8.07182143	0.13916933		
Corrected Total	66	9.06507463			

R-Square	C.V.	Root MSE	LENGTH Mean
0.109569	63.76179	0.373054	0.585075

General Linear Models Procedure

Dependent Variable: LENGTH

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	0.33378115	0.16689057	1.20	0.3088
AX	2	0.18920975	0.09460487	0.68	0.5107
VITE*AX	4	0.47026230	0.11756558	0.84	0.5026

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	0.34484199	0.17242099	1.24	0.2972
AX	2	0.20589107	0.10294553	0.74	0.4817
VITE*AX	4	0.47026230	0.11756558	0.84	0.5026

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	0.6826	23	400
A			
A	0.5375	24	600
A			
A	0.5300	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	0.6409	22	100
A			
A	0.6048	21	300
A			
A	0.5167	24	500

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

WEIGHT = Shrimp weight Gain (g)

LENGTH = Shrimp length Gain (mm)

Table 2A. Effects of vitamin E and astaxanthin supplementation on survival rates of male white shrimp *P. vannamei* during the experiment

General Linear Models Procedure

Dependent Variable: SURVIVAL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	642.3611111	80.2951389	2.23	0.0364
Error	63	2265.6250000	35.9623016		
Corrected Total	71	2907.9861111			

R-Square	C.V.	Root MSE	SURVIVAL Mean
0.220896	6.102809	5.996858	98.26389

General Linear Models Procedure

Dependent Variable: SURVIVAL

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	225.6944444	112.8472222	3.14	0.0502
AX	2	121.5277778	60.7638889	1.69	0.1928
VITE*AX	4	295.1388889	73.7847222	2.05	0.0978

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	225.6944444	112.8472222	3.14	0.0502
AX	2	121.5277778	60.7638889	1.69	0.1928
VITE*AX	4	295.1388889	73.7847222	2.05	0.0978

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	100.000	24	600
A			
B A	98.958	24	400
B			
B	95.833	24	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	100.000	24	500
A			
A	97.917	24	100
A			
A	96.875	24	300

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

SURVIVAL = Percentage of survival rate

Table 3A. Effects of vitamin E and astaxanthin supplementation on spermatophore weight of male white shrimp *P. vannamei* at the final of the experiment

General Linear Models Procedure

Dependent Variable: SPP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.00302955	0.00037869	2.39	0.0270

Error	58	0.00920887	0.00015877
Corrected Total	66	0.01223842	

R-Square	C.V.	Root MSE	SPP Mean
0.247544	30.47455	0.012601	0.041348

General Linear Models Procedure

Dependent Variable: SPP

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	0.00170346	0.00085173	5.36	0.0073
AX	2	0.00108496	0.00054248	3.42	0.0396
VITE*AX	4	0.00024112	0.00006028	0.38	0.8223

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	0.00182910	0.00091455	5.76	0.0052
AX	2	0.00102127	0.00051064	3.22	0.0474
VITE*AX	4	0.00024112	0.00006028	0.38	0.8223

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	0.046978	23	400
A			
B A	0.041756	24	600
B			
B	0.034383	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	0.045725	24	500
A			
B A	0.040841	22	100
B			
B	0.036876	21	300

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

SPP = Spermatiphore weight (g)

Table 4A. Effects of vitamin E and astaxanthin supplementation on sperm count of male white shrimp *P. vannamei* at the final of the experiment

General Linear Models Procedure

Dependent Variable: SPERMS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	3.78240E+12	4.72801E+11	2.42	0.0248
Error	58	1.13166E+13	1.95114E+11		
Corrected Total	66	1.50990E+13			

R-Square	C.V.	Root MSE	SPERMS Mean
0.250507	43.10416	441716.7	1024766

General Linear Models Procedure

Dependent Variable: SPERMS

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	2.62188E+12	1.31094E+12	6.72	0.0024
AX	2	6.81886E+10	3.40943E+10	0.17	0.8401
VITE*AX	4	1.09234E+12	2.73085E+11	1.40	0.2455

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	2.44150E+12	1.22075E+12	6.26	0.0035
AX	2	6.70092E+10	3.35046E+10	0.17	0.8426
VITE*AX	4	1.09234E+12	2.73085E+11	1.40	0.2455

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	1267452	23	400
B	1000816	24	600
B			
B	774417	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	1056443	24	500
A			
A	1030732	21	300
A			
A	984514	22	100

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

SPERMS = Amounts of total spermatozoa (cells/spermatophore)

Table 5A. Effects of vitamin E and astaxanthin supplementation on percentage of live sperm of male white shrimp *P. vannamei* at the final of the experiment

General Linear Models Procedure

Dependent Variable: LIVE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	330.2414294	41.2801787	1.39	0.2182
Error	58	1716.5559736	29.5957926		
Corrected Total	66	2046.7974030			

R-Square	C.V.	Root MSE	LIVE Mean
0.161345	5.831681	5.440202	93.28701

General Linear Models Procedure

Dependent Variable: LIVE

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	270.2325229	135.1162615	4.57	0.0144
AX	2	9.3935503	4.6967751	0.16	0.8536
VITE*AX	4	50.6153562	12.6538391	0.43	0.7881

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	288.4485313	144.2242656	4.87	0.0111
AX	2	14.6608347	7.3304174	0.25	0.7814
VITE*AX	4	50.6153562	12.6538391	0.43	0.7881

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	95.479	24	600
A			
B A	93.417	23	400
B			
B	90.508	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	93.475	24	500
A			
A	93.383	22	100
A			
A	92.971	21	300

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

LIVE = Percentage of live sperm

Table 6A. Effects of vitamin E and astaxanthin supplementation on percentage of abnormal body of sperm cell in male white shrimp *P. vannamei* at the final of the experiment

General Linear Models Procedure

Dependent Variable: AB_BODY

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	11.86594295	1.48324287	0.63	0.7480
Error	58	136.22770548	2.34875354		
Corrected Total	66	148.09364843			

R-Square	C.V.	Root MSE	AB_BODY Mean
0.080125	158.4032	1.532564	0.967508

General Linear Models Procedure

Dependent Variable: AB_BODY

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	3.51760067	1.75880034	0.75	0.4774
AX	2	0.91214561	0.45607280	0.19	0.8240
VITE*AX	4	7.43619667	1.85904917	0.79	0.5355

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	3.38645794	1.69322897	0.72	0.4906
AX	2	0.87827790	0.43913895	0.19	0.8300
VITE*AX	4	7.43619667	1.85904917	0.79	0.5355

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	1.2806	20	200
A			
A	0.9609	23	400
A			
A	0.7129	24	600

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	1.0570	21	300
A			
A	1.0299	22	100
A			
A	0.8320	24	500

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

AB_BODY = Percentage of abnormal body of sperm cell

Table 7A. Effects of vitamin E and astaxanthin supplementation on percentage of abnormal spike of sperm cell in male white shrimp *P. vannamei* at the final of the experiment

General Linear Models Procedure

Dependent Variable: AB_SPIKE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	2143.189814	267.898727	2.40	0.0262
Error	58	6480.617170	111.734779		
Corrected Total	66	8623.806984			

R-Square	C.V.	Root MSE	AB_SPIKE Mean
0.248520	34.06690	10.57047	31.02856

General Linear Models Procedure

Dependent Variable: AB_SPIKE

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	1.263181	0.631590	0.01	0.9944
AX	2	1711.626900	855.813450	7.66	0.0011
VITE*AX	4	430.299733	107.574933	0.96	0.4349

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	12.217177	6.108588	0.05	0.9468
AX	2	1717.609584	858.804792	7.69	0.0011
VITE*AX	4	430.299733	107.574933	0.96	0.4349

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	31.128	24	600
A			
A	31.107	23	400
A			
A	30.819	20	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	38.013	22	100
B	29.302	21	300
B			
B	26.137	24	500

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

AB_SPIKE = Percentage of abnormal spike of sperm cell

Table 8A. α-tocopherol acetate accumulation in muscle and hepatopancreas of male white shrimp *P. vannamei*

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	49.85515554	2.93265621	10.27	0.0001
Error	36	10.27696345	0.28547121		
Corrected Total	53	60.13211899			

R-Square	C.V.	Root MSE	VITE_CON Mean
0.829094	62.61868	0.534295	0.853252

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	2.29187078	1.14593539	4.01	0.0267
AX	2	0.76441793	0.38220897	1.34	0.2749
VITE*AX	4	1.35989029	0.33997257	1.19	0.3314
ORG	1	39.97796402	39.97796402	140.04	0.0001
VITE*ORG	2	3.24232030	1.62116015	5.68	0.0072
AX*ORG	2	0.84488413	0.42244207	1.48	0.2412
VITE*AX*ORG	4	1.37380810	0.34345202	1.20	0.3263

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	4.10023066	2.05011533	7.18	0.0024
AX	2	1.27360871	0.63680436	2.23	0.1221
VITE*AX	4	2.15273432	0.53818358	1.89	0.1342
ORG	1	39.97796402	39.97796402	140.04	0.0001
VITE*ORG	2	3.24232030	1.62116015	5.68	0.0072
AX*ORG	2	0.84488413	0.42244207	1.48	0.2412
VITE*AX*ORG	4	1.37380810	0.34345202	1.20	0.3263

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	1.1227	18	600
A			
B A	0.8144	18	400
B			
B	0.6226	18	200

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	1.0059	18	300
A			
A	0.8383	18	500
A			
A	0.7156	18	100

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	2.0701	18	2
B	0.2448	36	1

Table 8A. (Continued)

---------- ORG=1 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
VITE	3	200 400 600

Number of observations in by group = 36

---------- ORG=1 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.27300374	0.03412547	1.94	0.0944
Error	27	0.47448119	0.01757338		
Corrected Total	35	0.74748493			

R-Square	C.V.	Root MSE	VITE_CON Mean
0.365230	54.14361	0.132565	0.244839

---------- ORG=1 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	0.05455572	0.02727786	1.55	0.2301
AX	2	0.04086486	0.02043243	1.16	0.3278
AX*VITE	4	0.17758315	0.04439579	2.53	0.0640

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	0.05455572	0.02727786	1.55	0.2301
AX	2	0.04086486	0.02043243	1.16	0.3278
AX*VITE	4	0.17758315	0.04439579	2.53	0.0640

---------- ORG=1 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	0.29944	12	600
A			
A	0.22363	12	200
A			
A	0.21145	12	400

Table 8A. (Continued)

---------- ORG=1 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	0.27779	12	300
A			
A	0.25817	12	500
A			
A	0.19856	12	100

---------- ORG=2 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
VITE	3	200 400 600

Number of observations in by group = 18

---------- ORG=2 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	9.60418779	1.20052347	1.10	0.4399
Error	9	9.80248226	1.08916470		
Corrected Total	17	19.40667005			

R-Square	C.V.	Root MSE	VITE_CON Mean
0.494891	50.41504	1.043631	2.070078

---------- ORG=2 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	5.47963535	2.73981768	2.52	0.1356
AX	2	1.56843720	0.78421860	0.72	0.5128
AX*VITE	4	2.55611524	0.63902881	0.59	0.6806

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	5.47963535	2.73981768	2.52	0.1356
AX	2	1.56843720	0.78421860	0.72	0.5128
AX*VITE	4	2.55611524	0.63902881	0.59	0.6806

Table 8A. (Continued)

---------- ORG=2 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	2.7693	6	600
A			
A	2.0204	6	400
A			
A	1.4206	6	200

---------- ORG=2 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	2.4620	6	300
A			
A	1.9987	6	500
A			
A	1.7496	6	100

---------- VITE=200 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
ORG	2	1 2

Number of observations in by group = 18

---------- VITE=200 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	5.80650121	1.16130024	8.17	0.0014
Error	12	1.70475688	0.14206307		
Corrected Total	17	7.51125809			

R-Square	C.V.	Root MSE	VITE_CON Mean
0.773040	60.53794	0.376913	0.622606

Table 8A. (Continued)

---------- VITE=200 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AX	2	0.00325154	0.00162577	0.01	0.9886
ORG	1	5.73067741	5.73067741	40.34	0.0001
AX*ORG	2	0.07257226	0.03628613	0.26	0.7787

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AX	2	0.00420633	0.00210316	0.01	0.9853
ORG	1	5.73067741	5.73067741	40.34	0.0001
AX*ORG	2	0.07257226	0.03628613	0.26	0.7787

---------- VITE=200 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	0.6370	6	500
A			
A	0.6262	6	100
A			
A	0.6047	6	300

---------- VITE=200 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	1.4206	6	2
B	0.2236	12	1

---------- VITE=400 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
ORG	2	1 2

Number of observations in by group = 18

---------- VITE=400 ----------

General Linear Models Procedure

Dependent Variable: VITE_CON

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	13.46260478	2.69252096	8.24	0.0014
Error	12	3.91967100	0.32663925		
Corrected Total	17	17.38227579			

R-Square	C.V.	Root MSE	VITE_CON Mean
0.774502	70.17583	0.571524	0.814417

Table 8A. (Continued)

```
---------------------------------- VITE=400 ----------------------------------

                        General Linear Models Procedure

Dependent Variable: VITE_CON

Source                   DF       Type I SS    Mean Square   F Value     Pr > F

AX                        2      0.27674176     0.13837088      0.42     0.6641
ORG                       1     13.08847684    13.08847684     40.07     0.0001
AX*ORG                    2      0.09738618     0.04869309      0.15     0.8631

Source                   DF     Type III SS    Mean Square   F Value     Pr > F

AX                        2      0.35994593     0.17997297      0.55     0.5903
ORG                       1     13.08847684    13.08847684     40.07     0.0001
AX*ORG                    2      0.09738618     0.04869309      0.15     0.8631

---------------------------------- VITE=400 ----------------------------------

                        General Linear Models Procedure

                Duncan Grouping                Mean      N  AX

                                A            0.9527      6  500
                                A
                                A            0.8387      6  300
                                A
                                A            0.6519      6  100

---------------------------------- VITE=400 ----------------------------------

                        General Linear Models Procedure

                Duncan Grouping                Mean      N  ORG

                                A            2.0204      6  2
                                B            0.2115     12  1

---------------------------------- VITE=600 ----------------------------------

                        General Linear Models Procedure
                            Class Level Information

                             Class    Levels    Values
                             AX            3    100 300 500
                             ORG           2    1 2

                          Number of observations in by group = 18

---------------------------------- VITE=600 ----------------------------------

                        General Linear Models Procedure

Dependent Variable: VITE_CON
                                      Sum of                  Mean
Source                   DF          Squares                Square   F Value     Pr > F

Model                     5     28.29417877     5.65883575     14.60     0.0001

Error                    12      4.65253557     0.38771130

Corrected Total          17     32.94671434

                  R-Square             C.V.       Root MSE        VITE_CON Mean

                  0.858786         55.45971       0.622665              1.122733
```

Table 8A. (Continued)

```
---------------------------------- VITE=600 ----------------------------------

                        General Linear Models Procedure

Dependent Variable: VITE_CON

Source                   DF       Type I SS    Mean Square   F Value     Pr > F

AX                        2      1.84431492     0.92215746      2.38     0.1349
ORG                       1     24.40113006    24.40113006     62.94     0.0001
```

AX*ORG	2	2.04873379	1.02436689	2.64	0.1120

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AX	2	3.06219077	1.53109538	3.95	0.0481
ORG	1	24.40113006	24.40113006	62.94	0.0001
AX*ORG	2	2.04873379	1.02436689	2.64	0.1120

---------- VITE=600 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	1.5742	6	300
A			
A	0.9253	6	500
A			
A	0.8686	6	100

---------- VITE=600 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	2.7693	6	2
B	0.2994	12	1

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

ORG = Shrimp tissue: 1 = muscle and 2 = hepatopancreas

VITE_CONC = α-tocopherol acetate concentration (ppm)

Table 9A. Astaxanthin accumulation in muscle and hepatopancreas of male white shrimp *P. vannamei*

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	357.3538159	21.0208127	2.16	0.0259
Error	36	350.6071177	9.7390866		
Corrected Total	53	707.9609336			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.504765	45.94958	3.120751	6.791685

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	2.5870481	1.2935241	0.13	0.8761
AX	2	116.3696917	58.1848459	5.97	0.0057
VITE*AX	4	26.0616336	6.5154084	0.67	0.6177
ORG	1	144.8634867	144.8634867	14.87	0.0005
VITE*ORG	2	14.4940112	7.2470056	0.74	0.4823
AX*ORG	2	46.7787250	23.3893625	2.40	0.1049
VITE*AX*ORG	4	6.1992195	1.5498049	0.16	0.9576

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	3.5094286	1.7547143	0.18	0.8359
AX	2	149.7624868	74.8812434	7.69	0.0017
VITE*AX	4	29.4909137	7.3727284	0.76	0.5601
ORG	1	144.8634867	144.8634867	14.87	0.0005
VITE*ORG	2	14.4940112	7.2470056	0.74	0.4823
AX*ORG	2	46.7787250	23.3893625	2.40	0.1049
VITE*AX*ORG	4	6.1992195	1.5498049	0.16	0.9576

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	7.075	18	200
A			
A	6.757	18	600
A			
A	6.543	18	400

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	8.572	18	500
A			
B A	6.826	18	300
B			
B	4.977	18	100

Table 9A. (Continued)

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	9.1080	18	2
B	5.6335	36	1

---------- ORG=1 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
VITE	3	200 400 600

Number of observations in by group = 36

---------- ORG=1 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	49.62848122	6.20356015	1.38	0.2483
Error	27	121.11764975	4.48583888		
Corrected Total	35	170.74613097			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.290657	37.59598	2.117980	5.633528

---------- ORG=1 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	7.15695906	3.57847953	0.80	0.4607
AX	2	31.48501572	15.74250786	3.51	0.0442
AX*VITE	4	10.98650644	2.74662661	0.61	0.6574

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	7.15695906	3.57847953	0.80	0.4607
AX	2	31.48501572	15.74250786	3.51	0.0442
AX*VITE	4	10.98650644	2.74662661	0.61	0.6574

---------- ORG=1 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	6.1728	12	200
A			
A	5.6470	12	400
A			
A	5.0808	12	600

Table 9A. (Continued)

---------- ORG=1 ----------

General Linear Models Procedure

Duncan Grouping		Mean	N	AX
	A	6.9273	12	500
	A			
B	A	5.2242	12	300
B				
B		4.7491	12	100

---------- ORG=2 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
AX	3	100 300 500
VITE	3	200 400 600

Number of observations in by group = 18

---------- ORG=2 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	162.8618480	20.3577310	0.80	0.6192
Error	9	229.4894680	25.4988298		
Corrected Total	17	392.3513160			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.415092	55.44177	5.049637	9.108000

---------- ORG=2 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	9.9241003	4.9620502	0.19	0.8265
AX	2	131.6634010	65.8317005	2.58	0.1300
AX*VITE	4	21.2743467	5.3185867	0.21	0.9272

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	9.9241003	4.9620502	0.19	0.8265
AX	2	131.6634010	65.8317005	2.58	0.1300
AX*VITE	4	21.2743467	5.3185867	0.21	0.9272

Table 9A. (Continued)

---------- ORG=2 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	10.110	6	600
A			
A	8.881	6	200
A			
A	8.334	6	400

---------- ORG=2 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	AX
A	11.862	6	500
A			
A	10.030	6	300
A			
A	5.432	6	100

---------- AX=100 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
VITE	3	200 400 600
ORG	2	1 2

Number of observations in by group = 18

---------- AX=100 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	17.59693775	3.51938755	0.91	0.5074
Error	12	46.52021475	3.87668456		
Corrected Total	17	64.11715250			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.274450	39.56190	1.968930	4.976833

Table 9A. (Continued)

---------- AX=100 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	5.82077033	2.91038517	0.75	0.4929
ORG	1	1.86732225	1.86732225	0.48	0.5009
VITE*ORG	2	9.90884517	4.95442258	1.28	0.3139

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	11.02547006	5.51273503	1.42	0.2791
ORG	1	1.86732225	1.86732225	0.48	0.5009
VITE*ORG	2	9.90884517	4.95442258	1.28	0.3139

---------- AX=100 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	5.708	6	600
A			
A	4.902	6	400
A			
A	4.321	6	200

---------- AX=100 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	5.4323	6	2
A			
A	4.7491	12	1

---------- AX=300 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
VITE	3	200 400 600
ORG	2	1 2

Number of observations in by group = 18

---------- AX=300 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	98.22610994	19.64522199	2.77	0.0688
Error	12	85.10422100	7.09201842		
Corrected Total	17	183.33033094			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.535788	39.01352	2.663084	6.826056

Table 9A. (Continued)

---------- AX=300 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	1.68954444	0.84477222	0.12	0.8887
ORG	1	92.37772844	92.37772844	13.03	0.0036

VITE*ORG	2	4.15883706	2.07941853	0.29	0.7511

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	3.17702017	1.58851008	0.22	0.8026
ORG	1	92.37772844	92.37772844	13.03	0.0036
VITE*ORG	2	4.15883706	2.07941853	0.29	0.7511

---------- AX=300 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE
A	7.164	6	600
A			
A	6.892	6	200
A			
A	6.422	6	400

---------- AX=300 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	10.030	6	2
B	5.224	12	1

---------- AX=500 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
VITE	3	200 400 600
ORG	2	1 2

Number of observations in by group = 18

---------- AX=500 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	125.1610765	25.0322153	1.37	0.3016
Error	12	218.9826820	18.2485568		
Corrected Total	17	344.1437585			

R-Square	C.V.	Root MSE	AST_CONC Mean
0.363688	49.83376	4.271833	8.572167

Table 9A. (Continued)

---------- AX=500 ----------

General Linear Models Procedure

Dependent Variable: AST_CONC

Source	DF	Type I SS	Mean Square	F Value	Pr > F
VITE	2	21.13836700	10.56918350	0.58	0.5753
ORG	1	97.39716100	97.39716100	5.34	0.0395
VITE*ORG	2	6.62554850	3.31277425	0.18	0.8362

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VITE	2	18.79785206	9.39892603	0.52	0.6101
ORG	1	97.39716100	97.39716100	5.34	0.0395
VITE*ORG	2	6.62554850	3.31277425	0.18	0.8362

---------- AX=500 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	VITE

A	10.013	6	200
A			
A	8.304	6	400
A			
A	7.400	6	600

---------- AX=500 ----------

General Linear Models Procedure

Duncan Grouping	Mean	N	ORG
A	11.862	6	2
B	6.927	12	1

Note : VITE = Vitamin E concentrations: 200 = 200 ppm α-tocopherol acetate, 400 = 400 ppm α-tocopherol acetate, and 600 = 600 ppm α-tocopherol acetate,

Ax = Astaxanthin concentrations:100 = 100 ppm astaxanthin,300 = 300 ppm astaxanthin and 500 = 500 ppm astaxanthin

N = Amounts of shrimp (individuals)

ORG = Shrimp tissue: 1 = muscle and 2 = hepatopancreas

AST_CONC = Astaxanthin concentration (ppm)

APPENDIX B

VITAMIN E DETERMINATION

The determination of alpha-Tocopheryl Acetate in feeds and shrimp tissues by HPLC (Manz and Philipp, 1981)

Rang

Above 1000 mg/kg

Standard solution

The standard solution is prepared in n-hexane using pure dl-α-tocopheryl acetate. The final concentration solution should be about 100 mg/l.

Procedure

Sample Preparation and extraction

Coarse-grained samples are ground in a mortar and mixed well. To an accurately weighed amount of the test material in a 100-ml volumetric flask 20 ml of warm (65 ^{o}C) hydrochloric acid (0.01 mol/l) are added and placed with occasional shaking in an ultrasonic bath at a temperature of 65 ^{o}C for 3 minutes. After cooling under tap water, 70 ml of ethanol are added and mixture subjected to ultrasonic for further 3 minutes at room temperature. The flask is filled up to the mark with ethanol, the contents mixed well and left to stand for a few minutes to allow partial sedimentation. 2.0 ml of the supernatant are placed in a test tube, 4.0 ml of n-hexane are then added and the mixture is shaken mechanically for 2 minutes. Then 3.0 ml of distilled water are added and the tube is inverted gentle several times. After centrifugation at an equivalent force of 6,000 g for 5 minutes and the aliquot of the upper, organic phase is transferred into the sample flask of the injection sampler and subjected to HPLC

Oily concentrates are simply diluted to the appropriate concentrations with n-hexane (maximum sample weight 2.5 g/100 ml) and injected without further sample preparation into the HPLC system.

Test material containing larger amounts of gelatine are processed slightly differently. A maximum weight of 2.0 g of sample is treated with 20 ml of distilled water, 50 mg of pepsin and 50 mg of trypsin for 30 minutes at temperature of 50 ^{o}C in and ultrasonic bath. After cooling, 70 ml of ethanol are added slowly under vigorous shaking and by simultaneously dispersing the mixture in an ultrasonic bath. The further steps of sample preparation are performed according to the procedure described above.

Specifications for high-performance liquid chromatography

-Column: stainless steel, length 12.5 cm, inner diameter 4 mm

-Stationary phase: silica gel (e.g. HIBAR prepacked column filled with LiChrosorb Si 60, 5 μm, Merck, Darmstadt, FRG): pretreated with phosphoric acid

-Mobile phase: n-hexane containing 0.4% 1,4-dioxane, isocratic

-Flow rate: 1.5 ml/min

-Pressure: about 40 bar

-Temperature: ambient

-Injection volume: 20-50 μl

-Detection: UV detection at 285 nm
(instruments with varible wave lengths) or at 280 nm (instruments with filters)

-Retention time: 7.6-8 minutes

-Run time: 16 minutes

Calculation

The resulting peak area calculated by the integrator is compared with the corresponding peak area found for the standard solution, taking into account the weight of the sample and the dilution of the extract.

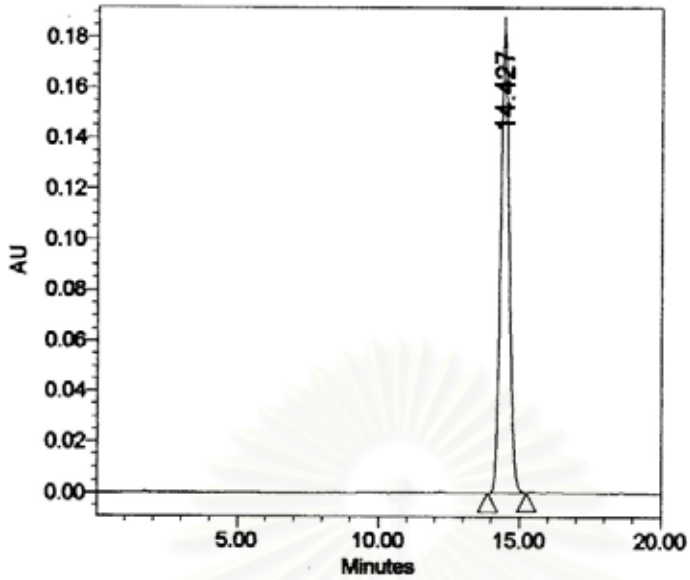

Figure 1B Chromatogram of standard DL-α-tocopherol acetate

APPENDIX C

ASTAXANTHIN DETERMINATION

The determination of astaxanthin in feed by HPLC (Weber, 1988)

Rang

Above 1 mg/kg

Standard solution

Astaxanthin (1.5 μg ml^{-1}): Dissolve approximate 3 mg pure astaxanthin cyst in 10 ml chloroform in a 100-ml volumetric flask and make up to volume with n-hexane. Mix 5 ml of this dilution with 4 ml of chloroform and dilute to 100 ml with n-hexane. To calculate the exact content of this standard solution and absorbance is measured in a spectrophotometer at 470 nm (at the maximum).

E(1%/1 cm) = 2100
= Standard absorbance of 1% astaxanthin solution
(weight/volume) in a 1-cm cuvette at 470 nm in n-hexane solvent.

The purity of standard is check by HPLC. This standard solution may be stored for 2 days if kept protected from light in a refrigerator

Procedure

Preparation of samples

Grind pellets in a coffee grinder. Mash feed and powdery premixes can be used as such. Mix well.

Extraction

Declared content above 20 mg astaxanthin/ kg

Weight approximate 10 g of feed or 1 g of premix in to a weighed 100-ml volumetric flask , add approximate 100 mg MAXATASE and approximate 80 ml of distilled water. Place the flask for 30 minutes in an ultrasonic water bath at 50 ^{o}C. Cool to ambient temperature and fill to the mark with distilled water. Weigh again, shake and weigh approximate 10 g of the mixture into a 250-ml volumetric flask. Add 100 ml absolute ethanol, shake, make up almost to volume with dichloromethane and shake. Leave in a dark place until room temperature is re-established. Make up to volume with dichloromethane and mix well.

Declared content lower than 20 mg astaxanthin/ kg

Weight approximate 5 g of final feed in to a tarred round-bottom flask, and approximate 200 mg MAXATASE and approximate 150 ml of distilled water, then weigh again. Place the flask for 30 minutes in an ultrasonic water bath at 50 ^{o}C. Cool to ambient temperature and weigh approximate 10 g of the mixture into a 250-ml volumetric flask. Add 100 ml absolute ethanol, shake, make up almost to volume with dichloromethane and shake. Leave in a dark place until room temperature is re-established. Make up to volume with dichloromethane and mix well.

Purification and the extract by open-column chromatography on silica gel

Preparation of the silica gel column:

Fill a chromatography tube with approximate 10 ml n-hexane/ether (1:1), add 5 g silica gel and bring the silica gel into suspension by a jet of n-hexane/ether (1:1) from a wash bottle. Let the silica gel sediment and keep it covered with solvent all the time.

Chromatography on the silica gel:

Transfer and aliquot (e.g. 25 ml) of the extract (from “Extraction:) on to the silica gel and elute with 100 ml-ml n-hexane/ether (1:1). Collect the eluate in a 250-ml round bottom flask and remove the solvent in a rotary evaporator. Dissolve the dry residue in 5 or 10 ml n-hexane containing 14% acetone.

High-performance liquid chromatography

Pretreatment of the stationary phase n the column:

A solution of phosphoric acid in a methanol (1g/100ml) is pumped through the column filled with silica gel for 1 hour at a flow rate of 10ml per minute.

HPLC procedure

Pump mobile phase through the column for a least 30 minutes to equilibrate the system. Determine the peak area (peak height) of the standard by repeated injection of 10 μl of standard solution. The retention time should be about 11 minutes. Inject 10 to 50 μl of sample solution. Use the net peak heights, or preferably the peak area, recorded by an integrator, for the calculation.

Specifications for high-performance liquid chromatography

-Column: stainless steel, length 25 cm, inner diameter 4 mm

-Stationary phase: silica gel (e.g. HIBAR prepacked column filled with LiChrosorb Si 60, 5 μm, Merck, Darmstadt, FRG): pretreated with phosphoric acid

-Mobile phase: n-hexane containing 14% acetone, isocratic

-Flow rate: 1.5 ml/min

-Pressure: approximate 80 bar

-Temperature: ambient

-Injection volume: 10 to 50 μl

-Detection: VIS-detection at 470 nm

-Retention time: canthaxanthin: about 4 minutes;
astaxanthin (all-E): about 11 minutes;
(Z)-isomers of astaxanthin: about 12 and 13 minutes

-Run time: 15 minutes

Calculation

The sum of the resulting areas from 11 to 13 minutes retention time, calculated by the integrator (or corresponding peak heights obtained on a record of the chromatograme), is compared with the corresponding peak area (height) for the standard solution.

mg total astaxanthin per kg of sample =

$$\frac{\text{Sum of the peak areas (test) x XF x standard conc. } (\mu g\ ml^{-1})}{\text{Peak area (standard) x Test portion (g)}}$$

XF = dilution factor

= theoretical volume in which the sample is dissolved

$$XF = \frac{V_{fin} \times 250 \times W_1 \times 10}{V_{eva} \times W_2 \times V_{inj}}$$

V_{fin} = Volume of the final test solution in n-hexane-acetone

V_{eva} = Volume of the evaporated ether solution

W_1 = Weight of aqueous suspension

W_2 = Weight of aqueous suspension transferred into the 250-ml flask

V_{inj} = Volume injected

If the extraction method is used for a content lower than 20 mg kg^{-1} (according to "Extraction"): $W_1 = W_2 = 1$

Determination of astaxanthin esters in animal tissues (Weber, 1988)

Acetone extraction

Cut the tissue in pieces of about 1 cm in length using a pair of scissors of a knife. Weight 10 to 20 g of the minced tissue into a 200 ml leaker and add approximate 5 g of magnesium sulfate hydrate (22.5-25%H_2O e.g. Merck No. 5885) For removing water. Add approximate 40 ml of acetone and homogenized the mixture with a rotation homogenizer. Filter the homogenate with partial vacuum to sintered glas. Scrape of the residue and homogenize again with a fresh portion of acetone. Repeat this procedure until the filtrate is colorless. Combine the filtrates in a volumetric flask and make up to volume with acetone.

Evaporate half of this extract under partial vacuum at 50 ^oC by means of a rotation evaporator. Dry the residue by repeated addition and evaporation of small of

ethanol. Take up the dry residue in a appropriate volume of n-hexane containing 14% of acetone and inject and aliquot of this extract into the HPLC system.

Saponification:

The other half of the acetone extract is saponified. For that purpose transfer the remaining half of the acetone extract into a 250 ml round-bottom flask and evaporate to dryness under partial vacuum at 50 ^{o}C. Redissolve the residue in 60 ml ethanol, 10 ml t-butyl methyl ether, and 5 ml 50% aqueous potassium hydroxide solution and reflux for 30 minutes (water bath, approximate 80 ^{o}C). After saponification, add approximate 20 ml of distilled water to the solution and cool down to ambient temperature.

Ether extraction:

Rinse the content of the flask into a 500 ml separating funnel with approximate 100 ml distilled water and with the first 100 ml of diethyl ether required for the extraction. Shake carefully and de-aerate the funnel several times. As soon as the overpressure is removed shake the content of the funnel vigorously. After complete separation of the phase transfer the aqueous one into a second separating funnel and extract again with fresh 100 ml diethyl ether as described above. Repeat the extraction the third time. Wash the combined organic phases with distilled water until their pH is neutral (check with pH-paper). Evaporate the neutral ether extract with a rotary evaporator under partial vacuum at 50 ^{o}C. Remove the last traces of water by addition and evaporation of some ethanol. Take up the dry residue in an appropriate volume eof n-hexane containing 14% of acetone and inject an aliquot of this extract in to the HPLC system.

Calculation:

In the HPLC chromatogram of the non-saponified extract the peaks of the astaxanthin esters occur with a retention time of 1.25-4.65 minutes. The peak areas in this chromatographic range are summarized and quantified with the response factor of

free astaxanthin. The result gives the “non-corrected amount of esterified astaxanthin”, calculated as free astaxanthin.

Peaks, which occur in HPLC chromatogram of the saponified extract with the retention time of astaxanthin esters (1.25-4.65 minutes) are in no case due to astaxanthin esters, since astaxanthin is readily liberated from the esters and oxidized to astacene (t_R = 5.1 minute) by the saponification process. These peaks are rather due to carotene (t_R = 1.30 minute) and the alkali-stable keto-carotenoid (echininone t_R = 2.0 minute, canthaxantin t_R = 4.1 minute). Therefore, such peaks are quantified with the response factor of free astaxanthin and subtracted from the “non-corrected amount of esterified astaxanthin”, determined by HPLC of the non-saponified acetone extract.

Esters of hydroxy-xanthophylls (as those of lutein and zeaxanthin) also cochromatographed with the esters of astaxanthin. If these esters are present in the tissue, the peaks of the free xanthophylls detected in the HPLC chromatograme of the saponified extract will be larger than the corresponding peaks produced by the non-saponified extract. In this case, the xanthophyll esters are quantified from the area difference between the peaks in the chromatograms of the saponified and non saponified extract using the response factor of free astaxanthin, and subtracted from the “non-corrected amount of esterified astaxanthin”.

In summary, the amount of astaxanthin esters is calculated as the “non-corrected amount of esterified astaxanthin” minus the amount of cochromatographing alkali-stable carotenoids and minutes the amount of other xanthophhyll esters.

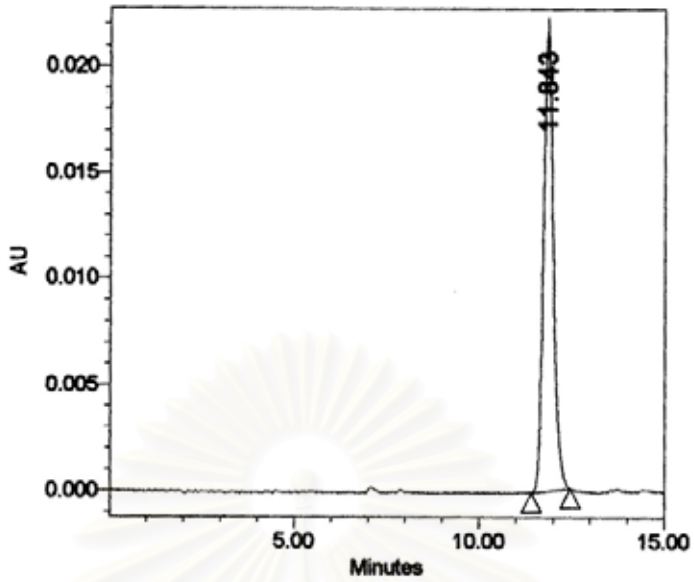

Figure 1C Chromatogram of standard astaxanthin

BIOGRAPHY

Ms. Suangsuda Supasai was born on July 12, 1979 in Surin Province. She graduated high school from Sirindhorn School in 1998. She received Sritrungtong Fund of Mahidol University to study Bachelor Degree of Science (Biotechnology) and graduated in 2002. Then, she studied for Master Degree in Biotechnology, Faculty of Science, Chulalongkorn University since 2002.