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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF ASTAXANTHIN FROM *PORTUNUS SANGUINOLENTUS* (THREE SPOTTED CRAB), *CALLINECTES SAPIDUS* (BLUE CRAB) AND *PARALITHODES BREVIPES* (SPINY KING CRAB)

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ABSTRACT

The goal of the present study was to isolate and characterize astaxanthin from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) using the three different types of solvent mixture such as Hexane: Isopropanol, DMSO: Acetone and acetone. The biochemical composition present in the shells of three crab varieties were evaluated and subjected to the isolation of astaxanthin. The moisture content of three crab varieties ranged between 72.58 ± 0.58 % to 87.67 ± 0.38 % which was higher than other biochemical compositions such as ash, lipid and protein. The yield of astaxanthin from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) using Hexane: Isopropanol was 31.23± 0.53µg/g, 48.41± 0.28µg/g and 20.15± 0.18µg/g. The yield of astaxanthin using DMSO: Acetone was found to be 29.01±10µg/g, 39.98 ± 0.43 µg/g and 18.06± 0.23µg/g and yield of astaxanthin using Acetone was found to be 19.24± 0.46µg/g, 30.05 ± 0.32µg/g and 12.13 ± 0.16 µg/g. The highest yield was obtained using Hexane: Isopropanol hence, the isolated compound was confirmed to be astaxanthin by TLC. The Rf values in the range of 0.33 to 0.73 was obtained by TLC which confirmed that the isolated extract contains astaxanthin and its esters which is compared with standard astaxanthin. Further, the standard astaxanthin and isolated astaxanthin were subjected to FT-IR, NMR, HPLC and GC-MS for determining the functional groups and predicting the structural conformation. The peak was obtained in FT-IR, NMR, HPLC and GC-MS analysis for isolated astaxanthin and standard astaxanthin which shows the presence of their functional groups. The resultssuggest that astaxanthin isolated from the shells waste of three crab varieties can be used as natural pigments in various field.

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INTRODUCTION

Seafood processing industry in India is contributing tonnes and tonnes of waste materials and among them crab and shrimp waste occupy more than a lakh tonnes every year (Saranya *et al.,* 2013). It has been estimated that solid waste from the crab industries is now in excess of a million kg/year in the USA (Kuo *et al.,* 1976). The proper utilization of these waste yields high economics value, as these wastes are rich in proteins, mucopolysaccharides like carotenoid, chitin, flavor compounds etc., (Saranya *et al.,* 2013). Astaxanthin is one of the most important carotenoid pigment which is ubiquitous in nature, especially in the marine environments such as salmon, trout, krill, shrimp, crayfish, crabs and lobsters (JyothikaDhankhar *et al.,* 2012), because it is synthesized by phytoplankton and zooplankton and bio-accumulates throughout the food web (Daly *et al.,* 2012). Astaxanthinfound in crabs will absorb the blue light from the environment and appears red, orange or yellow in color and gets incorporated with a protein called crustacyanin. The protein holds the pigment so tight and forms astaxanthin-crustacyanin complex to create a greenish blue coloration in blue crab. Astaxanthin is either conjugated with proteins or esterified with one or two fatty acids to form monoester and diester forms, displaying of colors in different organisms (Hussein *et al.,* 2006, Peng *et al.,* 2008). It consists of two oxygenated groups on each ring structure, which is responsible for its enhanced antioxidant features (Guerin *et al.,* 2003). The antioxidant activity of astaxanthin has been reported to be 10 times stronger than that of other carotenoids (NaguibYousry *et al.,* 2000).

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Astaxanthin has significant metabolic role in animals and humans ranging from protection against oxidation of essential polyunsaturated fatty acids, protection against UV light effects, pro-vitamin A activity and vision, immune response, pigmentation and communication to reproductive behavior and improved reproduction (Camera *et al.,* 2009). It is also used in the prevention and treatment of neural damage associated with age-related macular degeneration and effective in treating Alzheimer's disease, Parkinson's disease, spinal cord injuries and other central nervous system injuries. The biological activities of astaxanthin, includes anticancer, anti-inflammatory, anti-diabetic, immuno-modulatory activities and a Neuroprotective effect (Hussein *et al.,* 2006).

In all the tissues, astaxanthin was distributed and is able to cross the blood-brain and other biological barriers (Aoi *et al.,* 2003), which also protect the retina against photo oxidation and loss of photo receptor cells.

Astaxanthin is also widely used in aquaculture, cosmetic, and functional foods (Guerin *et al.,* 2003, Higuera- CiaparaI *et al.,* 2006). Most of the astaxanthin used in aquaculture feeds is synthetically derived, however, growing consumer demand for natural products has provided the opportunity for commercial production of astaxanthin from natural sources (John *et al.,* 2001). Synthetic astaxanthin has been banned into health food market by the U.S. Food and Drug Administration (FDA) due to its low bioavailability and security (Seabra *et al.,* 2010). Therefore, astaxanthin obtained from natural resources was approved by the FDA as dietary supplement in aquaculture in 1987 (Roche, 1987). The use of astaxanthin in the aquaculture industry is important for the pigmentation and consumer appeal but also as an essential nutritional component for adequate growth and reproduction.

Astaxanthin products are available in the form of capsule, soft gel, tablet, powder, biomass, cream, energy drink, oil and extract in the market (Ranga Rao Ambati *et al.,* 2014).

MATERIALS AND METHODS

Preparation of sample source

The *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab), *Paralithodes brevipes* (Spiny King Crab) were collected from Gandhi market, Trichy, Tamilnadu, India. The crabs were transported to the laboratory in a sterile container filled with ice. The shells from the crabs such as cephalothorax, abdominal portion and pincers were removed. They were washed under running water, air dried in the shade and powdered.

Determination of proximate composition

a) Moisture Content Determination

The moisture content was determined using AOAC method, 1990

$$\text{Percentage moisture} = \frac{W2 - W1}{W2 - W3} \times \frac{100}{1}$$

Where

W1 = Initial weight of empty dish
W2 = Weight of dish + undried sample
W3 = Weight of dish + dried sample

b) Ash Content Determination

Ash content was determined by AOAC method, 1990. The weight of the residual ash was then calculated as

$$\text{Ash Content Percentage} = \frac{\text{Weight of Ash}}{\text{Weight of original sample}} \times \frac{100}{1}$$

c) Fat Content Determination

Fat content was determined by AOAC method, 1990. The percentage oil content is percentage fat

$$= \frac{W2 - W1}{W3} \times \frac{100}{1}$$

Where

W1 = weight of the empty extraction flask
W2 = weight of the flask and oil extracted
W3 = weight of the sample

d) Crude Protein Determination

The micro kjeldahl method described by A.O.A.C (1990) was used.

This is given as percentage Nitrogen

$$= \frac{(100 \times N \times 14 \times VF)\ T}{100 \times Va}$$

Where

N= Normality of the titrate (0.1N)
VF= Total volume of the digest= 100ml
T= Titre Value
Va= Aliquot Volume distilled

Extraction of astaxanthin using various solvent mixtures

a) Hexane and Isopropanol

5gram of powdered crab shells were weighed and mixed with 50 ml of Hexane: Isopropanol solvent mixture in the ratio of 30: 20 v/v. It is placed in magnetic stirrer for proper mixing. Again 10 ml of solvent was added and kept in magnetic stirrer. This process was repeated until the color disappear. The solvent extract was washed with 50ml of saline and the hexane layer was separated and evaporated to dryness. The astaxanthin extracted was mixed with acetone, which was measured at 470

nm using UV-visible spectrophotometer (Sachindra N M*et al.,* 2007).

b) DMSO: Acetone

To 0.5 g of powdered crab shells, 2 ml of DMSO was added in centrifuge tube, pre-warmed at 55°C for 30 minutes, shaken well and vortexed for 1 min. It was then, kept undisturbed for 30 minutes. To this, 6 ml of acetone was added. The solution was centrifuged at 1745 x g for 10 min and the supernatant was collected. The same procedure was repeated for the precipitate. Supernatants were gathered for determining the astaxanthin specific concentration and the absorbance was measured at 470 nm (Persike *et al.,* 2002).

c) Acetone

1g of powdered crab shells was ground using 10 ml of acetone and vortexed for 1 min. The mixture was centrifuged and the pellet was re extracted with further 10ml of acetone until complete extraction, which was evaluated by the absence of color in the solvent. The extract was recorded at 400 – 500nm using UV- Visible spectrophotometer (Gouveia *et al.,* 1996).

Quantification of astaxanthin

The extracted astaxanthin is redissolved in 3 ml of acetone and read @ 470 nm (Uma Nath Ushakumari and Ravi Ramanujan 2012).

$$AST\ (\mu g/g) = \frac{A \times D \times 10^6}{100 \times G \times d \times E\ ^{1\%}{}_{1}cm}$$

Where,

AST is astaxanthin concentration in µg/g
A is absorbance
D is volume of extract in acetone
10^6 is dilution multiple
G is weight of sample in g
d is the cuvette width (1 cm)
E is extinction coefficient 2100
OD @470 nm

Confirmation of astaxanthin by TLC

Analysis of astaxanthin and its esters in the extract was confirmed using Thin Layer Chromatography (TLC). A small volume of the isolated astaxanthin was spotted on silica gel plate and developed using acetone: hexane 3:7 (v/v). The separated bands were identified using standard astaxanthin (NutraBio, USA) and internationally accepted Rf values for astaxanthin, astaxanthin monoester and astaxanthin diester (Uma Nath Ushakumari and Ravi Ramanujan 2012).

FT-IR analysis of standard astaxanthin and isolated astaxanthin

Astaxanthin extracted using Hexane: Isopropanol was subjected to FT-IR along with the standard in the range of 450 to 4500 $cm^{-1.}$ The range spectrum was evaluated to determine the functional groups present in different samples along with the standard (Nageswara Rao *et al.,* 2005).

NMR analysis of standard astaxanthin and isolated astaxanthin

Astaxanthin extracted using Hexane: Isopropanolwas subjected to NMR analysis along with the standard. $CDCl_3$ is used as a solvent in NMR analysis (Nageswara Rao *et al.,* 2005).

HPLC analysisof standard astaxanthin and isolated astaxanthin

Astaxanthin extract from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab), *Paralithodes brevipes* (Spiny King Crab) using Hexane: Isopropanol were subjected to High Performance Liquid Chromatography (HPLC) using C_{18} column with injection volume 20 µl, 25 cm × 4.6 mm with diameter 5 µm under room temperature. The mobile phase was combination of an isocratic solvent system consists of dichloromethane, acetonitrile and methanol in the ratio of 20:70:10 with low rate of 1.0 ml/min. All the separated astaxanthin were analyzed at 470 nm using UV visible detector. Retention times were used to identify peaks (Elumalai *et al.,* 2014).

GC-MS analysisof standard astaxanthin and isolated astaxanthin

Astaxanthin extract from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab), *Paralithodes brevipes* (Spiny King Crab) using Hexane: Isopropanol were subjected to GC-MS analysis to confirm the presence of astaxanthin (Elumalai *et al.,* 2014)

RESULTS

Proximate composition

Table 1. Determination of proximate composition

Sample components	*Portunus sanguinolentus* (Three Spotted Crab) MEAN ± SD	*Callinectus sapidus* (Blue Crab) MEAN ± SD	*Paralithodes brevipes* (Spiny King Crab) MEAN ± SD
MOISTURE %	81.56±0.48	87.67±0.38	72.58±0.58
ASH %	18.53±0.50	19.56±0.42	19.10±0.44
LIPID %	3.80±0.15	5.37±0.30	3.60±0.18
PROTEIN %	31.63±0.40	33.80±0.26	30.53± 0.42

The proximate composition of shells of *Portunus sanguinolentus* (Three Spotted Crab), *Callinectus sapidus* (Blue Crab), *and Paralithodes brevipes* (Spiny King Crab) are represented in the Table 1. The proximate compositions such as moisture content, ash content, lipid and protein content were performed.

Moisture content was found to be one of the major components. The study reveals the percentage of moisture content. Average value varied from 72.58±0.58 % to 87.67±0.38 % which is tabulated in Table 1. It is noticed that *Paralithodes brevipes* (Spiny King Crab) shows lower moisture

content i.e. 72.58±0.58 % than the *Portunus sanguinolentus* (Three Spotted Crab) i.e. 81.56±0.48 % and *Callinectus sapidus* (Blue Crab) i.e. 87.67±0.38 %, which reflected the good healthiness of the crab.

The ash content is a measure of the total amount of minerals present in the sample. Ash content is a useful parameter of the nutritional value of some food and feeds. The ash content of dried shell of the crab varied between 18.53±0.50 % to 19.56±0.42 %which is tabulated in the Table 1. The highest ash value was seen in the case of *Callinectus sapidus* (Blue Crab) and the lowest value was observed in *Portunus sanguinolentus* (Three Spotted Crab).

Lipids are considered as the source of energy. The energy output is greater than carbohydrates and proteins, they also play a vital role in the structural and biological function of the cells and transport fat soluble vitamin in the body. The average value of lipid ranged from 3.60±0.18 % to 5.37± 0.30 %which is shown in the Table 1.

Protein is one of the basic building blocks of body which enhance the normal function, growth and maintenance of body tissue. The protein content of the crabs therefore provide good nutrition. The concentration of protein was found to greater in the shells of the *Portunus sanguinolentus*(Three Spotted Crab) i.e. 31.63± 0.46 %, *Callinectus sapidus* (Blue Crab) i.e. 33.08± 0.26 % and *Paralithodes brevipes* (Spiny King Crab) i.e. 33.53± 0.42 shown in Table 1.

Quantification of astaxanthin

Table 2. Yield of astaxanthin using Hexane: Isopropanol

Source	Astaxanthin (µg/g)
Portunus sanguinolentus (Three Spotted Crab)	31.23 ± 0.53
Callinectus sapidus (Blue Crab)	48.41 ± 0.28
Paralithodus brevipes (Spiny King Crab)	20.15 ± 0.18

Table 3. Yield of astaxanthin using DMSO: Acetone

Source	Astaxanthin (µg/g)
Portunus sanguinolentus (Three Spotted Crab)	29.01 ±0.10
Callinectus sapidus (Blue Crab)	38.98 ±0.43
Paralithodus brevipes (Spiny King Crab)	18.06 ±0.23

Table 4. Yield of astaxanthin using Acetone

Source	Astaxanthin (µg/g)
Portunus sanguinolentus (Three Spotted Crab)	19.24 ± 0.46
Callinectus sapidus (Blue Crab)	30.05 ± 0.32
Paralithodes brevipes (Spiny King Crab)	12.13 ± 0.16

Extraction of astaxanthin, a carotenoid pigment from the shell waste of different varieties of crabs suchas *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) was investigated by the various organic solvent mixture (Hexane: Isopropanol, DMSO: acetone and acetone). The concentration of astaxanthin obtained from *Portunus sanguinolentus* (Three Spotted Crab), *Callinectes sapidus*(Blue Crab), and *Paralithodes brevipes* (Spiny King Crab) using Hexane: Isopropanol solvents was found to be 31.23 ± 0.53 (µg/g), 48.41 ± 0.28 (µg/g) and 20.15 ± 0.18 (µg/g) which is depicted in Table 2. Whereas, the yield of astaxanthin obtained from three crab varieties using other two alternative methods (DMSO: Acetone and Acetone alone) were found to be lesser (Table 3 and Table 4). The mixture of Hexane: Isopropanol gave the highest yield of astaxanthin.

Confirmation of astaxanthin using TLC

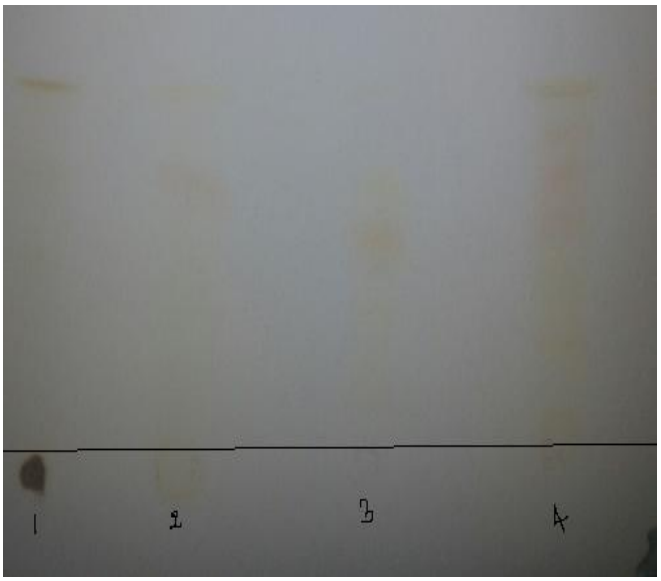


Figure 1. TLC for astaxanthin extracted using Hexane and Isopropanol

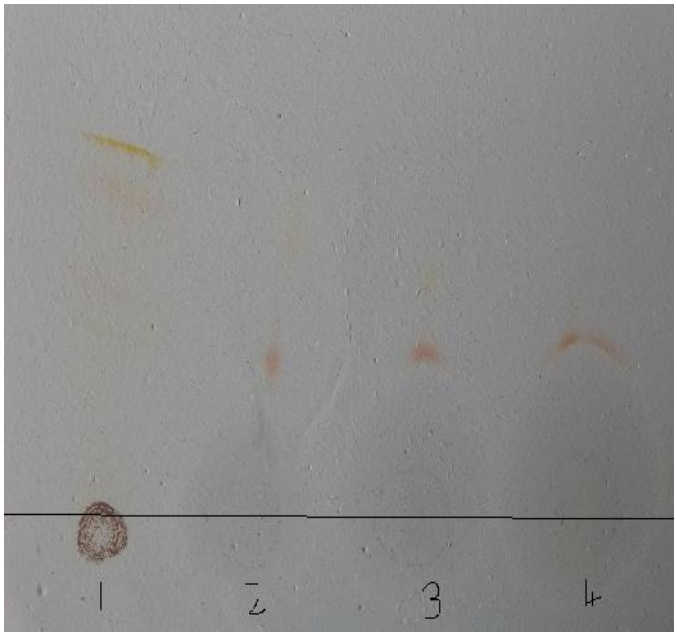


Figure 2. TLC for astaxanthin extracted using DMSO: Acetone

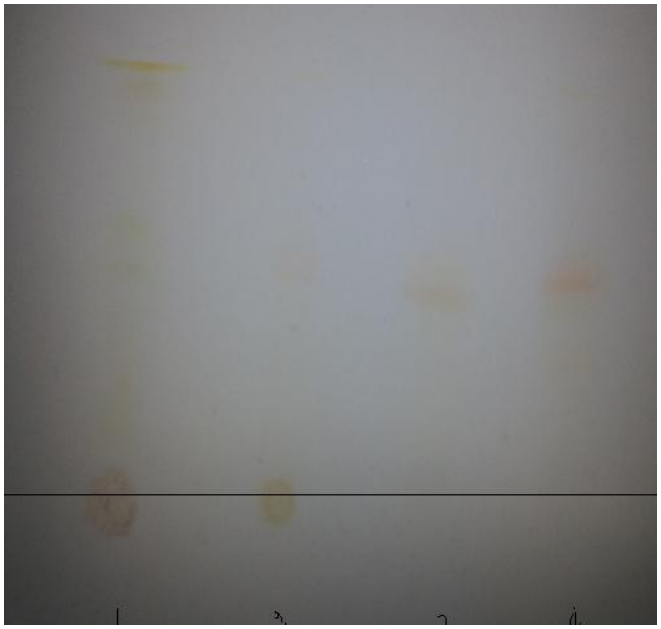


Figure 3. TLC for astaxanthin extracted using acetone

1. Astaxanthin standard, 2. Astaxanthin isolated from *Portunus sanguinolentus* (Three Spotted Crab), 3. Astaxanthin isolated from *Callinectus sapidus* (Blue Crab), 4. Astaxanthin isolated from *Paralithodes brevipes* (Spiny King Crab)

The isolated astaxanthin pigment which was in the form of the paste with an appearance of orange red color was subjected to TLC for confirmation and identification of various esters of astaxanthin. Results of TLC indicate the various bands obtained and it is picturized in Figure 1, 2 and 3.

Table 5. Rfvalues for astaxanthin isolated by Hexane: Isopropanol

Standard astaxanthin	*Portunus sanguinolentus* (Three spotted crab)	*Callinectus sapidus* (Blue Crab)	*Paralithodes brevipes* (Spiny King Crab)
0.33 (FREE ASTAXANTHIN)	0.38	0.36	0.33
0.50 (ASTAXANTHIN MONOESTER)	0.53	0.47	0.50
0.73 (ASTAXANTHIN DIESTER)	-	0.65	0.69

In hexane: isopropanol extraction method, the presence of free astaxanthin was confirmed by comparing with the standard astaxanthin with an Rf value of 0.33. The free form of astaxanthin was present in all the varieties of crabs. The monoester form of astaxanthin was found to be present in the three varieties of crab with an Rf value in the range of 0.47 – 0.53, which closely relates with the standard Rf values.

The diester form of astaxanthin was viewed in *Callinectus sapidus* (Blue Crab) and *Paralithodes brevipes* (Spiny King Crab) with an Rf value range of 0.65 – 0.69, which comparatively showed close relationship with the standard (Table 5).

Table 6. Rf Values for astaxanthin isolated by DMSO: Acetone

Standard astaxanthin	*Portunus sanguinolentus* (Three spotted crab)	*Callinectus sapidus* (Blue Crab)	*Paralithodes brevipes* (Spiny King Crab)
0.33 (FREE ASTAXANTHIN)	0.35	0.33	0.32
0.50 (ASTAXANTHIN MONOESTER)	-	0.48	-
0.73 (ASTAXANTHIN DIESTER)	-	-	-

In DMSO: acetone extraction method, the Rf value of free astaxanthin in the range of 0.32 – 0.35 were obtained in all the three crab varieties, when compared with the standard. Astaxanthin monoester was found to be present only in *Callinectus sapidus* (Blue Crab) with an Rf value of 0.48. It is excluded in other crab varieties. The diester form of astaxanthin was absent in all the three crabs varieties (Table 6).

Table 7. Rfvalues for astaxanthin isolated by Acetone

Standard astaxanthin	*Portunus sanguinolentus* (Three spotted crab)	*Callinectus sapidus* (Blue Crab)	*Paralithodes brevipes* (Spiny King Crab)
0.33 (FREE ASTAXANTHIN)	0.38	0.33	0.36
0.50 (ASTAXANTHIN MONOESTER)	-	-	-
0.73 (ASTAXANTHIN DIESTER)	0.73	-	0.73

In acetone extraction method, the Rf value of all the three crabs shows the presence of free astaxanthin which is correlated with the Rf value of standard astaxanthin. The diester form of astaxanthin was found to be lacking in all the three varieties of crabs. The Rf value in the range of 0.71 – 0.73 shows the presence of astaxanthin diester in *Portunus sanguinolentus* (Three Spotted Crab), *Paralithodes brevipes* (Spiny King Crab), but it is absent in *Callinectus sapidus* (Blue Crab) which is confirmed by relating with the Rf value of standard astaxanthin i.e. 0.73 (Table 7).

FT-IR analysis of standard astaxanthin and isolated astaxanthin

The pigment astaxanthin isolated from the three varieties of crabs was confirmed by analyzing the functional group using FT-IR techniques. Hydroxyl group (OH) and ketone group (C=O) are the major group used for identification of astaxanthin. The present investigation analyze the functional groups from the IR peaks obtained which picturized in Figure 4 to 7. The presence of OH group in the peak range 3500 – 3200, carboxylic group in the range of 1760 – 1690 was seen in all the IR graph along with the standard astaxanthin, also the presence of methyl group in the range 0f 1470-1450 were observed, which confirm the presence of aliphatic chain of

astaxanthin and the presence of alkene group in the range of 650-1000 was seen in all the graph which represents the presence of astaxanthin esters. The peak range at 1500-1400 confirm the presence of aromatic ring of benzene.

NMR analysis of standard astaxanthin and isolated astaxanthin

Astaxanthin extracted from various crabs were subjected to H-NMR to identify number of protons. 1° aliphatic (RCH_3), 2° aliphatic (R_2CH_2), aromatic (Ar-H), alcohols (HC-OH), carbonyl compounds (HC-C=O) and carboxylic (RCOOH) are the major types of protons used for the identification of astaxanthin. The NMR peaks obtained in the present study is picturized in Figure 8 to 11.

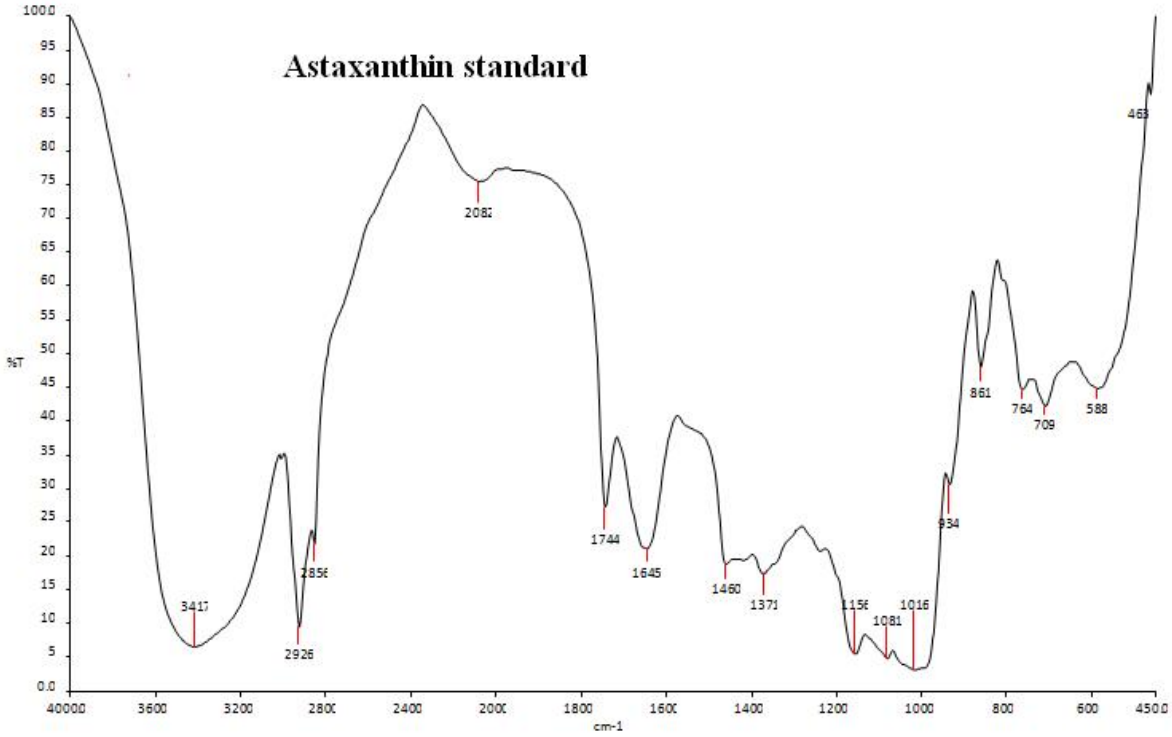


Figure 4. FT-IR analysis of standard astaxanthin

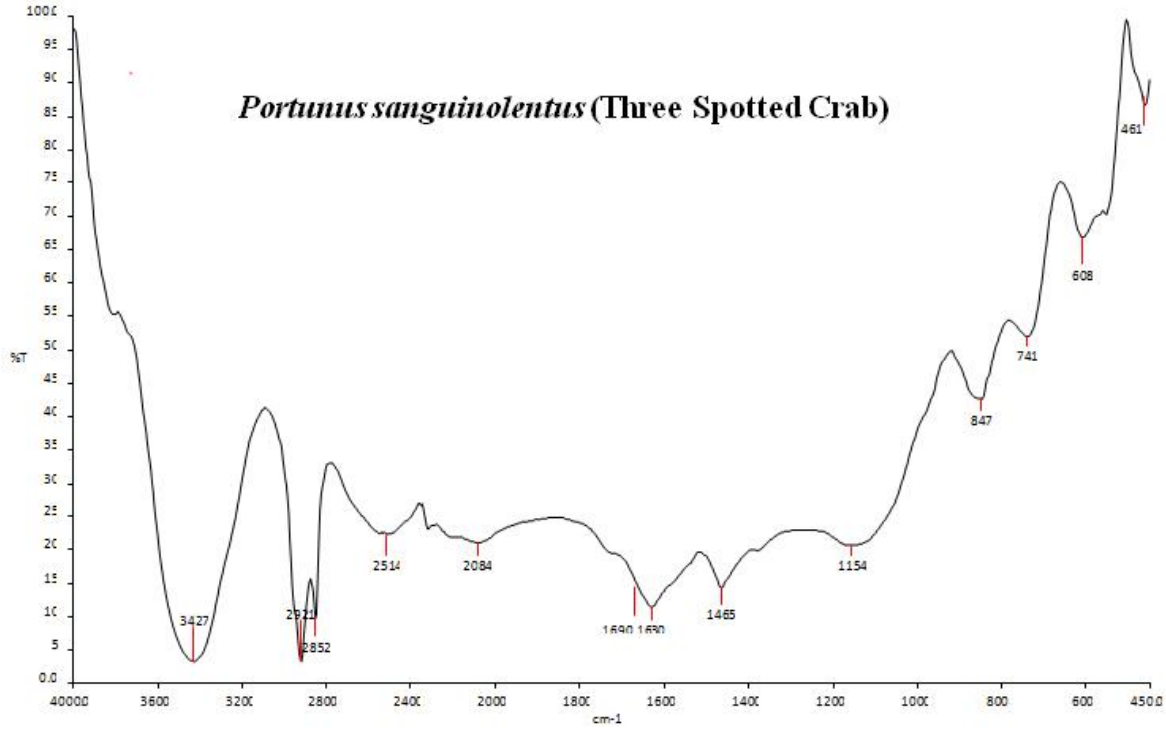


Figure 5. FT-IR analysis of *Portunus sanguinolentus* (Three Spotted Crab)

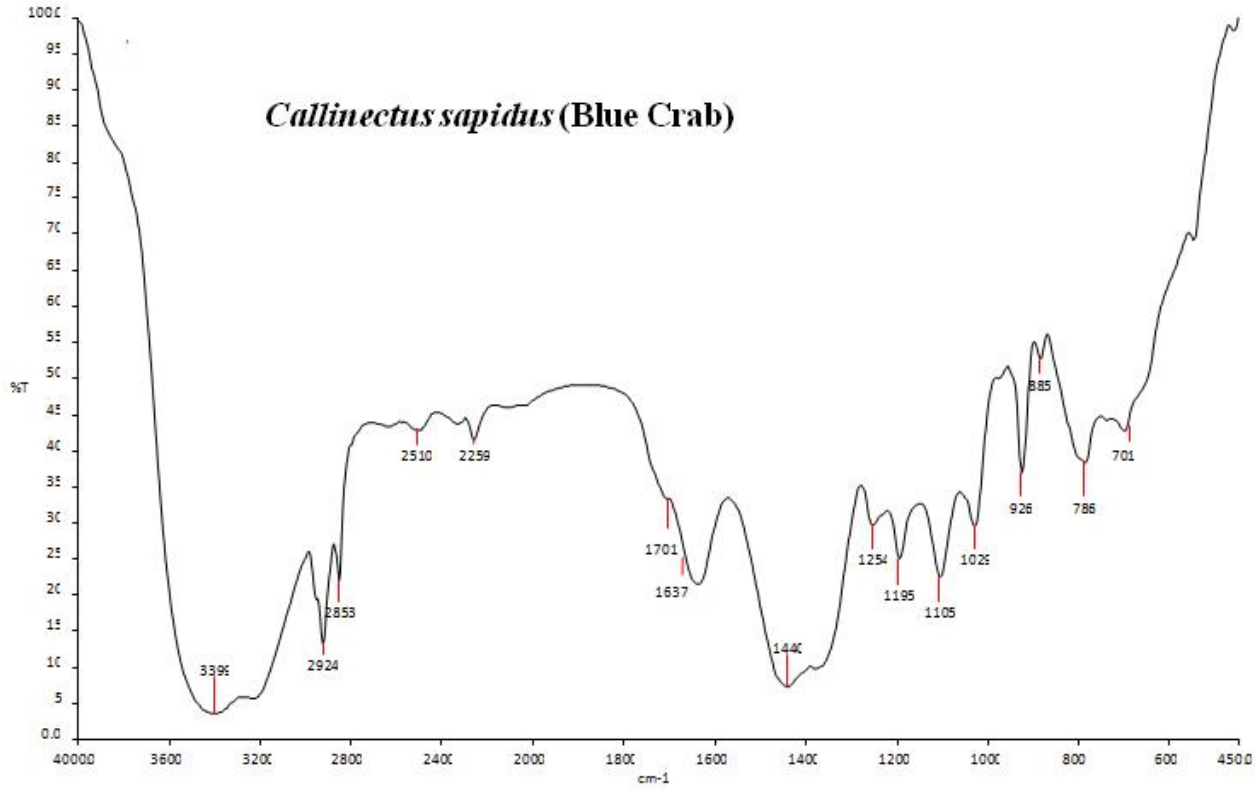


Figure 6. FT-IR analysis of *Callinectus sapidus* (Blue Crab)

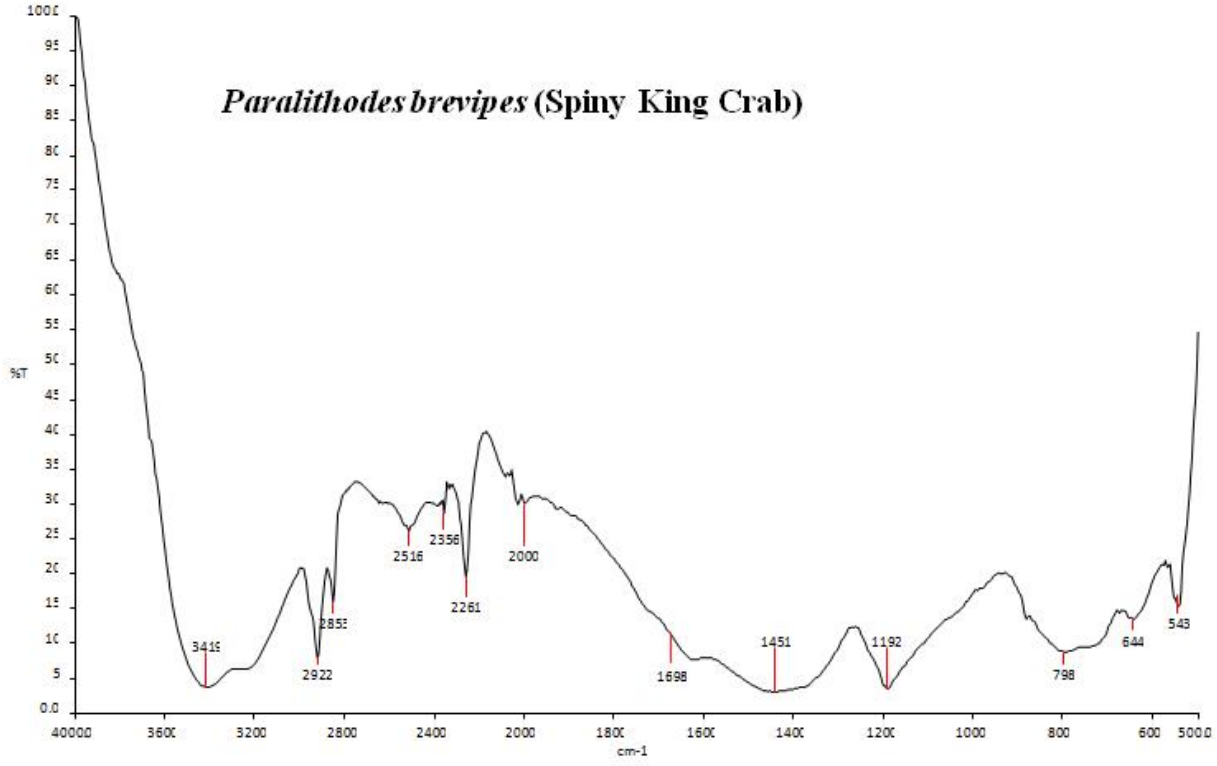


Figure 7. FT-IR analysis of *Paralithodes brevipes* (Spiny King Crab)

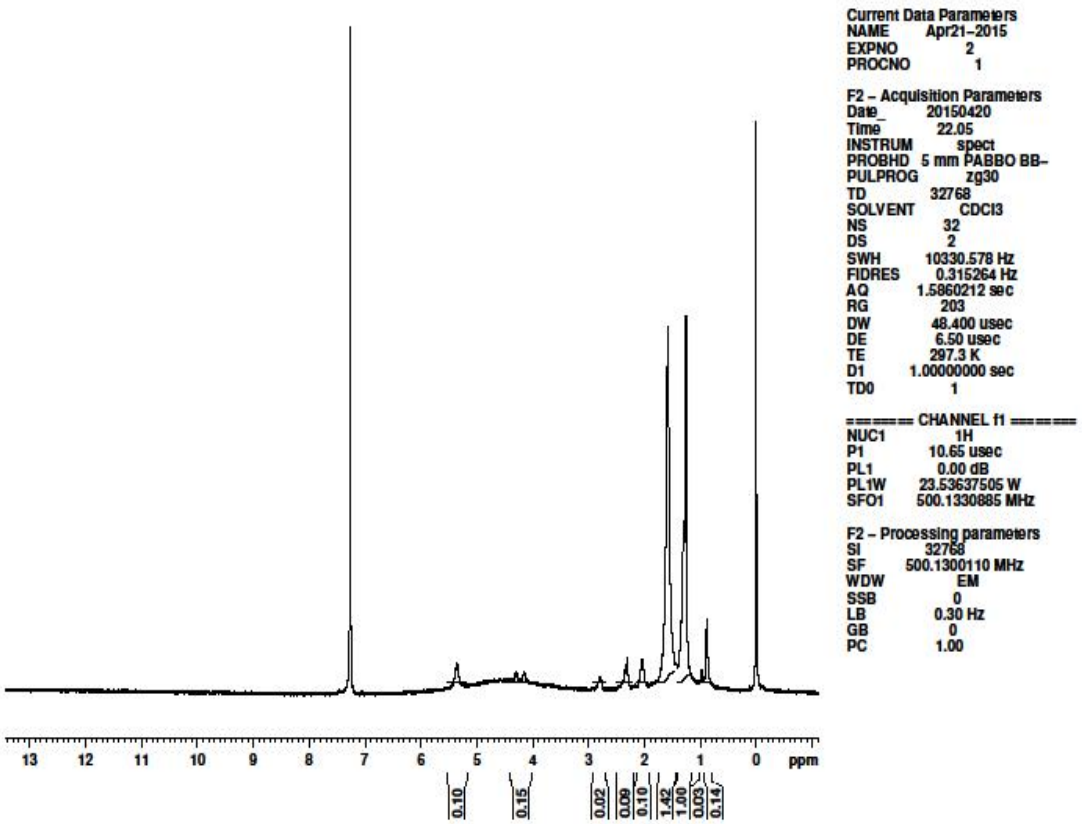


Figure 8. NMR analysis of astaxanthin standard

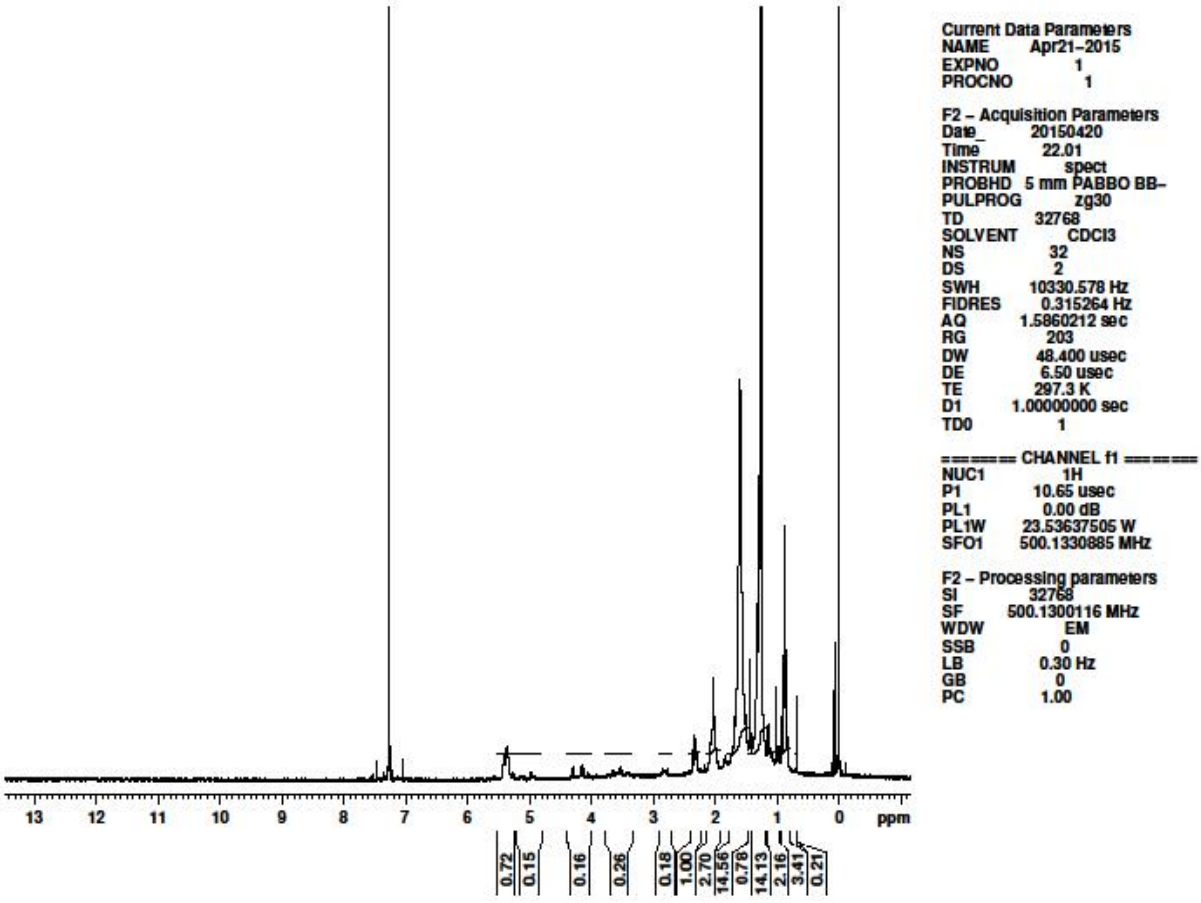


Figure 9. NMR analysis of astaxanthin isolated from *Portunus sanguinolentus* (Three Spotted Crab)

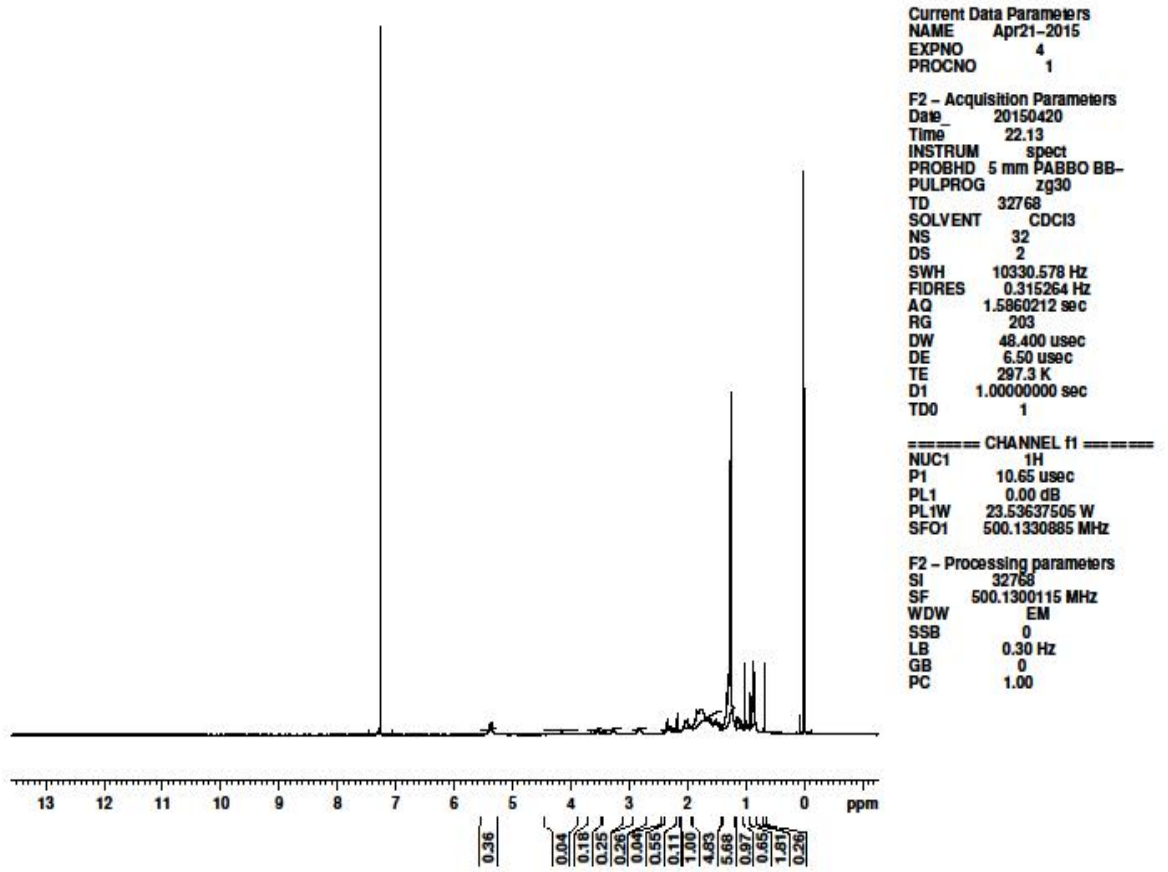


Figure 10. NMR analysis of astaxanthin isolated from *Callinectus sapidus*(Blue Crab)

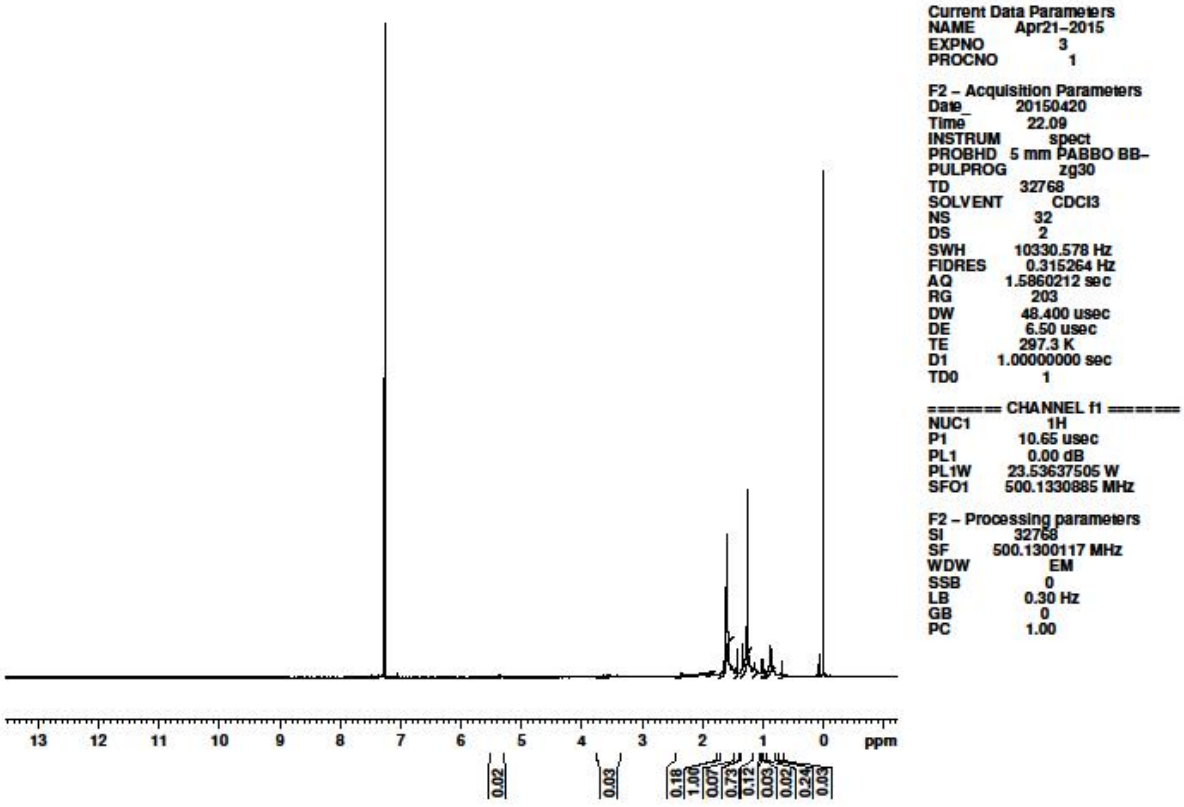


Figure 11. NMR analysis of astaxanthin isolated from *Paralithodes brevipes* (Spiny King Crab)

The chemical shift observed in the range of 0.9 ppm (1° aliphatic), 1.3 ppm (2°aliphatic), 6-8.5 ppm (aromatic compound), 3.4- 4 ppm (alcohols), 2-2.7 (carbonyl compounds), and 10- 13.2 ppm (carboxylic compound) for all the three crab varieties and standard astaxanthin.

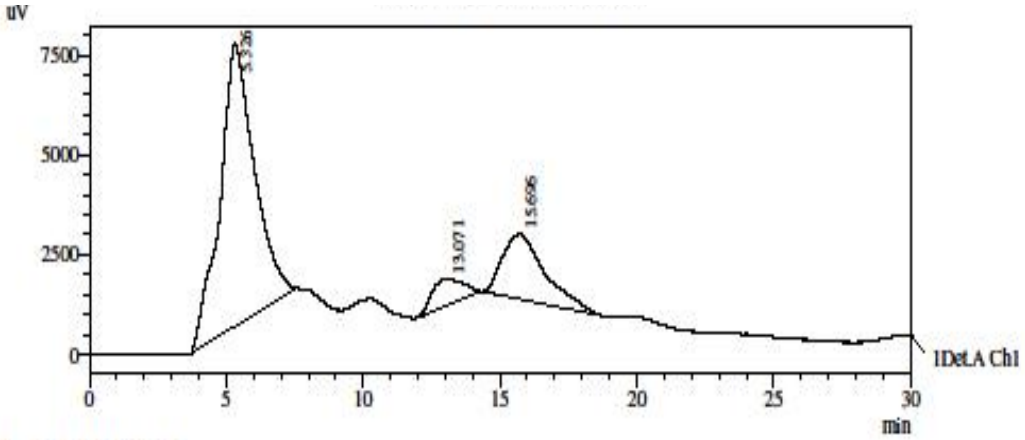
1. HPLC analysis of standard astaxanthin and isolated astaxanthin

HPLC analysis of the extracted astaxanthin and standard astaxanthin are picturized in Figure 12 to Figure 15. HPLC confirmed the presence of compound based on the retention time gained by each samples.

By analyzing the graphical representation for astaxanthin standard in Figure 12, it is noticed that, a retention time of 5.3 min peak was gained, which indicates the presence of astaxanthin.

Figure 13 indicates the HPLC analysis of *Portunus sanguinolentus* (Three Spotted Crab). A single retention time peak of 4.6 min was gained which predicts the presence of astaxanthin by comparing with the standard.

Figure 14 indicates the graphical representation of HPLC for *Callinectus sapidus* (Blue Crab), which indicates a retention time peak of 5.3, representing the astaxanthin by comparing with the standard.



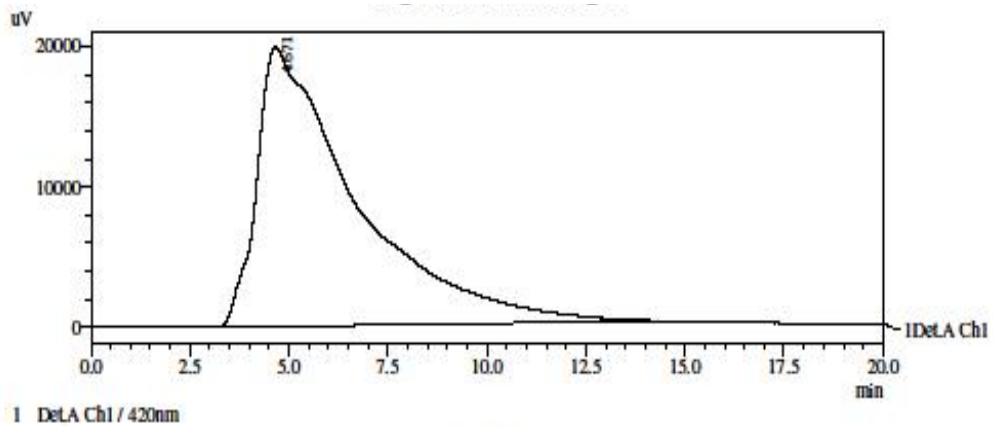
1 DetLA Ch1 / 420nm

PeakTable

Detector A Ch1 420nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.326	621220	7086	73.159	75.624
2	13.071	52316	659	6.161	7.031
3	15.696	175602	1625	20.680	17.345
Total		849138	9370	100.000	100.000

Figure 12. HPLC analysis of astaxanthin standard



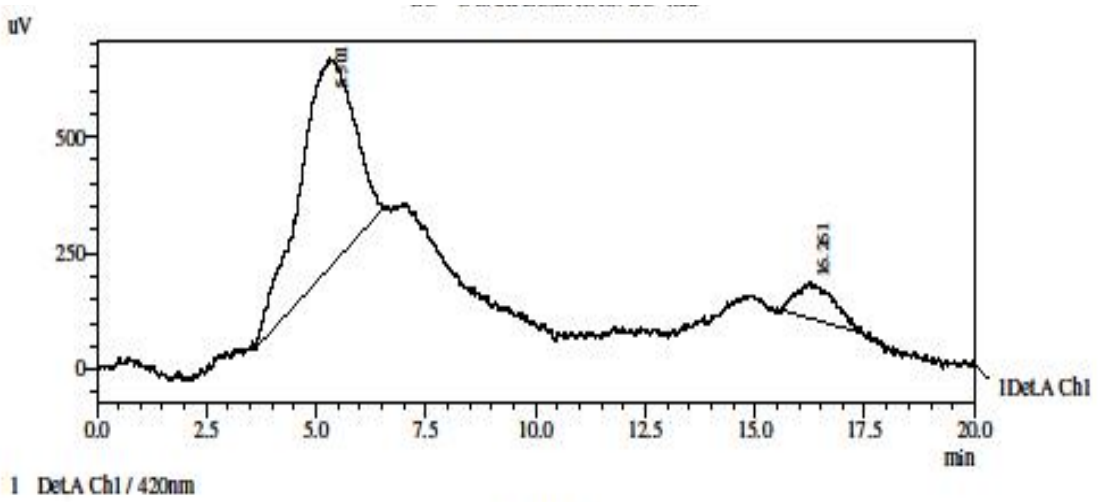
1 DetLA Ch1 / 420nm

PeakTable

Detector A Ch1 420nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.671	3478566	19876	100.000	100.000
Total		3478566	19876	100.000	100.000

Figure 13. HPLC analysis of astaxanthin isolated from *Portunus sanguinolentus* (Three Spotted Crab)



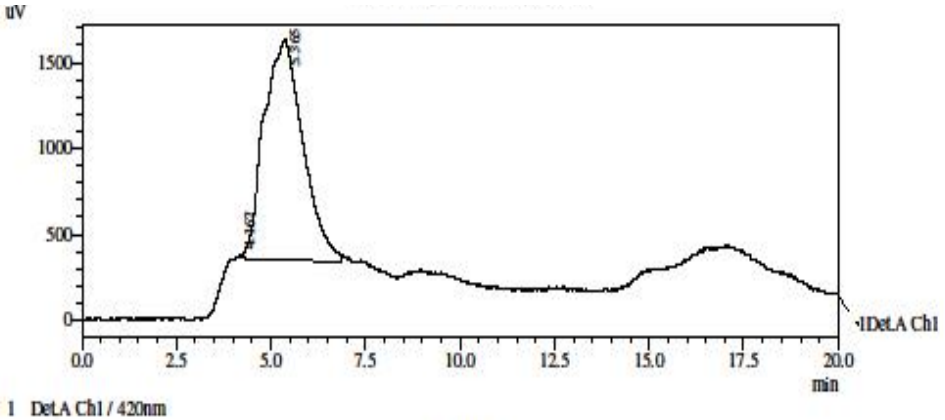
1 DetLA Ch1 / 420nm

PeakTable

Detector A Ch1 420nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.301	39313	452	89.813	85.907
2	16.261	4459	74	10.187	14.093
Total		43773	526	100.000	100.000

Figure 14. HPLC analysis of astaxanthin isolated from *Callinectus sapidus* (Blue Crab)



1 DetLA Ch1 / 420nm

PeakTable

Detector A Ch1 420nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.167	104	18	0.113	1.399
2	5.365	91702	1278	99.887	98.601
Total		91805	1296	100.000	100.000

Figure 15. HPLC analysis of astaxanthin isolated from *Paralithodes brevipes* (Spiny King Crab)

The HPLC chromatogram for *Paralithodes brevipes* (Spiny King Crab) is depicted in Figure 15 which shows 2 retention time peaks at 4.16 min and 5.3 min which indicates the presence of astaxanthin with reference to standard.

2. GC-MS analysis of astaxanthin standard and isolated astaxanthin

GC-MS analysis is a common confirmation test which provides a representative spectral output. The pigment astaxanthin isolated from three crabs was subjected to GC-MS analysis to confirm the presence of astaxanthin.

GC-MS spectrum of astaxanthin standard was dipected in Figure 16 a and Figure 16 b. the peak with the retention time of 32.32 mins was obtained which indicates the presence of astaxanthin.

Figure 17 a and Figure 17 b represents the GC-MS spectrum of astaxanthin isolated from *Portunus sanguinolentus* (Three Spotted Crab) with the retention time of 27.47 mins which represents the presence of astaxanthin when compared with the astaxanthin standard.

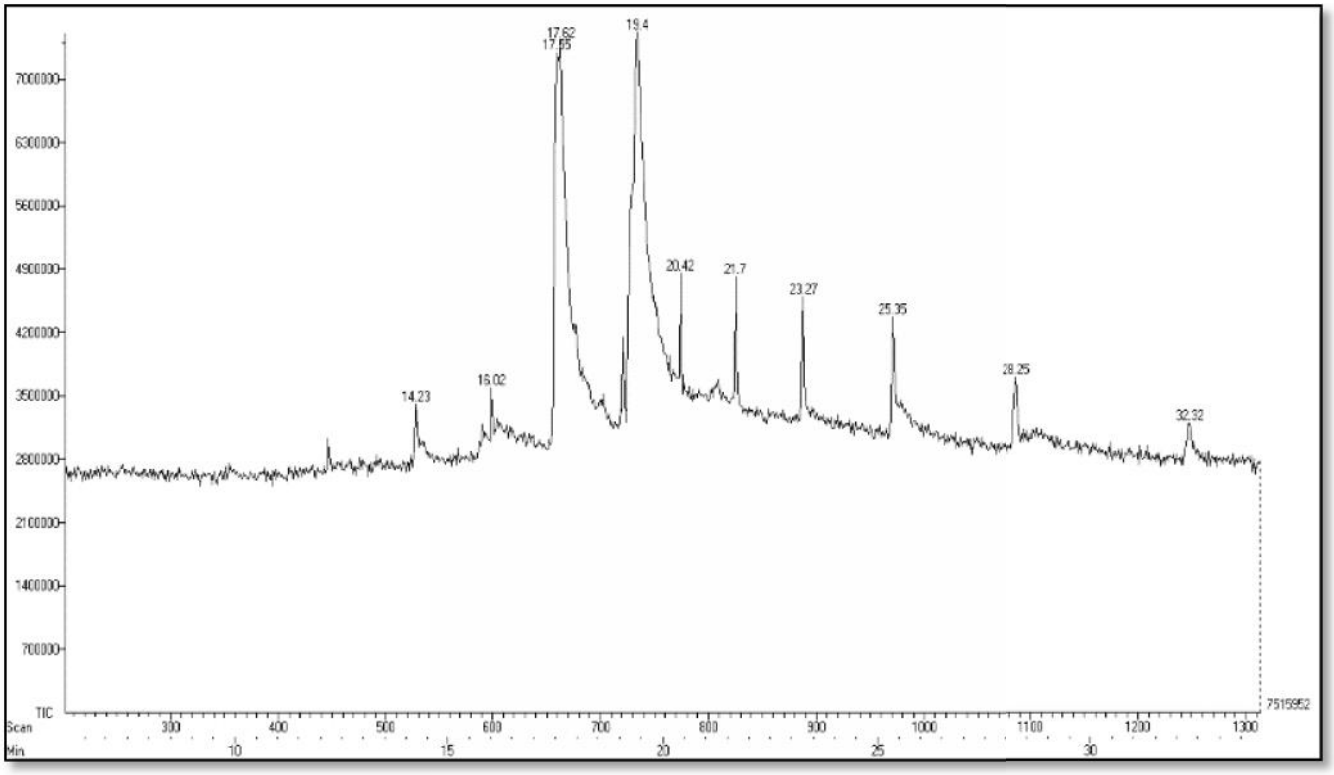


Figure 16 a GC - MS chromatography of astaxanthin standard

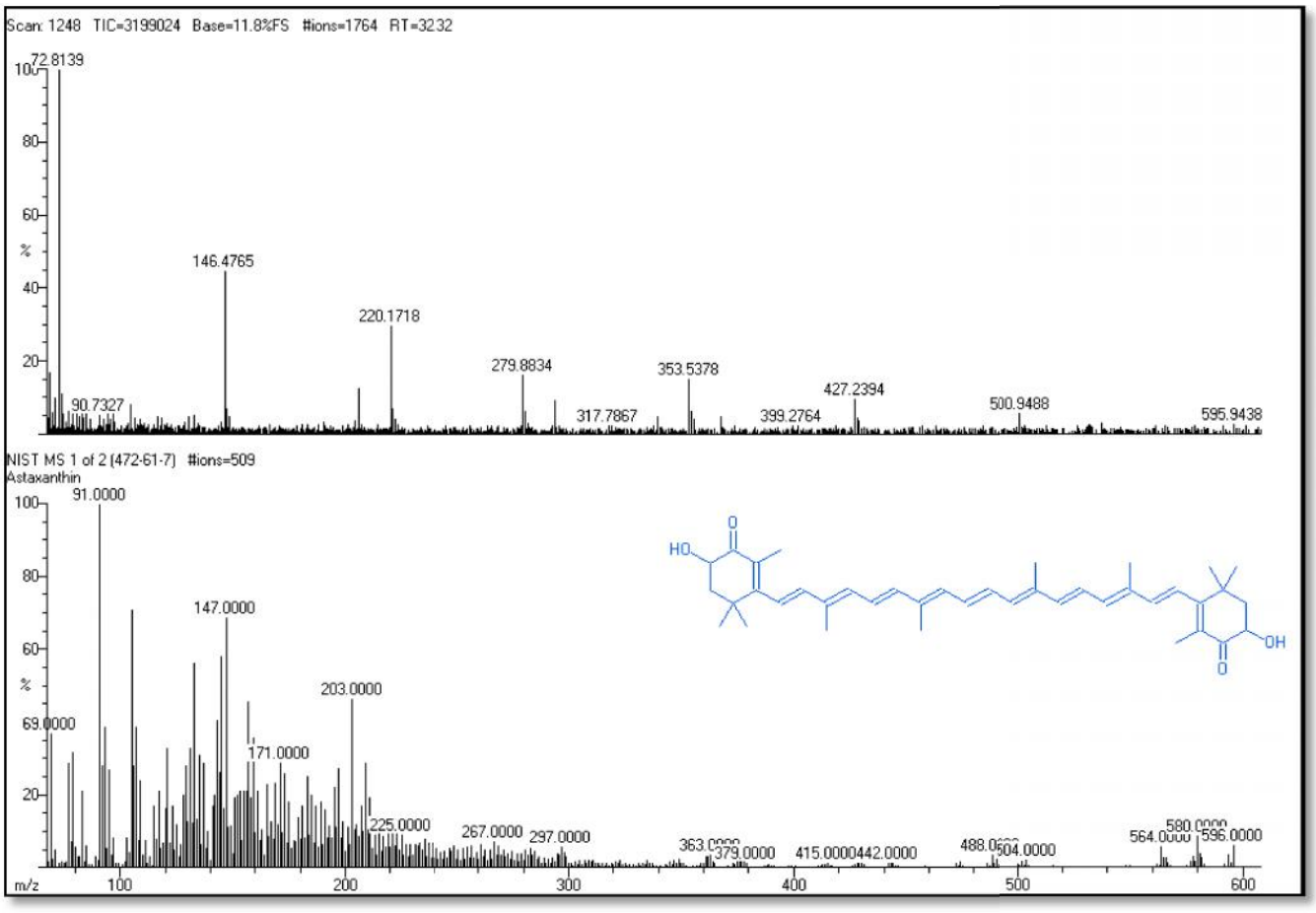


Figure 16 b GC - MS spectrum of astaxanthin from astaxanthin standard

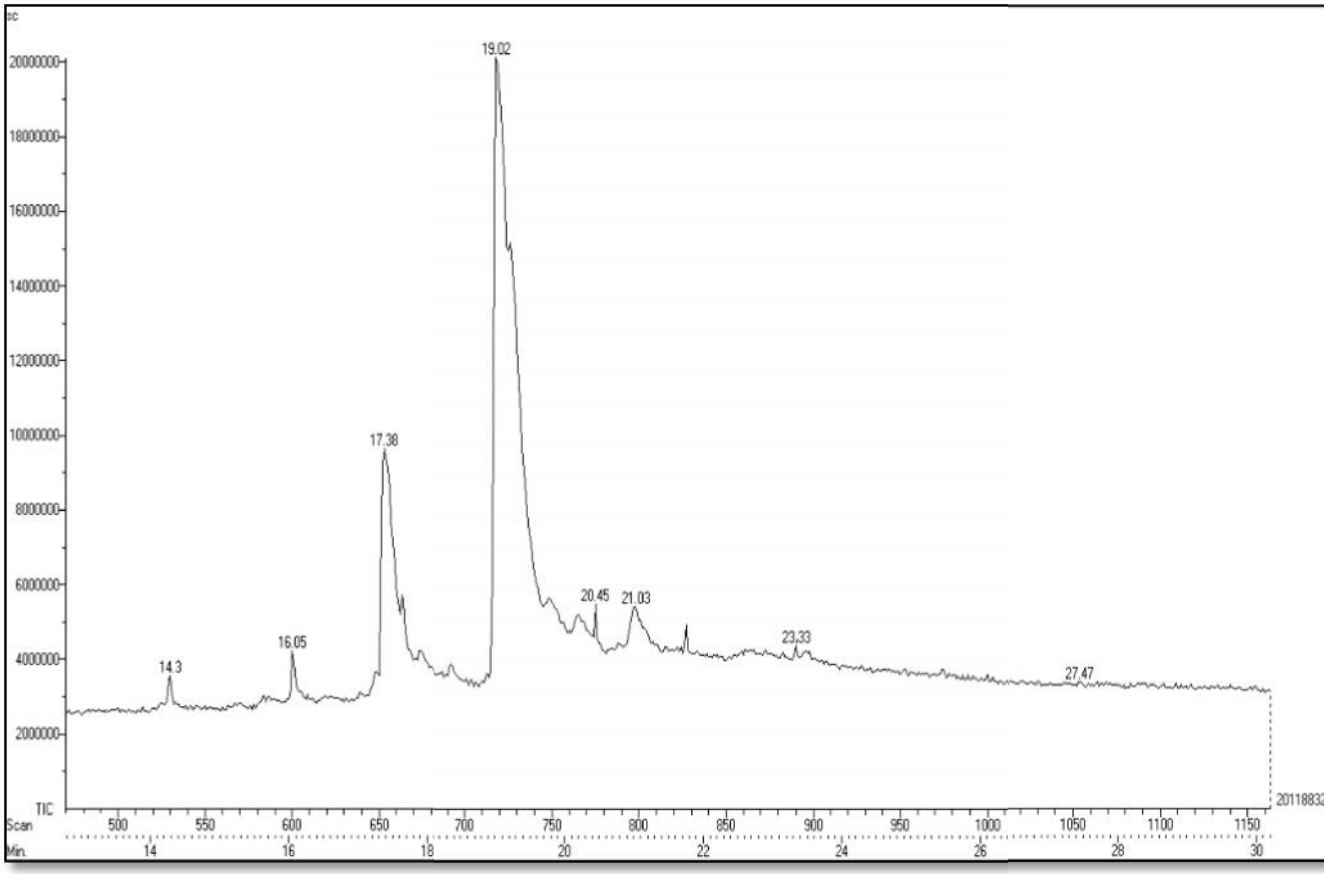


Figure 17 a GC - MS chromatography of astaxanthin extracted from *Portunus sanguinolentus* (Three Spotted Crab)

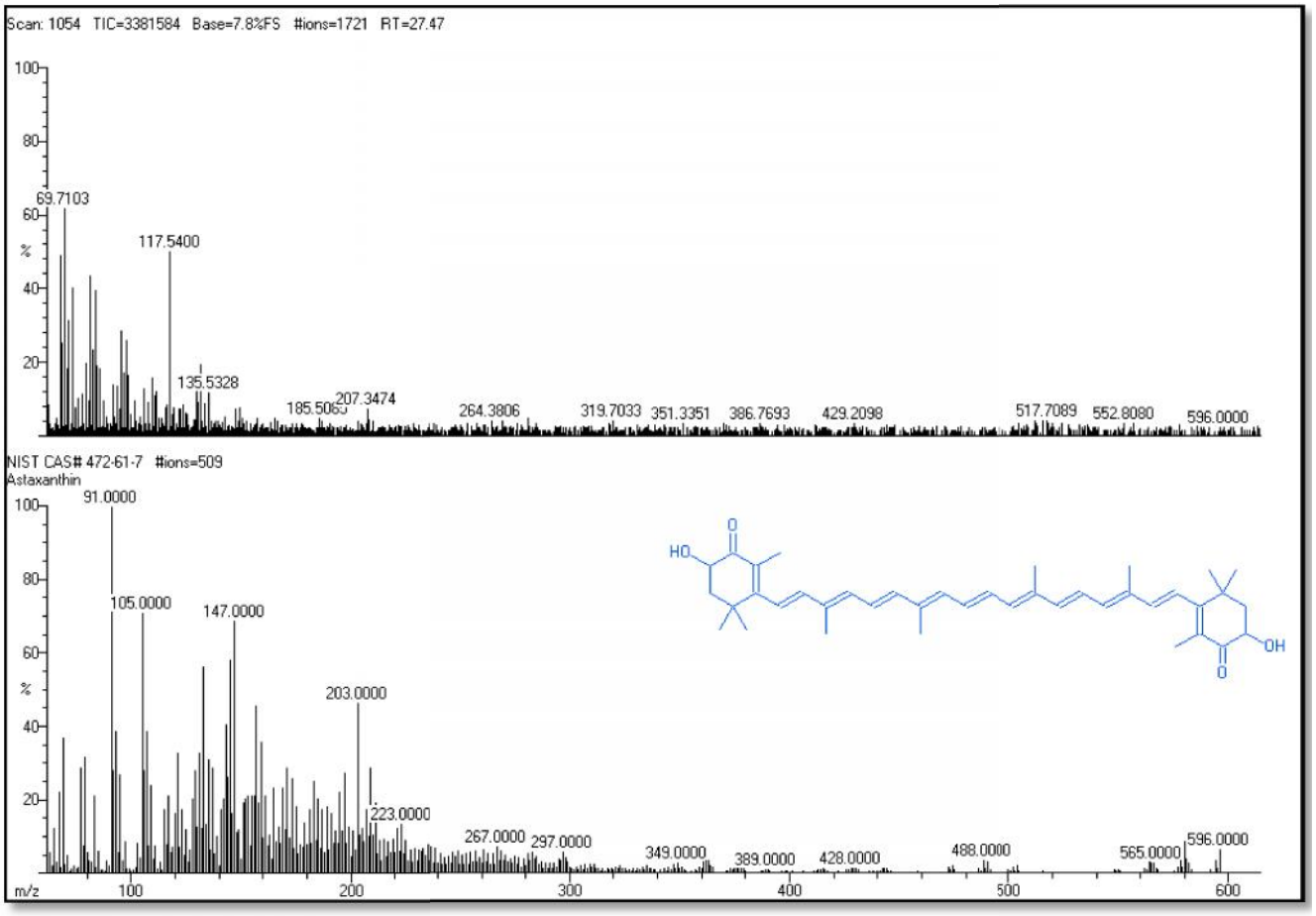


Figure 17 b GC - MS spectrum of astaxanthin from *Portunus sanguinolentus* (Three Spotted Crab)

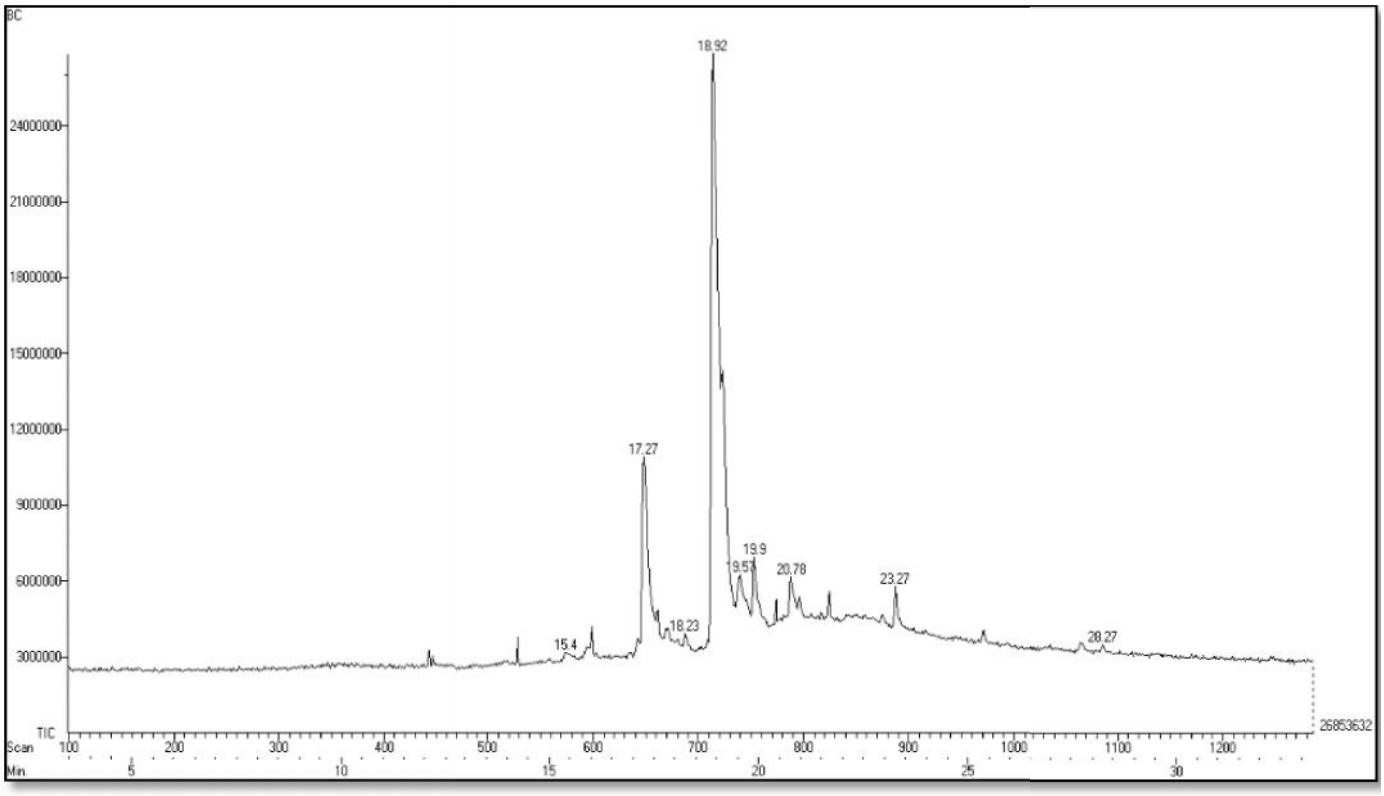


Figure 18 aGC - MS chromatography of astaxanthin extracted from *Callinectes sapidus* (Blue Crab)

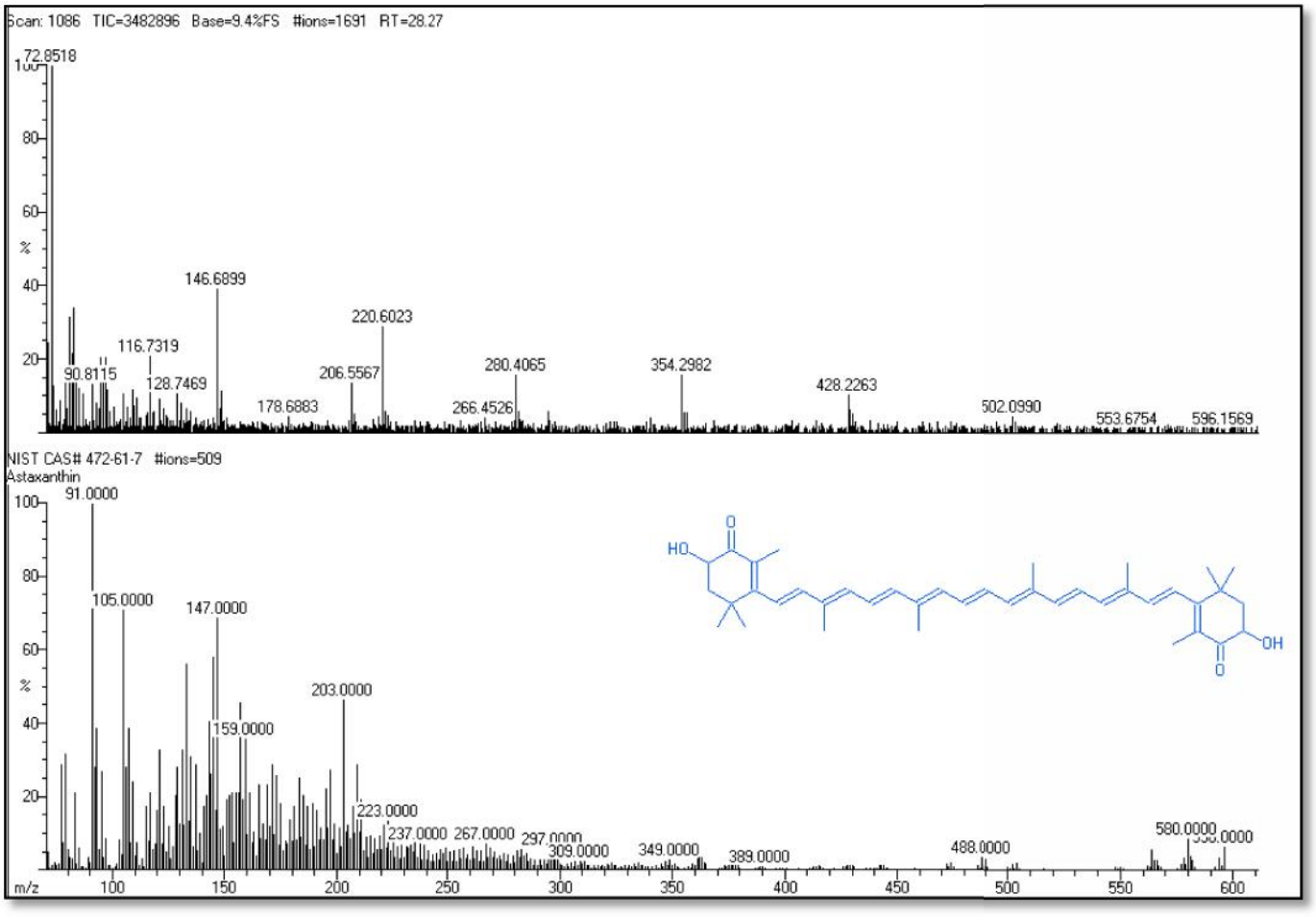


Figure 18 b GC - MS spectrum of astaxanthin from *Callinectes sapidus* (Blue Crab)

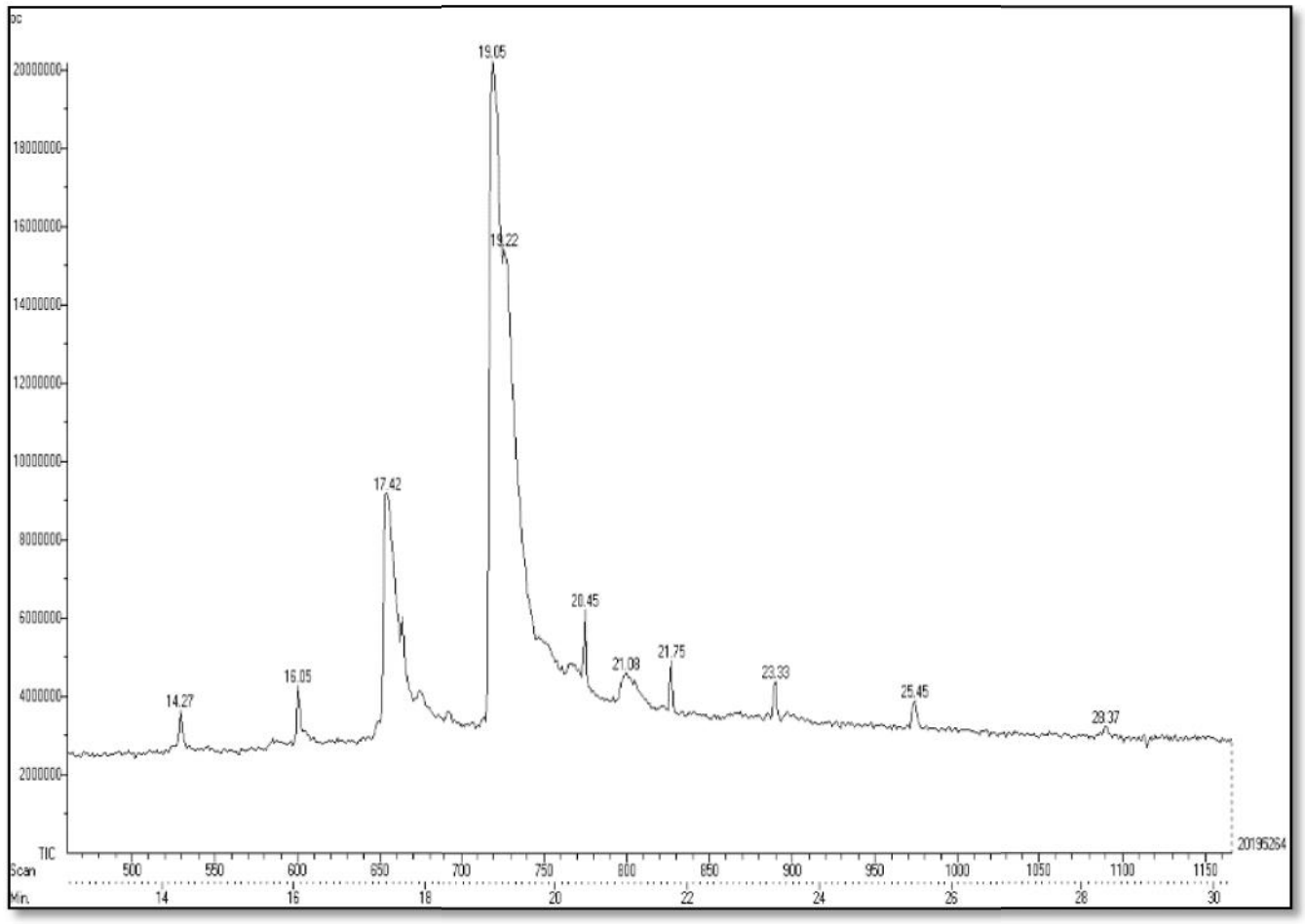


Figure 19 a GC - MS chromatography of astaxanthin extracted from *Paralithodes brevipes* (Spiny King Crab)

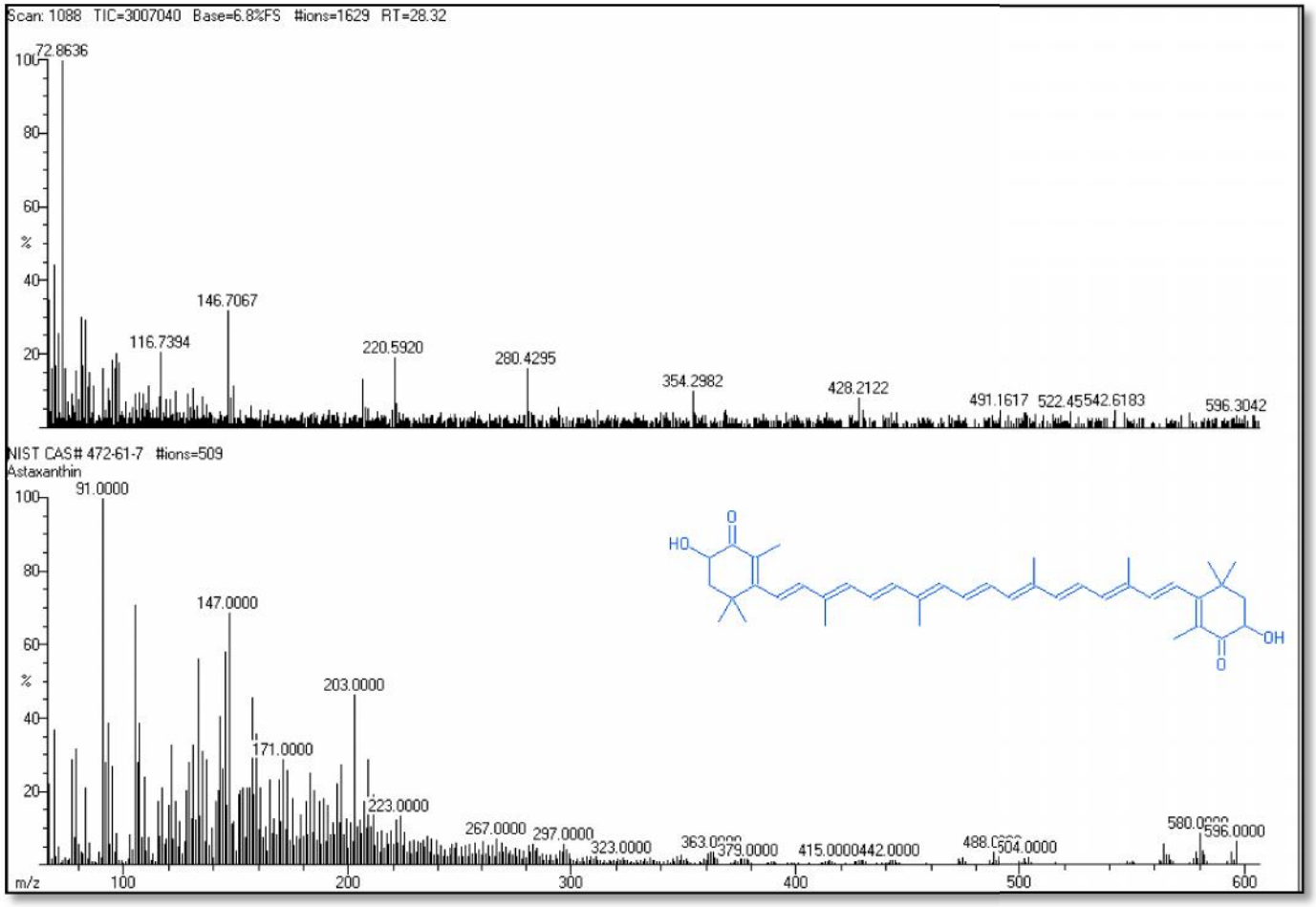


Figure 19 b GC - MS spectrum of astaxanthin from *Paralithodes brevipes* (Spiny King Crab)

Astaxanthin isolated from *Callinectes sapidus* (Blue Crab) subjected to GC-MS analysis was indicated in Figure 18 a and Figure 18 b which confirmed the presence of astaxanthin by the retention time of 28.27 mins.

The GC-MS spectrum for the isolated astaxanthin from *Paralithodes brevipes* (Spiny King Crab) with the retention time in the range of 28.32 mins was received which is represented in Figure 19a and Figure 19 b. When compared with the structural analysis received from standard library, it is confirmed that the structure predicted by the retention peak is astaxanthin.

DISCUSSION

The carotenoid content in *Callinectes sapidus*(Blue Crab), was 4.63 mg/g according to Coral-Hinostroza and Bjerkeng, 2002 which is interrelated with the present results. The present investigated was also correlated with the findings of Renata Aline dos Santos da Fonseca *et al.*, 2011 and Shahidi and Synowiecki, 1990.

The proximate composition of shell waste varies with species due to many other factors such as seasons, water, temperature and diet. The moisture content of present study was somewhat higher than the moisture contentreported by Akbar *et al.*, 1988; Soundarapandian and Singh R K, 2008 and similar to the report of Kucukgulmez *et al.*, 2006. The moisture content of *Callinectes Pallidus* was 53.56 ± 0.10 reported by Elegbede and Fashina-Bombata, 2013 which was found to be lower than the present results.

Comparing the present result with the other findings it is noticed that the percentage of ash value was greater. The ash content of present investigation were higher than the values reported for *Callinectes sapidus* 1.33% by Kuley *et al.*, 2008 and lower than the values of 39.11% reported by Ojewola and Udom, 2005.

It is evident from the present investigation that the concentration of lipid is greater in the shell of the crab compared to the meat of the crab by the findings reported by Elegdebe and Fashina Bombata, 2013. The present investigation also similar to the findings reported by Omotoso 2005.

By viewing the present investigation it is found that the concentration of protein is more or less the same in all the three varieties of crabs with slight increase noticed in *Callinectus sapidus* (Blue Crab). Hence, the present investigation suggest that the bony part of the crab can alsobe used as the source of protein in malnutrition conditions other than astaxanthin pigment extract. The present analysis was correlated with the outcomes of Moronkola *et al.*, 2011 and also compared with the study of Omotoso, 2005.

In present study the result of TLC was supported by the reports of Sindhu *et al.*, 2011. The results of present investigation, comparing with the standard astaxanthin graph and other research articles get confirmed that the isolated compound is astaxanthin. The present FT-IR analysis matches with the findings of Elumalai *et al.*, 2014 and Kim *et al.*, 2008. The NMR analysis of standard astaxanthin and isolated astaxanthin was compared with the previous findings by Hentschel *et al.*, 2006. The retention time of carotenoid extraction belongs to *Haematococcus pluvialis* showed astaxanthin esters in the range of 5.5 to 14.5 min was reported by Ranga rao, Ph.D., CFTRI, 2011 which is similar with the HPLC results of present study. The present investigation was also similar to Sarada *et al.*, 2006 study whose results shows the presence of free astaxanthin in the carotenoid extract from *Haematococcus pluvialis*within the range of retention time from 5 to 8 min. Katsuda *et al.*, 2004 and Miao *et al.*, 2006 showed the indication of astaxanthin and its esters in the range of 2.5 to 9 min.

In the present study, the presence of astaxanthin in all the extract isolated from three crabs were confirmed by GC-MS. Hence, the present results was correlated with the findings of Suvarna Lakshmi Ch *et al.*, 2014 who reported the presence of astaxanthin at the retention time 19.62 mins. Astaxanthin with the retention time of 32.42 mins was identified by Gopalakrishnan and Kalaiarasi which is also interrelated with the present investigation.

Conclusion

Thus, by viewing the results of the present study, it is concluded that Astaxanthin, a natural carotenoid pigments isolated from the waste shells of crabs can be suggested for use in Cosmetics, Food additives, Pharmaceuticals and Nutraceuticals industries.

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