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Characterisation of Astaxanthin from Shrimp shell waste of Sunflower oil extraction

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Abstract*: The study presents different methods for analysis of Astaxanthin isolated from the shrimp species Aristeus alcocki. The HPLC method is accomplished by using a C_{18} column and the mobile phase in the acetone : hexane in the ratio 9: 21v/v. . Astaxanthin is quantified by the detection at 470nm. The chromatogram showed three peaks, Astaxanthin as well as its monoesters and di esters. Further enzymatic hydrolysis was done to remove the esters. FTIR spectral bands in the range 4000 to 450 cm. The H1 NMR chemical shifts obtained at 1.3ppm and 2.3ppmwere due to the presence of CH_3 groups. The chemical shifts at 1.4 ppm, 1.6ppm, 2ppm, 2.5ppm and 2.6ppm were due to the presence of CH_2 groups. The chemical shift at 4ppm was due to the presence of CH-CH_2 groups and the chemical shift at 6.5ppm indicates the presence of aromatic CH. C_{13} NMR was also done to determine the C Skeleton of Astaxanthin. The peak obtained at 15 showed the presence of CH_3. Another peak was obtained at 24 was for CH_2. An aromatic ring presence was also confirmed by the peak seen at 150.*

Keywords *: Astaxanthin, extraction, isolation, characterisation*

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I. Introduction

Astaxanthin is a keto-carotenoid It belongs to a larger class of phytochemicals known as terpenes, which are built from five carbon precursors; Iso Pentenyl Diphosphate (IPP) and Dimethyl allyl Diphosphate (DMAPP). Astaxanthin is classified as a xanthophyll (originally derived from a word meaning "yellow leaves" since yellow plant leaf pigments were the first recognized of the xanthophyll family of carotenoids), but currently employed to describe carotenoid compounds that have oxygen-containing moities, hydroxyl (-OH) or ketone (C=O), such as zeaxanthin and canthaxanthin. Indeed, Astaxanthin is a metabolite of zeaxanthin and/or canthaxanthin, containing both hydroxyl and ketone functional groups. Similar to many carotenoids, astaxanthin be a vivid lipid-soluble pigment. This tint owe in the direction of the unlimited chain of conjugated (alternating double and single) double bonds at the centre of the compound. This chain of conjugated double bonds is also responsible for the antioxidant function of Astaxanthin (as well as other carotenoids) as it results in a region of decentralized electrons that can be donated to reduce a reactive oxidizing molecule.

Astaxanthin is found in microalgae, yeast, salmon, trout, krill, shrimp, crayfish, crustaceans, and the feathers of some birds. It provides the red colour of salmon meat and the red colour of cooked shellfish. Professor Basil Weedon's group was the first to prove the structure of Astaxanthin by synthesis, in 1975.
Isolation of Astaxathin

Sample Preparation (Sindhu *et al.*, 2011))

Shell waste from the deep sea shrimp *Aristeus alcocki.* The sample was collected from the processing plants, Cochin, Kerala, India. The shrimp waste was transported to the laboratory in a sterile container filled with ice. The organism was identified with the styles marketed under the trade name "Red Shrimp". The wastes generated from the shrimp are cephalothorax, abdominal shell and tail portion. Adhering meat from the cephalothorax was removed and the waste was washed under running water and dried under shade. They were packed in polyethylene bags and stored at -20 °C until use. A second set of sample was stored wet, after removing the adhering meat, was packed in polythene covers and stored at -20 °C.

Moisture Content Determination (AOAC Method, 1990)

Two grams of each of the sample was weighed into dried weighed crucible. The samples were put into a moisture extraction oven at 105 °C and heated for 3 h. The dried samples were put into desiccators, allowed to cool and reweighed. The process was repeated until constant weight was obtained. The difference in weight was calculated as a percentage of the original sample.

$$\text{Percentage moisture} = \frac{W_2 - W_1}{W_2 - W_3} \times 100$$

where, W_1 is initial weight of empty crucible
W_2 is weight of crucible + undried sample
W_3 is weight of crucible + dried sample.

3.3.2 Ash Content Determination (AOAC Method, 1990)

Two grams of each of the samples was weighed into crucible, heated in a moisture extraction oven for 3 h at 100 °C before being transferred into a muffle furnace at 550 °C, until it turned white and free of carbon. The sample was then removed from the furnace, cooled in a desiccator to a room temperature and reweighed immediately. The weight of the residual ash was then calculated as:

$$\text{Ash content percentage ash} = \frac{\text{Weight of ash}}{\text{Weight of original sample}} \times 100$$

3.3.3 Crude Protein Determination (AOAC Method, 1990)

The micro method described by AOAC (1990) was used. Two grams of each of the samples was mixed with 10ml of concentrated Sulphuric acid in a heating tube. One table of selenium catalyst was added to the tube and mixture heated inside a fume cupboard. The digest was transferred into distilled water. Ten mm portion of the digest mixed with equal volume of 45% sodium hydroxide solution and poured into a distillation apparatus. The mixture was distilled and the distillate collected into 4% boric acid solution containing 3 drops of methyl red indicator. A total of 50 ml distillate was collected and titrated as well. The sample was duplicated and the average value taken. The Nitrogen content was calculated and multiplied with 6.25 to obtain the crude protein content.

This is given as percentage Nitrogen,

$$= \frac{(100 \times N \times 14 \times V_f)\, T}{100 \times V_a}$$

where, N is normality of the titrate (0.1 N)
V_f is total volume of the digest = 100 ml
T is titer value
V_a is aliquot volume distilled.

3.3.4 Fat Content Determination (AOAC Method, 1990)

Two gm of the sample was loosely wrapped with a filter paper and put into the thimble which was fitted to a clean round bottom flask, which has been cleaned, dried and weighed. The flask contained 120 ml of petroleum ether. The sample was heated with a heating mantle and allowed to reflux for 5 h. The heating was then stopped and the thimbles with the spent samples kept and later weighed. The difference in weight was received as mass of fat and is expressed in percentage of the sample.

The percentage oil content is percentage fat,

$$= \frac{W_2 - W_1}{W_3} \times 100$$

where, W_1 is weight of the empty extraction flask
W_2 is weight of the flask and oil extracted
W_3 is weight of the sample.

Chitin Estimation

1 gm of dry sample and wet sample was ground to about 32 mm mesh and digested in 100 ml of 2% NaOH at 100 °C for one hour. The digest was filtered on a medium tared sintered glass crucible to refilter the residue. The digested residue was transferred to a beaker, treated for 12 h at room temperature with 100 ml of 5% HCl and refiltered on the sintered glass crucible. The residue was washed with hot distilled water until there was a negative test for chloride. The crucible was dried at 110 °C for 16 h and weighed.

$$\text{The percentage chitin} = \frac{W_2 - W_1}{W} \times 100$$

where, W_1 is weight of the crucible
W_2 is weight of the crucible and residue
W is weight of the sample in grams

Extraction of Shrimp Shell Waste by Using Different Solvents (Sindhu *et al.*, 2011)

3.4.1 Acetone Extraction (90%)

1g of wet shrimp shell waste was ground using 10 ml of acetone. The extract was filtered using Whatman filter paper. The sample was repeatedly extracted and filtered with the fresh solvent until the colorless filtrate (3 times) was gained. The pooled extract was collected in a separated conical flask and 9.4 ml of 0.73% NaCl were added. After thorough mixing the epiphase was collected. To the lower phase an equal amount of water was added, mixed well and then the epiphase was collected.

3.4.2 Extraction of Astaxanthin with Petroleum Ether: Acetone: Water (15:75:10 v/v/v) (Chen and Meyers, 1982)

Here the wet shrimp shell waste was extracted using 10ml of petroleum ether: acetone: water in the ratio (15:75:10 v/v/v). The extract was filtered using whatmann filter paper no. 42. The sample was repeatedly extracted using fresh solvent and filtered until the filtrate became colourless. The pooled extract was collected in a separated conical flask and 12.5 ml of petroleum ether (BP 40-60 °C) and 9.4 ml of 0.73% NaCl were added. After thorough mixing the epiphase was collected. To the lower phase an equal amount of water was added, mixed well and then the epiphase was collected. The pooled epiphase was kept in water bath at 60 °C for the evaporation of petroleum ether.

3.4.3 Extraction with Hexane: Isopropanol 3:2 (v/v)

1g of wet shrimp shell waste was ground using 10 ml of hexane: isopropanol (3:2). The extract was filtered using whatman no. 42 filter paper. The sample was repeatedly extracted and filtered with the fresh solvent until the colourless filtrate was obtained. The pooled extracted with hexane: isopropanol (3:2 v/v) was separated with equal volume of 1% (w/v) NaCl solution. The epiphase was collected and dehydrated with anhydrous sodium sulphate, and then evaporated to dryness under vacuum, and the residue was dissolved in 5 ml of hexane.

3.4.4 Extraction with Different Vegetable Oils (Coconut Oil, Palm Oil, Sunflower Oil)

The extraction was performed with shrimp shell waste. It was mixed with 10 ml of vegetable oil until the colourless sample was obtained and the solvent was removed under vacuum and re dissolved in 5 ml of hexane. The ratio of oil: waste used in vegetable oil extraction was 2:1 for wet sample, and 4:1 for dry samples. An antioxidant butyl hydroxy toluene (BHT) was added at 0.05% (w/v) and heated at 70 °C for 150 min, centrifuged, and the pigmented oil was recovered.

COMPOUND ISOLATION

Quantification of extraction for Astaxanthin

The extracted acetone extract is re dissolved in 3 ml of hexane and read at 470 nm.

$$\text{AST } (\mu g/g) = \frac{A \times D \times 106}{100 \times G \times d \times E_{12\,cm}}$$

where, AST concentration in µg/g
A is absorbance
D is volume of extract in hexane
106 is dilution multiple
G is weight of sample in g
D is the cuvette width (1 cm)
E is extinction coefficient 2100.
OD at 470 nm is 3.041

$$\text{AST } (\mu g/g) = \frac{3.041 \times 3 \times 106}{100 \times 1x \times 1x \times 2100} = 43.44\ \mu g/g$$

Identification of Astaxanthin in Shrimp Shell Waste Extract by Thin Layer Chromatography (TLC) (Sindhu *et al.*, 2011)

Analysis of different components in the shrimp shell extract was done using thin layer chromatography (TLC) based on the method of Kobayashi and Sakamoto, 1999. For this, a small volume of the extract was spotted on silica gel plate and developed using acetone: hexane [3:7 (v/v)]. The separated bands were identified using standard Astaxanthin (Source: green algae; Manufacturer: Sigma Chemicals, USA) and internationally accepted R_f values for Astaxanthin monoester and astaxanthin diester. The different fractions, Astaxanthin, Astaxanthin monoester, Astaxanthin diester were quantified by scraping out the respective bands in TLC plate. The Astaxanthin present in the scraped out sample was redissolved in 5 ml of hexane and quantified as described earlier.

Enzymatic Hydrolysis to Yield Free Astaxanthin

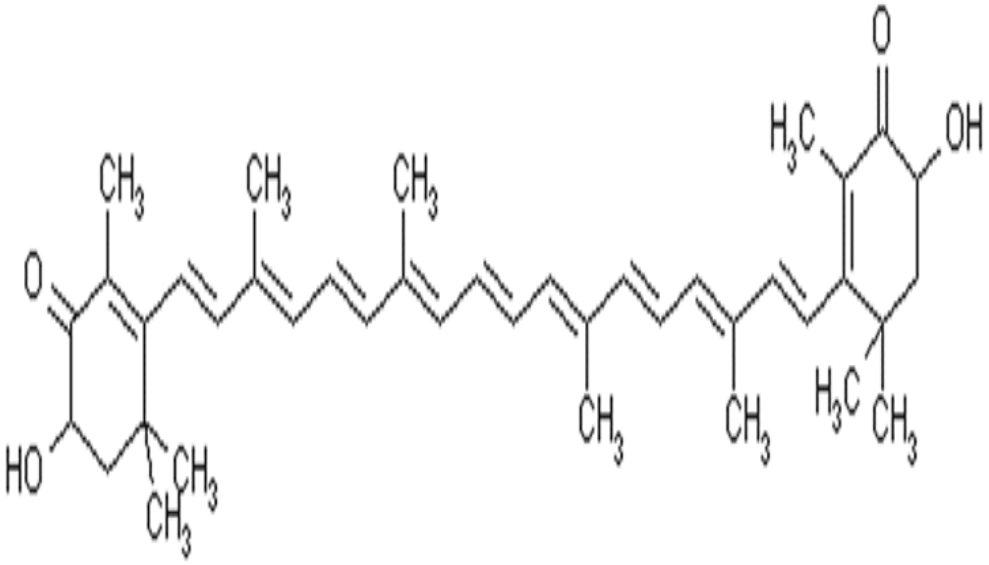
Esterified Astaxanthin must first be hydrolyzed by either a chemical or enzymatic procedure to yield all free Astaxanthin before proceeding with HPLC analysis. The enzymatic hydrolysis method is preferable as it is simple, complete and does not oxidize the Astaxanthin molecule when performed carefully.

Enzymatic hydrolysis of carotenoid esters for HPLC Analysis

Cholesterol esterase from *Pseudomonas flourescens* (Sigma C-9281, 10,000 units per gram) was purchased. A 3.4 unit/ml stock solution of enzyme in 0.05 M Tris-HCl pH 7.0 buffers in a 100 unit vial added entire contents of vial to 29.4 ml of the Tris buffer. Rinsed inside of vial well to remove all enzymes, mix solution well. Aliquot solution into 1 or 2 ml vials and store unused stock solutions frozen at -20 ^{o}C. Cholesterol esterase must be kept frozen at -20 ^{o}C until made into these working stock vials. The enzyme activity may be lost if not transported and stored properly.

3 ml of the extract is transferred to the glass test tube and 2 ml of 0.05 M Tris- HCl buffer is added. The tube is equilibrated in 37 ^{o}C water bath for 2 minutes. 600 µl of enzyme stock solution is added to the test tube and capped. Heated in 37 ^{o}C water bath for 45 minutes with gentle mixing on every 15 min. 1.0 g of sodium sulphate dehydrate and 4 ml of petroleum ether is added to the tube and vortexed vigorously for 30 seconds. The tube is centrifuged at 3500 rpm for 3 min. to separate the solvent and aqueous layers. The petroleum ether carotenoid mixture is removed with a pipette to a clean tube. Re-extracted with 2 more ml of petroleum ether, mixed well, and centrifuged until the petroleum ether is colorless. It was dried down under nitrogen gas. Re-dissolved in HPLC mobile phase.

II. Results and discussion



The Astaxanthin molecule has two asymmetric carbons located at the 3 and 3" positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the exact way that these hydroxyl groups (-OH) are attached to the carbon atoms at these centers of asymmetry. If the hydroxyl group is attached so that it projects above the plane of the molecule, it is said to be in the R configuration, and when the hydroxyl group is attached to project below the plane of the molecule, it is said to be in the S configuration. Thus the three possible enantiomers are designated R,R', S,S' and R,S' (meso).

Free Astaxanthin is the form in which the 3 and 3' hydroxyl groups are not bonded, the Astaxanthin monoester has one fatty acid bonded to a 3 position, and the Astaxanthin diester has one fatty acid bonded to each of the 3 positions. The mono-esters and di-esters are relatively non-polar, whereas free Astaxanthin is relatively polar.

Fig. 11: HPLC sample Astaxanthin

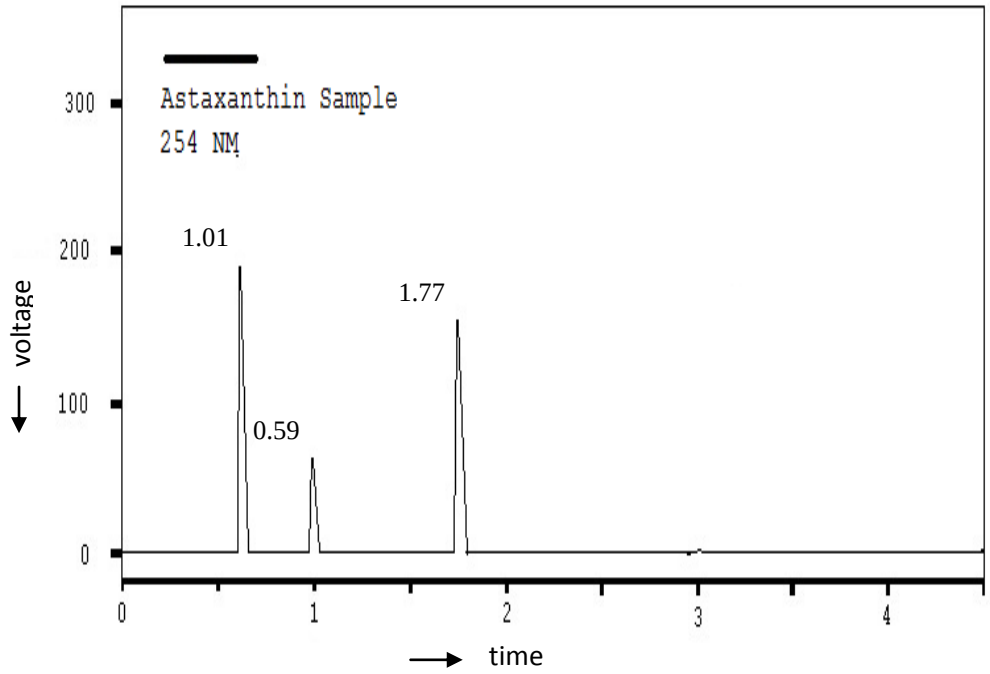


Table 6: HPCL spectra of Astaxanthin sample

Peak number	Retention time in min.	Peak area obtained under the curve
1	0.59	206
2	1.01	78
3	1.77	159

Fig. 12: HPLC standard Astaxanthin

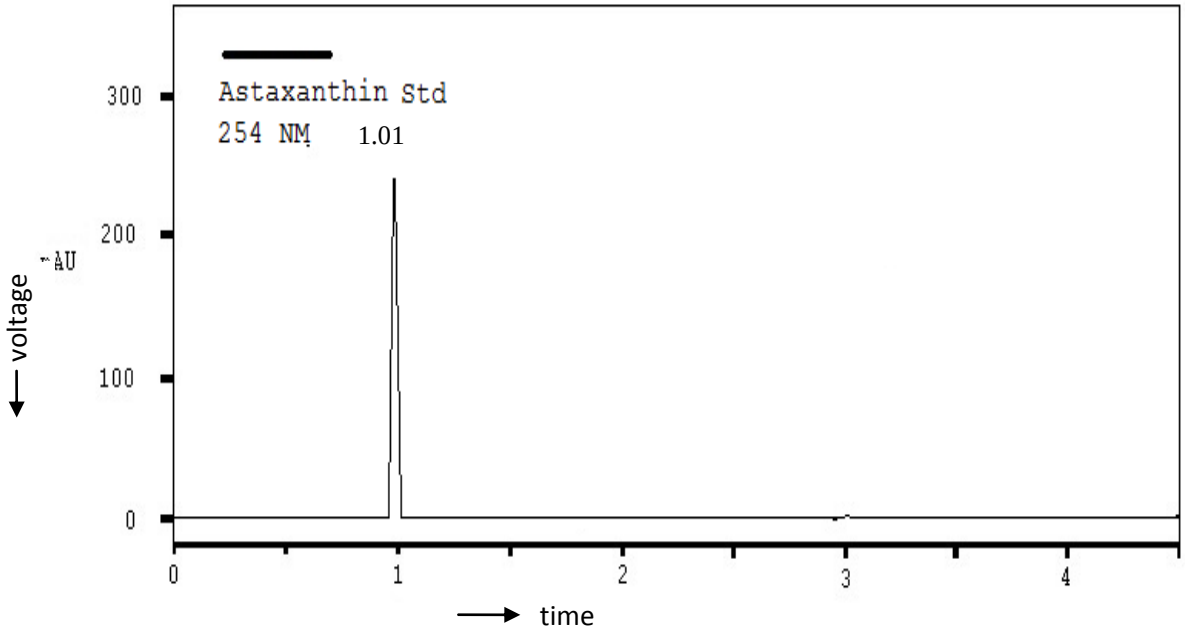


Table 7: Astaxanthin standard

Peak number	Retention time in min	Peak area obtained under the curve
1	1.01	244

4.4.2 IR Spectra

The sample was further analyzed in IR spectrum (using electromagnetic radiation) to predict the presence of Astaxanthin based on its molecular structure. FT-IR (Lark) was used to analyze the carotenoid sample at range of peaks measuring spectrum from 4000 to 450 cm; the peaks were measured in terms of percentage of transmittance and results were recorded.

Fig. 13: IR spectrum of Astaxanthin sample

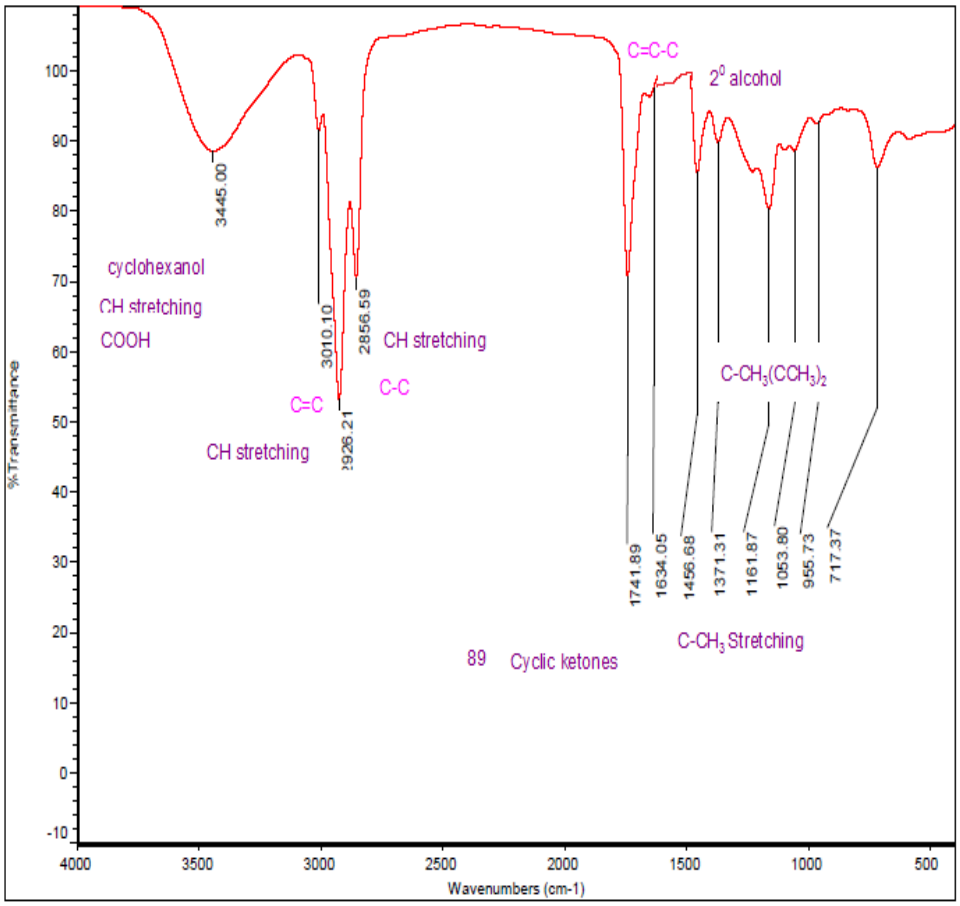


Table 8: Spectral characteristics of Astaxanthin

Peak cm^{-1}	Group	Types of vibrations
3445.0	C-H stretching, Cyclohexanol	Weak
3010.10	C-H stretching C=C	Broad
2926.21	C-H stretching C-C	Strong
1634.05	C=C-C	Weak

4.4.3 NMR Spectraof Astaxanthin Sample

Fig. 14: H_1NMR

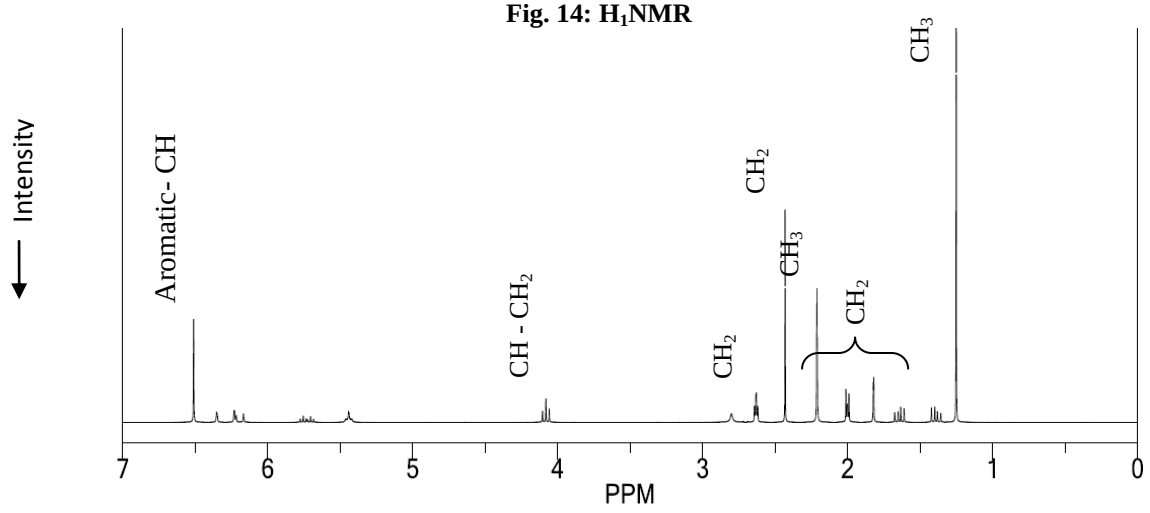


Table 9: H_1NMR

Signal	Chemical shift (ppm)	Functional group
1	1.3	CH_3
2	1.4	CH_2
3	1.6	CH_2
4	2	CH_2
5	2.3	CH_3
6	2.5	CH_2
7	2.6	CH_2
8	4	$CH\text{-}CH_2$
9	6.5	Aromatic- CH

Fig. 15: C_{13} NMR of Astaxanthin sample

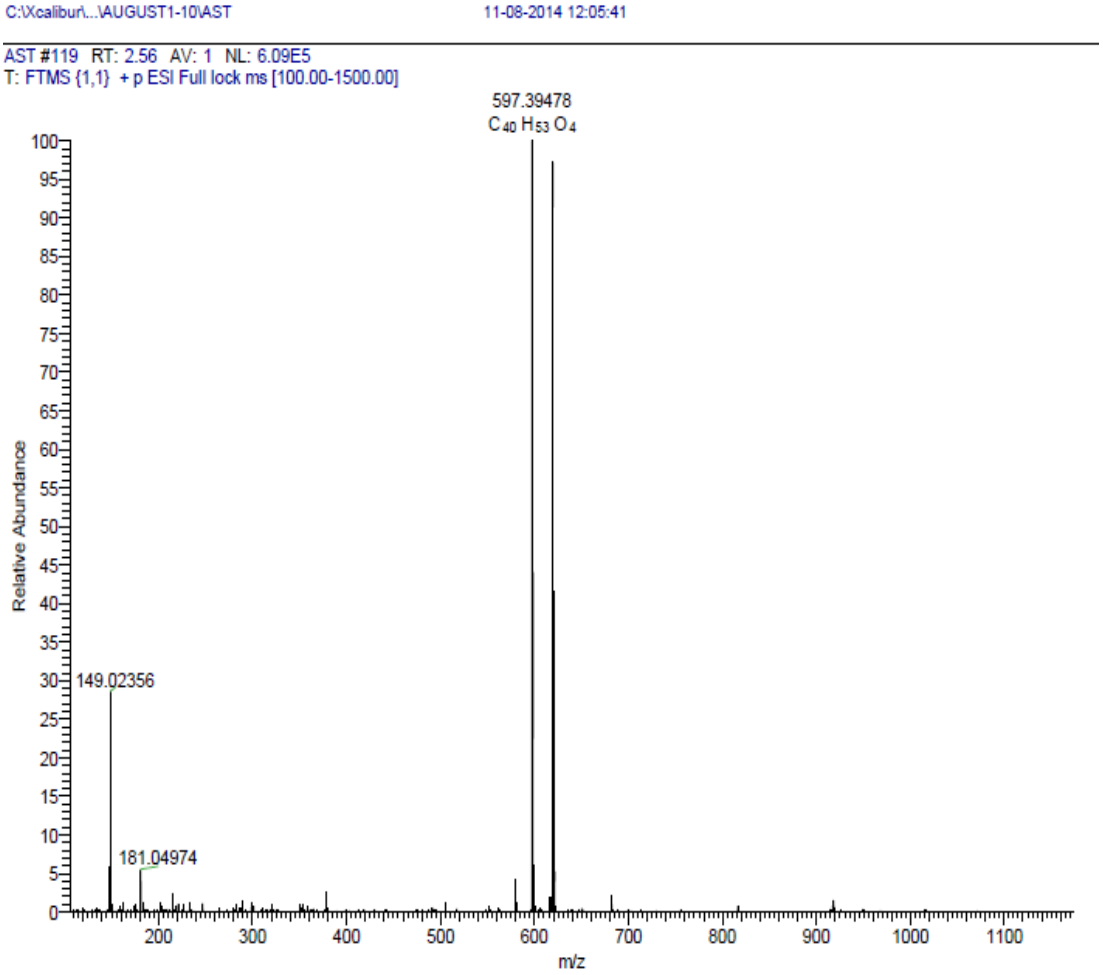
TABLE 10: C_{13} NMR

Signal	Chemical shift (ppm)	Functional group
1	15	CH_3
2	24	CH_2
3	25	CH_3, CH_2

4	50	CH_2
5	150	C in aromatic ring
6	2.5	CH_2
7	200	C=O

4.4.4 ESI Spectra

Fig. 16: Molecular mass: 596.84 g·mol^{-1}



Molecular mass is 596 gm mol $^{-1}$
Molecular formula C_{40} H_{53} O_4
n + 1 peak is 597.39

Reference

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