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OXIDATIVE STRESS IN ALGAE: METHOD DEVELOPMENT AND EFFECTS OF TEMPERATURE ON ANTIOXIDANT NUCLEAR SIGNALING COMPOUNDS

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OXIDATIVE STRESS IN ALGAE: METHOD DEVELOPMENT AND EFFECTS OF TEMPERATURE ON ANTIOXIDANT NUCLEAR SIGNALING COMPOUNDS

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Dedicated to

My wife, Farhana Islam

Son , Nashwan Yusha and to

My Parents

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Md Noman Siddiqui

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LIST OF ABBREVIATIONS

$^1O_2$ = Singlet oxygen

$O_2^{-\bullet}$ = Super oxide radical

ACN = Acetonitrile

AhR = Aryl hydrocarbon receptor

BHT = Butylated hydroxyl toluene

CRABP-II = Cellular retinoic acid binding protein-II

CRBP = Retinol binding protein

DAD = Diode array detector

DMRT = Duncan multiple range test

DRIP = Vitamin-D-receptor interacting proteins

EtOH = Ethanol

H_2O_2 = Hydrogen peroxide

HPLC/APPI-MS = High Performance Liquid Chromatography/ atmospheric photo ionization mass spectrometry

HPLC/ESI-MS = High Performance Liquid Chromatography/ electro spray ionization mass spectrometry

MDA = Malondialdehyde

MeOH = Methanol

MUFA = Mono unsaturated fatty acid

NCoA = Nuclear receptor coactivator

NCoR = Nuclear corepressor family

$OH^{\bullet}$ = Hydroxyl radical

OS = Oxidative stress

PCR = Polymerase chain reaction

PUFA = Polyunsaturated fatty acid

QQQ = Triple quadrupole

RA = Retinoic acid

RAR = Retinoic acid receptor

RO = Reverse osmosis

$ROO^{\bullet}$ = Peroxyl radical

ROS = Reactive oxygen species

RXR = Retinoic X receptor

SOD = Superoxide dismutase

TBA = Thiobarbituric acid

TCA = Trichloroacetic acid

THF = Tetrahydrofuran

VDR = Vitamin D receptor

ABSTRACT

Siddiqui, Md Noman M.S., Purdue University, May 2014. Oxidative stress in algae: method development and effects of temperature on antioxidant nuclear signaling compounds. Major Professor: Paul Brown.

Non-enzymatic antioxidants neutralize the oxidative stress through scavenging reactive oxygen species. The structural complexity of non-enzymatic nuclear signaling antioxidants poses are more challenges for traditional quantitative analysis as these compounds have multiple forms and structurally diverse than proteins and therefore, challenge traditional analytical techniques. Current analytical methods cannot distinguish the carotenoids, and vitamin A and D metabolites within a single sample. To find an appropriate method to measure all the nuclear signaling compounds in algae, *Isochrysis sp.,* under thermal stress, HPLC/ESI-MS and HPLC /APPI-MS was used. Standards of those compounds were run by QQQ HPLC/ ESI-MS and HPLC /APPI-MS methods by multiple reaction monitoring to optimize the most sensitive transition ion for each compound. Organic solvents (cold acetone and petroleum ether) were used for extraction. Neither APPI nor ESI method showed the sensitivity to detect all targeted non- enzymatic compounds. ESI method detects lower number of compounds than APPI method, but the sensitivity of this method was higher than APPI.

Isochrysis sp., was kept at 25 °C, 30 °C and 35 °C for 96 hrs to observe the changes of non-enzymatic antioxidants (carotenoids and vitamins) oxidative stress. Algae

were grown with continuous light and aeration in 70 ml test tube. Samples were collected from culture tubes (50 ml) at 0, 6, 12, 24, 48, 72 and 96 hrs *Isochrysis* showed best growth at 30 °C and lowest at 35 °C. Algae grown at 35 °C had significantly ($P<0.05$) higher concentrations of MDA compared to algae grown at lower temperatures and the MDA concentrations decreased over time. Fucoxanthin was the dominating pigment followed by diadinoxanthin for all treatment groups. The quantity of carotenoids (fucoxanthin, diadinoxanthin, diatoxanthin and antheraxanthin) and pro-vitamin A (retinol, retinal, retinoic acid) was higher in algae grown at 35 °C and algae grown at 30 °C had lower quantity of these carotenoids, .Algae grown at higher temperature had higher MDA. These data indicate that OS induced by thermal change alter carotenoids of antioxidants nuclear signaling compounds.

CHAPTER 1. INTRODUCTION

1.1 Introduction

The presence of oxygen in earth's atmosphere distinguishes this planet from others in our solar system and significantly influenced the flora and fauna that evolved. Oxygen is a necessary element for all life forms, but certain chemical species of oxygen are toxic to both plants and animals. In the process of routine metabolism, several reactive oxygen species (ROS) can be produced that are cytotoxic. These ROS include singlet oxygen ($^1O_2$), super oxide radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^\bullet$), hydrogen peroxide (H_2O_2) and peroxyl radical ($ROO^\bullet$) (Sirikhachornkit & Niyogi, 2010). A complex system developed to combat, or quench, ROS in both plants and animals (Sirikhachornkit & Niyogi, 2010), yet excessive production of ROS occurs. Excessive concentrations of ROS can lead to cellular oxidative stress (OS), and subsequent damage to DNA, proteins and lipids (Aro et al. 1993; Lesser, 2006; Martindale & Holbrook, 2002). This damage often results in cellular senescence and apoptosis (Sirikhachornkit & Niyogi, 2010), by chemically altering sugars and bases associated with DNA (Imlay & Linn, 1988). DNA damage includes both single and double strand breaks resulting in deletions and mutations in transcription products and gene expression (Lesser, 2006; Halliwell 2006). Abnormality in cell functions leads to genomic instability, and may be a cause of carcinogenesis (Britton, 1995). ROS can damage proteins through formation of carbonyls, which result

in inactivation and fatty acids and polyunsaturated fatty acid (PUFA) are oxidized to malondialdehyde (MDA) with guanine (Jeong et al. 2005). Damage to DNA and mistranslation of those repairs result in altered protein structure and function (Ling & Söll, 2010).

Despite the array of mechanisms to quench ROS, there are situations where production exceeds the ability to quench ROS produced and disease states occur. Oxidative stress has been implicated as one of the early steps in several human diseases such as cancer, atherosclerosis, malaria, arthritis and neurodegenerative diseases (Aruoma, 1998; Giustarini et al. 2009). OS has been an area of interest in medical research for several decades and is a significant consideration in space flight and colonization of the moon and other planets. Outside the protective barrier of Earth's atmosphere, gamma ray pulses from solar flares are regular events and considered one of the limiting factors for long-term habitation of space (Cucinotta and Durante, 2009). Gamma rays cause OS (Sangsuwan & Hangdoost, 2008; Kondo et al. 2010), yet the full impact of gamma ray-induced OS is not clear as the effects on gene expression have apparently not been considered. OS is also an area of interest in evolutionary biology (Peters et al. 2004) and life-history trade-offs (Dowling and Simons, 2009). This topic also has implications in trophic ecology and the flow of nutrients in food webs

Quenchers of ROS include both enzymatic and nonenzymatic approaches. Enzymes include multiple isoforms of superoxide dismutase, catalase, and ascorbate peroxidase. The non-enzymatic quenchers of ROS include glutathione, carotenoids, thiamin and vitamins A, D, C and E (Montserrat et al. 2012). Three of the nonenzymatic antioxidants (carotenoids, and vitamins A and D) are also regulators of gene expression.

There are relatively few nuclear receptor families; receptors on the outer membrane of the nucleus that can internalize targeted chemicals. The receptor families include thyroid hormone, retinoid (vitamin A), estrogen, nerve growth factor 1B, steroidogenic factor, germ cell nuclear receptor and a miscellaneous receptor family (Aranda and Pascual, 2001) (Table 1). Vitamin D acts through the thyroid receptor family, vitamin A through the retinoid receptor and carotenoids act through the miscellaneous receptor family (also known as the orphan nuclear receptors) via the aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003). Intracellular vitamin D initially binds with the vitamin D receptor (VDR) for translocation into the nucleus, where it can bind to as many as 2776 sites on DNA (Ramagopalan et al. 2012). Intracellular vitamin A binds with the cellular retinoic acid binding protein-II (CRABP-II) or cellular retinol binding protein (CRBP), then is transferred to the retinoic acid receptor (RAR) or the retinoic X receptor (RXR). There are 3 forms of both RAR and RXR (alpha, beta and gamma). Both RAR and RXR bind to DNA forming a heterodimer (McGrane, 2007). There are over 1800 vitamin A binding sites on DNA (Mahony et al. 2011). Further control of gene expression can occur via VDR-RXR heterodimer (Haussler et al. 2011). Once vitamins A and D bind to DNA, they are subject to regulation by additional proteins, either activators or repressors of gene transcription. Activators include the p160 family of proteins (nuclear receptor coactivator, NCoA, McGrane, 2007) and the vitamin-D-receptor interacting proteins (DRIP) (Rachez et al. 1999). Repressors of gene expression include the nuclear corepressor family (NCoR, Horwitz et al. 1996) and silencing mediator for retinoic acid (SMRT, Horwitz et al. 1996). Carotenoids include 17 separate chemicals and a distinct biochemical pathway (Fig 1.1). Mechanisms of carotenoid internalization, transport and

binding sites are not clear. Further, the specific carotenoids capable of binding to genomic DNA are unknown. Despite the available information on nuclear signaling in metazoans, the interaction between functions, quenching ROS and nuclear signaling, has not been explored and may be influencing several contemporary research topics.

Table 1: Identified exogenous and endogenous nuclear ligands

Exogenous	**Endogenous**
Retinol	Melatonin
Retinoic acid	Androstanol
Vitamin D	Ecdysone
Palmitic acid	17 β estradiol
Carotenoids	Cortisol
EPA/ DHA	Leukotriene B4
24(S),25-Epoxycholesterol	Thyroid hormone
22(R)-Hydroxycholesterol	Progesterone
Hyperforin	
Palmitoyl coenzyme A	

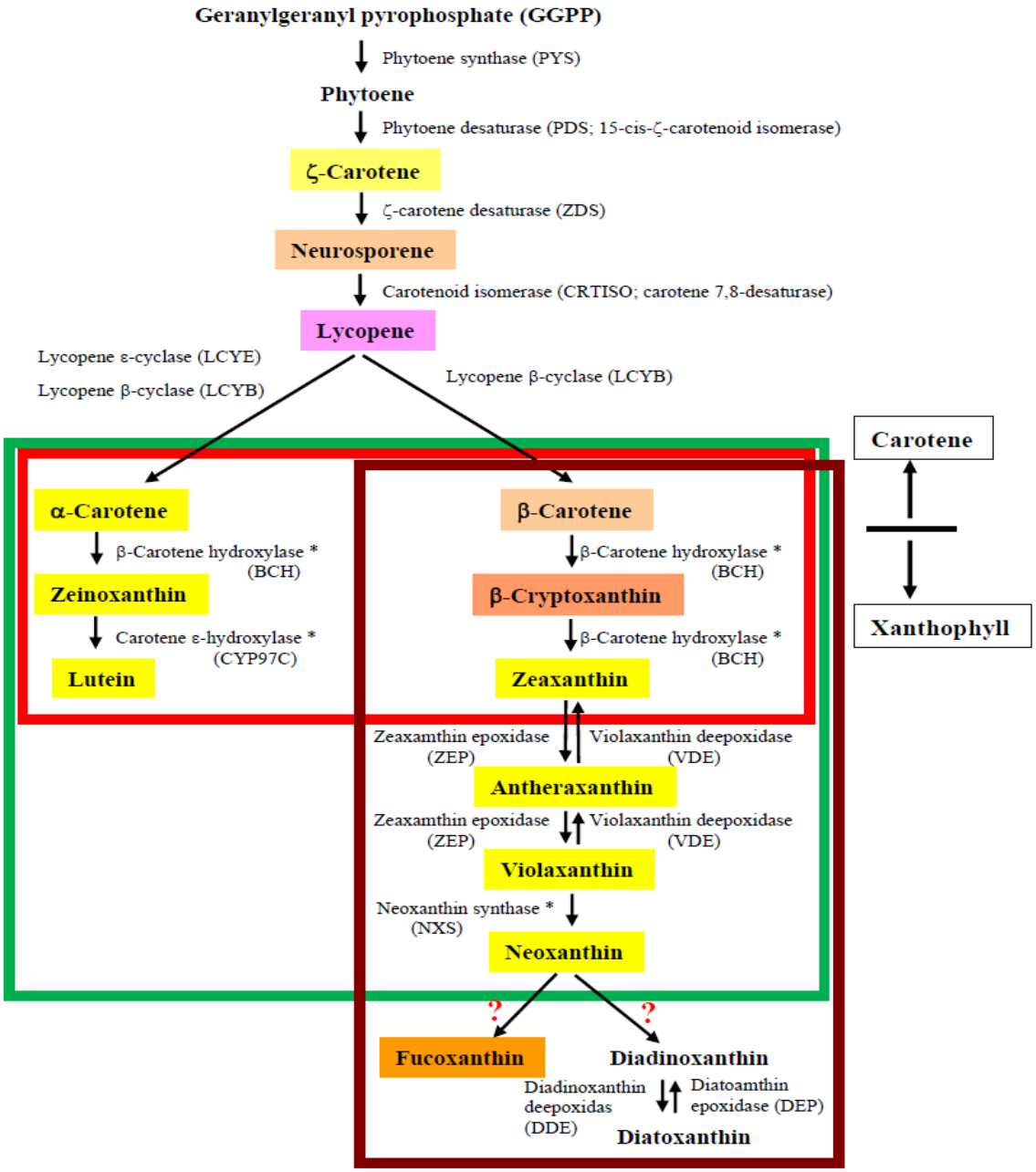

Figure 1.1 Carotenoid biosynthetic pathway in seaweeds. Red, green and brown boxes reveal the pathway in red, green and brown seaweeds, respectively. The symbol * indicates enzymes unidentified in seaweeds. Source: Mikami and Hosokawa, 2013.

Vitamins A and D and carotenoids are antioxidants. Synthetic antioxidants are important in the food industry as they serve as food preservatives (Nikki, 2002). Identification of antioxidant compounds in food, beverages and biological samples is an active area of investigation (Frankel and Meyer 2000; Nikki, 2002). Scientists have developed many methods and protocols to identify targeted antioxidants. However, there are no methods to determine nuclear signaling antioxidants (Frankel and Meyer, 2000; Nikki, 2002).

Isochrysis sp. is a free living marine unicellular phytoflagilllate. It is widely used in biological research as model algae (Brown, 1993). *Isochrysis sp*. has been used for nutritional studies (Brown, 1993), biodiesel production (Sanchez, 2013), and pollution studies (Campa -Córdova, 2006). However, studies of non-enzymatic antioxidant compounds in algae under temperature stress are not known. The current study was done to reveal the effects of different temperatures on non-enzymatic antioxidant compounds in brown algae *Isochrysis sp.*

1.2 Assay development

1.2.1 Introduction

Precision and effectiveness of a method depends on substrates, composition and analytical capabilities (Sánchez-Moreno, 2002). The structural complexity of antioxidants poses challenges for traditional quantitative analysis and, along with contemporary reductionist approaches in science have resulted in targeted experiments examining the roles of antioxidants, without a more thorough understanding of the interactions among antioxidants. There are several approaches for quantifying proteins in biological and botanical samples, from total protein concentrations to specific protein identification using electrophoretic methods to newer enabling technologies of polymerase chain reaction (PCR), proteomics and transcriptomics. However, the non-enzymatic quenchers of ROS are more structurally diverse than the proteins and challenge traditional analytical techniques. There are no analytical methods capable of identifying and quantifying the array of non-enzymatic nuclear signaling antioxidants, precluding a thorough understanding of their prioritized use in cells.

All of the non-enzymatic quenchers of OS occur in multiple forms. There are numerous carotenoid pigments and numerous metabolites of the antioxidant vitamins, also referred to as vitamers. Thus, classic quantitative analyses migrated from analyses of individual compounds to chromatographic separation of similarly structured compounds. Current analytical methods cannot distinguish the carotenoids, and vitamin A and D metabolites within a single sample. Newer enabling technologies and analytical instruments offer the possibility of quantifying diverse chemicals from complex matrices such as botanical and biological samples. Liquid chromatography coupled to tandem

mass spectrometers followed by multiple reaction monitoring (LC/MS/MS/MRM) has the ability to specifically monitor fragments dissociated through the tandem MS. Mass resolution is in the range of hundredths of a dalton, so it should be considered precise. However, there are no LC/MS/MS/MRM methods for the nuclear signaling antioxidants. Given the importance of OS in a variety of research areas, the abiotic factors exerting adaptive pressures on ecosystems and the importance of gene expression regulators, the antioxidant nuclear signaling compounds are a logical subset of antioxidant quenchers for development of improved chemical assays. Once developed, the method can be applied to the entire range of trophic levels, from primary producers to top carnivores

1.3 Materials and methods

1.3.1 Collection of standards

Standards for the target compounds were acquired from different vendors. Astaxanthin and zeaxanthin were purchased from Cayman (Ann Arbor, MI), retinol, retinal, fucoxanthin, canthaxanthin, 25 $(OH)D_3$, $1,25(OH_2)D_3$, $24,25(OH_2)D_3$, retinoic acid (RA), β-cyclocitral, D_2 , D_3 from Sigma Aldrich (St. Louis, MO); antheraxanthin, diadinoxanthin, diatoxanthin, violaxanthin and antheraxanthin from Chromadex (Irvine, CA), lycopene from TRC (Toronto, Canada), β -carotene from VWR (Radnor, PA), and β cryptoxanthin from Santacruz Biotechnology (Dallas, TX).

1.3.2 Selection of extraction solvent

Due to the complex structure and numbers of carotenoids, there is no single solvent to extract all carotenoids and vitamins from botanical or biological samples. Various types of organic solvents like acetone, tetrahydrofuran (THF), *n*-hexane, ethanol (EtOH), methanol (MeOH), acetonitrile (ACN) chloroform and combinations of different organic solvents have been used by different researchers to extract carotenoids from plants, fruits and biological samples. In this experiment, combinations of methanol (MeOH) and methyl tertbutyl ether (MtBE) (1:1; v:v), cold acetone and petroleum ether (1:1; v:v), methanol and hexane (1:1; v:v), and EtOH and hexane (1:3; v:v) were evaluated. Prepared samples were analyzed by atmospheric pressure photoionization (APPI).

1.3.3 Preparation of stock solution

Based on the chemical properties of the standards, stock solutions were developed in acetonitrile, chloroform or ethanol. Ethanol was used for retinoic acid (RA), retinol, retinal, D_2, D_3, 25 $(OH)D_3$,1,25$(OH_2)D_3$, and 24,25$(OH_2)D_3$. Chloroform was used for astaxanthin, fucoxanthin, canthaxanthin, β -cryptoxanthin and β -cyclocitral. The final concentration of astaxanthin, retinol, retinal, fucoxanthin, canthaxanthin, D_2, D_3, lycopene and β carotene and retinoic acid was 10 mg/mL, zeaxanthin 2.5 mg/mL, β -cryptoxanthin, and 25 $(OH)D_3$ 1 mg/mL, 1,25$(OH_2)D_3$ 0.1 mg/mL, 24, 25$(OH_2)D_3$ 0. 5 mg/mL, β -cyclocitral 94.3 mg/mL, antheranxathin 0.000587 mg/mL, violaxanthin 0.000752 mg/mL, diatoxanthin 0.000605 mg/mL and diadinoxanthin 0.001098 mg/mL. A dilution of 1:2 x, 1:5 x and 1:10 x for all the standards and mixtures of standards were prepared for quantification of analytes except for the 1,25$(OH_2)D_3$, 24,25$(OH_2)D_3$ mixture. For these standards, the dilution was 1:10 x, 1:100 x as the quantity of these standards was limited. The standard stock and mixed solutions were stored at −80 °C in sealed tubes and protected from light until further use. All extractions were carried out at temperatures at or below ambient and all extraction solvents were cooled in ice prior to use.

1.3.4 Sample extraction

Isochrysis sp. was used for method validation. HPLC-APPI/MS (high performance liquid chromatography- atmospheric pressure photo ionization/ mass spectrometry) and HPLC- ESI/MS (High performance liquid chromatography - electro spray ionization/ mass spectrometry) were used to analyze the samples. *Isochrysis sp*., was cultured in a 10 gallon tank with F/2 (Sigma Aldrich, St Louis, MO) enriched media, in reverse osmosis (RO) water at 30 ppt salinity and 30 °C temperature. Continuous aeration and light was provided by using two 40 W GE (2900 lumen) cool white florescent lamps. After 4 days, samples were collected through centrifugation at 4000 rpm for 10 min in 50 ml corning tubes. Water was drained and settled plankton was collected from the bottom using a pipette. Settled plankton from all the tubes was collected in a single corning tube. About 35 ml of samples was washed with RO water and centrifuged again at 4000 rpm for 15 min. The collected plankton sample was freeze dried for 72 hours. Dried samples of *Isochrysis* of 10 mg, 20 mg and 40 mg were placed in 2 ml glass bead tubes with 400 µm zirconium beads (Ops Diagnostics, Lebanon, NJ). 166 µl of dd H_2O, 500 µl of cold acetone and 500 µl petroleum ether was added to the sample. 10 µl *trans*-β-δ' carotenal as an internal standard was added (concentration 1 mg/mL). Oxidation of the sample was inhibited by addition of 10 µl of butylated hydroxyl toluene (BHT) (concentration 4 mg/mL). Samples were homogenized by Precellys @24 (Ann Arbor, MI) for 2 x 30 sec at 5000 cycles. After that, samples were centrifuged at 15000 rpm for 10 minutes, then the organic solvent (top phase) of the extraction was collected. This extraction protocol was repeated. After extraction, petroleum ether and acetone were evaporated with nitrogen gas. Finally, the collected

sample was preserved at -80 °C until analysis. All the extractions were done at or below ambient temperature and direct light to the samples was avoided.

For HPLC analysis, the samples were resuspended by using 200 µl Buffer A (water + 0. 1% formic acid), and vortexed for 5 minutes. After re-suspending the solution was centrifuged for 10 minutes at 15000 rpm. 100 µl of each sample was injected in HPLC glass vials for analysis. For analysis 10 mg, 20 mg and 40 mg samples was used in APPI and ESI methods.

1.3.5 Liquid Chromatography/APPI Mass Spectrometry Analysis

An Agilent 1200 series liquid chromatography (LC) platform (Agilent Technologies, Santa Clara, CA) equipped with a well plate auto sampler, binary pumping device, and diode array detector (DAD) coupled to an Agilent 6460 Triple Quadrupole (QQQ) mass spectrometer (MS) was used for the analysis. Reverse phase liquid chromatography was used to separate the samples before MS analysis. A Waters Xterra C18 column (Waters Corporation, Milford, MA) (2.1 x 150 mm, 5.0 µm) was used for the separation. Solvent A consisted of water + 0.1 % formic acid. Solvent B consisted of acetonitrile/methanol (50:50 v: v). The flow rate was 0.3 mL/minute. A sample volume of 20 µL was loaded onto the column. The linear gradient was as follows: time 0 minutes, 2 % B; time 1 minute, 2 % B; time 16 minutes, 95 % B; time 30 minutes, 95 % B; time 32 minutes, 2 % B; time 40 minutes, 2 % B. The UV spectra were collected by the DAD from 200-600 nm.

The MS analysis used positive and negative polarity atmospheric pressure photo-ionization (APPI). The source conditions were as follows, capillary voltage 3.5 kV for positive mode and 3.0 kV for negative mode, gas temperature 325 °C, drying gas 7 L/minute, nebulizer pressure 40 psi, and vaporizer temperature 250 °C. Data were acquired using multiple reaction monitoring mode (MRM) (Appendix, Table 1).

The samples were evaluated and processed with Agilent Masshunter Qualitative and Quantitative Analysis software (version B. 02.01).

1.3.6 Liquid Chromatography/ESI Mass Spectrometry Analysis

An Agilent 1200 series liquid chromatography (LC) instrument (Agilent Technologies, Santa Clara, CA) equipped with a well plate auto sampler, binary pumping device, and diode array detector (DAD) coupled to an Agilent 6460 Triple Quadrupole (QQQ) mass spectrometer (MS) instrument was used for the analysis. Reverse phase liquid chromatography was used to separate the samples before MS analysis. A YMC C30 carotenoid column (YMC Co., Ltd., Kyoto, Japan) with 2.1 x 150 mm, 3.0 µm dimensions were used for the separation. Solvent A consisted of methanol (MeOH) 88%, methyl *tert*-butyl ether (MtBE) 5%, water (ddH_2O) 5%, and 1 M ammonium acetate 2 % (NH_4OAc). Solvent B consisted of methanol (MeOH %) 20%, methyl *tert*-butyl ether (MtBE) 78%, and 1 M ammonium acetate 2% (NH_4OAc). The flow rate was 0.3 mL/ minute. A sample volume of 20 µL was loaded onto the column. The linear gradient was as follows. Time 0 minutes, 0 % B; time 1 minute, 0 % B; time 40 minutes, 85 % B; time

50 minutes, 100 % B; time 55 minutes, 0 % B; time 60 minutes, 0 % B. The UV spectra were collected by the DAD from 200-600 nm.

The MS analysis used positive and negative polarity electrospray ionization (ESI). The source conditions were as follows, capillary voltage 3.5 kV for positive mode and 3.5 kV for negative mode, gas temperature 325 °C, drying gas 7 L/minute, nebulizer pressure 40 psi, sheath gas temperature 250 °C, sheath gas 7 L/minute, and nozzle voltage 1000 V in positive mode and 500 V in negative mode. Data were acquired using multiple reaction monitoring mode (MRM) (Appendix- Table 2)

The samples were evaluated and processed with Agilent Masshunter Qualitative and Quantitative Analysis software (version B. 02.01).

1.4 Statistical analysis

Samples were analyzed by SPSS-16 software using ANOVA. Homogeneity of the samples was tested for every treatment group, using three replicates for each treatment Differences between mean values were analyzed by Duncan multiple range test (DMRT), with $\alpha = 0.05$.

1.5 Results

No single approach worked for the non-enzymatic compounds in *Isochrysis sp.* APPI detected β cyclocitral, fucoxanthin, astaxanthin, β cryptoxanthin, canthaxanthin, antheraxanthin, diadinoxanthin, diatoxanthin, retinal, retinol, violaxanthin and zeaxanthin (Table 2). ESI detected β cyclocitral, astaxanthin, β cryptoxanthin, canthaxanthin, diadinoxanthin, violaxanthin, zeaxanthin and retinoic acid (Table 2). Retinal, retinol, antheraxanthin, diadinoxanthin was detected only by APPI method and retinoic acid was detected by ESI method only. Compounds not detected by either approach were 25 (OH)D_3, D_2, D_3, 1,25 (OH)D_3, 24, 25 (OH)D_3, β carotene, and lycopene.

Table 2. MRM approaches that detected targeted compounds from *Isochrysis* samples, (D= detected, ND= Not detected)

Compounds	ESI	APPI
β-cyclocitral	D	D
Fucoxanthin	ND	D
Astaxanthin	ND	D
Canthaxanthin	D	D
β -Cryptoxanthin	D	ND
Diatoxanthin	D	D
Retinoic Acid	D	ND
Violaxanthin	D	D
Zeaxanthin	D	D
Antheraxanthin	ND	D
Diadinoxanthin	ND	D
Retinal	ND	D
Retinol	ND	D

1.5.1 Comparison of APPI and ESI method

β cyclocitral, canthaxanthin, diatoxanthin, violaxanthin and zeaxanthin were detected by both APPI and ESI platforms. Among these compounds, higher concentration of canthaxanthin, zeaxanthin and β cyclocitral were detected by ESI (Table 3). Amount of violaxanthin, β cycloictral and canthaxanthin detected by ESI and APPI was very small. Table 3 and figures below show the concentration of different analyte detected by APPI and ESI method.

Table 3. Concentration of compounds detected by APPI and ESI methods.

Compound	APPI ng/µL	ESI ng/µL
Zeaxanthin	0.540	5.045
β Cyclocitral	0.001	0.027
Canthaxanthin	0.001	0.007
Diatoxanthin	1.680	2.573
Violaxanthin	0.015	0.009

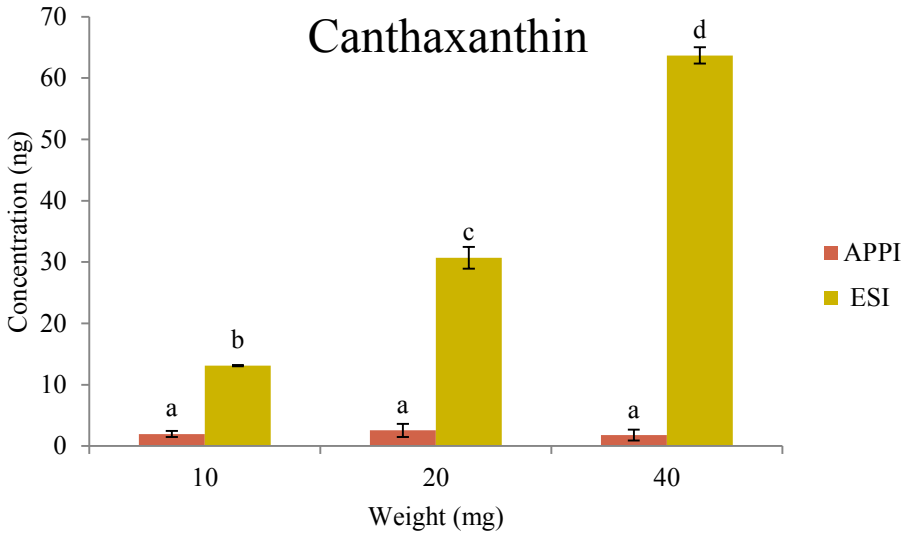

Figure 1.2 Comparison of canthaxanthin in *Isochrysis sp.* using APPI and ESI methods. Different letters at the top of the bar indicate significant ($P < 0.05$) difference (More detail in Appendix Table 3 & 4).

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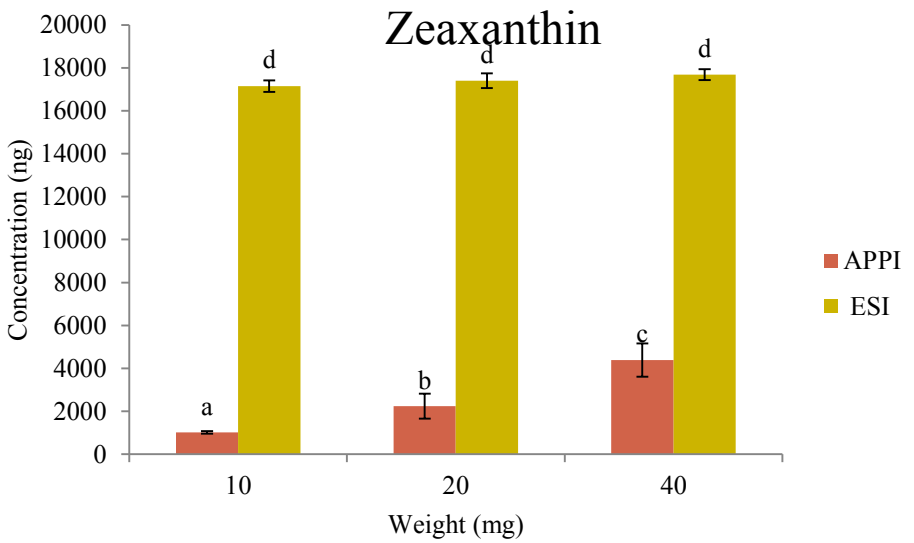

Figure 1.3 Comparison of zeaxanthin in *Isochrysis sp.* using APPI and ESI methods. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3 & 4).

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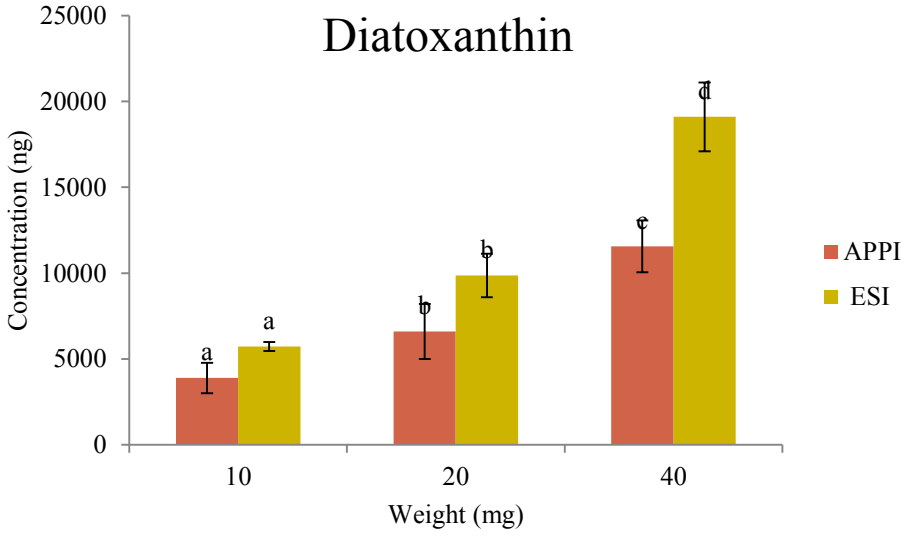

Figure 1.4 Comparison of diatoxanthin in *Isochrysis sp.* using APPI and ESI methods. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3 & 4).

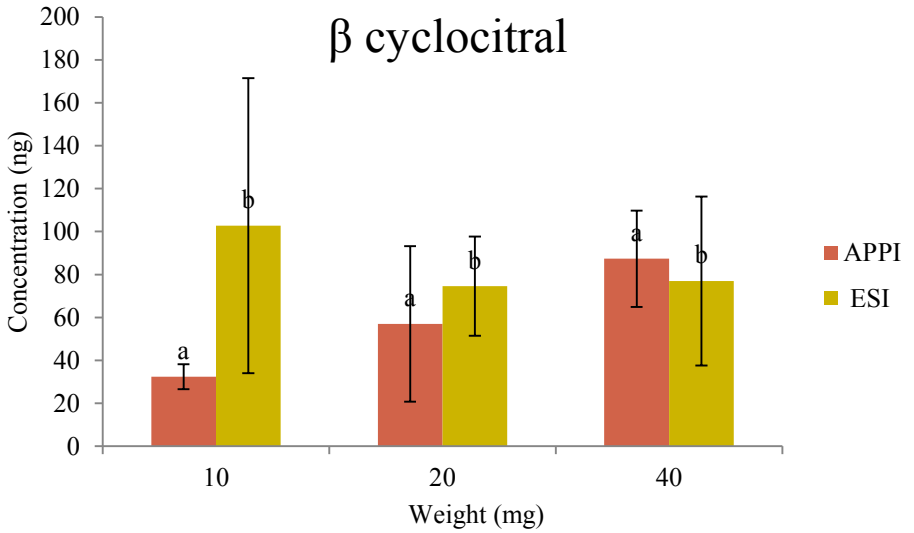

Figure 1.5 Comparison of β cyclocitral in *Isochrysis sp.* using APPI and ESI methods. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3 & 4).

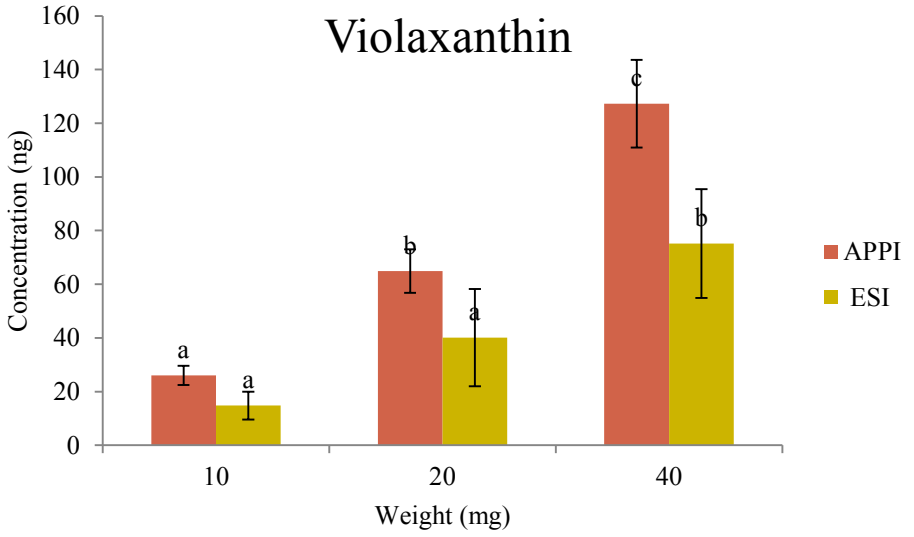

Figure 1.6 Comparison of violaxanthin in *Isochrysis sp.* using APPI and ESI methods. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3 & 4).

1.5.2 Compounds detected only by APPI method

Using APPI, fucoxanthin, astaxanthin, diadinoxanthin, antheraxanthin, retinal and retinol were detected. Very small quantities of β cyclocitral, β cryptoxanthin, canthaxanthin, retinal, retinol, violaxanthin were detected by this method.

All these carotenoids except astaxanthin, β cyclocitral had a positive relation with increasing weight. Concentration of fucoxanthin varied significantly ($P<0.05$) with each sample weight. No significant ($P>0.05$) difference was observed for astaxanthin with increasing weight.

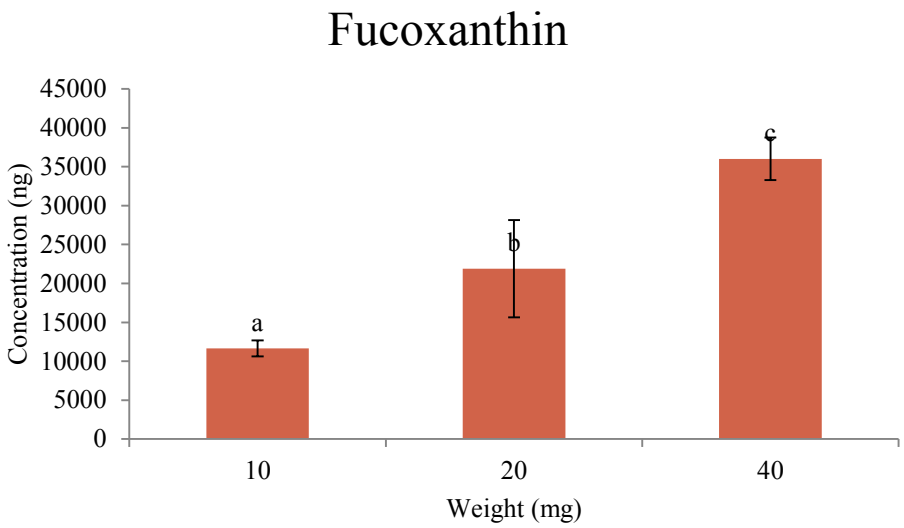

Figure 1.7 Concentration of fucoxanthin in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant ($P<0.05$) difference (More detail in Appendix Table 3).

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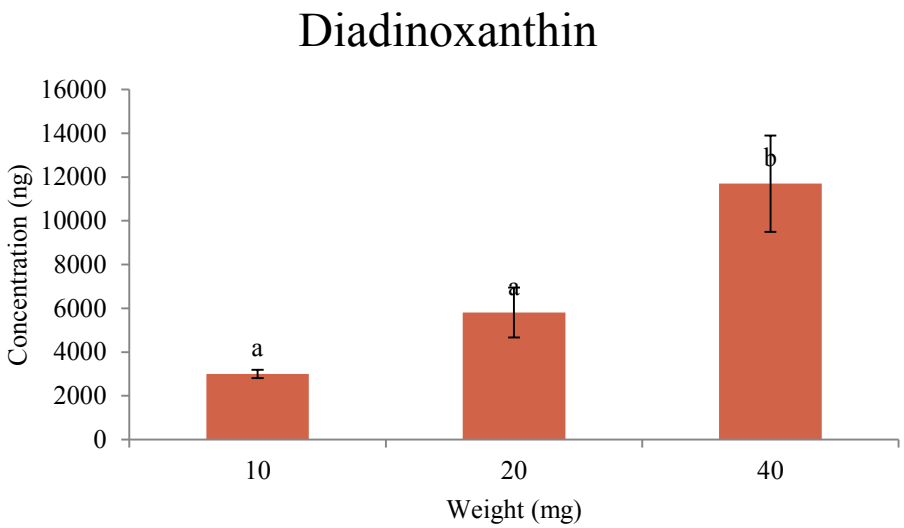

Figure 1.8 Concentration of diadinoxanthin in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3).

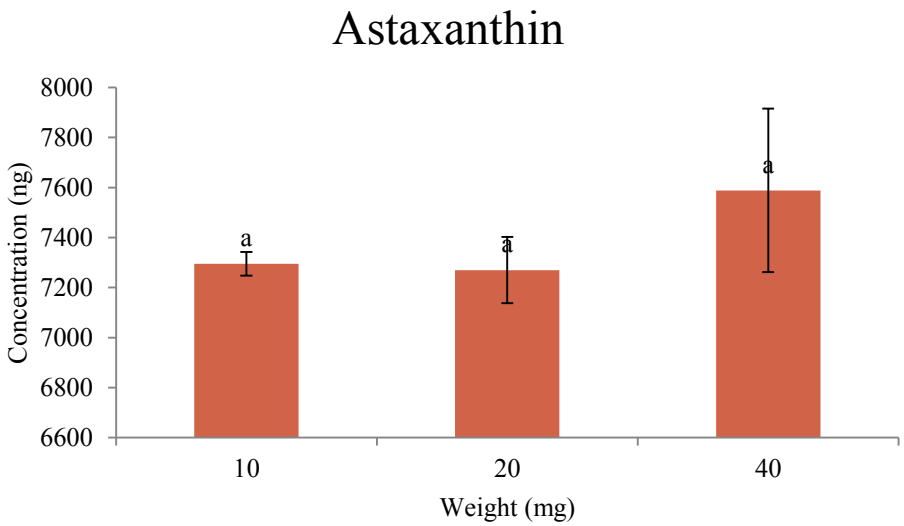

Figure 1.9 Concentration of astaxanthin in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3).

A positive relationship with weight was observed for this carotenoid and each weight group varied significantly compared to others.

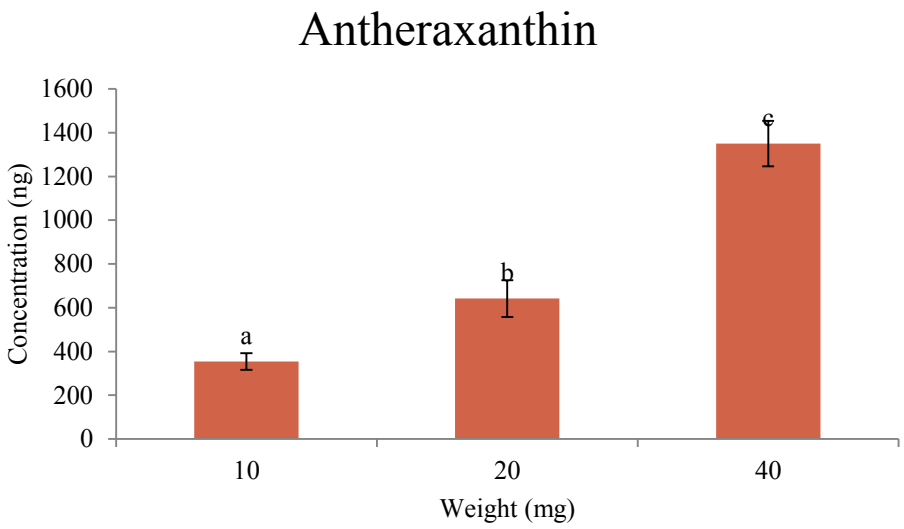

Figure 1.10 Concentration of antheraxantin in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3).

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Very small (<1 ng/µL) quantities of retinal and retinol were detected. Concentrations of these compounds increased with increasing weight. Every weight group significantly (P<0.05) differed from each other.

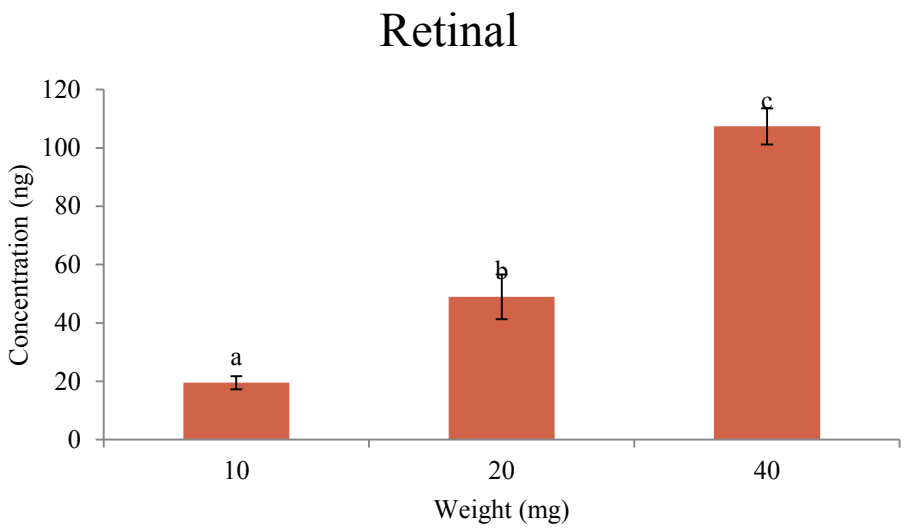

Figure 1.11 Concentration of retinal in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3).

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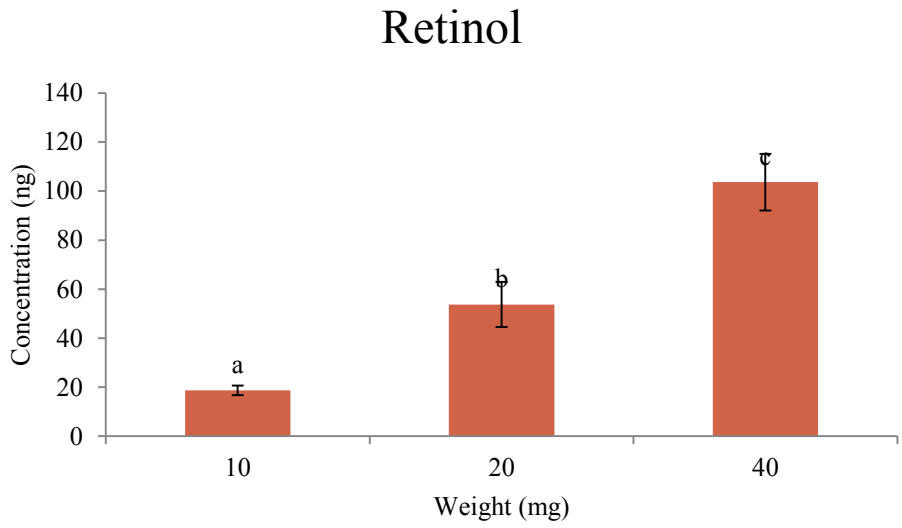

Figure 1.12 Concentration of retinol in *Isochrysis sp.* using APPI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 3).

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1.5.3 Compounds detected by only HPLC ESI/MS method

The ESI platform detected β cryptoxanthin, β cyclocitral, canthaxanthin, diatoxanthin, fucoxanthin, retinoic acid violaxanthin and zeaxanthin. Retinoic acid and β cryptoxanthin was detected only by ESI method. Concentrations of canthaxanthin ,diatoxanthin and violaxanthin, for each weight groups varied significantly ($P<0.05$) compared to 40 mg.

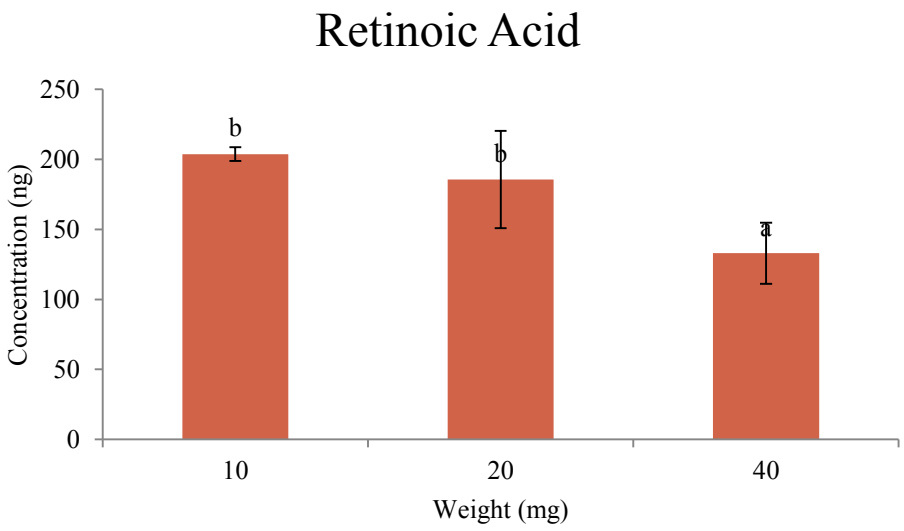

Figure 1.13 Concentration of retinoic acid in *Isochrysis sp.,* using ESI method. Different letters at the top of the bar indicate significant ($P<0.05$) difference (More detail in Appendix Table 4).

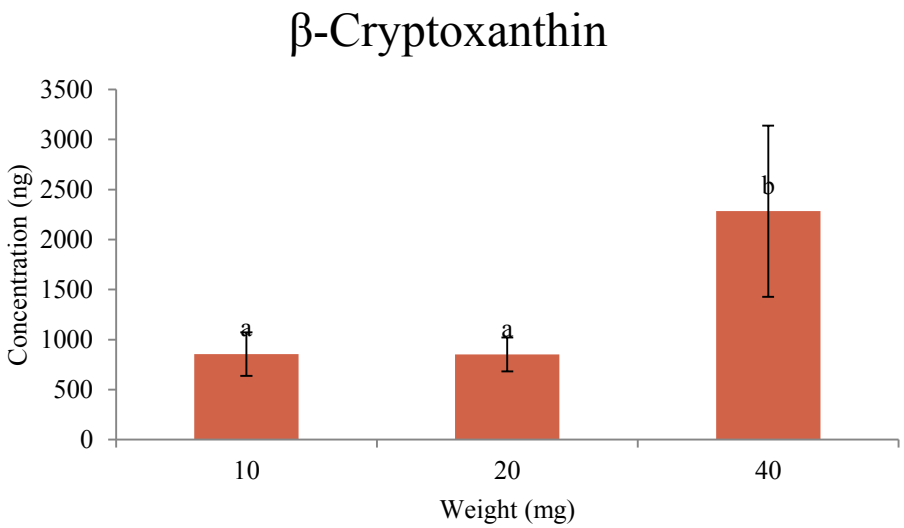

Figure 1.14 Concentration of β cryptoxanthin in *Isochrysis sp.*using ESI method. Different letters at the top of the bar indicate significant (P<0.05) difference (More detail in Appendix Table 4).

1.6 Discussion

Neither APPI nor ESI method were able to detect all the targeted compounds from *Isochrysis* samples. APPI can be recommended for analysis of diatoxanthin, fucoxanthin, diadinoxanthin, antheraxanthin, retinal and retinol. Higher concentrations of astaxanthin were detected by APPI, but it failed to accurately quantify with varying weight. Astaxanthin cannot be quantified by APPI. ESI was able to detect higher concentration of zeaxanthin, diatoxanthin and β cryptoxanthin. Quantity of these carotenoids (except zeaxanthin) had a constant ratio with weight. To identify and quantify these carotenoids, ESI can be used. Very small quantity of β cyclocitral, canthaxanthin and violaxanthin was detected by both APPI and ESI. These methods can be used to detect the presence or absence of these carotenoids. The concentration of provitamin A was very low (< 0.1

ng/μL). Retinal and retinol maintain the weight concentration ratio by APPI , and retinoic acid by ESI did not maintain concentration weight ratio. Roeck-Holtzhauer et al. (1991) detected 200 μm/gm of vitamin A from dired *Isochrysis galbana* by HPLC. That indicates this species had very low concentration of vitamin A (β carotene). It is established from different studies by HPLC that (Laza-Martinez et al.2007; Zapata et al. 2004;Fujiki and Taguchi, 2002), *I. galbana* has high concentration of fucoxanthin, followed by diadinoxanthin, ditatoxanthin and β carotene. Zapata et al. (2004) in their study noticed twelve unknown peaks of carotenoids in trace quantities. Findings from their study support current study. Only difference is β cryptoxanthin, which was not detected in the present study but β cyclocitral a derivative of β carotene was detected. HPLC- APPI/MS and HPLC- ESI/MS are considered more sensitive for detection of compounds from analyzed samples. Unknown peaks of Zapata et al. (2004) may belong to the new compounds detected by the current study. It can be assumed that these compounds are in very small amount in *Isochrysis*. It could be possible that APPI and ESI methods detect the presence of those, which were unidentified in previous studies. As APPI detects higher number of compounds, it can be used to detect the targeted compounds.

CHAPTER 2. ABIOTIC STRESS ON ALGAE

2.1 Introduction

All plants have fundamental requirements for growth. These include appropriate temperatures, light, water and nutrients. Chronic deficiencies or excesses of these abiotic factors can affect growth and reproduction (Jaleel et al. 2008; Tuteja et al. 2009). Temperatures below optimum damage photosynthetic pigments, protein synthesis and electron transfer capacity due to destruction of cell membranes (Guy et al. 1985; Holaday et al. 1991). Plants grown at lower temperatures had higher concentration of fatty acid and abcisic acid (Jun et al, 2001). Temperatures above optimum damage the thylakoid membranes, reduce the rate of respiration and photosynthesis, and disrupt tissue membranes, which results in destruction of cell arrangements and membrane integrity (Holaday et al. 1991). One of the common initial cellular responses to temperatures above or below optimum is development of oxidative stress**.** Normal physiological activities such as photosynthesis produce ROS. Chronic stress by abiotic factors like heat, light, salinity, or drought excite electrons that are transferred to molecular oxygen and form ROS (Mittler, 2002) ROS can be scavenged by antioxidant compounds. Any imbalance between ROS and antioxidants creates oxidative stress (OS). Despite the array of mechanisms to quench ROS, there are situations where production exceeds the ability to quench ROS produced and leads to diseased states. Vitamin A and

D, and carotenoids have the ability to neutralize the ROS. These compounds neutralize the extra energy and release that energy to their surrounding solvents and eliminate the toxic effect of ROS (Sthal and Sies, 2003).

Temperature affects the carotene composition of plants. Lefsrud et al (2005) observed that lutein and β carotene in kale has a positive relationship with increasing temperature and opposite phenomena was observed for spinach. Goodwin & Jamikorn (1952) found that synthesis of lycopene is prevented by higher temperature but β carotene production is enhanced in tomato. Ben-Amotz et al. (1982), and Borowitzka et al. (1984) reported that *Dunaliella salina* (a green algae), when exposed to environmental stress produced higher amounts of β carotene. Halfen and Francis (1972) found *Anacystis nidulans*, (a blue green alga) produce higher concentration of all carotenoids except canthaxanthin when grown at higher temperature. Zhang et al. (1997) in their study with *Chlorococcum sp.* (a chlorophyte), found that this species produced higher amount of astaxanthin when grown at higher temperatures. Garnier (1962) observed in *O. subbrevis* the amount of β carotene and xanthophyll increased with higher temperature.

2.2 Objective of the study

The current study was done to observe the effect of temperature in *Isochrysis* carotenoids.

2.3 Materials and methods

2.3.1 Stock culture of phytoplankton

Isochrysis sp (CCMP 463) was acquired from the Bigelow lab (East Boothbay, ME) for the experiment. The stock culture was incubated in a 2L Erlenmeyer flask at 30 °C to increase the number of cells for the experiment. Photoperiod during this phase of the experiment was 12 hrs light and 12 hrs dark. Artificial sea salt (Coral life, Carsol, CA) was dissolved in reverse osmosis water. Salinity was maintained at 30 ppt and the commercial F/2 (Sigma Aldrich, St Louis. MO) medium was used (Sánchez et al. 2013). Cells were transferred to experimental culture test tubes. 50 ml of water was used to culture the plankton in 70 ml test tube. The initial density of phytoplankton for the experiment was 959,211±181,470 cells/ml.

2.3.2 Experimental culture

Three temperatures were established for the experiment; 25 °C, 30 °C and 35 °C. Experimental test tubes were placed into 5 gallon aquaria. Each treatment had 35 replicates. Desired temperature was maintained by immersion water heaters. At the beginning of the experiment water temperature was 25 °C for all the tanks. Temperature was increased at the same rate for both treatments above 25 °C. Continuous light was provided by two 40 W GE (2900 lumen) cool white florescent lamps and continuous aeration was provided to every test tube by aquarium aerator.

2.3.3 Sample collection

The experiment was conducted for 96 hrs. The initial sample for analysis was collected as the temperature reached the target temperature (considered as 0 hr) of the respective treatments. Additional samples were collected at 6, 12, 24, 48, 72 and 96 hrs. Cells were counted in each tube before every sampling with a Neubaur hemacytometer (Bright line, Buffalo, NY). Samples were collected on Whatman glass fiber filter paper (4.2 cm) using suction pump in low light and preserved at -80 °C until analyzed

2.3.4 Preparation of stock solution

According to the properties of the standards, stock solutions of 1.0 mg/mL in acetone, chloroform or ethanol were prepared for each individual carotenoid, retinol and vitamin D metabolite. A vitamin and carotenoid standard mixture was prepared by mixing volumes of each of the individual standard stock solutions and was diluted with acetone. The standard stock and mixed solutions were aliquoted into cryovials, flushed with nitrogen gas, sealed and stored at −80 °C and protected from light until further use. Just prior to sample fraction analysis, an aliquot of standard mixture solution was thawed and serially diluted with methanol to provide standard curve. All extractions were carried out at temperatures at or below ambient temperature and all extraction solvents were cooled on ice prior to use.

2.3.5 Sample extraction

Collected samples on glass fiber filter paper were homogenized in 2 ml tubes with 400 µm zirconium beads (Ops Diagnostics, Lebanon, NJ) at 5000 rpm for 1 minute. 166 µl of dd H_2O, 500 µl of cold acetone and 500 µl petroleum ether was added to the sample.

10 µl *trans*-β-δ' carotenal as an internal standard was added (concentration 1mg/mL). To protect against oxidation 10 µl of butylated hydroxyl toluene (BHT) (concentration 4 mg/mL) was added. Samples were homogenized by Precellys @24, (Ann Arbor, MI) for 2 x 30 sec at 5000 cycles. Afterward, those samples were centrifuged at 15000 rpm for 10 minutes. Next, organic solvent (top phase) of the extraction was collected. Petroleum ether and acetone were evaporated by nitrogen flow. Finally, the collected sample was preserved at -80 °C until analysis. All extractions were done at or below ambient temperature and direct light to the samples was avoided.

The samples were resuspended in 200 µl Buffer A (water + 0. 1% formic acid), and vortexed for 5 minutes. Resuspended solutions were centrifuged for 10 minutes at 15000 rpm. 100 µl resuspended solution of each sample was injected in HPLC glass vial for analysis.

2.3.6 Liquid Chromatography/APPI Mass Spectrometry Analysis

It was done according to section 1.3.5

2.3.7 Liquid Chromatography/ESI Mass Spectrometry Analysis

It was done according to section 1.3.6

2.3.8 Thiobarbituric acid reactive substances assay (TBARS)

Malondialdehyde (MDA) value was measured to determine the oxidative stress of the plankton under temperature stress according to Buege and Aust (1978) using TBARS assay.

2.3.8.1 Reagents for TBARS methods

Stock solution of TCA-TBA-HCL was prepared. The recipe for the stock solution was 15% w/v of trichloroacetic acid (TCA), 0.375% w/v thiobarbituric acid (TBA), 0.25N HCL. The solution was kept in water bath at 120 °C until all the solids were dissolved.

2.3.8.2 Procedure

Samples with filter paper was homogenized by Precellys @24 (Ann Arbor, MI) for 2 x 30 sec at 6000 cycles with 1.2 ml of the stock solution of TBA. Samples were heated in water bath for 20 minutes at 120 °C. Then, the samples were placed in ice for cooling. Afterward, the samples were centrifuged at 1500 rpm for 10 min. The supernatant was collected in 10 ml cuvette and the absorption measured at 535 and 600 nm by (Thermo Scientific, Nanodrop 2000, Wilmington, DE). Blank sample was run in spectrophotometer at the beginning. MDA equivalents were calculated as.

MDA equivalents ($nmol.ml^{-1}$) = $[(A_{535}-A_{600})]/155000]10^6$

2.4 Statistical analysis

Samples were analyzed by SPSS-16 software using ANOVA. Homogeneity of the samples was tested for every treatment group, using three replicates for each treatment for carotenoids and vitamins and two replicates for oxidative stress measurements. Differences between mean values were analyzed by Duncan multiple range test (DMRT), with $\alpha = 0.05$.

2.5 Results

2.5.1 Number of cells at different temperatures

Number of cells at 96 hrs was influenced by test temperature, with the highest density in algae grown at 30 °C followed by those grown at 25 °C (Fig 2.1). Cell density in the 35 °C treatment was similar at all collection periods. There were no significant differences ($P>0.05$) among temperature groups at the first sampling. At 6 hours, cell density at 35 °C and at 30 °C significantly ($P<0.05$) differed from 25 °C. Algae grown at 30 °C were significantly denser compared with algae grown at 25 °C and 35 °C at 24 hours. The number of cells for all temperatures at 12, 48, 72 and 96 hrs were significantly ($P<0.05$) different from each other.

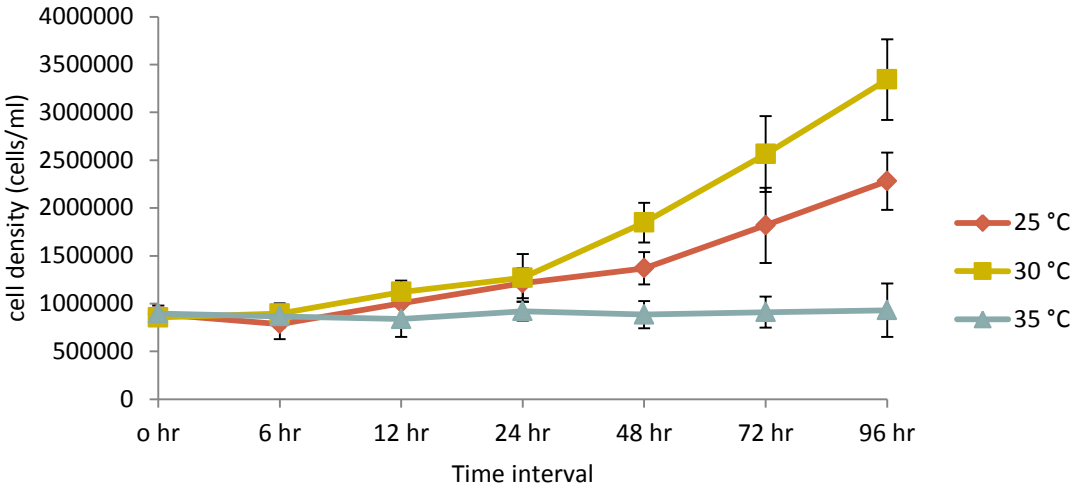

Figure 2.1 Number of *Isochrysis* cells over time (More detail in Appendix . Table 5)

2.5.2 Oxidative stress

MDA values decreased over time in the different treatment groups. MDA value was initially highest in algae grown at 35 °C, but lowest at 96 hr ($P<0.05$). Oxidative stress was higher for all treatment groups at the beginning. However the advancement of time that value decreased over time.

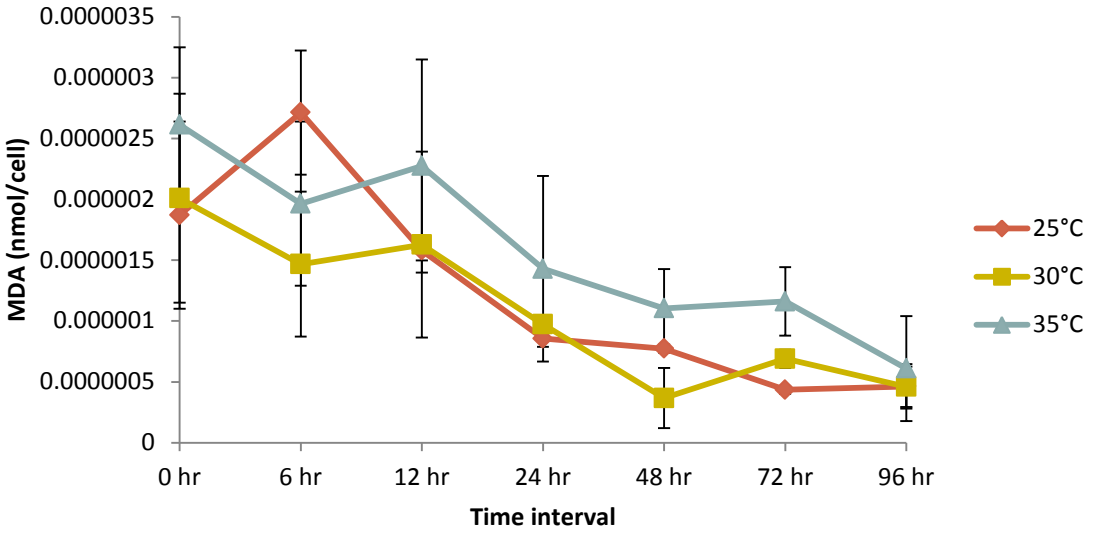

Figure 2.2 MDA values in *Isochrysis* at different temperatures over time (More detail in Appendix Table 6)

2.5.3 Results from APPI analysis

HPLC /APPI-MS method worked better for carotenoids rather than vitamins. Among the carotenoids the highest quantity was detected for fucoxanthin, and diadinoxanthin. Very small concentration of antheraxanthin, canthaxanthin, violaxanthin, retinal and retinol were also identified.

Among the carotenoids the concentration of fucoxanthin were the highest that was followed by diadinoxanthin. Highest concentration of fucoxanthin was found in samples from 35 °C at 72 hrs and lowest was found in samples from 30 °C at 96 hrs. Data indicates that the concentration of fucoxanthin decreased with time for each cell. No significant differences ($P>0.05$) among the samples were observed except for samples of

35 °C at 72 hrs, this sample significantly (P<0.05) differed from samples of all other treatment.

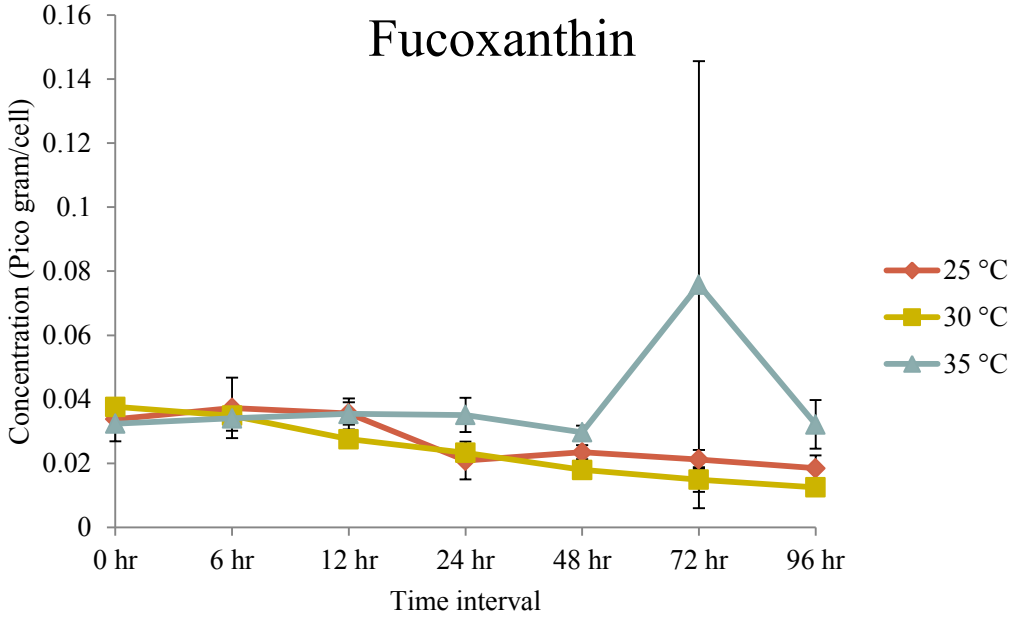

Figure 2.3 Concentration of fucoxanthin in *Isochrysis sp.* using APPI method (More detail in Appendix Table 7).

The second highest concentration of carotenoid was diadinoxanthin, detected by the APPI method. The lowest concentration for diadinoxanthin was in samples from 30 °C at 96 hrs and highest concentration was found in samples from 35 °C at 72 hrs, this group significantly (P<0.05) differed from other treatment groups. The changes over time diadinoxanthin were similar to those for fucoxanthin. The concentration of diadinoxanthin per cell decreased over time.

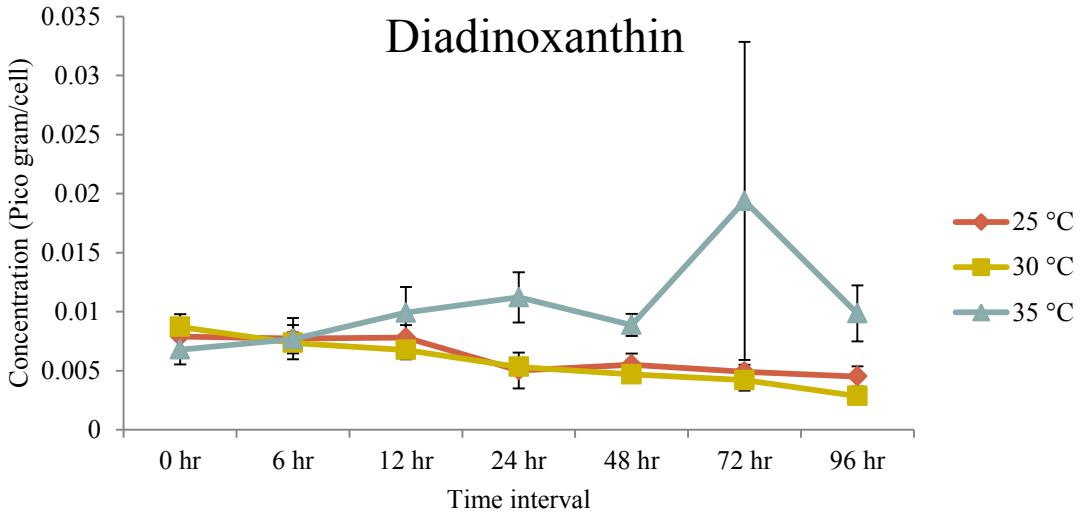

Figure 2.4 Concentration of diadinoxanthin in *Isochrysis sp.* using APPI method. (More detail in Appendix Table 7).

Diatoxanthin was found in very small quantity in all temperatures groups. The highest concentration was observed in samples from 35 °C at 96 hrs and that was followed by samples from 35 °C at 72 hrs. The lowest concentration was found in samples from 30 °C at 48 hrs. The highest concentration significantly ($P<0.05$) differed from other treatments.

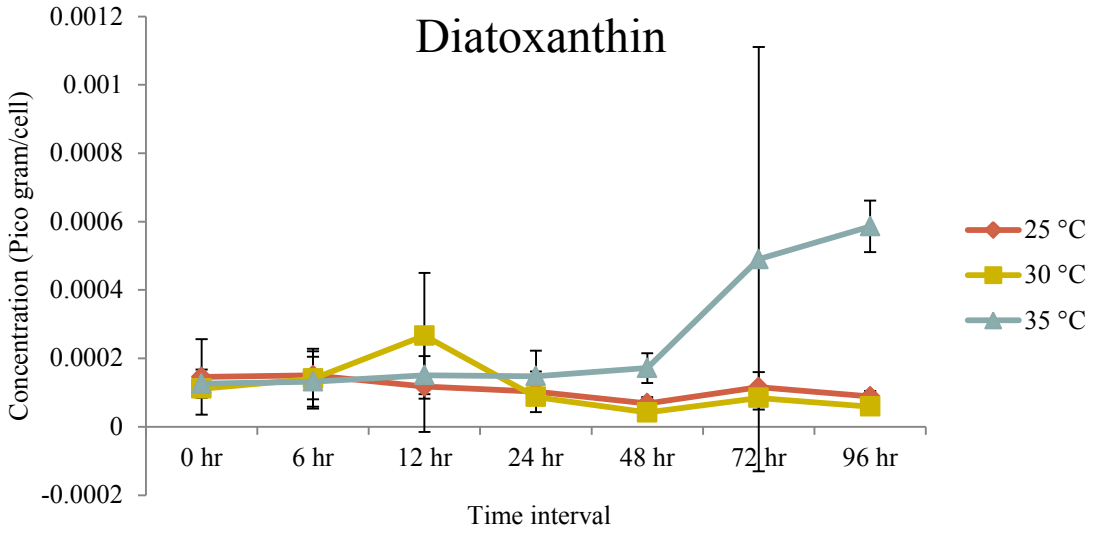

Figure 2.5 Concentration of diatoxanthin in *Isochrysis sp.* using APPI method. (More detail in Appendix Table 7).

Highest concentration of antheraxanthin was found in samples from 35 °C at 72 hrs that significantly ($P<0.05$) differed from other treatment groups. The lowest concentration was found in samples at 30 °C at 96 hrs The concentration of antheraxanthin decreased at 96 hrs in comparison with the beginning. However, there was no significant difference among these treatment groups. The concentration of antheraxanthin for algae grown at 35 °C was higher at the end of 96 hrs.

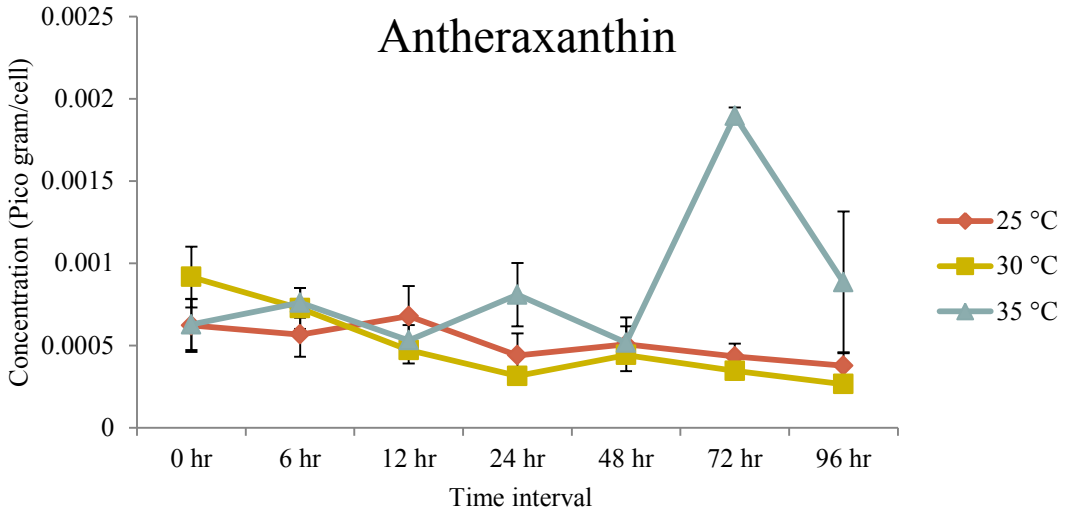

Figure 2.6 Concentration of antheraxanthin in *Isochrysis sp.* using APPI method. (More detail in Appendix Table 7).

Violaxanthin was weakly detected in algae grown at different temperatures. Algae samples from 25 °C at 6 hrs had the highest violaxanthin that differed significantly ($P<0.05$) from others. The concentration of violaxanthin decreased with time for all treatment groups.

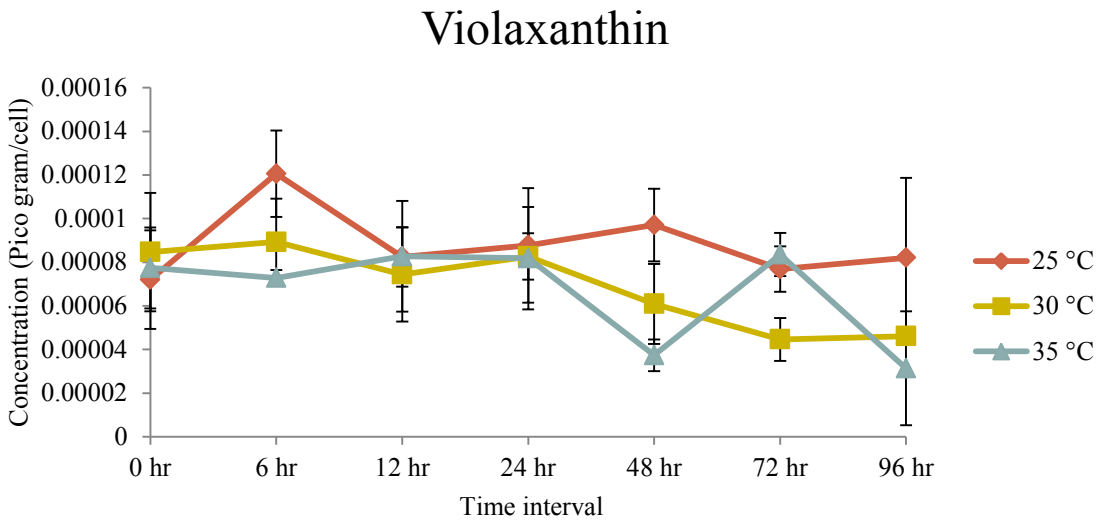

Figure 2.7 Concentration of violaxanthin in *Isochrysis sp.* using APPI method. (More detail in Appendix Table 7).

Very low concentrations of retinal were detected in all analyzed samples. The highest amount of retinal was found in samples from 35 °C at 72 hrs treatment that that varied significantly ($P<0.05$) with other treatments. On the contrary, the lowest concentration was observed in samples from 30 °C at 96 hrs. That was followed by samples from 25 °C at 96 hrs. They varied significantly ($P<0.05$) with initial sample for all treatments. It was observed that the concentration of retinal in algae grown at 25 °C, 30 °C and 35 °C temperature decreased with time.

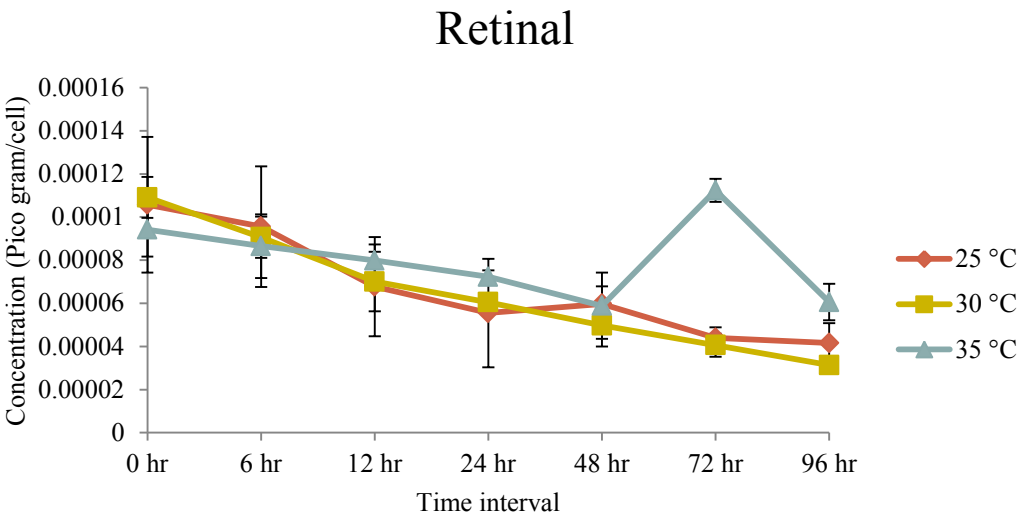

Figure 2.8 Concentration of retinal in *Isochrysis sp.* using APPI method. (More detail in Appendix Table 7).

Like retinal, very small amounts of retinol were identified for all temperatures groups. Highest amounts of retinol were in samples from 35 °C at 72 hrs that significantly ($P<0.05$) differed from other treatments. Lowest concentration was found in samples from 25 °C at 96 hrs. The concentration of the retinol decreased with time for all treatment groups.

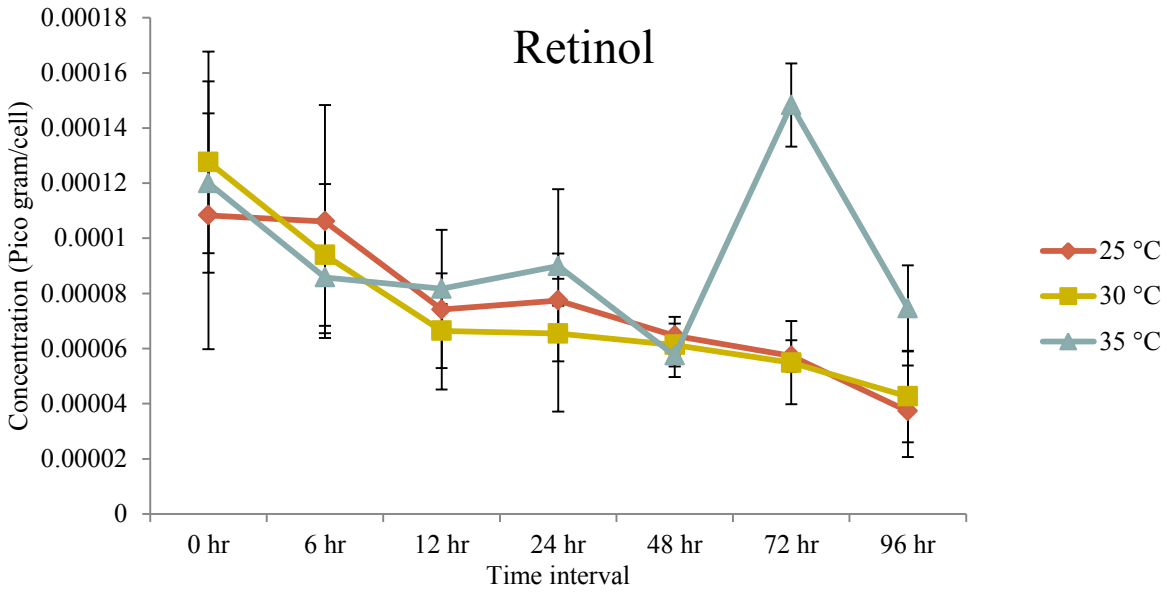

Figure 2.9 Concentration of retinol in *Isochrysis sp.* using APPI method (More detail in Appendix Table 7).

2.5.4 Results from ESI analysis

Only canthaxanthin was detected and quantified by ESI in *Isochryis* samples .The amount of canthaxanthin was higher for 25 °C temperature, that algae grown at 25 °C had the highest concentration of canthaxanthin and that was followed by algae grown at 35 °C and 30 °C. Samples from 35 °C at 72 hrs showed the highest amount of canthaxanthin. It significantly ($P<0.05$) differed from other treatment groups.

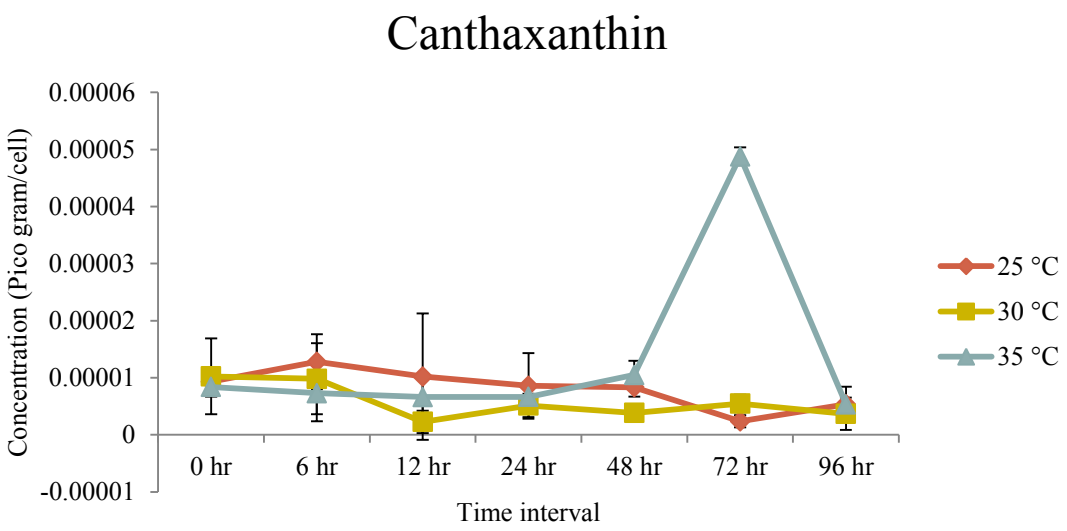

Figure 2.10 Concentration of canthaxanthin in *Isochrysis sp.* using ESI method. (More detail in Appendix Table 8).

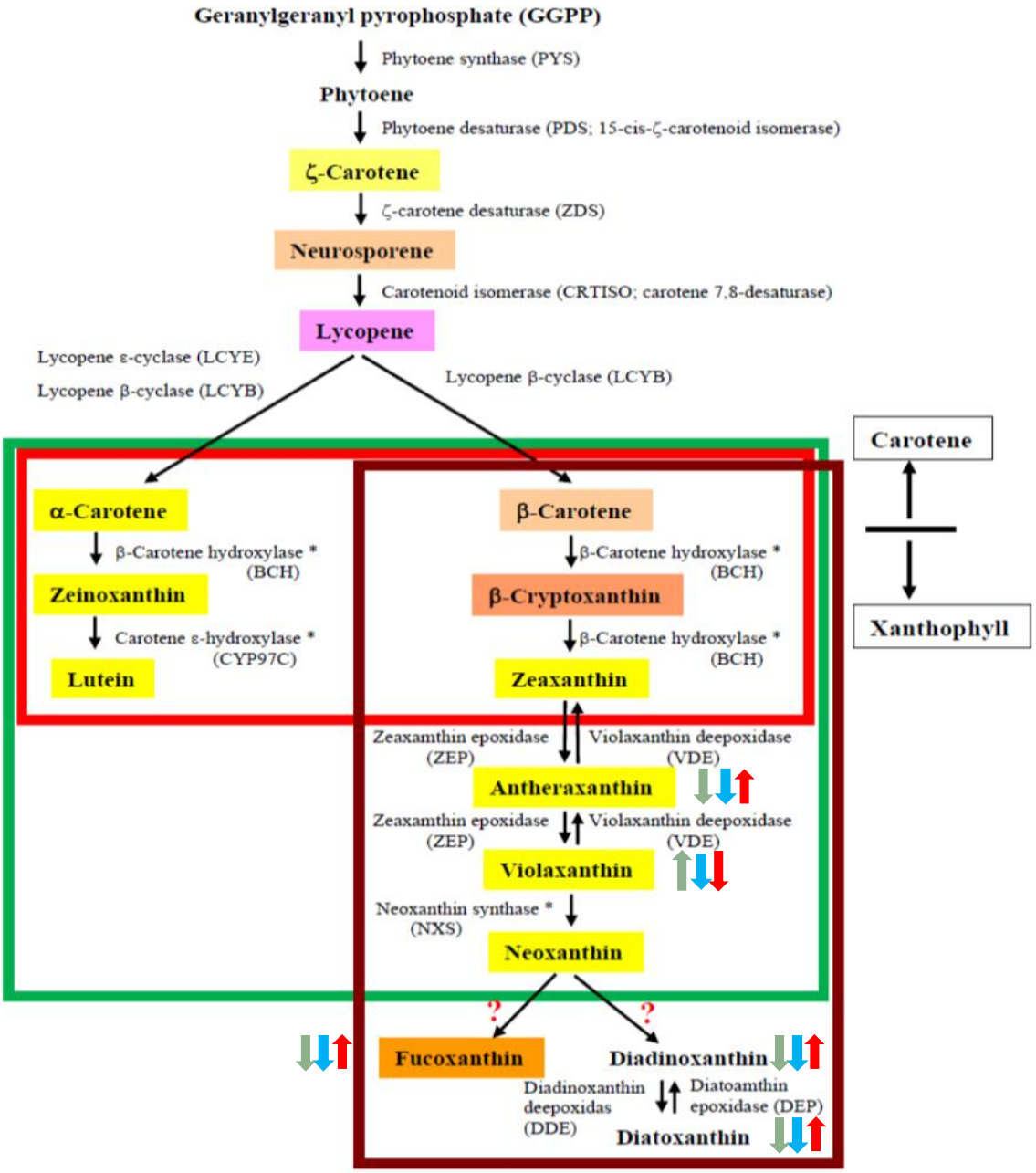

Figure 2.11 Carotenoid biosynthetic pathway shows the amount (arrow right side of the compound) of carotenoids of *Isochrysis sp.* after 96 hrs. (↓ = 25 °C, ↓ = 30 °C and ↓ = 35 °C)

2.6 Discussion

I. galbana (T ISO), a tropical algae, can tolerate wide ranges of temperature (Grima, 1992). Optimal temperature for *Isochrysis* growth 27 °C-30 °C, below 25 °C and above 35 °C is stressful. (Grima et al. 1992; Kaplan et al. 1985; Ewart and Pruder, 1981; Renaud et al, 2002; Renaud, 1995).

Findings of present study are similar to Reanud et al. (2002) and Renaud et al. (1995). They found *I. galbana* T-SIO grows better at 27 °C and that is followed by 30 °C and 25 °C, at 35 °C, the cell density declines. Similar results were also reported by Kapalan et al. (1986). They found *I. galbana* grows well at 27 °C and growth was retarded at 19 °C and 32 °C. Ewart and Pruder (1981) found this species grows better at 27 °C. They found positive relation with temperature and cell density for temperature 17.5 °C to 27.5 °C and no growth at 12.5 °C. Zhu et al., (1997) in their study on *I. galbana* TK species found that this species grows better at 30 °C and growth rate was about double for algae grown at 30 °C in comparison with algae grown at 15 °C. Abnormal metabolic activities because of higher temperature may reduce the number of cells at higher temperature as this temperature is not suitable for their growth and death of algal cells as well (Richmond, 1986).

Isochysis sp., is rich in polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA) and other fatty acids like 14:0, 16:0, 16:1, 18:4ω3, 20:5ω3 and 22:6ω3 (Bandarra et al. 2002). Higher concentration of fatty acids and higher temperature may be the cause of high MDA at the beginning. Continuous peroxidation can reduce the MDA for first 24 hrs. The MDA was higher for 30 °C after 48 hrs. Because of adaption of the cells with temperature, the MDA gradually declined (Barros et al. 2003).

Carotenoids frequently change their structure as they are highly unsaturated in nature and display isomerism (Fratianni et al. 2010). Guava fruits exposed to petroleum polycylclic hydrocarbons, an inducer of oxidative stress, exhibited reduced concentrations of vitamin C and carotene (Nwaogu & Ujowundu, 2010) suggesting quenching by these antioxidants, and use beyond the capability to restore concentrations in the fruit to pre-exposure concentrations. In aquatic ecosystems, the more thoroughly studied oxidative stressor has been photo-oxidative stress caused by ultraviolet (UV) radiation. Oxidative stress induced in the green alga *Dunaliella parva* by UV light led to reductions in 5 of 9 measured carotenoids (neoxanthin, violaxanthin, antheraxanthin, λ and ε carotene, and β carotene), no change in 2 carotenoids (lutein-5,6-epoxide and lutein) and increases in 2 carotenoids (zeaxanthin and β-carotene-5,6-epoxide) (Young & Britton, 1990). There is significant interconversion of carotenoids via the violaxanthin cycle and additional carotenoid interconversion pathways may exist involving lycopene, β carotene and cryptoxanthin (Ursi et al. 2003). It is observed from the current study that the amount of violaxanthin was lower than antheraxanthin and no zeaxanthin was detected. That may be because of thermal stress to *Isochrysis*. That is similar to the findings of above mentioned researchers.

Isochrysis galbana had higher amount of fucoxanthin, followed by diadinoxanthin and diatoxanthin (Zapata et al. 2004; Mulders et al. 2013; Dales 1960; Zapata and Garrido 1997). That supports current study. Mikami and Hosokawa (2013) in their proposed xanthophyll cycle for algae (Figure 1.1) showed that the end product of xanthophyll cycle is fucoxanthin and diadinoxanthin, where diadinoxanthin can be

converted to doatoxanthin and vice versa. The concentrations of these compounds were higher in algae grown at 30 °C and lower at 25 °C and followed by 35 °C. This may be due to thermal stress. Stability of fucoxanthin decreases with higher temperature. 80 % of fucoxanthin can be lost at 90 °C within 8 hrs. High temperature and irradiation speed up this process (Sugimura et al. 2012). Their conclusions concur with the current study. Algae grown at 35 °C synthesized lower amount of fucoxanthin whereas, 30 °C had the highest amount of fucoxanthin.

Diadinoxanthin is one of major compound of brown algae. Under high temperature and irradiance stress, the concentration of diadinoxanthin and diatoxanthin increases (Venn 2006, Sakshaug & Slagstad, 1991). That agrees with the current study. The concentration of diadinoxanthin and diatoxanthin increased with increasing temperature and algae grown at 35 °C showed the highest concentration of these compounds while algae grown at 25 °C showed the lowest concentration, in response to stress the concentration of diadinoxanthin increased. Environmental stress like high light trigger the production of diatoxanthin (Brunet et al. 1993). At the beginning of each treatment, the concentration was low, but over time the concentration increased, as the algae adapted with the changing environment so the production as higher with time progressed (Demers et al. 1991).

It is generally believed that vitamin A is heat resistant (Andersson & Öste, 1995), but in the current study the concentration decreased with increasing temperature. Very small amount of vitamin A compounds retinal, retinol and retinoic acid were detected in *Isochrysis* species. β carotene is the source of these compounds, which was absent in this species. The concentration of β cyclocitral a derivative of β carotene was also in very

small amount. As a result, the concentration of provitamin A was very low in algae grown at 35 °C and higher concentration was in algae grown at 30 °C. This may be due to senescence of the algae grown at 35 °C treatment group as the cell density increase. Another reason may be its higher oxidative stress, vitamin A compounds were used to minimize the stress.

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CHAPTER 3. SUMMARY AND CONCLUSIONS

Current study showed that neither APPI nor ESI method can detect all the non-enzymatic compounds in *Isochrysis* samples. Vitamin D and its metabolites are not detected by any of these methods. ESI is to detect β cyclocitral, β cryptoxanthin, retinoic acid and to quantify diatoxanthin is recommended. Concentration of violaxanthin was also low, but APPI is recommended because concentration maintained the ratio with increasing weight. APPI is recommended for quantification of fucoxanthin, diadinoxanthin, antheraxanthin, retinol, retinal and canthaxanthin because these compounds maintain weight -concentration ratio. Higher concentration of astaxanthin was detected by APPI but not suggested as it did not maintain weight -concentration ratio.

Isochrysis sp. showed better growth at 30 °C and lowest at 35 °C. The oxidative stress was higher for algae grown at 35 °C at the beginning and at the end it was lowest compared to other treatments. Concentration of fucoxanthin, diadinoxanthin, diatoxanthin, antheraxanthin, retinal and retinal were higher for algae grown at 35 °C. Algae grown at 35 °C had lower concentration of violaxanthin. Overall APPI gave better results for detection of antioxidants from *Isochrysis sp.* .

CHAPTER 4. SUGGESTED ADDITIONAL WORK

As it is observed from the current study that neither APPI nor ESI worked for detection of all carotenoids and vitamin compounds, further research is needed. Extraction method should be considered first. There are various combinations of organic solvent for either carotenoids or vitamin compounds but not for both, a suitable extraction solvent is needed to extract all these compounds from small quantities of samples.

Current results showed that higher temperatures reduce the nuclear signaling compound carotenoids and retinoid. Effect on primary consumer under oxidative stress should be studied. Metabolomic profiles, deformities of larvae, growth, reproduction and vulnerability to diseases should be included in research.

Oxidative stress on plankton and higher aquatic organisms because of oil spill and heavy metal pollution should be studied. Probability of nutrient flows from lower to higher organisms through food chain should be considered.

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APPENDIX

APPENDIX

Table 1. MRM table for analytes collected in APPI mode

Compound Name	**Precursor Ion**	**Product Ion**	**Dwell**	**Frag mentor**	**Collision Energy**	**Polarity**
Fucoxanthin	659.2	641.1	20	80	10	Positive
Fucoxanthin	659.2	109.1	20	80	20	Positive
Astraxanthin	597.3	173	20	80	30	Positive
Astraxanthin	597.3	147.2	20	80	30	Positive
Zeaxanthin	569.4	175.2	20	80	10	Positive
Zeaxanthin	569.4	135.1	20	80	10	Positive
Canthaxanthin	565.3	203.2	20	80	15	Positive
Canthaxanthin	565.3	133.1	20	80	40	Positive
B-carotene	537.4	445.3	20	80	10	Positive
B-carotene	537.4	177.2	20	80	10	Positive
lycopene	537.4	157.2	20	80	25	Positive
lycopene	537.4	69.2	20	80	40	Positive
1,25 $(OH)_2$-D_3	417.3	399.2	20	80	5	Positive
24,25 $(OH)_2$-D_3	417.3	381.2	20	80	5	Positive
24,25 $(OH)_2$-D_3	417.3	121.2	20	80	20	Positive
1,25 $(OH)_2$-D_3	417.3	95.1	20	80	30	Positive
25(OH)-D_3	401.3	383.3	20	80	5	Positive
25(OH)-D_3	401.3	365.3	20	80	5	Positive
D_2	397.3	379.3	20	80	5	Positive
D_2	397.3	69.2	20	80	15	Positive
D_3	385.3	367.3	20	80	5	Positive
D_3	385.3	259.2	20	80	15	Positive
7dehydrocholesterol	385.3	259.3	20	80	5	Positive

Table 1 continued

7dehydrocholesterol	385.3	367.3	20	80	5	Positive
Retinoic Acid	299.1	255.1	20	80	10	Negative
Retinoic Acid	299.1	119	20	80	10	Negative
Retinal	285.2	175.2	20	80	5	Positive
Retinal	285.2	161.2	20	80	5	Positive
Retinol	285.2	121.2	20	80	10	Positive
Retinol	285.2	93.2	20	80	15	Positive
Naringenin	273.1	174.1	20	80	15	Positive
Naringenin	273.1	153.1	20	80	15	Positive
B-cyclocitral	153.2	109.2	20	80	10	Positive
B-cyclocitral	153.2	95.2	20	80	10	Positive

Table 2. MRM table for analytes collected in ESI mode

Compound Name	Precursor Ion	Product Ion	Dwell	Frag mentor	Collision Energy	Polarity
Fucoxanthin	659.2	641.1	20	80	10	Positive
Fucoxanthin	659.2	109.1	20	80	20	Positive
Violaxanthin	601.1	221.1	30	80	15	Positive
Violaxanthin	601.1	93.1	30	80	30	Positive
Astraxanthin	597.3	173	20	80	30	Positive
Astraxanthin	597.3	147.2	20	80	30	Positive
Antheraxanthin	585.1	105.1	30	80	40	Positive
Antheraxanthin	585.1	93.1	30	80	25	Positive
Diadinoxanthin	583.1	565.2	30	80	10	Positive
Diadinoxanthin	583.1	121	30	80	25	Positive
Diadinoxanthin	583.1	119	30	80	25	Positive
Diadinoxanthin	583.1	109	30	80	20	Positive
Zeaxanthin	569.4	175.2	20	80	10	Positive
Zeaxanthin	569.4	135.1	20	80	10	Positive
Diatoxanthin	567.2	430.8	30	80	5	Positive
Diatoxanthin	567.2	227	30	80	25	Positive
Diatoxanthin	567.2	159	30	80	25	Positive
Canthaxanthin	565.3	203.2	20	80	15	Positive
Canthaxanthin	565.3	133.1	20	80	40	Positive
B-Crytoxanthin	552.3	460.3	30	80	10	Positive
B-Crytoxanthin	552.3	119.1	30	80	20	Positive
B-carotene	537.4	445.3	30	80	10	Positive
B-carotene	537.4	177.2	20	80	10	Positive
lycopene	537.4	157.2	30	80	25	Positive
lycopene	537.4	69.2	30	80	40	Positive
GGPP	449	431	30	80	15	Negative
GGPP	449	158.8	30	80	25	Negative
GGPP	449	79	30	80	40	Negative
1,25 $(OH)_2$-D_3	417.3	399.2	20	80	5	Positive
24,25 $(OH)_2$-D_3	417.3	381.2	20	80	5	Positive
24,25 $(OH)_2$-D_3	417.3	121.2	20	80	20	Positive
1,25 $(OH)_2$-D_3	417.3	95.1	20	80	30	Positive
25(OH)-D_3	401.3	383.3	20	80	5	Positive
25(OH)-D_3	401.3	365.3	20	80	5	Positive

Table 2 continued

D_2	397.3	379.3	20	80	5	Positive
D_2	397.3	69.2	20	80	15	Positive
D_3	385.3	367.3	20	80	5	Positive
7dehydrocholesterol	385.3	367.3	20	80	5	Positive
7dehydrocholesterol	385.3	259.3	20	80	5	Positive
D_3	385.3	259.2	20	80	15	Positive
Retinoic Acid	299.1	255.1	20	80	10	Negative
Retinoic Acid	299.1	119	20	80	10	Negative
Retinal	285.2	175.2	20	80	5	Positive
Retinal	285.2	161.2	20	80	5	Positive
Retinol	285.2	121.2	20	80	10	Positive
Retinol	285.2	93.2	20	80	15	Positive
Naringenin	273.1	174.1	20	80	15	Positive
Naringenin	273.1	153.1	20	80	15	Positive
B-cyclocitral	153.2	109.2	20	80	10	Positive
B-cyclocitral	153.2	95.2	20	80	10	Positive

Table 3: Amount (ng) of different compounds detected by APPI method in *Isochrysis sp.* at different weight

APPI	B-cyclocitral	Fucoxanthin	Astaxanthin	Canthaxanthin	Antheraxanthin	Diadinoxanthin	Diatoxanthin	Retinal	Retinol	Violaxanthin	Zeaxanthin
10mg	1.532575[a]	11641.03[a]	7295.347[a]	1.967446[a]	354.5284[a]	3003.389[a]	3892.263[a]	19.51987[a]	18.68159[a]	26.02537[a]	1020.174[a]
SD	0.441309	1022.316	46.94276	0.473924	37.72909	191.2255	886.9696	2.22787	1.940027	3.553913	62.08494
20mg	3.283085[a]	21893.4[b]	7270.154[a]	2.55992 [a]	642.1899[b]	5812.745[a]	6603.7[b]	48.94081[b]	53.75331[b]	64.90548[b]	2241.975 [b]
SD	0.529106	6255.379	133.01	1.07177	84.01326	1142.576	1601.916	7.65393	9.184661	8.155521	579.6473
40 mg	5.615792[a]	36011.67 [c]	7588.495[a]	1.784926 [a]	1350.349[c]	11699.09[b]	11552.2[c]	107.3801[c]	103.6413[c]	127.2427[c]	4387.703 [c]
SD	0.764445	2732.398	326.6451	0.903508	103.3774	2199.194	1506.766	6.205063	11.60429	16.33105	774.6461

Different superscript letter indicates significant ($P<0.05$) difference.

Table 4: Amount (ng) of different compounds detected by ESI method in *Isochrysis sp.* at different weight.

ESI	β-Cyclocitral (	Canthaxanthin	β -Cryptoxan	Diatoxanthin	Retinoic Acid	Violaxanthin	Zeaxanthin
10 mg	102.728[b]	13.09573 [a]	854.9832[a]	5727.846[a]	203.7603[b]	14.77043[a]	17149.53[d]
SD	68.73257	0.103622	218.8359	264.2293	4.877935	5.219121	268.0227
20 mg	74.57709 [b]	30.70493 [b]	851.4662[a]	9865.725[b]	185.5521[b]	40.08975[a]	17399.54 [d]
SD	23.11172	1.7618	169.0703	1266.247	34.80129	18.102	342.7981
40 mg	76.91656 [b]	63.6887 [c]	2283.319[b]	19098.15[d]	132.9486[a]	75.15009[b]	17683.6 [d]
SD	39.35253	1.336201	856.7831	2006.732	21.7546	20.23794	259.7216

Different superscript letter indicates significant (P<0.05) difference.

Table 5: Number of cells at different temperatures with time interval

	0 hr	6 hr	12 hr	24 hr	48 hr	72 hr	96 hr
25 °C	890000[a]	787083.3[a]	1004500[ab]	1213125[cd]	1369167[d]	1818750[e]	2280000[g]
SD	55480.85	159572.5	176329.8	156728.2	168757.7	392961.1	299034.9
30 °C	856000[a]	894583.3[a]	1121500[bc]	1270625[cd]	1847500[f]	2563750[h]	3342500[k]
SD	12196.31	107913.4	121058.7	249350.3	206814.7	396181.9	421455.8
35 °C	897500[a]	867083.3[a]	838500[a]	921250[ab]	885714.3[a]	911111.1[ab]	931250[ab]
SD	82632.47	116105.5	186254.9	98098.7	142002.9	160983.8	278107.4

Different superscript letter indicates significant ($P<0.05$) difference

Table 6: MDA values(nmol/cell) in *Isochrysis* at different temperatures over time

	0 hr	6 hr	12 hr	24 hr	48 hr	72 hr	96 hr
25°C	1.87024E-06^{cf}	2.71E-06f	1.58E-06 $^{b-f}$	8.56E-07^{abc}	7.73E-07 ^{abc}	4.35E-07 ^{ab}	4.63E-07 ^{ab}
SD	7.68873E-07	5.1E-07	8.17E-08	6.77E-08	3.33E-08	3.26E-08	1.82E-07
30°C	2.00859E-06 $^{a-d}$	1.47E-06 $^{c-f}$	1.63E-06 $^{c-f}$	9.72E-07 ^{abc}	3.67E-07 ^{ab}	6.9E-07 ^{ab}	4.59E-07^{ab}
SD	8.58036E-07	5.97E-07	7.65E-07	2.51E-08	2.47E-07	7.47E-08	1.67E-07
35°C	2.61299E-06 $^{e-f}$	1.96E-06 $^{c-f}$	2.27E-06 $^{d-f}$	1.43E-06 ^{ab}	1.1E-06 $^{a-d}$	1.16E-06 $^{a-d}$	6.1E-07^{a}
SD	6.35373E-07	6.73E-07	8.76E-07	7.63E-07	3.24E-07	2.8E-07	4.31E-07

Different superscript letter indicates significant (P<0.05) difference

Table 7: Amount (picogram/cell) of different compounds detected by APPI method in *Isochrysis sp.* at different temperature with varying time.

Name	Fuco	Diadino	Diato	Anthe	Viol	Retinal	Retinol
25-0	0.033814947 [a]	0.007898010 [abc]	0.000145561 [a]	0.000621742 [ab]	0.000071969 [a-e]	0.000105747 [d-e]	0.000108335 [a-d]
SD	0.003825126	0.001143766	0.000110334	0.000161396	0.000022587	0.000031486	0.000048568
25-6	0.037307515 [a]	0.007718052 [abc]	0.000150366 [a]	0.000566347 [ab]	0.000120559 [e]	0.000095597 [c-e]	0.000106097 [a-d]
SD	0.009434627	0.001758771	0.000070182	0.000135154	0.000019765	0.000027984	0.000042280
25-12	0.035578025 [a]	0.007805226 [abc]	0.000117727 [a]	0.000678525 [ab]	0.000082429 [b-e]	0.000067727 [a-e]	0.000074115 [a-d]
SD	0.003469525	0.001056071	0.000132424	0.000183118	0.000013701	0.000022944	0.000028916
25-24	0.020885495 [a]	0.005023895 [abc]	0.000102323 [a]	0.000439553 [ab]	0.000087709 [c-e]	0.000055496 [a-e]	0.000077477 [a-d]
SD	0.005891547	0.001524292	0.000059170	0.000133349	0.000026300	0.000025109	0.000040370
25-48	0.023481920 [a]	0.005515164 [abc]	0.000068264 [a]	0.000507482 [ab]	0.000097038 [d-e]	0.000059772 [a-e]	0.000064660 [a-c]
SD	0.002215754	0.000931842	0.000018536	0.000163561	0.000016635	0.000008136	0.000006850
25-72	0.021221267 [a]	0.004914358 [abc]	0.000115782 [a]	0.000433496 [ab]	0.000076837 [a-e]	0.000043912 [abc]	0.000057321 [a-c]
SD	0.002946293	0.000593526	0.000043921	0.000077923	0.000010410	0.000004950	0.000005722
25-96	0.018468607 [a]	0.004517122 [ab]	0.000088526 [a]	0.000376457 [ab]	0.000082053 [b-e]	0.000041540 [ab]	0.000037309 [a]
SD	0.003980415	0.000843479	0.000016505	0.000083225	0.000036546	0.000009339	0.000016590
30-0	0.037647677 [a]	0.008705053 [abc]	0.000111454 [a]	0.000916369 [ab]	0.000084670 [a-e]	0.000109087 [d-e]	0.000127633 [c-d]
SD	0.001937838	0.001087653	0.000016858	0.000184258	0.000027141	0.000009527	0.000040134
30-6	0.034883824 [a]	0.007362667 [abc]	0.000140265 [a]	0.000725467 [ab]	0.000089261 [c-e]	0.000090634 [b-e]	0.000093920 [a-d]
SD	0.000501362	0.000891927	0.000087469	0.000124767	0.000019827	0.000009518	0.000025724
30-12	0.027541388 [a]	0.006757477 [abc]	0.000266445 [ab]	0.000471756 [ab]	0.000074390 [a-e]	0.000070082 [a-e]	0.000066412 [a-c]
SD	0.001789923	0.000793214	0.000184001	0.000080511	0.000021543	0.000013855	0.000013446
30-24	0.023327887 [a]	0.005318198 [abc]	0.000087217 [a]	0.000314480 [ab]	0.000082596 [b-e]	0.000060477 [a-e]	0.000065452 [a-c]
SD	0.001947692	0.000454034	0.000016137	0.000028482	0.000010648	0.000003208	0.000010133
30-48	0.017978022 [a]	0.004692907 [ab]	0.000041909 [a]	0.000442073 [ab]	0.000060877 [a-d]	0.000049766 [abc]	0.000061290 [a-c]
SD	0.001348245	0.000453692	0.000005440	0.000045827	0.000018352	0.000009719	0.000007824

Table 7 continued

30-72	0.014920169 [a]	0.004204915 [ab]	0.000084274 [a]	0.000345312 [ab]	0.000044588 [abc]	0.000040616 [ab]	0.000054894 [a-c]
SD	0.003864242	0.000891924	0.000033586	0.000051091	0.000009879	0.000005350	0.000015073
30-96	0.012523667 [a]	0.002859598 [a]	0.000059235 [a]	0.000264997 [a]	0.000046092 [abc]	0.000031348 [a]	0.000042645 [ab]
SD	0.000753190	0.000248290	0.000008824	0.000025236	0.000003594	0.000000659	0.000016587
35-0	0.032400123 [a]	0.006789694 [abc]	0.000126081 [a]	0.000627378 [ab]	0.000077385 [b-e]	0.000094142 [b-e]	0.000119993 [b-d]
SD	0.005564162	0.001249985	0.000041016	0.000154483	0.000018597	0.000012422	0.000025357
35-6	0.034047526 [a]	0.007670539 [abc]	0.000132101 [a]	0.000760284 [ab]	0.000072754 [a-e]	0.000086503 [a-e]	0.000085795 [a-d]
SD	0.003847305	0.001193556	0.000073056	0.000090357	0.000003588	0.000014792	0.000020254
35-12	0.035451250 [a]	0.009917028 [abc]	0.000150865 [a]	0.000531796 [ab]	0.000082675 [b-e]	0.000079828 [a-e]	0.000081696 [a-d]
SD	0.004852270	0.002184006	0.000055777	0.000091752	0.000025353	0.000007464	0.000005605
35-24	0.035112437 [a]	0.011219402 [abc]	0.000147995 [a]	0.000808581 [ab]	0.000081836	0.000072321 [a-d]	0.000089941 [a-d]
SD	0.005351723	0.002124133	0.000074661	0.000192879	0.000023517	0.000002950	0.000004584
35-48	0.029725682 [a]	0.008880229 [abc]	0.000171964 [a]	0.000517035 [ab]	0.000037329 [ab]	0.000058861 [a-e]	0.000057469 [a-c]
SD	0.002089793	0.000924304	0.000043544	0.000099885	0.000007211	0.000015372	0.000007744
35-72	0.075785961 [b]	0.019375724 [d]	0.000490183 [bc]	0.001896230 [c]	0.000083513 [b-e]	0.000112353 [e]	0.000148322 [d]
SD	0.069776748	0.013475613	0.000620725	0.001266270	0.000070292	0.000105287	0.000150515
35-96	0.032147861 [a]	0.009853178 [abc]	0.000586124 [c]	0.000883273 [ab]	0.000031364 [a]	0.000060555 [a-e]	0.000074544 [a-d]
SD	0.007609587	0.002363870	0.000075253	0.000430965	0.000026077	0.000008457	0.000015632

Different superscript letter indicates significant ($P<0.05$) difference

Table 8: Amount (picogram/cell) of different compounds detected by ESI method in *Isochrysis sp.* at different temperature with varying time.

Name	Canthaxanthin (ng)
25-0	0.000009313 [a]
SD	0.000001958
25-6	0.000012779 [a]
SD	0.000004827
25-12	0.000010190 [a]
SD	0.000011080
25-24	0.000008578 [a]
SD	0.000005741
25-48	0.000008329 [a]
SD	0.000001632
25-72	0.000002352 [a]
SD	0.000001077
25-96	0.000005338 [a]
SD	0.000000385
30-0	0.000010241 [a]
SD	0.000006641
30-6	0.000009822 [a]
SD	0.000006212
30-12	0.000002264 [a]
SD	0.000001952
30-24	0.000005120 [a]
SD	0.000002044
30-48	0.000003828 [a]
SD	0.000000893
30-72	0.000005475 [a]
SD	0.000001560
30-96	0.000003681 [a]

Table 8 continued

SD	0.000002807
35-0	0.000008369 [a]
SD	0.000001682
35-6	0.000007290 [a]
SD	0.000004916
35-12	0.000006603 [a]
SD	0.000003657
35-24	0.000006632 [a]
SD	0.000002223
35-48	0.000010480 [a]
SD	0.000002499
35-72	0.000048786 [b]
SD	0.000060055
35-96	0.000005335 [a]
SD	0.000003074

Different superscript letter indicates significant ($P<0.05$) difference