THE IMPACT OF LIGHT IRRADIANCE, CHLOROPHYLL CONCENTRATION AND SOLVENT ON CHLOROPHYLL ABSORBANCE SPECTRUM

Vidya Sagar Kuncham

Department of Bioresource Engineering

McGill University

Montreal, Quebec, Canada

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

April 2021

ABSTRACT

The plant pigment chlorophyll (Chl) is considered as obligatory pigment in photosynthesis as it is used in the light harvesting process. Chl absorbs light energy predominately in the blue and red region of the visible spectrum with defined peaks at 433 nm and 665 nm for Chl *a* and at 458 nm and 646 nm for Chl *b*. Our first study was used to determine the impact that light irradiance had on Chl. Chl *a* and Chl *b* absorbance was measured under dark and after treating with different light intensities. It was identified that both Chl *a* and Chl *b* were sensitive to the light and the absorbance capacity (absorbance spectrum) of the chlorophyll changed based on the light intensity by which the chlorophyll pigments were treated. Chl *a* and Chl *b* absorbance level was inversely proportional to the light intensity supplied to the pigments. The Chl *a* absorbed significantly more light in the green region (between 500 nm and 530 nm) after treatment with higher light irradiance. Chl *b* absorbed more light in green region with distinct peaks at 518 nm and 570 nm after light treatments. The ability of Chl to return to a baseline absorbance based on a no light (dark) treatment was tested by storing Chl *a* and Chl *b* under dark conditions, however no significant recovery in absorbance was measured.

The second study investigated the effect of Chl concentration on the resultant absorbance spectrum. The absorbance of Chl was measured with four different Chl concentrations (3, 30, 300 and 3000 mg Chl in 10 ml of canola oil). The different absorbance spectra obtained indicate that the absorbance spectrum depends on the Chl concentration, with an increase in Chl concentration resulted an increase in Chl absorbance. The clear absorbance spectrum with distinct peaks in the blue (410 nm) and red (666 nm) regions of visible spectrum was identified only with 30 mg in 10 ml concentration while higher concentrations had an unclear spectrum and new peaks were identified at 350 nm and 382 nm.

The third study investigated the effect of solvent on the Chl absorbance spectrum. Three solvents were studied (canola oil, acetone and mineral oil). Different absorbance curves were observed between the solvents, with Chl having a higher absorbance with canola oil followed by acetone and mineral oil. The reason for the differences in Chl *a* and *b* absorbance spectrum under different light treatments was hypothesized to be due to photodegradation and the byproducts released. The Chl *a* and *b* absorbance differences in different Chl concentrations and in different solvents was due to aggregation of Chl molecules and Chl absorbance in monomer, dimer and polymer states. These studies indicate that the Chl cannot have a standard absorbance spectrum with absorbance peaks at specified wavelength, but it depends on factors like light irradiance, Chl concentration and solvent. This dynamic absorbance spectrum can only be used to determine Chl concentration but not to determine photosynthetic activity. It also indicates that Chl is highly sensitive to light which makes it hard to believe its primary function as light harvesting.

Key words: Chlorophyll, absorbance, light intensity, concentration, canola oil, acetone and mineral oil.

RÉSUMÉ

La chlorophylle (Chl) est un pigment essentiel à la photosynthèse chez les végétaux, impliquée dans les processus d'absorption de l'énergie lumineuse. Elle absorbe cette énergie dans la gamme du bleu et du rouge du spectre de la lumière visible, avec des pics d'absorption de 433 nm et 665 nm pour la Chl *a*, et de 458 nm et 646 nm pour la Chl *b*. L'objectif de la première étude a été de déterminer l'impact de l'intensité lumineuse sur la Chl. L'absorbance des pigments de Chl *a* et *b* fut mesurée pour des pigments adaptés à la noirceur, de même qu'exposé à différentes intensités lumineuses. Les résultats ont démontré que les Chl *a* et *b* sont toutes les deux sensibles à la lumière et que leur capacité d'absorbance (spectre d'absorption) peut être modifiée selon l'intensité lumineuse à laquelle ils sont exposés. Le niveau d'absorption a été inversement proportionnel à l'intensité lumineuse à laquelle les Chl *a* et *b* furent exposées. La Chl *a* a absorbé significativement plus de lumière verte (entre 500 nm et 530 nm) après le traitement à l'intensité lumineuse la plus élevée. La Chl *b* a aussi absorbé plus de lumière verte après l'exposition aux traitements lumineux, avec des pics distincts à 518 nm et 570 nm. Les pigments de Chl *a* et *b* furent placées à la noirceur afin de tester leur capacité de récupération, soit de revenir à leur état initial d'absorbance, mais les résultats n'ont pas démontré une capacité de récupération significative.

La deuxième étude s'est penchée sur l'effet de la concentration en Chl sur le spectre d'absorption. L'absorbance fut mesurée pour quatre concentrations de Chl différentes (3, 30, 300, 300 mg Chl dans 10 ml d'huile de canola). Les différents spectres d'absorption obtenus ont démontré que l'absorbance dépend de la concentration en Chl, et que plus celle-ci augmente, plus l'absorbance augmente. Un spectre d'absorption avec des pics distincts dans les régions de la lumière visible bleue (410 nm) et rouge (666 nm) fut clairement identifié, mais seulement pour la concentration en Chl de 30 mg dans 10 ml d'huile de canola. Aux concentrations plus élevées, les

spectres d'absorption obtenus furent plus confus, et de nouveau pics d'absorption furent identifiés à 350 nm et 382 nm.

La troisième étude visait à évaluer l'effet du solvant sur le spectre d'absorption de la Chl. Trois solvants ont été testés (huile de canola, acétone et huile minérale). Les courbes d'absorption obtenues furent différentes pour les trois solvants testés, la Chl ayant une absorbance plus élevée dans l'huile de canola, suivi par l'acétone, puis l'huile minérale. La photodégradation et la libération de sous-produits sont des hypothèses visant à expliquer les différences dans les spectres d'absorption des pigments de Chl *a* et *b* lorsque exposés à différentes intensités lumineuses. Les différences dans l'absorbance des Chl *a* et *b* liées à la concentration en Chl et au solvant employé, pourraient être dues, quant à elles, à l'agrégation des molécules de Chl et à son absorbance lorsqu'à l'état de monomère, dimère et polymère. Les résultats de ces études démontrent que la Chl n'a pas de spectre d'absorption standard, avec des pics d'absorbance à certaines longueurs d'ondes spécifiques, mais que celui-ci dépend de facteurs comme l'intensité lumineuse, la concentration en Chl et le type de solvant. Si ce spectre d'absorbance dynamique peut être employé pour déterminer la concentration en Chl, il ne peut pas l'être pour déterminer l'activité photosynthétique. Cela indique aussi que la Chl est hautement sensible à la lumière, ce qui met en doute sa fonction principale liée à la capture de l'énergie lumineuse.

Mots-clés : Chlorophylle, absorbance, intensité lumineuse, concentration, huile de canola, acétone et huile minérale

CONTRIBUTION OF AUTHORS

This thesis is written in traditional thesis format according to "Guidelines Concerning Thesis Preparation" of McGill University. It was authored by Vidya Sagar Kuncham and he was responsible for gathering the information, experimental design, collecting data and writing thesis. Dr Mark Lefsrud helped in designing the experiments, interpretation of experimental data and provided critical review of the thesis. Dr Alice Cherestes provided technical assistance, feedback on experimental results and also helped in critical review of the thesis. Bo-Sen Wu helped in data collection and reviewed the scientific writings.

ACKNOWLEDGEMENT

I would like to thank my supervisor Dr. Mark Lefsrud for making my Canada dreams come true by accepting me into his team. He was always been there for me to provide guidance, support and for showing me the right path in difficult times. I am very grateful to him for sharing his research knowledge and experiences that helped me to complete my research on time. I am equally thankful to my committee member, Dr. Alice Cherestes for her continued support and guidance by clarifying our research problems throughout my degree.

I would also like to thank Dr. Vijaya Raghavan and Dr. Jaswinder Singh of Bioresource Engineering and Plant Science departments respectively, for granting me access to their lab to use their spectrophotometer even during those critical pandemic times. I am thankful to Mr. Yvan Gariepy for training me to use the spectrophotometer.

I am very thankful to my lab mates and especially to Dr. Bo-Sen Wu for helping with my data collection and for peer reviewing my scientific writing without whom my experiments would have been very difficult.

I would like to thank my parents Venkateswarlu Kuncham and Vijaya Laxmi Kuncham who had seen my success far before everyone, for believing in me and providing all kinds of support. I will be indebted for the rest my life for all the sacrifices they made to make me what I am today. I am also thankful to my wife Latha Sree for providing moral support.

Finally, I am very thankful to Dr. Mark Lefsrud and his research collaborators for providing me financial assistance which helped me to complete my degree without any financial difficulties.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ALA	Aminolevulinic acid
ATP	Adenosine triphosphate
CAO	Chlorophyll(ide) *a* oxygenase
Chl	Chlorophyll
FCC	Fluorescent chlorophyll catabolites
FeCh	Ferrochelatase
GluTR	Glutamyl-tRNA reductase
GSA-AT	Glutamate 1-semialdehyde aminotransferase
H^+	Hydrogen ions
HMChl	Hydroxymethyl chlorophyll *a*
LED	Light emitting diode
LHC	Light harvesting complex
MgCh	Magnesium-chelatase
MgProtoMe	Magnesium-protoporphyrin IX monomethyl ester
NADPH	Nicotinamide adenine dinucleotide phosphate
NIR	Near infrared
nm	Nanometer

O^{2-}	Oxygen ions
POR	Pchlide oxidoreductase
PPX	Protoporphyrinogen oxidase
PQ	Plastiquinone
PSI	Photosystem I
PSII	Photosystem II
RCC	Red chlorophyll catabolites
UV	Ultraviolet
$\mu g\ L^{-1}$	Microgram per litter
$\mu mol\ m^{-2}\ sec^{-1}$	Micromoles per meter square per second

1. General Introduction

The chlorophyll molecules can be acknowledged as the preeminent photoreceptors in the photosynthetic process of plants and bacteria. Photosynthesis is the reduction-oxidation process that converts simple inorganic compounds into energy filled organic matter with the usage of sunlight and carbon dioxide and leads to the release of oxygen as a by-product, which makes higher life forms on our planet possible (Equation 1) (Taiz & Zeiger, 2010).

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (1)$$

This process occurs in specific cell organelles called chloroplast. In chloroplast, thylakoid membranes are stacked in grana where the photosynthetic process occurs, and these grana are surrounded by the stroma. The protein complexes required for electron transport and for the synthesis of ATP and NADPH are present within the thylakoid membranes (Cooper & Hausman, 2000). Photosystem I (PSI) and photosystem II (PSII) are two main complexes that perform the light reactions of photosynthesis, these complexes constitute light harvesting antenna complexes and reaction centers. In the reaction center, chlorophyll *a* is the prime pigment that is involved in the chemical reactions of photosynthesis (Farabee, 2007). PSI reduces $NADP^+$ and absorbs light at higher wavelength (<680 nm) while PS II helps in oxidizing water molecules and absorbs light at a wavelength of 680 nm (Taiz and Zeiger, 2002). The chlorophyll *a* pigment in the reaction center of PSI and PS II are referred to as P-700 and P-680, respectively, due to their absorption at that particular wavelengths (Mishra, 2004). The other plant pigments like chlorophyll *b*, β-carotene and other carotenoids (xanthophyll, zeaxanthin, lutein, violaxanthin, anteroxanthin, etc.) are considered as accessory pigments that help in harvesting light energy and funneling it into the

reaction center through the resonance effect (Lockstein, 2007). However, photosynthesis has only been observed by organisms that contain chlorophyll molecules, which highlights the importance of this pigment.

Chlorophylls are closely involved in the following events of photosynthesis: harvesting of light, transfer of energy and conversion of energy (Katz et al., 1978). In photosynthesis, the function of chlorophyll is a collective phenomenon as many chlorophyll molecules are involved in the conversion of a single photon (Emerson & Arnold, 1932). The light harvesting antenna, photoreaction center and the accessory pigments constitute the photosynthetic unit. An electron in the pigment Chl *a* gets excited by light absorption in PS II leading to start a chain of reactions. This electron loss in PS II is compensated through water splitting by the oxygen evolving complex which releases two hydrogen ions (H^+), an electron and free oxygen ions (O^{2-}). These hydrogen ions in the thylakoid membrane cause a proton gradient, driving ATP synthesis (Farabee, 2007). After the light energy is absorbed, the energy is transferred to the PS II reaction center, where electrons are transported to the plastiquinone (PQ) from which the electrons are then transported to the cytochrome *bf6* complex. Plastocyanin transports the electrons to the PS I reaction center (Cooper & Hausman, 2000). $NADP^+$ is reduced to NADPH by NADP reductase, which receive electrons from ferrodoxin and ADP is converted to ATP by ATP synthase (Taiz and Zeiger, 2002). Later these NADPH and ATP are used as part of dark reactions (Taiz and Zeiger, 2002).

As chlorophyll is treated as the primary initiator for the photosynthetic process, understanding chlorophyll's characteristics is an essential path for studying photosynthesis. It is clearly understood that chlorophyll is an obligatory pigment in a photosynthetic organism but the definite function of chlorophyll pigment in photosynthesis is still vague.

2. Literature review

2.1. Plant pigments

Plants contain various types of pigments. The most obvious and widely present pigment is chlorophyll. Some of the pigments and their occurrence is shown in Table 1.

Table 2.1: Important plant pigments and their presence in organisms (Davies, 2004).

Pigment	Common types	Occurrence
Chlorophylls	Chlorophyll *a* and *b*	All plants
Carotenoids	Carotenes Xanthophylls	Plants and photosynthetic bacteria
Flavonoids	Anthocyanins Aurones Chalcones Flavonols Proanthocyanidins	Most common in plants including gymnosperms, angiosperms, ferns and bryophytes.
Betalains	Betacyanins Betaxanthins	Caryophyllales and some fungi.

These plant pigments exist in different forms and it has been reported that carotenoids have over 600 naturally occurring structures with over 7000 structures for flavonoids, which includes over 500 anthocyanins (Britton, 1995). Chlorophylls have different structures, which are discussed in detail in this review.

2.2. Nomenclature of chlorophyll

All the chlorophyll molecules are complexes with a magnesium atom which are derived from porphyrin and its di- and tetrahydro-derivatives (Goodwin, 1976). The peripheral carbon atoms are numbered 1-8 and bridge carbon atoms are designated α-δ (Fischer et al., 1968). All chlorophylls unlike hemes which are iron complexes of porphyrin, contains one extra ring in addition to the four pyrrole rings (I-IV), this additional ring is referred as an isocyclic ring or ring V and it is biosynthetically derived by oxidation and cyclization of C-6 propionic acid onto the γ bridge atom (Cox et al., 1969). The following terms are used in this review to define chlorophyll and its derivatives:

Phyllins: The derivatives of chlorophyll that contain magnesium.

Pheophytin: These are magnesium free derivatives of chlorophylls.

Chlorophyllide: The chlorophyll without the phytol (tail), it's a result of the action of the chlorophyllase enzyme

Chlorophyllase: The enzyme which catalyzes hydrolysis of C_7 propionate ester.

Pheophorbides: The compounds having C_7 propionic acid after the removal of the magnesium atom and the phytol from the chlorophyll molecule.

Chlorins: Derivatives formed by cleavage of the isocyclic ring in pheophorbide *a*.

Purpurins: Chlorin derivatives in which C-10 has been oxidized.

Porphyrinogen: Hexahydro derivative of porphyrins formed by joining four pyrrole rings with saturated methylene bridges.

2.3. Chlorophyll profile and structure

Chlorophylls exist in all photosynthetic organisms. Its components, the macrocycle central metal ion and peripheral substituents, allows chlorophyll to have unique physical and chemical properties, which utilize sunlight as an energy source in photosynthesis (Grimm et al., 2007). In higher plants and algae (except blue green algae), chlorophyll molecules are found in separate cell organelle known as chloroplasts, while in the blue green algae and photosynthetic bacteria these chlorophyll molecules are arranged in intracellular lamellae (Goodwin, 1976). The constitution of pigments differs between organisms. For instance, algae and higher plants contain chlorophyll *a* and *b*; yet cyanobacteria comprise accessory photosynthetic pigments, phycocyanin, phycoerythrin and only chlorophyll *a* (Taiz & Zeiger, 2010).

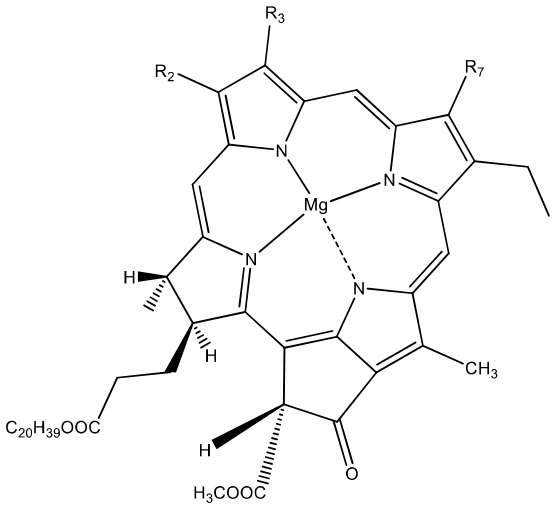
The structure and function of chlorophyll in photosynthetic organisms have been investigated for over two centuries, leading to a tremendous amount of knowledge on this essential pigment (Pelletier & Caventou, 1818; Rebeiz, 2014). The term chlorophyll was originally put to use by Pelletier and Caventou (1818) to report a pigment, later known to be the reason for the green color of leaves. The name chlorophyll is obtained from the Greek: "*chloros"* and "*phyllos"*, meaning green leaf. Since then, the research on chlorophyll has attracted researchers to better understand and characterize chlorophyll, including absorbance spectrum and its quantification method, fluorescent emission, and redox potential (Brown, 1977; Caesar et al., 2018; Connolly et al., 1982). Different forms of chlorophylls and accessory pigments (chlorophyll a & *b*, and carotenoids) were later isolated and discovered from plant pigment mixtures by using column chromatography (Goodwin, 1976; Tswett, 1906). Later other closely related chlorophylls (chlorophyll *c*, *d*, and *f*) have been found in algae and some photosynthetic bacteria (Chen et al., 2012; Dougherty et al., 1970; Kühl et al., 2005).

2.4. Chlorophyll *a* and *b*

Chlorophyll *a* ($C_{55}H_{72}O_5N_4Mg$) is the ubiquitous and most abundant pigment present in higher plants and algae (Schoefs, 2002). It is the main component in the reaction centers and light harvesting complex, which are responsible for collecting and converting light energy to chemical energy in the photosynthetic process (Boender et al., 1995; Grimm et al., 2007). The photosystems of all oxygenic photosynthetic organisms require chlorophyll *a* for their photochemistry except in *A. marinus* which need both chlorophyll *a* and *d*. (Tanaka & Tanaka, 2011; Tomo et al., 2007). Chlorophyll *a* is a macro aromatic compound containing tetra pyrrole ring (Figure 1). It has a porphyrin head in which four nitrogen atoms coordinate the central magnesium ion. The porphyrin head is connected to a long hydrocarbon tail called phytol. Some of the electrons in the porphyrin head are bound loosely and this portion of chlorophyll takes part in the transition of electrons and redox reactions .The hydrocarbon tail attach the chlorophyll to a hydrophobic membrane (Taiz & Zeiger, 1998).

Chlorophyll *b* ($C_{55}H_{70}N_4O_6Mg$) is present in the light harvesting system of vascular plants, euglenaceae, chlorophyta, and prochlorophyta. It is the second main components present in the photosystems after chlorophyll *a* and is approximately one-third of the amount of chlorophyll *a* (Burger-Wiersma et al., 1986). In chlorophyll *b,* the side chain of the porphyrin head contains a formyl group, whereas chlorophyll *a* contains a methyl group at that position. This structural difference makes chlorophyll *b* differ from chlorophyll *a* with respect to light absorbance properties (Goodwin, 1988). The advantage of plants having chlorophyll *b* is broadening the light harvesting range for example at 450 nm where chlorophyll *a* does not absorb. The carotenoids can help in the same way by absorbing in the blue region, but this overlap of spectrum absorbance with chlorophyll *b* suggests that Chl *b* may have some other functions (Tanaka & Tanaka, 2011a).

However, some recent studies have indicated that the chemical properties of chlorophyll *b* make it bond more tightly to the light-harvesting complex proteins than chlorophyll *a* and the biosynthesis of chlorophyll *b* is highly interlinked with light harvesting complex accumulation in plants (Horn et al., 2007).



	R_2	R_3	R_7
Chl *a*	CH_3	C_2H_3	CH_3
Chl *b*	CH_3	C_2H_3	CHO
Chl *d*	CH_3	CHO	CH_3
Chl *f*	CHO	C_2H_3	CH_3

Figure 2.1: Chemical structure of chlorophyll molecules (Niedzwiedzki & Blankenship, 2010).

2.5. Other chlorophylls (Chlorophyll *c*, *d* and *f*)

Chlorophyll *c* has two distinct forms: chlorophyll c_1 ($C_{35}H_{30}O_5N_4Mg$) and chlorophyll c_2 ($C_{35}H_{28}O_5N_4Mg$). Brown seaweeds, diatoms, and chrysomonads contain both these forms of chlorophyll *c* while dinoflagellates, symbiotic dinoflagellates and cryptomonads contain only chlorophyll c_2. Chlorophyll *c* was isolated and separated from *Sargassum flavicans* (brown seaweed) (Jeffrey, 1976). These two forms are the porphyrin derivatives, each consisting of four unsaturated rings with an acrylic acid at one of the carbon atoms and the phytol tail is absent in both types. Chlorophyll c_1 differs from chlorophyll c_2 by having an ethyl group at C_4 in the ring II of the tetrapyrrole head while chlorophyll c_2 have a vinyl group at that position (Jeffrey, 1976).

Chlorophyll *c* is one of the important pigments for aquatic plants because unlike autotrophic plants which have maximum photosynthetic efficiency in red light, the aquatic plants receive more yellow light because of the selective absorption of yellow by the water. Chlorophyll *c* helps in absorbing the light in the yellow region where there is weak absorption by chlorophyll *a.* Chlorophyll *c* is required as an accessory pigment for effective photosynthesis like chlorophyll *b* for higher plants (Dougherty et al., 1970).

Chlorophyll *d* ($C_{54}H_{70}O_6N_4Mg$) was first discovered in red algae, which belongs to *Rhodophyta* (Manning & Strain, 1943). The chlorophyll *d* differs from chlorophyll *a* by having a formyl group at the R_3, while chlorophyll *a* has a divinyl group at that position (Larkum & Kühl, 2005). It is believed that pigments like chlorophyll *b*, chlorophyll *c*, carotenoids and phycobilins only contribute to light harvesting in oxygenic photosynthesis; however, a prokaryotic organism (*Acaryochloris marina*) with oxygenic photosynthesis was isolated with the dominant pigment chlorophyll *d* instead of chlorophyll *a* (Hu et al., 1998). The cells possess chlorophyll *d* and *a* in 30:1 molar ratio. This chlorophyll *d* differs from all the other chlorophyll molecules by having its Q_y peak at ~720 nm (Larkum & Kühl, 2005). In *A. marina* the PSI driven by the chlorophyll *d* (P740) absorbs light in the far-red spectrum, which is between 680 nm to 700 nm absorbed by PSI and PSII reaction centers and 800 nm to 900 nm far red light absorbed by bacteriochlorophylls of anoxygenic photosynthesis. Therefore, *A. marina* photosynthesis may remain an intermediate between anoxygenic and oxygenic photosynthesis (Hu et al., 1998).

The existence of chlorophyll *e* is still a mystery because it has never been isolated and chemically characterized, as it was reported only in unpublished data of HH Strain's pigment extraction of *Tribonema bombycinum* and *Vaucheria hamata* (Chen et al., 2010). Even though the existence of chlorophyll *e* has not been proven, the next discovered chlorophyll was named

chlorophyll *f* , just to avoid confusion (Li & Chen, 2015). Chlorophyll *f* can be referred to as [2-formyl]-chlorophyll *a* and was first discovered in methanolic extract of shark bay stromatolites (Chen et al., 2010), but later it was also discovered in unicellular cyanobacterium which is isolated from Lake Biwa (Akutsu et al., 2011). It is the most red-shifted absorbing chlorophyll so far and was identified only when cultured under NIR LED light (Ohkubo et al., 2011). Chlorophyll *f* along with chlorophyll *d* are referred to as red shifted chlorophylls because they have the red shifted Q_y peaks when compared with chlorophyll *a* (Li & Chen, 2015).

2.6. Chlorophyll biosynthesis and cycle

Chlorophyll *a* and *b* are the prime components of photosystems in higher plants and green algae (Boender et al., 1995; Grimm et al., 2007). Chlorophyll *a* has two roles in oxygenic photosynthesis: harvesting light energy and in transforming light energy to chemical energy; these roles makes chlorophyll *a* very important among all the chlorophylls (Bjorn et al., 2009). An obvious question is, why would higher plants evolve the process to synthesize and maintain chlorophyll *b*? Chlorophyll *b* is made from chlorophyll *a* and is broken down after it is retransformed to chlorophyll *a*. This interconversion process between chlorophyll *a* and *b* is denoted as chlorophyll cycle. The chlorophyll cycle comprises of three phases: (I) chlorophyll a biosynthesis; (II) chlorophyll *b* biosynthesis; and (III) chlorophyll breakdown.

2.6.1. Chlorophyll *a* biosynthesis

The primary step in the chlorophyll biosynthesis is the synthesis of 5-aminolevulinic acid (ALA). This ALA is produced from glutamate by the function of enzymes like glutamyl-tRNA reductase (GluTR) and glutamate 1-semialdehyde aminotransferase (GSA-AT). Then eight

molecules of ALA condense to form uroporphyrinogen III, which is the first porphyrin molecule (Figure 2.2a). Later this uroporphyrinogen III molecule is oxidatively converted to protoporphyrin IX with an intermediate molecule called coproporphyrinogen III (Beale, 1999). The enzymes required until the production of coproporphyrinogen III are present in stroma or weakly attached to plastidal membranes. The protoporphyrinogen oxidase (PPX) is used to produce protoporphyrin IX through the dehydrogenation of protoporphyrinogen and is present in both mitochondrial and plastid membranes (Eckhardt et al., 2004).

The two metal chelatases called Mg-chelatase (MgCh) and ferrochelatase (FeCh) which have different biochemical properties, compete for protoporphyrin IX to form Mg-protoporphyrin or protoheme (Eckhardt et al., 2004). MgCh comprises of three subunits, CHLI, CHLD and CHLH (Papenbrock et al., 1997). The subunits, CHLI and CHLD, make an activation complex under ATP hydrolysis. It is believed that the first two subunits CHLI/CHLD complex provides Mg^{+2} while CHLH carries the protoporphyrin IX (Eckhardt et al., 2004). ALA synthesis and MgCh activity are at a maximum in light, while FeCh activity is highest at the start of the dark period (Papenbrock et al., 1999).

The cyclization of the fifth ring in the Mg-protoporphyrin IX monomethyl ester (MgProtoMe) leads to the formation of divinyl protochlorophyllide (Figure 2.2a), which is catalyzed by MgProtoMe cyclase (Eckhardt et al., 2004). This protochlorophyllide *a* with the reduction of C17/C18 double bond in one of its ring changes it into chlorophyllide *a*, and this reaction is catalyzed by NADPH-Pchlide oxidoreductase (POR). The enzyme, Chl synthetase, catalyzes the esterification of chlorophyllide *a* to form chlorophyll *a* (Figure 2.2b) (Eckhardt et al., 2004).

2.6.2. Chlorophyll *b* biosynthesis

In this phase, chlorophyll *b* is synthesized from chlorophyll *a* by the activity of chlorophyll(ide) *a* oxygenase (CAO), it was suggested that even chlorophyllide *a* is an intermediate compound of chlorophyll *b* biosynthesis (Tanaka et al., 1998; Tanaka & Tanaka, 2011a). In chlorophyllide *a* the 7-methyl group is converted into the formyl group by two oxygenation reactions which results in chlorophyllide *b* (Oster et al., 2000). This chlorophyllide *b* is ligated with a phytol tail to form chlorophyll *b* with the help of chlorophyll synthase. The biosynthesis of chlorophyll *b* is based upon the in-vivo experiment which states CAO reacts with chlorophyllide *a* but not with chlorophyll *a* because of the hydrophobic nature of phytol but there is a possibility for CAO to react with chlorophyll *a* when chlorophyll *a* binds to proteins that are available to CAO (Tanaka & Tanaka, 2011a). In the above process there is a need for the chlorophyllase enzyme to convert chlorophyll *a* to chlorophyllide *a* by removing the phytol but as of now the presence of this enzyme is still being debated (Shemer et al., 2008). So far, CAO is the only enzyme reported for catalyzing the reaction that transforms chlorophyll(ide) *a* to chlorophyll(ide) *b.*

Two independent mechanisms were proposed that regulate CAO activity (Tanaka et al., 1998). One mechanism suggest that chlorophyll *b* synthesis is controlled by CAO mRNA level. While another mechanism suggest that production of chlorophyll *b* is controlled at the protein level, particularly CAO protein stability. There are three domains in CAO named as A-, B- and C-domains (Nagata et al., 2004). The C-domain independently converts chlorophyll *a* to chlorophyll *b*. The A-domain of CAO protein senses the presence of chlorophyll *b* and controls level of CAO protein (Yamasato et al., 2005). The B-domain sequences has a PEST like motif with amino acids rich in Proline (P), Glutamate (G), Serine (S) and Threonine (T). This motif is known to mediate

proteolytic cleavage by proteasome. Even though the presence of proteasome is not found in plastids, the B-domain may have some specific function in CAO activity (Tanaka & Tanaka, 2006).

Chlorophyll *b* is capable of reconverting into chlorophyll *a* by the enzyme called chlorophyll *b* reductase which helps to reduce 7-formyl group of chlorophyll *b* to a hydroxyl group forming an intermediate compound called 7-hydroxymethyl chlorophyll *a* (HMChl). This HMChl is reduced to chlorophyll *a* by the action of HMChl reductase (HCAR) (Kusaba et al., 2007; Tanaka & Tanaka, 2011a). This interconversion of chlorophyll *a* and *b* is known as the chlorophyll cycle.

There are studies showing that this chlorophyll cycle controls the construction and destruction of the light harvesting complex (LHC). In a chlorophyll *b* lacking mutant (*chlorina f2*) of barley resulted in the absence of most of LHC (Highkin, 1950; Thornber & Highkin, 1974) but the expression of *lhc* genes and uptake of LHC apoproteins was not impacted by this mutation. The turnover rate of the LHC proteins was raised and mainly the subunits of LHC like Lhcb1, Lhcb2, Lhcb3,Lhcb4, Lhcb6, Lhca1, Lhca2, Lhca3 and Lhca4 except Lhcb5 was notably decreased (Bellemare et al., 1982; Krol et al., 1995). A similar result was observed in *Arabidopsis* which suggests a correlation between chlorophyll *b* and LHC proteins.

2.6.3 Chlorophyll breakdown

In the process of chlorophyll degradation, the first two steps are the removal of the phytol and removal of magnesium, catalyzed by chlorophyllase and magnesium dechelatase, respectively (Kräutler, 2008). All the catabolites found after the chlorophyll degradation are from chlorophyll *a* and none of them are from chlorophyll *b,* where chlorophyllide *b* is converted to chlorophyllide *a*. The removal of magnesium from chlorophyllide *a* turn it into pheophorbide *a*. The pheophorbide

a oxygenase opens the macrocycle of pheophorbide *a* to produce red chlorophyll catabolites (RCC) (Figure 2.2b). This RCC are reduced by ferredoxin dependent RCC reductase to light yellow fluorescent chlorophyll catabolites (pFCC). These pFCC are further converted to colorless and non-fluorescent compounds (Kräutler, 2008).

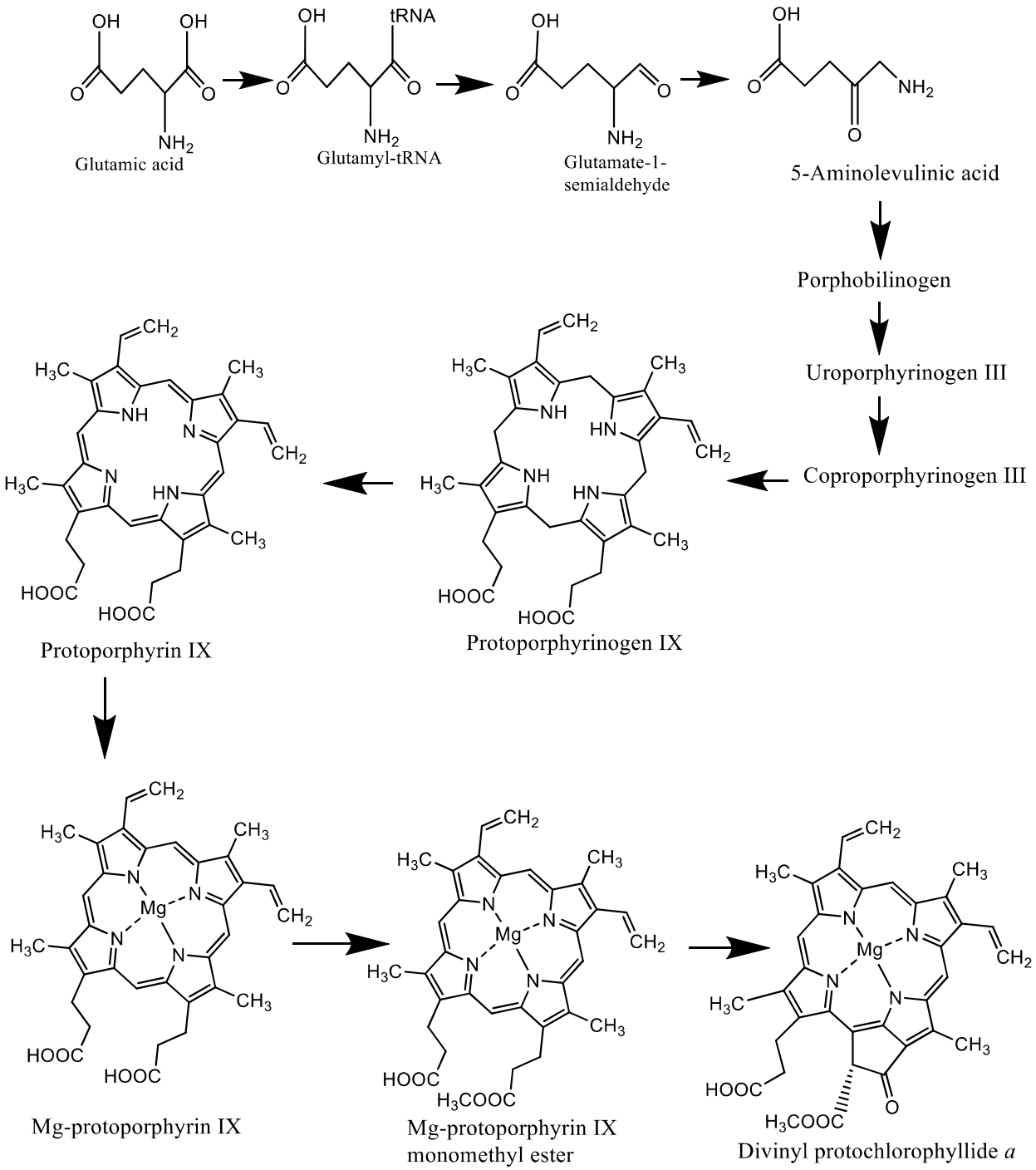


Figure 2.2a: Biosynthesis and breakdown of chlorophyll *a* and chlorophyll *b* (Tanaka & Tanaka, 2006).

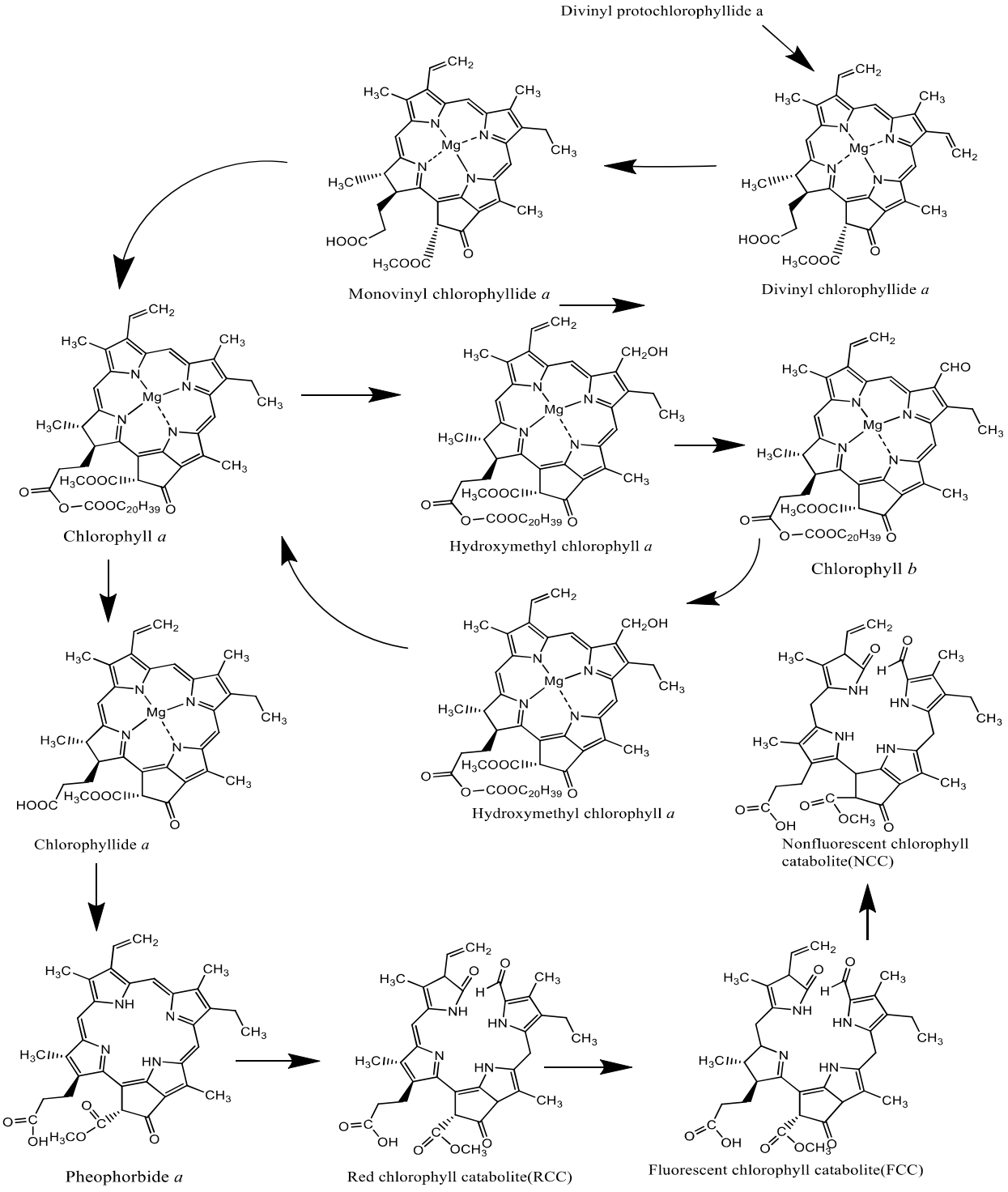


Figure 2.2b: Biosynthesis and breakdown of chlorophyll *a* and chlorophyll *b* (Tanaka & Tanaka, 2006)

2.7. Quantification of chlorophyll

There are different methods to quantify the chlorophyll extracted from plant materials including fluorometry, colorimetry, and spectrophotometry. Fluorometry is used to quantify chlorophyll concentration through its fluorescent emission, which allows to quantify chlorophyll concentration at 1 μg L^{-1}. The other two most commonly used methods are colorimetry and spectrophotometry in which light absorbed by the chlorophyll solutions is measured. These are widely used due to their accuracy and convenience. Both methods can detect 10 mg of chlorophyll per liter with a very high accuracy even in a few milliliters of solutions (Bruuinsma, 1963). With colorimetry, chlorophyll concentration can be determined by its magnesium content using different reagents like Titan yellow or Trilon B reagent. Spectrophotometry determines chlorophyll concentration based on its absorbance according to the Lambert-Beer law (Equation 2).

$$A = \log(I_0/I) = kcd \qquad (2)$$

Where A denotes absorbance, I_0 and I represent the intensities of incident and transmitted light respectively, *k* denotes specific absorption coefficient, which depends on the compound, solvent and wavelength, *c* represents the concentration in grams per liter while *d* is the optical pathlength in cm. There exists a linear relation between absorbance and concentration at a given wavelength with a constant light path. As chlorophyll *a* and chlorophyll *b* absorb the light independently at any given wavelength the equation can be written as;

$$A_\lambda = \log(I_0/I) = (k_a c_a + k_b c_b) d \qquad (3)$$

The indices *a* and *b* in the equation denote chlorophyll *a* and *b* respectively. The concentration of chlorophyll can be calculated based on measurements at a wavelength at which the values of specific absorption coefficients k_a and k_b are known; from these concentrations, total chlorophyll concentration can be computed. The specific absorption coefficient (*k*) values denotes the absorbencies of solutions at concentration of 1 g L^{-1} with 1 cm light path (Bruuinsma, 1963). Comar and Zscheile established the chlorophyll *a* and chlorophyll *b* absorption curves by using ethyl ether as the solvent. From these curves, values of specific absorption coefficient are derived. MacKinney derived these specific absorption coefficients by using 80% aqueous acetone as the solvent (Zscheile et al., 1942). The absorbance was measured at the wavelengths that show the highest possible difference between the *k* values of chlorophyll *a* and chlorophyll *b*. In aqueous acetone the greatest difference of k values was seen at peaks of chlorophyll *a* at 663 nm and chlorophyll *b* at 645nm (Bruuinsma, 1963). As per MacKinney the absorbencies in 1 cm cells are Equation 4, 5 (Mackinney, 1941);

$$A_{663} = 82.04\ C_a + 9.27\ C_b \qquad (4)$$

$$A_{645} = 16.75\ C_a + 45.6\ C_b \qquad (5)$$

Based on Mackinney work, Arnon derived equations for computing concentration in milligrams per liter (Equation 6 & 7) (Arnon, 1949);

$$C_a = 12.7\ A_{663} - 2.7\ A_{645} \qquad (6)$$

$$C_b = 22.9\ A_{645} - 4.7\ A_{663} \qquad (7)$$

$$C_{a+b} = 20.2\ A_{645} + 8.0\ A_{663} \qquad (8)$$

The two quantitative absorption curves intersect at 645 nm, having the same specific absorption coefficient. The total chlorophyll amount can be quantified in mg L^{-1} by measuring the absorbance at this wavelength (Equation 8). From the above information, the absorption spectrum and absorbance values of chlorophylls are most useful to determine the concentration of chlorophyll but the use of the absorption spectrum to define the photosynthetic activity is not clearly explained in any existing literature and thus requires further research.

2.8. Absorption spectrum of chlorophylls:

Among all the methods used to quantify concentration, absorption spectrophotometry is the most commonly accepted one. The porphyrin pigments are characterized by their absorption spectrum with pronounced bands in the yellow, red, near infrared and a strong absorption band in the blue wavelength region (400-500 nm), called the soret band. The absorption spectrum is affected by the dielectric properties of the solvent. The absorption bands of the chlorophyll are very sharp in ether as a solvent because of weak interaction between the solvent and the chlorophyll molecules. In polar solvents such as acetone, methanol, ethanol and these mixtures with water, the absorption maxima is displaced to the longer wavelength while the peak height and peak width decrease as a result of interaction between the solvent and chlorophyll (Mínguez-Mosquera et al., 2002).

In the blue wavelength region, chlorophyll *a* and chlorophyll *b* show maxima absorbance peak at 428 and 454 nm, respectively (Mínguez-Mosquera et al., 2002). In addition, there are four prominent bands in the red region between 500 and 700 nm. The difference in the absorption spectrum of chlorophyll *a* and chlorophyll *b* is due to their structural difference (Mínguez-Mosquera et al., 2002). The removal of Mg from the chlorophyll *a* molecule produces a

hypsochromic displacement from 428 to 408 nm and in the red region causes a bathochromic shift from 662 to 666nm. When the Mg atom is removed from the chlorophyll *b,* the soret band shifts from 454 to 430 nm and in the longer wavelengths the band shifts from 646 to 656 nm. When the chlorophyll molecule is de-esterified with phytol the absorption spectrum does not change because the phytol removal does not change the chromophore structure (Mínguez-Mosquera et al., 2002).

2.9. Aggregation and solubility of chlorophyll:

The spectral characteristics of chlorophyll are dependent on the nature of the solvent and upon the presence of polar or hydrogen bonding type impurities (Goodwin, 1976). In chlorophyll *a*, *b* the propionic acid side chain is esterified with a long chain aliphatic alcohol known as phytol, $C_{20}H_{39}OH$ (Katz & Norris, 1973). This esterifying alcohol separates the chlorophyll into polar and non-polar, lipophilic portions. The macrocycle of chlorophyll molecule is insoluble in water but is a polar entity, the presence of different polarity in chlorophyll has important effects on behavior of chlorophyll in organic solvents (Katz & Norris, 1973). The chlorophylls dissolved in polar solvents are intensely fluorescent while in non-polar solvents they are non-fluorescent (Livingston et al., 1949).

The chlorophyll donor-acceptor reactions can be divided into endogamous, where one chlorophyll molecule acts as donor and other chlorophyll molecule acts as acceptor and as exogamous, where one chlorophyll molecules act as an acceptor to another nucleophile donor (Katz et al., 1972). Endogamous reactions will generate chlorophyll dimers or chlorophyll oligomers linked together by keto C=O---Mg interactions. Exogamous reactions will form two series of chlorophyll- nucleophile adducts, based on the number of electron donors in nucleophile for example monofunctional donors like acetone, tetrahydrofuran form monomeric chlorophyll

species $Chl.L_1$ and $Chl.L_2$. Bifunctional ligands like dioxane, pyrazine or water are able to produce large polymeric by cross linking chlorophylls (Katz & Norris, 1973).

Water serves as both an electron donor and as a hydrogen bonding agent. In hydrocarbon solvents large polymers ($(Chl\text{-}H_2O)_n$, where n can be >100) may be formed (Katz et al., 1968). In benzene, chloroform and carbon tetra chloride water acts as a typical nucleophile as it binds to chlorophyll dimers to form monomer and dimer solvates.

$$Chl_2 + H_2O \rightarrow Chl_2.H_2O \quad (9)$$

$$Chl_2 . H_2O + H_2O \rightarrow 2Chl.H_2O \quad (10)$$

$$Chl_2 + 2H_2O \rightarrow 2Chl.H_2O \quad (11)$$

At low water and high chlorophyll concentrations, chlorophyll dimer monohydrate (Equation 9) seems to be the main species, but when the quantity of water is comparable to or greater than the quantity of chlorophyll it leads to Chl. H_2O formation (Equation 10 &11) (Katz et al., 1968). When the water concentration increases, or the temperature goes down below room temperature the A-663 monohydrate chlorophyll *a*. H_2O which absorb at 663 nm becomes A-678 anhydrous aggregate that absorb at 678 nm and in excess water conditions A-663 becomes A-743 (chlorophyll *a*. $2H_2O)_n$ (Fong & Koester, 1976). From the existing literature it is very clear that the absorption spectrum of chlorophyll pigment is very dynamic as it varies with the solvent, temperature, water content and intensity of light it is exposed to (Katz et al., 1968; Mínguez-Mosquera et al., 2002; Petrović et al., 2017; Sherman & Wang, 1966).

This review on chlorophyll emphasizes that Chl absorbance spectrum might be different and may not remain as a stable standard as it absorbs light at different regions of the visible

spectrum under different conditions. This sets our objective of the thesis as to determine the chlorophyll absorbance spectrum under different light conditions (dark, 30, 45, 60 and 100 µmol m^{-2} sec^{-1}), using different solvents (canola oil, acetone and mineral oil), and under different chlorophyll concentrations (3, 30, 300 and 3000 mg Chl in 10 ml of solvent).

In the chloroplast, lipids and chlorophyll (Chl) account for 18 to 37 % and 6 % respectively, of the total mass of chloroplast on dry mass basis (Photobiology, 1963). Based on this baseline, 20% of lipids and 4% of chlorophyll are in thylakoid membrane, assuming 2 % of free chlorophyll in the stroma. To mimic this natural concentration of chlorophyll in the chloroplast, 1 mg of chlorophyll was dissolved in 4 ml of canola oil to obtain a similar concentration as the chloroplast. However, to obtain the traditionally reported absorbance spectrum for chlorophyll, a concentration of 1 mg chlorophyll in 32 ml of canola oil was used. The reason for choosing canola oil as solvent is to use it as lipids in thylakoid membrane and light irradiance effect was studied as it is believed that the primary function of Chl is light harvesting. After treating with light irradiance Chl recovery was tested by storing in dark to see if it is capable to regain its absorbance after it was photodegraded.

3. Materials and methods

3.1 Chlorophyll *a* and *b* absorbance spectra under different light treatments

Chl *a* (C5753 Sigma-Aldrich, St Louis, USA) of 1 mg was dissolved in 4 ml of canola oil (Les Aliments Spectra, Montreal, Canada) in a test tube (Fisher Scientific, Waltham, MA, USA) that was wrapped with aluminum foil to prevent light exposure. This prepared solution was stored in dark conditions at -20°C for at least 24 hours for complete dissolution. After 24 hours, the solution was further diluted by adding 28 ml of canola oil to obtain a concentration of 1:32 (Chl *a*

: canola oil). A 3 ml volume of the prepared solution, was placed into a cuvette (Hellma Inc. NY, USA) and the absorbance was measured under dark conditions at defined wavelengths (350 nm-750 nm) using the Ultrospec 2100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, England) and using canola oil (without Chl *a*) as the reference blank. The absorbance data was recorded and used for statistical analysis.

After the above dark experiment, the same Chl *a* sample and blank canola oil were irradiated for 15 minutes at 30 µmol m^{-2} sec^{-1} light intensity emitted from cool white LED light (Luxeon Star LEDS, China), the light intensity was measured using light quantum meter (MQ-500 Apogee instruments Inc, Logan, UT, USA). After the light treatment, the Chl *a* sample absorbance was measured using the same procedure as the dark sample spectrophotometer measurement. The same sample and the canola oil blank were then irradiated for 15 minutes at 45 µmol m^{-2} sec^{-1} light intensity as reported above and the absorbance measurement of Chl *a* was repeated. After this experiment, the Chl *a* and blank canola oil were stored under dark conditions for 10 days at -20 ˚C after which time the absorbance of Chl a sample was measured again.

The above Chl *a* and blank canola oil was irradiated for 15 minutes at 60 µmol m^{-2} sec^{-1} light intensity and the absorbance was measured. Finally it was exposed to 100 µmol m^{-2} sec^{-1} light intensity and absorbance was measured. The samples were stored under dark conditions at -20 ˚C for 10 days and after the storage time the Chl *a* absorbance was measured again to monitor changes in the absorbance properties. The absorbance measurements of Chl *a* after all treatments were repeated thrice using three different Chl *a* samples. The absorbance data was recorded and statistically analyzed.

The Chl *b* (C5878 Sigma-Aldrich, St Louis, USA) of 1 mg was dissolved in canola oil and the final concentration was made to 1:32 (Chl *b* : canola oil), following the same process as for

Chl *a*, described above. A 3 ml volume of the prepared solution, was placed into a cuvette and the absorbance was measured under dark conditions at defined wavelength (350 nm- 750 nm) using the Ultrospec 1100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, England) and using canola oil (without Chl *b*) as the reference blank. The absorbance data was recorded and used for statistical analysis.

After the above dark experiment, the same Chl *b* sample and blank canola oil were irradiated for 15 minutes at 30 µmol m^{-2} sec^{-1} light intensity emitted from cool white LED light. After the light treatment, the Chl *b* sample absorbance was measured using the same procedure as the dark sample spectrophotometer measurement. The same sample and the canola oil blank were irradiated for 15 minutes at 100 µmol m^{-2} sec^{-1} light intensity as reported above and the absorbance of Chl *b* was measured. After this experiment, the Chl *b* and blank canola oil were stored under dark conditions at -20 °C for 15 days and after the storage time the Chl *b* absorbance was measured to monitor changes in the absorbance properties. The Chl *b* absorbance measurement after all treatments was repeated thrice using three different Chl *b* samples. The absorbance data was recorded and statistically analyzed.

3.2 Chlorophyll absorbance spectrum in different concentrations

The chlorophyll (C0780 TCI Chemicals, Portland, Oregon, USA) contains both Chl *a* and Chl *b* was dissolved in canola oil (Les Aliments Spectra, Montreal, Canada) at different concentrations (3 mg, 30 mg, 300 mg, and 3000 mg of Chl was dissolved in 10 ml of canola oil). These solutions were wrapped with aluminum foil to prevent light exposure and stored under dark conditions for at least 24 hours at -20 °C for complete dissolving. After 24 h of storage, 3 ml of each concentration solution was filled into a cuvette (Hellma Inc. NY, USA) and absorbance was

measured under dark conditions at wavelength from 350 nm to 750 nm with a Ultrospec 1100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, England) using blank canola oil as a reference. The absorbance data of Chl in all these concentrations was recorded and statistically analyzed. The Chl absorbance measurement of each concentration was repeated thrice with three different Chl samples.

3.3 Chlorophyll *a* and *b* absorbance spectrum in different solvents

Chl *a* (C5753 Sigma-Aldrich, St Louis, USA) of 1 mg was dissolved in 4 ml of canola oil (Les Aliments Spectra, Montreal, Canada) in a test tube (Fisher Scientific, Waltham, MA, USA) that was wrapped with aluminum foil to prevent light exposure. This prepared solution was stored in dark conditions at -20°C for at least 24 hours for complete dissolving. After 24 hours, the solution was further diluted by adding 28 ml of canola oil to obtain a concentration of 1:32 (Chl *a* : canola oil). The 3 ml of the prepared solution, was placed into a cuvette (Hellma Inc. NY, USA) and the absorbance was measured under dark conditions at defined wavelength (350 nm- 750 nm) using the Ultrospec 2100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, England) and using canola oil (without Chl *a*) as the reference blank. The absorbance data was recorded and used for statistical analysis.

The Chl *b* (C5878 Sigma-Aldrich, St Louis, USA) of 1 mg was dissolved in canola oil and the final concentration was made to 1:32 (Chl *b* : canola oil), following the same process as for Chl *a*. The 3 ml of the prepared solution, was placed into a cuvette and the absorbance was measured under dark conditions at defined wavelength (350 nm- 750 nm) using the Ultrospec 1100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, England) and using canola oil

(without Chl *b*) as the reference blank. The absorbance data was recorded and used for statistical analysis.

The same procedure and same concentration were used to determine Chl *a* and Chl *b* absorbance at the same wavelength range under dark conditions using acetone (Fisher Scientific, Waltham, MA, USA) and mineral oil (Paderno, Prince Edward Island, Canada) as solvents instead of canola oil. The treatments experiment of both Chl *a* and Chl *b* in canola oil, acetone and mineral oil was repeated thrice with three different Chl *a* and Chl *b* samples.

4. Statistical analysis

The effect of light intensity, concentration of chlorophyll and the effect of solvent on the chlorophyll light absorbance spectrum was measured. The data was recorded, and statistical analyses were conducted using the JMP software (JMP 4.3 SAS Institute Inc., Cary, NC, US) with a confidence level ($p < 0.05$) of 95 %. The data was analyzed using statistical tools (mean, standard deviation (SD), one-way analysis of variance and Tukey-Kramer HSD pairwise comparison test), to compare different absorbance spectra of chlorophyll.

5. Results

As well as under dark conditions, chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) absorbance spectrum in canola oil as the solvent (Figure 5.2 & 5.3) was found to be similar to the reported absorbance spectra (Figure 5.1) (Gross, 2012). The Chl *a* and Chl *b* absorbance spectra were characterized by having major absorbance peaks in the blue region at 430 and 450 nm and in

the red region at 640 and 660 nm, respectively (Figure 5.1). The absorbance spectra of all the light treated samples were compared between treatments at defined wavelength zones.

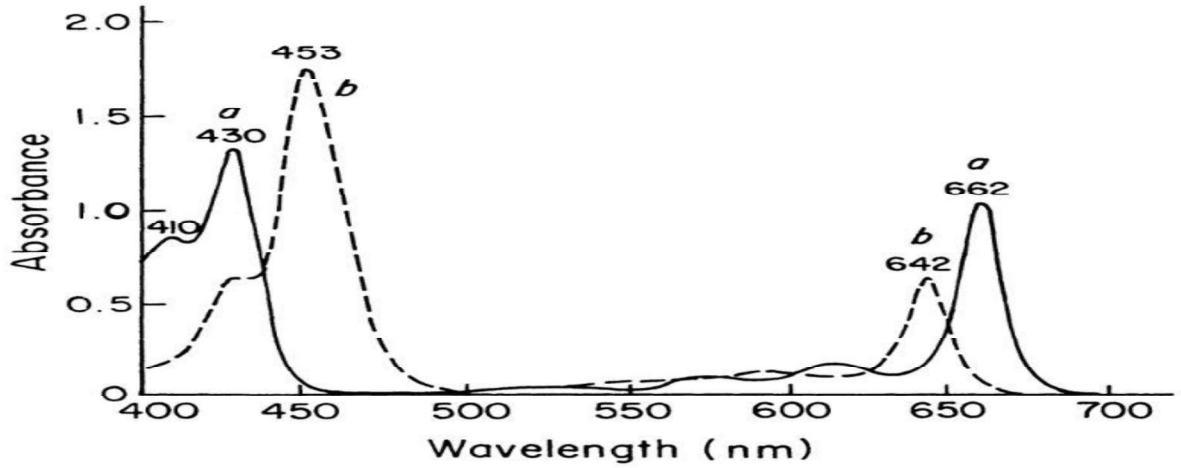


Figure 5.1: Absorbance spectra of chlorophyll *a* (——) and chlorophyll *b* (------)(Gross, 2012).

5.1. Effect of light irradiance on chlorophyll *a* absorbance

The various light pretreatments showed a significant ($p < 0.0001$) effect on Chl *a* absorbance in canola oil. The Chl *a* absorbance spectrum was normal with absorbance peaks in the blue region at 433 nm and in the red region at 665 nm under dark and low light intensity pretreatments. However, with higher light intensity treatments the Chl *a* absorbance at these peaks was reduced.

The Chl *a* absorbance at 350 nm (UV-A), was similar under the dark, and after 30 µmol m^{-2} sec^{-1} and 45 µmol m^{-2} sec^{-1} light treatment. The absorbance of Chl *a* was significantly decreased ($p < 0.05$, Appendix 1a) after treating with 60 and 100 µmol m^{-2} sec^{-1} light intensity (Figure 5.2) but no significant difference ($p > 0.05$, Appendix 1a) in absorbance was found between these two

treatments. The recovery treatment after the 45 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1} light treatments did not result any change in absorbance.

At both 380 and 410 nm, the dark, 30 µmol m^{-2} sec^{-1} and 45 µmol m^{-2} sec^{-1} Chl *a* absorbance spectrum showed a shoulder peak in the absorbance spectrum, but for the 60 µmol m^{-2} sec^{-1}, 100 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1} recovery treatments, the shoulder peak was not observed (Figure 5.2). At these two wavelengths, the dark absorbance spectrum had the highest absorbance while the Chl *a* 100 µmol m^{-2} sec^{-1} recovery had the lowest absorbance. When Chl *a* was exposed to the 30 µmol m^{-2} sec^{-1} treatment there was no significant ($p > 0.05$, Appendix 1a) effect on the Chl *a* absorbance at either 380 or 410 nm. When the intensity was increased to 45 µmol m^{-2} sec^{-1} there was a significant ($p < 0.05$, Appendix 1a) decrease in the absorbance at these wavelengths which was not regained even by storing in the dark. When Chl *a* was exposed to light intensities of 60 and 100 µmol m^{-2} sec^{-1}, the absorbance decreased significantly ($p < 0.05$, Appendix 1a) at 380 nm and 410 nm (Table 5.1). There was no significant difference ($p > 0.05$, Appendix 1a) in Chl *a* absorbance under 60 and 100 µmol m^{-2} sec^{-1} light treatments at either of these measured wavelengths. No recovery was observed for Chl *a* absorbance with recovery treatment after 100 µmol m^{-2} sec^{-1} light treatment.

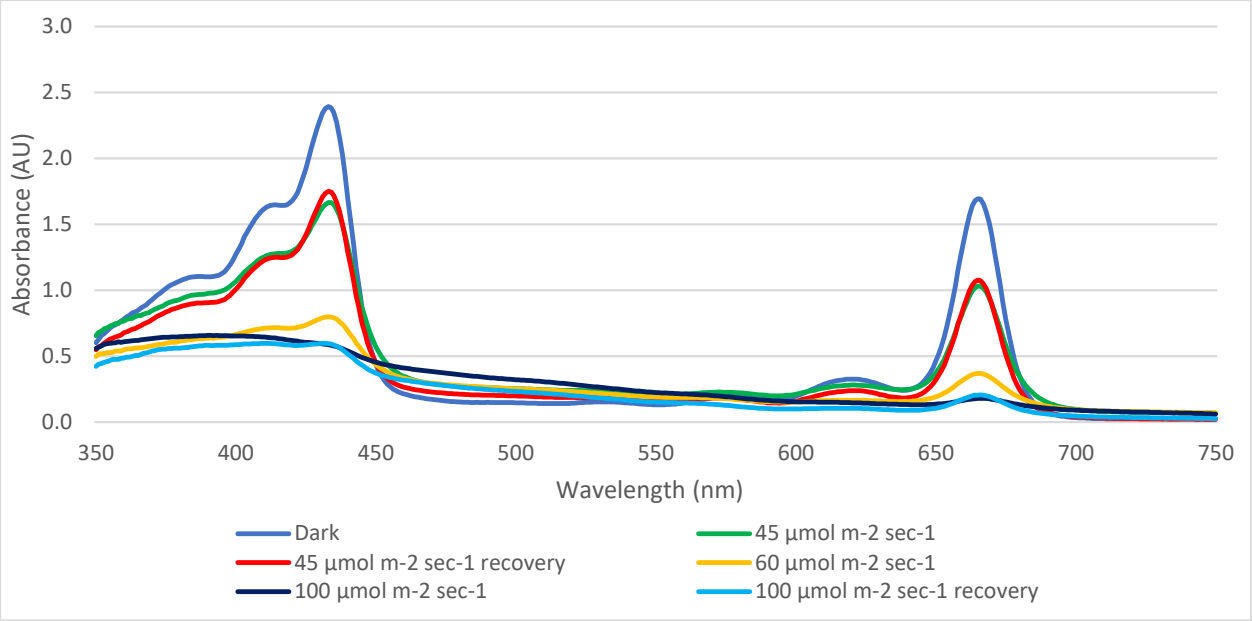


Figure 5.2: Absorbance spectra of Chl *a* in canola oil (1 mg Chl *a* in 32 ml of canola oil) under dark, 45 µmol m^{-2} sec^{-1}, 60 µmol m^{-2} sec^{-1}, 100 µmol m^{-2} sec^{-1} light intensities and the recovery of 45 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1}. The absorbance values are the means of three replicates

Chl *a* had one of the highest absorbance in the blue region with a peak at 433 nm, compared to the other wavelengths. At 433 nm, the dark absorbance spectrum has the highest absorbance value while the 100 µmol m^{-2} sec^{-1} showed the lowest absorbance (Table 5.1). When Chl *a* was treated with 30 µmol m^{-2} sec^{-1} there was a slight decrease in its absorbance, but it was not statistically significant ($p > 0.05$, Appendix 1a). When Chl *a* was treated with 45 µmol m^{-2} sec^{-1}, it resulted in a significant ($p < 0.05$, Appendix 1a) decrease in absorbance, and a slight recovery in absorbance was obtained when stored in the dark after treating with 45 µmol m^{-2} sec^{-1} light intensity but the recovery was not significant ($p > 0.05$, Appendix 1a). The 60 and 100 µmol m^{-2} sec^{-1} light intensities showed significantly ($p < 0.05$, Appendix 1a) different effect on Chl *a* at 433 nm. Chl *a* when treated with 100 µmol m^{-2} sec^{-1} light intensity the absorbance peak was significantly decreased (Figure 5.2). The 100 µmol m^{-2} sec^{-1} recovery treatment showed a slight recovery in absorbance at 433 nm, but it was not statistically significant ($p > 0.05$, Appendix 1a).

The Chl *a* absorbance at 450 nm, was similar with all the light treatments except 45 µmol m^{-2} sec^{-1} treatment. The Chl *a* absorbed significantly higher light ($p < 0.05$, Appendix 1a) when it was exposed to 45 µmol m^{-2} sec^{-1} light intensity (Table 5.1). The recovery treatment after both the 45 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1} treatments only resulted in a further decrease in absorbance indicating no recovery in its absorbance. The Chl *a* at 475 and 500 nm, absorbed light in the same manner, at these two wavelengths Chl *a* absorbance increased with an increase in light intensity treatment. The absorbance increased significantly and was equivalent when treated with 45 µmol m^{-2} sec^{-1} and 60 µmol m^{-2} sec^{-1} light intensity at both 475 and 500 nm. The Chl *a* absorbance decreased after the 45 µmol m^{-2} sec^{-1} recovery treatment at both 475 and 500 nm but the significant reduction in absorbance was observed only at 500 nm. Chl *a* when treated with 100 µmol m^{-2} sec^{-1} the resultant absorbance was significantly higher ($p < 0.05$, Appendix 1a) compared to previous light treatments, however it significantly decreased after the 100 µmol m^{-2} sec^{-1} recovery treatment.

The Chl *a* absorbance at 530 nm increased significantly with an increase in light intensity. The 45 and 60 µmol m^{-2} sec^{-1} light treatments resulted a similar and significant increase ($p < 0.05$, Appendix 1a) in absorbance. When treated with 100 µmol m^{-2} sec^{-1} light intensity the absorbance increased further, and it was significantly higher ($p < 0.05$, Appendix 1a) than lower light treatments. But when Chl *a* was treated with a recovery treatment, this absorbance was reduced back to its original state. The Chl *a* absorbed higher light at 575 nm after treatment with 45 µmol m^{-2} sec^{-1} light and the least absorbance was recorded after 100 µmol m^{-2} sec^{-1} recovery treatment. At this wavelength the absorbance was similar under the dark and after treatment with 30 µmol m^{-}

^{2} sec^{-1}, 60 µmol m^{-2} sec^{-1}, and 100 µmol m^{-2} sec^{-1} light intensities. No recovery in absorbance was found after the recovery treatments.

Table 5.1: Absorbance (AU) of Chl *a* in canola oil (1 mg Chl *a* in 32 ml of canola oil) under dark, 30, 45, 60 and 100 µmol m^{-2} sec^{-1} light treatments.

Wavelength / Treatment	350 nm (AU)	380 nm (AU)	410 nm (AU)	433 nm (AU)	450 nm (AU)	475 nm (AU)	500 nm (AU)	530 nm (AU)	575 nm (AU)	620 nm (AU)	665 nm (AU)	685 nm (AU)	700 nm (AU)
Dark	0.60 ± 0.00^{ab}	1.07 ± 0.02^{a}	1.61 ± 0.03^{a}	2.39 ± 0.02^{a}	0.43 ± 0.01^{b}	0.16 ± 0.00^{d}	0.14 ± 0.00^{d}	0.15 ± 0.00^{d}	0.19 ± 0.00^{b}	0.32 ± 0.00^{a}	1.69 ± 0.00^{a}	0.14 ± 0.00^{b}	0.03 ± 0.00^{b}
30 µmol m^{-2} sec^{-1}	0.61 ± 0.00^{ab}	1.05 ± 0.02^{a}	1.57 ± 0.05^{a}	2.30 ± 0.10^{a}	0.45 ± 0.02^{b}	0.18 ± 0.01^{d}	0.16 ± 0.01^{d}	0.16 ± 0.00^{d}	0.20 ± 0.00^{ab}	0.32 ± 0.01^{a}	1.59 ± 0.10^{a}	0.16 ± 0.01^{b}	0.04 ± 0.00^{b}
45 µmol m^{-2} sec^{-1}	0.65 ± 0.04^{a}	0.93 ± 0.03^{b}	1.25 ± 0.01^{b}	1.66 ± 0.03^{b}	0.56 ± 0.05^{a}	0.27 ± 0.04^{bc}	0.25 ± 0.03^{b}	0.23 ± 0.03^{ab}	0.22 ± 0.01^{a}	0.28 ± 0.00^{b}	1.03 ± 0.08^{b}	0.21 ± 0.01^{a}	0.09 ± 0.01^{a}
45 µmol m^{-2} sec^{-1} recovery	0.54 ± 0.06^{bc}	0.87 ± 0.09^{b}	1.22 ± 0.12^{b}	1.75 ± 0.17^{b}	0.44 ± 0.04^{b}	0.21 ± 0.02^{cd}	0.19 ± 0.02^{cd}	0.18 ± 0.01^{cd}	0.18 ± 0.01^{b}	0.23 ± 0.02^{c}	1.07 ± 0.10^{b}	0.12 ± 0.01^{cd}	0.03 ± 0.00^{b}
60 µmol m^{-2} sec^{-1}	0.49 ± 0.02^{cd}	0.61 ± 0.03^{c}	0.71 ± 0.06^{c}	0.79 ± 0.12^{c}	0.43 ± 0.01^{b}	0.28 ± 0.01^{b}	0.25 ± 0.01^{b}	0.21 ± 0.01^{bc}	0.17 ± 0.00^{b}	0.16 ± 0.01^{d}	0.36 ± 0.10^{c}	0.14 ± 0.00^{bc}	0.09 ± 0.00^{a}
100 µmol m^{-2} sec^{-1}	0.55 ± 0.01^{bc}	0.64 ± 0.01^{c}	0.64 ± 0.02^{c}	0.58 ± 0.03^{c}	0.45 ± 0.01^{b}	0.37 ± 0.00^{a}	0.32 ± 0.00^{a}	0.26 ± 0.00^{a}	0.19 ± 0.00^{b}	0.14 ± 0.00^{d}	0.17 ± 0.01^{c}	0.11 ± 0.00^{d}	0.09 ± 0.00^{a}
100 µmol m^{-2} sec^{-1} recovery	0.42 ± 0.00^{d}	0.56 ± 0.01^{c}	0.59 ± 0.04^{c}	0.59 ± 0.06^{c}	0.37 ± 0.00^{b}	0.27 ± 0.00^{bc}	0.23 ± 0.00^{bc}	0.18 ± 0.00^{cd}	0.12 ± 0.00^{c}	0.10 ± 0.00^{e}	0.20 ± 0.04^{c}	0.07 ± 0.00^{e}	0.04 ± 0.00^{b}

The absorbance values are the means of three replicates ± standard deviation. Values in the same column with same letter superscript are not significantly different ($p < 0.05$). Values in red show an increase during the recovery treatment. Values in blue show an increase in absorbance relative to the dark conditions.

In the red region at 620 and 665 nm, the Chl *a* absorbance was highest under the dark initial conditions. The 30 µmol m^{-2} sec^{-1} treatment did not show any impact on Chl *a* absorbance, while the 45 µmol m^{-2} sec^{-1} light intensity resulted in a significant ($p < 0.05$, Appendix 1a) decrease in absorbance at both 620 and 665 nm. The 60 and 100 µmol m^{-2} sec^{-1} light intensity had resulted in a similar and significant ($p < 0.05$, Appendix 1a) decrease in absorbance at both wavelengths. At 620 nm, the absorbance significantly decreased after both 45 and 100 µmol m^{-2} sec^{-1} recovery

treatments. At 665 nm, the Chl *a* absorbance after 45 and 100 µmol m^{-2} sec^{-1} recovery treatments were similar with their equivalent treatments, however a slight recovery in absorbance was identified at this wavelength for both the treatments.

At 685 nm wavelength, there was no significant difference between dark, 30 and 60 µmol m^{-2} sec^{-1} treatments. Chl *a* when treated with 45 µmol m^{-2} sec^{-1} light intensity had an absorbance that significantly ($p < 0.05$, Appendix 1a) increased but when treated with 100 µmol m^{-2} sec^{-1} no increase in absorbance was noticed. The recovery treatments did not show any recovery at 685 nm. The Chl *a* at 700 nm, response for the dark, 30 µmol m^{-2} sec^{-1}, 45 and 100 µmol m^{-2} sec^{-1} recovery treatments were similar and 45, 60 and 100 µmol m^{-2} sec^{-1} were similar, however there was a significant difference ($p < 0.05$, Appendix 1a) between these two groups.

5.2. Effect of light irradiance on chlorophyll *b* absorbance

The Chl *b* absorbance spectrum was similar to the Chl *a* spectrum (Figure 5.2), with two prominent absorbance peaks in the blue and red region at 458 nm and 646 nm respectively. However, the exact wavelength where these two chlorophylls absorb light varies between Chl *a* and *b*. The Chl *b* at 350 and 366 nm, absorbed more light under dark (Figure 5.3) and the light treatments resulted in a significant ($p < 0.05$, Appendix 1b) decrease in absorbance, but no significant difference was measured in absorbance between the 30 and 100 µmol m^{-2} sec^{-1} light treatments. At 350 nm, the Chl *b* absorbance was the same between the 100 µmol m^{-2} sec^{-1} light intensity and 100 µmol m^{-2} sec^{-1} recovery treatment but at 366 nm a significant ($p < 0.05$, Appendix 1b) difference was observed.

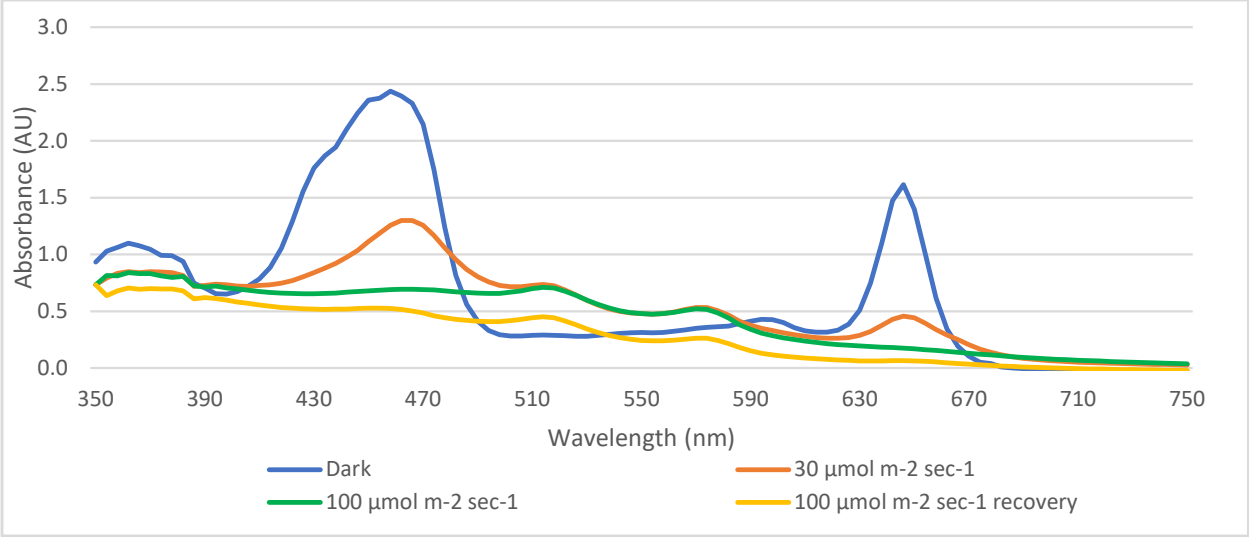


Figure 5.3: Absorbance spectra of Chl *b* in canola oil (1 mg Chl *b* in 32 ml of canola oil) under dark, 30 µmol m^{-2} sec^{-1}, 100 µmol m^{-2} sec^{-1} light intensities and the recovery of 100 µmol m^{-2} sec^{-1}. The absorbance values are the mean of three replicates.

The Chl *b* absorbance at 382 nm, was highest under the dark conditions (Table 5.2). The 30 and 100 µmol m^{-2} sec^{-1} light treatments resulted a similar and a significant ($p < 0.05$, Appendix 1b) decrease in Chl *b* absorbance at 382 nm when compared to the dark treatment. After the 100 µmol m^{-2} sec^{-1} light recovery treatment, Chl *b* did not show any recovery in absorbance and instead there was further decrease in absorbance (Figure 5.3).

The Chl *b* at 398 nm, showed higher absorption when treated with 30 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1} and a significant decrease ($p < 0.05$, Appendix 1b) in its absorbance was identified after the recovery treatment, indicating no recovery in its absorbance. At 410 nm the Chl *b* absorbance was similar under the dark and 30 µmol m^{-2} sec^{-1} light treatments. The 100 µmol m^{-2} sec^{-1} light treatment resulted in a significant decrease ($p < 0.05$, Appendix 1b) in absorbance and when treated for recovery, no recovery in absorbance was identified and there was further significant reduction in absorbance. The 458 nm wavelength was found to be the major light absorbing area for Chl *b* in the blue region of the spectrum (Figure 5.3). The 30 µmol m^{-2} sec^{-1}

light treatment resulted in a significant decrease ($p < 0.05$, Appendix 1b) in Chl *b* absorbance at 458 nm. The 100 µmol m^{-2} sec^{-1} light treatment, resulted in a further decrease in the absorbance peak at this wavelength (Figure 5.3). The recovery treatment did not show any significant ($p > 0.05$, Appendix 1b) difference from 100 µmol m^{-2} sec^{-1} , indicating no recovery in Chl *b* absorbance.

It was contradicting, to find that Chl *b* at 518 and 570 nm, absorbs more light after the treatment with 30 and 100 µmol m^{-2} sec^{-1} light intensity than under the dark conditions. It was observed that distinct absorbance peaks (Figure 5.3) at 518 nm and 570 nm were unidentified under the dark conditions and no significant difference ($p > 0.05$, Appendix 1b) was measured between 30 and 100 µmol m^{-2} sec^{-1} light intensity. At both these wavelengths, Chl *b* was unable to regain its absorbance after the recovery treatment. The Chl *b* at 586 nm, did not show any significant difference ($p > 0.05$, Appendix 1b) in its absorbance between dark and any of the light treatment and when treated for recovery it only had a significant decrease ($p < 0.05$, Appendix 1b) in its absorbance, indicating no increase in absorbance. At 610 nm, the Chl *b* absorbance under dark conditions was significantly different ($p < 0.05$, Appendix 1b) from100 µmol m^{-2} sec^{-1} but not with the 30 µmol m^{-2} sec^{-1} light treatment, and recovery was significantly different from all other treatments and no recovery in its absorbance was measured.

Table 5.2: Absorbance values (AU) of Chl *b* in canola oil (1 mg Chl *b* in 32 ml of canola oil) under dark, 30 and 100 µmol m^{-2} sec^{-1} light intensities.

Wavelength / Treatment	350 nm (AU)	366 nm (AU)	382 nm (AU)	398 nm (AU)	410 nm (AU)	458 nm (AU)	518 nm (AU)	570 nm (AU)	586 nm (AU)	610 nm (AU)	646 nm (AU)	666 nm (AU)	710 nm (AU)
Dark condition	0.93 ± 0.07^{a}	1.07 ± 0.06^{a}	0.93 ± 0.03^{a}	0.65 ± 0.03^{bc}	0.78 ± 0.04^{a}	2.43 ± 0.18^{a}	0.28 ± 0.03^{c}	0.34 ± 0.03^{b}	0.39 ± 0.03^{a}	0.32 ± 0.04^{a}	1.61 ± 0.03^{a}	0.19 ± 0.04^{ab}	0.00 ± 0.00^{b}
30 µmol m^{-2} sec^{-1}	0.73 ± 0.03^{b}	0.84 ± 0.02^{b}	0.81 ± 0.01^{b}	0.73 ± 0.02^{a}	0.72 ± 0.02^{ab}	1.25 ± 0.04^{b}	0.72 ± 0.01^{a}	0.53 ± 0.02^{a}	0.41 ± 0.02^{a}	0.28 ± 0.02^{ab}	0.45 ± 0.03^{b}	0.25 ± 0.02^{a}	0.05 ± 0.02^{ab}
100 µmol m^{-2} sec^{-1}	0.73 ± 0.03^{b}	0.83 ± 0.01^{b}	0.80 ± 0.02^{b}	0.70 ± 0.01^{ab}	0.67 ± 0.01^{b}	0.69 ± 0.01^{c}	0.70 ± 0.00^{a}	0.52 ± 0.00^{a}	0.38 ± 0.00^{a}	0.23 ± 0.01^{b}	0.17 ± 0.01^{c}	0.14 ± 0.01^{b}	0.06 ± 0.01^{a}
100 µmol m^{-2} sec^{-1} recovery test	0.73 ± 0.05^{b}	0.69 ± 0.03^{c}	0.67 ± 0.03^{c}	0.59 ± 0.02^{c}	0.55 ± 0.03^{c}	0.52 ± 0.02^{c}	0.44 ± 0.02^{b}	0.26 ± 0.02^{c}	0.18 ± 0.03^{b}	0.08 ± 0.03^{c}	0.06 ± 0.03^{d}	0.04 ± 0.03^{c}	0.00 ± 0.00^{ab}

Values are the means of three replicates ± standard deviation. Values in the same column with same letter superscript are not significantly different ($p < 0.05$). Values in red show a significant increase in absorbance from the dark conditions.

For Chl *b*, the other major absorbance peak was identified in the red region of the spectrum at 646 nm (Figure 5.3). At 646 nm, the Chl *b* absorbance significantly decreased ($p < 0.05$, Appendix 1b) under the 30 µmol m^{-2} sec^{-1} light treatment. When exposed to 100 µmol m^{-2} sec^{-1} the absorbance peak at 646 nm was further significantly decreased ($p < 0.05$, Appendix 1b) and it did not show any recovery in absorbance after the recovery treatment. At 666 nm the Chl *b* absorbance after 100 µmol m^{-2} sec^{-1} recovery treatment was significantly different ($p < 0.05$, Appendix 1b) from all other treatments. The Chl *b* absorbed significantly higher ($p < 0.05$, Appendix 1b) after 30 µmol m^{-2} sec^{-1} light treatment than after 100 µmol m^{-2} sec^{-1} light treatment. The Chl *b* absorbance at 710 nm, was similar under dark, 30 µmol m^{-2} sec^{-1} and 100 µmol m^{-2} sec^{-1} recovery treatments (Table 5.2). The absorbance of Chl *b* after 100 µmol m^{-2} sec^{-1}, treatment was significantly different ($p < 0.05$, Appendix 1b) from dark treatment.

5.3. Effect of chlorophyll (Chl) concentration on its absorbance spectrum

The total Chl absorbance was directly proportional to Chl concentration as the absorbance increased with an increase in Chl concentration for the majority of wavelengths across the

spectrum. Distinct peaks in the blue (410 nm) and red (666 nm) regions were identified only in the 30 mg in 10 ml concentration. At 350 and 382 nm, the absorbance of Chl which contains both Chl *a* and Chl *b* was found to be similar at 3 and 30 mg in 10 ml but there was a significant increase ($p < 0.05$, Appendix 2a) in absorbance at 300 and 3000 mg in 10 ml but there was no significant difference between these two concentrations (Table 5.3). At 410 nm, there was a significant increase ($p < 0.05$, Appendix 2a) in absorbance when the concentration was increased to 30 mg in 10 ml (Figure 5.4). The increase in concentration to 300 and 3000 mg in 10 ml showed a similar and significant ($p < 0.05$, Appendix 2a) increase in absorbance.

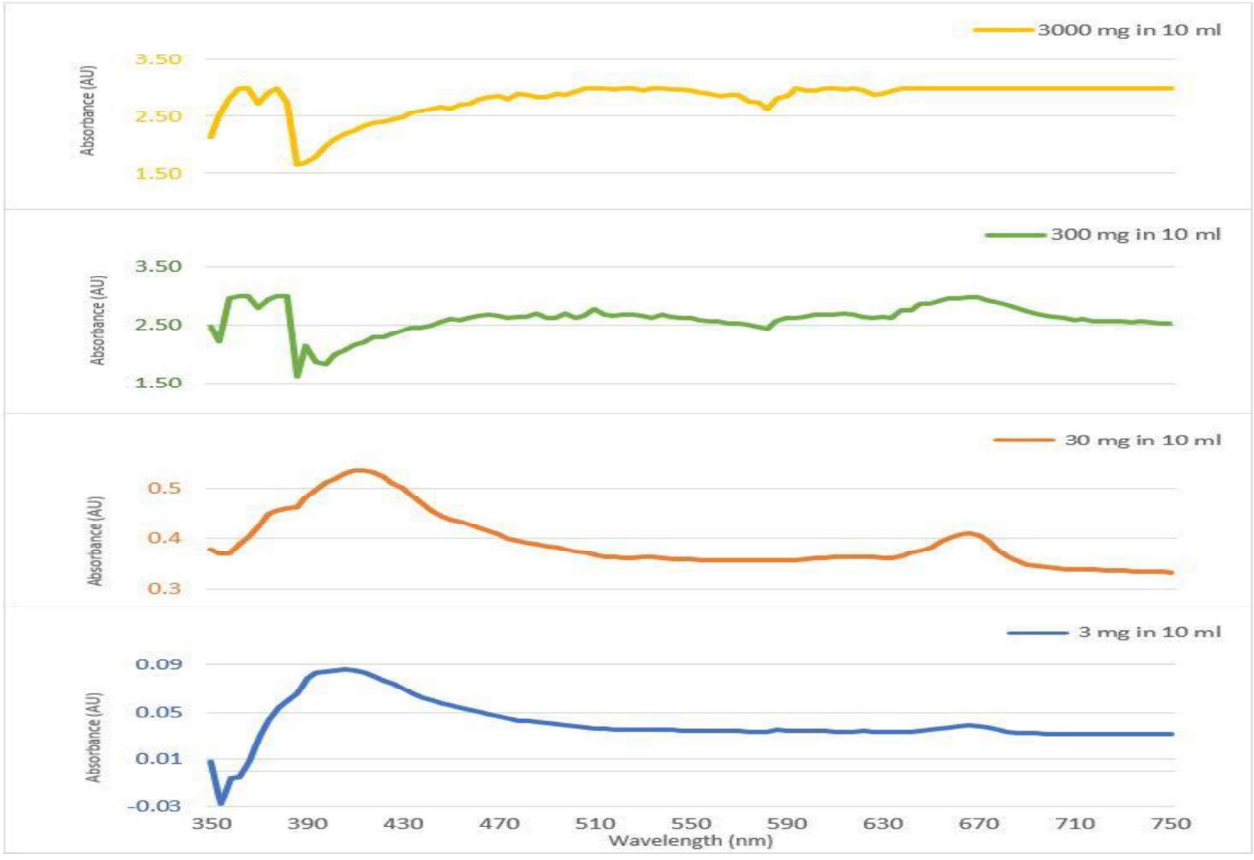


Figure 5.4: Dark absorbance spectra of total chlorophyll in different concentrations: 3, 30, 300 and 3000 mg Chl in 10 ml of canola oil. The absorbance values are the mean of three replicates.

The Chl absorbance at 510, 586 and 630 nm, was observed gradually increasing with an increase in concentration and there were significant differences ($p < 0.05$, Appendix 2a) between all the concentrations. Chl at 666 nm, showed an increase in absorbance with an increase in Chl concentration (Table 5.3), and the absorbance in all these concentrations were significantly different ($p < 0.05$, Appendix 2a) except 300 and 3000 mg in 10ml. The Chl absorbance at 750 nm significantly increased with increase in concentration and significant difference ($p < 0.05$, Appendix 2a) was found between all the treatments.

Table 5.3: Absorbance values (AU) of chlorophyll in different concentrations: 3, 30, 300 and 3000 mg in 10 ml of canola oil.

Wavelength / Concentration	350 nm (AU)	382 nm (AU)	410 nm (AU)	510 nm (AU)	586 nm (AU)	630 nm (AU)	666 nm (AU)	750 nm (AU)
3 mg in 10 ml	0.01 ± 0.02^{b}	0.06 ± 0.01^{b}	0.08 ± 0.02^{c}	0.03 ± 0.02^{d}	0.03 ± 0.02^{d}	0.03 ± 0.02^{d}	0.04 ± 0.01^{c}	0.09 ± 0.09^{d}
30 mg in 10 ml	0.37 ± 0.05^{b}	0.46 ± 0.03^{b}	0.53 ± 0.04^{b}	0.03 ± 0.03^{c}	0.35 ± 0.04^{c}	0.36 ± 0.03^{c}	0.41 ± 0.03^{b}	0.33 ± 0.02^{c}
300 mg in 10 ml	2.46 ± 0.92^{a}	3.00 ± 0.00^{a}	2.15 ± 0.10^{a}	2.76 ± 0.17^{b}	2.56 ± 0.15^{b}	2.63 ± 0.13^{b}	2.96 ± 0.04^{a}	2.52 ± 0.15^{b}
3000 mg in 10 ml	2.13 ± 0.80^{a}	2.74 ± 0.44^{a}	2.24 ± 0.06^{a}	3.00 ± 0.00^{a}	2.82 ± 0.02^{a}	2.90 ± 0.01^{a}	3.00 ± 0.00^{a}	3.00 ± 0.00^{a}

Values are the means of three replicates ± standard deviation. Values in the same column with same letter superscript are not significantly different ($p < 0.05$). Values in red indicate decrease in absorbance while concentration was increased.

5.4. Effect of solvent on chlorophyll *a* absorbance spectrum

The Chl *a* absorbance was found to be different in canola oil, acetone and mineral oil. The conventional Chl *a* absorbance spectrum with peaks in blue (434 nm) and red (666 nm) region was obtained only in canola oil while in acetone and mineral oil the absorbance spectrum was different. The Chl *a* at 350 and 362 nm had higher absorbance with distinct peaks in acetone than canola oil or mineral oil (Figure 5.5). There was significant difference ($p < 0.05$, Appendix 3a) between all the three solvents at both 350 and 362 nm. The Chl *a* at 382 nm, absorbed more light in acetone followed by canola and mineral oil. The absorbance of Chl *a* in these solvents (Table 5.4) was significantly different ($p < 0.05$, Appendix 3a).

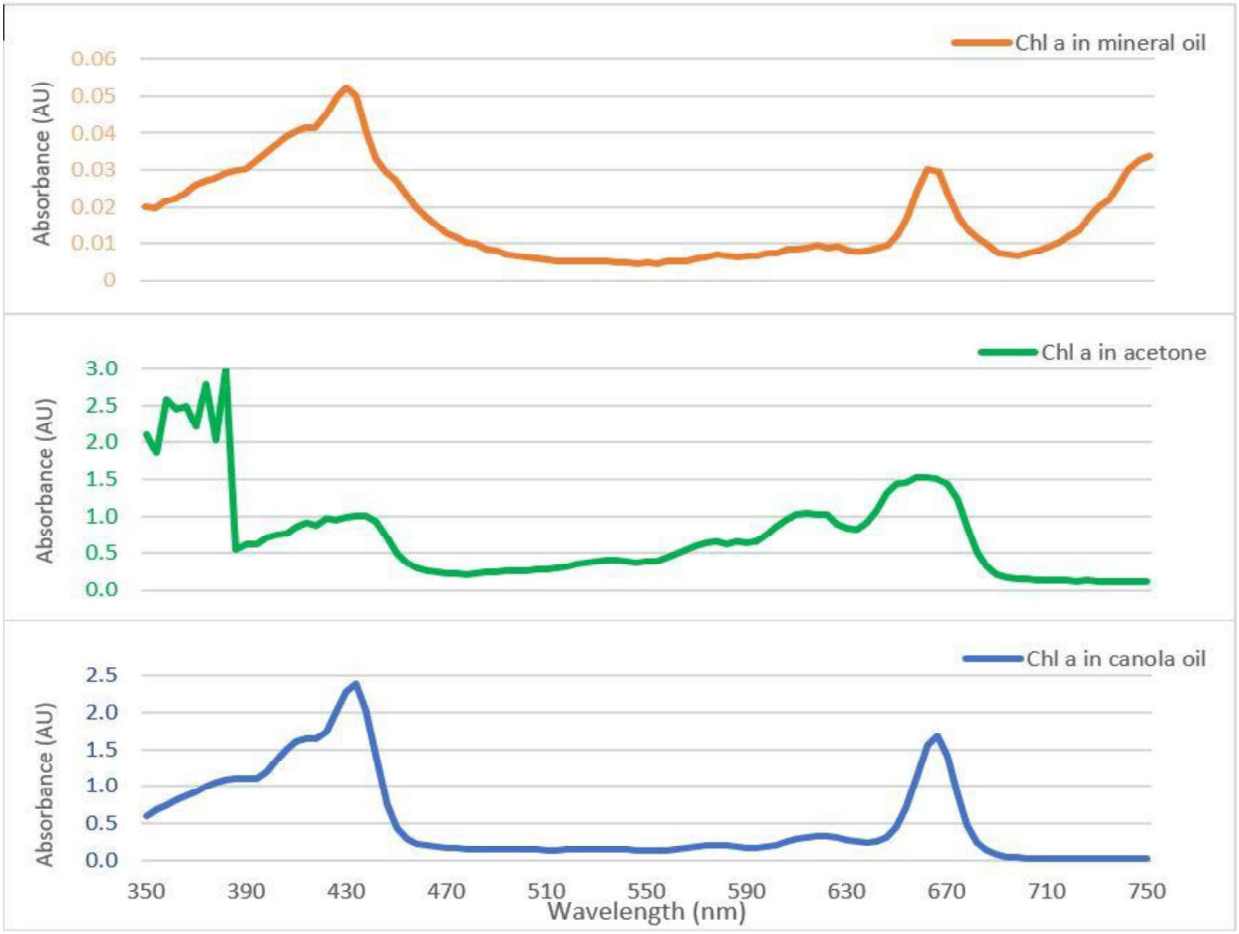


Figure 5.5: Dark absorbance spectra of Chl *a* in canola oil, acetone and mineral oil as solvents (1 mg Chl *a* in 32 ml of solvent). The absorbance values are the mean of three replicates.

At 394 nm Chl *a* absorbed more light in canola oil followed by acetone and mineral oil and a significant difference ($p < 0.05$, Appendix 3a) was found between all the three solvents. At 434 nm, which is the major light absorbing area in the blue region of spectrum reported for Chl *a* (Gross, 2012), the Chl *a* absorbed significantly different ($p < 0.05$, Appendix 3a) in these three solvents. Chl *a* absorbed higher light in canola oil and less in mineral oil, while the absorbance in acetone was between these two. The absorbance of Chl *a* at 574 and 618 nm, was significantly different ($p < 0.05$, Appendix 3a) from each solvent and was higher in acetone followed by canola oil and mineral oil (Table 5.4).

The Chl *a* absorbance at 630 nm, was high in acetone followed by canola oil and mineral oil and a significant difference ($p < 0.05$, Appendix 3a) was observed between each solvent. The 666 nm in the red region, at which the Chl *a* comprises one more major absorbance peak (Gross, 2012), at this wavelength the highest absorbance was identified in canola oil and acetone followed by mineral oil. At 710 and 750 nm, the Chl *a* had higher absorbance in acetone than canola and mineral oil, and there was no significant difference ($p > 0.05$, Appendix 3a) between the absorbance in canola and mineral oil (Figure 5.5). It was observed that Chl *a* in mineral oil absorbed light equally at both 666 and 750 nm equally (Figure 5.5) which was not observed in canola oil or acetone.

Table 5.4: Absorbance (AU) values of Chl *a* in canola oil, acetone and mineral oil as solvents.

Wavelength / Solvent	350 nm (AU)	362 nm (AU)	382 nm (AU)	394 nm (AU)	434 nm (AU)	574 nm (AU)	618 nm (AU)	630 nm (AU)	666 nm (AU)	710 nm (AU)	750 nm (AU)
Canola oil	0.60 ± 0.00^{b}	0.81 ± 0.00^{b}	1.08 ± 0.02^{b}	1.15 ± 0.03^{a}	2.38 ± 0.01^{a}	0.19 ± 0.00^{b}	0.32 ± 0.00^{b}	0.28 ± 0.00^{b}	1.69 ± 0.00^{a}	0.02 ± 0.00^{b}	0.02 ± 0.01^{b}
Acetone	2.10 ± 0.79^{a}	2.45 ± 0.64^{a}	3.00 ± 0.00^{a}	0.62 ± 0.17^{b}	0.99 ± 0.24^{b}	0.64 ± 0.05^{a}	1.02 ± 0.07^{a}	0.84 ± 0.05^{a}	1.50 ± 0.20^{a}	0.13 ± 0.05^{a}	0.11 ± 0.04^{a}
Mineral oil	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{c}	0.03 ± 0.00^{c}	0.05 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.02 ± 0.00^{b}	0.00 ± 0.00^{b}	0.03 ± 0.00^{b}

Values are the mean of three replicates ± standard deviation. Values in the same column with same letter superscript are not significantly different ($p < 0.05$).

5.5. Effect of solvent on chlorophyll *b* absorbance spectrum

The Chl *b* absorbance spectrum was found to be different in canola oil, acetone and mineral oil (Figure 5.6). The regular Chl *b* absorbance spectrum with peaks in blue (458 nm) and red (646 nm) was obtained only in canola oil but not in acetone or mineral oil. The Chl *b* at 350 and 362 nm had higher absorbance in acetone with distinct peaks which were not observed in the other two solvents (Figure 5.6). There was a significant difference ($p < 0.05$, Appendix 3b) in absorbance between each solvent at 362 nm but at 350 nm the absorbance was similar in canola oil and mineral oil (Table 5.5). The Chl *b* absorbance at 382 and 430 nm, was highest in acetone followed by canola oil and mineral oil and at 430 nm there was a significant ($p < 0.05$, Appendix 3b) difference between the absorbance of all the three solvents while at 382 nm significant difference ($p < 0.05$, Appendix 3b) was observed only between acetone and mineral oil (Table 5.5).

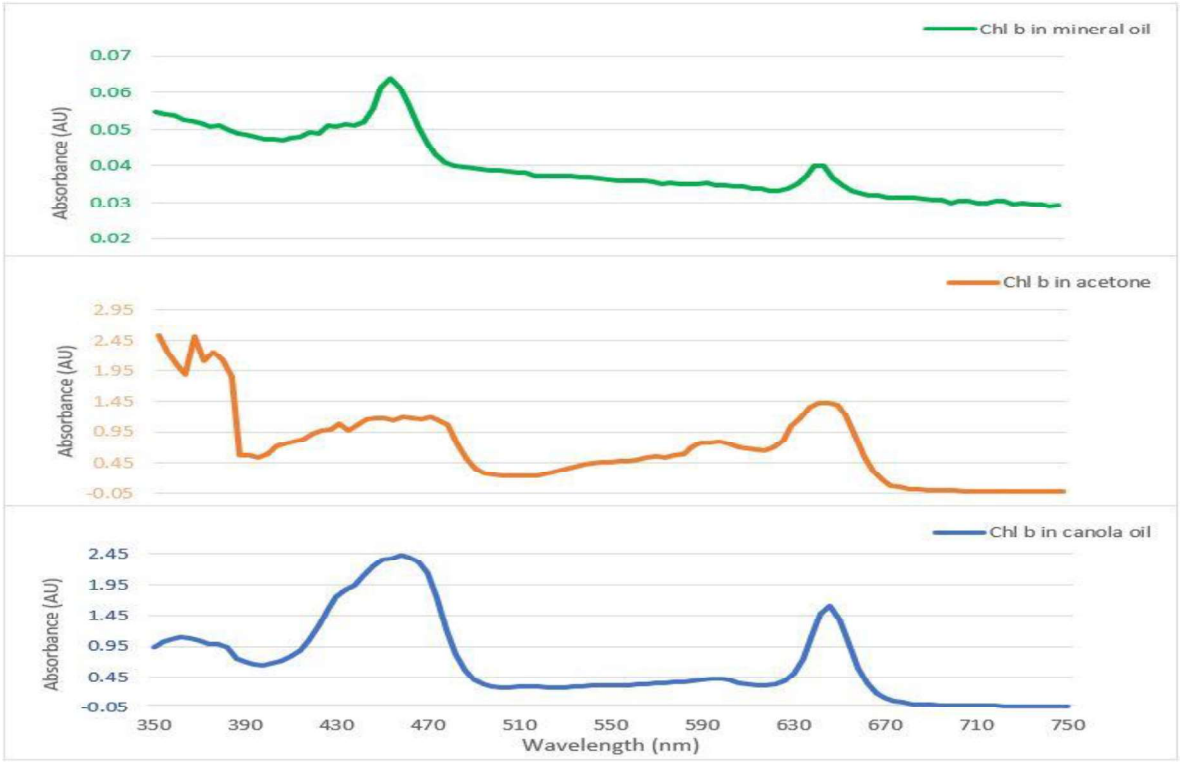


Figure 5.6: Dark absorbance spectra of Chl *b* in canola oil, acetone and mineral oil as solvents (1 mg Chl *b* in 32 ml of solvent). The absorbance values are the mean of three replicates.

At 458 nm wavelength, the highest absorbance by Chl *b* was identified in canola oil followed by acetone and mineral oil. The absorbance of Chl *b* in these solvents was significantly different ($p < 0.05$, Appendix 3b) from each other. At 518 nm, the Chl *b* absorbance was identified to be similar in canola oil and acetone and it was identified that very low light was absorbed by Chl *b* in mineral oil at this wavelength.

Table 5.5: Absorbance (AU) of chlorophyll *b* in canola oil, acetone and mineral oil solvents.

Wavelength / Solvent	350 nm (AU)	362 nm (AU)	382 nm (AU)	430 nm (AU)	458 nm (AU)	518 nm (AU)	598 nm (AU)	646 nm (AU)	710 nm (AU)	750 nm (AU)
Canola oil	0.93 ± 0.07^{b}	1.09 ± 0.09^{b}	0.93 ± 0.03^{ab}	1.76 ± 0.06^{a}	2.43 ± 0.18^{a}	0.28 ± 0.03^{a}	0.42 ± 0.04^{b}	1.61 ± 0.03^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Acetone	2.54 ± 0.79^{a}	1.89 ± 0.49^{a}	1.84 ± 1.00^{a}	1.08 ± 0.01^{b}	1.20 ± 0.10^{b}	0.24 ± 0.01^{a}	0.79 ± 0.04^{a}	1.43 ± 0.04^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Mineral oil	0.05 ± 0.00^{b}	0.05 ± 0.00^{c}	0.04 ± 0.00^{b}	0.05 ± 0.00^{c}	0.06 ± 0.00^{c}	0.03 ± 0.00^{b}	0.03 ± 0.00^{c}	0.04 ± 0.00^{c}	0.03 ± 0.00^{a}	0.02 ± 0.00^{a}

Values are the mean of three replicates ± standard deviation. Values in the same column with same letter superscript are not significantly different ($p < 0.05$).

The absorbance of Chl *b* at 598 nm, was significantly different ($p < 0.05$, Appendix 3b) in these three solvents and was highest in acetone followed by canola and mineral oil (Table 5.5). The other major light absorbing wavelength of Chl *b* was 646 nm, at this wavelength the Chl *b* absorbance was significantly different ($p < 0.05$, Appendix 3b) in these three solvents and it was found to be highest in canola oil followed by acetone and mineral oil. It was found that at both the wavelengths, where Chl *b* naturally contains major absorbance peaks i.e. 458 and 646 nm, the absorbance was highest in canola oil (Figure 5.6). The Chl *b* absorbance at 710 and 750 nm in canola oil, acetone and mineral oil was found to be negligible (Table 5.5).

6. Discussion

6.1. Interpretation of light irradiance effect on chlorophyll *a* and *b* absorbance spectra

In our study, Chl *a* under dark conditions, absorbed light in the blue (433 nm) and red (665 nm) regions of the visible spectrum, which confirms the existing literature (Taiz & Zeiger, 2010). The illumination by white light of Chl *a* in canola oil induced a gradual decrease in its average

absorbance across the full spectral range (350 - 750 nm). It was observed that Chl *a* absorbance at all wavelengths was inversely proportional to the light intensity, as the irradiance level increased the absorbance decreased. Our results show that, Chl *a* only has a slight decrease in absorbance up to 30 µmol m^{-2} sec^{-1} of light intensity but under high light intensities at 60 and 100 µmol m^{-2} sec^{-1} the absorbance peaks were significantly decreased ($P < 0.05$) and were almost unobservable at the higher irradiance levels.. A dark recovery tests after both the 45 and 100 µmol m^{-2} sec^{-1} light treatments did result a slight recovery in the absorbance, but the recovery was not significant ($P < 0.05$).

The gradual decrease in Chl *a* absorbance in methanol by UV and white light illumination was presented (Figure 6.1) and reported (Petrović et al., 2017). The two probable light induced reactions of Chl *a* are pheophytinization/demetalation in which the central magnesium atom is substituted by two hydrogen atoms which leads to the generation of hydroxy-pheophytin *a*, pheophytin *a* and the oxidative degradation process known as alomerization in which hydrogen atom at C-13 position of chlorophyll is replaced by OH or OCH_3 to generate hydroxy-lactone-pheophytin *a* (Petrović et al., 2017). These Chl *a* derivatives are seen in the thermal degradation of chlorophyll with virgin olive oil as a solvent indicating that these products are not just produced in methanol solvent and we assume the degradation of chlorophyll may follow the same process as any solvent including canola oil (Aparicio-Ruiz et al., 2010).

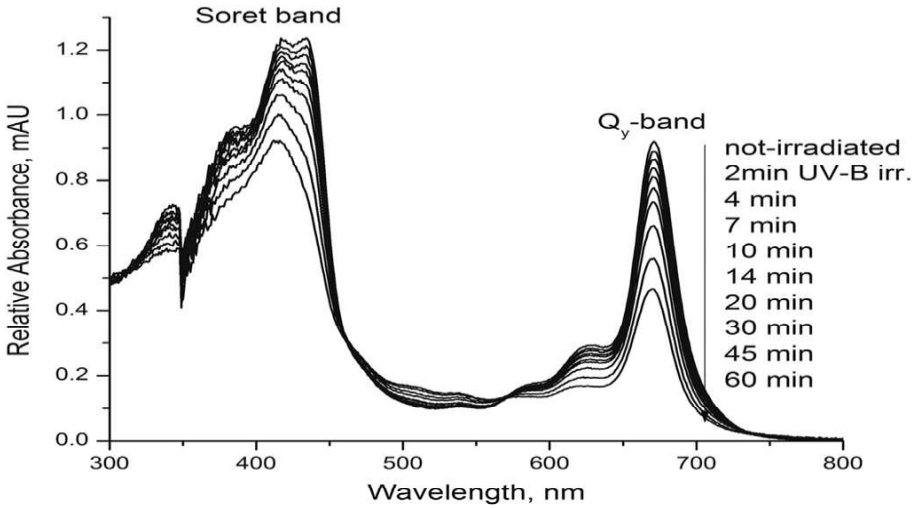


Figure 6.1: The absorbance spectrum of Chl *a* in aqueous methanol under UV and white light illumination (Petrović et al., 2017)**.**

The Chl *b* under dark conditions predominantly absorbs light in the blue (458 nm) and red (646 nm) region of the visible spectrum. The illumination of white light induced a gradual decrease in Chl *b* absorbance across the full spectral range (350 - 750 nm) indicating Chl *b* is highly sensitive to light. Chl *b* was found to be more sensitive to light compared to Chl *a*, as Chl *b* absorbance was significantly reduced ($P < 0.05$) at just 30 µmol m^{-2} sec^{-1} light intensity. Similar to Chl *a*, Chl *b* absorbance was inversely proportional to light intensity pretreatment. Chl *b* was not treated with the exact same light treatments as Chl *a* because our objective was to find the transition point (half reduction) in absorbance, which was achieved at 30 µmol m^{-2} sec^{-1} and a treatment level of 45 and 60 µmol m^{-2} sec^{-1} light intensity was not required. It was interesting to note that Chl *b* when illuminated at 30 µmol m^{-2} sec^{-1},the absorbance decreased the main peaks, but new peaks appeared at 518 and 578 nm. This results highlights that under light conditions above 30 µmol m^{-2} sec^{-1}, Chl *b* absorbs light in the green region of the spectrum. The dark recovery test showed that Chl *b* absorbance reduction and the increase in secondary peaks was irreversible, as no significant transition ($P < 0.05$) to the original state was observed.

The decrease in Chl *b* absorbance is expected to have occurred due to the Chl *b* degradation. There are many degradation mechanisms; the acid catalyzed degradation of chlorophyll to pheophytin is the most common but it is known that oxidation can cause chlorophyll degradation and form chlorophyll hydroperoxides. This oxidation might happen in our experiments because chlorophyll acts as a photosensitizer under the right light conditions and produce singlet oxygen to form atmospheric triplet oxygen (Choe & Min, 2006; Rontani et al., 2003). It is proposed that the singlet oxygen directly attacks the double bonds of chlorophyll molecule to form hydroperoxides followed by a shift in double bonds leading to a change in the absorbance spectrum. The produced hydroperoxides are cleaved by oxygen-oxygen linkages to form radicals (Chen & Huang, 1998).

Photodegradation effects both the porphyrin head and the phytol tail of chlorophyll but reacts more with the porphyrin head (Cuny et al., 1999). The major oxidation products of phytol of Chl *b* are phytone (6,10,14-trimethylpentadecan-2-one) and phytyldiol (3-methylidene-7,11,15-trimethylhexadecane-1,2-diol) (Rontani et al., 1994). Generally, Chl can be degraded at lower light intensities (25 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) as reported (Lee et al., 2014), however in our experiment it took a higher intensity (30 $\mu mol\ m^{-2}\ sec^{-1}$) to degrade because of the use of canola oil as the solvent and these lipids decrease the chlorophyll photodegradation by competing for the singlet oxygen with the chlorophyll (Lee et al., 2014).

6.2. Interpretation of chlorophyll concentration effect on its absorbance spectrum.

Our results confirm that Chl absorbance spectrum was highly influenced by the concentration of Chl. The absorbance of the sample increased gradually with an increase in Chl

concentration. The highest and lowest absorbance was identified in 3000 mg and 3 mg of chlorophyll in 10 ml of solvent, respectively. All the concentrations did not provide a clear absorbance spectrum, only 30 mg in 10 ml provided a clear spectrum with absorbance peaks in the blue (410 nm) and red (660 nm) region of the visible spectrum. The concentrations at 300 and 3000 mg in 10 ml had a very different spectrum, which might be due to Chl-Chl interaction leading to aggregation of Chl due to its high concentration. The aggregation of Chl results in spectral changes in the visible spectrum (Seliskar & Ke, 1968).

In nonpolar solvents, the Chl central magnesium atom's coordination unsaturation is satisfied by other chlorophyll's ketone oxygen function, this leading to the formation of Chl aggregates like dimers and oligomers through C=O---Mg bonding. This is the prime interaction to be accounted among Chl molecules while extraneous nucleophiles nonexistence, the amount of aggregation depends upon on several factors including the solvation characteristics of the solvent (Ballschmiter & Katz, 1969). Canola oil is a nonpolar solvent and similar interactions between Chl molecules was assumed to cause the changes in the absorbance spectrum.

6.3. Interpretation of solvent effect on chlorophyll *a* and *b* absorbance spectra

The Chl *a* and Chl *b* absorbance spectra were highly influenced by the solvent used. In our results, the spectrum shape and the absorbance were identified to be different in canola oil, acetone and mineral oil. The wavelength position of Chl *a* and Chl *b* absorbance peaks in the blue and red region was not impacted by the solvent. Both Chl *a* and Chl *b* absorbed more light in canola oil followed by acetone and mineral oil. The shoulder peak of Chl *a* at 410 nm was not observed in acetone solvent but a new peak appeared at 380 nm. It was interesting to note that in mineral oil a

new peak was observed for Chl *a* at 750 nm that absorbed light at the same intensity as with the red peak at 666 nm. In acetone, Chl *b* resulted a new peak at 380 nm, which was not observed in either canola oil or mineral oil.

For polar solvents, acetone acts as an electron donor (nucleophile) to the central Mg of the Chl molecule. When the nucleophile is bifunctional, crosslinking of Chl molecules occur through Chl-ligand-Chl interactions to form polynuclear entities. The Chl adducts formed with extraneous nucleophiles like polar solvents and have different structures from endogamous Chl-Chl aggregates formed in nonpolar solvents, thus spectral properties are highly solvent dependent (Katz et al., 1978).

The peak at 750 nm for Chl *a* in mineral oil was reported in earlier studies, it was suggested that Chl *a* in n-hexane solution exhibits a main red absorbance peak at 662 nm, a shoulder peak at 680 nm and the far-red absorption peak at 745 nm. It was concluded that peaks at 662, 680 and 745 nm are due to Chl *a* absorbance in monomer, dimer and polymer forms, respectively. It was explained that with an increase in temperature, these polymers (micro crystals) dissociate into dimers and monomers that results in the disappearance of the far-red absorption peak and can be regained when cooled (Tomita, 1968). Anderson and Calvin reported the 748 nm peak in iso-octane and claimed that on heating Chl *a* crystals to 50° C, the peak intensity at 748 nm was decreased and intensity of peak increased at 660 to 675 nm, which is due to the change from polymer to monomer and dimer (Anderson & Calvin, 1964). It was described that Chl *a* in mineral oil will aggregate with water and absorb at 743 nm and with the removal of water, Chl *a* exists in dimeric form and will have an absorbance peak at 673 nm (Sherman & Wang, 1966). These studies highlight that Chl *a* absorbs light in the far-red region (750 nm) by forming aggregates which are

solvent dependent. This unique feature of Chl *a* should be studied as it might had a specific role in the photosynthetic process by absorbing light in the far-red region.

7. Conclusion

The absorbance spectra of Chl *a* and Chl *b* in canola oil are highly impacted by the light intensity. This study showed significant difference in Chl *a* and Chl *b* absorbance spectra under different light treatments. The Chl *a* absorbed higher light energy in the blue (433 nm) and red (665 nm) region under dark conditions and after low light irradiances of 30 and 45 µmol m^{-2} sec^{-1} compared to high light irradiances of 60 and 100 µmol m^{-2} sec^{-1}. The Chl *a* absorbance in the green region (500 nm and 530 nm) increased after treatment with 45, 60 and 100 µmol m^{-2} sec^{-1}. The Chl *b* absorbance was higher in the blue (458 nm) and red region (646 nm) only under dark treatment conditions and was decreased by treating the Chl *b* with 30 µmol m^{-2} sec^{-1} light intensity, indicating that Chl *b* was more sensitive to light than Chl *a*. The Chl *b* absorbance in the green region (at 518 nm) was increased after treatment with 30 and 100 µmol m^{-2} sec^{-1} light irradiances. This study supports the conclusion that Chl *a* & *b* absorbance decreases with an increase in the intensity of light. After Chl losses its ability to absorb light, it cannot passively regain the light absorbance completely even by storing under dark conditions.

The Chl absorbance spectra appeared different under different Chl concentrations and the absorbance increased with an increase in Chl concentration. The Chl absorbance spectrum peaks occurred at roughly 430 (blue) and 660 nm (red) as measured with low Chl concentration (30 mg Chl in 10 ml canola oil). Under high concentrations, Chl appeared to absorb light almost in all regions of the visible spectrum with no distinct peaks in the blue and red region. Our research

revealed that Chl *a* and Chl *b* absorb light energy differently in canola oil, acetone and mineral oil. Chl absorbed a high amount of light in canola oil followed by acetone and mineral oil. In mineral oil, Chl *a* absorbed light in the far-red region (750 nm) along with the conventional peaks in red and blue region of visible spectrum. These studies prove that Chl absorbance spectrum is not set by having peaks in the blue and red region but rather the Chl absorbance spectrum is dynamic and determined by various factors including light conditions, Chl concentration and the solvent. The study also reveals there is no standard absorbance spectrum for chlorophyll and this absorbance spectrum can only be used to determine Chl concentration but the not photosynthetic activity. The study also raises questions like; that the chlorophyll primary function might not be light harvesting as it is highly sensitive to light.

8. Future Research

These experiments revealed that chlorophyll absorbance spectrum is dynamic and is affected by many factors like pre-light treatments, solvent and chlorophyll concentration. This emphasizes that further research is needed to find, if there are any other factors that might impact the chlorophyll absorbance like temperature or any other stress conditions. Extensive research is required to find the exact reason at molecular level that causes the shift in chlorophyll absorbance.

This research revealed that chlorophyll absorb light even in green region of spectrum when pretreated with light from a white LED. In the same manner chlorophyll pigment should be pre-light treated with red, blue, green and amber color LED's and see how chlorophyll respond to that. Further similar research should be done including other chlorophyll (*c*, *d*, e and *f*) and carotenoid pigments, as these findings help us to understand photosynthesis in a better way.

9. References

Akutsu, S., Fujinuma, D., Furukawa, H., Watanabe, T., Ohnishi-Kameyama, M., Ono, H., Kobayashi, M. (2011). Pigment analysis of a chlorophyll f-containing cyanobacterium strain KC1 isolated from Lake Biwa. *Photomedicine and Photobiology*, 33, 35-40.

Anderson, A., & Calvin, M. (1964). The aggregation of chlorophyll a. *Archives of Biochemistry and Biophysics*, 107(2), 251-259.

Aparicio-Ruiz, R., Mínguez-Mosquera, M. I., & Gandul-Rojas, B. (2010). Thermal degradation kinetics of chlorophyll pigments in virgin olive oils. Compounds of series a. *Journal of Agricultural and Food Chemistry*, 58(10), 6200-6208.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, 24(1), 1.

Ballschmiter, K., & Katz, J. J. (1969). Infrared study of chlorophyll-chlorophyll and chlorophyll-water interactions. *Journal of the American Chemical Society*, 91(10), 2661-2677.

Beale, S. I. (1999). Enzymes of chlorophyll biosynthesis. *Photosynthesis Research*, 60(1), 43-73.

Bellemare, G., Bartlett, S., & Chua, N. (1982). Biosynthesis of chlorophyll a/b-binding polypeptides in wild type and the chlorina f2 mutant of barley. *Journal of Biological Chemistry*, 257(13), 7762-7767.

Bjorn, L. O., Papageorgiou, G. C., Blankenship, R. E., & Govindjee. (2009). A viewpoint: Why chlorophyll a? *Photosynthesis Research*, 99(2), 85-98.

Boender, G., Raap, J., Prytulla, S., Oschkinat, H., & De Groot, H. (1995). MAS NMR structure refinement of uniformly 13C enriched chlorophyll a/water aggregates with 2D dipolar correlation spectroscopy. *Chemical Physics Letters*, 237(5-6), 502-508.

Britton, G. (1995). Carotenoids today and challenges for the future. In "Carotenoids" vol. by G. Britton, S. Liaaen-Jensen, and H. Pfadnar. In: Birkhäuser Verlag. Basel· Boston· Berlin.

Brown, J. S. (1977). Spectroscopy of chlorophyll in biological and synthetic systems. *Photochemistry and Photobiology*, 26(3), 319-326.

Bruuinsma, J. (1963). The quantitative analysis of chlorophylls a and b in plant extracts. *Photochemistry and Photobiology*, 2(2), 241-249.

Burger-Wiersma, T., Veenhuis, M., Korthals, H. J., Van de Wiel, C., & Mur, L. (1986). A new prokaryote containing chlorophylls a and b. *Nature*, 320(6059), 262.

Caesar, J., Tamm, A., Ruckteschler, N., Leifke, A. L., & Weber, B. (2018). Revisiting chlorophyll extraction methods in biological soil crusts–methodology for determination of chlorophyll a and chlorophyll a+ b as compared to previous methods. *Biogeosciences*, 15(5), 1415.

Chen, B., & Huang, J. (1998). Degradation and isomerization of chlorophyll a and β-carotene as affected by various heating and illumination treatments. *Food Chemistry*, 62(3), 299-307.

Chen, M., Li, Y., Birch, D., & Willows, R. D. (2012). A cyanobacterium that contains chlorophyll f, a red absorbing photopigment. *FEBS letters*, 586(19), 3249-3254.

Chen, M., Schliep, M., Willows, R. D., Cai, Z.-L., Neilan, B. A., & Scheer, H. (2010). A red-shifted chlorophyll. *Science*, 329(5997), 1318-1319.

Choe, E., & Min, D. B. (2006). Mechanisms and factors for edible oil oxidation. Comprehensive *Reviews in Food science and Food Safety*, 5(4), 169-186.

Connolly, J. S., Janzen, A. F., & Samuel, E. B. (1982). Fluorescence lifetimes of chlorophyll a: solvent, concentration and oxygen dependence. *Photochemistry and Photobiology*, 36(5), 559-563.

Cooper, G. M., & Hausman, R. (2000). A molecular approach. The Cell. 2nd edition. Sunderland, MA: Sinauer Associates.

Cox, M. T., Howarth, T., Jackson, A. H., & Kenner, G. W. (1969). Formation of the isocyclic ring in chlorophyll. *Journal of the American Chemical Society*, 91(5), 1232-1233.

Cuny, P., Romano, J.-C., Beker, B., & Rontani, J.-F. (1999). Comparison of the photodegradation rates of chlorophyll chlorin ring and phytol side chain in phytodetritus: is the phytyldiol versus phytol ratio (CPPI) a new biogeochemical index? *Journal of Experimental Marine Biology and Ecology*, 237(2), 271-290.

Davies, K. M. (2004). Plant pigments and their manipulation. Oxford: Blackwell.

Dougherty, R. C., Strain, H. H., Svec, W. A., Uphaus, R. A., & Katz, J. J. (1970). Structure, properties, and distribution of chlorophyll c. *Journal of the American Chemical Society*, 92(9), 2826-2833.

Eckhardt, U., Grimm, B., & Hörtensteiner, S. (2004). Recent advances in chlorophyll biosynthesis and breakdown in higher plants. *Plant Molecular Biology*, 56(1), 1-14.

Emerson, R., & Arnold, W. (1932). The photochemical reaction in photosynthesis. *The Journal of General Physiology*, 16(2), 191-205.

Farabee MJ (2007) Online biology book photosynthesis. Estrella Mountain Community College, Avondale, Arizona.

Fischer, H., Orth, H., & Stern, A. (1968). Die Chemie Des Pyrrols: Bd. Pyrrolfarbstoffe. (Vol. 2): Johnson Reprint Corp.

Fong, F. K., & Koester, V. J. (1976). In vitro preparation and characterization of a 700 nm absorbing chlorophyll-water adduct according to the proposed primary molecular unit in photosynthesis. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 423(1), 52-64.

Goodwin, T. W. (1976). Chemistry and biochemistry of plant pigments (2d edition.). London: Academic Press.

Goodwin, T. W. (1988). Plant Pigments. London: Academic Press.

Grimm, B., Porra, R. J., Rüdiger, W., & Scheer, H. (2007). Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications (Vol. 25): Springer Science & Business Media.

Gross, J. (2012). Pigments in vegetables: chlorophylls and carotenoids: Springer Science & Business Media.

Highkin, H. R. (1950). Chlorophyll studies on barley mutants. *Plant Physiology*, 25(2), 294.

Horn, R., Grundmann, G., & Paulsen, H. (2007). Consecutive binding of chlorophylls a and b during the assembly in vitro of light-harvesting chlorophyll-a/b protein (LHCIIb). *Journal of Molecular Biology*, 366(3), 1045-1054.

Hu, Q., Miyashita, H., Iwasaki, I., Kurano, N., Miyachi, S., Iwaki, M., & Itoh, S. (1998). A photosystem I reaction center driven by chlorophyll d in oxygenic photosynthesis. *Proceedings of the National Academy of Sciences*, 95(22), 13319-13323.

Jeffrey, S. (1976). The occurrence of chlorophyll c1 and c2 in algae 1. *Journal of Phycology*, 12(3), 349-354.

Katz, J. j., & Norris Jr, J. R. (1973). Chlorophyll and light energy transduction in photosynthesis. In Current Topics in Bioenergetics (Vol. 5, pp. 41-75): Elsevier.

Katz, J. J., Norris, J. R., Shipman, L. L., Thurnauer, M. C., & Wasielewski, M. R. (1978). Chlorophyll function in the photosynthetic reaction center. *Annual Review of Biophysics and Bioengineering*, 7(1), 393-434.

Katz, J. J., Shipman, L. L., Cotton, T. M., & Janson, T. R. (1978). Chlorophyll aggregation: coordination interactions in chlorophyll monomers, dimers, and oligomers. The Porphyrins, 5, 401-458.

Katz, J., Ballschmiter, K., Garcia-Morin, M., Strain, H., & Uphaus, R. (1968). Electron paramagnetic resonance of chlorophyll-water aggregates. *Proceedings of the National Academy of Sciences* of the United States of America, 60(1), 100.

Katz, J., Strain, H., Harkness, A., Studier, M., Svec, W., Janson, T., & Cope, B. (1972). Esterifying alcohols in the chlorophylls of purple photosynthetic bacteria. New chlorophyll, bacteriochlorophyll (gg), all-trans-geranylgeranyl bacteriochlorophyllide a. *Journal of the American Chemical Society*, 94(22), 7938-7939.

Kräutler, B. (2008). Chlorophyll breakdown and chlorophyll catabolites in leaves and fruit. *Photochemical & Photobiological Sciences*, 7(10), 1114-1120.

Krol, M., Spangfort, M. D., Huner, N. P., Oquist, G., Gustafsson, P., & Jansson, S. (1995). Chlorophyll a/b-binding proteins, pigment conversions, and early light-induced proteins in a chlorophyll b-less barley mutant. *Plant Physiology*, 107(3), 873-883.

Kühl, M., Chen, M., Ralph, P. J., Schreiber, U., & Larkum, A. W. (2005). A niche for cyanobacteria containing chlorophyll d. *Nature*, 433(7028), 820-820.

Kusaba, M., Ito, H., Morita, R., Iida, S., Sato, Y., Fujimoto, M., Nishimura, M. (2007). Rice non-yellow coloring is involved in light-harvesting complex II and grana degradation during leaf senescence. *The Plant Cell*, 19(4), 1362-1375.

Larkum, A. W., & Kühl, M. (2005). Chlorophyll d: the puzzle resolved. *Trends in Plant Science*, 10(8), 355-357.

Lee, E., Ahn, H., & Choe, E. (2014). Effects of light and lipids on chlorophyll degradation. *Food Science and Biotechnology*, 23(4), 1061-1065.

Li, Y., & Chen, M. (2015). Novel chlorophylls and new directions in photosynthesis research. *Functional Plant Biology*, 42(6), 493-501.

Livingston, R., Watson, W., & McArdle, J. (1949). Activation of the fluorescence of chlorophyll solutions. *Journal of the American Chemical Society*, 71(5), 1542-1550.

Lockstein, H., Grimm B. (2007). Chlorophyll binding proteins. Encyclopedia of Life Cycles. John Wiley & Sons, New York.

Mackinney, G. (1941). Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry*, 140(2), 315-322.

Manning, W. M., & Strain, H. H. (1943). Chlorophyll d, a green pigment of red algae. *Journal of Biological Chemistry*, 151(1), 1-19.

Mínguez-Mosquera, M. I., Gandul-Rojas, B., Gallardo-Guerrero, L., & Jarén-Galán, M. (2002). Chlorophylls. Methods of Analysis for Functional Foods and Nutraceuticals, 159-218.

Mishra, S. R. (2004). Photosynthesis in plants: Discovery Publishing House.

Nagata, N., Satoh, S., Tanaka, R., & Tanaka, A. (2004). Domain structures of chlorophyllide a oxygenase of green plants and Prochlorothrix hollandica in relation to catalytic functions. *Planta*, 218(6), 1019-1025.

Niedzwiedzki, D. M., & Blankenship, R. E. (2010). Singlet and triplet excited state properties of natural chlorophylls and bacteriochlorophylls. *Photosynthesis Research*, 106(3), 227-238.

Ohkubo, S., Usui, H., & Miyashita, H. (2011). Unique chromatic adaptation of a unicellular cyanobacterium newly isolated from Lake Biwa. Japan. *Journal of Phycology*.(Sorui), 59(52), A22.

Oster, U., Tanaka, R., Tanaka, A., & Rüdiger, W. (2000). Cloning and functional expression of the gene encoding the key enzyme for chlorophyll b biosynthesis (CAO) from Arabidopsis thaliana. *The Plant Journal*, 21(3), 305-310.

Papenbrock, J., Gräfe, S., Kruse, E., Hänel, F., & Grimm, B. (1997). Mg-chelatase of tobacco: identification of a Chl D cDNA sequence encoding a third subunit, analysis of the interaction of

the three subunits with the yeast two-hybrid system, and reconstitution of the enzyme activity by co-expression of recombinant CHL D, CHL H and CHL I. *The Plant Journal*, 12(5), 981-990.

Papenbrock, J., Mock, H.-P., Kruse, E., & Grimm, B. (1999). Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. *Planta*, 208(2), 264-273.

Pelletier, J., & Caventou, J.-B. (1818). Sur la matière verte des feuilles. *Annales de Chimie et de Physique*, 9, 194–196.

Petrović, S., Zvezdanović, J., & Marković, D. (2017). Chlorophyll degradation in aqueous mediums induced by light and UV-B irradiation: An UHPLC-ESI-MS study. *Radiation Physics and Chemistry*, 141, 8-16.

Photobiology, N. R. C. C. o. (1963). Photosynthetic mechanisms of green plants (Vol. 1145): *National Academies*.

Rebeiz, C. A. (2014). Some major steps in the understanding of the chemistry and biochemistry of Chl. In Chlorophyll Biosynthesis and Technological Applications (pp. 1-32): Springer.

Rontani, J. F., Rabourdin, A., Marchand, D., & Aubert, C. (2003). Photochemical oxidation and autoxidation of chlorophyll phytol side chain in senescent phytoplanktonic cells: potential sources of several acyclic isoprenoid compounds in the marine environment. *Lipids*, 38(3), 241-254.

Rontani, J., Grossi, V., Faure, R., & Aubert, C. (1994). “Bound” 3-methylidene-7, 11, 15-trimethylhexadecan-1, 2-diol: a new isoprenoid marker for the photodegradation of chlorophyll-a in seawater. *Organic Geochemistry*, 21(2), 135-142.

Schoefs, B. t. (2002). Chlorophyll and carotenoid analysis in food products. Properties of the pigments and methods of analysis. *Trends in Food Science & Technology*, 13(11), 361-371.

Seliskar, C., & Ke, B. (1968). Protochlorophyllide aggregation in solution and associated spectral changes. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 153(3), 685-691.

Shemer, T. A., Harpaz-Saad, S., Belausov, E., Lovat, N., Krokhin, O., Spicer, Eyal, Y. (2008). Citrus chlorophyllase dynamics at ethylene-induced fruit color-break: a study of chlorophyllase expression, posttranslational processing kinetics, and in situ intracellular localization. *Plant Physiology*, 148(1), 108-118.

Sherman, G., & Wang, S. F. (1966). Effect of water on the visible spectrum of Chlorophyll A in the solid state. *Nature*, 212(5062), 588-590.

Taiz, L., & Zeiger, E. (1998). *Plant Physiology* (2nd edition.). Sunderland, Mass.: Sinauer Associates.

Taiz, L., & Zeiger, E. (2010). *Plant physiology fifth edition*. Sunderland, Massachusetts, USA: Sinauer Assosiates inc., Publishers.

Taiz, L., Zeiger, E. (2002). Photosynthesis: The light reactions. *Plant Physiology* 7:130-148

Tanaka, A., & Tanaka, R. (2006). Chlorophyll metabolism. *Current Opinion in Plant Biology*, 9(3), 248-255.

Tanaka, A., Ito, H., Tanaka, R., Tanaka, N. K., Yoshida, K., & Okada, K. (1998). Chlorophyll a oxygenase (CAO) is involved in chlorophyll b formation from chlorophyll a. *Proceedings of the National Academy of Sciences*, 95(21), 12719-12723.

Tanaka, R., & Tanaka, A. (2011a). Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1807(8), 968-976.

Tanaka, R., & Tanaka, A. (2011b). Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. *Biochimica et Biophysica Acta (BBA) -Bioenergetics*, 1807(8), 968-976.

Thornber, J. P., & Highkin, H. R. (1974). Composition of the photosynthetic apparatus of normal barley leaves and a mutant lacking chlorophyll b. *European Journal of Biochemistry*, 41(1), 109-116.

Tomita, G. (1968). Dimer and polymers in solutions of chlorophyll a. *Biophysik*, 4(4), 296-301.

Tomo, T., Okubo, T., Akimoto, S., Yokono, M., Miyashita, H., Tsuchiya, T., Mimuro, M. (2007). Identification of the special pair of photosystem II in a chlorophyll d-dominated cyanobacterium. *Proceedings of the National Academy of Sciences*, 104(17), 7283-7288.

Tswett, M. (1906). Physikalisch-chemische Studien über das chlorophyll. Die Adsorptionen. 24(316-323), 20.

Yamasato, A., Nagata, N., Tanaka, R., & Tanaka, A. (2005). The N-terminal domain of chlorophyllide a oxygenase confers protein instability in response to chlorophyll b accumulation in Arabidopsis. *The Plant Cell*, 17(5), 1585-1597.

Zscheile, F., Comar, C., & Mackinney, G. (1942). Interlaboratory comparison of absorption spectra by the photoelectric spectrophotometric method. determinations on chlorophyll and weigert's solutions. *Plant Physiology*, 17(4), 666.

. 10. Appendix 1a: p-values of Tukey-Kramer HSD comparisons of all pairs for light effect on Chl *a*

(a = dark, b = 30 µmol m^{-2} sec^{-1}, c = 45 µmol m^{-2} sec^{-1} , d = 45 µmol m^{-2} sec^{-1} recovery, e =60 µmol m^{-2} sec^{-1}, f = 100 µmol m^{-2} sec^{-1}, g = 100 µmol m^{-2} sec^{-1} recovery)

350 nm			380 nm			410 nm			433 nm			450 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
c	g	<.0001*	a	g	<.0001*	a	g	<.0001*	a	f	<.0001*	c	g	<.0001*
b	g	<.0001*	b	g	<.0001*	b	g	<.0001*	a	g	<.0001*	c	a	0.0018*
a	g	0.0002*	a	e	<.0001*	a	f	<.0001*	b	f	<.0001*	c	e	0.0018*
c	e	0.0008*	b	e	<.0001*	b	f	<.0001*	b	g	<.0001*	c	d	0.0040*
f	g	0.0034*	a	f	<.0001*	a	e	<.0001*	a	e	<.0001*	c	f	0.0071*
d	g	0.0057*	b	f	<.0001*	b	e	<.0001*	b	e	<.0001*	c	b	0.0088*
b	e	0.0082*	c	g	<.0001*	c	g	<.0001*	d	f	<.0001*	b	g	0.0537
c	d	0.0234*	c	e	<.0001*	d	g	<.0001*	d	g	<.0001*	f	g	0.0661
a	e	0.0250*	d	g	<.0001*	c	f	<.0001*	c	f	<.0001*	d	g	0.1137
c	f	0.0395*	c	f	<.0001*	d	f	<.0001*	c	g	<.0001*	a	g	0.2369
e	g	0.1613	d	e	<.0001*	c	e	<.0001*	d	e	<.0001*	e	g	0.2369
b	d	0.2221	d	f	0.0003*	d	e	<.0001*	c	e	<.0001*	b	a	0.9678
b	f	0.3368	a	d	0.0011*	a	d	<.0001*	a	c	<.0001*	b	e	0.9678
f	e	0.3650	b	d	0.0019*	a	c	<.0001*	a	d	<.0001*	f	a	0.9839
c	a	0.4976	a	c	0.0196*	b	d	0.0001*	b	c	<.0001*	f	e	0.9839
a	d	0.5043	b	c	0.0349*	b	c	0.0003*	b	d	0.0001*	d	a	0.9990
d	e	0.5180	f	g	0.2647	e	g	0.3285	e	f	0.1806	d	e	0.9990
a	f	0.6716	c	d	0.6785	e	f	0.8411	e	g	0.2151	b	d	0.9993
c	b	0.8365	e	g	0.7667	f	g	0.9562	a	b	0.9014	f	d	0.9999
b	a	0.9952	f	e	0.9559	a	b	0.9663	d	c	0.9293	b	f	1.0000
f	d	0.9999	a	b	0.9999	c	d	0.9977	g	f	1.0000	e	a	1.0000

475 nm			500 nm			530 nm			575 nm			620 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
f	a	<.0001*	f	a	<.0001*	f	a	<.0001*	c	g	<.0001*	a	g	<.0001*
f	b	<.0001*	f	b	<.0001*	f	b	<.0001*	b	g	<.0001*	b	g	<.0001*
f	d	<.0001*	f	d	<.0001*	c	a	0.0002*	a	g	<.0001*	a	f	<.0001*
e	a	0.0001*	c	a	0.0001*	f	d	0.0002*	f	g	<.0001*	c	g	<.0001*
c	a	0.0002*	e	a	0.0001*	f	g	0.0002*	d	g	0.0002*	b	f	<.0001*
g	a	0.0003*	f	g	0.0008*	c	b	0.0016*	e	g	0.0005*	a	e	<.0001*
e	b	0.0009*	c	b	0.0008*	e	a	0.0022*	c	e	0.0005*	b	e	<.0001*
c	b	0.0015*	e	b	0.0009*	c	d	0.0128*	c	d	0.0011*	c	f	<.0001*
g	b	0.0018*	g	a	0.0011*	c	g	0.0128*	c	f	0.0130*	d	g	<.0001*
f	g	0.0023*	f	e	0.0078*	e	b	0.0198*	c	a	0.0391*	c	e	<.0001*
f	c	0.0028*	f	c	0.0088*	f	e	0.0291*	b	e	0.0522	d	f	<.0001*
f	e	0.0049*	g	b	0.0095*	e	d	0.1531	b	d	0.1206	a	d	<.0001*
e	d	0.0369*	c	d	0.0360*	e	g	0.1531	c	b	0.1906	b	d	<.0001*
c	d	0.0640	e	d	0.0404*	d	a	0.2715	a	e	0.2439	d	e	<.0001*
d	a	0.0751	d	a	0.0593	g	a	0.2715	a	d	0.4673	e	g	0.0003*
g	d	0.0775	g	d	0.3445	f	c	0.3042	f	e	0.5354	a	c	0.0049*
d	b	0.4065	d	b	0.3968	c	e	0.7898	b	f	0.7221	c	d	0.0086*
b	a	0.9205	c	g	0.8039	b	a	0.8747	f	d	0.8071	f	g	0.0092*
e	g	0.9994	e	g	0.8347	d	b	0.8949	b	a	0.9613	b	c	0.0152*
e	c	0.9999	b	a	0.8805	g	b	0.8949	a	f	0.9955	e	f	0.4733
c	g	1.0000	c	e	1.0000	g	d	1.0000	d	e	0.9987	a	b	0.9949

665 nm			685 nm			700 nm		
Treatment	**Treatment**	**p-Value**	**Treatment**	**Treatment**	**p-Value**	**Treatment**	**Treatment**	**p-Value**
a	f	<.0001*	c	g	<.0001*	c	a	<.0001*
a	g	<.0001*	c	f	<.0001*	e	a	<.0001*
b	f	<.0001*	c	d	<.0001*	c	d	<.0001*
b	g	<.0001*	b	g	<.0001*	f	a	<.0001*
a	e	<.0001*	a	g	<.0001*	e	d	<.0001*
b	e	<.0001*	e	g	<.0001*	c	b	<.0001*
d	f	<.0001*	c	e	<.0001*	c	g	<.0001*
d	g	<.0001*	c	a	<.0001*	e	b	<.0001*
c	f	<.0001*	c	b	<.0001*	f	d	<.0001*
c	g	<.0001*	d	g	<.0001*	e	g	<.0001*
d	e	<.0001*	b	f	0.0002*	f	b	<.0001*
a	c	<.0001*	f	g	0.0004*	f	g	<.0001*
c	e	<.0001*	b	d	0.0008*	g	a	0.4760
a	d	<.0001*	a	f	0.0075*	b	a	0.6756
b	c	<.0001*	e	f	0.0116*	c	f	0.9191
b	d	<.0001*	a	d	0.0335*	g	d	0.9358
e	f	0.1044	e	d	0.0517	d	a	0.9623
e	g	0.2018	b	e	0.2961	e	f	0.9863
a	b	0.7329	b	a	0.4097	b	d	0.9909
d	c	0.9889	d	f	0.9780	c	e	0.9998
g	f	0.9995	a	e	1.0000	g	b	0.9998

Appendix 1b: p-values of Tukey-Kramer HSD comparisons of all pairs for light effect on Chl *b*

(a = dark, b = 30 µmol m^{-2} sec^{-1}, c = 100 µmol m^{-2} sec^{-1}, d = 100 µmol m^{-2} sec^{-1} recovery)

350 nm			366 nm			382 nm			398 nm			410 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
a	d	0.0076*	a	d	<.0001*	a	d	<.0001*	b	d	0.0013*	a	d	<.0001*
a	b	0.0082*	a	c	0.0003*	b	d	0.0013*	c	d	0.0057*	b	d	0.0004*
a	c	0.0082*	a	b	0.0003*	a	c	0.0014*	b	a	0.0250*	c	d	0.0054*
c	d	0.9999	b	d	0.0074*	c	d	0.0022*	c	a	0.1510	a	c	0.0075*
b	d	0.9999	c	d	0.0102*	a	b	0.0024*	a	d	0.1510	b	c	0.1725
c	b	1.0000	b	c	0.9940	b	c	0.9683	b	c	0.5969	a	b	0.1792
458 nm			**518 nm**			**570 nm**			**586 nm**			**610 nm**		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
a	d	<.0001*	b	a	<.0001*	b	d	<.0001*	b	d	<.0001*	a	d	<.0001*
a	c	<.0001*	c	a	<.0001*	c	d	<.0001*	a	d	<.0001*	b	d	0.0002*
a	b	<.0001*	b	d	<.0001*	b	a	0.0002*	c	d	0.0001*	c	d	0.0011*
b	d	<.0001*	c	d	<.0001*	c	a	0.0003*	b	c	0.5527	a	c	0.0221*
b	c	0.0003*	d	a	0.0003*	a	d	0.0215*	b	a	0.7837	a	b	0.2711
c	d	0.2170	b	c	0.8265	b	c	0.9472	a	c	0.9733	b	c	0.3351
646 nm			**666 nm**			**710 nm**								
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value						
a	d	<.0001*	b	d	0.0001*	c	a	0.0291*						
a	c	<.0001*	a	d	0.0012*	c	d	0.0715						
a	b	<.0001*	b	c	0.0085*	b	a	0.0742						
b	d	<.0001*	c	d	0.0159*	b	d	0.1815						
b	c	<.0001*	b	a	0.1578	c	b	0.9065						
c	d	0.0133*	a	c	0.2240	d	a	0.9159						

Appendix 2a: p-values of Tukey-Kramer HSD comparisons of all pairs for concentration effect on Chl

(a = 3mg in 10 ml, b = 30 mg in 10ml, c = 300 mg in 10 ml, d = 3000 mg in 10 ml)

350 nm			382 nm			410 nm			510 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
c	a	0.0052*	c	a	<.0001*	d	a	<.0001*	d	a	<.0001*
d	a	0.0121*	d	a	<.0001*	c	a	<.0001*	c	a	<.0001*
c	b	0.0132*	c	b	<.0001*	d	b	<.0001*	d	b	<.0001*
d	b	0.0326*	d	b	<.0001*	c	b	<.0001*	c	b	<.0001*
b	a	0.8815	b	a	0.2041	b	a	0.0001*	b	a	0.0086*
c	d	0.9070	c	d	0.5283	d	c	0.4171	d	c	0.0481*
586 nm			**630 nm**			**666 nm**			**750 nm**		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
d	a	<.0001*	d	a	<.0001*	d	a	<.0001*	d	a	<.0001*
c	a	<.0001*	c	a	<.0001*	c	a	<.0001*	d	b	<.0001*
d	b	<.0001*	d	b	<.0001*	d	b	<.0001*	c	a	<.0001*
c	b	<.0001*	c	b	<.0001*	c	b	<.0001*	c	b	<.0001*
b	a	0.0066*	b	a	0.0018*	b	a	<.0001*	d	c	0.0010*
d	c	0.0254*	d	c	0.0065*	d	c	0.5957	b	a	0.0500*

Appendix 3a: p-values of Tukey-Kramer HSD comparisons of all pairs for solvent effect on Chl *a*

(a = canola oil, b = acetone, c = mineral oil)

350 nm			362 nm			382 nm			394 nm			434 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
b	c	0.0034*	b	c	0.0005*	b	c	<.0001*	a	c	<.0001*	a	c	<.0001*
b	a	0.0164*	b	a	0.0041*	b	a	<.0001*	b	c	0.0009*	a	b	<.0001*
a	c	0.3334	a	c	0.0933	a	c	<.0001*	a	b	0.0023*	b	c	0.0004*
574 nm			**618 nm**			**630 nm**			**666 nm**			**710 nm**		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
b	c	<.0001*	b	c	<.0001*	b	c	<.0001*	a	c	<.0001*	b	c	0.0054*
b	a	<.0001*	b	a	<.0001*	b	a	<.0001*	b	c	<.0001*	b	a	0.0110*
a	c	0.0006*	a	c	0.0004*	a	c	0.0001*	a	b	0.2166	a	c	0.7680
750 nm														
Treatment	Treatment	p-Value												
b	a	0.0209*												
b	c	0.0305*												
c	a	0.9431												

Appendix 3b: p-values of Tukey-Kramer HSD comparisons of all pairs for solvent effect on Chl *b*

(a = canola oil, b = acetone, c = mineral oil)

350 nm			362 nm			382 nm			430 nm			458 nm		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
b	c	0.0014*	b	c	0.0006*	b	c	0.0216*	a	c	<.0001*	a	c	<.0001*
b	a	0.0121*	a	c	0.0110*	b	a	0.2175	b	c	<.0001*	a	b	<.0001*
a	c	0.1243	b	a	0.0369*	a	c	0.2272	a	b	<.0001*	b	c	<.0001*
518 nm			**598 nm**			**646 nm**			**710 nm**			**750 nm**		
Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value	Treatment	Treatment	p-Value
a	c	<.0001*	b	c	<.0001*	a	c	<.0001*	c	a	0.1081	c	a	0.0636
b	c	0.0001*	a	c	<.0001*	b	c	<.0001*	c	b	0.1633	c	b	0.1147
a	b	0.1315	b	a	<.0001*	a	b	0.0015*	b	a	0.9446	b	a	0.8897