แคโรทีนอยด์และเมแทบอลิซึมของจุลสาหร่ายสีเขียว *Chlorococcum* sp. 8367RE
ภายใต้ภาวะเครียด

นางสาวกัณฐิมา จันทร์โชติ

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CAROTENOIDS AND METABOLISM OF THE GREEN MICROALGA *Chlorococcum* sp. 8367RE UNDER STRESS CONDITIONS

Miss Kantima Janchot

A Thesis Submitted in Partial Fulfillment of the Requirements
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Field of Study	Microbiology and Microbial Technology
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กัณฐิมา จันทร์โชติ : แคโรทีนอยด์และเมแทบอลิซึมของจุลสาหร่ายสีเขียว *Chlorococcum* sp. 8367RE ภายใต้ภาวะเครียด (CAROTENOIDS AND METABOLISM OF THE GREEN MICROALGA *Chlorococcum* sp. 8367RE UNDER STRESS CONDITIONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.รุ่งอรุณ วาดิถี สิริศรัทธา, หน้า.

จุลสาหร่ายสีเขียวเป็นหนึ่งในแหล่งทรัพยากรสำหรับการผลิตสารออกฤทธิ์ทางชีวภาพที่หลากหลาย และมีคุณประโยชน์ ตัวอย่างเช่น กลุ่มสารคัดกรองรังสียูวี กลุ่มสารแคโรทีนอยด์ เป็นต้น การศึกษานี้มุ่งเน้นศึกษาการเปลี่ยนแปลงชนิดของสารในกลุ่มแคโรทีนอยด์และเมแทบอลิซึมของจุลสาหร่ายสีเขียว *Chlorococcum* sp. 8367RE ภายใต้ภาวะเครียด ผลการทดลองแสดงให้เห็นว่าภาวะเครียดจากเกลือ ความเข้มแสง ความแห้งแล้ง และการขาดไนโตรเจน ทำให้ลักษณะสัณฐานวิทยาของจุลสาหร่ายสีเขียวชนิดนี้เปลี่ยนไป เป็นที่น่าสนใจว่าภาวะเครียดจากเกลือ โดยเฉพาะโพแทสเซียมคลอไรด์ส่งผลให้เซลล์มีขนาดใหญ่ขึ้นถึง 5 เท่า เมื่อวิเคราะห์ชนิดและปริมาณของแคโรทีนอยด์โดยใช้เทคนิค Ultra-high performance liquid chromatography (UHPLC) ร่วมกับ Mass spectrometry พบว่าแคโรทีนอยด์ชนิดหลักที่พบในสารสกัดจากจุลสาหร่ายที่เลี้ยงภายใต้ภาวะปกติมี 3 ชนิด ได้แก่ วิโอลาแซนธิน, แอนธีราแซนธิน และลูทีน ใน 3 ชนิดนี้พบปริมาณลูทีนสูงที่สุด คือ 2.24 ไมโครกรัม/มิลลิกรัมน้ำหนักแห้ง ส่วนในสารสกัดจากจุลสาหร่ายที่เลี้ยงภายใต้ภาวะเครียดจากโพแทสเซียมคลอไรด์พบแคโรทีนอยด์เพิ่มขึ้นอีก 2 ชนิด ได้แก่ แอสตาแซนธิน และแคนตาแซนธิน เป็นที่น่าสังเกตว่าภายใต้ภาวะเครียดดังกล่าวส่งผลให้ปริมาณลูทีนใกล้เคียงกับภาวะปกติ แต่ส่งผลกระตุ้นการผลิตแอสตาแซนธินได้ 0.12 ไมโครกรัม/มิลลิกรัมน้ำหนักแห้ง สำหรับภาวะเครียดจากการขาดไนโตรเจนพบว่าปริมาณแคโรทีนอยด์ทุกชนิดลดลง นอกจากนี้ยังได้วิเคราะห์การเปลี่ยนแปลงของกรดไขมัน โปรตีน กรดอะมิโน และคาร์โบไฮเดรต พบว่าภาวะเครียดจากโพแทสเซียมคลอไรด์ และการขาดไนโตรเจนส่งผลให้ปริมาณกรดไขมันทั้งหมดเพิ่มขึ้นประมาณ 2 เท่า (12.79 ± 0.03 % น้ำหนักแห้ง) และ 4 เท่า (28.07 ± 0.79 % น้ำหนักแห้ง) ตามลำดับ โดยกรดโอเลอิกเพิ่มขึ้นมากที่สุด จากการศึกษาครั้งนี้ชี้ให้เห็นว่าภาวะเครียดจากเกลือ ส่งผลทำให้เกิดการเปลี่ยนแปลงสัณฐานวิทยาของจุลสาหร่ายที่เด่นชัด กระตุ้นการสะสมแคโรทีนอยด์ทุติยภูมิชนิดที่มีมูลค่าสูง รวมถึงกรดไขมันโอเมก้า 9 ดังนั้นน่าจะเป็นประโยชน์ในอุตสาหกรรมที่เกี่ยวข้องกับสุขภาพ และอาหารเสริม

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สาขาวิชา จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KANTIMA JANCHOT: CAROTENOIDS AND METABOLISM OF THE GREEN MICROALGA *Chlorococcum* sp. 8367RE UNDER STRESS CONDITIONS. ADVISOR: ASSOC. PROF. RUNGAROON WADITEE-SIRISATTHA, Ph.D., pp.

Green microalgae are one of the natural resources for productions of various and beneficial bioactive compounds, such as UV-screening compounds and carotenoids. This study focuses on the changes of carotenoids and metabolisms in the green microalga *Chlorococcum* sp. 8367RE under stress conditions. The results showed that stresses by salt, light, drought, and nitrogen starvation affected on alterations of morphological characteristics. Interestingly, size of cells culturing under salt stresses dramatically increased. It should be noted that KCl stress caused the enlargement of cell sizes approximately 5 folds. Carotenoids were identified and quantified by using ultra-high performance liquid chromatography (UHPLC) and mass spectrometry. Three kinds of carotenoids namely violaxanthin, antheraxanthin and lutein were found in algal extracts culturing under control condition. Among these carotenoids, lutein was detected as the highest content 2.24 μg/mg DW. Upon KCl stress, two types of carotenoids; astaxanthin and canthaxanthin, were additionally identified. It is noteworthy that this stress condition, lutein content was very similar level as control while astaxanthin was accumulated 0.12 μg/mg DW. For nitrogen starvation, total carotenoids were decreased. Furthermore, fatty acids, proteins, amino acids, and carbohydrates were analyzed. KCl stress and nitrogen starvation led to the increment of fatty acids contents approximately 2 times (12.79 ± 0.03% DW) and 4 times (28.07 ± 0.79% DW), respectively. Oleic acid was found to be the predominant fatty acid. This study indicates that salt stress resulted in the remarkable alteration of cells morphology and the enhancement of valuable secondary carotenoids and omega-9 fatty acid accumulation. These would be advantageous in health and nutraceutical industries.

Department:	Microbiology	Student's Signature	.....
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	.....
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CHAPTER I
INTRODUCTION

Carotenoids are a group of red, orange or yellow pigments which are constituted by isoprene units and classed as tetraterpenes. These pigments typically contain 40 carbon atoms with conjugated double bond. Currently, over 750 carotenoids are discovered (Rivera Vélez, 2016). In the nature, carotenoids are found in plants, algae, yeast, some bacteria and animals. They are biosynthesized by photosynthetic organisms, mainly in plants and algae; however, carotenoids in animals or non-photosynthetic organisms are obtained from the dietary. The biological function of carotenoids in photosynthetic organism is accessory pigments to absorb solar energy at wavelengths between 400-500 nm (Erdoğan *et al.*, 2015). Besides, carotenoids also possess antioxidant and anti-inflammatory properties (Raposo *et al.*, 2015). Based on their structures, carotenoids are categorized into 2 groups, *i.e.* carotenes and xanthophylls (Kiokias *et al.*, 2016). Carotenes consist of only carbon and hydrogen in their structures. The pigments in carotenes group include lycopene, γ-carotene, **α**-carotene and β-carotene. Xanthophylls are oxygenated hydrocarbon and may include functional groups such as epoxy, carbonyl, hydroxy, methoxy, carbonyl and carboxyl. The pigments in this group include lutein, zeaxanthin, astaxanthin, neoxanthin and violaxanthin.

Green microalgae are photosynthetic organisms which is commonly found in all habitats, such as freshwater, marine, terrestrial and extreme environments. Therefore, green microalgae have ability to adapt well in the environments and can survive in stress conditions, such as light, salinity stress, heat stress, drought stress and osmotic stress (Borowitzka *et al.*, 2016). Various environmental stresses affect to microalgal cells. Thus, microalgae are necessary to adapt the several parts including

physiology, metabolism and the signaling system for environmental stresses responding (Vonshak and Torzillo, 2004). Moreover, microalgae have various strategies to alter their primary metabolite and produce secondary metabolite for cells survival (Skjånes *et al.*, 2013). Accumulating evidence revealed that, green microalgae are used as a source of bioactive compounds because they assimilate only light, carbon dioxide and water to produce the biomass and valuable biocompounds. It has been reported that green microalgae can also produce lipids that appropriate to use as an alternative energy source for biodiesel production (Chisti, 2007). Moreover, some microalgal strains rich in protein, carbohydrates and other compound such as organic acid and carotenoids which are high value products and have potential to use in nutraceutical, food and feed industries.

There are a number of studies to examine effects of abiotic stresses on the changes of bioproducts. For example, light stress stimulated the production of carotenoids and changed fatty acids metabolism in *Dunaliella salina* (Lamers *et al.*, 2010). Besides, nitrogen starvation impacted on starch and lipid synthesis in microalga *Chlorella zofingiensis* (Zhu *et al.*, 2014). In addition, it was reported that, green alga *Botryococcus braunii* responded to salt stresses by carotenoids, carbohydrates and fatty acid changing (Rao *et al.*, 2007). These reports reveal that stresses modulate to algal metabolism and carotenoids production. The production of carotenoids using microalgae are of interest because of varieties, compositions and proportions (Takaichi, 2011). Nowadays, *Haematococcus pluvialis*, a green microalga, is used as a model for carotenoids biosynthesis as this strain is capable of accumulating large amounts of carotenoids astaxanthin, in particular. However, the production of carotenoids in *H. pluvialis* has drawbacks by its slow growth rate, low cell density and low temperatures requirement. Therefore, the alternative potential strains have been increasingly explored.

Chlorococcum sp. is green microalga in family Chlorococcaceae. It is a unicellular microalga with a round shape found in freshwater and terrestrial habitats (Klochkova *et al.*, 2006; Liu *et al.*, 2000). This strain shows tolerancy to high temperature, high light intensity and high carbon dioxide concentration (Ota *et al.*, 2015; Zhang and Lee, 1997) Moreover, it can produce and accumulate carotenoids and neutral lipids (Kiran *et al.*, 2016). Liu and Lee (1999) reported that different nitrogen sources including ammonia, nitrates, urea, yeast and peptone extracts effected on carotenoids composition in *Chlorococcum* sp. (Liu *et al.*, 2000). In addition, it was found that temperature, pH and oxygen concentration affected the production of carotenoids in *Chlorococcum sp.* (Liu and Lee, 2000). Morphological observation and spectrophotometric analysis revealed that *Chlorococcum* sp. 8367RE induced carotenoids accumulation upon various stresses. Moreover, it can tolerate to the environmental stress (Janchot, 2016). Thus, this study aimed to investigate carotenoids compositions, metabolic alteration, *i.e.* fatty acid, amino acid, protein and carbohydrate content including morphological changing of the green microalga *Chlorococcum* sp. 8367RE under stress conditions.

The objectives of this research:

1. To observe morphological changing of the green microalga *Chlorococcum* sp. 8367RE under stress conditions.
2. To identify and quantify carotenoids composition in this algal strain.
3. To analyze fatty acid, amino acid, protein and carbohydrate content in this microalga.

CHAPTER II
LITERATURE REVIEW

2.1 Carotenoids

Carotenoids are a group of fat-soluble pigments that represent in red, oranges or yellow shading. They are classified in the group of terpenoids consisting of 8 isoprene units and also called tetraterpenoids (Forero, 2013). The structure typically contains 40 carbon atoms in manner of polyene chain backbone structure which have a chain of conjugated single and double bonds. Based on their structures, carotenoids are categorized into 2 groups namely carotene and xanthophylls (Kiokias *et al.*, 2016). Carotenes consist of only carbon and hydrogen in their structures. Among a group of carotenes, 3 typical structures are categorized. These are: (i) acyclic carotenes, which is pure hydrocarbon structure, such as lycopene, phytoene and phytofluene; (ii) monocyclic carotenes, which contain a ring at only one side of the end, such as γ-carotene and (iii) bicyclic structure of carotenes contain 2 rings at the both side of molecules, such as **α**-carotene and β-carotene (Othman *et al.*, 2017). Another class, xanthophylls, their structures compose of hydrocarbon which are oxygenated and contain functional groups, such as hydroxy group (*i.e.* zeaxanthin, lutein and β-cryptoxanthin), keto group (*i.e.* canthaxanthin and astaxanthin) and epoxy group (*i.e.* antheraxanthin, neoxanthin, and violaxanthin) (Bhosale and Bernstein, 2005). Chemical structures of representative carotenoids are exemplified in Figure 1.

To date, over 750 carotenoids are found in natural sources including plant, yeast, fungi, bacteria, algae and animal (Avalos and Limón, 2015; Khachatourians, 2017). Carotenoids possess a wide range of functions. In photosynthetic organisms, carotenoids act as accessory pigment which participated in light harvesting by light absorption at wavelengths between 400-500 nm (Erdoğan *et al.*, 2015). Moreover, these pigments show antioxidant and anti-inflammatory properties which are

interested in health care industries (Raposo *et al.*, 2015). In recent years, many studies of carotenoids are focused on their biological properties, particularly on human health. Both of carotenes and xanthophylls were shown their abilities to protect cells by acting as antioxidant. The well-known carotenoids are β-carotene, lycopene, lutein, zeaxanthin and astaxanthin.

β-carotene is orange shade pigment, which is mainly found in plants such as carrot, pumpkin and spinach (Figure 1). It is a precursor of vitamin A, called provitamin A, which can be converted to retinol and offer the benefits in eye care (Rasmussen and Johnson, 2013).

Lycopene, the red pigment that is massively produced in tomatoes. Its structure is unsaturated hydrocarbon with no β-rings (Figure 1). These non-provitamin A plays a vital role in many biological activities, such as antioxidant and anti-inflammatory activities (Hazewindus *et al.*, 2012).

Lutein and zeaxanthin are yellow pigment which are stereoisomer (Figure 1). They are mostly synthesized by plants and microalgae. These pigments are classified as xanthophylls group because the structure is unsaturated hydrocarbon with cooperating by hydroxyl groups added. They play important roles in antioxidant activity by either free radical scavenging or singlet oxygen quenching. They are also reported to delay the development of chronic diseases (Sun *et al.*, 2015). Moreover, they have capability to support eye health (Nwachukwu *et al.*, 2016).

Astaxanthin (known as the king of carotenoids) is red pigment which is found in plants, algae, some bacteria, yeast and animal. Astaxanthin is classified as ketocarotenoids in xanthophylls group (Figure 1). In nature, astaxanthin can be found in three forms: free astaxanthin, astaxanthin monoester and astaxanthin diester. Certain organisms can accumulate astaxanthin at different forms and ratios (Ambati *et al.*, 2014). Astaxanthin possess a strong antioxidant activity due to conjugated double bond of their structure which involve in electron donating when reacting with oxidants.

Moreover, it can act as singlet oxygen quenching and free radical scavenging. As a result, free radicals are in the stable form and chain reaction is inhibited. Astaxanthin is more effective in capturing oxygen, up to 550 times compared with vitamin E and also more effective in free radical damage than vitamin C, up to 65 times. Besides, it has anti-inflammatory properties (Capelli, 2007). The strong antioxidant property of astaxanthin is currently being used in the pharmaceutical and food industries.

It should be noted that, carotenoids are not stable compound (Boon *et al.*, 2010). From this reason, extraction of carotenoids from biological tissue are performed circumspectly. During the experiment, it is necessary to prevent the exposure to light. Alternatively, the samples should be wrapped with black fabric, aluminium foil or paper to avoid light exposure. In addition, samples should be avoided from high temperature exposure and need to keep at low temperatures.

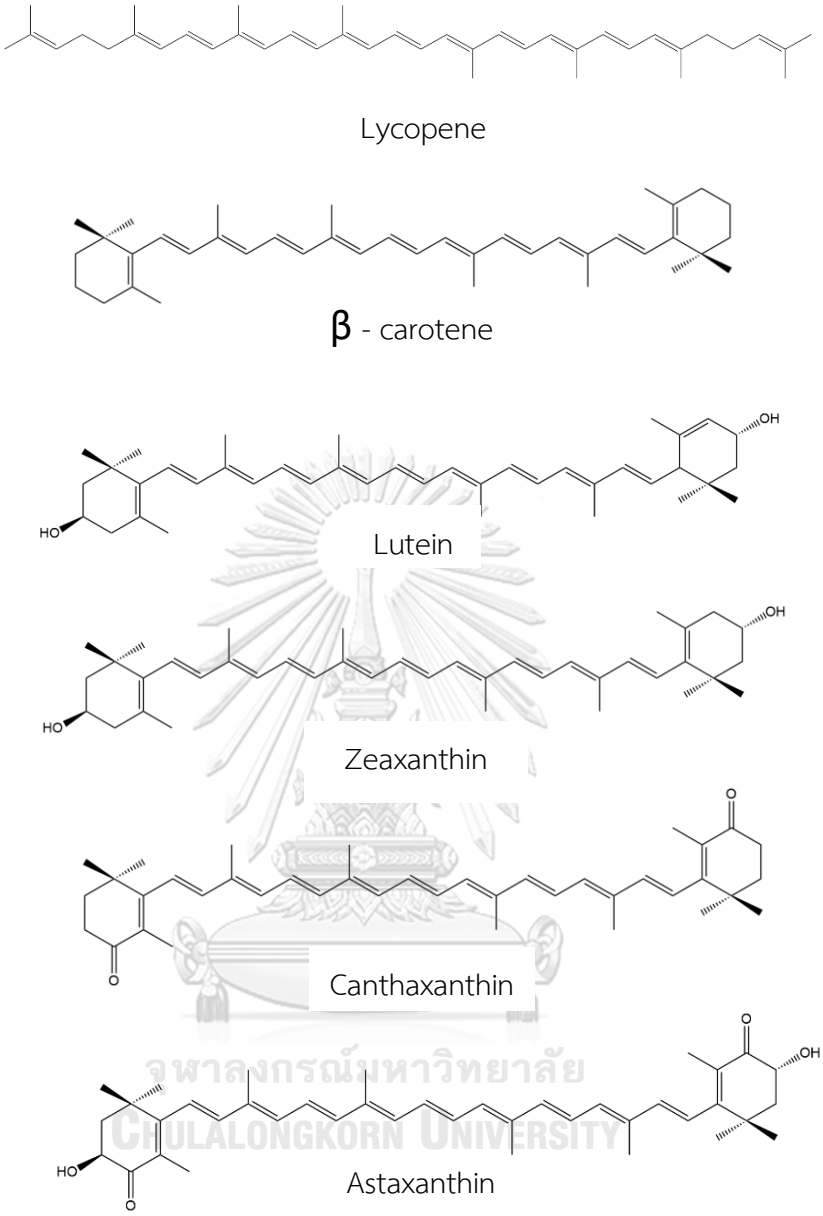


Figure 1 Structure of some well-known carotenoids. The structures were drawn by using ChemDraw Professional program.

2.2 Analysis of carotenoids

To analyze carotenoids qualification and quantification, chromatography and spectroscopy are commonly performed. For chromatography assay, thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are suggested. TLC is an easy method to separate and quantify carotenoids from biological samples. Silica is widely used as a stationary phase in TLC analysis and several organic solvent are used as the mobile phase such as acetone, petroleum ether, hexane and methanol (Zeb and Murkovic, 2010). In order to improve sensitivity of carotenoids quantitative HPLC was performed. This technique is appropriated for the quantitative and qualitative analysis of carotenoids. The carotenoids can be efficiently separated and can be compared to standard compounds to determine the type of carotenoids, including the calculation of carotenoids content. Hence, HPLC columns are important for carotenoids separation, type and proportion of solvents also have a significant effect on the analysis. It has been reported that several columns and solvents are used in the analysis of carotenoids, as shown in Table 1 (Rivera Vélez, 2016). The most commonly use is the C18 and C30 columns. The stationary phase of this column is made of silica. C30 column is more hydrophobic than C18 and more specific to long-structured substances as carotenoids.

For spectroscopy analysis, UV-Vis spectroscopy is a simple technique to measure the absorbance of a substance and used for quantitative measurement. Carotenoids can be scanned at the wavelength of 400-500 nm. However, the maximum absorption can be different and shifted, depending on type of spectrophotometer and solvent. In addition, accuracy of absorbances are concerned. It would be controlled the range between 0.3 to 0.85. The absorbance under 0.3 may interfere with non-zero baseline and cannot show the correct value. On the other hand, the absorbance over 0.85 or 0.9 may affect to the accuracy of the detector. After absorbance measurement, the absorbance value can be calculated by using the Lambert-Beer law. It should be

noted that, the maximum absorbance of carotenoids and chlorophyll b may interfere because both of them can absorb at the nearby wavelength. Especially, the samples having high chlorophyll b contents, such as algal or plant extracts should be concern to prevent the overestimation of carotenoids (Lichtenthaler and Buschmann, 2001). Nowadays, the high sensitivity detection method, mass spectrometry is improved. This technique is based on changing the injected substance into ion form and then ion moves into the analysis apparatus to analyze mass based on mass-to-charge ratio (m/z). This technique provides the information regarding the molecules of carotenoid. It can analyze mass of molecules, isotope ratio and others. These analytical methods can be combined with prediction program to interpret the data and suggest type of carotenoids (Rivera *et al.*, 2014). At present, carotenoids analysis using mass spectrometry couple with HPLC is becoming more popular. It has reported that HPLC-DAD-MS was perform to analyze ketocarotenoids in aquatic bacteria (Asker *et al.*, 2018). Likewise, three microalgae and cyanobacteria species were analyzed carotenoids structure by using HPLC-MS/MS method (Patias *et al.*, 2017). Nevertheless, this method is limited by expensive instrument and the user must have the knowledge to analyze the data.

Table 1 Column types and chromatographic conditions used in various biological samples (Rivera Vélez, 2016).

Column type	Chromatographic conditions	Carotenoids determined	matrix
ProntoSil C_{30}	Solvents A and B: MeOH-MTBE-H_2O (83:15:2, v/v) and (8:90:2, v/v), respectively; gradient elution; column temperature: 30 °C; flow rate 1 mL/min	3, 18, 19 and mono- and diesters of 18	*Haematococcus pluvalis*
C_{30}	Solvents A: MeOH-H_2O (75:25, v/v); solvent B: EtOAc; gradient elution; column temperature: 25 °C; flow rate 0.6 mL/min	3, 18, 19, 54, 55, and esters of 3, 18, and 54	*Haematococcus pluvalis*
C_{30} YMC	Solvents A: MeOH; solvent B: H_2O-MeOH (20:80, v/v) containing 0.2% ammonium acetate; solvent C: MTBE; gradient elution; column temperature: 25 °C; flow rate 1.0 mL/min	2, 15, 16, 17, 18, 19, 20, 22, 54, 55, 56, 57, 58, mono- and diesters of 18, and esters of 3 and 16	Transgenic tobacco and tomato
C_{30}	Solvents A, B, and C: MeOH, MTBE, and H_2O), respectively; gradient elution; column temperature: 20 °C; flow rate 1.0 mL/min	3, 4, 5, 15, 16, 17, 18, 19, 31, 58, and δ-carotene (59)	Carotenoid standards
C_{30} YMC	Solvents A and B: MeOH and MTBE, respectively; gradient elution; column temperature: not controlled; flow rate 0.4 mL/min	3, 15,16, 17, 18, 19,31, 54, 55, and 58	Transgenic carrots
C_{30} YMC	Solvent: MeOH-MTBE-H_2O-CH_2Cl_2 (77:13:8:2, v/v); column temperature: 30 °C; flow rate 0.8 mL/min	Four epoxides and di- and mono-*cis*-isomers of 18	Thermally treated astaxanthin
C_{30} YMC	Solvents A and B: MeOH and MTBE, respectively; gradient elution; column temperature: 29 °C; flow rate 0.9 mL/min	3, 16, 15, 17, 18, 19, 48, and crustaxanthin (60)	*Marthasterias glacialis*
Beckman Ultra-sphere C_{18}	Solvents A and B: CH_2Cl_2-MeOH-CH_3CN-H_2O (5.0:85.0:5.5:4.5, v/v) and (22:28:45.5:4.5, v/v), respectively; gradient elution; column temperature: not controlled; flow rate 1.0 mL/min	3, 15, 18, 19, 54, 58, and esters of 18	*Haematococcus pluvalis*
Hypersil HyPurity Elite C_{18}	Solvents: CH_3CN-MeOH-2-propanol (85:10:5, v/v); isocratic elution; column temperature: 32 °C; flow rate 1.0 mL/min	3, 4, 15, 16, 17, 18, 31, 55, 56, 58, α-crytoxanthin (61), and γ-carotene (62)	transgenic maize
Beckman Ultra-sphere C_{18}	Solvents A and B: CH_2Cl_2-MeOH-CH_3CN-H_2O (5.0:85.0:5.5:4.5, v/v) and (25:28:42.5:4.5, v/v), respectively; gradient elution; column temperature: 25 °C; flow rate 1.0 mL/min	3, 15, 18, 19, 55, esters of 18 and 55	*Chlorococcum* sp.
Supelco Discovery C_{18}	Solvents A and B: CH_2Cl_2-MeOH-CH_3CN-H_2O (5.0:85.0:5.5:4.5, v/v) and (22:28:45.5:4.5, v/v), respectively; gradient elution; column temperature: not controlled; flow rate 1.0 mL/min	3, 15, 18, 19, 20, 22, 32, 54, 55, 57, 58, and mono- and diesters of 18	*Scenedesmus obliquus*
Beckman Ultra-sphere C_{18}	Solvents A and B: MeOH-CH_2Cl_2-CH_3CN-H_2O (69:17:11.5:2.5, v/v); isocratic elution; column temperature: not controlled; flow rate 1.0 mL/min	3, 15, 18, 19, 54, 55, 56, 58, and esters of 18	*Chlorococcum* sp.
ReproSil 120 C_{18}-AQ	Solvents A and B: MeOH-H_2O-EtOAc (82:8:10, v/v) and (20:1:79, v/v), respectively; gradient elution; column temperature: 20 °C; flow rate 1.2 mL/min	3, 4, 15, 16,19, 22, 53, and 54	Transgenic *Arabidopsis thaliana*
Acquity UPLC C_{18} BEH	Solvent: CH_3CN-MeOH (7:3, v/v); solvent B: H_2O; gradient elution; column temperature: 32 °C; flow rate 0.4 mL/min	3, 4, 15, 16, 17, 18, 19, 21, 22, 54, 55, 56, 58, and β-zeacarotene (63)	Transgenic maize
Cosmosil $5C_{18}$-AR-II	Solvents A: MeOH-CH_2Cl_2-CH_3CN (90:5:5, v/v); solvent B: H_2O; gradient elution; column temperature: not controlled; flow rate 2.0 mL/min	*cis*-isomers and mono- and diesters of 18	*Parapenacopsis hardwickii*
Suplex pKb-100	Solvent A: CH_3CN-MTBE-H_2O (69.6:20.0:10.4, v/v); solvent B: CH_3CN-MTBE (70:30, v/v); gradient elution; column temperature: 20 °C; flow rate 0.5 mL/min	3, 4, 5, 15, 16, 18, 19, 54, 55, 58, astaxanthin dimethyl disuccinate (64), β-apo-8'-carotenal (65), β-apo-8'- carotenoic acid ethyl ester (66), and citranaxanthin (67)	Carotenoid standards
Spherisorb ODS-2	Solvent: CH_3CN-MeOH-0.1M Tris-HCl (pH 8.0) (84:2:14, v/v); solvent B: MTBE-EtOAc (68:32, v/v); gradient elution; column temperature: not controlled; flow rate 0.4 mL/min	4, 15, 18, 19, 54, 58, and esters of 18	Transgenic tomato
Luna 5 µm silica	Solvent A and B: hexane and acetone, respectively; gradient elution; column temperature: not controlled; flow rate 1.5 mL/min	18, 19, 53, 54, and papilioerythrinone (68)	*Oriolus cruentus*
Brownlee Spheri-5 silia 5 µm	Solvent A and B: acetone and hexane, respectively; gradient elution; column temperature: not controlled; flow rate 1.25 mL/min	3, 16, 18, 19, 55, 56, and 58	*Botryococcus brauni*
Spherisorb ODS-2	Solvent: hexane-CH_2Cl_2-isopropyl alcohol-TAE (88.5:10:1.5:0.1, v/v); isocratic elution; column temperature: not controlled; flow rate 1.5 mL/min	15, 16, 17, 18, 19, 54, 55, 57, and 58	Synechococcus sp. PCC7942 transformed strains
HIRPB-250AM	Solvent A, B, and C: CH_3CN, CH_2Cl_2, and MeOH, respectively; gradient elution; column temperature: 25 °C; flow rate 0.4 mL/min	esters and glycosides of 18	*Chlamydomonas nivalis*
Chiralcel(R) OD-RH	Solvent: CH_3CN-3.5 mM phosphoric acid (ratio not reported); isocratic elution column temperature: 25 °C; flow rate 0.5 mL/min	3*S*, 3'*S*, 3*R*, 3'*R*, and 3*R*, 3'*S*-astaxanthin (18, 69, and 70, respectively)	*Chlamydomonas nivalis*
Chiralpak 1C CSP	Solvent: MTBE-CH_3CN (1:1, v/v); isocratic elution column temperature: 22 °C; flow rate 1.0 mL/min	18, 69, and 70	astaxanthin standard

2.3 Microalgae as source of carotenoids

Microalgae are recognized as prolific sources of bioactive compounds with intricate chemical structures. Moreover, microalgal cells are also rich in proteins, carbohydrates, lipid and other biocompounds (Ördög *et al.*, 2012). Thus, they have potential to extract and use these compounds to supply in nutraceutical, food and feed industries. Among these, carotenoids are particularly interesting.

The green microalga *Haematococcus pluvialis* is used as a model for carotenoids production because it can accumulate large amounts of carotenoids, especially astaxanthin. Carotenoids production in *H. pluvialis* under sodium chloride and acetate stresses was investigated (Vidhyavathi *et al.*, 2008). This research suggested that salt stress resulted in enhanced accumulation of carotenoids. Expression of genes associated in carotenoids biosynthetic pathway were up-regulated. Many studies showed the effect of salinity stress and ethanol on carotenoids accumulation in *H. pluvialis* (Gao *et al.*, 2015; Wen *et al.*, 2015). However, the production of carotenoids in *H. pluvialis* was still limited by the slow growth rate, low cell density and low temperatures requirement. Therefore, it is interesting to survey the production of carotenoids using alternative green microalgae.

Chlorococcum sp. is green microalga in family Chlorococcaceae. It is unicellular microalga with a round shape, found in fresh water and terrestrial environments (Klochkova *et al.*, 2006; Liu *et al.*, 2000). *Chlorococcum* sp. grows considerably fast and easier to handle in out-door (Zhang and Lee, 1999). This strain shows tolerancy to high temperature and light intensity more than the green microalga *Haematococcus* sp. (Zhang and Lee, 1997). Moreover, it can produce and accumulate carotenoids and neutral lipids (Kiran *et al.*, 2016). Thus, *Chlorococcum* sp. has a potential to use as an alternative strain for carotenoids production.

2.4 Biosynthesis pathway of carotenoids

In higher plants, carotenoids biosyntheses are extensively studied; however, in microalgae remain elusive. Schematic model of carotenoids biosynthesis in chlorophytes was only proposed (Figure 2) (Varela *et al.*, 2015). It was found that carotenoids are synthesized from C5 building block, the precursor of isoprene. The biosynthesis pathway initiates with the synthesis of isopentenyl pyrophosphate (IPP) through the MEP pathway. Then, it is transformed to geranylgeranyl pyrophosphate (GGPP). After that, the first colorless carotenoids, phytoene, is occurred by a condensation of 2 GGPPs through phytoene synthase (PSY) activity. Next, phytoene desaturase (PDS) and **ζ**-carotene desaturase (ZDS) catalyze the dehydrogenation reactions and convert phytoene to pro-lycopene and lycopene, the first color carotenoids. After the lycopene synthesis, the pathway is divided into two pathways. First pathway, **α**-carotene is occurred by cyclization through lycopene **ε**-cyclase (LCYE) and lycopene **β**-cyclase (LCYB) catalyzation. After that, lutein is synthesized from **α**-carotene precursor. Second pathway, cyclization of lycopene is catalyzed by LYCB to form **β**-carotene. Subsequently, **β**-carotene is oxygenated and zeaxanthin was occurred by **β**-carotene hydroxylase (CHYB). Then zeaxanthin epoxidase (ZEP) catalyze the epoxy groups insertion on zeaxanthin and convert to violaxanthin. In some stress condition violaxanthin can be reconverted to zeaxanthin by violaxanthin de-epoxidase (VDE) activity. In some organisms, zeaxanthin and violaxanthin are converted to canthaxanthin and astaxanthin by the function of **β**-carotene ketolase (BKT) such as *H. pluvialis* (Jin *et al.*, 2006)

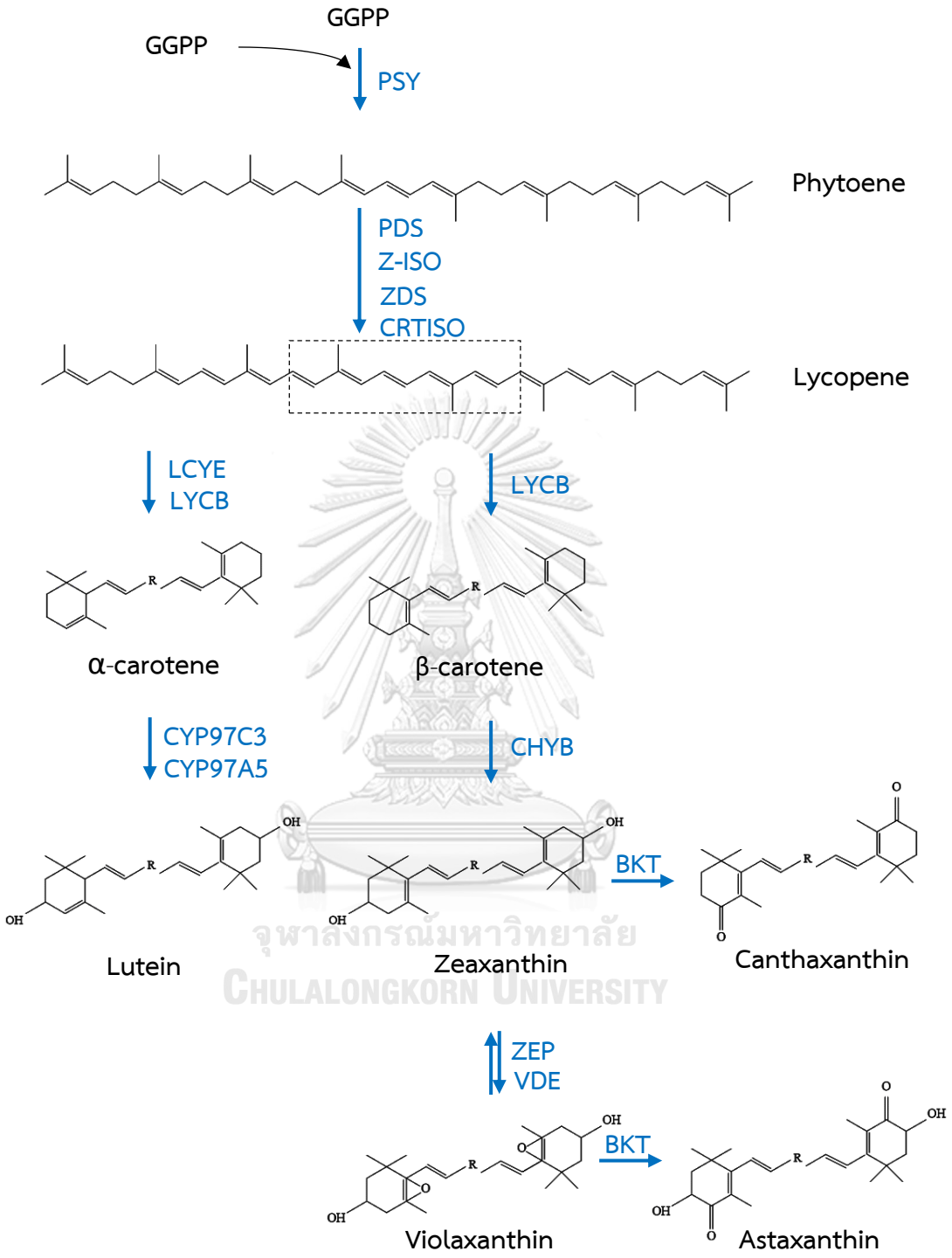


Figure 2 Schematic model for the biosynthetic pathway of carotenoids in chlorophytes (Varela *et al.*, 2015).

2.5 Effects of stresses on microalgal metabolisms and carotenoids accumulation

Microalgae can be found in several habitats including terrestrial, freshwater and marine. It can withstand to stresses from the environment, such as salinity-, light-, drought-, heat-, osmotic-, and oxidation stresses. These environmental stresses impacted on the survival of the green microalgae. It was found that the adaptive response was controlled by several parts including physiology, metabolism and the signaling system (Vonshak and Torzillo, 2004). Moreover, microalgae have various strategies to alter primary and secondary metabolites for their survivals (Skjånes *et al.*, 2013). Therefore, green microalgae can grow among environmental changes.

There are a number of studies to examine effects of abiotic stresses on the changes of biocompounds. For example, light stress stimulated the production of carotenoids and changed fatty acids metabolism in *Dunaliella salina* (Lamers *et al.*, 2010). Furthermore, nitrogen stress was widely used to stimulate carotenoids production. It was reported that the green algae *Coelastrella striolata* produced canthaxanthin as the main carotenoids under nitrogen deficiency (Abe *et al.*, 2007). Moreover, the depletion of nitrogen induced xanthophylls accumulation in various strains (Mulders *et al.*, 2015; Solovchenko *et al.*, 2008). Besides, nitrogen starvation impacted on starch and lipid syntheses in the microalga *Chlorella zofingiensis* (Zhu *et al.*, 2014). Additionally, effects of iron, magnesium and sulfur deprivations on carotenoids and lipid metabolism were examined (Shaker *et al.*, 2017). For salinity stress, it was reported that the green alga *Botryococcus braunii* responded to salt stresses by carotenoids, carbohydrates and fatty acids changing (Rao *et al.*, 2007). Moreover, the combination of these stresses led to metabolic changing in microalgae. For instance, the combination of nitrogen limitation and cadmium stress enhanced lipid, carbohydrate, protein and amino acid accumulation in the green microalga *Chlorella vulgaris* (Chia *et al.*, 2015). These evidences revealed that stresses modulate algal metabolisms and carotenoids productions.

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

Air pump: Model XP-60, Hiblow, USA

Analytical balances: Model AG285, Mettler Toledo, USA

Autoclave: Model SS-325, TOMY Digital Biology, Japan

Autoclave: Model ES-215, TOMY Digital Biology, Japan

Automated Critical Point Dryer: Model EM CPD300, Leica, Austria

C30 Reversed phase column: Model CT99S031546WT, YMC, Japan

Centrifugal vacuum concentrator: Model 5301, Eppendorf, Germany

Disposable syringe: Nipro, Japan

Freeze dryer: FlexiDry MP, Kinetics, USA

Gas chromatography: Model Agilent 6890N, Agilent, USA

Glass cuvette: Spectronic 401, Milton Roy, USA

Glass cuvette: Starna Scientific, UK

High Resolution Mass Spectrophotometer: Model X500R QTOF system, SCIEX, USA

High speed refrigerated centrifuge: Model 6500, Kubota, Japan

HP-INNOWax GC column: Agilent, USA

Laboratory glassware: Pyrex, USA

Laminar flow: Model H1, Labmicrotech, Thailand

Magnetic stirrer: Model MMS-3000, Biosan, Latvia

Micropipette: Eppendorf Research plus, Eppendorf, Germany

Microscope: Model BX51, Olympus, Japan

Mini centrifuge: Labnet International, USA

Orbital shaker: Model TT-20: Hercuvan Lab Systems, Malaysia

Precision Balance: Model ME3002, Mettler Toledo, USA

Refrigerated microcentrifuge: Model 5418 R, Eppendorf, Germany

Scanning electron microscope: Model JSM-6610LV, Jeol, Japan

Silica glass beads: Merck, Germany

Silicone tube, Dura, Thailand

Spectrophotometer: GENESYs 20, Thermo Fisher Scientific, USA

Sputter coater: Model SCD 040, Balzers, Germany

Ultra High Performance Liquid Chromatography (UHPLC): Model 1290 Infinity II LC Systems, Agilent Technologies, USA

Ultra High Performance Liquid Chromatography (UHPLC): Model Ultimate 3000, Thermo Fisher Scientific, USA

Ultrasonic bath: Bandelin, Germany

Ultraviolet lamp: Model UV-36W, Atman, China

Universal refrigerated centrifuge: Model 5922, Kubota, Japan

Vortex mixer: Model G560E: Scientific Industries, USA

3.2 Chemicals

Acetone for analysis: Merck, Germany

Acetonitrile for LC-MS: Merck, Germany

Astaxanthin authentic compound, Merck, Germany

β-carotene authentic compound, Merck, Germany

Bio-Rad protein assay (dye reagent concentrate), Bio-Rad Laboratories, USA

Boric acid: Merck, Germany

Butylated hydrotoluene, USA

Calcium chloride: Merck, Germany

Citric acid: Merck, Germany

Cobalt (II) nitrate: Ajax Finechem Pty Limited, Australia

Copper (II) sulfate: Ajax Finechem Pty Limited, Australia

Dipotassium phosphate: Ajax Finechem Pty Limited, Australia

EDTA (Ethylenediaminetetraacetic acid): Amresco, USA

Ferric ammonium nitrate: Merck, Germany

Hexane, USA

Hydrochloric acid, USA

Lutein authentic compound, Carbosynth, UK

Magnesium chloride: Merck, Germany

Magnesium sulfate: Merck, Germany

Manganese (II) chloride: Ajax Finechem Pty Limited, Australia

Methanol for HPLC: RCI Labscan, Thailand

Methyl-t-butyl ether for HPLC: RCI Labscan, Thailand

Phenol: Amresco, USA

Potassium chloride: Merck, Germany

Sodium carbonate: Merck, Germany

Sodium chloride: Ajax Finechem Pty Limited, Australia

Sodium molybdate: Carlo Erba, Italy

Sodium nitrate: Merck, Germany

Sodium sulfate, Japan

Sulfuric acid: Merck, Germany

Zeaxanthin authentic compound, Carbosynth, UK

Zinc sulfate: Ajax Finechem Pty Limited, Australia

3.3 Membrane

Nylon syringe filter: Membrane Solutions, USA

3.4 Microalga, culture condition and morphological observation

3.4.1 Microalga

The green microalga *Chlorococcum* sp. 8367RE was kindly provided from Thailand Institute of Scientific and Technological Research (TISTR). Stock culture was grown in Blue green medium (BG-11) (Stanier *et al.*, 1971) and incubated at 25 °C under light intensity of 30±5 μmoles/m^2/s until the absorbance at 730 nm reached to 0.4.

3.4.2 Morphological observation

Light microscope and scanning electron microscope (SEM) analyses were carried out to observe algal morphological changes. For light microscopic analysis, 20 μL of the cell culture was placed on to glass slide then covered with coverslip. The slide was examined under compound light microscope (100X objective, model BX51, Olympus, Japan). Images were captured by using the DP Controller microscope software.

For SEM, microalgal colony was resuspended and soaked in 2.5% of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for an hour. Cells were washed twice in phosphate buffer and followed by distilled water. Then sample was dehydrated by using 30%, 50%, 70%, 95% and absolute ethanol. Subsequently, sample was dried at critical point by critical point dryer (model EM CPD300, Leica, Austria). After that, gold coating was carried out by sputter coater (model SCD 040, Balzers, Germany) and sample was analyzed with SEM (Jeol, model JSM-6610LV, Japan).

3.5 Stress treatments

Stock culture from step 3.4.1 was subjected to 12-well plate under following conditions; BG-11 (control), BG-11 with 0-500 mM NaCl (NaCl stress), BG-11 with 0-500 mM KCl (KCl stress), BG-11 without $NaNO_3$ (nitrogen starvation), BG-11 illuminating at 50±5 and 80±5 μmoles/m^2/s (light stress), BG-11 with various volume from 1 mL to 3 mL, and 10 mL of cell culture were further grown in culture plate to increase water evaporation (drought stress). Cells were cultured in 12-well plate with 3 mL media unless otherwise stated. Algal cultures were grown at 25 °C under continuous illumination for 28 days. The observations under light microscope were performed at day 0, 7, 14 and 28, respectively.

Stress condition(s) that positively impact on carotenoids accumulation in this algae strain were selected for further experiments. Algal cultures were scaled up. For this step, cells from stock cultured were harvested by centrifugation at 8000 x g for 20 mins. Supernatants were discarded and cell pellets were washed by fresh BG-11. Then, the pellets were resuspended in the same original volume. Algal cultures were grown in glass bubble tubes with continuous aeration and illumination of 30±5 μmoles/m^2/s at 25 °C for 28 days. Lastly, cells were harvested by centrifugation at 8000 x g for 20 mins and cell pellets were washed by fresh BG-11 again. Cells pellets were kept at -40 °C for further analysis.

3.6 Extraction of carotenoids

Cell pellets harvesting from various stresses (as described in step 3.5) were extracted with 80% of acetone, acetonitrile, methanol or DMSO. Cells were broken by using several methods. The first method, cells were crushed using the freezed motar and pestle. The pellets were ground homogenously with solvent until cells debris were bleached. The second disruption method was employed by using silica bead together with vortexing. One milliliter of solvent and 0.5 mm. silica beads were added to the cell pellets and then vortexing. Lastly, silica bead combined with ultrasonic bath was performed. The ultrasonication was performed for 15 minutes intervals to avoid an increase in temperature. This step was repeated until the cell pellets were colorless. The supernatant was collected by centrifugation at 12,000 x g for 5 min. Thereafter, the carotenoids extract was concentrated by using centrifugal vacuum concentrator (Eppendorf, Germany). Finally, dry residue was resuspended in 1.0 mL of acetonitrile (LC-MS grade) and prepared for the analyses; HPLC and mass spectrometry. All procedures were carried out under dark and low temperature conditions to prevent carotenoids degradation.

3.7 Determination of pigment content

Five hundred microliters of the extracts were divided for pigment analysis. The extracts were measured absorbance at 470, 647 and 663 nanometers. Chlorophyll a, chlorophyll b and carotenoids contents were calculated as described previously (Lichtenthaler and Buschmann, 2001).

Chlorophyll a $= (12.25*A_{663}) – (2.79*A_{647})$

Chlorophyll b $= (21.50*A_{647}) – (5.10*A_{663})$

Total carotenoids $= [(1000* A_{647}) – (1.82Chla) – (85.02Chlb)]/198$

3.8 Analysis of carotenoids

Carotenoids extracts from step 3.6 were filtered through 0.22 µm nylon syringe filters and subjected to a reversed phase ultra-high performance liquid chromatography (UHPLC: Model Ultimate 3000, Thermo Fisher Scientific, USA). The carotenoids extracts were separated on C18 and C30 reversed phase column (5 µm, ID 4.6 mm x L 250 mm) (YMC, Japan) with four gradient solvent systems consisting of solvent A (methanol) and solvent B (Methyl-t-butyl ether). The flow rate was 0.4-0.9 mL/min, column temperature and sample chamber were maintained at 22 and 10 °C, respectively. The injection volume was 20 µL and the absorption spectra of carotenoids were monitored at 450, 642 and 665 nm by diode array detector. Astaxanthin, lutein, zeaxanthin and β-carotene were used as authentic compounds. Their contents in the extracts were calculated by comparing with standard calibration curves.

Identification of carotenoids in algal extracts were analyzed by high performance liquid chromatography-mass spectrophotometry (HPLC-MS/MS). To acquire the mass spectra between 450-1800 m/z, Sciex X500R QTOF system was equipped. Ten microliters were subjected and analyzed by Information Dependent

Acquisition (IDA) mode. The mobile phase consisted of solvent A (methanol) and solvent B (Methyl-t-butyl ether:methanol 50:50). The gradient system and time program were operated with the same conditions to UHPLC analysis. Mass spectrometry conditions followed by: ion source gas, 45 psi; source temperature, 500 °C; polarity, positive; spray voltage, 5500 V. Finally, data were processed by using Sciex OS software. Moreover, Identification of carotenoids was interpreted by using UV-Vis data combined with Mass spectra data.

3.9 Analysis of metabolite

3.9.1 Lipid analysis

3.9.1.1 Nile red staining

Algal cultures in step 3.5 were observed neutral lipid accumulation by using Nile red staining method (Chen *et al.*, 2009). Cells were harvested by centrifugation at 12,000 rpm for 5 mins and pellets were resuspended in Nile red stain and 15% of dimethyl sulfoxide at ratio 1:5 (v/v). Next, the mixture was spotted on glass slide then covered with coverslip. The slide was examined under fluorescent microscope (model BX51, Olympus, Japan). Images were captured by using the DP Controller microscope software and the relative intensity of fluorescence was calculated by Image J (https://imagej.nih.gov/ij/).

3.9.1.2 Fatty Acid Methyl ester analysis (FAME)

Cell pellets harvesting from 28 days of stress treatments (as described in step 3.5) were dehydrated by using freeze dryer (FlexiDry MP, Kinetics, USA) before fatty acid methyl ester analysis. The intracellular fatty acids were extracted from cells powder by methanol-hydrochloric acid (95:5 V/V). After that, mixed well and incubated at 85 °C for 90 min. Then, one milliliter of distilled water was added to the mixture. For FAMEs extraction, hexane containing 0.01% butylated hydrotoluene was used and

conducted at room temperature. The extracts were centrifuged and separated the organic and aqueous phase. For remaining water absorption, sodium sulfate was added to aqueous phase and mixed well. Next, FAMEs were evaporated and dry residues were redissolved in hexane. The extracts were injected to HP-INNOWax column (ID 0.25 μM) and analyzed by gas chromatography (Model Agilent 6890N, Agilent, USA) equipped with flame ionization detector (FID). The temperature of injection and detection were maintained at 250 °C. Helium gas was used as mobile phase. Fatty acids content in extract were calculated by comparing with authentic compound.

3.9.2 Amino acid and total protein analyses

Cell pellets that harvested from various stresses as described in step 3.5 were extracted with methanol. Cells were broken by using 0.5 mm. silica beads and ultrasonic bath. The ultrasonication was perform for 15 minutes intervals, avoiding an increase in temperature. This step was repeated until the cell pellets were colorless. The supernatant was collected by centrifugation at 12,000 x g for 5 min. After that, the extract was concentrated by using centrifugal vacuum concentrator (Eppendorf, Germany). Dry residue was dissolve in 400 μL of mobile phase (containing acetonitrile and methanol) and filtered through 0.22 μm nylon syringe filters. Forty microliters of extracts were subjected to amino acids analyzer (Hitachi L-8900, Japan) as manufacturer's instruction. The absorption spectra of amino acids were monitored at 570 and 440 nm. The profiles were compared with 17 amino acids as authentic compounds (aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine and proline) and ammonia. Amino acid analysis was done by Ms. Nozomi Takeda, Global Facility Center, Hokkaido University.

To analyze total protein, Bradford assay was performed. Standard curve was prepared by diluting 1 part of Bio-Rad dye reagent concentrate (Biorad, USA) with 4 parts of deionized water. This reagent was kept at 4 °C. Next, Bovine serum albumin (BSA) standard was prepared by diluting a range between 0-20 µg. One milliliter of diluted dye reagent was added to each tube and vortexed. Then the mixtures were incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm. and standard curve was plotted between BSA concentration and absorbance.

Cell pellets harvesting from various stresses as described in step 3.5 were extracted using 200 µL Tris-HCl buffer pH 8.0. Cells were broken by using 0.5 mm. silica beads and ultrasonic bath. The ultrasonication was perform for 15 minutes intervals, avoiding an increase in temperature. This step was repeated until the cell pellets were colorless. The supernatant was collected by centrifugation at 12,000 x g for 5 min. Various volume of extracts were mixed with one milliliter of diluted dye reagent. Then, the mixtures were incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm. Protein contents were calculated by comparing with standard curve (appendix 4).

3.9.3 Carbohydrate analysis

Cell pellets were hydrolyzed by using 1 mL of 2.5 M HCl and then the mixtures were boiled at 100 °C for 3 hours. After that, 1 mL of 2.5 M NaOH was added for neutralization. The supernatants were collected by centrifugation. Total carbohydrate content in supernatants was analyzed by phenol-sulfuric method (Dubois *et al.*, 1956). Five hundred microliters of 5% (w/v) phenol and 2.5 mL of sulfuric acid were added into 500 µL of microalgal extract. Then the reaction was mixed and incubated at room temperature for 30 mins. After that, the absorbance of mixture was determined at 490 nm. Total carbohydrate was calculated by compared with glucose standard curve (appendix 5).

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Morphology of the green microalga *Chlorococcum* sp. 8367RE

The green microalga *Chlorococcum* sp. 8367RE was streaked on BG-11 agar and kept as stock culture. The green colonies typically appeared on BG-11 agar after incubation at room temperature (25 ± 3 °C) for 3 days (Figure 3A). Its color transformed to orange colonies after 2 months (Figure 3B). The transformation from green to orange colonies presumably are pigments, such as carotenoids. Light microscope and SEM were performed to examine the algal morphology and the images were showed in Figure 3C and 3D. Under light microscope, the microalgal cells are green, unicellular with round shape. SEM image showed round shape of cells with smooth surface, no flagella. Cell sizes are in the range of 7-10 μm.

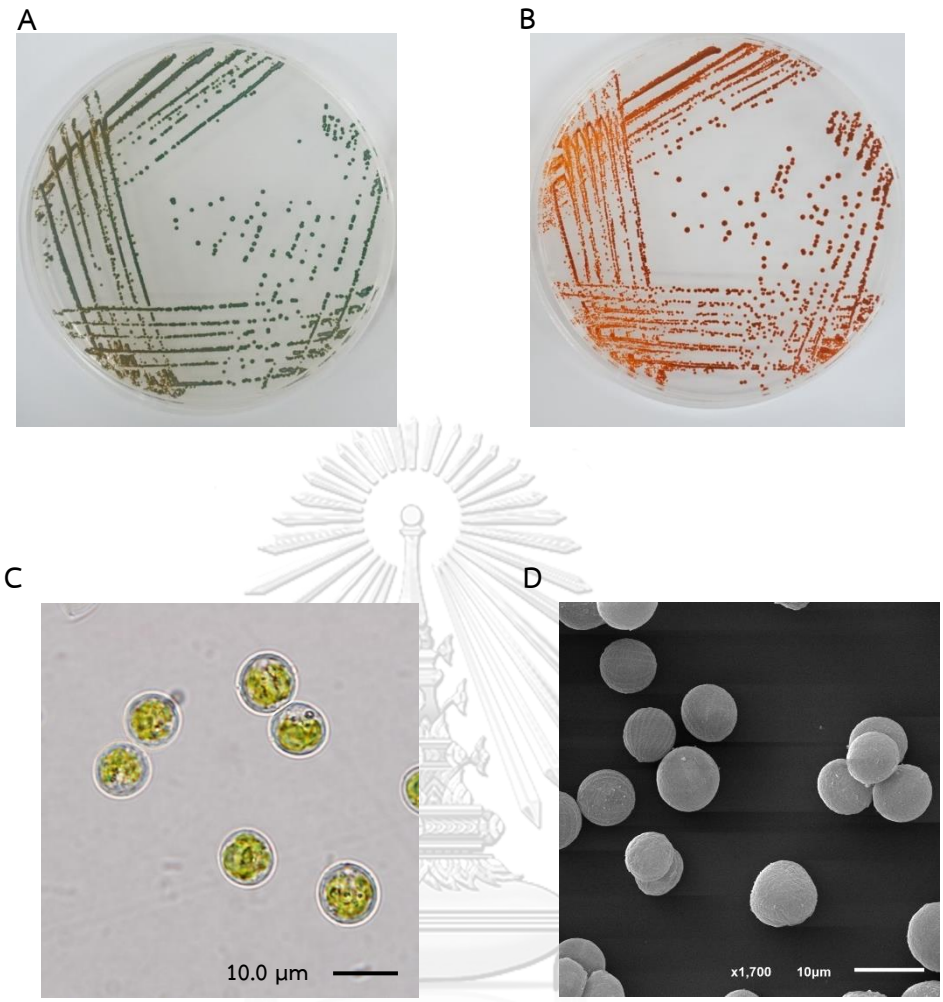


Figure 3 *Chlorococcum* sp. 8367RE cultured on BG-11 agar for 3 days (A) and a long time growing (B). Morphological of *Chlorococcum* sp. 8367RE observed under light microscope (C) and SEM (D).

4.2 Morphological observation under stress treatment

The green microalga *Chlorococcum* sp. 8367RE was cultured in 3 mL BG-11 media that placed in 12-well plate. Cells were grown for 28 days and the morphological observations under light microscope were performed at day 0, 7, 14 and 28, respectively. As shown in Figure 4, under control (nonstress) condition, microalgal cells are unicellular with round shape and greenish color. Cell sizes were slightly increased upon culturing time and remained the similar color. This is a typical pattern during algae growth under nonstress condition and assimilate nutrients for their growths.

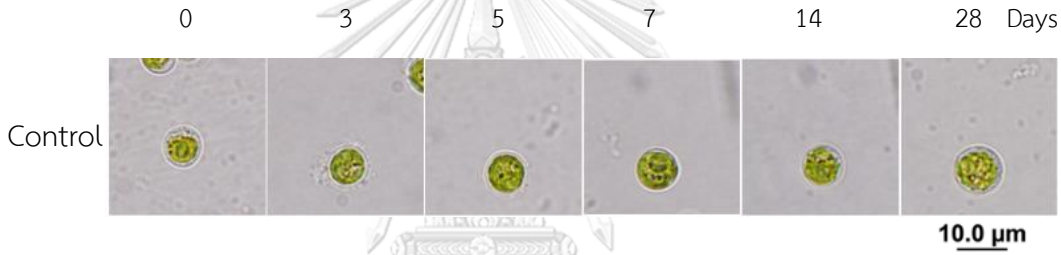


Figure 4 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under control (nonstress) condition. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

Next, we screened the conditions were applied to induce carotenoids accumulation in these algal cells. In this study, salts (NaCl and KCl), light, drought stresses including nitrogen starvation were performed. For NaCl stress, the concentrations from 100-500 mM were applied. Upon NaCl treatment, algal cell sizes were obviously increased compared with the control ones. Interestingly, high NaCl concentration could induce the enlargement of cells. For example, cell sizes become approximately 4.6 ± 0.5 folds increased upon 500 mM NaCl stress (Figure 5) (note: analysis of cell size and area was performed using SemAfore program, data not shown). It was obvious that the color of cells under NaCl stress transformed from green to yellow or orange shade.

From the observations, NaCl stress led the cell enlargement together with the cells color changing from green to yellow/orange colors. Based on morphological observations here, we hypothesized that yellow or orange color was the chromophores, carotenoids. Thus, NaCl treatment would relate to carotenoids accumulation in algal cells.

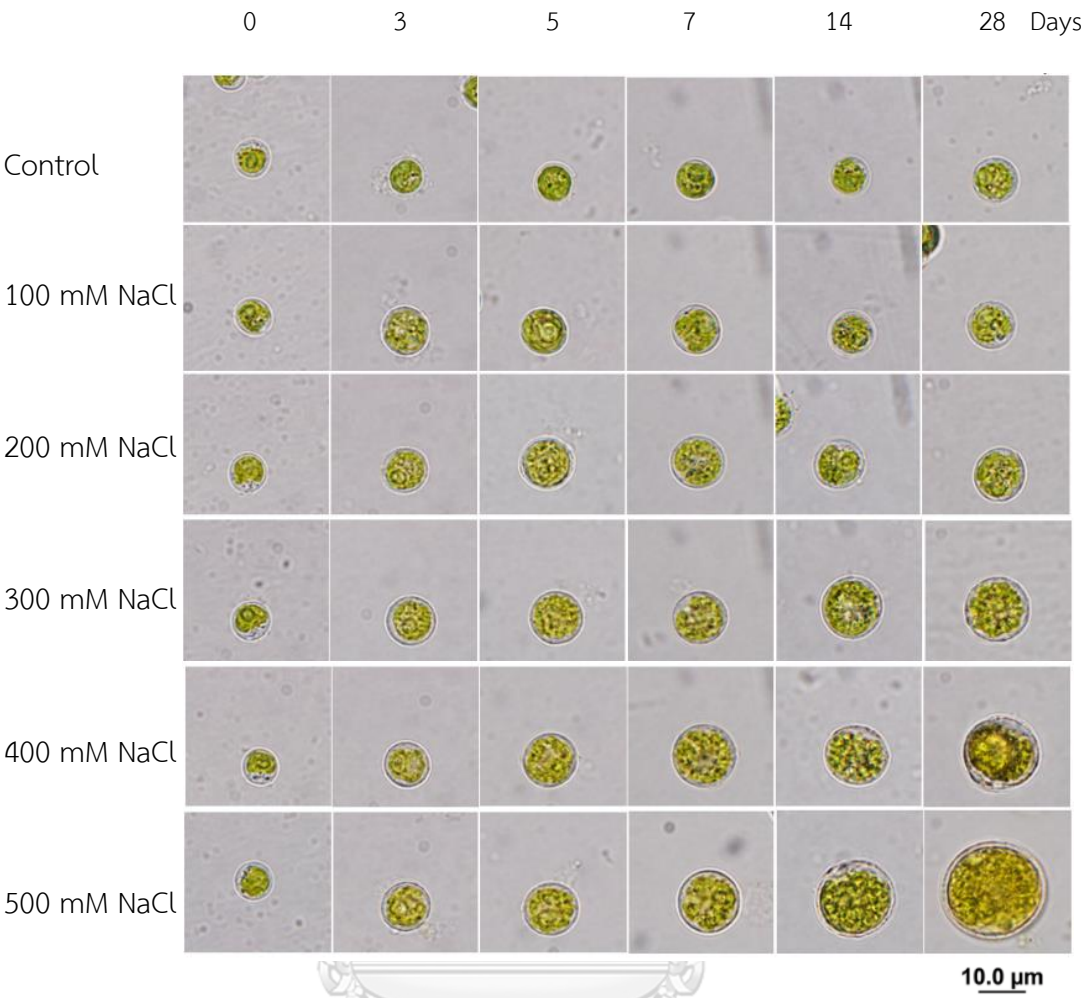


Figure 5 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under NaCl stress. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

Next, KCl stress was performed. The results are the similar trends as observing under NaCl stress. Size of cells under KCl stress increased approximately 5.1 ± 0.6 folds when compared with the control in the same period, especially at day 28, cells showed the largest in size. Comparing between cells under a high and low KCl concentrations, cells were observed to be larger under higher KCl concentration. The largest cell sizes were observed under 500 mM KCl stress. It should be note that color changes under this condition were much pronounced compared with NaCl stress (Figure 5 vs Figure 6). Additionally, algal cells were aggregated and colonized (*i.e.* under 300 mM KCl stress at day 14 and 28). The enlargement of *Chlorococcum* sp. 8367RE cells under salt stress (either NaCl or KCl) was similar as observing in a terrestrial green alga *Chlorococcum* sp. (Klochkova *et al.*, 2006). The authors showed the enlarged vacuoles under sea water culturing but no detailed analysis was clarified and discussed. Since, the color of algal cells under KCl stress transformed from green to yellow and orange faster, we next focused on the analysis of chromophores and carotenoids under this stress condition.

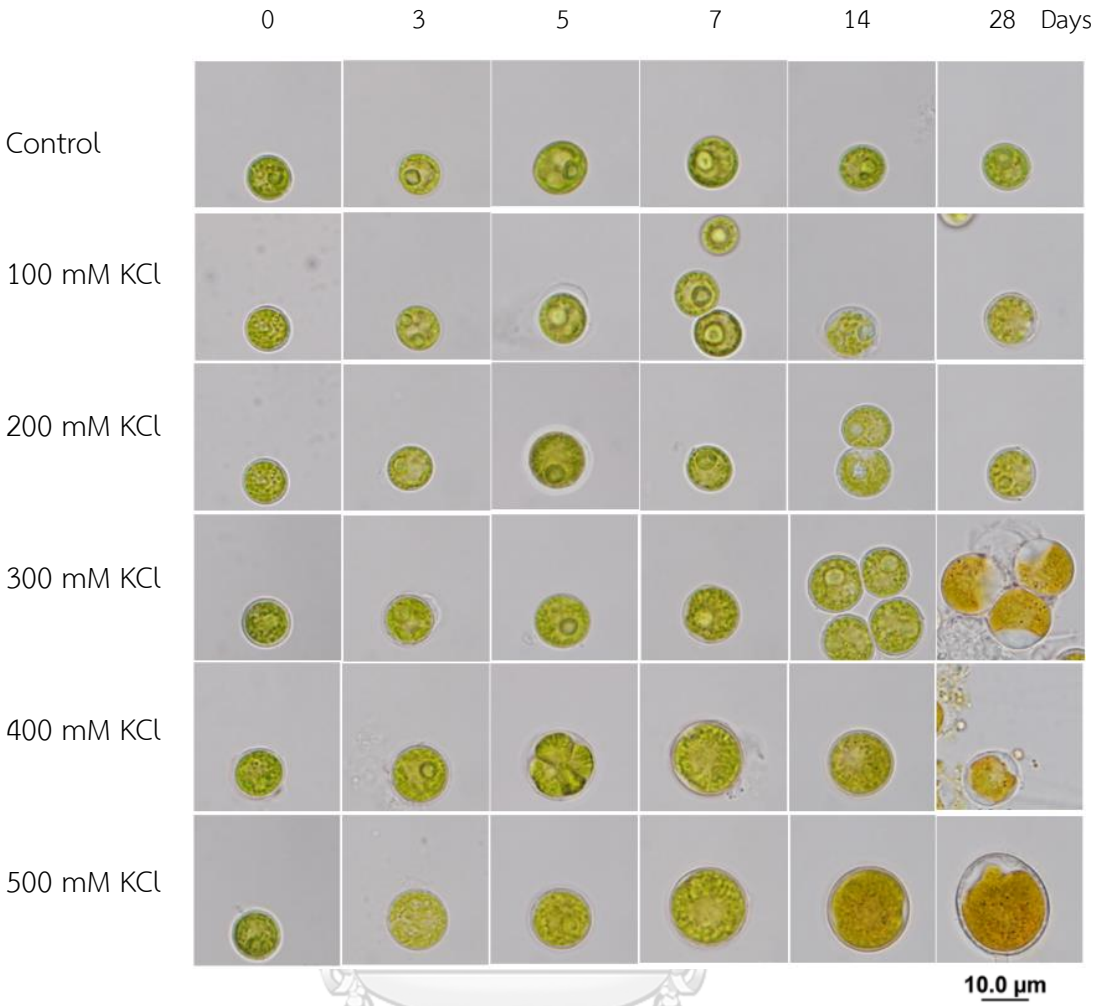


Figure 6 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under KCl stress. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

Nitrogen starvation is one of the effective conditions to induce carotenoids accumulation because chlorophylls generally decreased under this unflavored condition. On the contrary, nitrogen starvation resulted in accessory pigment accumulation, such as carotenoids (Markou and Nerantzis, 2013). We therefore examined morphological changes using nitrogen starvation condition to serve as a positive control. The results were shown in Figure 7. Size of cells were slightly small comparing with the control. It might cause by essential nutrient depleting because nitrogen is macronutrient which is important for algal growth and metabolism. Therefore, nitrogen depletion caused in morphological change (Juneja *et al.*, 2013). Furthermore, cell colors under nitrogen starvation seemed to be faint more than control cells. However, cells were changed from green to orange on day 28 of observation. Tranformed colors observing here assumed as a result from carotenoids accumulation induced by nitrogen starvation.

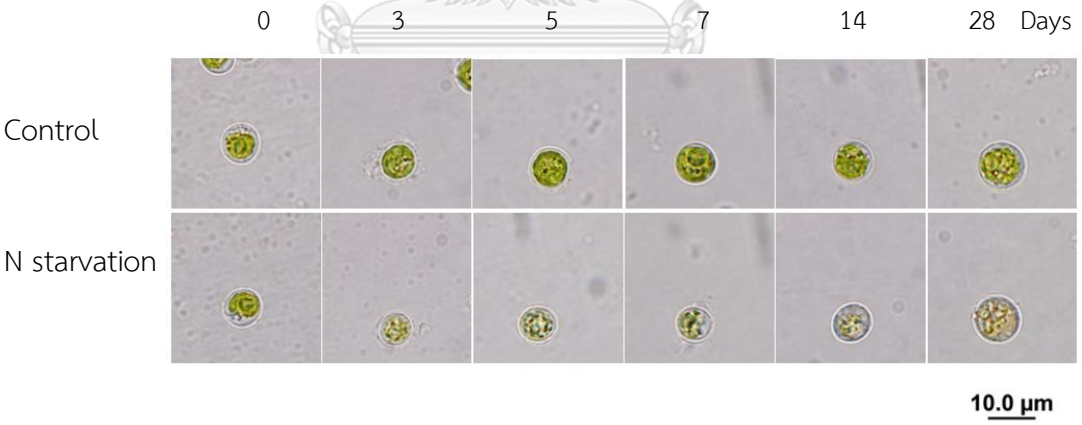


Figure 7 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under nitrogen starvation condition. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

To observe effect of light stress, cells were exposed to high light using light intensity at 50±5 and 80±5 μmoles/m^2/s. For this stress, the size of cells was gradually increased (Figure 8). This is likely due to sufficient nutrient and light for photosynthesis. Long-term of high light exposure; however, color of cells was changed from green to yellow on day 14 and to orange color on day 28 of treatment. Nevertheless, light intensity at 80±5 μmoles/m^2/s could induce color changing in cells faster than light intensity of 50±5 μmoles/m^2/s. Color changing in cells treated with high light would occur from carotenoids accumulation as well.

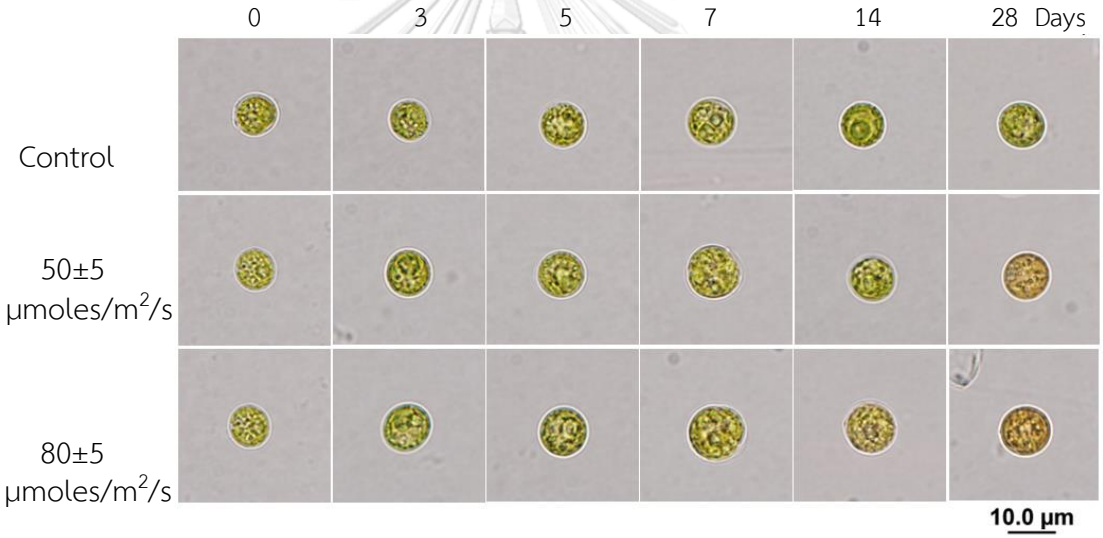


Figure 8 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under light stress. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

Chlorococcum sp. is the species that was reported the tolerancy to drought environment (Klochkova *et al.*, 2006). We therefore included drought stress in this study. To screen morphological changes under drought stress, cells were separated into 2 groups. The first group, cells were cultured in 1 to 3 mL BG-11 that placed in 12-well plate. The second group, ten milliliters of cells culture were grown in culture plate to increase surface area for higher water evaporation. Then cells were observed under light microscope and compared with control after 0, 3, 7, 14 and 28 days (Figure 9). Cell sizes in 12-well plates culturing were similar to the control ones. However, cells in 1 mL media changed from green to green-orange color at day 28. For cells that grown in culture plate, the size of cells was increased together with color of cells were transformed to orange on day 28 of observation. It should be noted that algal cell walls become thicken (Figure 10). This characteristic would cause the toughness to extract carotenoids from algal cells.

From morphological observations, all stresses led the cell colors changes from green to yellow or orange, which is hypothesized as carotenoids shade. Salinity stress enhanced in cell sizes together with orange shade color transformation, KCl stress in particular. As nitrogen served as macronutrient which is important for algal metabolism; thus, nitrogen starvation condition was also interested to examine. Taken together, KCl stress and nitrogen starvation were further analyzed to determine carotenoids compositions and other biocompounds in their cells.

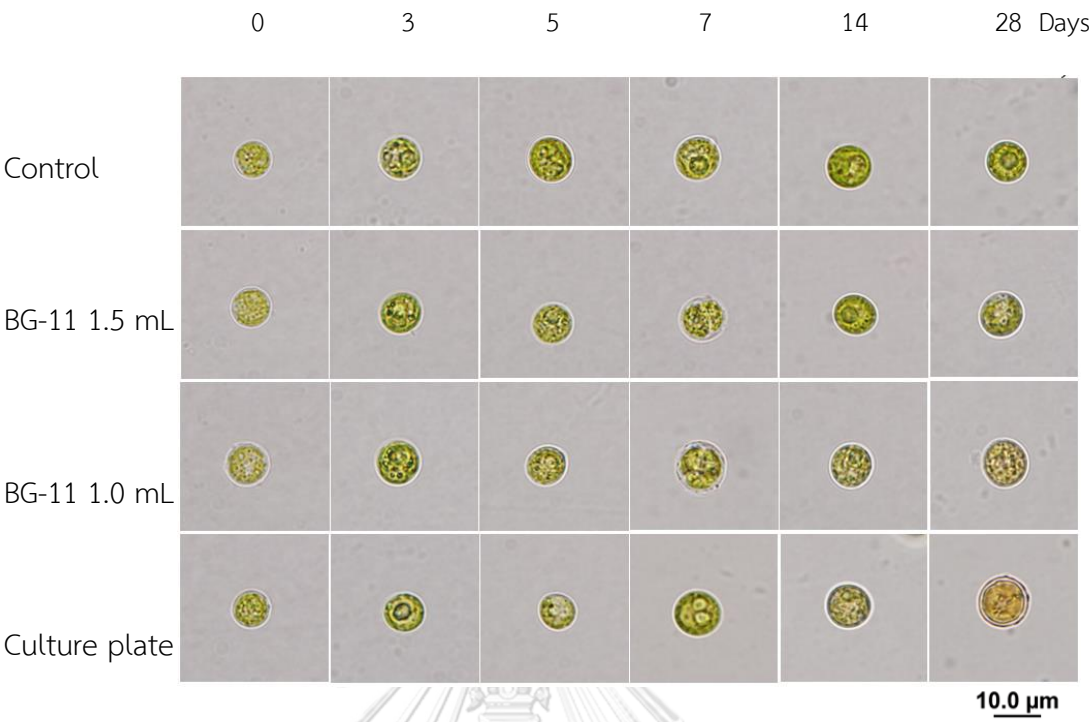


Figure 9 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under drought stress. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

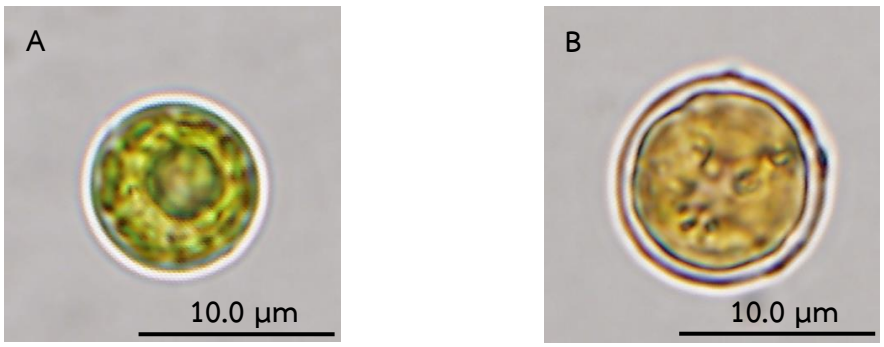


Figure 10 Morphological and color changes of *Chlorococcum* sp. 8367RE after culturing under control (A) and drought stress (B) for 28 days. Photographs were taken using light microscope (Olympus BX51, Japan) couples with DP controller program.

4.3 Extraction of carotenoids

To extract carotenoids from the green microalga *Chlorococcum* sp. 8367RE, cell pellets harvesting from control, KCl stress and nitrogen starvation were broken by using several disruption methods. The first disruption method, cells were crushed using the freezed motar and pestle. The pellets were ground homogenously with solvent until cells debris were bleached. However, this method was found a low efficacy to extract completely. It was observed that only a half part of the pellets was broken. The second disruption method was employed by using silica bead together with vortexing. By using this method, the pellets were mixed with solvent and 0.5 mm. silica beads were then added. Thereafter, the mixture was vortexed (Model G560E: Scientific Industries, USA). The pellets were broken by this procedure; nevertheless, it took a considerably long time to break a large amount of pellets. Thus, the ultrasonicator bath was used instead of a vortex mixer to disrupt the cells severely. This method was effective for microalgal cells disruption. In addition to disruption methods, various organic solvents were used to extract carotenoids. Solvents used in this study include methanol, acetone, acetonitrile and DMSO. It was reported that 80% acetone was selected in many studies. Here, we compared other three solvents; methanol, acetonitrile and DMSO. The algal pellets were extracted with these solvents by using 0.5 mm. silica beads combined with ultrasonicator until cells debris were bleached. After that, the supernatants were collected and filtered through syringe filter with a 0.22 μm. Then the extracts were analyzed by HPLC. Profiles were demonstrated (Figure 11). HPLC chromatograms showed that the highest signal of carotenoids peaks was detected by using 80% acetone extraction (Figure 11A). Thus, 80% acetone was the most effective solvent for carotenoids extraction from *Chlorococcum* sp. 8367RE.

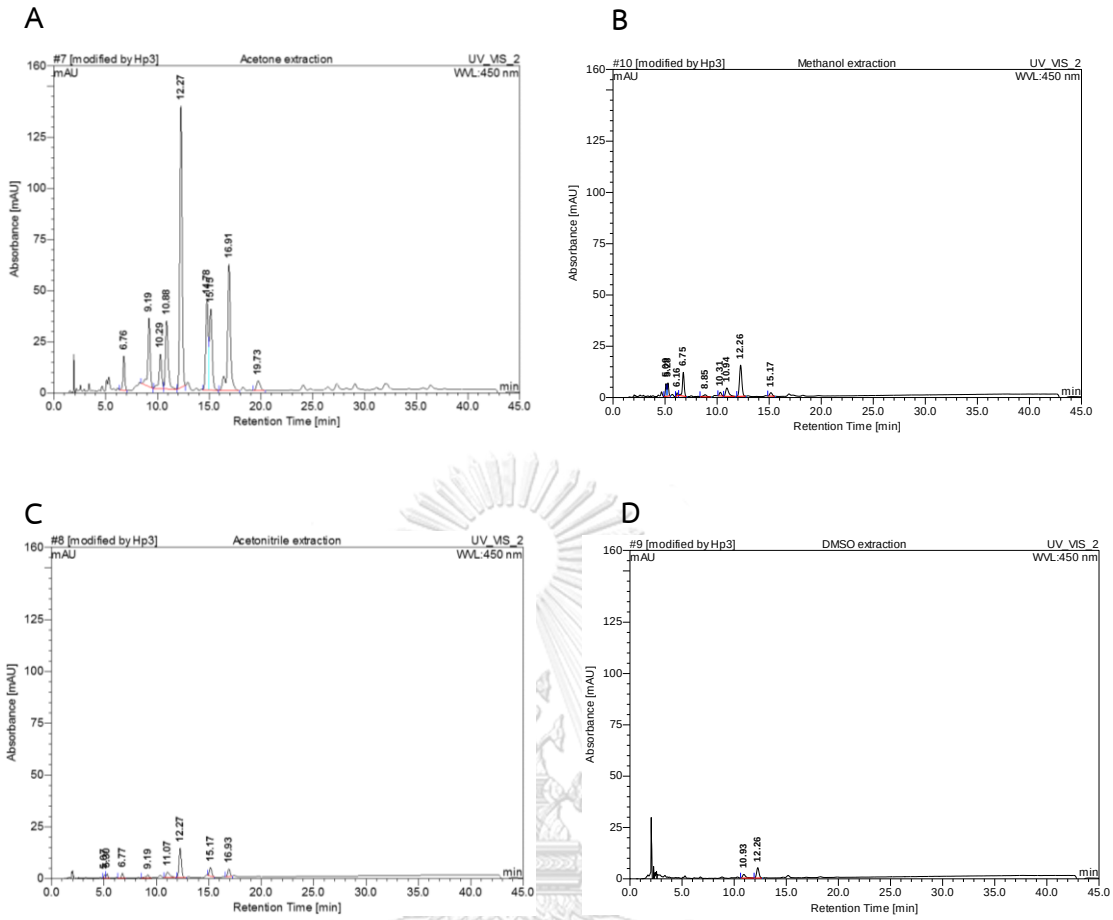


Figure 11 HPLC chromatograms of carotenoids extractions from *Chlorococcum* sp. 8367RE using 80% acetone (A), methanol (B), acetonitrile (C) and DMSO (D), respectively

4.4 Determination of pigment content

To observed UV absorption of the algal extracts, UV-Vis spectroscopy was performed. This method is a simple technique to measure the absorbance of substances and used for quantitative measurement. Thus, in this experiment the algal extracts were scanned at the wavelength in the range of 200-800 nm. The absorbance profiles were shown in Figure 12. From the absorbance profiles, absorbance peaks at around 400-500 nm were detected in all samples which are the range of carotenoids absorptions. From this pattern, it could be hypothesized that all of extracts samples contain carotenoids. In addition, the algal extracts from control and KCl stress conditions showed high spectrum at this range. Moreover, the absorbances between 350-400 and 650-700 nm were detected which is the absorption spectrum of chlorophylls.

To estimate chromophore contents, the absorbance value at 663, 647 and 470 nanometers were calculated by using the equations as described in Materials and Methods. Chlorophylls and carotenoids contents were represented in Figure 13. Under nitrogen starvation, pigment contents were dramatically reduced which is consistent with color of cells fading. However, carotenoids/chlorophylls ratio was increased. These results are in the similar trend as observing in *Scenedesmus* sp. CCNM 1077, which was cultured under nitrogen stress. The authors reported that when nitrate concentration in medium was reduced, pigment contents were also reduced except carotenoids/chlorophylls ratio were increased (Pancha *et al.*, 2014). It could explain that under nitrogen starvation, light harvesting pigments were decreased; however, microalgae attempt to balance all pigment for photosynthesis that result in pigment ratio changing. Moreover, it was reported that carotenoids/chlorophylls ratio increasing due to the antioxidative function against oxidative stress induced by nitrogen starvation (Zhang *et al.*, 2013). Upon KCl stress, it led to chlorophyll a reduction and carotenoids/chlorophylls ratio acceleration, which are elucidated as same as above. In addition, total carotenoids content equally estimated between control and KCl stress

which are higher than that measured under nitrogen starvation. These results revealed that pigments content, especially carotenoids in *Chlorococcum* sp. 8367RE were changed under stress conditions. Further, HPLC was performed to analyze and compare with authentic carotenoids.

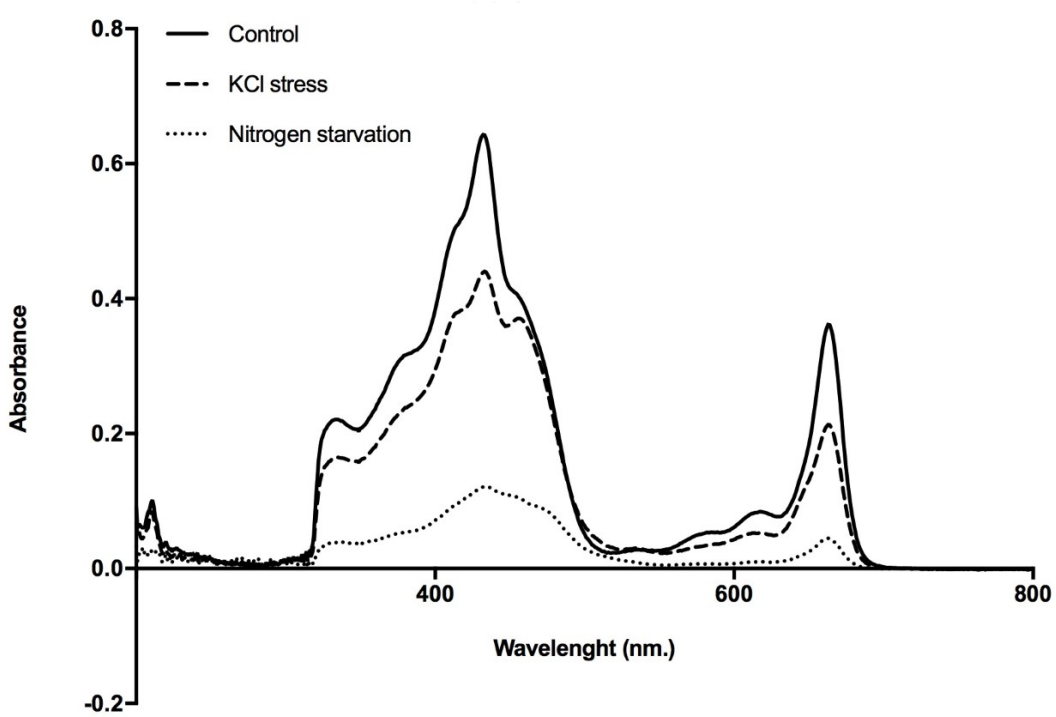
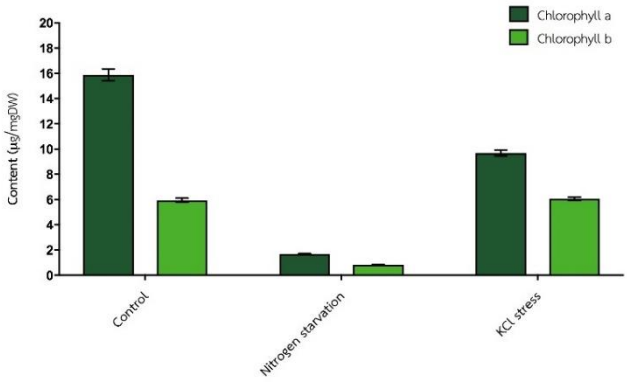
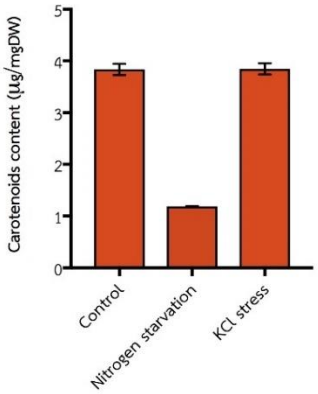


Figure 12 The absorbance profiles of the algal extracts culturing under control, KCl stress and nitrogen starvation conditions. The spectra were scanned from the wavelength between 200-800 nm.

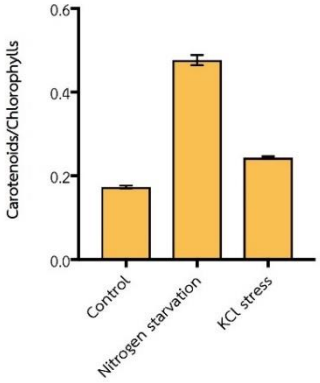
A



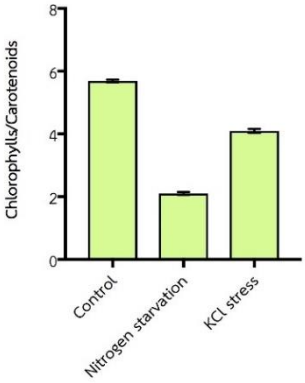
B



C



D



E

Chromophore	Pigment content (µg/mgDW)		
	Control	Nitrogen starvation	500 mM KCl stress
Chlorophyll a	15.89 ± 0.46	1.68 ± 0.03	9.68 ± 0.24
Chlorophyll b	5.95 ± 0.16	0.81 ± 0.03	6.06 ± 0.13
Total chlorophylls	21.80 ± 0.62	2.49 ± 0.06	15.70 ± 0.36
Total carotenoids	3.84 ± 0.11	1.18 ± 0.01	3.85 ± 0.11
Carotenoids/Chlorophylls	0.17 ± 0.00	0.48 ± 0.01	0.24 ± 0.00
Chlorophylls/Carotenoids	5.69 ± 0.04	2.10 ± 0.05	4.10 ± 0.07

Figure 13 Chlorophyll content (A), carotenoids content (B), carotenoids/chlorophylls (C), chlorophylls/carotenoids (D) and all pigment content (E) in *chlorococcum* sp. 8367RE culturing under control, nitrogen starvation and KCl stress for 28 days. (data were shown as mean ± SE)

4.5 Analysis of carotenoids

To determine carotenoids compositions in the algal extracts, reversed-phase high performance liquid chromatography was performed. Firstly, C18 column was used to separate the carotenoids and the mobile phase consist of acetonitrile and methanol (80:20); however, signals were not distinctly separated. Moreover, other mobile phase systems were tested but the results were not improved (data not shown). It was reported that, C30 column was employed in several studies, especially carotenoids analysis because C30 column are capable to discriminate the long chain molecules like carotenoids. Later, C30 YMC column was replaced for carotenoids analysis in this study.

To separate carotenoids on C30 column, various gradient systems were suggested as mentioned in the introduction (Table 1). In this study, methanol and methyl-t-butyl ether (MTBE) were selected as mobile phase. The gradient systems of these solvents were examined. Firstly, a gradient solvent system 1 was started at 5% solvent B (MTBE) for 5 mins, followed by a linear gradient to 30% of MTBE in 30 mins. Then, MTBE was increase to 50% in 35 mins. After 70 mins of running, MTBE was held at 5% until the end. This time program spent 90 mins running at 0.9 mL/min of flow rate. The algal extract profile showed the closely peaks and the sample was eluted out of column early (Figure 14). Additionally, after 30 mins of running, no signal was observed. It can be speculated that upon this gradient system only 30% of MTBE eluted all of samples out of the column. Thus, the gradient of MTBE was adjusted in the next experiment.

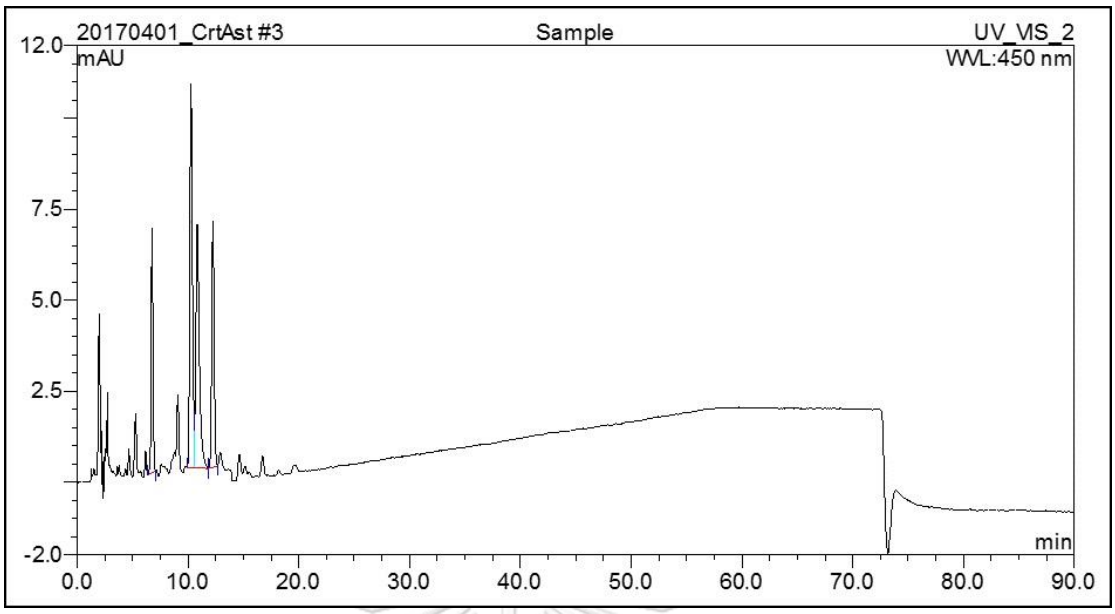


Figure 14 The chromatogram of the algal extracts which separated on C30 column by using gradient solvent system 1. The absorbance was detected at 450 nm.

Secondly, a gradient solvent system 2 was tested. This system began with 5% MTBE for 5 mins, followed by a linear gradient to 30% of MTBE in 30 mins. Then, 30% of MTBE was held for 5 mins. After that, MTBE was decreased to 5% until the end of experiment. This time program spent 45 mins running at 0.9 mL/min of flow rate. The chromatogram of algal extract showed the fine peak separation (Figure. 15A) with appropriate time running.

Moreover, other two gradient systems were performed. For the gradient system 3, the beginning and the last of running were adjusted to 0% MTBE replaced 5% MTBE in the gradient system 2. In addition, time for a linear gradient of MTBE was extended from 30 mins to 35 mins. The result showed a slight improve for separation. It should be noted that all the signals were delayed when compared with gradient solvent system 2 running (Figure. 15B). Lastly, a gradient solvent system 4 was determined by linear gradient of MTBE extended to 45 mins; however, the chromatogram showed a similar trend with gradient solvent system 3 unless peak elution was delayed (Figure 15C). Taken together, gradient solvent system 3 was selected to separate the algal extracts. Additionally, the flow rate was adjusted from 0.9 to 0.4 mL/min for gradually peak separation (Figure 15D).

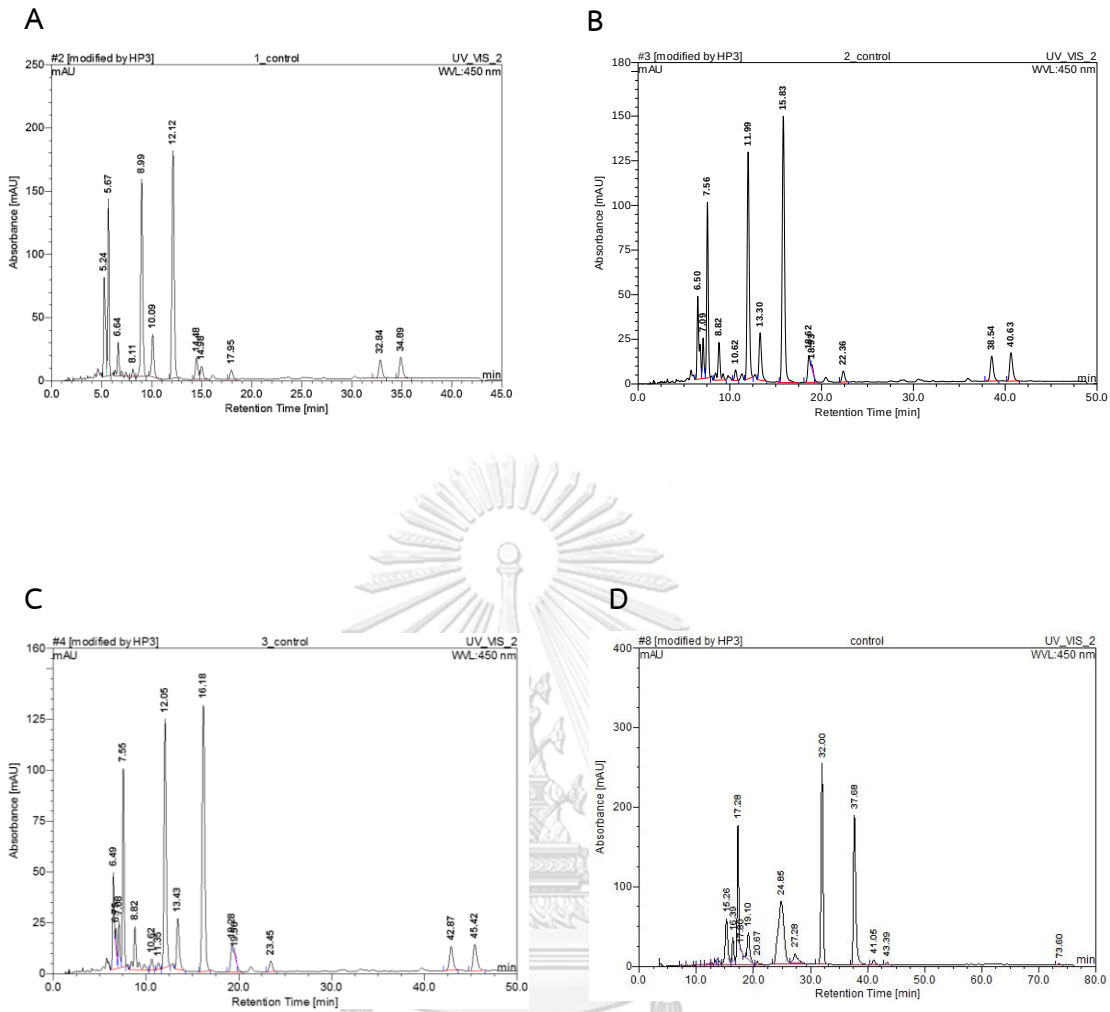


Figure 15 HPLC chromatograms of the algal extracts which separated on C30 column by using gradient solvent system 2 (A), 3 (B), 4 (C) at 0.9 mL/min of flow rate and gradient solvent system 3 at 0.4 mL/min (D). The absorbance was detected at 450 nm.

From chromatogram adjustment, the algal extracts were separated by using gradient solvent system 3 with 0.4 mL/min of flow rate. The results showed that algal extracts from control culturing represented six dominant peaks (Retention time (RT) 14.06, 15.89, 16.72, 22-23, 30.60 and 35.69, respectively) (detection at 450 nm.) (Figure. 16). However, chlorophylls can also absorb in this wavelength. To investigate chlorophylls peaks, the absorbance at 642 and 665 nms. (chlorophylls absorbance range) were investigated (Figure.17A and 17B). As shown in Figure.17, three peaks (RT 14.06, 22.53 and 35.69, respectively) were not carotenoids because these peaks could absorb at wavelength over 500 nm. In addition, lutein standard was also run and show the same retention time that detected in sample (RT 30.60). Thus, the highest peak of this chromatogram would be lutein. However, mass spectrometry was performed to further identify. For mass spectrometry, the result from IDA mode screening was shown in Figure. 18 (raw data of the analysis was shown in appendix 7). HPLC profile and mass spectrometry results were interpreted together and three types of carotenoids were detected including violaxanthin (1), antheraxanthin (2) and lutein (3). Lutein content was 2.24 µg/mg dry weight and represented as the dominant carotenoids in the algal extracted from control culturing. It should be noted that suspected steroid which is $C_{37}H_{60}O_5$ was detected (appendix 7).

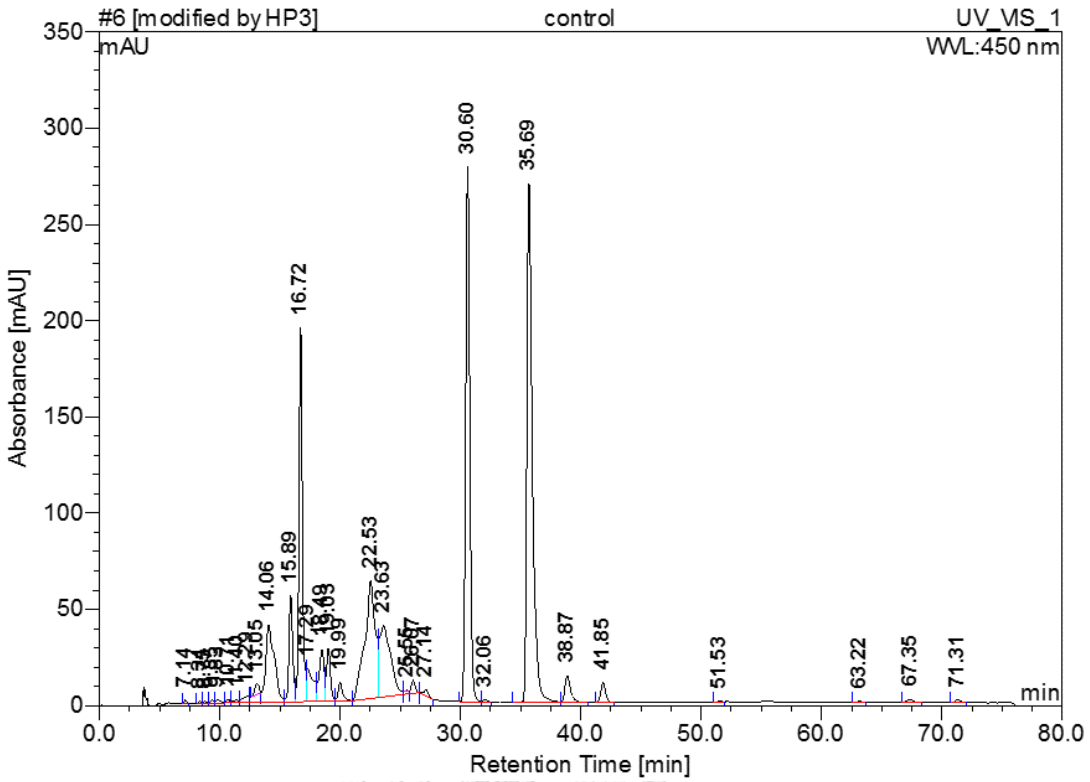


Figure 16 The chromatogram of the algal extracts from control culturing which separated on C30 column by using gradient solvent system 3 at 0.4 mL/min of flow rate. The absorbance was detected at 450 nm.

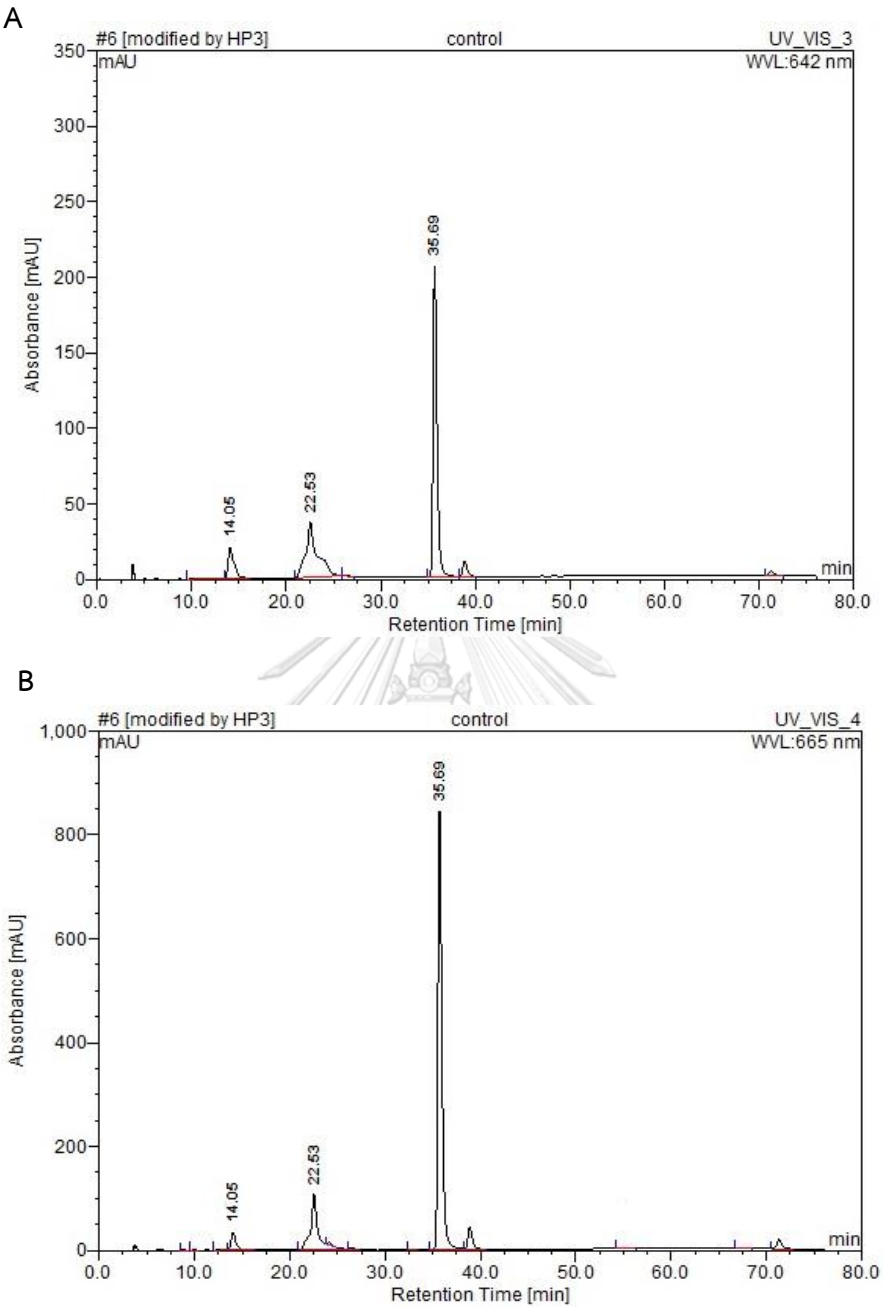


Figure 17 The chromatogram of the algal extracts from control culturing which separated on C30 column by using gradient solvent system 3 at 0.4 mL/min of flow rate. The absorbance was detected at 642 nm. (A) and 665 nm. (B)

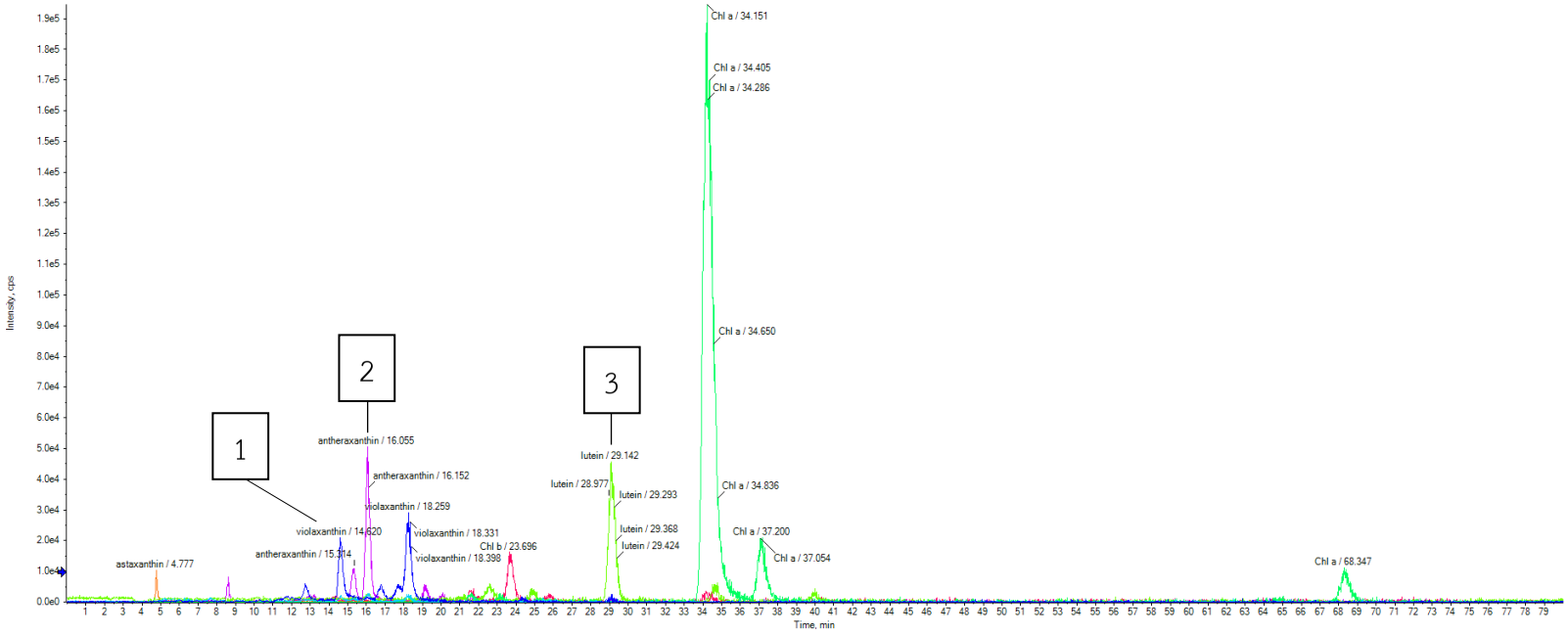


Figure 18 Spectrum of the algal extracts from control culturing that obtained from IDA screening mode. The spectrum represented violaxanthin (1), antheraxanthin (2) and lutein (3)

The algal extracts from KCl stress was analyzed. From HPLC profile (Figure.19), three dominant peaks (RT= 22-23, 30.62 and 35.77) were detected under 450 nm. Similarly with control condition, lutein (RT= 30.62) represented the dominant of carotenoids. It should be mentioned that, low signal of astaxanthin peak was detected (RT 27.93), when compared with astaxanthin standard. To further identify by mass spectrometry, the result from IDA mode screening was shown in Figure. 20 (raw data of the analysis was shown in appendix 7). HPLC profile and mass spectrometry results were interpreted together and five types of carotenoids were detected. These are violaxanthin (1), antheraxanthin (2), astaxanthin (3), lutein (4) and canthaxanthin (5). When comparing with authentic standards, lutein and astaxanthin contents in these algal extracts were 2.26 and 0.12 µg/mg dry weight, respectively Here, it was clearly elucidated that KCl stress led to carotenoids compositional changes in *Chlorococcum* sp. 8367RE. Moreover, KCl stress could induce astaxanthin which is a high value carotenoids. In *Chlorella zofingiensis,* astaxanthin and canthaxanthin accumulations increased upon salt stress (NaCl stress) (Pelah *et al.*, 2004). Furthermore, salinity stress could induce astaxanthin content in *H. pluvialis.* The authors reported that genes involve in astaxanthin synthesis were up regulated upon salinity stress (Gao *et al.*, 2015). From this study, KCl stress would be one of alternative ways to enhance high value carotenoids like astaxanthin which possess strong antioxidant activity to protect algal cells under unflavored conditions. Upon this stress also, the suspected steroid which is $C_{37}H_{60}O_5$ was detected.

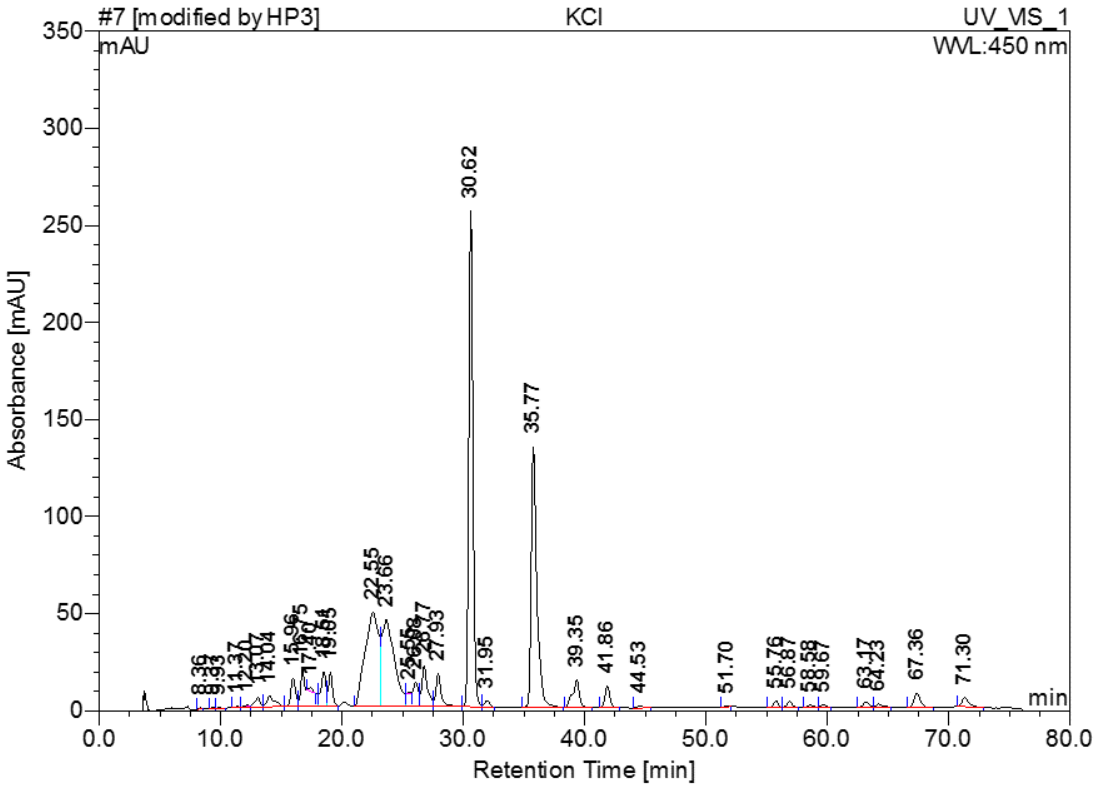


Figure 19 The chromatogram of the algal extracts from KCl stress culturing which separated on C30 column by using gradient solvent system 3 at 0.4 mL/min of flow rate. The absorbance was detected at 450 nm.

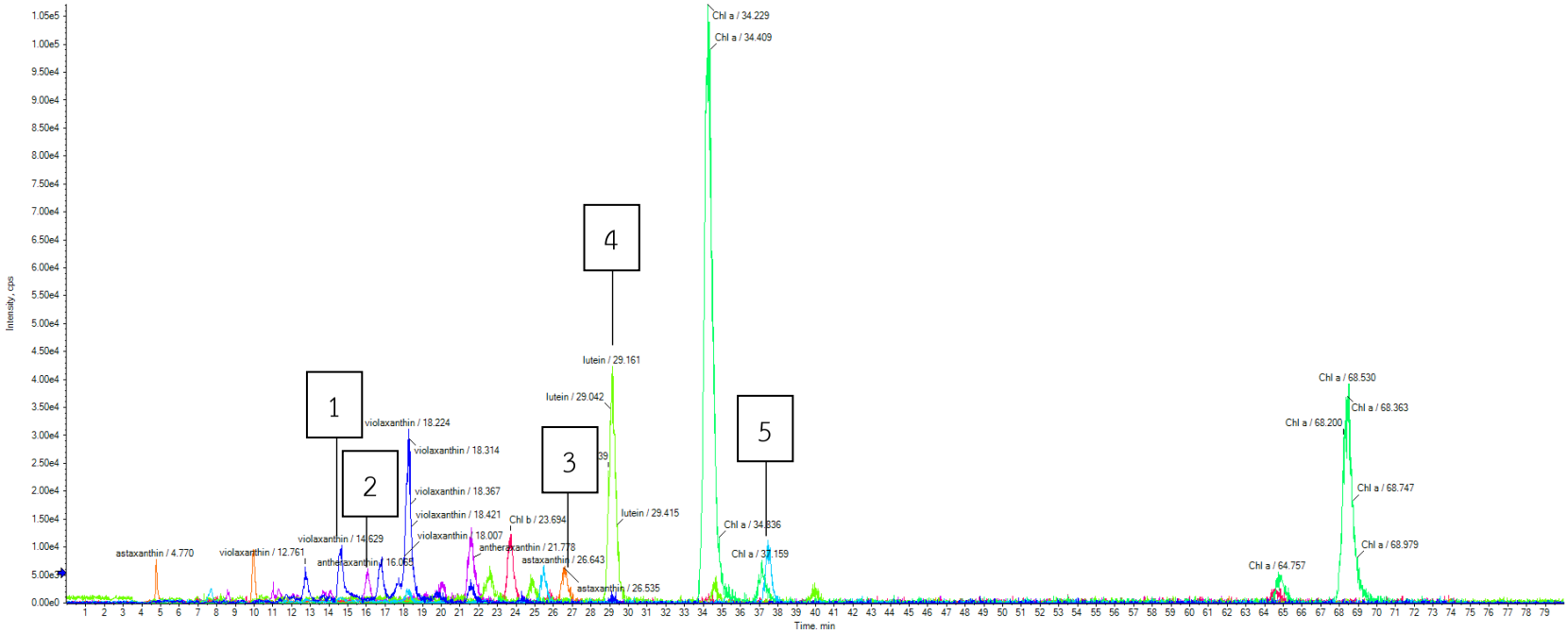


Figure 20 Spectrum of the algal extracts from KCl stress culturing that obtained from IDA screening mode. The spectrum represented violaxanthin (1), antheraxanthin (2), astaxanthin (3), lutein (4) and canthaxanthin (5).

The algal extracts from nitrogen starvation condition was further analyzed. From HPLC profile (Figure.21), the low signals were detected; however, lutein still showed as a dominant peak (RT=30.63). Lutein content in this extract was 0.44 µg/mg dry weight. Similary with KCl stress, astaxanthin was found. Trace amount of astaxanthin was 0.06 µg/mg dry weight. The result showed that nitrogen starvation affected on carotenoids and chlorophyll levels, which is agreeable with cell colors bleaching as observed under nitrogen starvation treatment. It was reported that nitrogen starvation effected on all photosynthetic pigments by decreasing chlorophyll a, chlorophyll b and carotenoids in *Scenedesmus* sp. CCNM 1077 and *Chlorella minutissima* (Ördög *et al.*, 2012; Pancha *et al.*, 2014) . Chlorophylls depletion would cause by nitrogen utilization under nitrogen starvation condition. As chlorophylls structure composed of nitrogen, it was utilized to nitrogen source for cells growth (Li *et al.*, 2008). Because of low carotenoids peaks, this condition was not further investigated by mass spectrometry.

From carotenoids analysis, stress conditions (salinity stress and nitrogen starvation) led to carotenoids composition changes in *Chlorococcum* sp. 8367RE including other chromophore like chlorophylls. Upon KCl stress, secondary carotenoids (astaxanthin and canthaxanthin) were additionally detected which possessed strong antioxidant activity to prevent cells damages from reactive oxygen species (Abe *et al.*, 2007). It should be mentioned that the microalga alters their morphological and pigments compositions for their survival under unsuitable conditions. Therefore, the other biocompounds such as lipid, protein and carbohydrate were further investigated.

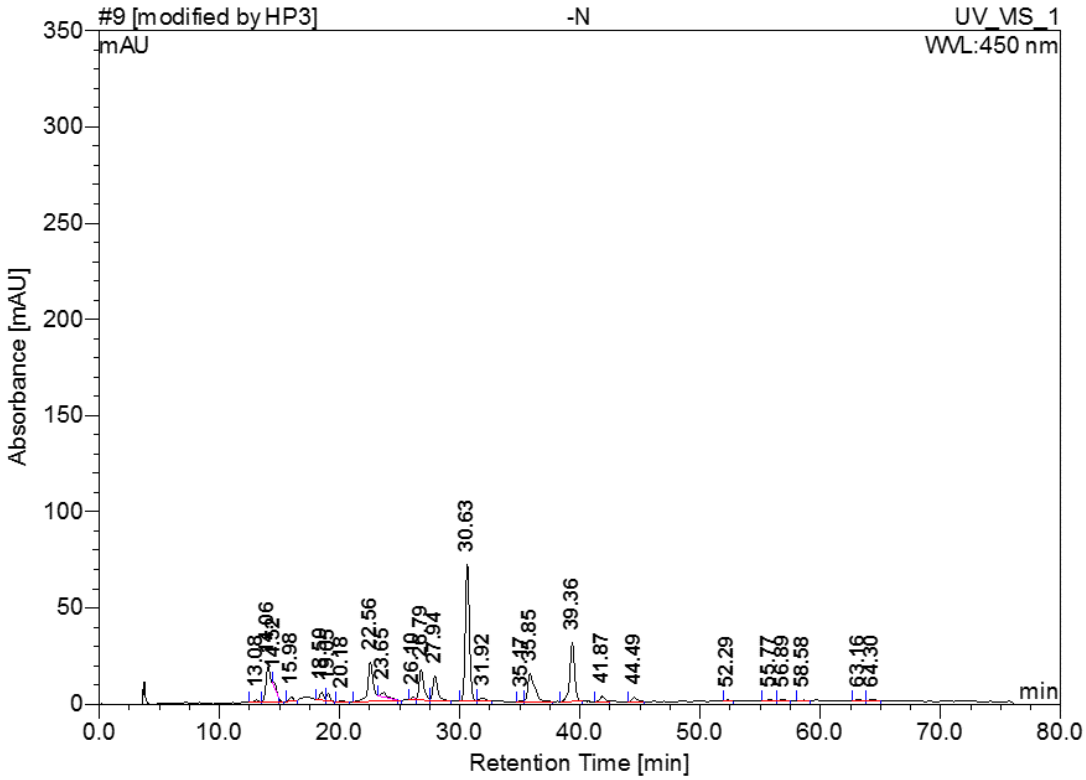


Figure 21 The chromatogram of the algal extracts from nitrogen starvation culturing which separated on C30 column by using gradient solvent system 3 at 0.4 mL/min of flow rate. The absorbance was detected at 450 nm.

4.6 Analysis of biocompounds

Microalgae are capable of producing a variety of high value products, such as carotenoids and polyunsaturated fatty acids (PUFAs) (Spolaore *et al.*, 2006). In addition, amino acids which can be used as a rich source for food and feed industries. We assumed that stress treatments would affect to biocompounds modulating in microalgae. In this study, we therefore analyzed lipid, protein and carbohydrate.

4.6.1 Lipid analysis

To examine neutral lipid accumulation in *Chlorococcum* sp. 8367RE under selected stress treatment, nile red staining method was performed and observed under fluorescent microscope as described in Materials and Methods. In this study, nile red was dissolved in 15% DMSO to serving as a stain carrier into the cells. After nile red permeated into cells, it binds to the neutral lipid droplets and exhibited a yellow fluorescence (Greenspan *et al.*, 1985). Nevertheless, the results from this study showed that control cells, KCl stress and nitrogen starvation treated cell exhibited red fluorescence over whole cells (data not shown). After 7 days of nitrogen starvation, cells exhibited low intensity of red fluorescence when compared with other conditions. From this point, nile red staining method seems to be low efficiency to permeate into the *Chlorococcum* sp. 8367RE cells. It might cause from properties of cell wall that effected nile red diffusion, resulting in low intensity of fluorescence (Huang *et al.*, 2009).

Fatty acid methyl ester analysis was further performed instead to analyze fatty acid composition. Cell pellets harvesting from 28 days of stress treatments were extracted and analyzed by gas chromatography (Model Agilent 6890N, Agilent, USA) equipped with flame ionization detector (FID) as described in Materials and Methods. Then fatty acids content in extracts were calculated by comparing with authentic compounds. Fatty acid composition of *Chlorococcum* sp. 8367RE under control, KCl stresses were summarized in Table 2 and Figure 22.

Total fatty acid in *Chlorococcum* sp. 8367RE after culturing under control, nitrogen starvation and KCl stress were 6.31 ± 0.06, 28.07 ± 0.79 and 12.79 ± 0.03 % dry weight. The dominant of fatty acids in control cells were linoleic acid (C18:2) (26.8 ± 0.01% of total fatty acids), linolenic acid (C18:3) (25.38 ± 0.05% of total fatty acids), palmitic acid (C16:0) (24.19 ± 0.02% of total fatty acids) and oleic acid (C18:1) (20.0 ± 0.08% of total fatty acids), respectively whereas under nitrogen starvation, oleic acid was considerably increased to 45.15 ± 0.01% of total fatty acids or more than 2 folds comparing with control. While palmitic acid, linoleic acid and linolenic acid were decreased. It was reported that fatty acids was enhanced under nitrogen starvation mainly oleic acid in *Chlorella vulgaris* NIES-227 (Shen *et al.*, 2015).

In case of KCl stress, it was similar trend with nitrogen starvation. Oleic acid was increased to 29.73 ± 0.08% of total fatty acids. In *Chlamydomonas reinhardtii*, it was reported that NaCl stress led the lipid content increasing (Hounslow *et al.*, 2016). Stress condition effect to growth limitation and microalgae could survive by increase accumulation of energy-rich storage including lipid accumulation (Pal *et al.*, 2011) .

Table 2 Fatty acids composition in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress (data were shown as mean ± SD). The unit was represented as % of total fatty acids.

Fatty acid (methyl ester)	**Control**	**Nitrogen starvation**	**500 mM KCl**	**Ratio -N/C**	**Ratio KCl/C**
Caprylic acid (C8:0)	0.05 ± 0.00	ND	0.03 ± 0.00	0.00	0.60
Capric acid (C10:0)	0.07 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.29	0.29
Lauric acid (C12:0)	ND	ND	0.02 ± 0.00	ND	0.02
Myristic acid (C14:0)	1.92 ± 0.01	0.39 ± 0.00	0.44 ± 0.02	0.20	0.23
Palmitic acid (C16:0)	24.19 ± 0.02	20.06 ± 0.04	19.26 ± 0.04	0.83	0.80
Palmitoleic acid (C16:1)	0.63 ± 0.00	0.17 ± 0.00	0.46 ± 0.00	0.27	0.73
Stearic acid (C18:0)	0.67 ± 0.08	4.20 ± 0.08	2.29 ± 0.25	6.27	3.42
Oleic acid (C18:1)	20.00 ± 0.08	45.15 ± 0.11	29.73 ± 0.08	2.26	1.49
Linoleic acid (C18:2)	26.80 ± 0.01	16.34 ± 0.01	21.79 ± 0.09	0.61	0.81
Linolenic acid (C18:3)	25.38 ± 0.05	12.67 ± 0.05	25.01 ± 0.06	0.50	0.99
Arachidic acid (C20:0)	0.10 ± 0.00	0.67 ± 0.01	0.36 ± 0.00	6.70	3.60
Behenic acid (C22:0)	0.20 ± 0.01	0.25 ± 0.00	0.41 ± 0.01	1.25	2.05
Erucic acid (C22:1)	ND	ND	ND	ND	ND
Lignoceric acid (C24:0)	ND	0.08 ± 0.00	0.18 ± 0.01	0.08	0.18
Total fatty acid (% dry weight)	6.31 ± 0.06	28.07 ± 0.79	12.79 ± 0.03	4.45	2.03

ND: not detected

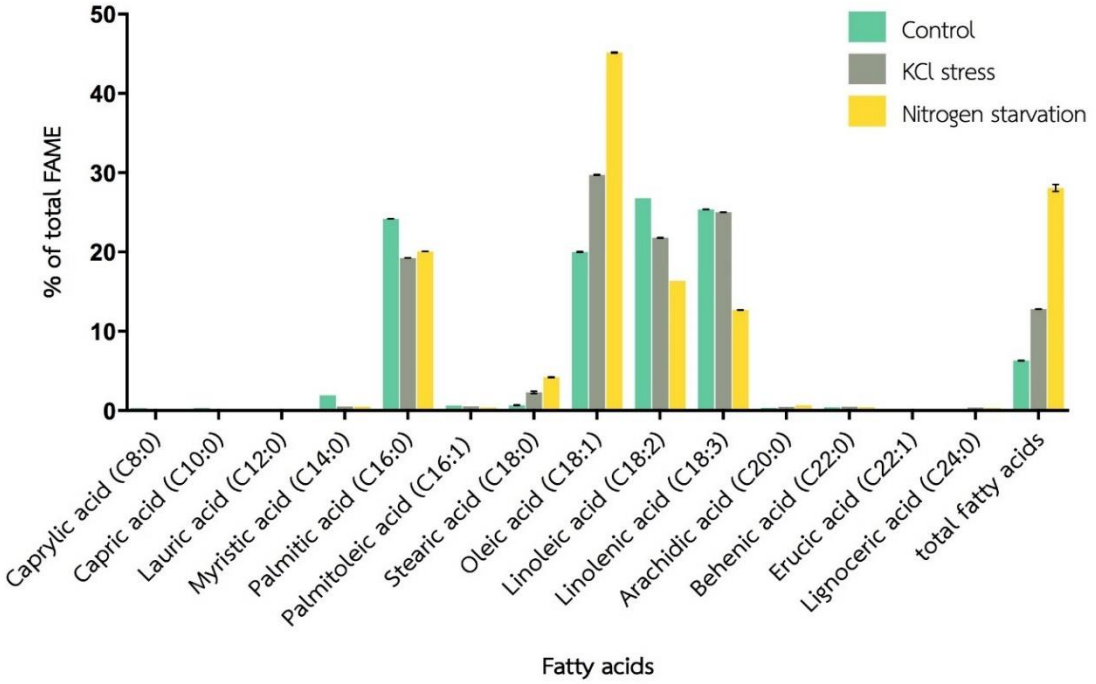


Figure 22 Fatty acid compositions in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress.

4.6.2 Amino acids and total protein analyses

For amino acid analysis, the algal cell pellets were extracted and subjected to amino acids analyzer. The profiles were compared with 17 authentic amino acids and ammonia. The results demonstrated in Table 3 and Figure 23. Under control condition, alanine was mainly found in *Chlorococcum* sp. 8367RE. Notably, methionine and histidine were not detected. The variety of amino acids composition in microalgae depended on strains that was reported in the literatures (Becker, 2007). Many microalgae mainly accumulate alanine, glutamic acid, aspartic acid and arginine as the main free amino acids. However, most of amino acids were decreased when the cells were exposed with nitrogen starvation and salt stress. Under nitrogen starvation, leucine was increased approximately 1.5 folds when compared with control. In addition, valine, isoleucine and phenylalanine were no difference between control and stress treatment. While ammonia was dramatically increased about 4 folds of control. Upon KCl stress, most of amino acids were decreased, except proline that was highly induced about 7.5 folds when compared with control and nitrogen starvation treated. It has been suggested that proline accumulation correlated with abiotic stress, especially salinity stress in plant and microorganisms (Szabados and Savoure, 2010). Upon salinity stress, proline acts as compatible osmolyte which assisted microalgal cells protection (Hiremath and Mathad, 2010).

Table 3 Amino acids composition in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress (data were shown as mean ± SD). The unit was represented as nmol/mg DW.

Amino Acid	Control (C)	Nitrogen starvation	500 mM KCl	Ratio -N/C	Ratio KCl/C
Aspartic acid	0.28 ± 0.01	0.02 ± 0.01	0.04	0.07	0.14
Threonine	0.45 ± 0.00	0.37 ± 0.06	0.36	0.82	0.80
Serine	0.70 ± 0.02	0.28 ± 0.05	0.10	0.39	0.14
Glutamic acid	1.74 ± 0.05	0.36 ± 0.08	0.48	0.21	0.28
Glycine	0.51 ± 0.01	0.26 ± 0.06	0.09	0.52	0.18
Alanine	7.98 ± 0.25	3.93 ± 0.25	3.80	0.49	0.48
Cystine	0.07 ± 0.04	0.02 ± 0.00	0.05	0.33	0.83
Valine	0.56 ± 0.02	0.58 ± 0.08	0.40	1.04	0.71
Methionine	ND	0.01 ± 0.01	0.01	0.01	0.01
Isoleucine	0.22 ± 0.01	0.28 ± 0.04	0.14	1.23	0.64
Leucine	0.40 ± 0.01	0.60 ± 0.07	0.22	1.54	0.56
Tyrosine	0.67 ± 0.00	0.22 ± 0.02	0.13	0.33	0.19
Phenylalanine	0.26 ± 0.00	0.26 ± 0.03	0.10	1.00	0.38
Lysine	0.54 ± 0.01	0.06 ± 0.01	0.11	0.11	0.20
Histidine	ND	ND	ND	ND	ND
Ammonia	3.06 ± 0.28	13.05 ± 0.02	0.86	4.26	0.28
Arginine	2.52 ± 0.01	0.08 ± 0.01	1.61	0.03	0.64
Proline	0.79 ± 0.01	0.49 ± 0.06	5.92	0.62	7.49

ND: not detected

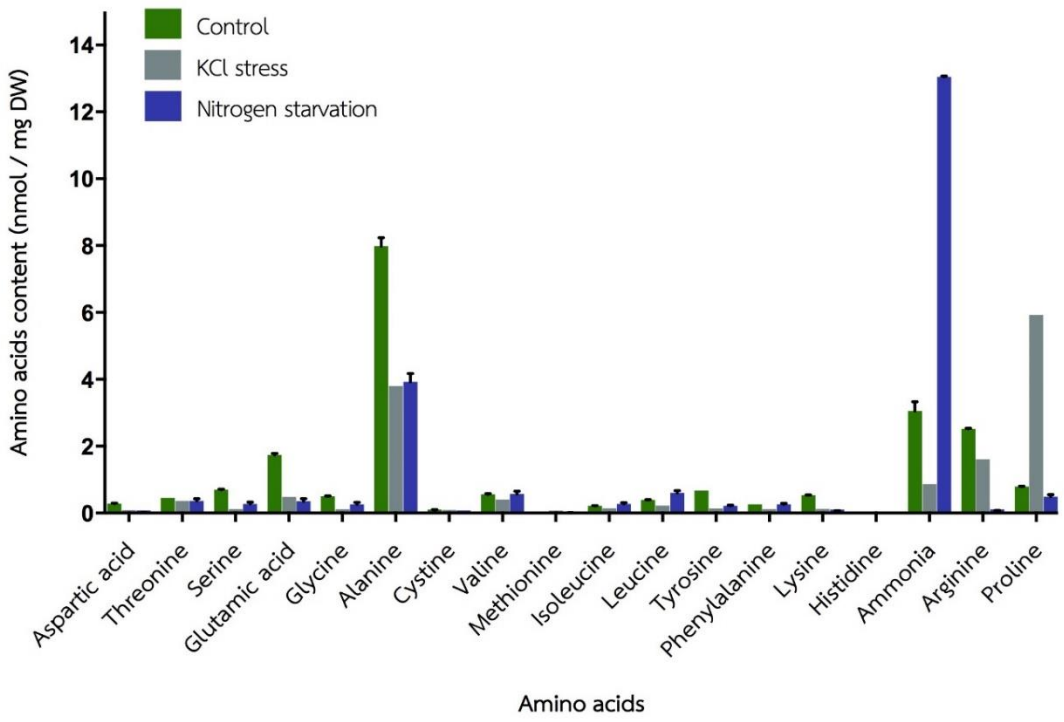


Figure 23 Amino acids composition in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress.

To estimated total protein content in *Chloroccocum* sp. 8367RE, Bradford assay was performed as described in Materials and Methods. The result revealed that total protein content that obtained from control, KCl stress and nitrogen starvation conditions were 16.73, 13.84 and 5.50% dry weight, respectively (Figure. 24). Stress conditions led to total protein content reduction especially under nitrogen starvation. Due to nitrogen served as a composition of protein, lack of nitrogen in medium could affect to protein synthesis in microalgal cells as same as *Acutodesmus dimorphus* (Chokshi *et al.*, 2017), *Chlorella vulgaris* (Agirman and Cetin, 2015) and *Spirulina platensis* (Uslu *et al.*, 2011). Upon KCl stress, total protein was also decreased from 16.73 to 13.84% dry weight which similar as observed in *Acutodesmus obliquus* and *Chlorella vulgaris*. It has been reported that salinity stress (NaCl stress) affected to protein content in both algae resulting in protein reduction from 23 to 13% dry weight in *A. obliquus* and 23 to 19% dry weight in *C. vulgaris as* compared between 0.06 and 0.4 M salt concentration (Pandit *et al.*, 2017). The reduction of protein under salinity stress was also reported in *Nannochloropsis occulate* CS 179 (Gu *et al.*, 2012).

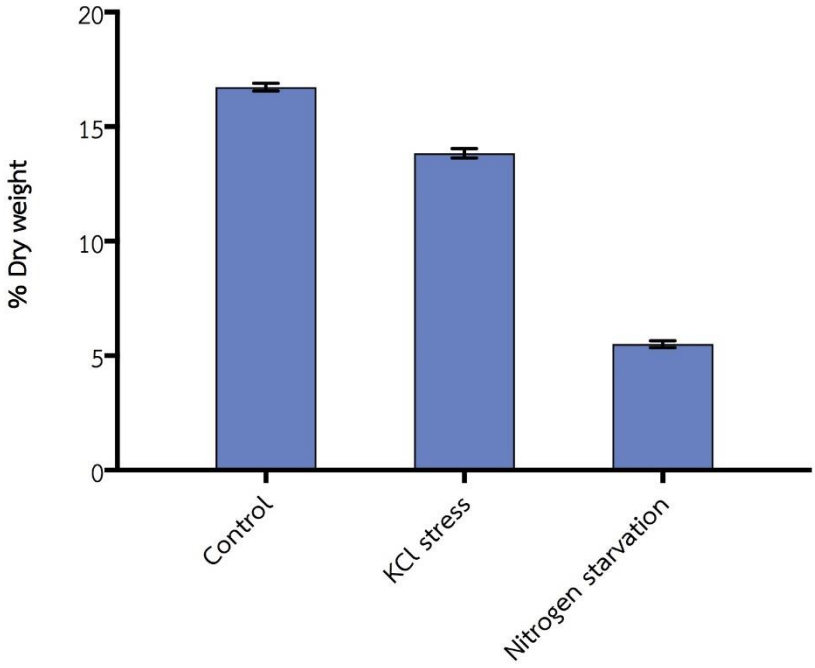


Figure 24 Total protein in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress. Data were shown as mean ± SE.

4.6.3 Carbohydrate analysis

Carbohydrate contents in *Chloroccocum* sp. 8367RE culturing under various conditions were determined by phenol-sulfuric assay as described in Materials and Methods. The total carbohydrate content in the algae that obtained from control, KCl stress and nitrogen starvation condition were 38.17, 35.23 and 29.52% dry weight, respectively (Figure. 25). Total carbohydrate contents decreased when the algal cells exposed to both stresses. It has been reported that five strains of *Chlorella* grown in low-nitrogen medium showed the difference of carbohydrate content. Four strains among these represented carbohydrate content reductions while one strain showed the enhancement of carbohydrate under low-nitrogen medium (Illman *et al.*, 2000). Hence, nitrogen starvation effected carbohydrate content depend on species. Likewise, salinity stress cause carbohydrate content decreasing in this study as same as carbohydrate contents in *Dunaliella salina* and *Dunaliella tertiolecta* decreased when growing under salt stress (Tammam *et al.*, 2011). Although many studies suggested that the limitation of nitrogen and salt stress could enhanced carbohydrate in microalgae, some studies revealed that the metabolic pathway of lipid and carbohydrate were closely linked due to the degradation of starch served as the precursor of fatty acid synthesis like acetyl-CoA. Thus, energy-rich molecule accumulation in microalgae depended on the precursor supplying pathways and strains (Chen *et al.*, 2013; Rismani-Yazdi *et al.*, 2011).

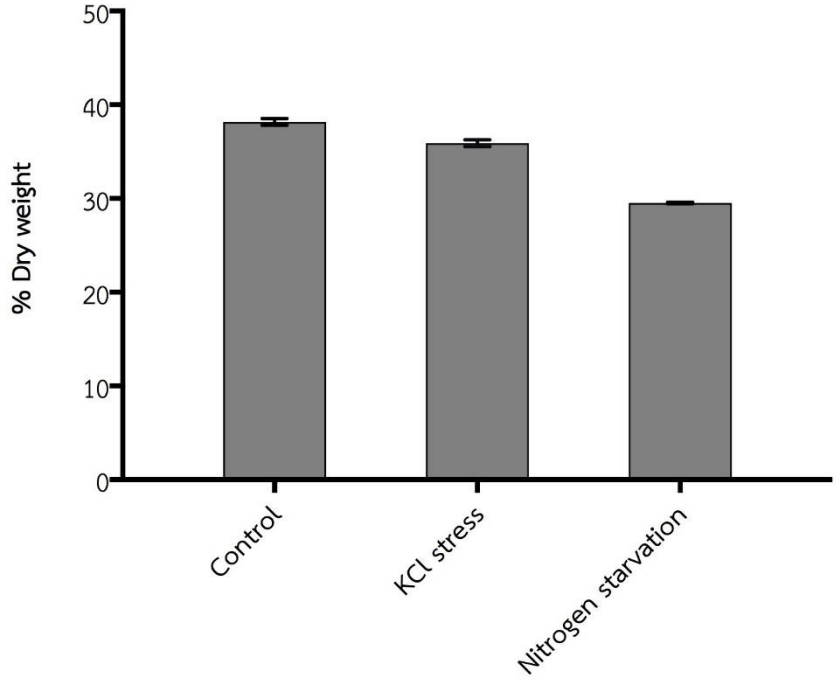


Figure 25 Total carbohydrate in *Chlorococcum* sp. 8367RE cultured under control, nitrogen starvation and 500 mM KCl stress. Data were shown as mean ± SE.

CHAPTER V
CONCLUSIONS

I) Salt, high light, drought stresses and nitrogen starvation altered the cell colors and morphologies in *Chlorococcum* sp. 8367RE. Interestingly, NaCl snd KCl stresses induced the enlargement of cells for 4.6 ± 0.6 and 5.1 ± 0.6 folds, respectively.

II) For extraction method, silica bead together with ultrasonicator bath and 80% acetone was the most effective disruption method for carotenoids extraction from this microalga.

III) For pigment analysis, total carotenoids in control, KCl stress and nitrogen starvation extracts were 3.84 ± 0.11, 3.85 ± 0.11 and 1.18 ± 0.01 μg/mg DW, respectively.

IV) Lutein was found as the majority carotenoid in *Chlorococcum* sp. 8367RE. Lutein levels were 2.24, 2.26 and 0.44 μg/mg DW in control, KCl stress and nitrogen starvation extracts, respectively.

V) KCl stress and nitrogen starvation enhanced accumulation of secondary carotenoids, such as astaxanthin and canthaxanthin.

VI) The fatty acid; oleic acid (omega-9) was dramatically increased under stress conditions, namely KCl and nitrogen starvation stresses. Total fatty acids under these conditions boosted for approximately 2- and 4- folds.

VII) Proline was significantly induced upon KCl stress (7.5 times increment), suggesting this amino acid functions as osmolyte.

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APPENDICES

Appendix 1

BG-11 medium

BG-11 solution

$NaNO_3$	1.5	g
K_2HPO_4	40	mg
$MgSo_4 \cdot 7H_2O$	75	mg
$CaCl_2 \cdot H_2O$	36	mg
Na_2CO_3	200	mg
EDTA*2Na	1	mg
Citric acid	6	mg
Ferric ammonium nitrate	6	mg
Trace element	1	mg

Dissilved all components with distrilled water to 1 liter.

Trace element solution

H_3BO_3	2.8	g
$MnCl_2 \cdot 4H_2O$	1.81	g
$ZnSO_4 \cdot 7H_2O$	0.22	g
$CuSO_4 \cdot 5H_2O$	0.079	g
$Co(NO_3)_2 \cdot 6H_2O$	0.049	g

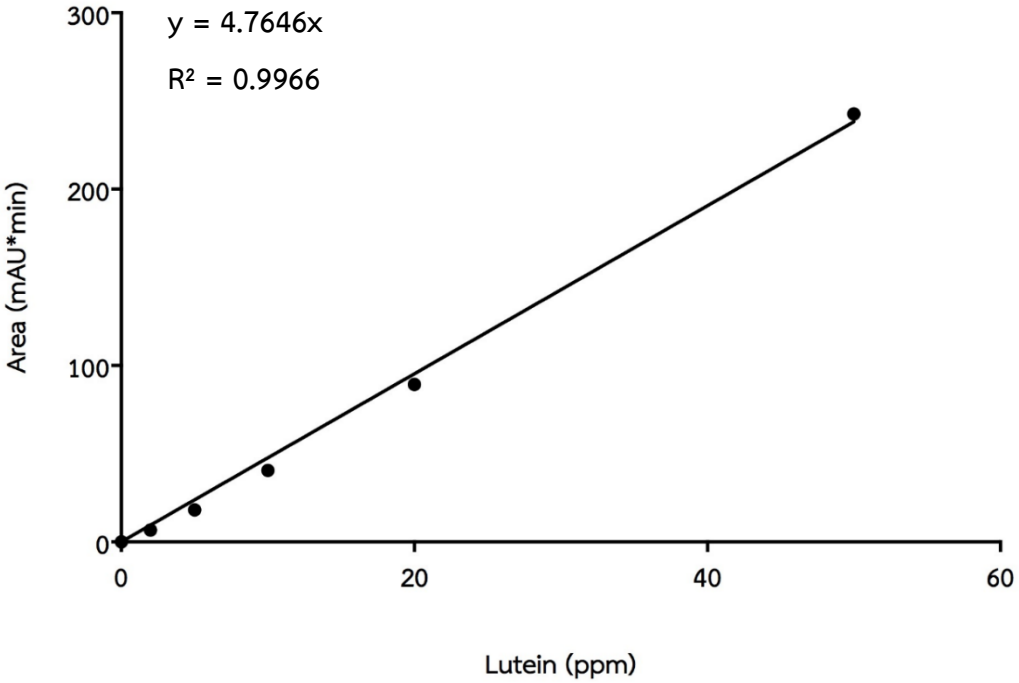
Dissilved all components with distrilled water to 1 liter

BG-11 agar

Agar powder	1.5 g/100 mL

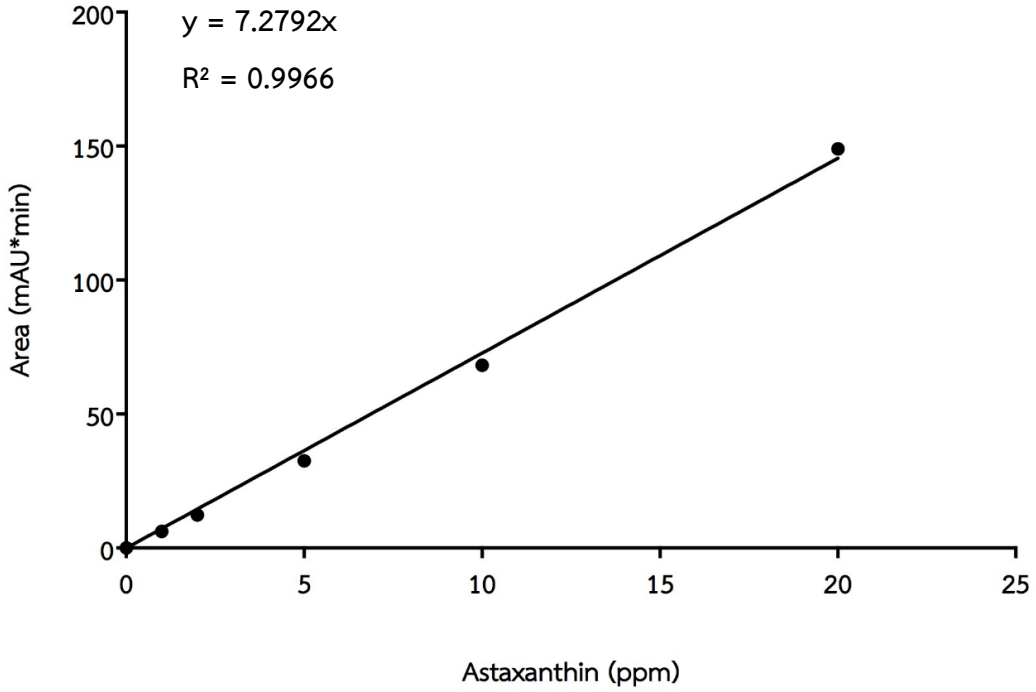
Appendix 2

Lutein standard curve



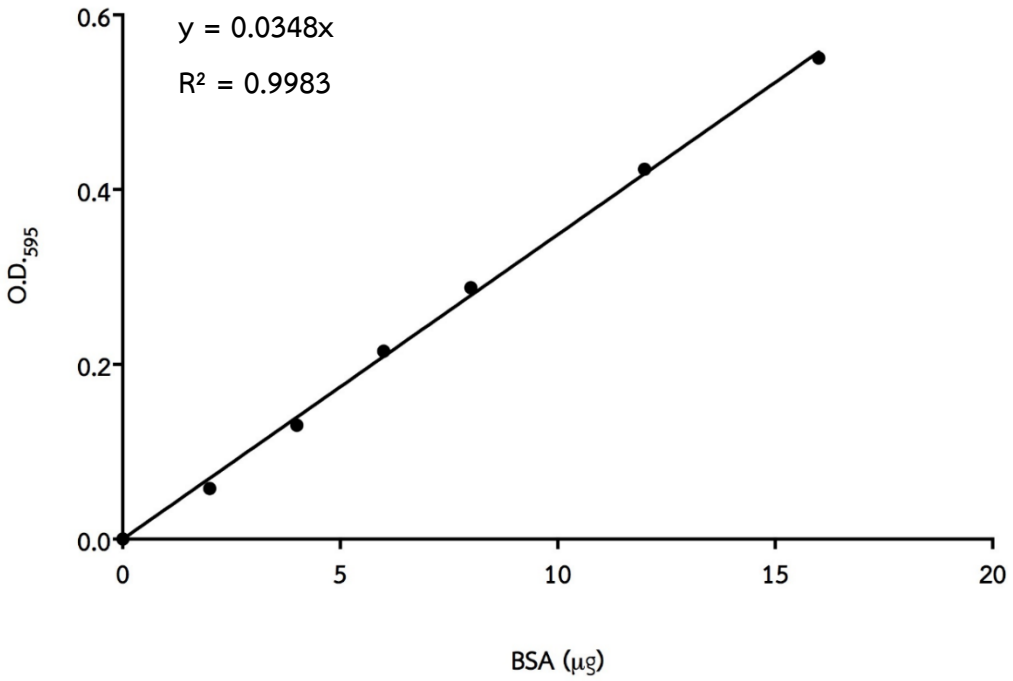
Appendix 3

Astaxanthin standard curve



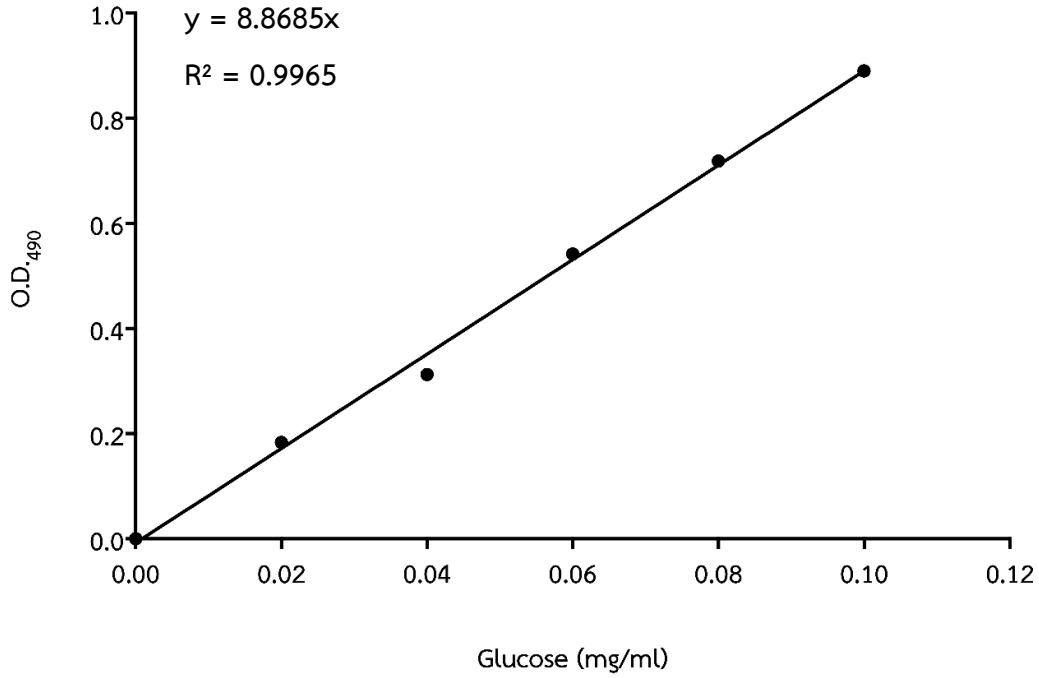
Appendix 4

BSA standard curve



Appendix 5

Glucose standard curve



Appendix 6

Mass spectrometry data of carotenoids standard

Sample Name	Component Name	Expected RT	Area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
Astaxanthin standard	Astaxanthin	26.48	6.39E+06	C40H52O4	597.3938	1.1493913	5.691053805
Astaxanthin standard	Astaxanthin	25.85	7.21E+06	C40H52O4	597.3938	2.282989959	1.908286711
Lutein standard	Lutein	28.47	2.88E+06	C40H56O2	569.4353	-2.192447169	19.08180497
Lutein standard	Lutein	29.11	3.47E+06	C40H56O2	569.4353	-2.661763723	23.46397821
Lutein standard	Lutein-1	22.04	1.19E+05	C40H56O2	569.4353	-1.964928045	3.559982353
Lutein standard	Lutein-1	22.53	1.10E+05	C40H56O2	569.4353	-0.31221281	23.08012618
Lutein standard	Lutein-2	33.55	1.34E+05	C40H56O2	569.4353	0.575147674	9.740754942
Lutein standard	Lutein-2	34.24	1.97E+05	C40H56O2	569.4353	-0.151207795	21.9737204
Zeaxanthin standard	Zeaxanthin	33.57	3.41E+06	C40H56O2	569.4353	-1.134737772	17.38313802
Zeaxanthin standard	Zeaxanthin	33.43	2.95E+06	C40H56O2	569.4353	-1.214144741	15.00297731
Zeaxanthin standard	Zeaxanthin-1	28.58	9.14E+05	C40H56O2	569.4353	-0.416572666	16.31809382
Zeaxanthin standard	Zeaxanthin-1	28.42	8.86E+05	C40H56O2	569.4353	-0.60304004	13.54183956

Appendix 7

Mass spectrometry data of samples

Astaxanthin								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Astaxanthin-3	N/A	N/A	N/A	C40H52O4	597.3938	N/A	N/A
control	Astaxanthin-3	N/A	N/A		C40H52O4	597.3938	N/A	N/A
KCl	Astaxanthin-3	26.60	1.33E+05	1.55E+05	C40H52O4	597.3938	0.941623559	3.234216033
KCl	Astaxanthin-3	25.95	1.78E+05		C40H52O4	597.3938	0.475454622	21.89322449
Astaxanthin monoester C12:0								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Astaxanthin C12:0-3	N/A	N/A	N/A	C52H74O5	779.5609	N/A	N/A
control	Astaxanthin C12:0-3	N/A	N/A		C52H74O5	779.5609	N/A	N/A
KCl	Astaxanthin C12:0-3	21.24	4.08E+04	3.09E+04	C52H74O5	779.5609	0.857111848	57.29484915
KCl	Astaxanthin C12:0-3	20.53	2.10E+04		C52H74O5	779.5609	-6.731695275	36.33704611
control	Astaxanthin C12:0-4	26.36	7.73E+04	8.13E+04	C52H74O5	779.5609	0.882369871	46.1813552
control	Astaxanthin C12:0-4	25.54	8.53E+04		C52H74O5	779.5609	0.96271063	5.477281265
KCl	Astaxanthin C12:0-4	26.38	1.20E+05	1.25E+05	C52H74O5	779.5609	-0.177358905	22.7139306
KCl	Astaxanthin C12:0-4	25.53	1.30E+05		C52H74O5	779.5609	1.959306613	7.779091624
Astaxanthin monoester C18:4								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Astaxanthin C18:4	N/A	N/A	N/A	C58H78O5	855.5922	N/A	N/A
control	Astaxanthin C18:4	N/A	N/A		C58H78O5	855.5922	N/A	N/A
KCl	Astaxanthin C18:4	28.97	4.26E+04	3.36E+04	C58H78O5	855.5922	-0.403427106	24.56034869
KCl	Astaxanthin C18:4	28.61	2.45E+04		C58H78O5	855.5922	0.154692396	22.0241225

Lutein or zeaxanthin								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	$C_{40}H_{56}O_2$ isomer-1	22.59	1.24E+05	7.47E+04	C40H56O2	569.4353	-0.963094025	27.00227138
control	$C_{40}H_{56}O_2$ isomer-1	22.01	2.51E+04		C40H56O2	569.4353	-0.551982512	14.70879572
KCl	$C_{40}H_{56}O_2$ isomer-1	22.59	1.38E+05	1.19E+05	C40H56O2	569.4353	-0.619257109	10.77799664
KCl	$C_{40}H_{56}O_2$ isomer-1	22.11	1.00E+05		C40H56O2	569.4353	0.508851199	18.16959554
control	$C_{40}H_{56}O_2$ isomer-2	24.89	9.19E+04	6.37E+04	C40H56O2	569.4353	-0.932209442	9.854643126
control	$C_{40}H_{56}O_2$ isomer-2	24.35	3.55E+04		C40H56O2	569.4353	-0.121238799	43.99467369
KCl	$C_{40}H_{56}O_2$ isomer-2	24.85	7.07E+04	6.70E+04	C40H56O2	569.4353	0.461052832	26.34679805
KCl	$C_{40}H_{56}O_2$ isomer-2	24.32	6.33E+04		C40H56O2	569.4353	-0.210653388	25.88911154
control	$C_{40}H_{56}O_2$ isomer-3	29.14	1.09E+06	7.19E+05	C40H56O2	569.4353	-1.776836126	24.12314876
control	$C_{40}H_{56}O_2$ isomer-3	28.52	3.44E+05		C40H56O2	569.4353	-0.219606163	14.88891063
KCl	$C_{40}H_{56}O_2$ isomer-3	29.14	9.76E+05	8.53E+05	C40H56O2	569.4353	-2.860494459	15.27715451
KCl	$C_{40}H_{56}O_2$ isomer-3	28.51	7.30E+05		C40H56O2	569.4353	-1.884186375	21.18230072
control	$C_{40}H_{56}O_2$ isomer-4	34.66	1.21E+05	7.24E+04	C40H56O2	569.4353	0.273260658	9.854643126
control	$C_{40}H_{56}O_2$ isomer-4	33.9	2.34E+04		C40H56O2	569.4353	-0.931563836	11.70792419
KCl	$C_{40}H_{56}O_2$ isomer-4	34.66	4.19E+04	3.38E+04	C40H56O2	569.4353	-0.667737472	15.33930257
KCl	$C_{40}H_{56}O_2$ isomer-4	33.91	2.58E+04		C40H56O2	569.4353	-1.362244435	9.854643126
control	$C_{40}H_{56}O_2$ isomer-5	39.97	6.53E+04	3.39E+04	C40H56O2	569.4353	0.968642047	43.99467369
control	$C_{40}H_{56}O_2$ isomer-5	39.19	2.52E+03		C40H56O2	569.4353	5.71895606	30.17051733
KCl	$C_{40}H_{56}O_2$ isomer-5	39.92	3.06E+04	1.81E+04	C40H56O2	569.4353	-0.970850493	43.99467369
KCl	$C_{40}H_{56}O_2$ isomer-5	39.23	5.62E+03		C40H56O2	569.4353	-7.754127472	20.11246878

Violaxanthin								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Violaxanthin-1	12.76	9.82E+04	8.71E+04	C40H56O4	601.4251	-0.949834788	12.43318938
control	Violaxanthin-1	12.4	7.60E+04		C40H56O4	601.4251	-0.73439057	5.554281532
KCl	Violaxanthin-1	12.76	1.51E+05	1.21E+05	C40H56O4	601.4251	-0.169444178	16.15158948
KCl	Violaxanthin-1	12.41	9.12E+04		C40H56O4	601.4251	-0.614462081	25.56886001
Red stage	Violaxanthin-1	12.76	5.00E+04	4.67E+04	C40H56O4	601.4251	0.319659552	44.07085882
Red stage	Violaxanthin-1	12.41	4.35E+04		C40H56O4	601.4251	-0.531471892	10.31405649
control	Violaxanthin-2	14.62	3.98E+05	4.05E+05	C40H56O4	601.4251	-1.693474696	13.70542446
control	Violaxanthin-2	14.25	4.13E+05		C40H56O4	601.4251	-0.793780112	12.69707357
KCl	Violaxanthin-2	14.63	1.92E+05	2.10E+05	C40H56O4	601.4251	-1.872654458	6.515398596
KCl	Violaxanthin-2	14.25	2.28E+05		C40H56O4	601.4251	-0.636225549	10.88310909
Red stage	Violaxanthin-2	14.61	9.76E+04	8.30E+04	C40H56O4	601.4251	-0.651232043	9.63129732
Red stage	Violaxanthin-2	14.25	6.85E+04		C40H56O4	601.4251	-0.8456947	2.624641344
control	Violaxanthin-3	18.26	6.42E+05	6.25E+05	C40H56O4	601.4251	-0.916270726	12.46812323
control	Violaxanthin-3	17.72	6.09E+05		C40H56O4	601.4251	-1.039620739	14.57298255
KCl	Violaxanthin-3	18.26	5.66E+05	5.86E+05	C40H56O4	601.4251	-1.794953568	17.64987006
KCl	Violaxanthin-3	17.73	6.06E+05		C40H56O4	601.4251	-1.549894491	16.60927983
Red stage	Violaxanthin-3	18.26	3.06E+05	3.11E+05	C40H56O4	601.4251	-1.421641672	20.442803
Red stage	Violaxanthin-3	17.7	3.17E+05		C40H56O4	601.4251	-0.756369874	6.786970545

Crytoxanthin 5,6,5',6'-diepoxide or antheraxanthin								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Antheraxanthin	16.07	7.93E+05	6.59E+05	C40H56O3	585.4302	-3.039422687	16.88778607
control	Antheraxanthin	15.62	5.25E+05		C40H56O3	585.4302	-2.171133435	16.04960704
KCl	Antheraxanthin	16.07	9.68E+04	1.02E+05	C40H56O3	585.4302	0.291195472	15.397677
KCl	Antheraxanthin	15.65	1.06E+05		C40H56O3	585.4302	0.101627397	20.56705

Canthaxanthin								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Canthaxanthin-4	N/A	N/A	N/A	C40H52O2	565.4040	N/A	N/A
control	Canthaxanthin-4	N/A	N/A		C40H52O2	565.4040	N/A	N/A
KCl	Canthaxanthin-4	37.5	2.30E+05	2.74E+05	C40H52O2	565.4040	0.78564192	2.948112791
KCl	Canthaxanthin-4	36.68	3.18E+05		C40H52O2	565.4040	0.936166765	1.242042823
Chlorophyll a								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Chlorophyll a-2	34.25	6.80E+06	7.02E+06	C55H72N4O5Mg	893.5426	-0.276835649	10.66903385
control	Chlorophyll a-2	33.64	7235577.102		C55H72N4O5Mg	893.5426	0.685419416	14.10935043
KCl	Chlorophyll a-2	34.26	3.22E+06	3.12E+06	C55H72N4O5Mg	893.5426	-0.388654961	10.45314675
KCl	Chlorophyll a-2	33.55	3.03E+06		C55H72N4O5Mg	893.5426	0.53595232	13.45738765
control	Chlorophyll a-3	37.13	6.24E+05	6.07E+05	C55H72N4O5Mg	893.5426	0.825905242	9.333134072
control	Chlorophyll a-3	36.51	5.89E+05		C55H72N4O5Mg	893.5426	-0.465959591	8.284946256
KCl	Chlorophyll a-3	37.14	1.47E+05	1.33E+05	C55H72N4O5Mg	893.5426	-0.504231918	34.32068964
KCl	Chlorophyll a-3	36.51	1.19E+05		C55H72N4O5Mg	893.5426	0.280914195	9.174124793
Chlorophyll b								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Chlorophyll b-1	21.64	1.06E+05	1.04E+05	C55H70N4O6Mg	907.5219	0.989158758	335.8636217
control	Chlorophyll b-1	21.29	1.03E+05		C55H70N4O6Mg	907.5219	0.358666379	231.91676
KCl	Chlorophyll b-1	N/A	N/A	N/A	C55H70N4O6Mg	907.5219	N/A	N/A
KCl	Chlorophyll b-1	N/A	N/A		C55H70N4O6Mg	907.5219	N/A	N/A
control	Chlorophyll b-2	23.7	3.83E+05	4.43E+05	C55H70N4O6Mg	907.5219	-0.002310519	6.54056827
control	Chlorophyll b-2	23.24	5.03E+05		C55H70N4O6Mg	907.5219	0.680123433	3.036783505
KCl	Chlorophyll b-2	23.69	2.73E+05	3.07E+05	C55H70N4O6Mg	907.5219	0.378928032	11.1858822
KCl	Chlorophyll b-2	23.22	3.42E+05		C55H70N4O6Mg	907.5219	0.301104591	5.347563663
control	Chlorophyll b-3	34.24	8.27E+04	7.62E+04	C55H70N4O6Mg	907.5219	0.878690046	100.2651382
control	Chlorophyll b-3	33.63	6.97E+04		C55H70N4O6Mg	907.5219	-0.363080093	16.04213236
KCl	Chlorophyll b-3	34.49	4.07E+03	8.66E+03	C55H70N4O6Mg	907.5219	4.151462096	26.85360993
KCl	Chlorophyll b-3	33.61	1.33E+04		C55H70N4O6Mg	907.5219	-0.832773405	90.65323426

Suspected steroid								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Suspected steroid-1	8.6	8.00E+05	7.13E+05	C37H60O5	585.4514	3.334410237	4.174651738
control	Suspected steroid-1	8.52	6.27E+05		C37H60O5	585.4514	3.479622879	10.59660107
KCl	Suspected steroid-1	8.6	3.40E+05	2.87E+05	C37H60O5	585.4514	2.611947994	3.659454577
KCl	Suspected steroid-1	8.51	2.35E+05		C37H60O5	585.4514	3.733550128	3.188424032
control	Suspected steroid-2	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-2	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-2	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-2	N/A	N/A		C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-3	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-3	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-3	13.71	2.09E+05	2.59E+05	C37H60O5	585.4514	-0.734133585	1.411364722
KCl	Suspected steroid-3	10.88	3.09E+05		C37H60O5	585.4514	1.706053008	5.093923231
control	Suspected steroid-4	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-4	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-4	13.71	2.09E+05	1.87E+05	C37H60O5	585.4514	-0.734133585	1.411364722
KCl	Suspected steroid-4	13.85	1.65E+05		C37H60O5	585.4514	0.185375488	3.223453085
control	Suspected steroid-5	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-5	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-5	14.07	1.99E+05	9.95E+04	C37H60O5	585.4514	-0.260235629	3.261541193
KCl	Suspected steroid-5	0	3.07E+02		C37H60O5	585.4514	-0.784272435	33.91018572
control	Suspected steroid-6	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-6	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-6	20.04	2.16E+05	2.02E+05	C37H60O5	585.4514	-0.098090882	3.638964914
KCl	Suspected steroid-6	19.41	1.88E+05		C37H60O5	585.4514	-0.070431707	4.73704292
control	Suspected steroid-7	N/A	N/A	N/A	C37H60O5	585.4514	N/A	N/A
control	Suspected steroid-7	N/A	N/A		C37H60O5	585.4514	N/A	N/A
KCl	Suspected steroid-7	21.61	2.03E+06	1.85E+06	C37H60O5	585.4514	-0.91833757	1.193999532
KCl	Suspected steroid-7	20.89	1.67E+06		C37H60O5	585.4514	-0.490627573	1.429378836

Unknown								
Sample Name	Component Name	Expected RT	Area	average area	Formula	Precursor Mass	Mass Error (ppm)	Isotope Ratio Difference
control	Unknown -1	8.05	5.18E+04	4.56E+04	C37H58O5	583.4357	2.415438922	17.23220926
control	Unknown -1	7.99	3.94E+04		C37H58O5	583.4357	2.020201321	10.13301033
KCl	Unknown -1	8.05	6.68E+04	6.39E+04	C37H58O5	583.4357	1.003129465	23.11789352
KCl	Unknown -1	7.98	6.09E+04		C37H58O5	583.4357	3.287761216	24.27009443
control	Unknown -2	N/A	N/A	N/A	C37H58O5	583.4357	N/A	N/A
control	Unknown -2	N/A	N/A		C37H58O5	583.4357	N/A	N/A
KCl	Unknown -2	10.03	1.77E+05	1.79E+05	C37H58O5	583.4357	-0.794494634	15.56029729
KCl	Unknown -2	9.96	1.82E+05		C37H58O5	583.4357	0.411677529	8.997707465
control	Unknown -3	N/A	N/A	N/A	C37H58O5	583.4357	N/A	N/A
control	Unknown -3	N/A	N/A		C37H58O5	583.4357	N/A	N/A
KCl	Unknown -3	11.32	6.48E+05	5.85E+05	C37H58O5	583.4357	-0.91833504	5.038769469
KCl	Unknown -3	11.19	5.22E+05		C37H58O5	583.4357	-0.513972041	5.370615212
control	Unknown -4	N/A	N/A	N/A	C37H58O5	583.4357	N/A	N/A
control	Unknown -4	N/A	N/A		C37H58O5	583.4357	N/A	N/A
KCl	Unknown -4	12.14	7.40E+05	5.63E+05	C37H58O5	583.4357	-0.839134887	8.407470035
KCl	Unknown -4	11.9	3.86E+05		C37H58O5	583.4357	0.462980947	2.1294967
control	Unknown -5	12.36	4.96E+04	4.40E+04	C37H58O5	583.4357	0.601308348	19.13539152
control	Unknown -5	12.18	3.85E+04		C37H58O5	583.4357	0.891730106	18.90815515
KCl	Unknown -5	N/A	N/A	N/A	C37H58O5	583.4357	N/A	N/A
KCl	Unknown -5	N/A	N/A		C37H58O5	583.4357	N/A	N/A
control	Unknown -6	18.3	4.09E+04	4.15E+04	C37H58O5	583.4357	-26.08905197	Infinity
control	Unknown -6	17.72	4.21E+04		C37H58O5	583.4357	-31.38458901	Infinity
KCl	Unknown -6	18.29	1.50E+05	1.44E+05	C37H58O5	583.4357	0.21635724	122.8733639
KCl	Unknown -6	17.73	1.38E+05		C37H58O5	583.4357	-1.149882851	66.91327065
control	Unknown -7	N/A	N/A	N/A	C37H58O5	583.4357	N/A	N/A
control	Unknown -7	N/A	N/A		C37H58O5	583.4357	N/A	N/A
KCl	Unknown -7	19.84	5.03E+05	5.12E+05	C37H58O5	583.4357	0.064088772	22.06801057
KCl	Unknown -7	19.16	5.22E+05		C37H58O5	583.4357	-0.017474323	8.498776617

VITA

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