การสกัดสารแอสตาแซนทินจากสาหร่าย *Haematococcus pluvialis*

นางสาวดวงกมล เรือนงาม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรดุษฎีบัณฑิต
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EXTRACTION OF ASTAXANTHIN FROM *Haematococcus pluvialis*

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A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Engineering Program in Chemical Engineering

Department of Chemical Engineering

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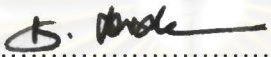
Thesis Title    EXTRACTION OF ASTAXANTHIN FROM
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By              Miss Duangkamol Ruen-ngam
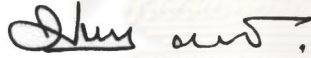Field of study  Chemical Engineering
Thesis Advisor  Associate Professor Prasert Pavasant, Ph.D.

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ดวงกมล เรืองงาม : การสกัดสารแอสตาแซนทินจากสาหร่าย *Haematococcus pluvialis*
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อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก: รองศาสตราจารย์ ดร.ประเสริฐ ภวสันต์, 93 หน้า.

งานวิจัยนี้ประกอบด้วยสองวัตถุประสงค์หลัก วัตถุประสงค์แรกเป็นการหาความสามารถในการละลาย
ของสารแอสตาแซนทินในสภาวะคาร์บอนไดออกไซด์วิกฤติยวดยิ่งซึ่งเป็นข้อมูลพื้นฐานที่สำคัญในการออกแบบ
ระบบการสกัด จากข้อมูลการละลายนี้ ทำให้ทราบว่ามีข้อจำกัดในแง่ของการถ่ายโอนมวลสารต่อการสกัดสารแอสตา
แซนทินด้วยกระบวนการที่ใช้ในปัจจุบัน ทำให้ต้องใช้เวลานานเพื่อให้ได้ปริมาณสารแอสตาแซนทินเท่ากับการสกัด
ด้วยวิธีทำละลาย ด้วยเหตุนี้จึงเป็นที่มาของวัตถุประสงค์ที่สองเพื่อทดลองหาวิธีอื่นที่เป็นไปได้เพื่อลดเวลาในการสกัด
สารต้านอนุมูลอิสระชนิดนี้จากสาหร่าย *Haematococcus pluvialis* ได้แก่วิธีใช้คลื่นอัลตราซาวน์ร่วมด้วยและ
ไมโครเวฟร่วมด้วยกับตัวทำละลายอินทรีย์

ในการศึกษาเรื่องความสามารถในการละลายของสารแอสตาแซนทินในสภาวะคาร์บอนไดออกไซด์
วิกฤติยวดยิ่งในส่วนแรก ใช้วิธีไดนามิกโดยใช้เครื่องมือสกัดสารที่มีอยู่ในห้องปฏิบัติการ ผลการศึกษาพบว่าเมื่อเพิ่ม
ความดันให้แก่ระบบในช่วง 20-40 เมกะพาสคาล ค่าการละลายของสารแอสตาแซนทินเพิ่มขึ้น เนื่องจากการเพิ่ม
ความดันให้แก่ระบบเป็นการเพิ่มความหนาแน่นของก๊าซคาร์บอนไดออกไซด์หรือเป็นการเพิ่ม solvent power ในขณะ
ที่การเพิ่มอุณหภูมิในช่วง 40-80°C ความหนาแน่นของก๊าซคาร์บอนไดออกไซด์ที่ให้ลดลงแต่กลับได้ค่าการละลายของ
สารแอสตาแซนทินมากขึ้น ทั้งนี้เนื่องมาจากการเพิ่มอุณหภูมิให้ระบบเป็นการเพิ่มความดันไอของตัวถูกละลาย และ
เมื่อนำค่าการละลายที่ได้จากการทดลองมาใช้ในการหาพารามิเตอร์สำหรับแบบจำลองที่เลือกมา 3 แบบจำลอง พบว่า
พบว่าแบบจำลองที่ใช้ equation of state (Peng Robinson) สามารถทำนายค่าการละลายของแอสตาแซนทินได้ดีที่สุด
และเมื่อประเมินประสิทธิภาพในการสกัดโดยเปรียบเทียบกับปริมาณสารแอสตาแซนทินที่ละลายต่อหน่วยปริมาณของ
คาร์บอนไดออกไซด์ในช่วงแรกของการสกัดกับค่าความสามารถในการละลายที่คำนวณได้จากแบบจำลอง พบว่าระบบ
การสกัดสารแอสตาแซนทินด้วยคาร์บอนไดออกไซด์วิกฤติยวดยิ่งจากสาหร่าย *Haematococcus pluvialis* ที่พบใน
รายงานวิจัยที่ผ่านมานั้น โดยทั่วไปแล้วมีข้อจำกัดเรื่องการถ่ายโอนมวลสาร ทำให้ใช้เวลาในการสกัดนาน

ส่วนที่สองของงานวิจัยนี้เกี่ยวข้องกับเรื่องการสกัดสารแอสตาแซนทินด้วยวิธีต่างๆ จากสาหร่าย
*Haematococcus pluvialis.* โดยปัจจัยที่สำคัญที่ศึกษาได้แก่ คุณสมบัติของตัวทำละลาย เวลา อุณหภูมิ มีอิทธิพลต่อ
ปริมาณสารแอสตาแซนทินที่สกัดได้ พบว่าสารดังกล่าวสามารถสกัดได้ดีด้วยตัวทำละลายอะซิโตน การใช้คลื่น
ไมโครเวฟช่วยร่วมกับการใช้ตัวทำละลายอะซิโตนในการสกัดที่อุณหภูมิ 75°C สามารถเพิ่มประสิทธิภาพในการสกัด
ได้ถึง 74% โดยใช้เวลาเพียง 5 นาที ด้วยประสิทธิภาพที่เท่ากันแต่เปลี่ยนวิธีการสกัดสารโดยใช้คลื่นเหนือเสียงเป็นตัว
ช่วยในการสกัดร่วมด้วยกับอะซิโตน พบว่าใช้เวลาในการสกัดนานถึง 45 นาที แต่ใช้อุณหภูมิต่ำกว่า คือ 45°C

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต ดวงกมล เรืองงาม
สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก
ปีการศึกษา 2553

# # 5071863321 : MAJOR CHEMICAL ENGINEERING

KEY WORDS: SOLUBILITY OF ASTAXANTHIN / DYNAMIC METHOD / PENG-ROBINSON/ EXTRACTION EFFICIENCY / ULTRASOUNDS-ASSISTED EXTRACTION/ MICROWAVE-ASSISTED EXTRACTION

DUANGKAMOL RUEN-NGAM: EXTRACTION OF ASTAXANTHIN FROM *Haematococcus pluvialis*. THESIS ADVISOR: ASSOC. PROF. PRASERT PAVASANT, Ph.D., 93 pp.

The astaxanthin extraction work comprised two main objectives. The first objective described the fundamental information of solubility of astaxanthin in supercritical carbon dioxide. From this first part we found that most supercritical $CO_2$ (SC-$CO_2$) extraction for astaxanthin extractions from *H. pluvialis* were operated at the mass transfer limitation, resulting in long extraction time requirement to obtained similar astaxanthin recovery, compared with other organic solvent extraction methods. This led us to the second objective to examine other extraction methods for extraction of astaxanthin from *Haematococcus pluvialis* such as y ultrasound-assisted and microwave-assisted organic solvent extractions.

In the first section, the dynamic method was employed to determine the solubility of astaxanthin in SC-$CO_2$. The results demonstrated that the solubility of astaxanthin increased with pressure in the range of 20-40 MPa because of an increasing density of $CO_2$ at higher pressure which allowed a better contact between the solute and solvent. An increase in temperature in the range of 40-80°C also enhanced the solubility of astaxanthin in spite of the drop in $CO_2$ density. This was because an increase in temperature raised the vapor pressure of solute which allowed a faster sublimation of astaxanthin, and this effect was believed to overcome the drop in $CO_2$ density. The experimental solubility data of astaxanthin was fitted to three selected models, among the, the equation of state (Peng Robinson) was found to best describe the solubility of astaxanthin over the entire range of temperature and pressure examined in this work. Such optimal solubility model was employed to evaluate the extraction efficiency of the previously reported experimental extraction data in literature. In SC-$CO_2$ extraction process, the solubility of astaxanthin from *H. pluvialis* was limited by to the mass transfer, causing long extraction time required for the process.

In the second section, the various extraction techniques were examined for astaxanthin extraction from *Haematococcus pluvialis*. The parameters such as solvent properties, time and temperatures were found to affect the level of astaxanthin recovery. Acetone was found to provide the highest astaxanthin content in the extract. The microwave-assisted extraction method with acetone as solvent achieved the highest amount of astaxanthin content (74% extraction efficiency at 75°C) within a very short extracting time (5 min). To reach a similar level of extraction efficiency, the ultrasound-assisted extraction with acetone as solvent required an extended extraction time (45 min), but this could lower the temperature down to 45°C.

Department   Chemical Engineering     Student's signature..........

Field of study Chemical Engineering     Advisor's signature..........

Academic year 2010

# ACKNOWLEDGEMENTS

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ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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# NOMENCLATURE

## Notations

| | |
|---|---|
| $a$ | constant parameter of Chrastil's model [-] |
| $a_1$ | energy parameter for component 1 in mixing rule |
| $a_2$ | energy parameter for component 2 in mixing rule |
| $A$ | constant parameter of enhancement and density based model [-] |
| $b$ | constant parameter of Chrastil's model [-] |
| $b_1$ | size parameter for component 1 in mixing rule |
| $b_2$ | size parameter for component 2 in mixing rule |
| $B$ | constant parameter of enhancement and density based model [-] |
| $C$ | solute concentration [g/L] |
| $exp$ | experiment |
| $cal$ | calculation |
| $E$ | enhancement factor [-] |
| $f$ | fugacity [-] |
| $k$ | Association number in Chrastil's model |
| $K_F$ | Fishtine constant, for sublimation calculation |
| $n$ | number of solubility data |
| $P$ | pressure [MPa] |
| $T$ | temperature [K] |
| R | gas constant [bar cm$^3$ mol$^{-1}$K$^{-1}$] |
| $v$ | molar volume [cm$^3$/mol] |
| $x$ | dissolved astaxanthin [mole of astaxanthin/mole of $CO_2$] |
| $y_2$ | solubility [mole of astaxanthin/mole of solent] |
| $Z$ | compressibility |

## Greek letter

| | |
|---|---|
| $\rho$ | density [mol/L, g/L] |
| $\phi$ | fugacity coefficient [-] |
| $\omega$ | acentric factor |

Subscript

| | |
|---|---|
| 1 | solvent |
| 2 | solute |
| 12 | interaction between solute and solvent |
| c | critical |
| s | saturation (vapor) or sublimation |

# CHAPTER I

## INTRODUCTION

## 1.1Motivations

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is one of the most effective dietary-supplement ingredients and is potentially the most efficient carotenoid in terms of antioxidant properties (Shimidzu et al., 1996) including immune enhancement, tissue damage prevention, anti-cancer, cardiovascular diseases prevention (Jyonouchi et al., 2000 Guerin et al., 2003 Ohgami et al., 2003, Tzu-Hua et al., 2006). Due to these extraordinary properties, astaxanthin could be priced as high as US$2500 per kg, and the annual market size of astaxanthin is around 200 million USD (Lorenz and Cysewski, 2000). There have been research attempts in synthesizing astaxanthin from natural sources such as krill oil and meal, crawfish oil, red yeasts ex. *Phaffia rhodozyma* (Sedmak et al., 1990, Gio-Bin et al., 2002), *Xanthophyllomyces dendrorhous* (Storebakken et al., 2004, Grewe et al., 2007)*, Dunaliella salina* (Herrero et al., 2006), *Scenedesmus sp*. (Grewe et al., 2007). However, these sources were only reported to contain low astaxanthin content. On the other hand, *Haematococcus pluvialis*, a single cell alga, could accumulate astaxanthin in the range between 1.5-3.0% by weight (Lorenz and Cysewski, 2000, Passos et al., 2006) and has attracted both research and commercial attentions as the potential source for astaxanthin biosynthesis.

Astaxanthin is an intracellular product which needs to be extracted before use. Extraction of astaxanthin is typically carried out by solvent extraction (Kobayashi, et al., 1997, Denery et al., 2004, Sarada et al., 2006, Kang and Sim, 2008). Problems with the use of solvents have stimulated research on new extraction techniques without solvent requirement such as high pressure supercritical fluid extraction (Valderrama et al., 2003, Machmuda et al., 2006, Nobre et al., 2006, Krichnavaruk et al., 2008, Thana et al., 2008). The supercritical fluid often used is supercritical carbon dioxide (SC-$CO_2$). Due to the exclusion of organic solvents in SC-$CO_2$ extraction, this technique is considered clean, safe, nonflammable, nontoxic, and environment-friendly. This technique is also quite attractive in the production of food and

pharmaceutical products as these products are heat intolerable, and tended to deteriorate at high temperature and oxygen condition. There have been some works on using co-solvents with SC-CO$_2$ extraction particularly edible vegetable oils (Krichnavaruk et al., 2008). For extraction astaxanthin from *Haematococcus pluvialis*, there have been little data regarding the solubility of astaxanthin in SC-CO$_2$, which makes it difficult to evaluate the extraction efficiency of this technique for such case. It is therefore fundamental to obtain reliable data of the astaxanthin solubility at supercritical conditions as these data would enable us to understand the SC-CO$_2$ extraction process and to better design the system.

Despite several advantages of SCCO2 extraction mentioned previously, economic feasibility of any extraction process requires the consideration of other factors such as energy consumption which largely translate to the cost of production. With this consideration, as a high-pressure technique, SC-CO$_2$ extraction would suffer several drawbacks from high energy requirement , not to mention the difficulty in the system scale-up. In addition for *H. pluvialis* extraction, it has often been the case where SC-CO$_2$ extraction process occurred at mass transfer limit, rather than at the solubility limit. As a result, in most operation of SC-CO$_2$ extraction of *H. pluvialis*, long extraction times are required to obtain comparable astaxanthin recoveries with other organic solvent extraction methods. For this reason, newly improved solvent-based techniques such as microwave-assisted extraction (MAE) or ultrasonic-assisted extraction (UAE) are gaining increasing interest and have been used for extraction of various plant compounds with high extraction efficiency (Hemvimon et al., 2007, Careri et al., 2001; Choi et al., 2007 and Rostagno et al., 2003). Although the use of solvents still cannot be avoided, the application of such techniques on the extraction of astaxanthin should also be thoroughly examined.

This research therefore has two main objectives. The first is to determine the solubility of astaxanthin in SC-CO$_2$. As the second objective, the newly potential astaxanthin extraction by accelerated methods such as UAE and MAE are examined to enable quantitative evaluation of the extraction recovery from each method.

## 1.2 Objectives

- To determine the solubility of astaxanthin employed in supercritical carbon dioxide.
- To evaluate the extraction recovery of astaxanthin from *Haematococcus pluvialis* using UAE and MAE and compare the results with various solvent extraction methods, i.e. maceration and soxhlet extraction.

## 1.3 Scopes of the study

- Dried samples of feed grade *Haematococcus pluvialis* were purchased from Cyanotech Corporation, Hawaii Ocean, Science and Technology Park, USA (supported by Professor Motonobu Goto, Kumamoto University, Japan). The samples were stored in a dry, tight aluminum pack at 5°C to prevent degradation until use.

- In part I, the dynamic method was used to quantify solubility of astaxanthin.

- The controlled parameters in solubility determination such as amount of astaxanthin standards and flow rate of carbon dioxide was priority examined.

- The variables as temperature, pressure are investigated the effect on solubility of astaxanthin.

- In the determination of appropriate solvent extraction conditions in part II, solvent extraction methods that were examined included maceration, soxhlet extraction and accelerated extraction techniques: UAE with power in range of 6.40–61.80 W; and MAE with power of 1200 W 2450 MHz.

- The effect of solvent and solid ratio (L/S) was investigated by maceration method.

- The variables of interest were: solvent type, extraction time and temperature.

- The four common solvent types, i.e. methanol, ethanol, acetonitrile and acetone were used in this experiment.

**1.4 Benefits from this work**

This research contributes to the fundamental knowledge on astaxanthin extraction methods such as maceration, soxhlet extraction, extraction UAE, MAE, as well as high pressure SC-$CO_2$ extraction. The research outcome should provide guideline for the selection and optimization of the extraction methods for astaxanthin recovery in many different applications. The success of such technology could contribute greatly to the development of extraction technology in Thailand.

# CHAPTER II

## BACKGROUND & THEORY

### 2.1 What is Astaxanthin?

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a ketocarotenoid with molecular formula $C_{40}H_{52}O_4$ with molecular weight of 596.82. The compound can be found invarious chemical structures such as, 3$S$,3'$S$, 3$R$,3'$R$ and 3$R$,3'$S$, as shown Figure 2.1.
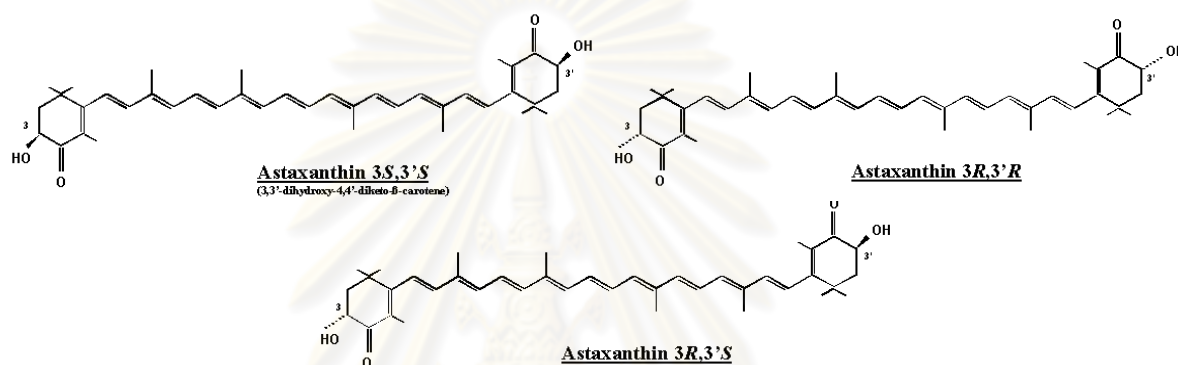


Figure 2.1 Various types of astaxanthin stereoisomers (Lorenz and Cysewski, 2000)

Astaxanthin is known to occur in marine and aquatic animal such as shellfish lobsters and shrimp, as well as in phytoplankton and algae. The content of astaxanthin in these biological sources differ, for examples, crustaceans byproduct (0-200 mg/kg), copepods small crustaceans (van Nieuwerburgh et al., 2005), crawfish oil (0.15% by weight), krill oil and meal, mold, bacteria; *Brevibacterium sp.* and *Mycobacterium lacticola,* fungi; *Peniophora* (*Hymenomycetes*) (Eric and Gil-Hwan, 1991). Yeast, *Xanthophyllomyces dendrorhous* formerly known as *Phaffia rhodozyma* could supply astaxanthin about 0.40% by weight (Bjerkeng et al., 1997). Some algae such as *Ankistrodesmus brauii* and *Chlorella sp.* can also produce astaxanthin but only in a quite low quantity. *Haematococcus pluvialis*, on the other hand, accumulates astaxanthin in high levels of 0.5-2% (87% are esterified form which has higher antioxidant activity, Margalith 1999, Kobayashi and Sakamoto, 1999). The amount of astaxanthin accumulation per unit cell mass of *H. pluvialis* is much higher than that in other strains, making it a potential source for industrial scale astaxanthin production, compared with other microorganisms. Generally, astaxanthin is biosynthesized

through the isoprenoid pathway. The pathway initiates at pyruvate and proceeds through phytoene, lycopene, β-carotene and canthaxanthin before the last oxidative steps to astaxanthin. However, the thick encysted form of cell wall limits the bioavailability of this pigment from *H. pluvialis* (Lorenz and Cysewski, 2000).

## 2.2 Abilities of astaxanthin

Astaxanthin is renowned for its antioxidant activity; e.g. immune enhancement, against tissue damage; photoprotectant eyes skin heart health, anti-cancer properties, against cardiovascular diseases, detoxification and liver function and neurodegenerative diseases (Jyonouchi et al., 2000 Guerin et al., 2003 Ohgami et al., 2003, Tzu-Hua et al., 2006). As the investigations of scavenging the ABTS+ radical cation due to astaxanthin has high number of increasing polarities such as carbonyl and hydroxyl groups, in the terminal rings, as well as by the number of conjugated double bonds (Miller et al., 1996). The α-hydroxyketocarotenoid astaxanthin proposed a high antioxidant activity causing from keto group activated the hydroxyl group hence facilitated hydrogen transfer to the peroxyl radicals as the activity of α-tocopherol (Vit E) (Naguib, 2000). Astaxanthin showed higher antioxidant activity than hydrocarbon carotenoids lycopene, α-carotene, β-carotene; and the hydroxy carotenoid lutein. Moreover Palozza and Krinsky (1992b) reported that astaxanthin was as effective as α-tocopherol and higher than β-carotene in habiting radical initiated in lipid oxidation of rate liver microcosms (Palozza and Krinsky, 1992b). Moreover astaxanthin can protect photosynthetic apparatus from light-mediated stress by quenching $^1O_2$ generated by photooxidation (Kobayashi and Sakamoto, 1999).

## 2.3 *Haematococcus pluvialis*

*Haematococcus pluvialis* is an important freshwater unicellular green microalgae. The taxonomy of *H. pluvialis* is classified as follows:

> Division : Chlorophyta
>
> Class : Chlorophyceae
>
> Order : Volvocales
>
> Family : Chlamydomondaceae
>
> Genus : *Haematococcus*
>
> Species : *Haematococcus pluvialis*

Generally there are two main stages for the growth of *H. pluvialis* depending on living condition. Under suitable conditions, *H. pluvialis* reproduces active vegetative cells by asexual cell division. The vegetative cell (zoospore) is green spherical or ellipsoid with two flagella for cell movement. In the starvation condition such as depletion of essential elements, e.g. carbon, nitrogen, phosphorous, light induction, etc, the green flagellated cells gradually transform into spherical immotile cyst cells (aplanospores) which could strive through for prolonged periods. The two main stages can be divided into sub four stages. The schematic diagram of model life cycle of *H. pluvialis* and the photographs in each growing stages appears in Figure 2.2.
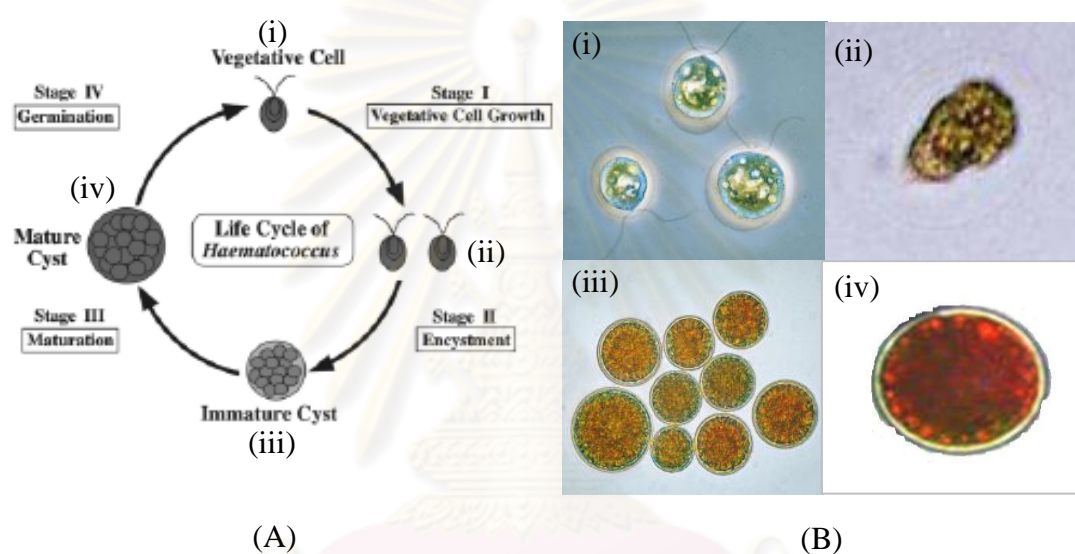


Figure 2.2 (A) Schematic diagram of model life cycle of *Haematococcus pluvialis* (Kobayashi et al., 1997), (B) Photographs of *H. pluvialis* (i) vegetative cell (ii) encyst cell with no flagella (iii) astaxanthin-poor immature cyst cell (iv) Mature cyst cell (Kobayashi, 2003)

Stage I, vegetative cell growth; cells produce chlorophylls a and b and primary carotenoids, especially β-carotene (10–20%) and lutein (75–80%) (Lorenz and Cysewski, 2000). Astaxanthin has not been generated in this stage. The shape of these cells is typically spherical or ellipsoid with a diameter of approximately 10-20 μm. The cells are enclosed with watery jelly-like cell walls consists of golgi apparatus, chloroplast, polysaccharidic envelope, pyrenoid, and fragility zone. Under stress conditions such as high light and/or nutrient deficiency (or Stage II), the growth rate of *H. pluvialis* decreases then the vegetative cells transform into immature cyst cells. Moreover, some cells loose flagella, generate extremely resistant thick-walled immotile spore with difficultly digest and larger size diameter from 10-20 to 40-50

µm. The diameter of cells increases dramatically in Stage III. Moreover, they produce secondary carotenoids such as echinenone, canthaxanthin and astaxanthin within cytoplasm causing enhanced the percent dry weight of astaxanthin up to 5% while the chlorophyll and primary carotenoids are decreasing. The cells in this stage are called aplanospore with spherical shape with increasing in astaxanthin content. In the last stage IV, germination if the surrounding condition becomes favorable mature cyst cell converses to vegetative cells again. (Hata et al., 2001)

## 2.4 Cultivation of *Haematococcus pluvialis* &Astaxanthin production

The cultivation of *H.pluvialis* is usually performed in two stages. The first stage is the cultivation of vegetative cells, and the second is the stage for the conversion of vegetative to cysts. The cultivation of vegetative cells has to be accomplished in close systems to avoid contamination. Pneumatic bioreactors such as bubble columns and airlift reactors are often employed for this purpose. The cultivation in bubble columns often achieved a lower growth rate when compared to airlift systems due to the limitation of the fluid flow in the bubble columns. Evidence of the actual cultivation of vegetative cells is: $4.8 \times 10^5$ cell ml$^{-1}$ day$^{-1}$ in bubble columns (Ranjbar et al., 2007) and $8.7 \times 10^5$ cell ml$^{-1}$ day$^{-1}$ in airlift photobioreactor (Issarapayup, 2007).

The starvation process of *Haematococcus pluvialis* for astaxanthin production is also the key procedure for improved astaxanthin yield. Recent records of such induction include the astaxanthin production rate of 16 mg L$^{-1}$ day$^{-1}$ in a novel double-layered photobioreactor (Suh et al., 2006) and the production of 14.4 mg L$^{-1}$ day$^{-1}$ in bubble column photobioreactor (Ranjbar et al., 2007). Recently commercial potential of the *H. pluvialis* cultivation is interesting continuously and try to enlarge the reactor of *H. pluvialis* cultivation for enhance level in astaxanthin production.

## 2.5 Harvesting of *Haematococcus pluvialis*

After cell cultivation, the subsequent process is to harvest the cell. Mostly suspending cells in the medium are settled down to the bottom by decanter. This sedimenting cells can be removed and dried with warm air and finally crushed by cracking mill. The extraction of astaxanthin from *Haematococcus pluvialis* is quite difficult as the cysts contain thick cell wall and it is primary concerns to break this wall without deteriorating the astaxanthin in it. Eight processes for breaking red-thick wall have

been reported: (i) autoclaved at 30 min, 121°C, 1 atm; (ii) spray-dried inlet 180°C outlet 115°C; (iii) mechanical disrupted cells by homogenizer; (iv) HCl 0.1 M 15; and 30 minute; (v) NaOH 0.1 M 15; and 30 minute; and (vi) mixing with 0.1% protease K + 0.5% driselase for 1 h. The highest astaxanthin obtained from spray drying, mechanical disruption and autoclave, respectively (Mendes-Pinto et al., 2001). Some reports breaking cell by using freeze drying and tissue homogenizer. (Jian-Ping and Feng, 1999, Abdolmajid and Choul-Gyun, 2006)

## 2.6 Astaxanthin extraction methods

After harvesting the *H. pluvilais* cells, one of the key significant processes is separation of astaxanthinfrom the cells. Quite a number of past reports revealed the development of appropriate extraction conditions for acquiring the highest yield of astaxanthin from various biological sources. This is summarized in Table 2.1. In fact, astaxanthin is a member of carotenoids therefore any relevant extraction methods could be possible. The well known extraction methods are described below.

### 2.6.1 Maceration extraction (ME)

Maceration method is an ordinary conventional organic extraction method by soaking the cell in the organic solvent. This method is the simplest and cheapest method compared to other extraction methods however it requires long extraction time.

### 2.6.2 Soxhlet extraction (SE)

Soxhlet extraction is conventional extraction method employing with soxhlet apparatus. The essential substance is released by dissolution into a refluxing liquid solvent. The material is weighed and placed in the thimble case. At a certain period of time the vapor of solvent condenses and fills into the upper cavity of the thimble case. This portion of liquid will dissolve and pull out the substance from the material. When the upper cavity is filled with solvent, the solvent will be extracted out of the cavity by means of siphon. The process works continuously and the concentration of the solute in the distillation flask continues to increase until the substance in material is virtually exhausted. The advantages of this method are 1) the newly or fresh solvent always contacts directly to the target material, and 2) the temperature in the distillation flask is near the boiling point of the solvent which can enhance the kinetics

of extraction. On the other hand, this method requires continuous supply of heat that might be harmful to the target compounds.

### *2.6.3 Ultrasound-assisted extraction (UAE)*

2.6.3.1 Introduction & Theory

Ultrasound is cyclic sound pressure with a frequency greater than the upper limit of human hearing (16 Hz - 16 kHz). Ultrasound frequencies can be divided into two areas as shown in Figure 2.4. The first, the higher frequency (2-10 MHz) and low amplitude (or low power) ultrasound is appropriate for medical applications and chemical analysis. The second area, the low frequency (20-100 kHz) and high energy wave, known as power ultrasound, is suitable for cleaning, plastic welding, and chemical reactivity or cell disruption (Mason and Lormier, 1988). Note that the wavelengths produced in liquid medium in chemical process (20-50 kHz) are in range 7.5-3.0 cm.
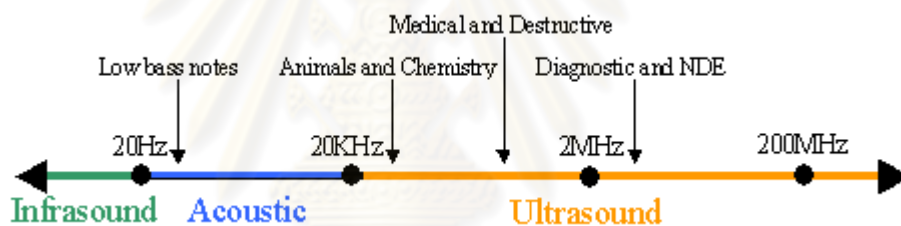
Figure 2.3 Frequency range of ultrasound

Ultraosund is produced by the vibration caused by a rapid alternating electrical potential of a synthetic piezoelectric crystal. The crystal expands or contracts when an electrical potential is applied. As the ultrasound crystal vibrates, it sends out ultrasound wave that consists of alternating compression (*positive pressure*) and rarefaction (expansion cycles exert *negative pressure* which can pull the substance away to another) zones into the media. If sufficient large negative pressure $P_c$, where $P_c = P_h-P_a$; $P_h$ is ambient pressure or hydrostatic pressure, $P_a$ is acoustic pressure $P_A\sin2\pi ft$; where $P_A$ acoustic amplitude, applies to liquid such as the average distance between molecules exceeds the critical molecules distance to hold the liquid intact, the liquid will break down and voids or cavities of microbubbles will be created. The small bubbles will absorb the energy from the sound waves and grow up during the expansion and recompressed during the compression cycles or collapse. This phenomenon occurs as the negative pressure exceeds the local tensile strength of the

liquid, which varies according to state of the medium such as intensity and frequency of the sound waves, temperature pressure properties and purity of liquid, kind and amount of dissolved gases.

2.6.3.2 Influence of operating parameters on UAE

There are various factors that affect to ultrasound wave performance. (Mason and Lormier, 1988)

- Frequency: Very high frequency is applied that cause to limit of time in the rarefaction and the compression cycle.
- Solvent: The formation of cavities or vapor-filled microbubbles requires the force during rarefaction must overcome the natural cohesive forces acting in the liquid, thus cavitation in liquid with high viscosity and surface tension requires greater sound intensity. Moreover vapor pressure ($P_V$) is significant factor as can be described as the effect of temperature below.
- Temperature: Loweravitation intensity is achieved at high temperature. At high temperature, the liquid vapor pressure increases, therefore the increasing the apparent hydrostatic, $P_h$-$P_a$, resulting in increased the number bubble cavities. Nevertheless, these cavities act as cushion to each other causing the reduction in the intensity of cavitational collapses. Therefore to increased mass transfer in UAE, it will of benefit to conduct extraction at low temperature, and in liquid of low vapor pressure.
- Gas type and content: During bubble collapse heat is generated, gas with high thermal conductivity will dissipate the heat and reduces the surrounding temperature. Existence of gases in the liquid undergoing ultrasound extraction could increases extractability by providing large number of nuclei and cavitation.
- External pressure ($P_h$): external pressure is found to influence both the cavitation threshold and the intensity of cavitational collapse.
- Intensity: Cavitation bubbles are initially difficult to produce at high frequency. When they collapse however, the bubble collapse will be more violent as the bubbles of large radius collapse more pronouncedly at higher pressure amplitude.

2.6.3.3 Literatures Reviews

Ultrasound has been applied for extraction of secondary metabolites from various plant tissues such as leaves of tea, mint, sage, chamomile, ginseng, arnica, and gentian. These investigators found that the use of ultrasound assisted methods can enhance the extraction efficiency generally by shortening the time of extraction processes. Most of these studies described and investigated the best conditions for obtaining the highest yield of essential substances as summarized in Table 2.2.

## 2.6.4 Microwave-assisted extraction (MAE)

2.6.4.1 Introduction & Theory

Microwaves are electromagnetic waves (energy, radiation) that consisted of electrical field and magnetic field. The range of frequency around 0.3 GHz to 300 GHz corresponding wavelengths ranging from 1m to 1mm as illustrated in Figure 2.5, while the general commercial used frequency is 2.450 MHz (2450 MHz).
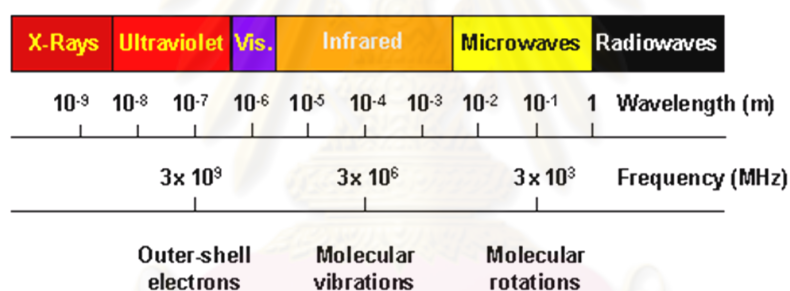
Figure 2.4 Electromagnetic spectrum. (www.anton-paar.com)
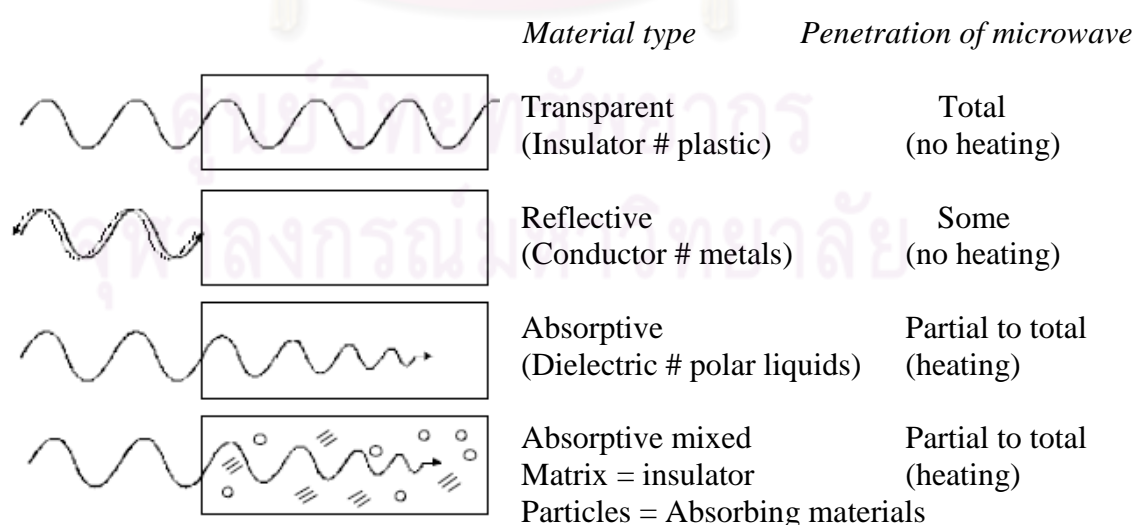
Figure 2.5 Interaction of microwave with different materials (www.anton-paar.com.)

Different materials have different characteristics on transmission, absorption or reflection microwave as depicted in Figure 2.6. Microwave generates heat internally within the material as opposed to originating from external heating sources. The effectiveness of heating mechanisms depends on coupling effects between components of the target material and the rapidly oscillating electrical field of the microwaves. Microwave possesses heat via two specific mechanisms: dipole interactions and ionic conduction, but that related to the heating effect in extraction study conducted in this research is the former, dipole interactions effect.

2.6.4.2 Influence of operating parameters on MAE

There are various typical parameters affecting the efficiency of extraction by microwave such asextraction time, temperature, microwaves power, material properties; moisture content, the stability of compounds, the nature of the solvent and the matrix. Especially nature of solvent is the typical parameter in microwave extraction. When microwave is applied, the microwave penetrates and propagates through a dielectric material where the internal field generated within the volume induces translational motions of free or bound charges such as electrons or ions, and rotated charge complexes such as dipoles. Inertial, elastic and frictional forces resist these induced motions and cause losses, and consequently heating. These interactions between the extracting solvent and electric fields (transmittance, reflection and absorbance) are characterized by two parameters defining the dielectric properties of the solvent. Generally the solvent with high dissipation factor ($\delta$) and dielectric constants ($\varepsilon'$) are appropriate for MAE process. Table 2.3 summarizes the properties of some common solvents for this technique.

2.6.4.3 Literature Reviews

The benefits of microwave are available in many fields as therapeutic diathermy treatment, satellite communications, destruction food spoilage microorganism, and also industrial heating. Microwave heating has been used in the past to reduce the moisture content of various fruits and vegetables such as bananas, apples, mushrooms and strawberries, carrots, corn, potatoes, and broad bean. Recently microwave has been used for peeling nutraceuticals from plant materials and extraction of environment samples. As microwave can interact with polar molecule in

biomaterialsm it can create heat from inside the biomasterials, which makes it more easily to pull out the valuable product such as antioxidant (Oufnac 2006, Wittayasinthana 2007) from such materials. Moreover microwave is well-known as accelerated extraction method for increasing in the yield of product in solvent extraction. The relevant microwave researches are summarized in Table 2.4.

### 2.6.5 Supercritical fluid extraction (SCF)

2.6.5.1 Introduction & Theory

The supercritical state is the condition positions in the upper region of the critical temperature and the critical pressure of the phase diagram. An example of the phase diagram of $CO_2$ is demonstrated in Figure 2.6. The coexistence of two phases represented as lines in the diagram. From the lower to upper line are solid-gaseous, solid-liquid, and liquid-gaseous. These 3 lines are equilibriums namely: sublimation, melting, and vaporization equilibrium, respectively, and the intersection of these three lines is the triple point. The terminal of liquid-gaseous equilibrium line breaks up at critical point which is the coordinate of critical temperature ($T_c$) and critical pressure ($P_c$). The properties at critical point are used to characterize the properties of each substance. Summaries of the properties of each gas at the critical point are shown in Table 2.5. Above this critical point occurs the single phase and this fluid is compressible and behaves like gas.

2.6.5.2 Influence of operating parameters on SCF (Brunner, 1994)

There are various factors that affect the extractability of supercritical extraction.

 ➢ Pressure: At extraction temperature of gas extraction, the solvent capacity raises up with pressure as at high pressure the solvent density, thus extracting capacity increases.

 ➢ Temperature: At suitable pressure not too low, the higher temperature often causes the higher extraction rate as the solute volatility increases. However at higher temperature, ther exracting capacity is low due to low solvent density.

 ➢ Density: At constant temperature, the extraction rate increases with density of solvent by enhancing the extracting capacity of solvent. Moreover, the density changes over wide range when alter temperature and pressure as previously described.

➢ Solvent ratio: This is the most important parameter for gas extraction and the changing of solvent and solid ratio enhances the extraction rate.

➢ Size of solid particles: In most case, extraction rate increases with decreasing particle size because it decreases the length of mass transport in the solid phase. However, in this work this parameter is not relevant.

➢ Diffusivity: The diffusivity properties of gas in supercritical fluids is in the range between those liquid and gas phases as shown in Table 2.6. The extractable substances can diffuse through supercritical fluid more easily than the liquid. Moreover, higher temperature can further enhance the diffusivity of the solute. On the other hand, higher pressure can reduce the solute diffusivity.

➢ Viscosity: At constant temperature, viscosity enhances with increasing pressure, hence, reducing solute diffusivity. Moreover, in supercritical fluids, pressure has critical influence on viscosity.

➢ Polarity: The property of atom or dipole-dipole intermolecular forces between the slightly positively-charged at one end of one molecule to the negative end of another or of the same molecule. This property is used for selecting solvent in extraction process. Generally dipole moment is used for measuring the polar of the polar covalence bond. Note that the supercritical $CO_2$ is non-polar.

➢ Dielectric constant: This is also a significant parameter because it affects the polar or non-polar of the solvent in extraction. This dielectric constant decreases with increasing temperature while increases with increasing pressure.
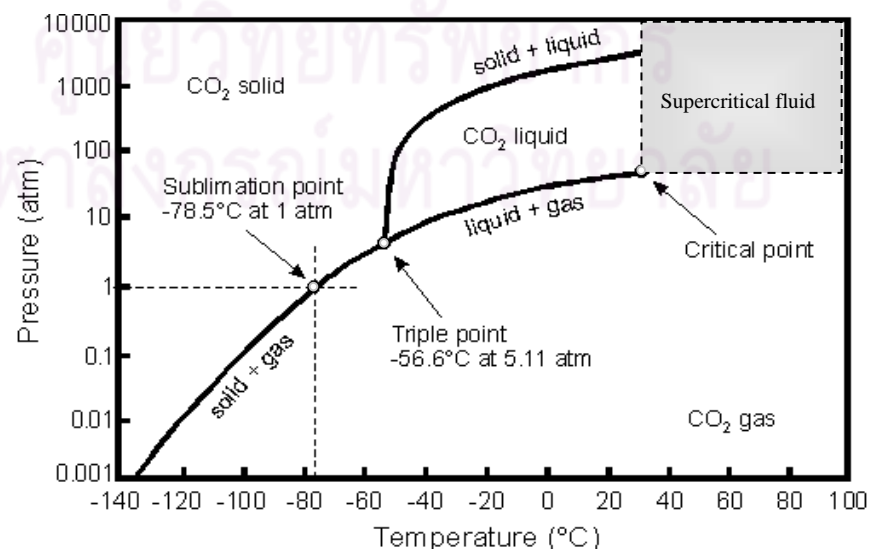


Figure 2.6 Phase diagram of supercritical carbon dioxide

2.6.5.3 Literature Reviews

Large scale supercritical extraction is used typically in industries such as decaffeination of coffee beans and black tea leaves (Brunner, 1994) whereas smaller scale is used for extraction of spices, flavoring compounds or other highly valued compounds such as antioxidants. There are large amount of available researches looking into the relevant parameters for acquiring the highest extraction yield and forecasting the supercritical extraction conditions. The additional review of research is summarized in Table 2.7.

**2.7 Solubility of high pressure extraction** (McHugh and Krukonis, 1994)

*2.7.1 Solubility measurement techniques*

The solubility is one of the key parameters determining the efficiency of an extraction process. In general, solubility of solute relies on the interaction between the molecules of solute and solvent, which is dictated by the molecular structures and the activity coefficient of the solution. Generally the solubility of substances in equilibrium at high pressure can be measured using two methods, i.e. static and dynamic methods. For the static method, the liquid $CO_2$ is mixed continuously with heavy solute by a stirrer or a circulating device in the extraction chamber at adequate contacting time to ensure equilibrium. The samples are then taken with a suitable technique. The dynamic technique on the other hand, is adapted from supercritical extraction, and the same equipment used for extraction could be used for the measurement of solute solubility. For dynamic solubility measurement, $CO_2$ flows continuously through the chamber filled with the solute loaded onto a matrix or support such as glasswool. The flow rate was kept low to ensure equilibrium. The equipment set-ups of these methods are illustrated in Figure 2.8 and the limitations and the advantages of each technique are concluded in Table 2.8.



(A)
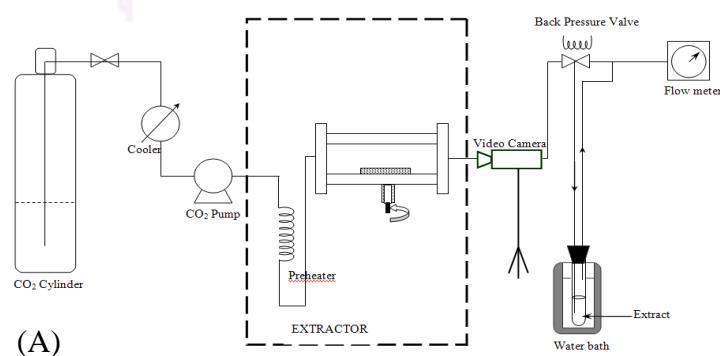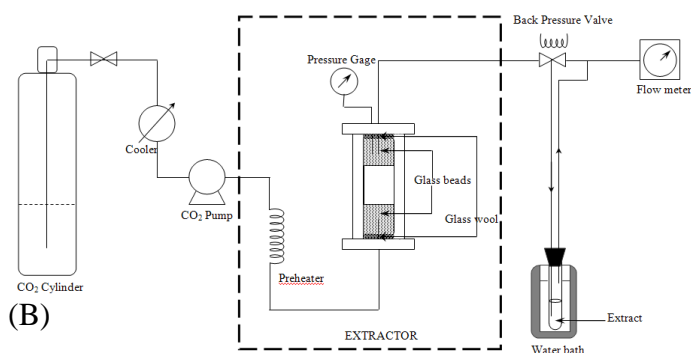
Figure 2.7 Schematic diagram
of solubility measurement
(A) Static technique method
(B) Dynamic technique
method

### 2.7.2 Solubility of astaxanthin in pure solvent

There are a few reports about astaxanthin solubility in high pressure $CO_2$. All of them employed either static (de la Fuente et al., 2006) or dynamic methods (Hyun-Seok et al., 2007). Literature illustrates that most operations were conducted in the temperature range of 303-333 K, 8-40 MPa. Solubility of astaxanthin at isothermal condition increased with pressure due to an increase in $CO_2$ density and associated with an increase in solvent power. The isobaric solubility of astaxanthin was found to increase with temperature in these works. The first article observed the minimum solubility of $2.8\times10^{-7}$ at 323K and 17MPa and the maximum mole fraction of $1.5\times10^{-6}$ at 333K and 40 MPa and the other obtained the minimum mole fraction of $0.42\times10^{-5}$ at 313K and 8 MPa and the maximum mole fraction of $4.89\times10^{-5}$ at 333K and 30 MPa. The inconsistency in such data can be illustrated in Figure 2.9.

### 2.7.3 Solubility in co-solvents

Organic solvents could enhance extractability of supercritical fluid extraction in various substances mentioned in previous section. This could be due to the solubility enhancement property of such solvents. Literature on this is summarized in Table 2.9. These data are useful for the design of the solubility measurement of various substances. Most of the reports concentrated on methanol and ethanol as co-solvents as they could enhance the extractability of polar molecule compounds. Nevertheless, ethanol is often preferred particularly in food industry because of its lower toxic effect. However the solubility of astaxanthin in supercritical fluid extraction with co-solvent has not been examined.
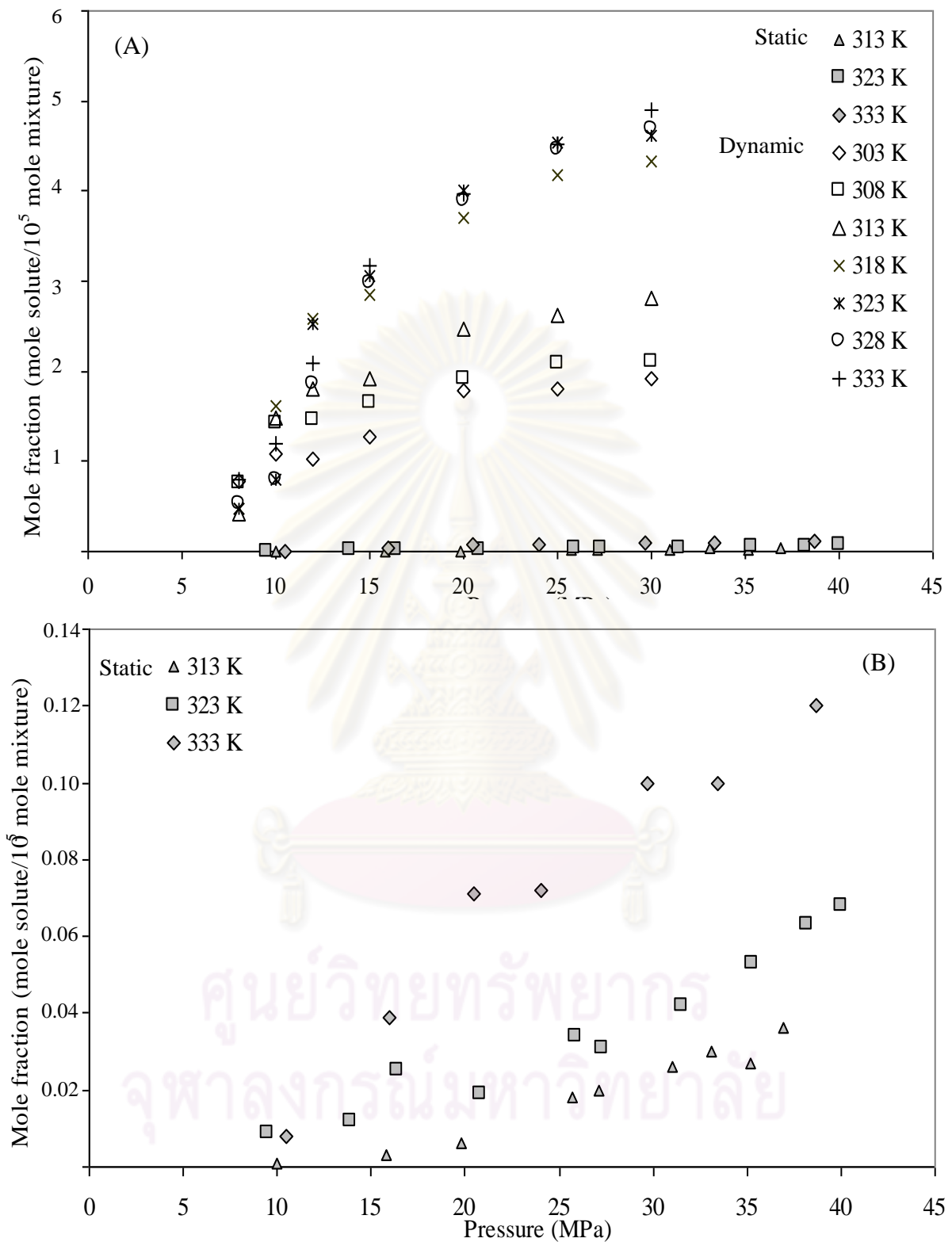
Figure 2.8 Solubility of astaxanthin measurement by (A) both static method and dynamic method (B) extension of static method

Table 2.1 Literature on astaxanthin extraction from biological sources

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|---|---|---|---|---|---|---|
| 1. | Sedmak, et al., 1990 | *Phaffia rhodozyma* (red yeast) | Organic solvent extraction | Glass beads and dimethysulfoxide (DMSO) | HPLC hexane:ethyl acetate 50% (v/v)+glacial acetic acid | N/A |
| | | | | | HPLC hexanes:ethyl acetate 62.5%/37.5% (v/v) | N/A |
| 2. | Jian-Ping and Feng, 1997 | *Haematococcus pluvialis* | Chromatographic separation and purification | N/A | Pigments extrated by dichloromethan:methane 25:75 (v/v) | |
| | | | | | HPLC: dichloromethane:ethanol:acetonitrite :water 6.5:82:7.5:4.6 | Increasing water, improve to separate of tran-astaxanthin |
| | | | | | HPLC: dichloromethane:ethanol:acetonitrite :water 5:85:5.5:4.5 | Increasing acetonitrile, improve to separate of lutein and cis isomer |
| 3. | Jian-Ping and Feng, 1999 | *Haematococcus pluvialis* | Hydrolysis astaxanthin esters | Grinding with pestle in mortar | Sequence step of extraction with dichloromethane:methanol 25:75 (v/v), | 5.02 |
| | | | | | saponification and separation by HPLC | trans-astaxanthin ester, free trans-, cis- 3.67, 3.47, 1.35 |
| | | | | | -Saponification at NaOH 0.021 M T=5-15°C | free trans- 3.4 (10°C) |
| | | | | | -Saponification at NaOH 0.018-0.032 M T=5°C | free trans- 3.25 (0.021 M) |
| 4. | Félix-Valenzuelia et al., 2001 | Blue crab (*Callinectes Sapidus*) | Supercritical $CO_2$ +ethanol co-solvent | Drying and ground and utilization | 100 ml chamber, 90:l0 M of CO2 and ethanol Flow rate 3.4-4.8 L/min | 57.11 |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|---|---|---|---|---|---|---|
| 5. | Hua-Bin and Feng, 2001 | *Chlorococcum sp.* | Isolation solvent and purification by high-sped counter-current (HSCCC) 1000 rpm | N/A | Crude preparing: n-hexane:ethanol 1:1 (v/v) -n-hexane-ethanol-water 10:8:2, 10: 8.5: 1.5 and 10:9:1 | 10:8:2, 10: 8.5: 1.5 too long retention time and 10:9:1 difficult to separate, the best=10:8:2 |
| | | | | | Two phase solvent for : HSCCC -n-hexane-ethyl acetate: ethanol-water 5:7:7:3, 5:5:7:3: 5:7:6.5:3.5 and 5:7:6.5:3 | Using with 5:7:7:3 difficult to separate and the best=5:7:6.5:3.5 |
| 6. | Gio-Bin et al., 2002 | *Phaffia rhodozyma* (red yeast) | SFE $CO_2$+ethyl alcohol | Bead mill | -Vary 102-500 bar with $CO_2$ =1.35 cm/min, 40 60 and 80°C | Dramatically increases rather than 200 bar to 550 bar 80>60>40°C |
| | | | | | -Vary CO2 0.32-1.0 g/cm3 with $CO_2$ = 1.35 cm/min, 40 60 and 80°C | |
| | | | | | -Vary pressure 400 ,500 and 600 bar and $CO_2$ 0.27, 0.54 cm/min with 60°C | Slightly increases with $CO_2$ flow rate for all pressures and double during initial 30 min for both flow rates |
| | | | | | -Ethyl alcohol , 5 10 and 15 volume% at 500 bar with 40 and 60°C $CO_2$ 0.27 cm/min -400, 500 and 600 bar with 40, 60 and 80°C $CO_2$ 0.27 cm/min | No significant difference |
| | | | | | 400 bar 40, 60 and 80°C | 0.2, 0.235, 0.225 |
| | | | | | 500 bar 40, 60 and 80°C | 0.22, 0.24, 0.22 |
| | | | | | 600 bar 40, 60 and 80°C | 0.2, 0.23, 0.225 |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|-----|--------|--------------|-------------------|---------------------|-----------|-----------------------------------|
| | | | | | -First step with 300 bar+ second 500 bar with 40 and 60°C | |
| | | | | | 40°C | 0.8 |
| | | | | | 60°C | 2.2 |
| 7. | Valderrma, et al, 2003 | *Haematococcus pluvialis* | SFE $CO_2$+ Ethanol | Cutting mill and grinding with dry ice | Vary $CO_2$/dry alga (kg/kg) 0-40 at 300 bar and 60°C | |
| | | | | | $CO_2$+ once ground | 1 |
| | | | | | $CO_2$+ twice ground | 1.3 |
| | | | | | $CO_2$+ twice ground+ ethanol 9.4% (w/w) | 1.6 |
| 8. | Denery, et al., 2004 | *Haematococcus pluvialis,* | Pressurized fluid extraction (PFE) | pulverized to pass 60 mesh particle sieve | Extraction condition: 1500 psi, 40°C, two 5 min extraction cycles, | High solubility in methylene chloride |
| | | *Dunaliella salina* and Piper methysticum (kavalactones) | | | Acetone | 0.95 |
| | | | | | Ethanol | 0.84 |
| | | | | | Acetone:ethanol (7:3, v/v) | 0.99 |
| | | | | | Acetone:methanol (7:3, v/v) | 1.03 |
| | | | | | Methylene chloride:methanol (1:3, v/v) | 1.09 |
| | | | | | Acetone: 1500 psi, three 5 min extraction cycles, T=20°C | Decompose at high temperature 1.14 |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|---|---|---|---|---|---|---|
| | | | | | T=40°C | 1 |
| | | | | | T=60°C | 0.95 |
| | | | | | T=100°C | 1.07 |
| 9. | López, et al., 2004 | Crustaceans (cray fish) | SFE $CO_2$+ Ethanol | N/A | Manual method | N/A |
| | | | | | The optimum value for SFE $CO_2$: | N/A Selective and precise |
| | | | | | Weight of ground crustacean sample 0.1 g | |
| | | | | | Weight of diatom earth 0.6 g to avoid reduce volume | |
| | | | | | Extraction time 15 min | |
| | | | | | Pressure 200 bar | |
| | | | | | Density 0.73 g/ml | |
| | | | | | Extraction chamber 60°C | |
| | | | | | Extraction flow rate 1.5 ml/min | |
| | | | | | Ethanol 15% (v/v) | |
| 10. | Abdolmajid and Choul-Gyun, 2006 | *Haematococcus pluvialis* | Organic solvent extraction | Tissue homogeniser | Water-miscible organic solvents (acetone:solvent=5/95 (v/v)) -acetone -methanol -hexane -chloroform ($CHCl_3$) | The first order derivative is limited detection 0.35 mg/l for chlorophyll and detection 0.25 mg/l for astaxanthin. |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|---|---|---|---|---|---|---|
| | | | | | -n-propanol | minimum |
| | | | | | -acetonitrile ($CH_3CN$) | 1.1 (maximum) |
| 11. | Machmuda et al., 2006 | *Haematococcus pluvialis* | SFE $CO_2$+ Ethanol | N/A | $CO_2$ 3 ml/min at 40-80°C, 55MPa 4 h | Increases with T, 2.52 (70°C) |
| | | | | | $CO_2$ 3 ml/min at 70°C, 20-55MPa 4 h | N/A |
| | | | | | $CO_2$ 2-4 ml/min at 50°C, 50MPa 4 h | N/A |
| | | | | | Ethanol 1.67% (v/v), $CO_2$ 3 ml/min at 40°C, 40MPa 4 h | N/A |
| | | | | | Ethanol 1.67% (v/v), $CO_2$ 3 ml/min at 20°C, 20MPa 4 h | N/A |
| | | | | | Ethanol 1.67% (v/v), $CO_2$ 2-3 ml/min at 20°C, 40MPa 4 h | N/A |
| | | | | | Ethanol 0-7.5% (v/v), $CO_2$ 2-3 ml/min at 70°C, 40MPa 4 h | 2.67 |
| 12. | Nobre, et al., 2006 | *Haematococcus pluvialis* | SFE $CO_2$+ Ethanol | Disk vibration | Acetone | 1.35 (75% recovery) |
| | | | | | Vary $CO_2$/dry alga (g/g) 0-400 | at 300 bar and 60°C |
| | | | | | at 200 and 300 bar, 40 and 60°C | Increased on increasing T and P |
| | | | | | $CO_2$+Crushing with one time | 0.6 (35.03% recovery) |
| | | | | | Ethanol/$CO_2$ 10% (v/v)+Crushing one time | 0.069 (44.03% recovery) |
| | | | | | Ethanol/$CO_2$ 10% (v/v)+Crushing two time | 1.2 (68.85% recovery) |
| 13. | Handayani et al., 2008 | *Panaeus monodon* (Giant tiger shrimp) | Palm oil extraction | Grinding and sieving | 50 g of shrimp | Increasing with size and T |
| | | | | | -40/60 mesh | |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|---|---|---|---|---|---|---|
| | | | | | $T = 50°C$ | 0.004 |
| | | | | | $T = 60°C$ | 0.0045 |
| | | | | | $T = 70°C$ | 0.05 |
| | | | | | -60/80 mesh | |
| | | | | | $T = 50°C$ | 0.06 |
| | | | | | $T = 60°C$ | 0.075 |
| | | | | | $T = 70°C$ | 0.085 |
| | | | | | -80/100 mesh | |
| | | | | | $T = 50°C$ | 0.01 |
| | | | | | $T = 60°C$ | 0.012 |
| | | | | | $T = 70°C$ | 0.013 |
| 14. | Kang and Sim, 2007 | *Haematococcus pluvialis* | Tandem organic solvent | N/A | Control: homogenized with dichloromethane:methanol 25:75 (v/v) 60 h<br><br>First with dodecane at room T then extracted with methanol:NaOH 1:1 (v/v) in dark at 4°C NaOH = 0-0.05 M | Decreased with increasing number of recycles (80% recovery) Dodecane (0.05 M of NaOH) 98% recovery |
| 15. | Thana et al., 2007 | *Haematococcus pluvialis* | SFE $CO_2$ | N/A | Acetone 6 h<br><br>$CO_2$ 3 ml/min at 40-80°C, 300-500 bar for 1-4 h | 2.75<br>Increases with pressure &time 2.28<br>83.05% recovery (80°C 500 bar and 4 h) |

| No. | Source | Raw Material | Extraction method | Cell Breaking Method | Condition | Yield% (dry basis of raw material) |
|-----|--------|--------------|-------------------|---------------------|-----------|-----------------------------------|
| 16. | Kang and Sim, 2008 | *Haematococcus pluvialis* | Direct extraction by vegetable oils | Without cell harvest | Cyst culture 30 ml mix with commercial vegetable oils 30 ml | |
| | | | | | -Soybean oil | N/A (91.7% recovery) |
| | | | | | -Corn oil | N/A (89.3% recovery) |
| | | | | | - Grape seed oil | N/A (87.5% recovery) |
| | | | | | - Olive oil | N/A (93.9% recovery) |

Table 2.2 Literature on ultrasound-assisted extraction of active components from biological sources

| No. | Source | Material | Compound | Frequency (MHz) & Power (W) | Solvent type | Temperature (°C) | Time (min) |
|---|---|---|---|---|---|---|---|
| 1. | Sališová et al., 1997 | *Salvia officinalis* | Pharmaceutical active compounds | 37-42 kHz, 130 W | 65% methanol | 20, 30 (opt.), 50 | 1, 3, 5, 12 (opt.), 24 h |
| 2. | Rostagno et al., 2003 | Soybeans | Isoflavones | 24 kHz, **2**00 W | Methanol, ethanol, MeCN | 10, 60 (opt. with 50% ethanol) | 10, 20 (opt.) |
| 3. | Elisandra et al,. 2003 | *Chresta exsucca* | Steroids | 60 Hz, 125 W | Dichloromethane, methanol | 30 | 30 |
|  |  | *Chresta scapigera* | trierpenoids |  |  |  |  |
| 4. | Hromádková and Ebringerová, 2003 | Buckwheat | Hemicelluloses | 20 Hz, 100 W | 3% NaOH, 5% NaOH (opt.) | 40, 60 (opt.) | 5, 10 (opt.) |
| 5. | Hromádková et al., 1999 | *Salvia officinalis* | polysaccharides | 20 kHz, 600 W | Water | 90 | 1 h |
| 6. | Sun et al., 2002 | Wheat straw | Hemicelluloses | 20 kHz, 100 W | 60% methanol | 60 | 5-35 (opt.) |
| 7. | Jianyong et al., 2001 | Ginseng root | Ginseng saponins | 38.5 kHz, 810 W | Pure ethanol, water-satuared, n-butanol, 10% methanol | 38-39 (opt.) | 1-2 h (opt.) |

Table 2.3 Properties of solvents used in microwave-assisted extraction (Zlotorzynski, 1995)

| Type of solvents | $\varepsilon'$ [a] (F/m) | $\varepsilon''$ [b] | tan $\delta$ |
|---|---|---|---|
| Methanol | 32.6 | 15.2 | 0.50 |
| Ethanol | 24.3 | 6.1 | 0.26 |
| Acetonitrile | 37.5 | 2.3 | 0.06 |
| Acetone | 20.7 | 11.5 | 0.62 |

[a] Determined at 20°C

[b] At 2450 MHz

Table 2.4 Literature on microwave-assisted extraction of active components from biological sources

| No. | Source | Material | Compound | Frequency (MHz) &Power (W) | Solvent type | Liquid/Solvent (ml/g) | Temperature (°C) | Time (min) | Pressure (kPa) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Pan et al., 2000 | Licorice root | Glycyrrhizic acid (GA) | 2450 MHz, 700W | Water, Ethanol, ethanol -water (opt.50-60%), Ammonia (opt.1-2%), ethanol-water- ammonia | opt.10:1 | 85 - 90 | 0.5-10 (opt. 4-5) | - |
| 2. | Guo et al,. 2001 | Radix puerariae | Puerarin | 2450 MHz, 700 W (10-100%) | Ethanol-water | 0, 30, 50, 70, 95 | 85, 90, 100, 115,130, 135 | 2, 5, 8, 12, 30 | 50-100 (opt.50) |
| 3. | Pan et al,. 2001 | *Salviamiltiorrhiza bunge* of root | Tanshinones | | *n*-butylacetate, ethanol (opt.95%), methanol, acetone, n-butanol , ethylacetate , tetrahydrofuran | opt.10:1 | 80 | 0.5 – 5 (opt.2) | - |
| 4. | Hao et al,. 2002 | *Artemisia annua* L | Artemisinin | 650 W | Ethanol, Trichloromethane , Cyclohexane, *n*-hexane, Petroleum ether, No. 120 solvent oil and No. 6 extraction solvent oil | opt. >11.3 | - | 2, 4, 6, 8, 10,12, 14, 18 (opt.12,diameter of material 0.125 mm) | - |
| 5. | Pan et al., 2002 | *Salviam iltiorrhiza bunge* | Tanshinones | 2450 MHz, 700 W (10-100%) | - | - | 80 | 0.5-5 min | - |

| No. | Author | Material | Compound | Frequency (MHz) &Power (W) | Solvent type | Liquid/ Solvent (ml/g) | Temperature (°C) | Time (min) | Pressure (kPa) |
|---|---|---|---|---|---|---|---|---|---|
| 6. | Shu et al., 2003 | Ginseng root | Ginsenosides | 2450 MHz, 30,150 W (opt.) | Water-Ethanol (opt.70%, 30%) | - | room temp. | 1, 2, 5, 10, 15 (opt.) | - |
| 7. | Li et al,. 2004 | *E.ulmodies* | Geniposidic | 2450 MHz, 700 W (90, 70, 50%) (opt.50%) | Methanol-Water (80%) | 20 | - | 0.10, 0.30, 50 (opt0.8) | - |
| | | | Chlorogenic acid | opt.5% | Methanol-Water (20%) | - | - | opt.0.3 | |
| 8. | Fulzele et al,. 2005 | *Nothapodytes foetida* | Camptothecin (CPT) 9-Me-CPT | 100W | Methanol, Ethanol | - | 80 | 3 | - |
| 9. | Zhou et al., 2006 | Tobacco leaves | Solanesol | 2450 MHz, 700W | Hexane, Ethanol, hexane:ethanol (3:1, 1:1, 1:3) opt.hexane-ethanol 1:3 with 0.05 mol/l NaOH | - | 60 | 5, 10, 20, 40, 60 | - |
| 10. | Martino et al., 2006 | *Melilotus officinalis* | Coumarin, *o*-coumaric and melilotic acids | 100W | Ethanol-water (opt.5%) | - | 50(opt.50), 110 | 5(opt.2heating cycles), 10 | - |
| 11. | Barbero et al., 2006 | Peppers | Capsaicinoids | 500W | Methanol, Ethanol (opt.100%), Acetone, Ethyl acetate and Water | opt.5:1 | 50-200 (opt.125) | 5-30 (opt.5) | - |
| 12. | Hemwimon et al,. 2006 | *Morinda citrifolia* of roots | Anthraquinones | 2450 MHz, 1200 W (60%) | Acetone, Methanol, Ethanol, Acetonitrile, ethanol:water (20:80, 50:50, 80:20) | 100 | 60, 80,100, 120 | 5, 10, 15, 20 | - |

| No. | Author | Material | Compound | Frequency (MHz) &Power (W) | Solvent type | Liquid/Solvent (ml/g) | Temperature (°C) | Time (min) | Pressure (kPa) |
|---|---|---|---|---|---|---|---|---|---|
| 13. | Chen et al., 2007 | Ganoderma atrum | Total triterpenoid saponins | 2450 MHz, 800 W (100%) | 95% ethanol, chloroform, ethyl acetate, n-butanol, acetone, and methylene chloride/methanol mixture (v/v,1:1) | 95% ethanol 25 | 60, 70, 78, 100, 120 | 20 | - |
| 14. | Mauricio et al., 2007 | Soybeans | Isoflavones | 500W (5%) | Ethanol or Methanol, (vary water 30–70%) (opt.50%ethanol) | 50 | 50, 75, 100, 125, 150 (opt.50) | 10, 15, 20, 25 and 30 (opt.20) | - |
| 15. | Mao et al., 2007 | *Rhodiola L.* | Salidroside and tyrosol | 2450MHz, 200, 400, 700 W (opt.400W) | methanol-water (10, 20, 30, 40, 50, 60, 80 and 90%) (opt.50%) | 5 | - | Soaked up the solution: 10, 30, 60, 120, 90, 120 and 150 (opt.60) Heated by a microwave: 1–8 min (opt.5) | - |
| 16. | Chen et al.,2008 | *Herba Epimedii* | Flavonoids | DMAE 20-100 W | ethanol and methanol | - | 80 | 10 | - |
| 17. | Wang et al., 2008 | Panax ginseng root | Ginsenosides | High pressure microwave assisted extraction (HPMAE) | methanol, 70% ethanol–water (opt.) and water | - | - | 2, 5, 10 (opt.), 15 and 30 | 100, 200, 300, 400 (opt.) and 500 |

Table 2.5 Characteristic of various solvents at the critical point

| Solvents | Critical Temperature (°C) | Critical Pressure (bar) | Critical Density (g/ml) |
|---|---|---|---|
| Inorganic | | | |
| $CO_2$ | 31.1 | 72.0 | 0.47 |
| $N_2O$ | 36.5 | 70.6 | 0.45 |
| Ammonia | 132.5 | 109.8 | 0.23 |
| Water | 374.2 | 214.8 | 0.32 |
| Helium | -268 | 2.2 | 0.07 |
| Hydrocarbons | | | |
| Methanol | -82 | 46.0 | 0.169 |
| Ethane | 32.3 | 47.6 | 0.2 |
| Propane | 96.7 | 42.4 | 0.22 |
| Alcohols | | | |
| Methanol | 239 | 78.9 | 0.27 |
| Ethanol | 243.4 | 72.0 | 0.276 |

Table 2.6 Properties of supercritical $CO_2$ of ordinary gases and liquids

| Phases | Density (g/cm$^3$) | Viscosity (g/cm s) | Diffusion coefficient (cm$^2$/s) |
|---|---|---|---|
| Gases | $0.1\times10^{-3}$-$2\times10^{-3}$ | $1\times10^{-4}$-$3\times10^{-4}$ | 0.1-0.4 |
| SC-$CO_2$ | 0.47 | $3\times10^{-4}$ | $7\times10^{-45}$ |
| Liquids | 0.6-1.6 | $0.2\times10^{-2}$-$3\times10^{-2}$ | $0.2\times10^{-5}$-$2\times10^{-5}$ |

Table 2.7 Literature on supercritical fluid extraction of active components from biological sources

| No. | Author | Material | Compound | Solvent type | Temperature (°C) | Pressure (bar) | Sample loading (g) | Solvent flow rate (ml/min) | Extraction chamber (ml) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Roy et al., 1996 | Peppermint leaves | Essential oils and cuticular wax | - | 40 | 100-300 optimal press. At 300 bar | 23-24 | 0.028-0.66 | ~177 |
| 2. | Cathy et al., 1999 | Fermentation broth of yeast and L-phynylalanine | Rose aroma | - | 32-42 (opt. 35-40) | 200 | 50 ml | N/A | 350 |
| 3. | Palma and Taylor et al., 1999 | Grape seed | Polyphenolic cmp. | 10% methanol (opt.) | 35-55 (opt. 55) | N/A | 0.03 | 1 | 1 |
| 4. | Tonthubthimt hong et al., 2001 | Neem seeds | Nimbin | | 35-60 (opt. 55) | 100-260 (opt. 230) | 2 | 0.24-1.24 | 10 |
| 5. | Huang-Chung et al., 2001 | Ginseng root hair | Ginseng root hair oil and ginsenosides | Ethanol as co- solvent | 35-60 (opt. 60) | 104-312 (opt. 312) | 80 | 5 | 300 |
| 6. | Mendes et al., 1995 | Algae *Chlorella vulgaris* | Carotenoids and other lipids | - | 40-55 (opt. 55) | 200-350 (opt.) | 5 | 400 | N/A |
| 7. | Matsuyama et al., 1998 | Yeast *Phaffia rhodozyma* | Astaxanthin | - | ~40 | 197 | 0.3 | N/A | 50 |
| 8. | Careri et al., 2001 | Spirulina Pacifica algae | Carotenoids | Ethanol as co- solvent | 40-80 (opt. 60, 76, 80 C for 3 carotenoids) | 150-350 (opt. 350) | 0.5 | 2 | 7 |
| | | | | | | | | 2-4 | 50 |

Table 2.8 Comparison of solubility measurement (Brunner 1994, McHugh and Krukonis, 1994, Taylor 1996)

| Type of solubility measurement | Advantages | Limitations |
|---|---|---|
| 1. Static Method | 1) Visual determining phase transition and inversion<br><br>2) Without sampling in solubility of binary mixtures of solid and liquid<br><br>3) Can measuring liquid, solid and polymers<br><br>4) Using minimum amounts of heavy components or supercritical fluid<br><br>5) Continue adjusting pressure at any fixed composition and temperature<br><br>6) Can sample of muticomponent mixtures in equilibrium phase | 1) Not easy obtaining the data of supercritical fluid stripping or fractionation<br>2) Typical one macrosized sample obtaining per cell loading |
| 2. Dynamic Method | 1) Using off-the-shelf<br><br>2) Rapid obtaining reasonably large amounts of solubility data<br>3) Obtaining equilibrium, stripping or fractionation data<br>4) Using straightforward sampling<br>5) Can measure very low solubility compound ($< 0.1\%$) | 1) Pushing liquid phase out of the column at high pressure liquid phase<br>2) Undetected phase change<br>3) Carefully designed for multicomponent<br>4) Sampling only lighter phase<br>5) High flow rates of liquid solute<br>6) Error from clogging of solid or liquid |

Table 2.9 Literatures on solubility measurement in various substances

| No. | Author | Compound | Measurement Method | Solvent type | Solute (g) | Temperature (°C) | Pressure (kPa) | Density of $CO_2$ (kg/m$^3$) | Yield |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Hyun-Seok, et al., 2007 | Astaxanthin | Dynamic (Semi-continuous flow) Dissolve in Chloroform and absorb on 1.0 g cotton wool | $CO_2$ | 0.2 | 30-60 | 80-300 | 191.71-982.04 | $0.42$-$4.89 \times 10^5$<br><br>Increasing with P & T and $CO_2$ density<br><br>Enhancement factor, η increasing with $CO_2$ density |
| 2. | Saldaña et al., 2006 | β-carotene &extraction others from carrots, a-carotene+ lutein | QCM (piezoelectric quartz crystal or quartz crystal microbalance) (invented cell)<br><br>Dissolve in Chloroform | $CO_2$ | 10mg | 40 and 50 | 12000 - 2000 bar | 718 - 784 | β-carotene At 12000 kPa $4.3 \times 10^{-7}$-$7.2 \times 10^{-7}$<br><br>At 20000 kPa $6.7 \times 10^{-7}$-$9.42 \times 10^{-8}$ Increasing with Temperature &pressure |
| 3. | Sakaki, 1992 | β-carotene | Dynamic method (Dissolve in Hexane merge alumina bead 2 mm-diameter) | $CO_2$ | NA/total of pure β-carotene | 35-55 | 9,600-30,000 | 616-928 | $0.085$-$5.3$ g/m$^3$ Mole fraction $0.022$-$0.50 \times 10^6$ |
| 4. | Škerget et al.,1995 | β-carotene& oleic acid | Static method | $CO_2$ | 0.7 g of Carotene 10 g of oleic | 25 and 40 | 10,000-30,000 | 616.8-966.3 | $0.6$-$5.5$ g/m$^3$ |
| 5. | Knez and Steiner, 1992) | capsaicin | Static method | $CO_2$ | | 25, 40 and 60 | 7750-36470 | 233.3-931.7 | $0.9$-$17.2$ g/m$^3$ |
| 6. | Subra et. al., 1997 | β-carotene | Dynamic method | $CO_2$ | 0.4 | 67, 57, 47, 37 | 9-28 | 0.842-0.502 | $10^{-8}$-$10^{-6}$ mol/mol in carbon dioxide and $10^{-7}$-$10^{-5}$ mol/mol |

| No. | Author | Compound | Measurement Method | Solvent type | Solute (g) | Temperature (°C) | Pressure (kPa) | Density of $CO_2$ ($kg/m^3$) | Yield |
|---|---|---|---|---|---|---|---|---|---|
| 7. | de la Fuente et al., 2006 | Carotenoid (lycopene& astaxanhtin) | New static method  12 h equilibration measuring with on-line HPLC | $CO_2$ | 0.2 g of astaxanthin | 40, 50, and 60 | 9,490-40,000 Increasing with T more than P | Above 330 | Mole fraction $0.20 \times 10^{-6}$-$0.50 \times 10^{-6}$ |
| 8. | de la Fuente et al., 2005 | Capsaicin | New static method | $CO_2$ | 0.3 g | 25 and 60 | 6,000-40,000 | ~607 | Mole fraction $\leq 2.88 \times 10^{-6}$ Increasing with Temperature& Pressure |
| 9. | de la Fuente, et al., 2005 | Antioxidant (Boldine) | New static method | $CO_2$ | 0.3 g | 25-60 | 8,000-40,000 | N/A (May according to Pressure) | Mole fraction, $4 \times 10^{-7}$-$6 \times 10^{-5}$ |
| 10. | Gómez-Prieto et al., 2007 | β-carotene and lutein from *Mentha spicata L.* | Extraction as dynamic method Hewlett-Packard 7680A extraction module (Wilmington, DE) | $CO_2$ | 0.6 of *Mentha spicata L.* | 40, 50, 60 | 8.6 - 25.9 | 250-800 | Increasing with Temperature &pressure all-*trans*-lutein $0.30 \times 10^{-3}$ g/L (at 60°C MPa CO2 25.9 MPa, 800 g/L) all-*trans*-β-carotene $2.0 \times 10^{-3}$ g/L (at 60°C MPa 25.9 MPa, 800 g/L) *cis*-lutein $0.6 \times 10^{-3}$ g/L (at 60°C MPa 25.9 MPa, 800 g/L) *cis*-β-carotene $0.3 \times 10^{-3}$ g/L (at 60°C MPa 25.9 MPa, 600 g/L) |

| No. | Author | Compound | Measurement Method | Solvent type | Solute (g) | Temperature (°C) | Pressure (kPa) | Density of $CO_2$ (kg/m³) | Yield |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Increasing with T (40 to 60°C) & P and $CO_2$ density |
| | | | | | | | | | β-Carotene could be extracted at 550 g/L whereas lutein at 600 g/L. |
| | | | | | | | | | all-*trans*-β-carotene extraction occurred at lower CO2 density than all-*trans* or *cis*-lutein. (estimate from k value) |
| | | | | | | | | | *cis*-form possesses higher solvation heat (estimate from a value) |
| 11. | Sovová et al., 2001 | *trans*-β-carotene | Dynamic method | $CO_2$, ethanol and vegetable oil | *trans*-β-carotene | 40, 50, 60 | 12-28 | 605.6-899.3 | pure $CO_2$, $0.32 \times 10^{-7}$-$5.80 \times 10^{-7}$ $CO_2$+ethanol, $4.62 \times 10^{-7}$-$14.0 \times 10^{-7}$ $CO_2$+vegetable oil, $2.28 \times 10^{-7}$-$7.47 \times 10^{-7}$ |
| 12. | Güçlü-Üstündağ et al., 2004 | β-carotene, α-tocopherol, stigmasterol, squalene from bio-sources | Dynamic method (Extraction) | $CO_2$ | Depending on each experiment Procedure | -15-80 | β-carotene 5-180 | Depend on pressure and estimated information in each paper used | β-carotene max. $5 \times 10^{-7}$ |

# CHAPTER III

## RESEARCH METHODOLOGY

### 3.1 Alga source

Red-powdered dried sample of feed grade *Haematococcus pluvialis*, were purchased from Cyanotech Corporation, Hawaii Ocean, Science and Technology Park, USA, (supported by Professor Motonobu Goto, Kumamoto University, Japan). The moisture content of samples was around 7% weight. The samples are stored in a dry, tight aluminium pack at 5°C to prevent degradation until use.

### 3.2 Chemicals and equipments

*Chemicals*:

Standard of astaxanthin (> 92%) (Sigma-Aldrich, USA) for extraction

Standard of astaxanthin (99.5%) (ALEXIS$^{®}$ Biochemicals) for determination of solubility

*Solvents*: all types of HPLC grade solvent are purchased from Wako Pure chemical Industries, Ltd.

acetone (99.0%)

ethanol (> 99.5%)

dichloromethane (99.5%),

methanol (99.7%),

acetonitrile (99.7%)

triethylamine (99.0%)

*General equipments*:

Glass Syringe 25 ml

Syringe filter diameter of 45 μm pore size PET (Whatman, USA)

*Equipment for maceration extraction*:

Water bath (XY-80, ITOCHU Corporations, JAPAN)

*Equipment for soxhlet extraction*: Figure 3.3

Soxhlet apparatus (SP glass, THAILAND)

Heater pocket (Electro mantle, USA)

*Equipment for ultrasound-assisted extraction*: Figure 3.4

Ultrasound baht (275DAE, Crest Ultrasonics, USA)

*Equipment for microwave-assisted extraction*: Figure 3.5

      Microwave reactor (CEM Corp., Mathews, NC, USA)

      Microwave safety membrane (XP-1200, USA)

## 3.3 Methodology for solubility determination (Part I)

### 3.3.1 *Preliminary experiments*

The control variables such as amount of astaxanthin standards and flow rate of carbon dioxide should be firstly considered. These are selected at the condition that offers the highest astaxanthin recovery, and are further used to determine the solubility of astaxanthin in various temperatures and pressures. The supercritical carbon dioxide extraction equipment is shown in Figure 3.1.

### 3.3.2 *Dynamic method for solubility determination*

      1)     Weigh astaxanthin standard 0.0500 g (50 mg) (measuring early to ensure excess amount of astaxanthin standard)

      2)     Mix astaxanthin standard with 15 g of 0.3 mm diameter glass bead then put inside 10 ml reactor vessel

      3)     Place reactor vessel into oven

      4)     Adjust the desired temperature (40, 60 and 80°C) with temperature controller

      5)     Pump liquified carbon dioxide from $CO_2$ cylinder tank. The liquefied was passed through cooler and entered the extraction unit at the bottom of reactor vessel

      6)     Adjust the desired pressure (20, 30 and 40 MPa) with back pressure regulator (BPR)

      7)     Entrain the sample from the vessel with $CO_2$ flow rate 0.5 ml/min (measured at pump to ensure equilibrium condition in reactor vessel) with interval time of 30 min

      8) The going solute was trapped in 10 ml dichloromethane with cold condition to prevent degradation of astaxanthin

      9)     Each sample was collected and kept at -20°C until analyzed by HPLC

      10)     Measure volume of the going out $CO_2$ with gas flow meter

      11)     Amount of accumulative astaxanthin was then plotted versus time of

extraction or volume of fluid as shown in Figure 3.2.

## 3.4 Calculation of solubility

The solute trapped by cold trap at the exit of supercritical fluid extraction chamber was then analyzed by HPLC technique using appropriate mobile phase. The amount of astaxanthin in the extract was analyzed by comparing the area under the peak with the standard astaxanthin in trap solvent. The mole fraction of astaxanthin was defined as the ratio between the accumulative mole of astaxanthin and the total mole of $CO_2$.

The astaxanthin solubility defined by the dynamic method was calculated from the initial period of overall extraction curve performed at equilibrium conditions. This curve presents amount of total solvent (solvent, x-axis) versus amount of accumulative solute (solute, y-axis) as showed in Figure 3.2.

During a initial linear part of this curve, the solute is readily available on sample surface which is solubilized in the solvent therefore it is assumed solubility controlled phase. (Ozlem Guclu Ustundag, 2003, Danielski et. al., 2007).

## 3.5 Accuracy evaluation of the models

The parameter of solubility model by EOS is estimated by comparing experimental solubility data to the calculated solubility data. The regression determination was carried out by minimizing the average absolute relative deviation (AARD) between experimental ($y_{exp}$) and calculated ($y_{cal}$) solubility data in following equation,

$$\text{AARD}(\%) = \left(\frac{100}{n}\right) \sum \frac{|y_{exp} - y_{cal}|}{y_{exp}} \tag{3.1}$$

where $n$ is the number of solubility data used in this evaluation. The suitable model which is use to predict solubility of astaxanthin is selected by the lowest amount of AARD (%).

## 3.6 Methodology for extraction (Part II)

### 3.6.1 Preliminary experiments

This preliminary experiment is aimed to determine the appropriate amount of liquid to solid ratio (L/S) for the extraction. Maceration at 30$^{\circ}$C and 45 minutes is employed as a method for this purpose in acetone is used as a solvent. The L/S ratio with the highest percent recovery is subsequently used in other extraction experiments, such as

maceration, ultrasound-assisted and microwave-assisted extractions except extraction by soxhlet.

### 3.6.2    Maceration extraction

1)    Weigh 0.1 g of *Haematococcus pluvialis* and place it in 125 ml flask

2)    Add 10 ml of acetone

3)    Cork the flask (with rubber cork)

4)    Perform extraction at various temperatures, i.e. 30, 45, and 60°C for 15, 30, 45, 60, 75 and 90 minutes in water bath

5)    Filtrate through a 0.45 μm pore size PET syringe filter (Whatman, USA)

6)    Measure the extract of astaxanthin by HPLC

7)    Change the solvent of the extraction in Step (2) to methanol, ethanol, and acetonitrile using the suitable extraction temperature and time which can provide the maximum of astaxanthin recovery as determined in Step (4)

All experiments are carried out in triplicate.

### 3.6.3    Soxhlet extraction

➢    Effect of solvent type

1)    Weigh 0.5 g of *Haematococcus  pluvialis* and place it in a thimble cartridge

2)    Keep it in 500 ml round-bottom flask

3)    Add 150 ml of acetone

4)    Connect each part of the equipment

5)    Place the equipment in the heater pocket (Electro mantle, USA)

6)    Carry out the experiment at boiling temperature for 4 hour or until the debris becomes colorless

7)    Change the solvent of the extraction in Step (3) to methanol, ethanol, and acetonitrile

All experiments are carried out in triplicate.

### 3.6.4    Ultrasound-assisted extraction

1)    Weigh 0.1 g of *Haematococcus  pluvialis* and place it in 30 ml gray glass bottle

2)       Add 10 ml of acetone

3)       Cork the bottle (with screw cap)

4)       Immerse in ultrasonic bath (275DAE, Crest Ultrasonics, USA) at 30, 45, and 60°C for 5, 15, 30, 45, 60, 75 and 90 minutes

5)       Filtrate through a 0.45 μm pore size PET syringe filter (Whatman, USA)

6)       Measure the extract of astaxanthin by HPLC

7)       Change the solvent of the extraction in Step (2) to methanol, ethanol, and acetonitrile using the suitable extraction temperature and time which can provide the maximum of astaxanthin recovery as determined from Step (4)

All experiments are carried out in triplicate.

### 3.6.5 *Microwave-assisted extraction*

1)       Weigh 0.1 g of *Haematococcus pluvialis* and place it in 100 ml vessel

2)       Add 10 ml of acetonel in each vessel

3)       Place the vessels symmetrically in the microwave reactor (CEM Corp., Mathews, NC, USA)

4)       Carry out experiment at 30, 45, 60, 70, 75, and 78°C for 3, 5, 15, 30, 45, and 60 minutes

5)       Filtrate the samples through a 0.45 μm pore size PET syringe filter (Whatman, USA)

6)       Measure the extract of astaxanthin by HPLC

7)       Change the solvent of the extraction in Step (2) to methanol, ethanol, and acetonitrile using the suitable extraction temperature and time which can provide the maximum of astaxanthin recovery as determined from Step (4)

All experiments are carried out in triplicate.

## 3.7 Analysis of astaxanthin with high performance liquid chromatography (HPLC)

The extracted solution of algae is analyzed for its astaxanthin content using the high performance liquid chromatography (HPLC, Venisep GES C18 4.6×150 mm, 5μm HPLC column) at 475 nm. The elution is fed at 1 ml/min with a mobile phase consisting of methanol and water at the volume ratio of 95:5. The amount of

astaxanthin in the extract is analyzed by comparing the area under the peak with the standard astaxanthin in each type of solvent. The recovery percentage of astaxanthin is defined as the ratio between the amount of astaxanthin obtaining from the extraction and the total amount of astaxanthin in the algae (Equation 3.2).

$$\% \text{ recovery} = \frac{\text{astaxanthin from the extraction}}{\text{total astaxanthin in the alga}} \times 100 \qquad (3.2)$$

The total amount of astaxanthin in the alga or the denominator in Eq. 3.2 is determined using the microwave extraction with the condition of the highest % recovery of astaxanthin (from Section 3.6.5) whereas the main astaxanthin in the debris is recovered by acetone until the debris appears white.
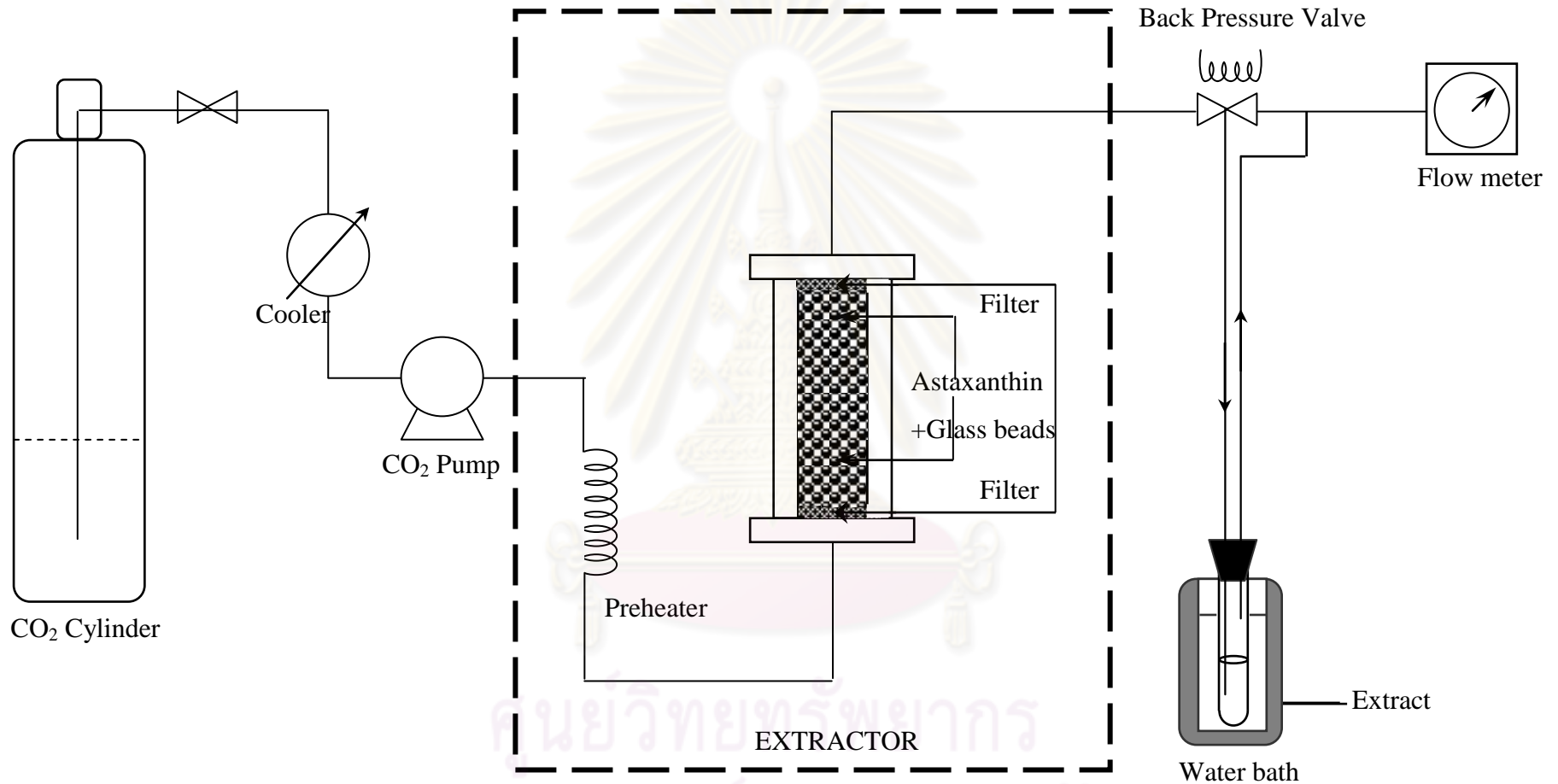
Figure 3.1 Schematic diagram of astaxanthin solubility in pure liquid $CO_2$ measurement by dynamic technique
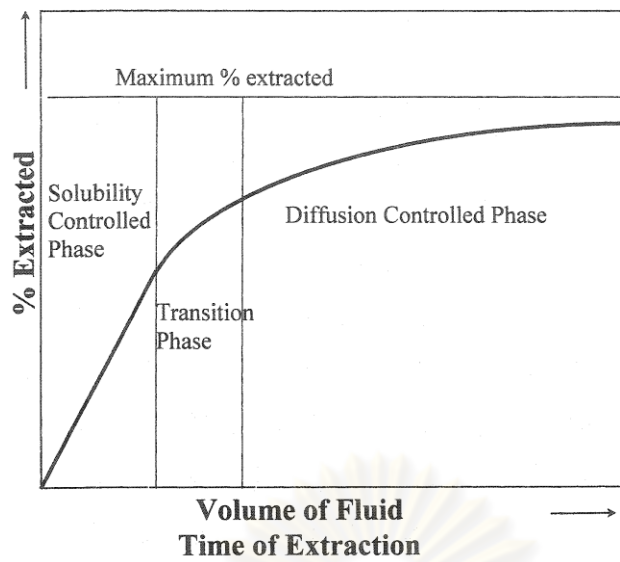
Figure 3.2 Overall extraction curve (OEC) as typical of supercritical fluid curve (Ozlem Guclu Ustundag, 2003)
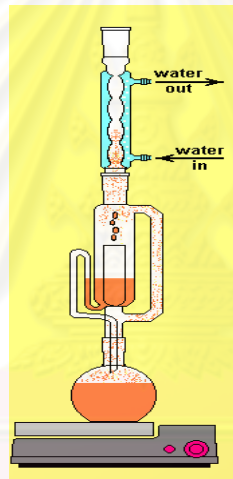


Figure 3.3 Apparatus of soxhlet extraction



Figure 3.4 Ultrasound bath

(a)                                                    (b)

Figure 3.5 (a) Microwave oven and (b) vessels

# CHAPTER IV

## ESTIMATION OF SOLUBILITY MODEL

### 4.1 Empirical models for solute solubility in supercritical $CO_2$

There are a number of correlations typically used to predict the solute solubility in supercritical $CO_2$. Among them, the most used semi-empirical models are 1) the famous simplicity density based correlation such as Chrastil's model which assumes the association between solute and solvent to form solvate-complex without the needs for solute property (Murga et al., 2002 and Gómez-Prieto et al., 2007) and 2) the linear correlation with enhanced factor and the density of the solvent (Murga et al., 2002). A more rigorous thermodynamic model (Gupta and Jae-Jin, 2007) is that employing cubic equation of state such as the Peng-Robinson equation of state (PR-EOS) which is used to describe the behavior of supercritical fluid phase.

### *4.1.1 Chrastil's model*

This model is based on the assumption that there is association between SC-$CO_2$ and solute molecules which results in formation of solvato-complex ($AB_k$, A is solute molecule, B is solvent molecule and k is association number) at equilibrium condition. This simple model relates the solubility of solute ($C$, g/l) in SC-$CO_2$ to the density ($\rho$, g/l) of the solvent and absolute temperature ($T$, K) of the fluid as shown below:

$$C = \rho^k \exp\left(\frac{a}{T} + b\right) \tag{4.1}$$

where $k$ is an association number which represents the average number of $CO_2$ molecules in the solvated complex, $a$ depends on heats of vaporization and solvation enthalpies of solute and, therefore, it indicates the influence of the temperature changes inside the extraction vessel. Consequently, a higher absolute value of $a$ associated with higher temperature influence. Another constant $b$ depends on the molecular weight of both $CO_2$ and solvent. We can estimate $k$, $a$ and $b$ from correlating experimental data with this model.

### 4.1.2 Solubility model by EOS

The rigorous methods, related to the fugacity of components in dense gas, were frequently employed earlier (Prausnitz et al., 1986, Sovová et al., 2001, Murga et al., 2002, Gupta and Shim 2007, Shi et al., 2009). This solubility model is conducted under assumption that (i) supercritical phase is dense phase, (ii) the gas in the solid is almost negligible, (iii) gas phase is considered pure and one-component solid is considered pure solid phase and (iv) a solid mixture is considered to behave like a heavy liquid phase and there is interaction between solvent (1) and solute (2). The general equation of equilibrium is

$$f_2^{solid} = f_2^v \qquad (4.2)$$

where subscript 2 referred to the solute, $f_2^{solid}$ and $f_2^v$ are the fugacity of pure solid and the fugacity of solute in the supercritical fluid phase. Because the solid phase is pure, thus the fugacity of component 2 is

$$f_2^{solid} = P_2^{sat}\emptyset_2^s exp\left(\int_{P_2^{sat}}^{P}\frac{v_2^s dP}{RT}\right) \qquad (4.3)$$

In supercritical phase, the fugacity is written in terms of solute mole fraction $(y_2)$ and fugacity coefficient$(\emptyset_2)$:

$$f_2^v = y_2\emptyset_2 P \qquad (4.4)$$

From Eq. (4.2), Eq. (4.3) and Eq. (4.4) the enhancement factor, $E$, can be defined as:

$$E = \frac{\emptyset_2^s}{\emptyset_2}exp\left[\frac{v_2^s(P-P_2^{sat})}{RT}\right] \qquad (4.5)$$

where $E$ is nearly greater than unity, and it is defined as the ratio between the observed equilibrium solution and that predicted by the ideal gas law at the same temperature and pressure as inferred in.

We can find the solubility of solute from the following equation:

$$y_2 = \left(\frac{P_2^{sat}}{P}\right)\left[\frac{\emptyset_2^s}{\emptyset_2}exp\left(\frac{v_2^s(P-P_2^{sat})}{RT}\right)\right] \qquad (4.6)$$

From the above assumptions, the solubility in Eq. (4.6) can be written in a simplified form as:

$$y_2 = (\frac{P_2^{sat}}{P})\left[\frac{1}{\emptyset_2}exp\left(\frac{v_2^s(P-P_2^{sat})}{RT}\right)\right] \tag{4.7}$$

$\frac{P_2^{sat}}{P}$ is the solubility in the ideal gas where $P_2^{sat}$ is the saturation (vapor) pressure of the pure solid. $E$ is enhancement factor as inferred in Eq. (4.5). $E$ contains the important terms, $\emptyset_2^s$ and $\emptyset_2$, which are fugacity coefficients at saturation pressure $P_2^{sat}$ and system pressure $P$, respectively, and $v_2^s$ is the solid molar volume, which can be assumed to be pressure independent for all the system temperature $T$. Due to low vapor pressure, the values of $\emptyset_2^s$ for most solutes can be taken as unity that do not have strong tendency of association and $\emptyset_2$ is calculated from Eq. (4.8).

$$RT\ln\emptyset_2 = \int_\infty^v \left[\left(\frac{\partial P}{\partial n_2}\right)_{T,v,n_1} - \frac{RT}{v}\right]dv - RTln\left(\frac{Pv}{RT}\right) \tag{4.8}$$

One way to find fugacity of solid solute in fluid phase $\emptyset_2$ in this equation is to use the widely known PR-EOS (Peng-Robinson equation of state) as defined in Eq. (4.9) which relates to interaction parameters as $a$ and $b$:

$$P = \frac{RT}{v-b} - \frac{a}{v(v+b)+b(v-b)} \tag{4.9}$$

where $v$ is the molar volume of the component. From equation (4.8) and (4.9) fugacity coefficient $\emptyset_2$ can be evaluated as derived as,

$$ln\emptyset_2 =$$
$$\frac{b_2}{b}\left(\frac{Pv}{RT}-1\right) - ln\left(\frac{P(v-b)}{RT}\right) - \frac{a}{2\sqrt{2}bRT}\left(\frac{2(y_1\sqrt{a_1a_2}(1-k_{12})+y_2a_2)}{a} - \frac{b_2}{b}\right)ln\left(\frac{v+2.414b}{v-0.414b}\right) \tag{4.10}$$

Mixture, size, and energy parameters can be evaluated using van der Waals one-fluid quadratic mixing rules and usual binary parameters $a$ and $b$ according to:

$$a = y_1^2a_1 + 2y_1y_2\sqrt{a_1a_2}(1-k_{12}) + y_2^2a_2 \tag{4.11}$$

$$b = y_1^2b_1 + y_1y_2(b_1+b_2)(1-c_{12}) + y_2^2b_2 \tag{4.12}$$

where $k_{12}$ and $c_{12}$ are adjustable parameters due to molecular interactions between solute-$CO_2$ in the mixture, and the different size of species. Other two parameters, $b_2$ and $a_2$, inferred to size and energy parameters for pure component 2 are described as follow:

$$b_1 = 0.07780 \frac{RT_c}{P_c} \qquad (4.13) \qquad ; \quad b_2 = 0.07780 \frac{RT_2}{P_2} \qquad (4.14)$$

$$a_1 = 0.45724 \frac{(RT_c)^2}{P_c}\left[1 + (0.37464 + 1.5423\omega_1 - 0.2699\omega_1^2)(1 - T_r^{0.5})\right]^2 \quad (4.15)$$

$$a_2 = 0.45724 \frac{(RT_{c2})^2}{P_{c2}}\left[1 + (0.37464 + 1.54226\omega_2 - 0.26992\omega_2^2)(1 - \sqrt{T/T_{c2}}\right]^2 (4.16)$$

where $T_c$ and $P_c$ are critical properties and $\omega_i$ is concentric factors of solute and solvent, and these properties were estimated by Joback group contribution method as detailed in previous researches (Reid et al., 1987, Poling et al., 2001, Ajchariyapagorn et al., 2008) or obtained from the sublimation of properties data of solute was shown in Eq. (4.17) - (4.32).

**Joback property function from Group Contribution** (Poling et al., 2001)

$$T_c(K) = T_b[0.584 + 0.965\{\sum_k N_k (tck)\} - \{\sum_k N_k (tck)\}^2]^{-1} \quad (4.17)$$

$$P_c(bar) = [0.113 + 0.0032N_{atoms} - \sum_k N_k (pck)]^{-2} \qquad (4.18)$$

$$V_c(cm^3 mol^{-1}) = 17.5 + \sum_k N_k (vck) \qquad (4.19)$$

$$T_{fp} = 122 + \sum_k N_k (tfpk) \qquad (4.20)$$

$$T_b = 198 + \sum_k N_k (tbk) \qquad (4.21)$$

where the value *N* of each group will be showed in Poling et al., 2001.

**Acentric Factor, $\omega$**

$$\omega = -\frac{\ln\left(\frac{P_c}{1.01325}\right) + f^{(0)}(T_{br})}{f^{(1)}(T_{br})} \qquad (4.22)$$

$$T_r = T_{br} = T_b/T_c \qquad (4.23)$$

$$f^{(0)} = \frac{-5.97616\tau + 1.29874\tau^{1.5} - 0.60394\tau^{2.5} - 1.06841\tau^5}{T_r} \qquad (4.24)$$

$$f^{(1)} = \frac{-5.03365\tau + 1.11505\tau^{1.5} - 5.41217\tau^{2.5} - 7.46628\tau^5}{T_r} \qquad (4.25)$$

$$\tau = (1 - T_r) \qquad (4.26)$$

where the dimension of *Pc* is in bars, $T_b$ and $T_c$ are K.

**Molar volume of component, $V_1$ or $v$**

$$v = \frac{Z_1 RT}{P} \tag{4.27}$$

**Solid Molar Volume, $(v_2^s)$** (Ajchariyapagorn et al., 2008, Poling et al., 2001)

The molar volume of a solid solute, $v_2^s$ is the volume per mole of a substance and it was estimated using Eq. (4.28) and (4.29):

$$\bar{v}_s = \sum_i m_i v_i \tag{4.28}$$

$$v_2^s = \frac{\bar{v}_s}{1.66} \tag{4.29}$$

where $\bar{v}_s$ is calculated crystal volume for a single molecule, $m_i$ is the relative stoichiometric multiplicity determined from the molecular structure and $v_i$ is the unit volume of an atomic element and estimated by Group Contributions of Constantinou/Gani property functions from. (Poling et al., 2001)

**The saturation (vapor) pressure of solute, $P_2^s$**
(Lyman et al., 1982, Ajchariyapagorn et al., 2008)

$$\ln P_2^s \approx \frac{\Delta H_{vb}}{\Delta Z_b R T_b}\left[1 - \frac{(3-2T_{\rho b})^m}{T_{\rho b}} - 2m(3-2T_{\rho b})^{m-1}\ln T_{\rho b}\right] \tag{4.30}$$

$$\frac{\Delta H_{vb}}{T_b} = K_F(8.75 + R\ln T_b) \tag{4.31}$$

where for all liquids, m = 0.19; and for all solids, the following values are recommended:

If $T_{\rho b} > 0.6$; m = 0.36

If $0.6 > T_{\rho b} > 0.5$; m = 0.8

If $T_{\rho b} < 0.5$; m = 1.19

$K_F$ is the so-called Fishtine constant and it depends on the dipole moments of polar and non-polar molecules. $\Delta H_{vb}$ is the enthalpy at the boiling temperature and m is a parameter dependent on

$$T_{\rho b} = \frac{T}{T_b} \tag{4.32}$$

The normal boiling point of solute is estimated using group-contribution methods. The parameter $\Delta Z_b$ is assumed to have the value of 0.97.

### 4.1.3 Enhancement and density based model

This model assumes a linear correlation between the enhancement factor ($E$) as estimate from Eq. (4.33) which will be defined in the next section, the equation of the solubility of solid in gas phase and the density ($\rho$) of the solvent is shown in Eq. (4.35):

$$y_2 = E(P_2^{sat}/P) \tag{4.33}$$

$$\ln E = A + B\rho \tag{4.34}$$

Replacing Eq. (4.33) to (4.34) modified to

$$\ln(y_2 P/P_2^{sat}) = A + B\rho \tag{4.35}$$

where $A$ and $B$ are constants at a constant temperature, $y_2$ is solubility or mole fraction between solute referred to (2) and solvent referred to (1), $E$ is enhancement factor over the ideal solubility, $P_2^{sat}$ is sublimation pressure of solute or the saturation (vapor) pressure of pure solid (2) and $P$ is operating pressure. However vapor pressure of nonvolatile solutes may not be always available therefore modified to,

$$\ln\left(\frac{y_2 P}{P_{\text{ref}}}\right) = A' + B(\rho - \rho_{\text{ref}}) \tag{4.36}$$

where $A' = A + B\rho_{\text{ref}}$, $A'$ and $B$ are constants at constant temperature, $P_{\text{ref}}$ is a reference pressure conventionally taken as 1 bar and $\rho_{\text{ref}}$ is reference density taken as 700 kg/m$^3$.

### 4.2 Effect of pressure and temperature on astaxanthin solubility

Figure 4.1 (a) demonstrates the effect of temperature and pressure in the range of 40 to 80°C and 20 to 40 MPa on the solubility of astaxanthin where the x-axis refers to the pressure of the system and the y-axis on the left hand side refers solubility of astaxanthin in mole fraction of solute whereas the y-axis on the right hand side shows the astaxanthin solubility in dimension of gram of astaxanthin per 1 liter of solvent. This figure demonstrates that the solubility of astaxanthin increased with increasing pressure. It is known that an increase in pressure enhances the density of $CO_2$ as

demonstrated in Figure 4.1 (b). This enhances the interaction between solute and solvent leading to an increase in a higher level of dissolution. This is reflected in a higher solubility of astaxanthin in $CO_2$ as indicated in Eq. 4.7.

Figure 4.1 also demonstrates the effect of temperature on the solubility of astaxanthin in $SCCO_2$ where an increase in tempersure seemed to steadily raise the solubility. This result, however, indicates that $CO_2$ density is not the only factor that influence the solubility as increasing temperssure resulted in a decreasing $CO_2$ density which should lower the solubility of astaxanthin. However, the solute vapor pressure was the other parameter that plays a significant role in this case. An increase in temperature led to a higher vapor pressure of astaxanthin which means that more astaxanthin is being extracted into the supercritical $CO_2$ phase, and this effect prevailed over that of $CO_2$ density which resulted in an increase in the solubility. (Roy C. B. et al., 1996, Machmudah et al., 2006, Shi et al., 2009)

## 4.3 Model Correlation

### 4.3.1 Chrastil's model

The results of astaxanthin solubility over range temperature of 40-80°C and pressure of 20-40MPa from previous section was further used to estimate the parmaters for correlations of solubility. The suitable correlations or models will be selected that best described solubility of astaxanthin. The easiest model to predict solubility is Chrastil's model which operates based on the assumption that solute and solvent as they dissolve form molecular structure, called solvato complex $AB_k$. (A is solute molecule, here is astaxanthin and B is solvent molecule, here is carbon dioxide) According to Eq. 4.1, linearization results in:

$$lnC = kln\rho + \frac{a}{T} + b \qquad (4.1a)$$

The density in Eq. (4.1a) is obtained from the handbook of solubility in supercritical carbon dioxide. (Gupta B. R. and Jae-Jin Shim, 2007), $k$ value is averaged over the three temperatures to get the mean value which is further used to calculate $a$ and $b$. The optimal parameters for Chrastil's correlation are summarized in Table 4.1. The solubility data of astaxanthin using the obtained Chrastil's model are compared to the experimental solubility data as illustrated in Table 4.1.

In this model, the meaning of $k$ value is the number of $CO_2$ molecule associated with one molecule of astaxanthin, and in this work, this has the value of

16.2. The different $k$ values for the various carotenoid derivatives such as lycopene and lutein are displayed as different values as they have different chemical structure and polarity (Cygnarowicz, 1990, Suba et al., 1997, Sovova´ et al., 2001, Topal et al., 2006, Gómez-Prieto et al., 2007).

### 4.3.2 Solubility model by EOS

This solubility determination model is more complex than the Chrastil's model as it involves the equation of state as mentioned in Section 4.1.2. This model allows a better adjustment of the solubility data as it explicitly includes the interaction between solute and solvent, $k_{12}$ and $c_{12}$, which can be optimized by minimizing absolute deviation value, %AARD in Eq. (3.1). The optimal parameters for the solubility equation of state model are summarized in Table 4.2.

### 4.3.3 Enhancement factor and the density based model

This model is designed for the determination of low volatile compounds with high molecular weight in absent of properties data particularly at the critical condition (Bartle et al., 1991). This model is derived based on the assumption that the system has the linear correlation between the enhancement factor and density as shown in Eq. (4.35) (Section 4.1.2). Experimental solubility data in Section 4.2 were employed to estimate the model parameters. The parameters, B and A′, are obtained from the plot between ln$E$ and $\rho$, and these values are reported in Table 4.3. The comparison between the solubility data obtained by this model and experimental data is shown in Table 4.3.

### 4.4 Consideration of optimal model

Comparison between solubility data of astaxanthin predicted by the three models mentioned above and experimental data are summarized in Figure 4.3 where the x-axis refers to pressure and y-axis refers to solubility of astaxanthin. This figure demonstrates that solubility data obtained by the equation of state can best fit with experimental data over the entire temperature and pressure ranges of 40-80°C and 20-40MPa. This could be due to the fact that the equation of state model is the only model that takes into account of the properties of solute at the critical condition such as vapor pressure, and the extraction conditions such as temperature and pressure, and

this renders the model to be more versatile than the other two models. Therefore it is concluded here that the equation of state is the most suitable model for the estimate of solubility of astaxanthin as it provided the minimum of % AARD, determined by Eq. (3.1). The values of % AARD for these three models are summarized in Table 4.1-4.3.

## 4.5 Comparison between solubility data and previous researches

The comparison of solubility of astaxanthin between this work and the past researches is shown in Figure 4.4, in which the solubilities of astaxanthin (y-axis) were plotted against pressure (x-axis). This figure demonstrates that solubility from this work lie between solubility from previous researches. To see which sets of data are reasonable data that present the actual solubility of astaxanthin, one may consider the data from actual experimental extraction of astaxanthin from *H. pluvialis* from previous work. Assuming that the solubility of astaxanthin can be estimated from initial slope of extraction curve, if the extraction flow rate is low enough, and that the mass transfer is not limited (astaxanthin can easily be leached out of the cells), then the initial extraction curve should be close to solubility.

The extraction results of Machmudah's and Krichnavaruk's published worksshow that the initial slopes of their extraction curve were lower than the solubility determined in this study, but were higher than those of de la Fuente. It is generally not likely that the solubility be lower than the slope of actual extraction curve as mass transfer is likely to play a big role in this system. From this reasoning, therefore, it should be concluded that the data obtained in this present study more reasonably represent the actual solubility data. On the other hand, the other set of data (Hyun-Seok et al., 2007) shows extremely high solubility data, which are 2 orders of magnitude higher than the data obtained in the present study and are are than the solubility of similar compounds like beta-carotene as shown in Figure 4.5. Therefore, it's likely that this is an over estimate of the solubility data of astaxanthin. When consider how their experimental results were obtained, we found that they use large amount of solvent (chloroform) to dissolve the astaxanthin before loading it into the cotton wool. If this solvent was not completely evaporates before the start of the extraction, it's most likely that it will act as cosolvent and thus causing over estimation in the solubility. If however that the experiment was conducted carefully to evpaporate chloroform entirely out of the loaded matrix prior to the experiment, which

is likely be the case, the overestimation of the solubility could be resulted from the fact that the compound could be carried over by the flow of CO2 passing through the system, particulary when dynamic method was used. Observed also from Figure 4.5 was that  the static method always gave lower solubility than the dynamic method. The exact reason for this still could not be given from the evidence available at this present time, but it was believed to be due to the experimental technique. In static method as employed in (de la Fuente et al., 2006), the samples were taken intermittently from the extractor at a certain time period whilst a continuous sampling was employed in the dynamic method. This way, the change (drastic drop) in the pressure during the sampling period could adversely affect the solubility and a lower solubility could be observed. It is usually the case where the data for solubility vary significantly from one study to another. It is therefore necessary that more experiments and in depth study would be conducted to verify these results.

**4.6 Evaluation of extraction efficiency of astaxanthin extraction from *H. pluvialis***

In this section, the evaluation of extraction efficiency using solubility data will be illustrated as using experimental extraction data from Machmudah et al. (2006) as case study. Figure 4.6 illustrates the results of solubility of astaxanthin calculated from the equation of state as explained in Section 4.3 over the temperature range 40 to 80°C. In this figure, x-axis is pressure of system and y-axis is the amount of astaxanthin in the dimension of mole astaxanthin dissolved in 1 mole of $CO_2$. The symbols "+" and "o" represent the data from the extraction of astaxanthin from the cell of *H.pluvialis* which is obtained from the work of Machmudah et al. (2006) whereas the different lines are the simulated solubility from the model which represents the data from the extraction of standard astaxanthin (from this work). The amount of dissolved astaxanthin from the extraction of *H. pluvialis* increased with pressure, i.e. $0.014 \times 10^{-7}$ (at 20 MPa) to $18.42 \times 10^{-7}$ (at 40 MPa) mole of dissolved astaxanthin per mole of $CO_2$ because an increase in pressure enhanced the density of $CO_2$ as stated earlier.

Let's now define the extraction efficiency (%) as the ratio between actual dissolved astaxanthin from the extraction of *H. pluvialis* and the solubility of astaxanthin (from the standard) multiplied by 100 at the same pressure and temperature. The extraction will be limited by the level of solubility when this extraction efficiency moves closer to 100%. On the other hand, if this extraction

efficiency becomes low, the extraction will not be solubility limited but will be regulated by some other mass transfer mechanism in the process, e.g. high mass transfer resistance between phases. To evaluate for the extraction efficiency of the extraction process provided by Machmudah et al. (2006), let's further focus on the extraction at $70^{o}C$ over the pressure range of 20 to 40 MPa. The extraction efficiency at 20 MPa was calculated to be around 0.41% whereas this was 29.4% and 21.8% at 30 and 40 MPa, respectively. This observation suggests that the solubility did not affect the extraction process at low pressure (20 MPa), rather, the solubility became more significant in controlling the extraction at higher pressure. Increasing the pressure from 30 to 40 MPa did not see much deviation in the extraction efficiency which could infer that solubility might not be the only one controlling mechanism in this process, and the other limiting factors such as mass transfer resistance also had a consistent share in manipulating the extraction process.

## 4.7 Conclusion remarks

This chapter reveals the results of solubility of astaxanthin in $SCCO_2$ which could be used to evaluate the extraction efficiency of astaxanthin extraction using $SCCO_2$. As demonstrated in the previous section, the extraction efficiency of such process (as reported in Machmudah et al., 2006) was still very low, and it seems that increasing solubility (by increasing pressure) will not have a positive effect on the extraction efficiency. Consequently, other operating parameters will need to be investigated in trying to improve the efficiency of the extraction by $SCCO_2$. For example, can the extraction be improved removing mass transfer limitation in such system such as using SCCO2 coupled with ultrasound extraction. As this remains to be examined, other questions regarding alternative extraction methods arises, that might be more economically feasible, such as microwave or ultrasound assisted solvent extraction technique. This issue will be examined in more detail in the next chapter.
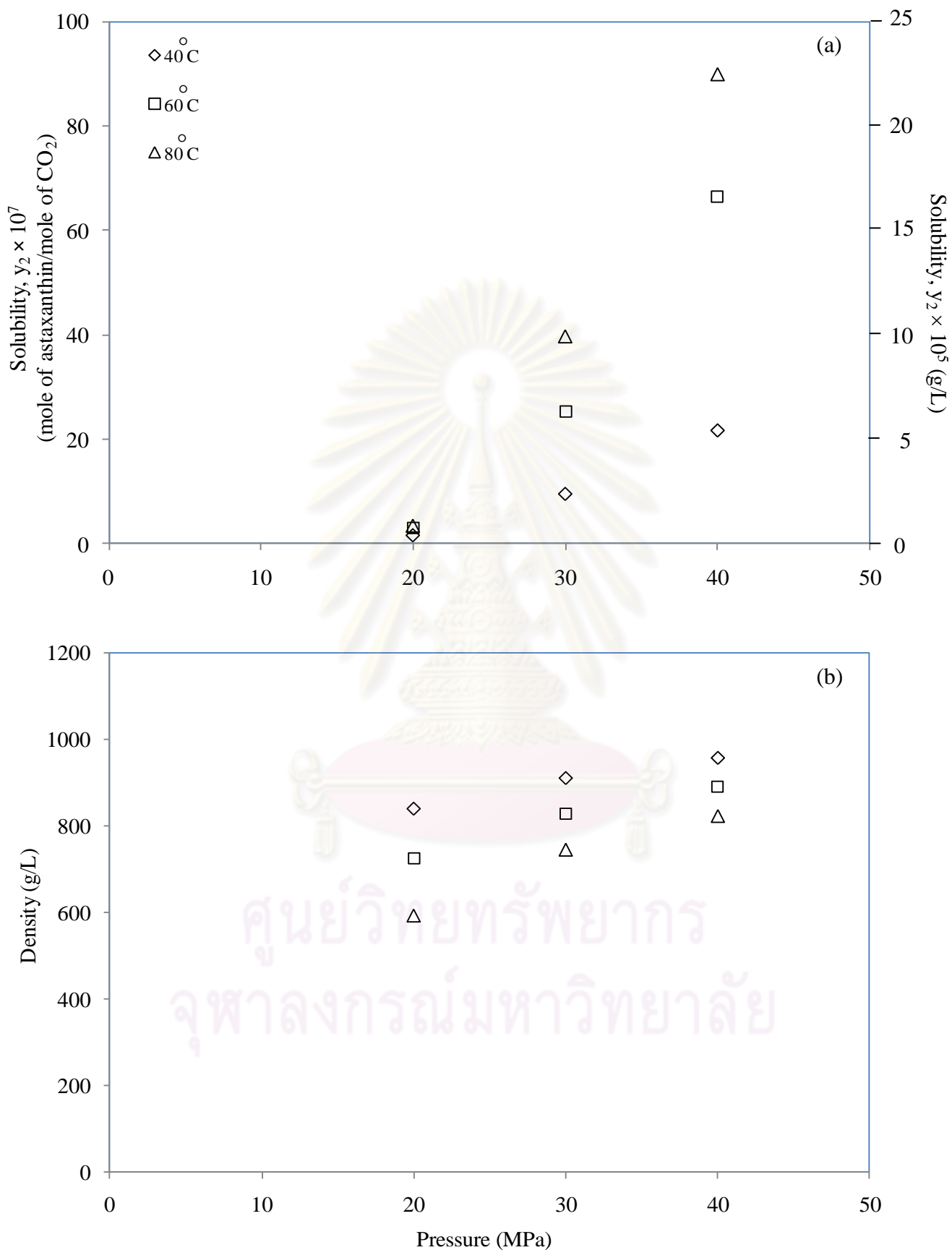
Figure 4.1 Astaxanthin solubility (a) Effect of temperature and pressure in SCCO$_2$ (b) Density of CO$_2$ as

a function of pressure at various temperatures
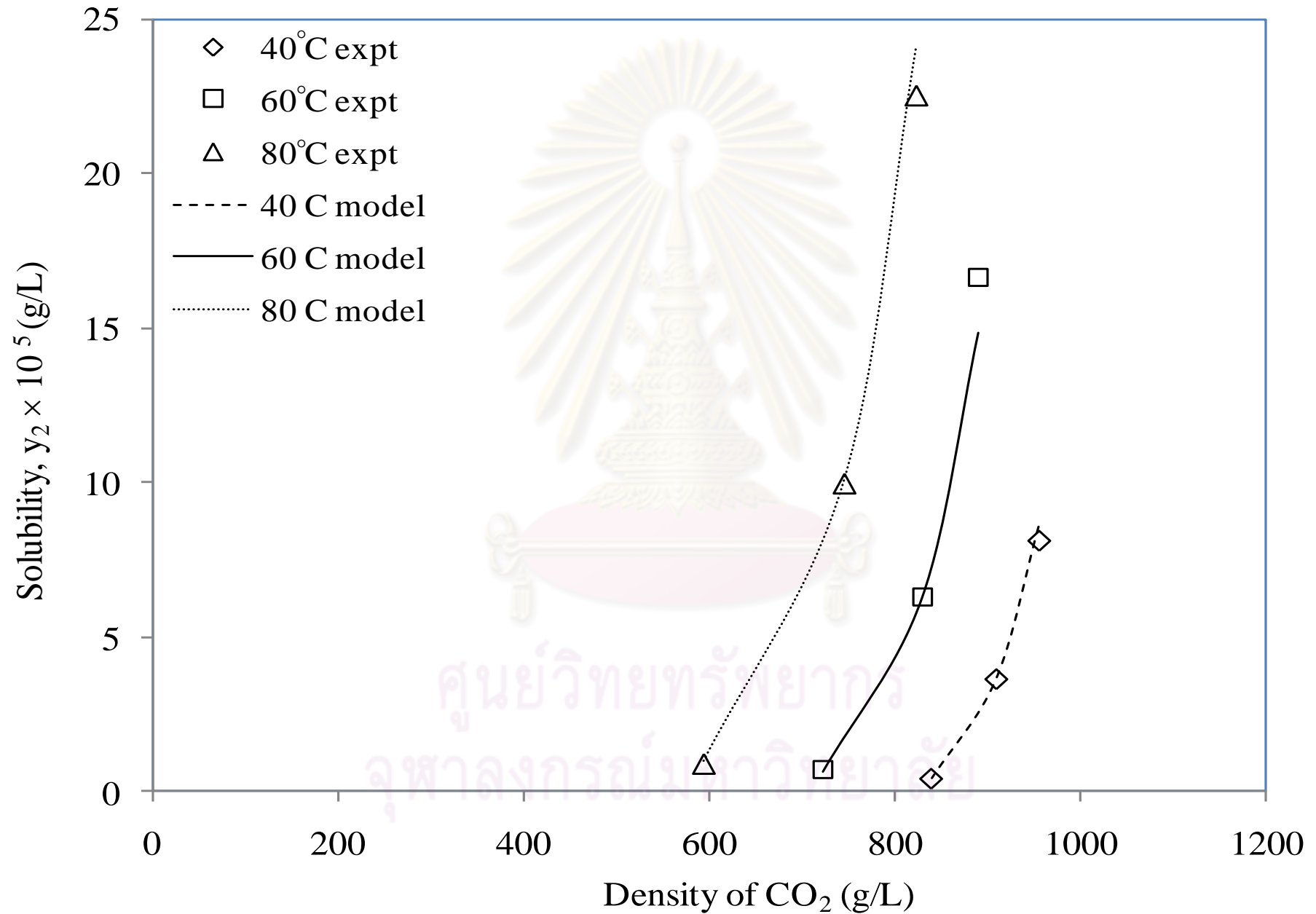
Figure 4.2 Correlation between solubility of astaxanthin and carbon dioxide density using Chrastil's model
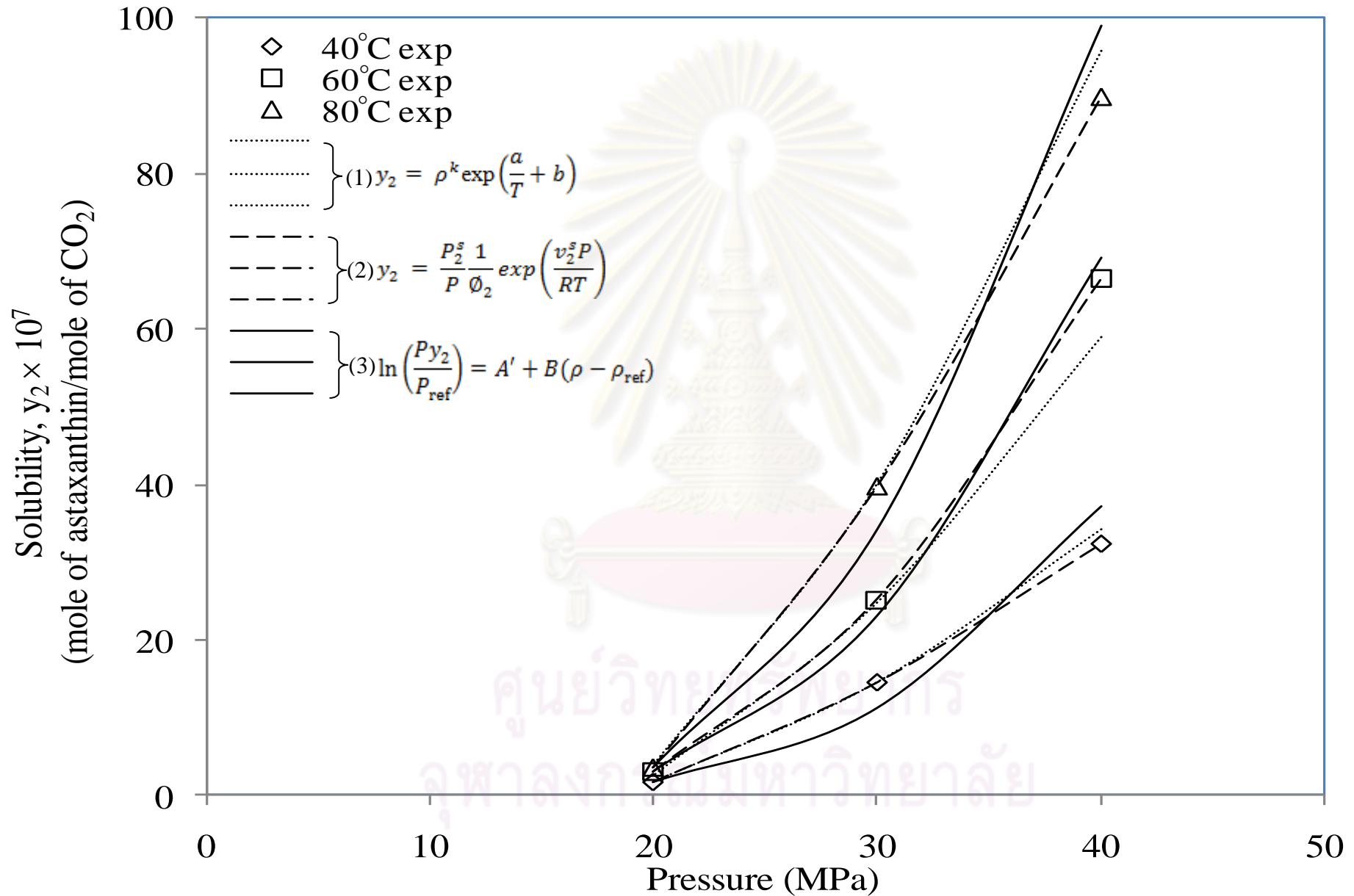
Figure 4.3 Correlation between solubility of astaxanthin and pressure using three models: (1) Chrastil's model, (2) enhancement factor

and the density based model and (3) solubility model by EOS
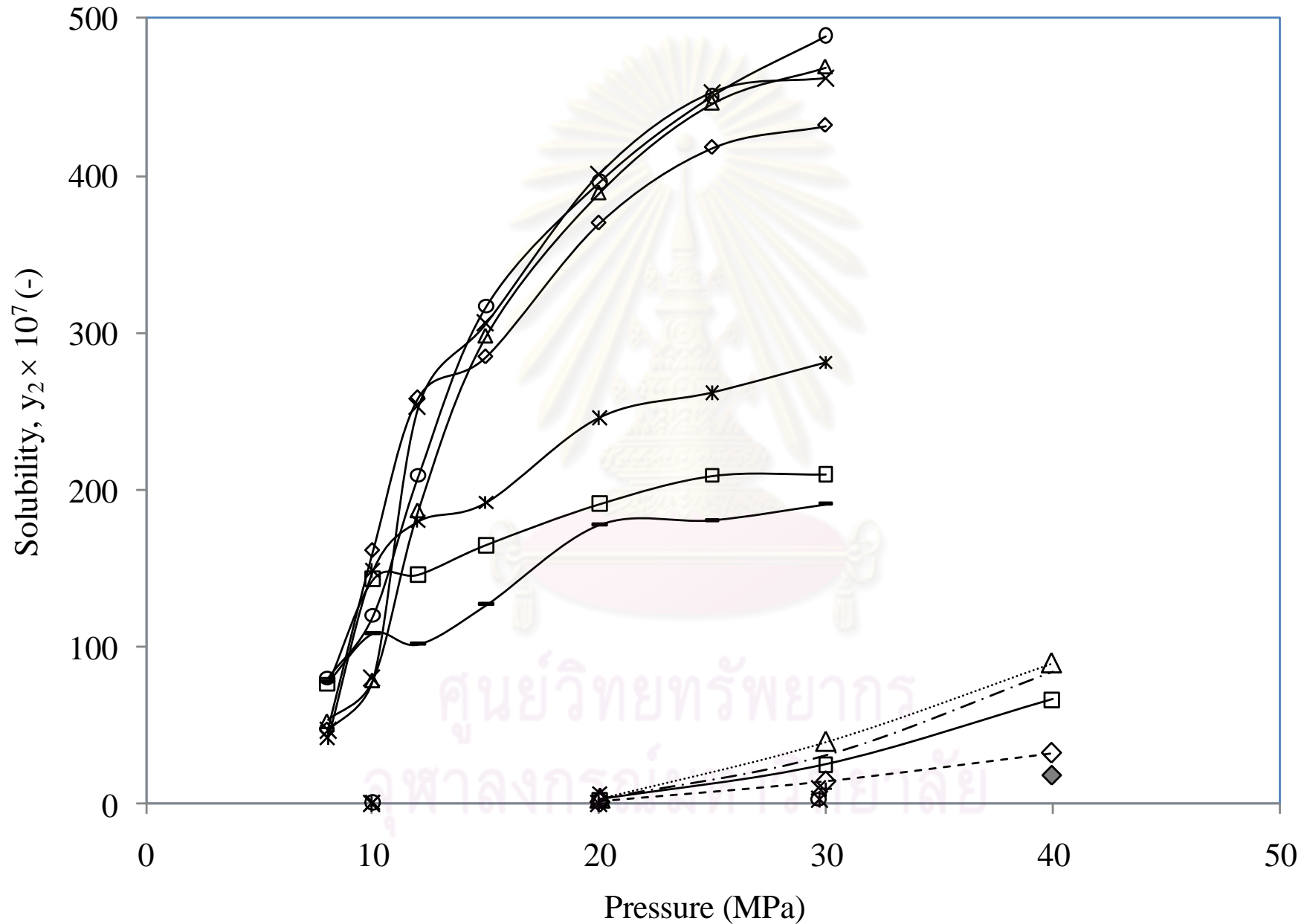
Figure 4.4 Solubility data of astaxanthin from various researches; pure astaxanthin (de la Fuente et al., 2006, Hyun-Seok et al., 2007, this work),

*H. pluvialis* using pure $CO_2$ (Machmudah et al., 2006, Krichnavaruk et al, 2008), symbols definition show in Table 4.4
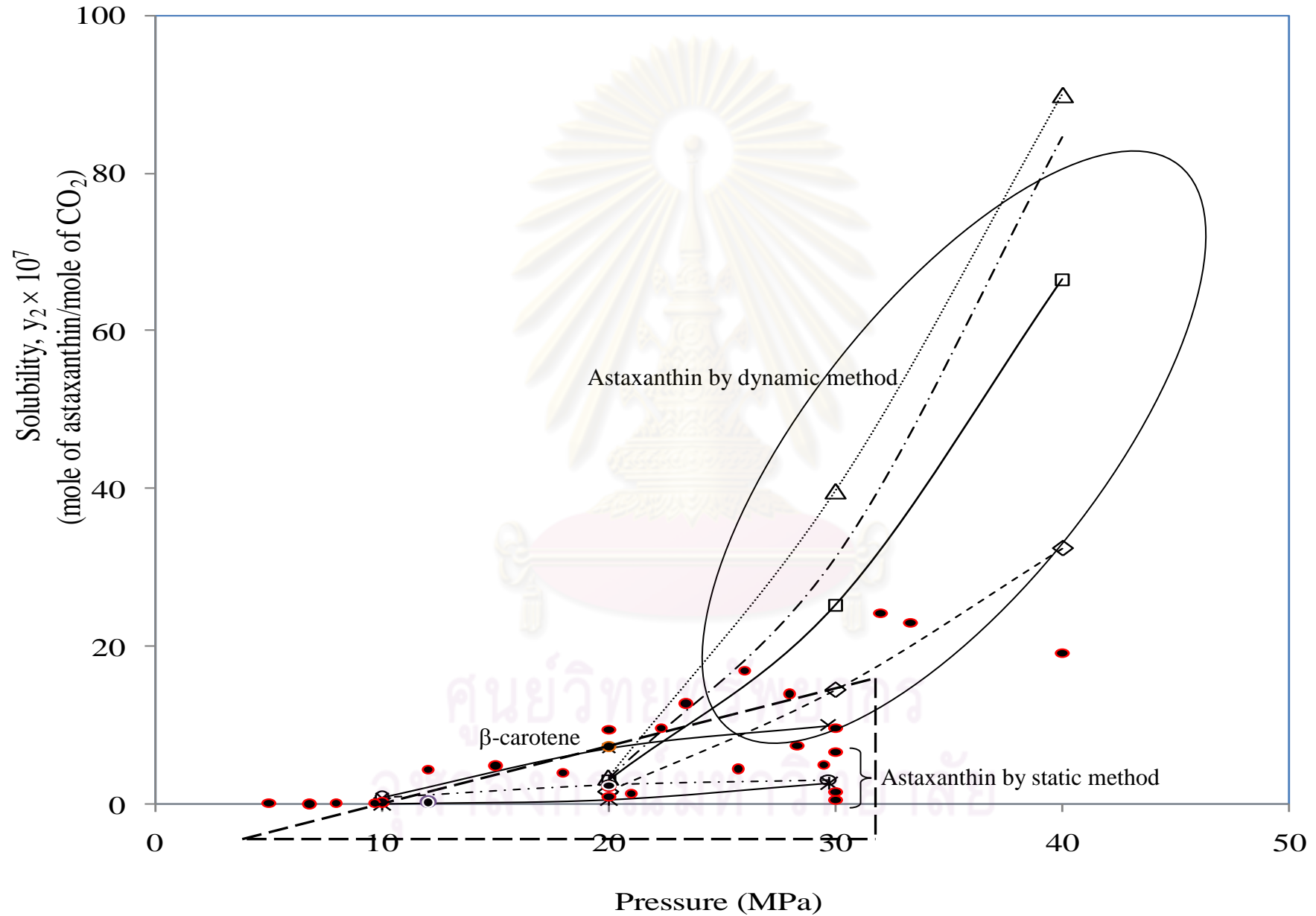
Figure 4.5 Solubility data of astaxanthin standard by dynamic and static method and β-carotene standard, symbols definition show in Table 4.4
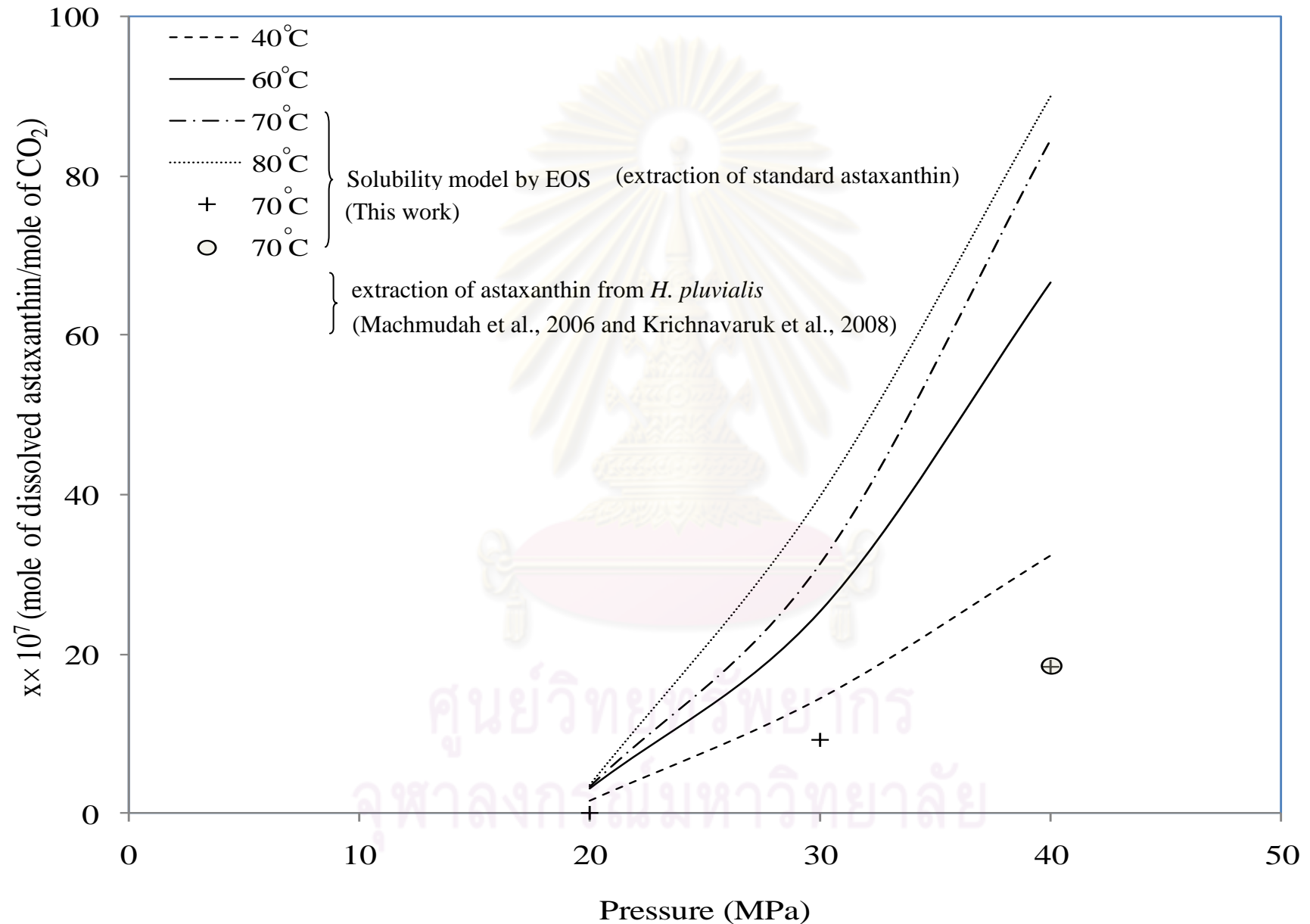
Figure 4.6 Comparison between dissolved astaxanthin standard and dissolved astaxanthin from *H. pluvialis* in carbon dioxide

(Machmudah et al., 2006, Krichnavaruk et al., 2008)

Table 4.1 Solubility data from Chrastil's model

| Temperature (K) | P (MPa) | k | A | b | Solubility, $y_2 \times 10^7$ (mole of astaxanthin/mole of $CO_2$) | %AARD |
|---|---|---|---|---|---|---|
| 353 | 40 | | -2835 | -108.9 | 95.8 | |
| | 30 | 16.2 | -2781 | -108.4 | 40.0 | 4.74 |
| | 20 | | -2104 | -109.0 | 3.7 | |
| 333 | 40 | | -2835 | -110.2 | 59.1 | |
| | 30 | 16.2 | -2781 | -110.1 | 24.9 | 8.17 |
| | 20 | | -2104 | -112.2 | 2.6 | |
| 313 | 40 | | -2835 | -111.4 | 34.3 | |
| | 30 | 16.2 | -2781 | -111.6 | 14.6 | 4.19 |
| | 20 | | -2104 | -114.6 | 1.7 | |

Table 4.2 Solubility data correlation from solubility model by EOS

| Temperature (K) | P (MPa) | $c_{12}$ | $k_{12}$ | Solubility, $y_2 \times 10^7$ (mole of astaxanthin/mole of $CO_2$) | %AARD |
|---|---|---|---|---|---|
| 353 | 40 | -0.656 | -0.09 | 89.96 | |
| | 30 | -0.656 | -0.06 | 39.76 | 0.00 |
| | 20 | -0.656 | -0.05 | 3.50 | |
| 333 | 40 | -0.656 | -0.11 | 66.55 | |
| | 30 | -0.656 | -0.07 | 25.24 | 0.03 |
| | 20 | -0.656 | -0.03 | 2.98 | |
| 313 | 40 | -0.656 | -0.16 | 32.46 | |
| | 30 | -0.656 | -0.12 | 14.51 | 0.07 |
| | 20 | -0.656 | -0.09 | 1.65 | |

Table 4.3 Solubility data from a linear correlation between the enhancement factor and solvent density

| Temperature (K) | P (MPa) | B | A' | Solubility, $y_2 \times 10^7$ (mole of astaxanthin/mole of $CO_2$) | %AARD |
|---|---|---|---|---|---|
| 353 | 40 | | | 99.17 | |
| | 30 | 0.017 | -4.45 | 34.31 | 9.68 |
| | 20 | | | 3.68 | |
| 333 | 40 | | | 69.31 | |
| | 30 | 0.023 | -7.02 | 23.17 | 5.09 |
| | 20 | | | 3.07 | |
| 313 | 40 | | | 37.17 | |
| | 30 | 0.032 | -11.48 | 11.32 | 15.38 |
| | 20 | | | 1.80 | |

Table 4.4 Symbols definition in Figure 4.4 and Figure 4.5

| Symbol | Sources | Conditions | | Solvents | Solubility, $y_2 \times 10^7$ (mole of astaxanthin/mole of $CO_2$) | References |
|--------|---------|------------|---|----------|----------------------------------------------------------------|------------|
| | | P (MPa) | T (°C) | | | |
| ◇ | Standard 99.5% | 20-40 | 313 | Pure-$CO_2$ | 1.65-21.70 | This work |
| □ | Standard 99.5% | 20-40 | 333 | Pure-$CO_2$ | 3.10-43.35 | This work |
| △ | Standard 99.5% | 20-40 | 353 | Pure-$CO_2$ | 5.28-90.21 | This work |
| ----- | Solubility Model | 20-40 | 313 | Pure-$CO_2$ | 1.65-32.46 | This work |
| —— | Solubility Model | 20-40 | 333 | Pure-$CO_2$ | 2.98-66.55 | This work |
| —·—· | Solubility Model | 20-40 | 343 | Pure-$CO_2$ | 3.36-84.62 | This work |
| ------- | Solubility Model | 20-40 | 353 | Pure-$CO_2$ | 3.50-89.96 | This work |
| ✳ | Standard >98% | 10-30 | 313 | Pure-$CO_2$ | 0.1-2.6 | Fuente de la et al., 2006 |
| ○ | Standard >98% | 10-30 | 323 | Pure-$CO_2$ | 0.90-3.10 | Fuente de la et al., 2006 |
| × | Standard >98% | 10-30 | 333 | Pure-$CO_2$ | 0.80-10.00 | Fuente de la et al., 2006 |
| —|— | Standard 98% | 8-30 | 303 | Pure-$CO_2$ | 78-191 | Hyun-Seok et al., 2007 |
| —⊟— | Standard 98% | 8-30 | 308 | Pure-$CO_2$ | 76-210 | Hyun-Seok et al., 2007 |
| —✳— | Standard 98% | 8-30 | 313 | Pure-$CO_2$ | 42-281 | Hyun-Seok et al., 2007 |
| —◇— | Standard 98% | 8-30 | 318 | Pure-$CO_2$ | 48-432 | Hyun-Seok et al., 2007 |
| —✳— | Standard 98% | 8-30 | 323 | Pure-$CO_2$ | 47-462 | Hyun-Seok et al., 2007 |
| —△— | Standard 98% | 8-30 | 328 | Pure-$CO_2$ | 53-469 | Hyun-Seok et al., 2007 |
| —○— | Standard 98% | 8-30 | 333 | Pure-$CO_2$ | 80-489 | Hyun-Seok et al., 2007 |
| + | *H. pluvialis* | 20-40 | 343 | Pure-$CO_2$ | 0.01-18.42 | Machmudah et al., 2006 |
| ▪ | *H. pluvialis* | 40 | 343 | Pure-$CO_2$ | 27.63 | Krichnavaruk et al., 2008 |
| ● | $\beta$-carotene | 8-40 | 288-353 | Pure-$CO_2$ | 0.02-24.18 | Remark[a] |

Remark[a]

References of solubility data of β-carotene

Stahl et al., 1987, Marshall, 1976, Cygnarowicz et al., 1990, Sovová et al., 1994, Johannsen et al., 1997, Subra et al., 1997, de França et al., 1999, Cocero et al., 2000, Hansen et al., 2001, Ambrogi et al., 2002, Kraska et al., 2002, Ambrogi et al., 2003, Saldaña et al., 2006, Sun et al., 2006, Shi et al., 2007, Škerget et al., 2007

Table 4.5 Model comparison; Chrastil's model, solubility model by EOS and linear relation between enhancement and the density

| no. | Conditions | Chrastil | EOS, Peng-Robinson | Linear correlation between enhancement factor and the density of the solvent |
|---|---|---|---|---|
| 1 | Equation | $y_2 = \rho^k \exp\left(\frac{a}{T} + b\right)$ | $y_2 = \frac{P_2^s}{P}\frac{1}{\phi_2}\,exp\left(\frac{v_2^s P}{RT}\right)$ | $\ln\left(\frac{P y_2}{P_2^{ref}}\right) = A' + B(\rho - \rho_{ref})$ |
| 2 | Number of phase | 1 (gas) | 1 (vapor or liquid) | 1 (gas) |
| 3 | Amout of substance in fluid phase | | | |
|  | -Solute, $y_2$ | / | / | / |
|  | -Solvent, $y_1$ | - | / | - |
| 4 | Condition parameter | | | |
|  | -Temperature, $T$ | / | / | / |
|  | -Pressure, $p$ | - | / | / |
| 5 | Physical Properties of substance | | It's hard to estimate properties accurately especially organic substance. | |
|  | -Gas constant, R | - | / | - |
|  | -Density of $CO_2$, $\rho$ | / | / | / |
|  | -Critical properties | | Mostly estimate by group distribution | |
|  |   - Temperature, $T_c$ | - | / Estimate by group distribution | - |
|  |   - Pressure, $P_c$ | - | / Estimate by group distribution | - |
|  |   - Density of $CO_2$, $\rho_c$ | - | / Include in van der Waal mixing rule | - |
|  |   - Molar volume of $CO_2$, $V_c$ | - | / Estimate by group distribution | - |
|  | -Binary interaction parameter, c12, k12 | - | / | - |
|  | -Molar volume of solute, $V_2^s$ | - | / Estimate by group distribution, literature data | - |
|  | -Solute vapor pressure, $P_2^s$ | - | / Estimate by group distribution, literature data | / Not estimate directly by group distribution but change equation in form of known pressure reference. |
|  | -Fugacity of solute, $\phi_2^s$ | - | / Assume to equal 1 because of low vapor | - |
|  | -Fugacity of solute in fluid phase, $\phi_2$ | - | / Estimate by cubic equation of state (Peng-Robinson,.etc..) | - |
|  | -Concentric factor of solute&solvent, $\omega_1\,\omega_2$ | - | / Estimate by group distribution | - |
|  | -Boiling temperature of solute&solvent, $T_b$ | - | / Estimate by group distribution, use for esimate concentric factor | - |
| 6 | Assumption | Equilibrium concentration from the law of mass action (macroscopic description)<br>There is association between solute and solvent in form of solvato-complex.<br>Vapor of solute is estimated from Clapeyron-Clausius equation.<br>It's dealt with equilibrium constant.<br>It's dealt with heat of solvation, vaorization. | Equilibrium<br>There is interaction between gas and solute.<br>Solid phase behaves like a heavy liquid phase (Subcooled liquid).<br>No gas dissolves in solid solute.<br>Gas and solid is considered pure.<br>Solute is incompressible.<br>Supecritical phase is dense phase.<br>Classical Peng-Robinson is not widely used in all range of pressure and temperature therefore adding some parameter.<br>Not suitable for solid solute, it's better for liquid-vapor system.<br>$P_2^s$ is very small<br>$P - P_2^s$ is equales to P or system pressure is greater than the vapor pressure of solute.<br>$\phi_2^s$ is equaled to 1 because of lower vapor pressure. | Equilibrium<br>Dilute solution<br>There is interaction between gas and solute.<br>Suitable for low volatility in SC-$CO_2$<br>Linear correlation between enhancement factor and the density of the solvent<br>It's modified from experimental data.<br>It's dealt with the partial vapor pressure of solute in SC-CO2 phase and the vapor pressure of pure solute.<br>Vapor of solute is estimated from Clapeyron-Clausius equation.<br>$P_{ref}$ is used in equation and equal to 0.1 Mpa.<br>$\rho_{ref}$ is assumed to be 700 kg/m$^3$.<br>It can't use with pressure lower than 10 MPa |
| 7 | Number of constant needs to estimate from data | 1 | 2 | 2 |
| 8 | Advantage | This can use to estimate the associated number between solute and solvent.<br>Linear relationship between ln$y_2$ and ln$\rho$ in a certain range of pressure and temperature. | This can use to estimate intermolecular interactions between solute and solvent in the mixture and the different size species.<br>Solubility consideration in high pressure mostly uses Peng-Robinson model.<br>This can use near critical point and in polar solvent.<br>This model can be modified for three components in mixture. | This equation can use to estimate enthalpy of vaporization of solid.<br>Linear relationship between lnE and $\rho$ in a certain range of pressure and temperature.<br>E value provides the meaning of solvent power and interactions in SCF. |

# CHAPTER V

## ASTAXANTHIN EXTRACTION FROM *Haematococcus pluvialis*

### 5.1 Background and motivations

The data of astaxanthin solubility in supercritical carbon dioxide determined in Part I of this dissertation and the evaluation of the actual extraction efficiency (29% maximum efficiency) previously reported reveal that most of the supercritical carbon extraction operations are mass transfer limiting, rather than solubility limiting. This is the reason why most of supercritical carbon dioxide extraction of *H. pluvialis* astaxanthin generally takes a long time to obtain reasonable compound recovery. Machmudah et al. (2006) reported that astaxanthin extraction from *H. pluvialis* by supercritical carbon dioxide using ethanol as co-solvent could increased the astaxanthin recovery from about 70% to as high as 80.6%. However, for this particular case of astaxanthin extraction of *H. pluvialis*, this method still suffered from a long operating time of around 4 hours and high energy consumption which might not be practical for a larger scale design at the current development. Consequently while the process is being improved, new accelerated extraction procedures such as ultrasound-assisted and microwave-assisted extractions should also be considered since past reports have shown that not only did these assisting methods significantly shorten the operation time, but they also did reduce the solvent requirement (Rostagno et al., 2003 and Hemwimon et al., 2007). The investigation of these methods along with the conventional maceration extraction becomes the main objective of Part II of the dissertation, presented in this chapter.

### 5.2 Effect of solvent types

Effect of solvent types was investigated with respect to each extraction method because different solvents played in different role in each extraction method. Organic solvent as methanol ethanol acetone and acetonitrile were chosen in the study to identify the best solvent giving the highest % astaxanthin recovery. Among these solvents, it was discovered that acetone gave the higest astxanthin recovery for all extraction methods. Methanol and ethanol gave comparable levels of % astaxanthin recovery, and the solvent giving the lowest astaxanthin recovery was acetonitrile. A common reason for acetone being the most suitable solvent for all methods is due to

its polarity suitable to astaxanthin, which is to be discussed in section 5.2.1. Other reasons for were given in detail in each of the following sections particularly for UAE and MAE since other factor than polarity properties such as vapor pressure and dissipation property of solvent could enhance % astaxanthin recovery in acetone when these methods were employed.

### 5.2.1 Maceration

The determination of the suitable solvent for maceration was conducted at 30°C for 30 min. As shown in Figure 5.1 (a), acetone resulted in the highest astaxanthin recovery, (44±1%), followed by methanol (18±5%), ethanol (17±5%) and acetonitrile (9±1%). Of all the solvents studied, acetone has the lowest polarity, indicated by dielectric constant (Table 5.1), making it the most appropriate solvent to dissolve the fat soluble molecules like astaxanthin. Moreover, the structure of astaxanthin is most similar to that of acetone, in as much as both feature carbonyl groups.

### 5.2.2 Soxhlet extraction

Soxhlet extraction in acetone gave as high as 70±2% astaxanthin recovery (Figure 5.1 (b)), compared with only 44±1% obtained by 30 min maceration at 30°C. The increased extraction efficiency was expected since Soxhlet extraction provides the algae continual contact with fresh solvent. Nevertheless, none of the other solvents showed similar improvement in the astaxanthin recovery. Since Soxhlet extractions must be done at the solvent boiling temperatures (56.5, 64.7, 78.5, and 81.6°C for acetone, methanol, ethanol, and acetonitrile respectively), possible thermal degradation of the astaxanthin in higher boiling point solvents could account for these unimproved recoveries.

### 5.2.3 Ultrasound-assisted extraction (UAE)

The results in Figure 5.1 (c) show the effects of extraction solvents on astaxanthin recovery for UAE carried out at 18.40 watt at 45°C and 60 min. The highest % astaxanthin recovery (73±3%) was again obtained with acetone. It should be noted that, in UAE, acoustic cavitation is an important phenomenon that is responsible for enhanced extraction recovery other than the solvent polarity. The degree of ultrasonic cavitation depends on various thermodynamic properties of the solvent. In solvents

with low vapor pressures, bubble collapses tend to be strong, facilitating the disruption of algal cells and the release of astaxanthin. However, the localized severe high temperatures and pressures could prompt compound degradation and thus have the opposite effect. Less severe bubble collapses in high vapor pressure solvents like acetone or methanol, by contrast, should therefore lead to minimal product degradation. This could explain the high % astaxanthin recovery obtained by UAE when using acetone. Methanol, which also has relatively high vapor pressure, was also observed to give rather high recovery (compared with ethanol) despite the greater differences in polarities and molecular structures between this solvent and the astaxanthin. These results suggest that, in UAE, acoustic cavitation significantly influences the extraction efficiency of the solvents.

Another solvent property generally known to affect the extraction recovery in UAE is surface tension. Specifically, bubble cavitation occurs more readily in solvents with higher surface tension (Zlotorzynski, 1995). In this study however, the effect of solvent surface tension was deemed negligible since the surface tensions of the selected solvents did not differ considerably (Zlotorzynski, 1995) (Table 5.1).

### 5.2.4 Microwave-assisted extraction (MAE)

To determine the effect of solvent type on MAE, the experiments were carried out at 75°C for 5 min. The extraction recoveries obtained with MAE followed the same order as the data obtained with maceration, that is, acetone ($74\pm4\%$) > methanol ($38\pm2\%$) > ethanol ($14\pm4\%$) > acetonitrile ($13\pm2\%$). Nevertheless, the degree of enhancement of astaxanthin recovery in methanol and acetone was higher than the other solvents. In general, MAE enhances the extraction capability of solvents because the electromagnetic field causes rapid heating of the solvent, the rate of which depends on a parameter called the dissipation factor (tan $\delta$), defined as follows.

$$\tan \delta = \frac{\varepsilon^{''}}{\varepsilon^{'}} \qquad (5.1)$$

where $\varepsilon'$ is the dielectric constant or relative permittivity and $\varepsilon''$ is the dielectric loss factor. $\varepsilon'$ describes the polarizability of the solvent molecule in an electric field, a measure of the ability of the solvent to store electromagnetic radiation. $\varepsilon''$ is a measure of the efficiency by which the absorbed microwave energy is converted into

heat when an electric field is applied. From this definition, the dissipation factor (tan $\delta$) therefore represents the ability of solvent to absorb the microwave energy and dissipate that energy into heat. The rate of heating under microwave irradiation is generally expected to be high if both the dielectric constant and dissipation factor of the solvent are high. It is likely, therefore, that the enhanced astaxanthin recoveries observed using acetone and methanol under microwave irradiation were due to the relatively high $\varepsilon'$ and $\varepsilon''$ values of these solvents (Zlotorzynski, 1995) (Table 2.3). By comparison, ethanol and acetronitrile are characterized by low dielectric loss factors, which explains their poorer uptake of algal astaxanthin.

In short, since acetone consistently gave the highest astaxanthin recovery, it was chosen for subsequent optimization studies.

## 5.3 Suitable extraction conditions

### 5.3.1 Maceration

The effects of time and temperature on maceration (Figure 5.2) indicate that for all extraction temperatures the astaxanthin recovery was rapid and essentially complete after 5 min. The high initial astaxanthin extraction rate was due to the high driving force of astaxanthin mass transfer between inside and outside the algal cell. Beyond this time, the rate of further astaxanthin extraction appears to drop markedly. The highest astaxanthin recovery (57 ± 4%) was obtained after 15 min at 45°C, whereas lower recoveries were observed beyond 60 min at 60°C. Higher extraction temperatures should increase astaxanthin solubility and decrease solvent viscosity, thereby increasing astaxanthin recovery. However, prolonged exposure to high temperatures could also lead to compound degradation, accounting for our observed drop in astaxanthin levels.

### 5.3.2 Ultrasound-assisted extraction (UAE)

For UAE, all extraction temperatures showed rapid astaxanthin recovery (up to 41±2 %) within the first 5 min, and thereafter, between 5 to 30 min, increasing only gradually (Figure 5.3). It should be noted that, despite the cavitation effect of UAE, astaxanthin recovery did not increase significantly, compared with maceration, for the first 30 min. This could be due to the fact that ultrasonic cavitation gives rise to localized hot spots that destroy astaxanthin molecules (Zhao et al., 2006, Toma et al.,

2001). After 30 min, however, the extraction recovery increased again until 60 min to around 70±3%. Following this maximum, the astaxanthin level finally dropped as the extraction time approached 90 min. The maximum astaxanthin recovery for UAE was found at the extraction temperature of 45°C and 60 min.

### 5.3.3 Microwave-assisted extraction (MAE)

In a closed, microwave-irradiated system, astaxanthin recovery was seen to increase instantly in the first 5 min at all extraction temperatures (Figure 5.4). For extraction temperatures of 30, 45, and 60°C, astaxanthin recovery remained relatively constant over time, whereas for extraction temperatures of 70 and 75°C, the highest % astaxanthin recovery was again reached at 5 min, immediately dropping thereafter. The highest recovery (74±4%) was obtained with MAE at 75°C after 5 min. The increase in temperature to 78°C did not further increase the astaxanthin recovery, possibly due to its structural decomposition (Zhao et al., 2006).

### 5.4 Comparison among extraction methods

The % astaxanthin recovery obtained by different methods at the most suitable conditions are summarized in Table 5.2. The highest % astaxanthin recovery (74±4%) was obtained by 5 min MAE at 75°C, followed by UAE at 45 °C (73±3%). The closed system used for MAE allowed high extraction temperature (above the boiling point of acetone), and because of rapid heating caused by microwave irradiation, shorter extraction time is required. Brief extraction times are considered favorable since they are expected to minimize compound degradation. Although comparable astaxanthin recovery could be achieved with UAE, it required much longer extraction time (60 min). When compared with the extraction recoveries by other nonconventional methods such as supercritical carbon dioxide extraction (60−83%) (Machmudah et al., 2006, Krichnavaruk, et al., 2008 and Thana et al., 2008) MAE achieves equally impressive recoveries but in a much shorter extraction time. Despite an enhanced recovery, careful considerations must be made when employing MAE and UAE as compound degradation can easily occur, either due to localized hot spots in UAE or by the elevated temperatures of MAE. The results of this study nevertheless are in good agreement with the detailed optimization study of MAE reported by Zhao et al.

(2009), thus supporting the potential use of MAE for astaxanthin recovery from *H. pluvialis* (Zhao et al., 2009).

Table 5.1 Properties of solvents used for extraction (Zlotorzynski, 1995)

| Type of solvents | Dielectric constant (D) | Surface tension (mN/cm) | Vapor pressure (mmHg) | Viscosity (cP) |
|---|---|---|---|---|
| Methanol | 32.6 | 22.6 | 127.05 | 0.6 |
| Ethanol | 24.6 | 23.7 | 59.02 | 1.2 |
| Acetonitrile | 37.5 | 19.1 | 88.47 | 0.38 |
| Acetone | 20.7 | 23.7 | 229.52 | 0.32 |

*Determined at 20˚C

Table 5.2 Comparison of astaxanthin recoveries obtained with various extraction methods

| Methods | Temperature (°C) | Time (min) | % astaxanthin recovery |
|---------|------------------|------------|------------------------|
| Maceration | 45 | 15 | 56.84±4.10 |
| Soxhlet | 56.5 | 4 hour | 70.11±1.65 |
| UAE | 45 | 60 | 73.33±2.89 |
| MAE | 75 | 5 | 74.32±3.83 |
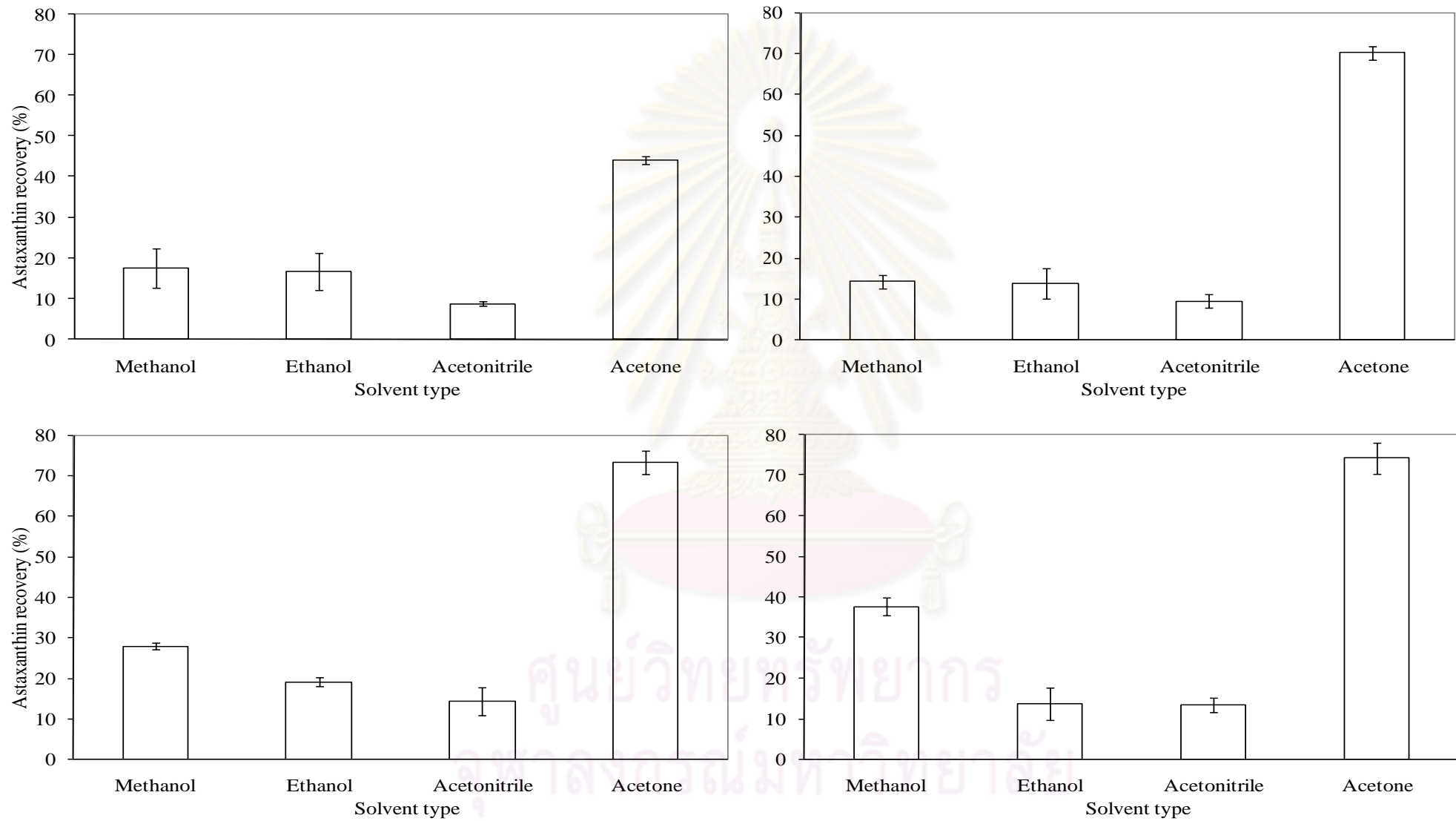
Figure 5.1 Effect of solvent type on astaxanthin extraction (a) maceration at 30°C, 30 min (b) Soxhlet extraction for 4 h (c) UAE at 45°C, 60 min, 18.40 W (d) MAE at 75°C, 5 min, power 720 W
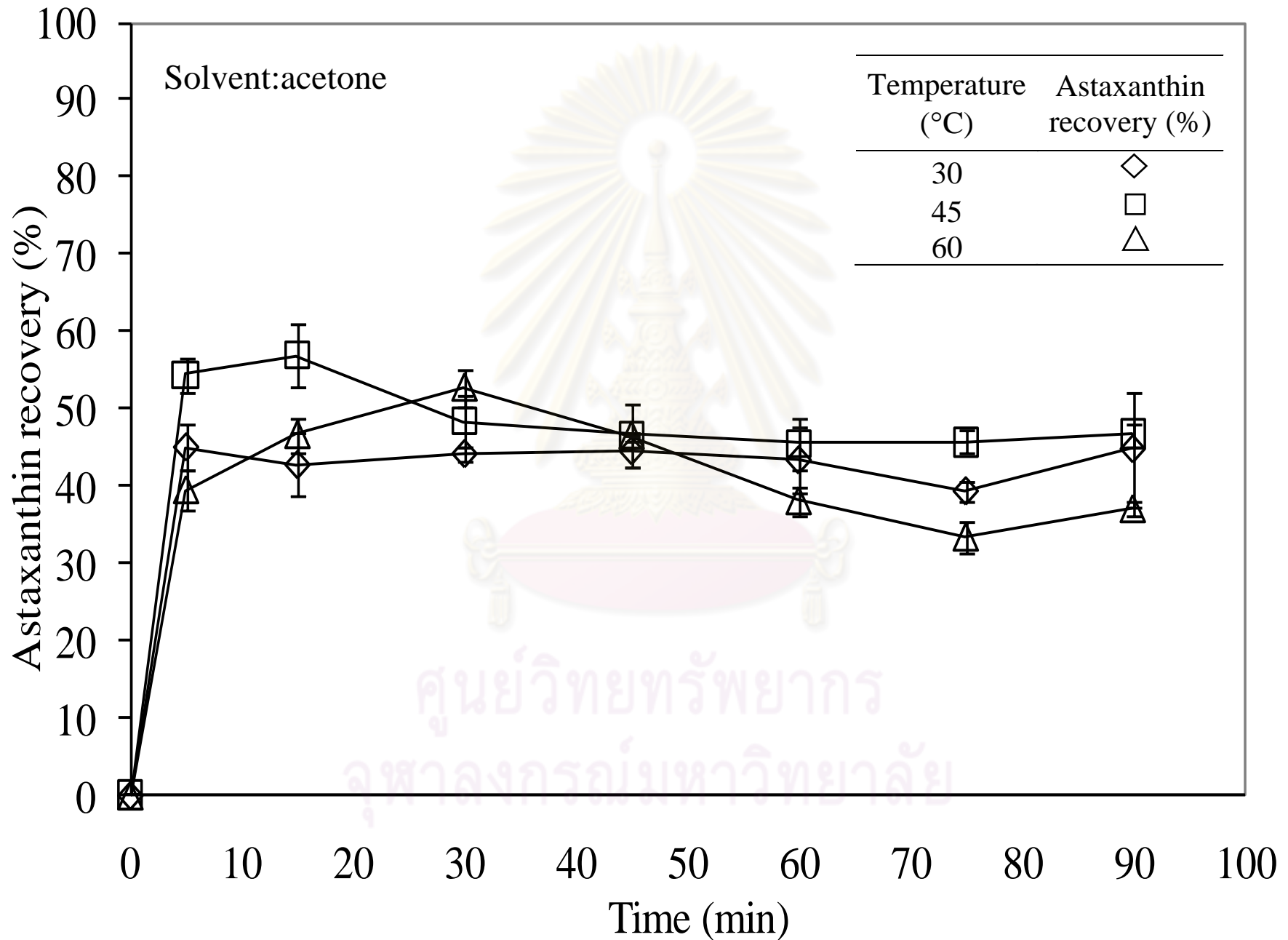
Figure 5.2 Effect of time and temperature on astaxanthin recovery by maceration
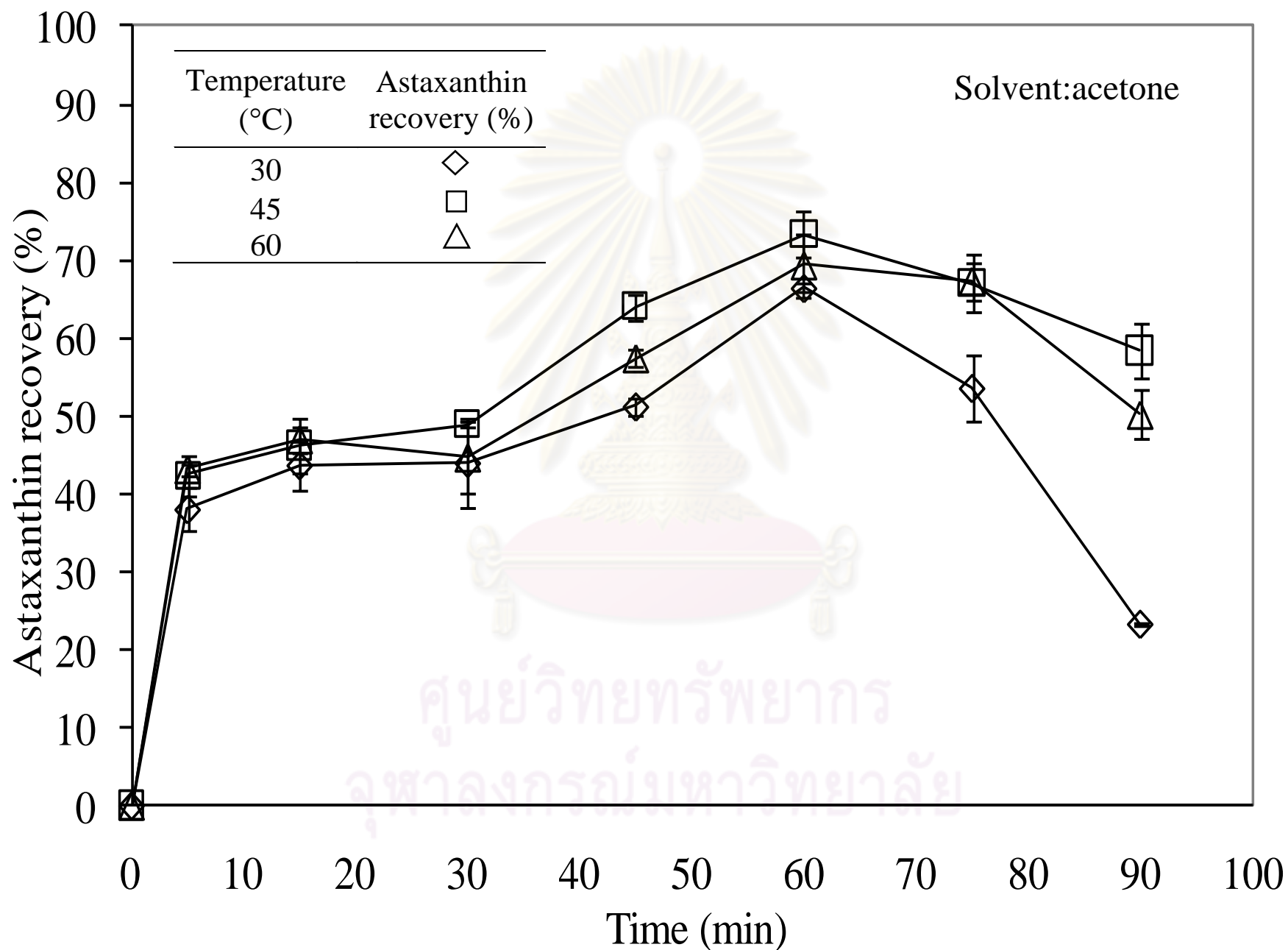
Figure 5.3 Effect of time and temperature on astaxanthin recovery by UAE (power 18.40 W)
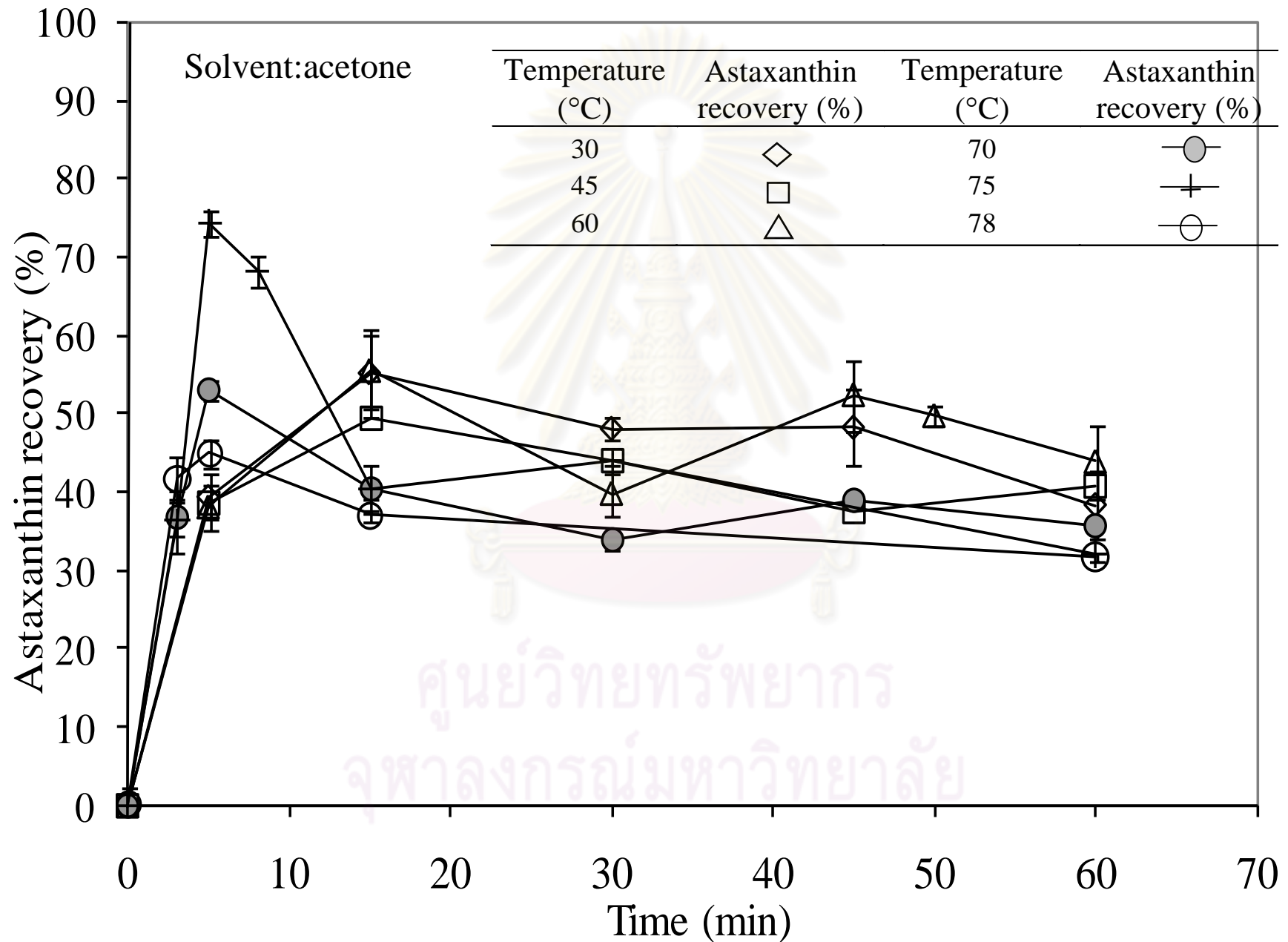
Figure 5.3 Effect of time and temperature on astaxanthin recovery by MAE (power 720 W)

# CHAPTER VI

## CONCLUSIONS AND RECOMMENDATIONS

### 6.1 Summary, achievements and contributions

This work evaluated the solubility of astaxanthin in supercritical carbon dioxide over temperature and pressure range of 40 to 80°C and 20 to 40 MPa. In addition, three different solubility models were fitted to the experimental data. In the temperature and pressure range of this study as summarized in Table 6.1, the solubility of astaxanthin was found to be in the range of $1.65 \times 10^{-7}$ to $89.96 \times 10^{-7}$ (mole solute/mole solvent). The solubility increased with temperature and pressure owing to the changes in density of $CO_2$ and sublimation vapor pressure of solute. Comparison among three candidate models, i.e. Chrastil's model, solubility by equation of state and linear correlation between enhancement factor and density, shows that solubility data by equation of state offered the best fit with experimental solubility data. This model was then used to evaluate the extraction efficiency of the astaxanthin extraction with supercritical $CO_2$ (Section 4.6) where it was illustrated that the extraction process was not confined by solubility at all pressures, particularly at low pressure (20 MPa). As displayed in Table 6.2, the extraction efficiency seemed to achieve the highest level of only around 29.36% at 30 MPa. Thus the solubility data indicated that extraction generally carried out as mass transfer liming process. It is this mass transfer limitation that should be of concern to improve the overall extraction efficiency for supercritical $CO_2$ extraction.

Due primarily to the unattractive time consuming operation and high energy consumption as well as the mass transfer limitation as already mentioned above, the $SCCO_2$ extraction process might not practical at this stage of development for extraction of astaxanthin from *H. pluvialis*. Other alternative assisting methods were therefore proposed such as ultrasound-assisted and microwave-assisted extractions. Solvent selection was a significant procedure in organic extraction. Acetone exhibited the highest efficiency by providing the highest yield of astaxanthin in the extract. Among all the extraction methods investigated in this work (i.e. ultrasound-assisted extraction, microwave-assisted extraction and conventional maceration and soxhlet methods), the microwave-assisted extraction method at 75°C could achieve the

highest astaxanthin content at around 74.32% in the extract within the first 5 minutes of extraction, such results are shown in Table 6.3.

The discovery about astaxanthin extraction from *H. pluvialis* in this dissertation greatly contributed to classical knowledge which facilitates modern extraction equipment design.

Table 6.1 Solubility of astaxanthin in $SCCO_2$

| Temperature (°C) | Solubility$\times 10^{-7}$ (mole solute/mole solvent) | | |
|---|---|---|---|
| Pressure (MPa) | 20 | 30 | 40 |
| 40 | 1.65 | 9.56 | 21.70 |
| 60 | 2.98 | 25.24 | 66.49 |
| 80 | 3.50 | 39.76 | 89.96 |

Table 6.2 %Extraction efficiency compared with Machmudah et al., 2006

| Pressure (MPa) | % Extraction Efficiency |
|---|---|
| 40 | 21.76 |
| 30 | 29.36 |
| 20 | 0.41 |

Table 6.3 Comparison of astaxanthin recoveries obtained with various extraction methods.

| Methods | Temperature (°C) | Time (min) | % astaxanthin recovery |
|---|---|---|---|
| Maceration | 45 | 15 | 56.84±4.10 |
| Soxhlet | 56.5 | 4 hours | 70.11±1.65 |
| UAE | 45 | 60 | 73.33±2.89 |
| MAE | 75 | 5 | 74.32±3.83 |

**6.2 Suggestions and recommendations**

The solubility model of astaxanthin in supercritical carbon dioxide could have great merit to the design of astaxanthin extraction by such process. The work of astaxanthin extraction by accelerated methods such as ultrasound-assisted extraction and microwave-assisted extraction is relatively new and catching a large content of research interest as the alternative extraction methods seem to be able to lower the extraction cost with minimal requirements of additional chemicals.

Further research focuses arose during the course of this research is substantial and those which are mostly concerned with this work are listed below.

1) The assisting extraction methods employed in this work using either microwave and ultrasound could bring about the concern regarding the degradation of astaxanthin as they could generate high localized hot spots as generally reported in UAE, and elevated temperature as in MAE. Hence, the test for the antioxidant activity in the extract at different extracting conditions would be necessary as the quantity of antioxidant compound extracted would have no meaning if it does not possess the antioxidant properties. The antioxidant activities of the extracts obtained with assisted solvent extraction methods should also be compared with those obtained with supercritical carbon dioxide extraction. This is the only way to know if the extraction condition really deteriorates the quality of the product.

2) Co-solvent could be used in supercritical carbon dioxide extraction to obtain higher extraction yield. From experimental results from the second part of the research, acetone was found to be the most suitable solvent for astaxanthin extraction, therefore it would thus be interesting if further experimentation could be conducted to determine the potential use of acetone as a cosolvent. Moreover, the examination of astaxanthin solubility of in supercritical carbon dioxide with various cosolvents might be further conducted.

3) The solubility of astaxanthin in the various solvents used in the solvent extraction processes should be examined. This will provide the extent of the extraction efficiency and suggest the potential improvement of the techniques.

# REFERENCES

[1]    Abdolmajid L., and Choul-Gyun L., 2006. Simultaneous Measurement of
       Chlorophyll and Astaxanthin in *Haematococcus pluvialis* Cells by First
       Order Derivative Ultraviolet-Visible Spectrophotometry. <u>Journal of
       Bioscience and Bioengineering</u> 101: 104-110.

[2]    Ajchariyapagorn A., Douglas L. P., Douglas S., Pongamphai S., and Teppaitoon
       W., 2008. Prediction of Solubility of Solid Biomolecules in Supercritical
       Solvents using Group Contribution Methods and Equations of State.
       <u>American Journal of Food Technology</u> 3: 275-293.

[3]    Anton Paar [Online]. Available from: http://<u>www.anton-paar.com</u> [2009,
       January 28]

[4]    Barbero G. F., Palma M., and Barroso C. G., 2006. Determination of
       Capsaicinoids in Peppers by Microwave-assisted Extraction–high-
       performance Liquid Chromatography with Fluorescence Detection.
       <u>Analytica Chimica Acta</u>
       578: 227–233.

[5]    Bjerkeng B., FØlling M., Lagocki S., Storebakken T., Olli J. J., Alsted N., 1997.
       Bioavailability of all-E-astaxanthin and Z-isomers of astaxanthin in rainbow
       trout (*Oncorhynchus mykiss*). <u>Aquaculture</u> 157: 63-82.

[6]    Brunner G., 1994. <u>Gas Extraction</u>. Steinkopff Darmstadt Springer, New York
       USA.

[7]    Careri M., Furlattini L., Mangia A., Musci M., Anklam E., Theobald A., and von
       Holst C., 2001. Supercritical Fluid Extraction for Liquid Chromatographic
       Determination of Carotenoids in *Spirulina* Pacifica algae: a chemometric
       approach. <u>Journal of Chromatography A</u> 912: 61–71.

[8]    Cathy E. F., Jean-Stéphane C., and Alain M., 1999. Extractive Fermentation of
       Aroma with Supercritical $CO_2$. <u>Biotechnology and Bioengineering</u> 64: 392-
       400.

[9]    Chen L., Jin H., Ding L., Zhang H., Li J., Qu C., and Zhang H., 2008. Dynamic
       Microwave-assisted Extraction of Flavonoids from *Herba Epimedii*.
       <u>Separation and Purification Technology 59</u>: 50–57.

[10]   Chen Y., Xie M. Y., and Gong X. F., 2007. Microwave-assisted Extraction used

83

for the Isolation of total Triterpenoid Aaponins from Ganoderma atrum. Journal of Food Engineering 81: 162–170.

[11] Choi Seok-Keun, Jeong-Hwan K., Young-Sam P., Young-Jin K., and Hyo-Ihl C., 2007. An Efficiency Method for the Extraction of Astaxanthin from the Red Yest *Xanthophyllomyces dendrorhous*. Journal of Microbiology and Biotechnology 17: 847-852.

[12] Cocero M.J., González S., Pérez S., and Alonso E., 2000. Supercritical Extraction of unsaturated Products. Degradation of β-carotene in Supercritical Extraction Processes. The Journal of Supercritical Fluids 19: 39–44.

[13] de França L.F., Reber G.; Meireles M.A. A., Machado, N. T., Brunner G., 1999. Supercritical Extraction of Carotenoids and Lipids from Buriti (*Maura flexuosa*), a Fruit from the Amazon Region. The Journal of Supercritical Fluids 14: 247–256.

[14] de la Fuente J. C., Quezada N., del Valle J. M., 2005. Solubility of boldo Leaf Antioxidant Components (Boldine) in High-pressure Carbon Dioxide. Fluid Phase Equilibria 235: 196–200.

[15] de la Fuente J. C., Oyarzún B., Quezada N., del Valle J. M., 2006. Solubility of Carotenoid Pigments (lycopene and astaxanthin) in Supercritical Carbon Dioxide. Fluid Phase Equilibria 247: 90–95.

[16] Danielski L., Campos M. A. S. L., Bresciani F. V. L., Hense H., Yunes A. R., and Ferreira R. S. S., 2007. Marigold (*Calendula officinalis* L.) oleoresin: Solubility in SC-$CO_2$ and composite profile. Chemical Engineering and Processing 46: 99-106.

[17] de la Fuente J. C., Valderrama J., and Bottini S. B., and del Valle J. M., 2005. Measurement and Modeling of Solubilities of Capsaicin in High-pressure $CO_2$. Journal of Supercritical Fluids 34: 195–201.

[18] Denery J. R., Dragull K., Tang C. S., and Li Q. X., 2004. Pressurized Fluid Extraction of Carotenoids from *Haematococcus pluvialis* and *Dunaliella salina* and Kavalactones from *Piper methysticum*. Analytica Chimica Acta 501: 175–181.

[19] Eric J. A., and Gil-Hwan A., 1991. Astaxanthin from Microbial Sources. Critical Reviews in Biotechnology 11(4): 297-326.

[20] Eskilsson C. S., and Bjőrklund E., 2000. Reviews: Analytical-scale Microwave-assisted Extraction. Journal of Chromatography A 902: 227–250.

[21] Félix-Valenzuelia L., Higuera-Ciapara H., and Goycoolea-Valencia F., 2001. Supercritical $CO_2$/Ethanol Extraction of Astaxanthin from Blue Crab (*Callinectes Sapidus*) Shell Waste. Journal of Food Process Engineering 24: 101-1 12.

[22] Fulzele D. P., and Satdive R. K., 2005. Comparison of Techniques for the Extraction of the Anti-cancer Drug Camptothecin from *Nothapodytes foetida*. Journal of Chromatography A 1063: 9–13.

[23] Gio-Bin L., Sang-Yun L., Eun-Kyu L., Seung-Joo H., and Woo-Sik K., 2002. Separation of Astaxanthin from Red Yeast *Phaffia rhodozyma* by Supercritical Carbon Dioxide Extraction. Biochemical Engineering Journal 11: 181–187.

[24] Grewe C., Menge S., and Griehl C., 2007. Enantioselective Separation of all *E*-Astaxanthin and its Determination in Microbial Sources. Journal of Chromatography A 1166: 97–100.

[25] Grung M., D'Souza F. M. L., Borowitzka M., and Liaaen-Jensen S., 1992. Algal Carotenoids 51. Secondary Carotenoids 2. *Haematococcus pluvialis* Aplanospores as a Source of (3S, 3'S)-astaxanthin Esters. Journal of Applied Phycology 4: 165-171.

[26] Guerin M., Huntley M. E., and Olaizola M., 2003. *Haematococcus* Astaxanthin: Applications for Human Health and Nutrition. Trends in Biotechnology 21: 210-216.

[27] Guo Z., Jin Q., Fan G., Duanb Y., Qin C., and Wen, M., 2001. Microwave assisted Extraction of Effective Constituents from a Chinese Herbal Medicine Radix Puerariae. Analytica Chimica Acta 436: 41–47.

[28] Gupta B. R., and Jae-Jin Shim, 2007. Solubility in Supercritical Carbon Dioxide. CRC Press Taylor&Francis Group, London.

[29] Güçlü-Üstündağ Ö, and Temelli F., 2004. Correlating the solubility behavior of minor lipid components in supercritical carbon dioxide. Journal of Supercritical Fluids 31: 235–253.

[30] Handayani A. D., Sutrisno, Indraswati N., and Ismadji S., 2008. Extraction of

Astaxanthin from Giant tiger (Panaeus monodon) Shrimp Waste using Palm oil: Studies of Extraction Kinetics and Thermodynamic. <u>Bioresource Technology</u> 99: 4414–4419.

[31] Hansen B.N.; Harvey A.H.; Coelho J.A.P.; Palavra A.M.F.; and Bruno, T.J., 2001. Solubility of Capsaicin and β-carotene in Supercritical Carbon Dioxide and in Halocarbons. <u>Journal of Chemical and Engineering Data</u> 46: 1054–1058.

[32] Hao J. U., Han W., Huang S. D., Xue B. Y., and Deng X., 2002. Microwave-assisted Extraction of Artemisinin from *Artemisia annua* L. <u>Separation and Purification Technology</u> 28: 191–196.

[33] Hata N., Ogbonna J. C., Hasegawa Y., Taroda H., Tanaka H., 2001. Production of Astaxanthin by *Haematococcus pluvialis* in a Sequential Heterotrophic-photoautotrophic culture. <u>Journal of Applied Phycology</u> 13: 395–402.

[34] Hemwimon S., Pavasant P., and Shotipruk, A., 2007. Microwave-assisted Extraction of Antioxidative Anthraquinones from Roots of *Morinda citrifolia*. <u>Separation and Purification Technology</u> 54: 44-50.

[35] Herrero M., Jaime L., Martián-Álvarez P. J., Alejandro C., and Ibáñes, 2006. Optimization of the Extraction of Antioxidants from *Dunaliella salina* Microalga by Pressurized Liquids. <u>Journal of Agricultural of Agricultural and Food Chemistry</u> 54, 5597-5603.

[36] Hemwimon S., Pavasant P., Shotipruk A., 2006. Ultrasound-assisted extraction of anthraquinones from roots of *Morinda citrifolia*. <u>Ultrasonics Sonochemistry</u> 13: 543–548.

[37] Hemwimon S., Pavasant P., and Shotipruk A., 2007. Microwave-assisted Extraction of Antioxidative Anthraquinones from Roots of *Morinda citrifolia*. <u>Separation and Purification Technology</u> 54: 44–50.

[38] Hua-Bin L., and Feng C., 2001. Preparative Isolation and Purification of Astaxanthin froom the Microalga *Chlorococcum sp.* by High-speed counter-current Chromatography. <u>Journal of Chromatography A</u> 925: 133–137.

[39] Hromádková Z., Ebringerová A., and Valachovič P., 1999. Comparison of Classical and Ultrasound-assisted Extraction of Polysaccharides from *Salvia officinalis* L. <u>Ultrasonics Sonochemistry</u> 5: 163–168.

[40] Hromádková Z., and Ebringerová A., 2003. Ultrasonic Extraction of Plant Materials–investigation of Hemicellulose Release from Buckwheat Hulls. Ultrasonics Sonochemistry 10: 127–133.

[41] Huang-Chung W., Chao-Ruey C., and Chiehming J. C., 2001. Analytical, Nutritional and Clinical Methods Section, Carbon dioxide Extraction of Ginseng Root hair Oil and Ginsenosides. Food Chemistry 72: 505-509.

[42] Hyun-Seok Youn, Myong-Kyun Roh, Andreas W., Gordon T. W., and Byung Soo C., 2007. Solubility of Astaxanthin in Supercritical Carbon Dioxide. Korean Journal of Chemical Engineering 24(5): 831-834.

[43] Issarapayup K., 2007. Flat Airlift Photobioreactor for Cultivation of Vegetative Cells of Microalga *Haematococcus pluvialis*. Master᾽s Thesis, Department of Chemical Engineering, Graduate School, Chulalongkorn University.

[44] Jian-Ping Y., and Feng C., 1997. Identification of astaxanthin isomers in *Haematococcus lacustris* by HPLC-photodiode array detection. Biotechnology Techniques 11: 455–459.

[45] Jian-Ping Y., and Feng C., 1999. Hydrolysis Kinetics of Astaxanthin Esters and Stability of Astaxanthin of *Haematococcus pluvialis* during Saponification. Journal of Agricultural and Food Chemistry 47: 31-35.

[46] Jianyong W., Lidong L., and Foo-tim C., 2001. Ultrasound-assisted Extraction of Ginseng Saponins from Ginseng Roots and Cultured Ginseng Cells. Ultrasonics Sonochemistry 8: 347–352.

[47] Johannsen M., and Brunner G., 1997. Solubilities of the Fat-Soluble Vitamins A, D, E, and K in Supercritical Carbon Dioxide. Journal of Chemical and Engineering Data 42, 106–111.

[48] Jyonouchi H., Sun S., Iijima K., and Gross M. D., 2000. Antitumor Activity of Astaxanthin and Its Mode of Action. Nutrition and Cancer 36(1), 59–65.

[49] Kang C. D., and Sim S. J., 2008. Direct Extraction of Astaxanthin from *Haematococcus* Culture using Vegetable Oils. Biotechnology Letters 30: 441–444.

[50] Kang C. D., and Sim S. J., 2007. Selective Extraction of Free Astaxanthin from *Haematococcus* Culture Using a Tandem Organic Solvent System. Biotechnology Progress 23: 866-871.

[51] Knez Ž., and Steiner R., 1992. Solubility of Capsaicin in Dense $CO_2$. The

Journal of Supercritical Fluids 5: 251-255.

[52] Kobayashi M., Kurimura Y., Sakamoto Y., and Tsuji Y., 1997. Selective Extraction of Astaxanthin and Chlorophyll from the Green Alga *Haematococcus pluvialis*. Biotechnology Techniques 11: 657–660.

[53] Kobayashi M., 2003. Astaxanthin Biosynthesis Enhanced by Reactive Oxygen Species in the Green Alga *Haematococcus pluvialis*. Biotechnology and Bioprocess Engineering 8: 322-330.

[54] Krichnavaruk S., Shotipruk A., Goto M., and Pavasant P., 2008. Supercritical Carbon Dioxide Extraction of Astaxanthin from *Haematococcus pluvialis* with Vegetable Oils as co-Solvent. Bioresource Technology 99: 5556–5560.

[55] Li H., Chen B., Zhang Z., and Yao, S., 2004. Focused Microwave-assisted Solvent Extraction and HPLC Determination of Effective Constituents in Eucommia ulmodies Oliv (*E. ulmodies*). Talanta 63: 659–665.

[56] Lorenz R. T., and Cysewski G. R., 2000. Commercial Potential for *Haematococcus* Microalgae as a natural Source of Astaxanthin. TIBTECH 18: 160-167.

[57] López M., Arce L., Garrido J., Ríos A., and Valcárcel M., 2004. Selective Extraction of Astaxanthin from Crustaceans by use of Supercritical Carbon Dioxide. Talanta 64: 726–731

[58] Lyman J. W., Reehl F. W., and Rosenblatt H. D., 1982. Handbook of Chemical Property Estimation Methods. McGraw-Hill Book Company: New York.

[59] Machmudah S., Shotipruk A., Goto M, Sasaki M., and Hirose T., 2006. Extraction of Astaxanthin from *Haematococcus pluvialis* Using Supercritical $CO_2$ and Ethanol as Entrainer. Industrial & Engineering Chemistry Research 45, 3652-3657.

[60] Mao Y., Li Y., and Yao N., 2007 Simultaneous Determination of Dalidroside and Tyrosol in Extracts of *Rhodiola* L. by Microwave assisted Extraction and High-performance Liquid Chromatography. Journal of Pharmaceutical and Biomedical Analysis 45: 510–515.

[61] Margalith P. Z., 1999. Production of Ketocarotenoids by Microalgae. Applied Microbiology and Biotechnology 51: 431-438.

[62] Martino E., Ramaiola I., Urbano M., Bracco F., and Collina S., 2006.

Microwave-assisted Extraction of Coumarin and related Compounds from *Melilotus officinalis* (L.) Pallas as an Alternative to Soxhlet and Ultrasound-assisted extraction. Journal of Chromatography A 1125: 147–151.

[63]    Mason T. J., and Lormier J. P., 1988. Sonochemistry: Theory, Applications and uses of Ultrasound in Chemistry. Ellis Horwood Limited, Chichester.

[64]    Matsuyama K., Mishima K., Taruta Y., Ezawa M., Nagatani M., and Park P. W., 1998. Extraction of Astaxanthin from Silver Nitrate Solution using Supercritical Fluid Extraction. Solvent Extraction Research and Development 5: 166-171.

[65]    Mauricio A., Rostagno, Miguel P., and Carmelo G. B., 2007. Microwave assisted Extraction of Soy Isoflavones. Analytica Chimica Acta 588: 274–282.

[66]    McHugh M. A. and Krukonis V. J, 1994. Supercritical Fluid Extraction. Butterworth-Heinemann, United States of America.

[67]    Mendes R. L., Fernandes H. L., Coelbo J. P., Reis E. C., Cabral J. M. S., Novais J. M., and Palavra A. F., 1995. Supercritical $CO_2$ Extraction of Carotenoids and other Lipids from *Chlorella vulgaris*. Food chemistry 53: 99-103.

[68]    Mendes-Pinto M. M., Raposo M. F. J., Bowen J., Young A. J., and Morais R., 2001. Evaluation of different Cell Disruption Processes on Encysted Cells of *Haematococcus pluvialis:* Effects on Astaxanthin Recovery and implications for Bio-availability. Journal of Applied Phycology 13: 19–24.

[69]    Miller N. J., Sampson J., Candeias L. P., Bramley P. M., and Rice-Evans C. A., 1996. Antioxidant Activities of Carotenes and Xanthophylls. FEBS Letters 384: 240-242.

[70]    Mukhopadhyay M., 2000. Natural Extracts Using Supercritical Carbon dioxide, CRS Press, United States of America.

[71]    Murga R., Sanz T. M., Beltrán S., Cabezas L. J., 2002 Solubility of some Phenolic Compounds cantained in Grape seeds, in Supercritical Carbon Dioxide. Journal of Supercritical Fluids 23, 113-121.

[72]    Naguib Y. M. A., 2000. Antioxidant Activities of Astaxanthin and Related Carotenoids. Journal of Agricultural and Food Chemistry 48: 1150-1154.

[73]   Nieuwerburgh L., Wänstrand I., Liu J., and Snoeijs P., 2005. Astaxanthin Production in Marine Pelagic Copepods Grazing on two Different Phytoplankton Diets. <u>Journal of Sea Research</u> 53: 147– 160.

[74]   Nobre B., Marcelo F., Passos R., Beirão L., Palavra A., Gouveia L., and Mendes R., 2006. Supercritical Carbon Dioxide Extraction of Astaxanthin and other Carotenoids from the Microalga *Haematococcus pluvialis*. <u>European Food Research and Technology</u> 223: 787–790.

[75]   Ohgami K., Shiratori K., Kotake S., Nishida T., Mizuki N., Yazaw K., and Ohno S., 2003. Effects of Astaxanthin on Lipopolysaccharide-Induced Inflammation In Vitro and In Vivo. <u>Investigative Ophthalmology & Visual Science</u> 44: 2694-2701.

[76]   Oufnac D. S., 2006. <u>Determination of Antioxidant of Antioxidant Capacity in Corn Germ, Wheat Germ and Wheat Bran using Solvent and Microwave-assisted Solvent Extraction.</u> Master's Thesis, Department of Food Science, Graduate School, Nicholls State University.

[77]   Palma M., and Taylor L. T., 1999. Extraction of Polyphenolic Compounds from Grape Seeds with near Critical Carbon Dioxide. <u>Journal of Chromatography A</u> 89: 117-124.

[78]   Palozza P., and Krinsky N. I., 1992. Astaxanthin and Canthaxanthin are potent Antioxidants in a Membrane Model. <u>Archives of Biochemistry and Biophysics</u> 297: 291-295.

[79]   Pan X., Liu H., Jia G., and Shu Y. Y., 2000. Microwave-assisted Extraction of Glycyrrhizic Acid from Licorice Root. <u>Biochemical Engineering Journal</u> 5: 173–177.

[80]   Pan X., Niu, G., and Liu H., 2001. Microwave-assisted Extraction of Tanshinones from *Salvia miltiorrhiza bunge* with Analysis by high-performance Liquid Chromatography. <u>Journal of Chromatography A</u> 922: 371–375.

[81]   Pan X., Niu G., and Liu H., 2002. Comparison of Microwave-assisted Extraction and Conventional Extraction Techniques for the Extraction of Tanshinones from *Salvia miltiorrhiza bunge*. <u>Biochemical Engineering Journal</u> 12: 71–77.

[82]   Poling E. B., Prausnitz M. J., and O'connell P. J., 2001 <u>The Properties of Gases</u>

and Liquids McGrawHill, London.

[83] Prausnitz M. J., Lichtenthaler N. R., and de Azevedo E.G., 1986 Molecular Thermodynamics of fluid-phase equilibria. Prentice-Hall, Englewood cliffs, New Jersey.

[84] Ranjbar R., Inoue R., Shiraishi H., Katsuda T., and Katoh S., 2007. High Efficiency Production of Astaxanthin by Autotrophic Cultivation of *Haematococcus pluvialis* in a Bubble Column Photobioreactor. Journal of Biochemical Engineering 39: 575–580.

[85] Reid C. R., Prausnitz M. J., and Poling E. B., 1987. The Properties of Gases and Liquids. McGraw-Hill, New York.

[86] Rostagno M. A., Palma M., and Barroso C. G., 2003. Ultrasound-assisted Extraction of Soy Isoflavones. Journal of Chromatography A 1012: 119– 128.

[87] Roy C. Bhupesh, Goto M., and Hirose T., 1996. Extraction of Ginger Oil with Supercritical Carbon Dioxide: Experiments and modeling. Industrial & Engineering Chemistry Research 35: 607-612.

[88] Roy C., Goto M., Kodama A., Hirose T., 1996. Supercritical $CO_2$ Extraction of Essential Oils and Cuticular Waxes from Peppermint Leaves. Journal of Chemical Technology Biotechnology 67: 21-26.

[89] Sakaki K., 1992. Solubility of β-Carotene in Dense Carbon Dioxide and Nitrous Oxide from 308 to 323 K and from 9.6 to 30 MPa. Journal of Chemical & Engineering Data 37: 249-251.

[90] Saldaña M. D. A., Sun Li, Guigard S. E., and Temelli F., 2006. Comparison of the Solubility of β-carotene in Supercritical $CO_2$ based on a Binary and a Multicomponent Complex System. Journal of Supercritical Fluids 37: 342– 349.

[91] Sališová M., Toma Š., and Mason T. J., 1997. Comparison of Conventional and Ultrasonically Assisted Extractions of Pharmaceutically Active Compounds from *Salvia officinalis*. Ultrasonics Sonochemistry 4: 131-134.

[92] Sarada R., Vidhyathi R., Usha D., and Ravishankar G. A., 2006. An Efficient Method for Extraction of Astaxanthin from Green Alga *Haematococcus pluvialis*. Journal of Agricultural Food Chemistry 54: 7585–7588.

[93] Sedmak J. J., Weerasinghe D. K., and Jolly S. O., 1990. Extraction and

Quantitation of Astaxanthin from *Phaffia rhodozyma*. Biotechnology Techniques 4: 107-112.

[94] Shi John, Yi Chun, Xue Jun Sophia, Jiang Yueming, Ma Ying, and Li Dong, 2009. Effects of Modifiers on the Profile of Lycopene extracted from Tomato Skins by SupercriticalCO$_2$. Journal of food engineering 93, 431-436.

[95] Shimidzu N., Goto M., and Miki W., 1996. Carotenoids as Singlet Oxygen Quenchers in Marine Organisms. Fisheries Science 62: 134.

[96] Shu Y. Y., Koa Y. M., and Chang Y. S., 2003. Microwave-assisted Extraction of Ginsenosides from Ginseng root. Microchemical Journal 74: 131–139.

[97] Škerget M., Knez Ž., and Habulin M., 1995. Solubility of β-carotene and Oleic acid in Dense CO$_2$ and Data Correlation by a Density Based Model. Fluid Phase Equilibria 109: 131-138.

[98] Škerget M., and Knez Ž., 1997. Solubility of Binary Solid Mixture β-Carotene Capsaicin in Dense CO$_2$. Journal of Agricultural and Food Chemistry 45: 2066-2069.

[99] Sovová H., Stateva P. R., Galushko A., and Anatolii, 2001 Solubility of β Carotene in Supercritical CO$_2$ and the Effect of Entrainers. Journal of Supercritical Fluids 21, 195-203.

[100] Sovová, H., 1994. Rate of the Vegetable Oil Extraction with Supercritical CO$_2$—I. Modelling of Extraction Curves. Chemical Engineering Science 4, 409–414.

[101] Storebakken T., Sørensen M., Bjerkeng B., Harris J., Monahan P., and Hiu S., 2004. Stability of Astaxanthin from Red Yeast, *Xanthophyllomyces dendrorhous*, during Feed Processing: Effects of Enzymatic Cell Wall Disruption and Extrusion Temperature. Aquaculture 231: 489–500.

[102] Subra P., Castellani S., Ksibi H., and Garrabos Y., 1997. Contribution to the Determination of the Solubility of β-carotene in Supercritical Carbon Dioxide and Nitrous oxide: Experimental Data and Modeling. Fluid Phase Equilibria 131: 269-286.

[103] Suh I. S., Joo H. N., and Lee C. G., 2006. A novel Double-layered Photobioreactor for Simultaneous *Haematococcus pluvialis* Cell Growth and Astaxanthin Accumulation. Journal of Biotechnology 125(4): 540-546.

[104]  Sun R. C., Sun X. F., and Ma X. H., 2002. Effect of ultrasound on the Structure and Physiochemical Properties of Organosolv Soluble Hemicelluloses from Wheat Straw. Ultrasonics Sonochemistry 9: 91-101.

[105]  Sun M., Temelli, F., 2006. Supercritical Carbon Dioxide Extraction of Carotenoids from Carrot Using Canola Oil as a Continuous Co-solvent. The Journal of Supercritical Fluids 37, 397–408.

[106]  Thana P., Machmudah S., Goto M., Sasaki M., Pavasant P., and Shotipruk A., 2008. Response Surface Methodology to Supercritical Carbon Dioxide Extraction of Astaxanthin from *Haematococcus pluvialis*. Bioresource Technology 99: 3110–3115.

[107]  Taylor L. T., 1996. Supercritical Fluid Extraction. John Wiley & Sons, Inc., United States of America.

[108]  Toma, M., Vinatoru, M., Paniwnyk, L., and Mason, J.T., 2001. Investigation of the Effects of Ultrasound on Vegetal Tissues Solvent Extraction. Ultrasonics Sonochemistry 8: 137-142.

[109]  Tonthubthimthong P., Chuaprasert S., Douglas P., and Luewisutthichat W., 2001. Supercritical $CO_2$ Extraction of Nimbin from Neem Seeds – an Experimental Study. Journal of Food Engineering 47: 289-293.

[110]  Ozlem G. U., 2003. Supercritical Carbondioxide Fractionation of Liquids: Solubility Behavior to Process Development, Doctor Dissertation of University of Alberta.

[111]  Tzu-Hua W., Jiahn-Haurliao L., Wen-Chi H., Fu-Yung H., Timothy J. M., and Chao-Chien H., 2006. Astaxanthin Protects against Oxidative Stress and Calcium-Induced Porcine Lens Protein Degradation. Journal of Agricultural and Food Chemistry 54: 2418-2423.

[112]  Valderrama J. O., Perrut M., and Majewski W., 2003. Extraction of Astaxantine and Phycocyanine from Microalgae with Supercritical Carbon Dioxide. Journal of Chemical & Engineering Data 48: 827-830.

[113]  Wang Y., You J., Yu Y., Qu C., Zhang H., Ding L., Zhang H., and Li X., 2008. Analysis of Ginsenosides in *Panax Ginseng* in High Pressure Microwave-assisted Extraction. Food Chemistry 110: 161–167.

[114]  Wittayasinthana W., 2007. Microwave-assisted Extraction of Anti-Cance

Damnacanthal from Roots of *Morinda Citrifolia*. Master's Thesis, Department of Chemical Engineering, Graduate School, Chulalongkorn University.

[115]  Zhao L., Chen G., Zhao G., and Hu X., 2009. Optimization of Microwave assisted Extraction of Astaxanthin from *Haematococcus Pluvialis* by Response Surface Methodology and Antioxidant Activities of the Extracts. <u>Separation and Purification Technology</u> 44: 243–262.

[116]  Zhao, L., Zhao, G., Chen, F., Wang, Z., Wu, J., Hu, X. (2006) Different Effects of Microwave and Ultrasound on the Stability of (*all-E*)-astaxanthin. <u>Journal of Africultural and Food Chemistry</u> 54: 8346-8351.

[117]  Zhou H. Y., and Liu C. Z., 2006. Microwave-assisted Extraction of Solanesol from Tobacco Leaves. <u>Journal of Chromatography A</u> 1129: 135–139.

[118]  Zlotorzynski A., 1995. The Application of Microwave Radiation of Analytical and Environmental Chemistry. <u>Critical Reviews in Analytical Chemistry</u> 25: 43–75.

# Appendix A

Table A-1 Experimental variables and ranges studied in solubility of astaxanthin
determination

| Temperature (°C) P (MPa) | 40 | 60 | 80 |
|---|---|---|---|
| 20 | / | / | / |
| 30 | / | / | / |
| 40 | / | / | / |

Table A-2 Experimental variables and ranges studied in astaxanthin extraction from
*Haematococcus pluvialis*

| Methods | Temperature (°C) | Time (min) |
|---|---|---|
| Maceration | 30, 45 and 60 | 0, 5, 15, 30, 45, 60, 75 and 90 |
| Soxhlet | Boiling point of solvent | 240 |
| Ultrasonic | 30, 45 and 60 | 0, 5, 15, 30, 45, 60, 75 and 90 |
| Microwave | 30, 45, 60, 75 and 78 | 0, 5, 15, 30, 45 and 60 |

# Appendix B

Table B-1 %Astaxanthin recovery obtain by maceration method

| Temperature (°C) Time (min) | 30 | 45 | 60 |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 5 | 45.00 | 54.33 | 39.45 |
| 15 | 42.73 | 56.85 | 46.56 |
| 30 | 44.14 | 48.39 | 52.66 |
| 45 | 44.52 | 46.59 | 46.48 |
| 60 | 43.33 | 45.54 | 38.03 |
| 75 | 39.33 | 45.67 | 33.35 |
| 90 | 44.78 | 46.67 | 37.01 |

Table B-2 %Astaxanthin recovery obtain by ultrasound-assisted extraction method

| Temperature (°C) Time (min) | 30 | 45 | 60 |
|---|---|---|---|
| 0 | 0.00 | 0.00 | 0.00 |
| 5 | 38.00 | 42.33 | 43.17 |
| 15 | 43.67 | 46.21 | 47.00 |
| 30 | 43.96 | 48.91 | 44.68 |
| 45 | 51.19 | 64.00 | 57.41 |
| 60 | 66.38 | 73.33 | 69.33 |
| 75 | 53.55 | 67.00 | 67.33 |
| 90 | 23.30 | 58.33 | 50.16 |

Table B-3 %Astaxanthin recovery obtain by microwave-assisted extraction

maceration method

| Temperature (°C) Time (min) | 30 | 45 | 60 | 70 | 75 | 78 |
|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 39.33 | 38.36 | 38.00 | 53.02 | 74.32 | 44.89 |
| 15 | 55.08 | 49.25 | 55.35 | 40.32 | 40.18 | 37.00 |
| 30 | 48.02 | 43.80 | 39.72 | 33.75 | 43.87 | - |
| 45 | 48.19 | 37.31 | 52.26 | 39.00 | 39.03 | - |
| 60 | 38.32 | 40.61 | 43.77 | 35.50 | 32.00 | 31.67 |

# Appendix C

# Paper Publication (Research Articles)

1. **Ruen-ngam, D.**, Shotipruk A., and Pavasant, P. Comparison of Extraction Methods for Recovery of Astaxanthin from *Haematococcus pluvialis*. Submitted to Separation Science and Technology, 30 December 2009**.**

2. **Ruen-ngam, D.**, Shotipruk A., Goto M., and Pavasant. Solubility and Extraction Efficiency of Astaxanthin in Supercritical Carbon dioxide. On preceding, draft.

# Appendix D

# Paper Publication (Research Articles)

1. **Duangkamol Ruen-ngam**, Artiwan Shotipruk and Prasert Pavasant, *Alternative Extraction Method of Astaxanthin from Haematococcus pluvialis*, **International COE Forum on Pulsed Power Engineering and young Researcher Training Camp**, Kumamoto, Japan, 14-16 September 2009 (Short Oral presentation+Poster presentation)

2. . **Duangkamol Ruen-ngam**, Siti Machmudah, Motonobu Goto, Mitsuru Sasaki, Artiwan Shotipruk and Prasert Pavasant, Solubility Consideration in Extraction of Astaxanthin from *Haematococcus pluvialis* using Supercritical Carbon dioxide, **International Conference on Supergreen Fluid (Supergreen 2009)**, Sendai, Japan, 15-17 October 2009 (Poster presentation, Proceeding paper)

3. **Duangkamol Ruen-ngam**, Siti Machmudah, Motonobu Goto, Mitsuru Sasaki, Artiwan Shotipruk and Prasert Pavasant, Solubility Consideration in Extraction of Astaxanthin from *Haematococcus pluvialis* using Supercritical carbon dioxide, **Asia Pacific Biochemical Engineering Conference 2009 (APBioChEC'09)**, Kobe, Japan, 24-28 November 2009 (Oral presentation, Proceeding paper)

4. **Duangkamol Ruen-ngam**, Motonobu Goto, Artiwan Shotipruk, and Prasert Pavasant, Dynamic Method for the determination of solubility of astaxanthin during the high pressure extraction from *Haematococcus pluvialis* using supercritical carbon dioxide, **RGJ-Ph.D. Congress XI**, Chonburi, Thailand 1-3 April 2010 (Poster Presentation, Proceeding paper)

# BIOGRAPHY

Miss Duangkamol Ruen-ngam was born on $4^{th}$ January, 1982 in Bangkok. Her native hometown was Ayutthaya, Thailand. She finished her secondary school from Triam Udom Suksa School in March 2000. After that she studied Food Technology, Faculty of Science, Chulalongkorn University and graduated her bachelor's degree in 2004. She continued to study more in master degree in Biochemical Engineering Research Group, Chemical Engineering, Faculty of Engineering, Chulalongkorn University and achieved her Master's degree in April, 2007. Because of looking forward to be teacher, she kept studying in the same year for Ph.D. During the Ph.D. program, she is granted a Royal Golden Jubilee Ph.D. scholarship (grant number PHD/0064/2550) by Thailand Research Fund and took this opportunity to be visiting researcher at Kumamoto University, Kumamoto, Japan for 11 months. She achieved Ph.D. degree in 2010.