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.

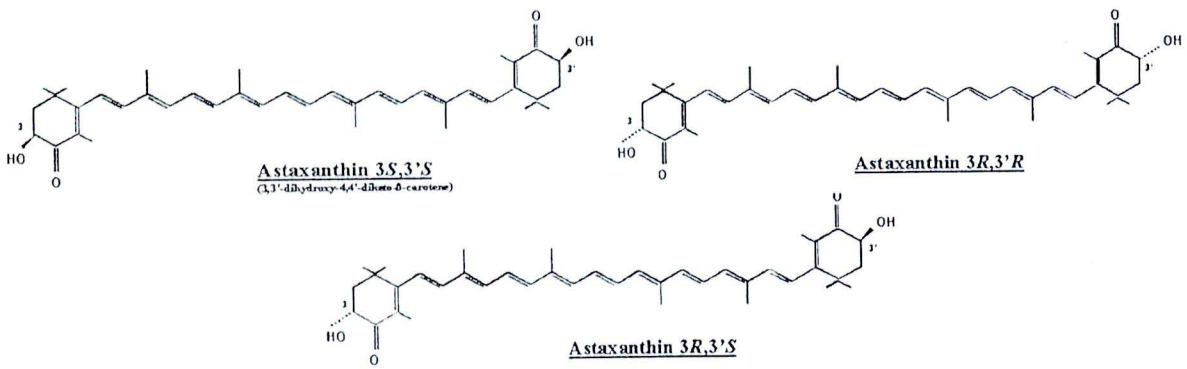


Figure 2.1 Various types of astaxanthin stereoisomers (Lorenz and Cysewski, 2000)

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.

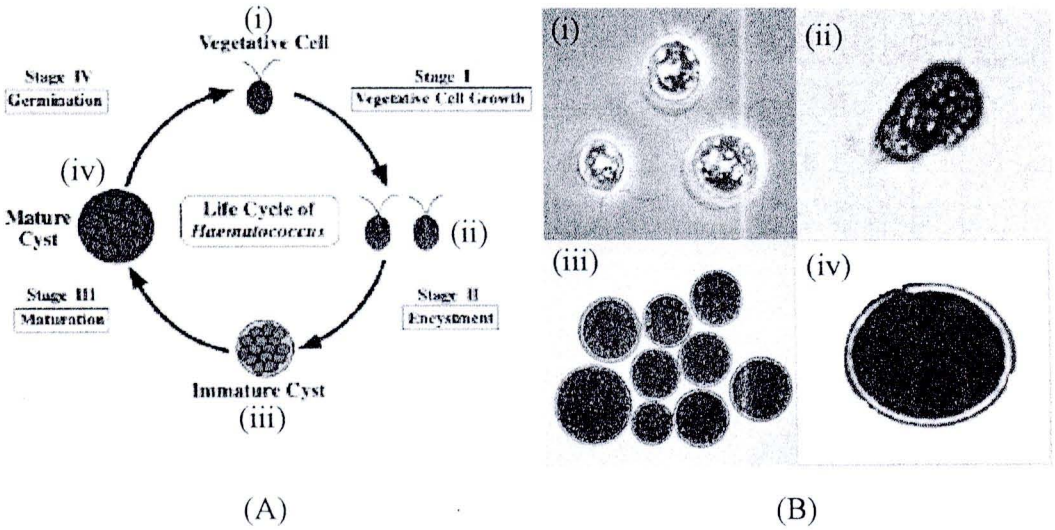


Figure 2.2 (A) Schematic diagram of model life cycle of *Haematococcus pluvialis* (Kobayashi et al., 1997), (B) Photographs of *H. pluvialis* (i) vegetative cell (ii) encyst cell with no flagella (iii) astaxanthin-poor immature cyst cell (iv) Mature cyst cell (Kobayashi, 2003)

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: 4.8×10^5 cell ml^{-1} day^{-1} in bubble columns (Ranjbar et al., 2007) and 8.7×10^5 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.

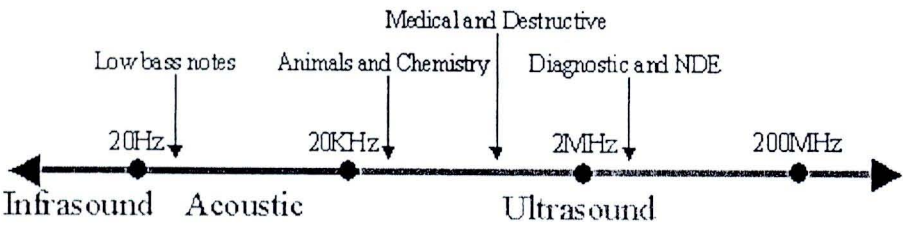


Figure 2.3 Frequency range of ultrasound

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 $P_A \sin 2\pi ft$; 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).

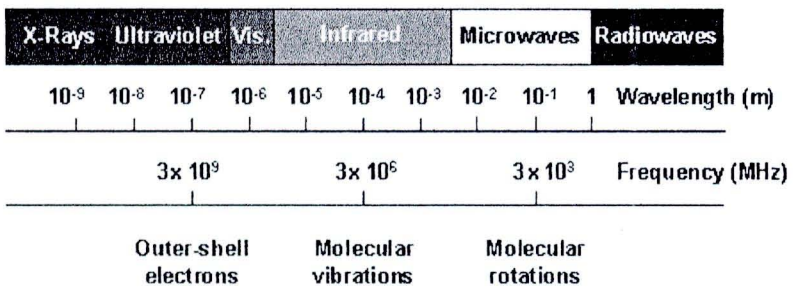


Figure 2.4 Electromagnetic spectrum. (www.anton-paar.com)

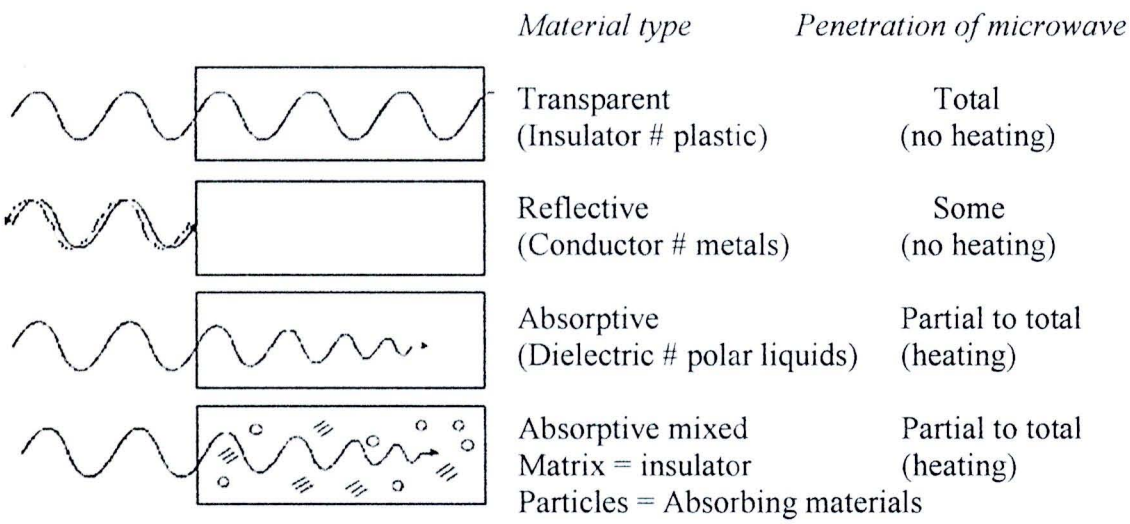


Figure 2.5 Interaction of microwave with different materials (www.anton-paar.com.)

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 (δ) and dielectric constants (ε') 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.

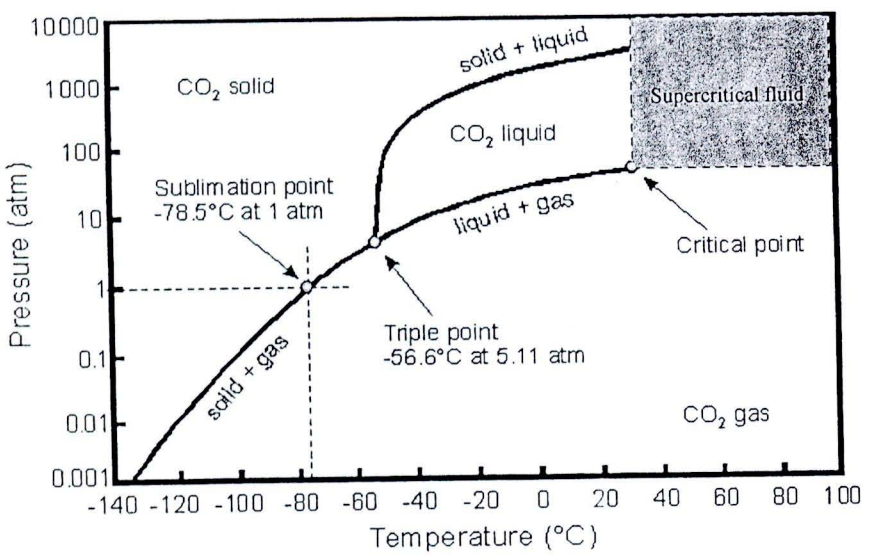


Figure 2.6 Phase diagram of supercritical carbon dioxide

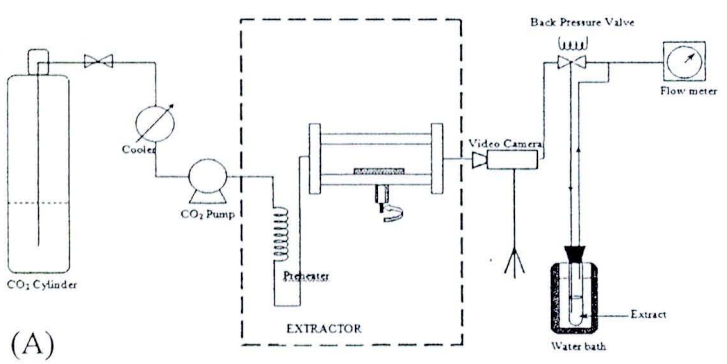
2.6.5.3 Literature Reviews

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.

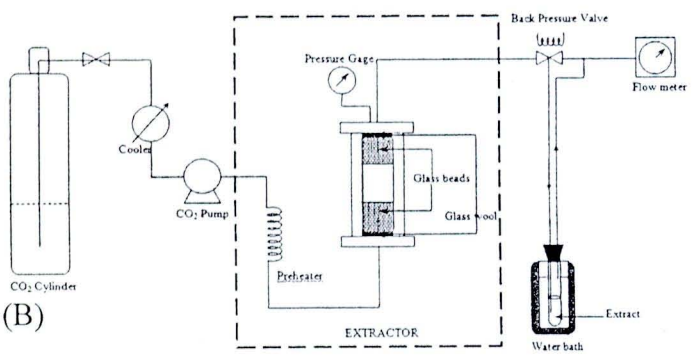
2.7 Solubility of high pressure extraction (McHugh and Krukonis, 1994)

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 glass wool. 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)



(B)

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 2.8×10^{-7} at 323K and 17MPa and the maximum mole fraction of 1.5×10^{-6} at 333K and 40 MPa and the other obtained the minimum mole fraction of 0.42×10^{-5} at 313K and 8 MPa and the maximum mole fraction of 4.89×10^{-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.

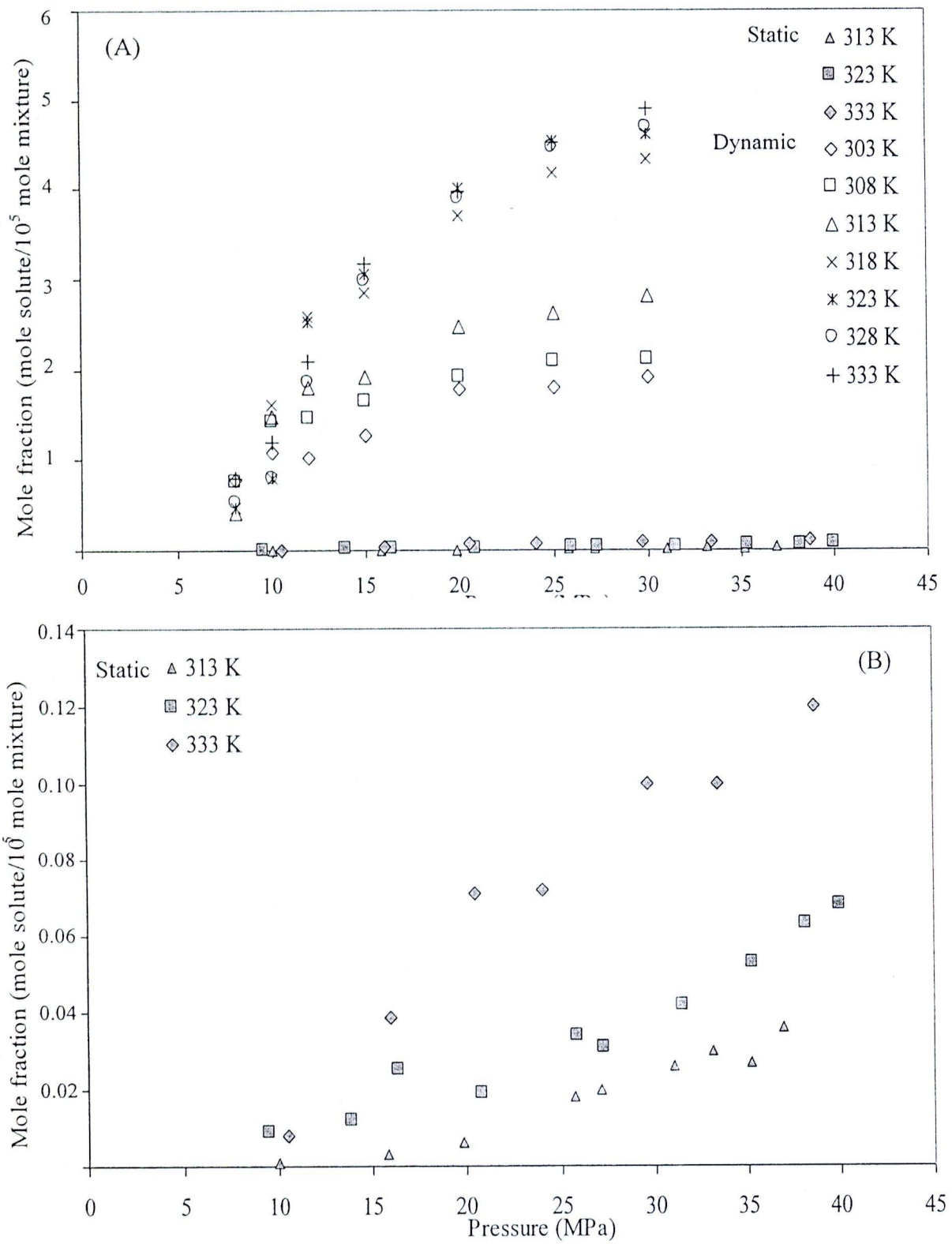


Figure 2.8 Solubility of astaxanthin measurement by (A) both static method and dynamic method (B) extension of static method

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
					-Vary CO2 0.32-1.0 g/cm3 with CO_2 = 1.35 cm/min, 40 60 and 80°C	80>60>40°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	No significant difference
					-400, 500 and 600 bar with 40, 60 and 80°C CO_2 0.27 cm/min	
					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,	Decompose at high temperature
					T=20°C	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)	The first order derivative is limited detection 0.35
					-acetone	mg/l for chlorophyll
					-methanol	and detection 0.25 mg/l
					-hexane	for astaxanthin.
					-chloroform ($CHCl_3$)	

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	Decreased with increasing number of recycles (80% recovery)
					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	Dodecane (0.05 M of NaOH) 98% recovery
15.	Thana et al., 2007	*Haematococcus pluvialis*	SFE CO_2	N/A	Acetone 6 h	2.75
					CO_2 3 ml/min at 40-80°C, 300-500 bar for 1-4 h	Increases with pressure &time 2.28 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, 200 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	ε' [a] (F/m)	ε'' [b]	tan δ
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*	Camptotheci n (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	Capsaicinoid s	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	Anthraquinon es	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	0.1×10^{-3}-2×10^{-3}	1×10^{-4}-3×10^{-4}	0.1-0.4
SC-CO_2	0.47	3×10^{-4}	7×10^{-45}
Liquids	0.6-1.6	0.2×10^{-2}-3×10^{-2}	0.2×10^{-5}-2×10^{-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.	Tonthubthimthong 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 2-4	7 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 2) Without sampling in solubility of binary mixtures of solid and liquid 3) Can measuring liquid, solid and polymers 4) Using minimum amounts of heavy components or supercritical fluid 5) Continue adjusting pressure at any fixed composition and temperature 6) Can sample of muticomponent mixtures in equilibrium phase	1) Not easy obtaining the data of supercritical fluid stripping or fractionation 2) Typical one macrosized sample obtaining per cell loading
2. Dynamic Method	1) Using off-the-shelf 2) Rapid obtaining reasonably large amounts of solubility data 3) Obtaining equilibrium, stripping or fractionation data 4) Using straightforward sampling 5) Can measure very low solubility compound (< 0.1%)	1) Pushing liquid phase out of the column at high pressure liquid phase 2) Undetected phase change 3) Carefully designed for multicomponent 4) Sampling only lighter phase 5) High flow rates of liquid solute 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	0.42-4.89×10^5 Increasing with P & T and CO_2 density 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) Dissolve in Chloroform	CO_2	10mg	40 and 50	12000 - 2000 bar	718 - 784	β-carotene At 12000 kPa 4.3×10^{-7}-7.2×10^{-7} At 20000 kPa 6.7×10^{-7}-9.42×10^{-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 0.022-0.50×10^6
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 $0.20\times10-6$-$0.50\times10-6$
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 $\leq 2.88\times10^{-6}$ 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, 4×10^{-7}-6×10^{-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 0.30×10^{-3} g/L (at 60°C MPa CO2 25.9 MPa, 800 g/L) all-*trans*-β-carotene 2.0×10^{-3} g/L (at 60°C MPa 25.9 MPa, 800 g/L) *cis*-lutein 0.6×10^{-3} g/L (at 60°C MPa 25.9 MPa, 800 g/L) *cis*-β-carotene 0.3×10^{-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/m^3)	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, 0.32×10^{-7}-5.80×10^{-7} CO_2+ethanol, 4.62×10^{-7} -14.0×10^{-7} CO_2+vegetable oil, 2.28×10^{-7}-7.47×10^{-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. 5×10^{-7}