EXTRACTION, IDENTIFICATION AND SEPARATION OF VITAMIN E AND DJENKOLIC ACID FROM *PITHECELLOBIUM JIRINGAN* (*JACK*) *PRAIN* SEEDS USING SUPERCRITICAL CARBON DIOXIDE

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by

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DEDICATION

This thesis is especially dedicated to my loved ones; mother, Hajjah Che Jah, wife, Rohaya Omar and children, Muhamad Afham, Adam Fikri and Nurul Izzah for their continuous love, support and prayer…….

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LIST OF SYMBOLS

		Unit
T_c	Critical Temperature	^{o}C
P_c	Critical Pressure	MPa
F	Solvent Flow Rate	mL/min
ΔT	Increment of Temperature	^{o}C
ΔP	Increment of Pressure	MPa
Δt	Increment of Time	min
dp	Particle Size	µm
m_0	Mass of Dish	g
m_1	Mass of Sample Before Drying	g
m_2	Mass of Dish and Sample After Drying	g
W_{oil}	Weight of Extracted Oil	mg
$W_{t,i}$	Weight of Initial Sample Before Extraction	g
$W_{t,f}$	Weight of Final Sample After Extraction	g
Y	Overall Extracted Oil Yield	%
Y_m	Overall Extracted Compound Yield	%
E_t	Extractability of Oil at Time of Extraction	mg oil/ g CO_2
S	Oil Solubility	mg oil/ g CO_2
$V_{CO2,\ t}$	Volume of CO_2 Used at Time of Extraction	mL
$\rho_{CO2,\ T,\ P}$	Density of CO_2 at Given Temperature and Pressure	g/mL
Q_{Dj}	Djenkolic Acid Yield	mg Dj/g sample
$Q_{Vit.\ E}$	Vitamin E Yield	mg Dj/g sample
Q_i	Area of Compounds I	%
A_i	Area Under the Peak Corresponding to Compound i	%
$\sum A_i$	Sum of the Areas Under Total Peaks	%
C*	Equilibrium Concentration of Oil	g oil/mL CO_2

C_o	Initial Concentration of Oil	g oil/mL CO_2
C_t	Concentration of Oil at Given Extraction Time	g oil/mL CO_2
Ap	Specific Surface Area of *P.jiringan* Seeds	m^{-1}
K	Overall Mass Transfer Coefficient	m/min
Rt	Retention Time	min

LIST OF ABBREVIATION

HPLC	High Performance Liquid Chromatogram
GC-TOFMS	Gas Chromatogram - Time of Flight Mass Spectrometry
UV	Ultraviolet-Visible
PLC	Paper Layer Chromatogram
SC-CO_2	Supercritical Carbon Dioxide
SCF	Supercritical Fluid
SEM	Scanning Electron Microscope
P.JIRINGAN	*Pithecellobium Jiringan (Jack) Prain*
DPPH	Diphenyl-2-Picrylhydrazyl Radical Scavenging Activity
BAW	Butanol-Acetic Acid-Water
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatogram
AIC	Analytical Ion Chromatogram
mAU	Mass Abundance Unit
FAME	Fatty Acids Methyl Ester
NIST	National Institutes of Standard and Technology
LOD	Limit of Detection
LOQ	Limit of Quantification
EPA	Environment Pollution Assessments
EC	European Country
FDA	Food Drug Association
WADA	World Anti-Doping Agency

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LIST OF PUBLICATIONS & SEMINARS

1.1 Mohd Azizi C. Y., Wahyu B. Setianto, Nik Norulaini N. A., Zhari I and Mohd Omar, A. K. (2005). Separation and Identification of *Pithecellobium Jiringan Jack* Seeds using Supercritical Fractionation Carbon Dioxide Extraction and Fast Gas Chromatography/Time of Flight Spectrometry, *Proceedings of the 2nd International Conference on Chemical and Bioprocess Engineering*, (ICCBPE), Kota Kinabalu Sabah, pp. 263-268

1.2 Mohd Azizi C. Y., Wahyu B. Setianto, Nik Norulaini N. A. and Mohd Omar, A. K. (2006). Supercritical Carbon Dioxide Extraction of Constituents of *Pithecellobium Jiringan Jack* Seeds and their Identification using Time of Flight Gas Spectrometry, *Proceedinds of 1st International Conference on Natural resources Engineering and Technology*, (INRET), Putrajaya Malaysia, pp. 616-625

1.3 Mohd Azizi C. Y., Wahyu B. Setianto, Nik Norulaini N. A. and Mohd Omar, A. K. (2006). Extraction of Djenkolic acid from *Pithecellobium Jiringan Jack* Seeds using Supercritical Carbon Dioxide Extraction, *Proceedings of 11th Asian Pacific Confederation of Chemical Engineering,* (APCChE), Kuala Lumpur Malaysia, paper ID. 350

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PENYARIAN, PENGENALPASTIAN DAN PEMISAHAN VITAMIN E DAN ASID DJENKOLIK DARI BIJI *PITHECELLOBIUM JIRINGAN (JACK) PRAIN* MENGGUNAKAN BENDALIR LAMPAU GENTING KARBON DIOKSIDA

ABSTRAK

Pelarut karbon dioksida dalam keadaan lampau genting telah digunakan sebagai satu kaedah untuk menyari dan seterusnya memisahkan komponen berubat dan beracun yang terdapat di dalam biji jering atau nama saintifiknya adalah *Pithecellobium Jiringan Jack (Prain)*, *P.jiringan*. Kaedah penyarian bendalir lampau genting, BLG, telah dipilih di dalam penyarian bahan berguna yang terdapat di dalam bahagian tumbuhan kerana ianya satu kaedah yang ringkas dan berteknologi bersih.

Dalam kajian ini, biji jering yang dikisar bergarispusat 215 µm dengan kandungan lembapan sebanyak 4.27 % telah digunakan. Keadaan lampau genting bagi pelarut karbon dioksida dipilih adalah pada nilai suhu 40°, 50°, 60°, 70° and 80° C manakala nilai tekanan adalah 20.68 MPa, 27.56 MPa, 34.47 MPa, 41.37 MPa, 48. 26 MPa dan 55.16 MPa. Proses penyarian dijalankan selama 60 minit. Komponen berubat yang dikenal pasti adalah vitamin E manakala komponen beracun adalah asid djenkolik. Kaedah kromatografi cecair prestasi tinggi (KCPT) bersama pengesan ultra lembayung telah dibangunkan untuk mengenalpasti dan mengesan kehadiran asid djenkolan di dalam minyak pati. Sementara itu, kaedah kromatografi gas masa penerbangan jisim spektrometri (KG-MSJS) digunakan untuk mengesan kehadiran vitamin E. Dalam kajian ini, tumpuan ialah untuk mendapatkan satu keadaan lampau genting yang boleh memisahkan vitamin E dan asid djenkolik serentak bagi menghasilkan produk akhir yang berkualiti dan sesuai untuk kegunaan awam.

Penemuan hasil kajian menunjukkan keseluruhan minyak pati paling maksimum dapat dihasilkan adalah 8.06 % pada suhu dan tekanan paling tinggi iaitu 80° C dan 55.16 MPa. Sebaliknya, pada pengoperasian rendah penghasilan minyak pati adalah paling minimum. Keseluruhan minyak pati bertambah dengan peningkatan suhu pada tekanan tetap, dan sebaliknya. Kelarutan minyak pati dan pekali pindah jisim keseluruhan minyak pati menunjukan kecendurungan yang sama seperti minyak pati keseluruhan. Kelarutan minyak pati paling maksimum adalah 2.60 mg minyak per g CO_2 (0.26 % berat) manakala pekali pindah jisim keseluruhan adalah 0.1466 $X10^{-6}$ m/minit juga diperoleh pada operasi suhu dan tekanan yang tinggi.

Penemuan hasil kajian kromatogram menunjukkan masa penahanan bahan mentah asid djenkolik ialah 3.503 minit manakala vitamin E ialah 1057.20 saat. Kehadiran asid djenkolik dan vitamin E di dalam minyak sari disahkan jika penyimpangan masa penahanan berada dalam julat yang dibenarkan mengikut garispanduan piawaian antarabangsa bagi penentuan bahan meruap dan tidak meruap secara KG/ KCPT-JS. Pengesahan kehadiran vitamin E juga dilakukan dengan membandingkan spektrum jisim pengionan vitamin E adalah melebihi 79.0 % dengan yang terdapat di perpustakaan NIST dan pengkalan data Willey.

Penemuan hasil kajian menunjukkan hasil asid djenkolik, kepekatan asid djenkolik di dalam minyak pati dan kemampuan sarian asid djenkolik didalam CO_2 bertambah dengan peningkatan suhu pada tekanan tetap, dan sebaliknya. Hasil asid djenkolik yang paling maksimum adalah 0.370 mg/ g sample (0.037 % berat), untuk kepekatan asid djenkolik adalah 4.587 mg/ g minyak pati (0.459 % berat) manakala kemampuan sarian adalah 0.0061 mg/ g CO_2 (0.00061 % berat) pada suhu dan tekanan paling tinggi iaitu 80° C dan 55.16 MPa. Walau bagaimana pun, pada suhu tetap yang rendah iaitu 40° C, asid djenkolik tidak dapat disari dari julat tekanan 20.68 MPa kepada 41.37 MPa dan suhu 50° C pada tekanan 20.68 MPa. Penyarian asid

djenkolic adalah rendah disepanjang suhu tetap 50° C pada julat tekanan 27.58 MPa kepada 48.26 MPa.

Penyarian vitamin E pula menunjukan kecendurungan berlawanan seperti yang terdapat dalam penyarian djenkolik asid. Jumlah vitamin E yang dihasilkan adalah bertambah dengan penurunan suhu pada tekanan tetap dan sebaliknya. Vitamin E boleh disari pada semua keadaan suhu dan tekanan penyarian yang telah dipilih. Hasil vitamin E yang paling maksimum adalah 0.046 mg/ g sampel (0.0046 % berat), untuk kepekatan vitamin E adalah 2.46 mg/ g minyak pati (0.246 % berat) manakala kemampuan sarian adalah 0.0014 mg/ g CO_2 (0.00014 % berat) pada suhu dan tekanan paling rendah, iaitu 40° C dan 20.68 MPa.

Penemuan hasil kajian menunjukkan jika hasil minyak pati digunakan sebagai produk akhir, maka keadaan terbaik pengoperasian BLG-CO_2 ialah pada 20.68 MPa dan 40° C. Sebaliknya, pengoperasian BLG-CO_2 pada 55.16 MPa 80° C adalah terbaik jika sisa sampel biji jering pula dipilih sebagai produk akhir.

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ABSTRACT

Carbon dioxide in supercritical region is employed as a method for extraction and separation of medicinal and toxic component in *Pithecellobium Jiringan Jack (Prain)* seeds, or namely as *P.jiringan* seeds. Supercritical fluid extraction was selected on the extraction of valuable compounds from plant parts because it is simple and environmental friendly process.

Ground *P.jiringan* seeds with particle size 215 μm (diameter) and moisture content of 4.27 % were used in this study. The supercritical carbon dioxide extraction conditions considered were temperatures of 40°, 50°, 60°, 70° and 80° C and pressures of 20.68 MPa, 27.56 MPa, 34.47 MPa, 41.37 MPa, 48.26 MPa and 55.16 MPa. The extraction process was conducted for 60 minutes. The vitamin E was identified as a medicinal compound, while djenkolic acid as toxic compound. Methods were developed for the separation, detection and quantification of extracted djenkolic acid by High Performance Liquid Chromatography (HPLC) with Ultra-Violet detector. Meanwhile, the separation and quantification of extracted vitamin E was done by Gas Chromatography Time of Flight Mass Spectrometry (GC-TOFMS). Thus, this study has focused on the determination of the supercritical carbon dioxide extraction conditions for the extraction and separation of vitamin E and djenkolic acid compounds simultaneously and to produce high quality finished products which are suitable for the human consumption.

The study shows that the maximum overall extraction yield was 8.06 %, at the highest pressure and temperature of 55.16 MPa and 80° C, respectively. In contrast, at

lower extraction conditions minimum oil yield was produced. The overall extracted oil yield was increased with increase in temperature at constant pressure. Likewise, the overall extracted oil yield also increased with increase in pressure at constant temperature. The solubility and overall mass transfer coefficient show the same trend as overall extracted oil yield. The maximum solubility and overall mass transfer coefficient are 2.60 mg oil per g CO_2 (0.26 wt %) and 0.147 X 10^{-6} m/min, respectively at the highest extraction conditions of 55.16 MPa and 80° C.

The chromatogram analysis showed that the retention time of djenkolic acid standard is 3.503 minute, while vitamin E standard is 1057.20 second. The presence of extracted djenkolic acid and vitamin E were confirmed if their tolerance for the peak about retention time of the standard is in accordance with international standard guideline for GC/HPLC-MS determination of volatile/non-volatile compounds. In addition, the confirmation of vitamin E also was done by similarity match of mass ionization spectrum and NIST library and Willey database with percentage similarity is above 79.0 %.

The study shows that extracted djenkolic acid yield, concentration of extracted djenkolic acid and extractability of djenkolic acid increased with increase in temperature for constant pressure and vice-versa. The maximum extracted djenkolic acid yield, concentration of extracted djenkolic acid and extractability of djenkolic acid are 0.37 mg/ g sample (0.037 wt %), 4.587 mg/ g oil (0.459 wt %) and 0.0061 mg/ g CO_2 (0.00061 wt %) respectively, at the higher extraction condition of 55.16 MPa and 80° C. However, no djenkolic acid was extracted at constant temperature of 40° C under pressure range 20.68 MPa to 41.37 MPa and also at extraction condition of 50° C and 20.68 MPa. At constant temperature of 50° C and under pressure range of 27.58 MPa to 48.26 MPa, the amount of djenkolic acid extracted is in trace amount.

On the other hand, the extraction of vitamin E shows a reverse trend as obtained in the extraction of djenkolic acid. The amount of vitamin E is increased with decrease in temperature for constant pressure and vice versa. Vitamin E can be extracted over extraction conditions studied. The maximum extracted vitamin E yield, concentration of extracted vitamin E and extractability of vitamin E were 0.0046 wt % (0.046 mg/ g sample), 0.246 wt % (2.46 mg/ g oil) and 0.00014 wt % (0.0014 mg/ g CO_2) respectively, at the lowest extraction conditions of 20.68 MPa and 40° C.

The findings of this study show that if the extracted oil is considered as finished product, the best SC-CO_2 conditions is 20.68 MPa and 40° C. On the other hand, the SC-CO_2 conditions of 55.16 MPa and 80° C is the best operational if residue sample is chosen as the finished product.

CHAPTER 1
INTRODUCTION

1.1 Introduction

The use of plant-based product for the treatment of diseases and maintaining healthcare started a long time ago. The conventional separation methods such as water extraction, steam distillation, evaporation and spray drying are some of common techniques used for the extraction of bioactive compounds from medicinal and aromatics plants. The demand and consumption of medicinal plants are expected to increase significantly in the coming years due to several reasons. The new approach in the production and marketing of the traditional medicinal products, the integration comprised modern and traditional medicine such as homeopathy and naturopathy and the increasing numbers of traditional medicine companies that have the urgent need for research in extraction of active compounds from natural products as medicines, food additives or natural pesticides.

In conjunction with the rapid demands natural medicinal product, the processing technologies used in its production have to make major technical and scientific improvements in their efficiencies and product quality. This is important in order to meet the basic requirement of hygiene and safety standards as well as to survive in this increasingly competitive and dynamic market product. The current processing methods are inefficient and the extract produced is low of quality. Majority of the local herbs medicinal products are still prepared in the traditional manners involving mainly hot or cold-water extraction, grinding to powder and conventional drying. By using the traditional processes of water extraction and conventional drying, the finished product is not guaranteed to be safe. Both the desired as well as undesired compounds are extracted and it is difficult to separate specific compounds of interest. Thus the finished products contain some undesired compounds such as polar toxic substances too. Most of factories that produce traditional medicine are aware of these limitations, but lack of

resources and new technologies and expertise forced them to still use these unconventional techniques.

In view of the above statement, the supercritical carbon dioxide extraction process has a promising field in the industrial application especially herbs industries where high quality product can be obtained. There are many reasons which make the use of supercritical carbon dioxide extraction as a very great potential method for extracting valuable compounds and at the same time removing of undesired compounds from plant materials and promising alternative in extraction process.

The first advantage of this technique is that the final product fulfills the expectations of health conscious users because it is free of solvent, does not contain toxic residues as well as undesired microorganisms. Supercritical fluid extraction using carbon dioxide as a solvent provides an excellent alternative to the use of chemical solvents. Since the carbon dioxide is non-toxic, non flammable and inert in nature, there is no risk of thermal degradation of processed product. In fact, the use of carbon dioxide has been accepted by most European Food and Drugs acts as an extraction medium for the isolation of food-related compounds (Starmans and Nijhuis, 1996). Over the past three decades, supercritical carbon dioxide extraction has been used for the extraction and isolation of valuable compounds from natural products.

On the issues of producing high added value products, supercritical carbon dioxide extraction is also capable to function as a sterilizer as studies by Banana (2005) showed that bacteria can be eliminated at pressure as low as 20.68 MPa and temperature 50° C. These results will contribute to the motives of choosing supercritical carbon dioxide as the extraction medium and to improve the extracts quality and compliance customer's awareness elsewhere.

Secondly, most of the medicinal plants extracts are usually in low concentration and exist in complex mixtures. Therefore the process selected to obtain the maximum valuable compounds as well as desired compounds must be effective, selective, and satisfy both economical requirement and environmental regulations. Bisunadan (1993) reported that SC-CO_2 is more efficient in extracting of trace amount of compounds such as carotenes and tocopherols from oil palm fruits at high pressure and temperature of 500 bars and 80° C compared with hexane extraction. On the other hand, the extraction of triglycerides it was sufficient to operate at pressure above 300 bars and temperature more than 40° C. This scenario indicates the ability of SC-CO_2 technique on the selectivity of target compounds from the heterogeneous mixture in nature, by adjusting the temperature and pressure of the extraction process. Gast et al. (2005) has studied on the purification of tocochromanols from the crude palm oil and soy oil deodorizer distillate. The results suggest that a tocochromanols enrichment starting from crude edible oil to very high concentration is possible by means of supercritical extraction. Kallio and Kerrola (1992) have studied the application of supercritical carbon dioxide extraction on the sample of coriander fruit. Their results show that at extraction temperature between 40 and 50 ^{o}C and extraction pressure lower than 100 bars, higher molecular weight compounds are not extracted. Another important aspect on comparing the different extraction methods is the quality of the resultant extracts where both the desired and undesired compounds could be extracted. For example, in the water extraction, the number of chemical constituents that could be extracted was more than supercritical fluid extraction. This is due to solubility of polar compounds in water is greater than in carbon dioxide. Therefore not all the compounds extracted using water could be considered because most of toxic compounds are present in polar compounds.

Thirdly, supercritical fluid has relatively lower viscosity and higher diffusivity therefore, it can penetrate into porous solid materials more efficiency than liquid solvents and consequently, it may render much faster mass transfer resulting a more rapid and efficient extraction. Norhuda (2005) used Transmission Electron Micrograph (TEM) to study the effect of pressure and temperature on the structure of the palm kernel after extraction with SC-CO_2. The comparison was made on the transmission electron micrograph of the cross section of a single palm kernel before extraction and after extraction at optimum conditions over a 30 minutes extraction. It was found, that the pore of the solid matrix filled with oil (before extraction) was voids after the completion of extraction. This argument was a good rationale for the use of supercritical carbon dioxide extraction in the separation of essential oils from the plants herb. Essential oil compounds are normally located within the cellular structure in vacuoles matrix. Therefore by adjusting the operating temperature and pressure; the penetration depth of solvent carbon dioxide into internal matrix structure could be achieved, and hence results in exploration of trapped essential oil compounds.

The fourth reason is to fulfill requirement of the end product quality. For Malaysian herbs, in order to meet the international herbal product standards and compete successfully in the international market, it is essential for the local manufacturers comply with the highest standards of manufacturing including the WHO-GMP (World Health Organization-Good Manufacturing Practices), which is globally accepted. Since the 7th Malaysian Plan, the government made major demands on the industry through implementation of new legislative requirement, such as more stringent approval criteria for new products and good manufacturing practice, GMP (Teik and Azmi, 1997). Since 1st January 1999, all herbal products registered with the Malaysian Government must comply with Malaysia GMP rulings, whereby the Malaysian GMP complies with the WHO-GMP guidelines. One of the aspects emphasized in GMP guidelines is processing method, referring to high quality and established product. In

fact, to make the Malaysian medicinal product viable, an extraction process plant has to be developed. Shortcomings of using crude products such as storage, standardizations, stability and a short shelf life can be overcomed by doing the extraction of natural compounds. The manufacturers should adopt modern concepts and technologies in processing and developing their products. Supercritical carbon dioxide extraction process is one of alternative method and also among the most recent technologies used in extraction and separation of valuable compounds from natural sources that have medicinal values.

A supercritical fluid (SCF) is defined as any substance at a temperature and pressure above the critical values. The critical temperature of a substance is defined as the temperature above which a pure, gaseous component cannot be liquefied regardless of the applied pressure. The critical pressure is then defined as the vapor pressure of the gas at the critical temperature. Another popular description of supercritical fluid is the substance in the intermediate state between gas and liquid; therefore it does inhibit the characteristics of high diffusivity comparable with a gas and high solubility as a liquid. Therefore, supercritical fluid extraction provides the extraction feasible, superior quality products and more efficient. In Malaysia, the use of supercritical fluid extraction for industrial application is still unpopular even though several local researchers have studied the feasibility of supercritical fluid extraction especially on the palm oil industries (Bisunadan, 1993; Hassan, 2000; Mastura et al., 2001; Anuar, 2002; Harcharan et al., 2002; Zaidul, 2003; Banana, 2005 and Norhuda, 2005).

The plant namely *Pithecellobium jiringan jack (P.jiringan)* seed was used for the exploration of chemical constituents as well as extraction of both, valuable and undesired compounds by using supercritical carbon dioxide extraction. The selection of *P.jiringan* seeds as a material used in the SC-CO_2 extraction is due to significant

reasons. Traditionally, the use of raw vegetables (ulam) is recommended for the prevention of disease and health care purposes. This plant seeds is categorized as unpopular plant than most of common plants like *eurycoma longifolia jack* (tongkat ali), *orthosiphon stamines benth* (misai kucing) and *andrographis paniculata* (hempedu bumi). The reasons *P.jiringan* seeds is considered unpopular medicinal plant for human consumption because the seeds contain of undesired compounds such as volatile oil consisting of an ally sulfur compounds and toxic acid namely djenkolic acid. The ally sulfur compounds, which is a group of short chain hydrocarbon with presence of sulfur have very strong noxious smell meanwhile excessive consumption of seeds may lead `kejenkolan disease', caused by crystallization of djenkolic acid in the kidneys and bladder with symptoms such as renal hyperemia, oliguria, no urination and painful of flow urine. These two groups of compounds have to be removed before consumption.

On the other side, *P.jiringan* seed also contains rich of bioactive compounds and it is possible that some of these compounds may be developed into medicinal drugs especially for anti hypertension and anti-diabetics. According to Vimala (2003), valuable compounds contained in *P.jiringan* seeds were protein, carbohydrate, fiber, calcium, phosphorus, vitamins and sterols. Also, the seeds were found to have high antioxidant potential to neutralize free radicals and balance the oxidative stress state. Therefore very important to exploit SC-CO_2 extraction method on the extraction and simultaneously separation of medicinal and toxic compounds from *P.jiringan* seeds in order to produce high quality finished product. In addition, to date there is no official document having the details of chemical constituents of *P.jiringan* seeds (Monograph Committee I, 1999; Monograph Committee II, 2001). Hence, the selection of SC-CO_2 extraction for exploration of possible compounds in *P.jiringan* seeds is an excellent approach, especially in unexplored medicinal plants. Detailed chemical constituents of *P.jiringan* seeds will be reported in this manuscript that can be evaluated for the therapeutic properties for their use as medicinal plant products.

1.2 Problem Statements

The ultimate goal of this research is to investigate the feasibility of removing the undesired compounds specifically the compound of djenkolic acid and lenthionine (noxious) from *P.jiringan* seeds by using SC-CO_2 extraction method. On the issues of problem statements, the points which should be highlighted are;

- Carbon dioxide is an excellent solvent for the extraction of non-polar compounds. Meanwhile extracting polar compound has proven to be more challenging. The chemical constituents in the *P.jiringan* seeds contain both polar as well as non-polar compounds. Therefore, in SC-CO_2 extraction technique, the solvent polarities of CO_2 are manipulated by the adjustments of temperature and pressure in order to match the polarity of compounds of interest.
- The studies on development of chromatographic method of djenkolic acid are rare in literature and particularly none in HPLC method. Therefore the development of HPLC conditions such as selection of mobile phase, column stationary phase, wavelength detector, solvent flow rate and injection volume are quite challenging.

1.3 Objectives of the Study

- To determine the supercritical carbon dioxide extraction conditions on the extraction and simultaneous separation of vitamin E and djenkolic acid compounds from *Pithecellobium jiringan* seeds to produce high quality finished product.
- To investigate the effects of temperature and pressure in 60 minute extraction period on the overall extraction oil yield, oil solubility, controlling step of extraction process and the overall mass transfer coefficient.
- To determine the cross over pressure under the ranges of pressures and temperatures of interest.

- To develop the method for separation and detection of djenkolic acid in the extracted oil of *P.jiringan* seeds by High Performance Liquid Chromatography with an UV detector.
- To develop the method for separation and detection of vitamin E in the extracted oil of *P.jiringan* seeds by Time-of-Flight Gas Chromatography Mass Spectrometry
- To investigate the effects of temperature and pressure in 60 minute extraction period on the amount of djenkolic acid and vitamin E extracted in the seed sample, concentration of djenkolic acid and vitamin E presence in extracted oils and extractability of djenkolic acid and vitamin E in supercritical carbon dioxide extraction.

1.4 Significant Contribution

The main contribution of this research can be looked at from two aspects, which are academic and professional contribution. On the view of academic contribution, the novel part were the manipulation of supercritical carbon dioxide extraction condition to remove polar compound of djenkolic acid from *P.jiringan* seeds in order to produce safe medicinal products and capability of supercritical carbon dioxide extraction to extract the low concentration of valuable compounds. The fundamental behavior of supercritical condition such as the solvating power of CO_2, solute vapor pressure, competing affect of temperature and pressure on the solubility, and compounds distribution due to their properties will be prime consideration. Nevertheless, method development and validation of djenkolic acid using High Performance Liquid Chromatography, HPLC, will be studied. In addition, the official document having the detailed chemical constituents of *P.jiringan* seeds will be reported in this thesis.

The professional contribution is the application of supercritical carbon dioxide extraction conditions for the extraction and separation of medicinal, toxic and noxious compounds from *P.jiringan* seeds. At particular SC-CO_2 condition, the extracted oil is considered as finished product (rich of vitamin E). On the other hand, the residue sample of seeds also could be considered as finished product if no djenkolic acid present at all. Therefore the manipulation of strength and polarity of CO_2 by changing the extraction conditions for extraction of specific compound can be done. This aspect becomes good prospects for the use of *P.jiringan* seeds as plant based product for disease prevention and treatment. On the other hand, the supply of raw material of *P.jiringan* seeds are available in sufficient quantity that contribute for the potential use of this plant as plant based product along with others well known plants.

1.5 Limitation of the Study

The limitation of the study was related to the laboratory SC-CO_2 equipment used (Model:ISCO,SFX^{TM220} Inc., Lincoln, Nebraska U. S. A). The high pressure syringe pump could not control the flow rate of CO_2 throughout the experiments. This limitation of the equipment will cause the measurement of solubility in supercritical fluids difficult when operating condition at high pressure and temperature conditions. In this study, dynamic approach method was preferable due to limitation of the equipment used.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

In order to apply SC-CO_2 extraction method for the removal of undesired compounds from *P.jiringan* seeds, several fundamental concepts and related subjects need to be reviewed. The information obtained is significant during selection of SC-CO_2 operating conditions as well as chromatogram analysis for identification and quantification of marker compounds in extracted oils. In this chapter, there are two main topics to be reviewed, the supercritical carbon dioxide extraction and the medicinal plants of *P.jiringan* seeds. On the fundamental concept of SC-CO_2 extraction, issues highlighted are the principles and properties of fluids, the competing effect of SC-CO_2 condition and mass transport and separation phenomenon for extracting *P.jiringan* seeds at selected supercritical conditions. Meanwhile, review of *P. jiringan* seeds will look on the plant description and review of chemical constituents of plant. Besides, review on the analysis of chromatogram will be focused for the identification and quantification of djenkolic acid in oil extract. As mentioned earlier, the djenkolic acid is considered as a new compound due to lack of chromatographic data, therefore the specific method development for its analysis will require studies of methods applied to others compound with the similar physical and chemical characteristics.

2.2 Overview of Supercritical Fluids

The discovery of supercritical fluids was evolved after the first observation report by Baron Cagniard (Stahl et al., 1988 and Mc Hugh and Krukonis, 1986). He visually noted that the gas liquid boundary disappeared when the temperature of certain materials was increased by heating each of them in a closed glass container. From these early experiments, the critical point of a substance was first discovered. Therefore, a high pressure test facility is required for a systematic study of fluids in or

near supercritical state. This might have been the main reason why studies in this field were comparatively few until recently. Research on supercritical fluids and its applications reached the initial peak during the period from the secondary half of the 1960s to the 1970s followed by the secondary peak about 15 years later.

In the last four decades, there has been a tremendous interest in the use of supercritical fluid as solvent for extraction, separation and removing purpose. The intensive study on extraction of food components by using supercritical fluid began in early 1970s. Many patents resulted from these studies such as for the extraction of hops, decaffeination of coffee and tea, and tobacco and spices. The first symposium on supercritical fluid was held in Essen, which was the result of rapid development of the SFE as a separation method (Stahl et al., 1988). Since this symposium, the supercritical fluid has been receiving interest as an alternative technique for extraction of solid and liquids. Also, supercritical fluid technology has become an interdisciplinary field utilized by chemical engineers, chemists, food scientists, materials scientists, agronomists and researcher in biotechnology and environmental control. As expected, the 21st century is a tertiary peak with mounting interest in the possible development of new material program, once is on the medicinal plant industry.

2.3 Properties of Supercritical Fluids

A supercritical fluid (SCF) is a substance that is gas like, which has been heated above its critical temperature (Tc) and simultaneously compressed above its critical pressure (Pc). Definition of critical temperature is the highest temperature at which a gas can be converted to a liquid by increase in pressure and the critical pressure is the highest pressure at which a liquid can be converted to a traditional gas by an increase in the liquid temperature. In other word, supercritical fluid can also be defined as a heavy gas with a controllable dissolving power or as form of matter in which the liquid and gaseous state are indistinguishable. This principle can be

explained more clearly by using pressure-temperature phase diagram as shown in Figure 2.1. In P-T diagram, there are lines describing the sublimation, melting and vaporization equilibria, respectively. The three curves intersect at the so-called triple point, TP, where the solid, liquid and gaseous phases coexist in equilibrium and ends at the critical point, CP. Above critical point, no liquefaction will take place on raising the pressure and no gas will form on increasing temperature. This region of pressure and temperature above Pc and Tc is called supercritical region, which is denoted by the yellowed area in P-T diagram.

In the case of carbon dioxide gas, the critical temperature is 31° C and critical pressure is 7.38 MPa. If the CO_2 gas is heated up to any temperature over 31° C, which would be over `super' the critical point, then it will not be turned into liquid no matter what ever pressure is applied. This region of supercritical fluid is unique because it is a phase that possesses some of the properties of both a gas and a liquid. At supercritical region, the characteristics of solvent is a strong function of temperature and pressure, can change the property sharply with very little change in pressure and temperature, able to control density, polarity, viscosity and other properties of the fluids continuously over a wide range. According to Mc Hugh and Krukonis (1986) these unique characteristics make it possible for extraction to take place rapidly. In supercritical fluid extraction, it is essential to discuss the properties of supercritical fluids in details such as solvent power, diffusivity/viscosity and competing factors effect hence it influence the solubility of desired compounds particularly those located remotely within solid matrices.

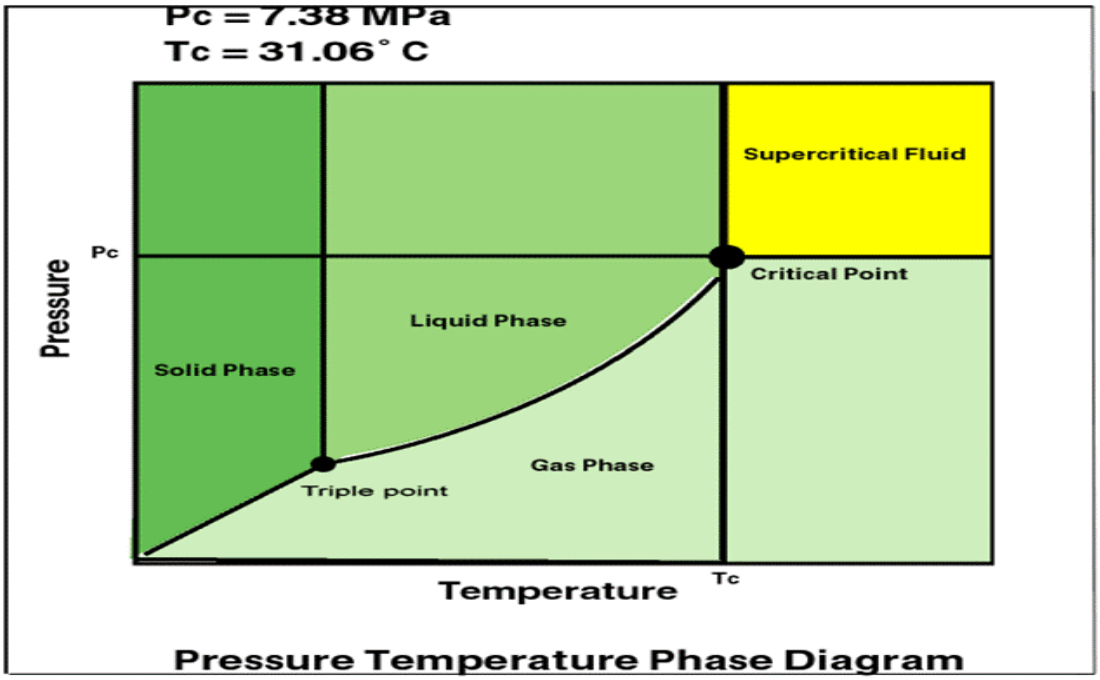

Figure 2.1: Pressure-temperature phase diagram of carbon dioxide.

Source: Dean, (1993)

2.3.1 Solvent Power Consideration

Basically at the beginning of supercritical fluid extraction, the process is controlled by the solubility followed by mass transfer controlled at the end of extraction. The solubility is influenced by solvent density and solute vapor pressure meanwhile mass transfer is influence by diffusivity and viscosity of supercritical fluids. In order to obtain maximum extracted mass, the solubility and mass transfer should be in higher magnitude. For utilization of supercritical fluids as an extraction solvent, the fluids must have characteristics of solvating power. Supercritical fluids begin to exhibit significant solvating power when they are compressed to liquid densities.

Table 2.1 shows the comparison of properties of supercritical CO_2 and typical liquid and gas properties. The density of CO_2 is 100 to 1000 times greater than gas and solvating characteristics approaching those of a liquid. The diffusivity of CO_2 is higher than that of a liquid by a factor of several hundreds. This means that mass transfer in a

supercritical fluid is faster than in a liquid by the same factor. The viscosity also shows a significant difference from that of a liquid, one hundred times lower. In addition, the Reynolds number becomes 30 to 100 times higher than that of a liquid for a given flow velocity. This facilitates to develop turbulent flow in tubing, reducing the laminar flow dispersion.

Table 2.1: Properties of supercritical carbon dioxide and those of ordinary gases and liquids

Region	Density (g/ml)	Viscosity (g/cm.s)	Diffusion Coefficient (cm^2/s)
Gases	$(0.1 – 0.2) \times 10^{-3}$	$(1 – 3) \times 10^{-4}$	0.1 – 0.4
Supercritical CO_2			
T_c, P_c	0.47	3×10^{-4}	7×10^{-4}
T_c, $6P_c$	1.0	1×10^{-3}	2×10^{-4}
Liquids	0.6 – 1.6	$(0.2 – 3.0) \times 10^{-2}$	$(0.2 – 2.0) \times 10^{-5}$

This solvating power that is referring to solvent density is directly related to the thermodynamic state of the solvent that is highly dependent on its temperature and pressure as shown in Figure 2.2. Indeed, adjusting the range of temperature and pressure interest can easily control the solvent strength.

At constant temperature, the solvent power or solvent density of a supercritical fluid increases as pressure increases. On the other hand, at constant pressure, as temperature increases, the solvent density will decrease. Meanwhile, increasing temperature will also lead to an exponential increase in the vapor pressure of the solute (solute volatility). The solubility of solute is increased as the solvent density and solute volatility is increased, hence the oil yield will also increase. As reported by Murga et al. (2005) the solubility of caffeic acid in supercritical carbon dioxide is increased with pressure and temperatures studied. As shown in Figure 2.3, the solubility of caffeic acid is high at higher operating pressure and temperature, 50 MPa and 333 K respectively. In other words, solubility depends on the pressure and temperature which is represented as solvent density and solute volatility respectively.

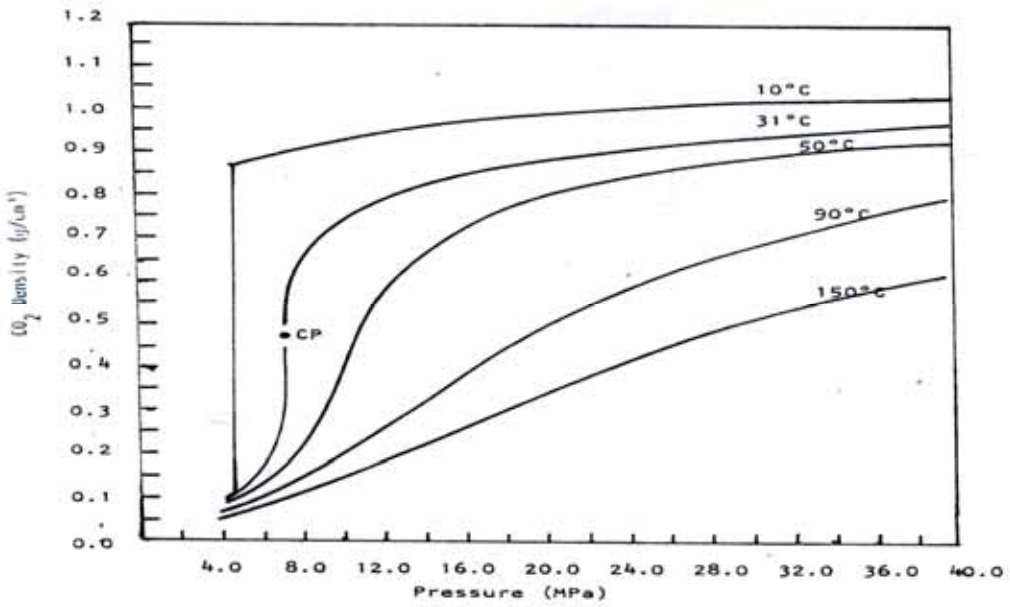

Figure 2.2: Density of carbon dioxide as a function of pressure at different temperature.

Source: Fattori et al. (1988)

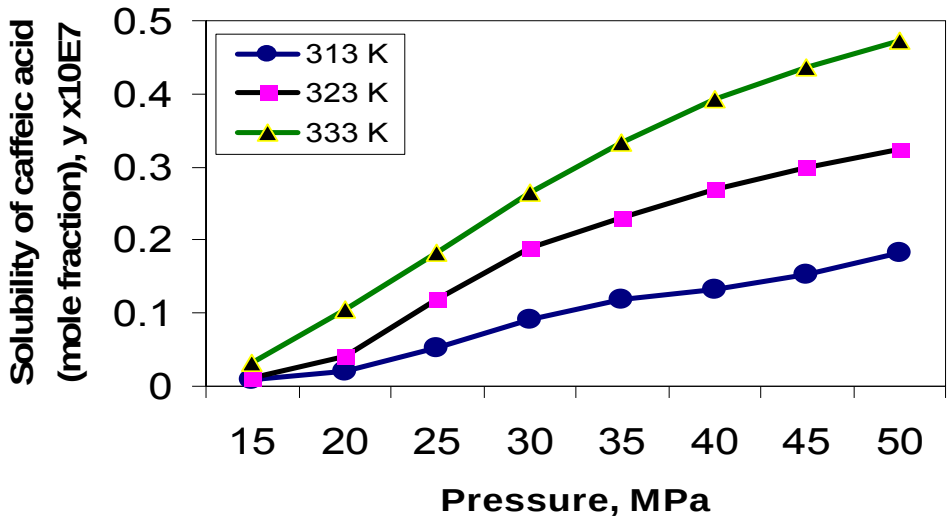

Figure 2.3: Effect of pressure on solubility of caffeic acid in SC-CO_2 at different temperature studied

Source: Murga et al. (2005)

2.3.2 Cross Over Pressure

An increase in either pressure or temperature while keeping the other parameter constant will result in enhanced solubility. These results can be easily explained by increasing the pressure that will increase solvent density to enhance solute-solvent interaction. Meanwhile increasing the temperature will make the vapor pressure of solute greater. On the overall comparison of the effect of pressure on solubility at different temperature studied or vice-versa, the results of solubility are clearly elaborated based on the points of competing effect and cross over pressure.

Cross over pressure or retrograde solubility is a point (graph solubility versus pressure at different temperature) where the behavior of solubility regarded due to the competing effects between the solvent density (referring to pressure) and solute volatility (referring to temperature). At pressure above cross over pressure, a CO_2 density is less sensitive to temperature and solute volatility effects dominate, so the solubility of compounds increases with a higher temperature. On the other hand, at pressure below the cross over pressure, the solubility decreases with increasing temperature due to the rapid decrease of CO_2 density. At the point of cross over pressure, the competing effects balance each other and the solubility remains relatively constant with increasing temperature. As reported by Murga et al. (2003), the behavior of cross over pressure occurs in the extraction of ferulic acid. At cross over pressure, the solubility of ferulic acid relatively constant even though temperature was increased up to 50° C. The phenomenon of cross over pressure was shown in Figure 2.4. Lee (1994) study on the extraction of oil from evening primrose seed using SC-CO_2 determined that the pressure of 200 bar is a pressure at cross over pressure.

Many researchers who have discussed the cross over pressure phenomenon include Taylor (1996), Kiriamiti et al. (2001), Perretti et al. (2003), Daood et al. (2002 and Gordillo et al. (2001). The significance for determination cross over pressure is

related to the selection of pressure of interest. If the pressure range selected is above cross over pressure, that means maximum extracted mass (yield) could be obtained at the higher interaction condition. Meanwhile at below cross over pressure, lower temperature will produce the maximum yield.

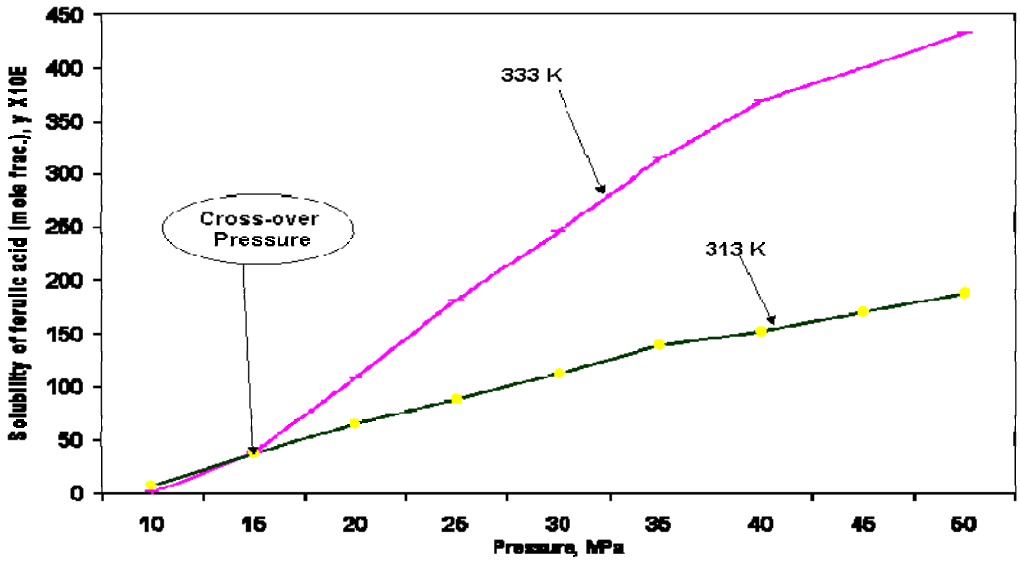

Figure 2.4: Solubility of ferulic acid as function of pressure at various temperatures. The crossover pressure located at the pressure of 13 MPa.

Source: Murga et al. (2005)

2.3.3 Diffusivity and Viscosity Consideration

As mentioned earlier, the extraction process is controlled by mass transfer properties, which are represented by diffusivity and viscosity of fluid solvent. The values of diffusivity and viscosity are function of temperature and pressure. Referring to Figure 2.5, the relationship of diffusivity function with temperature and pressure can be written as follows:

- at constant temperature, the diffusivity in a supercritical fluid decreases with increase in the pressure
- at constant pressure, the diffusivity increases with increasing temperature particularly, in the vicinity region of the critical point
- the slopes of diffusivity function of temperature isobar increase with decreasing temperature and pressure
- the diffusivity of a solute in a supercritical fluid always exceeds that in an ordinary liquid solvent

Figure 2.6 shows the variation of the CO_2 viscosity as a function of pressure at different temperature studied. Similarly, the relationship of viscosity with respect to pressure and temperature were:

- at constant temperature, viscosity increases with given pressure
- at constant pressure, viscosity decreases with increasing temperature particularly above critical pressure

As shown in Table 2.1 the properties of supercritical carbon dioxide with high diffusivity and low viscosity have quite significant properties for the purpose of extraction because their mass transfer properties are much more favorable. Another word, high diffusivity is a significant property as the rates of extraction are limited by the speed because solute molecules are transported by diffusion process from the matrix sample into the bulk fluid of CO_2. Meanwhile low viscosity will allow supercritical fluids to penetrate matrices of desired compounds with low permeability more rapidly than conventional solvents because it does not exhibit surface tension limitation (Dean, 1993).

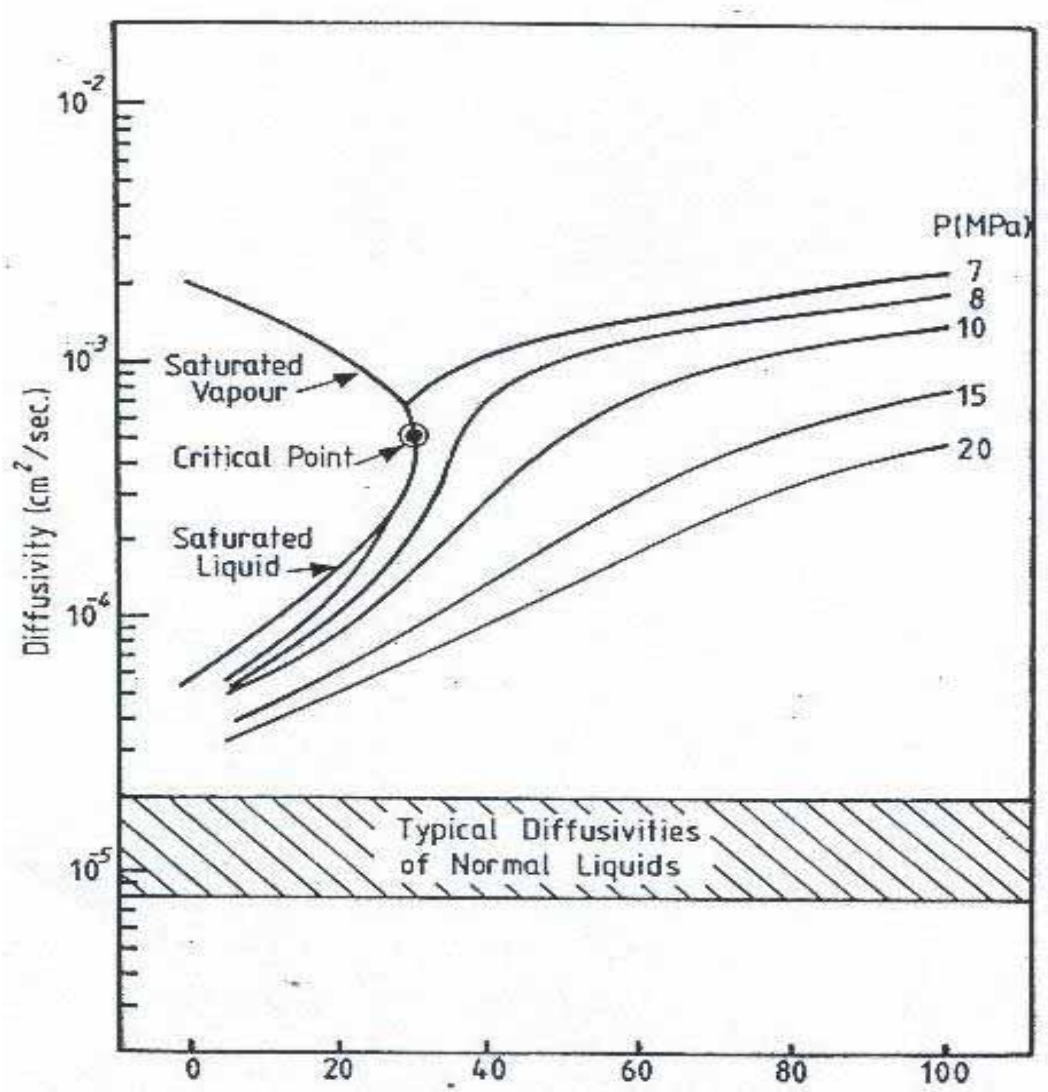

Figure 2.5: Diffusivity of CO_2 in the supercritical fluid and near critical region

Source: Weatherly (1994)

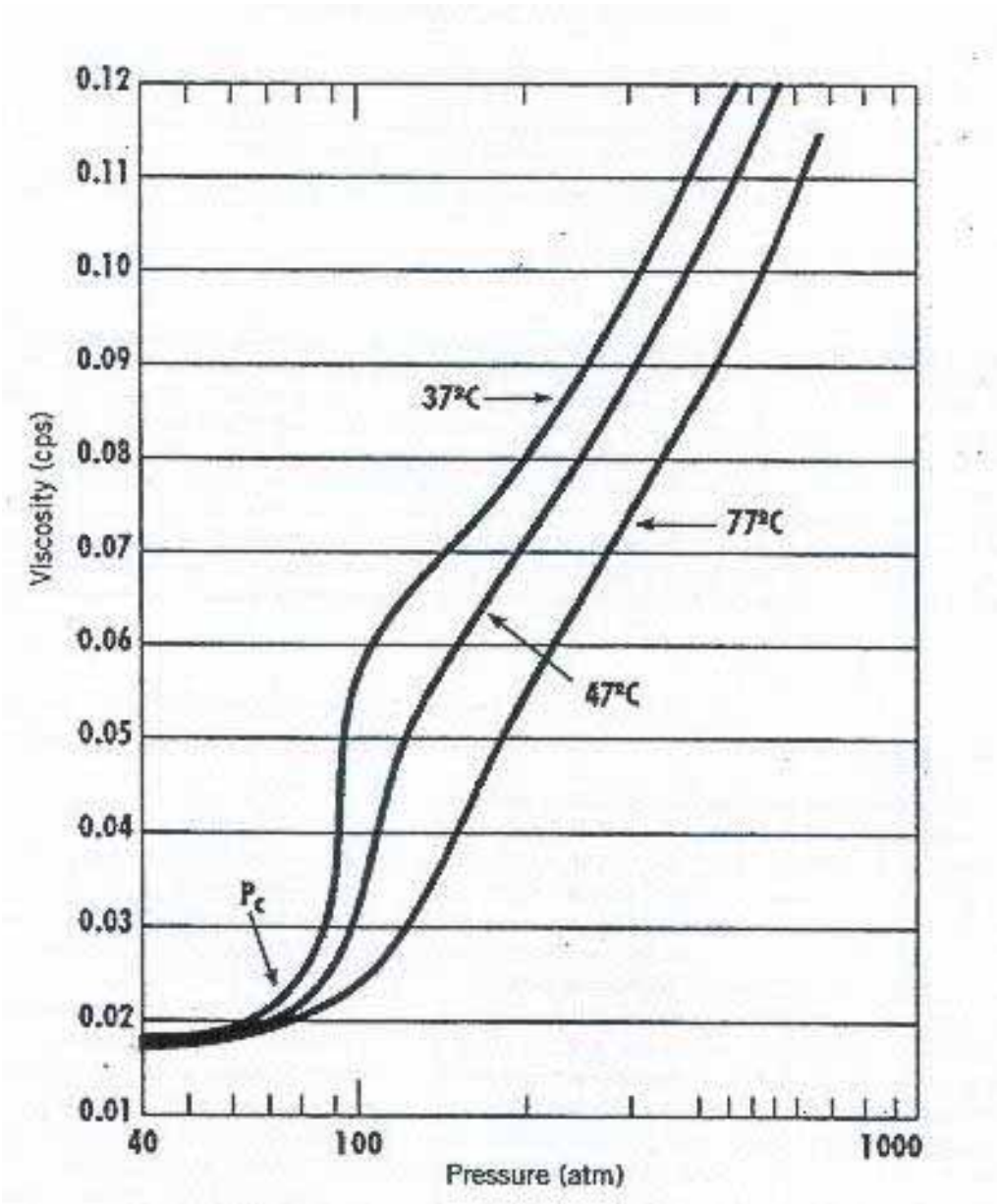

Figure 2.6: Variation of viscosity of CO_2 function of pressure at temperature studied

Source: Mc Hugh and Krukonis (1986)

2.3.4 Significance of Carbon Dioxide as a Solvent

The rationale of using carbon dioxide as a solvent in the supercritical fluid extraction has been discussed in many publications. The most important reason used of carbon dioxide is because it has a best critical temperature and pressure of 31.3 ^{o} C and 7.38 MPa, respectively. As mentioned by Dick and Herry (1996) no material with critical parameters as mild as carbon dioxide. This means that extraction can be conducted at temperatures that are low enough not to harm the physiochemical properties of the extract.

The clear advantages over other organic solvents are chemically stable, inflammable, radioactively stable, high purity, non-toxic and inert in nature. Thus, there is no risk of side reactions such as oxidation. It has been accepted by most European food and drugs acts as an extraction medium for the isolation of food related compounds. Moreover, there are no other residual solvents in any final extracted material since CO_2 is the only gaseous at room temperature and pressure. Thus, with CO_2, the extracted oil can be easily separated by decompression and subsequently, its recovery percentage is still high. Moreover, the characteristics of the finished products is also generally better than the conventional methods since it is free from inorganic salts, heavy metals and micro bacterial life.

On the chemical properties of carbon dioxide, it have a zero dipole moment is considered as non-polar. The polarity scale of CO_2 is closed to that pentane and hexanes which are solvents commonly used in liquid extraction procedures. Thus a similar range of compounds can be extracted using both techniques. Meanwhile, the polarity scale of CO_2 can be adjusted to reach positive dipole moment to form as mild polar or polar by increase the extraction pressure and temperature. As compared with other alternative solvent such as Freon-13 and SF_6, the rate of CO_2 to reach positive

dipole moment is high (Luque et al. 1994). In addition, CO_2 has low energy utilization due to low heat of vaporization at the critical point.

2.4 Application of Supercritical Fluids

Application of SC-CO_2 have been studied by numerous researchers on the different views such as the applications of SC-CO_2 in the extraction of medicinal components from plants, the comparison with conventional method, determination of SC-CO_2 properties behaviors such as oil solubility, mass transfer coefficient, extraction with reaction in supercritical fluids and thermodynamic modeling of phase equilibrium of solvent-solute mixture. The application of SC-CO_2 related to this work starting from 1991 was summarized in Table 2.2. In general, it was noticed that some of the conditions applied in the previous research contributed brilliant ideas for the selection of specific extraction conditions will be used in this study.

Table 2.2: Summary of application mode of SC-CO_2 used as separation technique for plant matrix

No.	Application of SC-CO_2 on Plant Matrices	SC-CO_2 Conditions	SC-CO_2 outcomes	References
A. Extraction process				
1	Extraction of evening primrose oil from the seed of *Oenothera biennis L*.	1.Temperature ranges=40 to 60° C 2.Pressure ranges = 20 to 70 MPa 3. CO_2 flow rate is 18 g/min	1) Over the temperature range, only a limited amount of oil could be removed at the lowest pressure of 20 MPa. 2) Recoveries above 90% were obtained when the pressure increased to 30 MPa and even higher yields were recorded when operating at 50 and 70 MPa	Favati et al. (1991)
2	Extraction of oil from Oil Palm fruits	1. Operating temperatures are 40°, 60° and 80° C. 2. Operating pressures are 300, 400 and 500 bar 3. F = 2.4 kg/hr	1) The optimum condition for extraction of oil was at highest pressure and temperature, 500 bar and 80° C. 2) The most suitable solvent flow rate was determined to be at 2.4 kg/hr.	Bisunadan (1993)

Table 2.2: Summary of application mode of SC-CO_2 used as separation technique for plant matrix (continued)

			3) The optimum condition for the extraction of α-tocopherol was at 500 bar and 80 ^{o} C with the maximum amount is 160 ppm 4) The efficient condition in extracting triglycerides at pressure above 300 bar and temperature above 40 ^{o} C.	
3	Extraction of oil from evening primrose seeds	1.100 ≤ P ≤ 300 bar, ΔP = 100 bar 2. 20 ≤ T ≤ 50 ^{o} C, ΔT = 15 ^{o} C 3. Sample = ground drier with particle size of 25 – 40 mesh	1) Under the range covered, the highest yield was 21 wt% at 300 bar and 50 ^{o} C. 2) The mass transfer value of the oil from the seeds to the CO_2 phase was found to increase linearly with the superficial velocity. 3) The oil extracted with SC-CO_2 was found to have comparable extraction yield and significantly less phosphorus content compared with n- hexane	Lee et al. (1994)
4	Extraction of tocopherols from soyn flakes, rice-bran and wheat germ	1. 250 bar from 40° to 80° C 2. 700 bar at 80° C	1) The soy fakes showed the better yields of vitamin E 2) The optimal condition is at 250 bar and 40° C with vitamin E in enriched is 0.6 wt/wt %	King et al. (1996)
5	Extraction of Vit. E and squalene from *Pandanus odorus Ridl./leaves*	1.40 ≤ T ≤ 80, ΔT= 10 C, 2. P = 80, 100, 200 kg/cm^2 , 3. T= 180 min; 4. Sample = 4 g of ground freeze dried leaves	1) The yield of vitamin E increases with an increase in pressure but decrease with increasing temperature 2) Maximum yield of Vit. E and squalene was obtained were 300 ppm and 1200 ppm, respectively at 200 kg/cm^2 and 40 ^{o} C during 3 hour extraction	Hassan et al. (1999)
6	Extraction of carotenoids and lipids from *buriti fruit (Mauritia flexuosa)*	1. P = 20MPa and 30 MPa 2. T = 313K and 328 K	1) Extraction with SC-CO2 was capable of removing about 80 % of the initial carotene content. 2) The extraction curves have the three distinct region with the largest amount of carotene was extracted in the diffusion controlled	De Franca et. al (1999)

Table 2.2: Summary of application mode of SC-CO_2 used as separation technique for plant matrix (continued)

			region 3) The constant extraction rate period was longer at 20 MPa than 30 MPa, while the mas transfer rate were about seven times larger at 30 MPa	
7	Extraction of lipids from *Pythirm irregulare*	1. Extraction temperatures are 40. 50 and 60° C 2. Extraction pressure are 13.8, 20.6 and 27.6 3. Flow rate are 20, 50, 60, 100 and 400 mL/min 4. Moisture content are 10, 20 and 30%	1) Extraction of oil showed success for moisture contents as high as 30% wet basis 2) The extraction rates decreased substantially after 40 to 50% of the lipids were removed, which indicated the transition to a diffusion controlled regime. 3) The extraction rate that lasted about 90 min for the 30% moisture and only 60 min for the 20% moisture 4) Higher molecular weight components appeared in greater amounts toward the end of extraction process	Walker et al. (1999)
8	Extraction of flavonoids of Scutellaria baicalensis from *Scutellaria e Radix*	1. Temperature was set at 40, 50, 60 and 70° C 2. Extraction pressures was set at 200, 300 and 400 bar 3. Methanol was used as modifier with concentration is 5 and 10 %	1) At 50° C, with modifier 10 % v/v, when the pressure increased from 200, 300 and 400 bar, the yield decrease 14.3, 15.2 and 18.6 %, respectively. 2) When 5% of modifier was used, as the pressure was increased, the decreased amounts were 48.8, 45.9 and 52.3 %.	Lin et al. (1999)
9	Extraction of oleic sunflower seeds	P = 250 bar; T = 40 and 60 ^{o} C.	At 40 ^{o} C and flow rate of 3.02 kg/hr, the rate of extraction is greater than 60 ^{o} C. The experimental operational pressure is below crossover pressure.	Kiriamiti et al. (2001)
10	Extraction of nimbin from Azadirachta indica A. Juss/seeds	10 ≤ P ≤ 26 MPa, 308 ≤ T ≤ 333 K 0.24 ≤ F ≤ 1.24 ml/min Sample = 2 g ground neem powder	Best extraction condition occurred at 23 MPa, 308 K and flow rate of 1.24 ml/min for producing 0.175 mg nimbin/ g neem seeds	Tonthubthimthong et al. (2001)