การสกัดสารแอนทราควิโนนส์จากรากยอด้วยน้ำสภาวะกึ่งวิกฤติ

นาย บุญชัย พงษ์นราวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตร์มหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547
ISBN 974-17-7130-4

SUBCRITICAL WATER EXTRACTION OF ANTHRAQUINONES FROM ROOTS OF *MORINDA CITRIFOLIA*

Mr. Boonchai Pongnaravan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2004
ISBN 974-17-7130-4

Thesis Title	SUBCRITICAL WATER EXTRACTION OF ANTHRAQUINONES FROM ROOTS OF *MORINDA CITRIFOLIA*
By	Boonchai Pongnaravan
Field of Study	Chemical Engineering
Thesis Advisor	Assistance Professor Artiwan Shotipruk, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Faculty of Engineering
(Professor Direk Lavansiri, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Prasert Pavasant, Ph.D.)

…….……. Thesis Advisor
(Assistance Professor Artiwan Shotipruk, Ph.D.)

…….. Member
(Assistance Professor Muenduen Phisalaphong, Ph.D.)

…….. Member
(Joongjai Panpranot, Ph.D.)

บุญชัย พงษ์นราวรรณ : การสกัดสารแอนทราควิโนนส์จากรากยอด้วยน้ำสภาวะกึ่งวิกฤติ. (SUBCRITICAL WATER EXTRACTION OF ANTHRAQUINONES FROM ROOTS OF *MORINDA CITRIFOLIA*) อ. ที่ปรึกษา: อ.ดร. อาทิวรรณ โชติพฤกษ์, หน้า 96. ISBN 974-17-7130-4.

งานวิจัยนี้ศึกษาการสกัดสารแอนทราควิโนนส์ที่มีคุณสมบัติต่อต้านอนุมูลอิสระจากรากต้นยอด้วยวิธีใช้ตัวทำละลาย โดยใช้น้ำสภาวะกึ่งวิกฤติหรือเรียกอีกชื่อว่าน้ำร้อนที่สภาวะความดันสูง การทดลองทำการสกัดที่สภาวะต่างๆ ที่อุณหภูมิ 150 170 และ 200 องศาเซลเซียส และอัตราการไหล 2 3 4 5 และ 6 มิลลิลิตรต่อนาที โดยใช้ความดันคงที่ที่ 40 บาร์ ผลการทดลองแสดงให้เห็นว่า ความสามารถการสกัดสูงขึ้นเมื่ออุณหภูมิเพิ่มขึ้น และอัตราการสกัดจะเร็วขึ้นเมื่อเพิ่มอัตราการไหลจนถึง 5 มิลลิลิตรต่อนาที แต่อัตราการสกัดจะไม่เพิ่มขึ้นเมื่อเพิ่มอัตราการไหลเป็น6 มิลลิลิตรต่อนาที โดยที่สภาวะการสกัดที่เหมาะสมที่สุดคือที่อุณหภูมิ 200 องศาเซลเซียส ที่อัตราการไหลในช่วง 3-5 มิลลิลิตรต่อนาที และจากการทดลองผลกระทบของอัตราการไหล สามารถสรุปได้ว่ากลไกการสกัดถูกควบคุมทั้งด้วยขีดจำกัดในการละลายและขีดจำกัดในการถ่ายเทมวลสาร นอกจากนี้ก็ได้ทำการทดลองหาความสามารถในการละลายของแอนทราควิโนนส์ในน้ำกึ่งวิกฤติที่อุณหภูมิต่างๆ และนำข้อมูลไปหาสมการแบบจำลองเพื่อใช้ทำนายความสามารถการละลายจากผลการเปรียบเทียบประสิทธิภาพในการสกัดด้วยน้ำกึ่งวิกฤติกับวิธีสกัดด้วยสารอินทรีย์แบบต่างๆ อันได้แก่ วิธีแช่ยุ่ย วิธีซอคเลต และวิธีใช้คลื่นอัลตราโซนิคช่วย พบว่า วิธีน้ำสภาวะกึ่งวิกฤติมีประสิทธิภาพการสกัดประมาณ 95 เปอร์เซ็นต์โดยใช้เวลาเพียง 2 ชั่วโมง ในขณะที่วิธีแช่ยุ่ยใช้เวลาถึง 72 ชั่วโมงได้ประสิทธิภาพ 80 เปอร์เซนต์ อนึ่ง วิธีสกัดแบบใช้คลื่นอัลตราโซนิคช่วยสกัดที่อุณหภูมิ 60 องศาเซลเซียส ใช้เวลาลดลงจาก 72 ชั่วโมง เหลือเพียง 2 ชั่วโมง อย่างไรก็ตาม ประสิทธิภาพยังคงต่ำกว่าแบบสกัดด้วยน้ำกึ่งวิกฤติและซอคเลท ณ เวลาที่ใช้สกัดเท่ากันอยู่ นอกจากจะวัดประสิทธิภาพในการสกัดในเชิงปริมาณแล้ว ยังวัดคุณภาพของสารที่สกัดได้ โดยจะวัดในรูปของความสามารถในการต่อต้านอนุมูลอิสระ โดยใช้วิธีดีพีพีเอช ซึ่งผลการทดลอง พบว่า ประสิทธิภาพในการต่อต้านอนุมูลอิสระของสารแอนทราควิโนนส์ที่สกัดด้วยวิธีน้ำกึ่งวิกฤติให้ประสิทธิภาพสูงใกล้เคียงกับสารที่สกัดได้จากวิธีซอคเลท และ วิธีแช่ยุ่ย แสดงให้เห็นว่าอุณหภูมิในช่วงที่ศึกษา ไม่ส่งผลต่อคุณภาพของสารที่สกัด ในขณะที่สารแอนทราควิโนนส์ที่ได้จากวิธีสกัดแบบใช้คลื่นอัลตราโซนิค ให้ผลในการต่อต้านอนุมูลอิสระที่ต่ำที่สุด จากผลการทดลองทั้งหมดสรุปได้ว่า วิธีการสกัดด้วยน้ำสภาวะกึ่งวิกฤติ เป็นวิธีการสกัดที่เหมาะสมในการสกัดสารแอนทราควิโนนส์จากรากต้นยอ

ภาควิชา.....วิศวกรรมเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....วิศวกรรมเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2547.....

4670357221 : MAJOR CHEMICAL ENGINEERING DEPARTMENT
KEY WORD: SUBCRITICAL WATER / *MORINDA CITRIFOLIA* / ANTHRAQUINONES / ANTIOXIDANT

BOONCHAI PONGNARAVAN : SUBCRITICAL WATER EXTRACTION OF ANTHRAQUINONES FROM ROOTS OF *MORINDA CITRIFOLIA*. THESIS ADVISOR : Dr. ARTIWAN SHOTIPRUK, pp 96. ISBN 974-17-7130-4.

A technique of solvent-free extraction of anthraquinones, a group of antioxidant active compound from roots of *Morinda citrifolia* was proposed. The tecqnique utilizes subcritical water, or sometimes called pressurize hot water extraction (PHWE) as extraction medium. A series of extraction experiments were carried out at different conditions, i.e., temperatures of 150, 170, and 200 °C and flow rates of 2, 3, 4, 5, and 6 ml/min, while the pressure remained fixed at 40 bars. The results of the study revealed that the extraction yield increases as the temperature increases and extraction rate increases as the flow rate increases up to 5 to 6 ml/min. The most suitable extraction condition was found to be at the temperature of 200 °C and the flow rate of between 3 and 5 ml/min. A set of experiments at various flow rates suggests that the overall extraction mechanism was influenced by both mass transfer and solubility. In addition to the extraction study, the solubility of anthraquinones in subcritical water at various temperatures was also determined and a mathematical model was proposed. Comparing the extraction yields of subcritical water extraction with conventional method including maceration, soxhlet extraction, and ultrasonic assisted extraction, subcritical water extraction was found to achieve approximately 95 % recovery within only 2 h, whereas it takes extended time period of 72 h to achieve the only 80 % recovery. Although the use of ultrasound at 60 °C was able to reduce the extraction time from 72 h to 2 h, it is still inferior to subcritical water extraction, which requires approximately the same extraction time as soxhlet extraction. Other than the quantitative anlaysis of anthraquinones extracted, the quality of the extract was also measured in term of antioxidant activity using a DPPH method. The results showed that antioxidant activity of the extracts obtained with subcritical water extraction was similar to that with soxhlet extraction and maceration and that there were no effects of the subcritical water temperature in the range tested. On the other hand, extracts obtained with ultrasonic assisted extraction showed the lowest antioxidant activity. All these have led to the conclusion that subcritical water extraction is a benign alternative for extraction of anthraqunones from the roots of *Morinda citrifolia*.

Department…….Chemical Engineering……Student's signature……..
Field of study…..Chemical Engineering……Advisor's signature……..
Academic year……2004……

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Prof. Dr. Artiwan Shotipruk for her encouragement and guidance throughout my work.

Special thanks for Motonobu Goto and Mitsuru Sasaki for any suggestion in my work

My thanks also go to Analytical Laboratory of Chemical Engineering, Chulalongkorn University for analysis and extraction equipment in my work.

Thanks for Thai-Japan Technology Transfer Program “TJTTP” and Faculty of Engineering, Chulalongkorn University for financial supports.

Sincere thanks are made to all members of the Biochemical Engineering Research Laboratory and all my friends in the Department of Chemical Engineering for their any assistance and warm collaborations.

Finally, I would like to express the highest gratitude to my parents, everyone in my family, and all of my friends for their help, their unfailing understanding and affectionate encouragements.

CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 Rationale

Natural products have long been used in folk remedies to treat various kinds of diseases. One of them is *Morinda citrifolia* (Noni) which originated in tropical Asia or Polynesia. During the past decades, whole parts, which include fruits, leaves, bark, and roots, have been shown to contain various biological activities. This work however focuses on extraction of the roots for a group of antioxidative compounds, namely anthraquinones, which possess several therapeutic properties. These include anti-viral *(Koyama et al., 2001; Talou et al., 2001)*, anti-bacterial *(Loy et al., 2001; Babu et al., 2003)*, anti-cancer activities (*Sadeghi-Aliabadi et al., 2004*), as well as analgesic effects. These properties make the compounds very useful in several medicinal applications *(Hiramatsu et al., 1993)*.

Conventionally, this compound is extracted with organic solvent (i.e. ethanol), followed by evaporation to separate solvent from the product. This process is simple but could leave organic solvent residue in the product, which is unacceptable for use on human. More benign alternative solvent needed for replace organic solvent. Supercritical fluids are becoming more attractive new solvents for several industrial application, and for extraction of natural product, supercritical carbon dioxide is the most popular because it is available, safe, and it leaves no solvent residue in the product. Despite these advantages, supercritical carbon dioxide is a non-polar solvent, thus it is not appropriate for extraction of anthraquinones which is a slightly polar compound.

For extraction of slightly polar compounds from natural materials, subcritical water has also been investigated as another benign solvent because of its inertness, non-toxicity, non-flammability and short extraction time *(Rogalinski et al., 2002)*. The term “subcritical water” refers to water at the temperatures between its boiling temperature (373.15 K) and its critical temperature (547.3 K), and at the pressure high enough to maintain in liquid state. At such conditions, water dielectric constant decreases, and thereby decreasing its polarity. As a result, the solubility of organic compounds in subcritical water increases. Despite its operation at rather high

temperature, subcritical water has been shown to give extraction efficiency comparable to conventional method for some thermal labile compounds such as berberine from *coptidis rhizoma*, glycyrrhizin from *radix glycyrrhizae*/liquorice and baicalein from scutlellaiae radix *(Ong and Len, 2003)*. However, *Rogalinski et al.* (*2002*) reported that at the operating temperature above 150 °C, the thermal degradation of some compounds in *Peumus boldus M.* occurs in subcritical water extraction. Thus, to avoid the loss of thermolabile compound, optimal temperature must be used for extraction process.

In this study, we investigated the extraction of antraquinones using subcritical water to determine the effect of extraction temperature and water flow rates. The solubility of anthraquinones in subcritical water at various temperatures was also determined experimentally. This fundamental information is necessary for selecting the optimal extraction conditions. Moreover, the antioxidant activity of the extract has been measured and compared to that of the extract obtained by the conventional solvent extraction techniques.

1.2 Objectives

1.2.1 To determine the solubility of anthraquinones in subcritical water.

1.2.2 To investigate the appropriate conditions for anthraquinones extraction with subcritical water

1.2.3 To compare the efficiency of subcritical water extraction with organic solvent extraction such as maceration ultrasonic assistance and soxhlet extraction.

1.2.4 To perform an antioxidant activity test on the extracts obtained with various extraction methods.

1.3 Working scopes

1.3.1 Subcritical water extraction of anthraquinones at various temperatures include 150, 170, and 200°C, pressures 40 bar, and flow rates 2, 3, 4, 5 and 6 ml/min.

1.3.2 Investigate solubility of anthraquinones in subcritical water at various condition

1.4 Expected benefits

1.4.1 This study provides an alternative for anthraquinones extraction with high product yield, quality, as well as safety.

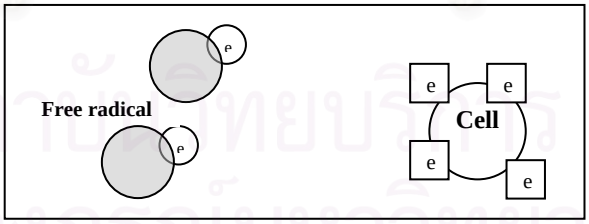
1.4.2 This study suggests fundamental information useful for the future operation and scale-up of the process.

CHAPTER II

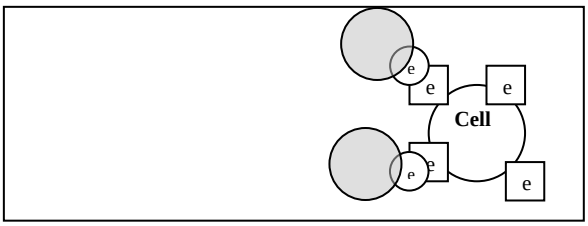
BACKGROUND AND LITERATURE REVIEW

Background

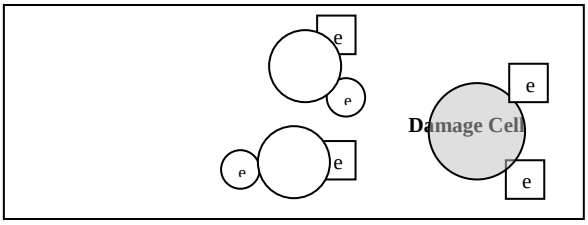
Scientific evidence suggests that a factor that increases the risk for chronic diseases in human is the formation of oxidative chemical species, called free radicals. Free radicals are atoms or molecules that have unpaired electrons. Because of this, they are highly unstable and tend to stabilize themselves by grabbing cellular molecules in order to donate their electrons to these molecules, thus causing oxidative damages to the cells, and resulting in failure of cellular functions. Free radicals are formed endogenously during the process of normal metabolism or under the influence of various environmental factors such as sunlight, smoking, drinking, strenuous exercises, and etc. The oxidative action of free radicals is illustrated in Figure 2.1. These actions result in a number of health problems such as cancer, tumors, heart disease, inflammation, shock, atherosclerosis, diabetes, and ischemia (*Jin & Chen et al., 1998; Kasai et al., 2000; Wallace, 1997*). However, studies have demonstrated that various plant-derived compounds exhibit the ability to trap free radicals, thus protecting cells from oxidative damages (Figure 2.2). Such ability is called anti-oxidant activity and the compounds possessing this activity are called antioxidants.



(a)

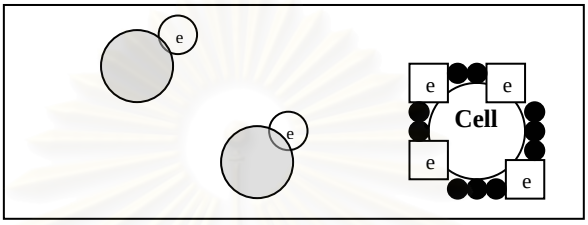


(b)

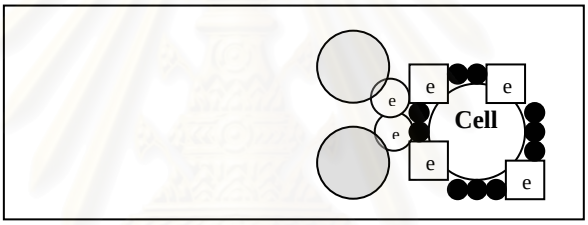


(c)

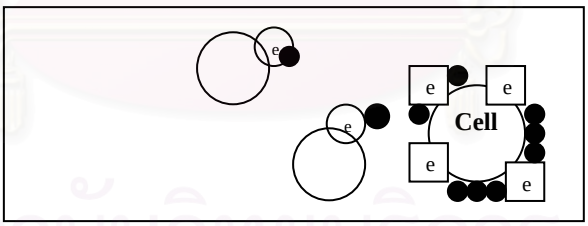
Figure 2.1 Schematic of free radical oxidative damage



(a)



(b)



(c)

Figure 2.2 Schematic of antioxidant mechanism (● is antioxidant molecule compound)

Up to now, it has been found that numerous products derived from various plants possess antioxidant activity. Some examples of these plants are *Hemerocallis fulva Kwanzo* (*Cichewicz et al., 2004*), *Salvia officinalis L.* (*Ollanketo et al., 2002*), and *Morinda citrifolia* (*Zin et al., 2002*). Because *Morinda citrifolia* is one of the several tropical plants grown widely in Thailand, this study, therefore, focuses on extraction of active compounds expressing antioxidant activity from M. *citrifolia*.

2.1 *Morinda citrifolia*

Morinda citrifolia, known as Noni (see figure 2.3), are grown widely in tropical areas. Various plant parts such as leaves, fruit, and bark, possess several therapeutic properties, and have been used for traditional remedies for a long period of time. The leaves were used to treat eye problems; heated leaves were used to relieve coughs, nausea, colic; juice of the leaves was taken for treatment of arthritis; and roots were found to relieve chronic diseases (cancer, diabetes, and cardiovascular diseases, and etc.). Scientific proof suggests that a group of antioxidative compounds in the roots of *Morinda citrifolia*, responsible for these therapeutic properties is anthraquinones (*Zin et al, 2002*). This work focuses on extraction of anthraquinones in roots of *Morinda citrifolia* plant.

Figure 2.3 *Morinda citrifolia* plant

2.2 Anthraquinones

Anthraquinones are the main constituent in root of *Morinda citrifolia*. It was first used by Polynesians as yellow dyestuff. Recent scientific studies however reported their more important role as anti-viral, anti-bacterial, and anti-cancer agents. There are great many varieties of anthraquinones derivatives, but all share the same basic molecular configuration as shown in Figure 2.4. Some these derivatives are show in Table 2.1.

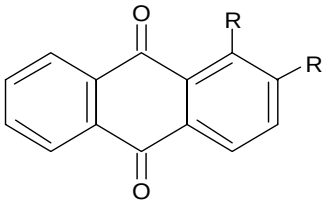
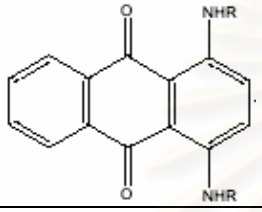
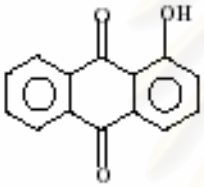
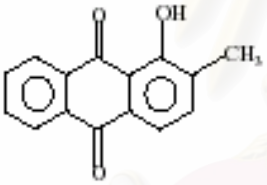
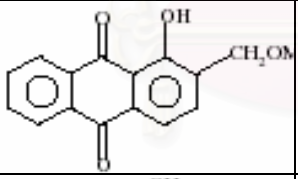
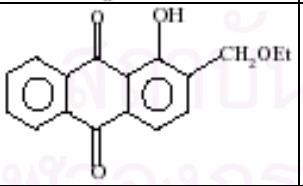
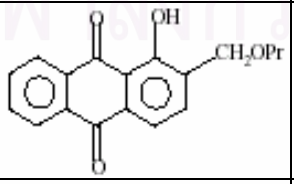
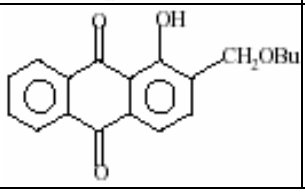
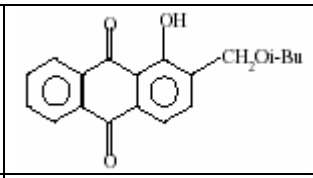
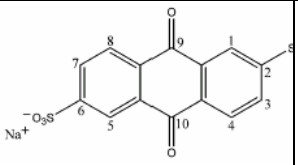
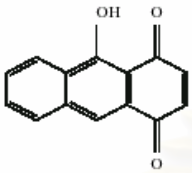
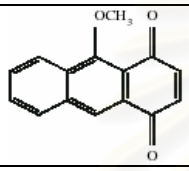
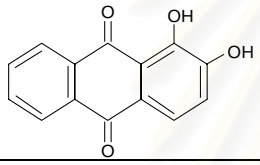


Figure 2.4 Molecular structures of anthraquinones

Table 2.1 Anthraquinones Derivatives

No.	Structural Formula	Name	Reference
1.		6-amino-hexanoic acid [4-(5-aminopentanoylamino)-9,10-dioxo-9,10-dihydro-anthracene-1-yl]-amide	Sadeghi-Aliabadi et al., 2004.
2.		1-hydroxy-9,10-anthraquinone	Shamsipur et al., 2004.
3.		1-hydroxy-2-methyl-9,10-anthraquinone	Shamsipur et al., 2004.
4.		1-hydroxy-2-(methoxymethyl)-9,10-anthraquinone	Shamsipur et al., 2004.
5.		1-hydroxyl-2-(ethoxymethyl)-9,10-anthraquinone	Shamsipur et al., 2004.
6.		1-hydroxy-2-(1-propoxymethyl)-9,10-anthraquinone	Shamsipur et al., 2004.
7.		1-hydroxy-2-(1-butoxymethyl)-9,10-anthraquinone	Shamsipur et al., 2004.

8.		1-hydroxy-2-(*n*-amyloxymethyl)-9,10-antheaquinone	Shamsipur et al., 2004.
9.		9,10-anthraquinone-2,6-disulfonic acid, disodium salt	Bruckard et al., 2004.
10.		9-hydroxy,1,4-anthraquinone	Jiménez et al., 2002.
11.		9-methoxy,1,4-anthraquinone.	Jiménez et al., 2002.
12.		1,2-dihydroxyanthraquinone (Alizarin)	Epstein, 2003

In this study, Alizarin (no.12 in Table 2.1) will be used as a representative compound for anthraquinones because of its standard compound is available. The compound has been commonly used as a representative in previous work for the determination and quantification of anthraquinones from natural extract (*Zenk et al, 1975*). Physical and chemical properties of alizarin are summarized in Table 2.2.

Table 2.2 Properties of alizarin

Name	1,2-Dihydroxyanthraquinone
Formula	$C_{14}H_8O_4$
Molecular weight	240.22
Melting point (°C)	287 – 289
Boiling point (°C)	430
Solubility at 25 °C (M)	$2.5x10^{-6}$

Conventionally, organic solvents are used to extract anthraquinones from roots of *Morinda citrifolia* plant. The mechanism of extraction process can be explained as follows.

2.3 Extraction of natural solid materials

2.3.1 Mechanism of Extraction

In solvent extraction of the desired solute constituent from the natural solid materials, the liquid solvent is brought into contact with the solid materials. The solute can then diffuse from the solid phase into the liquid phase, which causes a separation of the component originally in the solid. This process is sometimes called liquid-solid leaching or simply leaching or liquid-solid extraction. The mechanism of such process involves 5 steps as schematically shows in Figure 2.5. The description of each step is as follows.

Step 1: Solvent molecularly transfers from bulk fluid through thin layer of the fluid that covers the surface of solid materials. The driving force of this molecular transfer is the difference in solvent concentrations between the bulk fluid and the thin fluid film. This film acts as resistance to the molecular transfer of solvent, thus the transfer rate is affected by the film thickness. The thickness of the fluid film depends on the velocity of bulk liquid solvent. For example, higher solvent velocity gives thinner film, and thus, less resistance.

Step 2: Solvent molecularly transfers from the surface of materials through interconnected voids or pores in the solid materials. The transfer rate in this step is affected by the pore-size, porosity, and tortuosity of the solid matrix.

Step 3: Solvent dissolves the solute inside the porous solid materials. This step is sometime called solubilization step or solubility step, and the degree to which this step affect the overall extraction depends on chemical properties of the solute and the solvent.

Step 4: The solution of the desired solute molecularly diffuses out of the solid materials through the porous matrix to the surface of solid materials.

Step 5: Dissolved solute transfers from the surface of the solid through the

boundary layer of fluid to the bulk fluid.

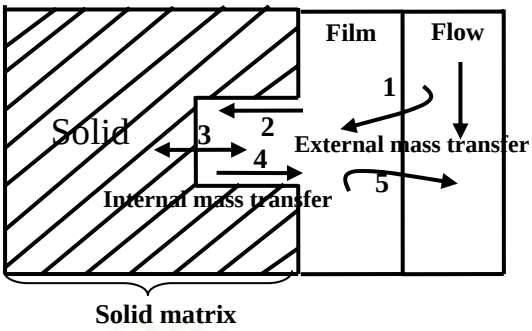
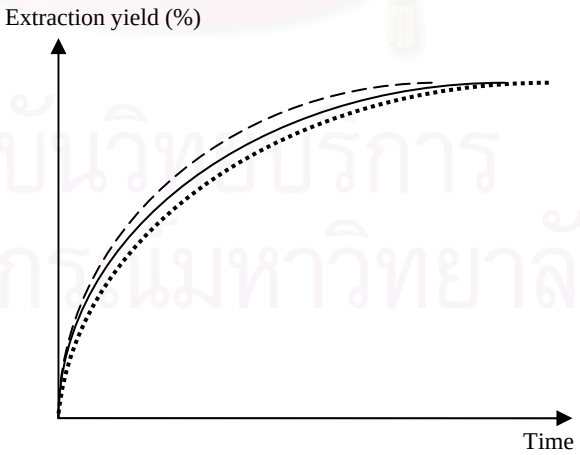


Figure 2.5 Five step of extraction mechanism

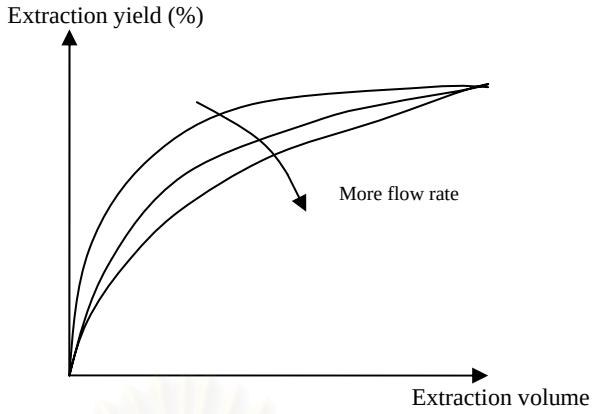
Generally step 1 and step 5 are considered unimportant because of volumetric flow rate in typical operation is fast enough for the film mass transfer resistance to be small compared with the resistance in the other steps.

Step 2 and step 4 are analogous but they progress in the opposite directions. These steps are sometimes called intraparticle diffusion steps. Either intra-particular diffusion (step 2 and 4) or solubilization (step 3) or both can be rate determining step.

If the rate of extraction is controlled solely by intra-particular diffusion, the plot between extraction yield (%) versus time and extraction yield (%) versus extraction volume are likely to follow the trends shown in figure 2.6 (a) and (b) respectively.



(a)

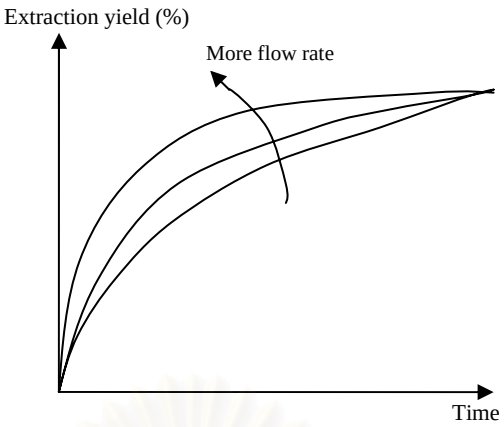


(b)

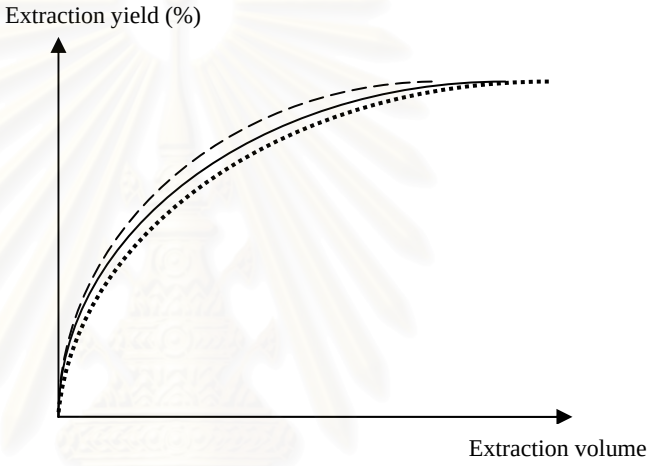
Figure 2.6: mass transfer limiting step (a) relation between extraction yield (%) versus time and (b) extraction yield (%) versus extraction volume

Figure 2.6 a indicates that increasing the flow rate does not increase the amount extracted at any given time of extraction. That is because the diffusion of solvent into the particle is limited. Thus, when consider the amount of product extracted by the same amount of solvent that passes the extractor, the amount extracted is higher at lower flow rate because at this rate, the solvent spend more time in the extractor and more effectively extract the product.

On the other hand, if the extraction rate is controlled by solubilization step (step 3), the plot between extraction yield (%) versus time and extraction yield (%) versus extraction volume are likely to follow the trends shown in figure 2.7 (a) and (b) respectively. When this is the case, higher amount of solvent is required to extract more product, thus higher flow rate will give higher yield at the same time of extraction (Figure 2.7 a). However, for the same volume of solvent passed the extractor the same amount of product would be extracted despite of the different flow rates employed (Figure 2.7 b).



(a)



(b)

Figure 2.7: solubility limiting step (a) extraction yield (%) versus time and (b) relation between extraction yield (%) versus extraction volume

In short, one can see that there are two main steps that control the efficiency of extraction process. The first is the transport of solvent into the porous solid materials, and of the dissolved solute out of the solid particles in to the bulk fluid. This mass transport is dependent upon many factors, such as viscosity, pore diameter, molecular size of solute and solvent, and tortuosity. The second is the solubility of the desired solute in the solvent, which depends on the polarity of the solvent and the dissolving solute. Thus in maximizing the extraction efficiency, one should understand the mass transfer mechanism and have knowledge about solute solubility in the solvent, in order to effectively select an appropriate extraction system and solvent.

2.3.2 Solubility

The solubility of a solute in a solvent is defined as the concentration of the solute in saturated solution at the temperature of interest. When the solute saturated, it is said to be in equilibrium with the solid solute. This is the state in which a concentration of solute in solution does not change with time, although there is continual exchange of solute between the solution and the solid. The solubility of solute typically depends on the relation between solute and solvent polarities, which is characterized by a specific parameter, namely dielectric constant (ε). The dielectric constant is sometimes called the relative permittivity and is defined as the material ability to reduce the electric force between two charges separated in space.

Selecting an appropriate solvent for extraction of particular compounds based on this characteristic will in part determines the success of any process extraction.

2.4 Extraction of natural product with near critical and supercritical solvents

Early 1970s, supercritical or near critical fluids have gained considerable attention for a variety of processes and technologies. Supercritical fluid is the fluid whose thermodynamic conditions are above the fluid critical region, defined as the area above both the critical pressure and critical temperature, as shown in figure 2.8.

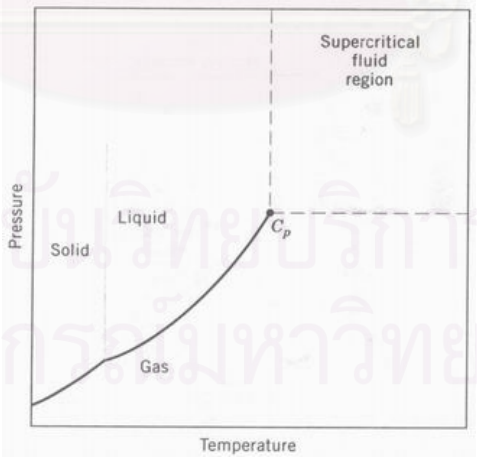


Figure 2.8: Schematic pressure-temperature diagram of supercritical fluid region

A particularly attractive and useful feature of supercritical fluids is that these fluids have properties some where between those of a gas and a liquid (see Table 2.3). A supercritical fluid has more liquid-like densities, and subsequent solvation

strengths, while possessing transport properties, ie, viscosities and diffusivities, that are more like gases. Thus, supercritical fluid may diffuses into a matrix more quickly than a liquid solvent, yet still possess a liquid-like solvent strength for extracting a component from the matrix.

Table 2.3 Comparison of properties of gases, supercritical fluids, and liquids

Physical property	gases	supercritical fluids	liquids
Density, g/cm^3	0.001	0.2-1.0	0.6-1.6
Diffusivity, cm^2/s	0.1	0.001	0.00001
Viscosity, g/ (cm·s)	0.0001	0.001	0.01

During the past decade, supercritical fluid carbon dioxide (SC-CO_2) is of particular interest. This compound has a mild (31°C) critical temperature (see Table 2.4). It is nonflammable, nontoxic, and, environmental friendly and CO_2 is the second least expensive solvent after water. Despite these advantages, supercritical carbon dioxide is a non-polar solvent, thus it is not appropriate for extraction of anthraquinones, which is a group of slightly polar compounds. Another benign alternative solvent need to be investigated.

Table 2.4 Critical properties for common supercritical fluids (Kirk-Othmer)

Solvent	T_c, °C	P_c, MPa	ρ_c, g/cm^3
Ethylene	9.3	5.04	0.22
Carbon dioxide	31.1	7.38	0.47
Ethane	32.2	4.88	0.20
Propane	96.7	4.25	0.22
Ammonia	132.5	11.28	0.24
Isopropanol	235.2	4.76	0.27
Methanol	239.5	8.10	0.27
Toluene	318.6	4.11	0.29
Water	374.2	22.05	0.32

Water, near its critical point, (supercritical and subcritical) is another benign alternative solvent because of its inertness, non-toxicity and non-flammability. The use of supercritical water for extraction of natural product has its drawbacks, (one being its high operating pressures and temperatures involved, and another difficulty being the extremely corrosive nature of water at supercritical conditions) thus, subcritical water has become of more interest for the purpose of natural product extraction.

Subcritical water extraction (SWE) is sometimes called hot water extraction, pressurised (hot) water extraction, high-temperature water extraction, superheated water extractions or hot liquid water extraction. This emerging technique is based on the use of water at temperatures between 100 and 374 °C (critical temperature of water) as an extracting solvent. The pressure of water is controlled so that it is high enough to keep it in the liquid phase. Organic compounds are much more soluble in water under these conditions than at room temperature. At high temperature, the increase in entropy causes solubility to rise with temperature as water becomes less polar at higher temperature as its structure breaks up. As a result, the dielectric constant of water, ε, i.e., its polarity, (and dramatically) is lowered. For example, pure water at ambient condition has a dielectric constant of 79. By increasing the temperature to 250 °C at a pressure of 5 MPa (necessary to maintain the liquid state) yields a significant reduction of this value to about 27. At this condition water has polarity similar to that of ethanol at 25 °C and 0.1 MPa. This value is low enough for the water to dissolve many compounds of intermediate or low polarity.

Literature review

Subcritical water extraction was first employed for the extraction organic pollutants such as polycyclic aromatic hydrocarbons (*Hawthorne et al., 1994; Yang et al., 1995, Kipp et al., 1998*) and pesticides (*Jiménez et al. 1997*) from environmental samples. Other application involves the extraction of inorganic metal pollutants, such as lead, copper, cadmium, arsenic, selenium and mercury from soil (*Priego-López et al., 2002*). Review of previous work on the environmental applications of subcritical water is summarized in Table 2.5.

Recently, subcritical water extraction was employed for the isolation of natural product for the production of fragrances, flavors, and pharmaceuticals. Successful cases have been reported for essential oils from majoram *(Jiménez-Carmona et al., 2002)*, savory and peppermint *(Kubátová et al., 2001)*, and oregano (*Ayala, et al., 2001*). Other than essential oils, other bioactive compounds have been extracted by this technique. They are hypericin and pseudohypericin from St. John's wort *(Mannila et al., 2002)*, iridoid glycosides from *Veronica lonifolia (Suomi et al, 2000)*, and kava lactones from kava roots *(Kubátová et al., 2001)*. Review of subcritical water extraction of natural product is summarized in Table 2.6. In comparison with conventional method, most study reports the same agreement of this technique as environmental friendly, inexpensive, and short extraction time at the same yields.

Until now, the study of subcritical water extraction of anthraquinones, an anticancer agent, from the roots of *Morinda citrifolia* is nonexistent, it is thus the focus of this research to carry out the detailed investigation of such process. Because therapeutic effects of anthraquinones have been demonstrated to be attributed to the compound antioxidant activity (*Zin et al., 2002*), the most appropriate extraction conditions not only should yield the high amount of anthraquinone but also should not degrade the extracted product. It is known that antioxidant activities of any compounds, including anthraquinones may be degraded at high temperature (*Rogalinski et al., 2002*), thus the extract should be assayed for any possible degradation under different extraction temperatures.

There are several tests commonly used for assaying the antioxidant activities from natural products. Some of these methods determine the resistance of lipid or lipid emulsions to oxidation in the presence of the antioxidant being tested. The example of this includes MAD, TBARS and FTC (Zin, et al., 2002), which use malondialdehyde, thiobarbituric acid, and ferric thiocyanate respectively as reactive substances. This type of assays has been used extensively since the 1950's to estimate the peroxidation of lipids in membrane and biological systems. These methods however can be time consuming because they depend on the oxidation of a substrate which is influenced by temperature, pressure, matrix, etc. and thus may not be practical when large numbers of samples are involved. Other methods measure the radical-scavenging activity of antioxidants against free radicals like 1,2-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O2) such as the xanthine / xanthine oxidase (AC/XOD)

system, the hydroxyl radical (OH) by using Fenton's reaction (Prakash, 2001), or the peroxyl radical (ROO) such as 2,2'-azobis (2-amidinopropane)dihydrochloride (AAPH) (Pellegrini, et al., 1999). For the detection of the radical-scavenging activity, these methods typically involve the use of special equipment such as electron spin resonance spectroscopy (ESRS) and chemiluminescence spectroscopy. Of these free radical scavenging methods, the simplest and most widely used method is the DPPH method. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. When the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H (see Figure 2.9). The resulting decloliraztion is stoichiometric with respect to number of electrons. Thus measuring the decolorization simply with visible spectrophotometer quantifies the antioxidant activity of the compound. Moreover, the method can be used for solid or liquid sample, and is not specific to any particular antioxidative component, but applies to the overall antioxidant capacity of the sample, making the methods very popular in recent years to quantify antioxidants in complex biological system.

The DPPH method was used in this study to determine the effect of various subcritical water extraction conditions (temperatures) on the possible degradation of antioxidant activity of the extracted antraquinones. The detailed experimental procedures of the proposed study are given in the following chapter.

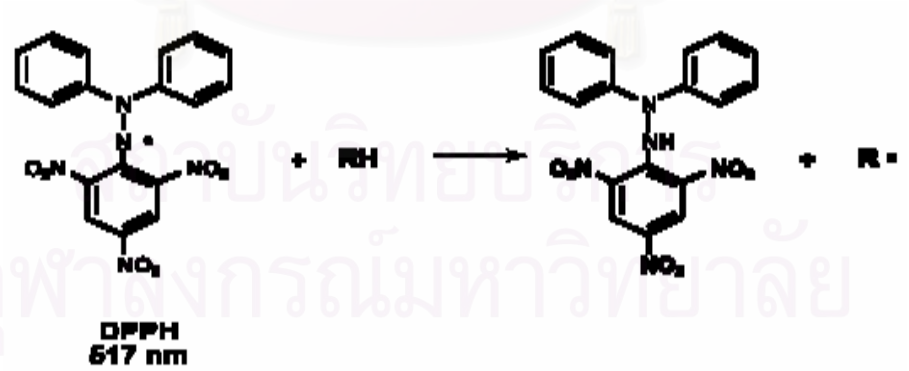


Figure 2.9 antioxidant scavenging of DPPH

Table 2.5 Review on Investigation of Subcritical Water Extraction of Environmental Pollutants from soil

Author	Condition	Sample size	contaminant	Objective
1. Miller et al., 1998	F = 0.1 ml/ min T = 25-225°C P = 30-60 bar t = 30 min	N/A	PAHs	To report solubility of contaminant in subcritical water.
2. Hawthorne et al., 2000	F = 1 ml/ min T = 250, 300°C P = 50 bar t = 30, 60 min	<6 mm	PAHs	To compare soxhlet extraction, PLE, SFE, and SWE extraction of PAH from ore.
3. Krieger et al., 2000	F = 0.4 to 3.5 ml/ min T = 50, 100, 150°C P = 65, 135, 500 atm t = 30 min	N/A	Cloransulam-methyl	To report the results of SWE of triazolopyrimidine sulfonanilide herbicides from soil.

Table 2.5 Review on Investigation of Subcritical Water Extraction of Environmental Pollutants from soil (continue)

Author	Condition	Sample size	contaminant	Objective
4. McGowin et al., 2001	F = 1 ml/ min T (PAH, Pesticide) = 110, 150, 250, 350°C 110, 130, 150, 250°C t = 20 min	<2 mm	PAHs & pesticide	To screen for PAH and pesticide by SWE in compost.
5. Dadkhah et al., 2002	F = 1 ml/ min T = 230, 250, 270°C P = 40 bar t = 45, 90 min	<4 mm	PAHs	To reporting the results of small-scale batch extraction of soils polluted with PAHs by using SWE.
6. Richter et al., 2003	F = 2 ml/ min T = 50 to 300°C P = 1200 psi t = 25 min	<2 mm	Pesticides	To evaluate efficiency of water at subcritical region from soils of a group of typical pesticides used in agriculture.

Table 2.5 Review on Investigation of Subcritical Water Extraction of Environmental Pollutants from soil (continue)

Author	Condition	Sample size	contaminant	Objective
7. Hashimoto et al., 2004	F = 2 ml/ min T = 25, 150, 300, 350°C P = 0.2 MPa t = 30 min	<0.1 mm	Dioxins	To understand of behavior of dioxins during SWE and optimize their efficiency.

Table 2.6: Review on Investigation of Subcritical Water Extraction of Natural Product

Author	Condition	Application	Plant	Parts used	Product	
1. Basile et al., 1998	F = 1, 2, 4 ml/ min T = 125-175°C P = 20 bar t = 200 min	Fragrance and flavor	*Rosmarinus officinalis*	Leaves	- α-Pinene - Camphene - Limonene - 1, 8-Cineole	- Camphor - Borneol - Verbenone - Isobornyl acetate

Table 2.6: Review on Investigation of Subcritical Water Extraction of Natural Product (continue)

Author	Condition	Type application	Plant	Parts used	Product
2. Miller et al., 2000	F = 0.1 ml/ min T = 25 to 200°C P = 70 bar t = N/A	Fragrance and Flavor	N/A	N/A	- *d*-Limonene - Carvone - Eugenol - Nerol - 1,8-Cineole
3. Kubatova et al., 2001	F = 1 ml/ min T = 100, 150, 175°C P = 65 bar t = 30 min	Fragrance and Flavor	Satureja hortensis and Menthe piperita	N/A	- Cymene - Thymol - Borneol - Linalool - etc.
4. Kubatova et al., 2001	F = 1 ml/ min T = 175°C P = 60 bar t = 20 min	Fragrance	*Piper methysticum*	root	- Dihydrokawain -Kawain - Yangonin - etc.

Table 2.6: Review on Investigation of Subcritical Water Extraction of Natural Product (continue)

Author	Condition	Type application	Plant	Parts used	Product
5. Pawlowski et al., 1998	F = 2-20 ml/ min T = 50, 75°C P = 50 atm t = 20 min	Food	Agriculture commudities e.g. banana, lemon, etc.	Fruit pulp	- Thiabendazole (TBZ) - Carbendazim (MBC)
6. Clifford et al., 1999	F = 2 ml/ min T = 150°C P = N/A t = 100 min	Essential oil	*Syzygium aromaticum*	Bud	- Eugenol - Eugenyl acetate - Caryophyllene
7. Jiménez-Carmona et al., 1999	F = 2 ml/ min T = 150°C P = 50 bar t = 15 min	Essential oil	*Thymus mastichina*	Leaves	- α-Pinene - β-Pinene - Linalool - Geraniol - etc.

Table 2.6: Review on Investigation of Subcritical Water Extraction of Natural Product (continue)

Author	Condition	Type application	Plant	Parts used	Product
8. Fernández-Perez et al., 2000	F = 2 ml/ min T = 150°C P = 50 bar t = 30 min	Essential oil	*Laurel*	Leaves	- 1, 8 Cineole - α-Phellandrene - β-Pinene - et al,
9. Gámiz-Gracia et al., 2000	F = 0.5-3.0 ml/ min T = 150°C P = 50 bar t = 50 min	Essential oil	*Foeniculum vulgare*	Fennel	- α Pinene - Limonene - β Pinene -Comphor - β Mircene - Linalyl propanoate
10. Ayala et al., 2001	F = 1-4 ml/ min T = 100-175°C P = 1.0-5.1 MPa t = 24 min	Essential oil	*Lippia graveolens*	Leaves	- 1, 3-Cyclohexadiene - α-Phellandrene - 3-Carene - etc.

Table 2.6: Review on Investigation of Subcritical Water Extraction of Natural Product (continue)

Author	Condition	Type application	Plant	Parts used	Product	
11. Ollanketo et al., 2002	F = 1 ml/ min T = 70, 100, 150°C P = 100 kg/cm^2 t = 60 min	Essential oil	*Salvia officinalis*	N/A	- Rosmarinic acid - Carnosic acid	- Carnosal - Methyl carnosate
12. Eng Shi Ong et al., 2003	F = 1 ml/ min T = 95-140 °C P = 10-20 bar t = 40 min	Essential oil	*Coptidis, Glycyrrhizae* and *Scutellariae radix*	Root	- Berberine	- Glycyrrhizin - Baicalein
13. Ozel et al., 2003	F = 2 ml/ min T = 100, 125, 150, 175°C P = 20, 60, 90 bar t = 40 min	Essential oil	*Thymbra spicata*	leaves	- Carvacrol - Thymol - E-3-caren-2-ol	- p-Cymene - Caryophyllene

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental setup

The diagram of the experimental setup is shown in Fig. 3.1. This system consists of two HPLC pumps (PU 980, JASCO, Japan), an oven (HARAEUS D63450), a stainless steel capillaries (1/16 inch inside diameter), extraction vessel (10 ml, Thar Design, USA), a back-pressure regulator (AKICO, Japan), and a collecting flask. The first pump is used for deliver water into the extractor, and the second pump connected to the outlet coil is used to deliver an organic solvent (absolute ethanol) to wash off any precipitated product inside the line. The oven is used to heat the extraction vessel installed inside it. The preheating coil in the oven at the inlet of the extractor and the cooling coil at the outlet of the extract outside the oven are used to heat the water to the set value and to cool down the product to prevent the loss of any thermal labile substances.

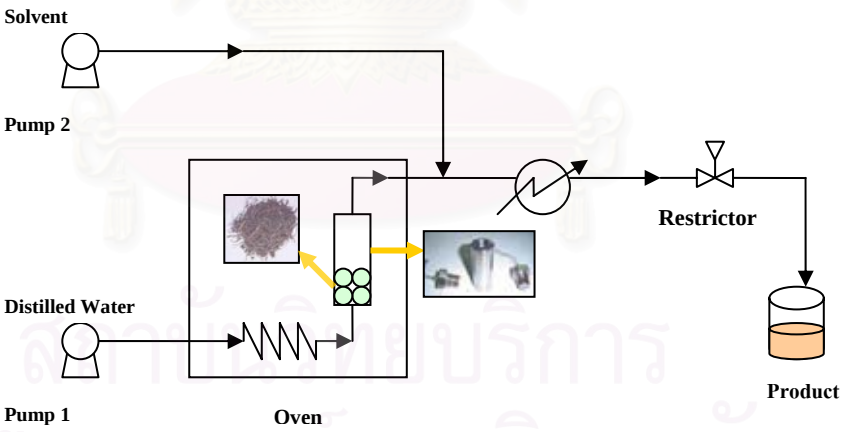


Figure 3.1 Diagram of experimental setup subcritical water extraction

3.2 Experiment

3.2.1 Preparation of solids materials

The hairy roots of *Morinda citrifolia* were harvested, washed, and oven dried at 50 °C for 2 days. The sample was then ground to small size using mortar and pestle with liquid nitrogen. The plant materials were stored in dry place until use.

3.2.2 Subcritical water extraction

Several extraction experiments was carried out to determine the effect of temperature, pressure, and water flow rates on the product yield and quality. The conditions tested are summarized in Table 3.1.

Prior to each extraction, distilled water was passed through a degassing equipment (ERC 3215, CE, Japan) to remove dissolved oxygen which may cause product oxidation during the extraction process. The degassed water was then delivered, at a constant flow rate with the first HPLC pump to the extractor preloaded with 0.5 g of ground Noni roots. The pressure of the system was adjusted to the desired condition by using the back-pressure regulator at the outlet coil. Before heating the extraction system, all connections were checked for possible leakage. The oven was turned on and the temperature set to the desired operating condition. At this point, the extraction started, the second pump delivering the wash solvent was turned on. The extract was then collected in fractions in collecting flasks and analyzed spectrophotometrically.

Table 3.1: Parameter condition in experiment

Parameters	condition
Temperature	150, 170, and 200°C
Flow rate	2, 3, 4, 5, and 6 ml/min
Pressure	40 bars
Approx. Materials size	0.2 mm

3.2.3 Organic solvent extraction

3.2.3.1 Maceration

Maceration was performed by placing 0.5 g roots into a 125 ml Erlenmeyer flask, containing 50 ml of ethyl alcohol. It was then allowed to release the products into the solvent overnight. The solution was then replaced with 30 ml of fresh ethanol daily for two days. All of extracts were collected to measure concentration of anthraquinones.

3.2.3.2 Soxhlet extraction

The 0.5 gram ground roots were placed into a thimble of a soxhlet apparatus and were extracted with 150 ml of solvent (ethanol). Extraction was carried out for 4 hours and the resulted extract was measured for anthraquinones concentration by a spectrophotometer

3.2.3.3 Ultrasonic assistance

Ultrasonic bath was used as an ultrasound source to extract anthraquinones. The bath, 275DAE (Crest Ultrasonics, USA), was basically a rectangular container (23.5 cm × 13.3 cm × 10.2 cm), to which two 38.5 kHz transducers were annealed at the bottom, and the bath power rating was 270 W on the scale of 0 to 9. 0.5 g of roots in 50 ml solvent contained in a 125 ml flask was extracted for 2 hrs at 60 °C and the power setting of 9. The extract concentration was measure spectrophotometrically.

3.2.4 Measurement of Anthraquinones concentration

The analysis method for determining the amount of anthraquinones was modified from that described by Zenk et al. (1975). Because antraquinones in the extract may not be in soluble form in ambient water after it exited the extraction system, ethanol was therefore added to the extract to keep the compound dissolved in the solvent mixture. The ratio of 1:4 (ethanol:water) was determined to be appropriate as this is the amount of ethanol that was just sufficient to keep the extract soluble. The concentrations of these solutions were analyzed spectrophotometrically by measuring

the absorbance at 435 nm, with Alizarin or 1, 2 dihydroxyanthraquinone as a standard, and with ethanol/water (1:4 v/v) as a reference. The standard calibration curve is shown in Figure A-1.1 and A-1.2 in Appendix A.

After each extraction, the amount of anthraquinones remained in the root residue was determined by solvent extraction with ethanol. The root residue was taken out of the extractor and placed into a 125 ml Erlenmeyer flask, containing 50 ml of ethyl alcohol. It was then allowed to release the products into the solvent overnight. The solution was then replaced with 30 ml of fresh ethanol daily for two days.

3.2.5 Measurement of alizarin solubility

As mentioned, knowledge of the solubility the compound in extraction solvent is crucial for the design of extraction process, the solubility of anthraquinones in subcritical water was therefore measured in this study using alizarin as a reference. The experiment was carried out in a batch system as shown in Figure 3.2. Instead of the root materials, 0.5 g of alizarin standard was loaded into a 100 ml pressure vessel containing 15 ml of water. The vessel was then tightened and heated to a desired temperature by means of a heating jacket around it, and was then allowed to reach equilibrium. This takes about 20 minutes. The solubility of alizarin was experimentally determined for the water temperatures at 125 °C, 150 °C, 175°C and 200 °C. The equilibrium concentrations of the alizarin solutions were measured using a spectrophotometer (Genesys 20, USA).

Figure 3.2 Batch extractor

3.2.6 Antioxidant activity measurement

Antioxidant activity of antraquinones extracts obtained using subcritical water extraction and other conventional methods was tested and compared, using DPPH method modified from that described in previous research (*Ollanketo et al., 2002*). For the purposes of comparing the antioxidant activity in various extracts, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. To find this value, the extract was diluted in series with ethanol and 1 ml of each diluted extract was added to 2 ml of 110 μM DDPH solution. The solutions were mixed using a vortex and the mixtures were then incubated for 2 hours in darkness at room temperature, after which the absorbance was measured at the wavelength of 517 nm using ethanol as a reference.

The values of percent inhibition (PI) was calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100 \qquad (3.1)$$

A_t and A_r are absorbance of test sample and absorbance of the DPPH reference, respectively. These values were plotted against the sample concentration and linear regression of the data were made and used to determine the value for IC_{50}.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the experimental results dealing with anthraquinones extraction with subcritical water. Firstly, the experimental results of solubility of anthraquinones standard in subcritical water at various temperatures was presented and discussed. Secondly the effect of temperature on extraction rate and yield was determined. In addition, the extraction profiles were obtained for various water flow rates, from which the mechanism of extraction could be suggested. Thirdly, the performance of subcritical water extraction was compared with other conventional methods of solvent extraction such as maceration, soxhlet extraction, and ultrasonic extraction. And lastly, the antioxidant activity of the extracts obtained with subcritical water extraction was compared with that of the extracts obtained by conventional extraction techniques.

4.1 Solubility of antraquinones in subcritical water

Knowledge of the solubility of the compound of interest in a solvent is important for the design of extraction process. In this research, the solubility of antraquinones in subcritical water at various tempereatures was determined using a static method in which the solubilization of the anthraquinones standard was carried out in a closed pressure vessel as described in chapter 3. The results are presented in Table 4.1.

Table 4.1 Solubility of standard alizarin in subcritical water

Temperature (°C)	Solubility (mg/ml)
125	0.143 ± 0.008
150	0.424 ± 0.057
170	0.521 ± 0.056
200	0.835 ± 0.071

As expected, the temperature has a dramatic effect on anthraquinones solubility as water polarity decreases with increasing temperature. This effect allows subcritical water to be used for extraction of natural compounds.

As shown in Figure 4.1, the results obtained from this study are in agreement with those from the previous research in which the solubility was measureed in a dynamic system (Shotipruk et al., 2004). At higher temperature however, the results differ considerably, possibly because the dynamic system encounters a problem with anthraquinones clogging within the apparatus tubing, thus giving underestimated values for solubility. Thus at higher temperature, a static determination of solubility yields higher accuracy.

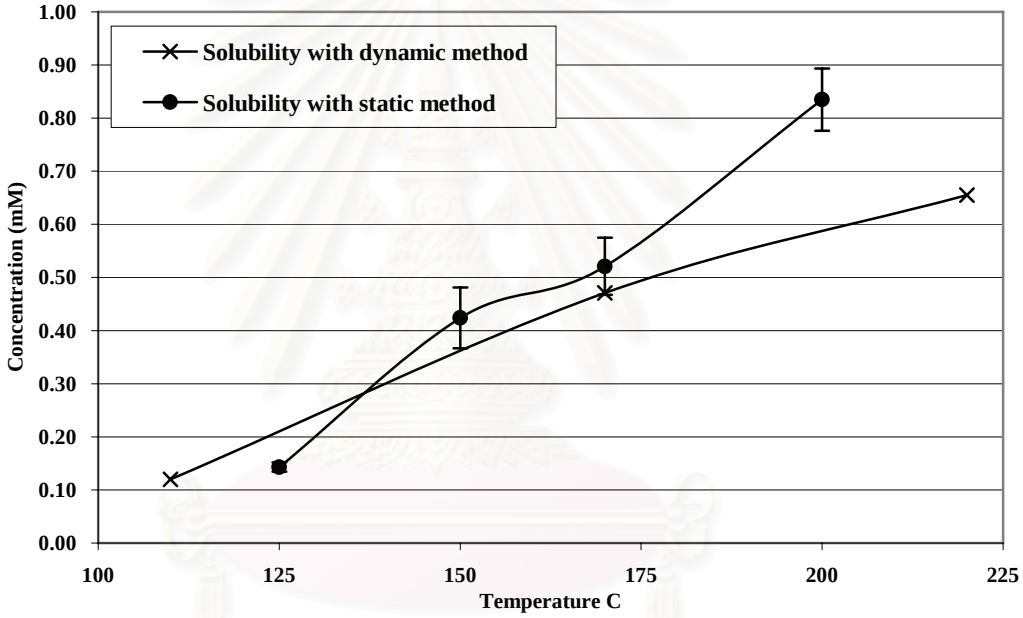


Figure 4.1 Solubility of anthraquinones in subcritical water measured in static and dynamic systems.

Solubility model approximation

Various approximation models for predicting the solubility of polycyclic aromatic hydrocarbon in subcritical water have been proposed (Miller D.J. et al., 1998, Mathis J. et al., 2004). To a zeroth approximation, the following model was proposed:

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) \qquad (1)$$

in which x_2 is the mole-fraction of organic compound of interest in water and T_0 represents ambient temperature. The development of this equation assumes the Gibbs function for solution does not change over the temperature range and there is no absorption of water by the solute. The assumption for the slight change in the Gibbs function was justified because the enthalpy of the solution for these insoluble molecules does not vary widely with temperature and is much greater than the entropy contribution. Equation (1) can be used to approximate mole fraction solubility at any temperature, T, from the known mole fraction solubility at ambient temperature. Our data for anthraquinones solubility can be fitted reasonably well to the zeroth approximation as shown in Figure 4.2.

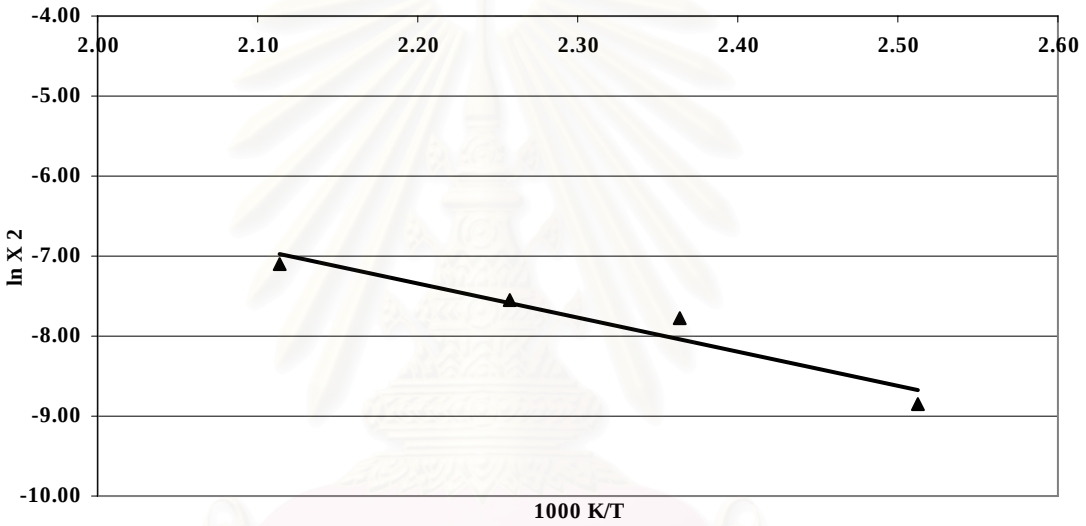


Figure 4.2 Solubility data fitted to a zero-order approximation model

However, when the validity of the model was checked by plotting $T\ln X_2(T)-T_0\ln X_2(T_0)$ versus temperature, the difference deviates significantly from zero. Miller et al. (1998) proposed a first approximate equation that better correlates the solubility data by adding a cubic term, $15[(T/T_0)-1]^3$ into the base equation, Eq (1). The first approximation becomes:

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) + 15[(T/T_0)-1]^3 \qquad (2)$$

It should be noted that both Equation (1) and Equation (2) do not contain the information regarding the molecular structure of the solute. That is, the equations give the same value of solubility in subcrical water for all organic compounds. Nevertheless, Equation (1) was found to predict their data reasonably well at temperature below 373 K, and Equation (2) for the temperature above 373 K.

Equation (2) was then used to estimate our experimental solubility data with T_0 taken to be the lowest temperature experimentally tested (125 ˚C or K), instead of ambient temperature. The values predicted by Equation (1) and Equation (2) are shown in Table 4.2. The two equations however do not show good agreement with the experimental data. Thus, we proposed an approximate equation in which the cubic term in Equation (2) is replaced by $56.67[(T/T_0)-1]^3$ as:

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) + 56.67[(T/T_0)-1]^3 \quad (3)$$

This equation is a better solubility predictor than the other two equations and the values calucated from this equation are listed in Table 4.2. Mathis et al. (2004) proposed a second approximation that contains an altered cubic term and use this model to fit with their experimental data for alkylbenzenes liquid organics. Their proposed model has the following form:

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) + 2[((T-T_0)/T_0)-1]^3 \quad (4)$$

For our anthraquinones solubility data, a model approximation was proposed by adding to the base equation, Equation (1), a different cubic term, which is $0.241[((T+T0)/T_0)-1]^3$. This modified equation becomes:

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) + 0.241[((T+T0)/T_0)-1]^3 \quad (5)$$

As can be seen in Table 4.2, Equation 4 gave good approximation of our experimental data except for the solubility at lower temperatures.

Table 4.2 Solubility of standard alizarin in subcritical water (mg/ml)

Temperature (°C)	10^{-1} mg/ ml					
	Experimental	Equation 1	Equation 2	Equation 3	Equation 4	Equation 5
125	1.43	1.43	1.43	1.43	0.19	1.82
150	4.24	2.41	2.42	2.45	0.47	3.22
170	5.21	3.51	3.59	3.81	0.87	4.90
200	8.35	5.82	6.43	8.50	2.00	8.72

Although Equation 4 may be used to reasonably predict the solubility of anthraquinones in subcritical water, more experimental data would be recommended to further develop solubility models with greater accuracy.

4.2 Effect of temperature on subcritical water extraction

In subcritical water extraction, temperature is considered a key variable affecting the extraction process. In this study, its influence on extraction of anthraquionones from *Morinda citrifolia* was determined for the temperatures of 150, 170 and 200°C using the apparatus explained in chapter 3. The results shown in Figure 4.3 for extraction at the flow rate of 2 ml/min indicates that extraction yield increases as the temperature increases. The yield of anthraquinones extracted at 150 °C was rather close to that extracted at °170 C and was lower than that obtained at 200°C. The increase in the amount of extracted product is due to the increase in the solubility of anthraquinones in subcritical water at higher temperature. (See section 4.1).

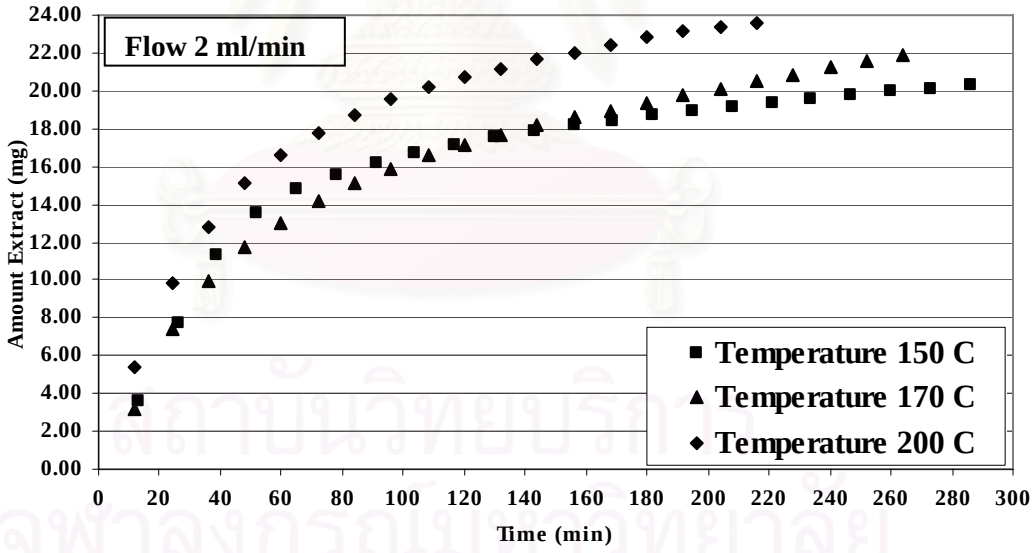


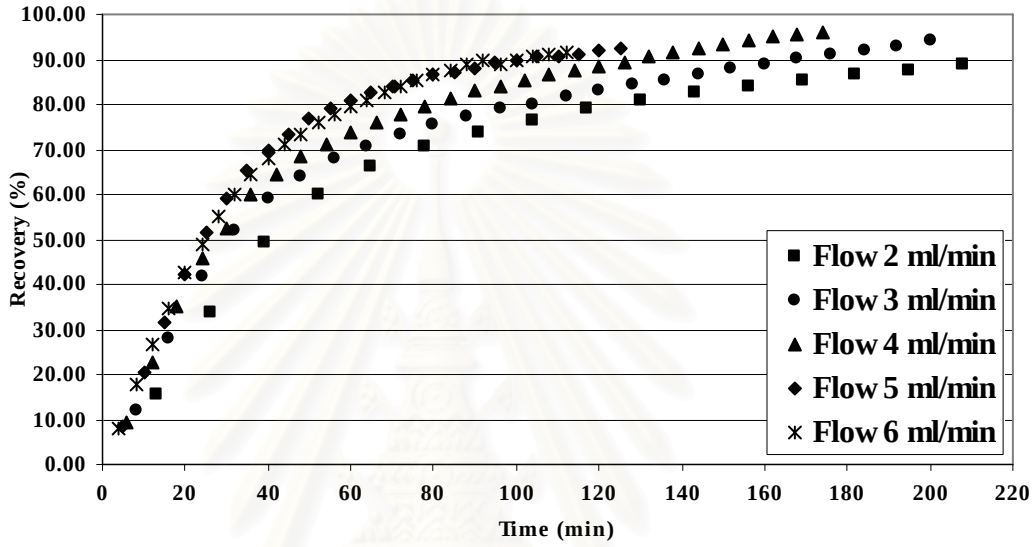
Fig 4.3 Effect of extraction temperature

4.3 Extraction profiles at various flow rates

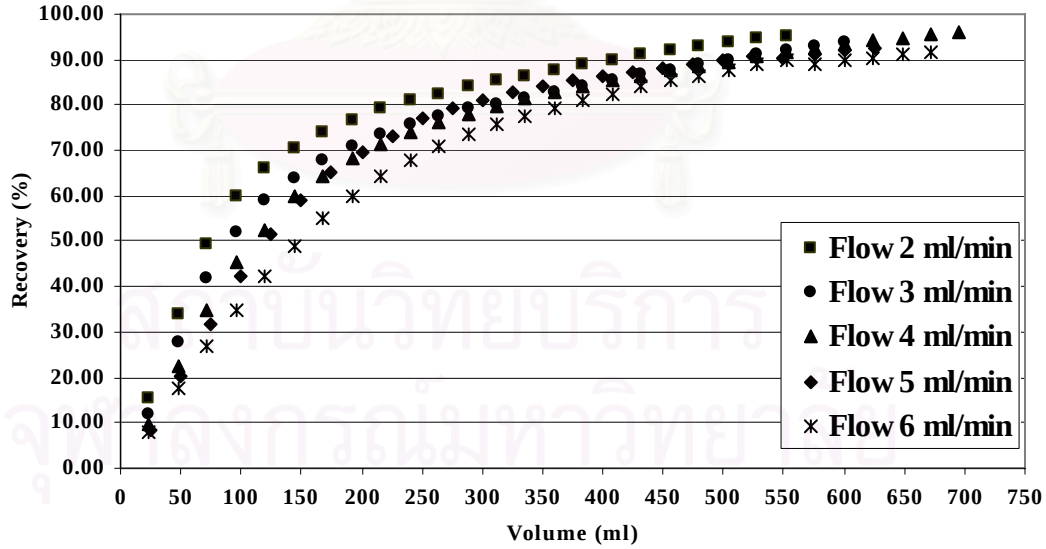
The effect of flow rate has been determined for the flow rate of 2, 3, 4, 5, and 6 ml/ min and for the temperatures of 150, 170, and 200°C while fixing the pressure at 4 MPa. The results are presented by plotting the percentage of anthraquinones extract versus time and versus volume of water used are shown in *Fig 4.4*, *4.*5, *and 4.*6, respectively.

Based on the plot of percent recovery of anthraquinones versus time shown in Figure 4.4 a, 4.5 a, and 4.6 a, it can be seen that the amount of antraquinones extracted increases with an increase of volumetric flow rate up to 5 ml/min. For the highest flow rate of 6 ml/min however, the curve was found to be similar to that of the flow rate of 5 ml/min. This means that the extraction rate was influenced by external mass transfer only up to the flow rate of 6 ml/min. When the flow rate increases from 5 to 6, the extraction process is no longer limited by external mass transfer. At such high flow rate, despite the large amount of solvent passed, the yield might be limited by other factors such as intraparticle diffusion. When consider the same set of data plotted as the percent recovery of anthraquinones versus volume of water shown in Figure 4.4 b, 4.5 b, and 4.6 b, the percent recovery data given for lower flow rates lie above those for lower flow rates. At lower flow rate, water residence time was higher, allowing the plant materials and water to come to close contact, thus, higher yield could be achieved with the same volume of water. At such higher flow rates on the other hand, there was not enough contact time between the solvent and the plant materials, the yield per unit volume was therefore inferior. This behavior was less obvious for extraction at 170 °C and 200 °C (Figure 4.5 b and 4.6 b, respectively), particularly at 170 ° C, in which all flow rates gave similar yields per unit volume of water used. At the first glance, these results might lead one to conclude that the amount of anthraquinones extracted was limited by the solubility. However, the solubilities determined in previous section for these temperatures are much higher than the initial slopes of these plots. The small values for the slopes could possibly be due to the low amount of anthraquinones initially loaded into the extractor. This turned out to be the case for this study as an increase in initial slope was observed in our separate experiment performed at 170 °C in which 1.0 g instead of 0.5 g of roots was loaded. Based on the above evaluation, it cannot be concluded from the above results at this point that extraction rate was limited by the solute solubility. Nevertheless, it can be

concluded that the overall behavior of subcritical water extraction of anthraquinones is controlled by both mass transfer and the solute solubility. The results obtained in this study were similar to those found in Shotipruk et al. (2004). The present results however show more uniformity due to the different sample preparation used. The preparation method used in the present study provides more uniformly distributed spherical sample, rather than non-uniform needle like the sample obtained in the previous study.

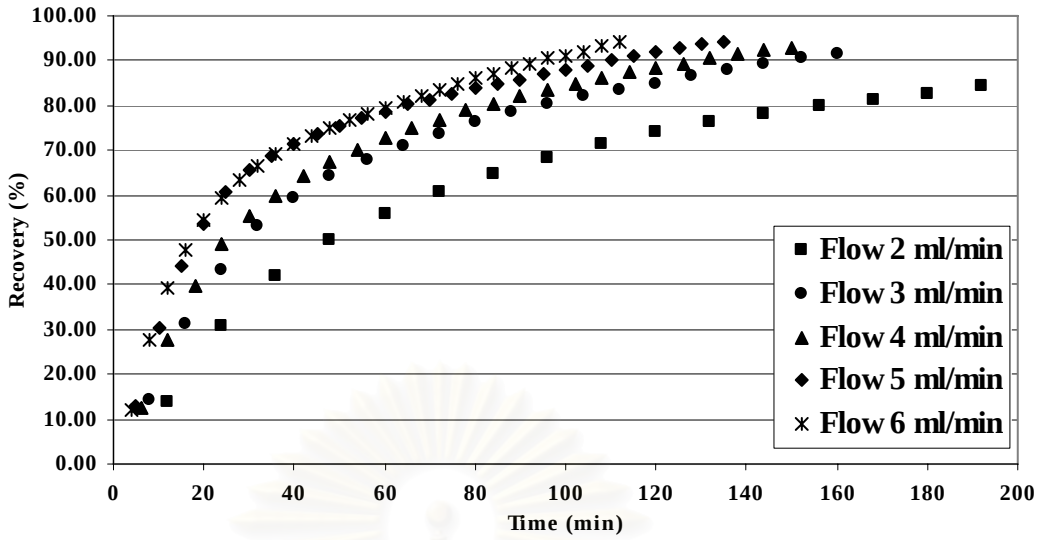


a)

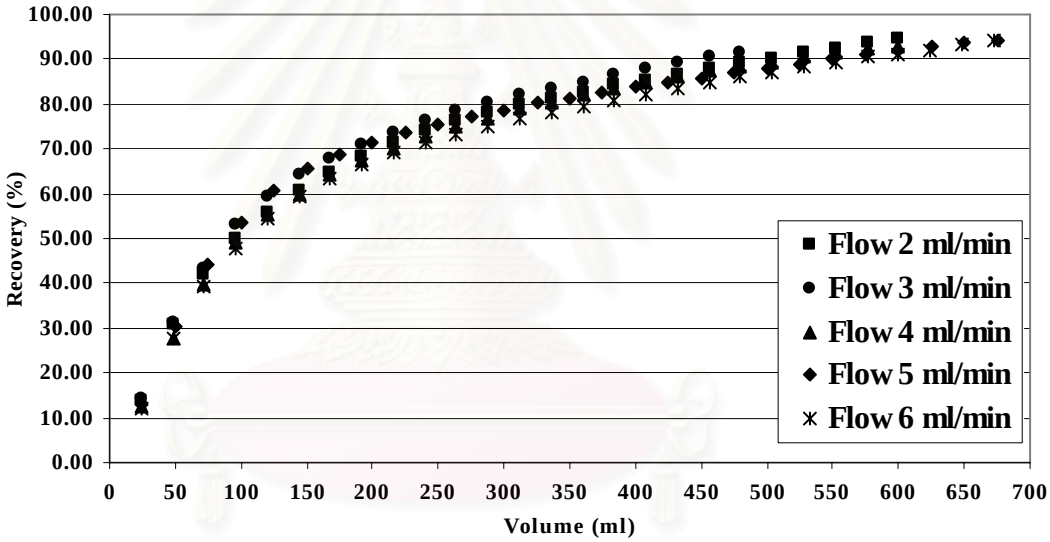


b)

Figure 4.4 a) Percent recovery versus extraction time, b) Percent recovery versus volume of water for subcritical water extraction at 150 °C

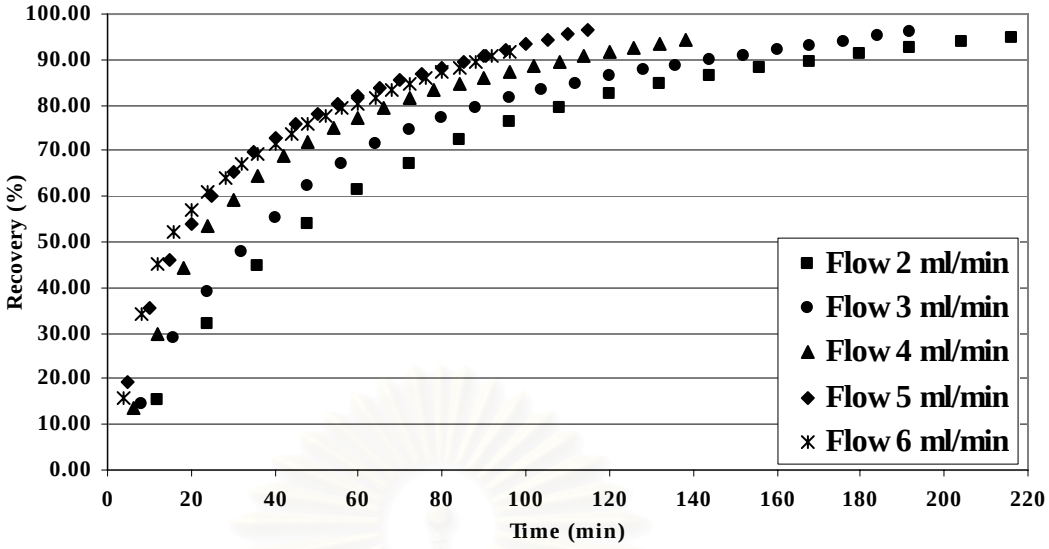


a)

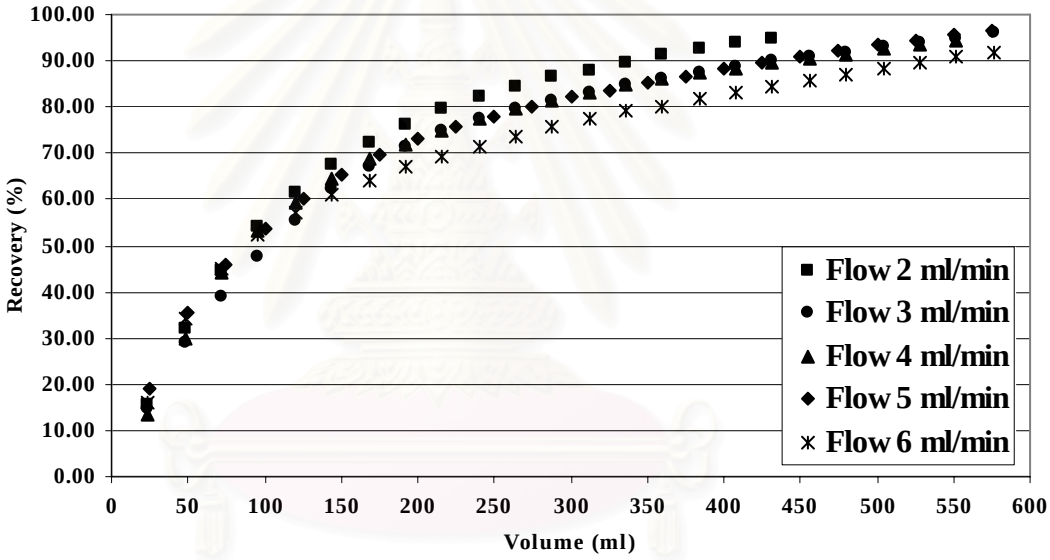


b)

Figure 4.5 a) Percent recovery versus extraction time, b) Percent recovery versus volume of water for subcritical water extraction at 170 °C



a)



b)

Figure 4.6 a) Percent recovery versus extraction time, b) Percent recovery versus volume of water for subcritical water extraction at 200 °C

4.4 Comparison of subcritical water and conventional extraction techniques

In this section, subcritical water extraction is compared with different extraction methods in terms of anthraquinones recovery and time required for extraction. The results are shown in Table 4.3. To achieve approximately 80 % recovery of anthraquionones, maceration requires extended time period (72 hr.). With ultrasound-assisted extraction at 60 °C, similar yield could be achieved within only 2 hrs due to enhanced mass transfer resulted from ultrasonic cavitation and the increase of solubility at higher temperature. More efficient extraction could be achieved using a soxhlet extractor in which continuous extraction with fresh solvent at near boiling temperature. For subcritical water extraction, the data presented in the table were taken from the experimental runs at 5 ml/min. This flow rate was found to be optimal as it allows efficient use of solvent and the extraction can be completed within a reasonable period of time.

Table 4.3 Comparison of percent recovery and extraction time for different extraction methods.

Extraction Method	Temperature	Time	Recovery (%)
Maceration	25 °C	72 hr.	81.16 ± 2.70
Ultrasonic extraction	60°C	2 hr	79.62 ± 0.56
Soxhlet extraction	78.3 °C	1 hr.	93.42 ± 0.58
		2 hr.	94.85 ± 0.85
		3 hr.	96.99 ± 0.77
		4 hr.	97.94 ± 0.70
Subcritical water extraction	150 °C	1 hr.	81.07 ± 2.78
		2 hr.	92.55 ± 1.60
	170 °C	1 hr.	78.79 ± 4.08
		2 hr.	91.89 ± 1.04
	200 °C	1 hr.	82.13 ± 3.77
		2 hr.	96.41 ± 1.14

Comparing the extraction yield resulted by subcritical water extraction and soxhlet extraction after the first hour, we found that soxhlet extraction gave highest

recovery (93.42 % versus 81.07%). However, beyond the first hour, recovery with subcritical water still increased, for example, at the extraction temperature of 200 °C, an increase from 82.13 to 96.41 % was achieved for the time increase from 1 to 2 hours. Although subcritcal water extraction and soxhlet extraction gave comparable results in term of recovery and time of extraction, subcritical water extraction does not involve the use of toxic organic solvents, making the method more favorable. Similar results were observed for subcritical water extraction at 150 and 170 °C in which the yield remained increase into the first two hours of extraction. However, at these conditions, it required longer time to reach a desired recovery compared to at 200 °C. Longer time period means higher amount of solvent required, thus extraction time longer than 2 hours is not recommended.

The above discussion suggests that subcritical water extraction is considered a benign alternative for extraction of anthraqunones from roots of *Morinda citrifolia*. The best condition for this method based is at temperature of 200 °C and flow rate between 3 ml/ min and 5 ml/ min. The yield obtained by this method is comparable to soxhlet extraction but superior to maceration and ultrasound assisted extraction. Nevertheless, subcritical water extraction requires high temperature conditions, thus the quality of the extract should be checked. In the next section, antioxidant activity of anthraquinones in the extracts obtained by various methods will be compared and discussed.

4.5 Antioxidant activity

The antioxidant activity of anthraquinones was analyzed to check the quality of the extract. The procedure for antioxidant test was explained in chapter 3 in which the radical-scavenging activity of antioxidants against free radicals, 1,2-diphenyl-2-picrylhydrazyl (DPPH) radicals, was measured at 517 nm after a 2 h of incubation. The antioxidant activity of the sample was quantified by the IC_{50} value, which is the concentration of the sample producing 50 % reduction of the radical absorbance. The values for the extract obtained by subcritical water extraction compared with that by conventional organic solvent extraction including maceration with magnetic stirrer, ultrasound-assisted extraction, and soxhlet extraction are shown in Table 4.4.

Table 4.4 Antioxidant activities of extracts obtained by different extraction method expressed as IC50 values

Extraction method	IC_{50} (mM)
Subcritical water extraction at 150°C	0.110 ± 0.009
Subcritical water extraction at 170°C	0.111 ± 0.015
Subcritical water extraction at 200°C	0.117 ± 0.012
Maceration	0.176 ± 0.025
Ultrasonic Assisted extraction	0.300 ± 0.003
Soxhlet extraction	0.102 ± 0.013

As seen from this table and also shown graphically in Figure 4.7, IC_{50} values of subcritical water extraction at the temperature of 150, 170 and 200°C are not statistically different as tested by analysis of variance (ANOVA) using a significance level of α=0.01. The highest IC_{50} value (lowest antioxidant activity) was obtained for the ultrasound assisted extraction in absolute ethanol, followed by maceration. The IC_{50} of the extract from each method was compared to that from subcritcal water extraction using analysis of means. The analysis shows that the antioxidant activity of the extracts obtained by soxhlet extraction appears similar to those obtained by subcritical water. There is no significant difference between the mean values using a significance level of α=0.01. From the graph, the IC_{50} of the extract obtained by maceration appears to be higher (lower antioxidant activity) compare with subcritical water extraction. However, the test-statistics does not allow the rejection of the null hypothesis. It is therefore concluded that the means of the IC_{50}'s obtained from both methods are the same. As for the ultrasonic assisted extraction however, the antioxidant activity was the most inferior and was found to be significantly different from the other methods. The detailed statistical analysis can be found in Appendix B.

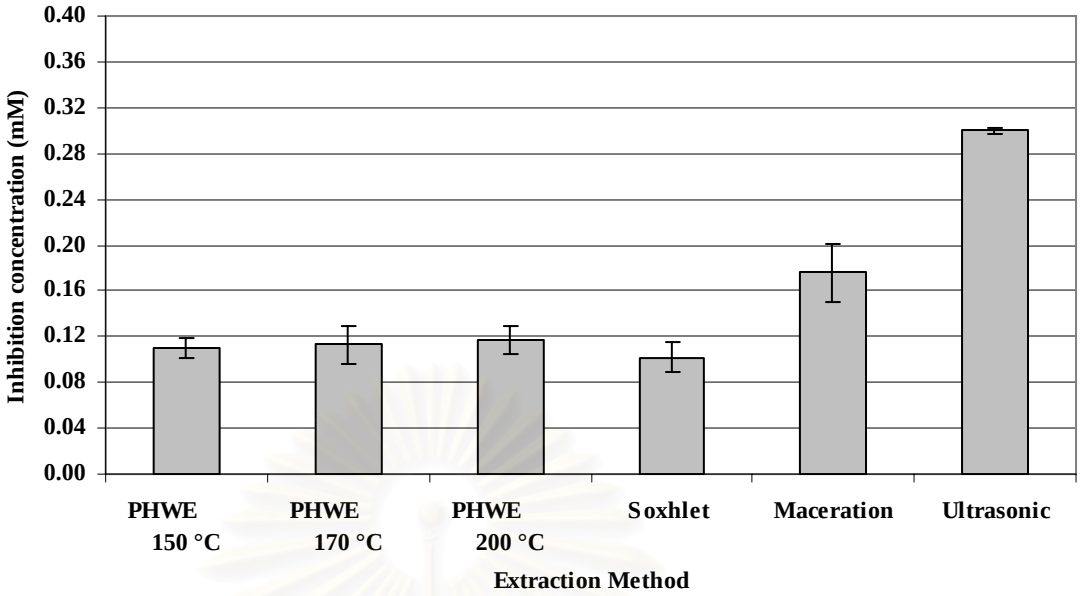


Figure 4.7 antioxidant measurement

This result suggests that subcritical water extraction gives comparable quality of the extracts compared with soxhlet extraction and maceration but without the use of toxic organic solvent. Ultrasound-assisted extraction gives the lowest activity even though the use of ultrasound may enhance the product release and reduce the time required for extraction, it may induce free-radical formation resulted by the existing dissolved O_2 within the medium, causing the oxidation reaction during ultrasonication, a phenomenon that leads to the degradation of antioxidant.

Besides the antioxidant activity of natural compound after extraction, we also test the stability of the extract when further kept for a longer time period. The antioxidation activity of anthraquinones extracts kept in darkness for zero, one, and two hour after extraction were determined.

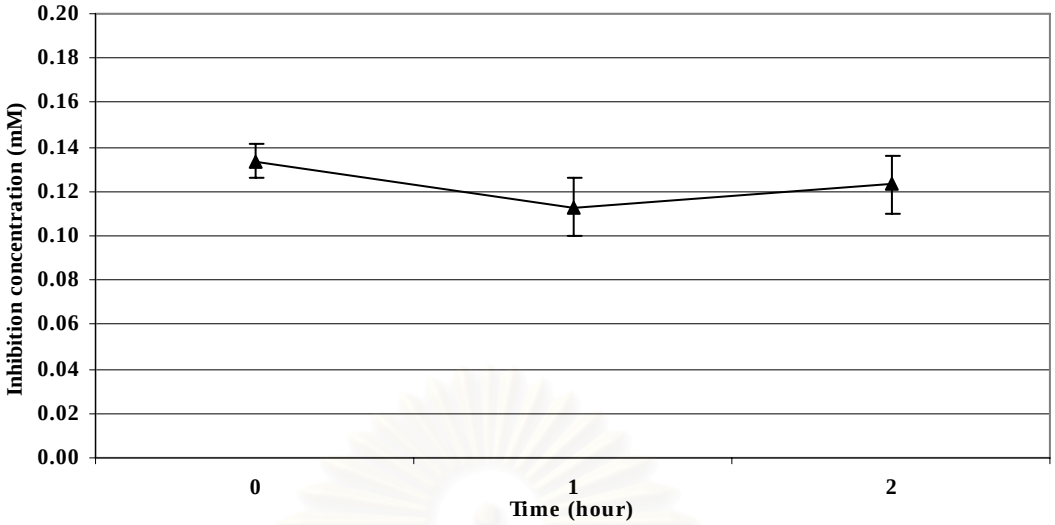


Figure 4.8 Effect of time

As shown in Figure 4.8, antioxidant activity of anthraquinones extracts obtained from subcritical water remains stable for at least two hours after extraction. Future experiments may be tested for the extended time period to determine the stability of the product extracted.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The amount of anthraquinones in the extract increases as temperature increases.
2. In this study, the most suitable condition for subcritical water extraction of anthraquinones is at the temperature of 200 °C and the flow rate of 3-5 ml/min.
3. Extraction profiles of anthraquinones recovery versus time and versus volume of water suggested that overall extraction mechanism was influenced by both mass transfer and solubility.
4. The solubility of anthraquinones in subcritical water increases when the temperature increases due to the decrease in water polarity.
5. The following approximate model is proposed for predicting anthraquionone solubility

$$\ln x_2 (T) \approx (T_0/T) \ln x_2(T_0) + 0.241[((T+T0)/T_0)-1]^3$$

6. With the best condition (200 °C, 5 ml/min), 90% of anthraquinones could be extracted with subcritical water after 2 hours. The yield was superior to maceration and ultrasonic extraction but similar to soxhlet extraction.
7. Higher antioxidant activity was achieved with subcritical water extract as well as soxhlet extraction and maceration compared to ultrasonic extraction. Ultrasound-assisted extraction gives the lowest activity, possibly due to the free-radical formation resulted by the existing dissolved O_2 within the medium, thus causing the product degradation. Moreover, antioxidant activity of anthraquinones extracts remains stable after 2 h that the compound had been extracted. Future experiments may be tested for the extended time period to determine the stability of the product extracted.

5.2 Recommendations

1. Higher solubility was obtained when the solubility measurements were carried out batchwise as compared to the continuous system. That is because the continuous system encounters a problem with anthraquinones clogging within the apparatus tubing. In the same way, this clogging problem may occur during the extraction process. It is therefore interesting to make a comparison of subcritical water extraction in a batch system and a continuous system.
2. More experimental data should be experimentally obtained to improve the mathematical model for solubility prediction.
3. Experiments in this study suggested that the reduction of particle size of the sample materials increases the surface area of extraction and influence the extraction behavior. Materials preparation was thus an important factor that affects to extraction efficiency. Therefore, the development of materials preparation techniques should be considered in the further work.
4. The yield of the extract was found to be higher at higher extraction temperature. The maximum extraction temperature in this study was chosen to be 200 °C because higher temperatures cause the sample to burn and may affect the product quality. Based on the study, the temperature in the range studied does not have a significant effect on the product quality, as measured by antioxidant activity. We thus tested an extraction at higher temperature (230 °C). The extract obtained with subcritical water at temperature 230 °C surprisingly gave much higher antioxidant activity than that obtained at temperature 150-200 °C. It is possible that at higher temperature the extract composition differs from that at lower temperatuare and may contain higher percentages of more active anthraquionones compounds. More extraction experiments and analysis of the extract composition with HPLC are recommended to confirm this interesting situation.

REFERENCES

Ayala, R.S., and M.D.L.D. Castro; "Continuous subcritical water extraction as a useful tool for isolation of edible essential oils," *Food Chemistry.*, 75 (2001), 109–113.

Aruna Prakash; "ANTIOXIDANT ACTIVITY," Medallion Laboratories ANALYTICAL PROGRESS, *Takes you into the Heart of a Giant Resource vol*.19 (2001)

Babu, K.S., Srinivas, P.V., Praveen, B., Kishore K.H., Murty, S.U., and J. M. Rao; "Antimicrobial constituents from the rhizomes of Rheum emodi," *Phytochemistry.*, 62 (2003), 203–207.

Basile, A., Jiménez-Carmona, M. M., and A.A. Clifford; "Extraction of rosemary by Superheated Water" J. Agric. Food Chem., 46 (1998), 5205-5209.

Bruckard, W.J., Calle, C., Fletcher, S., Horne, M.D., Sparrow, G.J., and A.J. Urban; "The application of anthraquinone redox catalysts for accelerating the aeration step in the becher process," *Hydrometallurgy.*, 73 (2004), 111-121.

Brunner G. "Gas Extraction" Steinkopff Darmstadt Springer: New York, 1994

Cichewicz, R.H., Zhang, Y., Seeram, N.P., and M.G. Nair; "Inhibition of human tumor cell proliferation by novel anthraquinones from daylilies," *Life Sciences.*, 74 (2004), 1791–1799

Clifford, A.A., Basile, A., and S.H.R. Al-Saidi; "A comparison of the extraction of clove buds with supercritical carbon dioxide and superheated water," Fresenius J Anal Chem., 364 (1999), 635-637.

Dadkhah, A.A., and A. Akgerman; "Hot water extraction with in situ wet oxidation: PAHs removal from soil," *Journal of Hazardous Materials B.* 93 (2002), 307–320.

Epstein M., and S. Yariv; "Visible-spectroscopy study of the adsorption of alizarinate by Al-montmorillonite in aqueous suspensions and in solid state," *Journal of Colloid and Interface Science.*, 263 (2003), 377–385.

Fernández-Perez, V., Jiménez-Carmona, M. M., and M. D.L.D. Castro; "An approach to the static-dynamic subcritical water extraction of laurel essential oil: comparison with conventional techniques," Anal. Chem., 125 (2000), 481-485.

Gámiz-Gracia, L., and M.D.L.D. Castro; “Continuous subcritical water extraction of medicinal plant essential oil: comparison with conventional techniques,” *Talanta.*, 51 (2000), 1179–1185.

Geankoplis, C.J. Transport Process and Unit Operations, 3th edition, New Jersey Prentice-Hall International,1995

Hashimoto, S., Watanabe, K., Nose, K., and M. Morita; “Remediation of soil contaminated with dioxins by subcritical water extraction,” *Chemosphere.,* 54 (2004), 89–96.

Hawthorne, S.B., Yang, Y., and D.J. Miller; “Extraction of organic pollutants from environmental solids with sub- and supercritical water,” Anal. Chem., 66 (1994), 2912-2920.

Hawthorne, S.B., Grabanski, C.B., Martin, E., and D.J. Miller; “Comparisons of Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix,” *Journal of Chromatography A.* 892 (2000), 421–433.

Hiramatsu, T., Imoto, M., Koyano, T. and K. Umezawa; “Induction of normal phenotypes in ras-transormed cells by damnacanthal from *Morinda citrifolia*,” *Cancer. Lett.*, 73 (1993), 161-6.

Jin, Z.Q, and Chen, X.; “A simple reproducible model of free radical-injured isolated heart induced by 1,1-diphenyl-2-picrylhydrazyl (DPPH),” *Journal of Pharmacological and Toxicological Methods.*, 39 (1998), 63–70.

Jiménez, P., Roux, M.V., Dávalos, J.Z., and M. T. Molina; “Thermochemistry of 9-hydroxy-1,4-anthraquinone and 9-methoxy-1,4-anthraquinone,”*J. Chem. Thermodynamics*., 34 (2002), 1117-1126.

Jiménez-Carmona M.M., Manclús, J.J., Montoya, A., and M.D.L.D. Castro; “Sub- and supercritical fluid extraction of trichloropyridinol from soil prior to immunoassay,”*Journal of Chromatography A.* 785 (1997), 329-336.

Jiménez-Carmona, M. M., Ubera, J. L. and L. D. Castro; “Comparison of continous subcrtical water extraction and hydrodistillation of majoram essential oil,” *J. Chromatography A*. 855 (1999), 625-632.

Kasai, H., Fukada, S., Yamaizumi, Z., Sugie, S., and Mori, H.; “Action of chlorogenic acid in vegetable and fruits as an inhibitor of 8-hydroxydeoxyguanosine

formation in vitro and in a rat carcinogenesis model," *Food and Chemical Toxicology.*, 38 (2000), 467–471.

Kipp, S., Peyrer, H., and W. Kleiböhmer; "Coupling superheated water extraction with enzyme immunoassay for an efficient and fast PAH screening in soil," *Talanta.*, 46 (1998), 385–393.

Kirk-Othmer. "Encyclopedia of Chemical Technology" Vol 23., New York; John wiley & Sons, 1997.

Koyama, J., Morita, I., Tagahara, K., Ogata, M., Mukainaka, T., Tokuda, H., and H. Nishino; "Inhibitory effects of anthraquinones and bianthraquinones on Epstein-Barr virus activation," *Cancer Letters.*, 170 (2001), 15-18.

Krieger, M.S., Wynn, J.L., and R.N. Yoder; "Extraction of cloransulam-methyl from soil with subcritical water and supercritical CO_2," *Journal of Chromatography A.* 897 (2000), 405–413.

Kubátová, A., Miller, D. J. and B. S. Hawthorne; "Comparison of subcritical water and organic solvents for extracting kava lactones from kava root," *J. Chromatography A*. 923 (2001), 187-194.

Kubátová, A., Lagadec, A. J. M., Miller, J. D. and S. B. Hawthorne; "Selective extraction of oxygenates from savory and peppermint using subcritical water," *Flavor Fragr. J.*, 16 (2001), 64-73.

Loy, G., Cottiglia, F., Garau, D., Deidda, D., Pompei, R., and L. Bonsignore; "Chemical composition and cytotoxic and antimicrobial activity of *Calycotome villosa* (Poiret) Link leaves," *Il Farmaco.*, 56 (2001), 433-436

Mannila, M. H., Kim, H. and C. M. Wai; "Supercritical carbon dioxide and high-pressure water extraction of bioactive compounds in St. John's wort," In. Proceedings of Supergreen 2002, Kyung Hee University, Korea (2002)

Mathis, J., Gizir, A. M. and Y. Yang; "Solubility of Alkylbenzenes and model for Predicting the Solubility of Liquid Organic in High-Temperature Water," *J. Chem. Eng. Data.*, 49 (2004), 1269-1272.

Mcgowin, A.E., Adom, K.K., and A.K. Obubuafo; "Screening of compost for PAHs and pesticides using static subcritical water extraction," *Chemosphere.*, 45 (2001), 857-864.

Miller, D.J., and A.A. Clifford; "Solubility of Polycyclic Aromatic Hydrocarbons in Subcritical Water from 298 to 498 K," *J. Chem. Eng. Data.*, 43 (1998), 1043-1047.

Ollanketo, M., Peltoketo, A., Hartonen, K., Hiltunen R. and M. L. Riekkola; "Extraction of sage (*Salvia officialis L.*) by pressurized hot water and conventional methods: antioxidant activity of the extracts," *Eur. Food. Res. Technol*., 215 (2002), 158-163.

Ong, E. S. and S. M. Len; "Pressurized hot water extraction of berberine, baicalein, and glycyrrhizin in medicinal plants," *Analytica. Chimica. Atcta*., 482 (2003), 81-89.

Ozel, M.Z., Gogus, F., and A.C. Lewisc; "Subcritical water extraction of essential oils from Thymbra spicata," *Food Chemistry.*, 82 (2003), 381–386.

Pawlowski, T.M.; "Extraction of Thiabendazole and Carbendazim from Foods Using Pressurized Hot (Subcritical) Water for Extraction: A Feasibility Study" J. Agric. Food Chem., 46 (1998), 3124-3132.

Pellegrini, N., Re, R., Yang, M., and C. Rice-Evans; "Method in Enzymology, vol. 299, 379-389

Priego-López, E., and M.D.L.D. Castro; "Demetalisation of soils by continuous acidified subcritical water extraction," *Talanta.*, 58 (2002), 377–385.

Richter, P., Sepúlveda, B., Oliva, R., Calderón, K., and R. Seguel; "Screening and determination of pesticides in soil using continuous subcritical water extraction and gas chromatography–mass spectrometry," *Journal of Chromatography A.* 994 (2003), 169–177.

Rogalinski, T., Valle, J.M.D., Zetzl, C., and G. Brunner; "Extraction of Boldo (*Peumus boldus* M.) leaves with hot pressurized water and supercritical CO_2," In. Proceedings of Supergreen 2002, Kyung Hee University, Korea (2002).

Sadeghi-Aliabadi, H., Tabarzadi, M., and A. Zarghi; "Synthesis and cytotoxic evaluation of two novel anthraquinone derivatives," *IL FARMACO* (2004).

Shamsipur, M., Karami, A.R. Yamini, Y., and H. Sharghi; "Solubilities of some 1-hydroxy-9,10-anthraquinone derivatives in supercritical carbon dioxide," *J. of Supercritical Fluids.*, (2004).

Suomi, J., Sirén, H., Hartonen, K. and M. L. Riekkola; "Extraction of iridoid glycosides and their determination by micellar electrokinetic capillary chromatography," *Journal of Chromatography A.* 868 (2000), 73-83.

Talou, J.R., Verberne, M. C., Muljono, R.A.B., Tegelen , L.J.P., Bernal, B.G., Linthorst, H.J.M., Wullems, G.J., Bol, J.F., and R. Verpoorte; "Isochorismate

synthase transgenic expression in *Catharanthus roseus* cell suspensions," *Plant Physiol. Biochem.,* 39 (2001), 595-602.

Wallace, K. B. Free radical toxicity; Washington, DC; Taylor & Francis, 1997.

Yang, Y., Bøwadt, S., Hawthorne, S.B., and D.J. Miller; "Subcritical water extraction of Polychlorinated biphenyls from soil and sediment," *Anal. Chem.,* 67 (1995), 4571-4576.

Zenk, M.H., El-Shagi, H., and U. Schulte; "Anthraquinone production by cell suspension cultures of Morinda citrifolia," *Planta Med. Suppl.,* 79-101 (1975).

Zin, Z.M., Abdul-Hamid, A. and A. Osman; "Antioxidative activity of extracts from Mengkudu (Morinda citrifolia L.) root, fruit and leaf," *Food Chemistry.,* 78 (2002), 227–231.

APPENDICES

APPENDIX A

EXPERIMENTAL AND DATA ANALYSIS

A-1 Standard calibration curve of alizarin

Table A-1 Standard calibration curve data

Concentration of Alizarin (M)	Absorbance at 435 nm.			
	Exp.1	Exp.2	Exp.3	Average
2.50E-06	0.013	0.012	0.011	0.01225
3.40E-06	0.017	0.014	0.012	0.0145
4.50E-06	0.019	0.018	0.018	0.01725
6.00E-06	0.025	0.023	0.023	0.02275
8.00E-06	0.029	0.026	0.032	0.0275
1.07E-05	0.036	0.032	0.035	0.03325
1.42E-05	0.045	0.043	0.039	0.04175
1.89E-05	0.057	0.049	0.053	0.052
2.53E-05	0.072	0.066	0.067	0.06725
3.38E-05	0.091	0.087	0.084	0.08575
4.50E-05	0.116	0.108	0.106	0.10825
6.00E-05	0.145	0.141	0.134	0.138

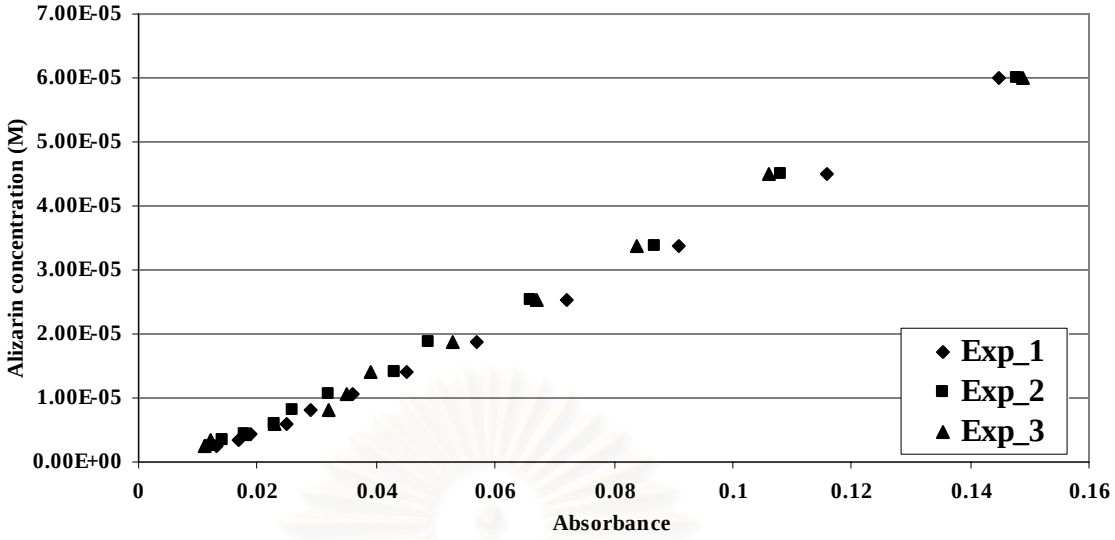


Figure A-1.1 Standard calibration curve of alizarin

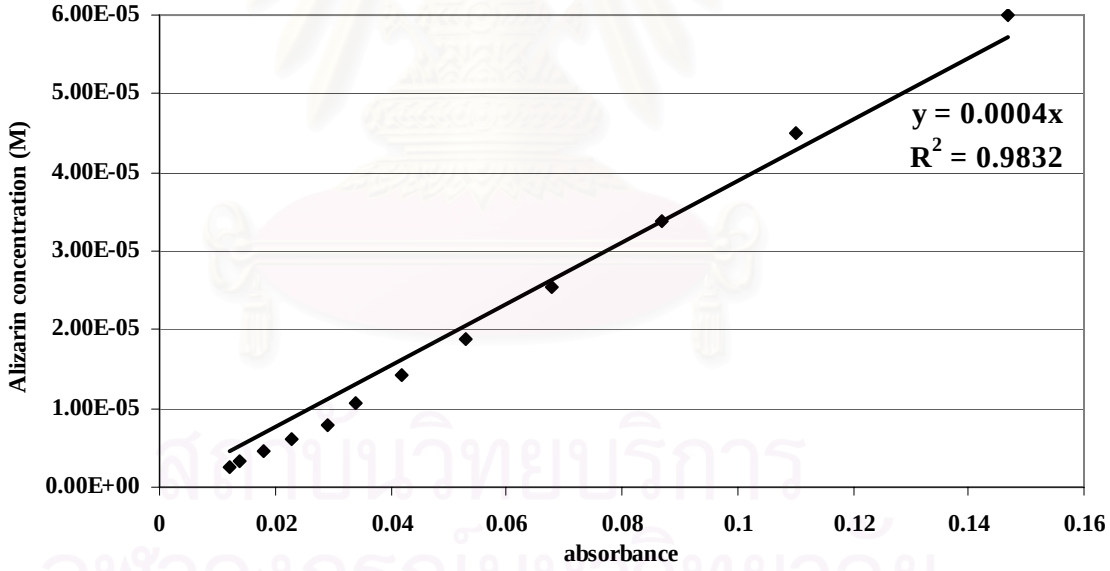


Figure A-1.2 Standard calibration curve of alizarin (average)

A-2 Solubility prediction

In general, the solute solubility depends on the interaction between the molecules of the solute and the solvent, which is dictated by the molecular structures and the activity coefficient of the solution. However, solubility does not only depend on the activity coefficient but also the ratio of fugacity of pure solid and the standard state fugacity according to the following equation.

$$X = \frac{f_{pure-solid}}{\gamma \; f^{o}_{subcooled-liquid}} \tag{A-2.1}$$

Where $f_{\text{pure solid}}$ is fugacity of solid at equilibrium and $f_{\text{subcooled liquid}}$ is standard state fugacity taken to be that of subcooled liquid.

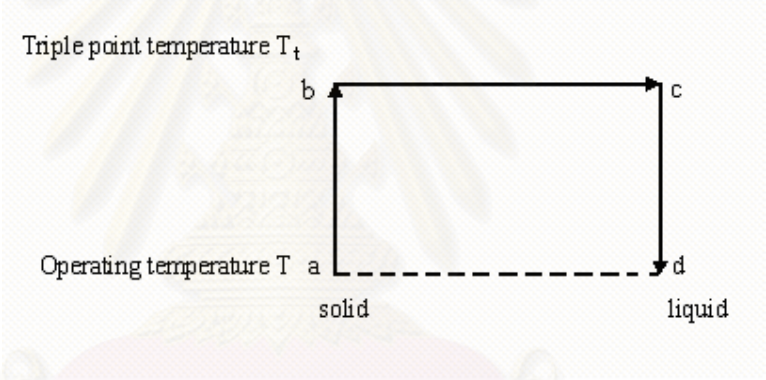


Figure A-2.1 Path independence thermodynamic properties

The ratio of the two fugacities are relate to the change of Gibbs energy in going from the state of solid (denoted as state a) to subcooled liquid (denoted as state d) following form equation:

$$\Delta G = RT\, ln\left(\frac{f_{subcooled-liquid}}{f_{pure-solid}}\right) \tag{A-2.2}$$

The change of Gibbs energy is related to the change of enthalpy and the change of entropy by the following equation:

$$\Delta G_{a\to d} = \Delta H_{a\to d} - T\Delta S_{a\to d} \tag{A-2.3}$$

Because the enthalpy and entropy are not dependent of the path, thus

$$\Delta H_{a\to d} = \Delta H_{a\to b} + \Delta H_{b\to c} + \Delta H_{c\to d} \quad \text{(A-2.4)}$$

The above equation becomes

$$\Delta H_{a\to d} = \Delta_{fus} H_{at\ T_t} + \int_{T_t}^{T} \Delta C_P dT \quad \text{(A-2.5)}$$

Where $\Delta_{fus}H$ is the enthalpy of fusion and $\Delta C_P = C_{P\ liquid} - C_{P\ solid}$, the difference between the heat capacity of liquid and the heat capacity of solid, and T_t is the triple point temperature of the solute.

Similarly, the entropy change from a to d can be determined as

$$\Delta S_{a\to d} = \Delta S_{a\to b} + \Delta S_{b\to c} + \Delta S_{c\to d} \quad \text{(A-2.6)}$$

Which can be written as follows:

$$\Delta S_{a\to d} = \Delta_{fus} S_{at\ T_t} + \int_{T_t}^{T} \Delta C_P dT \quad \text{(A-2.7)}$$

Where $\Delta_{fus}S$ is entropy of fusion which is related to $\Delta_{fus}H$ by the following equation:

$$\Delta_{fus} S = \frac{\Delta_{fus} H}{T_t} \quad \text{(A-2.8)}$$

substituting equation (A-2.7), (A-2.5) and (A-2.3) into equation (A-2.2), and assuming that ΔC_P is constant over the temperature range $T_t \to T$, we obtain the following equation.

$$ln\left(\frac{f_{subcooled-liquid}}{f_{pure-solid}}\right) = \frac{\Delta_{fus} H}{RT_t}\left(\frac{T_t}{T} - 1\right) - \Delta C_P\left(\frac{T_t}{T} - 1\right) + \Delta C_P\, ln\left(\frac{T_t}{T}\right) \quad \text{(A-2.9)}$$

Substitute equation (A-2.1) into equation (A-2.9) to give the expression for the solubility as follows.

$$ln\ X = -\frac{\Delta_{fus} S}{R}\left(\frac{T_t}{T} - 1\right) + \Delta C_P\left(\frac{T_t}{T} - 1\right) - \Delta C_P\, ln\left(\frac{T_t}{T}\right) - ln\ \gamma \quad \text{(A-2.10)}$$

As an approximation, the term of ΔC_P can be neglected and it is permissible to substitute melting temperature for triple point temperature then, equation (A-2.10) becomes:

$$ln\ X = \frac{-\Delta_{fus} S}{R}\left(\frac{T_m}{T} - 1\right) - ln\ \gamma \qquad \text{(A-2.11)}$$

This is the basic equation that used to determine solubility. Firstly, developed an approximate equation is based on derived this equation with assumes the Gibbs function for solution does not change over the temperature range and there is no absorption of water by the solute. The assumption for the slight change in the Gibbs function was justified because the enthalpy of the solution for these insoluble molecules does not vary widely with temperature and is much greater than the entropy contribution and assumed an ideal solution ($\gamma = 1$). The equation become

$$T_1 \ln X_1 = \frac{T_2 \ln X_2}{(T_m - T_2)}(T_m - T_1) \qquad \text{(A-2.12)}$$

Where, X_1 and X_2 are solubility at temperature T_1 and T_2, respectively.
For the simplest calculation, we assume that $T_m >> T_1$ and T_2. Thus $(T_m - T_1)$ assumed equal to $(T_m - T_2)$. Equation (2.12) become

$$\ln X_1 \approx \frac{T_2}{T_1} \ln X_2 \qquad \text{(A-2.13)}$$

This called zeroth approximation model of solubility prediction (Miller, D.J., et al, 1998). This basic equation was developed for more accuracy predicts with add an appropriate cubic equation which give minimum error compare with experimental result.

A-3 Antioxidant activity measurement

Antioxidant activity of natural compound may be lost due to some factors such as high temperature or exposure to light and chemicals. To investigate the effect of these factors on compound antioxidant activity, measurement of radical-scavenging activity of antioxidants against free radicals like 1, 2-diphenyl-2-picrylhydrazyl (DPPH) radical is one of the simplest methods used.

Generally, the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The Figure of reference sample of DPPH solution is show in Fig. A-3.1. In this study, DPPH solutions in ethanol with the spectroscopic absorbance of 1.13 at 517 nm (110 μM) were prepared as a initial reference sample. The percent reduction in absorbance from this value as a result of adding to the solution the antioxidantive agent of known concentration would be used to calculate the percentage inhibition or PI%.

Figure A-3.1 DPPH solution absorbance value at 1.13

To determine the activity in terms of IC_{50}, series of sample antraquinones solutions were prepared. One milliliter from each prepared solution was added with 2 milliliter

of DPPH solution and the mixture was vortexed until completely mixed, and it was incubated at 37 °C for 2 h in darkness.

As a result of antioxidant agent added, the color of solution changes from the dark purple to the bright yellow with high degree of bleaching seen with higher antioxidant concentration. The most concentrated antioxidant solution was that on the left hand side of Fig. A-3.2. On the other hand, the color of solution does not change for when low concentration antioxidant solution or non-antioxidative solutions were added (see the sampling vial on the right hand side in Fig. A-3.2).

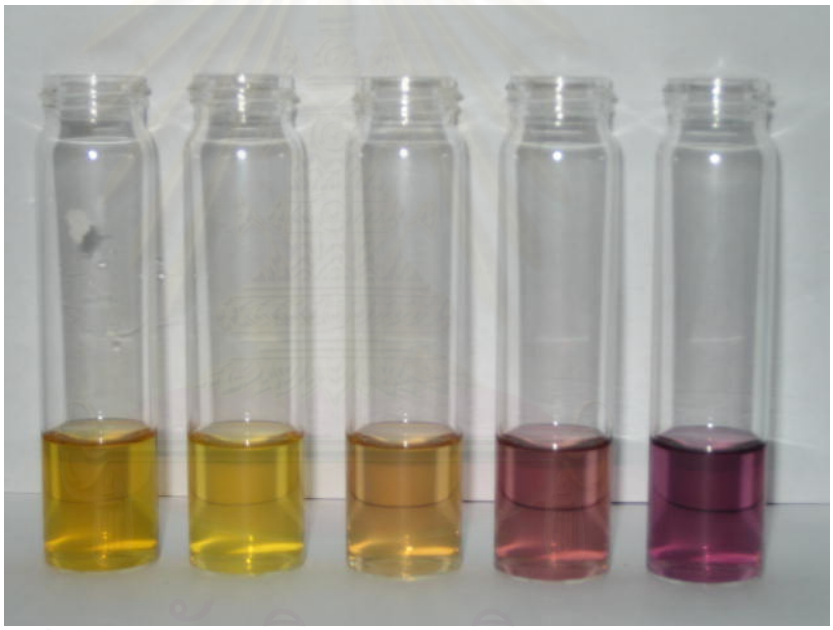


Figure A-3.2 Antioxidant develop for dilute sample

The antioxidant activity of anthraquinones extracts was determined by measuring the absorbance of the mixture solutions at 517 nm. After the 2 h of incubation, the absorbance of each vial was measured and the PI % can be calculated for each solution from Eq (3.3).

All the data of antioxidant activity measurement are summarized in Table below.

Table A-3.1 PI % for each sample solution prepared from PHWE extract at 150 °C

No.	Sample														
	1			2			3			4			5		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.235	0.359	68.34	0.286	0.376	66.84	0.190	0.450	60.32	0.165	0.463	59.17	0.179	0.476	58.02
2	0.176	0.450	60.32	0.214	0.494	56.44	0.143	0.542	52.20	0.124	0.546	51.85	0.134	0.550	51.49
3	0.132	0.522	53.97	0.161	0.528	53.44	0.107	0.571	49.65	0.093	0.575	49.29	0.100	0.577	49.12
4	0.099	0.567	50.00	0.121	0.572	49.56	0.080	0.610	46.21	0.069	0.625	44.89	0.076	0.600	47.09
5	0.077	0.602	46.91	0.09	0.600	47.09	0.06	0.643	43.29	0.052	0.645	43.12	0.057	0.646	43.03
6	0.056	0.635	44.00	0.068	0.620	45.33	0.045	0.656	42.15				0.043	0.666	41.27

Table A-3.2 PI % for each sample solution prepared from PHWE extract at 170 °C

No.	Sample														
	1			2			3			4			5		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.314	0.319	71.79	0.272	0.400	64.63	0.314	0.309	72.53	0.272	0.389	65.42	0.173	0.447	60.27
2	0.235	0.438	61.27	0.204	0.492	56.49	0.235	0.420	62.67	0.204	0.489	56.53	0.129	0.527	53.16
3	0.176	0.477	57.82	0.153	0.550	51.37	0.176	0.506	55.02	0.153	0.528	53.07	0.097	0.570	49.33
4	0.132	0.554	51.02	0.115	0.571	49.51	0.132	0.536	52.36	0.115	0.554	50.76	0.073	0.600	46.67
5	0.099	0.575	49.16	0.086	0.604	46.59	0.099	0.564	49.87	0.086	0.590	47.56	0.055	0.627	44.27
6	0.074	0.587	48.09	0.065	0.623	44.92	0.074	0.596	47.02				0.041	0.643	42.84

Table A-3.3 PI % for each sample solution prepared from PHWE extract at 200 °C

No.	Sample														
	1			2			3			4			5		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.349	0.316	71.79	0.325	0.426	61.96	0.244	0.440	61.09	0.185	0.470	59.13	0.282	0.410	65.66
2	0.262	0.407	63.66	0.244	0.455	59.38	0.183	0.522	53.85	0.144	0.557	51.69	0.204	0.496	56.59
3	0.196	0.469	58.13	0.183	0.503	55.09	0.137	0.586	48.19	0.110	0.587	50.11	0.162	0.550	52.36
4	0.147	0.508	54.64	0.137	0.521	53.48	0.103	0599	47.04	0.079	0.611	48.89	0.121	0.576	49.52
5	0.110	0.546	51.25	0.103	0.560	50.00	0.077	0.629	44.39	0.062	0.656	44.02	0.096	0.603	46.69
6	0.083	0.587	47.59	0.077	0.592	47.14	0.058	0.636	43.77	0.048	0.677	41.46	0.068	0.626	44.97

Table A-3.4 PI % for each sample solution prepared from soxhlet extraction

No.	Sample								
	1			2			3		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.118	0.562	50.31	0.118	0.498	55.97	0.177	0.456	59.68
2	0.088	0.587	48.09	0.088	0.554	51.02	0.133	0.529	53.23
3	0.066	0.628	44.47	0.066	0.612	45.89	0.099	0.591	47.74
4	0.049	0.666	41.11	0.049	0.660	41.64	0.075	0.618	45.36
5	0.037	0.690	38.99	0.037	0.706	37.58	0.056	0.653	42.26

Table A-3.5 PI % for each sample solution prepared from maceration

No.	Sample								
	1			2			3		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.277	0.506	54.00	0.277	0.507	53.91	0.122	0.590	47.32
2	0.208	0.536	51.27	0.208	0.535	51.36	0.092	0.625	44.19
3	0.156	0.559	49.18	0.156	0.557	49.36	0.069	0.668	40.36
4	0.117	0.591	46.27	0.117	0.588	46.55	0.052	0.677	39.55
5	0.088	0.611	44.46	0.088	0.612	44.36	0039	0.692	38.21
6	0.066	0.634	42.36	0.066	0.627	43.00			

Table A-3.6 PI % for each sample solution prepared from ultrasonic

No.	Sample					
	1			2		
	Conc.(mM)	Abs.	PI%	Conc.(mM)	Abs.	PI%
1	0.422	0.531	53.17	0.443	0.536	52.76
2	0.317	0.551	51.41	0.332	0.547	50.44
3	0.237	0.583	48.59	0.249	0.578	47.69
4	0.178	0.601	47.00	0.187	0.615	46.00
5	0.134	0.628	44.62	0.140	0.605	40.39
6	0.100	0.660	41.79	0.105	0.670	41.19

Using the data PI can be plotted against the concentration of the antioxidant sample and the data are fitted with a linear equation which is then used to determine the value of $IC_{50.}$ The sample determination was shown in Figure A-3.3.

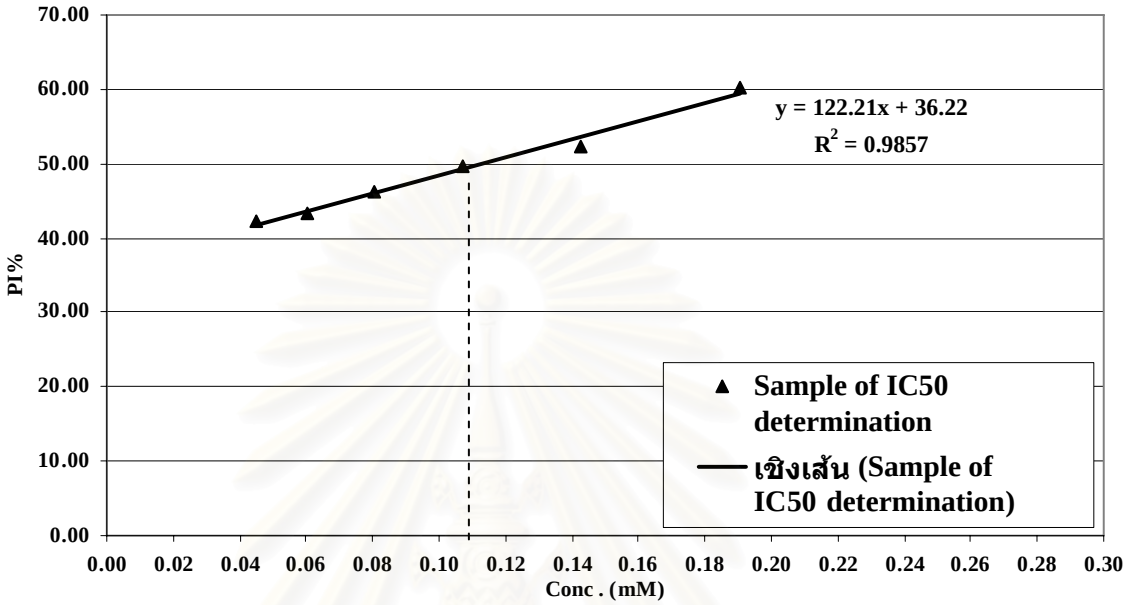


Figure A-3.3 Sample of IC_{50} determination

An example to determine IC_{50} for the PHW extract obtained at 150°C can be seen in this Figure, the fitted equation was y = 122.21x + 36.22, which give the value of IC_{50} defined as the concentration of compound that can inhibit free radical by 50 percents. In other words it is the concentration that gives the PI % equal to 50%. Thus, from the above example, we get an IC_{50} of 0.103 mM.

APPENDIX B

STATISTICAL ANALYSIS

In this study, the antioxidant activities of the extracts from PHWE and other conventional methods are compared. In addition, the effect of temperature of PHW on antioxidant activity of anthraquinones extract was determined for the range of temperature between 150-200 °C. Statistical analysis of the data was conducted to determine the significance of these factors. This section describes how this was done. To test the temperature effect on the extract antioxidant activity, we use analysis of variance by means of F-statistics and to test the effect of various extraction techniques, we use analysis of means by mean of student t-test. The methods of analysis will be described here.

B-1 Analysis of means

To test the equality of the means μ_1 and μ_2 of two normal distributions where the variances are unknown, we test the following null (H_0) and alternative hypotheses (H_1):

$$H_0: \quad \mu_1=\mu_2 \tag{B-1.1}$$

$$H_1: \quad \mu_1\neq\mu_2$$

We then calculate a test statistics, t_0^*, then be calculated from the following equation:

$$t_0^* = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \tag{B-1.2}$$

We accept the null hypothesis if $-t_{\alpha/2,\,\nu} \leq t_0^* \leq t_{\alpha/2,\,\nu}$, in which the degree of freedom, ν, is calculated from:

$$\nu = \frac{(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2})^2}{\frac{(S_1^2/n_1)^2}{n_1+1} + \frac{(S_2^2/n_2)^2}{n_2+1}} - 2 \tag{B-1.3}$$

When the null hypothesis is true, we conclude that the difference between the two means is negligible. If, however, the null hypothesis is rejected, the means are significantly different.

B-2 Analysis of variance (ANOVA)

Suppose we have a different levels of a single factor that we wish to compare. The observed response at each of the factor levels is a random variable. The data would appear in general form as in Table B-2.1. Each entry of the table, denoted by y_{ij}, represents the j_{th} observation taken under treatment *i*. We consider the case where there is an observation, *n*, for each factor level.

Table B-2.1 Typical data for One-Way classification analysis of variance

Treatment	Observation				Total	Average
1	y_{11}	y_{12}	⋯	y_{1n}	$y_{1.}$	$\bar{y}_{1.}$
2	y_{21}	y_{22}	⋯	y_{2n}	$y_{2.}$	$\bar{y}_{2.}$
.	.	.	...	.	.	.
.	.	.	...	.	.	.
.	.	.	...	.	.	.
a	y_{a1}	y_{a2}	⋯	y_{an}	$y_{a.}$	$\bar{y}_{a.}$

We may describe the observations in Table B-2.1 by the linear statistical model.

$$y_{ij} = \mu + \tau_i + \epsilon_{ij} \qquad \text{(B-2.1)}$$

Where y_{ij} is the $(ij)^{th}$ observation, μ is a parameter common to all factors called the oveall mean, τ_i is a parameter associated with the i^{th} factor level called the i^{th} factor effect, and ϵ_{ij} is a random error component. We would like to test certain hypotheses about factor effects and to estimate them. For hypothesis testing, the model errors are assumed to be normally and independently distributed random variables with mean zero and variance σ^2 is assumed constant for all levels of factor

The factor effect τ_i are usually defined as deviation from the overall mean, so that

$$\sum_{i=1}^{a} \tau_i = 0 \qquad \text{(B-2.2)}$$

Let

$y_{i.}$ = the total of observations under the i_{th} treatment

$\bar{y}_{i.}$ = the average of observations under the i_{th} treatment

$y..$ = the grand total of all observations

$\bar{y}_{..}$ = the grand mean of all observations

Which expressed mathematically, as

$$y_{i.} = \sum_{j=1}^{n} y_{ij} \qquad \bar{y}_{i.} = y_{i.} / n \qquad i = 1, 2, \ldots, a \tag{B-2.3}$$

$$y.. = \sum_{i=1}^{a}\sum_{j=1}^{n} y_{ij} \qquad \bar{y}_{..} = y_{..} / N \tag{B-2.4}$$

where $N = an$ is the total number of observations. Thus the "dot" subscript notation implies summation over the subscript that it replaces.

We are interested in testing the equality of the a treatment effects. The appropriate hypotheses are

$$H_0: \quad \tau_1 = \tau_2 = \ldots = \tau_a = 0 \tag{B-2.5}$$

$$H_1: \quad \tau_i \neq 0 \text{ for at least one } i$$

That is, if the null hypothesis is true, then conclude that treatment effect insignificantly affects the result. The total corrected sum of squares, which is a measure of total variability in the data, and may be written as

$$\sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{..})^2 = \sum_{i=1}^{a}\sum_{j=1}^{n}[(\bar{y}_{i.} - \bar{y}_{..}) + (y_{ij} - \bar{y}_{i.})]^2 \tag{B-2.6}$$

or

$$\sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{..})^2 = n\sum_{i=1}^{a}(\bar{y}_{i.} - \bar{y}_{..})^2 + \sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{i.})^2 + 2\sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{i.})(\bar{y}_{i.} - \bar{y}_{..})$$

Note that the cross-product term in above equation is zero, since

$$\sum_{j=1}^{n}(y_{ij} - \bar{y}_{i}.) = y_{i.} - n\bar{y}_{i.} = y_{i.} - n(y_{i.} / n) = 0$$

Therefore, we have

$$\sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{..})^2 = n\sum_{i=1}^{a}(\bar{y}_{i.} - \bar{y}_{..})^2 + \sum_{i=1}^{a}\sum_{j=1}^{n}(y_{ij} - \bar{y}_{i.})^2 \tag{B-2.7}$$

This equation shows that the total variability in the data, measured by the total corrected sum of squares, can be partitioned into a sum of squares of differences between treatment means and the grand mean and a sum of squares of differences of observations within treatments and the treatment mean. Differences between observed treatments means and the grand mean measure the differences between treatments, while difference of observations within a treatment from the treatment mean can be due only to random error. Therefore, we write equation as

$$SS_T = SS_{treatment} + SS_E \quad \text{(B-2.8)}$$

Where

SS_T = The total sum of squares

$SS_{treatment}$ = The sum of squares due to treatment

SS_E = The sum of squares due to error

As mentioned earlier, there are $an = N$ total observations; thus SS_T has N-1 degree of freedom. There are a levels of the factor, so $SS_{treatment}$ has a-1 degree of freedom. Finally, within any treatment there are n replicates providing n-1 degree of freedom. Since there are a treatments, we have $a(n\text{-}1) = an\text{-}a = N\text{-}a$ degree of freedom for error.

We now present test for comparing variances with suppose that the F statistic

$$F_0 = \frac{SS_{treatment}/(a-1)}{SS_E/(N-a)} = \frac{MS_{treatment}}{MS_E} \quad \text{(B-2.9)}$$

Efficient computational formulas for the sums of squares may be obtained by equations

$$SS_T = \sum_{i=1}^{a}\sum_{j=1}^{n} y_{ij}^2 - \frac{y_{..}^2}{N} \quad \text{(B-2.10)}$$

and

$$SS_{treatment} = \sum_{i=1}^{a} \frac{y_{i.}^2}{n} - \frac{y_{..}^2}{N} \quad \text{(B-2.11)}$$

The error sum of squares is obtained by

$$SS_E = SS_T - SS_{treatment} \quad \text{(B-2.12)}$$

Therefore, we would reject H_0 if $F_0 > F_{\alpha,\, a\text{-}1,\, N\text{-}a}$. Where $F_{\alpha,\, a\text{-}1,\, N\text{-}a}$ is the upper percentage point of the *F* distribution with *a*-1 and *N*-*a* degrees of freedom. The test procedure is summarized in Table B-2. This is called an analysis of variance table.

Table B-2.2: The analysis of variance for the one-way classification fixed effects model

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F_0
Between Treatments	$SS_{treatments}$	a-1	$MS_{treatment}$	$F_0 = \frac{MS_{treatment}}{MS_E}$
Error (within Treatments)	SS_E	N-a	MS_E	
Total	SS_T	N-1		

B-3 Effect of temperature on antioxidant activity: analysis of variance

Initially, statistical analysis was performed to determine the effect of temperatures on the antioxidant activity of the PHW extract. Here only one factor (temperature) was investigated. Three levels of temperature (150, 170, and 200 °C) were experimentally tested each with 5 observations or replicates. The experiment was in completely randomized order and the data for IC_{50} values are summarized in Table B-3.1 below.

Table B-3.1: IC_{50} of anthraquinones from subcritical water extraction.

Temperature	Observations					Totals	Averages
(°C)	1	2	3	4	5		
150	0.099	0.112	0.123	0.103	0.112	0.549	0.110
170	0.125	0.099	0.115	0.125	0.093	0.557	0.111
200	0.112	0.102	0.121	0.116	0.135	0.586	0.117
						1.692	0.113

From the data in Table B-3, the sums of squares for analysis of variance are computed.

$$SS_T = \sum_{i=1}^{a}\sum_{j=1}^{n} y_{ij}^2 - \frac{y_{..}^2}{N} = [(0.099)^2 + (0.112)^2 + \ldots + (0.135)^2] - (1.692)^2/15$$

$$= 0.0019$$

$$SStreatment = \sum_{i=1}^{a} \frac{y_{i.}^2}{n} - \frac{y_{..}^2}{N} = [((0.5497)^2 + (0.557)^2 + (0.586)^2)/\ 5] - (1.692)^2/15$$

$$= 0.00015$$

$$SS_E = SS_T - SS_{treatment} = 0.0019 - 0.00015$$

$$= 0.0018$$

These results summarized as the analysis of variance table in Table B-3.2.

Table B-3.2 Analysis of variance for the antioxidant with subcritical water extraction data

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F_0
Between Treatments	0.00015	2	0.000075	$F_0 = 0.000075/0.00015 = 0.5$
Error (within treatments)	0.0018	12	0.00015	
Total	0.0019	14		

From Table V, the percentage points of the *F* Distribution (Montgomery D.C.) with 99% confidence interval:

$$F_{\alpha, a-1, N-a} = F_{0.01, 2, 12} = 6.93$$

F_0 is less than 6.93, thus subcritical water temperature in the range studied does not significantly affect to antioxidant activity of anthraquinones extract.

B-4 Effect of different extraction methods on antioxidant activity: analysis of means

As mentioned above, temperature in the range studied does not significantly affect to antioxidant activity of anthraquinones extract, thus all specimens was used as the antioxidant data of anthraquinones. The average value for this method was 0.113 mM and the variance was 0.000139. These two values, respectively, are used as the $\overline{X}_1$ and S_1^2 for t-statistical calculation in section B-1. The mean $\overline{X}_1$ was compared with the mean of each case ($\overline{X}_2$). The values for $\overline{X}_1$ and S_2^2 for each case are summarized in Table B-4.1, from which the t-statistic, t_0^*, and the degree of freedom, *v*, for each pair can then be calculated.

For example, for the comparison of antioxidant activity of the extracts from PHWE and soxhlet extraction, this is the t_0^* value calculation for soxhlet extraction case.

The given data are 0.102 in mean and 0.000169 in variance of this case.

$$t_0^* = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

$$t_0^* = (0.113\text{-}0.102) \ / \ \sqrt{(0.000139/15) + (0.000169/3)}$$

$$= 0.011 / 0.0081$$

$$= 1.358$$

v is calculated from:

$$\nu = \frac{(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2})^2}{\frac{(S_1^2/n_1)^2}{n_1+1} + \frac{(S_2^2/n_2)^2}{n_2+1}} - 2$$

$$= \frac{[(0.000139/15) + (0.000169/3)]^2}{\frac{(0.000139/15)^2}{15+1} + \frac{(0.000169/3)^2}{3+1}} - 2$$

$$= \quad (4.305/\ 0.798) - 2$$

$$= \quad 3.39$$

$$\approx \quad 3$$

For a confidence level $\alpha = 0.01$, $t_{\alpha/2,\ \nu} = 5.841$ (*Montgomery D.C., 1990*).

Table B-4.1 *t* statistical analysis

Method	Average ($\overline{X}$)	Variance (σ^2)	t_0^*	ν	$t_{\alpha/2,\ \nu}$	*P*-value
Soxhlet extraction	0.102	0.000169	1.358	3	5.841	0.40
Maceration	0.176	0.000625	-4.27	2	9.925	0.10
Ultrasonic	0.300	7.84 E-6	-51.46	23	2.807	< 0.0001

From the analysis, $-\ t_{\alpha/2,\ \nu} < t_0^* < t_{\alpha/2,\ \nu}$, we therefore accept null hypothesis for the comparison of PHWE with soxhlet extraction. Same conclusion was reached with the comparison of PHWE and maceration. However, we reject the null hypothesis in the case of the comparison with ultrasonic extraction. In addition, *P*-values in the right-most column represent the smallest level of significance that would lead to rejection of H_0. *P*-values are determined from the the following equation

$$P = 2[\Phi(|t_0^*|)] \qquad \text{(B-13)}$$

Where, $\Phi(|t_0^*|)$ is the probability that the test statistic will take on a value at least as extreme when H_0 is true.

Generally, if *P* is less than or equal toα, we would reject H_0, whereas if *P* exceeds α we would fail to reject H_0. In this study, we chose a confidence level $\alpha = 0.01$ for all statistic analysis. The *P*-values thus agreed with the result derived from the t-test that led to the conclusion that the antioxidant activity of anthraquinones extract obtained with soxhlet extraction and maceration was not different from that with subcritical water extraction but was the activity of the extract derived from ultrasound assisted extraction was significantly different.

APPENDIX C

EXPERIMENTAL DATA

C-1 Experimental data of anthraquinones extract with subcritical water

Temperature effect experiment

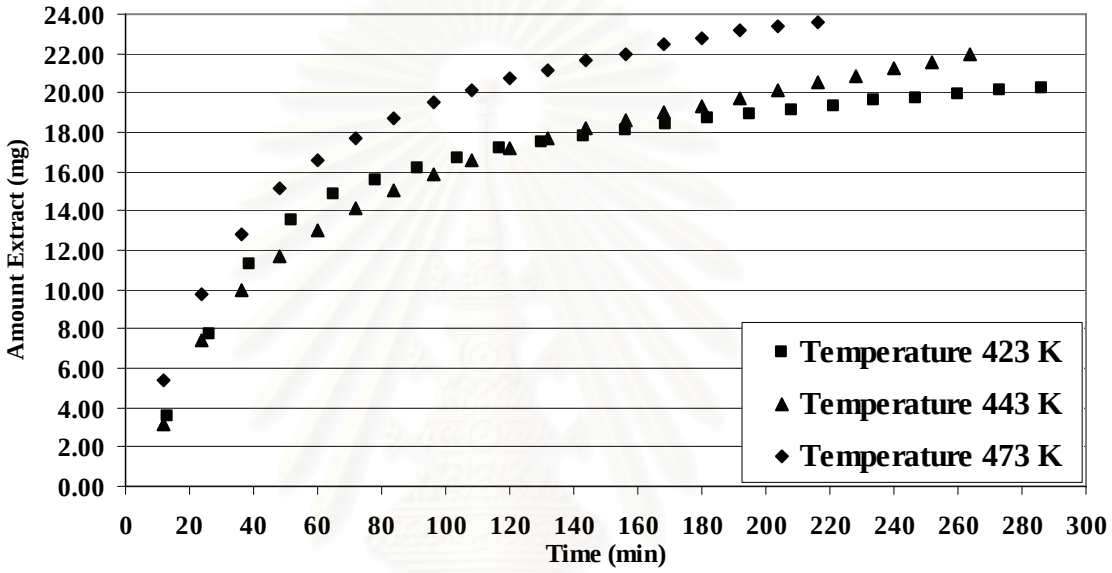


Figure C-1.1: Effect temperature with flow rate of 2 ml/min

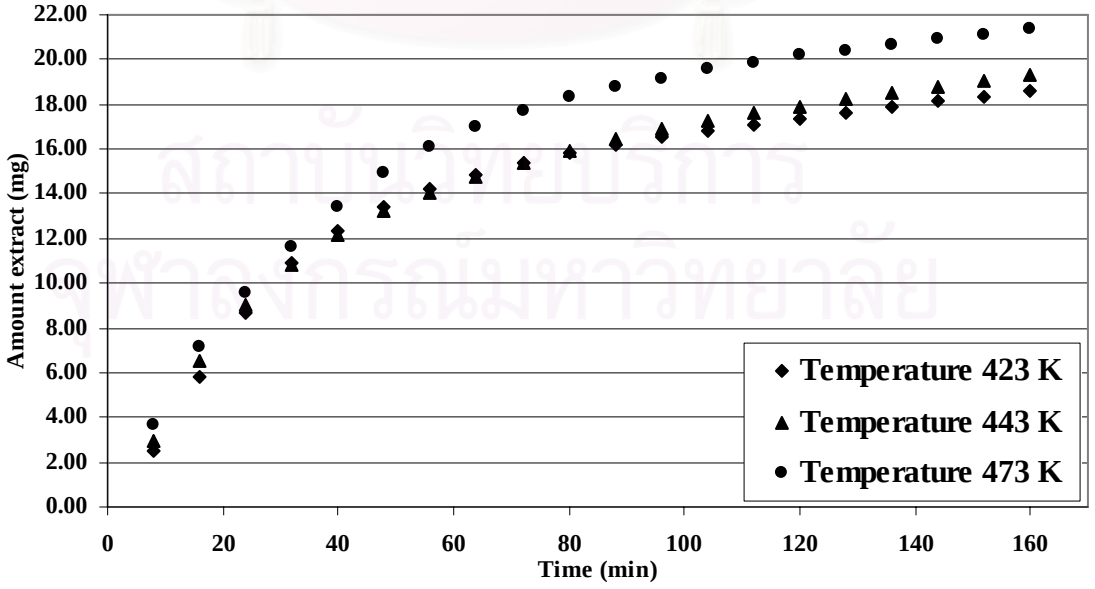


Figure C-1.2: Effect temperature with flow rate of 3 ml/min

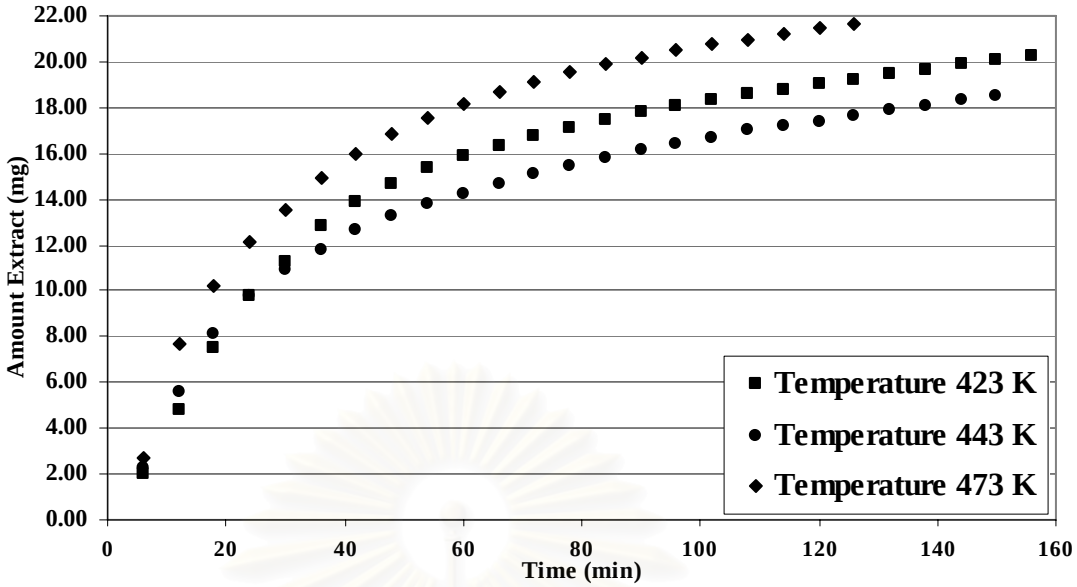


Figure C-1.3: Effect temperature with flow rate of 4 ml/min

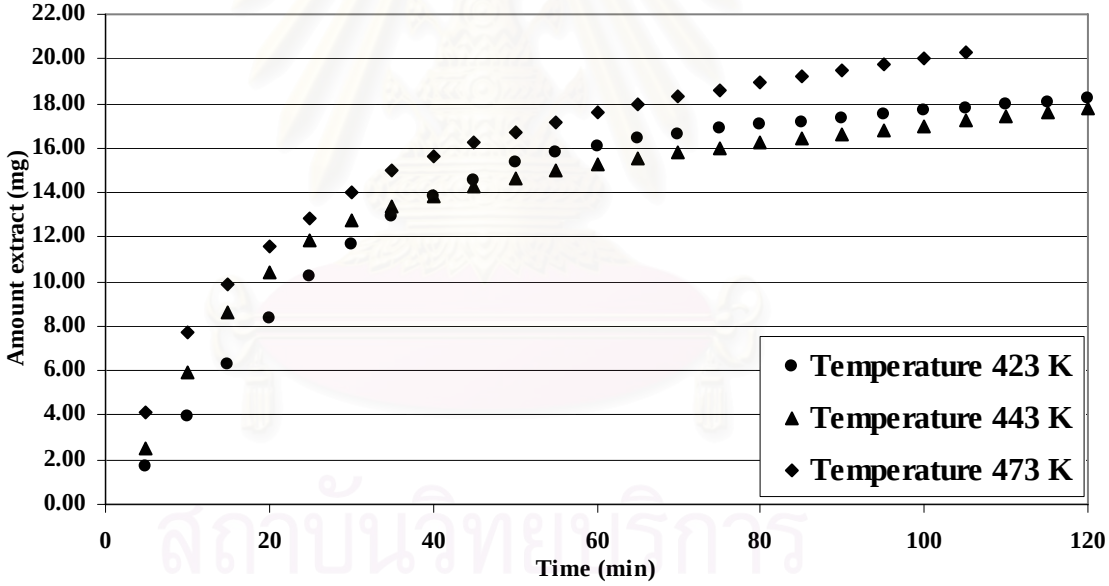


Figure C-1.4: Effect temperature with flow rate of 5 ml/min

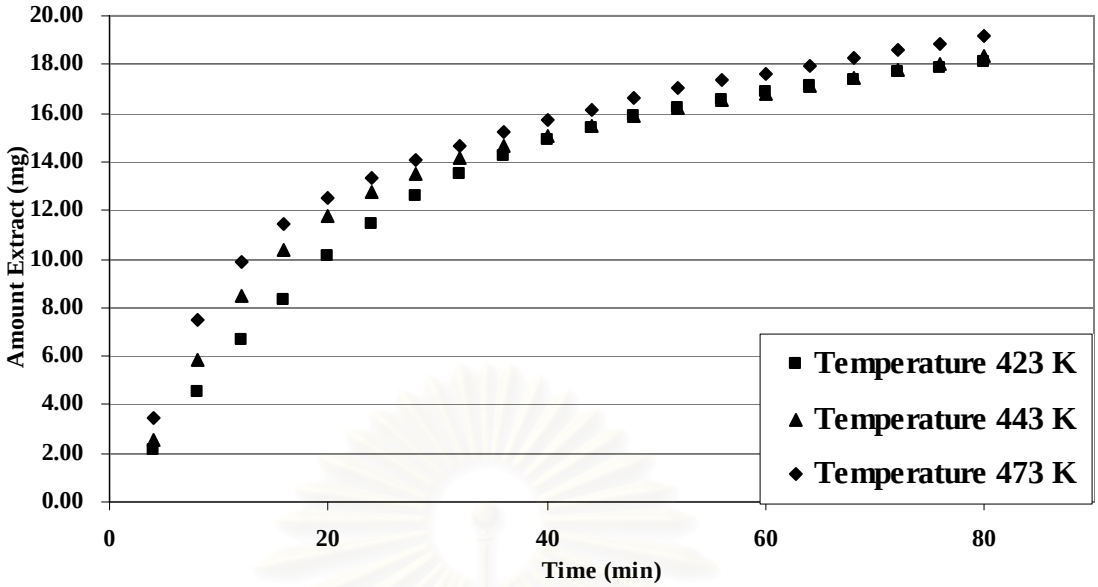


Figure C-1.5: Effect temperature with flow rate of 6 ml/min

Flow rate effect experiment

Table C-1.1: Subcritical water extraction of temperature 150 °C for flow rate 2 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
13	24	16.46	16.75	12.99	15.39	2.092
26	48	39.39	32.29	29.62	33.77	5.052
39	72	53.29	53.35	41.15	49.26	7.027
52	96	60.90	68.42	50.17	59.83	9.169
65	120	66.33	75.59	56.84	66.26	9.378
78	144	70.18	78.58	63.09	70.62	7.755
91	168	73.35	81.11	67.53	73.99	6.811
104	192	75.68	83.02	71.28	76.66	5.931
117	216	77.54	84.74	75.03	79.10	5.042
130	240	79.27	86.12	77.39	80.93	4.595
143	264	80.77	87.36	79.47	82.53	4.226
156	288	82.09	88.45	81.69	84.08	3.790
169	312	83.31	89.44	83.13	85.24	3.589
182	336	84.82	90.27	84.45	86.51	3.258
195	360	86.15	91.06	85.73	87.65	2.962
208	384	87.46	91.80	87.29	88.85	2.557
221	408	88.68	92.45	88.76	89.97	2.152
234	432	89.91	93.07	90.10	91.03	1.771
247	456	91.06	93.63	91.36	92.02	1.402
260	480	92.09	94.12	92.55	92.92	1.063
273	504	93.08	94.68	93.74	93.83	0.803
286	528	93.97	95.26	94.79	94.68	0.655
299	552	94.82	95.79	95.94	95.52	0.611

Table C-1.2: Subcritical water extraction of temperature 150 °C for flow rate 3 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
8	24	7.13	14.64	14.40	12.06	4.267
16	48	21.39	30.21	32.00	27.86	5.682
24	72	37.22	42.98	45.41	41.87	4.202
32	96	48.79	53.91	53.81	52.17	2.921
40	120	57.65	59.75	59.61	59.00	1.173
48	144	64.02	64.06	63.81	63.96	0.138
56	168	68.59	67.32	67.43	67.78	0.705
64	192	72.36	70.00	70.15	70.84	1.319
72	216	75.13	72.31	72.62	73.35	1.549
80	240	77.49	74.29	75.26	75.68	1.639
88	264	79.45	76.09	77.13	77.55	1.720
96	288	81.19	77.75	78.75	79.23	1.769
104	312	81.34	79.16	80.16	80.22	1.094
112	336	82.75	80.52	81.71	81.66	1.117
120	360	84.12	81.63	83.13	82.96	1.252
128	384	85.29	83.13	84.27	84.23	1.087
136	408	86.49	84.52	85.35	85.46	0.994
144	432	87.56	85.79	86.46	86.60	0.890
152	456	88.87	87.05	87.45	87.79	0.955
160	480	90.12	88.25	88.42	88.93	1.036
168	504	91.31	89.37	89.34	90.01	1.132
176	528	92.49	90.45	90.26	91.07	1.239
184	552	93.64	91.53	91.10	92.09	1.359
192	576	94.78	92.55	91.93	93.09	1.498
200	600	95.85	93.54	92.73	94.04	1.619

Table C-1.3: Subcritical water extraction of temperature 150 °C for flow rate 4 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
6	24	7.39	10.89	10.35	9.54	1.882
12	48	19.52	24.59	23.52	22.54	2.673
18	72	32.21	36.42	36.39	35.00	2.420
24	96	43.39	46.18	47.11	45.56	1.934
30	120	51.26	53.87	52.64	52.59	1.307
36	144	58.36	60.07	61.05	59.82	1.360
42	168	64.14	64.24	65.22	64.53	0.596
48	192	68.69	67.62	68.72	68.34	0.627
54	216	72.17	70.20	71.62	71.32	1.013
60	240	74.95	72.49	74.00	73.81	1.244
66	264	77.14	74.77	76.08	75.99	1.187
72	288	78.98	76.96	77.99	77.97	1.008
78	312	80.59	78.88	79.65	79.71	0.854
84	336	82.04	80.57	81.63	81.41	0.758
90	360	83.39	82.06	83.22	82.89	0.723
96	384	84.67	83.45	84.52	84.21	0.664
102	408	85.70	84.74	85.56	85.34	0.518
108	432	86.76	86.00	86.62	86.46	0.402
114	456	87.68	87.19	87.56	87.48	0.253
120	480	88.55	88.32	88.43	88.43	0.112
126	504	89.62	89.45	89.52	89.53	0.086
132	528	90.61	90.56	90.52	90.56	0.046
138	552	91.54	91.60	91.45	91.53	0.074
144	576	92.39	92.57	92.32	92.43	0.127
150	600	93.24	93.51	93.17	93.31	0.177
156	624	94.03	94.40	93.97	94.14	0.234
162	648	94.78	95.22	94.73	94.91	0.272
168	672	95.49	96.01	95.45	95.65	0.309

Table C-1.4: Subcritical water extraction of temperature 150 °C for flow rate 5 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
5	25	7.96	8.35	9.02	8.44	0.538
10	50	21.18	18.08	21.43	20.23	1.867
15	75	32.61	28.68	33.61	31.63	2.607
20	100	43.01	40.03	43.77	42.27	1.976
25	125	52.76	48.76	53.47	51.66	2.541
30	150	60.15	56.41	60.77	59.11	2.359
35	175	66.15	61.99	67.77	65.30	2.984
40	200	70.34	66.27	72.15	69.59	3.012
45	225	74.11	69.57	76.10	73.26	3.344
50	250	77.07	73.96	80.04	77.02	3.040
55	275	79.44	76.20	82.05	79.23	2.928
60	300	81.47	78.11	83.62	81.07	2.778
65	325	83.18	79.77	84.94	82.63	2.624
70	350	84.69	81.34	86.03	84.02	2.416
75	375	86.17	82.67	86.99	85.28	2.296
80	400	87.53	83.96	87.90	86.46	2.177
85	425	88.74	84.07	88.68	87.16	2.676
90	450	89.88	85.25	89.39	88.18	2.546
95	475	90.94	86.32	90.07	89.11	2.455
100	500	91.04	87.31	90.71	89.69	2.062
105	525	92.05	88.28	91.29	90.54	1.997
110	550	92.63	89.15	91.86	91.21	1.825
115	575	93.29	90.00	92.39	91.90	1.697
120	600	93.95	90.80	92.90	92.55	1.603
125	625	94.61	91.59	93.39	93.20	1.517

Table C-1.5: Subcritical water extraction of temperature 150 °C for flow rate 6 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
4	24	7.35	10.60	5.67	7.87	2.504
8	48	15.36	21.83	16.28	17.82	3.498
12	72	22.98	31.17	25.95	26.70	4.145
16	96	30.86	39.25	34.25	34.78	4.218
20	120	37.82	47.07	42.55	42.48	4.624
24	144	44.19	53.13	49.58	48.96	4.502
28	168	50.64	58.43	55.79	54.95	3.959
32	192	56.55	62.63	60.97	60.05	3.140
36	216	61.19	66.34	65.22	64.25	2.710
40	240	65.37	69.48	68.67	67.84	2.175
44	264	68.65	72.30	71.83	70.92	1.987
48	288	71.27	74.81	74.42	73.50	1.940
52	312	73.58	77.08	76.71	75.79	1.925
56	336	75.42	79.06	78.57	77.69	1.974
60	360	77.12	80.87	80.37	79.45	2.035
64	384	78.62	82.45	82.01	81.02	2.095
68	408	79.97	83.85	83.55	82.46	2.158
72	432	81.16	85.09	85.51	83.92	2.396
76	456	82.27	86.26	87.31	85.28	2.655
80	480	83.24	87.32	88.99	86.52	2.955
84	504	84.35	88.33	90.56	87.75	3.149
88	528	85.35	89.30	92.05	88.90	3.368
92	552	86.29	90.13	93.44	89.95	3.578
96	576	87.17	90.85	94.11	90.71	3.467
100	600	88.01	91.51	94.65	91.39	3.319
104	624	88.81	92.14	95.10	92.02	3.145

Table C-1.6: Subcritical water extraction of temperature 170 °C for flow rate 2 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
12	24	14.41	16.49	10.34	13.75	3.131
24	48	31.75	36.38	24.51	30.88	5.981
36	72	44.36	47.00	34.52	41.96	6.578
48	96	53.82	53.75	42.03	49.87	6.789
60	120	60.80	58.35	48.53	55.89	6.493
72	144	65.53	62.34	53.84	60.57	6.044
84	168	69.59	65.73	58.31	64.54	5.730
96	192	73.79	68.68	62.56	68.34	5.624
108	216	76.71	71.15	66.01	71.29	5.348
120	240	79.41	73.33	68.99	73.91	5.236
132	264	81.64	75.35	71.49	76.16	5.125
144	288	83.87	77.12	73.58	78.19	5.228
156	312	85.32	78.78	75.58	79.90	4.963
168	336	86.51	80.32	77.31	81.38	4.691
180	360	87.64	81.66	78.86	82.72	4.484
192	384	88.76	83.25	80.47	84.16	4.220
204	408	89.78	84.72	81.93	85.48	3.978
216	432	90.77	86.11	83.36	86.75	3.747
228	456	91.74	87.43	84.75	87.97	3.527
240	480	92.64	88.70	86.02	89.12	3.329
252	504	93.54	89.85	87.36	90.25	3.111
264	528	94.45	90.98	88.66	91.36	2.915
276	552	95.40	92.09	89.99	92.49	2.723
288	576	96.31	93.09	91.28	93.56	2.545
300	600	97.18	94.06	92.51	94.58	2.376

Table C-1.7: Subcritical water extraction of temperature 170 °C for flow rate 3 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
8	24	11.00	17.38	14.63	14.34	3.201
16	48	23.80	39.15	30.20	31.05	7.711
24	72	35.67	51.70	42.98	43.45	8.028
32	96	45.64	59.56	53.90	53.03	7.002
40	120	53.13	64.73	59.74	59.20	5.821
48	144	59.58	68.68	64.06	64.10	4.550
56	168	64.37	71.71	67.31	67.80	3.698
64	192	68.31	74.41	70.00	70.91	3.153
72	216	72.01	76.86	72.30	73.72	2.722
80	240	75.24	78.99	74.29	76.17	2.484
88	264	78.13	80.93	76.08	78.38	2.431
96	288	80.41	82.70	77.74	80.28	2.478
104	312	82.54	84.31	79.15	82.00	2.620
112	336	84.30	85.77	80.52	83.53	2.712
120	360	86.01	87.08	81.63	84.91	2.890
128	384	87.57	88.60	83.12	86.43	2.912
136	408	89.04	90.04	84.51	87.86	2.944
144	432	90.32	91.42	85.79	89.17	2.983
152	456	91.60	92.70	87.05	90.45	2.994
160	480	92.55	93.77	88.24	91.52	2.904

Table C-1.8: Subcritical water extraction of temperature 170 °C for flow rate 4 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
6	24	15.22	14.16	8.26	12.55	3.749
12	48	27.39	33.51	22.26	27.72	5.632
18	72	38.25	46.12	34.98	39.78	5.726
24	96	49.11	54.18	44.01	49.10	5.084
30	120	57.0	59.71	49.61	55.44	5.228
36	144	61.90	63.76	54.35	60.00	4.983
42	168	65.90	66.80	59.50	64.07	3.983
48	192	69.78	69.43	63.14	67.45	3.736
54	216	72.89	71.73	65.98	70.20	3.702
60	240	75.60	73.76	68.72	72.69	3.564
66	264	77.82	75.70	71.14	74.89	3.411
72	288	79.94	77.33	73.46	76.91	3.260
78	312	82.08	78.87	75.63	78.86	3.225
84	336	83.63	80.32	77.68	80.54	2.981
90	360	85.04	81.57	79.65	82.08	2.733
96	384	86.45	82.72	81.52	83.56	2.568
102	408	87.81	83.85	83.26	84.97	2.476
108	432	88.84	84.86	85.24	86.31	2.193
114	456	89.82	85.81	86.31	87.31	2.187
120	480	90.77	86.80	87.39	88.32	2.146
126	504	91.71	87.99	88.64	89.45	1.985
132	528	92.62	89.16	89.81	90.53	1.840
138	552	93.50	90.28	90.93	91.57	1.707
144	576	94.36	91.36	92.01	92.58	1.580

Table C-1.9: Subcritical water extraction of temperature 170 °C for flow rate 5 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
5	25	13.36	11.24	13.78	12.79	1.363
10	50	33.29	24.26	33.00	30.18	5.130
15	75	48.12	36.04	47.94	44.03	6.924
20	100	56.40	44.60	59.78	53.59	7.968
25	125	62.01	52.99	67.74	60.91	7.437
30	150	65.79	58.59	71.82	65.40	6.623
35	175	68.64	62.71	74.68	68.68	5.987
40	200	70.97	66.08	77.01	71.35	5.474
45	225	72.74	68.97	78.95	73.55	5.043
50	250	74.34	71.44	80.65	75.47	4.712
55	275	75.88	73.69	82.08	77.22	4.354
60	300	77.29	75.66	83.39	78.78	4.075
65	325	78.57	77.50	84.51	80.19	3.774
70	350	79.72	79.09	85.53	81.45	3.548
75	375	80.81	80.54	86.50	82.62	3.366
80	400	82.12	81.91	87.34	83.79	3.072
85	425	83.39	83.15	88.08	84.87	2.780
90	450	84.61	84.34	88.79	85.91	2.495
95	475	85.81	85.48	89.47	86.92	2.212
100	500	86.92	86.55	90.12	87.86	1.963
105	525	88.02	87.96	90.92	88.97	1.691
110	550	89.05	89.22	91.70	89.99	1.480
115	575	90.07	90.45	92.40	90.97	1.251
120	600	91.07	91.56	93.06	91.89	1.036
125	625	92.04	92.56	93.70	92.77	0.849
130	650	92.94	93.52	94.28	93.58	0.672
135	675	93.86	94.43	94.86	94.38	0.505

Table C-1.10: Subcritical water extraction of temperature 170 °C for flow rate 6 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
4	24	12.91	10.67	11.96	11.85	1.123
8	48	29.28	26.68	26.46	27.47	1.570
12	72	41.97	39.02	37.33	39.44	2.347
16	96	50.04	47.69	45.12	47.62	2.458
20	120	57.88	53.53	51.69	54.37	3.176
24	144	63.17	58.21	56.85	59.41	3.327
28	168	67.71	61.85	60.53	63.36	3.824
32	192	71.11	64.62	63.76	66.49	4.016
36	216	73.68	66.87	66.53	69.03	4.033
40	240	76.06	68.78	69.08	71.31	4.117
44	264	78.01	70.62	71.39	73.34	4.062
48	288	79.72	72.29	73.43	75.15	4.002
52	312	81.35	73.82	75.13	76.77	4.020
56	336	82.73	75.12	76.85	78.23	3.989
60	360	84.11	76.31	78.37	79.60	4.042
64	384	85.28	77.81	79.77	80.95	3.874
68	408	86.37	79.40	81.09	82.29	3.633
72	432	87.40	80.92	82.44	83.58	3.389
76	456	88.43	82.41	83.66	84.83	3.180
80	480	89.27	83.84	84.85	85.99	2.888
84	504	90.04	85.25	86.22	87.17	2.535
88	528	90.75	86.57	87.66	88.33	2.172
92	552	91.47	87.89	89.05	89.47	1.825
96	576	92.16	89.11	90.30	90.52	1.536
100	600	92.46	90.32	91.52	91.43	1.071
104	624	92.76	91.53	92.69	92.33	0.693
108	648	93.07	92.65	93.75	93.16	0.555

Table C-1.11: Subcritical water extraction of temperature 200 °C for flow rate 2 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
12	24	22.25	12.10	11.98	15.44	5.898
24	48	40.54	25.98	29.82	32.11	7.548
36	72	52.93	37.83	43.07	44.61	7.669
48	96	62.48	47.16	52.76	54.13	7.750
60	120	68.48	55.49	59.89	61.29	6.610
72	144	73.36	63.37	65.23	67.32	5.311
84	168	77.43	70.09	69.91	72.48	4.289
96	192	80.83	74.70	73.65	76.39	3.879
108	216	83.35	78.55	76.85	79.58	3.370
120	240	85.58	81.62	79.79	82.33	2.960
132	264	87.61	83.76	82.00	84.46	2.871
144	288	89.50	85.66	83.94	86.36	2.846
156	312	91.01	87.36	85.69	88.02	2.724
168	336	92.76	88.85	87.31	89.64	2.807
180	360	94.25	90.29	88.84	91.13	2.796
192	384	95.65	91.66	90.18	92.50	2.828
204	408	96.70	92.96	91.54	93.73	2.665
216	432	97.69	94.16	92.75	94.87	2.543

Table C-1.12: Subcritical water extraction of temperature 200 °C for flow rate 3 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
8	24	17.41	14.73	11.76	14.63	2.826
16	48	32.40	29.81	24.55	28.92	4.001
24	72	42.40	40.58	33.83	38.94	4.514
32	96	50.43	50.53	42.50	47.82	4.608
40	120	58.28	57.99	49.93	55.40	4.741
48	144	64.85	65.04	57.03	62.31	4.571
56	168	70.18	69.72	61.92	67.27	4.639
64	192	73.87	73.68	66.50	71.35	4.203
72	216	76.99	76.94	70.13	74.69	3.948
80	240	79.06	79.72	73.28	77.35	3.542
88	264	81.01	81.91	75.83	79.58	3.281
96	288	82.71	83.63	77.95	81.43	3.046
104	312	84.33	85.24	80.08	83.22	2.755
112	336	85.65	86.70	82.03	84.79	2.448
120	360	86.99	87.98	83.73	86.23	2.223
128	384	88.10	89.07	85.33	87.50	1.938
136	408	89.24	90.03	86.83	88.70	1.670
144	432	90.30	90.96	88.21	89.82	1.435
152	456	91.31	91.85	89.53	90.90	1.212
160	480	92.41	92.67	90.75	91.94	1.044
168	504	93.49	93.62	91.87	92.99	0.977
176	528	94.51	94.52	92.95	93.99	0.908
184	552	95.56	95.43	93.94	94.98	0.897
192	576	96.54	96.33	94.88	95.92	0.906

Table C-1.13: Subcritical water extraction of temperature 200 °C for flow rate 4 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
6	24	10.55	12.93	17.07	13.52	3.299
12	48	28.14	37.70	23.59	29.81	7.199
18	72	41.29	46.74	44.70	44.24	2.750
24	96	49.49	54.56	55.88	53.31	3.368
30	120	54.96	61.51	61.47	59.31	3.765
36	144	59.60	68.46	65.81	64.62	4.547
42	168	63.57	73.50	69.05	68.70	4.974
48	192	67.09	77.33	71.76	72.05	5.125
54	216	69.99	80.45	74.02	74.82	5.275
60	240	72.59	83.24	76.15	77.32	5.416
66	264	74.96	85.41	78.14	79.50	5.354
72	288	77.03	87.13	79.99	81.38	5.191
78	312	79.00	88.66	81.73	83.13	4.980
84	336	80.64	89.98	83.32	84.64	4.808
90	360	82.25	91.13	84.79	86.06	4.573
96	384	83.61	92.15	86.21	87.32	4.374
102	408	84.98	93.05	87.51	88.51	4.123
108	432	86.23	93.91	88.65	89.59	3.925
114	456	87.39	94.74	89.70	90.61	3.753
120	480	88.50	95.53	90.63	91.55	3.604
126	504	89.79	96.22	91.45	92.49	3.337
132	528	91.04	97.04	92.16	93.41	3.188
138	552	92.27	97.81	92.75	94.28	3.068

Table C-1.14: Subcritical water extraction of temperature 200 °C for flow rate 5 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
5	25	15.04	18.27	24.39	19.23	4.750
10	50	32.89	32.49	41.63	35.67	5.167
15	75	43.92	42.91	51.27	46.03	4.566
20	100	51.55	51.81	58.22	53.86	3.777
25	125	56.81	58.65	64.50	59.99	4.014
30	150	61.27	64.78	69.66	65.24	4.210
35	175	65.11	70.06	74.37	69.84	4.632
40	200	68.25	73.66	77.12	73.01	4.470
45	225	71.16	76.73	79.43	75.77	4.216
50	250	73.71	78.96	81.41	78.03	3.932
55	275	76.03	81.05	83.40	80.16	3.764
60	300	78.03	82.89	85.45	82.12	3.765
65	325	79.89	84.40	86.98	83.76	3.593
70	350	81.63	85.77	88.56	85.32	3.491
75	375	83.21	87.04	89.97	86.74	3.390
80	400	85.02	88.20	91.21	88.14	3.096
85	425	86.71	89.30	92.32	89.44	2.811
90	450	88.29	90.76	93.49	90.85	2.597
95	475	89.80	92.13	94.60	92.18	2.397
100	500	91.21	93.44	95.68	93.44	2.231
105	525	92.57	94.63	96.11	94.44	1.779
110	550	93.89	95.78	96.75	95.47	1.452
115	575	95.12	96.81	97.28	96.41	1.134

Table C-1.15: Subcritical water extraction of temperature 200 °C for flow rate 6 ml/min

Time (min)	Vol. (ml)	Recovery (%)				
		1	2	3	Average	Std.
4	24	12.17	19.07	16.23	15.82	3.465
8	48	27.19	37.97	37.26	34.14	6.028
12	72	37.46	47.09	50.37	44.97	6.714
16	96	45.02	52.96	58.67	52.22	6.855
20	120	50.59	56.64	64.10	57.11	6.767
24	144	54.96	59.61	68.36	60.98	6.802
28	168	58.48	62.30	71.66	64.14	6.780
32	192	61.69	64.48	74.49	66.89	6.733
36	216	64.51	66.49	76.92	69.30	6.667
40	240	67.14	68.46	78.99	71.53	6.493
44	264	69.69	70.59	80.83	73.70	6.189
48	288	71.93	72.61	82.53	75.69	5.934
52	312	74.08	74.48	84.02	77.53	5.629
56	336	75.85	76.31	85.33	79.17	5.346
60	360	77.63	76.48	86.54	80.22	5.506
64	384	79.22	78.08	87.92	81.74	5.386
68	408	80.76	79.62	89.20	83.20	5.231
72	432	82.19	81.04	90.43	84.55	5.121
76	456	83.53	82.41	91.55	85.83	4.986
80	480	84.78	83.81	92.59	87.06	4.813
84	504	86.29	85.21	93.60	88.37	4.563
88	528	87.73	86.60	94.57	89.63	4.309
92	552	89.06	88.00	95.47	90.84	4.040
96	576	89.18	89.39	96.34	91.64	4.073

Table C-1.16: Maximum concentration of anthraquinones with subcritical water extraction

Temperature (°C)	Maximum concentration (mg/ml)	
	Flow rate 2 ml/min	Flow rate 6 ml/min
150	0.135	0.104
170	0.218	0.184
200	0.272	0.227

C-2 Experimental data of anthraquinones extract with conventional method

Method	Extraction time (hr)	Recovery (%)				
		1	2	3	Average	Std.
Maceration	72	80.96	84.31	78.20	81.16	2.703
Soxhlet extraction	4	97.50	97.57	98.75	97.94	0.698
Ultrasonic	2	79.22	80.01	-	79.62	0.555

VITA

Mr. Boonchai Pongnaravan was born on 25 April, 1981 in Bangkok, Thailand. He received a Bachelor's Degree of Chemical Engineering from the Faculty of Engineering, Mahidol University in 2001. After then he subsequently completed the requirements for a Master's Degree in Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University in 2005.