MICROWAVE ASSISTED EXTRACTION: THE EFFECTS, MECHANISMS AND APPLICATIONS ON SELECTED PLANT MATERIALS

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Microwave Assisted Extraction: the Effects, Mechanisms and Applications on Selected Plant Materials

by

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CERTIFICATE OF ORIGINALITY

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Hu Zhuoyan

June, 2010

Abstract

The application of solvent free microwave extraction (SFME) and microwave assisted extraction (MAE) of the effective compounds from plant matrices was investigated. The diffusion coefficient of saikosaponins through the solid matrix under MAE was determined. The effect of microwave irradiation on microstructure of plant tissues was observed using scanning electronic microscopy (SEM) technique.

SFME was employed to obtain essential oil from pomelo fruit peels, and sequential MAE of pectin from oil extracted peels was also performed. SFME was superior to the conventional hydrodistillation (HD) method in terms of extraction efficiency and the essential oil yield. The chemical composition analysis by GC-MS shows that SFME did not affect the quality of essential oils compared with HD. In extracting pectin from oil extracted pomelo peels, the extraction time of MAE was significantly shorter than that of the conventional method. The sequential extraction of essential oil and pectin from pomelo fruit peels by SFME and MAE was a feasible processing method.

In the study of MAE for extraction of saikosaponins from *Radix Bupleuri,* the individual effects of microwave power, irradiation time, temperature, ethanol concentration, solvent-to-sample ratio, and sample particle size were evaluated. It was found that the extraction of saikosaponins a, c, and d by MAE with 300 to 500 W power level for 5 min at 75 °C with 30–70 % ethanol in water, 30:1 solvent-to-sample ratio, and 0.30 to 0.45 mm particle size were the favorable extraction conditions. Compared with conventional extraction methods, MAE can significantly reduce the extraction time, resulting in better extraction efficiency.

In the optimization of MAE for saikosaponins, microwave power, time, temperature and ethanol concentration were optimized using response surface methodology (RSM) with desirability function approach. The optimum MAE conditions for extracting saikosaponin a, c, and d simultaneously were found to be at the microwave power of 360-400 W, ethanol concentration of 47-50 %, temperature of 73-74 ^{o}C and time of 5.8-6.0 min. At these conditions, the yields from the verification experiments were 96.18-96.91 % for saikosaponin a, 95.05-95.71 % for saikosaponin c, and 97.05-97.25 % for saikosaponin d, which were in good agreement with the predicted values from the fitted models.

In the mechanism studies, the diffusion coefficients of saikosaponins through the solid matrix under MAE were determined using a Fick's second law-based model. It was found that the effective diffusion coefficients (D_{eff}) under different microwave heating conditions increased as a result of the increase in microwave power, and were significantly higher than those extracted with the conventional extraction method. SEM results indicated that microwave heating produced distinguishable microstructure changes on pomelo peels and *Radix Bupleuri*. Microwave irradiation caused the explosion of oil glands of pomelo peels and rupture of parenchymal cells; therefore the target compounds within the cell were rapidly released into the surrounding extraction solvents. As the liquid phase absorbed the microwaves, the kinetic energy of the molecules increased, and consequently, the diffusion rate accelerated. As a result, better extraction efficiency and significantly reduced extraction time for extraction of the effective compounds from two plant matrices were obtained using MAE.

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Chapter 1. Introduction

The use of traditional herbs for treatment of various diseases has a long history in China. In recent years the interest in research and development of traditional Chinese medicine (TCM) has been raised enormously, since herbal extracts and prescriptions contain multiple effective components, which can provide unique therapeutic properties with minimal or no undesirable side effects and can act in a synergistic manner within the human body.

The extraction and characterization of effective components from medicinal herbs have resulted in the development of new drugs with high therapeutic value. For example, Artemisinin (qing-hao-su in Chinese) is an anti-malarial drug used for treatment of malaria and regarded as a breakthrough in the history of anti-malarial drug. It was isolated from a traditional Chinese medicinal plant 'qing-hao' (*Artemisia annua* L.) and its structure had been defined correctly in 1971 in China [1]. Another example reported by Talebi et al. (2004) is paclitaxel (taxol), which proved to have unique antitumor activities. It was first isolated from the bark of the pacific yew tree (*Taxus brevifolia* Nutt.) [2]. At present, the standardization and quality control of TCM by using modern science and technology is the key to development of modern herbal products. Therefore, extraction of the effective components from plant matrix is a crucial and initial step [3].

Since the effective compounds often exist inside the plant cells, extraction of such intracellular compounds requires that they have to be released into the surrounding medium first. The conventional extraction method is based on a liquid-solid extraction process, in which the solid matrix is placed into water or organic solvent to extract the target compounds. In conventional extraction of TCM, herbal matrices were boiled with water for about 30-60min to prepare

"herbal drinks" for oral intake, or leached with solvent (ethanol or aqueous ethanol) under moderate temperature for a relatively long time to extract the target compounds for preparation of samples, which were used for the herbal prescription medicine, or/and for analysis of analytes. However, this conventional extraction procedure is not only time consuming and low efficiency, but also accompanied by the degradation of some heat-sensitive compounds and uncertainties in analyte recovery. Current tendencies tend to overcome these problems either by the development of new methods, or the improvement of old solvent extraction methods. Therefore, great attention has been paid to develop more efficient methods for the rapid extraction, isolation and analysis of extract from medicinal plants.

There have been numerous publications showing that the use of modern extraction techniques for sample preparation of medicinal plants or herbal materials leads to cleanup, higher efficiency and increase of the extraction yields of effective components [3-4]. These modern extraction techniques include accelerated solvent extraction (ASE), supercritical-fluid extraction (SFE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). However, some extraction techniques have their own limitations. For example, SFE method operated with high system pressure, high capital input for equipment and has the limitation of selectivity on the target compounds.

In recent years, application of microwave energy in extraction has attracted growing interests [5]. MAE has been successfully used for the extraction of effective compounds from different medical matrices in the past 10 years [6-21]. The results from these publications have shown that MAE can significantly reduce extraction time and solvent consumption, while can offer better extraction

efficiency. However, the previous works mainly focused on the search of optimum values for a given system (mainly using a domestic microwave oven or modified domestic microwave oven), while few were on the fundamental kinetics and mechanisms. Also, MAE technique was mainly used in laboratory scale, and no industrial application was reported.

The purpose of this study is to investigate the applicability of MAE for effective compounds from Chinese medicinal plants. The objectives include (1) studying the influence of MAE parameters such as the microwave power, irradiation time, extraction temperature, solvent concentration, matrix load and particle size on the effectiveness and efficiency of the extraction; (2) optimizing the MAE conditions for extraction of effective compounds from Chinese medicinal plants; (3) investigating the effects of microwave irradiation and conventional heating on microstructure changes of plant tissues and proposing a useful model for MAE, thus revealing the fundamental mechanisms and further enriching the theoretical understanding of MAE. Such a project will result in the development of an efficient and economical method to separate effective components from medicinal plants.

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Chapter 2. Literature Review

Extraction is the crucial and initial step for analysis and preparation for botanical and plant matrices. The aim of extraction is to make target compounds suitable for isolation from the matrices or/and analysis.

Several review papers on sample preparation techniques for the extraction plant materials were given by Ong (2004) [1], Zygmunt and Namiesnik(2003) [2], and Huie (2002) [3]. To date, several different techniques have been used for extraction of target compounds from plant matrices. These methods include conventional solvent extraction, Soxhlet extraction, and modern extraction techniques such as accelerated solvent extraction (ASE), supercritical-fluid extraction (SFE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE).

Most of the conventional extractions are time consuming, laborious, additionally, they involve lengthy operation procedures, large solvent consumption and ultimately thermal degradation of the target compounds at high temperature. Current tendencies aim at overcoming these problems either by the development of new extraction methods, or the improvement of old extraction methods.

2.1 Conventional Extraction Methods

2.1.1 Solvent Extraction

Solvent extraction is one of the conventional methods for the extraction of target components from plant matrices. Solvent extraction uses an organic solvent or water as an extractant to extract and separate the target compounds from the plant matrices. The solvent is mixed with plant matrices in an extraction device. The extraction process is sometimes performed at an elevated

temperature in order to improve recovery or extraction yield. The extracted solution then is filtered or passed through a separator, where the target compounds and extractant are separated from the matrix. The extracted solution then is subjected to concentration for further use.

This method is commonly used for the extraction of various active components in TCM preparation..

The shortcomings of this method involve not only time consuming and low efficiency, but also accompanied by degradation of some heat sensitive compounds and uncertainties in analyte recovery.

2.1.2 Soxhlet Extraction

Soxhlet extraction is an old extraction technique for extraction of target compounds and is still widely used in analysis. In a conventional Soxhlet extraction device, the matrix of solid phase, or a solid-liquid mixture sample is placed in a porous cellulose thimble; then the thimble is placed in an extraction chamber that is gradually filled with fresh solvent by condensation of vapors from a distillation flask. When the liquid reaches an overflow level, a siphon aspirates the content of the cavity and unloads it back into the distillation flask, by carrying the extracted analytes in the bulk liquid (Figure 2.1) [4]. This operation is repeated until complete separation is achieved and the analytes are all in the flask. The solvent in the flask is then evaporated and the mass of the remaining components is collected. The advantages of Soxhlet extraction are as follows: the sample phase is repeatedly brought into contact with fresh portions of the solvent and the temperature of the system is higher than room temperature since the heat applied to the distillation flask reaches the extraction cavity to some extent, thereby enhancing the displacement of the analyte from the matrix;

no filtration is required. However, Soxhlet extraction also has significant drawbacks as the long time required for the extraction and the large amount of organic solvent consumed, which are not only expensive to dispose off but also can cause environmental pollution. Soxhlet extraction has been applied for extraction of various compounds from different matrices [5-6]. This method takes a few hours, even more than 24 h for extraction and large amount of solvents are wasted.

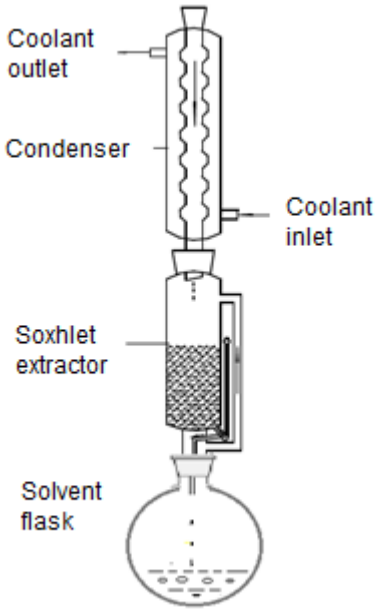


Figure 2.1 Schematic diagram of the Soxhlet extraction (from Wang, et al., 2006) [4]

Although the literatures display good recoveries using Soxhlet extraction for a broad range of compounds, the long extraction time reduces sample throughput and makes Soxhlet extraction an unattractive technique when a large number of samples must be analyzed.

2.2. Modern Extraction Methods

2.2.1 Accelerated Solvent Extraction (ASE)

Richter et al. (1996) described a new technique for sample preparation, accelerated solvent extraction (ASE), which combines elevated temperatures and pressures with liquid solvents [7]. ASE is a liquid–solid extraction process performed at elevated temperature, usually between 50-200^{o}C and pressures of 1000-2000 psi. It also named as pressurized solvent extraction (PSE) or pressurized liquid extraction (PLE). Figure 2.2 shows a schematic view of an ASE system.

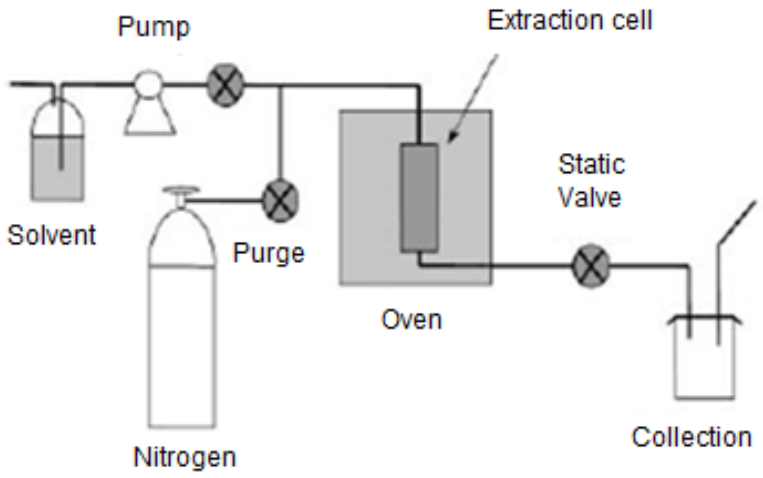


Figure 2.2 Schematic of Accelerate Solvent Extraction system (Richter, et al. 1996) [7]

The solvent is pumped into the extraction cell containing the sample, which is then brought to an elevated temperature and pressure. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the

liquid state, thus enabling safe and rapid extractions. High pressure forces the solvent into the matrix pores and hence facilitates extraction of analytes. High temperatures decrease the viscosity of the liquid solvent, allowing a better penetration of the matrix and weakened solute–matrix interactions. In addition, elevated temperatures enhance diffusivity of the solvent, leading to an increase in extraction speed.

ASE has been used since 1995 and is currently used most extensively in environmental analysis.

2.2.2 Supercritical Fluid Extraction (SFE)

SFE is defined as extraction of a material using a supercritical fluid. The extracted material is usually recovered by reducing the pressure or increasing the temperature of the extraction fluid and allowing the volatile components of the mobile phase to evaporate [8].

SFE has been developed since the 1980s to avoid the use of organic solvents and to increase the speed of extraction for volatile compounds, such as essential oils or aroma compounds from plant matrices. Generally, SFE instrumentation incorporates supercritical carbon dioxide with and without organic solvent modifiers (co-solvents). The extraction efficiency of SFE depends on the supercritical fluid density, temperature and pressure used in extraction (Figure 2.3) [9]. The technique uses supercritical fluids that have similar densities to liquids, but lower viscosities and higher diffusion coefficients. Carbon dioxide (CO_2) is frequently used as a supercritical fluid because of its suitable critical temperature and pressure.

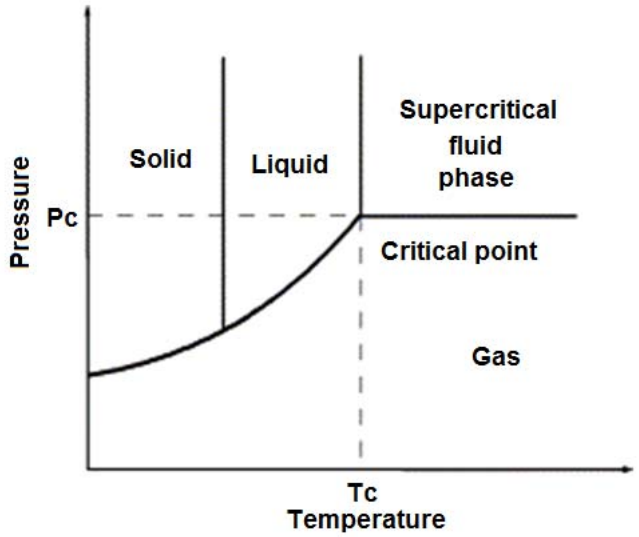


Figure 2.3 Pressure-temperature phase diagram for a pure substance (from Riera et al., 2004) [9]

An important benefit of applying SFE to the extraction of effective compounds from medicinal plants is that degradation as a result of lengthy exposure to elevated temperatures and atmospheric oxygen are avoided. However this method is limited due to its cost and selectivity (non-polar to low polar components), which requires advanced optimization.

2.2.3 Ultrasound Assisted Extraction (UAE)

Application of ultrasonic energy to aid the extraction of medicinal compounds from plant material has been found in the literature as early as the 1950s [3]. Extraction with UAE, which provides a more efficient contact between the solid and solvent, usually results in a greater yield of extraction. Ultrasound can be successfully employed to enhance extraction when low boiling point solvents are used, and the temperature of the extraction mixture is kept below its boiling point.

The effects of ultrasound on the cell walls of plants can be described as follows (Vinatoru, 2001): a characteristic of external glands of plant cell, which was filled essential oil, is that their skin is very thin and can be easily destroyed by sonication, thus facilitating release of essential oil contents into the extraction solvent; and ultrasound can also facilitate the swelling and hydration of plant materials to cause enlargement of the pores of the cell wall. Better swelling improves the rate of mass transfer and, occasionally, breaks the cell walls, thus results in increased extraction efficiency and/or reduced extraction time [10].

2.2.4 Microwave Assisted Extraction (MAE)

2.2.4.1 Overview

Microwave is a form of electromagnetic energy and is generally regarded as occupying the frequency from 300MHz to 300GHz, with corresponding wavelengths ranging from 1mm to 1m [11]. On the electromagnetic spectrum, as is shown in Figure. 2.4, the microwaves region lies between the infrared and radio waves region. Unlike X-rays and gamma rays, microwaves are non-ionizing radiations, do not break chemical bonds or cause molecular changes in a compound by removal of electrons. However, use of microwave frequencies is controlled by governmental regulations. In order to avoid interfering with communication service, the two frequencies mostly used in food/chemical engineering processing are 915MHz and 2450MHz. Of these two, the 2450 MHz frequency is used for household microwave ovens, and both are used in industrial heating.

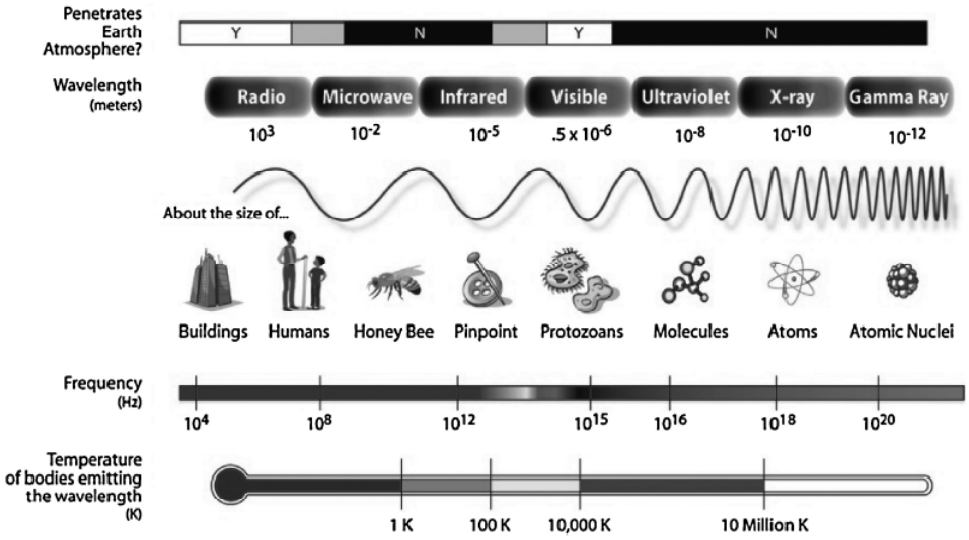


Figure 2.4 Electromagnetic spectrum (from Gupta and Wong, 2007) [11]

Originally, microwaves were principally used for communication. Since World War II, interest has been raised in the use of microwaves for heating applications. In 1946, Dr. Percy Spencer, while conducting laboratory tests for a new vacuum tube called a magnetron, accidentally discovered that a candy bar in his pocket melted upon exposure to microwave radiation. Dr. Spencer developed the idea further and established that microwaves could be used as a method of heating. Subsequently, he designed the first microwave oven for domestic use in 1947. Nevertheless, technological reasons and high costs of investment slowed down the development of applications until the beginning of the 1960s. By the 1970s, technological advances and further developments led more and more people to realize that microwaves had the potential to provide rapid, energy-efficient heating for materials [11-12].

Microwave technology has been applied in chemistry since the late 1970s. A domestic microwave oven was first used by Abu-Samra et al, (1975) to heat acid rapidly to digest biological matrices reducing conventional sample digestion

times from 1-2 h to 5-15min and resulting in a net reduction in analysis time [13]. These works spawned the research and development of a new sample preparation technique. In 1986, Ganzler and co-workers reported applications using a microwave irradiation to enhance extraction of organic compounds from solid matrices such as soils, seeds, food, and feeds as a novel sample preparation method for chromatography analysis [14-16]. Onsuka and Terry (1993) used microwave to extract organochlorine pesticides from sediment samples. They reported nearly 100% recovery for most compounds and no degradation as a result of exposure to microwave energy [17]. In 1994, Paré and co-workers reported microwave-assisted process (MAP^{TM}) as a sample preparation technique for the analytical laboratory [18-19]. Since this date, numerous researchers have studied the analytical possibilities of this new technique; the number of publications related to MAE has increased significantly (Figure 2.5). The use of MAE is a continuously expanding area of research at present.

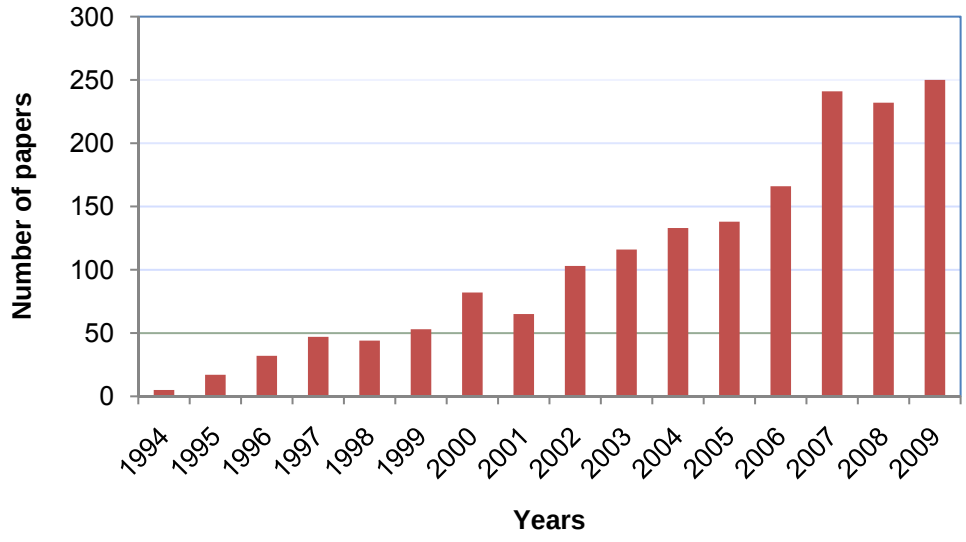


Figure 2.5 Growth in the number of published papers on microwave assisted extraction from 1994 to 2009 (based on a search in SCI databases).

2.2.4.2 Characterization of microwave heating

When material was irradiated with microwaves, there are three possible modes of interaction depending on the type of materials: (a): absorption of microwaves by the material (e.g., polar solvents), (b): reflection of microwaves by the material (e.g., non-polar metals) and (c): transmission of microwaves through the material (e.g., ceramic, glass), as showed in Figure 2.6 [20]. The materials must absorb a portion of the microwave energy for heating to occur.

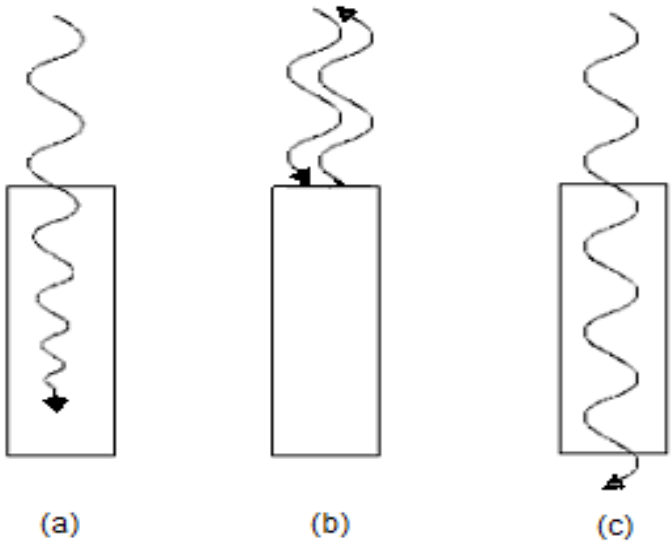


Figure 2.6 The interaction of microwaves with different materials (Ku,et al. 2001) [20]

The mechanism of heating with microwave involves primarily two mechanisms: dipolar rotation and ionic polarization [21]. This provides a qualitative understanding of microwave heating mechanisms. The dipole rotation mechanism of a polar molecule is illustrated in Figure 2.7(a). In the presence of a microwave field, the polar molecule of the material continually realigns itself with the changing field, like a microscopic magnet, which attempts to align with

the field by rotating around its axis. As the polarity of the electric field changes, the rotation also changes. The molecule thus absorbs microwave energy by rotating back and forth billions of times at the frequency of microwave. However, owing to inter-molecular forces, polar molecules experience inertia and are unable to follow the field. This results in the random motion of particles, and this random interaction generates heat. This causes electromagnetic energy to get converted into heat energy. Water in the material is often the primary component responsible for dipolar rotation. Due to their dipolar nature, water molecules try to follow the electric field associated with electromagnetic radiation as it oscillates at the very high frequencies. Such oscillations of the water molecules produce heat.

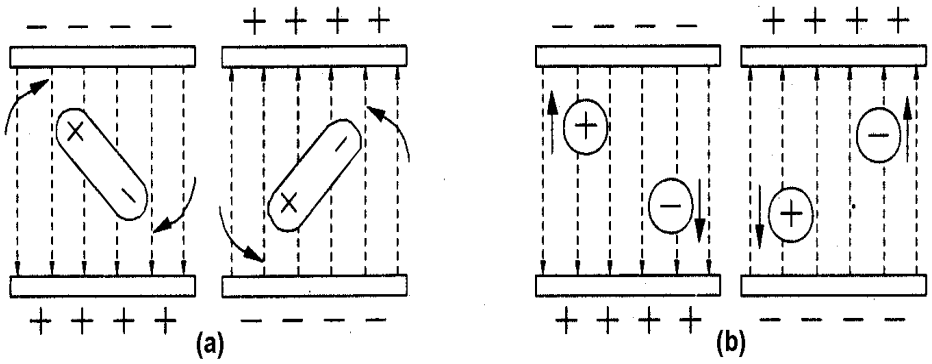


Figure 2.7 Microwave heating mechanisms: (a) dipolar rotation and (b) ionic polarization (from Yam, 2006) [22].

The second mechanism of heating with microwaves is through the oscillatory migration of ions in the material that generates heat under the influence of the oscillating electric field. As is shown in Figure 2.7(b), in the presence of an electric field, the ions move in the direction of the field, as the polarity of the electric field changes, the ions move in the opposite direction. The ions absorb

microwave energy by oscillating at microwave frequencies. Any ions present in the material (for example, sodium and chloride from salt) will be driven by the electric field and give rise to resistance heating.

Knowledge of the dielectric properties of materials is essential for proper understanding of the heating pattern during microwave irradiation [20-24]. The dielectric properties of the material provide a quantitative characterization of the interactions between microwave electromagnetic energy and material. The dielectric properties can be divided into the dielectric constant (ε') and the dielectric loss factor (ε''). The dielectric constant (ε'), which describes the ability of a molecule to be polarized by an electric field, expresses the capacity of the material to get heated with microwave irradiation. The dielectric loss factor (ε''), which describes the ability of the material to dissipate electrical energy, expresses the efficiency of converting microwave into heat. Thus, polar solvents such as water, ethanol, and methanol get heated easily; and the microwaves have no effect on non-polar solvents such as hexane, toluene and diethyl ether. The dissipation factor, often called as the loss tangent (tan δ), is a ratio of the dielectric loss (ε'') to the dielectric constant (ε'). It is a measure of how well a material absorbs the electromagnetic energy and dissipates that energy in the form of heat to which it is exposed. The dielectric constant, dipole moment and dissipation factor of some solvents commonly used in MAE are shown in Table 2.1. Comparison between ethanol and water shows that ethanol has a lower dielectric constant but a higher dielectric loss than water, this indicates that ethanol has lower ability to obstruct the microwave as they pass through, but a higher ability to dissipate the microwave energy into heat.

Table 2.1 Dielectric constant, dipole moment and dissipation factor of some solvents (from Jassie, 1997; Zlotorzynski. 1995) [21, 24]

Solvent	Dielectric constant, ε' (20 ^{o}C)	Dipole moment (25 ^{o}C)	Dissipation factor, tan δ ($\times 10^{4}$)
Hexane	1.88	<0.1	0.1
Acetone	20.7	2.69	5555
Ethanol	24.3	1.69	2500
Methanol	32.7	2.87	6400
Water	78.3	1.87	1570

The microwave heating is different from conventional heating. With microwaves, heat is generated internally within the material. As a result, the thermal gradients and flow of heat is reversed compared to conventional heating and the heating is volumic (Figure 2.8).

To understand how microwave heating can have effects that are different from conventional heating, one must focus on what in the material (solvent) is actually absorbing the microwave energy. Differential absorption of microwaves leads to differential heating and localized thermal in homogeneities that cannot be duplicated by conventional heating [25]. It may be worth mentioning that microwave irradiation has been found useful in extraction of specific target substances from plant matrices.

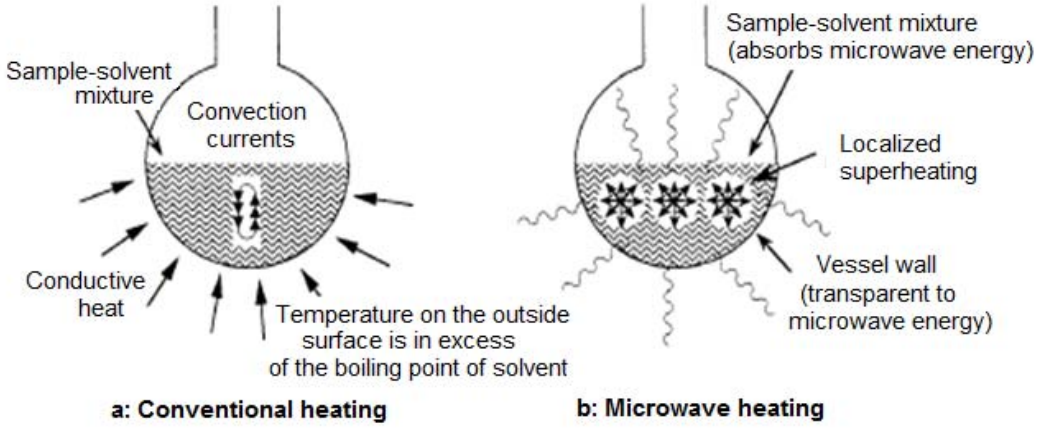


Figure 2.8 The modes of conventional heating and microwave heating (from Neas and Collins, 1988) [25]

2.2.4.3 Mechanisms of microwave assisted extraction

As mentioned by Aguilera (2003), in general, extraction process may consist of more than one step: solubilization/desorption at the matrix-solvent interface followed by diffusion of the solute into the solvent. Figure 2.9 is the schematic diagram of the steps in solvent extraction of solid plant particle. Solvent, microstructure of sample and other extraction operating parameters may affect the solubilization/desorption and diffusion of the target compounds [26].

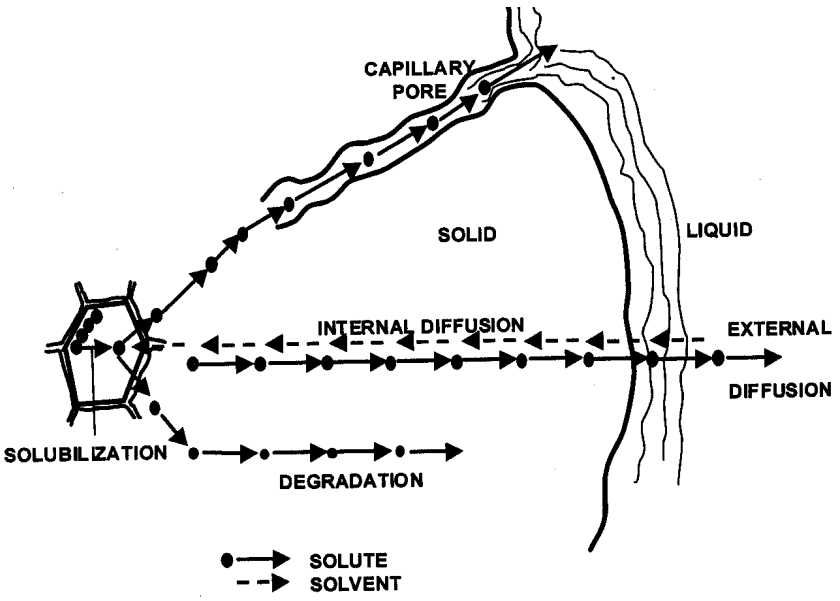


Figure 2.9 Schematic diagram of the steps in solvent extraction of solid plant particle (Aguilera, 2003) [26]

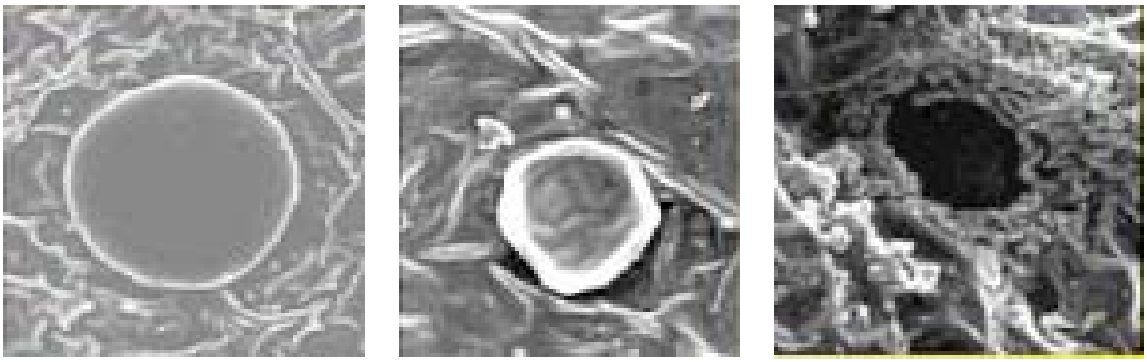
Previous works of MAE focused on investigation of the effect of parameters and search of optimum values of effective factors for a given system, but few on the fundamental kinetics and mechanisms.

Microwave assisted extraction of target compounds may occur by any one of the following three heating solvent mechanisms or as a combination [21]:

Mechanism I: the sample could be immersed in a single solvent or mixture of solvents that have high dielectric loss coefficients. Such highly polar solvents are coupled with microwave, and through dielectric relaxation mechanisms transfer heat to solvent medium, resulting in the elevated bulk temperature and accelerated extraction. These solvents such as water, ethanol and mixture of them have been used for extraction of puerarin from *Radix puerariae* by Guo et al. (2001) [27] and ginsenosides from ginsenroot by Shu et al. (2003) [28].

Mechanism II: the sample could be extracted in a solvent mixture containing solvents with both high and low dielectric losses mixed in various proportions. One sample of this is using solvent mixture of hexane-water/ethanol for extraction of ginger by Alfaro, et al, (2003) [29]. Hexane will not heat but by mixing it with water/ethanol heating will take place in a second.

Mechanism III: samples that have a high dielectric loss can be extracted with a microwave transparent solvent. One example of this is the microwave-assisted extraction of essential oils from peppermint leaves by Pare, et al, (1994) [19]. The solvent is hexane, which is a microwave transparent solvent and will not be heated in microwave field. The glandular and vascular systems are microwave-heating targets. The microwaves interact with the free water molecules present in the glands and vascular systems. Thus such systems undergo a dramatic expansion, which subsequent rupture of the tissue, allowing the essential oil to flow towards the hexane solvent, as is shown in Figure 2.10.



Fresh peppermint leaf (untreated) | Soxhlet extraction (6 h in hexane) | MAE (at 625W 30 s in hexane)

Figure 2.10 SEM of untreated, Soxhlet-extracted and MAE-treated fresh peppermint (Mentha piperita L. Mitchum) leaves. (from Pare et al, 1994) [19]

The effects of the microwave radiation on cell microstructure of plant material which was extracted in solvents with high dielectric loss (mechanism I) or mixtures of high and low dielectric loss (mechanism II) are still not fully understood.

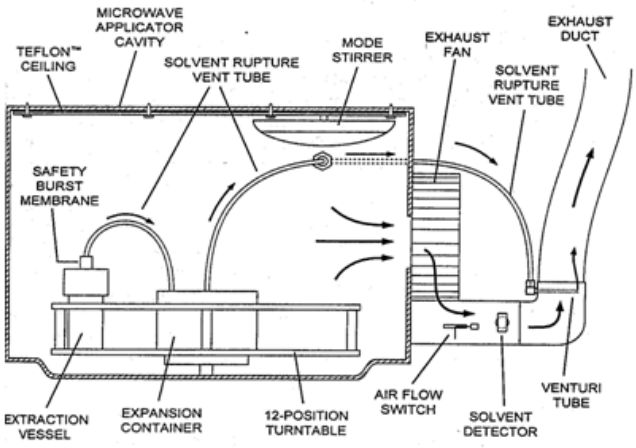
Further work on MAE is necessary to study on the fundamental kinetics and mechanisms

2.2.4.4 Application

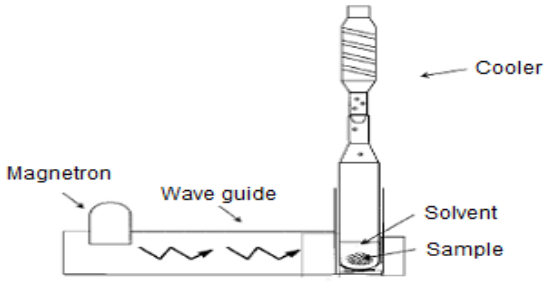
2.2.4.4.1 The MAE system

The application of microwave energy for extraction may be performed using two technical/instrumental systems (Figure 2.11): closed vessels under controlled pressure and temperature, and open vessels at atmospheric pressure.

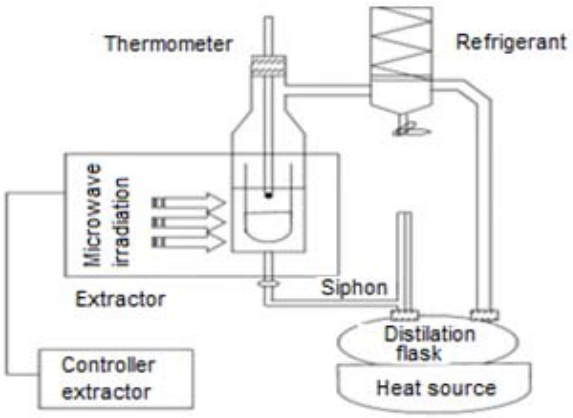
Generally, closed vessels are of multi-mode system and open vessels are of the focused mode system. In the closed vessels system, the microwave radiation is allowed to disperse randomly in a cavity, so every zone in the cavity and the sample it contains is evenly irradiated. The main parameters to be considered when using the closed systems are: solvent, temperature, pressure, power and extraction time.



(a)



(b1)



(b2)

Figure 2.11 Schematic view of a closed vessel system (a) and open focused vessel system (b1) of microwave assisted extraction [21] and open vessel (b2) system of focused microwave-assisted Soxhlet extraction [30]

The feature of closed-vessel MAE is that losses due to volatilization are minimized because the samples are allowed to cool before the vessels are opened. Also, most closed-vessel systems can extract up to 12 or 24 samples at the same time, thereby increase sample throughput.

The disadvantage of closed systems is that sample size should be limited to 0.5-1.0 g and the technical drawback is that it takes time for the samples to cool and depressurize. Additionally, there are some safety concerns when employing closed-vessel extractions, for example, the possibility of explosion.

For the open vessel system where the microwave radiation is focused on a restricted zone, the sample is subjected to a much stronger electrical field than in the closed vessel. The open vessel system is ease to make reagent additions, efficient in solvent/matrix heating, offering large sample capacity, and speed. The open vessel systems are simple and usually safe, the optimization parameters involve the solvent, power and time.

The drawback of open systems is the losses of volatile compounds such as benzene and lower molecular weight hydrocarbons when compare open systems to closed systems. To overcome this drawback, an open vessel system of focused microwave-assisted Soxhlet extraction) has developed by Luque-Garcia, et al. (2004) [30], as it was showed in Figure 2.11(b_2).

In both cases of closed and open vessels, the solvent, power and time are dependent on the type of matrix and the target analyte.

2.2.4.4.2 Application of MAE for extraction of natural product

Microwave-assisted extraction has been applied to the extraction of natural products from plant matrices or biomass. Ganzler et al. (1986) demonstrated the first report on MAE for extraction of target compounds was more effective than

conventional extraction methods [14]. MAE can offer shorter time, less solvents, higher extraction rate and better products with lower costs.

The target compounds extracted by both of closed vessel system and open vessel system of MAE were involved in alkaloids, saponins, glycosides, terpenes, essential oils, carotrnoids, steroids and flavonoids etc**.,** such as extraction of taxanes (paclitaxel) from *Taxus baccata L.* by Mohammad Talebi, et al. (2004) [31], extraction of azadiractine related limonoids from *Azadirachta indica* seed kernel by Dai, et al. (2001) [32], extraction of glycyrrhizic acid from *Glycyrrhizia glaubra* root by Pan, et al. (2000) [33], extraction of tanshinones from *Salvia miltorrhiza bung* by Pan, et al. (2001) [34], extraction of artemisinin from *Artemisia annua* by Hao, et al. (2002) [35], extraction of ginsenosides from *Panax ginseng* root by Shu, et al. (2003) [28], and extraction of saponins from chickpea (*Cicer arietinum*) by Zohar Kerem, et al. (2005)[36]. The system and extraction conditions for MAE of different target compounds from different matrices are showed in Table 2.2.

Table 2.2 Selected applications of MAE for extraction of natural product

Target compounds	Matrix	System	Extraction condition	Reference
Vicine, convicine (pyrimidine glycosides)	Faba beans (*Vicia faba*)	Domestic oven	Methanol:water (1:1); two successive irradiations (30 s) with an intermediate cooling step	Ganzler et al. (1986a,1986b) [14-15]
Sparteine (alkaloid)	Lupine seeds	Domestic oven	Four cycle (30 s) with cooling steps in between	Ganzler et al. (1986b,1990) [15-16]

(Continued)

Table 2.2 Selected applications of MAE for extraction of natural product

Target compounds	*Matrix*	System	Extraction condition	Reference
Essential oils	*Monarda fistulosa, Allium sp*.; Peppermint leaves	Modified domestic oven	Hexane, alkanes (transparent solvents)	Paré et al. (1990,1994) [18-19]
Ergosterol	Fungal contaminations	Domestic oven	375 W; 35 s	Young (1995) [37]
Terpens (linalool, terpineol, citronellol, nerol and geranol)	Must (*Vitis vinifera)*	Closed vessels	10 mL dichloromethane; 475 W; 10 min; 90^{o}C Hexane	Carro et al. (1997) [38]
Tananes (Paclitaxel)	Needles of *Taxus* sp.*; Taxus baccata L.*	Closed vessels	5g needles, 10 mL of 95% ethanol; 1.5 g sample, 20 mL methanol : water (9:1); 85-95^{o}C	Incovia Mattine et al. (1997) [39]; Mohammad Talebi, et al. (2004) [31]
Glycyrrhizic acid	Licorice, the roots of *Glycyrrhizia glaubra*,	Modified microwave oven (open vessel)	100 mL ethanol /water/ammonia; three cycles of power on (15s) and off (15s) to 85-90^{o}C; then 3s power on for heating and 15s power off for cooling.	Pan, et al. (2000) [33]
Puerarin	*Radix puerariae*	Closed vessel	0.5 g with 15 mL ethanol in water (30-95%); 5 min	Guo, et al.(2001) [27]
Withanolides	Leaves of *Iochroma gesnerioides***,**	Open vessel system, focused	100 mg; extracted with 5 mL methanol and 0.6 mL water for 40 s at 25 W.	Kaufmann, et al,.(2001) [40]
Tanshinones	*Salvia miltiorrhiza bunge*	Modified microwave oven (open vessel)	100mL; 95%ethanol; power on (25s) to 80^{o}C, then 2s power on for heating and 10s power off for cooling	Pan, et al. (2001) [34]

(Continued)

Table 2.2 Selected applications of MAE for extraction of natural product

Target compounds	Matrix	System	Extraction condition	Reference
Azadirachitin	*Neem (Azadirachta indica)*	Open vessel	30 mL methanol; 150 W; 30s on30s off; 10min	Dai, et al. (2001) [32]
Cocaine and benzoylecgonine	Leaves of *Erythroxylum coca* var. *coca*	Open vessel system, focused	100mg sample with 5-30 mL Methanol; 125 W; 30 s	Brachet, et al.,(2002) [41]
Artemisinin	*Artemisia annua* L.	Modified microwave oven (open vessel)	60s power on; water cooling; cycle.	Hao, et al. (2002) [35]
Piperine	Black pepper (*Piper nigrum*)	Modified microwave oven	Glass vessel with continuous nitrogen sparging to maintain an inert atmosphere inside the vessel; petroleum ether; 150 W; 120 s.	Raman, et al. (2002) [42]
Ginger extracts	Ginger (*Zingiber officinale*)	Open vessel system, focused	5 g sample; 1-2 mL water or enthanol +30 mL ethanol or hexane; 150-300 W;30-120 s	Alfaro, et al. (2003) [29]
Ginsenosides	Ginseng root	Open vessel system, focused	Ethanol-water; 150 W; 15 min	Shu, et al. (2003) [28]
Ginsenosides	Panax ginseng	Closed vessel	1g sample; 40 mL 70% enthanol in water; 400 kPa; 10 min	Wang,et al.(2008) [46]
Tea polyphenols and tea caffeine	Green tea leaves	Modified microwave oven (open vessel)	100 mL ethanol (0-100% in water); power on (45s) to 90 ^{o}C, then 3s power on for heating and 10 s power off for cooling; 0.5-8 min	Pan, et al. (2003) [43]

(Continued)

Table 2.2 Selected applications of MAE for extraction of natural product

Target compounds	Matrix	System	Extraction condition	Reference
Berberine	*Mahonia bealei* (Fort.)	Modified microwave oven (open vessel)	Methanol, ethanol:water (v/v=9:1), ethanol; power on-power off for cycle irradiation	Gao, et al. (2004) [44]
Saponins	Chickpea (*Cicer arietinum*).	Modified microwave oven (closed vessel)	4 g sample with 16 mL 70%ethanol in water; 300 W; 60 ^{o}C; 20 min.	Zohar Kerem, et al. (2005) [36]
Anthraquinones	Morinda citrifolia	Closed vessel, CEM system	0.1 g sample with 10 mL 80% ethanol in water, 60 ^{o}C; 30 min.	Hemwimon, et al.(2007) [45]
Tetrahydropalmatine; imperatorin; isoimperatorin	Yuanhu Zhitong prescription	MAE testing system	70% entahnol in water,,500 W, 27 min	Liao, et al. (2008) [47]

Previous works were mainly focused on investigating effects of operation parameters such as solvent, microwave power, irradiation time and extraction temperature etc., on the yields of extraction, and searched for the optimized conditions. Findings from published literatures demonstrated that MAE had shorter extraction times (typically 15 min), less use of solvent (10mL for MAE versus 250 mL for Soxhlet) and offered comparable or better extraction rate to that of conventional extraction. But very few investigations of the fundamental kinetics and mechanisms of MAE have been published.

MAE technique has been mostly used to isolate polar components from complex matrices, but MAE does have applicability to non-polar compounds as well. This is accomplished by using a non-polar solvent like hexane and adding

some of the polar solvents like water that absorbs the microwave energy and transfers the heat produced to the bulk solution.

2.3. Conclusion

The application of MAE has increased rapidly in the last decade, and for most cases it has proven to be effective compared to conventional extraction method. The major advantages are the reduction in decreased extraction times, solvent consumption, as well as a better extraction rate. MAE is a viable candidate for performing extractions of effective compounds from TCM. It is also a strong competitor to other recent sample preparation techniques.

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Chapter 3. Solvent Free Microwave Extraction of Essential Oil and Sequential MAE of Pectin from *Pomelo Fruit Peels*

Abstract: The pomelo fruit peels were processed for the extraction of essential oils by solvent free microwave extraction (SFME), and the oil extracted peels were continuously treated for pectin extraction by microwave-assisted extraction (MAE). To evaluate the effect, conventional hydrodistillation (HD) method for essential oil extraction and acidic solution pectin extraction were also performed. The results indicated that SFME under microwave power levels of 130 W, 260 W, and 390 W were all superior to HD in terms of extraction efficiency and essential oil yield. The chemical composition analysis by GC-MS showed that SFME did not affect the quality of essential oils compared with HD. The MAE extraction of the oil extracted pomelo peels significantly shorten the pectin extraction time compared to the traditional extraction method. Response surface methodology was employed to optimize the MAE extraction condition. The sequential extraction of essential oils and pectin from pomelo fruit peels by SFME and MAE was a feasible processing method.

3.1 Introduction

The pomelo (*Citrus grandis*) is a citrus fruit native to South East Asia, which is pale green to yellow when ripe, with sweet white flesh and very thick spongy rind. Pomelo is a major fruit in southern China with large amount of consumption. The pomelo peel is of a spongy nature, representing about 40% of the fruit mass and the fact that it is an agricultural by-product. This remains as a problem looking for solution to turn the wasted peel into useful products [1].

Essential oil in pomelo peels is responsible for the typical citrus-like aroma of the fruit. Essential oil is defined as a complex mixture of volatile constituents biosynthesized by living organisms [2]. Essential oil is widely used in many food products including alcoholic and non-alcoholic beverages, candy, gelatins, and so on. In pharmaceutical industry, essential oil serves as flavoring agents to mask unpleasant tastes of drugs; in perfumery and cosmetic, it is used in many preparations [3]. Since citrus essential oils have been recognized for their antimicrobial properties, it is also suggested to be the source to providing natural antimicrobials for food industry [4]. Generally, essential oil is extracted by hydrodistillation (HD). Recently, a new technique called solvent-free microwave extraction (SFME) is developed [5-7]. SFME combines microwave heating with dry distillation at atmospheric pressure for the isolation and concentration of the essential oil in fresh plant materials [6]. The internal heating of in situ water of the plant material distends it and makes the glands and oleiferous receptacles burst to allow the essential oil free to be entrained by the vapor [8]. For extraction of essential oil, SFME is reported to be advantageous to conventional distillation in terms of rapidity, efficiency, cleanliness, substantial saving of energy, and is environmentally friendly [9].

Pomelo peels also contain rich content of pectin. Pectin is a group of complex polysaccharides localized in the middle lamella, intercellular crevices, and primary cell walls of most the higher plants, and is known for the possession of pharmacological, hypoglycemic, and cholesterol-lowering effects [10]. According to the value for methoxyl content and degree of esterification, pectin isolated from pomelo peels can be classified as low methoxyl pectin and is of potential use in the manufacture of low sugar products such as low sugar jam and jellies [11]. Conventionally, pectin is extracted in acidic solution at about 90 °C for at least 1h. Microwave-assisted extraction (MAE) is a process of using microwave energy to heat solvent in contact with a sample in order to partition analytes from the sample matrix to the solvent. Due to the nature of microwave heating, MAE for plant materials can remarkably reduce extraction time and solvent consumption, while offering better extraction efficiency [12]. MAE has been successfully applied for the extraction of pectin from apple pomace [13] , orange skin [14], and lime [15].

The objective of this work is to establish a two step microwave extraction process for the utilization of pomelo peels. The first step is to extract the essential oil using SFME. The extracted peels were then used for extraction of pectin by MAE. Extraction effects of SFME and MAE were compared to the conventional water-based extraction method. The condition of MAE for pectin extraction was also optimized using the response surface methodology.

3.2 Materials and methods

3.2.1 Materials

Fresh pomeloes were provided by an orchard in Guangdong Province. Before treatment, the pomeloes were washed and manually peeled. The moisture contents of the peels were determined to be 81.90 ± 0.23 %. The peels were cut into particles at the size of 5 mm × 5 mm × 10 mm for extraction of essential oil by SFME and HD. The extracted peels by SFME were continued to be used as material for extraction of pectin.

3.2.2 Microwave apparatus

Two different forms of microwave extraction methods used the same microwave apparatus with modification for each step. SFME was used for the extraction of essential oil, and the extracted peels were then treated by MAE for extraction of pectin. A 2450 MHz microwave oven with full power of 1300 W (NN-S760WA, Panasonic, Japan) was modified for the microwave treatment. The microwave oven was linked to a personal computer installed with a computer control program (Fiso technologies Inc., Canada). The program precisely controlled the microwave power generated.

3.2.3 Solvent free microwave extraction of essential oil

3.2.3.1 Solvent free microwave apparatus

For SFME treatment, a flat bottom flask with the capacity of 500 mL was placed in the microwave oven and connected to the Clevenger apparatus. A hole was drilled on the top of the microwave oven initially with the purpose of installing the OSR system for on-line temperature measurement. In this study,

the hole allowed the Clevenger apparatus to go through the microwave oven. Fresh pomelo peels of 130 g were treated at each trial. The distillates passed through the condenser outside the microwave oven for collection in the Clevenger apparatus. The schematic diagram of experiment set-up is shown in Figure 3.1.

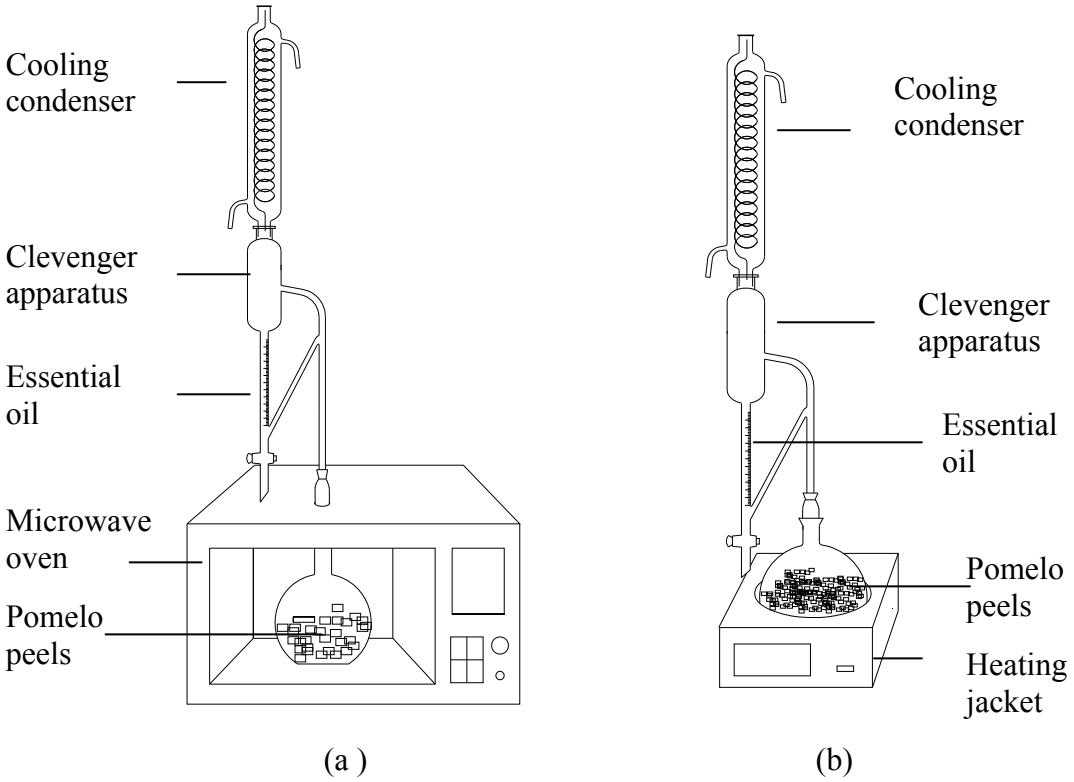


Figure 3.1 The schematic diagram of experiment set-up of (a) solvent free microwave extraction and (b) conventional hydrodistillation for essential oil extraction from pomelo peel

The essential oil was collected in amber colored vials, dried with anhydrous sodium sulfate, and stored under 4 °C until being analyzed.

Three microwave power levels including 130 W, 260 W, and 390 W were selected to evaluate the effect of microwave energy on extraction of essential oils. For each condition, the experiments were replicated twice.

3.2.3.2 Hydrodistillation

The same Clevenger apparatus used for SFME was employed for conventional hydrodistillation (HD) with the change of heat source from microwave oven to a heating jacket at 100 °C for 90 min, as shown in Figure 3.1(b). Peel material : water ratio of 1:10（w（db）/w）was employed. The collected essential oil was also dried and stored at 4 °C until being analyzed. The experiments were duplicated for each condition,

3.2.3.3 Gas chromatography-mass spectrometry identification

The components of essential oil from pomelo peels by different extraction method were analyzed by GC-MS (6890N-5973I, Agilent Technology, USA) using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The operation conditions were as follows: helium carrier gas flow rate 1.0 mL / min; split 5:1; injection volume 1.0 μL; injection temperature 260℃; oven temperature program from 50 °C to 150 °C at 15 °C/min, holding for 4 min, and to 260 °C at 10 °C /min followed by a holding for 4 min; the ionization mode used was electronic impact at 70 eV; ionization temperature 230 °C; MSE quadrupole temperature 150 °C; Transfer line temperature 260 °C; Solvent delay was for 3 min. The compounds of the extracted essential oil were identified by comparing their mass spectral fragmentation patterns with those of similar compounds from a database library (NIST98, Wiley 7n) purchased from Agilent Technologies Inc..

3.2.4 Microwave-assisted extraction of pectin

3.2.4.1 Microwave-assisted extraction

MAE was conducted using the extraction system of SFME with the modification of removing the Clevenger apparatus. 10 g of oil extracted pomelo peels were dispersed in 180 mL of HCl with pH value adjusted according to the experiment design and placed in a 500 mL flask.

To study the optimized condition of MAE by Response Surface Methodology (RSM), a Box-Behnken experiment design with three independent variables was employed. The variables studied included pH value of solvent, microwave power level, and extraction time of MAE. The low, high and central levels for pH value were set at 1.0, 3.0, and 2.0, 390 W, 650 W, and 520 W for microwave power levels, and 3 min, 7 min and 5 min for extraction time. According to the Box-Behnken design generated by Design-Expert (Version 7.0, Stat-Ease, Inc., USA), 15 experiments including 12 factorial points with three replicate at the center point for estimation of pure error sum of squares were employed. The order of the experiments was fully randomized.

3.2.4.2 Conventional extraction

The same apparatus used for MAE was employed for pectin conventional extraction with the change of heat source from microwave oven to a hot water bath. 10 g of oil extracted pomelo peels were dispersed in 180 mL of HCl with pH 2.0 and placed in a 500 mL flask at about 90 °C for 90 min. The experiment was repeated for three times.

3.2.4.3 Yield of pectin

After extraction, the samples were hot-filtered, and precipitated with 180 mL of 95 % (v/v) ethanol for 3 h. The coagulated pectin mass was separated and

rinsed with 75 % (v/v) ethanol and anhydrous ethanol. The treated samples were dried under 60 °C till the weight remained constant. The yield of pectin (%) was expressed in term of the weight of pectin collected in gram per 100 gram of oil extracted pomelo peel weight.

3.2.5 Statistical analysis

Yields of essential oil and pectin by different extraction procedure were statistically evaluated by analysis of variance (ANOVA) using SAS (SAS version 9.0, SAS Institute Inc., USA). The statistical analysis of the MAE optimization through regression model and plotting the response surface graphs was achieved by Design-Expert.

3.3 Results and discussion

3.3.1 Essential oil yield

The yield of essential oil was expressed in terms of the volume of essential oil collected in mL per 100 g of the pomelo peel weight.

Yields of essential oil obtained by SFME at 190 W, 260 W, and 390 W and by HD are shown in Figure 3.2. The extraction started much earlier for SFME at 260 W and 390 W (4.33 and 3.33 min, respectively) than that for HD (13 min). Significant higher final yields of essential oil were observed for SFME at 260 and 390 W, which were 0.15 mL/100 g and 0.16 mL/100 g, than that for HD (0.077 mL/100 g). This means an increase of yield by 89.6 % and 94.8%, respectively. The extraction time for SFME at 130 W (10 min) also began earlier than that for HD, but with a similar final yield. The efficiency of yield is also improved. The times for reaching a yield of 0.08 mL/100 g, which was the

maximum yield obtained by HD, was shorten by 75.0 % and 76.6 % for SFME at 260 and 390 W respectively than that for HD..

Microwave heating is known for its capacity to heat the entire sample almost simultaneously and at a higher rate [16], therefore it provides a more efficient extraction in comparison with the conventional heating method. Bayramoglu et al. (2008) performed a experiment of extraction of essential oil from oregano by SFME and stated that the lower yield in HD and SFME at lower microwave power level could be attributed to the loss of some of the volatile compounds due to longer processing time [6].

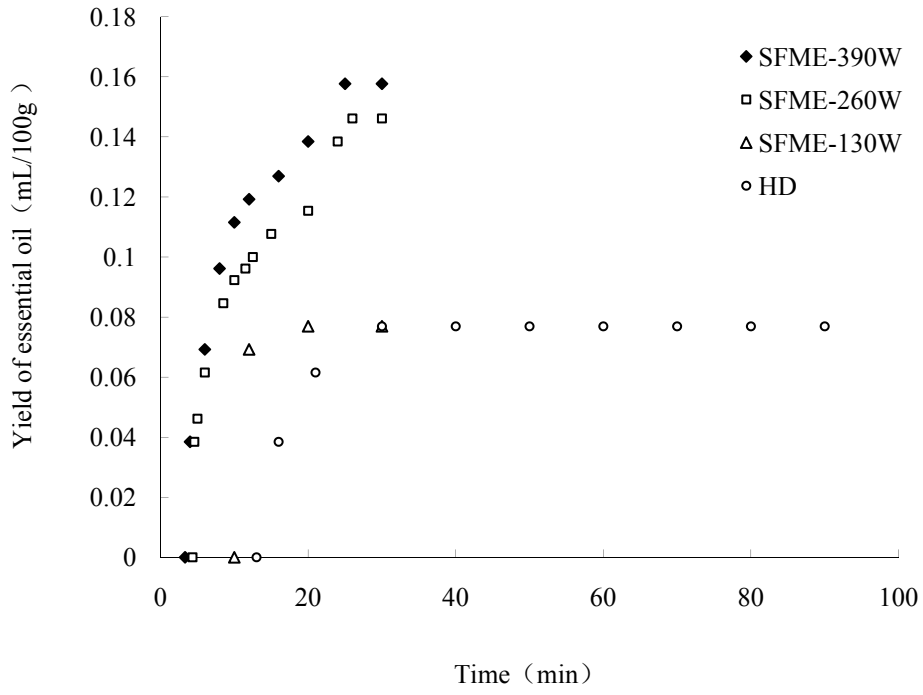


Figure 3.2 Yield of essential oil extracted from pomelo peels by SFME at three power levels (390 W, 260 W, and 130 W) and by HD

3.3.2 Essential oil composition

The chemical compositions of the essentials oil were studied using GC-MS. The results are displayed in Figure 3.3 and Table 3.1.

Sum of total fractions of obtained essential oil from SFME at 390 W, 260 W, 130 W, and HD were 95.62 %, 99.15 %, 98.71 %, and 95.54 %, respectively. From Table 3.1, it can be seen that the components of essential oils by different extraction methods remained generally the same. For SFME at 390 W, 260 W, and 130 W, the numbers of compounds identified were 22, 20, and 25; for HD, 23 compounds were confirmed. The result indicated that SFME did not affect the quality of essential oil. Therefore, SFME can be introduced as a valid method for the extraction of essential oil with shortened extraction time.

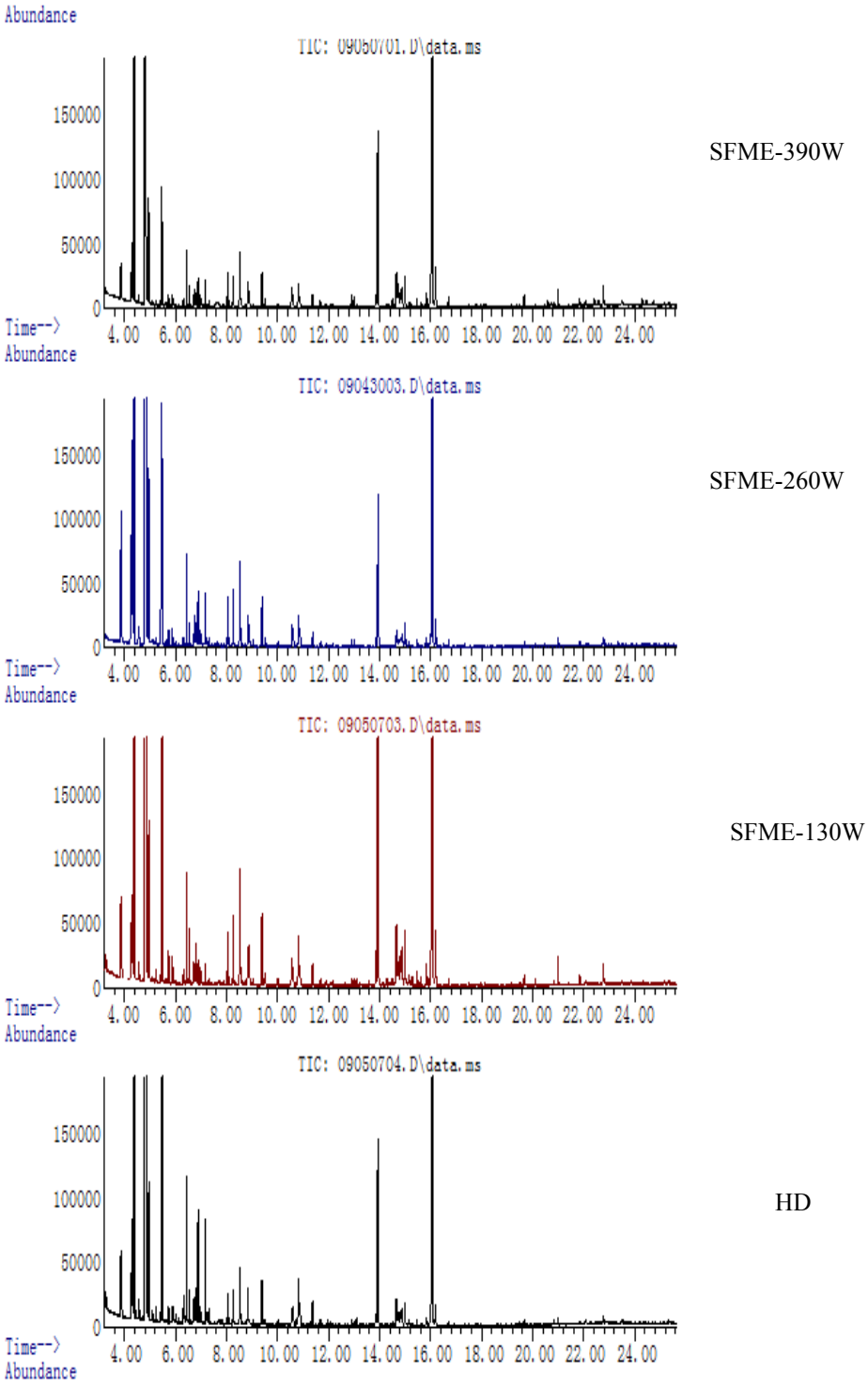


Figure 3.3 GC-MS total ion chromatograms of essential oil extracted from pomelo peels by SFME at three power levels (390 W, 260 W, and 130 W) and by HD.

Table 3.1 Chemical compositions of essential oil obtained by SFME at three power level (390 W, 260 W, and 130 W) and by HD

No.	RT	Compounds	Mol. form.	Content (%)			
				SFME-390 W	SFME-260 W	SFME-130 W	HD
1	3.86	α-Pinene	$C_{10}H_{16}$	0.28	0.67	0.43	0.45
2	4.26	β-Phellandrene	$C_{10}H_{16}$	0.17	0.34	0.21	0.20
3	4.31	β-Pinene	$C_{10}H_{16}$	0.35	0.65	0.36	0.53
4	4.38	β-Myrcene	$C_{10}H_{16}$	2.07	3.69	1.53	2.49
5	4.83	Limonene	$C_{10}H_{16}$	80.70	86.53	78.06	82.58
6	4.95	Cis-ocimene	$C_{10}H_{16}$	0.39	0.55	0.39	0.46
7	5.44	Undecane	$C_{11}H_{24}$	0.27	—	3.36	—
8	5.46	β-Linalool	$C_{10}H_{18}O$	0.46	0.78	0.85	—
9	5.73	6-Isopropenyl-3-methyl-1-cyclohexen-1-ol	$C_{10}H_{16}O$	—	—	0.08	—
10	6.31	Terpene-4-ol	$C_{10}H_{18}O$	—	—	0.06	0.14
11	6.43	α-Terpineol	$C_{10}H_{18}O$	0.20	0.20	0.27	—
12	6.80	(-)-Carveol	$C_{10}H_{16}O$	0.07	—	0.13	0.13
13	6.88	β-Citral	$C_{10}H_{16}O$	0.11	0.12	—	0.46
14	7.17	α-Citral	$C_{10}H_{16}O$	0.14	0.13	0.07	0.41
15	7.30	Perillaldehyde	$C_{10}H_{14}O$	—	—	—	0.07
16	8.04	δ-elemene	$C_{15}H_{24}$	0.17	0.13	0.18	—
17	8.04	(+)-4-Carene	$C_{10}H_{16}$	—	—	—	0.13
18	8.25	neryl acetate	$C_{12}H_{20}O_2$	0.16	0.15	0.22	0.16

(Continued)

Table 3.1 Chemical composition of essential oil obtained by SFME at three power level (390 W, 260 W, and 130 W) and by HD

No.	RT	Compounds	Mol. form.	Content (%)			
				SFME-390 W	SFME-260 W	SFME-130 W	HD
19	8.52	geranyl acetate	$C_{12}H_{20}O_2$	0.31	0.26	0.44	0.30
20	8.83	β-Elemene	$C_{15}H_{24}$	—	—	0.15	0.17
21	8.88	methyleugenol	$C_{11}H_{14}O_2$	0.12	0.08	0.18	—
22	9.38	Caryophyllene	$C_{15}H_{24}$	0.22	0.17	0.29	0.24
23	10.57	Germacrene D	$C_{15}H_{24}$	—	0.15	—	0.11
24	10.60	β-Cubebene	$C_{15}H_{24}$	0.23	—	—	—
25	10.61	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	$C_{15}H_{24}$	—	—	—	0.13
26	10.82	Valencene	$C_{15}H_{24}$	0.19	0.09	—	—
27	10.82	β-Patchoulene	$C_{15}H_{24}$	—	—	—	0.31
28	10.82	β-Panasinsene	$C_{15}H_{24}$	—	—	0.26	—
29	10.87	Selinene	$C_{15}H_{24}$	0.06	—	—	0.14
30	11.37	(-)-α-Panasinsen	$C_{15}H_{24}$	—	0.06	0.11	0.15
31	13.93	β-Neoclovene;	$C_{15}H_{24}$	—	0.54	1.67	0.98
32	15.99	Solavetivone	$C_{15}H_{22}O$	—	—	0.09	—
33	16.05	Nootkatone	$C_{15}H_{22}O$	8.90	3.86	9.28	4.80
34	19.67	Osthole	$C_{15}H_{16}O_3$	0.05	—	0.04	—
% of total				95.62	99.15	98.71	95.54

3.3.3 Effect of parameters of MAE on the yield of pectin extraction

The advantages of using MAE for extraction of pectin from fruit processing wastes had been confirmed by Wang et al. (2007) [13] and Fishman et al. (2006) [15]. In order to determine the effect of each variable on the response, individual effect of the three variables, namely microwave power, extraction time, and pH of solvent on the pectin yield from the oil extracted pomelo peels were studied. Response surface methodology, as a tool for optimization, was employed to determine an optimum condition for pectin extraction of the oil extracted pomelo peels. Based on the preliminary study, a design based on Box-Behnken experiment design using Design-Expert was developed as listed in Table 3.2. A central point was selected as microwave power level at 520 W, MAE extraction time at 5 min, and pH of solvent at 2.0. Experiment under this condition was repeated for three times in order to make the estimation of pure error possible [17]. The experimental values obtained for the pectin yields at the designed points are shown in Table 3.2.

As can be seen in Table 3.2, the yield of pectin was found within the range of 0.05-2.93 %. Based on the data, a second-order regression model was built as Eq. (1):

$$\textit{Pectin yield (\%)} = 2.85 + 0.24\,x_1 + 0.34\,x_2 - 1.04\,x_3 - 0.34\,x_1 x_2 - 0.099 x_1 x_3 - 0.38\,x_2 x_3 - 0.39 x_1^2 - 0.68\,x_2^2 - 1.19\,x_3^2 \quad (1)$$

where x_1 is the microwave power level, x_2 and x_3 are extraction time of MAE and pH value of solvent, respectively.

Table 3.2 Experimental setting for MAE of pectin yield from oil extracted pomelo peels at various microwave power levels, treatment times, and solvent pH

Run order	x_1 microwave power level (W)	x_2 extraction time (min)	x_3 solvent pH	Y pectin yield (%)
1	650	5	3.0	0.18
2	650	3	2.0	2.15
3	390	3	2.0	0.85
4	520	7	1.0	2.67
5	390	5	3.0	0.05
6	650	5	1.0	2.69
7	390	7	2.0	2.10
8	520	3	1.0	1.15
9	520	5	2.0	2.71
10	520	7	3.0	0.06
11	520	5	2.0	2.93
12	520	3	3.0	0.06
13	390	5	1.0	2.17
14	520	5	2.0	2.92
15	650	7	2.0	2.06

Analysis of variance (ANOVA) for the model was also presented in Table 3.3. As can be seen in Table 3.3, the coefficient of determination (R^2) of this model was 0.9887, and the lack of fit was 0.2216, which suggests a goodness of fit, and that the regression models can reasonably represent the observed values. Based on the results of ANOVA, the significance of each coefficient was evaluated by *F*-test and *p*-value. The result indicated that the variables with the extremely significant ($p < 0.01$) effect were extraction time (x_2) of MAE, pH (x_3) and the quadratic term of these two variables. All the other variables showed

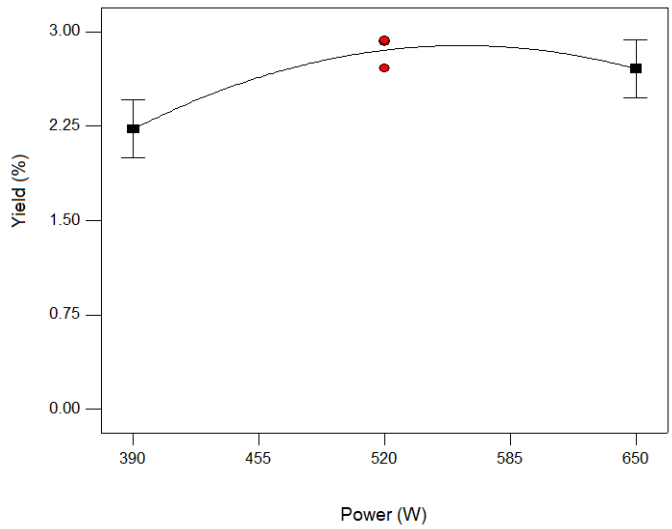
significant effect on the yield of pectin from oil extracted pomelo peels ($p < 0.05$), with the only exception of the interaction effect of microwave power level and pH ($x_1 \times x_3$) ($p > 0.05$).

Table 3.3 ANOVA for regression model built for pectin yield

Source	SS	DF	MS	*F*	*p*
Model	17.86	9	1.98	49.44	0.0002***
x_1-power	0.45	1	0.45	11.31	0.0200*
x_2-time	0.90	1	0.90	22.45	0.0052**
x_3-pH	8.68	1	8.68	216.16	< 0.0001***
$x_1 \times x_2$	0.45	1	0.45	11.18	0.0205*
$x_1 \times x_3$	0.039	1	0.039	0.97	0.3706
$x_2 \times x_3$	0.57	1	0.57	14.30	0.0129*
x_1^2	0.55	1	0.55	13.76	0.0139*
x_2^2	1.69	1	1.69	41.11	0.0013**
x_3^2	5.26	1	5.26	130.94	< 0.0001***
Residual	0.20	5	0.040		
Lack of Fit	0.17	3	0.057	3.67	0.2216
Total	18.06	14			
R^2			0.9889		

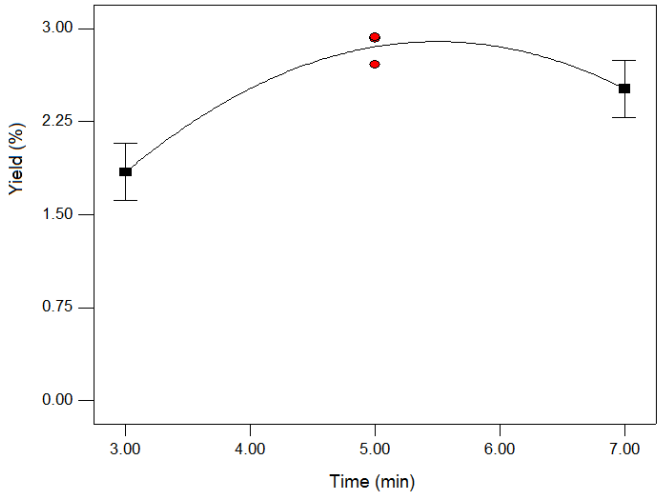
* $p<0.05$, ** $p<0.01$, *** $p<0.001$

The individual effects of microwave power, extraction time, and solvent pH on the pectin yield are showed on Figure 3.4. As can be seen on Figure 3.4 (a), pectin yield increased with the increase of microwave power in the power level ranging from 390 W to 550 W, and slightly decreased with further rise of power level to 650 W. The same trend was shown for the effect of extraction time and pH value of solvent, with the highest pectin yield at the extraction time of 5.5 min and the solvent pH value of 1.5 respectively.



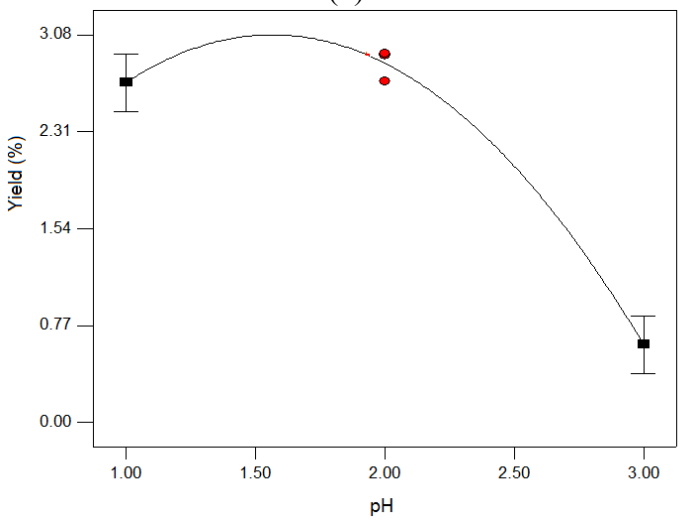
time=5min;
pH=2.0

(a)



power=520W
pH=2.0

(b)



time=5min:
power=520W

(c)

Figure 3.4 Individual effects of (a) microwave power level, (b) MAE time, and (c) solvent pH on pectin yield from oil extracted pomelo peels

The interaction effects of the variables are presented on Figures 3.5-3.7. From Figure 3.5, a significant interaction effect can be seen between microwave power level and MAE time ($p < 0.05$).

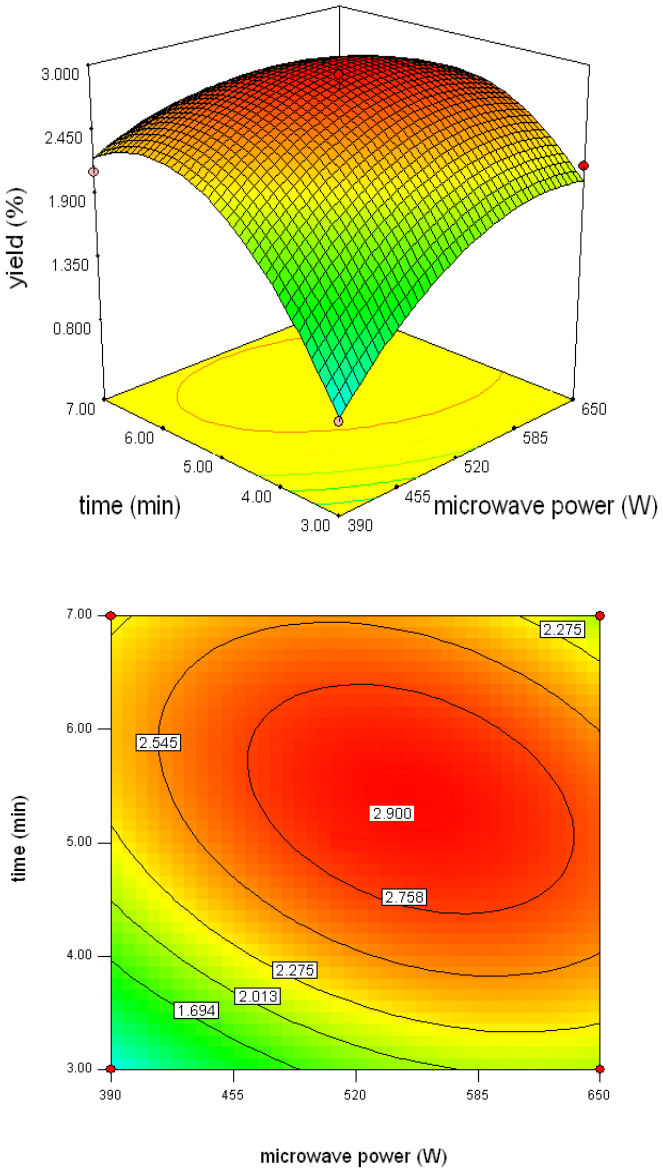


Figure 3.5 Response surface and contour plot for effects of microwave power level and extraction time on pectin yield from oil extracted pomelo peels

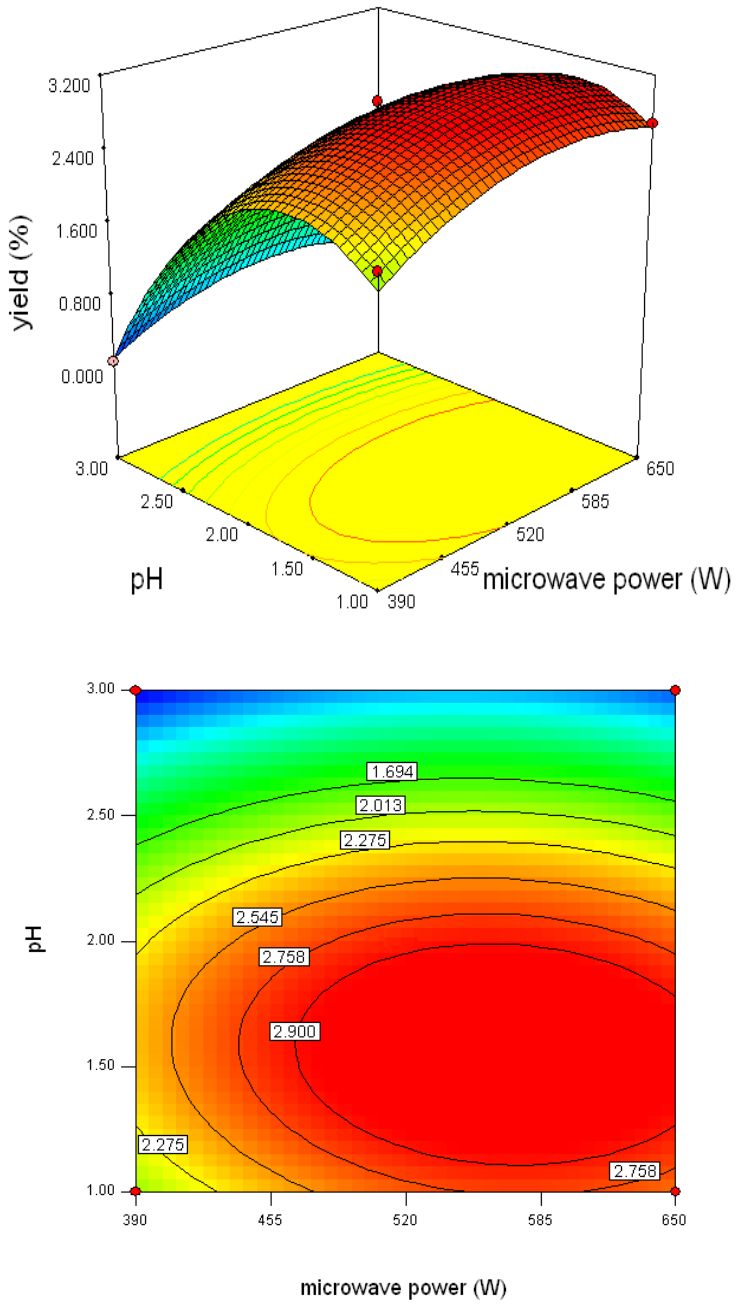


Figure 3.6 Response surface and contour plot for effects of microwave power level and pH value on pectin yield from oil extracted pomelo peels

Figure 3.6 showed the interaction effect of microwave power level and solvent pH, the cross effect of which is not significant based on the result of ANOVA ($p > 0.05$).

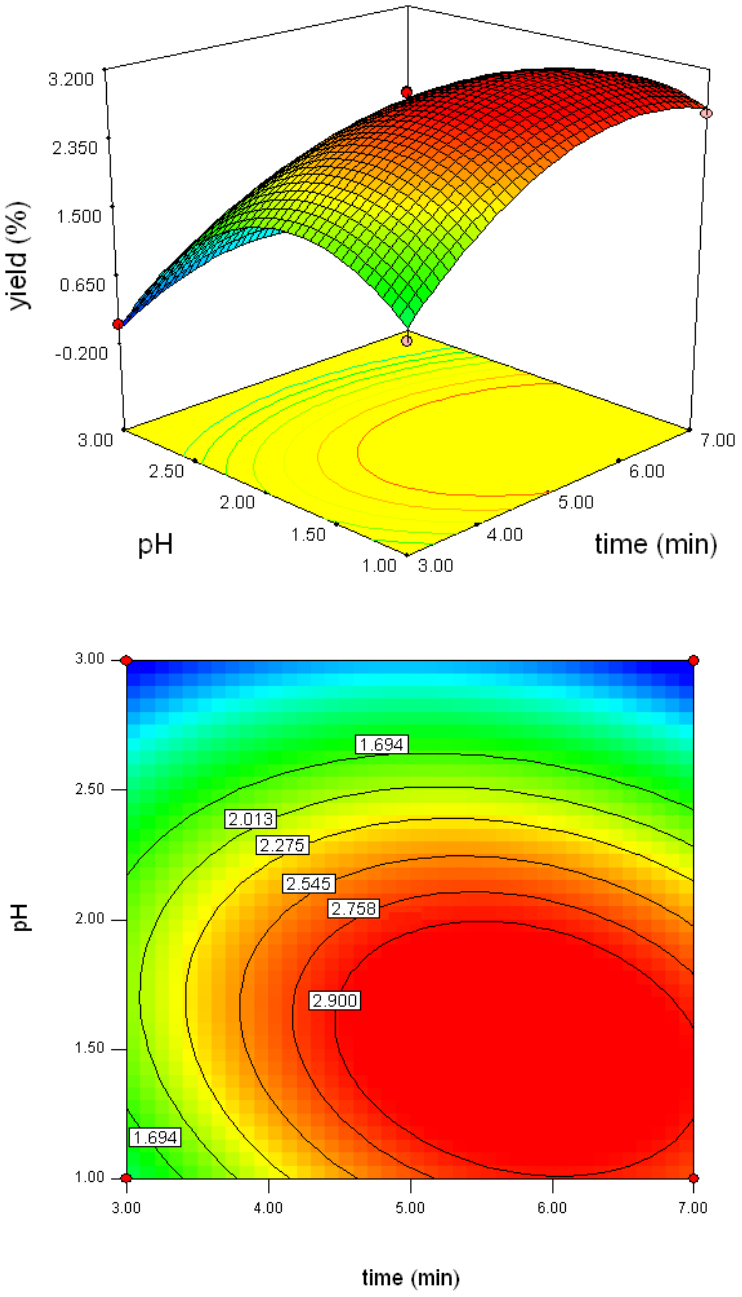


Figure 3.7 Response surface and contour plot for effects of extraction time and pH value on pectin yield from oil extracted pomelo peels

Figure 3.7 displayed a significant interaction effect of the extraction time and pH value of solvent on the pectin yield ($p < 0.05$). Similar results on pectin extraction from apple pomace with the use of MAE had been reported by Wang et al., (2007) [13]. In this study, the vary of the pH of solvent and the extraction

time showed dramatic effects on pectin extraction,while the microwave power displayed a significant quadratic effect.

3.3.4 Optimization of MAE condition for pectin yield

The optimization of MAE condition for pectin yield was performed using graphical technique approach. The optimum region (shaded) was obtained by superimposing contour plots of pectin yield as function of time and solvent pH, solvent pH and microwave power, microwave power and time, as shown in Figure 3.8.

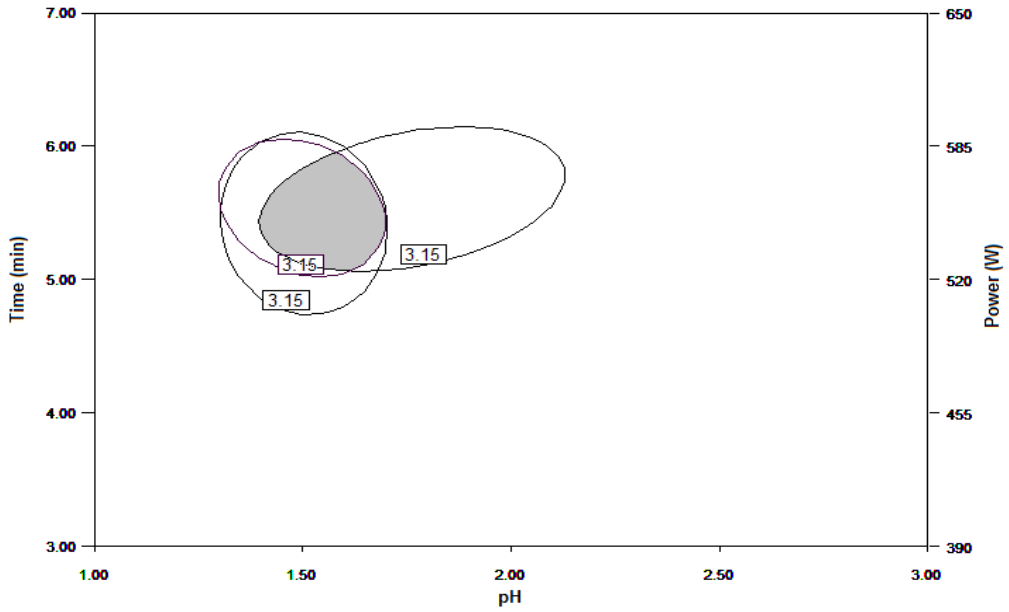


Figure 3.8 Superimposed contour plot showing optimum region (shaded) for pectin yield by MAE.

The results indicated that it is possible to obtain a higher extraction yield of pectin under the circumstance with the microwave power level at 520-585 W, the

treatment time at 5-6 min, and the pH value at 1.3-1.7. Under these conditions, the yields were expected to be more than 3.15 %. Verification experiments were carried out at the selected optimum condition of microwave power level at 520 W, time at 5.6 min and pH value at 1.5. The results indicated that the average yield was 3.29±0.15 %, which was found to be in good agreement with the results obtained by graphical method.

3.3.5 Comparison of MAE and conventional method for pectin extraction

Conventional acidic solution extraction was performed for extraction of pectin from oil extracted pomelo peels. After 90 min of extraction time, the yield of pectin was 3.11 %, which is lower than the yield of 3.29 % extracted by MAE under optimum condition for 5.6 min. Therefore, considering the significant shortening of extraction time, MAE is preferable over the conventional acidic solution extraction method.

3.4 Conclusion

SFME showed a superior performance than HD in essential oil extraction from pomelo fruit peels in terms of extraction efficiency and essential oil yield. At the same time, SFME would not affect the quality of essential oil. The oil extracted pomelo fruit peels could be used for extraction of pectin by MAE. Among the variables, MAE time, pH value of solvent and quadratic term of these two variables showed to have extremely significant effect on the pectin yield. The optimized MAE conditions were verified by experiments. The established sequential microwave extraction of essential oil and pectin by SFME and MAE was considered to be a feasible processing method to improve the utilization of biowaste from pomelo processing industry.

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Chapter 4. Microwave-Assisted Extraction and Characteristics of Saikosaponins from *Radix Bupleuri*

Abstract: In the present work, the individual effects of microwave power, irradiation time, temperature, ethanol concentration, solvent-to-sample ratio, and sample particle size were evaluated. The characterization and quantification of extracted saikosaponins were carried out by high-performance liquid chromatography. Results indicated that high extraction yields of saikosaponins a, c, and d with only trace amounts of saikosaponin b_2 were obtained by MAE with a 300 to 500 W power level for 5 min at 75 °C with 30–70 % ethanol in water, 30:1 solvent-to-sample ratio, and 0.30 to 0.45 mm particle size. With regard to obtaining saikosaponin b_2 with conventional hot solvent extraction (50 % ethanol, 75 °C) for 120 min and hot water reflux extraction for 60 min, the detected concentrations of saikosaponin b_2 were 0.62 mg/g and 1.59 mg/g, respectively, which were much higher than that obtained by MAE. In the extracts of conventional hot water reflux extraction, saikosaponin d decreased to undetectable level. The degradation of saikosaponin d could be minimized by MAE. Moreover, MAE can significantly reduce the extraction time, resulting in better extraction efficiency.

4.1 Introduction

The dried root of *Bupleurum chinense* D.C., known as *Radix Bupleuri*, has been recognized as one of the most important traditional Chinese medicines. It is adopted for treating fever, pain, and inflammation-associated diseases such as influenza, common cold, allergic activities, hepatic diseases, and autoimmune diseases [1–4], and considered as an alternative medicine in prevention and treatment of severe acute respiratory syndrome [5, 6]. *Radix Bupleuri* contains various structures of saikosaponins, including saikosaponin a, c, d, and b_2, in different compositions. Studies have confirmed the physiological and pharmacological activities of the saikosaponins, including immunomodulatory, hepatoprotective, anti-tumor, and anti-virus [2–4, 7].

In traditional Chinese medicine, the conventional approaches to the extraction of bioactive components normally involved boiling the herbs in water for approximately 30–60 min to produce herbal drinks for oral intake. Alternatively, soaking the herbs in ethanol or aqueous ethanol solution under moderate temperature can gradually leach out the target compounds. The active compounds can be manufactured in advance as herbal prescription medicines in various forms, such as pills, tablets, or injection solutions. Several studies have investigated the processing of extraction, isolation, and analysis of saikosaponins that were carried out in the conventional methods [8–10], and the studies had revealed several inherent limitations and problems of the traditional methods: they were labor intensive and time consuming, with high energy costs, while resulting in low degree of purity. Therefore, development of more efficient methods for rapid extraction and isolation of the bioactive components from the herbal matrix was a necessary for industrial applications.

Since the original application of microwave energy for acid digestion in 1975 [11] and sample preparation in 1986 [12], microwave-assisted extraction (MAE) has attracted growing interests [13]. There were numerous reports on the application of MAE for extraction of target components from different matrices, with typical practices reported for the extraction of bioactive compounds from Chinese herbs such as ginsenosides from different parts and ages of ginsengs [14–18], flavonoid-enriched extract from Folium Eucommiae [19, 20], and tetrahydropalmatine, imperatorin, and isoimperatorin from Yuanhu Zhitong formula [21]. MAE has proven to be a promising technique used widely in the extraction of pigments from plants [22, 23], essential oils from herbal seeds [24–26], total phenols from black tea powder [27], chlorogenic acid from flower buds of *Lonicera japonica* Thunb. [28], free amino acids from vegetables [29], and fat from chocolate and cocoa products [30]. In general, the results from the published works confirmed that, compared with conventional extraction approaches, MAE can remarkedly reduce the extraction time and solvent consumption as well as increase the extraction yield rate and extraction efficiency.

In our previous work [31], response surface methodology and a central composite rotatable design approach were adopted to predicatively define the optimum MAE condition for obtaining desirable extraction yields for all saikosaponins. The objectives of this work were to validate the optimized conditions of saikosaponin MAE from *Radix Bupleuri* and analyze the active compounds by high-performance liquid chromatography (HPLC). The efficacy of MAE for the extraction process and the parameters controlling the extraction, including microwave power, irradiation time, temperature, solvent composition

(ethanol concentration), solvent-to-sample ratio, and sample particle size are evaluated.

4.2 Experimental design

4.2.1 Apparatus

All microwave extractions were carried out in a custom-made microwave reactor with a cylindrical cavity (model 961, Microwave Power Consultants, VIC Australia). This microwave reactor has a continuously variable microwave power system with power output up to 1000 W and a fiber-optical temperature controller. The system was equipped with a glass extraction vessel topped by a cooler, and it worked at atmospheric pressure. Each sample was subjected to focused microwave irradiation to provide rapid heating, and the solvent was circulated through the sample at a fixed flow rate of 4 mL/s by a pump (Masterflex, IL, USA). A water bath (JULABO-F10, Julabo Labortechnik GMBH, Germany) was used to cool the extraction solvent in order to keep the microwave working at the correct setting power when the extraction temperature was achieved during extraction. The schematic diagram of the microwave extractor is shown in Figure 4.1.

A centrifuge (Eppendorf 5415D, Germany) was used to separate the extract from the matrix residue, and a rotary evaporator (RE-52, Shanghai Qingpu Huxi instruments, China) was applied to concentrate the extract. A Hewlett Packard HP 1100 HPLC equipped with a Quaternary pump (G1311A), a variable wavelength detector (G1314A), and a Rheodyne model 7255 manual injector with a fixed 20 μL sample loop, a Hypersil ODS C_{18} (200 × 4.6 mm; 5 μm) reverse phase column (Hewlett Packard, USA), and a guard column (PN 96013, Alltech) was used for the analysis.

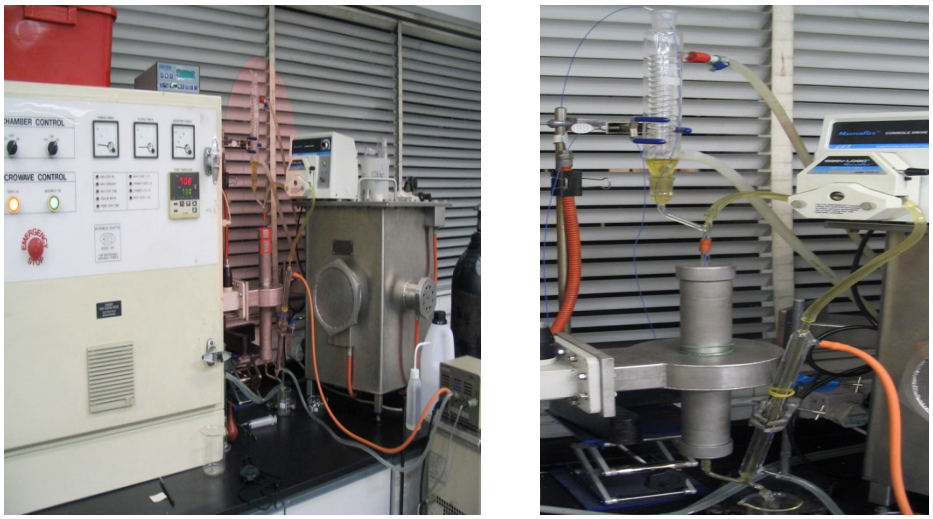

Figure 4.1 Experiment set-up of MAE

4.2.2 Materials and chemicals

Radix Bupleuri (dried root of *B. chinense* D.C.; Beichaihu) of 10.35 % moisture content was obtained from Guangzhou Zhisheng Medicinal Co. (Guangzhou, PR China). The *Radix Bupleuri* was ground with a grinder and sieved to separate components measuring <0.30, 0.30–0.45, 0.46–0.90, and 0.91–2.00 mm, respectively, which were packed into separate polyethylene bags and stored at room temperature (23 °C) for use.

Reference standards were saikosaponins a and c (Nacalai Tesque Inc., Tokyo, Japan), saikosaponin d (Wako Pure Chemical Industries Ltd., Tokyo, Japan), and saikosaponin b_2 (Chengdu Cogon Bio-tech Co., Ltd., Chengdu, PR China). The solvents used were methanol and acetonitrile (HPLC grade, Tedia Company, Inc., USA), ethanol (analytical grade, Merck KgaA, Germany), and deionized distilled water.

4.2.3 Extraction procedures

4.2.3.1 Microwave-assisted extraction

Pre-weighted *Radix Bupleuri* was transferred to the extraction vessel carefully. The vessel was placed in the microwave cavity and fitted with a cooler, and 60 mL of solvent was added in the vessel. The extraction of 2 g of *Radix Bupleuri* with particle sizes of 0.30–0.45 mm was carried out at various levels of microwave power (100, 200, 300, 500 W), extraction times (0.5, 1, 3, 5, 10, 15 min), temperatures (25, 45, 55, 65, 75 °C), and ethanol concentrations (0 %, 30 %, 50 %, 70 %, 90 %). In addition, various sample weights (6.00, 3.00, 2.00, 1.50, 1.20 g) and particle sizes (<0.30, 0.30–0.45, 0.46–0.90, and 0.91–2.00 mm) were used to study the effects of solvent-to-sample ratio and sample particle size on extraction yields.

4.2.3.2 Hot solvent extraction

Two grams of *Radix Bupleuri* with particle sizes of 0.30–0.45 mm were placed in the extraction vessel (same as used for MAE), and then 60 mL of 50 % ethanol was heated and circulated at 75 °C. Extractions were carried out for 10, 30, 60, and 120 min, respectively.

4.2.3.3 Hot reflux extraction

Two grams of *Radix Bupleuri* with particle sizes of 0.30–0.45 mm were placed with 60 mL of water in a 250 mL flask, which was fitted with a top cooler and placed into a hot bath at 100 °C. Reflux extraction was carried out for 60 min.

4.2.3.4 Collection of extracts

The extracts from all methods were filtered and collected in volumetric flasks. The residue in the vessel was washed twice with ~30 mL of extract solvent and the washings were collected and combined with the extracts. The final volume of the combined filtrate was adjusted to 100 mL, and then 25 mL of this extract solution was evaporated under vacuum conditions at 45 °C with the

use of a rotary evaporator. After evaporation, the residue was dissolved in 5 mL of HPLC-grade methanol, centrifuged at 12,000 r/min for 10 min, and filtered through a PTFE syringe filter (0.45 μm) for HPLC analysis.

4.2.4 HPLC analysis of saikosaponins

The HPLC conditions were based on Park and Kanazawa's work [32, 33] with some modifications. The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II), and separation was achieved by using the following gradients: 0–10 min, 30 % I, 70 % II; 10–18 min, 30–40 % I, 70–60 % II; 18–28 min, 40–45 % I, 60–55 % II; 28–35 min, 45 % I, 55 % II; 35–40 min, 45–30 % I, 55–70 % II. The flow rate was fixed at 0.8 mL/min, and the absorbance was measured at a wavelength of 203 nm at room temperature. Ten microliters of sample solution obtained from the extraction was injected to the HPLC for analysis.

4.2.5 Statistical analysis

Multiple range statistical analysis (Duncan's test) at a 0.05 significance level was adopted to determine the significant differences between the means by the Statistical Analysis System software package ver. 6.12 (SAS Inc., Cary, NC, USA).

4.3 Results and discussion

4.3.1 Identification and quantitative analysis by HPLC

The chromatographic peaks of saikosaponins were identified by comparing the retention times with those of the reference standards, and quantitative

analysis was performed by comparing the peak area with that of the reference standard using the external standard method. As shown in Figure 4.2**,** peaks of saikosaponins a, b_2, c, and d were obtained with an acceptable resolution from neighboring compounds. Standard curves were obtained for saikosaponins a, b_2, c, and d with correlation coefficients of 0.9998, 0.9983, 0.9997, and 0.9998, respectively.

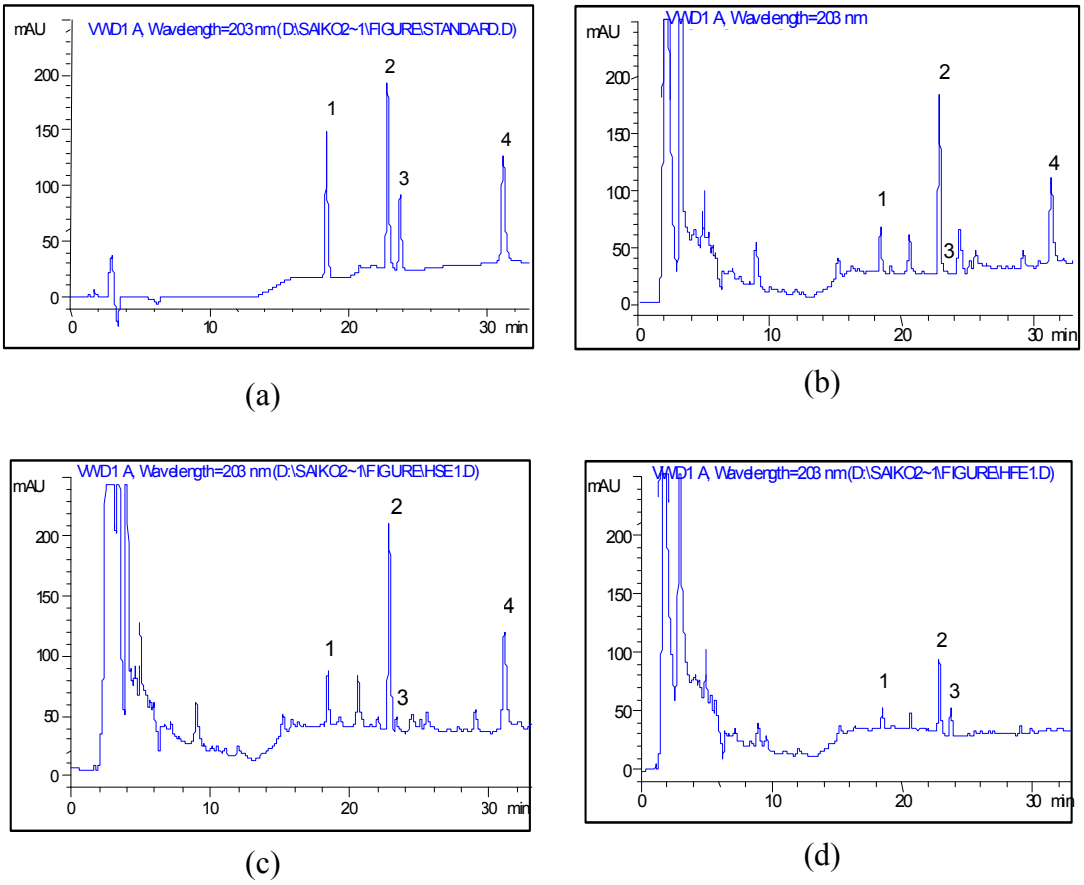

Figure 4.2 HPLC Chromatograms of saikosaponins (key to peak identify: 1, saikosaponin-c; 2, saikosaponin-a; 3, saikosaponin-b_2; 4, saikosaponin-d). (a) Standard of saikosaponins; (b) MAE: t=5 min, T = 75 °C, I = 300 W, and C = 50 % (ethanol to water, v/v); (c) HSE: t= 120 min, T = 75 °C, and C = 50 % (ethanol to water, v/v); (d) HWRE: t= 60 min, T = 100 °C, and C = 0 % (water)

The results indicated that the RSD% values for the peak area of three injections of each sample were less than 10%, and a good precision of the method was obtained. Furthermore, chromatographic patterns of the samples obtained by MAE, by hot solvent extraction, and by hot reflux extraction appeared to be similar, except that saikosaponin d was not obtained by hot reflux extraction at 100 °C for 60 min (Figure. 4.2d), saikosaponin b_2 was found in only trace amounts in the extract obtained by MAE at 75 °C for 5 min compared with that by hot solvent extraction at 75 °C for 120 min and that of hot reflux extraction at 100 °C for 60 min (Figure. 4.2b–d). Based on this observation, saikosaponins b_2 was left out of the analysis of MAE efficiency.

4.3.2 Effect of microwave power and irradiation time

The effect of microwave power at 100, 200, 300, and 500 W on the extraction yields was studied with MAE for 5 min. the results showed that a power level between 100 and 300 W had a significant influence on the efficiency of extraction of saikosaponins (Figure.4.3). Although an increase in power level led to better extraction efficiency, as the power level was increased from 300 to 500 W, there was no significant increase in extraction yields of total saikosaponins (p>0.05).

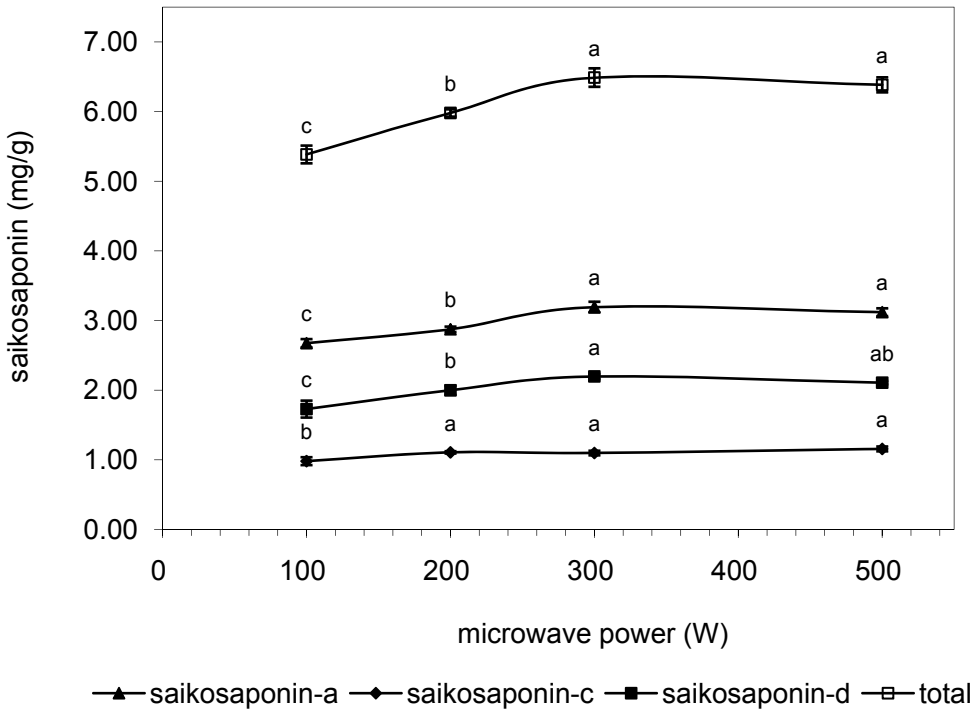

Figure 4.3 Effect of microwave power level on the yield of saikosaponins by MAE (t = 5 min, T= 75 °C, C = 50 % (ethanol to water, v/v)). Data with different superscript letters within a same series are significantly different, $p<0.05$)

The effect of irradiation time on the extraction yields at 300 W of microwave power was investigated. The extraction yields increased significantly by increasing irradiation time from 0.5 to 3 min (Figure. 4.4). When irradiation time was extended from 3 to 15 min, there was no significant increase in extraction yields ($p>0.05$). The result suggested that a period of 5 min was sufficient to extract the saikosaponins from the matrix, and longer irradiation time made no contribution to the extraction efficiency. The lack of an increase in extraction efficiency with a prolonged irradiation period was also observed in the extraction of amino acids from food by Kovacs et al. [29].

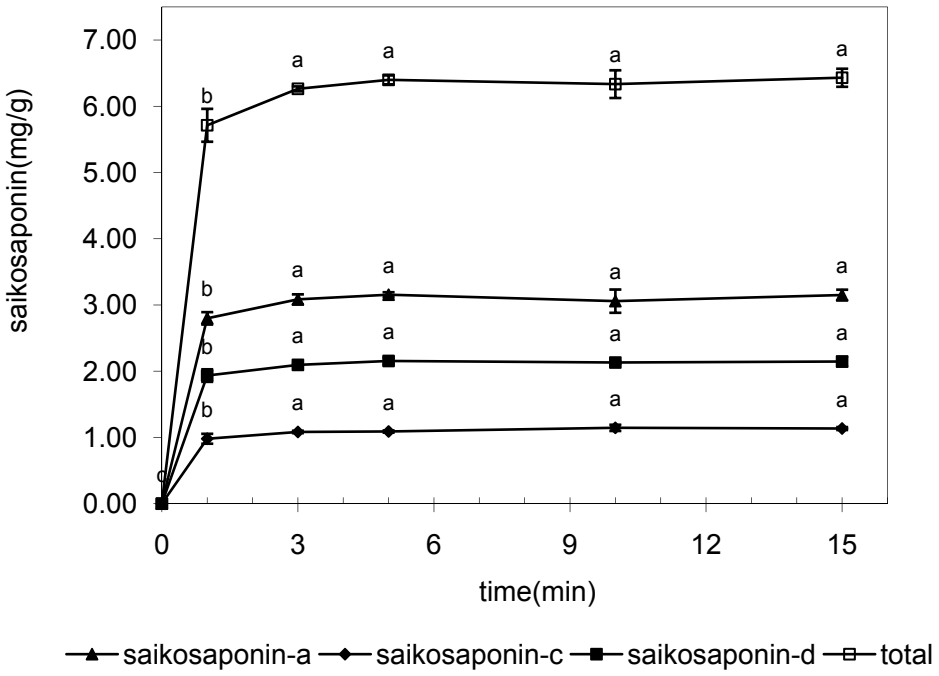

Figure 4.4 Effect of microwave irradiation time on the yield of saikosaponins by MAE (T = 75 °C, I = 300 W, C = 50 % (ethanol to water, v/v)). Data with different superscript letters within a same series are significantly different ($p<0.05$).

With regard to the effect of power on the efficiency of MAE, Chen and Spiro (1994) reported that an increase of irradiation power led to better extraction efficiency of essential oils from plants [34]. In contrast, Kaufmann et al. (2001) reported that the power of irradiation had no influence on withanolides recovery from *Iochroma gesnerioides* [35]. Alfaro et al. (2003) found that extra energy (power × time) output did not result in greater extraction once sufficient energy had been applied to rupture the plant material structure and release the chemicals contained therein[36]. They suggested that the energy density (power per mass for a given unit of time) was a more important parameter than simple power level. The effects of time and power level on extraction efficiency obtained in this

study supported the previous findings. Based on our findings, the condition of 300 W of microwave power with 5 min of irradiation was chosen for the investigation of other parameters.

4.3.3 Effect of solvent composition

The solvent selected for MAE was mainly determined by the solubility of the target compound, the interaction between the solvent and matrix, and the microwave absorbing properties of the solvent by its dielectric constant [37]. Saikosaponins a, c, and d are glycosides of pentacyclic triterpenes with the sugar moieties of glucose, fucose, and rhamnose as well as hydroxyl groups attached to the molecular backbones. They were quite soluble in polar solvent, such as water and diluted ethanol, but were insoluble in nonpolar solvents.

Considering that an ethanol-water mixture was commonly used to extract saponins [38], five ethanol concentrations were tested: 0 %, 30 %, 50 %, 70 %, and 90 % ethanol in water. The extraction yields of saikosaponins were influenced by the concentration of ethanol. Higher extraction yields were obtained with 30–70 % ethanol in water, while significantly lower yields were observed in both pure water and 90 % ethanol (p<0.05) (Figure.4.5). This result supported previous findings that ethanol concentration played a significant role in the extraction of soluble components from different natural products [25, 38]. For further analysis, 50 % ethanol in water was selected as the solvent concentration due to its good heating capacity by microwaves and desirable saikosaponin solubility.

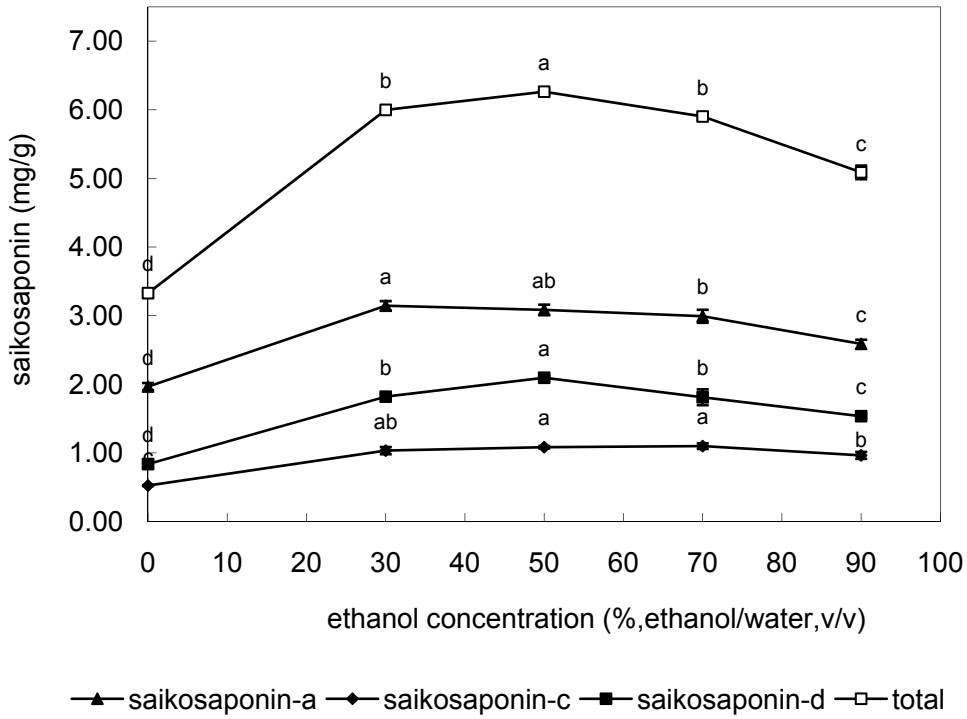

Figure 4.5 Effect of ethanol concentration on the yield of saikosaponins by MAE (T = 75 °C, I = 300 W, t = 5 min). Data with different superscript letters within a same series are significantly different ($p<0.05$).

4.3.4 Effect of temperature

The effect of temperature on extraction yields was studied using 25, 45, 55, 65 and 75 °C. Extraction at high temperature (75 °C) below the boiling point of the solvent resulted in significantly higher yields ($p<0.05$) (Figure 4.6). The improvement of extraction at elevated temperature might be due to the surface tension and viscosity of solvent being decreased at high temperature, thus accelerating the diffusion of solvent into the matrix, enhancing desorption of the analytes from the matrix, and promoting the yields. Thus, 75 °C was considered to be a suitable extraction temperature.

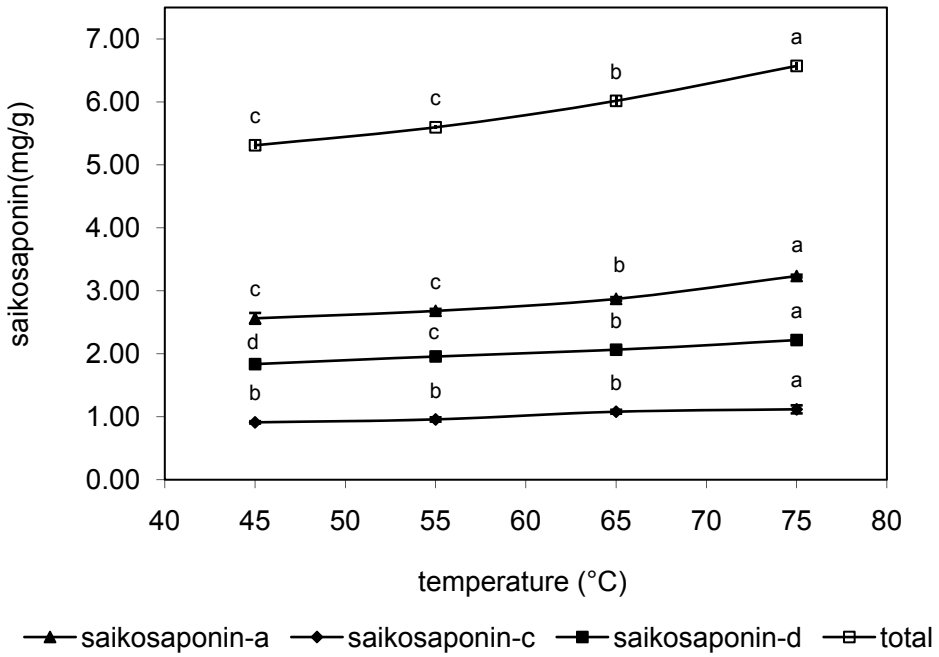

Figure 4.6 Effect of temperature on the yield of saikosaponins by MAE (I = 300 W, t = 5 min, C=50 % (ethanol to water, v/v)). Data with different superscript letters within a same series are significantly different ($p<0.05$)

4.3.5 Effect of solvent-to-sample ratio

The effects of various solvent-to-sample ratios on the extraction yields were studied with 50% ethanol at 300 W of microwave power and 75 °C for 5 min. The extraction yields of saikosaponins a and d and the sum of saikosaponins a, c, and d increased significantly ($p<0.05$) as the solvent-to-sample ratio increased from 10:1 to 30:1 (Figure 4.7). However, there was no significant increase in yield ($p>0.05$) as the solvent-to-sample ratio increased from 30:1 to 50:1. Thus, 30:1 was considered to be a suitable solvent-to-sample ratio.

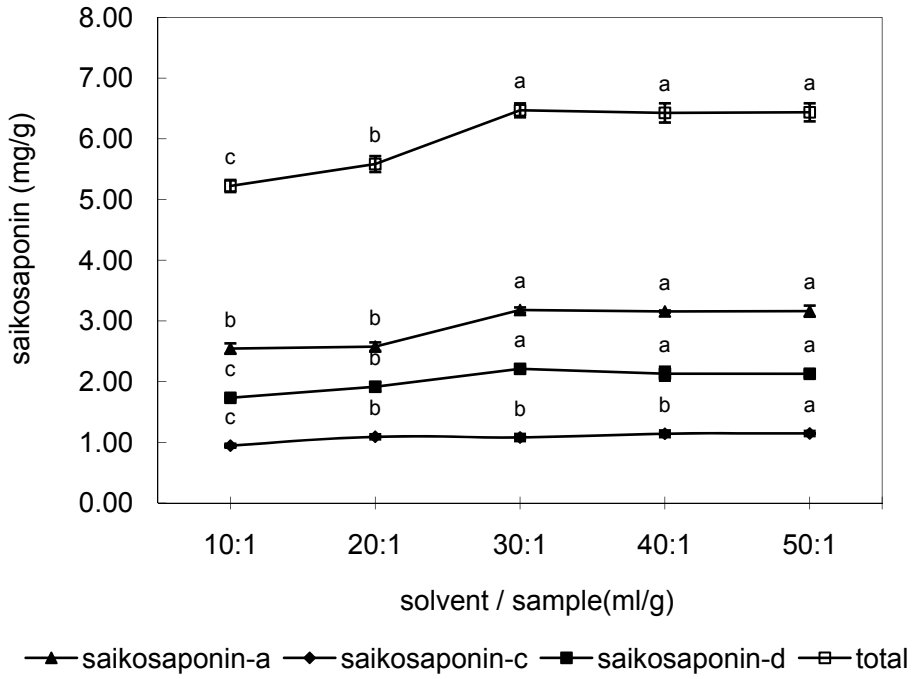

Figure 4.7 Effect of solvent to sample ratio on the yield of saikosaponins by MAE (t = 5 min, T= 75 °C, C = 50 % (ethanol to water, v/v)). Data with different superscript letters within a same series are significantly different, $p<0.05$)

4.3.6 Effect of sample particle size

The effects of four particle sizes on the extraction yields were studied. Samples with a particle size distribution between 0.30 and 2.0 mm had a significant influence ($p<0.05$) on the efficiency of extraction (Figure 4.8), However, extraction yields were slightly decreased if the particle size was less than 0.30 mm. The results indicated that the greater the particle size, the lower the yields of saikosaponins a, c, and d and their sum. Likewise, in studies of cocaine extraction from coca leaves [39] and withanolide extraction from *Iochroma gesnerioides* [35], smaller particle sizes were more favorable for higher extraction yield. The larger surface area and less mass transfer resistance

likely provided the analyte more access to the solvent. The particle with a size of 0.30–0.45 mm was therefore chosen as the most suitable one.

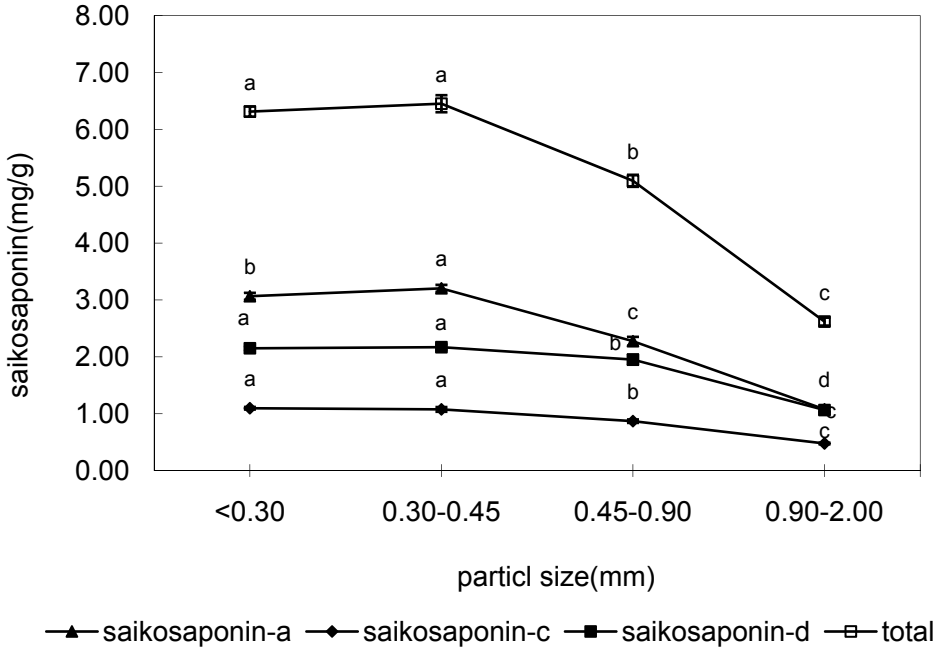

Figure 4.8 Effect of particle size on the yield of saikosaponins by MAE (t = 5 min, T= 75 °C, C = 50 % (ethanol to water, v/v)). Data with different superscript letters within a same series are significantly different, $p<0.05$)

4.3.7 Comparison of conventional and MAE methods

Results from hot solvent extraction and hot reflux extraction were compared with those from the MAE method. The comparison indicated that the yields of total saikosaponins extracted by MAE for 5 min were similar to those obtained by hot solvent extraction for 120 min. The yields of saikosaponins extracted in water with the use of hot reflux extraction at 100 °C for 60 min were lower than those extracted in 50 % ethanol at 75 °C with the use of either heat solvent extraction or MAE. In addition, saikosaponin b_2 in extracts from MAE was below the detection limit. In contrast, hot solvent extraction with the same

solvent and temperature for 10–120 min increased the concentration of saikosaponin b_2 significantly (p<0.05) to 0.62 mg/g, while hot reflux extraction with water at 100 °C for 60 min yielded 1.59 mg/g. On the other hand, saikosaponin d extracted by hot solvent extraction was less than that extracted by MAE, and no increase was obtained by prolonging the time of the heat solvent extraction. No saikosaponin d was observed in extract obtained by hot water reflux extraction at 100 °C for 60 min (Table 4.1; Figure 4.2). This could be explained by the fact that saikosaponin d was easily degraded and converted to saikosaponin b_2 under high temperature and prolonged extraction time.

Our results indicated that with the application of MAE for extraction of saikosaponins from *Radix Bupleuri*, the extraction time could be significantly reduced (less than 10 min) and the degradation of saikosaponin d could be eliminated compared with conventional methods.

As mentioned earlier, several research works have been published on the comparison of MAE with other conventional techniques. Findings from the published literatures have demonstrated that MAE could shorten the extraction time, reduce the solvent usage and increase the extraction yields.

Saikosaponin a, c, and d contain an unstable allyloxide linkage and are readily converted into diene saponins by mild acid treatment or on heating [40]. Zhang et al. (1985) performed a study on saikasaponin composition changes during extraction, the results shown that the saikosaponin d was degraded and converted to saikosaponin b_2 after hot water extraction at 100 °C for 30 min [41].

In our study, it was found that saikosaponin d extracted by hot solvent extraction was less than that extracted by MAE, no saikosaponin d was obtained by hot water reflux extraction at 100 °C for 60 min. On the other hand,

saikosaponin b_2 was found in extract and increased with prolonged extraction time in both hot solvent extraction and hot water reflux extraction, as shown in Table 4.1 and Figure 4.2.

In view of the limitations of MAE for extraction of natural products, the scale-up of the system for industrial application may be the concern. MAE has basically been used on the extraction of limited amount of sample at laboratory scale for analysis. The use of MAE for extraction of natural products at industrial scale was rarely reported. Issues with scale-up and uncertainty on the applicability of the scale-up system have hampered the wide applications of MAE. Furthermore, the microwaves penetration depth depends on the dielectric constant of target matrix, the loss factor of the compound is also important and is related to the transparency to microwaves and the ability to dissipate the absorbed energy. Since microwaves have low penetration depth (~1.5 cm in water at 2450 MHz), the sample layers should be less than 1.5 cm thick, and should be uniformly spread [42]. This must be taken in to account carefully in the design of large-scale commercial MAE system.

Table 4.1 Comparison of saikosaponins extracted by microwave assisted extraction with hot solvent extraction and hot reflux extraction

Method	Time (min)	saikosaponins extracted from *Radix Bupleuri* (mg/g; (RSD %))				
		a	b_2	c	d	total
MAE (50%EtOH, 300 W, 75℃)	5	3.20 (1.91)[1]a	-[2]	1.08 (3.82) a	2.17 (2.22) a	6.45 (2.32) a
	5	3.18 (1.37) a	-	1.08 (3.39) a	2.21 (1.64) a	6.47 (1.77) a
	5	3.24 (1.76) a	-	1.12 (2.44) a	2.22 (0.88) a	6.57 (0.90) a
	5	3.19 (2.40) a	-	1.10 (3.00) a	2.20 (1.67) a	6.49 (2.04) a
	5	3.15 (1.21) a	-	1.09 (1.52) a	2.15 (2.34) a	6.40 (1.05) a
Hot Solvent Extraction (50%EtOH, 75℃)	10	2.53 (1.21) d	-	0.89 (1.35) b	1.73 (2.58) b	5.15 (1.12) d
	30	2.77 (2.46) c	0.40 (7.14) d	0.90 (4.65) b	1.69 (4.01) b	5.77 (1.98) c
	60	2.86 (2.19) bc	0.55 (2.43) c	1.06 (2.49) a	1.70 (5.29) b	6.17 (2.80) b
	120	2.95 (3.14) b	0.62 (2.74) b	1.10 (4.38) a	1.77 (2.37) b	6.43 (3.00) a
Hot Reflux Extraction (Water, 100℃)	60	1.50 (0.40) e	1.59 (1.94) a	0.53 (1.93) c	-	3.63 (0.83) e

1. Means (RSD %) within a column followed by the different letters are significantly different ($p<0.05$).

2. Dash indicates below detection limit.

4. 4 Conclusions

Our results confirmed that the extraction efficiency of saikosaponins by MAE was influenced by the parameters of microwave power, irradiation time, temperature, solvent composition, solvent-to-sample ratio, and sample particle size. High yield was obtained with 300–500 W of power for 5 min at 75 °C with 30–70 % ethanol in water, a 30:1 solvent-to-sample ratio, and 0.30–0.45 mm particle size. Our findings indicated that MAE was a good technique for the rapid extraction of saikosaponins a, c, and d from *Radix Bupleuri* for it required less than 10 min to extract. In comparison to conventional extraction methods, the degradation of saikosaponins during extraction can be minimized because MAE can significantly reduce extraction time, resulting in better extraction efficiency.

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Chapter 5. Optimization of Microwave-Assisted Extraction of Saikosaponins from *Radix Bupleuri*

Abstract: Microwave-assisted extraction (MAE) was applied in the extraction of saikosaponin a, c and d from *Radix Bupleuri*. Several operating parameters, namely microwave power, time, temperature and ethanol concentration, were optimized using response surface methodology (RSM) with a central composite rotatable design (CCRD). The ethanol concentration and time were found to be the most significant factors (P<0.0001) for the extraction of all three saikosaponins. By using the desirability function approach, the optimum MAE conditions to obtain desirable extraction yields for all these saikosaponins simultaneously were found to be at the microwave power of 360-400 W, ethanol concentration of 47-50 %, temperature of 73-74 ^{o}C and time of 5.8-6.0 min. At these conditions, the yields from the verification experiments were 96.18-96.91 % for saikosaponin a, 95.05-95.71 % for saikosaponin c and 97.05-97.25 % for saikosaponin d, which were in good agreement with the predicted values from the fitted models

5.1 Introduction

In recent years, interest in the research and development of herbal medicine has risen enormously, especially in the field of modernization of traditional Chinese medicines (TCM), which normally requires the extraction of effective components from herbal matrix. The dried root of *Bupleurum chinense D.C.*, which is known as *Radix Bupleuri*, is one of the well-known TCM herbs and is used as a key ingredient for many Chinese multi-herb remedies, such as xiao-chai-hu-tang, a famous haematopoietic remedy in oriental medicine. It contains different forms of saikosaponins, such as saikosaponin a, c and d, which have been recognized as pharmacologically active compounds and possess immuneomodulatory, hepatoprotective, anti-tumour and anti-viral activities [1-2]. The quality of *Radix Bupleuri* medicine is generally determined by the contents of saikosaponins.

There were several studies focusing on the extraction, separation and analysis of saikosaponins from *Radix Bulerui* by conventional methods [3-7]. The conventional methods for the extraction of effective components usually involves boiling the herbal matrices with water for 30-60min for the preparation of "herbal drinks" for oral intake, or soaking with ethanol or aqueous ethanol under moderate temperature for a relatively long time to leach the target compounds for being used in herbal prescription medicine or for analysis purpose. However, these conventional methods are very time consuming and low efficient. Attention has therefore been drawn to the development of more efficient methods for rapid extraction, isolation and analysis of the effective components from medicinal plants.

Microwave-assisted extraction (MAE) has been successfully used for the extraction of effective compounds from different plant matrices in the past 5 years [8-18]. In a number of our previous work, MAE was also employed for the extraction of effective components from various TCM herbs, including saikosaponins from *Radix Buplerui*. The results from these applications indicated that MAE can remarkably reduce the extraction time and solvent consumption, while offering better extraction efficiency. Our previous studies also showed that, when using the one-variable-at-a-time approach, the extraction yields of saikosaponin a, c and d were influenced by the levels of parameters such as the microwave power, irradiation time, temperature, ethanol concentration, solvent to sample ratio and sample particle size. More than 90 % of relative extraction yields were obtained by using 2.00 g samples with a particle size of 0.3-0.45 mm in 60 mL of 30-70 % of aqueous ethanol, 300-500 W of microwave power and 75 ^{o}C for 3-5 min. But there is no information about the interaction effects among the operating parameters on the extraction efficiency. Therefore, it is necessary to perform a systematic optimization to find the "best" conditions for MAE of saikosaponins from *Radix Bupleuri*.

Response surface methodology (RSM) with the desirability function approach has been shown to be a useful statistical tool to solve multi-variable problems and optimize one or several responses [19, 20]. Moreover, personal computer, statistical software and computer graphics for desired function methodology implementation are now available and have been successfully applied in various industrial processes and researches optimising conditions for sample preparation and analysis of analytes. Kwon and co-workers [15] used RSM to optimize the conditions for MAE of saponin components from ginseng

roots; Lee et al. [21] optimized the extraction procedure for the quantification of Vitamin E using RSM; Carro and Lorenzo [22] used the desirability function to simultaneously optimize the solid-phase extraction of organochlorine and organophosphorus pesticides; Jimidar et al [23] applied the desirability function for the selection of optimum separation conditions in capillary zone electrophoresis. Bourguignon and Massart [24] simultaneously optimized several chromatographic performance goals using the desirability function. The main advantage of RSM is its ability to decrease the experimental trials required to evaluate multiple parameters and their interactions. Therefore, it is less laborious and less time-consuming than other approaches to optimize a process with one or more responses.

The objectives of this study are to better understand the relationships between the yields of saikosaponins and the extraction parameters such as microwave power, time, temperature and ethanol concentration, and to find a set of optimum conditions for MAE of saikosaponin a, c and d from *Radix Buplerui* using RSM with the desirability function approach

5.2 Materials and Methods

5.2.1 Materials

Radix Bupleuri (dried root of *Bupleurum chinense* D.C.; Beichaihu) of 10.35% moisture content was obtained from Guangzhou Zhisheng Medicinal Company (Guangzhou, PR China). The *Radix Bupleuri* was grounded with a grinder and sieved to 0.30–0.45 mm, then packed in a polyethylene bag and stored at room temperature (23 °C) until used.

5.2.2 Reagents and Apparatus

Chemicals used in this study included standards of saikosaponin a, c (Nacalai Tesque Inc., Tokyo, Japan) and d (Wako Pure Chemical Industries Ltd., Tokyo, Japan), methanol and acetonitrile (HPLC-grade, Tedia Company, Inc., USA), ethanol (analytical grade, MERCK KgaA, Germany) and de-ionized distilled water.

All MAE experiments were carried out in a custom-made Microwave Reactor with cylindrical cavity (model 961, Microwave Power Consultants, VIC, Australia). This Microwave Reactor is a continuously variable microwave power system with power output up to 1000 W and a fibre optical temperature controller. The system is equipped with a glass extraction vessel topped by a cooler and works at atmospheric pressure. The sample was subjected to focused microwave irradiation to provide rapid heating and the solvent was circulated through the sample by a pump (Masterflex, IL, USA). A water bath (JULABO-F10, Julabo Labortechnik GMBH, Germany) was used for cooling the extraction solvent in order to keep the microwave work with the setting power when the extraction temperature was achieved during extraction. The schematic diagram of the microwave extractor was shown in Figure 5.1.

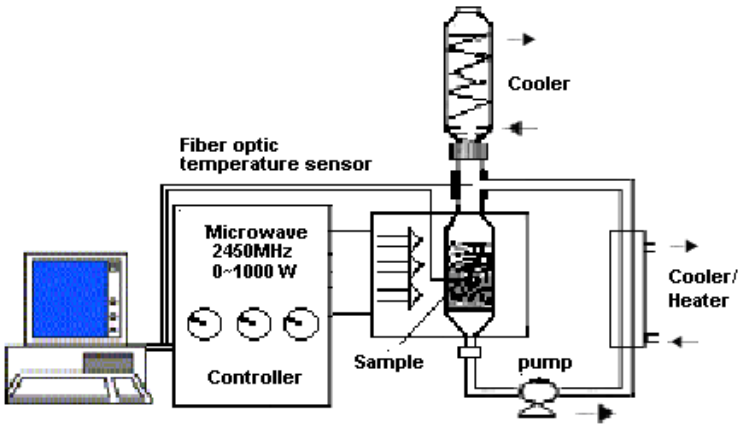


Figure 5.1 The schematic diagram of the microwave extractor

A centrifuge (Eppendor 5415D, Germany) was used for the separation of the extract from the matrix residue. A rotary evaporator (RE-52, Shanghai, China) was used for the concentration of extract.

A Hewlett Packard HP 1100 HPLC, equipped with a Quaternary pump (G1311A), a variable wavelength detector (G1314A) and a Rheodyne model 7255 manual injector with a fixed 20 μL sample loop, a Hypersil ODS C_{18} (200×4.6 mm; 5 μm) reverse phase column (Hewlett Packard, USA) and a guard column (PN 96013, Alltech), was used for the analysis.

5.2.3 Experimental Design

Based on our previous work, four operating factors, namely the microwave power (X_1), irradiated time (X_2), extraction temperature (X_3) and ethanol concentration (X_4, %, v/v, ethanol in water) with five levels respectively were considered to be the independent variables, and the dependent variables were the

extraction yields of saikosaponin a (Y_1), saikosaponin c (Y_2) and saikosaponin d (Y_3). As shown in Table 5.1, total of 30 runs based on a central composite rotatable design (CCRD) with 6 center points were performed in random order with triplicates for each run.

5.2.4 Microwave-Assisted Extraction

2.00 g *Radix Bupleuri* samples were accurately weighed in the extraction vessel. The vessel was placed into the microwave cavity and 60 mL aqueous ethanol of different concentration was poured into the system. MAE with different levels of operating factors was carried out.

The extracts were then filtered and collected in a volumetric flask. The residue in the vessel was washed twice with ~30 mL solvent and the washings were collected and combined with the extracts. The final volume of the combined filtrate was adjusted to 100 mL. 50 mL of these extracts was evaporated under vacuum at 45 °C using a rotary evaporator. The evaporated residue was dissolved in 5 mL of HPLC-glade methanol, centrifuged at 12000 r/min for 10 min, and filtered through a PTFE syringe filter (0.45 μm) for HPLC analysis.

5.2.5 Conventional Extraction

A conventional solvent extraction based on Park's work [7] was carried out as control for comparative purpose. 2.00 g of samples were extracted in a flask with 60 mL of 70 % ethanol at 45 °C for 8h with agitation. The extraction matrix was re-extracted twice using fresh solvent. The extracts were then combined and prepared for HPLC analysis in the same procedure as above.

5.2.6 HPLC Analysis

The HPLC conditions were based on Kanazawa [4] and Park's work [7] with modifications. The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II) and separation was achieved using the following gradient procedures: 0-10 min, 30 % I, 70 % II; 10-18 min, 30-40 % I, 70-60 % II; 18-28 min, 40-45 % I, 60-55 % II; 28-35 min, 45 % I, 55 % II; 35-40 min, 45-30 % I, 55-70 % II. The flow rate was fixed at 0.8 mL/min and the absorbance was measured at a wavelength of 203 nm at room temperature. 10 μL of the sample solution obtained from the extraction step was injected to the HPLC for analysis.

The analytes were quantified by comparing the chromatographic peak area with linear calibration at five concentration levels in the range from 0.05 to 1.0 μg/μL using saikosaponin a, c and d as external standards. The relative percentage extraction yield of each saikosaponin was defined as (mg saikosaponin obtained by MAE per g sample / mg saikosaponin obtained by conventional extraction per g sample) × 100 %.

5.2.7 Statistical Analysis and Optimization

The mean values of the triplicate trials were fit to a second order polynomial of the following form by the response surface regression procedure (RSREG) in the Statistical Analysis System (SAS 6.12):

$$Y_k = b_{k0} + \sum_{i=1}^{4} b_{ki} X_i + \sum_{i=1}^{4} b_{kii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{kij} X_i X_j \qquad (1)$$

where Y_k are the responses or dependent variables, with Y_1 for the extraction yield of saikosaponin a, Y_2 for saikosaponin c, and Y_3 for saikosaponin d; $b_{k0,}$ b_{ki},

b_{kii} and b_{kij} are the regression coefficients; and Xs are the coded independent variables (MAE operating factors). The fit of the model is evaluated by the R^2 and the lack of fit.

In order to obtain a maximum yield for saikosaponin a, c and d simultaneously, the desirability function approach [20] was used. Derringer's approach is first to convert each response, Y_k, into an individual desirability, d_k. The desirability scale ranges from 0 to 1, where if the response is outside an acceptable region, sets $d_k = 0$, and if the response is fully desirable (at its goal or target), sets $d_k = 1$. Considering the situation in the extraction of the saikosaponins by MAE, we wanted all yields to be as high as possible. Thus, a one-sided transformation was applied:

$$
\begin{aligned}
&d_k = 0 && \text{if} \quad Y_k \le Y_k^{(\min)} \\
&d_k = \left(\frac{Y_k - Y_k^{(\min)}}{Y_k^{(\max)} - Y_k^{(\min)}} \right)^r && \text{if} \quad Y_k^{(\min)} < Y_k < Y_k^{(\max)} \qquad (2) \\
&d_k = 1 && \text{if} \quad Y_k \ge Y_k^{(\max)}
\end{aligned}
$$

where $Y_k^{(\min)}$ is the minimum acceptable value of Y_k, $Y_k^{(\max)}$ is the maximum value that is considered desirable and r is a positive constant. If $r = 1$, the d_k increases linearly as Y_k increases; if $r < 1$, the d_k increases rapidly as Y_k increases; if $r > 1$, the d_k increases slowly as Y_k increases.

The individual desirability functions from the considered responses are then combined to obtain the overall desirability D, defined as the geometric average of the individual desirability:

$$D = (d_1, d_2, \ldots, d_k)^{1/k} \qquad (3)$$

where $0 \leq D \leq 1$, a high value of D shows that all d_ks are toward the target value, which is considered as the optimal solutions of the system.

5.2.8 Computer Program and Software

Statistical analysis and the search for optima were performed by a subroutine written in SAS (6.12). The Pareto chart and 3-D plots of the responses were drawn using the software Statistica (6.0).

5.3. Results and discussion

5.3.1 Modeling the responses

The experimental data obtained from MAE given in Table 5.1 were analyzed by using the RSREG procedure in SAS (6.12).

The effects of independent variables on the relative percentage extraction yields (Y_1, Y_2 and Y_3, respectively) were tested for adequacy and fitness by the analysis of variance (ANOVA). As shown in Table 5.2, the R^2 of each second-order polynomial regression was 0.9178, 0.9016 and 0.9374 for Y_1, Y_2 and Y_3 respectively, with no significant lack of fit at $p>0.05$. The results indicated that the models used to fit the response variables were significant ($p<0.01$ for all) and adequate to represent the relationship between the response and the independent variables.

Table 5.1 Experimental conditions from the central composite rotatable design and experimental measurements of response variables

run	power (W) X_1	time (min) X_2	temp (^oC) X_3	ethanol (%) X_4	relative extraction yield (%)[a] saikosaponin a Y_1	saikosaponin c Y_2	saikosaponin d Y_3
1	-1 (200)	-1 (3)	-1 (65)	-1 (35)	87.43 ± 1.68	81.79 ± 1.56	84.97 ± 0.46
2	-1 (200)	-1 (3)	-1 (65)	1 (65)	85.74 ± 1.51	81.26 ± 0.40	83.27 ± 1.04
3	-1 (200)	-1 (3)	1 (75)	-1 (35)	87.49 ± 2.21	84.41 ± 4.87	90.09 ± 2.31
4	-1 (200)	-1 (3)	1 (75)	1 (65)	84.91 ± 2.05	84.10 ± 3.08	85.70 ± 1.54
5	-1 (200)	1 (5)	-1 (65)	-1 (35)	91.16 ± 2.60	89.40 ± 4.56	92.82 ± 0.65
6	-1 (200)	1 (5)	-1 (65)	1 (65)	88.36 ± 1.18	90.94 ± 2.60	92.25 ± 0.93
7	-1 (200)	1 (5)	1 (75)	-1 (35)	92.58 ± 0.06	90.20 ± 0.76	93.39 ± 2.45
8	-1 (200)	1 (5)	1 (75)	1 (65)	88.08 ± 1.77	88.43 ± 4.49	91.25 ± 1.52
9	1 (400)	-1 (3)	-1 (65)	-1 (35)	87.30 ± 1.72	88.15 ± 2.97	86.21 ± 2.00
10	1 (400)	-1 (3)	-1 (65)	1 (65)	84.17 ± 0.74	86.61 ± 0.59	85.58 ± 0.11
11	1 (400)	-1 (3)	1 (75)	-1 (35)	90.49 ± 5.13	91.71 ± 1.69	91.08 ± 2.94
12	1 (400)	-1 (3)	1 (75)	1 (65)	87.35 ± 4.99	89.46 ± 0.71	89.31 ± 4.76
13	1 (400)	1 (5)	-1 (65)	-1 (35)	93.94 ± 0.79	90.83 ± 1.70	93.54 ± 1.38
14	1 (400)	1 (5)	-1 (65)	1 (65)	87.34 ± 2.16	90.30 ± 1.10	92.28 ± 1.49
15	1 (400)	1 (5)	1 (75)	-1 (35)	94.29 ± 4.09	92.33 ± 2.50	94.70 ± 2.25
16	1 (400)	1 (5)	1 (75)	1 (65)	93.25 ± 3.27	93.35 ± 1.71	92.82 ± 0.65
17	-2 (100)	0 (4)	0 (70)	0 (50)	90.20 ± 1.02	86.93 ± 2.59	90.32 ± 3.26
18	2 (500)	0 (4)	0 (70)	0 (50)	92.26 ± 1.77	92.48 ± 1.55	92.91 ± 2.39
19	0 (300)	-2 (2)	0 (70)	0 (50)	88.64 ± 1.35	83.49 ± 1.58	89.68 ± 0.22
20	0 (300)	2 (6)	0 (70)	0 (50)	94.23 ± 1.97	94.37 ± 1.23	95.43 ± 0.86
21	0 (300)	0 (4)	-2 (60)	0 (50)	88.95 ± 1.31	82.68 ± 0.37	85.65 ± 0.32
22	0 (300)	0 (4)	2 (80)	0 (50)	93.53 ± 1.65	93.33 ± 1.70	93.81 ± 0.57
23	0 (300)	0 (4)	0 (70)	-2 (20)	86.07 ± 4.05	74.08 ± 4.46	81.25 ± 0.95
24	0 (300)	0 (4)	0 (70)	2 (80)	84.72 ± 2.74	74.35 ± 0.28	80.52 ± 0.79
25	0 (300)	0 (4)	0 (70)	0 (50)	92.25 ± 1.09	91.34 ± 1.59	92.69 ± 1.56
26	0 (300)	0 (4)	0 (70)	0 (50)	94.02 ± 2.11	92.18 ± 2.98	93.29 ± 0.45
27	0 (300)	0 (4)	0 (70)	0 (50)	93.21 ± 2.00	92.24 ± 1.76	93.72 ± 0.82
28	0 (300)	0 (4)	0 (70)	0 (50)	93.78 ± 1.15	92.57 ± 0.83	94.40 ± 0.88
29	0 (300)	0 (4)	0 (70)	0 (50)	93.87 ± 1.98	94.91 ± 0.76	94.98 ± 0.87
30	0 (300)	0 (4)	0 (70)	0 (50)	94.39 ± 2.95	94.02 ± 0.96	93.95 ± 0.47

[a] Mean ± standard deviation (n=3), relative yield of saikosaponin extracted by MAE to that by control extraction (conventional extraction for 8h)

Table 5.2 Analysis of variance for the second-order polynomial models fitted to the responses Y_k

response	source	degrees of freedom	sum of square	F-value	p-value
Y_1 (saikosaponin a)	model	14	304.801	11.968***	<0.0001
	residual	15	27.288		
	lack of fit	10	24.453	4.313	0.0602
	pure error	5	2.835		
	R^2	0.9178			
Y_2 (saikosaponin c)	model	14	785.851	9.813***	<0.0001
	residual	15	85.806		
	lack of fit	10	77.0181	4.382	0.0583
	pure error	5	8.788		
	R^2	0.9016			
Y_3 (saikosaponin d)	model	14	490.762	16.045***	<0.0001
	residual	15	32.772		
	lack of fit	10	29.507	4.519	0.0549
	pure error	5	3.265		
	R^2	0.9374			

* p<0.05, **p<0.01, ***p<0.001

The regression coefficients of the models for Y_1, Y_2 and Y_3 are given in Table 5.3. The significance of each coefficient was determined by the p-value. The smaller the p-value is, the more significant is the corresponding coefficient. The results indicated that the linear terms were all significant for all Y_k (p<0.05), except X_4 for Y_2 (p>0.05). The quadratic terms were all significant for Y_1 (p<0.05), especially X_4^2 for all Y_k (p<0.0001) and X_3^2 for Y_3 (p<0.05). The cross terms were not significant for all Y_k (p>0.05), except of X_1X_3 for Y_1 (p<0.05) and X_2X_3 for Y_3 (p<0.05).

Table 5.3 Estimated coefficients from the fitted models for the responses Y_k

source	Y_1(saikosaponin a)		Y_2(saikosaponin c)		Y_3(saikosaponin d)	
	coefficient	*p*-value	coefficient	*p*-value	coefficient	*p*-value
b_0	93.5717		92.8767		93.8383	
b_1(X_1: power)	0.6875*	0.0246	1.8046**	0.0022	0.7100*	0.0327
b_2(X_2: time)	1.8875***	<0.0001	2.5021***	0.0001	2.4342***	<0.0001
b_3(X_3: temp.)	0.9233**	0.0044	1.5004**	0.0077	1.4025***	0.0003
b_4(X_4: ethanol)	-1.1742***	0.0007	-0.1596	0.7483	-0.6550*	0.0464
b_{11}	-0.7242*	0.0131	-0.2736	0.5580	-0.3471	0.2377
b_{22}	-0.6729*	0.0196	-0.4674	0.3223	-0.1121	0.6969
b_{33}	-0.7217*	0.0134	-0.6985	0.1469	-0.8183*	0.0110
b_{44}	-2.1829***	<0.0001	-4.1461***	<0.0001	-3.0296***	<0.0001
b_{12}	0.3062	0.3781	-1.0331	0.1045	-0.2875	0.4487
b_{13}	0.7662*	0.0382	0.4506	0.4627	0.2037	0.5895
b_{14}	-0.1462	0.6707	-0.1394	0.8186	0.1987	0.5986
b_{23}	0.1125	0.7433	-0.5644	0.3602	-0.9250*	0.0244
b_{24}	-0.2750	0.4275	0.3056	0.6167	0.1600	0.6712
b_{34}	0.1850	0.5913	-0.1406	0.8172	-0.3712	0.3310

* p<0.05, **p<0.01, ***p<0.001

5.3.2 Effect and mutual relationship of variables

The overall effects of each independent variable within the experimental range are given in Table 5.4. The results showed that Y_1 was affected most significantly by ethanol concentration (X_4) (p=0.0001), followed by time (X_2) (p=0.0001), temperature (X_3) (p=0.01) and power (X_1) (p=0.05). Y_2 was affected most significantly by ethanol concentration (X_4) (p=0.0001), followed by time (X_2) (p=0.01), power (X_1) (p=0.05), and temperature (X_3) (p=0.1). Y_3 was affected most significantly by both ethanol concentration (X_4) (p=0.0001) and time (X_2) (p=0.0001), followed by temperature (X_3) (p=0.001).

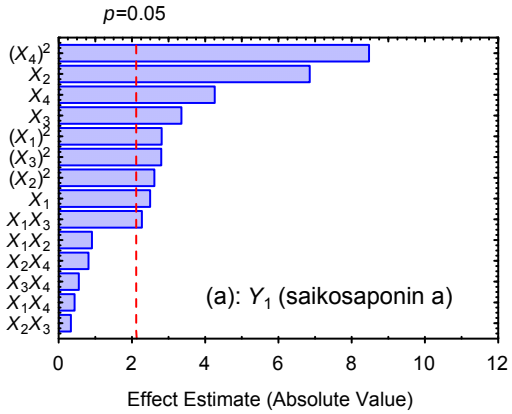
Table 5.4 Analysis of variance for the overall effect of the independent variables on the response variables

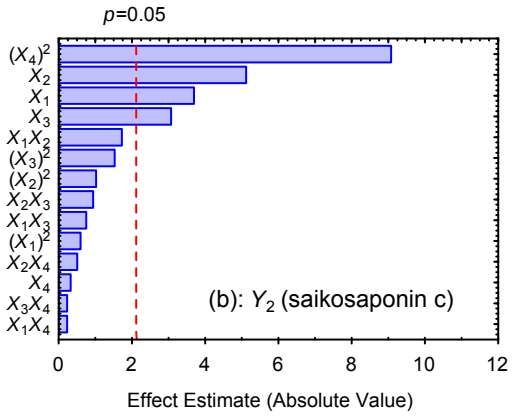
response	independent variable	degrees of freedom	sum of square	F-value	*p*-value
Y_1 (saikosaponin a)	X_1: power (W)	5	36.965	4.064*	0.0156
	X_2 time (min)	5	100.837	11.086***	0.0001
	X_3: temperature (^oC)	5	44.890	4.935**	0.0072
	X_4: ethanol (%)	5	165.888	18.237***	<0.0001
Y_2 (saikosaponin c)	X_1: power (W)	5	100.848	3.526*	0.0263
	X_2 time (min)	5	179.910	6.290**	0.0024
	X_3: temperature (^oC)	5	76.080	2.660	0.0649
	X_4: ethanol (%)	5	474.244	16.581***	<0.0001
Y_3 (saikosaponin d)	X_1: power (W)	5	18.021	1.650	0.2074
	X_2 time (min)	5	157.971	14.461***	<0.0001
	X_3: temperature (^oC)	5	82.136	7.519***	0.0010
	X_4: ethanol (%)	5	265.293	24.285***	<0.0001

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

The linear, quadratic and cross effects of each independent variable plotted in the form of Pareto chart are illustrated in Figure 5.2, in which the bar lengths are proportional to the absolute values of the estimated effects and are used for comparing their relative importance. The effect is significant if its corresponding bar crosses the vertical line at the p=0.05 level.

It can be seen in Figure 5.2 that the quadratic term of ethanol concentration (X_4^2) gives the most important effect on all responses of Y_1, Y_2 and Y_3, followed by the linear term of time (X_2). The third most important effect on Y_k is as X_4 for Y_1, X_1 for Y_2, and X_3 for Y_3.





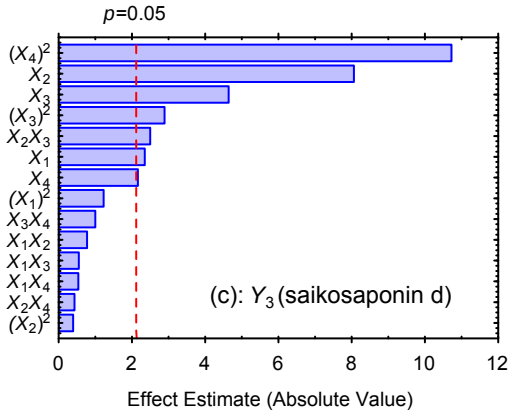


Figure 5.2 Pareto charts of standardized effects for the relative extraction yield of saikosaponins. (a): Y_1, saikosaponin a; (b): Y_2, saikosaponin c; and (c): Y_3, saikosaponin d

Figure 5.2 also indicates other significant effects on Y_k. Figure 5.2(a) shows the effects of the linear term of X_3 and X_1, the quadratic term of X_1^2, X_2^2, X_3^2, and the cross term of X_1X_3 on Y_1. Figure 5.2 (b) shows the effects of the linear term of X_3 on Y_2, and Figure 5.2(c) shows the effects of the quadratic term of X_3^2, the cross term of X_2X_3 and both the linear terms of X_1 and X_4 on Y_3.

The effects of the independent variables and their mutual interaction on the extraction yields of Y_1, Y_2 and Y_3 can also be visualized on the response surface and contour plots shown in Figures 5.3-5.5.

As can be seen in Figure 5.3, Y_1, Y_2 and Y_3 increased significantly with increasing ethanol concentration from 20 % to around 50 %, then decreased significantly with increasing ethanol concentration from around 50 % to 80 % at any time range from 2.0 to 6.0 min (Figure 5.3(a)), temperature from 60 to 80 ^oC (Figure 5.3(b)), and power from 100 to 500 W (Figure 5.3(c)). This indicates that ethanol concentration is one of the critical factors taken into consideration in MAE for saikosaponins, owing to its important implications on the solubility to the target compound, the interaction between the solvent and matrix, and the microwave absorbing properties.

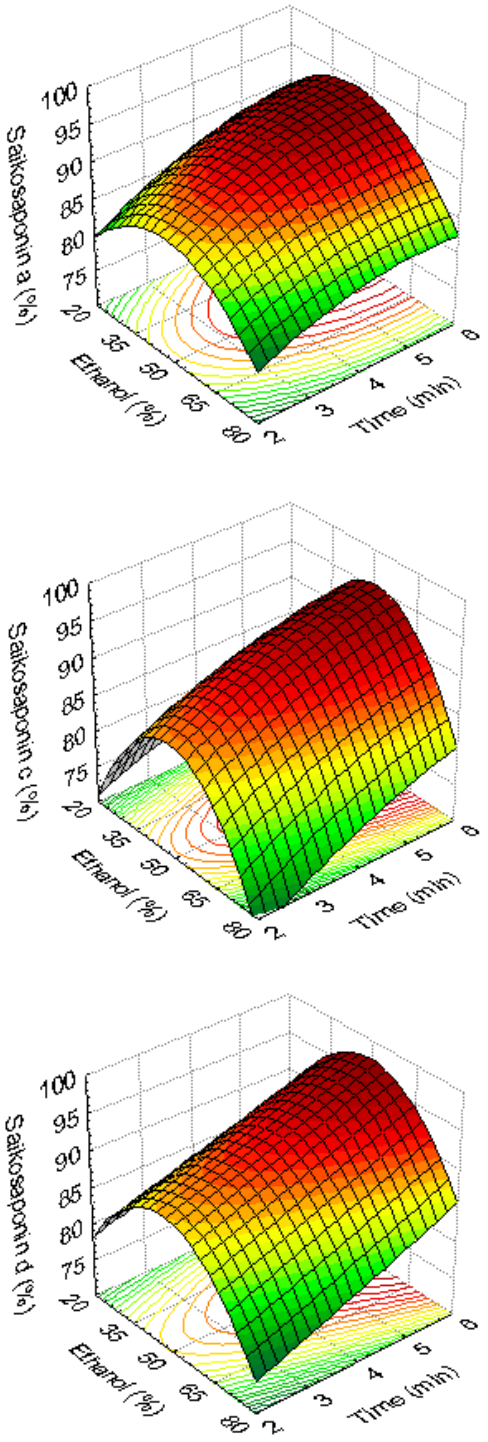
The effect of time on Y_k can be seen in Figures 5.3(a), 5.4(a) and 5.4(b), in which Y_1, Y_2 and Y_3, increased correspondingly with increasing time from 2.0min to 6.0min for all levels of other variables.

The effect of temperature on Y_k can be seen in Figure 5.3(b), in which Y_1, Y_2 and Y_3 increased with increasing temperature from 60 to 70 ^oC for all ethanol concentration levels. No improvement in yields with a high level of temperature (>75 ^oC) was observed.

Figure 5.4(a) and Figure 5.5 show that Y_1, Y_2 and Y_3 increased with increasing

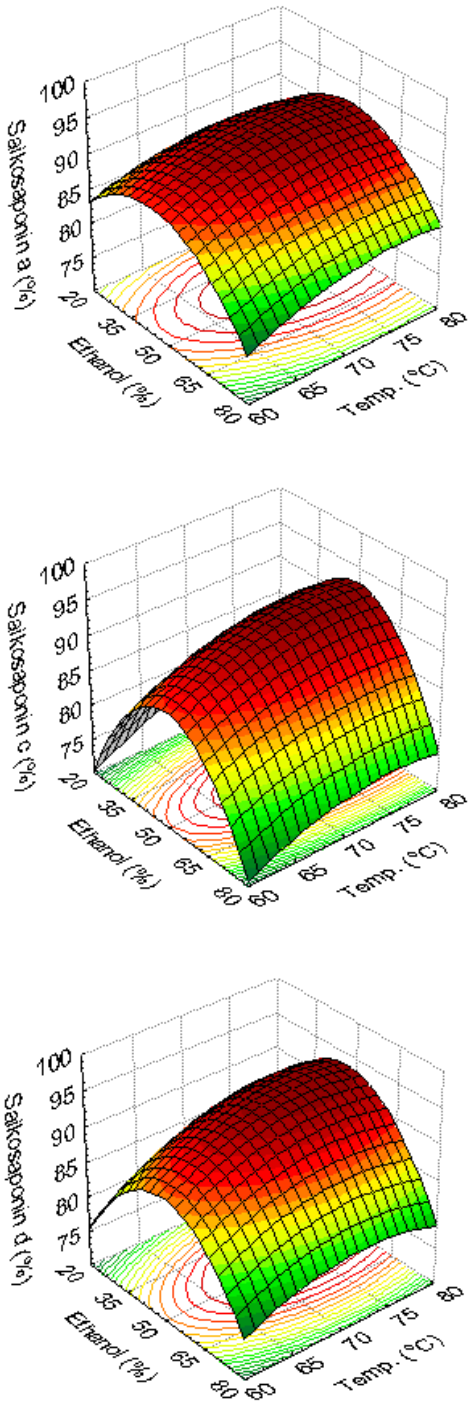
temperature from 60 to 80 ^{o}C for levels of time from 2.0 to 6.0 min and power from 100 to 500 W, except that at time longer than 5.0 min and temperature higher than 75 ^{o}C, no increase in yield for saikosaponin d was observed. This might be explained by that the saikosaponin d was easily subjected to thermal degradation at a high temperature for a long time [25].

The effect of power is shown in Figure 5.3(c). Increase in power from 100 to 500 W at any level of ethanol concentration led to no significant increases in Y_1, Y_2 and Y_3. Similar results were observed in Figure 5.4(b) at a high level of time from 4.0 to 6.0 min. Figure 5.5 shows that high levels of power and temperature led to higher Y_1, Y_2 and Y_3.



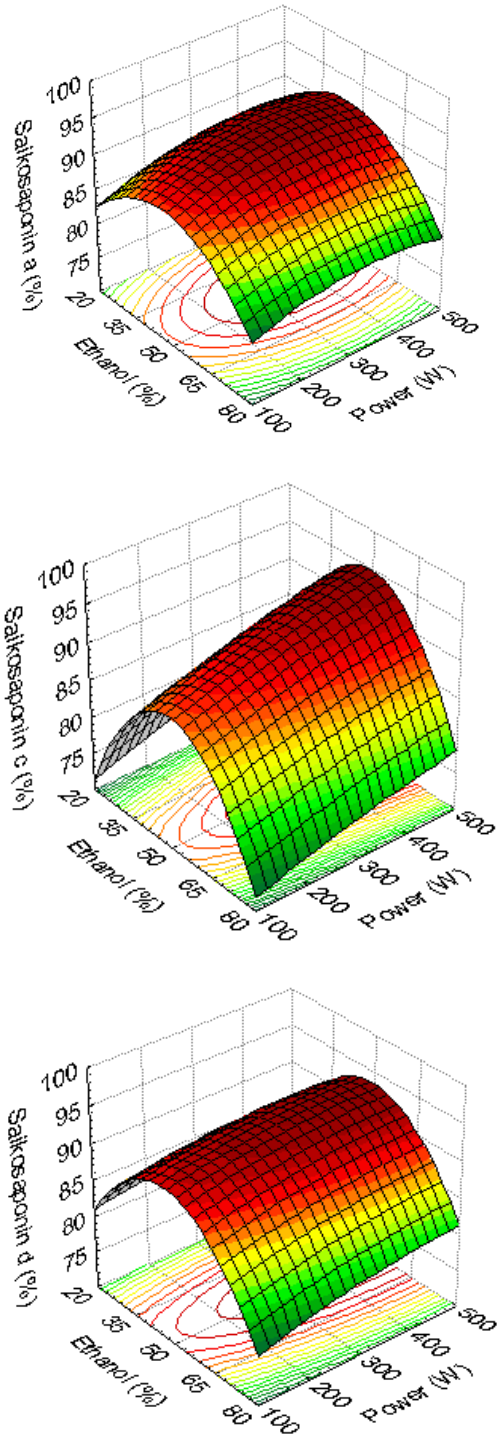
(a)

Figure 5.3a Response surface and contour plots showing the effect of ethanol concentration and time on yields of saikosaponin a, c and d. Other variables are constant at zero levels.



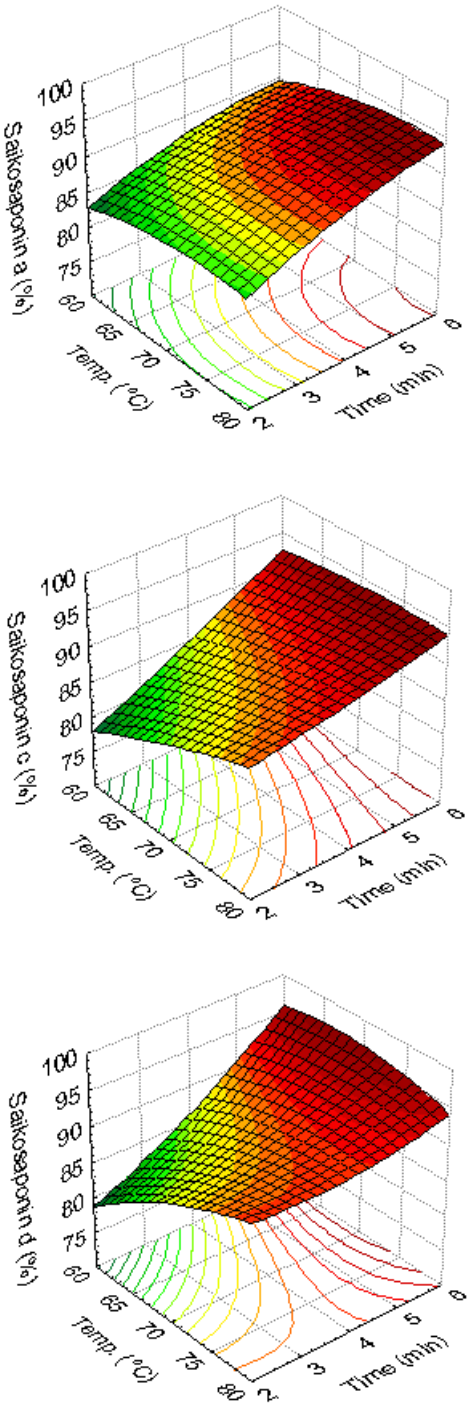
(b)

Figure 5.3b Response surface and contour plots showing the effect of ethanol concentration and temperature on yields of saikosaponin a, c and d. Other variables are constant at zero levels.



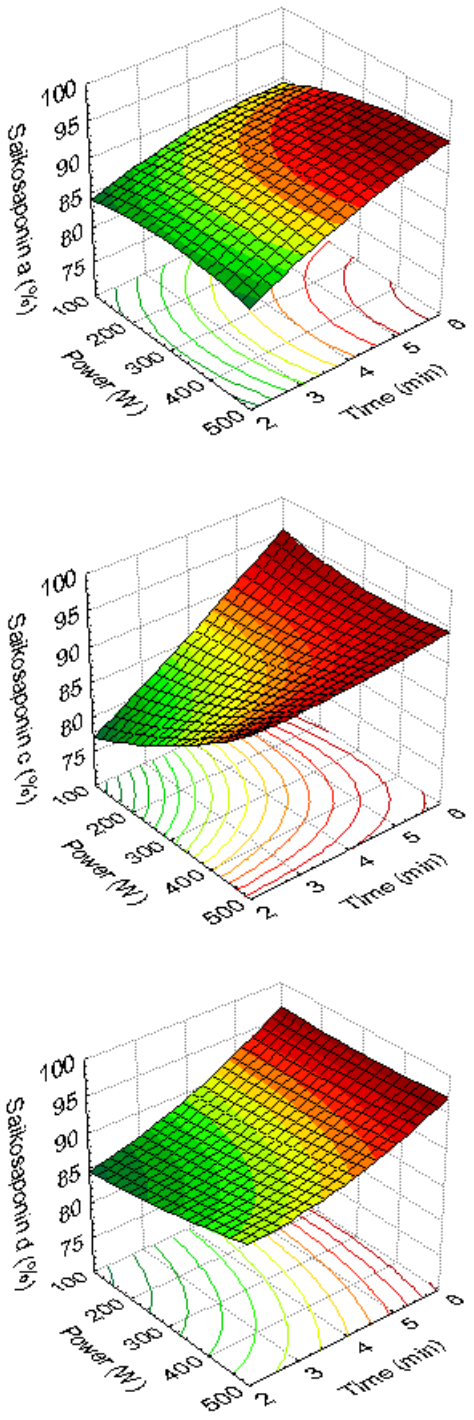
(c)

Figure 5.3c Response surface and contour plots showing the effect of ethanol concentration and power on yields of saikosaponin a, c and d. Other variables are constant at zero levels.



(a)

Figure 5.4a Response surface and contour plots showing the effect of time and temperature on yields of saikosaponin a, c and d. Other variables are constant at zero levels



(b)

Figure 5.4b Response surface and contour plots showing the effect of time and power on yields of saikosaponin a, c and d. Other variables are constant at zero levels

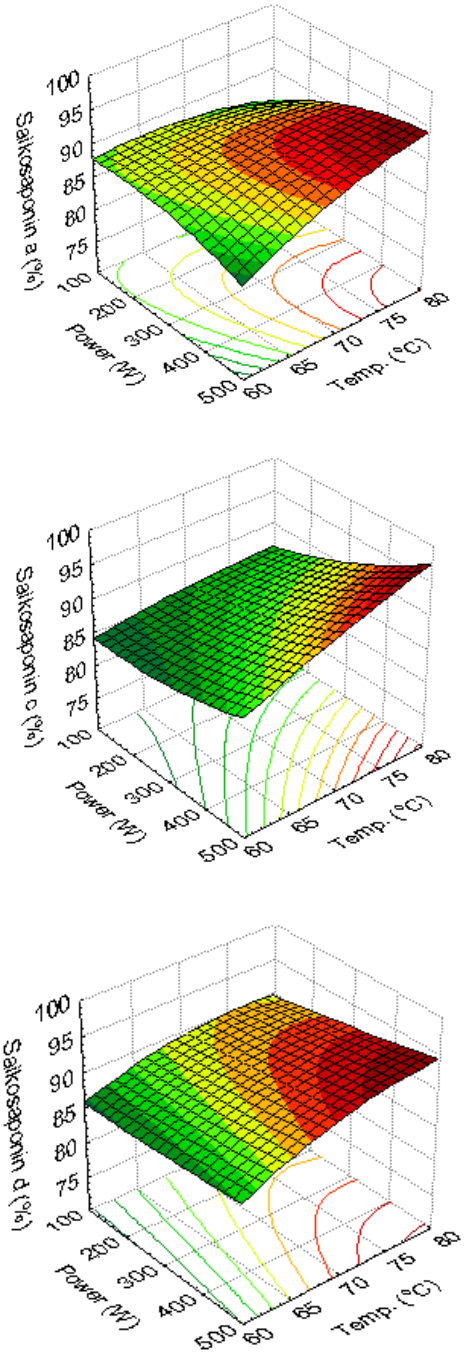


Figure 5.5 Response surface and contour plots showing the effect of temperature and power on yields of saikosaponin a, c and d. Other variables are constant at zero levels.

5.3.3 Optimization

The objective of optimization was to look for the MAE conditions which give the maximum extraction yields of each saikosaponin simultaneously. The desirability function approach was employed in the optimization procedure. For all responses, $Y_k^{(\min)}$ =93 % and $Y_k^{(\max)}$ =100 %, which were considered as the minimum and maximum values of the responses, were chosen for the optimization. The positive constant r=0.3 was chosen. For each independent variable, the coded level was taken from -2.0 to 2.0 with a 0.2 interval. Using these values as constrains, a computer program was written first to calculate the individual desirability for each response by using Eq (2), then to combine the individual desirability to obtain the overall desirability D by using Eq (3), and finally to sort the maximum values of D and produce the optimum solutions for all responses. The nine results were obtained by running this program at the overall desirability D>0.805, as shown in Table 5.5.

The results indicated that the coded levels of the optimized conditions were 0.6-1.0 for power (360-400 W), 1.8-2.0 for time (5.8-6.0 min), 0.6-0.8 for temperature (73-74 ^{o}C), and -0.2-0.0 for ethanol concentration (47-50 %), with the predicted yield of 95.87-96.43 % for Y_1, 95.60-95.91 % for Y_2 and 97.13-97.69 % for Y_3.

5.3.4 Verification

In accordance with the optimization results obtained from RSM with the desirability function, verification experiments were carried out at the selected cases described in Table 5.6. The high, low and middle levels of each variable covered in the selected cases were taken into consideration.

Table 5.5 The predicted extraction yield of saikosaponins from optimization process

case	*D* values	optimized condition (coded level)				predicted yield (%)		
		power	time	temp.	ethanol	saikosaponin		
						a	c	d
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	0.805	0.6	2.0	0.6	-0.2	96.13	95.67	97.69
2	0.805	0.6	2.0	0.6	0.0	95.87	95.89	97.73
3	0.805	1.0	1.8	0.8	0.0	96.35	95.84	97.13
4	0.805	1.0	1.8	0.6	-0.2	96.43	95.65	97.33
5	0.806	1.0	1.8	0.6	0.0	96.17	95.85	97.37
6	0.806	0.8	2.0	0.6	-0.2	96.28	95.60	97.64
7	0.806	0.8	1.8	0.6	-0.2	96.34	95.70	97.40
8	0.806	0.8	2.0	0.6	0.0	96.02	95.82	97.68
9	0.806	0.8	1.8	0.6	0.0	96.10	95.91	97.44

The results indicated that the experimental values were 96.18-96.91 % for Y_1, 95.05-95.71 % for Y_2 and 97.05-97.25 % for Y_3 at the selected optimum conditions of 360-400 W for power, 5.8-6.0 min for time, 73-74 ^{o}C for temperature and 47-50 % for ethanol concentration. These experimental yields were in good agreement with the predicted values. Thus, it can be seen that the second-order models were adequate to describe the influence of the selected MAE operating variables on the relative extraction yields of saikosaponins.

Table 5.6 The experimental extraction yield of saikosaponins at the selected optimized conditions

case	description of case		optimized condition (true value)				experimental yield (%)[a]		
	level	variable	power (W)	time (min)	temp. (^{o}C)	ethanol (%)	saikosaponin		
							a	c	d
			X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	low mid. high	X_4, X_3, X_1 - X_2	360	6.0	73	47	96.52 (±1.72)	95.71 (±1.12)	97.05 (±0.48)
3	low mid. high	X_2 - X_4, X_3, X_1	400	5.8	74	50	96.78 (±1.06)	95.05 (±0.97)	97.22 (±1.50)
5	low mid. high	X_2, X_3 - X_1, X_4	400	5.8	73	50	96.18 (±1.27)	95.29 (±0.81)	97.22 (±1.13)
6	low mid. high	X_3, X_4 X_1 X_2	380	6.0	73	47	96.91 (±0.88)	95.57 (±0.37)	97.25 (±1.48)
9	low mid. high	X_2, X_3 X_1 X_4	380	5.8	73	50	96.61 (±1.01)	95.09 (±1.40)	97.16 (±1.08)

[a] Means from triplicate experiments (±SD)

5.4 Conclusions

The saikosaponin a, c and d can be efficiently extracted using MAE. The variables of ethanol concentration and time showed a significant effect on the extraction yield for all three saikosaponins. The overall effect of temperature was more significant for saikosaponin d than for saikosaponin a, and was the least

significant for saikosaponin c. The optimum conditions from the RSM with the desirability function approach were found to be 360-400 W of microwave power, 47-50 % of ethanol concentration, 73-74 ^{o}C of temperature and 5.8-6.0 min of time, and were verified by experiments. At these conditions, the relative extraction yields of 96.18-96.91 % for saikosaponin a, 95.05-95.71 % for saikosaponin c and 97.05-97.25 % for saikosaponin d were obtained simultaneously from the verification experiments. The experimental results were in good agreement with the predicted values from the fitted models.

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Chapter 6. Mechanisms Studies: Effect of Microwave Irradiation on Diffusion Coefficient and Microstructure of Plant Tissues

Abstract: The diffusion coefficient of saikosaponins through the solid matrix in microwave assisted extraction was determined with the use of a Fick's second law-based model. The effect of microwave irradiation on microstructure changes of plant tissues was observed by scanning electronic microscopy technique. Results indicated that the increase in the microwave power, led to a higher effective diffusion coefficient D_{eff} than that found in conventional extraction method. Microwave irradiation produced distinguishable microstructure changes in the tissues of *Radix Bupleuri* and pomelo peels, and caused oil glands of pomelo peels to explode and parenchymal cells of plant tissues to rupture, thus the target compounds within the cells were rapidly released into the surrounding extraction solvents. While the liquid phase absorbed the microwave energy, the kinetic energy of its molecules increased, and consequently, the diffusion rate increased. Better efficiency and significant reduction in extraction time were obtained with the use of MAE.

6.1 Introduction

Mass transfer can be defined as the migration of a substance through a mixture under the influence of a concentration gradient in order to reach chemical equilibrium. Extracting some interesting intracellular components from plant tissues was based on solid-liquid extraction [1-2]. In the case of the microwave-assisted extraction of saikosaponins from *Radix Bupleuri*, the solid-liquid extraction process may be considered as a diffusion process in the liquid state since the solute transfer, even inside of a solid, existed as a dilute solution [2]. The microwave assisted extraction can be considered as an unsteady mass transfer process, in which diffusion fluxes and concentrations were time-dependant [3].

The amount of the compounds that released into the external liquid was characterized by the degree of cell disintegration, which influenced the efficiency of the extraction process [4]. For a better understanding of the effect on microstructure, the application of scanning electron microscopy technique which microscopically explored the complex changes at the cell level in real biological systems would be helpful. In food and chemical processing, the majority of the researches that related to plant cell structure put the focus on understanding cell wall or cell membrane in relation to the texture and mass transfer in plant materials under different processing treatments such as conventional heating, freezing, high pressure, pulsed electric field (PEF), microwave irradiation, etc..

From an engineering point of view, understanding of mass transfer phenomenon at the solid-liquid interface was crucial for scaling-up processes from analytical to pilot scale, which in turn could enhance the development of industrial applications [5]. The objective of this chapter was to study the

extraction mechanism of MAE. In the first part, a Fick's second law-based model was used to fit with the experimental data and determine the diffusion coefficient of saikosaponins through the solid matrix under different microwave assisted extraction conditions. Then, in the second part, the samples were examined by scanning electron microscopy in order to reveal the structural changes of plant tissues caused by the different extraction methods.

6.2 Materials and methods

6.2.1 Extraction condition

The material and extraction methods were the same as described in sections 3.2, 4.2 and 5.2.

6.2.2 Scanning Electron Microscope

Microstructure analyses were carried out with the use of the scanning electron microscopy technique with a Scanning Electron Microscope (Philips FEI XL-30, The Netherlands) at an accelerating voltage of 20 kV. Samples fixation were done by immersion in glutaraldehyde (3 %) for 12 h in phosphate buffer (pH 7.2) at 4 °C. A second fixation was made with 1 % osmium tetraoxide solution for 3 h in phosphate buffer at 4 °C. Samples were dehydrated by immersion in ethanol solutions (30 %, 50 %, 70 % and 100 %) for 15 min each. The dehydrated samples were observed by SEM after coated with a thin layer of gold.

6.2.3 Mathematical modeling

A series of phenomenological steps would occur during the period of interaction between the solute-containing particles and the solvent affecting the separation, as the followings are described by Aguilera (2003) [2]:

1) Entrance of the solvent into the solid matrix;
2) Solubilization and/or breakdown of components;
3) Transport of the solute to the exterior of the solid matrix;
4) Migration of the extracted solute from the external surface of the solid into the bulk solution;
5) Movement of the extract with respect to the solid (i.e., extract displacement), and
6) Separation and discharge of the extract and solid.

The rate limiting step is the diffusion of the dissolved solute within the solid into the solvent [6-7].

According to the Fick's second law, the general diffusion model of solid-liquid extraction has been applied for understanding of mass transfer in natural products extraction by Wongkittipong (2004) [8] and Spigno (2009) [9]:

$$\frac{\partial C(t,x)}{\partial t} = D\frac{1}{x^{\varphi-1}}\frac{\partial}{\partial x}(x^{\varphi-1}\frac{\partial C(t,x)}{\partial x}) \qquad (1)$$

where C is the concentration of solute, kg / L;

x is the position, while in this case the distance for diffusion, m;

t is time, s;

φ is geometric shape factor values;

and D is the constant diffusion coefficient D, m^2 / s.

For spherical condition as in extraction of saikosaponin, x is considered as the radius of the particle r, therefore, the above equation can be expressed as:

$$\frac{\partial C_{saikosaponin}}{\partial t} = D\frac{1}{r^2}\frac{\partial}{\partial r}(r^2\frac{\partial C_{saikosaponin}}{\partial r}) \qquad (2)$$

which says that the flow of solute is directly proportional to the change of the concentration gradient with position [10]. The estimation of constant diffusion coefficient D in liquid phase is based on the Stokes-Einstein equation [2]:

$$D = \frac{k_B T}{f} = \frac{k_B T}{6\pi\mu R_0} \qquad (3)$$

where k_B is the Boltzmann's constant, T is the absolute temperature, f is the friction factor, R_0 is the solute radius, and μ is the viscosity of the solvent. In the case of microwave assisted extraction of saikosaponin, matrices in extraction solvent can be considered as a porous diffusion media, due to the presence of porosity at the microstructure level, the diffusion coefficient should be corrected by accommodating structural effects by introducing tortuosity and porosity so as to form an effective diffusion coefficient D_{eff} [11]:

$$D_{eff} = \frac{\varepsilon}{\tau}\frac{k_B T}{6\pi\mu R_0} \qquad (4)$$

where ε is a factor which compensates for the path through longer pores, ranging between 1.5 and 5; τ is porosity, which is the ratio of volume of pores to the total volume.

$$\frac{\partial C_{saikosaponin}}{\partial t} = D_{eff}\frac{1}{r^2}\frac{\partial}{\partial r}(r^2\frac{\partial C_{saikosaponin}}{\partial r}) \qquad (5)$$

The model is with the following initial and boundary conditions for well-agitated unlimited volume of the bulk solution :

initial conditions: $C_{saikosaponin} = C_0$ at $t = 0,\ 0 \leqslant r \leqslant R$

boundary conditions: $C_{saikosaponin} = 0$ at $t > 0\ \ r = R$

$$\partial C_{saikosaponin} / \partial r = 0 \text{ at } t \geq 0 \quad r = 0$$

Solving the differential equation and defining C as a dimensionless variable, a series of equations is obtained [12]. The mass transferred from the sphere at time t, relative to the total amount transferred after infinite time (M_∞) is expressed as [5, 10]:

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\{-\frac{D_{eff}n^2\pi^2 t}{r^2}\} \qquad (6)$$

where M_t is the total amount of saikosaponins (mg/g) removed from *Radix Bupleuri* at time t; M_∞ is maximum amount (mg/g) of saikosaponin extracted after infinite time.

When time becomes larger, only one term in the series is significant, and Eq.(6) becomes [13]:

$$1 - \frac{M_t}{M_\infty} = \frac{6}{\pi^2}\exp\left(-\frac{D_{eff}\pi^2 t}{r^2}\right) \qquad (7)$$

When the logarithm of $(1-(M_t/M_\infty))$ is plotted against time, a straight line should be obtained and the diffusity can be assessed from its slope.

$$\ln(1 - \frac{M_t}{M_\infty}) = \ln(\frac{6}{\pi^2}) - \frac{D_{eff}\pi^2}{r^2}t \qquad (8)$$

D_{eff} can be calculated from Eq. (8):

$$D_{eff} = \frac{[\ln(\frac{6}{\pi^2}) - \ln(1 - \frac{M_t}{M_\infty})]\times r^2}{\pi^2 t} \qquad (9)$$

The temperature dependence of D_{eff} was fitted to the Arrhenius type equation:

$$D_{eff} = D_0 \exp\left(-\frac{E_a}{RT}\right) \qquad (10)$$

Where D_0 is the initial diffusion coefficient, E_a is the energy of activation for diffusion (kJ/mol), R is the universal gas constant (kJ / mol K), and T is the absolute temperature (K).

6.3 Results and discussion

6.3.1 Effects of microwave irradiation on the diffusion coefficient

The solution of Fick's second law was used to find the concentration of a solute as a function of time and position, and was mainly applicable to diffusion in solids [3]. The values of effective diffusion coefficient D_{eff} under different extraction methods were calculated from the experimental data by Eq. (9) and displayed in Table 6.1.

The results indicated that the values of effective diffusion coefficient D_{eff} under different microwave heating condition were increased as the addtion of microwave power. At the same extraction time of 1min, 3 min and 5 min, when using MAE with 100 W of power level, the values of D_{eff} ranged from 12.21×10^{-12} m^2/s to 62.64×10^{-12} m^2/s; under 200 W of power level condition, the values of D_{eff} ranged from 18.25×10^{-12} m^2/s to 65.08×10^{-12} m^2/s; under 300 W of power level condition, the values of D_{eff} ranged from 19.91×10^{-12} m^2/s to 70.74×10^{-12} m^2/s; and under 500 W of power level condition, the values of D_{eff} ranged from 23.53×10^{-12} m^2/s to 83.42×10^{-12} m^2/s.

Table 6.1 Values of coefficients D_{eff} obtained under different extraction methods for saikosaponins

Extraction methods	T(°C)	Time(s)	$D_{eff} \times 10^{-12}$ (m^2/s)
Microwave power (W)			
100	75	60	62.64
	75	180	21.69
	75	300	12.21
200	75	60	65.08
	75	180	25.83
	75	300	18.25
300	75	60	70.74
	75	180	30.21
	75	300	19.91
500	75	60	83.42
	75	180	42.10
	75	300	23.53
Hot solvent extraction			
	75	600	4.86
	75	1800	2.40
	75	3600	1.57
	75	7200	0.97

However, in the case of conventional extraction, the values of D_{eff} ranged from 0.97×10^{-12} m^2/s to 4.86×10^{-12} m^2/s for hot solvent extraction with 10, 30, 60

and 120 min, which were enormously lower than those with the use of microwave-assisted extraction.

The Arrhenius plot of ln D_{eff} vs. 1/T for saikosaponons extracted by MAE was showed in Figure 6.1.

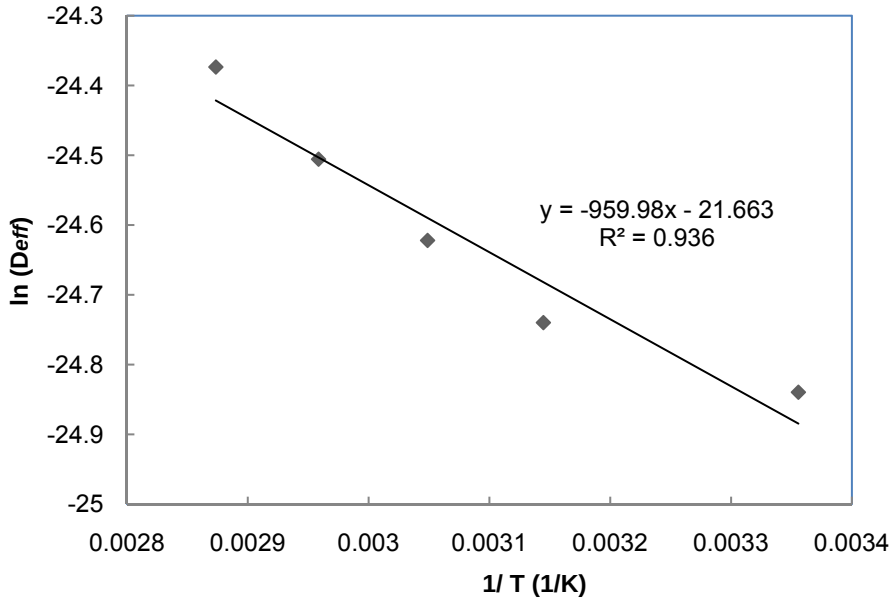


Figure 6.1 The Arrhenius plot of ln D_{eff} vs. 1/T for saikosaponons extracted by MAE

The results showed the regression coefficient R^2 of 0.936. Activation energy for microwave-assisted extraction of saikosaponins from *Radix Bupleuri* was calculated to be 7.98 kJ/mol. In the cases of conventional extraction, Cacae and Mazza (2003) reported the activation energy value for diffusion of phenolic from berries was 70-90 kJ/mol [6]; Ho et al. (2008) reported the activation energy value for pressurized low polarity water extraction of lignans from flaxseed meal at pH 9 and pH 4 was 51 and 56 kJ/mol respectively [13]. The data indicated that microwave energy considerably reduced the energy needed for the diffusion

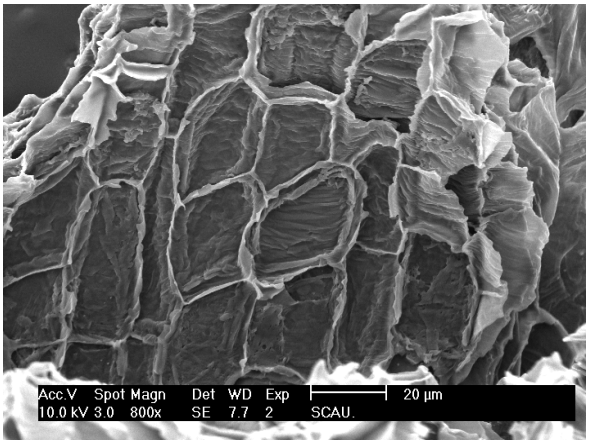
process, making it easier for the solute diffusion. This may be mainly related to the alteration of internal microstructure of the plant tissues by microwave irradiation.

6.3.2 Effects of microwave irradiation on microstructure changes of plant tissue

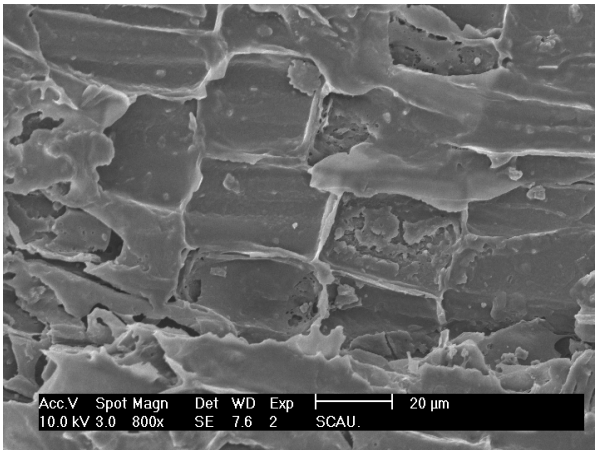
Figure 6.2 and Figure 6.3 showed the micrographs of tissues of raw *Radix Bupleuri* (RM), treated RM by hot solvent extraction (HSE), and by MAE, respectively. It is observed that different extraction treatments produced distinguishable changes on the microstructure of *Radix Bupleuri*

In MAE, the damage of parenchymal cells and sieve tubes on the surface of sample was obviously severer than that done by HSE method, as shown on Figure 6.2 and Figure 6.3. The structure of tissue or cell was affected by the sudden temperature rise and the increase of internal pressure increase caused by microwave irradiation. During MAE, the target compounds within the cell rapidly released into the surrounding extraction solvents.

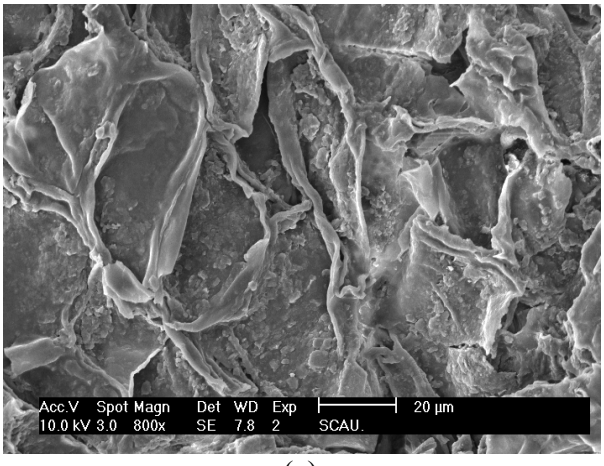
In HSE, little destruction of the microstructure of sample was observed and only a few of slight ruptures presented on the surface of the sample. In this process, the solvent transfers into the sample and the compounds were extracted by solubilization and permeation processed under higher temperature with longer extraction time.



(a)

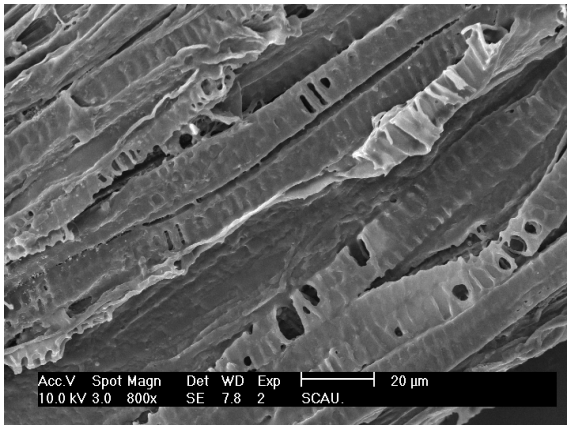


(b)

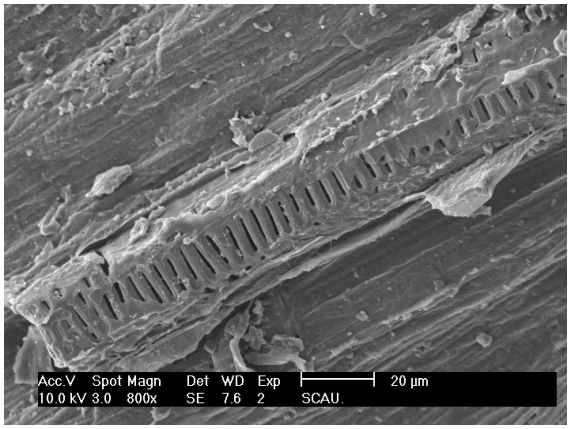


(c)

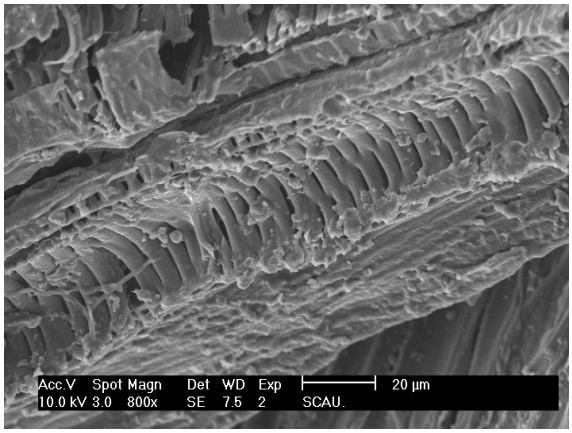
Figure 6.2 Scanning electron micrographs of praenchymal cells of *radix bupleuri*: (a) raw sample, (b) after hot solvent extraction (t =120min, T= 75°C, C = 50% ethanol to water, v/v), and (c) after MAE (t =10 min, P= 300W, T= 75°C, C = 50% ethanol to water, v/v)



(a)



(b)



(c)

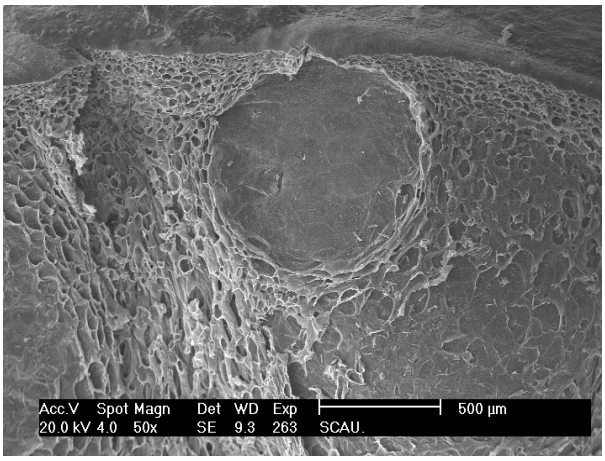
Figure 6.3 Scanning electron micrographs of sieve tubes of *radix bupleuri*: (a) raw sample, (b) after hot solvent extraction (t =120 min, T= 75 °C, C = 50 % ethanol to water, v/v), and (c) after MAE (t =10 min, P= 300 W, T= 75 °C, C = 50 % ethanol to water, v/v)

The micrographs of the surface of pomelo peels obtained by SEM before and after the extraction were shown on Figure 6.4 and Figure 6.5, respectively.

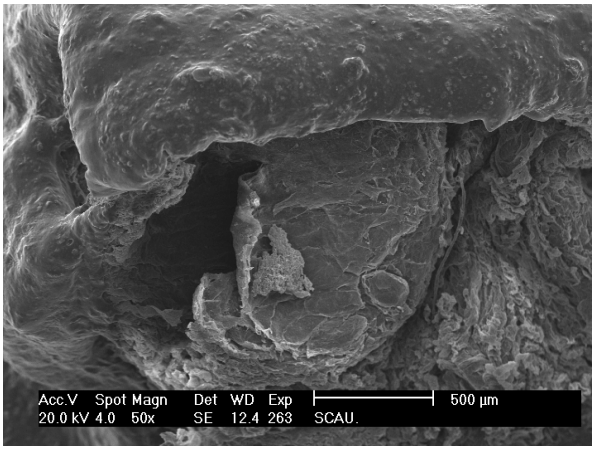
Figure 6.4a and Figure 6.5a were the micrographs of the untreated peels containing an oil gland and parenchymal cells. Images from the pomelo peels undergone a 90 min HD (Figure 6.4b and Figure 6.5b) and a 30 min SFME (Figure 6.4c and Figure 6.5c) were also shown for comparison.

Both extraction methods resulted in apparent structure changes in the pomelo peels tissues. The oil gland and parenchymal cells undergone SFME (Figure 6.4c and Figure 6.5c) were destroyed, while that undergone HD (Figure 6.4b and Figure 6.5b) exhibited shrinkage. This indicated that microwaves irradiation causes the glandular walls to rupture more rapidly and more efficiently. Such differences could be attributed to the difference in the way of heat transfer between the two extraction methods.

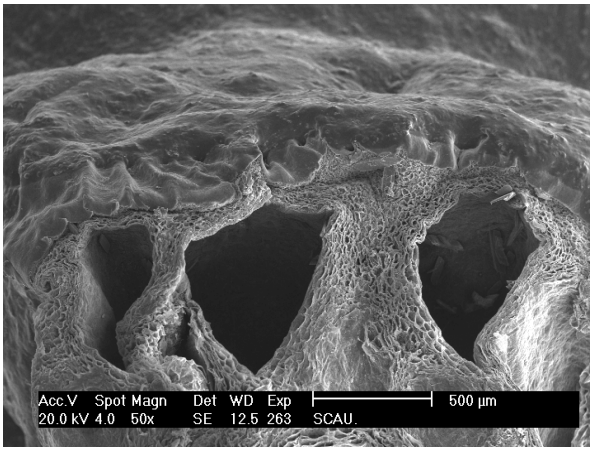
For extraction of essential oil from pomelo peels by SFME, when the glands were subjected to microwave irradiation, polar molecules within the glands/cells were initial targeted, heated up rapidly and began to evaporate, Thus, a severe thermal stress and a localized high pressure were built-up within the glands/cells and exceeded the capacity of the glands/cells for expansion. As a result, oil glands exploded and parenchymal cells ruptured, and the intracellular contents were rapidly spilled out. While the liquid phase absorbed the microwave energy, the kinetic energy of its molecules increased, and consequently, the diffusion rate accelerated.



(a)

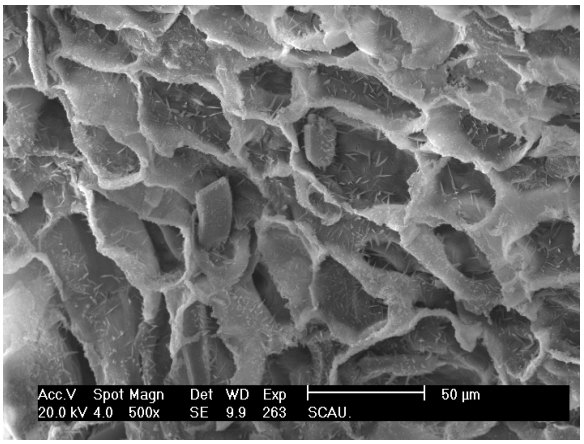


(b)

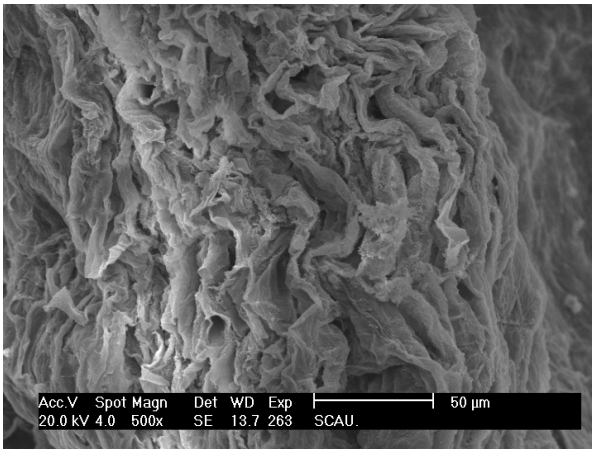


(c)

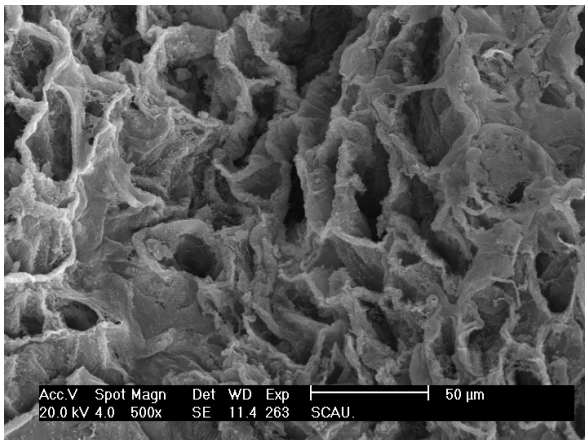
Figure 6.4 Scanning electron micrographs of oil glands of Pomelo peels: (a) raw material, (b) after hydrodistillation for 90 min and (c) after SFME-390 W for 30 min



(a)



(b)



(b)

Figure 6.5 Scanning electron micrographs of parenchymal cells of Pomelo peels: (a) raw material, (b) after hydrodistillation for 90 min and (c) after SFME-390 W for 30 min

In contrast, oil gland or parenchymal cells of pomelo peel were deeply shrunk (Figure 6.5b) after HD Therefore it was conceivable that, during HD, essential oils had to permeate through the shrunk tissues to be extracted. Thus the oils were easy to be trapped by the surrounding non-grandular tissues and difficult to transfer [14]. As a result, longer extraction time was required and better yields were rarely obtained.

Similar observation were reported in studies on the microwave extraction of essential oil from Lavandula flowers by Iriti et al. (2006) [14] and by Farhat et al. (2009) [15], these authors concluded that compared with the conventional extraction, the microwave extraction could result in better yields and dramatic reduction in extraction time.

6.4 Conclusions

A Fick's second law-based model which was used to fit with the experimental data and to determine the diffusion coefficient of saikosaponins through the solid matrix under microwave assisted extraction. The effective coefficients of diffusion D_{eff} under different microwave conditions were all increased by the increase of microwave power, and they were significantly higher than those extracted by the conventional extraction method.

SEM images indicated that microwave assisted extraction produced distinguishable changes on the microstructure of *Radix Bupleuri* and pomelo peels. Microwave irradiation caused oil glands of pomelo peels to explode and parenchymal cells of plant tissues to rupture. Thus the target compounds within the cell were rapidly released into the surrounding extraction solvents. While the liquid phase absorbed the microwave energy, the kinetic energy of its molecules

increased, and consequently, the diffusion rate increased. As a result, better efficiency and extremely reduced extraction time were obtained with MAE.

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Chapter 7. Conclusions and Recommendations

The objective of this study was to investigate the application of solvent free microwave extraction (SFME) and microwave assisted extraction (MAE) for extraction of the effective compounds from plant matrices. This investigation included (1) to study the effects of various factors for solvent free microwave extraction of the essential oil from pomelo peels and sequential MAE of pectin from oil extracted pomelo peels, (2) to find out the effects of MAE parameters on extraction of saikosaponins from *Radix Bupleuri* and optimization by using response surface methodology (RSM) with desirability function approach, (3) to explore the mechanisms of MAE, and determine the diffusion coefficient of saikosaponins through the solid matrix under MAE with the use of a Fick's second law-based model. The effect of microwave irradiation on microstructure changes of plant tissues was observed by the scanning electronic microscopy technique. The detailed conclusions for each part of the study were as follows.

Applicability of SFME for extraction of essential oil from pomelo peels was investigated, and conventional hydrodistillation (HD) method and acidic solution pectin extraction were also performed. The results indicated that SFME under microwave power level of 130 W, 260 W, and 390 W were all superior to the HD in terms of extraction efficiency and the essential oil yield. The chemical composition analysis by GC-MS showed that SFME did not affect the quality of essential oil compared with HD. In extracting pectin from oil extracted pomelo peels, the extraction time of MAE was significantly shorter than that of the conventional method. RSM was employed to optimize the MAE extraction condition of pectin. The sequential extraction of essential oils and pectin from pomelo fruits by SFME and MAE was a feasible process.

In the study of MAE for extraction of saikosaponins from *Radix Bupleuri,* the individual effects of microwave power, irradiation time, temperature, ethanol concentration, solvent-to-sample ratio, and sample particle size were extensively evaluated. Results indicated that high extraction yields of saikosaponins a, c, and d with only trace amounts of saikosaponin b_2 were obtained by MAE with a 300 to 500 W power level for 5 min at 75 °C with 30–70 % ethanol in water, 30:1 solvent-to-sample ratio, and 0.30 to 0.45 mm particle size. With regard to obtaining saikosaponin b_2 with conventional hot solvent extraction (HSE) (50 % ethanol, 75 °C) for 120 min and HSE for 60 min, the detected concentrations of saikosaponin b_2 were 0.62 mg/g and 1.59 mg/g, respectively, which were much higher than that obtained by MAE. In the extracts of conventional hot water reflux extraction, saikosaponin d decreased to undetectable level. The degradation of saikosaponin d could be minimized by MAE. Moreover, MAE can significantly reduce the extraction time, resulting in better extraction efficiency.

In the study of optimization of MAE for saikosaponins, microwave power level, time, temperature and ethanol concentration, were optimized using RSM with a central composite rotatable design (CCRD). By using the desirability function approach, the optimum MAE conditions to obtain desirable extraction yields for all these saikosaponins simultaneously were found to be at the microwave power of 360-400 W, ethanol concentration of 47-50 %, temperature of 73-74 ^{o}C and time of 5.8-6.0 min. At these conditions, the yields from the verification experiments were 96.18-96.91 % for saikosaponin a, 95.05-95.71 % for saikosaponin c and 97.05-97.25 % for saikosaponin d, which were in good agreement with the predicted values from the fitted models.

In the mechanism studies, A Fick's second law-based model was used to determine the diffusion coefficient of saikosaponins through the solid matrix under microwave assisted extraction. The values of effective diffusion coefficient (D_{eff}) under different microwave heating conditions were increased as a result of the increase in microwave power, and were extremely significantly higher than those extracted with the conventional extraction method. SEM results indicated that the MAE produced distinguishable changes on microstructure of *Radix Bupleuri* and pomelo peels. Microwave irradiation caused the explosion of oil glands in pomelo peels and rupture of parenchymal cells in plant tissues, and the target compounds within the cell thereby rapidly released into the surrounding extraction solvents. While the liquid phase absorbed the microwaves, the kinetic energy of its molecules increased, and consequently, the diffusion rate increased. As a result, better efficiency and significantly reduced extraction time were obtained with MAE.

As it was mentioned above, in order to understand the mechanism of MAE, the effective diffusion coefficients of MAE were calculated by Fick's second law based on the experimental data, and microstructure changes of plant cells after MAE were observed by SEM images. However, these may not be sufficient to describe the extraction process accurately for MAE. As a further work to this project, the following works are recommended for further investigation:

(1) It is needed to build a comprehensive model of MAE which would couple the key parameters of microwave and degree of disintegration of plant cells;

(2) Development of a pilot scale MAE system, by integrating all the findings from the previous steps, for extracting effective components from dried plant materials.

Appendices

Appendix 1: Calibration Line for Determination of Saikosaponin a

A Hewlett Packard HP 1100 HPLC was used for analysis.

The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II), and separation was achieved using the following gradient: 0–10 min, 30 % I, 70 % II; 10–18 min, 30–40 % I, 70–60 % II; 18–28 min, 40–45 % I, 60–55 % II; 28–35 min, 45 % I, 55 % II; 35–40 min, 45–30 % I, 55–70 % II. The flow rate was fixed at 0.8 mL/min, and the absorbance was measured at a wavelength of 203 nm. 10 µL of sample solution was injected to the HPLC for analysis.

Calibration equation for saikosaponin a was obtained for the range from 0.53 to 10.6 µg / 10 µL solvent, as shown in Figure A1. .

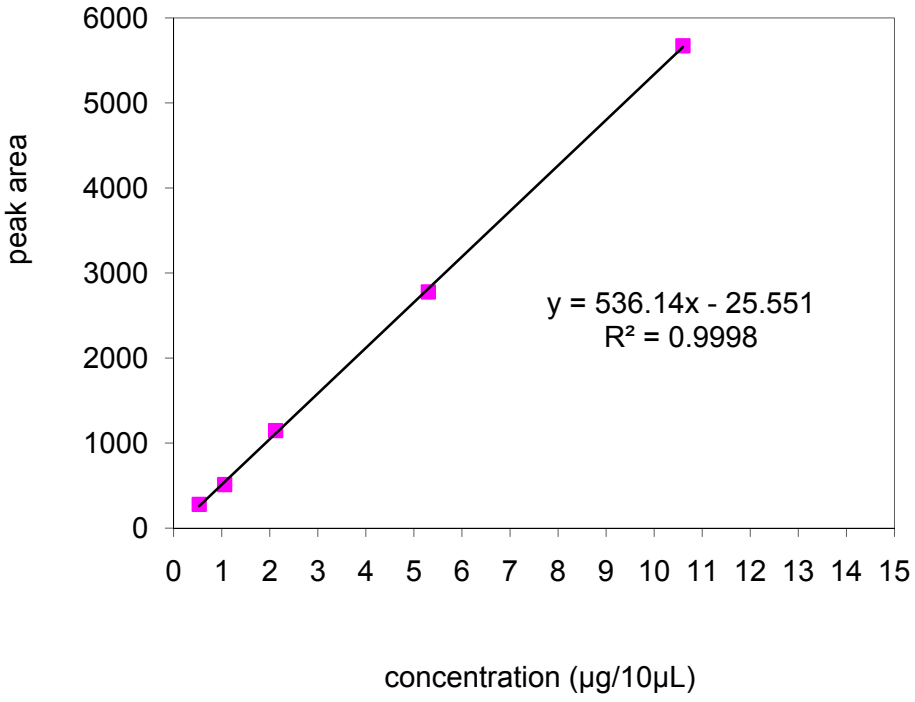

Figure A1 Calibration line for determination of sikosaponin a

Appendix 2: Calibration Line for Determination of Saikosaponin b_2

A Hewlett Packard HP 1100 HPLC was used for analysis.

The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II), and separation was achieved using the following gradient: 0–10 min, 30 % I, 70 % II; 10–18 min, 30–40 % I, 70–60 % II; 18–28 min, 40–45 % I, 60–55 % II; 28–35 min, 45 % I, 55 % II; 35–40 min, 45–30 % I, 55–70 % II. The flow rate was fixed at 0.8 mL/min, and the absorbance was measured at a wavelength of 203 nm. 10 µL of sample solution was injected to the HPLC for analysis.

Calibration equation for saikosaponin b_2 was obtained for the range from 0.52 to 10.4 µg /10 µL solvent, as shown in Figure A2. .

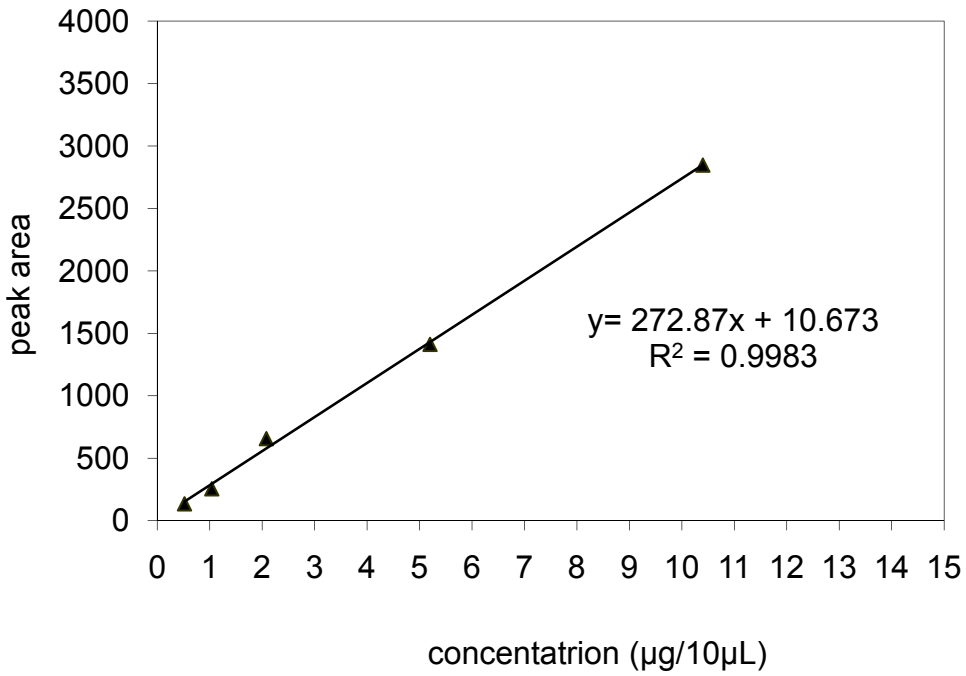

Figure A2 Calibration line for determination of sikosaponin b_2

Appendix 3: Calibration Line for Determination of Saikosaponin c

A Hewlett Packard HP 1100 HPLC was used for analysis.

The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II), and separation was achieved using the following gradient: 0–10 min, 30 % I, 70 % II; 10–18 min, 30–40 % I, 70–60 % II; 18–28 min, 40–45 % I, 60–55 % II; 28–35 min, 45 % I, 55 % II; 35–40 min, 45–30 % I, 55–70 % II. The flow rate was fixed at 0.8 mL/min, and the absorbance was measured at a wavelength of 203 nm. 10 µL of sample solution was injected to the HPLC for analysis.

Calibration equation for saikosaponin c was obtained for the range from 0.49 to 9.8 µg / 10 µL solvent, as shown in Figure A3. .

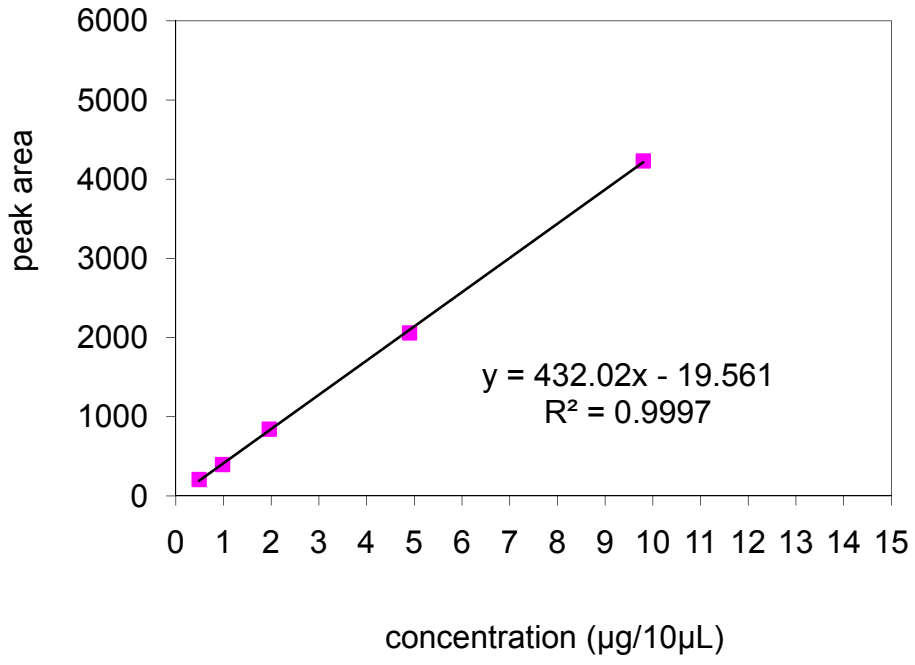

Figure A3 Calibration line for determination of sikosaponin c

Appendix 4 Calibration Line for Determination of Saikosaponin d

A Hewlett Packard HP 1100 HPLC was used for analysis.

The gradient elution system consisted of acetonitrile (solvent I) and water (solvent II), and separation was achieved using the following gradient: 0–10 min, 30 % I, 70 % II; 10–18 min, 30–40 % I, 70–60 % II; 18–28 min, 40–45 % I, 60–55 % II; 28–35 min, 45 % I, 55 % II; 35–40 min, 45–30 % I, 55–70 % II. The flow rate was fixed at 0.8 mL/min, and the absorbance was measured at a wavelength of 203 nm. 10 µL of sample solution was injected to the HPLC for analysis.

Calibration equation for saikosaponin d was obtained for the range from 0.50 to 10.0 µg /10 µL solvent, as shown in Figure A4.

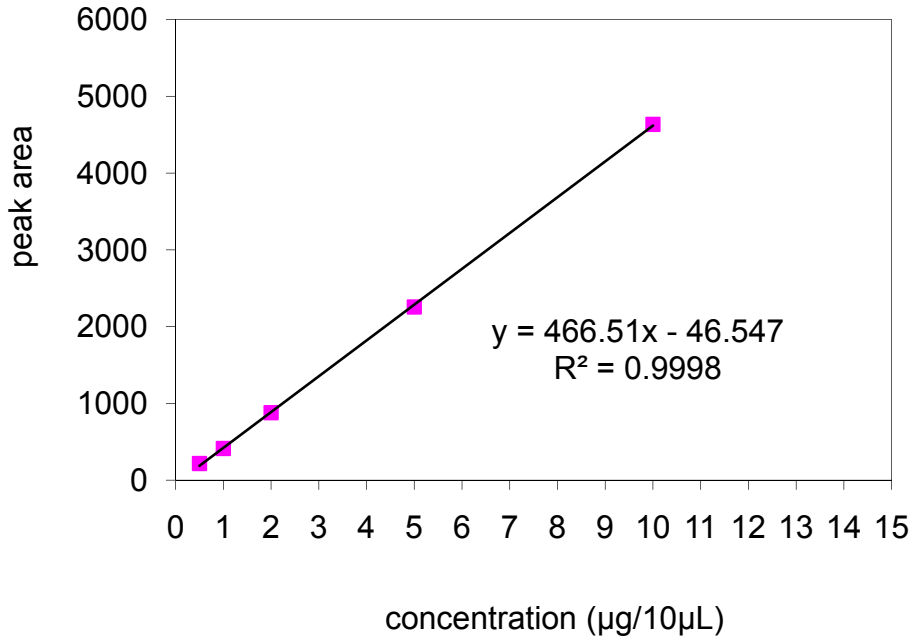

Figure A4 Calibration line for determination of sikosaponin d