MICROWAVE ASSISTED EXTRACTION OF *CERIOPS DECANDRA SP.* LEAVES FOR HYPOGLYCEMIC ACTIVE COMPOUNDS

MUHAMMAD SYADDAD BIN ZAINOL

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Faculty of Chemical Engineering & Natural Resources
University Malaysia Pahang

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ABSTRACT

In this paper, a method of microwave-assisted extraction (MAE) was for the first time used to extract total triterpenoid from *Ceriops Decandra*, which have been reported to have hypoglycaemic activities towards human bodies. The extracts were directly determined by colorimetric method without any further treatment. Compared with Soxhlet extraction method and ultrasonic extraction method, MAE only need 20 min to give the highest yield of triterpenoids at 1.1785%, while the Soxhlet extraction methods need several hours and give lower yield. Several factors affecting the MAE extraction rate were also discussed, such as extraction time, temperature and ratio of solvent to material. Optimal conditions of MAE from this research can be concluded as follows: 20 min at 80 ^{o}C, the ratio of solvent to material is 25 by using 95% ethanol as the solvent.

ABSTRAK

Dalam projek ini, pengekstrakan yang dibantu oleh gelombang mikro adalah pertama kali digunakan untuk mengekstrak oleanolik asid yang merupakan ahli kumpulan triterpenoid yang terdapat di dalam spesis bakau *Ceriops Decandra,* dimana ia dikatakan dapat membantu menurunkan kadar gula di dalam darah manusia. Ekstrak yang diperolehi dianalisis terus oleh teknik pengkoloran tanpa perlu melalui teknik rawatan yang lain. Jika dibandingkan dengan teknik Soxhlet dan teknik Ultrasonik, pengekstrakan yang dibantu oleh gelombang mikro hanya memerlukan 5 minit untuk memperolehi hasil triterpenoid yang optimum iaitu pada kadar 1.1785% dan teknik yang lain memerlukan masa yang lebih lama untuk memperolehi ekstrak dan hasil yang diperolehi adalah lebih sedikit. Faktor-faktor yang mempengaruhi pengekstrakan yang dibantu oleh gelombang mikro juga diambil kira termasuklah masa yang dilalui untuk pengekstrakan berlangsung, suhu dan kadar pelarut terhadap bahan. Keadaaan optima untuk, pengekstrakan yang dibantu oleh gelombang mikro adalah seperti berikut: 20 minit pada suhu 80 ^{o}C, kadar pelarut terhadap bahan adalah 25 dengan menggunakan 95% etanol sebagai bahan pelarut.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

P	-	Pressure
m	-	Mass
ΔH	-	Enthalpy change of reaction
ΔS	-	Entropy change of reaction
ΔG	-	Energy change of reaction
T	-	Temperature
ρ	-	Density
μ	-	Viscosity of liquid (Pa.s)
h	-	Heat transfer coefficient
^oC	-	Degree Celsius
kg	-	Kilogram
K	-	Degree Kelvin
m	-	Meter
n	-	Number of moles
L	-	Litre

CHAPTER 1

INTRODUCTION

1.0 Introduction

The design of environmentally friendly process for isolation of naturally occurring triterpenoids via extraction and means of quantitative analysis of the yields conceived for current research was among a few of its kind that is reported in current literature. There have been a few publications along very close lines as discussed in the literature survey. The field of triterpenoids is vast and there appears to be great potential for inter-disciplinary systems research. The current research was carried out to determine the optimum yield of triterpenoids from specific mangrove species under certain condition which will be experimented. This chapter briefly introduces the background of study, problem statement, objective, expected outcome, scope of research and research contribution to the environment and society.

1.1 Background of Study

Mangrove trees from *Rhizophoracae* family are famous for its medicinal purposes. Scientists around the world have conduct research and experiment on almost all part of the mangrove from its root to the leaves on the sprouting branch and it have been proven that each part of mangrove have its own distinctive medicinal properties. Mangrove root, leaf and stem extracts have its own distinctive inhibitory properties that can affect the growth of various human pathogenic organisms such as bacteria, fungi and viruses (Hernandez and Perez, 1978). Gargles of mangrove bark can also cure throat cancer (Garcia-Barriga, 1975). Leaf of *Rhizophora apiculata* polysaccharide extracts said to blocked the expression of HIV-1 antigen in MT-4 cells and abolished the production of HIV-1 p24 antigen in peripheral blood mononuclear cells (PBMC). It also reduced the production of viral mRNA when added before virus adsorption. These results suggest that *Rhizophora apiculata* Polysaccharide (RAP) may inhibit AIDS virus in an early stage of its life cycle. It reported that extracts of *Rhizophora mangle,* which is also known as red mangrove that is usually found in subtropical and tropical areas in both hemispheres, extending to approximately 28°, had anti-diabetic and anti-hyperglycemic property (Alarcon-Aguilara et al., 1998).

Recent research conduct on anti-hyperglycemic effect of the crude extract and four fractions of *Ceriops Tagal* (Rhizophoracae family) were evaluated in normal healthy sucrose-loaded and STZ-induced diabetic rats. The crude extract improved the glucose tolerance of the sucrose-loaded rats significantly while in STZ-induced diabetic rats the extract caused a fall in hyperglycemia. All of the four isolated fractions improved the glucose tolerance of normal rats. Research conduct by Sur, Tapas Kumar and his colleague (Sur et al, 2004) reveal that leaves of *R.Apiculata* extracts has potential hypoglycemic action. Alcoholic extract of the leaves of this plant was prepared and hypoglycemic activity was studied in fed rats, glucose loaded rats and streptozotocin (STZ) induced diabetic rats.

Research conduct by V. Lakhsmi and her colleagues (V.Lakhsmi et al, 2006) also reveals the hypoglycemic activity of the ethanolic extract of the roots of the *Rhizophora apiculata* in rats (GLM and STZ models). On further fractionation of the ethanolic extract into four fractions, the activity was localized in the chloroform and aqueous fractions. These on purification led to the isolation of 7 pure compounds - lupeol (1), oleanolic acid (2), β-sitosterol (3), palmitic acid (4), β-sitosterol-β-D-glucoside (5), inositol (6), and pinitol (7). The inositol and pinitol, two of the pure compounds, showed promising activity in STZ model of rat at 100 mg kg-1 dose level.

1.2 Problem Statement

Commercially, local mangroves did not being fully use. The most common use of mangrove in Malaysia is just for charcoal production and as tannin agents. Local mangrove is not being use widely in pharmaceutical industry or in simple word let just said that it is being use only for the research purpose, not for mass production. It is quite important for mangrove to be introduced to another type of industry besides charcoal production and tannins as it can generate the economy of this country. When the purpose of mangrove plantation reach peoples thought, they will suddenly notice the importance of mangrove not just for their own benefit but also for local soil for example, mangrove can reduce the impact of big ocean wave such as tsunami that had strike this country and other Asian country last three year.

It is common in not just pharmaceutical but other industry also that price of medicine or products are depend on how much that being spent to produce the products. The price of diabetic medicine that is available in the market nowadays is quite expensive, for example, it can cost $145 for a 60-capsule bottle of D-Chiro Inositol. At that price, society with average to low monthly income that has diabetic condition will have a hard time just to keep theirself on prescription. Usually, diabetic medicine that is available in the market nowadays is produce as a result of synthesizing drugs that is human-made so the price of production of these synthesis drugs will increase and that is why it is quite expensive to make these diabetic remedy.

As being explained above, modern diabetic medicine is produce by synthesizing human-made drugs. As we all know, synthesis drugs have several bad side effects to our body systems. For example synthesize D-Chiro Inositol and Pinitol that is being use to reduce insulin level in blood can also reduce testosterone level in human body and this effect is vary for each human body. If one body already produces less testosterone and that person is having diabetic condition, he cannot take this kind of medicine to reduce sugar level in his blood because it will result him to be unfruitful once he take the medicine continuously. So, it is important to produce diabetic medicine from natural product as it will eliminate these bad side effects. There is a few companies in Malaysia that had taken opportunities to make diabetic remedy from natural products such as from *Orthosiphon stamineus, Benth* that is known for its medicinal purpose but usually the medicine is make into a tea. Some party had try to make a tablet or capsule out of the plant but it seem it cost is quite expensive so that is why we are trying a new alternative of plant which is mangrove.

1.3 Objective

The main objectives of this project are to extract and identify how much naturally occurring triterpenoids resides in mangrove leaves of *Ceriops Decandra sp*. and to optimize the condition for triterpenoids extractions from that particular species.

1.4 Scope of Research

Regarding this particular research, there are several scopes that need to be fulfilling to ensure that this experiment will achieve the objective that had been set. Scopes of research are also important as it will be the benchmark for the research to be further advanced in the future in the same manner.

First scope of this research is the experimental study on the extraction process of Oleanolic Acid from *Ceriops Decandra sp.* Leaves. This research will only focus on extraction process alone in the first stage. Second scope of this study is to analyze and compare results obtain in the experiments based on parameters that are to be experimented including duration of the extraction process, temperature that is being apply during the experiments and also ratio of solvent that being use to the materials. The third scope is to optimize the extraction condition based on the parameters that being use whereas from each of the designated experiment, the parameter that give the highest yield will be use as constants for the next experiment and at the end of the experiments, the optimum condition for the yield extraction can be obtained.

1.5 Research Contribution

Research will be meaningless at all if it got no contribution to be provided. So it must able to provide a benefit to the surrounding in order for the research to be useful and conducive. For this research, it got contribution whether to the society or the environment so it meant to be further advanced in the future.

This particular research provide a sum of benefit to the society, for Malaysian citizen's specifically and for other country globally as it provide an alternative resource for diabetic medicine. As for today, the natural source of diabetic medicine is quite limited and people tend to rely on synthesize drug which have more side effect than the natural sources. Hopefully through this research, the production of diabetic medicine will be more focused on the natural resources as it has fewer side effects to human body.

In today's market, the price of diabetic-inducing medicine is quite high so with this research the price of diabetic medicine will be reduced as there are more sources for raw material in producing the medicine, especially in countries with a vast mangrove population such as Malaysia, Philippines, Thailand, Australia and Indonesia and individuals with low to moderate income can also purchase the medicine if they have diabetic condition as the price will be affordable.

Environmental problem have been quite an issues in this modern world. Mangrove habitat is among the other wild habitats that have been threatened by modern world development. People tend to see that mangrove habitat is not important and many developments have been initializing on top of the mangrove area which cause deforestation to the area. So hopefully from this research, society will acknowledge the important of mangrove and realize that mangrove plantation is compulsory and they will not only preserve the natural mangrove habitat, instead they will provide and renew it for the benefit of the environment and themselves.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, among things that are to be elaborately explained are the microwave extraction process as how it operates including its mathematical aspects, microwave extraction of biologically active compound, Soxhlet extraction method, colorimetric analysis approach, acid hydrolysis principle, triterpenoids and mangrove *Ceriops Decandra Sp.*

2.2 Introduction to Extraction

By definition, extraction best define as the process of obtaining something from a mixture or compound by chemical or physical or mechanical means. Solvent extraction is the removal of one or more components from a liquid mixture by intimate contact with a secondary liquid that is nearly insoluble in the first liquid and which dissolves the impurities and not the substance to be purified. But as any other methods, there are advantages and disadvantages of solvent extractions. For example direct solvent extraction is preferred in industry because it is simple, has no complex equipment, controlled recovery, large selectivity and nice flexibility but the disadvantages is quite many compare to other solvent extractions method such as emulsion formation, not efficient, loss of compounds, complicated, laborious and pre-concentration step required.

Furthermore, almost all natural products cannot tolerate with high temperature condition. So, alternative method is required to reduce these unwanted situations from occurring. Therefore Microwave Assisted Extraction is chosen in this experiment. Although there are small disadvantages of this method as it requires high technical skills and high installation cost, that is not going to be a problem in this research as installation cost is not under student jurisdiction and technical skills can be acquire if much quality time spent on the equipment in order to become familiar with it.

2.2.1 An Overview of Microwave Technology

Electronic and electromagnetic technologies, which are familiar today, were invented during the dismal and desperate early days of World War II. Sir Henry Tizard representing the British Forces was instrumental in setting the pace for radio detection and ranging research by the US defense, most of it at the MIT's Radiation Laboratory in 1940. The cavity magnetron revealed to the US by the Tizard mission has been described, as among the most valuable cargo to ever cross the Atlantic. The new magnetron was a breakthrough, in that it could produce microwave pulses many orders of magnitude than could anything else then in existence (Pound 1999). RADAR, NMR and microwave ovens were the outcome of the 1940s radio frequency research.

Microwave energy for heating has been in commercial use since 1950 (Edgar 2001) But it is only recently that its benefits as an environmentally-friendly source of thermal energy has been widely appreciated. Thermal technology dictates the quality, economics and environmental impact of any processing plant. It is by far the most sensitive aspect of food processing. The rising numbers of Green engineering regulations (Figure) call for more efficient energy usage and more environment friendly raw materials as well as effluents (Allen 2002).

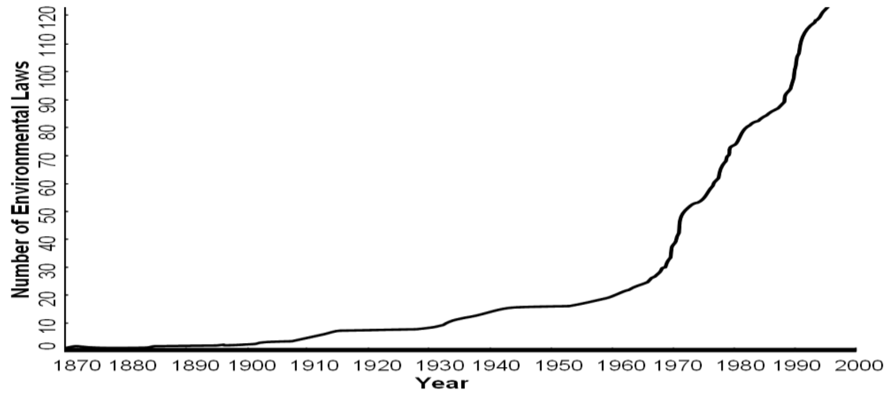

Figure 2.1: The Ever Tightening Environmental Regulations (Allen 2002).

Efficiency demands a bare minimal processing of materials especially nutritive foods. Hence, electric heating technologies such as radio frequency, microwave, ohmic and infrared are fast emerging; among them microwave shows a highly promising future (Chang et. al., 2000). They are energy efficient and can operate in combination with other 18 heating technologies. Table 2.1 shows a comparison of the conversion efficiency of different types of heating systems.

Table 2.1: Comparison of Conversion Efficiencies of Various Heating Source (Wilson et al, 2003).

Appliance	Temperature (^{o}C)	Appliance Rating (W)	Time (min)	Energy Used (kWh)	Energy Cost (RM)
Electric Oven	177	2000	60	2	0.60
Convection Oven	163	1853	45	1.39	0.42
Gas Oven	177	36	60	3.57	0.25
Frying Pan	216	900	60	0.9	0.25
Toaster Oven	218	1140	50	0.95	0.28
Crockpot	93	100	420	0.7	0.21
Microwave Oven	High	1440	15	0.36	0.11

The volumetric heating or heating of the bulk as opposed to transferring heat from the surface, inwards, is more efficient, uniform and less prone to overkill. Controllability is by far the greatest advantage of microwaves over conventional thermal technologies. In processing applications, the ability to instantaneously shut the heat source makes enormous difference to the product quality and hence the production economics. The very nature of heating through the involvement of the raw material under processing (instead of using fossil fuels or less efficient, indirect electrical heating systems) brings about quality consistency as well as positive environmental impact. Specifically in the triterpenoids extraction, microwave mediated processes are highly desirable due to their small equipment size (portability) and controllability through mild increments of heating. However, so far the microwave technology has found application in very few industrial bio-processing installations due to the lack of available data on microwave interaction with heterogeneous natural raw materials. The sensing and close control of microwave process is a challenging science. There is insufficient literature on microwave process sensing. Therefore, further research has to be done on this topic to ensure that microwave technologies can be fully utilized in the industrial arena.

In the current work, it is proposed to study a procedure for microwave extraction of triterpenoids and compare the extraction under different conditions of temperature, ratio of solvent to material and extraction time using single beam UV-Vis as final analytical instrument. The daunting challenge remains in measurement and comprehending the precision and accuracy of the data obtained. Data on microwave processes still remain, to a great extent, of an empirical nature. This chapter introduces the technological areas involved in the microwave processing, the empirical approaches attempted and the various challenges they pose. Therefore, the microwave extraction of triterpenoids is discussed in this section, starting from the materials as well as industrial perspective of the process, based on current literature.

2.2.2 Microwave energy in terms of Electromagnetic Fields

Microwaves are electromagnetic fields in the frequency range 300 MHz to 300 GHz or between wavelengths of 1 cm and 1m (Singh et. al., 2001). The electromagnetic field plays a central role in any attempt to describe physical reality. They are as real as the physical substances we ascribe to everyday experience. Therefore fields and particles of matter must be put on the same footing: both carry energy and momentum, and both interact with the observable world (Rothwell et. al., 2001). Electromagnetic field is an oscillating electric and magnetic disturbance that spreads as a harmonic wave through space. The very low end of electromagnetic fields are felt at the charged double layers, direct current (DC) batteries and electrical conduction phenomena, whereas the higher end lead us to the realms of particle physics where matter transforms into radiation and vice versa.

Electromagnetic fields interact with matter resulting in energy transfer. Photosynthesis and human vision are examples of such interactions. There are numerous effects across the whole spectrum of electromagnetic frequencies most of which are not so obvious and appear only in numerical solutions. The classical electromagnetism is described by Maxwell equations (Rothwell 2001). These describe essentially three electromagnetic properties viz., complex electrical permittivity and permeability (ability to store electrical and magnetic inductive capacity) and electrical conductivity. Electromagnetic waves interact with matter in energy transfers that are quantized. Predominantly, waves in the microwave region excite molecular rotational energy levels (about 10-3 eV) and their energies fall just short of inducing Brownian motion in liquids and gases. They have an orienting effect on polar molecules present in microwave susceptible materials. This orienting effect, however, marginally falls short of synchrony with the alternating applied microwave field. This difference in synchrony, called dielectric relaxation results in a net absorption of energy, which manifests as heat.

2.2.3 Microwave Heating – Mathematical Aspects

The depth of the sample where the attenuation is 1/e (or in other words 1/α), can be approximately expressed as (λ / 2π)(ε ' /ε ") . This is the depth where effectively, the heat generated by an applied microwave field in a lossy medium is most likely to be volumetric. In very basic sense, the heat generated by microwaves can be represented by the conduction equation as given in Eq. 2.2.3(A):

$$\rho c_p \frac{\partial T}{\partial t} = \lambda \nabla^2 T + Q \qquad \text{(Eq. 2.2.3(A))}$$

This simplistic case has to be expanded with terms for convection and transport in order to get an accurate numerical solution. The source term for heat generation being in the form of electromagnetic field (microwave region), is a function of field frequency and absorbed power by foods. The two equations used for deriving the field equations for microwave are the Ampere's law (Eq. 2.2.3(B)) and Faraday's Law (Eq. 2.2.3(C)) both of which are Maxwell's electromagnetic equations.

$$\nabla \times H = \sigma E + \frac{\partial D}{\partial t} \qquad \text{(Eq. 2.2.3(B))}$$

Relates magnetic field **H** to the electric flux density **D**

$$\nabla \times E = \frac{\partial B}{\partial t} \qquad \text{(Eq. 2.2.3(C))}$$

Relates electric field **E** to the flux density **B**

However, a mode of the microwave can be chosen such that the magnetic component need not be considered. For a dielectric material, the propagation can be given as (Eq. 2.2.3(D));

$$\nabla^2 E = \mu \varepsilon \frac{\partial^2 E}{\partial t^2} \qquad \text{(Eq. 2.2.3(D))}$$

Here μ is the permeability representing interaction with magnetic field and ε is the dielectric constant representing the interaction of non-conducting material with electric field. The power dissipated per unit volume is manifested as heat Q. (Eq. 2.2.3(E)) is a concise statement for the heat generated in microwave (Meredith et. al., 1998):

$$Q = \frac{1}{2}\sigma_e |E|^2 \qquad \text{(Eq. 2.2.3(E))}$$

Q in the above equation appears as dissipation density in classical treatment of electromagnetism (where it is ohmic and applies equally to capacitive coupling as well as magnetic induction). The conductivity term σ includes the direct current conductivity (zero in this case) and an imaginary part of permittivity ε or the dielectric loss factor.

This is the starting point for any industrial microwave heating calculation, may it be for food or non-food application. However when foods are considered, there is a need to include innumerable influencing factors and corrections for unknowns. It is easier to model non-food processing. When it comes to foods, the basic difference is the fact that foods come in infinite variety, no two compositions and dimension remaining alike.

The rise in temperature is obtained by dividing the heat term Q by density and specific heat (Chang et. al., 2000). The functional density is difficult to measure for two reasons: (i) the density used in the permittivity measurements may not reflect the density of material exposed to multi-mode microwave radiation; and (ii) the density of the bulk may not be evenly and completely exposed to microwaves. Microwave penetrates to a limited depth, which is inversely proportional to the operating frequency as well as dielectric loss of the substrate.

Biomaterials heat up in the presence of microwaves owing to their chemical constituents that have dielectric relaxation. The heat generated by microwave interaction with biomaterials can be fully quantified by a measuring the frequency dependent relative permittivity (in a vector network analyzer) or as an impedance spectrum, characterizing amplitude and time scale (via the relaxation time) of the charge-density fluctuations within the sample (in dielectric relaxation spectroscopy). The microwave processing literature uses relative permittivity measured using network analyzers as the indicator of the heat that can be generated in a sample when subjected to microwave.

In order to utilize microwave for unit processes, quantitative characterization has to be successfully carried out for: (a) electric field profile in the processing chamber; as well as, (b) the resulting changes occurring in the substrate. However, these are complex tasks, seldom achievable in industrial microwave equipments due to their poor reproducibility and thus giving rise to poor control over process quality.

Interpreting from the current literature on microwave processing, the complexity in controlling microwave process quality may arises from the following:

1. The theoretical modeling of an empty multimode oven cavity has little predicting capability for events in a partially loaded cavity due to enormous perturbation of electric field by the very presence of a load.

2. The complexity of modeling is compounded by the fact that permittivity varies spatially depending on the chemical composition and bulk density of the load as well as temperature. Biomaterials can be by far the most complex substrates for microwave.

3. Low penetration depth of microwaves giving rise to competing heat transfer mechanisms. Uniform heating is rarely achievable in conventional microwave systems, often giving rise to both unprocessed and severely over heated spots.

4. Various mixing operations applied in mainstream thermal technologies become inapplicable in microwave systems due to material selection issues imposed by microwave environment.

2.2.4 Microwave Extraction of Biological Active Compounds

Extraction is what brings us wake-up coffee. It is a trite but poorly understood engineering process. Solutes within the powdered raw material move or partition into the solvent phase and diffuse out of the solid matrix and eventually out of the particulate bulk. Traditionally, plant materials were subjected to mechanical shear to release the volatiles in virgin state. There are today various closely controlled sophisticated methods of extraction from distillation, through leaching to super- or sub-critical solvent extraction. Among the various available methods, microwave assisted extractions show the highest promise. Even among the microwave methods there are several variations. The literature survey covers some of the latest reported methods of microwave extraction.

Many proposed microwave based methods often adopt comminution for pretreatment (Gaikar et. al., 2002). In industrial scale, Annatto (which gives cheddar cheese its golden hue), for instance, is extracted by particle attrition and impact using a ball mill (Mendonça et.al., 2001). Solvent extraction remains the most important methods of volatile extraction. From macerated species, there have been attempts to use various solvents to effect extraction. Among several new technologies, microwave assisted solvent extraction is also well-reported (Nélida et. al., 1999; Gaikar et. al., 2002). Microwave ovens have gained acceptance as a mild and controllable processing tool. Microwaves allow simple, rapid and low solvent consuming processes. (Bernard et al, 2002; Amer et al, 1998). In the current research a study of extraction method from dry raw materials with addition of organic solvents was studied.

2.3 Soxhlet Extraction

Usually, Soxhlet extraction is required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapor cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.