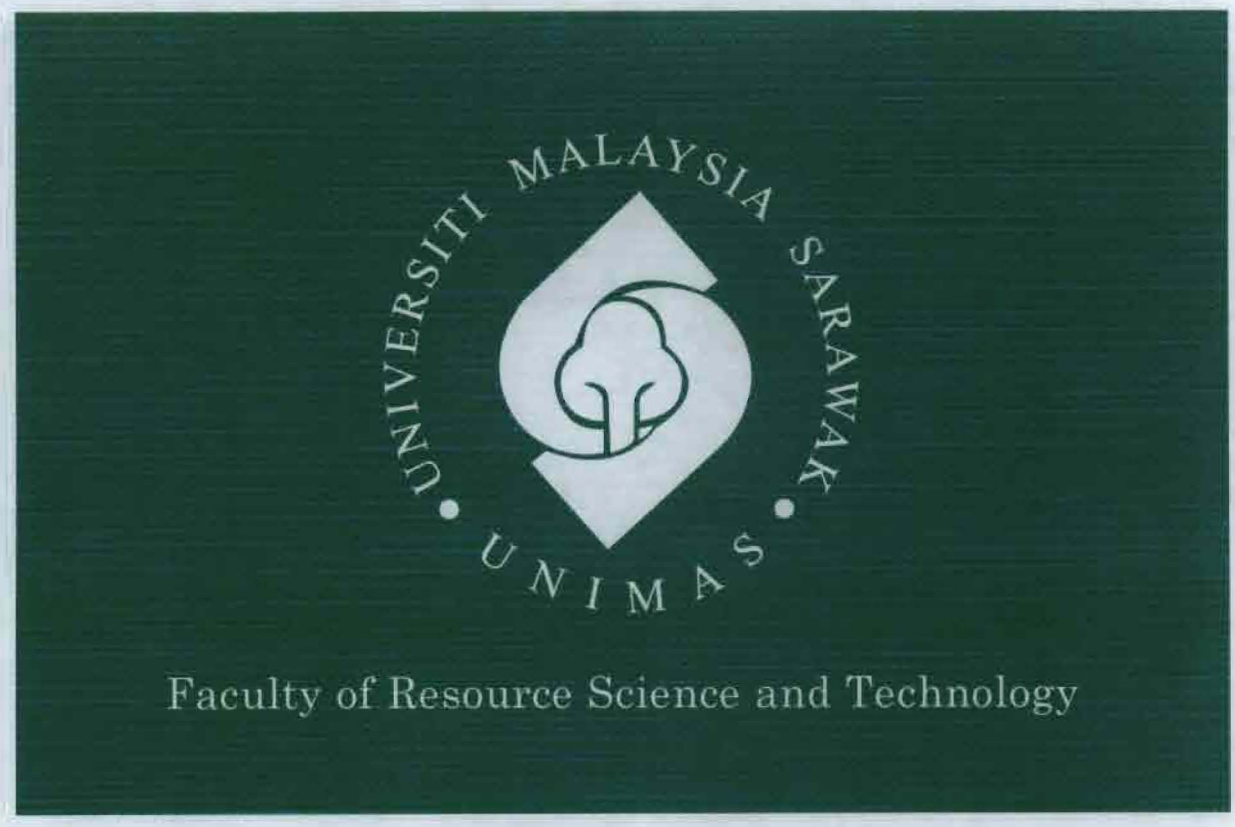

OPTIMIZATION OF METABOLITE EXTRACTION FROM SAGO LEAVES TISSUE

Farhana Binti Aktar

Bachelor of Science with Honours
(Resource Biotechnology)
2013

Acknowledgements

First of all, I would like to express my deepest appreciation and sincere gratitude to my supervisor, AP Dr Mohd Hasnain Hussain for his advice, support and encouragement all this while. It has been a honor for me to work on this project under his supervision. His guidance and comments have been a great help for me throughout this project.

I would also like to thank AP Dr Zainab Ngaini who gives approval for me to use the equipments in the Organic Chemistry Laboratory which is necessary and very helpful in this project. I wish to express my cordial appreciation to members of Organic Chemistry Laboratory, and Molecular Plantation Laboratory as well for allowing me to use the equipments and facilities in their laboratories.

I am grateful and appreciate to post graduate in the Department of Molecular Biology especially Yan Wei Jie for all his guidance and advices, not forget to Nikson, Siti Izyan Liyana, Izzuddin, Vannessa, Wan Syarifatun Handayani, Dayang Nurul Qalila and Muhd Farid for their generous help and guidance as well. I wish to thank lab assistant, Saiful as well for always providing me enough materials and reagents and kept reminding to lock laboratory when I do my lab work for overtime.

Special thanks to all my labmates in Proteomic Laboratory and Organic Chemistry Laboratory especially to Hassekeen, Allysya, Anizavilla, Wani and Ain for their support and for all the fun we had in the laboratory. I am glad and thankful too to Dollyna for leniently to share with me software which I used it for my analysis.

Last but not least, I am thankful to all my family members for never give up encourage me and give their love and support.

Declaration

I hereby declare that this dissertation is the result of my own work and no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

Signature : ……....

Name : Farhana binti Aktar

Date : 12 June 2013

Table of content

Acknowledgements I

Declaration II

Table of contents IV

List of Abbreviations V

List of Tables and Figures VI

Abstract 1

1.0 Introduction 2

2.0 Literature Review 4

2.1 *Metroxylon sagu* 4

2.1.1 Characteristics of *Metroxylon sagu* 5

2.2 Metabolites diversity and their roles 6

2.3 Quenching 7

2.4 Extraction 7

2.5 Sample concentration 8

2.6 Solvent extraction method 9

2.7 Chemical and physical properties of solvent 10

2.7.1 Roles of methanol chloroform co-solvent 11

2.8 Factorial design 12

3.0 Materials and methodology 16

3.1 Sample storage 16

3.2 Chemical preparation 16

3.3 Parameters 16

3.4 Pre-treatment and grinding 16

3.5 Extraction 17

3.6 Phase separation 17

3.7 Sample concentration 18

3.8 Statistical analysis …… 18

3.9 Hypothesis ……18

4.0 Results …… 21

4.1 Quantitative measurement of replication …… 21

4.2 Factorial analysis …… 26

5.0 Discussion ……... 36

5.1 Effect of temperature …….. 36

5.2 Effect of extraction period ……... 36

5.3 Effect of extraction times …… 37

5.4 Interaction of temperature with extraction period ……... 38

5.5 Interaction of temperature with extraction times …… 38

5.6 Interaction of extraction period with extraction times …… 39

5.7 Interaction of temperature with extraction period and extraction times ……. 39

6.0 Recommendations for future study...……..…….. 41

7.0 Conclusion....…….. 42

8.0 References ……..…… 43

9.0 Appendixes ……..…… 46

List of Abbreviations

ANOVA	Analysis of variance
cm	Centimeter
cPoise	Centipoise
E	East
g	Gram
h	Hour
ha	Hectare
Kg	Kilogram
kPa	Kilopascal
M	Molar
mbar	Milibar
mL	Mililiter
N	North
pH	Potential of hydrogen
rpm	Revolution per minute
SPSS	SPSS software system
^{o}C	Degree celcius
%W/W	Percentage weight over weight

List of Tables

Table 1: Physical properties of methanol, chloroform and water …….. 11

Table 2: Formulation for statistical analysis of factorial design ……..……. 14

Table 3: The weight of replication after minus blank …… 22

Table 4: Descriptive statistic of analysis data of Table 1 ……... 23

Table 5: Main effects and interaction of three parameters with 0.05 significance level; 95% confidence level ……... 26

Table 6: Replication of extraction factors …….. 46

List of Figures

Figure 1: Estimated marginal means of Replication at Extraction time =1……..……. 29

Figure 2: Estimated marginal means of Replication at Extraction time =2 ……...…….31

Figure 3: Estimated marginal means of Replication at Extraction time =3 ……...….32

Figure 4: The interaction of temperature with extraction period ...……..... 33

Figure 5: The interaction of temperature with extraction time/s …..…….. 34

Figure 6: The interaction of extraction period with extraction time/s ……...… 35

Figure 7: The colour of solvent in 40^{o}C for 4 hours extraction after third times extraction... 46

Figure 8: The homogenize leaves powder size ……...…….…...……..…. 47

Figure 9: Protein deposited was seen after transfer evaporated chloroform contained extracts into the vial ……..……...… 47

Optimization of metabolic extraction from sago leaves tissue

Farhana Binti Aktar

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Metabolic profilling have been widely studies in plants especially for highly commercial product. Currently, the study of metabolites in *Metroxylon sagu* becomes significantly interesting as it is one of the starch-rich sources for many potential industries. Extraction is the most critical step in metabolites profiling. This study was conducted to optimize several parameters in the extraction method of metabolites of sago leaves. The parameters were temperature for extraction, extraction period and extraction times which were tested using solvent extraction method. Methanol chloroform co-solvent was used in extraction with ratio 2 : 1.The data was measured in quantity by weighing the metabolites extracted. From factorial analysis, the result showed there was significant correlation between temperature, extraction period and extraction time towards metabolites extracted in leaves tissue of *M. sagu*. Optimal condition towards metabolites extraction was at temperature 30^{o}C for 2 hours at first batch extraction.

Keywords: metabolic profiling, *Metroxylon sagu*, factorial analysis, methanol chloroform co-solvent

ABSTRAK

Metabolik profil telah digunakan secara meluas dalam kajian tumbuh-tumbuhan terutamanya dalam produk yang tinggi nilai komersial. Baru-baru ini, kajian metabolik dalam Metroxylon sagu mendapat tarikan sebagai salah satu sumber yang kaya dengan kanji untuk pelbagai potensi dalam industri. Kajian ini dijalankan untuk mengoptimumkan beberapa parameter dalam kaedah pengekstrakan metabolik daripada daun sagu. Parameter-parameter tersebut adalah suhu pengekstrakan, jangka masa pengekstrakan dan pengulangan pengekstrakan yang diuji menggunakan kaedah pengekstrakan pelarut. Pelarut methanol klorofom digunakan dalam pengekstrekan dengan ratio 2 : 1. Data diukur dalam kuantiti dengan penimbangan ekstrak metabolik. Daripada analisis faktorial, keputusan menunjukkan hubungan antara suhu, jangka masa mengekstak dan kekerapan mengekstrak terhadap pengekstrakan metabolik dalam daun tisu M. sagu. Keadaan yang optimal untuk mengekstrak metabolik adalah pada suhu 30^{o}C selama 2 jam pada pengekstrakan kali pertama.

Kata kunci: metabolik profil, Metroxylon sagu, analisis faktorial, pelarut metanol klorofom

1.0 Introduction

Metabolite is defined as the product of enzyme-catalyzed reactions (Harris, 2003) which take part in metabolic process. A metabolomic study is a technique that helps in measuring and elucidating the functions of uncharacterized genes (Bais *et al*, 2010). In addition, it is aimed for understanding of these metabolic networks and the subsequent biochemical composition of plants, other biochemical composition of plants, and other biological organisms (Dixon *et al*, 2006). The plant metabolome is highly complex, dynamic assortment of primary and secondary compound. The suitable methods in approaching the genuine metabolomic have not been developed due to vast diversities in primary and secondary metabolites. Isolation of metabolites is an initial of chemometric methods to extract, separate out and display systematic variation (Hagel & Facchini, 2008). The study of metabolites in plant is important as there is little understanding of the interaction between metabolites, metabolites signaling, interaction with development and the role of metabolism in genotype to phenotype relationships (Stitt *et al.*, 2010). Moreover, metabolites studies are performed to discover new products with medicinal especially drug. In addition, metabolites are profitable for marketing of flavor and fragrance, dye and pigments, pesticides and food addictives in secondary metabolites (Hussain *et al*., 2012).

In order to extract a vast amount of quality metabolites, a rapid, efficient and reproducible technique has become a consideration for extraction method. An ideal extraction method is the main problems faced by many researchers because the efficient method for metabolites extraction in plants is different in each species. Solvent extraction is one of the major approaches for plant metabolite extraction. Some of the challenges to extract metabolites are due to long period of incubation, repeating process, high variability of abundance and quenching process (Savita, 2009). In this research study, metabolites studies of sago palm species has not been discovered yet. However, no specific extraction

method is suitable to obtain a large amount of metabolites rapidly with minimal degradation of them. Therefore in this study, the optimization of solvent extraction method was carried out by adjusting some parameters of the procedure taken from other journals to fit the most appropriate extraction procedure for *Metroxylon sagu.* The study of *M. sagu* metabolites is profitable as their species is the largest exporter of starch production in Sarawak (May-Chiun, 2008). Furthermore, the species is valuable because of its availability, characteristics and many uses for industries development as well. Regarding of these matter, there are more growing interest for the investment on the species, marketing and scientific findings.

There are three parameters for optimizing the extraction method which are the temperature for incubation, extraction time and extraction period. The basic steps of extraction method are mainly from Sobolev *et al*., (2005) with little modification according to availability of instrument and equipments in the laboratory. The quantity analysis will be performed by weighing the extracts obtain at the end of experiment. Each parameter (factors) is subdivided into five variables (5 levels) as a subject of study. The data obtain was analyzed by using Factorial design and SPSS system to measure the overlapping effects of parameters. The specific objectives of this research were to:

1) identify the overlapping effects of parameters by testing each of them.
2) develop a rapid, efficient and reproducible extraction method for sago plant species.
3) provide a current data useful in metabolites profiling research of sago plants.

2.0 Literature Review

2.1 *Metroxylon sagu*

Sago is a valuable starch product in the market produced from sago palm tree which abundantly can be found in Malaysia, Indonesia, Philippines, New Guinea, Pacific Islands and some part of South India (Kerala and Tamilnadu). Starch is significantly important for industries development mainly in the Asia Pacific region and South East Asia. In Malaysia, more than 90% of all sago-planting areas are found in the state of Sarawak in East Malaysia (Bujang, 2011). Sarawak is one of the world's largest exporters about 40,000 tons sago a year (May-Chiun, 2008). Sago palm plantation is well distributed at Pusa-Saratok, Oya-Dalat, Mukah, Balingan, Igan, Matu-Daro, Maradong, Bintulu-Tatau, and Limbang. Oya-Dalat is the largest sago palm area with 6,410 hectares (ha) land area located in Sarawak (Adrina, 2004). Based on Department of Statistic (2011), the total land area of sago from smallholders and mini estates in Sarawak were estimated around 54,905 hectares.

The sago palm is rich in starch production, approximately 150 to 300kg of starch for one healthy palm (May-Chiun, 2008). Starch found in the pith of sago plant is initially synthesed by leaves tissue of sago plant. It produces starch used by the plant in flowering and fruiting as a food supply. Sago starch then accumulates at the pith core of the stem of sago tree due to the massive size and lengthy vegetative phase of stems. Based on thesis from Adrina (2004), the vegetative phase of sago palm can last for 7 to 15 years during which time, excess photosynthate from the leaves is transported to the trunk and stored as starch. Therefore, the study of metabolites was preferred collected from leaves tissues as starch was initially produced from there.

2.1.1 Characteristics of *Metroxylon Sagu*

M. sagu is the earliest foodplant and reached western country at 18th century (Flach, 1997). The local name of *Metroxylon sagu* is 'Rumbia' (Department of Agriculture Sarawak, 2013). It can be found mainly in Indonesia, Malaysia, and Papua New Guinea. *Metroxylon* genus of sago species is the only species which are both hepaxanthic (once flowering) and soboliferous, possess a massive rhizome for tillering and suckering (Singhal *et al*.,2008). Originally, sago plant is belongs to family *Palmae jussieu*, subfamily *Calamoidea Griffith*, tribe *Calameae drude*, subtribe *Metroxylinae blume*, and genus *Metroxylon rottboell* (Flach, 1997). There are four characteristics of *M. sagu* spines which including 1) Sago leaf sheath and petioles are unarmed at all different ages of plant, 2) *Tuberosum* with the base of sheath covered with knob-life structures at all ages of plant, 3) *Micracanthum* , the leaf sheath, petiole and rachis are covered with spines at early age, 4cm or shorter and lastly. 4) *Longispinum* with the leaf sheat, petiole and rachis are covered with spines with 4 cm long and up to 20 cm.

In Sarawak, *M. sagu* is the preferred sago palm to be planted by the local farmer as the smoothed sheathed and thorn-less nature of the palm makes it easier to manage. Mostly, *M. sagu* are well distributed at Malay island. Under good condition, a healthy palm can carry approximately 24 leaves or fronds. The old frond of *M. sagu* is replaced with a new one in each month. Sago starch is stored in the bole during flowering and fruiting. After that, the trunk decays and the suckers are taking over (Flach, 1997).

2.2 Metabolites diversities and their roles

Metabolites produced naturally within cells as the products of enzyme-catalyzed reaction (Harris, 2003). Metabolites categorized from two different groups according to their chemical pathways in plant growth. Primary metabolite is a basic cell an intermediate or precursors in the essential metabolic compound production. In higher plant as such compound usually located in seeds and vegetative storage organs, needed for physiological development. In other hand, secondary metabolites are compounds biosynthetically from primary metabolites which also called as end-products produced such as nicotine, the pyrethrins and rotenone. Secondary metabolites commonly accumulated in plants in small quantities than primary metabolites, and these compound tend to be synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult (Balandrin *et al.*, 1985). The study of metabolites in plants is important as there is little understanding of interaction between metabolites, metabolites signaling, interaction with development and the role of metabolism in genotype to phenotype relationships (Stitt *et al.*, 2010). The level of metabolites is determined by the concentration and the properties of the enzymes. The functions of metabolites are rather playing a role as complex functions in many different regulatory processes inside the cell such as regulation of transcription and translation, regulation of protein-protein interactions, and allosteric regulation by interaction of enzymes with metabolites. Thus, the phenotype of all cell or tissue in response to genetic or environmental changes can be defined by the level of metabolites which represents integrative information of the cellular functions (Villas-Boas *et al.*, 2005). Moreover, metabolomic studies aimed for understanding of these metabolites networks and the subsequent biochemical composition of plants, other biochemical composition of plants and other biological organisms (Dixon *et al.*, 2006).

2.3 Quenching

Intracellular metabolites extractions are accessible in many different analytical methods but usually are time-consuming and it is virtually impossible due to many factors particularly because of the large chemical variability of the different metabolites. Mostly, available extraction methods produce high sample dilutions which lowering metabolites concentration. There are several basic steps require for metabolites extraction. Quenching is one of the earliest steps for inactivation of metabolism. It frequently involves rapid changes in temperature or pH. The commonly use of different applications are freeze clamping, snap-freeze with liquid nitrogen oracidic treatments which perchloric acid and nitric acid are the main quenching methods used for plant and animal. For plant tissue samples, snap freeze with liquid nitrogen is the most effective way to stop enzymatic activity and help in quenching cellular metabolism. Snap-freeze with liquid nitrogen is a rapid and easiest but it does not allow separation between intra- and extracellular metabolites. Quenching with methanol is another rapid method and allows separation of intra- and extracellular metabolite, but the adaptations of method is developed for yeast on short time-scales (Villas-Boal *et al*., 2005).

2.4 Extraction

Extraction method is followed after quenching. The main objective of extraction method is to extract for maximum number of metabolites in their original state and manner. Solvent extraction supposedly is able to avoid any further physical and chemical alterations of the molecules. Commonly, two or more solvent are used in extraction procedure comprises of polar and non-polar solvents. The chemical nature of the plant sample and the polarity of the solvents used determine the solubility of phenolic compounds (Dai & Mumper, 2010). Extraction method with boiling solvent is another of the simple, fast and

accurate and reliable method. However, the method is not suitable for many intracellular metabolites extraction. (Villas-Boas *et al*, 2005). In other hand, acidic and alkaline extractions are a traditional method widely used for metabolites extraction from animal and plant tissues. However, some degradation of metabolites has been reported when using low pH for extraction. Besides, Villas-Boas *et al*. (2005) said that microwave and ultrasound-assisted extraction methods are another efficient way to extract metabolites compare to classical solvent extraction such as acidic and alkaline extraction method. According to Faijes *et al*. (2007), perchloric acid is efficiency in extraction of intracellular metabolites from cell cultures. Weak organic acids such as acetic acid, formic acid, citric acid, tartaric acid and phosphoric acid give the best yield of anthocyanin extraction.

2.5 Sample concentration

Subsequently, extraction method is followed by sample concentration steps. Frequently, low amount of metabolites are extracted and large volume of solvents used. Solvent evaporation under vacuum is one of alternative but cannot be concentrated simultaneously. Freeze-drying or evaporation avoids thermal degradation by removing water from aqueous samples. In addition, most metabolites show good stability allowing storage almost indefinitely in a cold dry and neutral atmosphere and in dark for some metabolites. Solid –phase extraction (SPE) and Solid-phase micro-extraction (SPME) can be applied to concentrate the diluted sample. However, these methods require different trapping materials for different classes of metabolites. Thus, not all metabolites are suitable concentrated with SPE or SPME method (Villas-Boas *et al*., 2005)

2.6 Solvent extraction method

Solvent extraction method can be categorized as continuous or discontinuous method. Examples of continuous methods such as percolation and soxhlet extraction in which solvent is continuously flow through the plant material (flower, root, leave or bark). The plant material is diffuse into surrounding solvent and increase the solvent saturation. The saturated solvent is replaced with less saturated solvent. In other case, discontinuous method refers to a method which the solvent is added and removes in batches (Sakshat Virtual Labs, 2013). The resulting solution is filtered to remove any remaining particulate matter. Solvent extraction method in the study is one of the discontinuous methods (Gamse, 2007).

A discontinuous solvent extraction method is a process used two immiscible solvents which are vigorously shaken in an attempt to disperse one in the other so that the solute can migrate from one solvent to another. When the two solvents are vigorously shaken, they become intimately dispersed in each other in the form of droplets. The more vigorous shaken, the smaller the droplets are produced and then, more surface area between the two solvents. The smaller linear distance for the molecules to travels to reach to other solvent and migrate into it. Therefore, more rapid extraction is if shorter the linear distance travelled by the molecules (U.S. EPA., 2011).

One of the advantages of solvent extraction method is using relative solubility of solute in immiscible solvents. If the solutes are in aqueous solution, an organic solvent that is immiscible with water is added where the solute dissolves in either water or organic solvent. If the relative solubility of the solutes differs in the two solvents, a partial separation occurs. Besides, solvent extraction method has been widely used and applicable for many different plant samples due to their ease of use, reproducible and efficiency

(Sakshat Virtual Labs, 2013). However, there are also limitations regarding the method where it largely dependent on the type of solvent used with varying polarities. The method is laborious needed long time for extraction.

2.7 Chemical and physical properties of solvent

Methanol is a colorless liquid, completely miscible with water and other organic solvents. It has an distinctive odor, flammable and poisonous (O'Leary, 2000). At room temperature, methanol is a polar liquid. It boils at 64.96^{o}C and solidifies at -93.9^{o}C. It is toxin and should not be ingested, where consume quantities of methanol can cause blindness and severe damage to the central nervous system. Besides, it can be inhaled and absorbed through the skin which harmful to the body. In vapour form, methanol causes irritation to eyes, skin, lungs and respiratory tract. It may affect central nervous system and liver as well (Cetiner Engineering Corporation, 2013).

As for chloroform is a colorless liquid with a characteristic odor. It is denser than water and slightly-soluble in water, thus sink in water. Chloroform is flammable but burns under extreme conditions. It can cause illness by inhalation, skin absorption or ingestion. Chloroform classified as moderately toxic. If our body is exposed to chloroform suspected at possibility to get cancer. In addition, it is a central nervous system depressant and a gastrointestinal irritant. It also cause rapid death due to cardiac arrest and delayed death from liver and kidney damage (Anonymous, 2010). Therefore, laboratories wearing and precautions is not safe to be ignore. Our mind as well should always be alert when handling these two solvents or if the solvents are close to our surroundings. Table 5 below shows physical properties of methanol, chloroform and water:

Table 1 : Physical properties of methanol, chloroform and water

Solvents	Polarity index	Boiling point (^{o}C)	Viscosity (cPoise)	Solubility in water (%w/w)
Chloroform	4.1	61	0.57	0.815
Methanol	5.1	65	0.60	100
Water	9.0	100	1.00	100

2.7.1 Role of methanol chloroform co-solvent

According to Seidel (2012), the ideal extraction method should be exhaustive where many compounds or desired metabolites can be extracted as much as possible. Furthermore, it should be economical, fast, simple, safe, environmental-friendly and reproducible if repeating process is performed. Likewise, an ideal solvent should have low toxicity, a low flammability, a low risk explosion and low potential for artifact formation. Nevertheless, there are many metabolites which are associated with other plant components such as carbohydrates, lipids and proteins. Thus, it is almost impossible to develop an ideal extraction procedure using only one solvent suitable for extraction of various plant metabolites (Dai & Mumper, 2010).

The solvent system used during extraction depending on the solubility of the desired metabolites from plant to be extracted. A few years ago, chloroform-methanol-water mixture was developed to extract as many as possible metabolites. The mixture give a good result where both polar and non-polar can extracted together in the same operation (Verpoorte *et al.*, 2007). The advantage of extracting using mixture of chloroform-methanol-water is the generation of biphasic sample. Therefore, it is easier way to analyze samples extracted by the fractionation of the metabolites into polar aqueous and lipophilic organic fractions (Dettmer *et al.*, 2007). Methanol is a polar organic solvent which mixed

with water to extract hydrophilic metabolites whereby chloroform can be used to extract hydrophobic metabolites (Lin *et al.*, 2006). By using chloroform-methanol-water mixture is a time consuming method and requiring careful transfer of solvents from biphasic solution. However, it resulted in a high yield and reproducibility due to both hydrophilic and hydrophobic properties (Lin *et al.*, 2006). As mentioned by Verpoorte and his coworkers (2007), they experienced buffered-water (1:1) is an excellent solvent for studying a wide range of both primary and secondary metabolites. In addition, Dai and Mumper (2010) stated that methanol was found to be more efficient in extraction of lower molecular weight metabolites such as polyphenols

2.8 Factorial Design

Factorial design analysis is a design uses to find the effects of two or more factors encountered and all the interaction factors are investigated in each replicate of the experiment (ReliaSoft Corporation, 2008). Factorial design can be used with both within or between participants' designs, or a combination of both. The purpose of the design is to determine the unique effect of each categorical variable has on the dependent variable. Moreover, the factorial design is also better in the attempts to explain behavior for example, the dependent variable as a function of two or more influences (i.e., the categorical variables).

There are many factorial design including 2-way, 3-way and so on. The main effect of one categorical variable had on the dependent variable, without account for the other categorical variable(s). However, an interaction effect occurs when the effect that one categorical variable has on the dependent variable varies, depending on the level of the other categorical variable(s).Plotting the mean scores on the dependent variable using a line graph is one of the way to determine the interaction effect. The interaction effect IS is

present whenever the lines are not parallel (lines diverge or cross). Another way is by computing the difference scores between two levels of one categorical variable at one level of the other categorical variable(s). An interaction IS is present if the difference score calculated at other levels of categorical variable are unequal (Laman, 2010)

For estimation of main effects and interaction in a two-factor completely randomized design, there is an equation use as shown below:

For example, 2 variables of classification we can define:

A_i = Main effect of level $_i$ of factor A = $\overline{X}_i - \bar{\bar{X}}$

B_i = Main effect of level $_j$ of factor B = $\overline{X}_j - \bar{\bar{X}}$

AB_{ij} = Interaction effect for cell $_{ij}$ = $\overline{X}_{ij} - A_i - B_j + \bar{\bar{X}}$

In order to find the interaction between these 2 variables, the following equation is use:

$$Y_{ijk} - \mu = A_i + B_j + AB_{ij} + \ell_{ijk}$$

Where

$Y_{ijk} - \mu$ = Deviation of observed value from the grand mean μ

A_i = Main effect of level $_i$ of factor A

B_i = Main effect of level $_j$ of factor B

AB_{ij} = Interaction term between A_i and B_j

ℓ_{ijk} = Experimental error

As we are investigating some variables involving replicates, the observation can be described by the linear statistical model:

$$Y_{ijk} = \mu = A_i + \tau_i + B_j + (\tau B)_{ij} + \varepsilon_{ijk} \qquad \begin{cases} i = 1,2,3,\ldots a \\ j = 1,2,3,\ldots b \\ k = 1,2,3,\ldots h \end{cases}$$

Where

μ = the overall mean effect

τ_i = effect of $_i$th level of the row factor A

B_j = effect of jth level of the column factor B

$(\tau B)_{ij}$ = effect of interaction between B_j and τ_i

ε_{ijk} = a random error component (Laman, 2010).

The statistical test can be concluded by the calculation using the following table:

Table 2: Formulation for statistical calculation of Factorial design

Sorces of Variance	Sum of Squares	Degree of Freedom	Mean Square	F_o
A Treatment	SS_A	**a - 1**	$MSA = \frac{SSA}{a-1}$	$F_o = \frac{MS_A}{MSE}$
B Treatment	SS_B	**b - 1**	$MSB = \frac{SSB}{b-1}$	$F_o = \frac{MS_A}{MSE}$
Interaction	SS_{AB}	**(a – 1)(b – 1)**	$MSAB = \frac{SSAB}{(a-1)(b-1)}$	$F_o = \frac{MSAB}{MSE}$
Error	SS_E	**ab(n – 1)**	$MSB = \frac{SSB}{ab(n-1)}$	
Total	SS_T	**abn – n**		**(Laman, 2010)**

Where,

$$SST = \sum_a i = 1, \sum_b j = 1, \sum_n k = 1\ y^2_{ijk} - \frac{y2}{abn}$$

$$SS_A = \frac{1}{bn}\sum_{i=1}^{a} y\text{^}2_{\ i} - \frac{y\text{^}2}{abn}$$

$$SSsubtotals = \frac{1}{n}\sum_a i = 1 \sum_b j = 1\ y^2 ij - \frac{y\text{^}2}{abn}$$

$$SS_{AB} = SS_{SUBTOTALS} - SS_A - SS_B$$

$$SS_E = SS_T - SS_A - SS_B - SS_{AB}$$

From table calculation above, we can conclude whether there is a significant interaction or not between two or more types of variables tested by referring to F table, we can find the F-value corresponding to the statistic, based on the F distribution refers of degree of freedom in numerator and denominator respectively. Finally, we can conclude the significantly important of these two or more variable interaction effects in the factorial experiments (Laman, 2010).

3.0 Materials and methodologies

3.1 Sample Storage

The study used leave tissue samples collected from Kg. Pinang, Kota Samarahan at GPS location (1^{o}25.501min (N)) for longitude and (110^{o}26.523 min (E)) for latitude scale. All the leaves samples were taken from non-trunking tree of *M. sagu* and snap-freeze with liquid nitrogen, stored under -80^{o}C (Maier *et al.*, 2010).

3.2 Chemical preparation

Methanol and chloroform with ratio (2:1) was used as the extraction solvent. The volume of the solvent used in the experiment is 15mL in each tube with the triplicates samples used for every combination parameter tested.

3.3 Parameters

There were three parameters considered in this experiment to get the optimal result for extraction method. All the combination of parameters were tested one by one with the same extraction procedure. However, there were different temperature levels for incubation which were tested for the effects of metabolites quality and quantity. The temperatures were tested one by one with 10^{o}C, 20^{o}C, 30^{o}C and 40^{o}C. The second parameter was the extraction times which the samples were treated for once, twice, and finally for the third time. Lastly, the incubation period is recorded for 1 hour, 2 hours, 3 hours and 4 hours.

3.4 Pre-treatment and grinding

The leaves samples from *M. sagu* were cleaned with 70% ethanol. In this experiment, triplicates samples were used for each type of test. Several leaves samples were taken adequately and snap-freeze with liquid nitrogen. Then, the samples were grinded

immediately using mortar and pestle until become powder form. The mortar and pestle were rinsed with 5mL chloroform. The powder leaves were weighted for 2g and placed into every soda lime culture tubes on a weight balancer. 3mL of 0.15M of acetic acid is added in each lime culture tube.

3.5 Extraction

After extraction solvent (chloroform with methanol co-solvent) was added, the suspensions was shaken in platform shaker in four different condition (10 ^{o}C, 20 ^{o}C, 30 ^{o}C and 40 ^{o}C) which were tested at different time for different period of incubation as 1 hour, 2 hours, and 3 hours in the experiment (time incubation change in next experiment). The supernatant was filtered using filter paper and collected in an evaporating flask. The supernatant was collected before dried in rotary evaporator.

In order to test for several times, the material needs to be extracted to gain almost quantitative extraction of metabolites, leaf powdered (pellet) were extracted for 1, 2, and 3 times in methanol: chloroform (2:1). The suspensions were incubated at temperature 10^{o}C, and then followed by 20^{o}C, 30^{o}C and 40^{o}C in the next experiment.

3.6 Phase separation

This phase was conducted if only the two phases of methanol and chloroform do not separate completely. Ultra pure water was added into the pool of supernatant until three layers were seen. The upper phase was methanol, the middle phase was ultra pure water and at the bottom phase was chloroform. The chloroform phase was only collected for next step, evaporating step in evaporating flask. The other phases were thrown away in a waste bottle.