ULTRASONIC EXTRACTION OF ANTHOCYANINS FROM *CLITORIA TERNATEA* FLOWERS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

This thesis deals with the anthocyanins extraction from *Clitoria ternatea* flowers using ultrasonic extraction with response surface methodology (RSM). The objective of this thesis were to investigate a simple, sequential and standardized method to obtain a high yield of anthocyanins from *Clitoria ternatea* flowers by ultrasonic extraction compared to conventional solvent extraction, evaluate the effect of extraction factors which were extraction temperature (30-50 °C), extraction time (30-150 min), ratio of liquor to solid (2-15 ml/g) and sonication power (96-240 W) on the extraction efficiency by performing first level optimization in two-level factorial design with Design Expert 7 software and measure the antioxidant activity (AA) using radical scavenging activity (RSA) method of 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay. This study will benefit consumers and food industry where healthier alternatives could be introduced in their diet and into the production of food. Ultrasonic extraction using water solvent was used to extract the anthocyanins where its screening test experimental variables were optimized via Design Expert 7 software using RSM. Ultrasonic extraction showed a 246.4789 % better efficiency than conventional solvent extraction. The anthocyanins extracts exhibited a DPPH activity of 68.48 % at the optimized experimental variables. In conclusion, ultrasonic extraction is a viable extraction method for extracting anthocyanins with high antioxidant activity from *Clitoria ternatea* flowers. It is recommended to further optimize the screening test results in a two-level factorial design.

PENGEKSTRAKAN CARA ULTRASONIK BAGI ANTHOCYANINS DARIPADA BUNGA *CLITORIA TERNATEA* MENGGUNAKAN METODOLOGI TINDAK BALAS PERMUKAAN

ABSTRAK

Tesis ini membentangkan penyelidikan menggunakan ultrasonik sebagai cara pengekstrakan anthocyanins daripada bunga *Clitoria ternatea* menggunakan metodologi tindak balas permukaan (RSM). Objektif tesis ini adalah untuk menyiasat satu cara yang senang, teratur dan seragam untuk mendapatkan hasil tinggi untuk anthocyanins daripada bunga *Clitoria ternatea* menggunakan cara ultrasonik dibandingkan dengan cara pelarut, menilai faktor pengekstrakan ultrasonik iaitu suhu (30-50 °C), masa (30-150 min), nisbah pelarut kepada berat bunga (2-15 ml/g), kuasa ultrasonik (96-240 W) dalam kecekapan pengektsrakan dalam tahap 1 dalam reka bentuk dua-tahap faktorial menggunakan perisian Design Expert 7 dan mengukur aktivit antioksidan menggunakan aktiviti reduksi radikal menggunakan cara 2,2-diphenyl-2-picrylhydrazyl (DPPH). Tesis ini akan memanfaatkan pengguna dan industry makanan di mana alternatif lebih sihat boleh digunakan dalam diet mereka dan penghasilan makanan. Pengekstrakan ultrasonik menggunakan pelarut air diaplikasikan untuk mengekstrak anthocyanins di mana optimasi faktor dijalankan menggunakan perisian Design Expert 7 dengan RSM. Pengekstrakan ultrasonik menunjukkan hasil 246.4789 % lagi bagus daripada pengekstrakan pelarut. Anthocyanins yang diekstrak menunjukkan aktiviti DPPH sebanyak 68.48 % menggunakan nilai optimum faktor pengekstrakan ultrasonik. Kesimpulannya, pengekstrakan ultrasonik ialah satu cara pengekstrakan yang bagus untuk ekstrak anthocyanins yang mempunyai aktiviti antioksidan yang tinggi daripada bunga *Clitoria ternatea*. Adalah dicadangkan untuk mengoptimumkan nilai tahap 1 reka bentuk dua-tahap faktorial dalam tahap 2.

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LIST OF ABBREVIATIONS

A	Absorbance
AA	Antioxidant activity
ACOCSE	Anthocyanins content of conventional solvent extraction
ACOUE	Anthocyanins content of ultrasonic extraction
ANOVA	Analysis of variance
CCD	Central composite design
DPPH	2,2-diphenyl-1-picrylhydrazyl
DF	Dilution factor
DNA	Deoxyribonucleic acid
DOE	Design of experiment
EtOH	Ethanol
FRAP	Ferric reducing antioxidant power
HPLC-MS	High performance liquid chromatography-mass spectrometry
MCC	Metal-chelating capacity
MW	Molecular weight
ORAC	Oxygen radial absorption capacity
RSA	Radical scavenging activity
RSM	Response surface methodology
SE	Solvent extraction
ST	Screening test

TAC	Total anthocyanins content
UE	Ultrasonic extraction
VT	Validation test

LIST OF SYMBOLS

° C	SI unit for temperature
%	percentage
e	molar absorbance
g	unit of weight, gram
mg	unit of weight, milligram
µg	unit of weight, microgram
kHz	unit of frequency, kilohertz
l	unit of volume, liter
ml	unit of volume, milliliter
µl	unit of volume, microliter
M	unit of concentration, molar
N	unit of concentration, normality
mM	unit of concentration, millimolar
W	unit of power, Watt

CHAPTER 1

INTRODUCTION

1.1 Background Information

Food additives have been used extensively in the food and beverage industry. It is added because people tend to associate certain colors with certain flavors, and the color of food can influence the perceived flavor in anything from candy to wine, (Delwiche, 2003). Thus addition of food additive is paramount in attracting customers. However, consumers nowadays are interested in using natural pigments as opposed to synthetic additives used in food industry due to the increasing health awareness of consumers for a healthier diet (Montes et al., 2005). Commonly found in plants, bioflavonoids anthocyanin is the functional pigments that produce the colors orange, violet, blue, red or purple of plants. Besides that, Bridle and

Timberlake (1997) stated that anthocyanins are the most crucial water-soluble pigments in plants. Moreover, Mazza and Miniati (1993) mentioned that with bright colors and high water solubility, anthocyanins are considered an alternative natural pigment to replace artificial food colorants.

Interest has surged on the usage of anthocyanin in the food industry due to them being natural food colorants and their budding health-promoting properties. *Clitoria ternatea* flowers are commonly known as blue pea flowers or butterfly pea flowers due to its distinctive colors. *Clitoria ternatea* flowers have long been recognized to be highly beneficial especially in Ayurveda (disease prevention and health-promoting in Indian medicine) approach and in many regions of the world (Mukherjee et al., 2008). *Clitoria ternatea* flowers have a vivid blue or white color and are normally associated as food colorant in Southeast Asia due to its high stability (Mukherjee et al., 2008). Anderson and Jordheim (2006) mentioned that cyanidin, delphinidin, peonidin, malvidin, pelargonidin, petunidin are commonly found in plants. The petals of blue pea flowers contain ternatins which are blue anthocyanin (Srivastava and Pande, 1977). Ternatins are a group of 15 delphinidin 3-*O*-(6''*O*-malonyl)-β-glucoside-3',5'-glucosides which are *p*-coumaroylated or variously glucosyl-*p*-coumaroylated at 3'- and/or 5'-glucosyl groups (Kogawa et al., 2006) . Honda and Saito (2002) stated that one of the factors for *Clitoria ternatea* blue flowers petal is the polyacylation of ternatins with *p*-coumaroyl groups due to polyacylation with aromatic acyl groups generally contribute to make anthocyanin bluish under a physiological pH by intramolecular co-pigmentation among aromatic acyl groups and an anthocyanidin chromophor.

The benefits of anthocyanin pigments are tremendous. They are strong antioxidants, anti-inflammatory with cancer chemopreventive and antimutagenic property (Kong et al., 2003). Anthocyanin is known to have a high free radical scavenging properties which will minimize the risk of cardiovascular diseases due to their pro-cardiovascular properties (Bonerz et al., 2006) and shield cells from oxidative damage (Bae and Suh, 2007). As mentioned earlier, anthocyanin is instrumental in reducing risk of cancer by being anticarcinogenic (Lee et al., 2009) and reducing the risk of progression of tumors to malignant state by being anti-angiogenic (Bagchi et al., 2004).

Conventional extraction methods of active compounds using solvent extraction (SE) or thermal extraction involves long extraction hours, low extraction efficiency and could result in the degradation of anthocyanin and a decrease of the antioxidant activity of the extracts (Camel, 2000; Lapornik et al., 2005). In the process of ultrasonic extraction (UE), the concept utilized is the production of acoustic cavitation that causes molecular movement of solvent and sample which could result in the breakdown of sample micelle or matrix to the intracellular hydrophobic compounds due to the frequency of ultrasonic. This means that there is no chemical used in UE, thus reducing the possibility of chemical degradation of the targeted compounds. Advantages that are brought by UE are improved extraction efficiency, low solvent usage, high level of automation and reduced extraction time (Wang and Curtis, 2006).

Response surface methodology (RSM) is commonly used for optimization of a process. It is efficient as it reduces the number of experimental trials required to evaluate the interactions of multiple parameters, less time-consuming and less taxing (Giovanni, 1983). Due to this, RSM is widely applied in optimizing the extraction process variables like anthocyanin, phenolic compounds and polysaccharides (Cacace and Mazza, 2003; Chandrika and Fereidoon, 2005; Liyana-Pathirana and Shahidi, 2005; Qiao et al., 2009).

Anthocyanin antioxidant properties need to be assessed due to their potential important uses in medicine, food and cosmetics. Living system generates various reactive species namely free radicals and reactive oxygen species (ROS). These free radicals and ROS could increase oxidative stress and cause diseases such as cancer and cardiovascular diseases (Noguchi and Niki, 2000; Grune et al., 2001). 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a commonly applied standard to assess antioxidant properties of a compound from plants in different solvent system (Cheng et al., 2006). DPPH is a stable radical and appears in purple color absorbing at 517 nm in ethanol. DPPH will change to yellow with concomitant decrease in absorbance at 517 nm when it is reacted with antioxidant.

1.2 Problem Statements

Delgado-Vargas and Paredes-López (2003) have stated that there is a growing interest in further use of natural food colorant. This means *Clitoria ternatea flowers* could show a very promising alternative natural food colorant to synthetic food colorant in the food industry. Anthocyanin from *Clitoria ternatea* flowers as natural food colorant could present more health benefits as compared to synthetic food colorant (Mukherjee et al., 2008). Though the usage of synthetic food colorant in the food industry is long established, the introduction of *Clitoria ternatea* flowers as synthetic food additive alternative at best is still at its infant stage. This is due to the fact that there is no extraction process or guideline that has been done on *Clitoria ternatea* flowers. Thus, efforts need to be done to encourage *Clitoria ternatea* flowers as a replacement or alternative to synthetic food colorant owning to its many advantages.

Researches have shown that UE is suitable for extracting bio-compounds from plants (Wang and Curtis, 2006). UE is a process that produces yield that requires shorter extraction time, high level of automation, low solvent consumption and increased efficiency compared to conventional method like solvent extraction and thermal extraction (Chen et al., 2007). Suitable parameters for UE setting point need to be known for extraction of anthocyanin from *Clitoria ternatea* flowers due to its (UE process) many benefits. Hence, this research put the theory into test by proving that a high yield of anthocyanin from *Clitoria ternatea* flowers could be (was) obtained by UE process. Anthocyanins extracts were further subjected for antioxidant activity test by DPPH assay to cement its reputation of an antioxidant compound.

1.3 Research Objectives

1.3.1 To investigate a simple, sequential and standardized method to obtain a high yield (>80%) of anthocyanins from *Clitoria ternatea* flowers by ultrasonic extraction compared to conventional solvent extraction.

1.3.2 To perform first level optimization of two level factorial design for ultrasonic extraction.

1.3.3 To measure the antioxidant activity (AA) of anthocyanins extract using radical scavenging activity (RSA) method of DPPH assay.

1.4 Scope of Research

Flowers of *Clitoria ternatea* (blue pea flower) was used in this research. The proper selection of process variables was needed to obtain high efficiency in terms of high yields on anthocyanin extraction. Hence, the optimum UE process parameters conditions which were extraction temperature (°C), extraction time (min), ratio of liquor to solid (ml/g) and sonication power (W) were determined. This was done by performing first level optimization on Design Expert 7 software in two-level factorial design to obtain the highest yield of anthocyanin possible. Measurement of anthocyanin extracts yield was done on UV-VIS spectrophotometer. Then, this study only studied two extraction methods, namely ultrasonic extraction and conventional solvent extraction. This research limited the parameters setting to the ultrasonic benchtop cleaner machine as the laboratory only has this ultrasonic machine. Then, only RSA method of DPPH assay was used to measure the anthocyanins extracts AA to validate the claim that natural food colorant has health benefits.

1.5 Research Outcome

This study claims to produce first level optimization for ultrasonic extraction of anthocyanin from *Clitoria ternatea* flowers of over 80% as compared to the conventional solvent extraction. This serves as model verification for first level optimization which could be used for second level optimization in two-level factorial design. The percentage of DPPH anthocyanins extracts was >50%.

1.6 Significance of the Research

There are two biggest beneficiaries from this proposed research, consumers and food industry. Consumers would benefit as better food colorant in term of nutrients can be used in their food which would bring greater benefit to their health. Consumers nowadays who are health conscious would welcome better nutrient food colorant in their food. Food industry would benefit as more consumers would prefer product that is healthy. Food producers that apply this product should see significance growth in their sales as consumers prefer healthier products. Thus the significance of this proposed research would also be instrumental encouraging the addition of healthy natural colorants into commercial food products that would present greater health benefits for consumers and encourage the food industry to replace synthetic food colorant with natural food colorant due to the increasing health awareness among consumers.

1.7 Conclusion

This chapter has explained on the background information of the research itself in terms of current food colorant, promising benefits of *Clitoria ternatea* flowers, ultrasonic extraction, RSM and DPPH assay. Problem statement, research objectives and significance of research were discussed to explain the purposes, significance and benefits of this research. Lastly, scope of the research and research outcomes were stated to show that the research objectives has been achieved.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter discusses in detail about the *Clitoria ternatea* plants, health benefits of anthocyanin, commercialization of anthocyanin, non-thermal extraction, ultrasonic extraction method and DPPH radical scavenging activity.

2.2 Blue Pea Flower (*Clitoria Ternatea* Flower)

2.2.1 Plant Morphology

Clitoria ternatea flowers can grow up to 2-3 m in height with its perennial climber characteristic as shown in Plate 2.1. It is usually found growing in the wild and also cultivated domestically bearing white or blue flowers with shape of a conch-shell in a humid tropics of the old and new world below 1600 m elevation (Morton, 1981). It is widely distributed in tropical Asian countries like India, Philippines and South and Central America as well (Sivaranjan and Balachandran, 1994). *Clitoria ternatea* seeds have subglobose or oval in shape with either blackish or yellowish-brown color. Its root system is made up of many slender lateral roots and a fairly stout taproot with few branches. Karandikar and Satakopan (1959) mentioned that the plant grows papilionaceous, solitary and axillary flowers in bright blue or white with orange or yellow center as shown in Plate 2.2 with bright blue petals and yellow center. Pillai (1976) supported this by reinforcing the description with the pods being flat, sharply beaked and 5-10 cm long.