OPTIMIZATION OF EXTRACTION PARAMETERS OF TOTAL PHENOLIC COMPOUND FROM *Cosmos caudatus*

HAZWANI BINTI ZULKIPLY

Thesis submitted in fulfillment of the requirements for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2012

SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of degree of Bachelor of Chemical Engineering (Biotechnology).

Signature
Name of Supervisor:
Position:
Date:

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature

Name: HAZWANI BINTI ZULKIPLY

ID Number: KE08039

Date:

Dedicated to my parents

Zulkiply Bin Yope & Su Bashitah Binti Ali

ACKNOWLEDGEMENT

First and foremost, I am most thankful to The Almighty for granting me such a lifetime opportunity. I am heartily thankful to my supervisor, Miss Rohana Binti Abu, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. This thesis would not have been possible without her review and correction of my many mistakes, accompanied by the countless valuable suggestions.

I would like to acknowledge the staff of Faculty of Chemical and Natural Resources Engineering of Universiti Malaysia Pahang for providing the physical necessities of completing the project as well as generous access to unlimited support, either of internal or external resources. It is an honor for me to thank the educators and professors for all the theoretical and life lessons learnt this whole time. Special thanks to my Academic Advisor, Dr. Mimi Sakinah Abdul Munaim for all the time spent guiding me through these memorable years.

I owe my deepest gratitude to my parents for their undeniable love, understanding and sacrifice throughout my life. It is a pleasure to thank my beloved family for their continuing encouragement and moral support whenever needed. I am sincerely grateful to my fellow colleagues for the willingness of going through all the ups and downs together. Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

ABSTRACT

The wide ranges of extraction parameters used being identified from previous studies derives the need to find the best conditions to yield optimum extraction of total phenolic compounds from *Cosmos caudatus*. The objective of this research is to determine the optimum extraction parameters, namely ultrasonic frequency (from 30 to 70 kHz), sample-to-solvent ratio (from 2 to 10 w/v %) and extraction time (from 30 to 300 minutes) of total phenolic compound from *Cosmos caudatus*. The experimental design was first generated from Response Surface Methodology by using Design Expert 7.1.6 with three independent variables, namely ultrasonic frequency, sample-to-solvent ratio (SSR) and extraction time. Results showed that the optimization of extracting total phenolic compounds (TPC) from *Cosmos caudatus* can be accomplished by employing ultrasonic frequency of 70 kHz, 2g dry sample/100mL ethanol and extraction time of 300 minutes with yield of 7.7395 mg GAE/g dw which is in close agreement with the predicted value (7.5359 mg GAE/g dw). Analysis of variance showed significant ultrasonic frequency and sample-to-solvent ratio, but insignificant extraction time. This might be partly due to phenolic oxidation during the extraction itself. Since previous and present studies suggests that the extraction of total phenolic compounds can be further optimized, upcoming studies need to be directed at varying the significant extraction parameters including the extraction temperature, types of solvent used and extraction methods.

ABSTRAK

Julat faktor pengekstrakan yang luas telah digunakan dalam pelbagai kajian terdahulu menimbulkan keperluan untuk menentukan kondisi terbaik untuk menghasilkan pengekstrakan optimum kandungan keseluruhan fenol daripada ulam raja. Jadi, tujuan kajian ini adalah untuk menentukan nilai optimum bagi faktor pengekstrakan kandungan keseluruhan fenol daripada ulam raja iaitu frekuensi ultrasonic (30 hingga 70 kHz), nisbah sampel : pelarut (2 hingga 10 w/v%) dan tempoh pengekstrakan (30 hingga 300 minit). Corak eksperimen diperoleh terlebih dahulu daripada simulasi Response Surface Methodology menggunakan Design Expert versi 7.1.6 dengan tiga pembolehubah tidak bergantung, iaitu frekuensi ultrasonik, nisbah sampel : pelarut dan tempoh pengekstrakan. Keputusan yang diperolehi menunjukkan pengoptimuman mengekstrak kandungan keseluruhan fenol daripada ulam raja boleh dicapai menggunakan frekuensi ultrasonik 70kHz, nisbah 2 g sampel kering dalam 100mL etanol dan tempoh pengekstrakan selama 300 minit yang menghasilkan 7.7395 mg GAE/g dw dan didapati hampir kepada nilai yang dijangka (7.5359 mg GAE/g dw). Analisis varians menunjukkan frekuensi ultrasonik dan nisbah sampel : pelarut sebagai signifikan manakala tempoh pengekstrakan adalah tidak signifikan. Ini mungkin disebabkan pengoksidaan fenol ketika pengekstrakan itu sendiri. Memandangkan kajian terdahulu dan kini mencadangkan bahawa pengekstrakan kandungan keseluruhan fenol boleh terus dioptimumkan, kajian pada masa akan datang harus difokuskan untuk mempelbagaikan faktor pengekstrakan yang penting seperti suhu pengekstrakan, jenis pelarut yang digunakan dan teknik pengekstrakan.

TABLE OF CONTENTS

CHAPTER 3 METHODOLOGY

CHAPTER 4 RESULTS AND DISCUSSION

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

APPENDICES

LIST OF TABLE

LIST OF FIGURE

LIST OF SYMBOLS

℃	degree Celcius
kHz	kilo Hertz
min	minute
w/v %	weight of sample / volume of solvent %
mL	mili Liter
mg/L	mili gram / Liter

LIST OF ABBREVIATIONS

C.Caudatus	*Cosmos caudatus*
TPC	total phenolic compound
RSM	Response Surface Methodology
SSR	sample-to-solvent ratio
GAE	Gallic acid equivalent
fw	fresh weight
dw	dry weight

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Cosmos caudatus (ulam raja) is an annual, short-lived, perennial, aromatic herb found to be containing extremely high antioxidant capacity (Shui *et al*., 2005). *Cosmos caudatus* originated from tropical Central America and is now widespread in almost all tropical regions. Its young leaves are often eaten raw with chilli or coconut paste and are used in dishes such as kerabu. They are also used as an appetiser and food flavouring due to their unique taste and aroma. Several bioactive components in ulam raja have been reported. For instance, Ragasa *et al*. have reported several antimutagen and antifungal compounds from ulam raja, e.g. cotunolide, stigmasterol, lutein and 4,4′-bipyridine; Zanariah *et al*. have reported protein and amino acid compositions of ulam raja.

Total phenolic compounds (TPC) are common dietary phytochemicals found in fruits, vegetables and grains. Most of the beneficial characteristics of phenolic compounds have been ascribed to their antioxidant activity which is a fundamental property important to life (Rice-Evans *et al*., 1997). Among the phytochemicals, phenolic compounds are reputed to be the main contributor of antioxidant activity in plant extracts due to their higher value in total content (Hodzic *et al*., 2009), interaction and redox property of an individual or combination of their diverse chemical structures with assay used (Teixeira *et al.*, 2005) and their synergistic effectiveness as hydrogen donors, reducing agents and free radical scavengers (Vattem *et al*., 2005; Zhou *et al*., 2009).

Figure 1.1: *Cosmos caudatus*

Phenolics are phytochemicals extensively distributed among plants that have been receiving great deal of attention for their functionality. Although chemicals are commonly employed to isolate phenolics, the use of physical treatments such as sonication is still limited. Study done by Department of Food Science, University of Arkansas was conducted to optimize a procedure to isolate phenolics from rice bran using sonication as a preextraction treatment. Sonication was optimized by varying output, time, and temperature. Extraction was optimized by varying solvent, extraction time, temperature, and sample-to-solvent ratio (Onofre and Hettiarachchy, 2007).

Sonication has numerous effects, both chemical and physical. The chemical effect of ultrasound, i.e., sonochemistry is concerned with understanding the effect of sonic waves on chemical systems. The chemical effects of ultrasound do not come from a direct interaction with molecular species. Studies have shown that no direct coupling of the acoustic field with chemical species on a molecular level can account for sonochemistry or sonoluminescence. Instead, sonochemistry arises from acoustic cavitation: the formation, growth, and implosive collapse of bubbles in a liquid. As liquids cannot flow as fast as crystals oscillate, during the contraction small vacuum cavities are formed. When the crystals expand, the cavities rapidly implode and create microscopic shock waves. This process, known as cavitation, is extremely powerful when the collective energy of all the imploding cavities is combined. The cavities are formed and collapse in

microseconds which releases tremendous energy within the liquid (Suslick and Flannigan, 2008).

Figure 1.2: Sonication bath

1.2 PROBLEM STATEMENT

There have been a number of researches on the extraction of total phenolic compounds, from various plants such as *Murraya koenigii, Citrus hysrix* and *Pandanus odurus* as well as *Cosmos caudatus*. Unlimited to this only, extraction of numerous compounds from *Cosmos caudatus* such as flavanoid, polyphenols, polypropane and total phenolic compounds too have been extensively carried out. Thus, the wide ranges of extraction parameters used being identified, derives the need to find the best conditions to yield optimum extraction of total phenolic compounds from *Cosmos caudatus*. Therefore, this study was conducted to determine the optimum value of ultrasonic frequency, sample-to-solvent ratio and extraction time based on the minimum and maximum limits obtained from the previous studies.

1.3 RESEARCH OBJECTIVE

The objective of this research is to determine the optimum extraction parameters (ultrasonic frequency, sample-to-solvent ratio and extraction time) of total phenolic compound from *Cosmos caudatus*.

1.4 SCOPE OF STUDY

There are three scopes of this research which are;

1.3.1. Determining the linear effect of extraction parameters on total phenolic compound yield from *Cosmos caudatus* extract.

1.3.2. Determining the interaction effect between the extraction parameters on total phenolic compound yield from *Cosmos caudatus* extract.

1.3.3. Determining the optimum extraction parameters on total phenolic compound yield from *Cosmos caudatus* extract.

1.5 SIGNIFICANCE OF STUDY

Identifying the optimum extraction parameters for total phenolic compounds from *Cosmos caudatus* would definitely be beneficial in the large-scale industries in terms of saving on the operational cost and time. Furthermore, the extraction and purification of phytochemicals from natural sources is needed, since these bioactives are often used in the preparation of dietary supplements, nutraceuticals, functional food ingredients, food additives, pharmaceutical and cosmetic products (Gao and Mazza, 1996). High content of antioxidants contained in ulam raja could be partly responsible for its ability to reduce oxidative stress. This is in addition to the major role played by *Cosmos caudatus* as a natural supplement which is undeniably much better than chemicals as they could go a long way ahead of pharmaceutical medications in enhancing health and vitality.

CHAPTER 2

LITERATURE REVIEW

2.1 TOTAL PHENOLIC COMPOUNDS

Phenolics, which are widely distributed in plant kingdom, appear to have desirable medicinal properties and play a major role in both plant and animal health. Some have been reported to be antitumor agents and to exhibit antiviral and antimicrobial activities, hypotensive effects and antioxidant properties. These compounds, either as isolates or in conjunction with other compounds, may be used for various health benefits (P Jamal *et al*., 2010).

Antioxidant treatments are thought to offset radical damage to biomolecules, thereby slowing or delaying the onset of the diseases by preventing oxidative stress. Phenolic compounds, as major natural antioxidants of many fruits and vegetables, are currently the focus of nutritional and therapeutic interest. Foods and beverages rich in phenolic compounds have been associated with decreased risk of age-related diseases in some epidemiologic studies (Shui *et al*., 2005).

C. caudatus is believed to promote the formation of healthy bones and is said to be useful in 'cleansing the blood' (Burkill, 1966; Ismail, 2000). The methanol extracts of *C. caudatus* have been reported to show moderate antioxidant activity when tested using the xanthine–xanthine oxidase enzymatic assay (Norhanom *et al*., 1999). Recently, antioxidative and radical-scavenging activities of compounds isolated from this plant have been reported (Abas *et al*., 2003).

Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans *et al*., 1995). The phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Kähkönen *et al*., 1999). The importance of natural phenolic compounds from plants materials is also raising interest among scientists, food manufacturers, and consumers due to functional food with specific health effects (Löliger, 1991).

Phenolics are carbon-based compounds present in many plants. They are of general interest because of their wide ranging ecological effects from the organism to ecosystem level (Appel, 1993). They are perhaps most noted for their ability to bind proteins in vitro, forming soluble and insoluble complexes (Goldstein and Swain, 1965; Feeny, 1976; Hagerman and Butler, 1980; Mc Manus *et al.*, 1981; Hagerman and Klucher, 1986; Hagerman and Robbins, 1987). These phenolic-protein interactions are thought to be, inpart, responsible for the putative function of phenolics as plant defense compounds (Feeny, 1976; Rhoades and Cates, 1976; Coley, 1983; Mole and Waterman, 1987).

Research done by Arbianti *et al.* (2007) showed that phenolic compounds and most other reported bioactive compounds are generally more soluble in polar solvents and the presence of phenolic compound in extract determined that the presence of antioxidant compound. Antioxidant activity of an extract from plants can be related with its phenolic content. The majority of the antioxidant activity of fruits and vegetables may be from phenolic compounds rather than vitamin C and E, or ß-carotene since some phenolic compounds have much stronger antioxidant activities against peroxyl radicals. Phenolic compounds had reported to possess antioxidant activity that allows them to scavenge both active oxygen species and electrophiles, to inhibit nitrosation and to chelate metal ions, to have the potential for auto-oxidation and the capability to modulate certain cellular enzyme activities.

Several studies had been conducted to evaluate the correlation between phenolic compounds and antioxidant activity. The antioxidative properties of some vegetables and fruits are partly due to the low molecular weight phenolic compounds, which are known to be potent as antioxidants (Wang *et al*., 1999).

2.2 EXTRACTION PARAMETERS

Numerous studies have been conducted on the extraction of total phenolic compounds from various plant materials and extraction of several phytochemicals from *Cosmos caudatus* employing a very wide range of extraction parameters. Extraction of 50mg dried *Cosmos caudatus* using 95 v/v% ethanol and centrifugation of 5 minutes yields 1.52 mg gallic acid extract (GAE)/g fresh weight sample (Andarwulan *et al*.,2010). Meanwhile, Sulaiman *et al*. (2010) used 70v/v% methanol to extract 10g *C.caudatus* at 27°C for 1 hour and 27.7 mg GAE/g dried weight sample was obtained. According to Abas *et al*. (2006), extracting 4mg dried *C.caudatus* leaves using 99.5% ethanol at 40°C would result in 0.23w/w% yield. On the other hand, Shui *et al*. (2005) stated that extracting 100g fresh *C.caudatus* using 50% ethanol at 80°C for 45 minutes would yield 12 mg GAE/ g fw. Using 80% methanol to extract 500g dried *C.caudatus* roots for 10 minutes at room temperature yields 73 mg GAE/mg dw (N. Fuzzati *et al.,* 2000).

According to Wong *et al.* (2006), extracting 0.5g dried *C.caudatus* leaves using 25mL deionised water at room temperature for 1 hour with occasional agitation will produce the highest antioxidant capacity. Huda-Faujan *et al*. (2007) soaked samples with methanol for seven days, where methanol was later completely removed by vacuum evaporator at 50°C yielding 18.83 mg TAE/100g fw. On the other hand, Sukrasno *et al*. (2011) stated that extracting 1g dried *C.caudatus* with 70% methanol at 60°C yielded 11.4 mg/g. Meanwhile, extracting 1g dried *C.caudatus* using 10mL ethanol for 24 hours with 50rpm agitation speed yields 4480 mg GAE/L (P. Jamal *et al*., 2010).

Accumulating evidence has suggested that the recovery, yield and type of polyphenolics in an extract are influenced by the type and polarity of extracting solvents,

time and temperature of extractions as well as physical characteristic of the samples (Naczk and Shahidi, 2006). The selection of solvent systems for this study was made on the basis of their reported efficiency in extracting polyphenols and other antioxidant compounds from fresh sample matrix (Luthria *et al.,* 2006; Sun *et al.*, 2007; Alothman *et al.,* 2009). The details on the extraction parameters are summarized in Table 2.1.

Table 2.1: Various extraction parameters on different plant extracts

Authors	**Research**	**Findings**
Chukwumah *et al.*, 2009	Extraction of selected isoflavones and trans-resveratrol from peanuts (*Arachis hypogaea*)	Ultrasonication extraction depends on frequency, duration of sonication, and the combination of both the frequency and duration (time) of sonication.
Melecchi *et al.*, 2006	Optimization of the sonication extraction method of *Hibiscus tiliaceus L*. flowers	The most influential parameters are solvent polarity and extraction time.
Arbianti *et al.*, 2007	Comparison of antioxidant activity and total phenolic content of *Dillenia indica* leaves extracts obtained using various techniques	The total phenolic content of extracts affected by the extraction method and operating conditions performed. High pressure extraction method with circulation produces extracts that have total phenolic content higher than sonication and soxhlet extraction method.
Fuzzati *et al.*, 1994	Phenylpropane derivatives from roots of *Cosmos caudatus*	Although phenylpropane derivatives are well known antifungal compounds, activity against *C. albicans* suggests that the epoxy moiety is an important structural element. On the contrary, for activity against *C. cucumerinum* it is not possible to make any comment on structure-activity relationships.
Naczk and Shahidi, 2006	Solvent extraction systems to procure antioxidants from oilseed	Yield of polyphenols depend on type and polarity of extracting solvents, time and temperature of extractions

Total phenolic content of an extract can be evaluated with spectrophotometer method using Folin-Ciocalteu reagent. The principle of this method is reduction ability of phenol functional group. Oxidation and reduction reaction of phenolat ion takes place at base condition. The reduction of fosfotungstat-fosfomolibdenum complex (Folin-Ciocalteu reagent) by phenolat ion will change its color to be blue. The reduction of complex will increase when the extract contain more phenolic compounds. Thus, the color will be darker and the absorbance will be higher (Arbianti *et al.*, 2007).

2.3 SONICATION

Plant derived phytochemicals have been the focus of recent research due to their health promoting effects. Previous studies to estimate the levels of these bioactive compounds made use of traditional solvent extraction procedures such as homogenization and soxhlet (reflux) methods. Recently, the ultrasonication technique has been shown to be an efficient non-thermal extraction method (Chukwumah *et al*., 2009).

Ultrasonication involves the use shear force created by the implosion of cavitation bubbles of ultrasonic waves (sound waves in the kHz range) to alter material properties thereby further disrupting plant tissues and facilitating extraction. It however requires a medium such as water for radiation of the sound waves. Its improvement on extraction efficiency is as a result of the enhancement of cell disruption, solvent penetration and mass transfer (Chukwumah *et al*., 2009).

In the research done by Arbianti *et al.* (2007), sonication method was applied by mixing two grams of *Dillenia indica* leaves powder with ethanol as the solvent and extracted using sonication (room temperature, 42 kHz, 50 minutes). The parameters that varied in this method are solution concentration followed by extraction time variation. The optimum condition to obtain extract with highest antioxidant activity using sonication method is at concentration 2/100 with extraction time 50 minutes. Meanwhile, total phenolic content of extract with highest antioxidant activity from each variation was determined using Folin-Ciocalteu reagent. Gallic acid was used as standard. Sample was

diluted in ethanol (200 ppm). Standard solution was made with concentration 5, 10, 15, 20, 40, 60, 80, 100, 125 and 150 ppm. Each solution was pipette 1 ml and putted into flask. Each solution was added 9 ml aquades and 1 ml Folin-Ciocalteu. After 5 min, each mixture was added 10 ml Na_2CO_3 (7%) and was diluted with aquades until 25 ml. After 90 min, absorbance was read at 750 nm. Results were expressed as gallic acid equivalents (mg GAE/L).

2.4 RESPONSE SURFACE METHODOLGY

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods (e.g. finite element method or CFD analysis) and their associated numerical noise. The problem can be approximated with smooth functions that improve the convergence of the optimization process because they reduce the effects of noise and they allow for the use of derivative-based algorithms (Van Keulen *et al*., 2000).

Taking the combined interactions among various physical and chemical parameters into consideration, RSM presented a methodology for the construction of responses using both function values and derivatives on a weighted least-squares formulation. For example, the statistical response surface methodology (RSM) is a useful model for simultaneously studying the effect of several factors influencing the process of enzyme production. This also reduces the number of experiments required in growth medium optimization. Use of factorial designs and regression analyses for generating empirical models makes RSM a good statistical tool. To analyze the effect of various factors in better way, a number of statistical approaches with response surface methodology are attempted for the optimization of enzyme production (Singh *et al*., 2011).

CHAPTER 3

METHODOLOGY

3.1 MATERIALS USED

3.1.1 Plant materials

Cosmos caudatus (ulam raja) was purchased from local markets in Kuantan, Pahang. The edible portion of fresh samples were cleaned and washed under running tap water. The samples were dried in the oven at 60°C for 48 hours. Then, the samples were weighed and blended using dry blender before being stored at 4°C until further use (Huda-Faujan *et al.*, 2007).

3.1.2 Chemicals

All chemicals and reagents used in this study were of analytical grade. 95% ethanol, Folin-Ciocalteu phenol reagent, gallic acid and anhydrous sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich Chemicals (Sulaiman *et al.,* 2011). 95% ethanol was used since it was found to be the most efficient solvent to extract total phenolic compounds from plant extracts (Andarwulan *et al.*, 2010) with sample-to-solvent ratio based on 100mL ethanol.

3.2 EXPERIMENTAL PROCEDURES

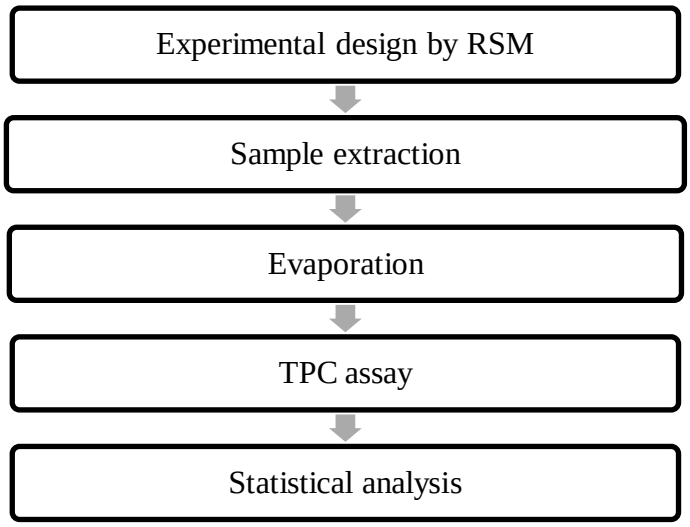


Figure 3.1: Experimental procedures

3.2.1 Experimental design by Response Surface Methodology

The upper and lower limits obtained from previous studies as shown in Table 3.1 were used in Response Surface Methodology (three-variable Central Composite Design) using Design Expert 7.1.6 to determine the optimum values of each process variables.

Table 3.1: Upper and lower limits of extraction parameters

Parameters	**Lower limit**	**Upper limit**
Ultrasonic frequency (kHz)	30	70
Sample-to-solvent ratio (w/v %)	2	10
Extraction time (min)	30	300

3.2.2 Sample extraction and evaporation

Employing modified method by Melecchi *et al.* (2005), the sonication bath was kept at constant temperature (25°C) during all the extraction processes and then evaporated to dryness under vacuum at 78°C using a rotary evaporator according to the simplified specifications outlined from previous step as shown in Table 3.2.

Table 3.2: Experimental design by Response Surface Methodology

Sample	**Ultrasonic frequency (kHz)**	**Sample-to-solvent ratio (w/v %)**	**Extraction time (min)**
1	20	6.0	165
2	30	10.0	30
3	30	10.0	300
4	30	2.0	30
5	30	2.0	300
6	70	10.0	30
7	70	10.0	300
8	70	2.0	30
9	70	2.0	300
10	80	6.0	165
11	50	6.0	165
12	50	6.0	165
13	50	6.0	165
14	50	6.0	165
15	50	6.0	165
16	50	6.0	165
17	50	6.0	165
18	50	6.0	165
19	50	6.0	395
20	50	13.0	165

3.2.3 Total Phenolic Assay

TPC of the extracts were measured using Folin-Ciocalteu method as described by Amin *et al*. (2004). All samples and readings were prepared and measured in triplicate.

Gallic acid was used as standard. 500 mg/L stock standard solution of gallic acid was prepared by dissolving 250 mg of dry gallic acid in 500 mL of extracting solvent. The stock solution was stored at 4°C. Working standards of between 100 and 500 mg/L were prepared by diluting the stock solution with distilled water. The extract was prepared at concentration of 1 mg/L. 100 mL of extract was transferred into a test tube and 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with deionised water) was added and mixed. The mixture was allowed to stand at room temperature for 5 min. Then, 0.75 mL of 6% (w/v) sodium carbonate was added to the mixture and mixed gently. After standing at room temperature for 90 min, the absorbance was read at 760 nm using UV/Vis spectrophotometer. The standard calibration curve of gallic acid (100–500 mg/L) was plotted (Almey *et al*., 2010).

Total phenolic content was determined using Folin–ciocalteu reagent following the method of Singleton and Rossi (1965) with slight modification using gallic acid as a standard. Briefly, 1 ml of extract solution was added in a 100 ml volumetric flask that contained about 60 ml distilled water. Then, 5 ml of Folin–ciocalteu reagent was added and the content of the flask was thoroughly mixed. After 1-8 minutes, 15 ml $Na2CO_3$ (20%) was added and the volume was made up to 100 ml using distilled water. The mixture was allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer. Total phenolic content was determined as mg of gallic acid equivalent (GAE) using an equation obtained from the standard tannic acid calibration graph (Huda-Faujan *et al.*, 2007).

3.2.4 Statistical analysis

Optimal conditions for the extraction of phenolic compounds from *Cosmos caudatus* depending on ultrasonic frequency, sample-to-solvent ratio (SSR) and extraction time course were obtained using the predictive equations of Response Surface Methodology using Design Expert 7.1.6. The experimental and predicted values were compared in order to determine the validity of the model (Chandrika and Fereidon, 2004).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 CALIBRATION CURVE

In order to determine the concentration of total phenolic compound, a calibration curve was first generated using gallic acid as the standard, yielding a linear calibration curve with R-squared value of 0.987.

.

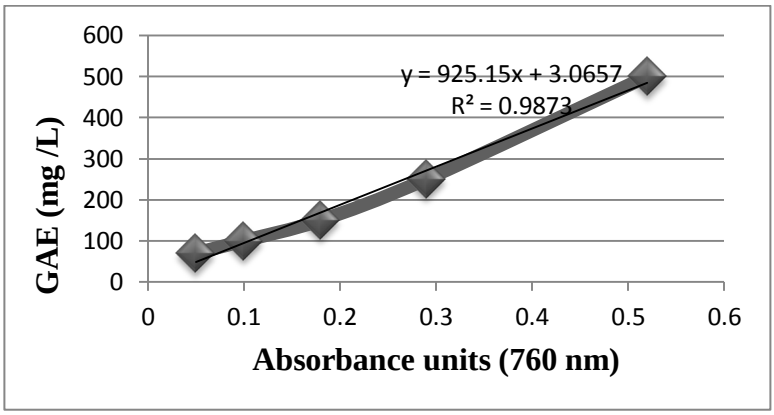


Figure 4.1: Gallic Acid Calibration Curve

Figure 4.1 shows mean total phenolic compounds (TPC) of the *Cosmos caudatus*' leave extracts measured using GAE (Equation 4.1)

$$y = 925.15x + 3.0657 \quad \textbf{(Equation 4.1)}$$

where x = absorbance at 760nm and

y = concentration of total phenolic compounds in mg per liter of the extract.

4.2 EXTRACTION YIELD

Table 4.1 lists the extraction parameters used for each run according to the experimental design by Response Surface Methodology, namely the ultrasonic frequency, sample to solvent ratio, extraction time and their respective concentration of total phenolic compounds yield.

Table 4.1: Total phenolic compounds yield from *Cosmos caudatus* extract

Trial no.	Factors			Response
	Ultrasonic frequency (kHz)	Sample to solvent ratio (w/v %)	Time (min)	TPC yield (mg GAE/g dw)
1	20	6.0	165	1.7162
2	30	10.0	30	1.1037
3	30	10.0	300	1.1964
4	30	2.0	30	5.5650
5	30	2.0	300	6.0163
6	70	10	30	1.4924
7	70	10	300	1.6682
8	70	2.0	30	7.5081
9	70	2.0	300	7.7395
10	80	6.0	165	2.4257
11	50	6.0	165	2.0710
12	50	6.0	165	2.0671
13	50	6.0	165	2.0594
14	50	6.0	165	2.0681

15	50	6.0	165	2.0659
16	50	6.0	165	2.0633
17	50	6.0	165	2.0837
18	50	6.0	165	2.0810
19	50	6.0	395	3.0271
20	50	13.0	165	1.0348

4.3 MODEL FITTING FROM RSM

Total phenolic compounds yield from *Cosmos caudatus* extract obtained were evaluated using Response Surface Methodology. The independent and dependent variables were then fitted to the second-order model equation (Equation 4.2).

$$y = 2.06 + 0.45\,A - 2.74\,B + 0.048\,C - 0.35\,AB - 0.017\,AC - 0.052\,BC + 0.10\,A^2 + 1.34\,B^2 + 0.42\,C^2 \quad \textbf{(Equation 4.2)}$$

Table 4.2 shows the analysis of the R-squared values. On the other hand, evaluation of the goodness of fit and the results of analysis of variance were shown in Table 4.3.

Table 4.2: R-squared values for TPC yield of *Cosmos caudatus* extract

Std. Dev.	0.29	R-squared	0.9899
Mean	2.85	Adj R-squared	0.9808
C.V.%	10.07	Pred R-squared	0.8494
PRESS	12.31	Adeq. precision	31.852

Table 4.3: ANOVA for TPC yield of *Cosmos caudatus* extract

Source	**Sum of Squares**	**df**	**Mean Square**	**F value**	**p-value Prob>F**	
Model	80.86	9	8.98	108.85	<0.0001	significant
A-frequency	2.50	1	2.50	30.30	0.0003	
B-SSR	65.03	1	65.03	787.92	<0.0001	
C-time	0.020	1	0.020	0.24	0.6337	
AB	0.98	1	0.98	11.92	0.0062	
AC	2.339E-003	1	2.339E-003	0.028	0.8697	
BC	0.021	1	0.021	0.26	0.6213	
A^2	0.10	1	0.10	1.23	0.2928	
B^2	15.19	1	15.19	183.99	<0.0001	
C^2	1.43	1	1.43	17.27	0.0020	
Residual	0.83	10	0.083			
Lack of fit	0.83	3	0.27	3882.54	<0.0001	significant
Pure error	4.957E-004	7	7.082E-005			
Cor total	81.69	19				

The analyses of variance (ANOVA) were performed to determine the lack of fit and the significance of the linear, quadratic and interaction effects of the independent variables on the dependent variables. The Model F-value of 108.85 implies the model is significant. Values of 'Prob > F' less than 0.05 indicate model terms are significant. In this case A, B, AB, B^2 and C^2 are significant model terms.

The lack of fit test is a measure of the failure of a model to represent data in the experimental domain at which points were not included in the regression (Varnalis *et al.*, 2004). The 'Lack of Fit F-value' of 3882.54 implies the Lack of Fit is significant. This situation is mainly contributed by the insignificance of the parameter extraction time. Failure to identify the relation of the parameter to the extraction yield then cause this 'Lack-of-Fit' to occur.

Meanwhile, coefficient of determination or r^2 is the proportion of the variation in the response attributed to the model rather than to random error and was suggested that for good fit model, r^2 should be at least 80%. The model ANOVA of regression model demonstrated that r^2 is 0.9899, which means 98.99% variability in the response could be explained by this model. The 'Pred R-Squared' of 0.8494 is in reasonable agreement with the 'Adj R-Squared' of 0.9808 with the higher value of adjusted r^2 indicates greater significance of the model (Singh *et al.*, 2011).

'Adequate Precision' measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 31.852 indicates an adequate signal. This model can be used to navigate the design space. On the other hand, a very low value of coefficient of variation (C.V. %) indicates better precision and reliability of the experiments executed (Singh *et al.*, 2011).

4.4 EFFECT OF EXTRACTION PARAMETERS ON TOTAL PHENOLIC COMPOUNDS YIELD FROM *Cosmos caudatus*

4.4.1 Linear effect of extraction parameters on total phenolic compounds (TPC) yield from *Cosmos caudatus*

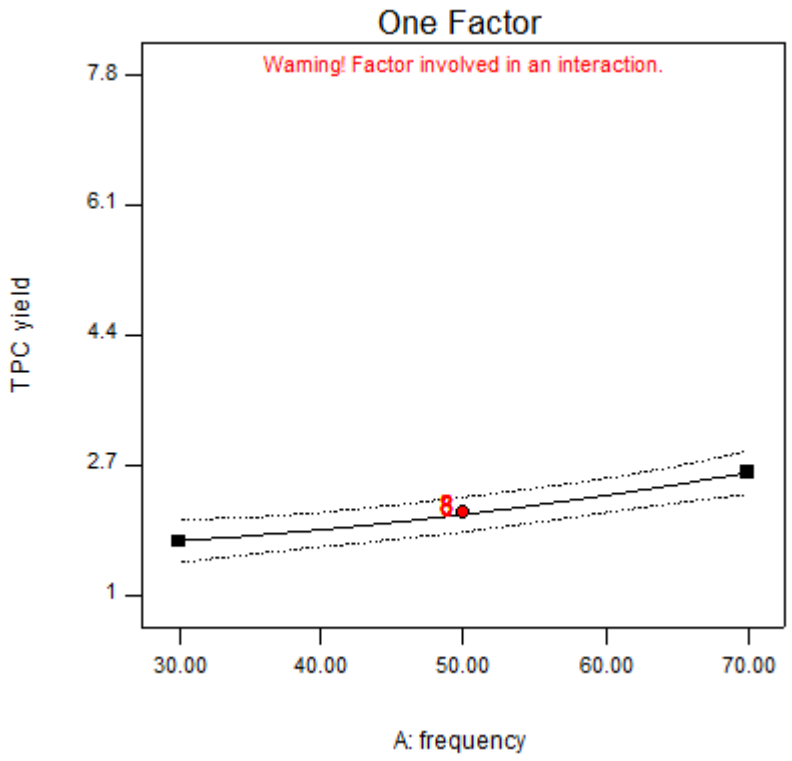


Figure 4.2: Linear effect of ultrasonic frequency (kHz) on TPC yield from *Cosmos caudatus* (mg GAE/g dw)

Common belief of a more vigorous mixing between the sample and solvent encourages better decomposition of compounds in the sample can be further proved by this finding. This can be reasonably justified with the direct correlation obtained from Figure 4.2, as ultrasonication which involves the use of shear force created by the implosion of cavitation bubbles of ultrasonic waves to alter material properties, thereby further disrupting plant tissues and facilitating extraction, as proposed by Chukwumah *et al*.

(2009). Other advantages of this technique are its high reproducibility, the possibility of using a wide range of sample sizes and the low cost of the whole process (Melecchi *et al.*, 2006).

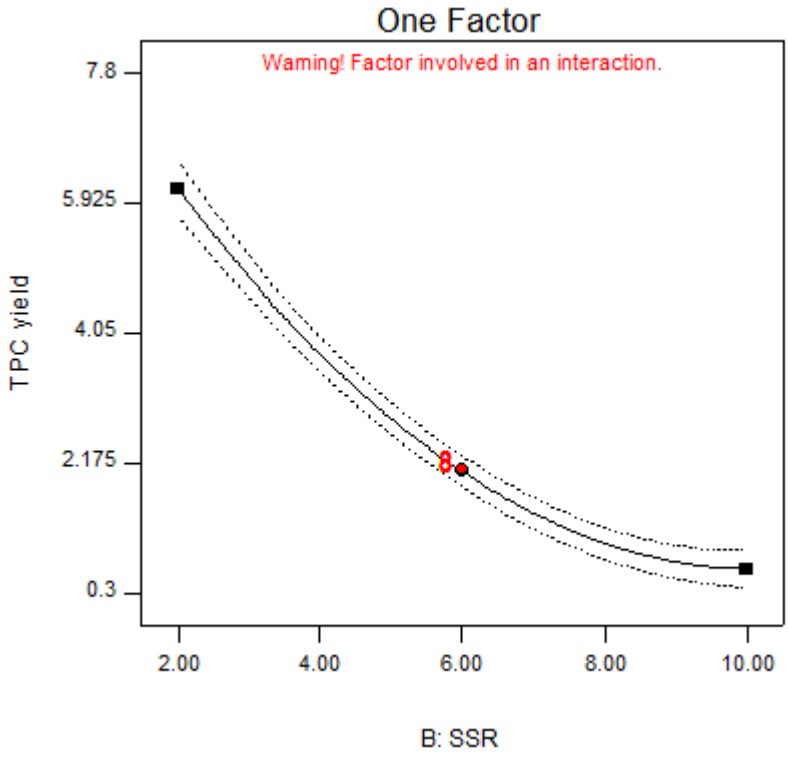


Figure 4.3: Linear effect of sample-to-solvent ratio, SSR (w/v %) on TPC yield from *Cosmos caudatus*

On the other hand, previous studies suggest that the extraction of polyphenols from plant material can be influenced by the sample-to-solvent ratio (SSR). Naczk *et al.* (1992) found that changing the SSR from 1:5 to 1:10 increased the extraction of condensed tannins from commercial canola meals. The same can be said for the extraction of total phenolic compounds from *Cosmos caudatus* as depicted by Figure 4.3. Furthermore, the analysis of variance (ANOVA) results from Response Surface Methodolgy finds the parameter to be significant. This is in accordance to the study done by Herodez *et al.* (2003), who found that the percentage of extraction yields will increase with the particle size of sample, temperature extraction and the ratio of solvent and sample extraction.

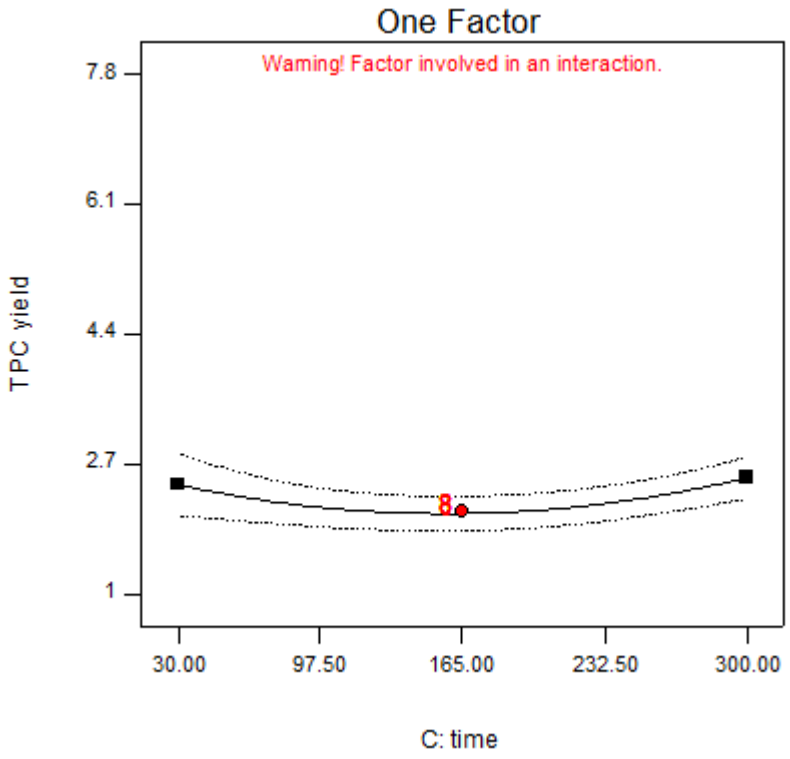


Figure 4.4: Linear effect of extraction time (min) on TPC yield from *Cosmos caudatus* (mg GAE/g dw)

Moving on to the effect of extraction time on total phenolic compounds yield from *Cosmos caudatus* extract, Figure 4.4 shows that the extraction yield can be optimized unnecessarily on the higher level of time, yet longer duration of exposure to ethanol (solvent) is likely to result in a higher extraction yield of TPC from *C.caudatus*. This is corresponding to the efficiency of sonication bath used compared to the other traditional solvent extraction methods such as leaching, homogenization and soxhlet (reflux) extractor since the estimation of the amounts of bioactive compounds from plant sources using homogenization with distilled water for an instance yields the lowest TPC extract from *Cosmos caudatus* (Huda-Faujan *et al*., 2007).

4.4.2 Interaction effect between extraction parameters on total phenolic compounds (TPC) yield from *Cosmos caudatus*

The 3D response surface plots were used to understand the interaction effects of extraction parameters and optimum value of each component required for maximum total phenolic compounds extract. In each set, two variables varied within their experimental range, while the other two variables remained constant at zero level.

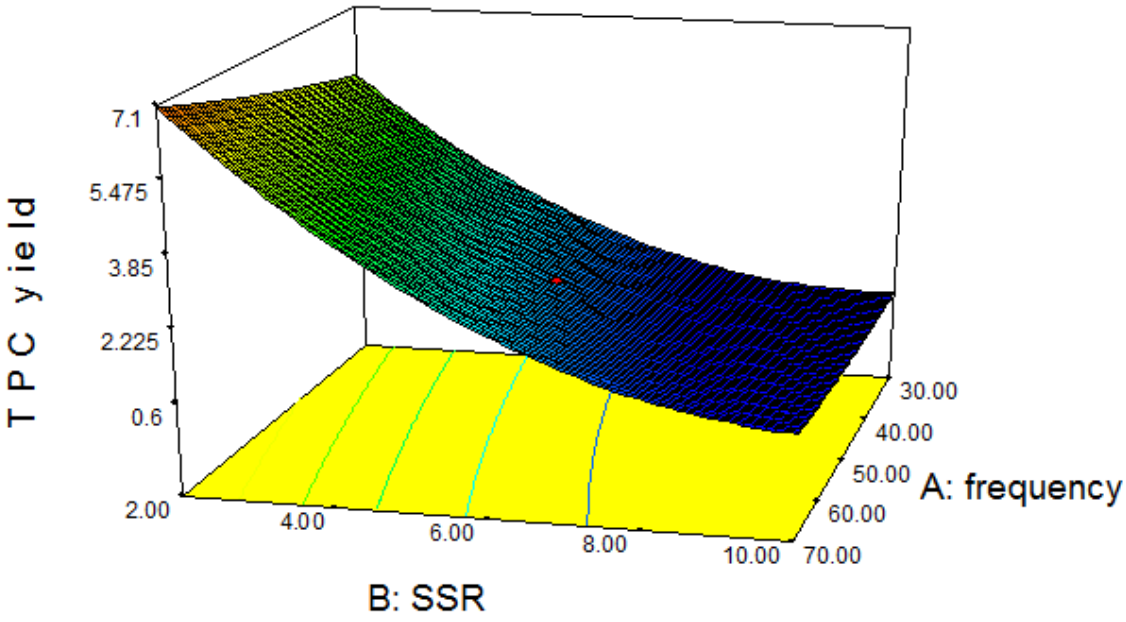


Figure 4.5: Interaction effect between ultrasonic frequency (kHz) and sample-to-solvent ratio, SSR (w/v %) on TPC yield of *Cosmos caudatus* extract (mg GAE/g dw)

Figure 4.5 reveals the interaction between sample-to-solvent ratio and ultrasonic frequency in the design range at constant extraction time of 165 minutes which shows that extraction time is directly proportional to TPC yield up to the positive alpha level. It is generally accepted that a longer exposure of sample to solvent would encourage better decomposition of compounds in the plant into the solvent. There is no exception for this study where extended duration yields higher total phenolic compounds but after a certain period, the rate of extraction degrades. This occurrence might be partly due to phenolic

oxidation during the extraction itself after an excessive mixing with the organic solvent (Torti *et al.*, 1994).

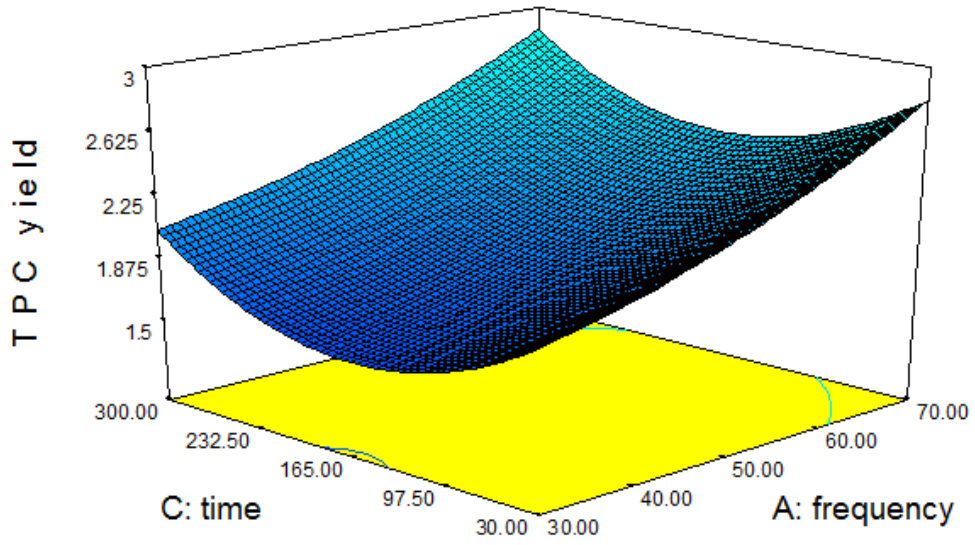


Figure 4.6: Interaction effect between ultrasonic frequency (kHz) and extraction time (min) on TPC yield of *Cosmos caudatus* extract (mg GAE/g dw)

Meanwhile, Figure 4.6 which illustrates the interaction between ultrasonic frequency and extraction time at constant sample-to-solvent ratio of 6w/v % indicates that SSR plays a significant role on the overall yield of extraction. This may due to the chemical effect of ultrasound on aqueous solutions and organic solvents. Previous studies on the sonolysis (breaking of chemical bonds or formation of radicals using ultrasound) of water and organic liquids have shown that sonication of these solvents generates free radicals such as superoxide ions, hydroxyl ions, solvated electrons, and atomic hydrogen that cause secondary oxidation-reduction reactions. It is therefore possible that the reduction in the concentrations of these phytochemicals with increased sonication time may be as a result of the oxidation of these compounds by the free radicals since these bioactive compounds have antioxidant properties and are free radical scavengers (Chukwumah *et al.*, 2009).

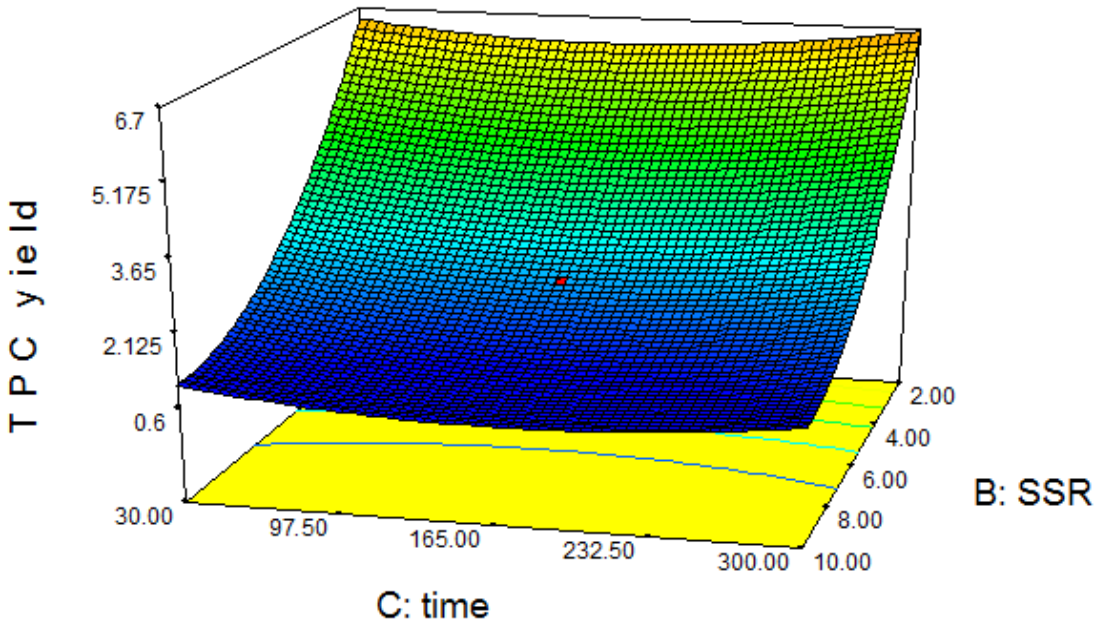


Figure 4.7: Interaction effect between sample-to-solvent ratio, SSR (w/v %) and extraction time (min) on TPC yield of *Cosmos caudatus* extract (mg GAE/g dw)

Last but not least, Figure 4.7 which depicts the relationship between sample-to-solvent ratio and extraction time implies that ultrasonic frequency is directly proportional to the TPC yield, as greater ultrasonic frequency cause greater effects of the collapse of cavitation bubbles produced by ultrasound on the cell walls of plants. The mechanisms can be described by the fact that some plant cells have glands filled with essential oil and one characteristic of external glands 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. On the other hand, ultrasound can also facilitate the swelling and solvatation of plant materials causing enlargement of the pores of the cell wall. Better swelling will improve the rate of mass transfer and, occasionally, break the cell walls, thus resulting in increased extraction efficiency and/or reduced extraction time (Melecchi *et al.*, 2006).

4.5 OPTIMUM EXTRACTION PARAMETERS OF TOTAL PHENOLIC COMPOUNDS YIELD FROM *Cosmos caudatus*

Based on the analysis by Response Surface Methodology, it is clear that only ultrasonic frequency and sample-to-solvent ratio are significant for the process, while extraction time plays an insignificant role with dependent effects on the other factors. Table 4.3 shows that the optimum experimental values for each extraction parameters are exactly the same with the predicted values, resulting in a very close difference between the two. Results obtained are supporting previous findings such as Herodez *et al.* (2003), who found that the percentage of extraction yields will increase with the ratio of solvent and sample extraction as well as that the actual factors responsible for the effective extraction of the target compounds by ultrasonication were frequency and duration of sonication (Chukwumah *et al*., 2009).

Table 4.4: Optimum extraction parameters of experimental and predicted values for total phenolic compounds yield from *Cosmos caudatus*

Optimum conditions	**Experimental value**	**Predicted value**	**Difference (%)**
Ultrasonic frequency (kHz)	70.00	70.00	0.00
Sample-to-solvent ratio (w/v %)	2.00	2.00	0.00
Extraction time (min)	300.00	300.00	0.00
TPC (mg GAE/g dw)	7.74	7.54	2.58

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The study was aimed to show that the optimization of extracting total phenolic compounds (TPC) from *Cosmos caudatus* can be accomplished by manipulating the extraction parameters, namely the sample-to-solvent concentration, ultrasonic frequency and the extraction time. Inevitably, longer extraction time and higher sample-to-solvent ratio with high ultrasonic frequency for extraction would suggest higher yield of TPC. However, alongside with the evaluation of predicted statistical analysis by Response Surface Methodology, this research has proven the previous theory to be unreasonably applicable, especially for mass productions as in the industrial scale. The optimum conditions for the extraction of total phenolic compounds (TPC) from *Cosmos caudatus* were found to be ultrasonic frequency of 70 kHz, sample-to-solvent ratio of 2 w/v% and extraction time of 300 minutes which yields 7.74 mg GAE/g dw, which is only 2.58% different with the predicted value.

Results obtained support the previous study which found that the actual factors responsible for the effective extraction of the target compounds by ultrasonication were frequency, duration of sonication, and the combination of both the frequency and duration (time) of sonication (Chukwumah *et al*., 2009). It is also in accordance to the study done by Herodez *et al.* (2003), who found that the percentage of extraction yields will increase with the particle size of sample, temperature extraction and the ratio of solvent and sample extraction. The identified optimal medium for maximized total pheolic compounds from

Cosmos caudatus extract is very beneficial since the extraction and purification of phytochemicals from natural sources is needed, since these bioactives are often used in the preparation of dietary supplements, nutraceuticals, functional food ingredients, food additives, pharmaceutical and cosmetic products (Gao and Mazza, 1996).

5.2 RECOMMENDATIONS

Having identified the optimum parameters of extraction of total phenolic compounds from *Cosmos caudatus*, scale-up process should not be a big obstacle for maximizing the production of phenol. Since previous and present studies suggests that the extraction of total phenolic compounds can be further optimized, upcoming studies need to be directed at varying the significant extraction parameters including the extraction temperature, types of solvent used (acetone, water or methanol) and extraction methods (maceration, high pressure or microwave method). This is due to the possible effect of the other factors as described by Wong *et al*., (2006).

In addition to this, further studies should be done to characterize, isolate and purify the extracted phenolic compounds from *Cosmos caudatus*. Besides, the study on other phytochemical compounds available in *Cosmos caudatus* such as lactones, polyacetylenes, flavonoids and phenylpropanoids (Fuzzati *et al.,* 1994) may also be considered in terms of their toxicology properties, antioxidant and antifungal activities for pharmaceutical applications.

REFERENCES

Abas F., N. H. Lajis, D. A. Israf, S. Khozirah and Y. U. Kalsom (2006), Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables, *Food Chemistry 95*, 566-573

Alothman, M., Bhat, R., Karim, A.A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry 115*, 785-788.

Amin, I., Zamaliah, M. M. and Chin, W. F. (2004). Total antioxidant activity and phenolic content in selected vegetables. *Food Chemistry*, *87(4):* 581-586.

Andarwulan N., R. Batari, D. A, Sandrasari, B. Bolling and H. Wijaya (2010), Flavanoid content and antioxidant activity of vegetables from Indonesia, *Food Chemistry 121*, 1231–1235

Aqil, F., Ahmad, I., & Mehmood, Z. (2006). Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. *Turkish Journal of Biology*, *30*, 177–183.

Arbianti R., Utami T.S., Kurmana A. and Sinaga A (2007)Comparison of antioxidant activity and total phenolic content of *dillenia indica* leaves extracts obtained using various techniques *14th Regional Symposium on Chemical Engineering 2007*

Azlim Almey, A. A., Ahmed Jalal Khan, C, Syed Zahir, I., Mustapha Suleiman, K., 'Aisyah, M. R. and Kamarul Rahim, K. (2010), Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leaves, *International Food Research Journal 17*: 1077-1084

Chanwitheesuk, A., Teerawutgulrag, A., Rakariyatham, N. (2005). Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chemistry 92*, 491-497

Choi, E. M., & Hwang, J. K. (2005). Screening of Indonesian medicinal plants for inhibitor activity on nitric oxide production of raw 264.7 cells and antioxidant activity. *Fitoterapia*, *76*, 194–203.

Chu, Y.-H., Chang, C.-L., & Hsu, H.-F. (2000). Flavonoid content of several vegetables and their antioxidant activity. Journal of the Science of Food and Agriculture, 80, 561–566.

Chukwumah Y. C., Lloyd T. Walker, Martha Verghese, O. Simon (2009) Effect of frequency and duration of ultrasonication on the extraction efficiency of selected

isoflavones and trans-resveratrol from peanuts (*Arachis hypogaea*) Ultrasonics Sonochemistry 16 (2009) 293–299

Escarpa, A., & Gonzalez, M. C. (2001). Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta, 427, 119–127.*

Gan, H. E., Karim R., Muhammad S.K.S, Bakar J.A., Hashim D.M. and Rahman R. (2005), Optimization of the basic formulation of a traditional baked cassava cake using response surface methodology, *LWT 40 611-618*

Gil, M. I., Tomas-Barberan, F. A., Hess-Pierce, B., & Kader, A. A. (2002). Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and pulm cultivars from California. *Journal of Agricultural and Food Chemistry, 50, 4976–4982.*

Han, X., Shen, T., Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences 8*, 950-988

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *Journal of Nutritional Biochemistry, 13, 572–584.*

Hodzic, Z., Pasalic, H., Memisevic, A., Srabovic, M., Poljakovic, M. (2009). The influence of total phenols content on antioxidant capacity in the whole grain extracts. *European Journal of Scientific Research 28*, 471-477.

Huda-Faujan N., Noriham A., Norrakiah A. S. and Babji, A. S. (2007), Antioxidative activities of water extracts of some Malaysian herbs, *ASEAN Food Journal 14 (1)* : 61-68

Huda-Faujan N., Noriham A., Norrakiah A. S. and Babji, A. S. (2009), Antioxidant activity of plants methanolic extracts containing phenolic compounds, *African Journal of Biotechnology Vol. 8 (3)*, pp. 484-489, 4 February, 2009

Ismail, A., Marjan, Z.M., Foong, C.W. (2004). Total antioxidant activity and phenolic content in selected vegetables. *Food Chemistry 87*, 581-586

Ismail, A., Tiong, N-W., Tan, S-T., Azlan, A. (2009). Antioxidant properties of selected nonleafy vegetables. *Nutrition and Food Science 39*, 176-180

Jamal, P., A.A. Barkat and A. Amid (2010). Distribution of phenolics in various Malaysian medicinal plants. *J. Applied Sci*., 10: 2658-2662.

Kaur, C., Kapoor, H.C. (2001). Antioxidants in fruits and vegetables – The millennium's health. *International Journal of Food Science and Technology 36*, 703-725

Laurence M. Harwood, Christopher J. Moody. Experimental organic chemistry: Principles and Practice (Illustrated edition ed.). pp. 122–125

Maisuthisakul, P., Pasuk, S., Ritthiruangdej, P. (2008). Relationship between antioxidant properties and chemical composition of some Thai plants. *Journal of Food Composition and Analysis 21,* 229-240

Melecchi M.I.S., Peres, V.F.,Dariva C., Zini C.A, Abad F.C,Martinez M.M. and Caramao E.B, (2006) Optimization of the sonication extraction method of *Hibiscus tiliaceus L*. flowers, Ultrasonics Sonochemistry 13 (2006) 242–250

N. Fuzzati, Sutarjadi, W. Dyatmiko, A. Rahman and K. Hostettmann (1994), Phenylpropane derivatives from roots of Cosmos caudatus

Naczk, M., Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis 41*, 1523-1542.

Prior, R.L., Wu, X.L., Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry 53*, 4290-4302.

Robards, K. (2003). Strategies for determination of bioactive phenols in plants, fruit and vegetables. *Journal of Chromatography A 1000*, 657-691.

Samy, J., Sugumaran, M., Lee, K.L.W. (2005). Herbs of Malaysia: An introduction to medicinal, culinary, aromatic and cosmetic use of herbs. *Federal Publications Sdn. Bhd., Selangor*. Page 23.

Santana C. M., Ferrea Z.S., Padron M.E.T. and Rodriguez J.J.S. (2009) Methodologies for the extraction of phenolic compounds from environmental samples: new approaches, *Molecules* **2009**, *14*, 298-320

Shui,G. L. P. Leong and S. P. Wong (2005), Rapid screening and characterization of antioxidants of Cosmos caudatus using liquid chromatography coupled with mass spectrometry, *Journal of Chromatography B, 827*, 127-138

Singh, S. K., Tripathi, V. R., Khare, S. K.,and Garg, S. K, (2011) Comperative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychotropic *Pseudomonas putida* SKG-I isolate, *Microbial Cell Factories 2011, 10:114*

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology 299*, 152-178

Song, W., Derito, C.M., Liu, M.K., He, X., Dong, M., Liu, R.H. (2010). Cellular antioxidant activity of common vegetables. *Journal of Agricultural and Food Chemistry 58*, 6621-6629

Sukrasno, S., I. Fidriany, K. Anggadiredja, W.A. Handayani and K. Anam (2011). Influence of drying method on flavonoid content of Cosmos caudatus (kunth) leaves. *Res. J. Med. Plant, 5*: 189-195.

Sulaiman, S. F., Sajak, A. A. B., Ooii, K. L. and Seow, E. M. (2010), Effect of solvent in extracting polyphenols and antioxidants of selected raw vegetables, *Journal of Food Compositon and Analysis*

Sumazian Y., A Syahida, M Hakiman and M Maziah (2010), Antioxidant activities, flavonoids, ascorbic acid and phenolic contents of Malaysian vegetables, *Journal of Medicinal Plants Research Vol. 4 (10)*, pp. 881-890, 18 May 2010

Torti, S. D., Dearing, M.D. and Kursar T.A. (1994), Extraction of phenolic compounds from fresh leaves: a comparison of methods, *Journal of Chemical Ecology, Vol. 21, No. 2, 1995*

Wong, S. P., L. P. Leong and J. H. W. Koh (2006), Antioxidant activities of aqueous extracts of selected plants, *Food Chemistry 99*, 775-783

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry, 49, 5165–5170.*

APPENDIX A

ABSORBANCE AND TPC YIELD

Trial no.	Absorbance (760nm)	GAE (mg/L)	TPC yield (mg GAE/g dw)
1	0.108	102.973	1.7162
2	0.116	110.374	1.1037
3	0.126	159.416	1.1964
4	0.117	111.299	5.5650
5	0.128	152.940	6.0163
6	0.158	149.239	1.4924
7	0.177	166.817	1.6682
8	0.159	150.165	7.5081
9	0.164	154.790	7.7395
10	0.154	145.539	2.4257
11	0.131	119.635	2.0710
12	0.131	119.635	2.0671
13	0.130	119.635	2.0594
14	0.131	119.635	2.0681
15	0.131	119.635	2.0659
16	0.130	119.635	2.0633
17	0.132	119.635	2.0837
18	0.132	119.635	2.0810
19	0.193	181.620	3.0271
20	0.142	124.260	1.0348

$y = mx + c$ where y = GAE concentration (mg GAE/L)

$m = 925.15$

x = absorbance (760nm)

$c = 3.0657$

$$\text{TPC yield (mg GAE/g dw)} = \frac{\text{GAE concentration (mg GAE/L)} \times \text{volume of ethanol (0.1L)}}{\text{Sample's dry weight (g dw)}}$$

APPENDIX B

DESIGN SUMMARY

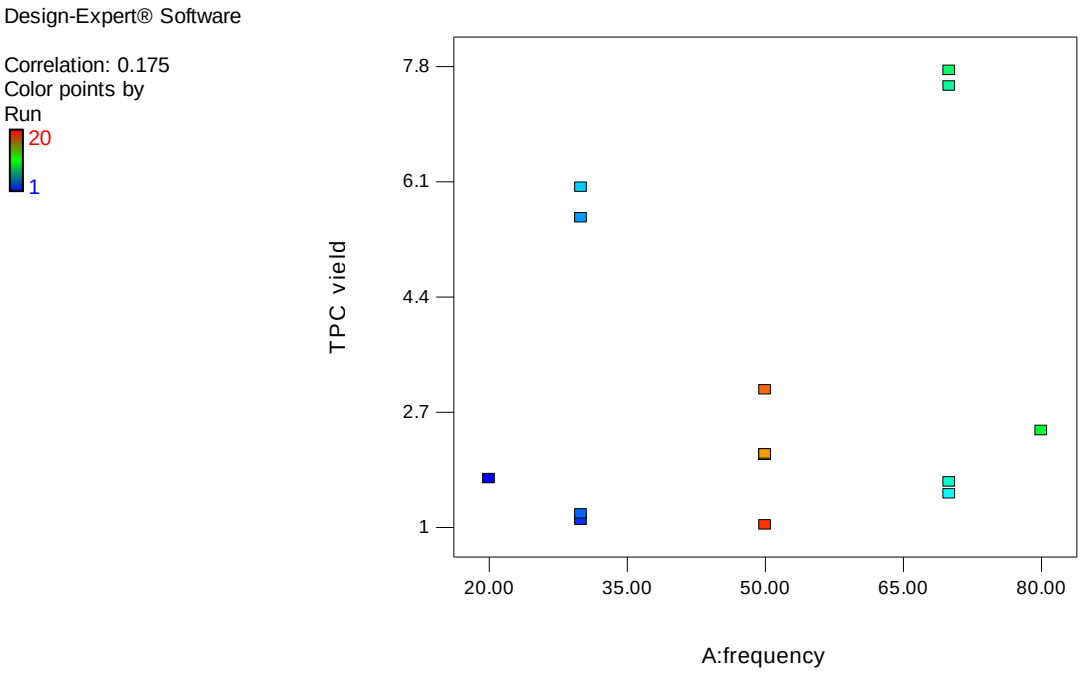
Study Type	Response Surface	**Runs**	20
Initial Design	Central Composite	**Blocks**	No Blocks
Design Model	Quadratic		

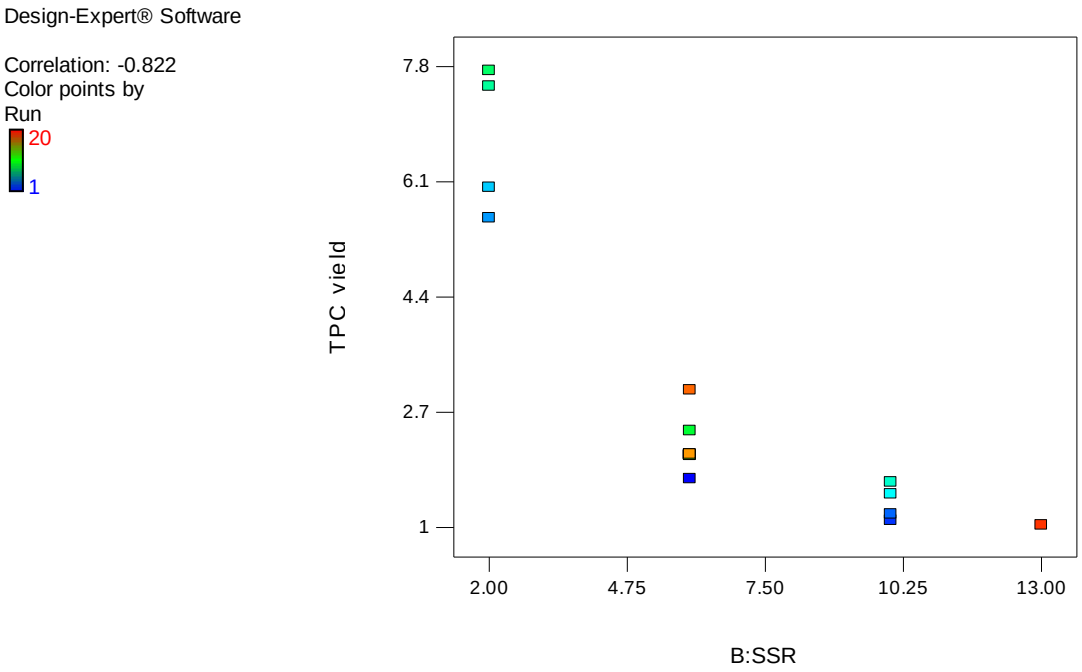
Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.
A	frequency	kHz	Numeric	30.00	70.00	-1.000	1.000	50.000	15.811
B	SSR	w/v%	Numeric	2.00	10.00	-1.000	1.000	6.350	2.954
C	time	min	Numeric	30.00	300.00	-1.000	1.000	176.500	99.009

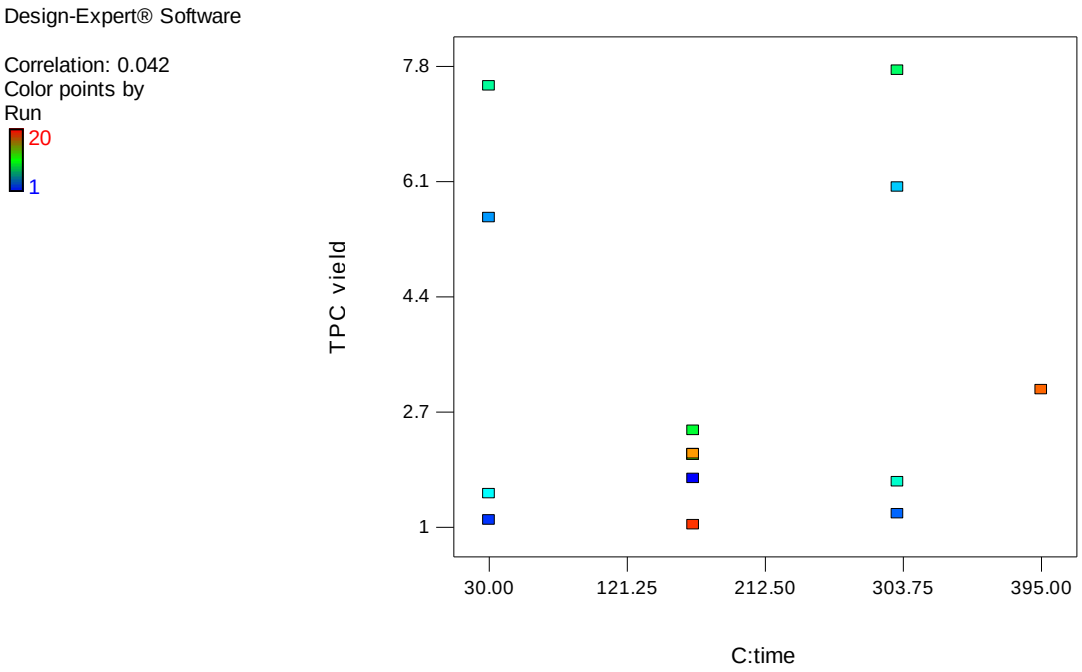
Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans
Y1	TPC yield	mg GAE/g dw	20	Polynomial	1.0348	7.7395	2.85264	2.07346	7.4792	None

APPENDIX C

GRAPH COLUMNS






Design-Expert® Software
Correlation: 0.042
Color points by
Run
20
1
7.8
6.1
4.4
2.7
1
TPC vield
30.00
121.25
212.50
303.75
395.00
C:time

APPENDIX D

DESIGN MATRIX EVALUATION FOR RESPONSE SURFACE QUADRATIC MODEL

3 Factors: A, B, C

No aliases found for Quadratic Model

Aliases are calculated based on your response selection,
taking into account missing datapoints, if necessary.
Watch for aliases among terms you need to estimate.

Degrees of Freedom for Evaluation

Model	9
Residuals	10
Lack 0f Fit	*3*
Pure Error	*7*
Corr Total	19

A recommendation is a minimum of 3 lack of fit df and 4 df for pure error.
This ensures a valid lack of fit test.
Fewer df will lead to a test that may not detect lack of fit.

				Power at 5 % alpha level to detect signal/noise ratios of		
Term	**StdErr****	**VIF**	**Ri-Squared**	**0.5 Std. Dev.**	**1 Std. Dev.**	**2 Std. Dev.**
A	0.28	1.00	0.0000	12.6 %	35.9 %	88.9 %
B	0.34	1.26	0.2061	10.2 %	26.2 %	75.6 %
C	0.34	1.24	0.1912	10.3 %	26.7 %	75.8 %
AB	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
AC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
BC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
A^2	0.32	1.06	0.0564	29.2 %	80.2 %	99.9 %
B^2	0.34	1.32	0.2439	26.2 %	74.9 %	99.9 %
C^2	0.35	1.31	0.2343	25.0 %	72.4 %	99.9 %

****Basis Std. Dev. = 1.0**

Standard errors should be similar within type of coefficient. Smaller is better.

Ideal VIF is 1.0. VIF's above 10 are cause for alarm,
indicating coefficients are poorly estimated due to multicollinearity.

Ideal Ri-squared is 0.0. High Ri-squared means terms are correlated with each other,
possibly leading to poor models.

If the design has multilinear constraints multicollinearity will exist to a greater degree.
The presence of multicollinearity increases the VIF's and the Ri-squareds.
Due to imposed constraints, the design is only valid for a limited set of combinations.
High VIF's and high Ri-Squareds are less of a concern.

Measures Derived From the $(X'X)^{-1}$ Matrix

Std	Leverage	Point Type
1	0.1246	Center
2	0.5876	Axial
3	0.1246	Center
4	0.1246	Center
5	0.7780	Axial
6	0.5876	Axial
7	0.6938	Fact
8	0.8006	Axial
9	0.8017	Fact
10	0.8017	Fact
11	0.1246	Center
12	0.1246	Center
13	0.8293	Fact
14	0.1246	Center
15	0.1246	Center
16	0.8293	Fact
17	0.1246	Center
18	0.7999	Fact
19	0.7999	Fact
20	0.6938	Fact
Average =	**0.5000**	

Watch for leverages close to 1.0. Consider replicating these points
or make sure they are run very carefully.

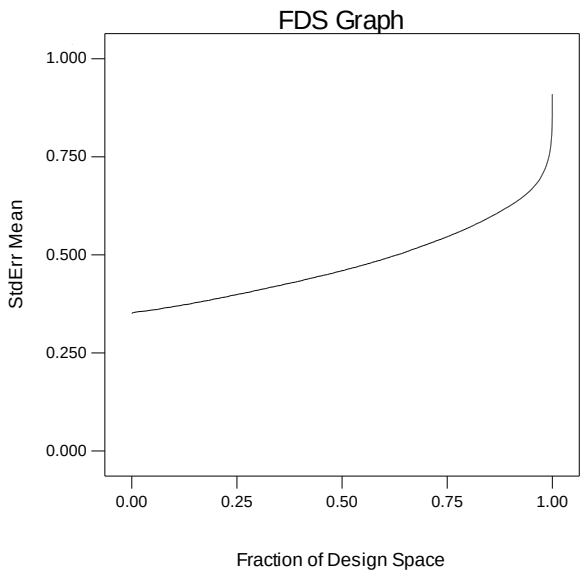
APPENDIX E

FRACTION OF DESIGN SPACE

Mean

Design-Expert® Software

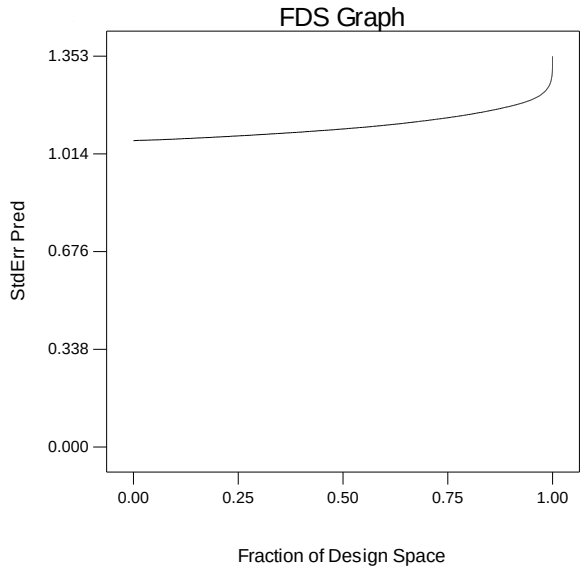
Min StdErr Mean: 0.351
Max StdErr Mean: 0.911
Cuboidal
radius = 1
Points = 50000
t(0.05/2,10) = 2.22814



Predicted

Design-Expert® Software

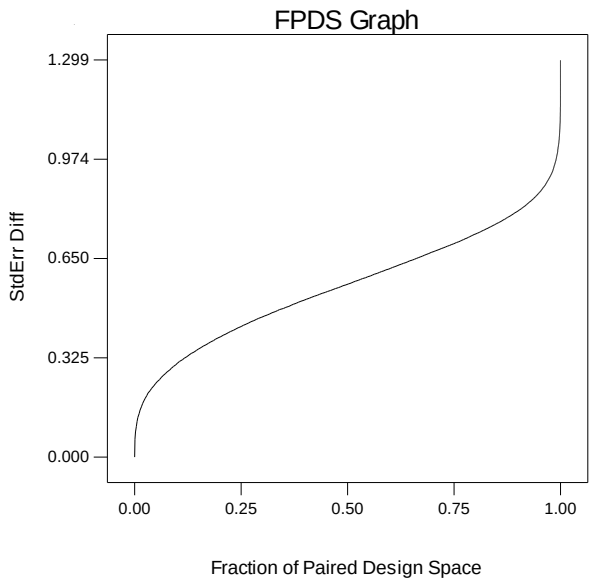
Min StdErr Pred: 1.060
Max StdErr Pred: 1.353
Cuboidal
radius = 1
Points = 50000
t(0.05/2,10) = 2.22814



Difference

Design-Expert® Software

Min StdErr Diff: 0.000
Max StdErr Diff: 1.299
Cuboidal
radius = 1
Pairs = 50000
t(0.05/2,10) = 2.22814



APPENDIX F

FIT SUMMARY

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	162.75	1	162.75			
Linear vs Mean	57.82	3	19.27	12.92	0.0002	
2FI vs Linear	1.01	3	0.34	0.19	0.9006	
Quadratic vs 2FI	22.03	3	7.34	88.97	< 0.0001	Suggested
Cubic vs Quadratic	0.82	3	0.27	3882.54	< 0.0001	Aliased
Residual	4.957E-004	7	7.082E-005			
Total	244.44	20	12.22			

"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	23.86	9	2.65	37439.55	< 0.0001	
2FI	22.86	6	3.81	53787.46	< 0.0001	
Quadratic	0.82	3	0.27	3882.54	< 0.0001	Suggested
Cubic	0.000	0				Aliased
Pure Error	4.957E-004	7	7.082E-005			

"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	1.22	0.7079	0.6531	0.4761	42.80	
2FI	1.33	0.7202	0.5911	-0.5621	127.60	
Quadratic	0.29	0.9899	0.9808	0.8494	12.31	Suggested
Cubic	8.415E-003	1.0000	1.0000		+	Aliased

+ Case(s) with leverage of 1.0000: PRESS statistic not defined

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

APPENDIX G

ANOVA for Response Surface Quadratic Model

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.06	1	0.10	1.83	2.28	
A-frequency	0.45	1	0.081	0.27	0.63	1.00
B-concentration	-2.74	1	0.098	-2.96	-2.52	1.26
C-time	0.048	1	0.097	-0.17	0.26	1.24
AB	-0.35	1	0.10	-0.58	-0.12	1.00
AC	-0.017	1	0.10	-0.24	0.21	1.00
BC	-0.052	1	0.10	-0.28	0.17	1.00
A^2	0.10	1	0.092	-0.10	0.31	1.06
B^2	1.34	1	0.098	1.12	1.55	1.32
C^2	0.42	1	0.10	0.20	0.65	1.31

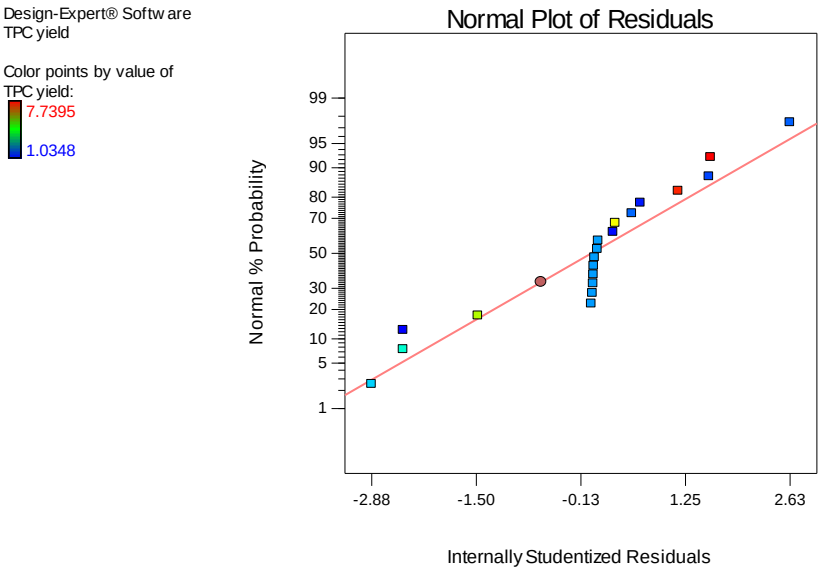
Final Equation in Terms of Actual Factors:

TPC yield	=
+7.80083	
+0.024146	* frequency
-1.45171	* SSR
-6.38325E-003	* time
-4.38406E-003	* frequency * SSR
-6.33333E-005	* frequency * time
-9.58796E-005	* SSR * time
+2.55677E-004	* $frequency^2$
+0.083474	* SSR^2
+2.31207E-005	* $time^2$

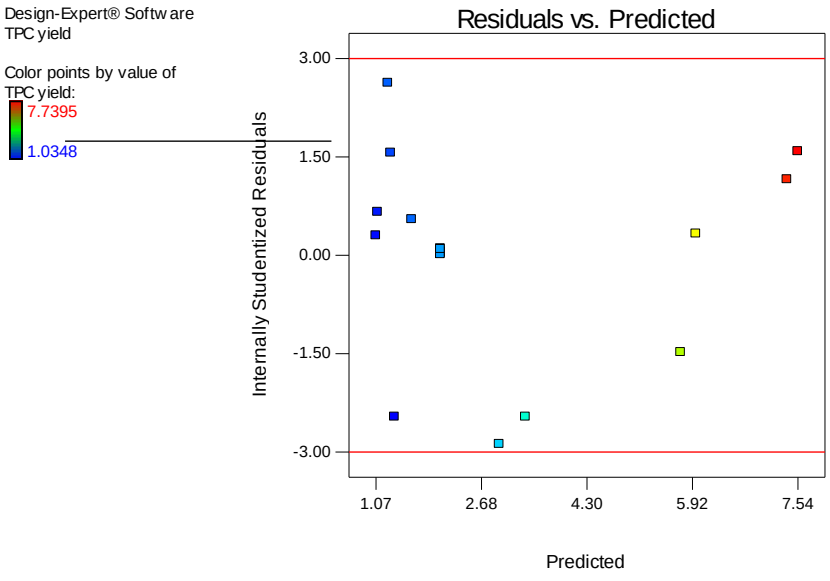
APPENDIX H

DIAGNOSTICS

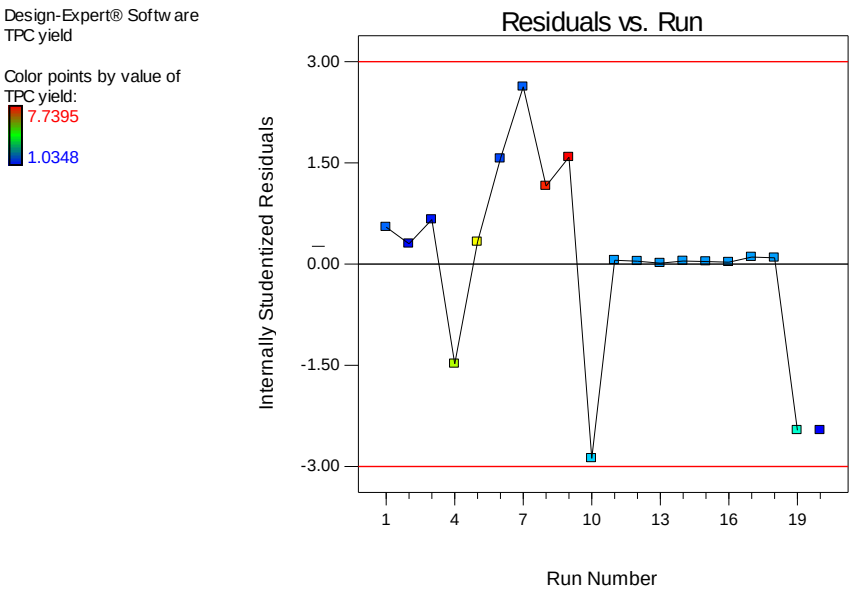
Normal plot



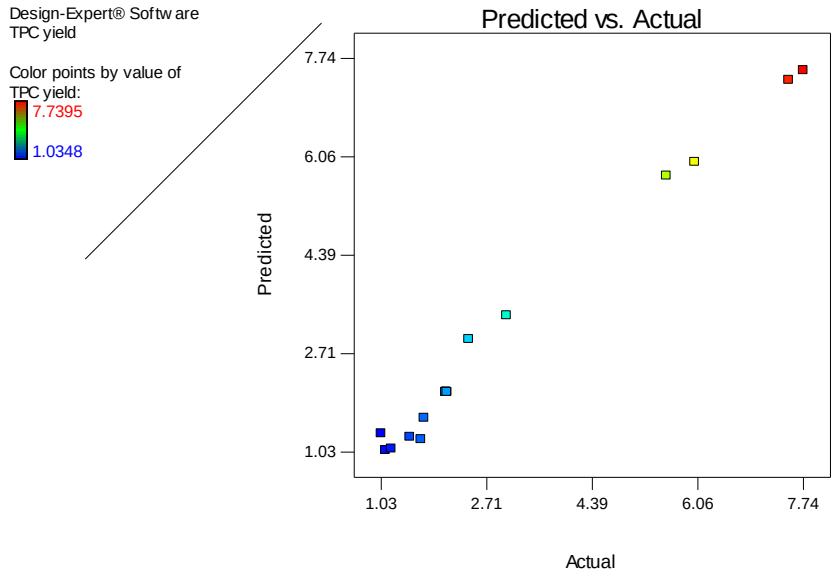
Residuals vs. predicted



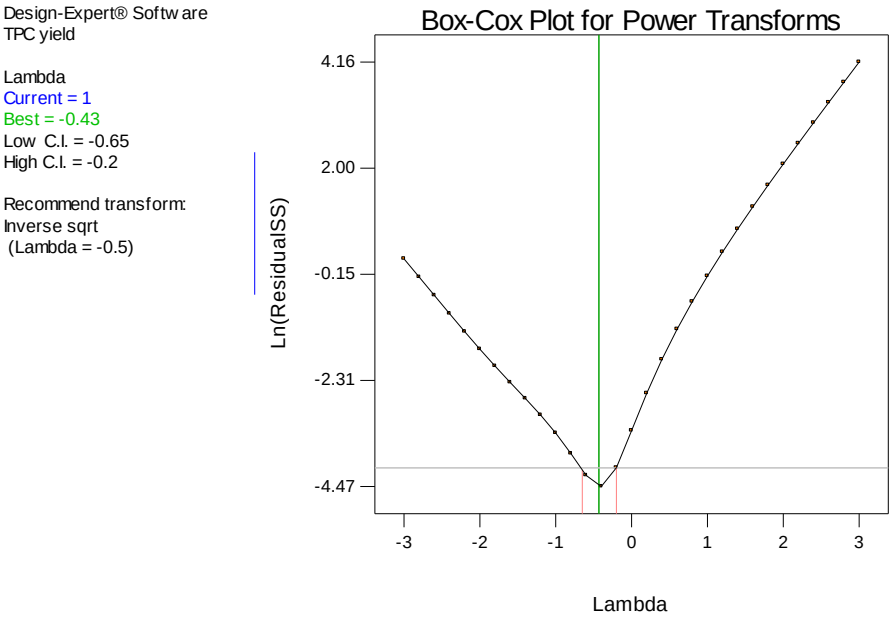
Residuals vs. run



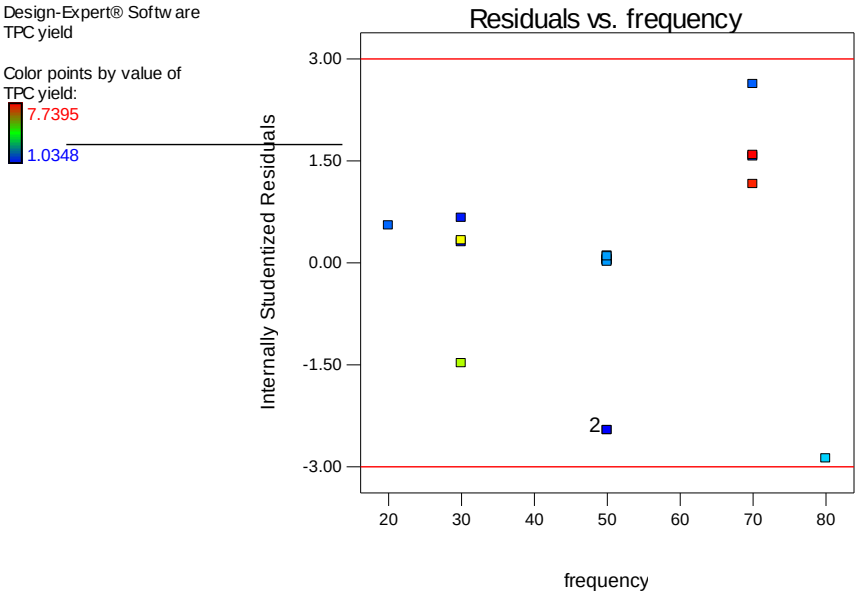
Predicted vs. actual



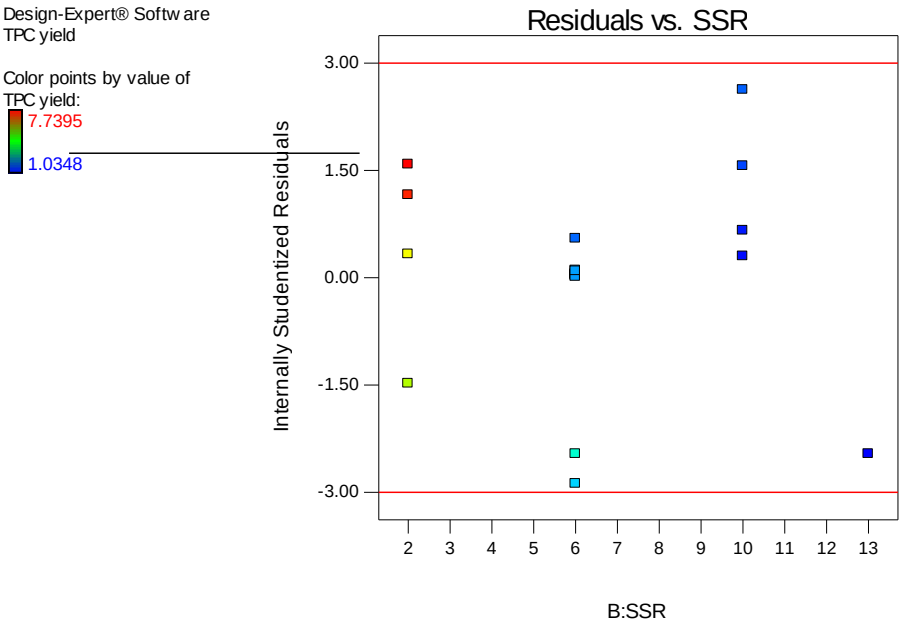
Box-Cox plot



Residuals vs. frequency



Residuals vs. SSR



Residuals vs. time