



EXPERIMENTAL DESIGN FOR OPTIMIZATION OF BIOACTIVE COMPOUND
EXTRACTION FROM PEANUT KERNELS



By
MR. Kritamorn JITRANGSRI

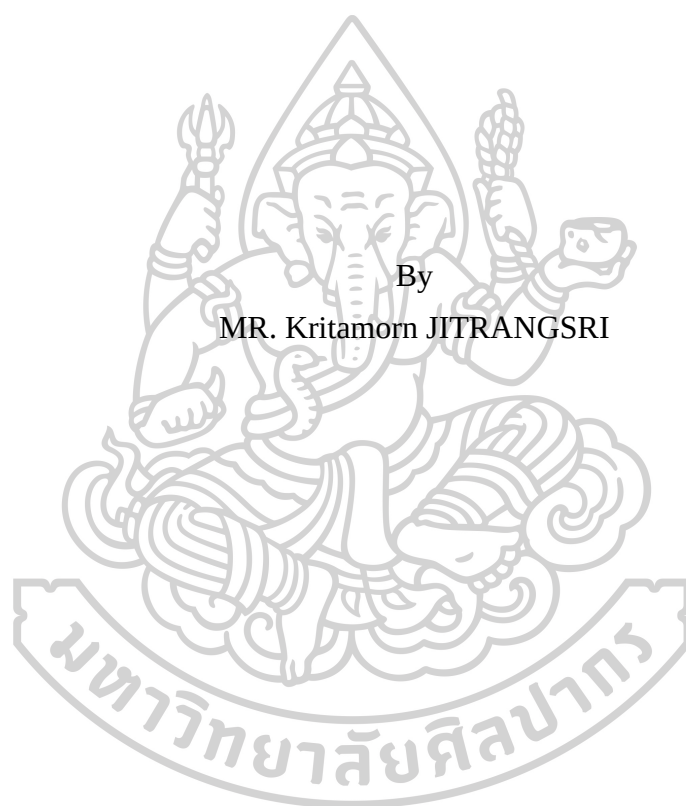
A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES
Graduate School, Silpakorn University
Academic Year 2022
Copyright of Silpakorn University

การออกแบบการทดลองเพื่อให้ได้สภาวะที่เหมาะสมของการสกัดสารออกฤทธิ์ทาง
ชีวภาพจากเมล็ดถั่วลิสง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
สาขาวิชาวิทยาการทางเภสัชศาสตร์ แบบ 1.2 ปรัชญาดุษฎีบัณฑิต
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2565
ลิขสิทธิ์ของมหาวิทยาลัยศิลปากร

EXPERIMENTAL DESIGN FOR OPTIMIZATION OF BIOACTIVE COMPOUND EXTRACTION FROM PEANUT KERNELS



A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES
Graduate School, Silpakorn University
Academic Year 2022
Copyright of Silpakorn University

Title	Experimental design for optimization of bioactive compound extraction from peanut kernels
By	MR. Kritamorn JITRANGSRI
Field of Study	PHARMACEUTICAL SCIENCES
Advisor	Assistant Professor MALAI SATIRAPHAN, Ph.D.
Co advisor	Assistant Professor AMORNRAT CHAIDECHGUMJORN, Ph.D.

Graduate School Silpakorn University in Partial Fulfillment of the
Requirements for the Doctor of Philosophy

.....	Dean of the Graduate School
(Assistant Professor Sathit Niratisai, Ph.D)	(Acting)

Approved by

.....	Chair person
(Associate Professor Theerasak Rojanarata, Ph.D.)	

.....	Advisor
(Assistant Professor MALAI SATIRAPHAN, Ph.D.)	

.....	Co advisor
(Assistant Professor AMORNRAT CHAIDECHGUMJORN, Ph.D.)	

.....	Committee
(Associate Professor Lawan Srattaphut, Ph.D.)	

.....	External Examiner
(Assistant Professor Samarwadee Plianwong, Ph.D.)	

57356806 : Major PHARMACEUTICAL SCIENCES

Keyword : Solid-Liquid Extraction, Ultrasound-Assisted Extraction, Microwave-Assisted Extraction, Antioxidant activity, *Arachis hypogaea*, Full Factorial Design, Central-Composite Designs

MR. KRITAMORN JITRANGSRI : EXPERIMENTAL DESIGN FOR OPTIMIZATION OF BIOACTIVE COMPOUND EXTRACTION FROM PEANUT KERNELS THESIS ADVISOR : ASSISTANT PROFESSOR MALAI SATIRAPHAN, Ph.D.

To reveal the accurate bioactivity of natural products, extraction is a critical step. The aim of this study was to optimize the extraction conditions by different extraction methods solid-liquid extraction (SLE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) using experimental designs approach. The interested variables were screened for signification by two levels full factorial design. Then, the significant factors were optimized by three levels center-faced central composite design. The optimized conditions were obtained at maximum responses of antioxidant capacities. The responses at optimized conditions were repeatedly determined to verify the validity of calculated models, and it was compared between extraction methods and evaluated the correlation between responses by Pearson's analysis. The results found that all generated models were significant and reliable ($p < 0.05$). The optimized conditions for SLE were 30%v/v ethanol, extraction solvent 68 mL, extraction temperature 78°C and extraction time 153 min, for UAE were 30%v/v ethanol, extraction solvent 75 mL, extraction temperature 65°C and extraction time 180 min, and for MAE were 30%v/v ethanol, extraction temperature 80°C and extraction time 90 s. Most of experimental values were fell within 95% Predictive Interval (PI) of those predicted values. The responses obtained from SLE and UAE usually provided higher responses than MAE ($p < 0.05$) while they were not much different between SLE and UAE. Pearson's correlation revealed the strong positive correlations between total phenolic content (TPC) with all antioxidant tests, and among the antioxidant tests. The moderate positive correlations were found between *t*-resveratrol with TPC, and *t*-resveratrol with all antioxidant tests, which may imply the higher contribution of TPC to antioxidant activity rather than *t*-resveratrol alone. The TPC and *t*-resveratrol obtained from SLE in this study (3.5915 mg gallic acid equivalent (mgGAE)/g for TPC, 3.4718 µg/g for *t*-resveratrol) were higher than the previous study (0.4067 mgGAE/g for TPC, 1.40 µg/g for *t*-resveratrol) in the same of materials and extraction method. This affirmed the advantage of using experimental designs in optimization of extraction conditions in terms of improved extraction efficiency.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my academic advisor, Assist.Prof.Dr.Malai Satiraphan. She always supports me and believes in me in everything that I have chosen. She knows her students very well and treats them nicely and kindly. She has never put me on edge or make me feel struggle during my study. Every time I got the problems, she always finds the solutions without increasing works. She is one of the keys that make me successful in my double doctorate degree study by encouraging and let me taking my time so I can put all of my attempts on that study without any concerns. Secondly, I would like to give my gratitude to my co-advisor, Assist.Prof.Dr.Amornrat Chaidedgumjorn. She is also kind and never put any pressure on me. She has suggested some works that I could earn some money to cover my expenses during the study. I am so grateful being a student under their supervisions.

I also thank you to the scientists; Ms.Pattaravadee Luangthuwapranit, Ms.Anchalee Kumsum, Ms.Siriporn Jenwithaya-amornwech who always help me solving out any problems on analytical instruments in the faculty. I also would like to pay my gratitude to all the examination committee; Assoc.Prof.Dr.Theerasak Rojanarata, Assoc.Prof.Dr.Lawan Srattaphut and Asst.Prof.Dr.Samarwadee Plianwong who gave the valuable comments on my thesis that made it flawless. I am also thankful to all teachers in Department of Pharmaceutical Chemistry, Silpakorn University for their dedications in teaching me during my coursework. I also thank you to the Faculty of Pharmacy, Silpakorn University for their support in facility and study environments. I have met up with good people both local and international friends who are the important supporters as well. I also would like to acknowledge my friend, Assist.Prof.Dr.Sureewan Duangjit who was the first person that recommended me to study at Faculty of Pharmacy, Silpakorn University. I also thank you to the Graduate School, Silpakorn University for the publication fund which help to disseminate this work. Lastly, I would like to thank my mother, brothers, nieces, friends who never give up on me and drag me up when I am down.

MR. Kritamorn JITRANGSRI

TABLE OF CONTENTS

	Page
ABSTRACT.....	D
ACKNOWLEDGEMENTS.....	E
TABLE OF CONTENTS.....	F
LIST OF TABLES.....	K
LIST OF FIGURES	M
CHAPTER 1	1
INTRODUCTION	1
1.1 Statements and significance of the problems.....	3
1.2 Objectives of research.....	3
1.3 Hypothesis	4
1.4 Scope of the study.....	4
1.5 Expected or Anticipated Benefit Gain	4
CHAPTER 2	5
LITERATURE REVIEW	5
2.1 Introduction.....	5
2.2 Free radicals.....	5
2.2.1 Reactive Oxygen Species (ROS).....	6
2.2.2 Reactive Nitrogen Species (RNS)	7
2.2.3 Free radical-mediated chain reaction	8
2.3 Antioxidants.....	9
2.4 Measurement of antioxidant activity	10
2.4.1 DPPH scavenging assay	12
2.4.2 ABTS method.....	13
2.4.3 Ferric reducing antioxidant power (FRAP) method.....	14
2.4.4 Oxygen radical absorption capacity (ORAC) method	15

2.5 TPC and antioxidant capacity in peanuts.....	16
2.6 Extraction of phytochemicals from plant materials.....	18
2.6.1 Solid-Liquid Extraction (SLE)	19
2.6.2 Ultrasound-assisted extraction (UAE).....	20
2.6.3 Microwave-assisted extraction (MAE)	22
2.7 Experimental designs in phytochemicals extraction.....	24
2.8 Terminology of experimental design (52)	26
2.9 Experimental designs.....	27
2.9.1 Screening design.....	27
2.9.1.1 Two-level full factorial designs.....	28
2.9.1.2 Two-level fractional factorial designs	29
2.9.1.3 Plackett-Burman designs.....	29
2.9.2 Optimization designs	30
2.9.2.1 Central composite designs (CCD).....	30
2.9.2.1.1 Circumscribe CCD (CCC).....	31
2.9.2.1.2 Inscribe CCD (CCI).....	31
2.9.2.1.3 Face centered CCD (CCF).....	32
2.9.2.2 Box-Behnken design (BBD)	32
2.10 Desirable properties for response surface designs (56)	34
2.11 Experimental designs methodology (54).....	34
CHAPTER 3	36
MATERIAL AND METHODS	36
3.1 Materials	36
3.2 Preparation of peanut samples	36
3.3 Extraction procedures	36
3.3.1 Solid-Liquid Extraction (SLE)	36
3.3.2 Ultrasound-Assisted Extraction (UAE).....	37
3.3.3 Microwave-Assisted Extraction (MAE).....	37
3.4 Experimental designs.....	37

3.4.1 Screening design.....	37
3.4.2 Optimization design	38
3.4.3 Model verification	40
3.4.4 Statistical analysis	40
3.5 Determination of %yield.....	40
3.6 Determination of <i>t</i> -resveratrol	40
3.7 Determination of Total Phenolic Content (TPC) (61)	42
3.8 Determination of Total Flavonoid Content (TFC) (62).....	43
3.9 DPPH radical scavenging activity determination (31)	45
3.10 ABTS radical scavenging activity determination (31)	46
3.11 Ferric reducing antioxidant power (FRAP) determination (63)	47
3.12 Oxygen radical absorbance capacity (ORAC) determination (25, 64).....	49
CHAPTER 4	52
RESULT AND DISCUSSION	52
4.1 Development of HPLC analytical method for determination of resveratrol in peanut kernels	52
4.1.1 Method development.....	52
4.1.2 Method validation.....	53
4.1.2.1 Specificity	54
4.1.2.2 Linearity and range.....	54
4.1.2.3 Precision	54
4.1.2.4 Accuracy	55
4.1.2.5 Limit of detection (LOD) and Limit of quantification (LOQ)	55
4.1.2.6 System suitability	56
4.1.3 Conclusion.....	56
4.2 Optimization of extraction conditions from peanut kernels using solid-liquid extraction (SLE)	56
4.2.1 Results	56
4.2.1.1 Screening design.....	56
4.2.1.2 Optimization study	60

4.2.1.3 Model verification	73
4.2.2 Discussion	73
4.2.2.1 Screening design.....	73
4.2.2.2 Optimization design	75
4.2.2.3 Model verification	78
4.2.3 Conclusion.....	79
4.3 Optimization of extraction conditions from peanut kernels using ultrasound-assisted extraction (UAE).....	80
4.3.1 Results	80
4.3.1.1 Screening design.....	80
4.3.1.2 Optimization study	83
4.3.1.3 Model verification	96
4.3.2 Discussion	96
4.3.2.1 Screening design.....	96
4.3.2.2 Optimization design	97
4.3.2.3 Model verification	100
4.3.3 Conclusion.....	101
4.4 Optimization of extraction conditions from peanut kernels using microwave-assisted extraction (MAE).....	102
4.4.1 Results	102
4.4.1.1 Screening design.....	102
4.4.1.2 Optimization study	104
4.4.1.3 Model verification	113
4.4.2 Discussion	113
4.4.2.1 Screening design.....	113
4.4.2.2 Optimization design	114
4.4.2.3 Model verification	117
4.4.3 Conclusion.....	117
4.5 Comparison of SLE, UAE and MAE methods for extraction of peanut kernels	118

4.5.1 Introduction	118
4.5.2 Results	119
4.5.3 Discussion	121
CHAPTER 5	124
CONCLUSION.....	124
REFERENCES	125
VITA.....	132



LIST OF TABLES

	Page
Table 1 Dielectric constant of commonly used solvents (5).....	23
Table 2 Two-level full factorial design for 3 factors and contrast coefficients for the interactions.....	28
Table 3 Fractional factorial design for 4 factors and column of contrast coefficients	29
Table 4 Plackett-Burman design for 7 factors	30
Table 5 Structural Comparisons of CCC (CCI), CCF, and Box-Behnken Designs for Three Factors (53).....	33
Table 6 Independent and dependent variables in screening and optimization designs	39
Table 7 The developed HPLC analytical conditions from determination of t-resveratrol in peanut kernels	52
Table 8 Precision results from assaying two different analysis day	55
Table 9 Accuracy results of three different concentration spiked samples	55
Table 10 System suitability of t-resveratrol from replicate injections of standard solution.....	56
Table 11 SLE full factorial experimental design table and its responses	57
Table 12 ANOVA table of SLE-FFD study	58
Table 13 Experimental design table for SLE-CCF study in uncoded unit	61
Table 14 ANOVA table for all responses	62
Table 15 Predicted and experimental values at optimized conditions of SLE-CCF study.....	73
Table 16 UAE full factorial design table and its responses	80
Table 17 ANOVA table of UAE-FFD study	82
Table 18 Experimental design table for UAE-CCF study in uncoded unit	84
Table 19 ANOVA table for all responses	85
Table 20 Predicted and experimental values at optimized conditions of UAE-CCF study.....	96
Table 21 MAE full factorial design table and its responses	102

Table 22 ANOVA table of UAE-FFD study	103
Table 23 Experimental design table for MAE-CCF study in uncoded unit.....	105
Table 24 ANOVA table for all responses	106
Table 25 Predicted and experimental values at optimized conditions of MAE-CCF study.....	113
Table 26 Responses at optimization point of SLE, UAE and MAE of 3 peanut varieties: Kalasin-I, Kalasin-II and Thainan-IX	119
Table 27 Pearson's correlation coefficients of all responses	121



LIST OF FIGURES

	Page
Figure 1 Unpaired electron in radical atom	5
Figure 2 Diseases caused by Oxidative Stress (22)	8
Figure 3 DPPH radical scavenging activity reaction	12
Figure 4 ABTS.+ generated by potassium persulfate ant its reaction (29).....	13
Figure 5 FRAP reaction	14
Figure 6 Generation of peroxy radical and ORAC reaction with antioxidants	15
Figure 7 Cavitation process caused by ultrasonication (43)	21
Figure 8 Optimization strategies.....	25
Figure 9 A graphical representation of 2-level, 3 factors full factorial designs (53)...	28
Figure 10 A graphical representation of 2-level, 3 factors fractional factorial designs (either dark or white spot is performed) (53).....	28
Figure 11 Generating of Central Composite Designs by combining factorial points, center point and star points (53).....	31
Figure 12 Comparison of the Three Types of Central Composite Designs (53)	32
Figure 13 A Graphical Picture of Box-Behnken Design for Three Factors (53).....	33
Figure 14 HPLC Chromatograms of t-resveratrol standard (a) peanut samples (b; t- resveratrol at 26.0 min) and UV spectrum of t-resveratrol standard (c) and t- resveratrol in peanut samples (d)	53
Figure 15 Standard curve of resveratrol standards (0.2-10 µg/mL)	54
Figure 16 Pareto Chart of variables (a) and main effect plot for %yield (b)	58
Figure 17 Pareto Chart of variables (a) and main effect plot for t-resveratrol (b).....	58
Figure 18 Pareto Chart of variables (a) and main effect plot for TPC (b)	58
Figure 19 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on %yield response.....	65
Figure 20 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on t-resveratrol content response.....	66
Figure 21 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on TPC response.....	67

Figure 22 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on TFC response.....	68
Figure 23 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on DPPH response.....	69
Figure 24 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on ABTS response.....	70
Figure 25 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on FRAP response.....	71
Figure 26 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on ORAC response.....	72
Figure 27 Pareto Chart of variables (a) and main effect plot for %yield (b)	81
Figure 28 Pareto Chart of variables (a) and main effect plot for t-resveratrol (b).....	81
Figure 29 Pareto Chart of variables (a) and main effect plot for TPC (b)	81
Figure 30 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on %yield response	88
Figure 31 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on t-resveratrol response	89
Figure 32 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on TPC response	90
Figure 33 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on TFC response	91
Figure 34 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on DPPH response.....	92
Figure 35 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on ABTS response	93
Figure 36 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on FRAP response.....	94
Figure 37 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on ORAC response.....	95
Figure 38 Pareto Chart of variables (a) and main effect plot for %yield (b)	103
Figure 39 Pareto Chart of variables (a) and main effect plot for t-resveratrol (b).....	103
Figure 40 Pareto Chart of variables (a) and main effect plot for TPC (b)	103

Figure 41 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on %yield response.....	107
Figure 42 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on t-resveratrol content response.....	108
Figure 43 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on TPC response.....	109
Figure 44 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on TFC response.....	110
Figure 45 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on DPPH response	111
Figure 46 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on ABTS response	111
Figure 47 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on FRAP response	112
Figure 48 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on ORAC response	112
Figure 49 Comparing all responses between SLE, UAE and MAE of Kalasin-I.....	120
Figure 50 Antioxidant activity of peanut extracts from var. Kalasin-I, Kalasin-II and Thainan-IX by DPPH, ABTS, FRAP and ORAC assays	120



CHAPTER 1

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is one of common food in Thailand that is widely consumed as boiled, roasted or one of food components. For nutritional aspect of peanut, per 100 g of nuts, it contains water 1.55 g, carbohydrates 21.51 g, fibers 8.0 g, lipids 49.66 g and proteins 23.68 g. It also contains a large group of vitamins and minerals including fat soluble vitamins; A, D, E, and K, water-soluble vitamins; B-Complex and vitamin C, as well as magnesium, calcium, potassium, phosphorus, zinc, iron, copper and selenium (1). Moreover, other bioactive phytochemicals are also available in peanut including polyphenols, mainly stilbenes and flavonoids (2). These compounds are defined as antioxidants which are able to delay, inhibit, or prevent the oxidation of oxidizable materials and lessen oxidative stress which is known to play an important role in the initiation of many illnesses such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions (3, 4).

Several extraction techniques were used to extract phytochemicals from medicinal plants. Maceration, a traditional Solid-Liquid Extraction (SLE) method, is the easiest and simplest extraction method but it requires a lot of time and solvent to finish extraction (5, 6). Some authors used this technique by macerating sample with different aqueous organic solvent together with shaking or vortex (7, 8). Sonication or Ultrasound-Assisted Extraction (UAE) is a green technique that has been reported to extract phytochemicals from plants. The extraction processes start by the disruption of plant cells and facilitating mass transfer to the solvent layer (9). The advantage of UAE is to offer faster extraction process with less phenolic compounds degradation (3). Phan-Thien et al. reported on using UAE to extract and analyze *p*-coumaric acid, salicylic acid, *t*-resveratrol, and daidzein (10). Microwave-Assisted Extraction (MAE) is another green extraction technique that the microwave radiation help accelerating the partition of analytes from sample matrix to the medium (5). It damages cell structure by making severe thermal stress and localizing internal high pressure toward the cell wall which results in cell wall disruption (11). Ultimately, the mass of phytochemicals is transferred toward cell walls which could improve extraction

efficiency and shorten extraction time subsequently (12, 13). MAE was used for extraction of phenolic antioxidants from peanut skins (12). It is benefit for small molecule phenolic compounds such as gallic acid, ellagic acid, quercetin, isoflavone and *t*-resveratrol (3). A study found that small molecule phenolic compounds were stable up to 100°C for 20 minutes using MAE (14).

In order to obtain the best extraction condition, the experimental design was employed to locate the optimal extraction condition. It is mainly categorized into screening designs and optimization designs. Screening design is used to indicate the important factors that influence potentially to the considered responses which two-level screening designs such as factorial or Plackett-Burman design are commonly used. Then, the important factors from screening step will be optimized by Response Surface Methodology (RSM), generally central composite design (CCD) or Box-Behnken designs, for finding out optimal extraction condition (15). The extraction of curcumin from *Curcuma longa* rhizomes using dynamic maceration was optimized by 25 full factorial design and the response surface methodology (16). Another study extracted natural antioxidants from *Piper betle* using UAE together with optimization by Box-Behnken design. The result showed that the optimized condition provided better yield, total phenolic content, total flavonoid content and antioxidant activity than conventional extraction method (17). RSM was used to optimize phenolic antioxidants extraction from peanut skins using MAE. The utilization of response surface experimental design with MAE was found significantly reduce an extraction time when compared to conventional extraction method (12). Another study employed experimental design in optimization of phenolic antioxidants using SLE and found that a similar recovery of phenolic compounds could obtain with only single extraction stage allowing a reduction in solvent consumption and required time for extraction (18). Moreover, applying Box-behnken design in extraction of mulberry leaf polysaccharide (MLP) by hot water and enzyme-assisted extraction demonstrated that threefold higher in MLP yield could be obtained (19).

According to the advantages of experimental design that have been mentioned, the utilizing of experimental design for optimization of bioactive compound extraction is interesting. Moreover, the study about optimization of bioactive

compound extraction from Thai peanut kernels has not been established which is probably useful for depicting the awareness of Thai peanut.

1.1 Statements and significance of the problems

Peanut is one of popular consumed fruit in Thailand. Some people take it for granted since it can be afforded easily at very low price. Moreover, the production was not sufficient to the market demand and its economic value was underrated. To increase commercial appeal, the benefits of peanut should be exposed more than the nutritional values. Polyphenols is one of antioxidant classes that benefits to human health and possesses many biological activities such as anti-inflammatory and anticancer properties. However, the efficient extraction method is required to extract these phytochemicals and expose the actual available content of these biologically active compounds in peanuts. More researches are needed to improve the extraction procedures that provide higher extraction efficiency and lower extraction time. This dissertation focused on optimization of extraction condition by comparing different extraction methods. Moreover, the antioxidant powers of peanut extracts were determined by various antioxidant mechanism assays and content of *t*-resveratrol which is known as a precious food supplement nowadays was also quantified.

1.2 Objectives of research

1. Primary objective

To employ experimental designs for investigation and comparison the optimal extraction condition for bioactive compounds in peanut between solid-liquid extraction (SLE), ultrasound-assisted extraction (UAE) and microwave assisted extraction (MAE).

2. Secondary objectives

2.1 To develop a reliable analytical method for determination of *t*-resveratrol in Thai peanut extract using low particle size HPLC column.

2.2 To determine total phenolic content (TPC) and total flavonoid content (TFC) in Thai peanut extract.

2.3 To determine antioxidant capacity and study antioxidant mechanism of Thai peanut extract using radical scavenging antioxidant test, DPPH and ABTS, Ferric reducing antioxidant power (FRAP) assay and Oxygen radical absorbance capacity (ORAC) assay.

1.3 Hypothesis

1. The generated optimization model can accurately predict the total phenolic, flavonoid, *t*-resveratrol content and antioxidant capacity at optimized condition.
2. A developed HPLC method for determination of *t*-resveratrol from Thai peanut extract using low particle size packing HPLC column is accurate, reliable and reproducible.
3. Thai peanut contains *t*-resveratrol and bioactive compounds.
4. There are correlations among TPC, TFC, *t*-resveratrol and antioxidant activities.

1.4 Scope of the study

Thai peanut sample (var. Kalasin-1) was bought from a local market in Bangkok and processed pre-treatment steps. The extraction methods in the study involved SLE, UAE and MAE. The important variables of each extraction techniques were screened by full factorial design. The significant variables will be carried on optimization step using response surface methodology, central composite design (CCD). The responses for screening step include %yield, *t*-resveratrol content and TPC. Then, TFC and antioxidant activity tests, DPPH, ABTS, FRAP and ORAC, were additionally determined in an optimization step. The statistically significant mathematical model is generated to locate the optimal extraction condition for each extraction technique by statistical software, MinitabTM18. The model is verified for checking validity at optimized condition.

1.5 Expected or Anticipated Benefit Gain

When this research done, some results can be compiled using easily understandable language and disseminated through the approachable media. In this way, people can create awareness of peanut benefits and enormous value would be added to the peanuts, finally. Moreover, the research can be further developed by creating some peanut extracts incorporated products which is another way to add value to peanuts as well.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Polyphenols are the major class of natural antioxidants found in peanut and possesses many pharmacological activities that are beneficial to human health. However, to get the highest polyphenols recovery, the optimal extraction techniques and conditions should be explored. This review highlights the theory and current state of research that relates to free radicals and antioxidants, conventional (solid-liquid extraction) and green (ultrasound-assisted extraction and microwave-assisted extraction) methods for the extraction of antioxidant compounds and also analytical methods for determination of antioxidant capacities of the extracts and *t*-resveratrol content in the extracts. In addition, this review discussed about the experimental designs utilized in natural antioxidants extraction.

2.2 Free radicals

Free radicals are molecules or molecular fragments that contain one or more unpaired electron in molecular orbitals (Figure 1) (20). The uncoupled electron leads to the reactivity of the free radical to the adjacent molecules such as lipids, proteins and carbohydrates (21). The free radicals can be broadly divided into reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both reactive species can act as either beneficial or noxious substance to the living cells (4).

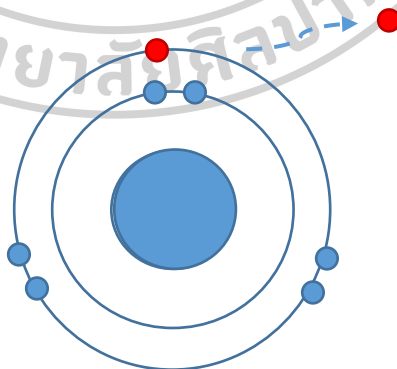


Figure 1 Unpaired electron in radical atom

Generally, free radicals are synthesized from two major pathways: cellular metabolism and external induction (pollution, cigarette smoke, radiation and medication, heavy or transition metal). At low concentration, free radicals exert cell

responses and immune system but at high concentration, it can generate the oxidative stress which can damage the cells (22) and causes different types of disease including cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, aging and atherosclerosis (23).

2.2.1 Reactive Oxygen Species (ROS)

The free radicals that oxygen involved in generating are called reactive oxygen species (ROS) (21). The reactive oxygen species include hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\cdot-}$), singlet oxygen, hydrogen peroxide (H_2O_2), peroxy radical ($\text{ROO}\cdot$) and alkoxy radical ($\text{RO}\cdot$).

Superoxide anion ($\text{O}_2^{\cdot-}$) is an oxygen molecule that contains an extra electron. It is considered as the least reactive ROS (21) but can react with two proton via superoxide dismutase (SOD) and form hydrogen peroxide (4).

Hydroxyl radical ($\cdot\text{OH}$) is the most reactive radical in ROS and has very short half-life approximately of 10^{-9} s. Hence, it is very dangerous radical in living cells. Hydroxyl radical can be produced under Fenton's reaction. At stress condition, the overproduction of superoxide will release free ions (Fe^{2+}) and then participate in Fenton's reaction which result to the generating of hydroxyl radical (20). Moreover, the superoxide anion can react with hydrogen peroxide and makes the hydroxyl radical via Haber-Weiss reaction as well (20). The hydroxyl radical takes part in damaging of cell membranes and lipoproteins by lipid peroxidation process (21).

Peroxy radical ($\text{ROO}\cdot$) is another radical which is typically found in living systems. The conjugate acid of superoxide ($\text{HOO}\cdot$) is the simplest form of peroxy radicals. The biochemistry of peroxy radical is dependent on the R group substitution and local environment. The peroxy radicals involve in lipid peroxidation, DNA cleavage, protein backbone modification and synergistic of superoxide induced DNA damage (4).

Hydrogen peroxide (H_2O_2) is non-radical itself but easily convert to radical molecule. It is mostly produced from peroxisomes because it has highly consumption of oxygen and used to oxidize molecules. Inside the organelles, the catalases enzyme is used to decompose the overproduced hydrogen peroxide to water and oxygen molecule. The peroxisomes have to balance between the production and

decomposition of hydrogen peroxide otherwise it can contribute to oxidative stress (20).

2.2.2 Reactive Nitrogen Species (RNS)

Nitric oxide ($\text{NO}\cdot$) is a plentiful RNS radical that possess many physiological functions in biological system including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. It can be synthesized by enzyme nitric oxide synthases (NOSs) which is used to metabolize arginine to citrulline and derive $\text{NO}\cdot$ as by-product. The half-life of $\text{NO}\cdot$ is only a few seconds in aqueous but very stable and has longer half-life in low oxygen concentration environment. Nitric oxide can react with superoxide to form peroxynitrite in cells immune system during the inflammatory process which can cause DNA fragmentation and lipid oxidation (4).

Benefits of free radicals (22)

- Vital for maturation process of cellular structures
- Destroying invading pathogenic microbes
- Function of several cellular signaling systems
- Induction of a mitogenic response

Disadvantages of free radicals and oxidative stress (22)

- Alteration of cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA)
- Induction of variety of chronic and degenerative diseases

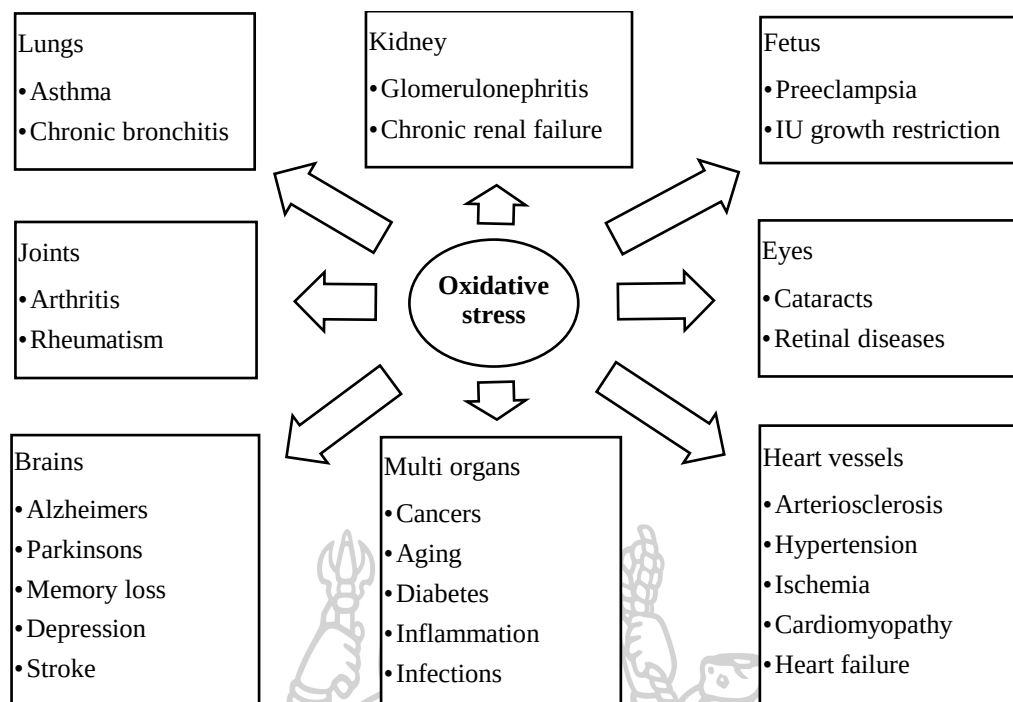


Figure 2 Diseases caused by Oxidative Stress (22)

2.2.3 Free radical-mediated chain reaction

The free radical can lead to a rapid destruction of cells by creating oxidative chain reaction. The oxidation of a free radical-mediated chain reaction comprises of 4 steps including initiation, propagation, branching and termination (24).

Initiation: $\text{LH} + \text{R}\cdot \rightarrow \text{L}\cdot + \text{RH}$

Whereas LH is substrate molecule (e.g., lipid), $\text{R}\cdot$ is initiating oxidizing radical. The $\text{R}\cdot$ radical oxidizes lipid and generates a highly reactive allyl radical ($\text{L}\cdot$).

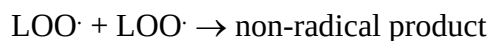
Propagation: $\text{L}\cdot + \text{O}_2 \rightarrow \text{LOO}\cdot$
 $\text{LOO}\cdot + \text{LH} \rightarrow \text{L}\cdot + \text{LOOH}$

The allyl radical reacts to oxygen molecule and make lipid peroxy radical. Then, further oxidizes another lipid molecule producing lipid hydroperoxides (LOOH) which can be broken down in branching step.

Branching: $\text{LOOH} \rightarrow \text{LO}\cdot + \text{HO}\cdot$
 $2\text{LOOH} \rightarrow \text{LOO}\cdot + \text{LO}\cdot + \text{H}_2\text{O}$

In branching step, the lipid hydroperoxides can be decomposed to lipid peroxy and lipid alkoxyl radicals but need transition metal ion as catalyst in this reaction.

Termination: $\text{LO}\cdot + \text{LO}\cdot \rightarrow \text{non-radical product}$



For termination step, the radicals will react with radical itself to make non-radical products.

2.3 Antioxidants

To deal with the overproduction of free radical, the body needs antioxidants to counteract the oxidative stress, either natural (endogenous antioxidants) or external antioxidants (exogenous antioxidants) (22). The dictionary has given the definition of antioxidants that “a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging)”. In terms of biochemistry and medicine, antioxidants can be defined as “enzymes or other organic substances, such as vitamin E or beta-carotene that are capable of counteracting the damaging effects of oxidation in animal tissues” (25). In food science, the antioxidant is defined as a substance in foods that when present at low concentrations, compared to those of an oxidizable substrate, significantly decreases or prevents the adverse effects of reactive species such as reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans (26).

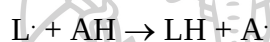
The antioxidants can be broadly divided into endogenous and exogenous antioxidants. Endogenous antioxidants which usually found in cells can be categorized as enzymatic antioxidants and non-enzymatic antioxidants. The enzymatic antioxidants which directly counteract to ROS and RNS radicals include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). The non-enzymatic antioxidants which are naturally produced by metabolism in the body include lipid acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins and transferrin (22).

The exogenous or nutrient antioxidants are antioxidants that cannot be produced in the body and must be derived by taken from foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc),

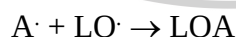
flavonoids, omega-3 and omega-6 fatty acids, etc. The nutrient antioxidants are needed in situation that body cannot balance between generated free radicals and the endogenous antioxidants (22).

Antioxidants can act in several ways such as being physical barriers to prevent ROS passing to important biological sites, absorption energy and electrons or quenching ROS, neutralization or diversion of ROS, binding or inactivation of metal ions to prevent generation of ROS and free radical chain-breaking (26).

According to the free radical-mediated chain reaction, if these reactions occur among the trace level of antioxidants, the antioxidants can reduce the chain reaction process by two mechanisms. Firstly, the antioxidants (AH) can directly react with a lipid radical in initiation step or peroxy or alkoxy radicals in propagation step. The antioxidants that can inhibit or delay the reaction by these mechanisms are called primary antioxidants (27).



An antioxidant radical can further counteract to the lipid radical in propagation step by forming peroxy antioxidant compound which is non-radical product. The efficiency of the antioxidant depends on the dissociation energy between AH bond and LH bond. The compound has higher antioxidant activity when it has lower AH bond strength (24).



Some antioxidants can retard the rate of oxidation in many ways including removal of substrate or singlet oxygen quenching. These antioxidants are called secondary or preventive antioxidants (27).

2.4 Measurement of antioxidant activity

The evaluation of antioxidant capacity can be performed through *in vitro* and *in vivo* assay. The *in vitro* methods are carried out in tube and generally determine the free radical traps while *in vivo* methods usually administer the tested samples to testing animals and after the designated time, the blood or tissues are collected from

sacrificed animal and analyzed. In this review, only the *in vitro* methods will be mentioned since it is frequently used because of its simple and reasonable cost of analysis (23).

For *in vitro* methods, the antioxidant assays can be classified into two types depending on the reactions; hydrogen atom transfer (HAT)-based assays and electron transfer (ET)-based assay (28).

HAT-based assays

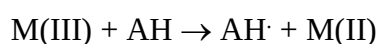
The HAT-based methods consider the ability of antioxidants to donate their hydrogen atom to radical molecules and form a stable compound.



The relative reactivity of HAT methods depends on the bond dissociation energy of H-donating group in antioxidant compounds. The HAT reaction methods are quick, only seconds or minutes to complete the test. It usually contains a free radical generator such as AAPH, 2,2'-azobis (2amidinopropane)dihydrochloride (ABAP), 2,2'-azobis(2,4dimethylvaleronitrile) (AMVN), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dichlorofluorescein (DCFH) and fluorescein. However, the HAT-based assay methods may have a limitation on the difference between tested conditions and biological conditions. In other words, the concentration of substrates is usually smaller than the concentration of antioxidant in HAT-assays but in fact, the concentration of antioxidants is less than the substrates (26).

ET-based assays

The ET-based methods discover the capability of antioxidant to transfer electron for reducing metals, carbonyls or radicals.



The relative reactivity of antioxidants depends on deprotonation and ionization potential of reactive functional group. Therefore, the pH is the critical parameter that can affect ionization potential. Moreover, the solvent which is used in the analysis should be considered to prevent solvent stabilization of charged species. ET-assays are slow and take long time to complete the reaction. The ET-assays consist of 2 composition, probe and antioxidant. Probe can act as oxidant or withdrawing an

electron from antioxidant compound which results in changing in probe color. The reaction completed when the probe color stopped changing. The limitation of ET-based assays is that no oxygen radical involved in the reaction (26).

2.4.1 DPPH scavenging assay

1,1-diphenyl-2-picrylhydrazyl or DPPH $\cdot$ is one of the stable radical molecules because of its capability in delocalization of spare electron throughout the molecule. This makes compounds visualized as deep purple color which can absorb UV at 515 nm. DPPH radical can react with both electron and hydrogen donors and resulting in discoloration of blue color which is a non-radical product of DPPH (Figure 3) (23, 28).

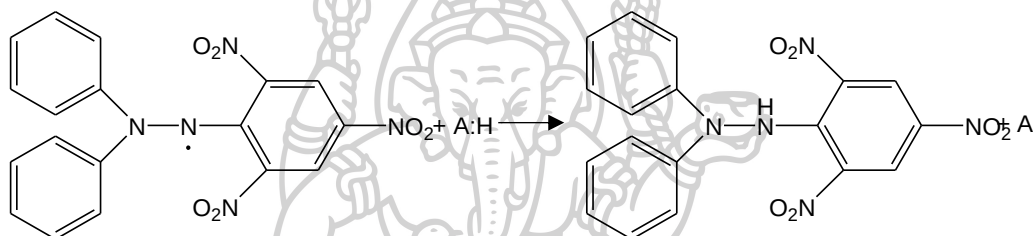


Figure 3 DPPH radical scavenging activity reaction

Advantages (26)

- Simple method because only UV-VIS spectrophotometer is required.
- High throughput screening with UV-VIS microplate reader

Disadvantages (26)

- DPPH is dissolved in methanol and ethanol but not dissolved in aqueous media which may lead to underestimation of total antioxidant activity because lack of hydrophilic antioxidants determination.
- The control sample and reagent blank must be prepared to avoid the overlapping the spectra with DPPH radical.
- The reaction may be influenced by steric effects of DPPH rather than the chemical characteristics and makes underestimation of antioxidant capacity for large antioxidant molecule.
- Trace metals can affect the radical reactions so the assays should be performed in double distilled or ultra-purify water only.
- The DPPH radical is not really existed in our body.

2.4.2 ABTS method

ABTS or TEAC assay is widely used in testing antioxidant capacity in food samples. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) is a peroxidase substrate that can be oxidized by peroxy radicals or other oxidants and generates a metastable radical cation ($\text{ABTS}^{\cdot+}$) (26). The $\text{ABTS}^{\cdot+}$ radicals give a bluish-green color which can absorb UV at 743 nm. ABTS is oxidized by potassium persulphate or manganese dioxide and give rise to the ABTS cation radical. The antioxidant reduces $\text{ABTS}^{\cdot+}$ to non-radical ABTS (decolorize) by quenching hydrogen atom of nitrogen atom that can be monitored by the decreasing of absorbance at 743 nm (Figure 4) (27).

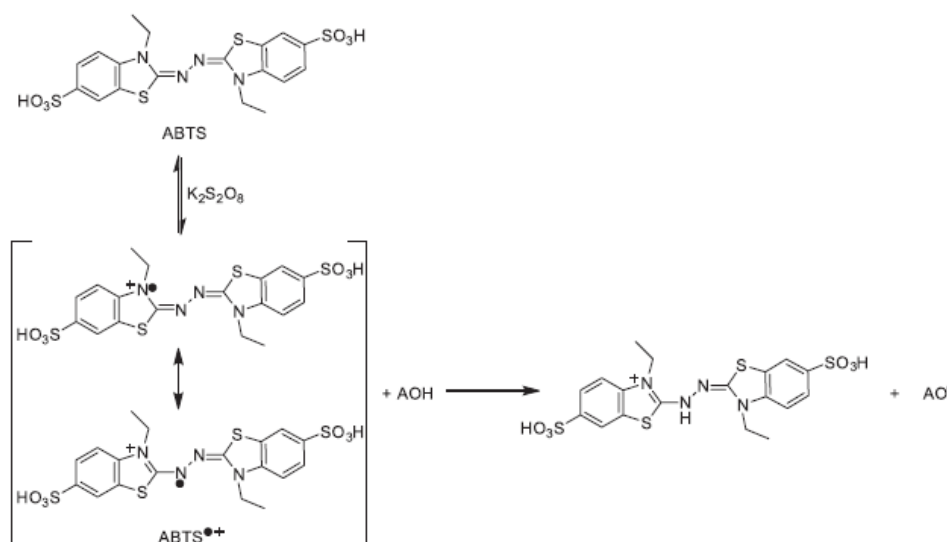


Figure 4 $\text{ABTS}^{\cdot+}$ generated by potassium persulfate and its reaction (29)

Advantages (26, 28)

- ATBS cation radical is soluble in both aqueous and organic solvent.
- Reaction is not affected by ionic strength.
- Simple operation
- Perform over a wide range of pH but frequently use at pH 7.4 because trolox and ascorbic standard show reliable end-point value after 10 min.
- Compatible with high-throughput screening by using microplate reader

Disadvantages (26)

- Phenolics in natural products may take a long time to complete the reaction.
- ABTS cation radical is not found in biological system.

2.4.3 Ferric reducing antioxidant power (FRAP) method

TPTZ or 2,4,6-tri(2-pyridyl)-1,3,5-triazine forms complex with ferric (Fe^{3+}) to ferric tripyridyltriazine (Fe(III)-TPTZ) which show yellow color. In present of antioxidant, it is reduced by antioxidants to ferrous tripyridyltriazine complex (Fe(II)-TPTZ) which show blue color and can be determined by UV absorption at 593 nm (26). The redox potential of Fe(III)-TPTZ ($<0.7\text{ V}$) is comparable to ABTS^+ (0.68 V) so TEAC and FRAP may give comparable relative values. The difference is that TEAC is tested at neutral pH while the FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility (30). The reaction monitors the increasing of ferrous 2,4,6-tripyridyl-s-triazine (Fe(II)-TPTZ) which shows blue colored product (Figure 5).

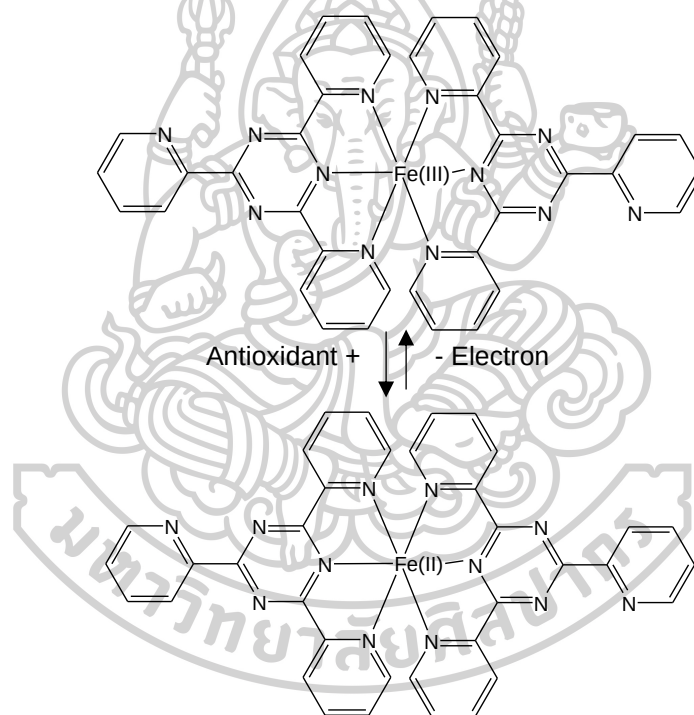


Figure 5 FRAP reaction

Advantages (26)

- Simple method, rapid, inexpensive and robust
- Can be automatic, semi-automatic or manual operation.

Disadvantages (26)

- Not all reductants that can reduce Fe(III) are antioxidants.
- FRAP value and the number of electrons in antioxidants is not correlated.

- FRAP assay cannot detect antioxidants that act by H transfer, especially thiols and proteins.
- The determination of FRAP activity by using fixed end point may not represent a completed reaction because some polyphenols do not stop the reaction at minutes but increase even after several hours of reaction time.

2.4.4 Oxygen radical absorption capacity (ORAC) method

This method focus on the activity of antioxidants to scavenge the peroxy radical (27) which is decomposed from AAPH (2,2-azobis 2-amidopropane dihydrochloride) in aqueous buffer by heating. The fluorescein, an oxidizable molecule which can be quantitated by fluorescence, will react with peroxy radical and form non-fluorescent product (26). Antioxidant capacity is determined by a decreased rate and amount of non-fluorescent product formed over time (30) (Figure 6). The loss of fluorescence indicates the extent of damage from its reaction with the peroxy radical. The fluorescence intensity will be measured every minute for minutes or hour at temperature of 37°C and wavelength 485 nm (ex)/525 nm (em) (26). Trolox is used as a compared standard and the ORAC value will be expressed in uM of Trolox equivalents (TE) per g dry weight of sample (µMTE/g) (23).

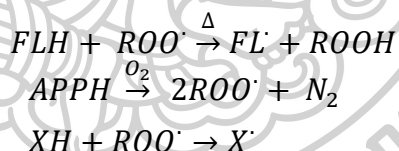


Figure 6 Generation of peroxy radical and ORAC reaction with antioxidants

Advantages (26)

- The ORAC assay is measured the net integrated area under the decay curves which stands for lag time, initial rate and total inhibition. This avoids the underestimation of antioxidant capacity and account for potential effects of secondary antioxidant products.
- Employing many types of radical
- Peroxy radical is really existed in biological system.

Disadvantages (26)

- Fluorescein is a non-protein probe which is more stable and less reactive but it is pH sensitive so the pH value should be carefully monitored during the run.

- The ORAC test with fluorescein probe is limited to the measurement of hydrophilic chain-breaking antioxidant only because the solubility of fluorescein in nonpolar organic solvent is very low.

2.5 TPC and antioxidant capacity in peanuts

Several authors have reported the availability of polyphenols in peanut parts that have significant antioxidant properties. Chuenchom et al. (31) studied antioxidant activities of peanut skin extracts. Polyphenols were extracted by stirring in methanol for 2 hours and results found that peanut skin extracts contained 79.97 to 188.52 mg gallic acid equivalent (mgGAE)/g dry peanut skins and exhibited antioxidant capacities of DPPH, ABTS and FRAP assay at 116.10, 227.95 and 97.65 mg trolox equivalent (mgTE)/g dry skin weight for Valencia peanuts.

Win et al. (32) performed quantitatively analysis of total phenolic contents (TPC) and antioxidant properties of peanut's skin, hull, raw kernel and roasted kernel flour. Peanut extracts were prepared by shaking in methanol on a water bath for 2 hours at room temperature. Results indicated that antioxidant activity and phenolic compounds were highest in peanut skin extract followed by peanut hull, roasted kernel flour (RKF) and raw kernel. The TPC and % DPPH scavenging activity were 0.92 mgGAE/g dry sample and 11.74% respectively. Moreover, a strong correlation was found between TPC and DPPH radical scavenging capacity with r^2 of 0.8436 using bivariate Pearson procedure.

Antioxidant activity by the hydrophilic oxygen radical absorbance capacity (H-ORAC) assay and TPC of peanut skin extract were determined by Lewis et al. (33) Peanut skins were extracted with 50%v/v acetone and 90%v/v ethanol at room temperature by stirring for 120 min. The H-ORAC and total phenolic values of the acetone peanut skin extract (A-PSE) were comparable to the ethanol peanut skin extract (E-PSE) (1836 μ mol Trolox/100 g and 67.9 mg GAE/g, and 1830 μ mol Trolox/100 g and 51.8 GAE/g respectively). Additionally, the anti-inflammatory activity of PSE was also determined and found that COX-2 protein expression was significantly inhibited by A-PSE and E-PSE at 2.5% and 5.0% concentrations.

The bioactive polyphenols in peanuts including phenolics, flavonols, and proanthocyanidins were reported by Chukwumah et al. (34). Extraction of phytochemicals was performed in 80%v/v methanol using ultrasound-assisted

extraction (UAE). Dry peanut extracts were suspended with 80%v/v methanol and passed through solid-phase extraction (SPE) before analyzed with HPLC-UV-MS. The group of compounds, genistein, daidzein, biochanin A, *trans*-resveratrol, rutin and quercetin were identified in all cultivars by comparing UV spectra and retention time with reference standard. *t*-Resveratrol found in this study ranged from 0.02–0.20 mg/100 g.

To study effect of germination, Rusydi et al. (8) have studied the effect of germination on TPC and found that TPC were decreased significantly in 48 hours germinated peanut (43.79 and 18.777 mgGAE/mg dry weight for non-germinated and germinated peanut extract respectively). The possible reason on decreasing of TPC was probably from the enzymatic changes during germination period in seeds. Another study, Limmongkon et al. (35) reported TPC, antioxidant capacity and *t*-resveratrol content in 5 peanut cultivars. TPC range from 15.65 to 36.85 µg gallic acid/g dry weight and antioxidant capacities by DPPH and FRAP assays were reported at 29.36 to 73.74 mmol Trolox/g dry weight, and 30.63 to 101.07 mmol ascorbic acid/g dry weight respectively. *t*-Resveratrol content was recovered in the range of 1.20 to 3.69 µg/g dry sample. The highest TPC, antioxidant capacity and *t*-resveratrol content were found highest mostly in first day of germination and declined along the germination time extension. Khang et al. (36) studied phenolic profile and antioxidant activity of peanut extract. In ungerminated grains, TPC were found at 18.21 mgGAE/g dry weight and identified including protocatechuic, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid, sinapic acid, ellagic acid and cinnamic acid. Antioxidant activities after five days of germination, peanuts showed highest antioxidant activities among black beans, mung beans, adzuki beans, soybeans and white cowpeas by DPPH method and reducing power assay.

Attree et al. (7) examined TPC, total flavonoid content (TFC) and antioxidant activity by DPPH scavenging capacity and ferric reducing antioxidant power (FRAP) assay and found that phytochemicals and antioxidant activities were contributed mainly by seed coat, followed by raw kernel and then cotyledon. It is more interesting that boiling process made significant losses in the phytochemicals and antioxidant capacity.

Antioxidant capacity and TPC of peanut skins from 9 different varieties were studied by Swatsitang et al. (37) Peanut skins were extracted with methanol by stirring for 2 hour at room temperature. The average TPC that found in this study was $8,554.97 \pm 1,264.80$ mg GAE/100 g dry peanut skin. Antioxidant activities were tested by DPPH and deoxyribose assay. Negative correlations were obtained between TPC and IC₅₀ values of both antioxidant assays which could be implied that higher phenolic content exhibited higher antioxidant activity.

Chukwumah et al. (38) investigated the relationship between peanut skin color and polyphenol content and antioxidant capacity. Pearson's correlation analysis revealed the interesting correlations between the redness and hue angle of the peanut skin with TPC and antioxidant capacity by Trolox Equivalent Antioxidant Capacity (TEAC) assay. Therefore, hue angle of peanut skin may be advantage for being a biomarker for TPC and antioxidant capacities.

Yu et al. (39) studied effects of processing methods and extraction solvents on TPC and antioxidant activity of peanut skin extract. The best recovery of phenolic was obtained by direct skin peeling method or blanching together with roasting and ethanol extraction. TPC value of 90–125 mgGAE/g dry sample was recovered. Comparing extraction conditions, ethanol extraction samples showed slightly higher total antioxidant activity than water extraction while demonstrated comparable TPC values.

2.6 Extraction of phytochemicals from plant materials

Extraction is a key for quality of the extracts. It is defined as a process of isolation of interested compounds and discard the insoluble cellular matrix behind (5). Before extraction, samples usually are passed pre-extraction step by milling, grinding and homogenizing which sometimes are preceded by drying process (3).

Several of extraction methods are available to extract phytochemicals from natural materials. A conventional extraction method, Soxhlet extraction, is one of solid-liquid and liquid-liquid extraction method. The samples are extracted by repetition of vaporizing and condensing of the extraction solvent through Soxhlet thimble. However, this method has been associated with large amount of solvent consumption and long extraction time. Moreover, it has high risk in explosion of flammable liquid organic solvents (3, 5). Later on, the novel green extraction

techniques have been proposed to improve extraction efficiency, reduce extraction time and solvent consumption. Some of these novel methods include subcritical water extraction (SWE), accelerated solvent extraction (ASE), solid-phase microextraction (SPME), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE). The details of each extraction method will be discussed.

2.6.1 Solid-Liquid Extraction (SLE)

Solid-liquid extraction is a process for removal of desired components (solute) from a food matrix using a liquid (solvent) that is capable to dissolve the solute (40). During the extraction, solutes from the food material have transferred to the solvent by employing three steps: 1) the solute dissolves in the solvent, 2) the solution moves through the particle of food to its surface and 3) the solution becomes dispersed in the bulk of the solvent (40). The conventional solvent extraction procedures include maceration, infusion, percolation and decoction (5). A lot of variables affect the yield of chemical extraction such as type of solvents, extraction time and temperature, sample-to-solvent ratio and chemical nature and physical characteristics of the plant samples (3).

Solvents including methanol, ethanol, acetone, ethyl acetate, and their combinations with different proportions of water have been utilized for the extraction of phenolics antioxidant from plant materials. Generally, methanol is effective in extraction of lower molecular weight polyphenols while acetone is found efficient for the higher molecular weight like flavanols extraction (3). Ethanol is also another good solvent for polyphenol extraction since it is safe for human consumption. At higher extraction temperature, the viscosity and the surface tension of the solvents are decreased which helps solvents reach the sample matrices and results in improving the extraction rate. This phenomenon promotes analyte solubility by increasing solubility and mass transfer rate. However, the degradation of phenolic compounds by using long extraction times and high temperature should be concerned because it increases the chance of oxidation and hydrolysis of phenolics (3).

SLE has been adopted by many researchers for extraction of phytochemicals from natural matrices. Aqueous organic solvent have been used in extraction of phenolic antioxidants together with facilitation by shaking and heating (18). The

maceration was used in phenolic antioxidants extraction and found that optimized SLE conditions were between 50 % and 70 % v/v aqueous ethanol (41). Yu et al. extracted phytochemical from peanut skin by putting 250 mg of sample in 20 mL of water, 80%v/v ethanol or 80%v/v methanol and stirred overnight at room temperature (39). Another study made a peanut extract by stirring a gram of ground peanut testae in 50 mL methanol for 2 hours at room temperature and evaporated the supernatant until it had become dryness (37) and there were some more studied that used SLE method to extract the phytochemical compounds (7, 33, 42). This indicated the benefits of SLE in ease of use, efficiency, and wide applicability (3). However, there are some drawbacks of this SLE conventional extraction method such as consuming large volume of solvents so it needs proper waste management before discard or using too high extraction temperature can result in degradation of phenolic antioxidants or sometimes the alteration of temperature is unachievable because of the limitation of boiling temperature of extraction solvents (5).

2.6.2 Ultrasound-assisted extraction (UAE)

Ultrasound is the mechanical waves at a frequency above the limitation of human hearing (>16 kHz) which can be categorized into two ranges according to their frequencies. If the frequencies are higher than 100 kHz at intensities below 1 Wcm^{-2} , it will be categorized to Low-energy ultrasound but if the frequencies fall between 20 and 500 kHz and intensities higher than 1 Wcm^{-2} , it was high-energy ultrasound (43).

UAE technique is usually implemented with solid-liquid extraction system because the transmission of ultrasound wave can occur easily. The acoustic wave will induce a cavitation in a liquid medium and help facilitating the mass transfer tremendously. When mechanical waves are transmitted through a fluid, there is changing in the average distance within the molecules by oscillating around their equilibrium position and it changes according to the cycle types. The intermolecular distance shortens during the compression cycle and lengthens in the rarefaction cycle. During the rarefaction cycle, when the pressure decrease is enough to exceed the critical distance between molecules, cavities will generate in the bulk liquid. Those beginning bubbles are growing until the minimal pressure is reached, the subsequent compression cycle starts again. After that, those cavities may start another rarefaction cycle or break down adiabatically following by a violent implosion at the end of a

compression cycle with high pressure shock waves at several hundred atmospheres and 5,000 K of temperature (44).

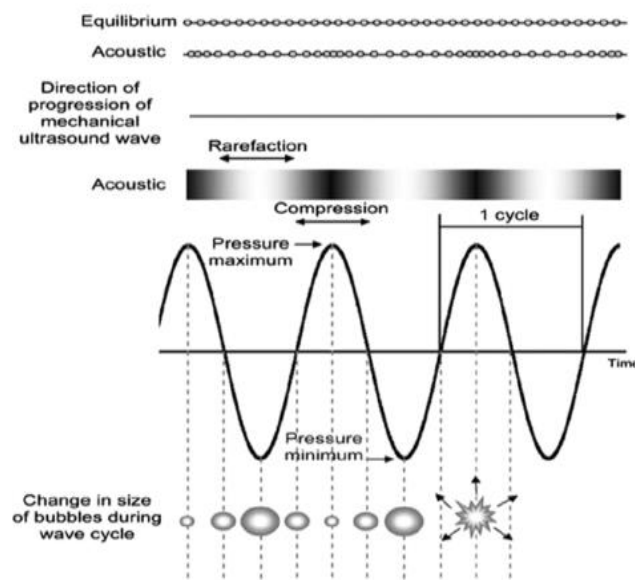


Figure 7 Cavitation process caused by ultrasonication (43)

Another mechanical effect that involves in sonochemical extraction process apart from shockwave-induced damages is microjet impacts. The microjet impacts cause by the collapsing of cavitation bubbles. The potential energy of the expanded bubble is altered to the kinetic energy of a liquid jet that extends inside the bubble and explodes to the opposite side of the bubble wall with the velocities of hundreds of meters per second. Moreover, the formation of radical species is also another principle that helps in extraction process. The dramatic changes in temperature or pressure could result in the scission of water molecules as a component in extraction solvent and lead to the formation of hydroxyl radicals which can enhance the cell walls destruction and mass transport processes (44).

The benefit of UAE is mainly reduction in extraction time and solvent consumption. Furthermore, the equipment is simpler, and the total extraction cost is lower. However, one of the main drawbacks of UAE is that it is not totally a green extraction method, sometimes the organic solvents usage is unavoidable (5, 43). From the benefits of UAE mentioned so far, UAE has been used extensively as an efficient extraction method in many studies. It has been used to extract phenolics antioxidant from the flower of *Limonium sinuatum* with appropriate volume of aqueous ethanol.

The optimum condition was found at 60%v/v ethanol, solvent to solid ratio of 56.9:1 mL/g, sonication time 9.8 min and temperature 40°C. It could reduce the extraction time and give higher antioxidants yield when compared to conventional maceration and Soxhlet extraction methods (45).

Horžic et al. used ultrasonic probe to extract bioactive content from yellow tea. The highest total flavonoid content was obtained by 75%v/v ethanol, 30 min of extraction and 50% ultrasonic amplitude. The prolonged extraction time increased the TFC yield. However, highest antioxidant capacity was obtained after 15 min of extraction and then slightly decreased. Conventional extraction method also succeeded the extracts with 75%v/v aqueous ethanol or water but it needed high extraction temperature so it can be thought as energy consuming method (46).

Trusheva et al. compared different extraction methods on the extraction efficiency of biologically active components from propolis and found that UAE was the most efficient extraction method among maceration and microwave-assisted extraction (MAE). The highest phenolics content was obtained at 70%v/v ethanol, solid to solvent ratio of 1:10 and sonication time 30 min. MAE and UAE methods demonstrated higher extraction yield, shorter extraction time and lesser work than maceration. However, higher amount of non-phenolics and non-flavonoids had been extracted by MAE which indicated the superior of UAE in selectivity of bioactive compounds in the extracts (6).

Orio et al. studied the extraction of psychoactive compound from *Mitragyna speciosa* leaves. Five grams of dry leaf powder in 50 mL of aqueous methanol solvent were extracted by ultrasonic probe at room temperature for one hour. The results found that UAE gave the highest yield for mitragynine while MAE gave the highest alkaloid amount (47).

2.6.3 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is an extraction process that uses microwave energy to facilitate partition analytes from the sample matrix into the solvent (3). The microwave energy produces heat directly in the molecules by ionic conduction and dipole rotation (48). Microwave radiates electromagnetic wave that induces the dipole rotation of the molecules and results in disruption of hydrogen bonding in the molecule. This phenomenon enhances the migration of dissolved ions

and promotes solvent penetration into the matrix (5). Therefore, MAE is a selective extraction method for polar molecules and solvents with high dielectric constant only (Table 1). In non-polar solvents, MAE is poor heating source as the energy is transferred by dielectric absorption only (5). The advantages of MAE are decreased extraction time and solvent consumption when compared to conventional extraction techniques (3).

Table 1 Dielectric constant of commonly used solvents (5)

Solvent	Dielectric constant (20°C)
Hexane	1.89
Toluene	2.4
Dichloromethane	8.9
Acetone	20.7
Ethanol	24.3
Methanol	32.6
Water	78.5

However, the extraction with MAE should be cautioned with the thermal degradation of phenolic compounds when using improper conditions. The degradation of small-molecule polyphenols such as gallic acid, ellagic acid, quacertin, isoflavin and *trans*-resveratrol have been reported. These molecules were found stable up to 100°C for only 20 minutes under microwave heating conditions and it has been serious decrease in the later cycles of MAE (5).

MAE has been utilized in many studies to extract phytochemicals from different plants. *Rhizma Polygoni Cuspidati* was extracted by MAE to optimize condition for *trans*-resveratrol extraction. At optimized conditions (the sample size of 0.30–0.45mm, the liquid/solid ratio of 20:1, the extraction temperature of 60°C and the extraction time of 10 min), the extraction yield of *trans*-resveratrol was 92.8% in a single extraction (49).

Swamy et al. studied continuous and intermittent MAE for pectin extraction from banana peels. The highest pectin yield from banana peels was derived at optimized condition of 2.18% and 2.58% for continuous and intermittent method respectively. The optimum condition was microwave power of 900 W and pH 3.00

and time 100 s for continuous and pulse ratio of 0.5 for intermittent method (50). However, this study has not compared to the conventional extraction method.

Ionic liquid-based microwave assisted extraction (IL-MAE) method has been proposed by Bhan et al. in extraction of andrographolide from *Andrographis paniculata*. The optimum MAE conditions were [bmim]Cl at concentrations of 2.84mol/L, microwave power 600W, extraction time 6 min, extraction temperature 70 °C, solid to liquid ratio of 1:10. The andrographolide yield at optimized conditions was increased from 1.5% w/w in 7 days (maceration) and 1.8% w/w in 3 h (soxhlet) to 4.71%w/w in 6 min (IL-MAE) (51). This indicated the better extraction efficiency and the shorter extraction time using MAE. Moreover, both IL and MAE are environmentally friendly since it is reusable and non-created pollution.

For extraction of essential oils using MAE, leaves of lemongrass were extracted with 850 W of microwave power, 200 mL of water, 30 min of rehydration time, and 45 min of extraction time. The results found that extraction time was the most influential factor affecting the extraction process and MAE was better than hydro-distillation in terms of yield, extraction time, biological activity, energy efficiency and environmental friendliness (11).

2.7 Experimental designs in phytochemicals extraction

In an optimization, the efficiency of scientific discovery is maximized while minimized waste and cost. The researchers would do smarter experiments by doing a fewest experiments which is enough to provide the most information (52). The optimal settings for several factors are going to figure out. Factors are parameters that can be set and affect the responses of the method procedure. If only one factor is optimized, the univariate procedure is performed. However, two or more factors are usually involved in study so either univariate or multivariate optimization strategies can be approached (15). The optimization strategies are shown in figure 8.

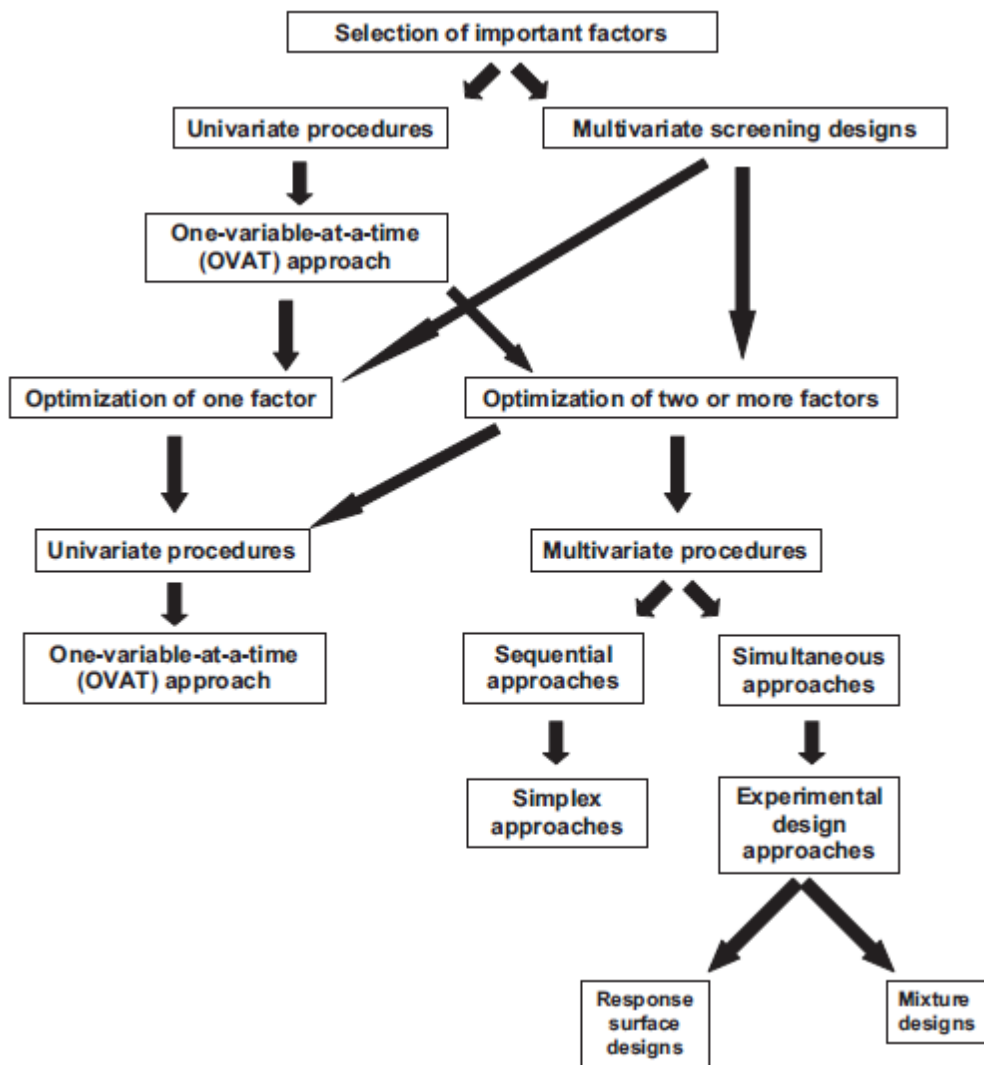


Figure 8 Optimization strategies

A univariate procedure is the one-variable-at-a-time (OVAT) approach which means only one factor at a time is varied and optimized. The OVAT approach is easy to operate, although, there is some disadvantages such as the interaction effects between factors are not taken into account, the number of experiments is increased when the number of factors increases, only a small part of the experimental domain is tested, the actual optimum might not be found, and the optimal conditions might depend on the starting conditions (15).

A multivariate approach will vary several factors at the same time of analysis and can be divided to sequential and simultaneous approaches. The sequential approach can be used when the experimental domain containing the optimum is unknown, but it is limited to one response optimization only. The simultaneous

approach will be done according to a well-defined experimental set-up and follows to an experimental orders which are predefined by the experimental design (15).

2.8 Terminology of experimental design (52)

- Experimental design: Statistical technique for planning, conducting, analyzing and interpreting data from experiments
- Response: Measured or observed quantity that is the subject of study or optimization, For example: peak area, retention time, number of theoretical plates, extraction yield
- Factor: Quantity that affects responses
 - Factor can be either controlled or uncontrolled, depends on the designs.
 - Factor can be discrete or continuous values. For example: column temperature, concentration of organic solvent, type of stationary phase
 - Some papers might mention variable, predictor and parameter instead of factor.
- Level of factor: Value of factor that is defined in an experimental design. The numbers of levels were created by the design, e.g., two-level, three-level design.
- Response surface: Relationship of a response to values of one or more factors which is usually plot in two- or three-dimensions graphic that is fitted to the experimental data.
- Response surface methodology (RSM): RSM is used to describe the use of experimental designs that give response surfaces from which information about the system is inferred.
- Model: The equation that relates a response to factors

A model can be empirical, which is chosen for the mathematical form, or is based on a theoretical understanding of the process that gives the response. Empirical models based on polynomials of the factors are described by the order of the polynomial, e.g., first order, second order.
- Effect: Coefficient of a term in a model
 - Main effect is the coefficient of the term in the first order of a factor.

- Interaction effects are coefficients of products of linear terms such as two-way interaction, three-way interaction.

Example:

$$\log(k) = \beta_0 + \beta_1 P + \beta_2 c_1 + \beta_3 c_1^2 + \beta_4 c_u + \beta_5 c_u^2 + \beta_6 g + \beta_7 g^2 + \beta_8 c_1 P + \beta_9 c_1 P + \beta_{10} g P + \beta_{11} c_u P + \beta_{12} c_1 g + \beta_{13} c_u g + \varepsilon$$

- This is second-order model in g , c_1 and c_u and first-order in P .
- β_1 , β_2 , β_4 and β_6 are main effect, β_3 , β_5 and β_7 are second-order effects, and β_8 to β_{13} are two-way interaction effects.

2.9 Experimental designs

The experimental designs can be categorized to screening design (e.g., full factorial, fractional factorial and Plackett-Burman designs), response surface designs (e.g., central composite designs, Box-behnken design, Taguchi design) and mixture designs. Screening designs use to screen many factors and identify the most influencing factors. Generally, the factors in screening designs will be evaluated at two levels. The response surface designs are used to optimize the most important factors and find the optimal condition. The factors or variables are usually examined at three levels and the optimal condition is obtained from response surface plot. Mixture designs are one of response surface designs but used when all factors studied relate to the mixture, for instances, the organic modifiers in a mobile phase or the excipients in a tablet or other formulations (15).

2.9.1 Screening design

The purpose of screening design is to indicate the most important factors that influence potentially to the considered responses. Apart from OVAT strategy, two-level screening designs including factorial or Plackett-Burman design are commonly used. When the number of factors are small, the two-level full factorial designs might be performed because the number of experiments are not too much to operate (15).

Factorial designs

Factorial designs create experiments at every combination of factor levels. The number of experiments created equal to L^k when L is number of levels and k is number of factors (52). For example, if we perform 2-leveled, 3-factors full factorial design, the number of experiments will be $2^3 = 8$. In full factorial design, every

experiment is performed while in fractional factorial design, only a specific subset of full is performed (Figure 9 and 10).

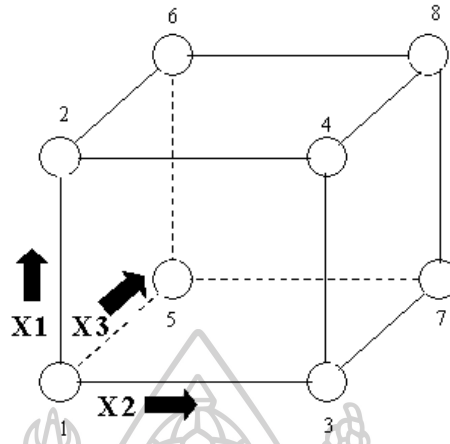


Figure 9 A graphical representation of 2-level, 3 factors full factorial designs (53)

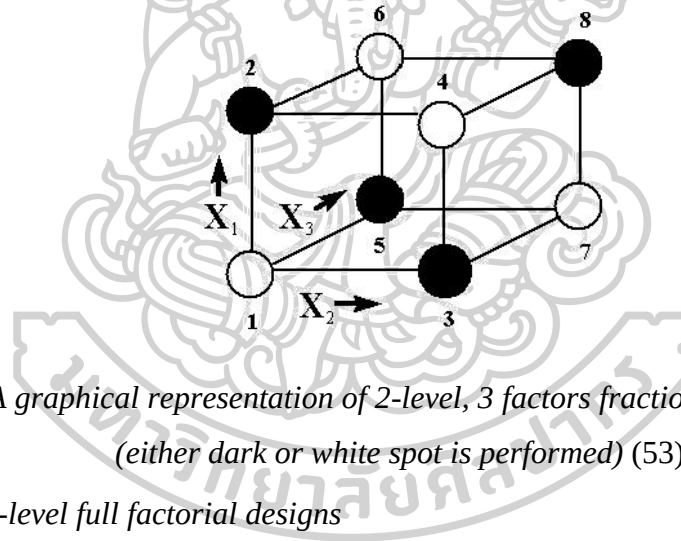


Figure 10 A graphical representation of 2-level, 3 factors fractional factorial designs (either dark or white spot is performed) (53)

2.9.1.1 Two-level full factorial designs

The all-possible combinations between the factors and levels are performed. $L=2$ and number of factors = k led to $N=L^k=2^k$ experiments. This design is capable to estimate all main and interaction effects. The interaction effects are calculated from the contrast coefficient columns (Table 2) (15).

Table 2 Two-level full factorial design for 3 factors and contrast coefficients for the interactions

Experiment	Factors			Contrast coefficients			
	A	B	C	AB	AC	BC	ABC
1	-1	-1	-1	1	1	1	-1

2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1

2.9.1.2 Two-level fractional factorial designs

A specific fraction of full factorial designs is performed. Number of experiments equal to $N=L^{k-v}=2^{k-v}$ when v is a fraction of the full factorial ($v=1, 2, 3, \dots$) (Table 3). In this design, N is power of two ($N=8, 16, 32, \dots$) because only half of full factorial designs is performed. Unfortunately, not all main and interaction effects can be estimated separately since some effects are confounded. For example in half-fraction factorial design, the estimation effect is confounding of two effects (15).

Table 3 Fractional factorial design for 4 factors and column of contrast coefficients

Experiment	Factors				Contrast coefficients		
	A	B	C	D	I_1	I_2	I_3
1	-1	-1	-1	-1	1	1	1
2	1	-1	-1	1	-1	-1	1
3	-1	1	-1	1	-1	1	-1
4	1	1	-1	-1	1	-1	-1
5	-1	-1	1	1	1	-1	-1
6	1	-1	1	-1	-1	1	-1
7	-1	1	1	-1	-1	-1	1
8	1	1	1	1	1	1	1

2.9.1.3 Plackett-Burman designs

The number of factors which are allowed in Plackett-Burman (PB) design equal to $N-1$ factors when N is number of experiments. For example, there are 8 experiments in the 7-factors PB design (Table 4). When k exceeds the number of real factors to be tested, the remaining columns are defined as dummy factor columns and

has no physicochemical meaning (15). These designs are useful for detecting large main effects and assuming all interactions are negligible.

Table 4 Plackett-Burman design for 7 factors

Experiment	Factors						
	A	B	C	D	E	F	G
1	1	1	1	-1	1	-1	-1
2	-1	1	1	1	-1	1	-1
3	-1	-1	1	1	1	-1	1
4	1	-1	-1	1	1	1	-1
5	-1	1	-1	-1	1	1	1
6	1	-1	1	-1	-1	1	1
7	1	1	-1	1	-1	-1	1
8	-1	-1	-1	-1	-1	-1	-1

2.9.2 Optimization designs

The important factors and ranges which either are obtained from screening step or previous knowledge will be further examined by response surface designs. Generally, only 2 to 3 factors are explored because too high number of experiments may employ if too many factors are investigated, and the entire response surfaces cannot be visualized which make it difficult to indicate the optimal point (15). In extraction processes, mostly central composite design (CCD) and Box-Behnken designs are used for optimization.

2.9.2.1 Central composite designs (CCD)

In the general two-level designs, only linear models of responses are obtained. It cannot provide information of maximum area or non-linear relationships. In another way, if more than two levels are studied by factorial designs, the great number of experiments should be carried out. The CCD can reduce the number of experiments by performing experiments at factorial points, star points and alpha-value points (Figure 11). The alpha-value depends on number of factors. The experiments that required by CCD equal to $L^k + Lk + n_c$ where L is number of levels, k is number of factors and n_c is number of replications at center point (52). CCD allows to estimate the constant, linear terms, quadratic terms and interactions between variables (54).

There are three types of CCD including circumscribe, inscribe and face-center designs (53).

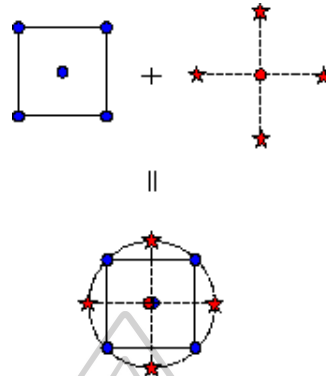


Figure 11 Generating of Central Composite Designs by combining factorial points, center point and star points (53)

2.9.2.1.1 Circumscribe CCD (CCC)

These designs are general type of CCD. Some journals might call rotatable CCD (55) since the position of alpha-value or star points represents new extremely low and high values for each factors in the designs and the distances from the center points to any positions of star points are the same (Figure 12). Rotatable property indicates the quality of variance at any experimental levels. CCC analyzes the largest process space and has circular, spherical, or hyper-spherical symmetry upon the number of factors. This design requires 5 levels for each factor.

2.9.2.1.2 Inscribe CCD (CCI)

Sometimes CCC designs cannot be achieved because of the setting limitations (too low or high values). The CCI is in consideration because those factorial points, center points and star points are still in the designs as same as CCC but the star points are reduced to ± 1 levels and creates new factorial points within those limits (Figure 12). Therefore, CCI seems to be a reduction scale of CCC design. Although, it has rotatable property as same as CCC, the smallest process space is examined by CCI. It cannot provide the same high quality prediction over the entire space when compared to the CCC (53). The highest and lowest levels in CCI are ± 1 and require 5 levels for each factor.

2.9.2.1.3 Face centered CCD (CCF)

The star points of CCF are at the center of factorial points. The alpha-values are ± 1 . Three levels are required for each factor. CCF design is not rotatable since the distances from center point to any experimental points are not the same (Figure 12). The advantages of CCF are providing relatively high-quality predictions over the entire design space and no experimental points outside the original factor range. However, it demonstrates poor precision for estimating pure quadratic coefficients (53).

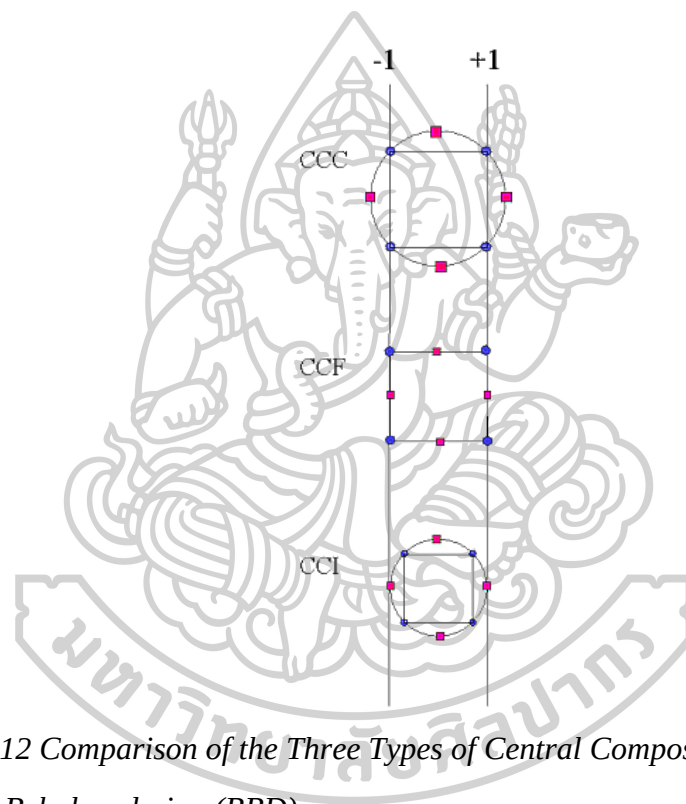


Figure 12 Comparison of the Three Types of Central Composite Designs (53)

2.9.2.2 Box-Behnken design (BBD)

In BBD, there is no factorial or extreme points as in CCC and this design require 3 levels for each factor. The number of experiments of BBD equal to $2k(k-1) + n_c$ when k represents number of factors and n_c represents replications at center point (52). In this design, the experiments are at the midpoints of edges of the process space and center point (Figure 13) and require fewer experiments than CCD (for 3 - 4 factors). BBD is a rotatable design but contains regions of poor prediction quality as same as CCI (53).

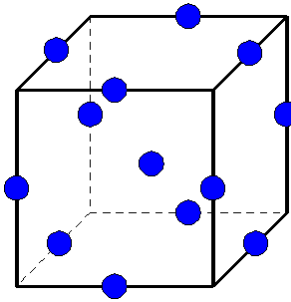


Figure 13 A Graphical Picture of Box-Behnken Design for Three Factors (53)

Comparisons of response surface designs for three factors (Table 5)

- Box-behnken designs give fewer experiments than CCC (CCI) and CCF but more than 4 factors, this advantage is disappeared.
- The structures of CCC and CCI are the same but different in alpha-value.
- The alpha-value of CCF equal to ± 1 .
- CCF and Box-behnken designs require 3 levels for each factor while CCC (CCI) requires 5 levels for each factor.

Table 5 Structural Comparisons of CCC (CCI), CCF, and Box-Behnken Designs for Three Factors (53)

CCC (CCI)				CCF				Box-Behnken			
Rep	X_1	X_2	X_3	Rep	X_1	X_2	X_3	Rep	X_1	X_2	X_3
1	-1	-1	-1	1	-1	-1	-1	1	-1	-1	0
1	+1	-1	-1	1	+1	-1	-1	1	+1	-1	0
1	-1	+1	-1	1	-1	+1	-1	1	-1	+1	0
1	+1	+1	-1	1	+1	+1	-1	1	+1	+1	0
1	-1	-1	+1	1	-1	-1	+1	1	-1	0	-1
1	+1	-1	+1	1	+1	-1	+1	1	+1	0	-1
1	-1	+1	+1	1	-1	+1	+1	1	-1	0	+1
1	+1	+1	+1	1	+1	+1	+1	1	+1	0	+1
1	-1.682	0	0	1	-1	0	0	1	0	-1	-1
1	1.682	0	0	1	+1	0	0	1	0	+1	-1
1	0	-1.682	0	1	0	-1	0	1	0	-1	+1
1	0	1.682	0	1	0	+1	0	1	0	+1	+1
1	0	0	-1.682	1	0	0	-1	3	0	0	0

1	0	0	1.682	1	0	0	+1				
6	0	0	0	6	0	0	0				
Total Runs = 20				Total Runs = 20				Total Runs = 15			

2.10 Desirable properties for response surface designs (56)

- Satisfactory distribution of information across the experimental region (rotatability)
- Fitted values are as close as possible to observed values.
- Good lack of fit detection
- Internal estimate of error
- Constant variance check
- Transformations can be estimated.
- Suitability for blocking
- Sequential construction of higher order designs from simpler designs
- Minimum number of treatment combinations
- Good graphical analysis through simple data patterns
- Good behavior when errors in settings of input variables occur

2.11 Experimental designs methodology (54)

1. Define the goal of the experiments.
2. Detect all the factors that can have an effect (Screening).
3. Plan the experiments and perform follow to the plan (Optimizing).
4. Analyze the data obtained by the experiments (Modeling).
5. Model verification (Predictive & Experimental value)
6. Compare to a standard method and perform add-on experiments (if any).

Several of related articles have used experimental design to optimize extraction conditions. Wang et al. used Box-behnken designs to optimize subcritical ethanol extraction of flavonoids from *Moringa oleifera* leaf. The optimization variables included ethanol concentration, extraction temperature and time. At the optimized condition, flavonoids yield was tremendously increased by 26.7% compared with the traditional ethanol reflux method while the extraction time was reduced from 3 hours to only 2 hours (57).

Iranian basil was extracted by UAE together with CCD to locate an optimal extraction condition. Three independent variables including temperature, methanol to water ratio, and sonication time were studied. The results found that %methanol and extraction temperature were the most influenced parameters on antioxidant capacity and TPC values while extraction time was insignificant variable. The optimum conditions were reported at temperature of 59°C, 65.2% v/v methanol and extraction time of 20 min (58).

Xu et al. optimized UAE conditions for natural antioxidants extraction from the flower of *Limonium sinuatum* using CCD. The ethanol concentration, solvent to solid ratio, sonication time and temperature were investigated and found that optimum extraction conditions were 60%v/v ethanol, solvent to solid ratio of 56.9:1 mL/g, sonication time 9.8 min and temperature 40°C. The experimental values at optimized condition matched with the predicted values within a 95% confidence level (45).

The extraction of lutein and chlorophyll from spinach was optimized using Box-behnken designs. The optimum extraction condition was at 93%v/v ethanol, solvent to raw material ratio of 1/66, extraction time 4.3 h and temperature 43°C. Under optimum condition, the experimental recovery of these molecules was close to those predicted (96% for chlorophyll and 70% for lutein). Hence, the model was adequate to optimize extraction of lutein and chlorophyll from spinach (59).

UAE of essential oils from clove was carried out using CCD. Three independent variables were investigated such as extraction temperature, time, and plant concentrations. The dependent variable was clove extract. The results showed the validity of the model used in the experiment and the extraction temperature was the most significant influence on the clove extract yield (60).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

Peanuts in this study were purchased from a local market on Phutamonthon Sai II rd. It consisted of three varieties: Kalasin-I, Kalasin-II and Thainan-IX. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, Trolox[®] and *trans*-resveratrol were purchased from Sigma-Aldrich, China or US. Gallic acid, Folin-Ciocalteu's (FC) reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2-2'-azobis(2-amino-propane) dihydrochloride (AAPH) and fluorescein sodium (FL) were procured from Sisco Research Laboratories (SRL, India). Other chemicals such as sodium carbonate (Na₂CO₃), aluminium chloride, potassium persulphate, sodium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate, sodium acetate trihydrate, iron(III) chloride hexahydrate were used analytical grade from Ajax Finechem.

3.2 Preparation of peanut samples

Peanuts were washed three times with tap water to remove residues and unpeeled. The kernels were blended with household blender (Tefal[®]) at highest speed for 3 minutes. The coarse powder was dried at 60°C until the weight was constant. It was crushed into fine powder with mortar and pestle and kept in refrigerator at 8°C until extraction.

3.3 Extraction procedures

3.3.1 Solid-Liquid Extraction (SLE)

The SLE samples were performed on J.P.Selecta[®] temperature-controlled water bath, Spain. One gram of dry peanut sample was accurately weighed and transferred into 50-mL conical tubes (in screening step) or 250-mL Duran bottle (in optimization step). The percentage of ethanol content, volume of extraction solvent, extraction temperature and extraction time were set follow to the experimental design tables. After finishing each extraction, the crude extracts were filtered, and the filtrates were collected. It was dried under rotary evaporator (Buchi[®], Japan) at 38°C. The dry extracts were kept at -20°C until analysis. Each extraction was performed once.

3.3.2 Ultrasound-Assisted Extraction (UAE)

One gram of dry peanut sample was accurately weighed and put into 50-mL conical tubes (in screening step) or 250-mL Duran bottle (in optimization step). The percentage of ethanol content, solvent volume, extraction temperature and extraction time were assigned according to the experimental design tables. The sample was sonicated at the highest sonication power of 230D Crest Ultrasonics Powersonic, 160 W, 45 kHz, USA. The media level and extraction position in sonication bath was constant. A tube was fixed to control the distance from the bottom of the tank. After complete sonication, the samples were filtered, and supernatant were separated. It was dried under rotary evaporator (Buchi®, Japan) at 38°C. The dry extracts were kept at -20°C until analysis. Each extraction was performed as a single replicate.

3.3.3 Microwave-Assisted Extraction (MAE)

MAE experiments were performed with CEM Discover® microwave synthesis system, USA using opened-vessel mode. A gram of dry peanut sample was transferred to 125-mL round bottom flask and 15 mL of extraction solvent was added. The condenser was connected at the top of the round bottom flask. %Ethanol content, extraction temperature and time were set according to the experimental design tables. The power of the microwave was temperature dependent. After microwave radiation, the samples were filtered, and supernatants were collected. It was dried under rotary evaporator (Buchi®, Japan) at 38°C. The dry extracts were kept at -20°C until analysis. All extraction experiments in screening and optimizing were done single replicate.

3.4 Experimental designs

3.4.1 Screening design

FFD were selected for the screening of 4 interested variables, each at 2 levels with 3 replications at center points. The independent variables were the percentage of ethanol (X_1), volume of extraction solvent (X_2), extraction temperature (X_3), and extraction time (X_4). The dependent variables were %yield (Y_1), resveratrol content (Y_2) and TPC (Y_3). The range and levels of independent variables were assigned by reviewing literatures (12, 18, 35, 37), and shown in Table 6. ANOVA was employed by Minitab18™ software and multiple regression method was used to generate the

mathematical models for each response. A linear polynomial regression was obtained as following equation:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i<j=1}^k \beta_{ij} x_i x_j$$

Whereas Y is the dependent variable; β_0 is the constant term; k number of variables; β_i is the coefficients of linear terms; β_{ij} is the coefficients of interaction terms.

The significance of variables for each response was evaluated by F-test. The factors with significance level above 5% ($p < 0.05$) were considered and carried on optimization step.

3.4.2 Optimization design

The independent variables were the same as in the FFD study since they were all significant factors, or they are required to preserve the rotatable design. The three levels center-faced CCD of significant variables from screening and 6 center points were utilized for optimization of extraction conditions. The dependent variables included %yield (Z_1), resveratrol content (Z_2), TPC (Z_3), TFC (Z_4), and antioxidant values by DPPH (Z_5), ABTS (Z_6), FRAP (Z_7) and ORAC (Z_8) assays (Table 6). A second-order polynomial equation fitted to the dependent variables data as equation:

$$Z_i = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i<j=1}^k \beta_{ij} x_i x_j$$

Where, Z_i is the dependent variables; β_0 is the constant term; k number of variables; β_i is the coefficients of linear terms; β_{ii} is the coefficients of quadratic terms; β_{ij} is the coefficients of interaction terms.

F-test and multiple regression analysis was conducted by Minitab18™, and the optimized extraction conditions were located at the highest antioxidant activity since the positive correlation between antioxidant activity and TPC (from screening study) has been evidenced (7, 31).

Table 6 Independent and dependent variables in screening and optimization designs

Screening design	Independent variables	Dependent variables
• SLE	The percentage of ethanol content, volume of extraction solvent, extraction temperature, extraction time	% yield, resveratrol content, TPC
• UAE	The percentage of ethanol content, volume of extraction solvent, extraction temperature, extraction time	% yield, resveratrol content, TPC
• MAE	The percentage of ethanol content, extraction temperature, extraction time	% yield, resveratrol content, TPC
Optimization		
• SLE	The percentage of ethanol content, volume of extraction solvent, extraction temperature, extraction time	% yield, resveratrol content, TPC, TFC, FRAP, ABTS, DPPH, ORAC
• UAE	The percentage of ethanol content, volume of extraction solvent, extraction temperature, extraction time	% yield, resveratrol content, TPC, TFC, FRAP, ABTS, DPPH, ORAC
• MAE	The percentage of ethanol content, extraction temperature, extraction time	% yield, resveratrol content, TPC, TFC, FRAP, ABTS, DPPH, ORAC

3.4.3 Model verification

The optimized models were verified by performing the experiments at optimized conditions for 6 replications and determining the responses. The outcomes should be within the 95% prediction interval (95%PI) values computed from the generated mathematical models. Moreover, the different varieties of peanut, Kalasin-II and Thainan-IX, were also examined at optimized conditions for checking model validity and comparing the responses across genotypes.

3.4.4 Statistical analysis

One-way ANOVA was employed to investigate significant differences between groups with Tukey HSD's post-hoc analysis ($p < 0.05$). Correlations between responses were determined by Pearson's correlation analysis at 95% confidence interval ($p = 0.05$).

3.5 Determination of %yield

The percentage of extraction yield (%yield) is calculated as follow:

$$\% \text{yield} = [\text{weight of dry extract (g)} / \text{weight of peanut powder (g)}] \times 100$$

3.6 Determination of *t*-resveratrol

Mobile phase preparation

- 1) Preparation of 0.1%formic acid in ultra-purified water (UPW)

Each 1 mL of Formic acid AR (Merck[®], USA) is adjusted to 1000 mL with UPW.

- 2) Preparation of 0.1%formic acid in methanol

Each 1 mL of Formic acid AR (Merck[®], USA) is adjusted to 1000 mL with methanol HPLC (VWR[®], UK).

The mobile phase is a mixture of *a*) and *b*) using gradient elution program as follow: 5 min; 22%B, 12 min; 23%B, 28 min; 63%B, 29 min; 80%B, 30 min; 5%B and 36 min; 5%B.

Standard preparation

- 1) Accurately weigh 10 mg of *t*-resveratrol working standard into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with methanol.
- 3) Pipette 100 μ L of standard stock solution into 10-mL volumetric flask.
- 4) Adjust to volume with methanol HPLC and mix well to obtain intermediate standard stock solution.
- 5) Pipette 20, 50, 100, 200, 500 and 1000 μ L of the intermediate standard stock solution into 10-mL volumetric flask.
- 6) Make up to 10 mL with methanol HPLC and mix well to receive *t*-resveratrol calibration curve standard solutions in the range of 0.2 – 10.0 μ g/mL.

Sample preparation

Peanut extracts are dissolved with 10 ml of methanol HPLC grade (VWR[®], UK) and centrifuge at 10,000 rpm for 5 minutes. The supernatant is injected to HPLC.

HPLC methods

HPLC:	Agilent [®] 1260 series, Germany
Column:	Poroshell 120 EC-C18, 2.7 μ m particles size, 4.6 x 150 mm diameter equipped with 2.0 mm guard column
Mobile phase (MP):	0.1%formic acid in UPW (MP-A), 0.1%formic acid in methanol (MP-B)
Gradient elution program:	5 min; 22%B, 12 min; 23%B, 28 min; 63%B, 29 min; 80%B, 30 min; 5%B and 36 min; 5%B
Column temperature:	40°C
Flow rate:	0.3 ml/min
Injection volume:	5 μ L
Detector:	UV 306 nm
Run time:	36 min

Validation of HPLC methods

The analytical method is validated through Specificity, Linearity and Range, Precision, Accuracy, Limit of Detection (LOD) and Limit of Quantitation (LOQ) and system suitability parameters.

1) Specificity

t-Resveratrol peak in sample preparation chromatogram is well separate from other peaks and peak purity factor should be more than 0.995.

2) Linearity and Range

The coefficient of determination (r^2) from a linear regression by plotting between *t*-resveratrol standard concentrations and peak area should be more than 0.999.

3) Precision

- Repeatability is performed by assaying 6 determinations at 100% of the sample concentration and %RSD of its 6 determinations should be less than 11% (AOAC guideline) and reported as repeatability of the method.
- Intermediate precision is carried out by assaying 6 determinations at 100% of the sample concentration on different day and the assay results between two analytical runs should not show difference using F-test.

4) Accuracy

Three levels of *t*-resveratrol standard are spiked into peanut samples and assayed. The mean recovery percentage of *t*-resveratrol is calculated and should be within 80-110% (AOAC guideline).

5) Limit of Detection (LOD) and Limit of Quantitation (LOQ)

t-Resveratrol standard is diluted to the concentrations that provided a signal-to-noise ratio about 3:1 and 10:1, and report as LOD and LOQ respectively.

6) System suitability

%RSD of 5 consecutive injections of *t*-resveratrol standard solutions is calculated and reported as system suitability of the method.

3.7 Determination of Total Phenolic Content (TPC) (61)

Preparation of 5% Na₂CO₃ solution

Accurately weigh 2.5 g of Na₂CO₃ into 50-mL volumetric flask and adjust to volume with UPW.

Standard preparation

- 1) Accurately weigh 10 mg of gallic acid reference standard into 10-mL volumetric flask.

- 2) Dissolve and adjust to volume with UPW.
- 3) Pipette 10, 20, 40, 60, 80 and 100 μL of the gallic acid standard stock solution into 1.5-mL micro-centrifuge tubes.
- 4) Adjust to 1 mL with UPW and mix well to obtain gallic acid calibration curve standard solutions in the range of 0.01 – 0.1 mg/mL.

Sample preparation

- 1) Dry peanut extract is dissolved with 10 mL of methanol HPLC and centrifuged at 4500 rpm for 5 minutes.
- 2) Pipette 200 μL of the supernatant into 1.5-mL micro-centrifuge tube.
- 3) Add 800 μL of UPW and mix well using Vortex[®].

TPC procedures

- 1) Pipette 20 μL of sample or standard preparation solutions into 96-well plate.
- 2) Pipette 20 μL of FC reagent into previously pipetted sample or standard wells.
- 3) Cover plate lid and leave for 5 minutes.
- 4) Pipette 160 μL of 5% Na_2CO_3 solution and keep it for 20 minutes at 40°C.
- 5) Measure UV-Vis absorbance at 700 nm using microplate reader.

Calculation

The result is expressed as milligram gallic acid equivalent per gram dry sample (mgGAE/g) using this equation:

$$\text{mgGAE/g} = C \times V \times \text{DF} / m$$

Whereas C = Conc. of gallic acid equivalence obtained from standard curve regression

V = Volume of first sample dilution

DF = Dilution Factor

m = Mass of dry sample used in extraction

3.8 Determination of Total Flavonoid Content (TFC) (62)

Preparation of 2% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution

Accurately weigh 1.0 g of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ into 50-mL volumetric flask and adjust to volume with UPW

Standard preparation

- 1) Accurately weigh 10 mg of quercetin reference standard into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with methanol.
- 3) Pipette 100 μ L of the quercetin standard stock solution into 1.5-mL microcentrifuge tube.
- 4) Adjust to 1 mL with methanol and mix well to obtain the highest concentration of quercetin calibration curve at concentration of 0.1 mg/mL.
- 5) Perform a serial dilution for more five concentrations until the lowest quercetin standard concentration at 0.003125 mg/mL is obtained. The quercetin calibration curve standard set includes 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.003125 mg/mL.

Sample preparation

Dissolve dry peanut extract with 10 mL methanol and centrifuge at 4500 rpm for 5 minutes and collect the supernatant.

TFC procedures

- 1) Pipette 50 μ L of sample or standard preparation solutions into 96-well plate.
- 2) Pipette 50 μ L of 2% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ solution into previously pipetted sample or standard wells.
- 3) Cover plate lid and leave for 60 minutes.
- 4) Measure UV-Vis absorbance at 420 nm using microplate reader.

Calculation

The result is expressed as milligram quercetin equivalent per gram dry sample (mgQE/g) using this equation:

$$\text{mgQE/g} = C \times V \times \text{DF} / m$$

Whereas C = Conc. of quercetin equivalence obtained from standard curve regression

V = Volume of first sample dilution

DF = Dilution Factor

m = Mass of dry sample used in extraction

3.9 DPPH radical scavenging activity determination (31)

Preparation of 0.2 mM DPPH solution

Accurately weigh 4.0 mg of DPPH into 50-mL volumetric flask and adjust to volume with methanol.

Standard preparation

- 1) Accurately weigh 2.5 mg of Trolox[®] reference standard into 1.5-mL micro-centrifuge tube.
- 2) Dissolve with 1 mL of methanol.
- 3) Pipette 50 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tube and 950 μ L of methanol into the same tube and mix well.
- 4) Pipette 10, 20, 50, 100, 150 and 200 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tubes.
- 5) Adjust to 1 mL with methanol and mix well to obtain Trolox[®] calibration curve standard solutions in the range of 0.00125 – 0.025 mg/mL.

Sample preparation

- 1) Dry peanut extract is dissolved with 10 mL of methanol HPLC and centrifuge at 4500 rpm for 5 minutes.
- 2) Pipette 100 μ L of the supernatant into 1.5-mL micro-centrifuge tube.
- 3) Add 900 μ L of methanol and mix well using Vortex[®].

DPPH procedures

- 1) Pipette 100 μ L of sample or standard preparation solutions into 96-well plate.
- 2) Pipette 100 μ L of 0.2 mM DPPH solution into previously pipetted sample or standard wells.
- 3) Cover plate lid and leave for 30 minutes.
- 4) Measure UV-Vis absorbance at 515 nm using microplate reader.

Calculation

The result is expressed as milligram Trolox[®] equivalent per gram dry sample (mgTE/g) using this equation:

$$\text{mgTE/g} = C \times V \times \text{DF} / m$$

Whereas C = Conc. of Trolox[®] equivalence obtained from standard curve regression

V = Volume of first sample dilution

DF = Dilution Factor

m = Mass of dry sample used in extraction

3.10 ABTS radical scavenging activity determination (31)

Preparation of 7 mM ABTS solution

- 1) Accurately weigh 3.8 mg of ABTS into 15-mL centrifuge tube.
- 2) Dissolve with 1 mL of UPW by Vortex[®].

Preparation of 2.4 mM potassium persulfate solution

- 1) Accurately weigh 6.7 mg of $K_2S_2O_8$ into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with UPW.

Preparation of ABTS^{•+} working solution

- 1) Pipette 1 mL of 2.4 mM $K_2S_2O_8$ solution into 7 mM ABTS solution.
- 2) Keep in the dark for 14-16 hours for radical generation.
- 3) Dilute the stock ABTS^{•+} with methanol until the absorbance at 734 nm was 0.8 ± 0.02 AU.

Standard preparation

- 1) Accurately weigh 2.5 mg of Trolox[®] reference standard into 1.5-mL micro-centrifuge tube.
- 2) Dissolve with 1 mL of methanol.
- 3) Pipette 50 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tube and 950 μ L of methanol into the same tube and mix well.
- 4) Pipette 10, 20, 50, 100, 150 and 200 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tubes.
- 5) Adjust to 1 mL with methanol and mix well to obtain Trolox[®] calibration curve standard solutions in the range of 0.00125 – 0.025 mg/mL.

Sample preparation

- 1) Dry peanut extract is dissolved with 10 mL of methanol HPLC and centrifuge at 4500 rpm for 5 minutes.
- 2) Pipette 20 μ L of the supernatant into 1.5-mL micro-centrifuge tube.
- 3) Add 980 μ L of methanol and mix well using Vortex[®].

ABTS procedures

- 1) Pipette 50 μ L of sample or standard preparation solutions into 96-well plate.
- 2) Pipette 100 μ L of ABTS⁺ working solution into previously pipetted sample or standard wells.
- 3) Cover plate lid and leave for 30 minutes.
- 4) Measure UV-Vis absorbance at 734 nm using microplate reader.

Calculation

The result is expressed as milligram Trolox[®] equivalent per gram dry sample (mgTE/g) using this equation:

$$\text{mgTE/g} = C \times V \times \text{DF} / m$$

Whereas C = Conc. of Trolox[®] equivalence obtained from standard curve regression

V = Volume of first sample dilution

DF = Dilution Factor

m = Mass of dry sample used in extraction

3.11 Ferric reducing antioxidant power (FRAP) determination (63)

Preparation of 300 mM acetate buffer, pH 3.6 solution

- 1) Weigh 0.31 g of sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) into 100-mL volumetric flask and dissolve with UPW.
- 2) Add 1.6 mL of glacial acetic acid and adjust to volume with UPW.
- 3) Measure and adjust pH to 3.6 ± 0.05 .

Preparation of 40 mM HCl solution

- 1) Pipette 170 μ L of 37%w/w HCl into 50-mL volumetric flask.
- 2) Adjust to volume with UPW.

Preparation of 10 mM TPTZ solution

- 1) Accurately weigh 31 mg of TPTZ into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with 40 mM HCl solution.

Preparation of 20 mM FeCl₃ solution

- 1) Accurately weigh 54 mg of ferric chloride hexahydrate (FeCl₃.6H₂O) into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with UPW.

Preparation of FRAP reagent solution

- 1) Mix these reagents at defined ratio:

- 20 mM FeCl ₃ solution	2 mL
- 10 mM TPTZ solution	2 mL
- UPW	2.2 mL
- 300 mM acetate buffer, pH 3.6 solution	20 mL
- 2) Keep at 37°C until used.

Standard preparation

- 1) Accurately weigh 2.5 mg of Trolox[®] reference standard into 1.5-mL micro-centrifuge tube.
- 2) Dissolve with 1 mL of methanol.
- 3) Pipette 100 µL of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tube and 900 µL of UPW into the same tube and mix well.
- 4) Pipette 20, 40, 60, 80, 100 and 150 µL of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tubes.
- 5) Adjust to 1 mL with UPW and mix well to obtain Trolox[®] calibration curve standard solutions in the range of 0.005 – 0.0375 mg/mL.

Sample preparation

- 1) Dry peanut extract is dissolved with 10 mL of methanol HPLC and centrifuge at 4500 rpm for 5 minutes.
- 2) Pipette 100 µL of the supernatant into 1.5-mL micro-centrifuge tube.
- 3) Add 900 µL of UPW and mix well using Vortex[®].

FRAP procedures

- 1) Pipette 50 μL of sample or standard preparation solutions into 96-well plate.
- 2) Pipette 150 μL of FRAP reagent solution into previously pipetted sample or standard wells.
- 3) Cover plate lid and leave for 30 minutes.
- 4) Measure UV-Vis absorbance at 593 nm using microplate reader.

Calculation

The result is expressed as milligram Trolox[®] equivalent per gram dry sample (mgTE/g) using this equation:

$$\text{mgTE/g} = C \times V \times \text{DF} / m$$

Whereas C = Conc. of Trolox[®] equivalence obtained from standard curve regression

V = Volume of first sample dilution

DF = Dilution Factor

m = Mass of dry sample used in extraction

3.12 Oxygen radical absorbance capacity (ORAC) determination (25, 64)

Preparation of 75 mM phosphate buffer, pH 7.4 solution

- 1) Weigh 0.238 g of sodium dihydrogen phosphate (NaH_2PO_4) and 1.556 g of disodium hydrogen phosphate (Na_2HPO_4) into 100-mL volumetric flask.
- 2) Dissolve and adjust to volume with UPW.
- 3) Measure and adjust pH to 7.4 ± 0.05 .

Preparation of 0.0042 mM Fluorescein stock solution

- 1) Accurately weigh 0.8 mg of fluorescein sodium into 50-mL volumetric flask.
- 2) Dissolve and adjust to volume with 75 mM phosphate buffer, pH 7.4 solution.
- 3) Pipette 1 mL of the previous solution into 10-mL volumetric flask.
- 4) Adjust to volume with 75 mM phosphate buffer, pH 7.4 solution.

Preparation of Fluorescein working solution

- 1) Pipette 1 mL of 0.0042 mM Fluorescein stock solution into 50-mL volumetric flask.
- 2) Adjust to volume with 75 mM phosphate buffer, pH 7.4 solution.

Preparation of 153 mM AAPH solution

- 1) Accurately weigh 414 mg of AAPH into 10-mL volumetric flask.
- 2) Dissolve and adjust to volume with 75 mM phosphate buffer, pH 7.4 solution.

Standard preparation

- 1) Accurately weigh 2.5 mg of Trolox[®] reference standard into 1.5-mL micro-centrifuge tube.
- 2) Dissolve with 1 mL of methanol.
- 3) Pipette 100 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tube and 900 μ L of 75 mM phosphate buffer, pH 7.4 solution into the same tube and mix well.
- 4) Pipette 10, 20, 40, 60, 80 and 100 μ L of the Trolox[®] standard stock solution into 1.5-mL micro-centrifuge tubes.
- 5) Adjust to 1 mL with 75 mM phosphate buffer, pH 7.4 solution and mix well to obtain Trolox[®] calibration curve standard solutions in the range of 0.0025 – 0.025 mg/mL.

Sample preparation

- 1) Dry peanut extract is dissolved with 10 mL of methanol HPLC and centrifuge at 4500 rpm for 5 minutes.
- 2) Pipette 50 μ L of the supernatant into 1.5-mL micro-centrifuge tube.
- 3) Add 950 μ L of 75 mM phosphate buffer, pH 7.4 solution and mix well using Vortex[®].
- 4) Pipette 200 μ L of the previous solution into 1.5-mL micro-centrifuge tube.
- 5) Add 800 μ L of 75 mM phosphate buffer, pH 7.4 solution and mix well using Vortex[®].

ORAC procedures

- 1) Pipette 25 μ L of sample, standard preparation solutions or 75 mM phosphate buffer, pH 7.4 solution as blank into opaque 96-wells plate.
- 2) Pipette 150 μ L of Fluorescein working solution into previously pipetted sample or standard wells.
- 3) Cover plate lid and incubate at 37°C for 15 minutes.

- 4) Pipette 25 μL of 153 mM AAPH solution into sample or standard wells.
- 5) Measure fluorescence kinetically at Ex.485/Em.535 nm every minute for 60 cycles using microplate reader (previously warm at 37°C).

Calculation

The result is expressed as milligram Trolox[®] equivalent per gram dry sample (mgTE/g) using this equation:

$$\text{mgTE/g} = ((C \times (\text{AUC}_{\text{sam}} - \text{AUC}_{\text{blank}}) \times \text{DF}) / (\text{AUC}_{\text{std}} - \text{AUC}_{\text{blank}})) / m$$

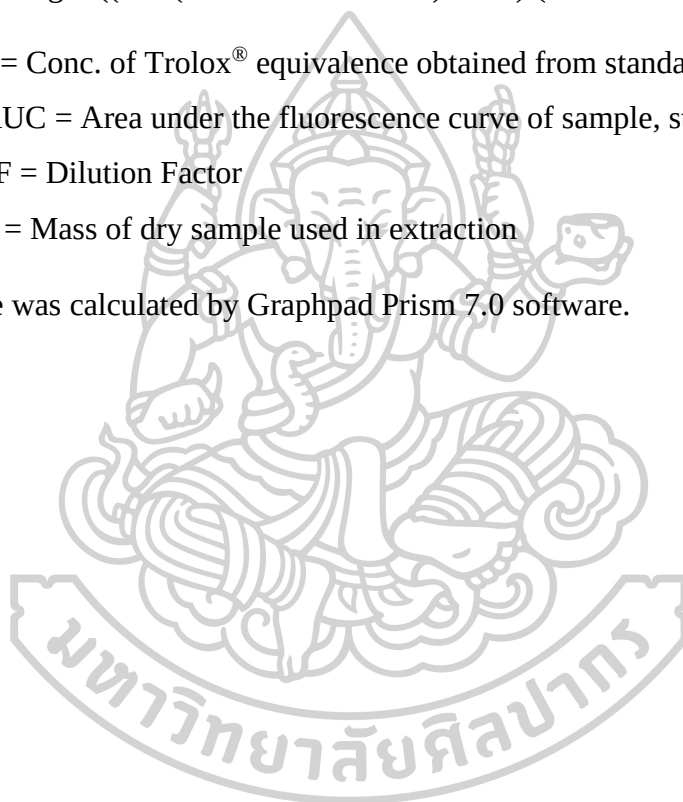
Whereas C = Conc. of Trolox[®] equivalence obtained from standard curve regression

*AUC = Area under the fluorescence curve of sample, standard or blank

DF = Dilution Factor

m = Mass of dry sample used in extraction

*AUC value was calculated by Graphpad Prism 7.0 software.



CHAPTER 4

RESULT AND DISCUSSION

4.1 Development of HPLC analytical method for determination of resveratrol in peanut kernels

4.1.1 Method development

The HPLC conditions for determination of *t*-resveratrol from peanut kernels were successfully developed and reported in Table 7. It was modified from the analytical method of Sales et al., 2009 (65) by changing organic solvent from acetonitrile to methanol and using smaller size of packing column (2.7 μ m). These resulted in slower elution of interested compound, increase of separation efficiency, decrease of overall runtime, and flow rate that finally reduced solvent consumption. The 0.1% of formic acid was added to the mobile phase to suppress the ionic dissociation of three hydroxyl groups of resveratrol and decreased peak tailing (66).

Table 7 The developed HPLC analytical conditions from determination of t-resveratrol in peanut kernels

Parameters	Conditions
HPLC	Agilent® 1260 series, Germany (PDA @306 nm)
Column	Poroshell 120 EC-C18, 2.7 μ m particles size, 4.6 x 150 mm diameter equipped with 2.0 mm guard column
Column temperature	40°C
Injection volume	5 μ L
Mobile phase	0.1%formic acid in UPW (MP-A) 0.1%formic acid in methanol (MP-B)
Flow rate	0.3 ml/min
Gradient program	5 min; 22%B, 12 min; 23%B, 28 min; 63%B, 29 min; 80%B, 30 min; 5%B and 36 min; 5%B
Runtime	36 min

The chromatograms of standard and sample preparation analysis were shown in figure 14. The using of small particle size column or Poroshell column indicated the high quality of *t*-resveratrol peak with K' value 11.23, peak width 0.085 min and theoretical plates above 500,000. The *t*-resveratrol peak showed up at 26.0 min. The runtime of this method was 36 minutes which was shorter than the previous methods (39, 67). Moreover, the developed method has advantages over previous methods as no sample purification steps is required prior to injection on HPLC (10, 34, 39).

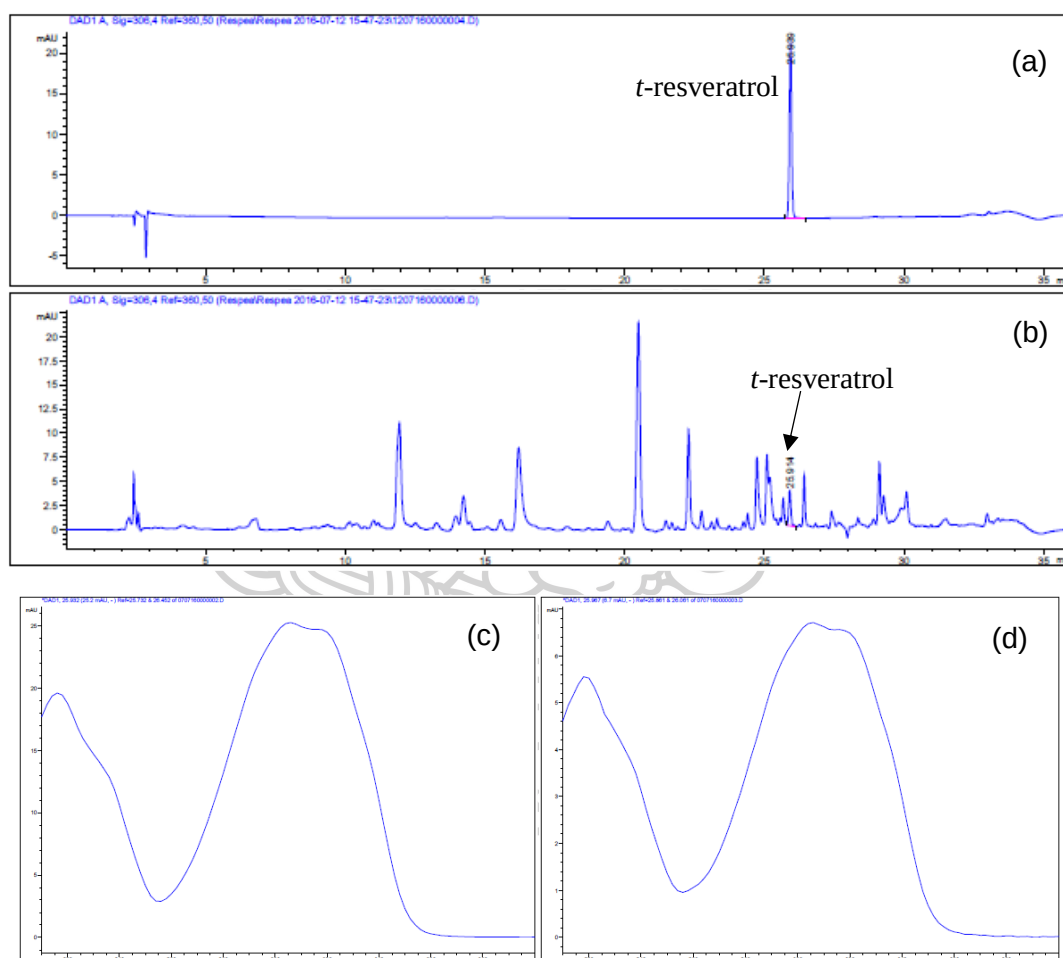


Figure 14 HPLC Chromatograms of *t*-resveratrol standard (a) peanut samples (b; *t*-resveratrol at 26.0 min) and UV spectrum of *t*-resveratrol standard (c) and *t*-resveratrol in peanut samples (d)

4.1.2 Method validation

The developed analytical method was validated through the ICH guidelines, and the results were shown in table 8-10. The validation procedures and acceptance criteria were shown in 3.6.

4.1.2.1 Specificity

The developed analytical method was specific to analyze *t*-resveratrol in peanut samples. The *t*-resveratrol peak was well separated from the nearby peaks with the resolution value 1.9. The peak purity factor of *t*-resveratrol was obtained at 0.998. The UV spectrum of *t*-resveratrol peak in standard (Fig.14(c)) and sample solution (Fig.14(d)) were comparable.

4.1.2.2 Linearity and range

The range of 0.2 – 10.0 µg/mL of *t*-resveratrol standard solution and the UV absorption responses were found a linear correlation with the regression equation of $y=145.2312x - 0.5374$ and the determination coefficient of 1.000 (Figure 15).

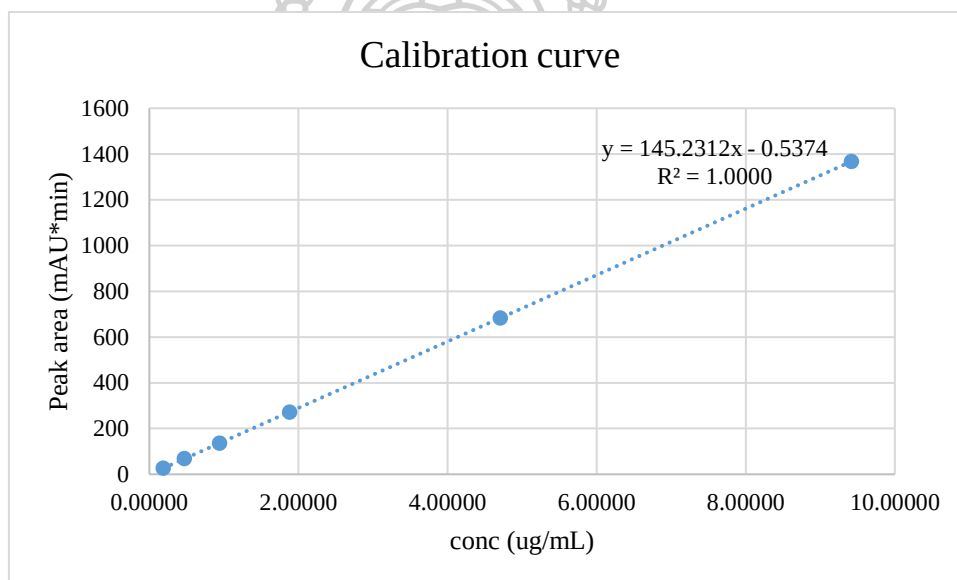


Figure 15 Standard curve of resveratrol standards (0.2-10 µg/mL)

4.1.2.3 Precision

The assay of six samples in two different analysis days were used to calculate %RSD for intra- and inter-day precision. It showed that the quantitative substance was not significant different between two analysis days by F-test at confidential value 0.05, and the %RSD of the same analysis day (6.67%, 7.06%) was passed the criteria of AOAC guideline, and less than the study of Lee et al. (68) (Table 8).

Table 8 Precision results from assaying two different analysis day

Replications	Content of <i>t</i> -resveratrol (µg/g)	
	Day 1	Day 2
1	2.1757	2.1539
2	2.1814	2.2298
3	2.4485	2.1259
4	2.5158	2.1633
5	2.1667	2.4197
6	2.2658	1.9469
Mean	2.2923	2.1733
SD	0.1528	0.1535
%RSD	6.67	7.06
F _{cal}	1.01	
F _{critical} 0.05(5,5)	5.05	

4.1.2.4 Accuracy

The recovery of the spiked samples at three different concentrations was determined. The %recovery was 90.84, 97.11 and 106.46 at spiked samples of 0.4, 0.5 and 0.6 µg/mL, respectively. The mean recovery at 98.14% was in the acceptable range of AOAC guideline (80%-110%) (Table 9).

Table 9 Accuracy results of three different concentration spiked samples

Sample	Peak area	conc total (ug/mL)	Calc. conc added (ug/mL)	Actual conc added (ug/mL)	% Recovery	average
Blank	56.83346	0.36017	0.00000	0.00000	0.00	
0.4-1	113.78746	0.69077	0.33060	0.37670	87.76	
0.4-2	118.92281	0.72058	0.36041	0.37670	95.68	
0.4-3	114.64615	0.69576	0.33559	0.37670	89.09	90.84
0.6-1	137.05286	0.82582	0.46565	0.47090	98.89	
0.6-2	136.55281	0.82292	0.46275	0.47090	98.27	
0.6-3	133.23279	0.80365	0.44348	0.47090	94.18	97.11
0.8-1	162.59384	0.97408	0.61391	0.56510	108.64	
0.8-2	165.34195	0.99003	0.62986	0.56510	111.46	
0.8-3	153.49063	0.92124	0.56107	0.56510	99.29	106.46

4.1.2.5 Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ values was reported at the concentrations that provide the signal-to-noise ratio about 3:1 and 10:1, respectively. They were reported at 0.002 and 0.005 µg/mL for LOD and LOQ respectively which were lower than many previous reports (0.01-0.25 µg/mL for LOD, and 0.04-0.77 µg/mL for LOQ) (66, 68-70).

4.1.2.6 System suitability

The system suitability of the method was determined by injecting 5 replications of *t*-resveratrol standard solution. The results showed that the %RSD of replicate injections was less than 1%, and the theoretical plates of *t*-resveratrol peaks were >500,000 which indicated the high-quality peak and suitability of the analytical method (Table 10).

Table 10 System suitability of *t*-resveratrol from replicate injections of standard solution

Injection order	Peak area (mAU*min)	Retention time (min)	Theoretical Plates (USP)
1	134.54988	26.039	515911
2	133.25362	26.045	516167
3	135.34184	26.041	526321
4	135.48450	26.047	516019
5	135.34827	26.060	524088
Mean	134.87482	26.046	519701
%RSD	0.69	0.03	0.98

4.1.3 Conclusion

The developed analytical method was suitable to analyze *t*-resveratrol in peanut kernels. Using of small particle size HPLC column provided the superior chromatogram with shorter analysis time and lesser solvent consuming. The validation results indicated that the developed method is precise, accurate and reliable to quantify the *t*-resveratrol in peanut samples.

4.2 Optimization of extraction conditions from peanut kernels using solid-liquid extraction (SLE)

4.2.1 Results

4.2.1.1 Screening design

The screening study was performed using two-leveled full factorial design of 4 variables with 3 replications at center point. The interested variables included solvent volume, percentage of ethanol content, extraction temperature and time. The interesting outcomes were %extraction yield, *t*-resveratrol and total phenolic content.

Nineteen experiments were generated and performed according to the experimental design. The results of each experiment were shown in table 11.

Table 11 SLE full factorial experimental design table and its responses

Run Order	Independent variables				Responses		
	Solvent vol (mL)	%EtOH (%)	Temp (°C)	Time (min)	%yield (%)	Resv (µg/g)	TPC (mgGAE/g)
1	10	80	70	30	6.86	1.5093	0.8688
2	50	80	30	30	2.52	0.7202	0.3443
3	50	80	70	30	11.96	2.3129	1.0871
4	10	80	70	120	11.07	2.4875	1.0562
5	50	20	30	30	4.15	0.5188	1.0069
6	30	50	50	75	6.65	2.0427	1.6410
7	50	20	30	120	5.00	0.6564	1.2033
8	10	20	30	120	3.57	0.4138	0.8244
9	10	80	30	30	2.42	0.5467	0.1476
10	10	20	30	30	2.34	0.2620	0.5367
11	50	80	70	120	15.13	2.4306	1.3051
12	50	20	70	30	9.78	2.3696	2.6081
13	50	80	30	120	4.40	1.0002	0.3658
14	30	50	50	75	5.92	1.5629	1.3436
15	30	50	50	75	7.75	1.9826	1.5976
16	10	20	70	30	6.87	1.7782	1.7399
17	10	20	70	120	7.25	1.8079	1.7508
18	50	20	70	120	10.69	2.6641	2.4762
19	10	80	30	120	3.91	1.0760	0.4955

The Pareto chart and main effect plots of variables obtained from model were shown in Figure 16-18. It was found that all variables were important for SLE. Additionally, the most influential factor for all responses was extraction temperature (Figure 16(a), 17(a), 18(a)). All of variables showed positive effect to %yield and *t*-resveratrol content except total phenolic content (TPC) that negatively correlated to %ethanol content (Figure 18(b)). The ANOVA table of FFD study was shown in table 12. The linear equation in uncoded unit obtained from full factorial study for %yield, *t*-resveratrol and TPC was in A.1, A.2 and A.3 respectively.

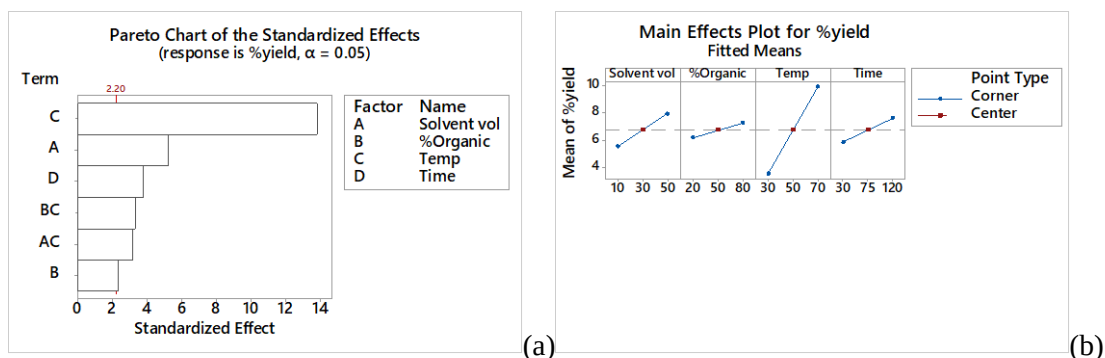


Figure 16 Pareto Chart of variables (a) and main effect plot for %yield (b)

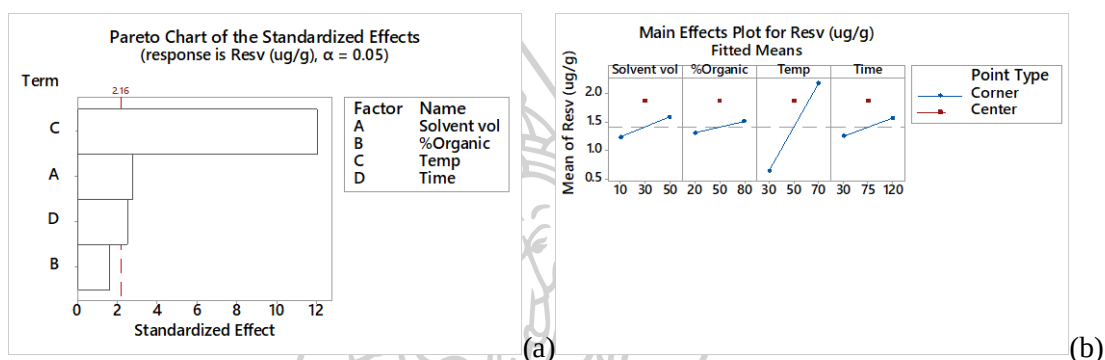


Figure 17 Pareto Chart of variables (a) and main effect plot for t-resveratrol (b)

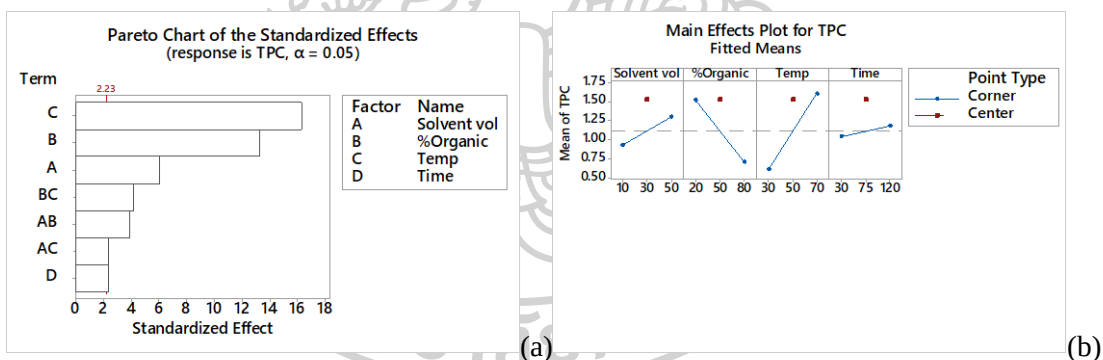


Figure 18 Pareto Chart of variables (a) and main effect plot for TPC (b)

Table 12 ANOVA table of SLE-FFD study

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	222.855	31.836	36.98	0.000
	Solvent vol	23.377	23.377	27.16	0.000
	%EtOH	4.644	4.644	5.39	0.040
	Temp	164.481	164.481	191.08	0.000
	Time	12.461	12.461	14.48	0.003
	Solvent vol*Temp	8.526	8.526	9.91	0.009
	%EtOH*Temp	9.364	9.364	10.88	0.007
	Lack-of-Fit	0.864	1.020	0.589	0.864
	$R^2(\text{adj}) = 93.33\%$				
	$R^2(\text{pred}) = 87.78\%$				

<i>t</i> -resveratrol	Model	10.8154	2.16308	33.95	0.000
	Sol vol	0.4870	0.48699	7.64	0.016
	%EtOH	0.1625	0.16253	2.55	0.134
	Temp	9.2507	9.25072	145.21	0.000
	Time	0.3965	0.39652	6.22	0.027
	Lack-of-Fit	0.6915	0.06286	0.92	0.629
	R ² (adj) = 90.15%				
	R ² (pred) = 84.79%				
TPC	Model	8.22601	1.02825	68.72	0.000
	Sol vol	0.55393	0.55393	37.02	0.000
	%EtOH	2.62128	2.62128	175.19	0.000
	Temp	3.96762	3.96762	265.17	0.000
	Time	0.08090	0.08090	5.41	0.042
	Solvent vol*%EtOH	0.22766	0.22766	15.22	0.003
	Solvent vol*Temp	0.08187	0.08187	5.47	0.041
	%EtOH*Temp	0.26001	0.26001	17.38	0.002
	Lack-of-Fit	0.09801	0.01225	0.47	0.816
	R ² (adj) = 96.78%				
	R ² (pred) = 93.93%				

Regression equation in uncoded units

- A.1) %yield = 0.47 - 0.0308 Solvent vol - 0.0458 %Organic + 0.0418 Temp
+ 0.01961 Time + 0.001825 Solvent vol*Temp
+ 0.001275 %Organic*Temp + 0.028 Ct Pt
- A.2) Resveratrol = -1.183 + 0.00872 Solvent vol + 0.00336 %Organic
+ 0.03802 Temp + 0.00350 Time + 0.453 Ct Pt
- A.3) TPC = -0.416 + 0.01030 Solvent vol + 0.00310 %Organic
+ 0.03016 Temp + 0.001580 Time -
0.000199 Solvent vol*%Organic + 0.000179 Solvent vol*Temp
- 0.000212 %Organic*Temp + 0.4139 Ct Pt

Although the opposite direction of %ethanol content on *t*-resveratrol and TPC was observed, the antioxidant activity of the peanut extracts should be contributed from TPC rather than only *t*-resveratrol alone. Furthermore, a group of studies demonstrated a positive correlation between TPC and antioxidant activities (7, 38, 71). Therefore, at 20%ethanol content that provided higher TPC level was selected for optimization study together with at solvent volume 50 mL, extraction temperature 70°C and time 120 min.

4.2.1.2 Optimization study

The suitable conditions derived from screening study (solvent volume 50 mL, 20%v/v ethanol content, extraction temperature 70°C and extraction time 120 min) were assigned as center point for optimization study. The 4 factors, 3-leveled center-faced central composite design (CCF) was used to optimize extraction conditions which generated 31 run orders (table 13). The variables of interest included %yield, *t*-resveratrol content, TPC, Total flavonoid content (TFC) and antioxidant capacity such as DPPH and ABTS antiradical activity, Ferric reducing antioxidant power (FRAP) and Oxygen radical absorbance capacity (ORAC). The results of these variables for each experimental run of optimized model were shown in table 13. The ANOVA tables of all responses were demonstrated in table 14. The optimized polynomial equations obtained from CCF study for all responses were equation B.1-B.8. The Pareto charts, main effect plots, contour plots and response surface plots of each response were shown in Figure 19-26.

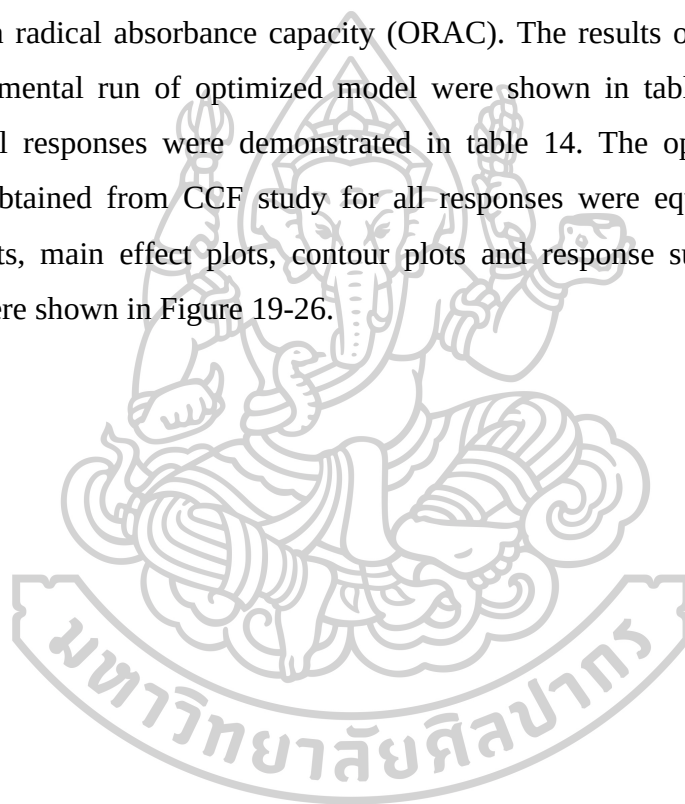


Table 13 Experimental design table for SLE-CCF study in uncoded unit

RunOrder	Independent variables					Responses						
	EtOH (%)	Sol vol (mL)	Temp (°C)	Time (min)	Yield (%)	Resv (µg/g)	TPC (mgGAE/g)	TFC (mgQE/g)	(mgTE/g)			
									FRAP	DPPH	ABTS	ORAC
1	10	75	80	60	10.10	2.9749	3.4730	0.0251	1.5493	0.9802	6.4038	14.3965
2	10	25	80	180	9.91	2.2889	2.9488	0.0299	1.3658	0.8769	6.2069	12.9682
3	10	75	80	180	11.25	3.1314	3.4499	0.0193	1.5814	1.0768	6.5382	15.7068
4	10	25	60	60	8.57	1.3470	2.8738	0.0504	1.1185	0.7657	6.0205	11.8995
5	30	75	60	60	8.84	2.2983	2.9017	0.1339	1.3205	1.0548	6.4601	11.7425
6	30	25	80	60	9.11	2.8584	3.0793	0.1481	1.3969	1.0860	6.5269	14.5162
7	30	50	70	120	10.13	2.9337	3.3343	0.1561	1.6419	1.2582	6.7509	17.5218
8	10	25	60	180	9.51	1.7981	3.0441	0.0328	1.2560	0.8394	6.1413	12.9554
9	30	75	80	180	11.79	3.7114	3.3995	0.2530	1.8235	1.3664	6.8888	17.7787
10	30	75	80	60	10.34	3.0575	3.6934	0.2438	1.6691	1.2878	6.6458	15.0488
11	10	50	70	120	10.50	2.7056	3.5947	0.0291	1.5984	0.9870	6.6959	15.1708
12	20	50	70	120	9.58	2.8180	3.4584	0.0338	1.5464	1.0696	6.7227	13.9065
13	20	50	70	120	9.80	2.8223	3.4465	0.0385	1.5505	1.1438	6.7227	14.6275
14	30	25	60	60	7.29	2.1024	2.7433	0.0305	1.2654	0.9338	6.3805	13.5717
15	20	50	80	120	10.63	3.3756	3.1759	0.0423	1.5804	1.1671	6.6195	15.0847
16	10	25	80	60	8.94	2.1467	3.0810	0.0154	1.3193	0.8431	6.1205	15.4365
17	20	50	70	120	10.06	2.9825	3.6179	0.0340	1.5721	1.0647	6.5632	14.5516
18	30	25	60	180	8.96	2.7766	3.1356	0.0535	1.4048	1.0180	6.2102	13.7678
19	10	75	60	60	9.67	1.9233	2.9344	0.0287	1.4210	0.9238	6.4258	9.926
20	20	50	70	60	9.25	2.6780	3.4166	0.0299	1.5304	1.1082	6.5506	11.246
21	20	50	70	180	10.10	3.0150	3.3820	0.0465	1.5577	1.1318	6.7359	12.9469
22	20	50	70	120	9.70	2.8732	3.5894	0.0395	1.6146	1.0872	6.7415	12.9076
23	20	50	70	120	9.75	2.9619	3.4613	0.0374	1.5836	1.1103	6.8078	12.7981
24	20	50	70	120	9.76	3.2497	3.6021	0.0362	1.6391	1.1296	6.5846	12.2018
25	30	25	80	180	10.30	3.2194	3.1541	0.1296	1.5124	1.1396	6.4362	14.9876
26	30	75	60	180	9.86	2.9189	3.5484	0.1281	1.5220	1.1156	6.3926	12.3682
27	20	25	70	120	8.81	2.5832	3.1555	0.0136	1.4562	0.9597	6.5656	10.6331
28	20	75	70	120	10.36	3.1984	3.7378	0.0398	1.6281	1.1163	6.9521	11.9614
29	10	75	60	180	10.96	2.2208	3.5449	0.0313	1.5772	0.9355	6.2065	11.2126
30	20	50	70	120	9.47	2.8422	3.4526	0.0336	1.5417	1.0572	6.5221	11.0186
31	20	50	60	120	9.52	2.8249	3.2364	0.0285	1.5164	1.0035	6.3781	9.4519

Table 14 ANOVA table for all responses

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	21.1824	3.02605	78.16	0.000
	%EtOH	0.4325	0.43245	11.17	0.003
	Sol vol	7.6963	7.69627	198.79	0.000
	Temp	4.692	4.69201	121.19	0.000
	Time	6.16	6.16005	159.11	0.000
	%EtOH*%EtOH	0.3653	0.36531	9.44	0.005
	Sol vol*Sol vol	0.5707	0.57070	14.74	0.001
	%EtOH*Temp	1.6256	1.62562	41.99	0.000
	Lack-of-Fit	0.6844	0.04026	1.17	0.453
	R ² (adj) = 94.74%				
	R ² (pred) = 92.98%				
t-resveratrol	Model	7.31149	0.52225	26.97	0.000
	%EtOH	1.58414	1.58414	81.80	0.000
	Sol vol	1.03402	1.03402	53.39	0.000
	Temp	2.38631	2.38631	123.22	0.000
	Time	0.75809	0.75809	39.15	0.000
	%EtOH*%EtOH	0.10352	0.10352	5.35	0.034
	Sol vol*Sol vol	0.0429	0.04290	2.22	0.156
	Temp*Temp	0.01697	0.01697	0.88	0.363
	Time*Time	0.07756	0.07756	4.00	0.063
	%EtOH*Sol vol	0.16818	0.16818	8.68	0.009
	%EtOH*Temp	0.01576	0.01576	0.81	0.380
	%EtOH*Time	0.0996	0.09960	5.14	0.038
	Sol vol*Temp	0.06561	0.06561	3.39	0.084
	Sol vol*Time	0.00063	0.00063	0.03	0.860
	Temp*Time	0.03329	0.03329	1.72	0.208
	Lack-of-Fit	0.16902	0.01690	0.72	0.692
	R ² (adj) = 92.38%				
	R ² (pred) = 86.83%				
	Model	1.85279	0.264684	19.54	0.000
	%EtOH	0.00011	0.000112	0.01	0.928
	Sol vol	0.66798	0.667975	49.31	0.000
TPC	Temp	0.12372	0.123720	9.13	0.006
	Time	0.11058	0.110575	8.16	0.009
	Temp*Temp	0.21593	0.215933	15.94	0.001
	Time*Time	0.01100	0.011000	0.81	0.377
	Temp*Time	0.30091	0.300907	22.21	0.000
	Lack-of-Fit	0.27326	0.016074	2.52	0.129
	R ² (adj) = 81.22%				
	R ² (pred) = 71.63%				
	Model	0.119929	0.008566	79.49	0.000
	%EtOH	0.057190	0.057190	530.66	0.000
	Sol vol	0.008853	0.008853	82.15	0.000
TFC	Temp	0.008398	0.008398	77.93	0.000
	Time	0.000018	0.000018	0.17	0.685
	%EtOH*%EtOH	0.008281	0.008281	76.84	0.000

	Sol vol*Sol vol	0.000230	0.000230	2.13	0.164
	Temp*Temp	0.000001	0.000001	0.01	0.914
	Time*Time	0.000011	0.000011	0.11	0.750
	%EtOH*Sol vol	0.011088	0.011088	102.89	0.000
	%EtOH*Temp	0.014520	0.014520	134.73	0.000
	%EtOH*Time	0.000013	0.000013	0.12	0.737
	Sol vol*Temp	0.000251	0.000251	2.33	0.146
	Sol vol*Time	0.000000	0.000000	0.00	0.977
	Temp*Time	0.000000	0.000000	0.00	0.947
	Lack-of-Fit	0.001689	0.000169	29.00	0.000
	R ² (adj) = 97.34%				
	R ² (pred) = 91.27%				
DPPH	Model	0.508938	0.063617	94.23	0.000
	%EtOH	0.229345	0.229345	339.72	0.000
	Sol vol	0.108113	0.108113	160.14	0.000
	Temp	0.084570	0.084570	125.27	0.000
	Time	0.014826	0.014826	21.96	0.000
	Sol vol*Sol vol	0.016166	0.016166	23.95	0.000
	Temp*Temp	0.001524	0.001524	2.26	0.147
	%EtOH*Temp	0.012377	0.012377	18.33	0.000
	Sol vol*Temp	0.005358	0.005358	7.94	0.010
	Lack-of-Fit	0.007987	0.000499	0.44	0.913
	R ² (adj) = 96.13%				
	R ² (pred) = 95.14%				
ABTS	Model	1.3419	0.33547	29.75	0.000
	%EtOH	0.2075	0.20750	18.40	0.000
	Sol vol	0.29519	0.29519	26.17	0.000
	Temp	0.17425	0.17425	15.45	0.001
	Temp*Temp	0.66496	0.66496	58.96	0.000
	Lack-of-Fit	0.22309	0.01115	0.95	0.575
	R ² (adj) = 79.31%				
	R ² (pred) = 74.38%				
FRAP	Model	0.563499	0.062611	20.63	0.000
	%EtOH	0.032905	0.032905	10.84	0.003
	Sol vol	0.221512	0.221512	72.97	0.000
	Temp	0.108314	0.108314	35.68	0.000
	Time	0.056717	0.056717	18.68	0.000
	%EtOH*%EtOH	0.001123	0.001123	0.37	0.550
	Sol vol*Sol vol	0.008491	0.008491	2.80	0.109
	Temp*Temp	0.006736	0.006736	2.22	0.151
	Time*Time	0.007936	0.007936	2.61	0.121
	%EtOH*Temp	0.012438	0.012438	4.10	0.056
	Lack-of-Fit	0.055535	0.003702	2.71	0.113
	R ² (adj) = 85.48%				
	R ² (pred) = 75.45%				
ORAC	Model	101.067	11.2296	10.53	0.000
	%EtOH	7.516	7.5156	7.05	0.015
	Sol vol	0.02	0.0196	0.02	0.893
	Temp	46.814	46.8138	43.88	0.000

	Time	2.652	2.6515	2.49	0.130
	%EtOH*%EtOH	31.485	31.4852	29.51	0.000
	Sol vol*Sol vol	8.46	8.4597	7.93	0.010
	Time*Time	2.434	2.4336	2.28	0.146
	Sol vol*Temp	8.951	8.9512	8.39	0.009
	Sol vol*Time	2.803	2.8034	2.63	0.120
	Lack-of-Fit	12.058	0.8039	0.47	0.892
	R ² (adj) = 74.08%				
	R ² (pred) = 66.71%				

Equation

B.1) %yield = $8.212 - 0.3684 \%EtOH + 0.0910 \text{ Sol vol} - 0.0127 \text{ Temp} + 0.009750 \text{ Time} + 0.00324 \%EtOH* \%EtOH - 0.000649 \text{ Sol vol}*\text{Sol vol} + 0.003187 \%EtOH*\text{Temp}$

B.2) Resveratrol = $0.65 + 0.1363 \%EtOH + 0.0199 \text{ Sol vol} - 0.074 \text{ Temp} + 0.01743 \text{ Time} - 0.001997 \%EtOH* \%EtOH - 0.000206 \text{ Sol vol}*\text{Sol vol} + 0.000809 \text{ Temp}*\text{Temp} - 0.000048 \text{ Time}*\text{Time} - 0.000410 \%EtOH*\text{Sol vol} - 0.000314 \%EtOH*\text{Temp} + 0.000131 \%EtOH*\text{Time} + 0.000256 \text{ Sol vol}*\text{Temp} + 0.000004 \text{ Sol vol}*\text{Time} - 0.000076 \text{ Temp}*\text{Time}$

B.3) TPC = $-12.01 + 0.00025 \%EtOH + 0.00771 \text{ Sol vol} + 0.3850 \text{ Temp} + 0.02106 \text{ Time} - 0.002495 \text{ Temp}*\text{Temp} - 0.000016 \text{ Time}*\text{Time} - 0.000229 \text{ Temp}*\text{Time}$

B.4) TFC = $0.471 - 0.04349 \%EtOH - 0.00082 \text{ Sol vol} - 0.00363 \text{ Temp} - 0.000129 \text{ Time} + 0.000565 \%EtOH* \%EtOH - 0.000015 \text{ Sol vol}*\text{Sol vol} - 0.000007 \text{ Temp}*\text{Temp} + 0.000001 \text{ Time}*\text{Time} + 0.000105 \%EtOH*\text{Sol vol} + 0.000301 \%EtOH*\text{Temp} + 0.000001 \%EtOH*\text{Time} + 0.000016 \text{ Sol vol}*\text{Temp} - 0.000000 \text{ Sol vol}*\text{Time} - 0.000000 \text{ Temp}*\text{Time}$

B.5) DPPH = $-0.468 - 0.00818 \%EtOH + 0.00890 \text{ Sol vol} + 0.0270 \text{ Temp} + 0.000478 \text{ Time} - 0.000109 \text{ Sol vol}*\text{Sol vol} - 0.000210 \text{ Temp}*\text{Temp} + 0.000278 \%EtOH*\text{Temp} + 0.000073 \text{ Sol vol}*\text{Temp}$

B.6) ABTS = $-9.02 + 0.01074 \%EtOH + 0.00512 \text{ Sol vol} + 0.4254 \text{ Temp} - 0.002968 \text{ Temp}*\text{Temp}$

B.7) FRAP = $-1.85 - 0.0236 \%EtOH + 0.01359 \text{ Sol vol} + 0.0735 \text{ Temp} + 0.00462 \text{ Time} + 0.000208 \%EtOH* \%EtOH - 0.000092 \text{ Sol vol}*\text{Sol vol} - 0.000509 \text{ Temp}*\text{Temp} - 0.000015 \text{ Time}*\text{Time} + 0.000279 \%EtOH*\text{Temp}$

B.8) ORAC = $14.54 - 1.266 \%EtOH + 0.032 \text{ Sol vol} + 0.0117 \text{ Temp} + 0.0541 \text{ Time} + 0.03325 \%EtOH* \%EtOH - 0.002758 \text{ Sol vol}*\text{Sol vol} - 0.000257 \text{ Time}*\text{Time} + 0.00299 \text{ Sol vol}*\text{Temp} + 0.000279 \text{ Sol vol}*\text{Time}$

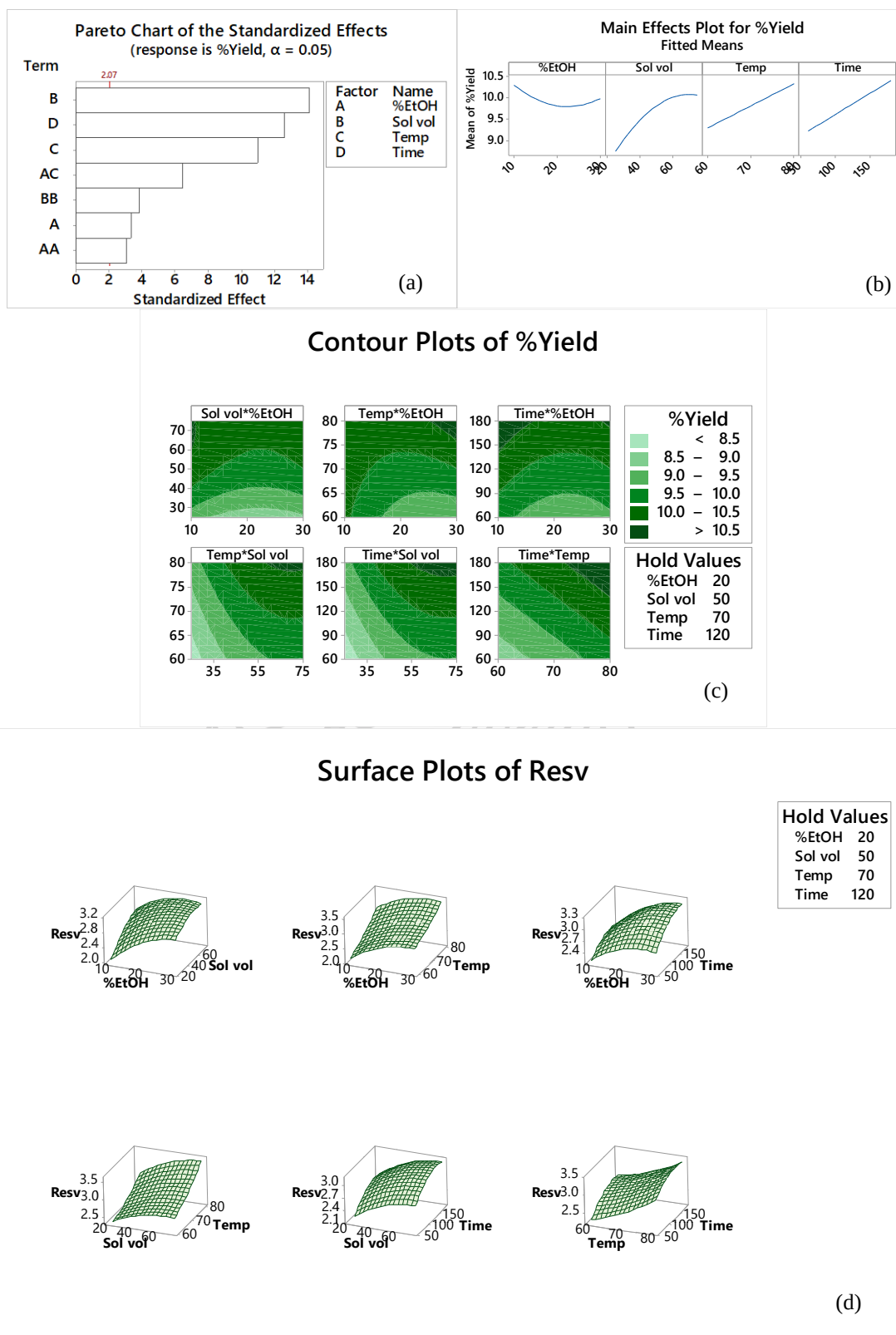


Figure 19 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on %yield response

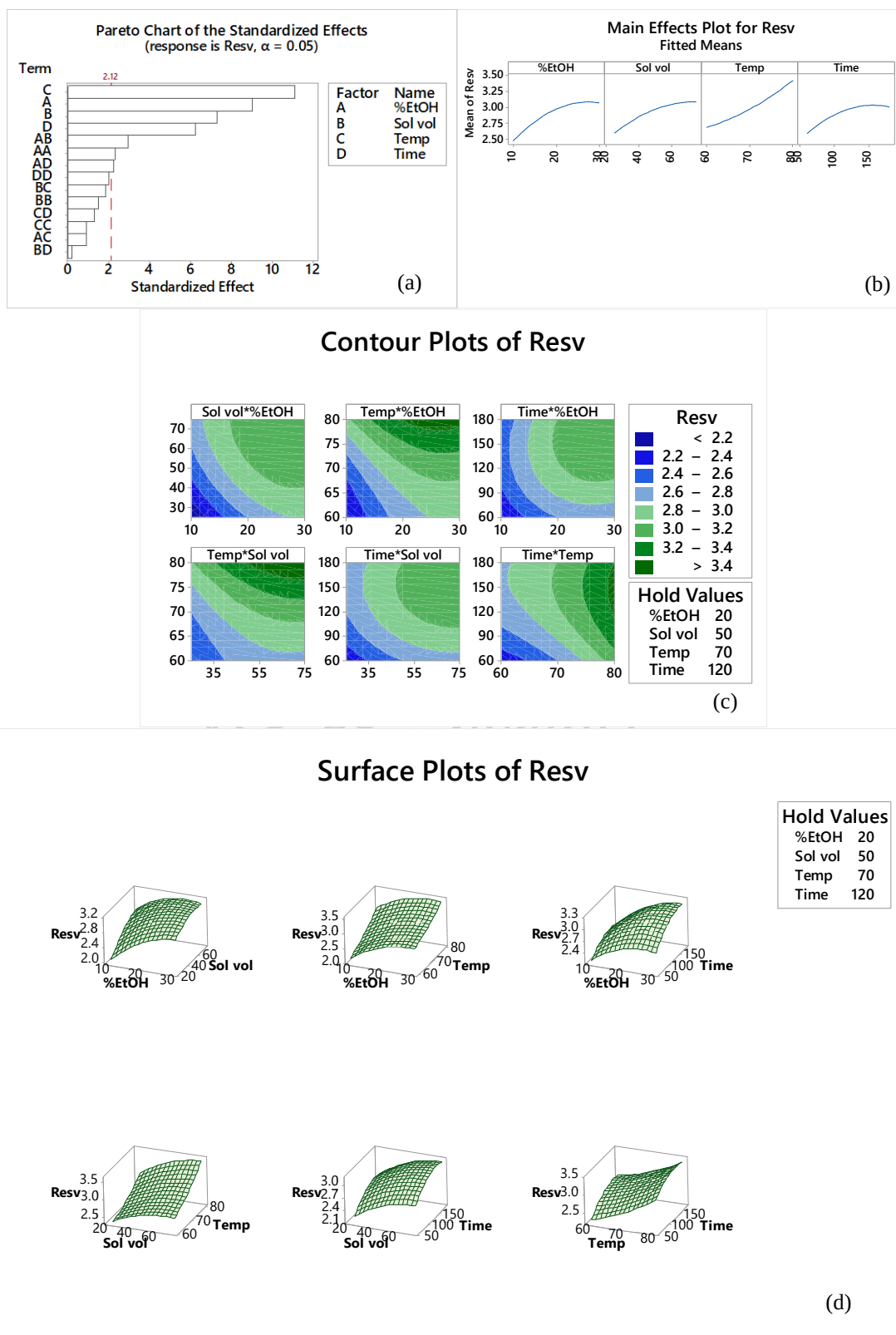


Figure 20 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on *t*-resveratrol content response

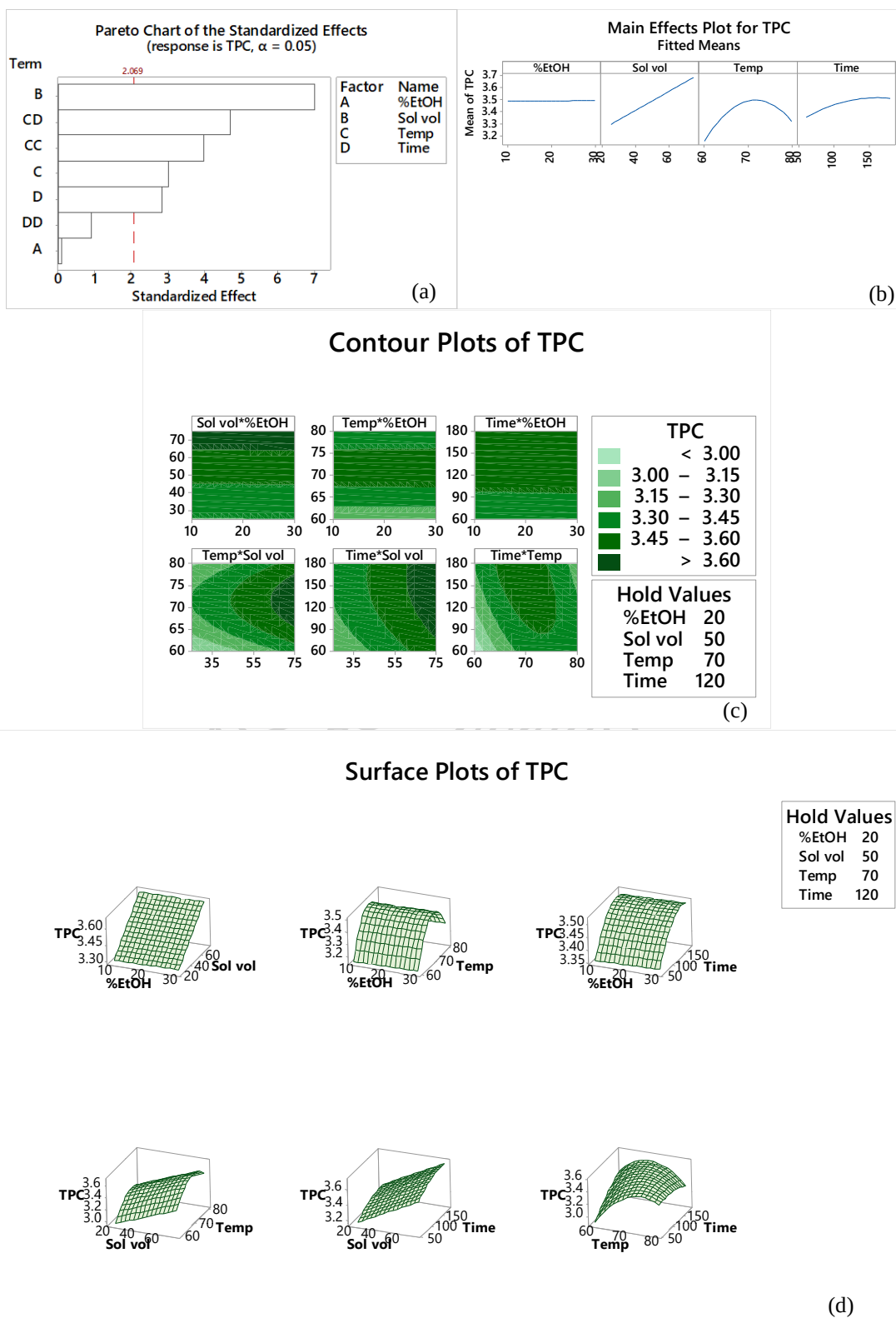


Figure 21 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on TPC response

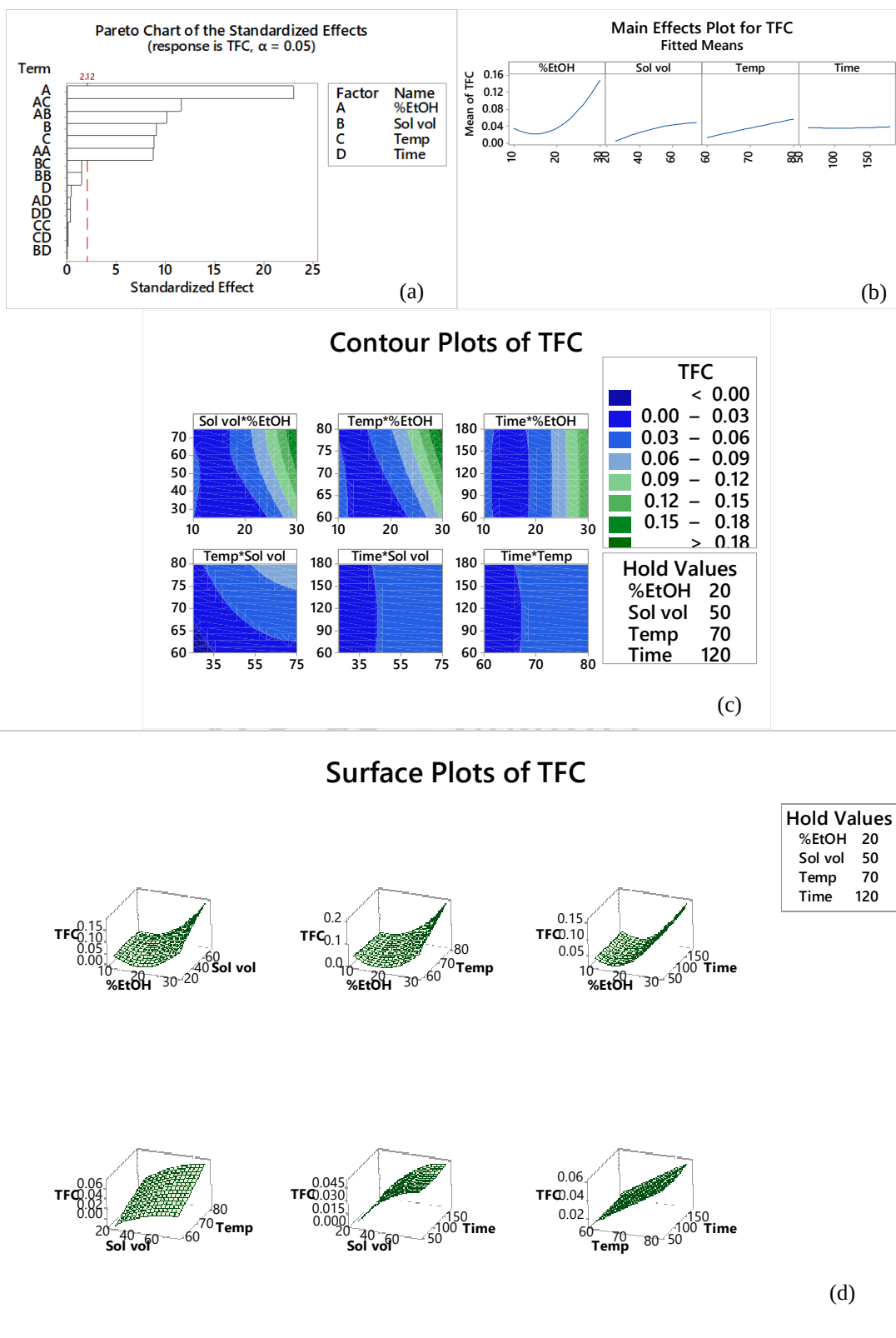


Figure 22 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on TFC response

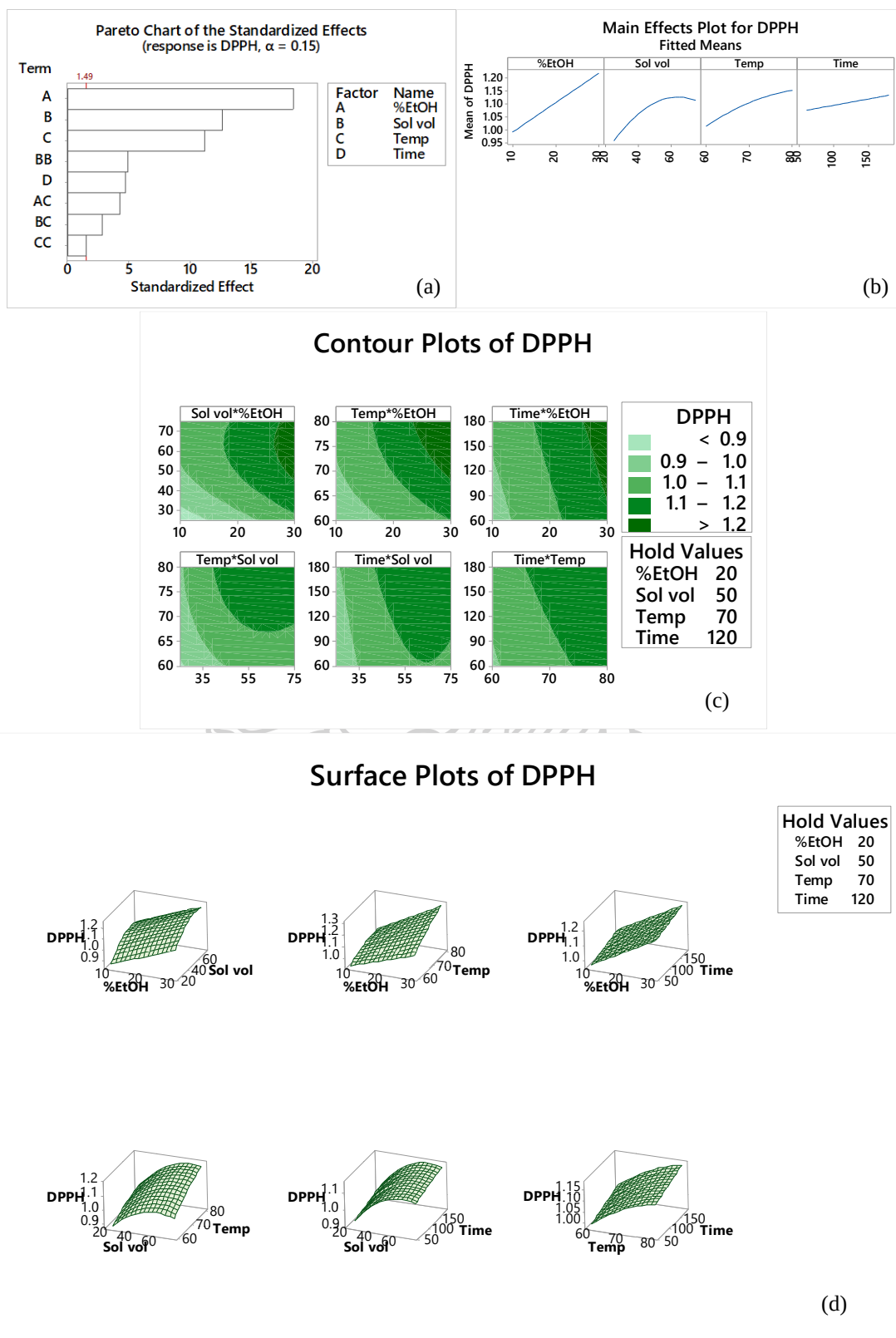


Figure 23 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on DPPH response

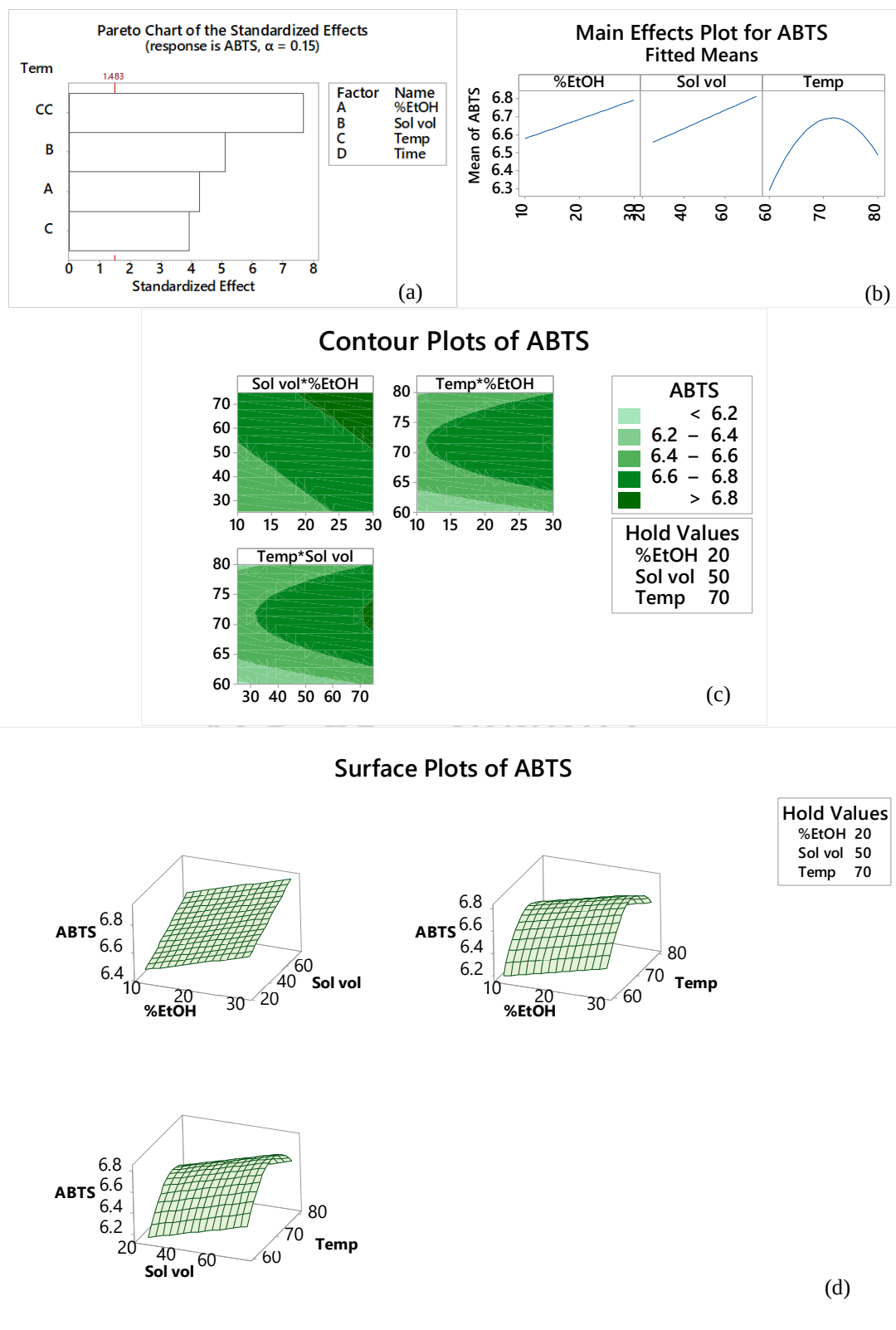


Figure 24 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on ABTS response

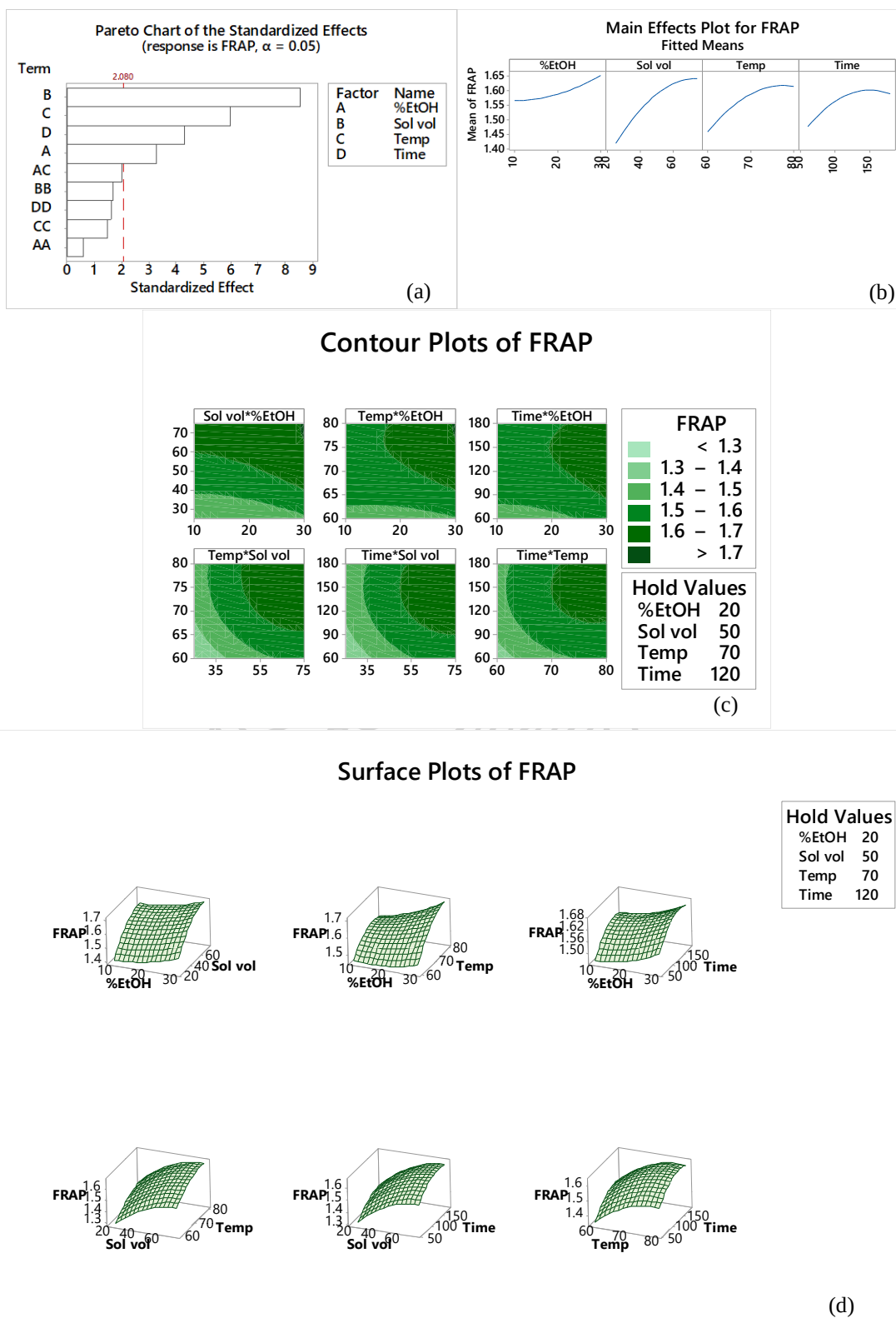


Figure 25 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on FRAP response

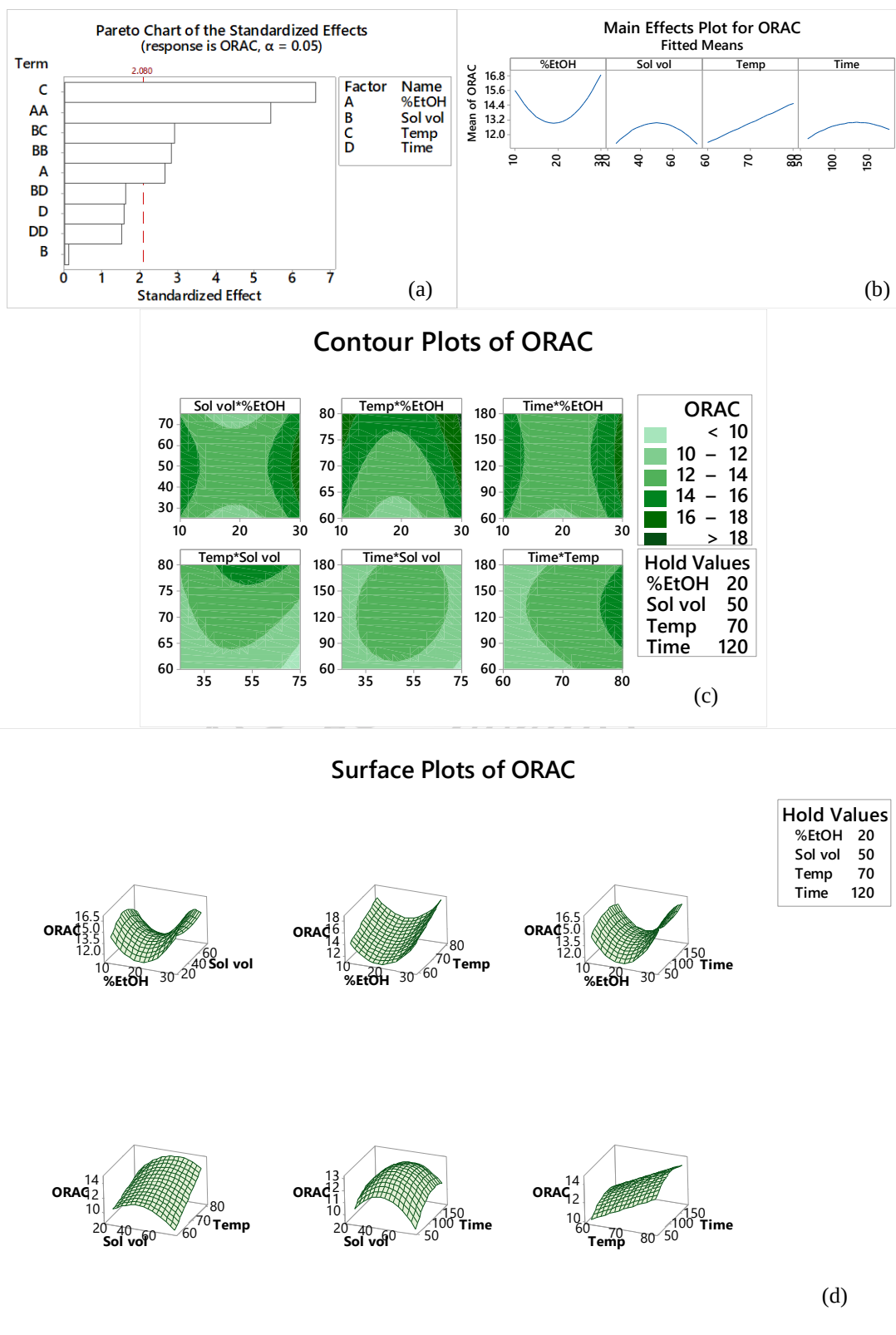


Figure 26 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of SLE-CCF on ORAC response

4.2.1.3 Model verification

The optimized extraction condition was selected at conditions that provided maximum antioxidant activity of the extract by DPPH, ABTS, FRAP and ORAC assay. At 30%ethanol, extraction solvent 68 mL, extraction temperature 78°C and extraction time 153 min was reported to be optimized condition with highest antioxidant capacity and used for model verification. The predicted and actual values obtained from calculation and experiments were shown in table 15. All experimental values were fallen within 95%CI of predicted values which indicated the validity of the model.

Table 15 Predicted and experimental values at optimized conditions of SLE-CCF study

Response	Predicted	Actual ($\pm$ SD)	95% Prediction Intervals
%yield	11.2307	11.3320 ($\pm$ 0.1440)	(10.7717, 11.6897)
<i>t</i> -resveratrol (μ g/g)	3.5445	3.4718 ($\pm$ 0.1126)	(3.1916, 3.8974)
TPC (mgGAE/g)	3.5030	3.5915 ($\pm$ 0.0720)	(3.2380, 3.7680)
TFC (mgQE/g)	0.2238	0.2207 ($\pm$ 0.0197)	(0.1975, 0.2501)
ORAC (mgTE/g)	17.742	17.957 ($\pm$ 0.954)	(15.275, 20.209)
FRAP (mgTE/g)	1.7663	1.7669 ($\pm$ 0.0676)	(1.6343, 1.8983)
ABTS (mgTE/g)	6.7914	6.7708 ($\pm$ 0.9540)	(6.5575, 7.0252)
DPPH (mgTE/g)	1.3244	1.3469 ($\pm$ 0.0244)	(1.2649, 1.3839)

4.2.2 Discussion

4.2.2.1 Screening design

In screening design, 4 factors, 2-leveled full factorial experimental design with 3 center points was used to determine the significant response-affected factors. The variable of interests and their levels were solvent volume (10, 50 mL), %ethanol (20, 80%), extraction temperature (30, 70°C) and extraction time (30, 120 min). The levels were assigned by reviewing previous literatures (18, 41, 72, 73). Sixteen plus three experimental runs were generated and performed. The experimental outcomes of each run order were recorded and shown in table 11. ANOVA was performed using MinitabTM 18 and the linear polynomial equation was applied to determine the significant variables and explained the relationship between factors and responses.

Parameters that significantly affected to extraction yield included the linear terms of all variables and interaction terms between solvent volume and temperature,

and %ethanol and temperature ($p < 0.05$) (Figure 16(a)). The calculated model was fitted to predict the response with $R^2_{(adj)} = 93.33\%$ and $R^2_{(pred)} = 87.78\%$ (Table 12). The maximum %yield obtained from screening study was 15.13%. The main effect plot for %yield indicated the positive correlation between %yield and all variables but different weight (Figure 16(b)). %Yield was impacted mostly by extraction temperature, followed by solvent volume, extraction time and %ethanol content respectively (Figure 16(a)). This meant the increasing of factor levels, extraction temperature, especially, resulted in the increasing of %yield.

For *t*-resveratrol content, only main effects of linear terms were included in the model. All main effects were significantly impacted on *t*-resveratrol level except %ethanol content. Either increase or decrease %ethanol content would not affect the extracted *t*-resveratrol level. The generated model demonstrated a satisfactory fitting of the experimental data with *t*-resveratrol content ($R^2_{(adj)} = 90.15\%$, $p = 0.000$). The most impact factor on *t*-resveratrol level was also extraction temperature (Figure 17(a)). The relationship between all factors and *t*-resveratrol content showed a curvature characteristic with a positive direction relationship (Figure 17(b)). This meant that increasing of all factors' level resulted in increasing of *t*-resveratrol level.

The linear regression model for prediction of TPC was successfully calculated with $R^2_{(adj)} = 96.78\%$ and $R^2_{(pred)} = 93.93\%$. All main effects and some interaction terms were important on TPC value ($p < 0.05$). Extraction temperature was the most influential factor on TPC, followed by %ethanol, solvent volume and extraction time respectively (Figure 18(a)). All factors showed positive correlation on TPC level except %ethanol content that showed negative relationship on TPC value (Figure 18(b)). Although, the relationship direction of %ethanol content likely contradicted to *t*-resveratrol content model, %ethanol content in *t*-resveratrol model was insignificant factor, and the antioxidant capacity of peanut extract should be contributed from TPC rather than *t*-resveratrol alone. Therefore, the lower range of %ethanol content (20%ethanol) should be chosen as optimal point for optimization study. In conclusion, the optimum conditions obtained from screening study were solvent volume 50 mL, 20%v/v ethanol content, extraction temperature 70°C and extraction time 120 min which were carried on as center point or “0” level in optimization study.

4.2.2.2 Optimization design

In optimization study, the center-faced central composite design (CCF) was utilized in order to obtain the optimal extraction condition. All significant factors from screening study were divided into three levels (-1, 0, 1) to generate the response surfaces. The 3 levels of un-coded (-1, 0, 1) of interested factors included 10, 20, 30%v/v of ethanol content, 25, 50, 75 mL of solvent volume, 60, 70, 80°C of extraction temperature and 60, 120, 180 min of extraction time. Total 31 run orders were generated and performed (Table 13). The responses of interested factors were %yield, *t*-resveratrol content, TPC, TFC and antioxidant capacities by FRAP, DPPH, ABTS, and ORAC assay. The results of all response values were shown in table 13. The calculated mathematical models for each response were derived as equation B.1-B.8. The signification of terms and variables was evaluated using ANOVA table in table 14.

For %yield, all of linear terms, quadratic terms of %ethanol content and solvent volume and interaction term between %ethanol content and temperature were found significant with $p < 0.01$. The created model was able to predict %yield value with the power of prediction ($R^2(\text{pred})$) 92.98% while $R^2(\text{adj})$ was 94.74%. The most impactful factor on %yield was solvent volume, followed by extraction time and temperature respectively while %ethanol content was found to be the least important factor on %yield (Figure 19(a)). Most of factors showed a positive relationship to %yield either linear (extraction temp, time) or polynomial direction (solvent volume) which meant that increasing of these factor levels resulted in increasing of %yield while %ethanol content showed negative polynomial relationship to %yield which meant that from 10%-20%v/v, the increasing of %ethanol content in extraction solvent would decrease the obtained %yield but at above 20%v/v, the obtained %yield was higher (Figure 19(b)-(d)). This finding corresponded to the study of De Zordi et al. (2014) which concluded that extraction time was a higher impactful factor than extraction temperature on yield of Propolis extract (74). By %ethanol content, this agreed with the reported by Ameer et al (2017) who mentioned that yield was affected by ethanol concentration in quadratic effect, and it was higher at beyond 20%v/v of ethanol concentration (75).

Full quadratic polynomial regression was fitted to predict *t*-resveratrol content with $R^2(\text{adj})$ of 92.38%. In addition, the developed model was able to predict the response with $R^2(\text{pred})$ of 86.83%. The non-significant 'lack of fit' was implied by a *p*-value of 0.692, suggesting quadratic model validity for optimized *t*-resveratrol response. All main effects were found significant on *t*-resveratrol value while only a quadratic effect of %ethanol*%ethanol and some interaction effects between %ethanol*sol volume and %ethanol*time were found significant to response, the rest of it were non-significant terms but included in the model to preserve the prediction ability. From main effect plot and response surface plot of *t*-resveratrol (Fig. 20(b)-(d)) revealed that all factors showed positive correlation to *t*-resveratrol level which meant that increasing of all factor levels resulted in increasing of extracted *t*-resveratrol. The maximum *t*-resveratrol level was probably obtained at about 25% of ethanol content, 75 mL of extraction solvent, extraction temperature 80°C and extraction time 155 min which were the highest levels for most of factors. The effect of %ethanol and extraction temperature on *t*-resveratrol was supported by the study of Karacabey et al. (73) who concluded that at lower %ethanol, a linear temperature effect was obtained, however, at higher concentration of ethanol, the linear effect of extraction temperature disappeared.

For TPC, the main effects of all factors were significant on TPC level except %ethanol content. The quadratic terms of extraction temperature and time, and the interaction term between Temp*Time were also important to predict TPC level ($p < 0.05$). The calculated model had the ability to predict the response at 71.63% ($R^2(\text{predict})$) and it fitted the TPC value with $R^2(\text{adj})$ of 81.22%. The validity of the model was observed by lacking significant on the Lack-of-Fit term ($p = 0.129$). Solvent volume was the most significant factor on TPC level in positive direction while extraction temperature and time correlated with TPC in quadratic direction (Fig.21(a), (b)). The response surface plots showed that increasing of solvent volume resulted in increasing of TPC level while increasing of extraction temperature and time also increased TPC level but up to only specific values and the TPC value seemed to decline afterward. This finding was in the agreement of Ali et al. (2008) that increasing of extraction temperature led to increasing of extracted TPC but after the

specific temperature, the TPC level declined (17). The reason behind was that increasing of extraction temperature would decrease the solvent viscosity and facilitated the solvent solubility into plant matrix (45). The TPC found in this study (3.5915 mgGAE/g) was higher than Limmonkon et al. (2017) study who reported TPC level in a range of 0.01565 – 0.03685 mgGAE/g dry sample (35).

The calculated model for prediction of TFC level was obtained using a full quadratic regression. The data was fitted the model with $R^2(\text{adj}) = 97.34\%$ and was able to predict the response with $R^2(\text{pred})=91.27\%$. The model contained both significant and non-significant terms. The linear term of extraction time was found to be non-significant term including some other quadratic and interaction terms. However, the lack-of-fit (LOF) indicated lacking of fit between input data and model ($p<0.05$) which meant that the data were unreliable. Although the significant lack-of-fit has been reported, the model was still able to predict the TFC response in 95% Prediction Intervals. The reason of significant lack-of-fit might be from the very low variation obtained from repeated experiments (0.0361 ± 0.0024 mgQE/g) which was necessary for pure error calculation and resulted in pretty much lower Adjust MS value of pure error than lack-of-fit (0.000006 vs 0.000169, pure error and LOF respectively) and led to the significant p-value of lack-of-fit parameter. The relationship between %ethanol content, solvent volume and extraction temperature and TFC value was in positive direction while changing in extraction time had no impact on TFC level (Fig. 22(b)). The %ethanol content and solvent volume demonstrated a quadratic relation with TFC but the extraction temperature exhibited almost linear relation with TFC response (Fig. 22(d)). This finding was also reported by another study that ethanol content had significant effect on TFC and antioxidant capacity of crude extract in parabola shape (76). The increasing of %ethanol content in extraction medium could increase the solubility of TFC because ethanol will react to flavonoids by non-covalent interaction and promote the diffusion of flavonoids into the medium (77).

Antioxidant capacity of the extract was determined by DPPH, ABTS, FRAP and ORAC assay. It was optimized and studied the relationship to those extraction parameters. All calculated models were statistically significant ($p<0.05$) and data were

able to explain the variations with non-significant lack-of-fit ($p>0.05$). For antiradical assay, DPPH and ABTS, all factors were found significant in extraction process except extraction time for ABTS assay. In FRAP study, all factors were also significant factors in extraction process but for ORAC study, only %ethanol content and extraction temperature was the significant factors. From main effects plot, most of factors related to antioxidant capacity of the extracts in positive direction either by linear or quadratic effect except %ethanol content in ORAC assay that related in negative direction. %Ethanol content and extraction temperature was the most significant factor for DPPH and ORAC assay respectively while solvent volume was the most influential factor for ABTS and FRAP assay (Fig. 23-26(a)). These implied all factors were important to cover up all antioxidant activities of the peanut extract.

4.2.2.3 Model verification

There were several reports on correlation between antioxidant activity and anti-inflammatory activity of plant extracts (78-80). In this study, therefore, the optimal extraction conditions were located at the maximum antioxidant activity of the extracts by DPPH, ABTS, FRAP and ORAC determination methods. The optimal extraction conditions were at 30%v/v ethanol, extraction solvent 68 mL, extraction temperature 78°C and extraction time 153 min. The predicted values and experimental values for all responses were in table 15. All experimental values were found within 95% prediction intervals which meant all of calculated models contained validity to predict the responses. The experimental antioxidant values in our study were 1.3469, 6.7708, 1.7669 and 17.957 mgTE/g for DPPH, ABTS, FRAP and ORAC respectively. The higher %ethanol content, solvent volume, extraction temperature and extraction time mostly provided higher antioxidant capacity of the extracts as well. This was in an accordance with previous literatures. The increasing of %ethanol content would increase soluble TPC and resulted in increasing antioxidant activity of the extracts (76). For solvent volume, it is known that the volume of solvent has to be enough for a good hydration and swelling of the solid phase which helps increasing yield recovery and increasing antioxidant activity of the extracts finally (81). As same as extraction temperature and time, increasing of extraction temperature generates higher solubility of phenolics, higher diffusivities in the solvent and facilitates mass transfer

while increasing extraction time will enhance a hydration and swelling of the matrix which gives rise to better phenolics yield and antioxidant activity (81, 82).

4.2.3 Conclusion

The optimal extraction conditions were figured out by experimental designs approach. Extraction parameters including %ethanol content, solvent volume, extraction temperature and time were screened by 4 factors and 2 levels full factorial designs. The extraction conditions that provided highest TPC were chosen as center points in optimization study. A 4 factors and 3 levels center-faced central composite design was acquired in optimization study. Thirty-one experiments were generated and performed as well as collected the responses such as %yield, *t*-resveratrol, TPC, TFC, DPPH, ABTS, FRAP and ORAC. The optimal extraction conditions were at 30%v/v ethanol, extraction solvent 68 mL, extraction temperature 78°C and extraction time 153 min which provided maximum antioxidant activity of the extracts. The data fitted well to the model and were able to predict the responses. The generated models were valid and reliable since the experimental values were not different to those predicted values. Generally, all factors were significant in extraction process by SLE but different impact on individual response. Ethanol content was a highly influential factor on antioxidant capacity of the extract, followed by solvent volume or extraction temperature while extraction time was a least impact factor on antioxidant capacity. The relationship between factors and antioxidant activity was mostly found to be positive either in linear or quadratic effect. Increasing of all factors' level usually resulted in higher antioxidant capacity of the extracts which was in the same manner as previous literatures. The higher TPC yield in this study was obtained if compared to the previous study that used the same extraction technique. This implied the advantage of using experimental design for finding the optimal extraction conditions in terms of better extraction efficiency.

4.3 Optimization of extraction conditions from peanut kernels using ultrasound-assisted extraction (UAE)

4.3.1 Results

4.3.1.1 Screening design

The 4 factors and 2 levels full factorial design with 3 replications at center point was used for screening study. The independent and dependent variables were the same as in screening design in 4.2. Totally, 19 experiments were performed, and the results of all dependent variables were shown in table 16.

Table 16 UAE full factorial design table and its responses

Run Order	Independent variables				Responses		
	Solvent vol (mL)	%EtOH (%)	Temp (°C)	Time (min)	%yield (%)	Resv (µg/g)	TPC (mgGAE/g)
1	10	80	70	30	11.79	1.7343	1.3634
2	50	80	30	30	21.30	1.8914	1.3250
3	50	80	70	30	22.50	2.0540	2.5031
4	10	80	70	120	13.55	1.5550	1.3722
5	50	20	30	30	8.40	0.9480	1.7676
6	30	50	50	75	10.03	2.2926	2.7515
7	50	20	30	120	11.17	2.8017	2.7245
8	10	20	30	120	7.07	1.0702	1.8154
9	10	80	30	30	9.70	1.4704	0.9374
10	10	20	30	30	6.15	3.7815	1.1772
11	50	80	70	120	26.60	3.5828	2.4754
12	50	20	70	30	9.83	2.7715	2.2406
13	50	80	30	120	21.14	3.1350	1.7912
14	30	50	50	75	11.27	3.6757	2.7172
15	30	50	50	75	10.68	3.5116	2.5291
16	10	20	70	30	6.76	1.4973	1.7069
17	10	20	70	120	7.64	1.4762	2.0249
18	50	20	70	120	11.16	3.6537	2.9465
19	10	80	30	120	12.61	1.9805	1.1386

The Pareto chart and main effect plots of variables were shown in Figure 27-29. All factors were important for UAE. %Ethanol content was the most influential factor for %yield while solvent volume was the most significant factor for *t*-resveratrol and TPC (Figure 27(a)-29(a)). The main effect plots revealed the positive

effect of all factors to %yield and *t*-resveratrol content, except for %ethanol content that showed negative correlation to total phenolic content (TPC) (Figure 29(b)). The ANOVA table of full factorial designs (FFD) study was shown in table 17. The linear equation in uncoded unit obtained from full factorial study for %yield, *t*-resveratrol and TPC was in equation A.1, A.2 and A.3, respectively.

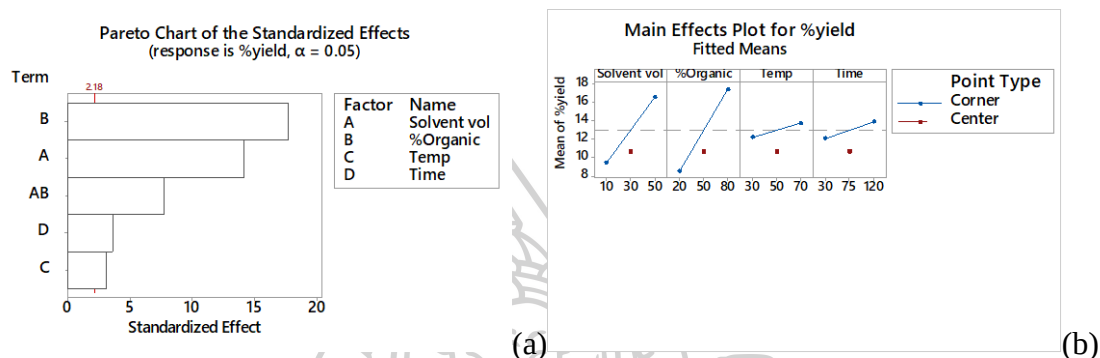


Figure 27 Pareto Chart of variables (a) and main effect plot for %yield (b)

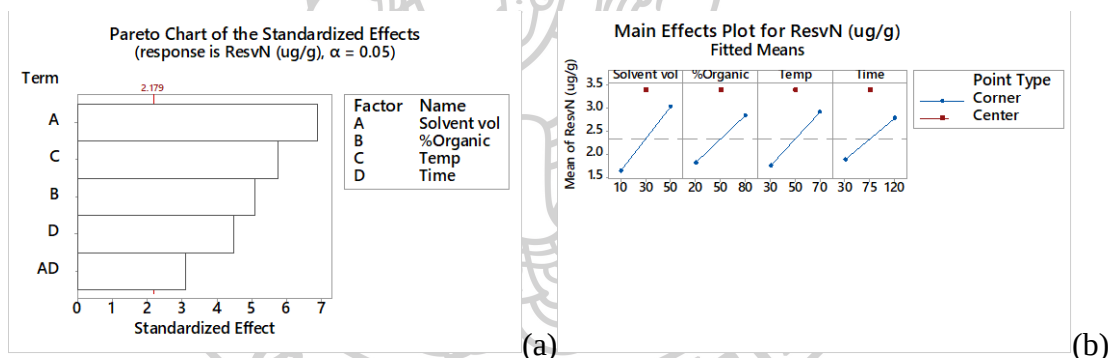


Figure 28 Pareto Chart of variables (a) and main effect plot for *t*-resveratrol (b)

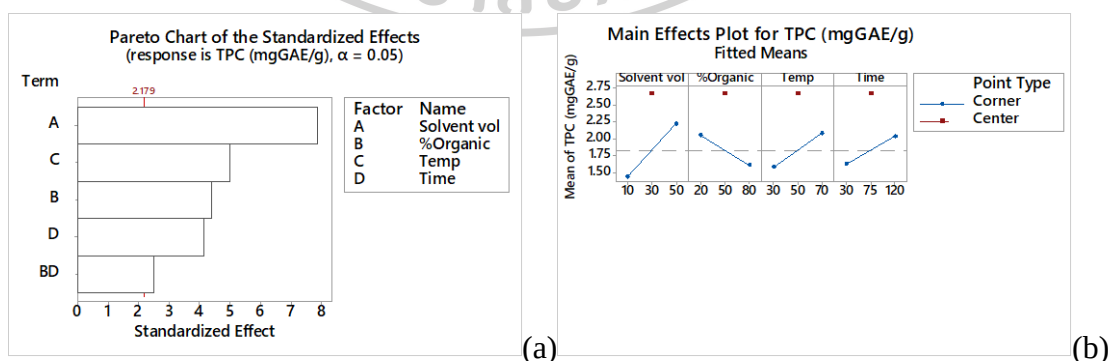


Figure 29 Pareto Chart of variables (a) and main effect plot for TPC (b)

Table 17 ANOVA table of UAE-FFD study

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	612.844	102.141	100.97	0.000
	Solvent vol	201.853	201.853	199.55	0.000
	%EtOH	315.151	315.151	311.55	0.000
	Temp	9.440	9.440	9.33	0.010
	Time	13.159	13.159	13.01	0.004
	Solvent vol*%EtOH	59.869	59.869	59.19	0.000
	Lack-of-Fit	11.369	1.137	2.96	0.279
	R ² (adj) = 97.09%				
	R ² (pred) = 95.07%				
<i>t</i> -resveratrol	Model	25.0549	4.17581	25.57	0.000
	Sol vol	7.7448	7.74481	47.43	0.000
	%EtOH	4.2191	4.21912	25.84	0.000
	Temp	5.3984	5.39842	33.06	0.000
	Time	3.2781	3.27809	20.07	0.001
	Solvent vol*Time	1.5629	1.56288	9.57	0.009
	Lack-of-Fit	1.8771	0.18771	4.55	0.194
	R ² (adj) = 89.12%				
	R ² (pred) = 81.53%				
TPC	Model	6.84207	1.14034	29.05	0.000
	Sol vol	2.43196	2.43196	61.96	0.000
	%EtOH	0.76449	0.76449	19.48	0.001
	Temp	0.97807	0.97807	24.92	0.000
	Time	0.66728	0.66728	17.00	0.001
	%EtOH*Time	0.24265	0.24265	6.18	0.029
	Lack-of-Fit	0.44233	0.04423	3.09	0.269
	R ² (adj) = 90.34%				
	R ² (pred) = 83.63%				

Regression equation in uncoded units

- A.1) %yield = $1.64 + 0.0164 \text{ Solvent vol} + 0.0512 \% \text{Organic} + 0.0384 \text{ Temp} + 0.02015 \text{ Time} + 0.003224 \text{ Solvent vol} * \% \text{Organic} - 2.301 \text{ Ct Pt}$
- A.2) Resveratrol = $-0.998 + 0.00874 \text{ Solvent vol} + 0.01712 \% \text{Organic} + 0.02904 \text{ Temp} - 0.00036 \text{ Time} + 0.000347 \text{ Solvent vol} * \text{Time} + 1.062 \text{ Ct Pt}$
- A.3) TPC = $0.311 + 0.01949 \text{ Solvent vol} - 0.00044 \% \text{Organic} + 0.01236 \text{ Temp} + 0.00910 \text{ Time} - 0.000091 \% \text{Organic} * \text{Time} + 0.834 \text{ Ct Pt}$

As same as SLE screening study results in 4.2, increasing %ethanol content resulted in increasing *t*-resveratrol but decreasing TPC level. However, the studies indicated the positive relationship between TPC and antioxidant activities (7, 38, 71), and antioxidant activity of peanut extract should not be contributed from *t*-resveratrol alone. Therefore, 20%ethanol content, solvent volume 50 mL, extraction temperature

70°C and time 120 min, which provided higher TPC level was chosen for optimization study.

4.3.1.2 Optimization study

The solvent volume 50 mL, 20%v/v ethanol content, extraction temperature 70°C and extraction time 120 min obtained from screening study were used as center point for optimization study. Total 31 orders were generated from the 4 factors and 3 levels center-faced central composite design (CCF) with 6 replications at center points (table 18). The dependent variables in this study were %yield, *t*-resveratrol content, TPC, total flavonoid content (TFC) and antioxidant capacity by DPPH, ABTS, FRAP and ORAC assays. The results of experimental run and the ANOVA tables of all responses were shown in table 18 and 19 respectively. The optimized polynomial equations obtained from CCF study for all responses were expressed as equation B.1-B.8. The Pareto charts, main effect plots, contour plots and response surface plots of each response were shown in Figure 30-37.

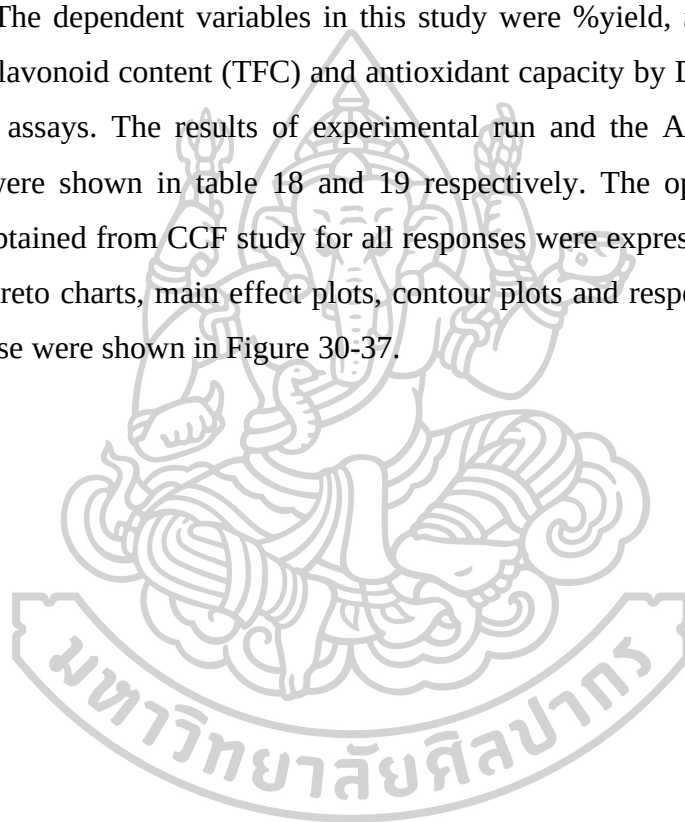


Table 18 Experimental design table for UAE-CCF study in uncoded unit

RunOrder	Independent variables					Responses						
	EtOH (%)	Sol vol (mL)	Temp (°C)	Time (min)	Yield (%)	Resv (µg/g)	TPC (mgGAE/g)	TFC (mgQE/g)	(mgTE/g)			
									FRAP	DPPH	ABTS	ORAC
1	10	75	80	60	10.89	3.7337	3.5010	0.0306	1.2968	5.0239	1.7496	11.6233
2	10	25	80	180	11.76	2.9538	2.8441	0.0146	0.8468	4.3379	1.2893	7.2663
3	10	75	80	180	12.48	3.6210	3.1492	0.0242	1.1450	5.0377	1.5591	11.7766
4	10	25	60	60	9.31	3.2063	2.9205	0.0115	1.0676	4.8306	1.4759	5.0089
5	30	75	60	60	9.89	3.6031	3.4490	0.1051	1.4525	5.5905	1.7280	12.0063
6	30	25	80	60	9.18	3.9315	2.9518	0.0465	1.5040	5.2302	1.5177	8.8580
7	30	50	70	120	10.51	4.2609	3.2997	0.0866	1.6069	5.4863	1.6689	12.5316
8	10	25	60	180	9.83	2.9487	3.0907	0.0128	0.9421	4.7010	1.3777	7.4938
9	30	75	80	180	11.79	4.1710	3.3114	0.1548	1.8470	5.4804	1.7268	12.6715
10	30	75	80	60	10.28	3.7023	3.4660	0.1299	1.6787	5.4022	1.7038	11.7412
11	10	50	70	120	11.47	3.6736	3.3152	0.0164	1.1440	5.2653	1.5407	8.2141
12	20	50	70	120	10.42	3.8222	3.2599	0.0232	1.3579	5.1618	1.6329	10.8387
13	20	50	70	120	10.75	4.0875	3.3624	0.0345	1.3454	5.1595	1.7097	10.3303
14	30	25	60	60	8.52	3.7285	2.9038	0.0481	1.4387	5.5119	1.6206	8.8845
15	20	50	80	120	10.78	3.7541	3.2699	0.0309	1.3918	5.3816	1.6439	10.4432
16	10	25	80	60	10.00	3.0833	2.9585	0.0166	0.9971	4.7691	1.4370	6.0892
17	20	50	70	120	10.27	3.8546	3.2581	0.0308	1.3489	5.4136	1.6580	9.7039
18	30	25	60	180	9.37	4.0423	3.2244	0.0512	1.6188	5.6059	1.5873	8.6097
19	10	75	60	60	10.53	3.4175	3.3875	0.0250	1.3755	5.5111	1.7437	11.2202
20	20	50	70	60	10.06	3.7759	3.2597	0.0229	1.3775	5.5318	1.6518	11.5119
21	20	50	70	180	12.04	3.9281	3.2699	0.0304	1.3690	5.5005	1.6208	9.8860
22	20	50	70	120	10.62	3.7421	3.3518	0.0199	1.4167	5.4646	1.5868	11.5106
23	20	50	70	120	10.06	3.8973	3.1760	0.0207	1.4006	5.5478	1.6791	12.4659
24	20	50	70	120	10.77	4.0659	3.2759	0.0273	1.4442	5.3288	1.6645	11.2100
25	30	25	80	180	11.00	3.7675	3.1400	0.0625	1.9087	5.0461	1.5351	8.8147
26	30	75	60	180	10.97	4.1186	3.6366	0.1373	1.7800	5.6353	1.8522	13.6225
27	20	25	70	120	9.92	3.5756	3.0501	0.0186	1.2886	5.0403	1.5867	9.5341
28	20	75	70	120	12.01	4.0919	3.3843	0.0403	1.4366	5.2980	1.6981	13.3918
29	10	75	60	180	11.16	3.6273	3.5254	0.0218	1.2421	5.2428	1.7293	9.6284
30	20	50	70	120	10.49	3.9499	3.1508	0.0197	1.3962	5.4972	1.6674	10.2993
31	20	50	60	120	10.34	3.6986	3.3528	0.0169	1.3894	5.3397	1.6436	8.1505

Table 19 ANOVA table for all responses

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	20.2335	5.05838	33.06	0.000
	%EtOH	1.9470	1.94702	12.73	0.001
	Sol vol	6.8573	6.85734	44.82	0.000
	Temp	3.7721	3.77209	24.66	0.000
	Time	7.6571	7.65709	50.05	0.000
	Lack-of-Fit	3.5768	0.17884	2.68	0.113
	R ² (adj) = 81.04%				
	R ² (pred) = 76.51%				
t-resveratrol	Model	3.05328	0.38166	28.30	0.000
	%EtOH	1.42270	1.42270	105.50	0.000
	Sol vol	0.45090	0.45090	33.44	0.000
	Temp	0.00595	0.00595	0.44	0.513
	Time	0.05513	0.05513	4.09	0.056
	Temp*Temp	0.61221	0.61221	45.40	0.000
	%EtOH*Sol vol	0.27097	0.27097	20.09	0.000
	%EtOH*Time	0.12674	0.12674	9.40	0.006
	Sol vol*Time	0.10867	0.10867	8.06	0.010
	Lack-of-Fit	0.20050	0.01253	0.78	0.679
	R ² (adj) = 87.92%				
	R ² (pred) = 80.25%				
TPC	Model	1.01432	0.144903	29.88	0.000
	%EtOH	0.02650	0.026496	5.46	0.028
	Sol vol	0.77149	0.771489	159.10	0.000
	Temp	0.04488	0.044880	9.26	0.006
	Time	0.00862	0.008620	1.78	0.195
	%EtOH*Time	0.03062	0.030616	6.31	0.019
	Sol vol*Time	0.03474	0.034736	7.16	0.013
	Temp*Time	0.09748	0.097484	20.10	0.000
	Lack-of-Fit	0.07341	0.004318	0.68	0.754
	R ² (adj) = 87.08%				
	R ² (pred) = 81.12%				
TFC	Model	0.043993	0.006285	142.32	0.000
	%EtOH	0.023364	0.023364	529.09	0.000
	Sol vol	0.008303	0.008303	188.03	0.000
	Temp	0.000364	0.000364	8.23	0.009
	Time	0.000299	0.000299	6.78	0.016
	%EtOH*%EtOH	0.006547	0.006547	148.27	0.000
	%EtOH*Sol vol	0.004648	0.004648	105.25	0.000
	%EtOH*Time	0.000468	0.000468	10.59	0.003
	Lack-of-Fit	0.000811	0.000048	1.40	0.358
	R ² (adj) = 97.06%				
	R ² (pred) = 95.43%				
DPPH	Model	1.72036	0.24577	153.83	0.000
	%EtOH	1.26845	1.26845	793.95	0.000
	Sol vol	0.14975	0.14975	93.73	0.000
	Temp	0.00531	0.00531	3.32	0.081
	Time	0.01451	0.01451	9.08	0.006

	%EtOH*Sol vol	0.05265	0.05265	32.95	0.000
	%EtOH*Temp	0.06126	0.06126	38.34	0.000
	%EtOH*Time	0.16843	0.16843	105.42	0.000
	Lack-of-Fit	0.02829	0.00166	1.18	0.448
	R ² (adj) = 97.21%				
	R ² (pred) = 95.10%				
ABTS	Model	2.3062	0.46125	24.09	0.000
	%EtOH	1.0127	1.01265	52.89	0.000
	Sol vol	0.5509	0.55087	28.77	0.000
	Temp	0.2837	0.28368	14.82	0.001
	Sol vol*Sol vol	0.3253	0.32533	16.99	0.000
	%EtOH*Sol vol	0.1337	0.13370	6.98	0.014
	Lack-of-Fit	0.3307	0.01741	0.71	0.741
	R ² (adj) = 79.37%				
	R ² (pred) = 73.41%				
FRAP	Model	0.356870	0.059478	27.28	0.000
	%EtOH	0.059870	0.059870	27.46	0.000
	Sol vol	0.236511	0.236511	108.48	0.000
	Temp	0.019734	0.019734	9.05	0.006
	Time	0.006825	0.006825	3.13	0.090
	%EtOH*Sol vol	0.012752	0.012752	5.85	0.024
	%EtOH*Time	0.021178	0.021178	9.71	0.005
	Lack-of-Fit	0.043388	0.002410	1.62	0.287
	R ² (adj) = 84.07%				
	R ² (pred) = 77.40%				
ORAC	Model	110.349	22.0699	25.82	0.000
	%EtOH	20.950	20.9503	24.51	0.000
	Sol vol	76.560	76.5604	89.59	0.000
	Temp	1.206	1.2060	1.41	0.246
	Time	0.444	0.4437	0.52	0.478
	Temp*Temp	11.189	11.1889	13.09	0.001
	Lack-of-Fit	16.325	0.8592	1.02	0.533
	R ² (adj) = 80.54%				
	R ² (pred) = 75.12%				

Equation

- B.1) %yield = $5.478 - 0.03289 \%EtOH + 0.02469 \text{ Sol vol} + 0.04578 \text{ Temp} + 0.01087 \text{ Time}$
- B.2) Resveratrol = $-11.00 + 0.03634 \%EtOH + 0.01015 \text{ Sol vol} + 0.4005 \text{ Temp} - 0.00479 \text{ Time} - 0.002848 \text{ Temp*Temp} - 0.000521 \%EtOH*\text{Sol vol} + 0.000148 \%EtOH*\text{Time} + 0.000055 \text{ Sol vol*Time}$
- B.3) TPC = $1.953 - 0.00491 \%EtOH + 0.01201 \text{ Sol vol} + 0.01062 \text{ Temp} + 0.00957 \text{ Time} + 0.000073 \%EtOH*\text{Time} - 0.000031 \text{ Sol vol*Time} - 0.000130 \text{ Temp*Time}$
- B.4) TFC = $0.0788 - 0.01267 \%EtOH - 0.000504 \text{ Sol vol} + 0.000449 \text{ Temp} - 0.000112 \text{ Time} + 0.000295 \%EtOH* \%EtOH + 0.000068 \%EtOH*\text{Sol vol} + 0.000009 \%EtOH*\text{Time}$
- B.5) DPPH = $1.539 - 0.02581 \%EtOH + 0.008237 \text{ Sol vol} - 0.01066 \text{ Temp} - 0.002947 \text{ Time} - 0.000229 \%EtOH*\text{Sol vol} + 0.000619 \%EtOH*\text{Temp} + 0.000171 \%EtOH*\text{Time}$
- B.6) ABTS = $4.249 + 0.04200 \%EtOH + 0.04753 \text{ Sol vol} - 0.01255 \text{ Temp} - 0.000332 \text{ Sol vol*Sol vol} - 0.000366 \%EtOH*\text{Sol vol}$
- B.7) FRAP = $1.581 + 0.00414 \%EtOH + 0.00684 \text{ Sol vol} - 0.00331 \text{ Temp} - 0.001537 \text{ Time} - 0.000113 \%EtOH*\text{Sol vol} + 0.000061 \%EtOH*\text{Time}$
- B.8) ORAC = $-57.2 + 0.1079 \%EtOH + 0.08249 \text{ Sol vol} + 1.730 \text{ Temp} + 0.00262 \text{ Time} - 0.01217 \text{ Temp*Temp}$



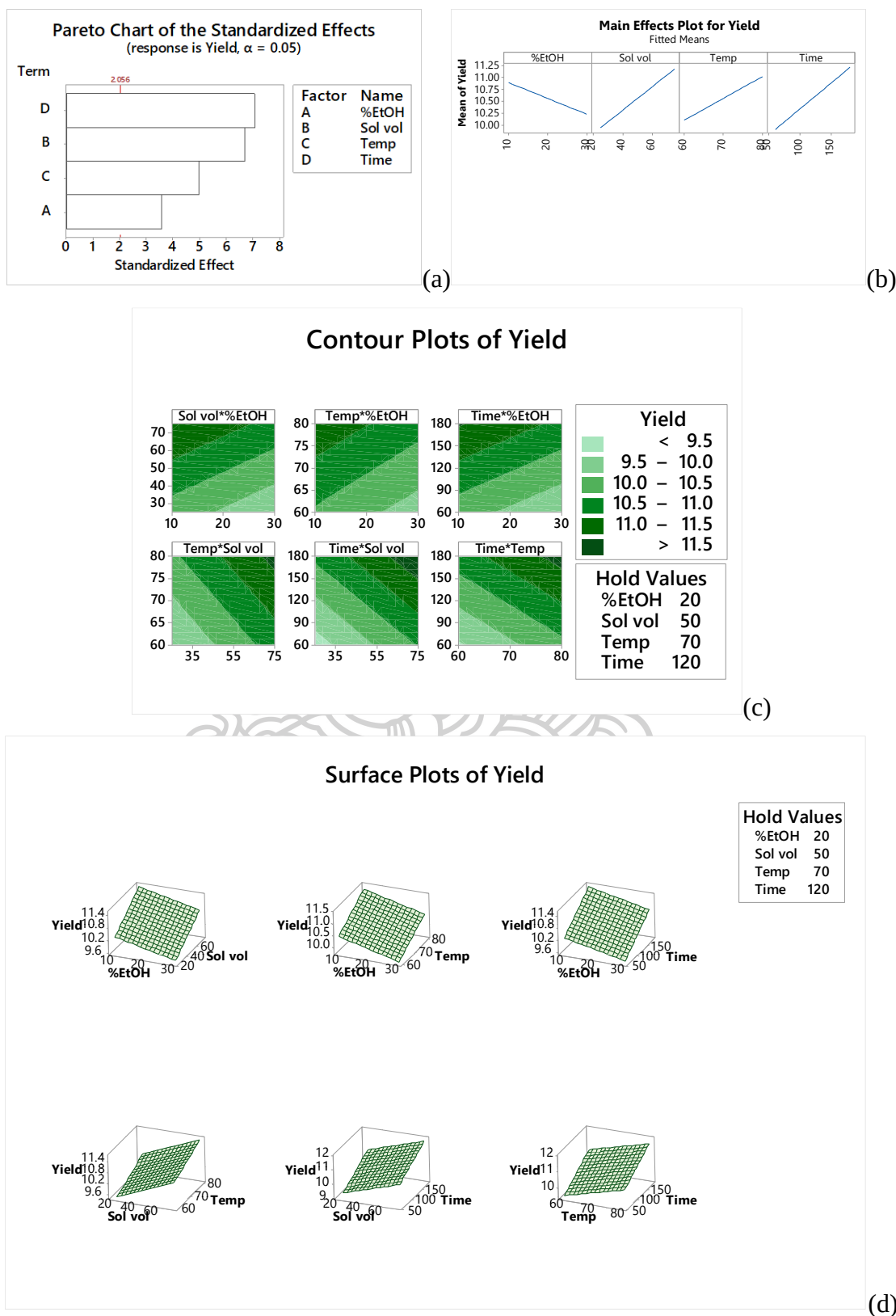


Figure 30 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on %yield response

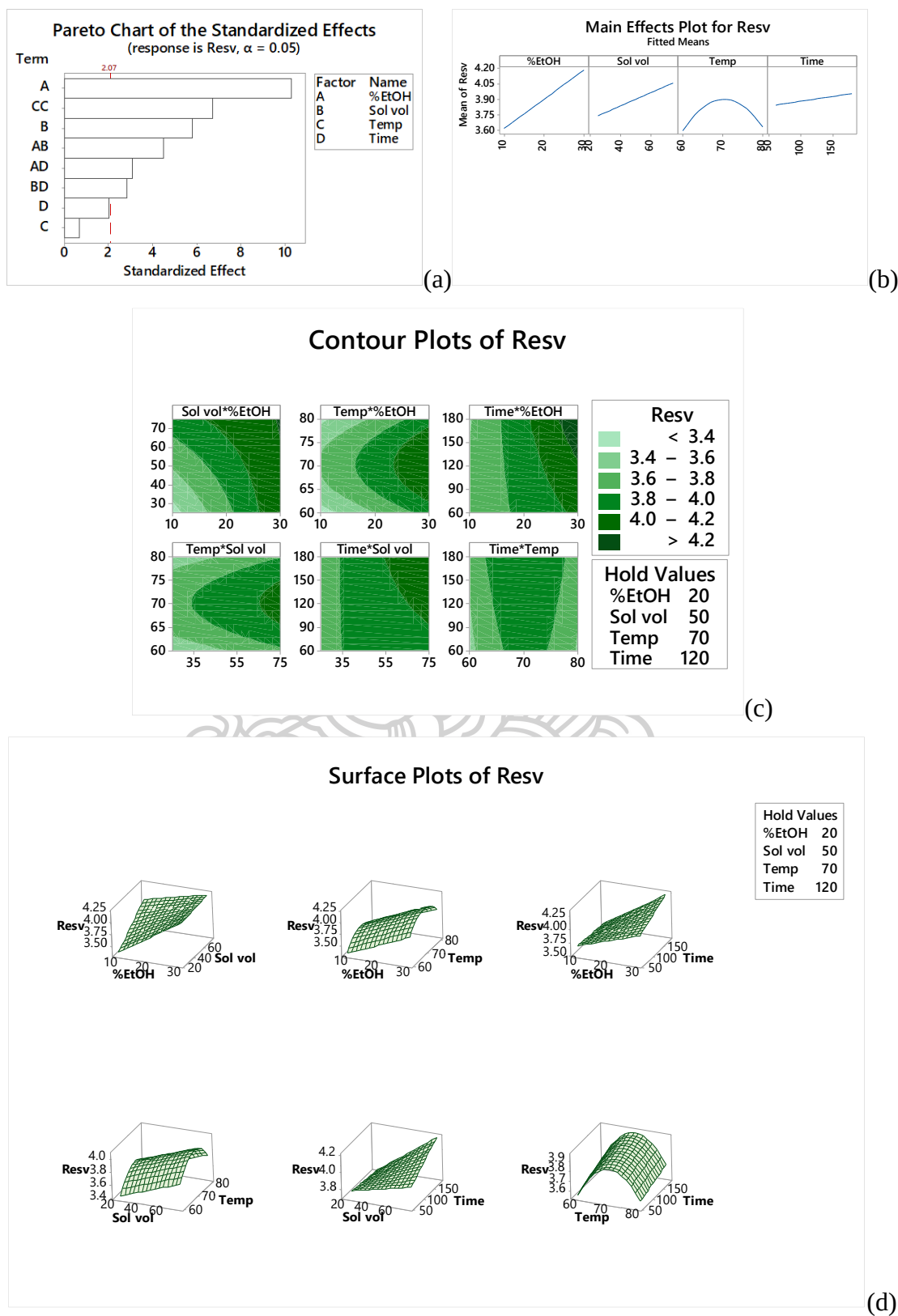


Figure 31 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on *t*-resveratrol response

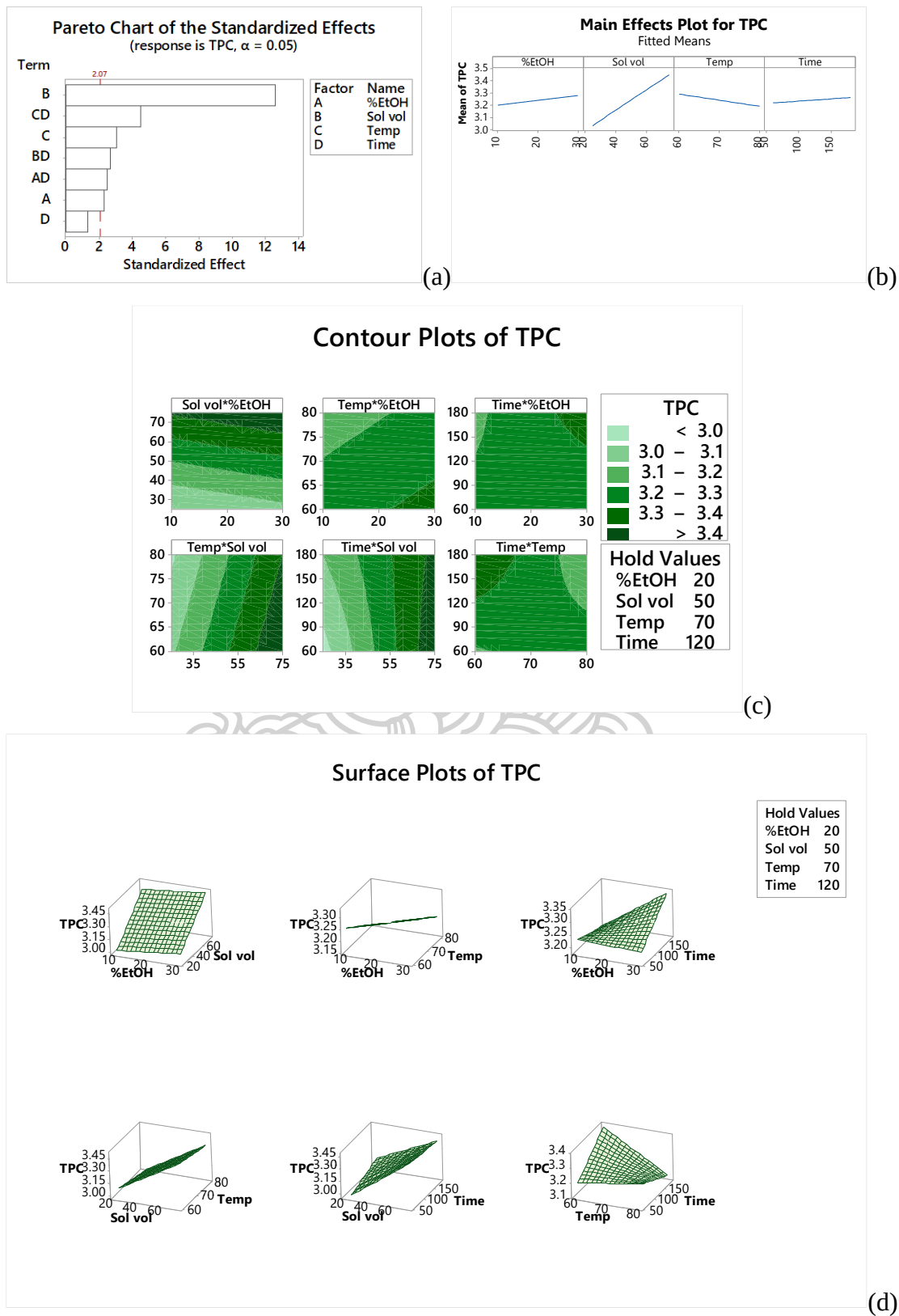


Figure 32 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on TPC response

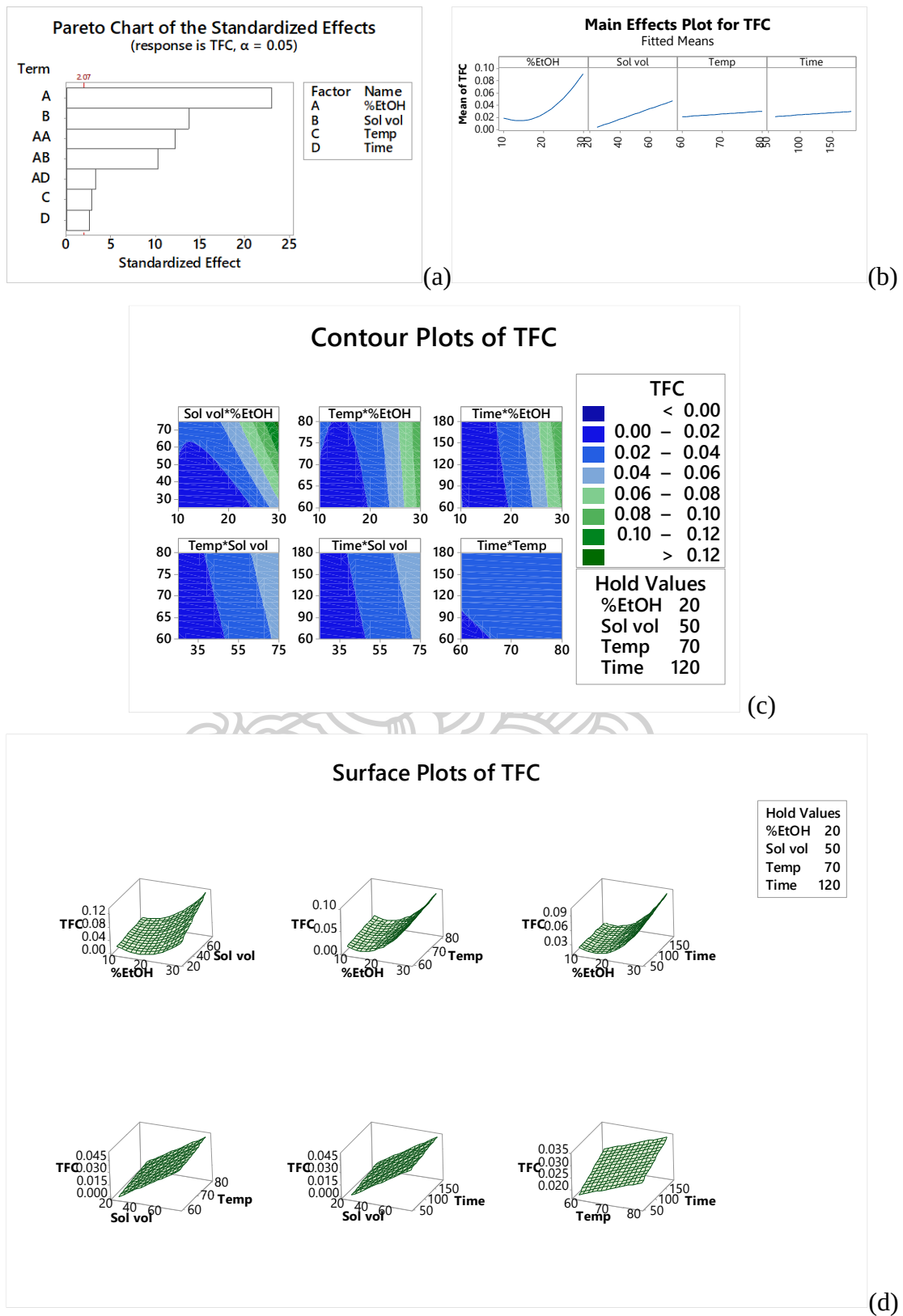


Figure 33 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on TFC response

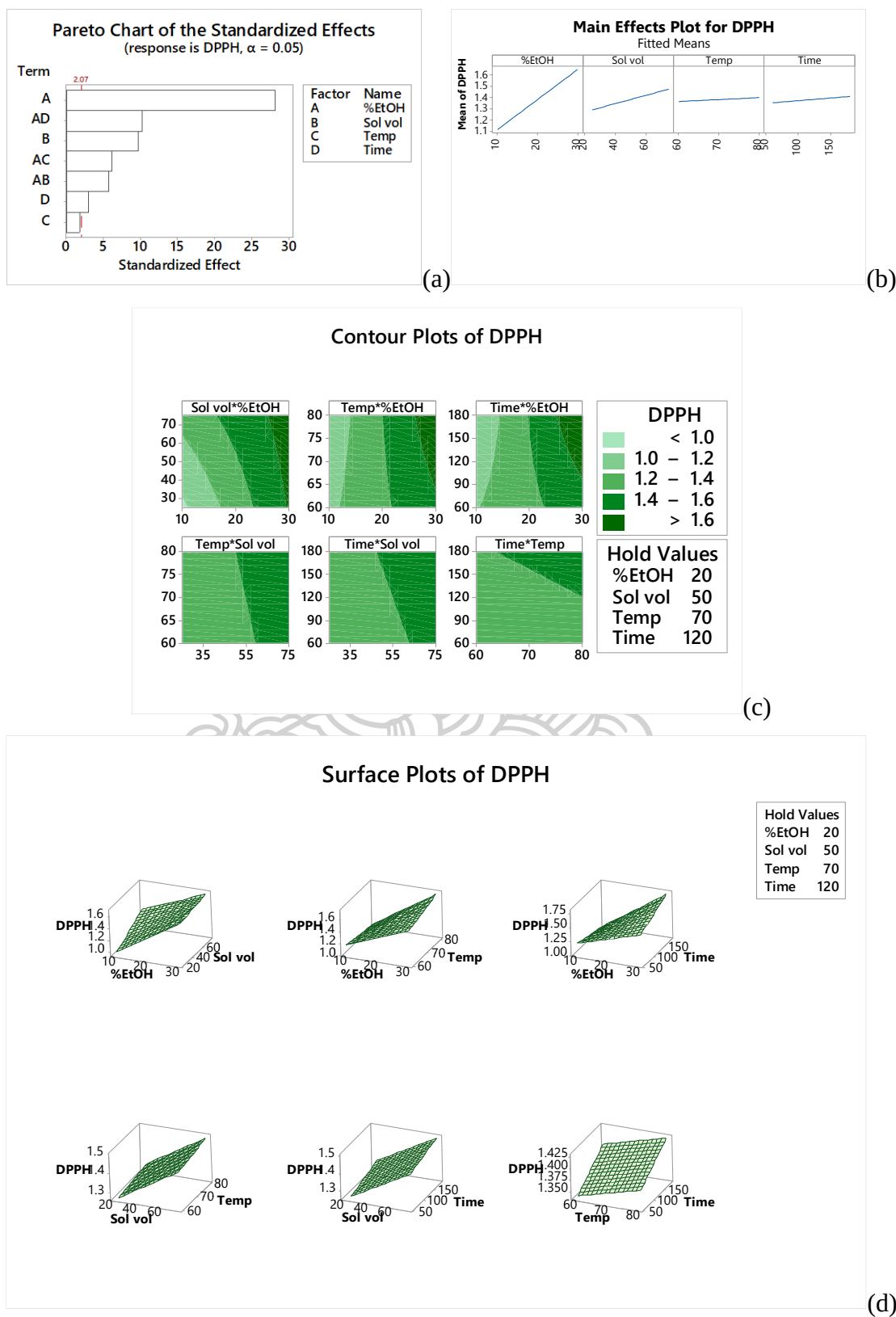


Figure 34 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on DPPH response

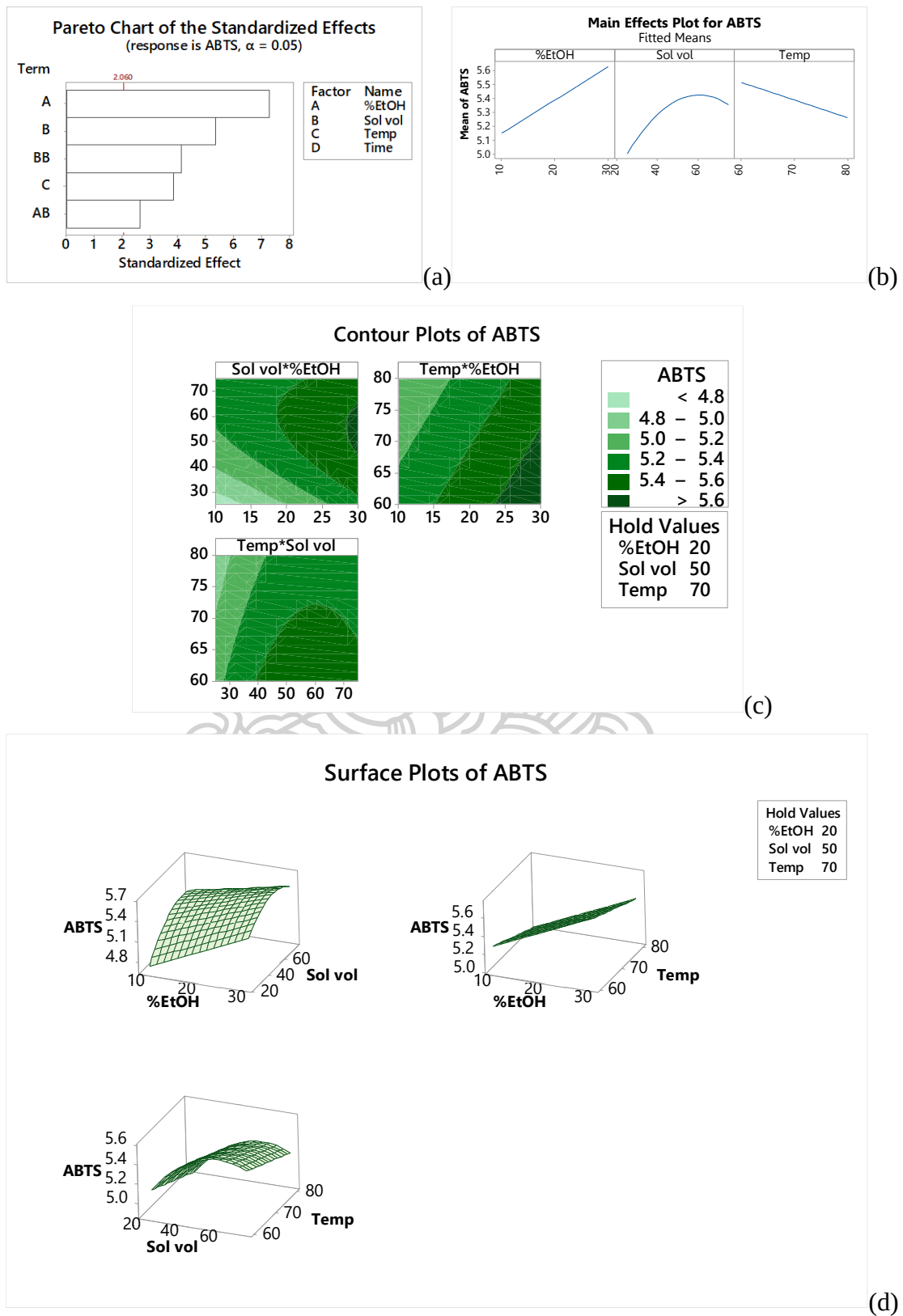


Figure 35 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on ABTS response

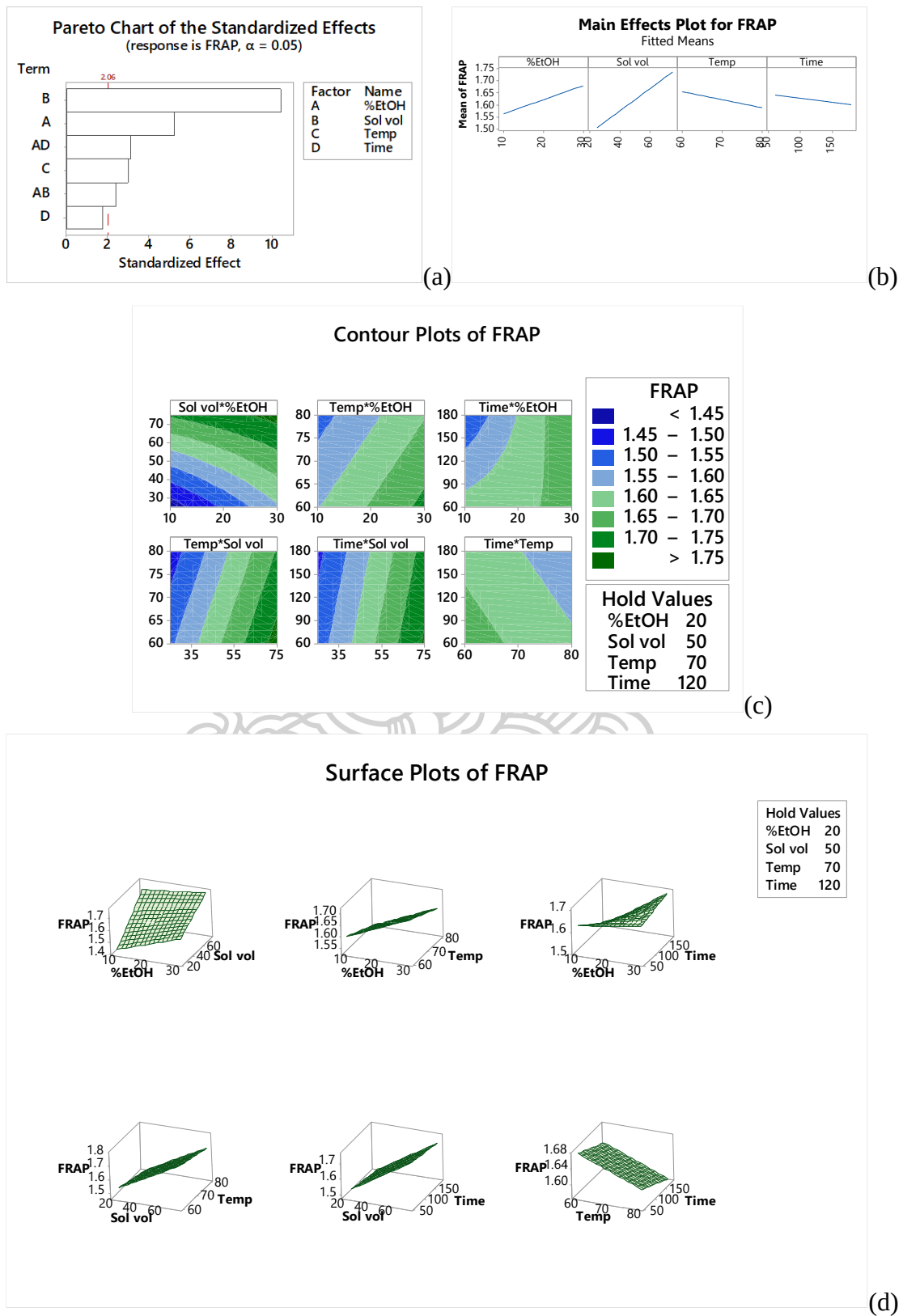


Figure 36 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on FRAP response

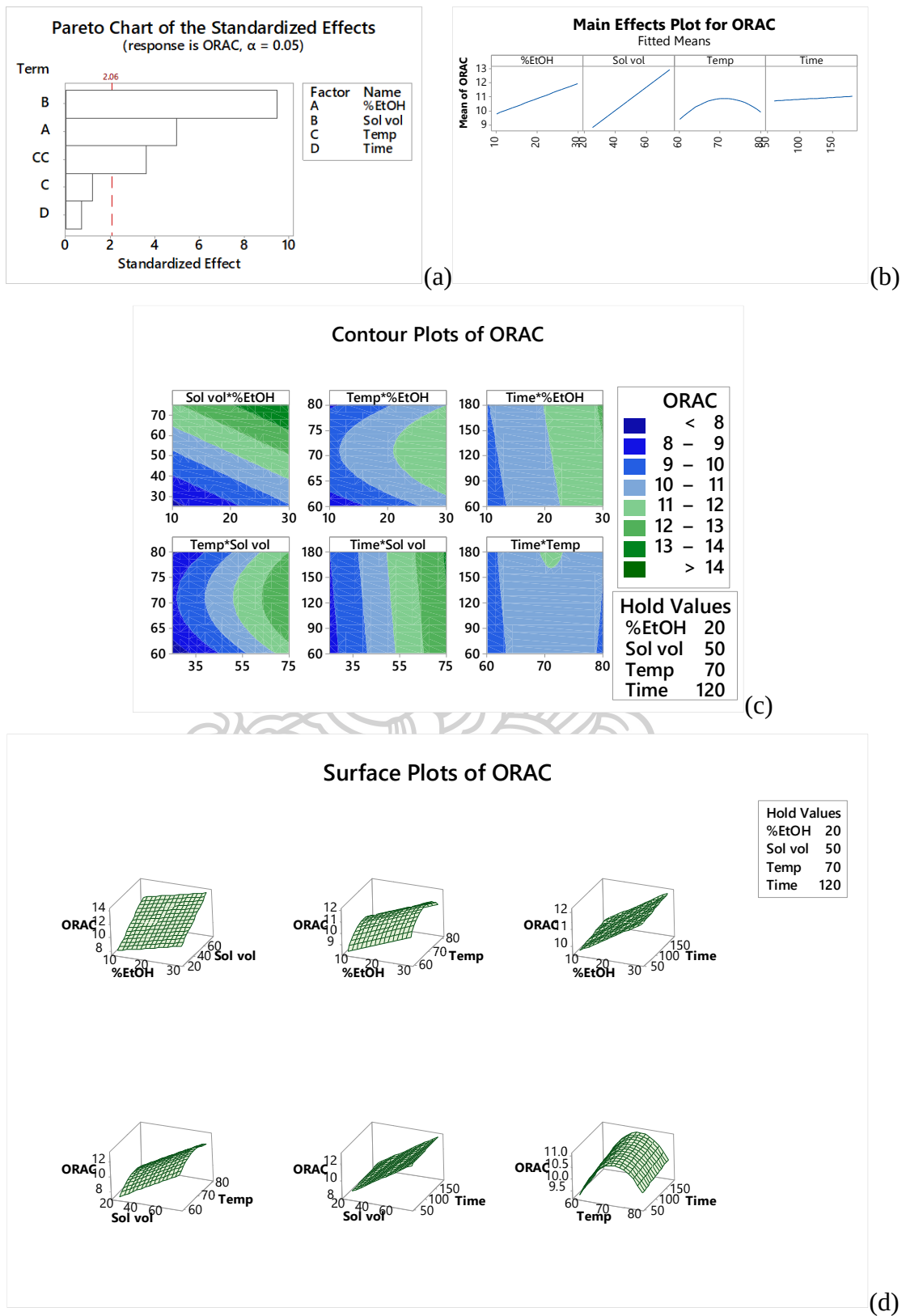


Figure 37 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of UAE-CCF on ORAC response

4.3.1.3 Model verification

The conditions that provided maximum antioxidant activity of the extract by DPPH, ABTS, FRAP and ORAC assay were selected as optimal conditions which were 30%ethanol, extraction solvent 75 mL, extraction temperature 65°C and extraction time 180 min. Model verification was performed six replications at optimized conditions. The predicted and actual values were shown in table 20. All experimental values were fallen within 95%CI of predicted values which indicated the validity of the model.

Table 20 Predicted and experimental values at optimized conditions of UAE-CCF study

Response	Predicted	Actual ($\pm$ SD)	95% Prediction Intervals
%yield	11.2752	10.80 ($\pm$ 0.20)	(10.9043, 11.6461)
<i>t</i> -resveratrol (μ g/g)	4.3577	3.2060 ($\pm$ 0.2830)	(4.20259, 4.51281)
TPC (mgGAE/g)	3.5030	3.5702 ($\pm$ 0.1276)	(3.48460, 3.65588)
TFC (mgQE/g)	0.1371	0.1382 ($\pm$ 0.0059)	(0.128831, 0.145352)
ORAC (mgTE/g)	13.625	14.1610 ($\pm$ 2.0950)	(12.720, 14.529)
FRAP (mgTE/g)	1.8022	1.7449 ($\pm$ 0.0660)	(1.7458, 1.8586)
ABTS (mgTE/g)	5.5760	5.6140 ($\pm$ 0.0788)	(5.4342, 5.7178)
DPPH (mgTE/g)	1.7672	1.7363 ($\pm$ 0.0557)	(1.7174, 1.8170)

4.3.2 Discussion

4.3.2.1 Screening design

The signification of variables was determined using 4 factors and 2 levels full factorial design with 3 center points. The variable of interests including solvent volume (10, 50 mL), %ethanol (20, 80%), extraction temperature (30, 70°C) and extraction time (30, 120 min) were assigned by reviewing previous literatures (18, 41, 72, 73). Totally, 19 experimental runs were generated and performed (Table 16). The ANOVA and multiple linear regression were applied to determine the significant variables and the relationship between factors and responses.

The linear terms of all variables were significant for every response ($p < 0.05$) (Figure 27-29). The interaction terms of solvent volume and %ethanol (AB), solvent volume and time (AD), and %ethanol and time (BD) were significant for %yield, *t*-resveratrol and TPC, respectively ($p < 0.05$) (Table 17), while other terms were not included in the model. For %yield, the main effect plot indicated the positive

correlation between %yield and all variables (Figure 27(b)). The variable that affected %yield the most was %ethanol content, followed by solvent volume, extraction time and temperature respectively (Figure 27(a)). It implied that increase of all factor levels resulted in the increase of %yield.

For *t*-resveratrol content, the most influential factor was solvent volume, followed by extraction temperature, %ethanol content and extraction time, respectively (Figure 28(a)). The main effect plots indicated the curvature characteristic between variables and response, and it revealed the positive effect for all variables on *t*-resveratrol content (Figure 28(b)). This meant that increasing of all factors' level resulted in increasing of *t*-resveratrol level.

For TPC, the calculated linear regression model was fitted to the response with $R^2(\text{adj}) = 90.34\%$ and $R^2(\text{pred}) = 83.63\%$. All main effects were important on TPC value ($p < 0.05$). Solvent volume was the most important factor on TPC, followed by extraction temperature, %ethanol content and extraction time respectively (Figure 29(a)). Solvent volume, extraction temperature and time had positive effect on TPC level, except for %ethanol content that had negative effect on TPC (Figure 29(b)). As same as 4.2, the inverse direction was found between %ethanol content on *t*-resveratrol and TPC level. To select the optimal range of %ethanol content for optimization study, the antioxidant capacity of peanut extract should be contributed from TPC rather than *t*-resveratrol alone (7, 38, 71). Hence, 20%ethanol was selected as middle point for optimization study. In conclusion, solvent volume 50 mL, 20%v/v ethanol content, extraction temperature 70°C and extraction time 120 min were obtained from screening study and were the center point in optimization study.

4.3.2.2 Optimization design

The center-faced central composite design (CCF) was employed for optimization study. The significant factors from screening study were divided into three levels (-1,0,1) which were 10, 20, 30%v/v of ethanol content, 25, 50, 75 mL of solvent volume, 60, 70, 80°C of extraction temperature and 60, 120, 180 min of extraction time. Total 31 experimental orders were created and performed (Table 18). The studied responses included %yield, *t*-resveratrol content, TPC, TFC and antioxidant capacities by FRAP, DPPH, ABTS, and ORAC assay (Table 18). The

optimized mathematical models for each response were shown as equation B.1-B.8. All terms that were significant were shown in Table 19.

For %yield, the calculated model explained the relationship between variables and responses with $R^2(\text{adj})=81.04\%$ and $R^2(\text{pred})=76.51\%$. Only linear terms were significant to %yield with $p<0.01$ (Table 19). The order of impact of variables on %yield was extraction time, solvent volume, extraction temperature and %ethanol content respectively (Figure 30(a)). Most of factors showed a positive effect to %yield including solvent volume, extraction temp and time while %ethanol content showed negative effect to %yield (Figure 30(b)-(d)). It was consistent with the study of Chairerk et al. (2021) which stated that the extraction yield decreased with increasing of %ethanol in the aqueous solvent (83).

For *t*-resveratrol, the quadratic polynomial regression was fitted the model with $R^2(\text{adj})=87.92\%$ and was able to predict the response with $R^2(\text{pred})=80.35\%$. The non-significant ‘lack of fit’ indicated the validity of the calculated quadratic model. All main effects with a quadratic effect of Temp*Temp and some interaction effects between %EtOH*Solent volume, %EtOH*Time, and Solvent volume*Time were found significant to the response, other terms were excluded from the model (Table 19). The main effect plot and response surface plots of *t*-resveratrol (Figure 31(b)-(d)) demonstrated the positive relationship of %ethanol content, solvent volume and extraction time, while extraction temperature showed the quadratic relationship to *t*-resveratrol level. This implied that the increase of extraction temperature to 70°C resulted in increase of *t*-resveratrol level, but it would be lower when the temperature was higher than 70°C. This may be explained by the instability of *t*-resveratrol at high temperature, especially in alkaline conditions (69).

For TPC, the main effects of all factors except extraction time were significant in the model, together with the interaction terms of %EtOH*Time, Solvent volume*Time and Temp*Time. The calculated model had prediction ability of 81.12% ($R^2(\text{pred})$) with $R^2(\text{adj})=87.28\%$. The validity of the model was observed from non-significant Lack-of-Fit ($p=0.754$) (Table 19). Solvent volume had the biggest effect on TPC level than other factors in positive direction, whereas only the extraction temperature showed negative effect on TPC (Figure 32(a), (b)). It was in an

agreement of previous study that mentioned the volatility of ethanol at too high temperature, and was not conducive in extraction of TPC (79). The response surface plots showed that increasing of solvent volume resulted in increasing of TPC level while increasing of %ethanol content or extraction temperature had little effect on TPC level, and extraction time was negligible factor (Figure 32(d)). The effect of solvent volume on TPC level could be explained by the greater of the concentration gradient between the solid and the liquid is achieving when higher amount of solvent is used, and it has to be adequate for the sufficient mass transfer during extraction process (9). The TPC recovered from UAE was 3.5702 mgGAE/g which was higher than previous study (35).

The calculated model for TFC response exhibited the signification of all linear terms, a quadratic term of %EtOH*%EtOH and the interaction terms of %EtOH*Solvent volume, and %EtOH*Time. The data was fitted the model with $R^2(\text{adj}) = 97.06\%$ and prediction ability of $R^2(\text{pred})=95.43$. The validity of the model was indicated by the non-significant lack-of-fit ($p=0.358$) (Table 19). The most influential factor of TFC was %ethanol content, followed by solvent volume, extraction temp and time respectively (Figure 33(a)). The relationship between variables and response was in positive linear direction, except for %ethanol content that was in parabola shape (Figure 33(b)), which meant that increasing of %ethanol content to 15% led to decrease TFC level but it would drastically increase beyond 15%v/v ethanol (Figure 33(b)). This finding was in the same manner of SLE study (in 4.2) and supported by the literature that ethanol content has significant effect on TFC and antioxidant capacity of crude extract in parabola shape (76). Increasing of ethanol content, the solubility of flavonoids will increase since the ethanol will react to flavonoids by non-covalent interaction and promote the diffusion of flavonoids into the medium (77).

Antioxidant capacity of the extract was determined by DPPH, ABTS, FRAP and ORAC assay. It was optimized and studied the relationship to those extraction parameters. The calculated models of all determinations were statistically significant ($p<0.05$) and valid to explain the variations ($p_{LOF}>0.05$). For DPPH radical-scavenging activity, the order of factor's impact on response was %ethanol content,

solvent volume and extraction time, while extraction temperature was not significant for DPPH assay. For ABTS assay, %ethanol content and solvent volume still were the impactful factors for ABTS value, followed by extraction time, whereas extraction temperature was not significant factor and was excluded from the model. In FRAP study, solvent volume influenced mostly on the response, followed by %ethanol content and extraction temperature, but extraction time was not a significant factor. Only %ethanol content and solvent volume were significant for ORAC study, while extraction temperature and time were not significant factors (Figure 34(a)-37(a)). From all antioxidant tests, %ethanol content and solvent volume were the important factors in UAE. The main effects plots indicated the positive correlation of %ethanol content and solvent volume on the responses either in linear or quadratic effect (Figure 34(b)-(d)-37(b)-(d)). This was in accordance with previous studies that %ethanol content and solvent volume had the quadratic effect on antioxidants extraction (17, 76, 84).

4.3.2.3 Model verification

The optimal extraction conditions were located at the maximum antioxidant activity of the extracts by DPPH, ABTS, FRAP and ORAC determinations. The optimal extraction conditions were derived at 30%v/v ethanol, extraction solvent 75 mL, extraction temperature 65°C and extraction time 180 min. The created model was verified by six determinations at optimized conditions. The predicted and experimental values for all responses were in table 20. All experimental values were fallen within 95% prediction intervals which indicated the validity of calculated models to predict the responses, except for %yield and *t*-resveratrol that the values were not within 95%PI. This might be from lacking the predictability at tested conditions because the model was optimized based on antioxidant capacity. The antioxidant capacities of peanut extract in our study obtained from DPPH, ABTS, FRAP and ORAC assays were 1.7363, 5.6140, 1.7449, 14.1610 mgTE/g respectively. The higher %ethanol content and solvent volume was found obtained higher antioxidant capacity of the extracts. The increasing of %ethanol content would increase soluble TPC and resulted in increasing antioxidant activity of the extracts (76). For solvent volume, it is be adequate for a good hydration and swelling of the

solid phase which helps increasing yield recovery and increasing antioxidant activity of the extracts (81).

4.3.3 Conclusion

Extraction factors including %ethanol content, solvent volume, extraction temperature and time were screened by 4 factors and 2 levels full factorial designs. The extraction conditions that provided highest TPC were selected for the center points in optimization study. The center-faced central composite design was used to optimize the extraction conditions. The responses including %yield, *t*-resveratrol, TPC, TFC, DPPH, ABTS, FRAP and ORAC were determined. The optimal extraction conditions were obtained at maximum antioxidant activity of the extracts; 30%v/v ethanol, extraction solvent 75 mL, extraction temperature 65°C and extraction time 180 min. The data fitted well to the model and were able to predict the responses. The generated model was verified and was able to predict the responses since the experimental values were not different to those predicted values, except for %yield and *t*-resveratrol. Most of single factors were significant in extraction process by UAE. %Ethanol content and solvent volume highly influenced on antioxidant capacity of the extract in positive direction either in linear or quadratic effect, followed by extraction temperature, while extraction time was a non-significant factor for most of antioxidant tests. Increasing of %ethanol content and solvent volume resulted in higher antioxidant capacity of the extracts which was in the same manner of previous literatures. The TPC obtained from this study was higher than the previous study that implied the advantage of using experimental design together with UAE in improvement of extraction efficiency. However, the antioxidant capacities of the extract at optimized conditions were not able to compare with literatures since different parts of peanut or different standardized units have been used.

4.4 Optimization of extraction conditions from peanut kernels using microwave-assisted extraction (MAE)

4.4.1 Results

4.4.1.1 Screening design

Due to the instrumental limitation, the solvent-to-solid ratio was fixed at 15:1 to prevent the overflow of solvent during the extraction (12). Therefore, 3 factors and 2 levels full factorial design with 3 center points was used for screening study. The other interested variables were the same as in screening design in 4.2, but the level was changed according to literatures (12). Then, 11 run orders were generated and performed. The results of all dependent variables were shown in table 21.

Table 21 MAE full factorial design table and its responses

Run Order	Independent variables			Responses		
	%EtOH (%)	Temp (°C)	Time (second)	Yield (%)	Resv (µg/g)	TPC (mgGAE/g)
1	80	30	600	4.66	1.1482	0.1102
2	20	70	600	8.35	2.1206	1.7792
3	20	30	60	3.18	1.0577	0.9651
4	50	50	330	5.99	2.3907	1.6930
5	80	30	60	4.48	1.0981	0.1403
6	80	70	600	13.53	2.4710	1.0447
7	20	70	60	6.79	2.2344	1.9126
8	80	70	60	11.54	2.2442	1.2952
9	50	50	330	4.73	1.8209	1.3475
10	50	50	330	4.82	1.8865	1.6408
11	20	30	600	4.65	1.0285	1.1326

The Pareto chart and main effect plots of variables were shown in Figure 38-40. The ANOVA table of full factorial designs study was shown in table 22. It showed that only extraction temperature was significant for all responses, %ethanol content was a significant factor for %yield and TPC, while extraction time was not an important factor for all responses. From main effect plots, %ethanol content and extraction time showed positive effect to all factors, except for %ethanol content that showed negative effect to total phenolic content (TPC) (Figure 40(b)). The linear equation in uncoded unit obtained from full factorial study for %yield, *t*-resveratrol and TPC was in equation A.1, A.2 and A.3, respectively.

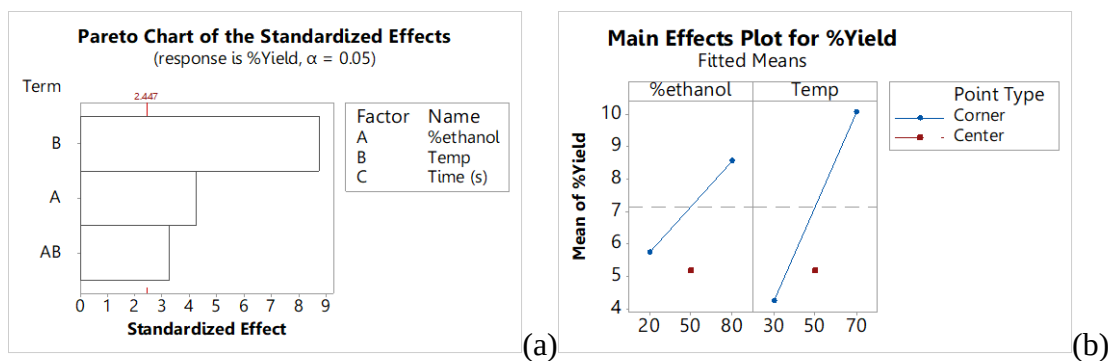


Figure 38 Pareto Chart of variables (a) and main effect plot for %yield (b)

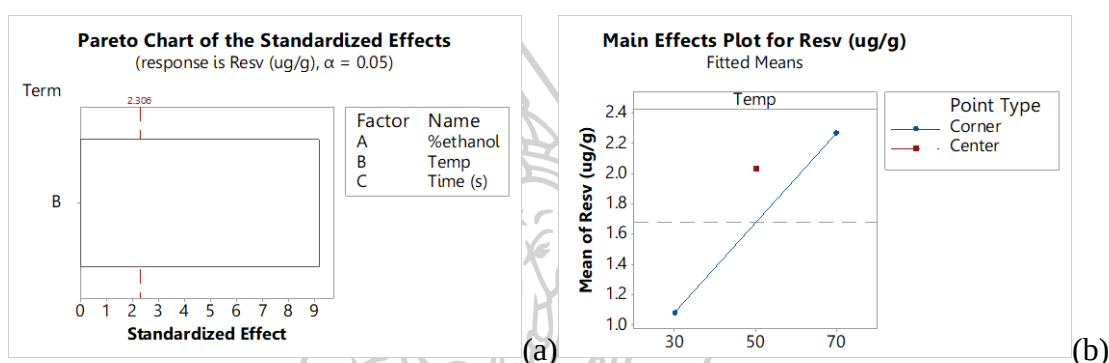


Figure 39 Pareto Chart of variables (a) and main effect plot for t-resveratrol (b)

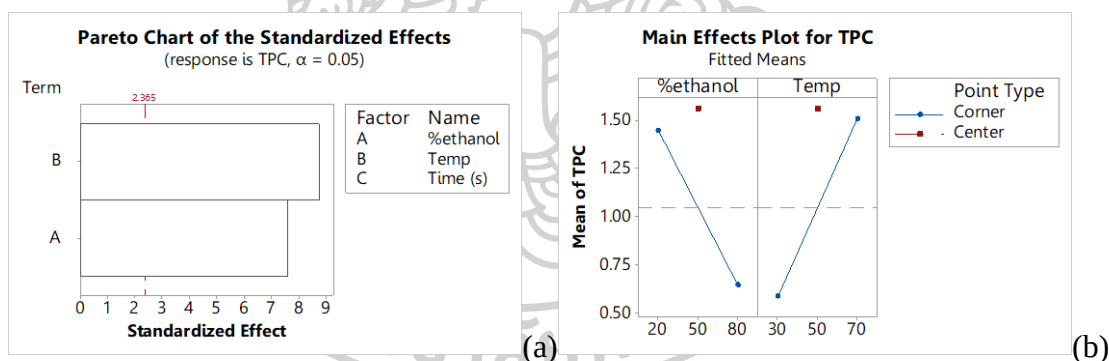


Figure 40 Pareto Chart of variables (a) and main effect plot for TPC (b)

Table 22 ANOVA table of UAE-FFD study

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	101.038	25.2596	28.69	0.000
	%EtOH	15.792	15.7922	17.94	0.005
	Temp	67.512	67.5122	76.69	0.000
	%EtOH*Temp	9.288	9.2880	10.55	0.018
	Lack-of-Fit	4.293	1.0734	2.17	0.339
	R ² (adj) = 91.72%				
	R ² (pred) = 81.76%				
t-resveratrol	Model	3.08436	1.54218	46.19	0.000
	Temp	2.80573	2.80573	84.03	0.000
	Lack-of-Fit	0.07272	0.01212	0.12	0.980
	R ² (adj) = 90.04%				
	R ² (pred) = 83.09%				

TPC	Model	3.54946	1.18315	53.50	0.000
	%EtOH	1.27932	1.27932	57.85	0.000
	Temp	1.69607	1.69607	76.69	0.000
	Lack-of-Fit	0.08542	0.01708	0.49	0.774
	R ² (adj) = 94.03%				
	R ² (pred) = 89.88%				

Regression equation in uncoded units

$$\text{A.1) \%yield} = 2.03 - 0.0430 \text{ \%ethanol} + 0.0555 \text{ Temp} + 0.001796 \text{ \%ethanol*Temp} - 1.967 \text{ Ct Pt}$$

$$\text{A.2) Resveratrol} = 0.195 + 0.02961 \text{ Temp} + 0.357 \text{ Ct Pt}$$

$$\text{A.3) TPC} = 0.563 - 0.01333 \text{ \%ethanol} + 0.02302 \text{ Temp} + 0.513 \text{ Ct Pt}$$

As same as in 4.2 and 4.3, increasing %ethanol content resulted in increasing *t*-resveratrol but decreasing TPC level. Although the contradicted direction between %ethanol content and responses have been observed, the studies indicated the positive relationship between antioxidant activities and TPC (7, 38, 71), and antioxidant activity of peanut extract should not be contributed from *t*-resveratrol alone. Therefore, 20%ethanol content, extraction temperature 70°C that provided highest TPC level was selected for optimization study, while extraction time that was not significant factor were selected at 60 second to preserve the rotatable design, and solvent volume was fixed at 15 mL.

4.4.1.2 Optimization study

The obtained conditions from screening study (20%v/v ethanol content, extraction temperature 70°C and extraction time 60 s) were assigned as center point for optimization study. The 3 factors and 3 levels center-faced central composite design (CCF) was used to optimize extraction conditions and generated 20 run orders (Table 23). The dependent variables included %yield, *t*-resveratrol content, TPC, Total flavonoid content (TFC) and antioxidant activities by DPPH, ABTS, FRAP and ORAC. The results of each experimental run were also shown in table 23. The ANOVA tables of all responses were exhibited in table 24. The optimized polynomial equations obtained from CCF study for all responses were demonstrated as equation B.1-B.8. The Pareto charts, main effect plots, contour plots and response surface plots of each response were shown in Figure 41-48.

Table 23 Experimental design table for MAE-CCF study in uncoded unit

RunOrder	Independent variables				Responses						
	%EtOH	Temp (°C)	Time (min)	Yield (%)	Resv (µg/g)	TPC (mgGAE/g)	TFC (mgQE/g)	FRAP	DPPH	ABTS	ORAC
								(mgTE/g)			
1	30	70	60	4.006	0.9400	1.7148	0.9238	4.5271	0.0387	2.5133	6.0297
2	10	60	90	4.482	0.9444	1.8677	0.6459	4.3134	0.0091	2.0834	5.9210
3	20	70	30	4.771	0.9582	1.7610	0.9403	4.6742	0.0347	2.3682	6.9056
4	20	70	60	3.961	0.9605	1.6808	0.8679	4.4167	0.0235	2.2819	5.7922
5	10	70	60	5.104	1.0028	1.8287	0.7842	4.3952	0.0110	2.2234	6.9159
6	30	80	90	5.710	1.1781	2.2336	1.0056	5.0100	0.0489	2.6746	8.3942
7	20	70	60	4.680	1.0228	1.8849	0.9649	4.5470	0.0379	2.3631	7.2330
8	20	70	60	4.972	1.0566	1.8748	0.8882	4.6653	0.0277	2.4157	6.3584
9	20	70	90	4.654	1.0480	2.0393	0.8281	4.4086	0.0303	2.3619	6.8169
10	20	60	60	3.711	0.8634	1.6712	0.6979	4.3384	0.0266	2.1834	4.5482
11	10	80	30	5.979	0.9922	1.6468	0.9307	4.7372	0.0091	2.2930	8.5323
12	30	60	30	4.108	0.8516	1.5344	0.8698	4.5188	0.0568	2.5202	5.1037
13	20	70	60	4.530	0.9664	1.8147	0.7986	4.5698	0.0286	2.3154	6.3779
14	30	80	30	5.497	0.9681	1.7480	0.9631	4.9842	0.0315	2.5231	7.3673
15	20	70	60	4.992	1.0259	1.8384	0.9456	4.6475	0.0258	2.3827	7.4861
16	20	80	60	8.94	2.1467	3.0810	0.0154	1.3193	0.8431	6.1205	15.4365
17	20	70	60	10.06	2.9825	3.6179	0.0340	1.5721	1.0647	6.5632	14.5516
18	10	80	90	8.96	2.7766	3.1356	0.0535	1.4048	1.0180	6.2102	13.7678
19	30	60	90	9.67	1.9233	2.9344	0.0287	1.4210	0.9238	6.4258	9.926
20	10	60	30	9.25	2.6780	3.4166	0.0299	1.5304	1.1082	6.5506	11.246

Table 24 ANOVA table for all responses

Response	Source	Adjusted sum of squares	Adjusted mean square	F-value	P-value
%yield	Model	6.40165	2.13388	17.24	0.000
	Temp	5.66858	5.66858	45.80	0.000
	Time	0.05595	0.05595	0.45	0.511
	Time*Time	0.67712	0.67712	5.47	0.033
	Lack-of-Fit	1.08645	0.09877	0.55	0.809
	R ² (adj) = 71.95%				
	R ² (pred) = 66.91%				
t-resveratrol	Model	0.460791	0.115198	38.46	0.000
	%EtOH	0.331204	0.331204	110.57	0.000
	Temp	0.104040	0.104040	34.73	0.000
	Time	0.007134	0.007134	2.38	0.144
	%EtOH*Temp	0.018413	0.018413	6.15	0.026
	Lack-of-Fit	0.023897	0.002390	0.57	0.791
	R ² (adj) = 88.75%				
TPC	Model	0.609543	0.152386	20.40	0.000
	%EtOH	0.005212	0.005212	0.70	0.417
	Temp	0.239785	0.239785	32.10	0.000
	Time	0.342805	0.342805	45.88	0.000
	%EtOH*%EtOH	0.021740	0.021740	2.91	0.109
	Lack-of-Fit	0.079316	0.007932	1.21	0.441
	R ² (adj) = 80.33%				
TFC	Model	0.003406	0.000568	17.06	0.000
	%EtOH	0.003094	0.003094	92.98	0.000
	Temp	0.000007	0.000007	0.22	0.645
	Time	0.000028	0.000028	0.85	0.374
	%EtOH*%EtOH	0.000015	0.000015	0.45	0.512
	%EtOH*Temp	0.000171	0.000171	5.14	0.041
	Temp*Time	0.000090	0.000090	2.70	0.124
DPPH	Model	0.000296	0.000037	1.36	0.384
	R ² (adj) = 83.53%				
	R ² (pred) = 69.76%				
	Model	0.17655	0.058849	22.96	0.000
	%EtOH	0.05869	0.058691	22.90	0.000
	Temp	0.09626	0.096256	37.55	0.000
	%EtOH*Temp	0.02160	0.021601	8.43	0.010
ABTS	Lack-of-Fit	0.02334	0.002122	0.60	0.777
	R ² (adj) = 77.62%				
	R ² (pred) = 73.13%				
	Model	1.00908	0.336360	28.61	0.000
	%EtOH	0.2075	0.20750	18.40	0.000
	Temp	0.79524	0.795240	67.65	0.000
	Temp*Temp	0.08150	0.081498	6.93	0.018
FRAP	Lack-of-Fit	0.14897	0.013543	1.73	0.283
	R ² (adj) = 81.34%				
	R ² (pred) = 74.67%				
	Model	0.12387	0.061936	41.38	0.000
	Temp	0.08219	0.082192	54.92	0.000

	Time	0.04168	0.041680	27.85	0.000
	Lack-of-Fit	0.01220	0.001016	0.38	0.919
	R ² (adj) = 80.96%				
	R ² (pred) = 77.68%				
ORAC	Model	23.0267	11.5134	37.63	0.000
	%EtOH	0.8341	0.8341	2.73	0.117
	Temp	22.1927	22.1927	72.54	0.000
	Lack-of-Fit	2.7897	0.2325	0.48	0.861
	R ² (adj) = 79.41%				
	R ² (pred) = 74.96%				

Equation

- B.1) %yield = $0.599 + 0.0753 \text{ Temp} - 0.0466 \text{ Time (s)} + 0.000409 \text{ Time (s)*Time (s)}$
- B.2) Resveratrol = $0.550 + 0.01979 \text{ Temp} + 0.000890 \text{ Time (s)} + 0.0518 \text{ %EtOH} - 0.000480 \text{ Temp*%EtOH}$
- B.3) TPC = $0.073 + 0.01548 \text{ Temp} + 0.006172 \text{ Time (s)} + 0.0287 \text{ %EtOH} - 0.000659 \text{ %EtOH*%EtOH}$
- B.4) TFC = $-0.0276 + 0.000169 \text{ Temp} - 0.000726 \text{ Time (s)} + 0.00569 \text{ %EtOH} - 0.000017 \text{ %EtOH*%EtOH} + 0.000011 \text{ Temp*Time (s)} - 0.000046 \text{ Temp*%EtOH}$
- B.5) DPPH = $-0.692 + 0.02020 \text{ Temp} + 0.0440 \text{ %EtOH} - 0.000520 \text{ Temp*%EtOH}$
- B.6) ABTS = $8.59 - 0.1505 \text{ Temp} + 0.01150 \text{ %EtOH} + 0.001277 \text{ Temp*Temp}$
- B.7) FRAP = $0.2194 + 0.00907 \text{ Temp} + 0.002152 \text{ Time (s)}$
- B.8) ORAC = $-3.32 + 0.1490 \text{ Temp} - 0.0289 \text{ %EtOH}$

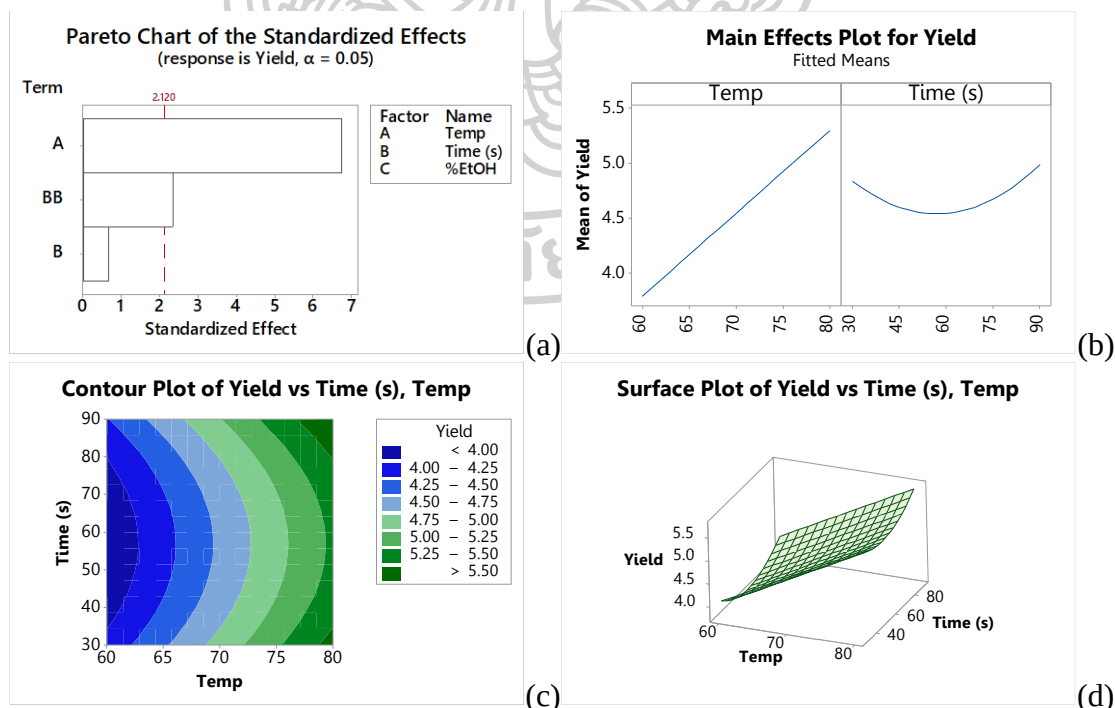


Figure 41 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on %yield response

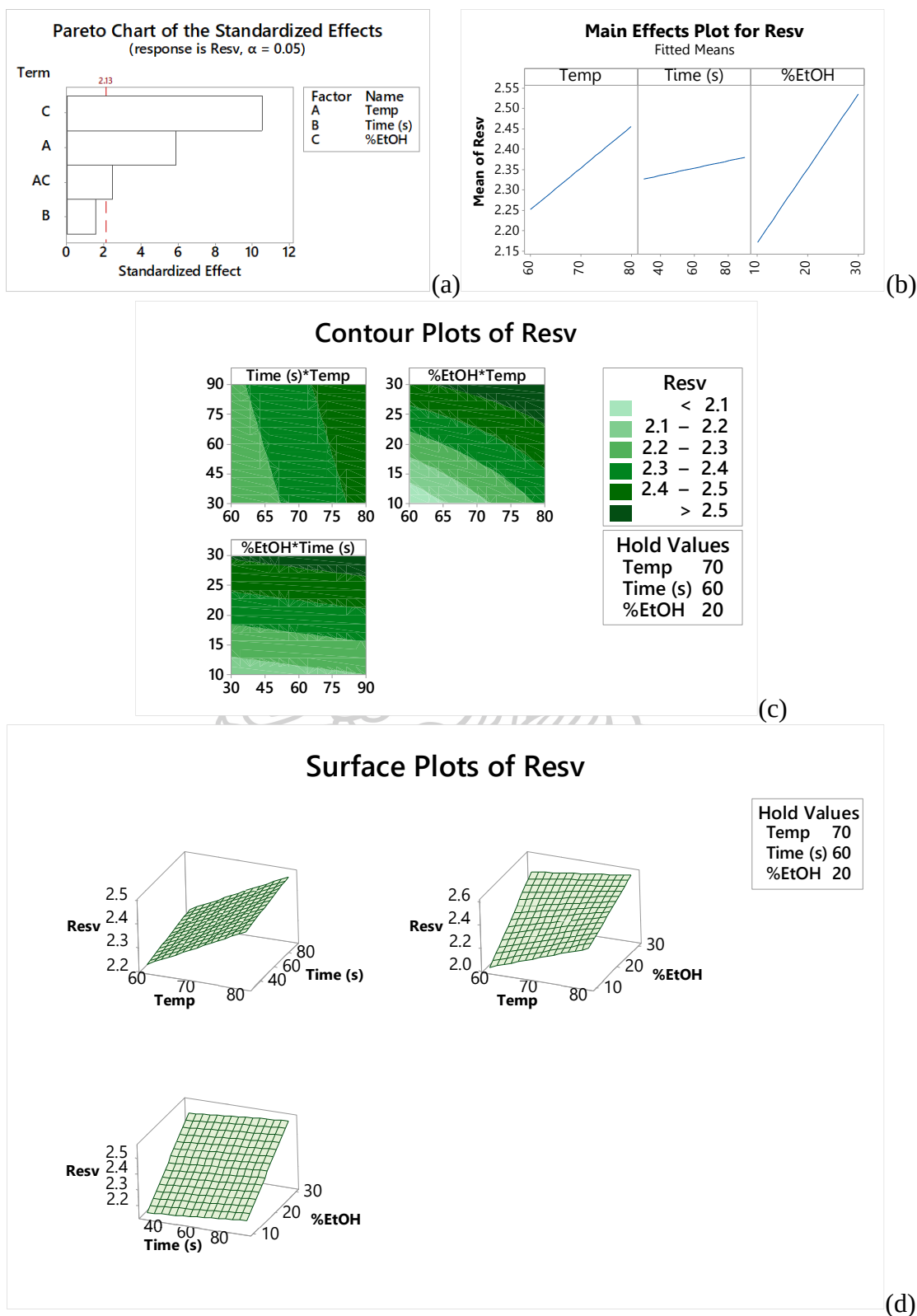


Figure 42 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on *t*-resveratrol content response

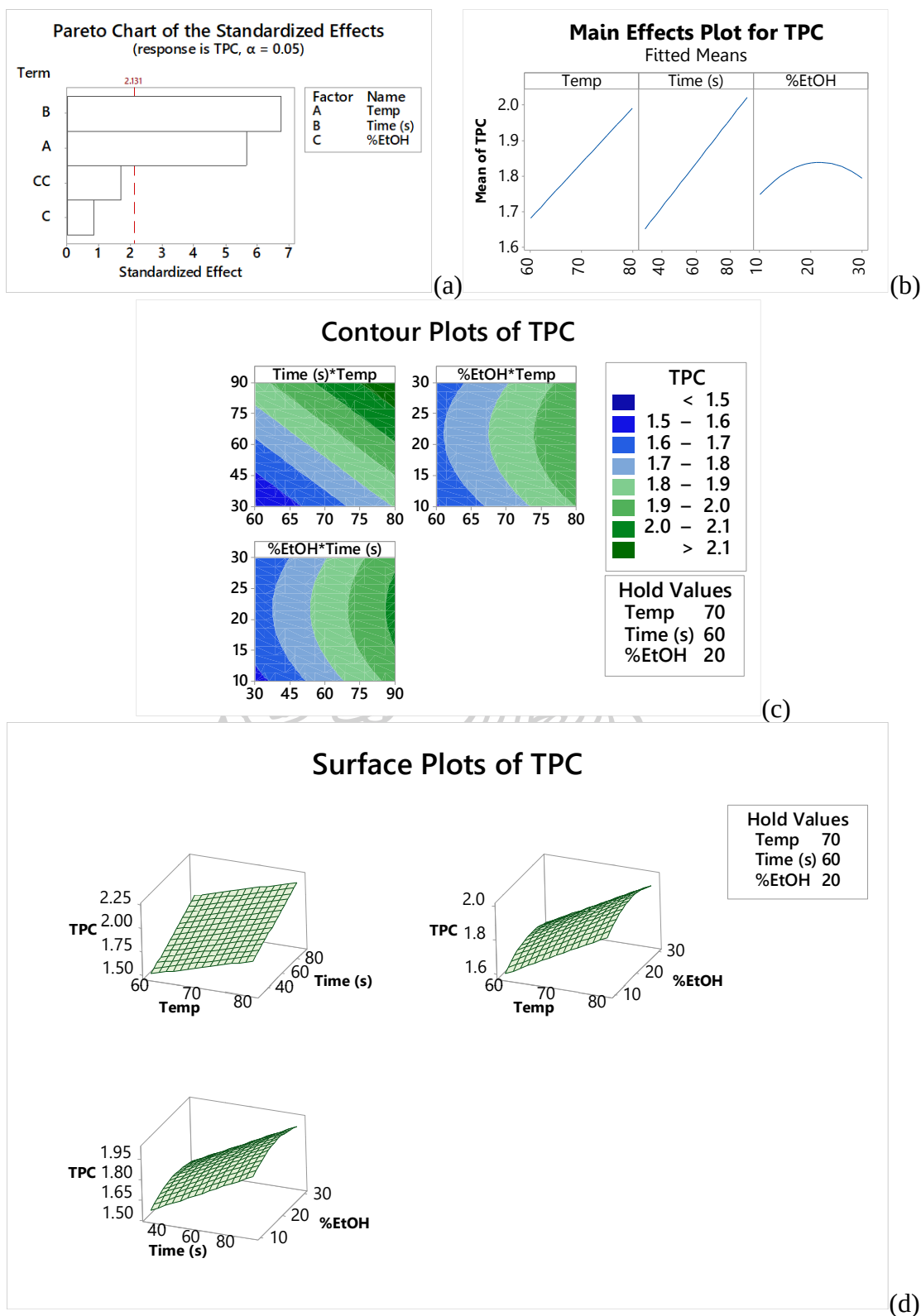


Figure 43 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on TPC response

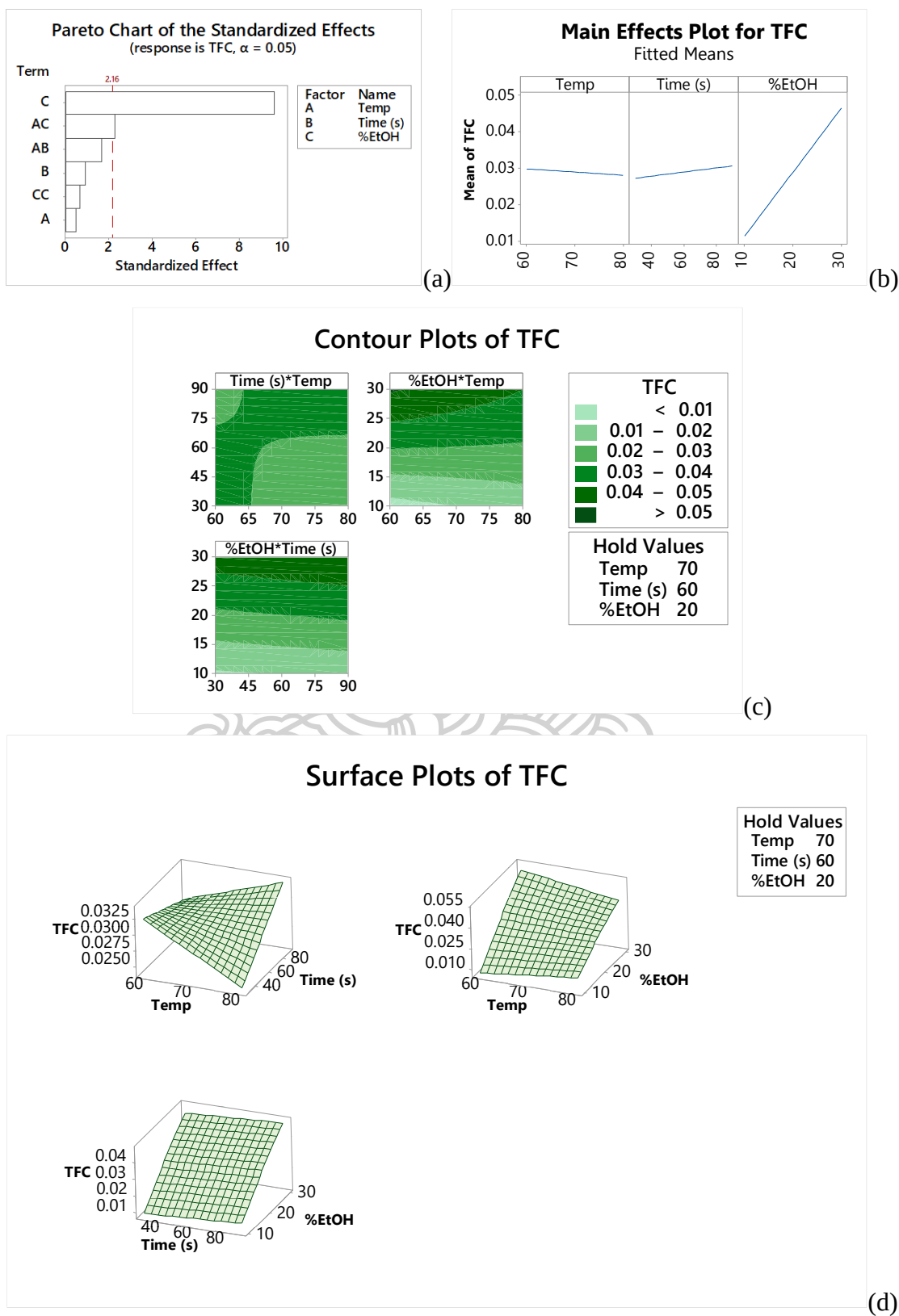


Figure 44 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on TFC response

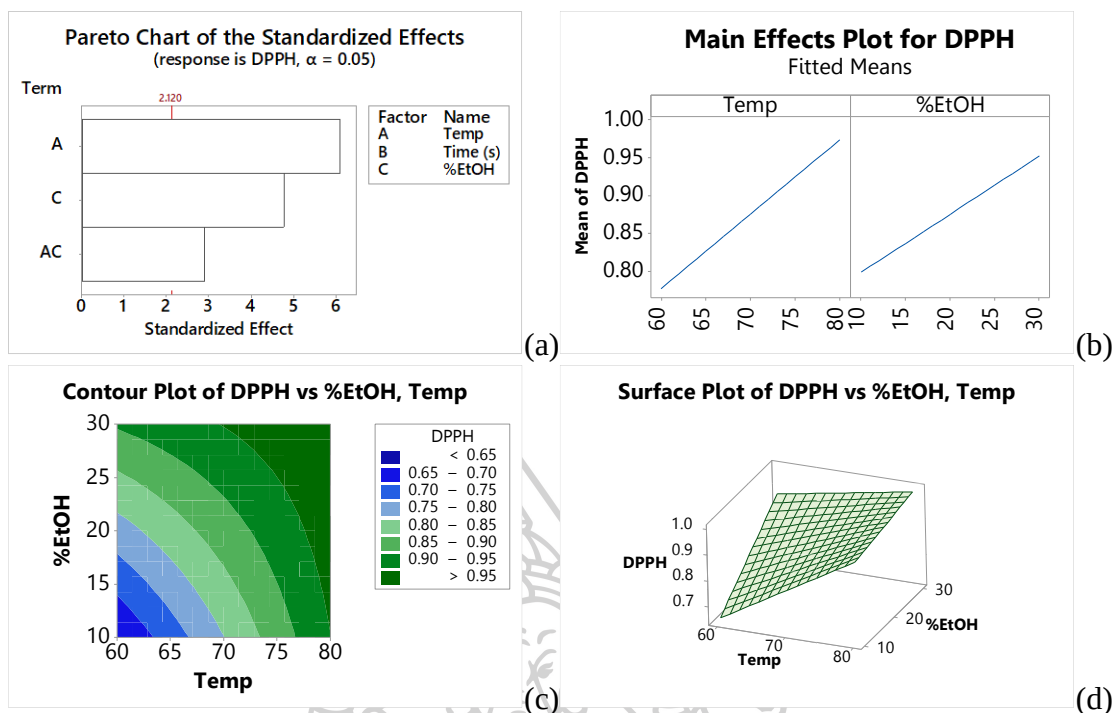


Figure 45 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on DPPH response

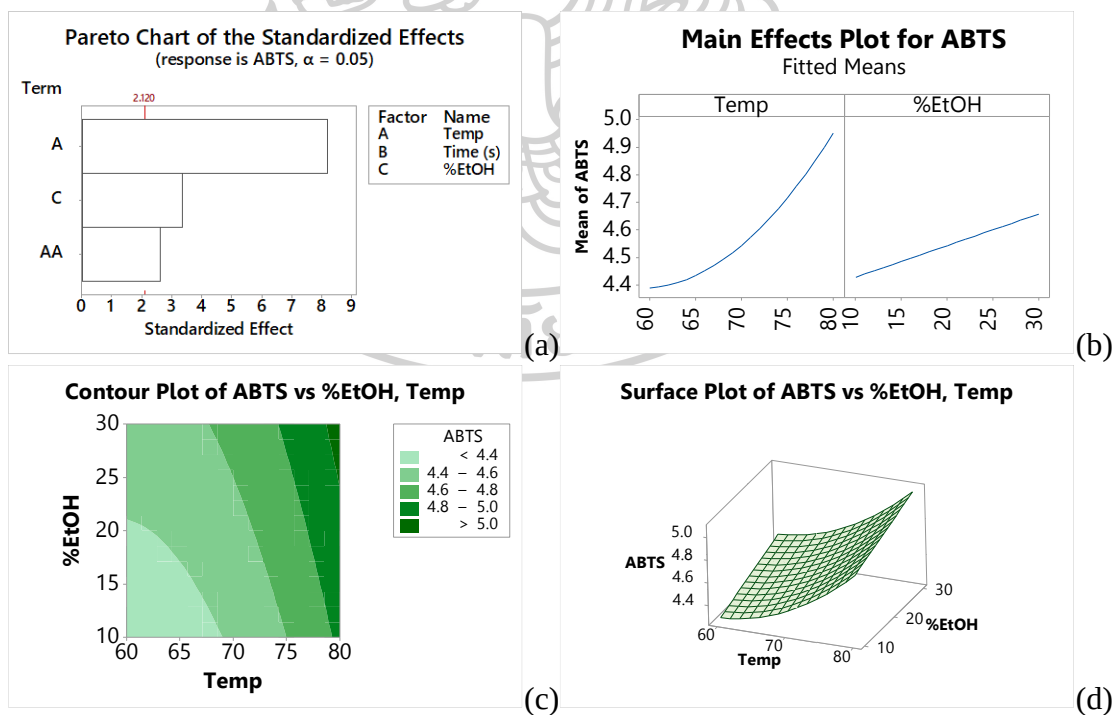


Figure 46 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on ABTS response

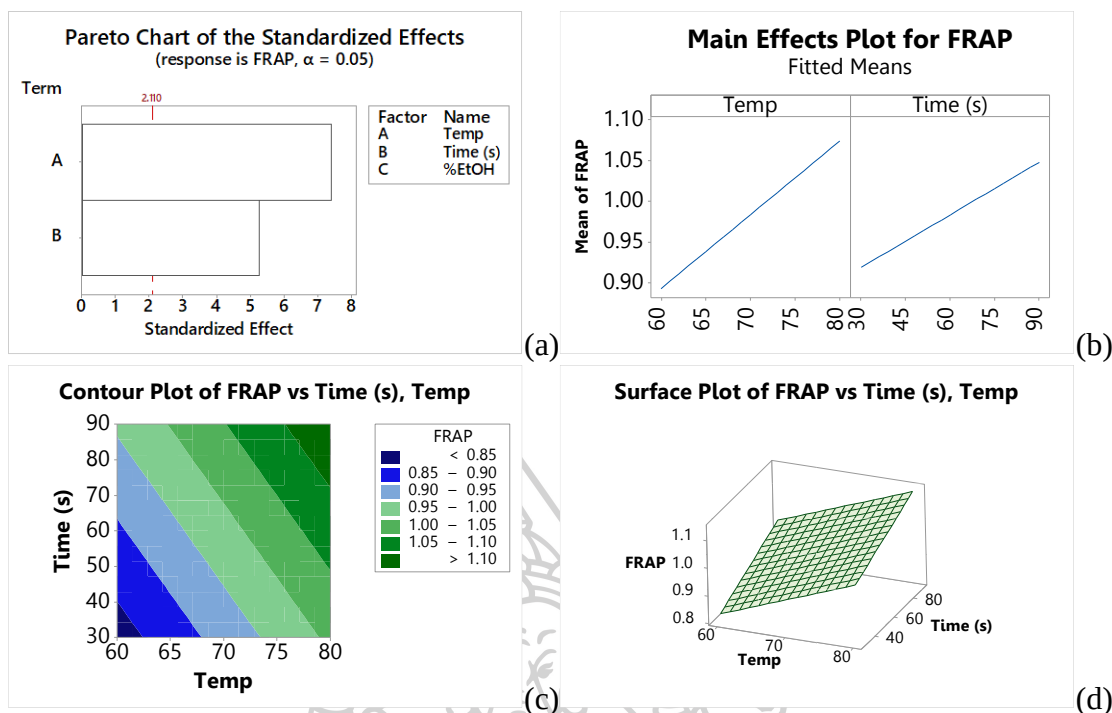


Figure 47 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on FRAP response

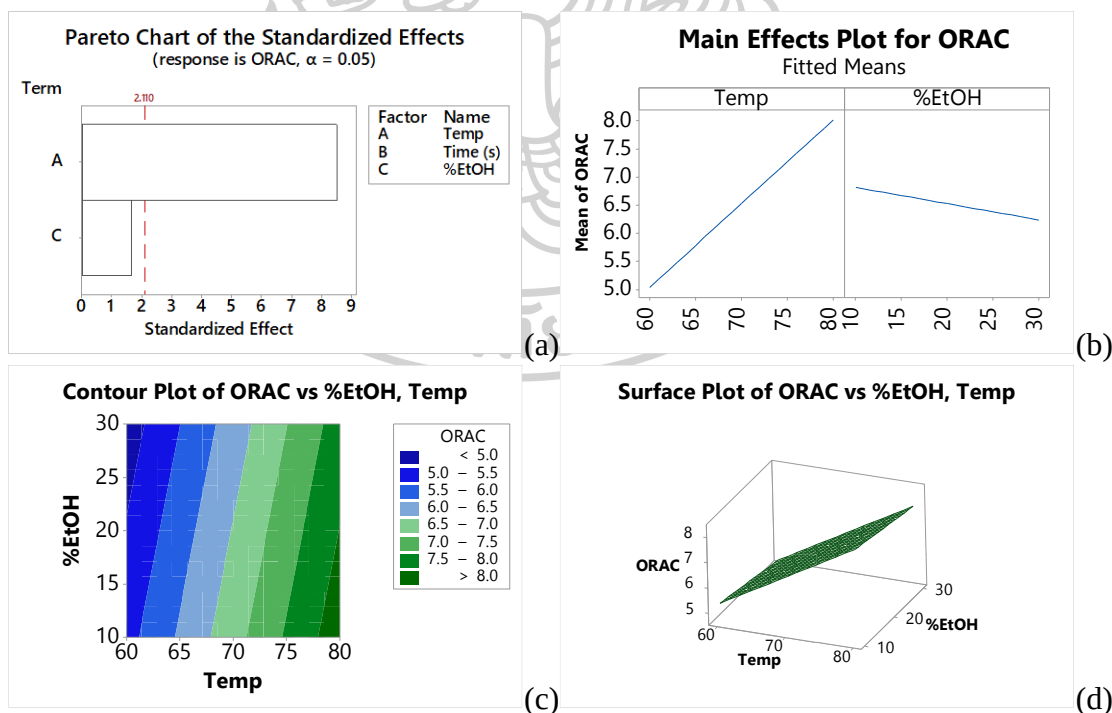


Figure 48 Pareto chart of effect plot (a), Main effect plot (b), Contour plots (c) and Response surface plots (d) of MAE-CCF on ORAC response

4.4.1.3 Model verification

The optimized extraction condition was selected at conditions that provided maximum antioxidant activity of the extract by DPPH, ABTS, FRAP and ORAC assay which were at 30%v/v ethanol, extraction temperature 80°C and extraction time 90 s. It was used for model verification by determining the responses for 6 replications. The predicted and experimental values were shown in table 25. All experimental values were fallen within 95%CI of predicted values which indicated the validity of the model, except for *t*-resveratrol that the experimental value was not in 95%PI.

Table 25 Predicted and experimental values at optimized conditions of MAE-CCF study

Response	Predicted	Actual ($\pm$ SD)	95% Prediction Intervals
%yield	5.7425	5.59 ($\pm$ 0.76)	(4.89220, 6.59280)
<i>t</i> -resveratrol (μ g/g)	2.61598	1.2844 ($\pm$ 0.1208)	(2.47430, 2.75766)
TPC (mgGAE/g)	2.13388	2.1962 ($\pm$ 0.1892)	(1.91589, 2.35187)
TFC (mgQE/g)	0.0452	0.0419 ($\pm$ 0.0053)	(0.0292, 0.0612)
ORAC (mgTE/g)	7.727	7.3630 ($\pm$ 1.6880)	(6.422, 9.031)
FRAP (mgTE/g)	1.072	1.0768 ($\pm$ 0.0954)	(0.9168, 1.2271)
ABTS (mgTE/g)	5.0667	5.0537 ($\pm$ 0.1285)	(4.8047, 5.3288)
DPPH (mgTE/g)	0.998	1.0287 ($\pm$ 0.0588)	(0.8721, 1.1238)

4.4.2 Discussion

4.4.2.1 Screening design

The 3 factors and 2 levels full factorial design with 3 center points was employed for screening of significant factors. The variable of interests including %ethanol (20, 80%), extraction temperature (30, 70°C) and extraction time (60, 600 s) were assigned according to the literature (12). A total of 11 experimental orders was obtained and performed (Table 21). The ANOVA and multiple linear regression were applied to determine the significant variables and the relationship between factors and responses.

The linear term of extraction temperature was significant for all responses ($p < 0.05$), while %ethanol content was significant for %yield and TPC, but extraction time was not a significant factor for any response (Table 22). In other words, the

extraction temperature was the most important factor for MAE, followed by %ethanol content, and it was the only factor that affected to *t*-resveratrol content. The main effect plot revealed the positive correlation between variables and responses [Figure 38(b)-40(b)], except for %ethanol content vs TPC that showed negative and no correlation, respectively (Figure 40(b)). This implied that increase of extraction temperature resulted in the increase of all responses, while increase of %ethanol content resulted in increase of %yield but decreased TPC level. To select the optimal range for optimization study, the high level of extraction temperature and low level of %ethanol content was selected to maximize the TPC level since there were several studies indicated the correlation between TPC and antioxidant activity (7, 38, 71). Therefore, at 20%v/v ethanol and extraction temperature 70°C that provided highest TPC level were selected for optimization study. Moreover, although the extraction time was not a significant factor, it was selected at 60 s in optimization study to preserve the rotatable characteristic of the design. In conclusion, at 20%v/v ethanol content, extraction temperature 70°C and extraction time 60 s were obtained from screening study and were the center point in optimization study, while the solvent volume was fixed at 15 mL.

4.4.2.2 Optimization design

The 3 factors and 3 levels center-faced central composite design (CCF) was utilized for optimization study. At 10, 20, 30%v/v of ethanol content, 60, 70, 80°C of extraction temperature and extraction time 30, 60, 90 s were assigned as level -1, 0, 1 for each variable. A total of 20 run orders was generated and performed (Table 23). The responses including %yield, *t*-resveratrol content, TPC, TFC and antioxidant capacities by FRAP, DPPH, ABTS, and ORAC assay were collected and shown in Table 23. The optimized mathematical models for each response were shown as equation B.1-B.8, and all terms included in the model were shown in Table 24.

For %yield, the data were fitted well to the calculated model ($p_{LOF} > 0.05$), and it was able to explain the variations with $R^2(\text{adj}) = 71.95\%$ but it showed moderate predictability with $R^2(\text{pred}) = 66.91\%$. Only linear term of extraction temperature and quadratic term of time*time were significant to %yield ($p < 0.05$) while the linear term of extraction time was not significant ($p > 0.05$) (Figure 41(a)). The extraction

temperature is the only factor that positively influenced the %yield (Figure 41(b)-(d)). It could be explained by the increase of vapor pressure and decrease of the viscosity and the surface tension of the liquid phase at high temperature, which leads to the ultrasonic cavitation forming and accelerates the mass transfer between solute and solvent (60).

For *t*-resveratrol, the model was valid with non-significant Lack-of-fit ($p=0.791$). It could explain the variations by the fitted model with $R^2(\text{adj})=88.75$ and be able to predict the response with $R^2(\text{pred})=84.16\%$. It included the significant linear terms of %ethanol content and extraction temperature, and the interaction term of %EtOH*temp ($p<0.05$) with a non-significant term of extraction time ($p>0.05$). (Table 24). The Pareto chart indicated the most influential factor was %ethanol content, followed by extraction temperature (Figure 42(a)). The main effect plot and response surface plots revealed the positive relationship of %ethanol content, and extraction temperature on *t*-resveratrol level (Figure 42(b)-(d)). This implied that the increase of %ethanol content and extraction temperature resulted in an increase of *t*-resveratrol level. This contradicted the result in 4.2. The reason might be from the shorter extraction time (90 s) at high temperature in MAE, while it was a long exposure time of sample (120 min) at extreme temperature ($>80^\circ\text{C}$) in SLE and UAE, which may result in degradation of *t*-resveratrol (69).

For TPC, the linear terms of extraction temperature and time were significant while the linear term and the quadratic term of %ethanol content and %EtOH*%EtOH were not significant in the model. The calculated model was valid with $p_{LOF}=0.441$. The adjusted and predicted determination coefficients were $R^2(\text{adj})=80.33\%$ and $R^2(\text{pred})=70.43\%$ (Table 24). Extraction time had the greatest effect on TPC level, followed by extraction temperature in positive direction, whereas %ethanol content was a not significant factor for MAE (Figure 43). This implied that increase of extraction temperature and time would increase TPC recovery. Although the previous studies reported the dropping of TPC level at high extraction temperature (76, 77) which was the same as in UAE study (in 4.3), the MAE required shorter extraction time so the degradation of phenolics may not be occurred. The TPC found in this

study was 2.1962 mgGAE/g. Despite it was lower than in SLE and MAE study, it was higher than the previous report (32).

The model for TFC was significant ($p < 0.001$) and was able to explain the variations with $R^2(\text{adj}) = 83.53\%$, but it had low prediction ability with $R^2(\text{pred}) = 69.76$. The validity of the model was indicated by the non-significant lack-of-fit ($p = 0.441$) (Table 24). Only linear term of %ethanol content and interaction term of %EtOH*temp were significant ($p < 0.05$) while the linear terms of extraction temperature and time and the quadratic term of %EtOH*%EtOH were not significant. The Pareto Chart revealed that the greatly impact factor on TFC was %ethanol content (Figure 44(a)). The relationship between %ethanol content and response were in positive direction (Figure 44(b)). The response surface plot showed that increasing of %ethanol content led to increase of TFC level (Figure 44(c)-44(d)). It could be explained by the increase of non-covalent binding between increased ethanol and flavonoids which promotes the diffusion of flavonoids into the medium (77).

The calculated models of all antioxidant tests including DPPH, ABTS, FRAP and ORAC were statistically significant ($p < 0.05$) and valid ($p_{\text{LOF}} > 0.05$). The adjusted coefficient of determinations [$R^2(\text{adj})$] were 77.62%-81.34% while the predicted coefficient of determinations [$R^2(\text{pred})$] were 73.17%-77.68%. From main effect plots, the order of impact factors on DPPH and ABTS values was extraction temperature and %ethanol content (Figure 45(a), 46(a)). In addition, extraction temperature and time were the significant factors for FRAP assay (Figure 47(a)), while only extraction temperature was important for ORAC assay (Figure 48(a)). All significant factors influenced on antioxidant values in positive direction (Figure 45(b)-48(b)), which meant that higher of all factor levels, particularly extraction temperature, result to higher antioxidant activities of peanut extract. This finding is opposed to the previous study where antioxidant capacity by DPPH and ABTS decreased after 45°C (76). The reason might be from longer extraction time used in conventional extraction which resulted to degradation of antioxidants while it was much shorter in MAE. This was supported by a group of authors who concluded that longer incubation time, lesser antioxidant activities (85).

4.4.2.3 Model verification

The maximum antioxidant activity of the extracts by DPPH, ABTS, FRAP and ORAC determinations were reported as optimized conditions which were at 30%v/v ethanol, extraction temperature 80°C and extraction time 90 s. It was performed six determinations at optimized conditions to check validity of the model. The predicted and experimental values for all responses were in table 25. All experimental values were fallen within 95% prediction intervals which indicated the validity of calculated models to predict the responses, except for *t*-resveratrol that was lower than the predicted value which may cause by model optimization through antioxidant capacity, and about 15% that calculated model for *t*-resveratrol could not explain the response. The antioxidant capacities of peanut extract in our study obtained from DPPH, ABTS, FRAP and ORAC assays were 1.0287, 5.0537, 1.0768, 7.3630 mgTE/g respectively. The most important factor for MAE was extraction temperature. The increasing of extraction temperature would increase in TPC and antioxidant activity of the extracts. Previous study suggested the same as increasing polyphenols yield when increased extraction temperature (86). However, the antioxidant activity tended to decrease when increased extraction temperature (45) which contradicted to our study. It could be explained by shorter period of extraction time in MAE that reduces the degradation of polyphenols at high temperature (85).

4.4.3 Conclusion

%Ethanol content, extraction temperature and time were screened by 3 factors and 2 levels full factorial designs. Solvent volume was fixed at 15 mL. The extraction conditions that provided highest TPC were chosen as center points in optimization study by center-faced central composite design. The responses for optimization study such as %yield, *t*-resveratrol, TPC, TFC, DPPH, ABTS, FRAP and ORAC were determined. The optimal extraction conditions were obtained at maximum antioxidant activity of the extracts: 30%v/v ethanol, extraction temperature 80°C and extraction time 90 s. The data fitted well to the model and were able to predict the responses. The model verification at optimized conditions revealed the validity of the model by showing the non-different experimental values from the predicted values, except for *t*-resveratrol. Extraction temperature was the most important factor in MAE. The

%ethanol content and extraction time were significant only for some specific tests. All factors influenced the antioxidant capacity of the extract in a positive direction. Increasing of all factor levels, especially extraction temperature resulted in higher TPC and antioxidant capacity of the extracts. Although the TPC obtained from this study was lower than in SLE and UAE study, it was higher than the previous study that implied the advantage of optimization of extraction conditions using experimental designs and MAE. However, the antioxidant capacities of the extract at optimized conditions could not be compared to literatures since different parts of peanut or different standardized units have been used.

4.5 Comparison of SLE, UAE and MAE methods for extraction of peanut kernels

4.5.1 Introduction

Extraction is a crucial step to reveal the actual phytochemicals in plants (5). Solid-liquid-extraction (SLE) is the simplest method by maceration samples in extraction solvent for a certain time, but it needs large amount of solvent and not a green extraction method (77). Ultrasound-assisted-extraction (UAE) promotes the extraction by creating the cavitation bubbles that result to cell disruption and increase mass transfer (3). UAE may require lesser extraction time and solvent volume, but it may end up with extremely high extraction temperature that is harmful for thermo-sensitive compounds (5). One of the green extraction methods, Microwave-assisted-extraction (MAE) facilitates the extraction by using microwave energy to increase the dissolved-ions and solvent penetration into the matrix. MAE is known to be a safe method and require small amount of extraction solvent (5). However, it has a limitation on solvent types that only polar solvents that can transfer the microwave energy are compatible with MAE (3).

In this session, the results of all bioactivity determinations at optimized conditions including *t*-resveratrol, TPC, TFC, DPPH, ABTS, FRAP and ORAC were compared between extraction methods. In addition, peanut var. Kalasin-II and Thainan-IX were also determined the bioactivities and compared to Kalasin-I that was used in optimization study. Lastly, the correlation among bioactivity tests were determined by Pearson's correlation analysis.

4.5.2 Results

The results of all determinations of all varieties were shown in Table 26. To compare between extraction methods, the responses of Kalasin-I were separately plotted and shown in Figure 48. It could be seen that SLE and UAE significantly provided higher level of all responses than MAE ($p<0.05$) (Figure 48). Concerning antioxidant results of the extracts by ABTS and ORAC assays, SLE provided higher antioxidant capacity than UAE and MAE, respectively, while it gave lower DPPH antioxidant capacity than UAE but still higher than MAE ($p<0.05$) (Table 26). In addition, FRAP values obtained from SLE and UAE were not significant different, but they were significantly higher than that from MAE. The highest antioxidant capacity of peanut extracts was obtained by ORAC assay, followed by ABTS, whereas DPPH and FRAP provided lowest antioxidant values of peanut extracts.

Table 26 Responses at optimization point of SLE, UAE and MAE of 3 peanut varieties: Kalasin-I, Kalasin-II and Thainan-IX

	SLE			UAE			MAE		
	Kalasin-I	Kalasin-II	Thainan-IX	Kalasin-I	Kalasin-II	Thainan-IX	Kalasin-I	Kalasin-II	Thainan-IX
Yield (%)	11.33 ^c	16.16 ^b	18.05 ^b	10.80 ^c	16.82 ^b	38.45 ^a	5.59 ^e	9.10 ^{c,d}	6.57 ^e
(SD)	(0.14)	(0.46)	(0.62)	(0.20)	(0.70)	(4.59)	(0.76)	(0.25)	(1.20)
Resv (µg/g)	3.4718 ^a	3.1689 ^a	2.6380 ^b	3.2060 ^a	3.1062 ^a	2.4550 ^b	1.2844 ^d	1.8095 ^c	1.5290 ^{c,d}
(SD)	(0.1126)	(0.2062)	(0.1974)	(0.2830)	(0.2164)	(0.2880)	(0.1208)	(0.1655)	(0.1449)
TPC (mgGAE/g)	3.5915 ^a	2.9855 ^b	3.4071 ^a	3.6233 ^a	3.1195 ^b	3.4826 ^a	2.1962 ^c	1.8784 ^d	2.2285 ^c
(SD)	(0.0720)	(0.0971)	(0.0623)	(0.1276)	(0.0993)	(0.1099)	(0.1892)	(0.2325)	(0.2022)
TFC (mgQE/g)	0.2143 ^d	0.2795 ^c	0.3066 ^c	0.1382 ^e	0.3628 ^b	0.5025 ^a	0.0419 ^f	0.0210 ^f	0.1029 ^e
(SD)	(0.0236)	(0.0162)	(0.0188)	(0.0059)	(0.0732)	(0.0271)	(0.0053)	(0.0116)	(0.0231)
DPPH (mgTE/g)	1.3469 ^f	1.7076 ^{c,d}	2.2631 ^a	1.7363 ^{b,c}	1.5666 ^{d,e}	1.8935 ^b	1.0287 ^g	1.0254 ^g	1.4265 ^{e,f}
(SD)	(0.0244)	(0.0642)	(0.1103)	(0.0557)	(0.0638)	(0.1540)	(0.0588)	(0.0797)	(0.1195)
ABTS (mgTE/g)	6.7708 ^{b,c}	6.4698 ^c	7.6204 ^a	5.614 ^d	6.6572 ^{b,c}	7.8053 ^a	5.0537 ^e	5.9250 ^d	7.0600 ^b
(SD)	(0.1176)	(0.2281)	(0.2057)	(0.0788)	(0.1631)	(0.2045)	(0.1285)	(0.335)	(0.3340)
FRAP (mgTE/g)	1.7669 ^{b,c}	1.5591 ^{d,e}	2.0440 ^a	1.7449 ^{b,c}	1.6968 ^{c,d}	1.8967 ^{a,b}	1.0768 ^f	1.0678 ^f	1.4230 ^e
(SD)	(0.0676)	(0.0653)	(0.1185)	(0.0660)	(0.1017)	(0.1135)	(0.0954)	(0.0988)	(0.1049)
ORAC (mgTE/g)	17.9570 ^{b,c}	13.1560 ^d	28.1270 ^a	14.1610 ^d	14.8840 ^{c,d}	20.6700 ^b	7.3630 ^e	9.4560 ^e	14.2270 ^d
(SD)	(0.9540)	(1.8400)	(1.4430)	(2.0950)	(1.9580)	(3.0600)	(1.6880)	(1.4750)	(1.9160)

In the same row, means that do not share a letter are significantly different ($p<0.05$).

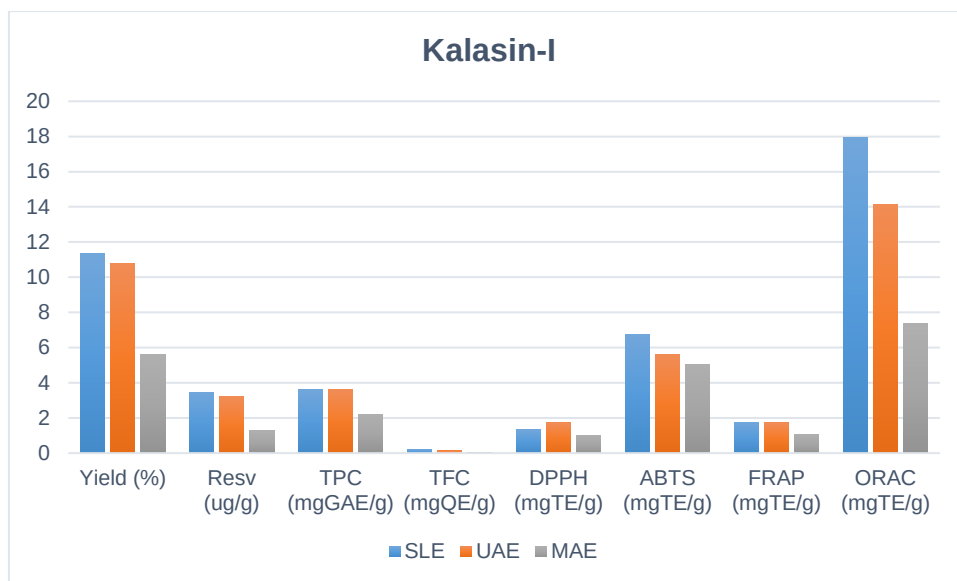


Figure 49 Comparing all responses between SLE, UAE and MAE of Kalasin-I

Considering of peanut varieties, the antioxidant activity of peanut extracts obtained from different tests and extraction methods were shown in figure 50. It could be observed that Thainan-IX possessed higher antioxidant capacity of the extract than Kalasin-I and -II for all tests ($p < 0.05$), while the antioxidant capacity between Kalasin-I and -II was not significant different in most of tests.

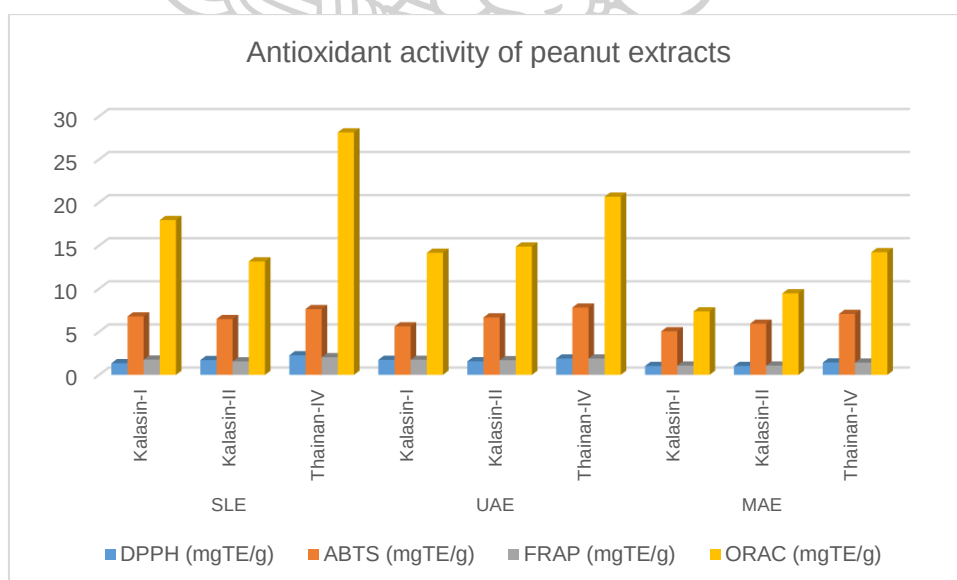


Figure 50 Antioxidant activity of peanut extracts from var. Kalasin-I, Kalasin-II and Thainan-IX by DPPH, ABTS, FRAP and ORAC assays

Pearson's correlation was determined to find the correlation between responses. All values regardless of varieties and extraction methods were gathered and calculated Pearson's correlation, and the Pearson's values and p-value were shown in Table 27. The significant strong positive correlations were found between TPC with all antioxidant tests, and among the antioxidant tests. The moderate positive correlations were found between *t*-resveratrol with TPC, and *t*-resveratrol with all antioxidant tests. The strong negative correlations were obtained between TPC with TFC, and TFC with antioxidant tests by ABTS and DPPH, while the moderated negative correlations were found between *t*-resveratrol with TFC, and TFC with antioxidant tests by FRAP and ORAC.

Table 27 Pearson's correlation coefficients of all responses

	%Yield	Resv	TPC	TFC	FRAP	ABTS	DPPH
Resv	0.092						
	0.464						
TPC	0.195	0.683					
	0.116	0.000					
TFC	0.064	-0.577	-0.801				
	0.610	0.000	0.000				
FRAP	0.289	0.614	0.945	-0.677			
	0.018	0.000	0.000	0.000			
ABTS	0.142	0.528	0.882	-0.899	0.861		
	0.257	0.000	0.000	0.000	0.000		
DPPH	0.241	0.599	0.922	-0.800	0.941	0.919	
	0.051	0.000	0.000	0.000	0.000	0.000	
ORAC	0.317	0.517	0.836	-0.635	0.918	0.830	0.906
	0.010	0.000	0.000	0.000	0.000	0.000	0.000

Cell Contents

Pearson correlation

p-Value

4.5.3 Discussion

Comparison of extraction methods, SLE and UAE gave higher responses than MAE ($p < 0.05$) (Figure 48). This was in an agreement of Orio et al. (47) who concluded that UAE showed the best yield for mitragynine extraction, compared to MAE and SFE, as well as Garcia et al. (87) who suggested that UAE was more effective than MAE and maceration in extraction of *t*-resveratrol from *Arachis repens*

leaves. It may be caused by the cell disruptions because of sonication power that facilitate mass transfer of the cellular components (87). Several studies suggested the benefits of UAE in reduction of extraction time and energy consumption, compared to conventional extraction methods (45, 81). However, the extraction efficiency between SLE and UAE in our study was not much different. The reason was that the compared responses in our study were from optimized conditions while in other studies were from assigned conditions (45, 87). The MAE showed lowest extraction efficiency among others in our study (Figure 49), although, it has shorten the extraction time from 120 min in SLE and UAE to 90 s in MAE which is the major advantage of MAE as also found in other studies (12, 51). It should be considered as alternative extraction method due to lowest extraction time, solvent volume and energy consumption.

Concerning peanut varieties, the antioxidant capacities obtained from different peanut varieties and extraction methods were shown in Figure 50. Thainan-IX possessed highest antioxidant activities of the extract than Kalasin-I and -II by all extraction methods, while the antioxidant activities of the extract between Kalasin-I and -II was not significant different. It may be from the different types of peanuts; Kalasin-I and -II were Valencia type while Thainan-IX was Spanish type. However, our finding was contradicted to the previous paper which claimed that Kalasin-I expressed higher antioxidant values than Kalasin-II and Thainan-IX varieties (35). It could be explained by unoptimized extraction conditions used in that paper. They were extracted by maceration in 80%ethanol at room temperature for 24 h, and they also studied the effect of germination in peanuts for 4 days (35), which affected to the level of bioactive compounds and antioxidant activities of the extracts. In our study, the higher antioxidant activity of Thainan-IX may be from higher polyphenols than Kalasin-I and -II (Table 26) that are the major antioxidants in peanuts (7).

Pearson's correlation and significant values that represented the correlation between responses were shown in Table 27. The strong correlations were found in positive direction between TPC with all antioxidant tests, and among the antioxidant tests. These were in an accordance with previous literatures that indicated the strong correlation between TPC and antioxidant activity (32, 88, 89). The moderate

correlations were found between *t*-resveratrol with TPC, and *t*-resveratrol with all antioxidant tests, which may imply that the antioxidant activity of the peanut extracts may not be contributed by *t*-resveratrol alone. The negative correlations were found mostly between TFC with antioxidant tests, which may imply that antioxidant activity of peanut extracts should be contributed by TPC rather than TFC. This was supported by the study of Fidrianny et. al. (90) who also found the moderate negative correlation between TFC and antioxidant activity by FRAP assay of peanut shell extract.

Comparing the same extraction method SLE, TPC and *t*-resveratrol found in our study (3.5915 mgGAE/g for TPC, 3.4718 µg/g for *t*-resveratrol) were higher than the study of Limmongkon et. al. (0.4067 mgGAE/g for TPC, 1.40 µg/g for *t*-resveratrol) for var. Kalasin-I (35). This affirmed the advantage of using experimental designs in optimization of extraction conditions in terms of improved extraction efficiency. The highest antioxidant values were obtained by ORAC, followed by ABTS which were higher than DPPH and FRAP assays. The reason may be from that ORAC is the only method that complete the measurement by measuring both inhibition time and degree of inhibition, while the others measure only at a specific time (91). The higher of ABTS than DPPH and FRAP values could be caused by the high content of *p*-coumaric in peanut extracts that greater contributes antioxidant activity by ABTS than DPPH assay (31). Unfortunately, the antioxidant values from our study could not been compared to previous literatures due to different standardized compounds or units.

In conclusion, at optimized conditions, SLE and UAE provided higher level of all response than MAE. The values were not greatly different between SLE and UAE. Thainan-IX var. showed higher antioxidant values than Kalasin-I and -II. The TPC and *t*-resveratrol level found in this study was higher than previous report, which indicated the benefits of using experimental designs in optimization of extraction conditions.

CHAPTER 5

CONCLUSION

From all study results, it can be concluded that the developed mathematical models were reliable to predict the responses for all extraction methods: SLE, UAE and MAE, particularly the antioxidant activity of the extracts. The resveratrol content found at optimized conditions was higher than previous reports. The developed analytical method for determination of resveratrol was accurate, reliable, and reproducible. In addition, the correlation between TPC with antioxidant activity has been observed which was stronger than resveratrol with antioxidant activity, while TFC showed negative correlation with antioxidant activity. This implied the antioxidant activity of peanut extracts was mostly contributed by TPC.

This study may have limitation on controlling the variation during growing or harvesting period since they were purchased through the merchant. However, to reduce the variation, a single batch of peanut samples was used throughout the study and all varieties of peanuts were purchased from the single merchant.

Lastly, although the developed models in our study were reliable enough to predict the responses, the author would like to suggest of using other experimental design approach such as artificial neuron network (ANN) to improve the model accuracy since it is more powerful to create the non-linear model and explain the complex relationships.

REFERENCES

1. Settaluri VS, Kandala CVK, Puppala N, Sundaram J. Peanuts and Their Nutritional Aspects—A Review. *Food and Nutrition Sciences*. 2012;03:1644-50.
2. Lopes RM, Da Silveira Agostini-Costa T, Gimenes MA, Silveira D. Chemical composition and biological activities of *Arachis* species. *Journal of Agricultural and Food Chemistry*. 2011;59:4321-30.
3. Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*. 2010;15:7313-52.
4. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*. 2006;160:1-40.
5. Nn A. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Medicinal & Aromatic Plants*. 2015;04:3-8.
6. Trusheva B, Trunkova D, Bankova V. Different extraction methods of biologically active components from propolis; a preliminary study. *Chemistry Central Journal*. 2007;1:1-4.
7. Attree R, Du B, Xu B. Distribution of phenolic compounds in seed coat and cotyledon, and their contribution to antioxidant capacities of red and black seed coat peanuts (*Arachis hypogaea* L.). *Industrial Crops and Products*. 2015;67:448-56.
8. Megat Rusydi MR, Azrina A. Effect of germination on total phenolic, tannin and phytic acid contents in soy bean and peanut. *International Food Research Journal*. 2012;19:673-7.
9. Katsampa P, Valsamedou E, Grigorakis S, Makris DP. A green ultrasound-assisted extraction process for the recovery of antioxidant polyphenols and pigments from onion solid wastes using Box-Behnken experimental design and kinetics. *Industrial Crops and Products*. 2015;77:535-43.
10. Phan-Thien K-Y, Wright GC, Lee NA. Peanut antioxidants: Part 2. Quantitation of free and matrix-bound phytochemicals in five selected genotypes with diverse antioxidant capacity by high performance liquid chromatography (HPLC). *LWT - Food Science and Technology*. 2014;57:312-9.
11. Desai MA, Parikh J. Extraction of essential oil from leaves of lemongrass using microwave radiation: Optimization, comparative, kinetic, and biological studies. *ACS Sustainable Chemistry and Engineering*. 2015;3:421-31.
12. Ballard TS, Mallikarjunan P, Zhou K, O'Keefe S. Microwave-assisted extraction of phenolic antioxidant compounds from peanut skins. *Food Chemistry*. 2010;120:1185-92.
13. Wang H, Li Y, Ren Z, Cong Z, Chen M, Shi L, et al. Optimization of the microwave-assisted enzymatic extraction of *Rosa roxburghii* Tratt. polysaccharides using response surface methodology and its antioxidant and α -D-glucosidase inhibitory activity. *International Journal of Biological Macromolecules*. 2018;112:473-82.
14. Liqid A, Palma M, Brigui J, Barroso CG. Investigation on phenolic compounds stability during microwave-assisted extraction. *Journal of chromatography A*. 2007;1140:29-34.
15. Dejaegher B, Vander Heyden Y. Experimental designs and their recent advances in set-up, data interpretation, and analytical applications. *Journal of Pharmaceutical and Biomedical Analysis*. 2011;56:141-58.

16. Paulucci VP, Couto RO, Teixeira CCC, Freitas LAP. Optimization of the extraction of curcumin from *Curcuma longa* rhizomes. *Brazilian Journal of Pharmacognosy*. 2013;23:94-100.
17. Ali A, Lim XY, Chong CH, Mah SH, Chua BL. Optimization of ultrasound-assisted extraction of natural antioxidants from Piper betle using response surface methodology. *LWT - Food Science and Technology*. 2018;89:681-8.
18. Ballard TS, Mallikarjunan P, Zhou K, O'keefe SF. Optimizing the Extraction of Phenolic Antioxidants from Peanut Skins Using Response Surface Methodology. *J Agric Food Chem*. 2009;57:3064-72.
19. Yang S, Li Y, Jia D, Yao K, Liu W. The synergy of Box-Behnken designs on the optimization of polysaccharide extraction from mulberry leaves. *Industrial Crops and Products*. 2017;99:70-8.
20. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*. 2007;39:44-84.
21. Scheibmeir HD, Christensen K, Whitaker SH, Jegaethesan J, Clancy R, Pierce JD. A review of free radicals and antioxidants for critical care nurses. *Intensive and Critical Care Nursing*. 2005;21:24-8.
22. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *International journal of biomedical science : IJBS*. 2008;4:89-96.
23. Alam MN, Bristi NJ, Rafiquzzaman M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*. 2013;21:143-52.
24. Antolovich M PP, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *The Analyst*. 2002;127:183-98.
25. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry*. 2005;53:1841-56.
26. Karadag A, Ozcelik B, Saner S. Review of methods to determine antioxidant capacities. *Food Analytical Methods*. 2009;2:41-60.
27. Pisoschi AM, Negulescu GP. Methods for Total Antioxidant Activity Determination: A Review. *Biochemistry & Analytical Biochemistry*. 2012;01:1-10.
28. Apak R, Gorinstein S, Böhm V, Schaich KM, Özyürek M, Güçlü K. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report)*. *Pure Appl Chem*. 2013;85:957-98.
29. Oliveira Calil N, Senra Goncalves de Carvalho G, Farah da Silva A, David da Silva A, Rezende Barbosa Raposo N. Antioxidant Activity of Synthetic Resveratrol Analogs: A Structure- Activity Insight. *Letters in Drug Design & Discovery*. 2012;9:676-9.
30. Prior RL, Wu X, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*. 2005;53:4290-302.
31. Chuenchom P, Swatsitang P, Senawong T, Jogloy S. Antioxidant Capacity and Phenolic Content Evaluation on Peanut Skins from 3 Peanut Types. *Chiang Mai Journal of Science*. 2016;43:123-37.
32. Win MM, Abdul-Hamid A, Baharin BS, Anwar F, Sabu MC, Pak-dek MS. Phenolic compounds and antioxidant activity of peanut's skin, hull, raw kernel and roasted kernel flour. *Pakistan Journal of Botany*. 2011;43:1635-42.
33. Lewis WE, Harris GK, Sanders TH, White BL, Dean LL. Antioxidant and Anti-

- Inflammatory Effects of Peanut Skin Extracts. *Food and Nutrition Sciences*. 2013;4:22-32.
34. Chukwumah Y, Walker L, Vogler B, Verghese M. Profiling of bioactive compounds in cultivars of Runner and Valencia peanut market-types using liquid chromatography/APCI mass spectrometry. *Food Chemistry*. 2012;132:525-31.
35. Limmongkon A, Janhom P, Amthong A, Kawpanuk M, Nopprang P, Poohadsuan J, et al. Antioxidant activity, total phenolic, and resveratrol content in five cultivars of peanut sprouts. *Asian Pacific Journal of Tropical Biomedicine*. 2017;7:332-8.
36. Khang D, Dung T, Elzaawely A, Xuan T. Phenolic Profiles and Antioxidant Activity of Germinated Legumes. *Foods*. 2016;5:27.
37. Swatsitang P, Chuendchom P, Jogloy S. Antioxidant capacity and total phenolics of peanut testae. *Khon Kaen Agricultural Journal*. 2011;39:48-52.
38. Chukwumah Y, Walker LT, Verghese M. Peanut skin color: A biomarker for total polyphenolic content and antioxidative capacities of peanut cultivars. *International Journal of Molecular Sciences*. 2009;10:4941-52.
39. Yu J, Ahmedna M, Goktepe I. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chemistry*. 2005;90:199-206.
40. Fellows P. Water Activity. *Food Processing Technology Principles and Practice*. 2000:153-7.
41. Nepote V, Grosso NR, Guzmán CA. Optimization of extraction of phenolic antioxidants from peanut skins. *Journal of the Science of Food and Agriculture*. 2005;85:33-8.
42. Nepote V, Grosso NR, Guzman CA. Antioxidant activity of methanolic extracts from peanut skin. *Molecules*. 2000;5:487-8.
43. Majid I, Nayik GA, Nanda V. Ultrasonication and food technology: A review. *Cogent Food & Agriculture*. 2015;1:1-11.
44. Esclapez MD, García-Pérez JV, Mulet A, Cárcel JA. Ultrasound-Assisted Extraction of Natural Products. *Food Engineering Reviews*. 2011;3:108-20.
45. Xu DP, Zheng J, Zhou Y, Li Y, Li S, Li HB. Ultrasound-assisted extraction of natural antioxidants from the flower of *Limonium sinuatum*: Optimization and comparison with conventional methods. *Food Chemistry*. 2017;217:552-9.
46. Horžić D, Jambrak AR, Belščak-Cvitanović A, Komes D, Lelas V. Comparison of Conventional and Ultrasound Assisted Extraction Techniques of Yellow Tea and Bioactive Composition of Obtained Extracts. *Food and Bioprocess Technology*. 2012;5:2858-70.
47. Orio L, Alexandru L, Cravotto G, Mantegna S, Barge A. UAE, MAE, SFE-CO₂ and classical methods for the extraction of *Mitragyna speciosa* leaves. *Ultrasonics Sonochemistry*. 2012;19:591-5.
48. Shirsath SR, Sonawane SH, Gogate PR. Intensification of extraction of natural products using ultrasonic irradiations-A review of current status. *Chemical Engineering and Processing: Process Intensification*. 2012;53:10-23.
49. Du FY, Xiao XH, Li GK. Application of ionic liquids in the microwave-assisted extraction of trans-resveratrol from *Rhizma Polygoni Cuspidati*. *Journal of Chromatography A*. 2007;1140:56-62.
50. Swamy GJ, Muthukumarappan K. Optimization of continuous and intermittent

- microwave extraction of pectin from banana peels. *Food Chemistry*. 2017;220:108-14.
51. Bhan M, Satija S, Garg C, Dureja H, Garg M. Optimization of ionic liquid-based microwave assisted extraction of a diterpenoid lactone-andrographolide from *Andrographis paniculata* by response surface methodology. *Journal of Molecular Liquids*. 2017;229:161-6.
 52. Hibbert DB. Experimental design in chromatography: A tutorial review. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2012;910:2-13.
 53. NIST/SEMATECH. Process improvement. NIST/SEMATECH e-Handbook of Statistical Methods. 2012.
 54. Leardi R. Experimental design in chemistry: A tutorial. *Analytica Chimica Acta*. 2009;652:161-72.
 55. Oyejola BA, Nwanya JC. Selecting the Right Central Composite Design. *International Journal of Statistics and Applications*. 2015;5:21-30.
 56. Box GEP, Draper NR. *Empirical Model Building and Response Surfaces*. 1987:477.
 57. Wang Y, Gao Y, Ding H, Liu S, Han X, Gui J, et al. Subcritical ethanol extraction of flavonoids from *Moringa oleifera* leaf and evaluation of antioxidant activity. *Food Chemistry*. 2017;218:152-8.
 58. Izadiyan P, Hemmateenejad B. Multi-response optimization of factors affecting ultrasonic assisted extraction from Iranian basil using central composite design. *Food Chemistry*. 2016;190:864-70.
 59. Derrien M, Badr A, Gosselin A, Desjardins Y, Angers P. Optimization of a green process for the extraction of lutein and chlorophyll from spinach by-products using response surface methodology (RSM). *LWT - Food Science and Technology*. 2017;79:170-7.
 60. Tekin K, Akalin MK, Şeker MG. Ultrasound bath-assisted extraction of essential oils from clove using central composite design. *Industrial Crops and Products*. 2015;77:954-60.
 61. Cicco N, Lanorte MT, Paraggio M, Viggiano M, Lattanzio V. A reproducible, rapid and inexpensive Folin-Ciocalteu micro-method in determining phenolics of plant methanol extracts. *Microchemical Journal*. 2009;91:107-10.
 62. Chandra S, Khan S, Avula B, Lata H, Yang MH, Elsohly MA, et al. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-based Complementary and Alternative Medicine*. 2014;2014:1-9.
 63. Rebaya A, Belghith SI, Baghdikian B, Leddet VM, Mabrouki F, Olivier E, et al. Total Phenolic, Total Flavonoid, Tannin Content, and Antioxidant Capacity of *Halimium halimifolium* (Cistaceae). *Journal of Applied Pharmaceutical Science*. 2015;5:052-7.
 64. Kenny O, Brunton NP. In vitro Protocols for Measuring the Antioxidant Capacity of Algal Extracts. *Methods in molecular biology*. 2015;1308.
 65. Sales JM, Resurreccion aVa. Maximising resveratrol and piceid contents in UV and ultrasound treated peanuts. *Food Chemistry*. 2009;117:674-80.
 66. Rudolf JL, Resurreccion aVa, Saalia FK, Phillips RD. Development of a reverse-phase high-performance liquid chromatography method for analyzing trans-resveratrol in peanut kernels. *Food Chemistry*. 2005;89:623-38.

67. Liu C, Wang L, Wang J, Wu B, Liu W, Fan P, et al. Resveratrols in Vitis berry skins and leaves: their extraction and analysis by HPLC. Food chemistry. 2013;136:643-9.
68. Lee SS, Lee SM, Kim M, Chun J, Cheong YK, Lee J. Analysis of trans-resveratrol in peanuts and peanut butters consumed in Korea. Food Research International. 2004;37:247-51.
69. Zupančič Š, Lavrič Z, Kristl J. Stability and solubility of trans-resveratrol are strongly influenced by pH and temperature. European journal of pharmaceuticals and biopharmaceutics : official journal of Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik eV. 2015;93:196-204.
70. Pascual-Martí MC, Salvador A, Chafer A, Bernal A. Supercritical fluid extraction of resveratrol from grape skin of Vitis vinifera and determination by HPLC. Talanta. 2001;54:735-40.
71. Shian TE, Abdullah A, Musa KH, Maskat MY, Ghani MA. Antioxidant properties of three banana cultivars (Musa acuminata 'Berangan', 'Mas' and 'Raja') Extracts. Sains Malaysiana. 2012;41:319-24.
72. Bucić-Kojić A, Planinić M, Tomas S, Jokić S, Mujić I, Bilić M, et al. Effect of Extraction Conditions on the Extractability of Phenolic Compounds from Lyophilised Fig Fruits (Ficus Carica L.). Polish Journal of Food and Nutrition Sciences. 2011;61:195-9.
73. Karacabey E, Mazza G. Optimization of Solid–Liquid Extraction of Resveratrol and Other Phenolic Compounds from Milled Grape Canes (Vitis vinifera). Journal of Agricultural and Food Chemistry. 2008;56:6318-25.
74. De Zordi N, Cortesi A, Kikic I, Moneghini M, Solinas D, Innocenti G, et al. The supercritical carbon dioxide extraction of polyphenols from Propolis: A central composite design approach. The Journal of Supercritical Fluids. 2014;95:491-8.
75. Ameer K, Bae SW, Jo Y, Lee HG, Ameer A, Kwon JH. Optimization of microwave-assisted extraction of total extract, stevioside and rebaudioside-A from Stevia rebaudiana (Bertoni) leaves, using response surface methodology (RSM) and artificial neural network (ANN) modelling. Food Chem. 2017;229:198-207.
76. Chew KK, Khoo MZ, Ng SY, Thoo YY, Aida WMW, Ho CW. Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of Centella asiatica extracts. International Food Research Journal. 2011;18:1427-35.
77. Jovanović AA, Đorđević VB, Zdunić GM, Pljevljakušić DS, Šavikin KP, Gođevac DM, et al. Optimization of the extraction process of polyphenols from Thymus serpyllum L. herb using maceration, heat- and ultrasound-assisted techniques. Separation and Purification Technology. 2017;179:369-80.
78. Shaikh RU, Pund MM, Gacche RN. Evaluation of anti-inflammatory activity of selected medicinal plants used in Indian traditional medication system in vitro as well as in vivo. Journal of Traditional and Complementary Medicine. 2015:1-7.
79. Yu Y, Wang L, Fu X, Wang L, Fu X, Yang M, et al. Anti-oxidant and anti-inflammatory activities of ultrasonic-assisted extracted polyphenol-rich compounds from Sargassum muticum. Journal of Oceanology and Limnology. 2019;37(3):836-47.
80. Marrassini C, Peralta I, Anesini C. Comparative study of the polyphenol content-related anti-inflammatory and antioxidant activities of two Urera aurantiaca specimens from different geographical areas. Chin Med. 2018;13:22.

81. Pradal D, Vauchel P, Decossin S, Dhulster P, Dimitrov K. Kinetics of ultrasound-assisted extraction of antioxidant polyphenols from food by-products: Extraction and energy consumption optimization. *Ultrasonics Sonochemistry*. 2016;32:137-46.
82. Boonkird S, Phisalaphong C, Phisalaphong M. Ultrasound-assisted extraction of capsaicinoids from *Capsicum frutescens* on a lab- and pilot-plant scale. *Ultrason Sonochem*. 2008;15(6):1075-9.
83. Chairerk N, Pongyeela P, Chungsiriporn J, Rakmak N. Ethanol extraction of active ingredients and antioxidants from germinated sangyod rice. *Applied Science and Engineering Progress*. 2021;14(1):52-9.
84. Singh B, Sharma HK, Sarkar BC. Optimization of extraction of antioxidants from wheat bran (*Triticum spp.*) using response surface methodology. *Journal of Food Science and Technology*. 2012;49:294-308.
85. Ruenroengklin N, Zhong J, Duan X, Yang B, Li J, Jiang Y. Effects of various temperatures and pH values on the extraction yield of phenolics from litchi fruit pericarp tissue and the antioxidant activity of the extracted anthocyanins. *International Journal of Molecular Sciences*. 2008;9:1333-41.
86. Du FY, Xiao XH, Luo XJ, Li GK. Application of ionic liquids in the microwave-assisted extraction of polyphenolic compounds from medicinal plants. *Talanta*. 2009;78:1177-84.
87. Garcia L, Garcia R, Pacheco G, Sutili F, Souza RD, Mansur E, et al. Optimized Extraction of Resveratrol from *Arachis repens* Handro by Ultrasound and Microwave: A Correlation Study with the Antioxidant Properties and Phenol Contents. *Scientific World Journal*. 2016;2016.
88. Sun Y, Pan Z, Yang C, Jia Z, Guo X. Comparative assessment of phenolic profiles, cellular antioxidant and antiproliferative activities in ten varieties of sweet potato (*ipomoea batatas*) storage roots. *Molecules*. 2019;24(4476):1-13.
89. Lou SN, Lin YS, Hsu YS, Chiu EM, Ho CT. Soluble and insoluble phenolic compounds and antioxidant activity of immature calamondin affected by solvents and heat treatment. *Food Chemistry*. 2014;161:246-53.
90. Fidrianny I, Puspitasari N, Marlia Singgih W. Antioxidant activities, Total flavonoid, Phenolic, Carotenoid of various shells extracts from four species of legumes. *Asian Journal of Pharmaceutical and Clinical Research*. 2014;7:42-6.
91. Tabart J, Kevers C, Pincemail J, Defraigne JO, Dommes J. Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry*. 2009;113:1226-33.



VITA

NAME	Kritamorn Jitrangsri
DATE OF BIRTH	21 February 1985
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Ubonratchathanee university
HOME ADDRESS	94 Soi Sirikasem 2 Bangpai Bangkae Bangkok 10160
PUBLICATION	Kritamorn Jitrangsri, Amornrat Chaidedgumjorn, Malai Satiraphan. Effect of Ethanol Percentage upon Various Extraction Methods of Peanut Based on Antioxidant Activity with Resveratrol and Total Phenolic Contents. Pharmaceutical Sciences Asia 2020;47(2):164-172.
AWARD RECEIVED	Silver Medal Award (Oral presentation) in the title of Determination of trans-Resveratrol in Peanut using Poroshell Column and Microwave-Assisted Extraction, The 2nd International Conference on Herbal and Traditional Medicine (HTM 2017). Asia Hotel, Bangkok, Thailand

