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Full Paper

Optimisation of ultrasonic-assisted extraction of gamma-aminobutyric acid from mulberry leaves (*Morus alba* L.) using response surface methodology

Tinakhom Wigromsakulwong, Prukraya Pongyeela, Nirana Chairerk, Tanya Thamsee and Juntima Chungsiriporn*

Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand

* Corresponding author, e-mail: juntima.c@psu.ac.th

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Abstract: Ultrasonic-assisted extraction of gamma-aminobutyric acid (GABA) from mulberry leaves was investigated by response surface methodology (RSM) using central composite design and a numerical optimisation technique. Effects of extraction time (0-60 min.), ethanol solvent concentration (30-80%) and ratio of solvent to mulberry leaf powder (5-20 mL/g) on GABA content were studied with ultrasonic wave frequency fixed at 35 kHz. Optimised conditions for GABA extraction at 450.36 ± 3.50 mg/100 g dry leaf powder (R^2 = 0.9645) were extraction time of 60 min., ethanol concentration of 54.75% and ratio of solvent to mulberry leaf powder of 20 mL/g.

Keywords: gamma-aminobutyric acid, ultrasonic-assisted extraction, mulberry leaves, response surface methodology

INTRODUCTION

Mulberry is a tropical plant native to Asian countries and belongs to the same family as blueberry and raspberry. Mulberry is used as a food for silkworm production and is also a well-known herbal medicine, especially in China. Mulberry leaves have gained popularity in recent years due to their high quantities of gamma-aminobutyric acid (GABA) [1]. GABA is an important neurotransmitter that helps the brain to relax, reduces stress and ameliorates the risk of Alzheimer's disease [2]. Under anaerobic (oxygen-free) conditions, GABA level in some plants significantly increases [3]. When oxygen in the fermentation chamber was replaced with nitrogen, the amount of

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GABA increased and substantial accumulation of GABA was found in green tea after 6-12 hr of fermentation [4].

Jianrong et al. used ultrasonic-microwave synergistic extraction to extract GABA from mulberry leaves [5]. Response surface methodology (RSM) was used to optimise the ultrasonic parameters for GABA extraction from pumpkin seed [6]. In the present study GABA was extracted from mulberry leaves by an ultrasonic-assisted extraction technique. A three-variable, five-level central composite design (CCD) was used to simultaneously enhance total GABA content based on optimal conditions of ultrasonic-assisted extraction time, concentration of ethanol and ratio of solvent to mulberry leaf powder. Based on a single factor experiment, CCD was used to optimise the GABA extraction process. The objectives of this study are to (1) investigate the effects of ultrasonic-assisted extraction parameters on total GABA content and antioxidant agents, (2) solve the response surface model and optimisation problem, and (3) predict the optimal conditions for GABA extraction using the model.

MATERIALS AND METHODS

Materials and Reagents

Fresh mulberry leaves (*Morus alba* L., c.v. Chiang Mai 60) were collected from Phatong sub-district, Hat Yai district, Songkhla province, Thailand. They were washed with water and dried at room temperature in the shade to constant weight. To accumulate GABA, the leaves were incubated in an anaerobic state by feeding nitrogen gas to replace air for 16 hr at 40°C. Then they were dried at 60°C in a hot air oven for 24 hr. The dried leaves were ground to powder using a food grinder, sieved through a 60-mesh screen to obtain a homogeneous sample and stored in a desiccated box.

GABA, Na_2CO_3, $AlCl_3$, NaOH and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Alfa Aesar Chemical Co. (China). Ethanol, Folin-Ciocalteu reagent, gallic acid, $NaNO_2$, quercetin, acetonitrile, potassium phosphate dibasic and potassium phosphate monobasic of HPLC grade were obtained from Sigma -Aldrich (USA).

Ultrasonic-assisted Extraction of GABA

Twenty grams of mulberry leaf powder in a 1000-mL beaker were extracted with ethanol in an ultrasonic bath (Type: TI-H-20 MF3, Elma Schmidbauer GmbH, Germany) at 35 kHz and 950 W. The beaker was placed at the centre of the ultrasound bath to allow equal effect of the ultrasonic waves during extraction on all sides of the container. The ultrasonic-assisted extraction parameters were extraction time (0-60 min.; 0 min. means extraction using conventional extraction for 60 min. to eliminate the effect of ultrasonic wave), ethanol solvent concentration (30-80%) and ratio of solvent to mulberry leaf powder (5-20 mL/g). The extract solution was separated from the residue by centrifugation at 4,000 rpm for 5 min. and then concentrated using a rotary evaporator at 40°C under reduced pressure. Experiments were performed with different extraction time, ethanol concentration and ratio of solvent to mulberry leaf powder. Each experimental parameter was repeated three times.

Conventional Extraction

Extraction was performed using the solvent extraction method. Udachan and Sahoo [7] showed that the use of a spindle stirring machine during solvent extraction increased extraction

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efficiency. Thus, 20 g of mulberry leaf powder was extracted by ethanol using a spindle stirring machine at a speed of 200 rpm and optimal extraction time of 60 min., ethanol concentration of 54.75% and ratio of solvent to mulberry leaf powder of 20 mL/g. The results were compared with the ultrasonic-assisted extraction method.

Experimental Designs

RSM was used to investigate the effects of three independent variables, viz. extraction time (X_1), ethanol concentration (X_2) and ratio of solvent to mulberry leaf powder (X_3), on GABA content. Table 1 shows the impact of these three independent variables as '-α (-1.68),' '-1,' '0,' '+1' and '+α (+1.68)' for low, moderate and high levels. The axial or star points were statistically investigated using CCD. Table 2 lists the independent CCD variables with 20 experimental runs. Multiple regressions were used to fit the following quadratic polynomial model to the data from the experimental design:

$$Y = b_0 + \sum_{i=1}^{i=3} b_i X_i + \sum_{i=1}^{i=3} b_{ii} X_i^2 + \sum_{i=1}^{i=2} \sum_{j=i+1}^{j=3} b_{ij} X_i X_j \quad (1)$$

where Y is the predicted GABA content and b_0, b_i, b_{ii} and b_{ij} are the regression coefficients for model intercept, linear, quadratic and interaction terms, with the linear coefficients of extraction time (X_1), ethanol concentration (X_2) and ratio of solvent to mulberry leaf powder (X_3). The settings of the independent variables were represented as X_i and X_j.

Table 1. Three independent variables and their CCD levels

Independent variable	Coded symbol	Factor level				
		- α (-1.68)	**-1**	**0**	**+1**	**+α (+1.68)**
X_1: Extraction time (min.)	X_1	0	12	30	48	60
X_2: Ethanol concentration (%)	X_2	30	40.14	55	69.86	80
X_3: Ratio of solvent to mulberry leaf powder (mL/g)	X_3	5	8.04	12.5	16.96	20

The CCD design was completed using Minitab software (version 19.1, Pine Hall Road, State College, PA, USA). The model's adequacy was assessed using the correlation coefficient (R^2), the adjusted correlation coefficient (R^2 adj) and the lack-of-fit test. To determine the optimal condition for GABA extraction, regression analysis and three-dimensional (3D) response surface plots were used.

Determination of GABA

The GABA content of each sample was analysed by an Agilent 1200 HPLC with a Hypersil 5-µm ODS 250 x 4.0 mm C_{18} column and an Agilent Multi λ fluorescence detector (EX:330, EM:440 nm). The mobile phase consisted of acetonitrile: 0.1 M phosphate buffer pH 7 (13:87) and the flow rate was 1 mL/min. at 30°C. The injection amount of the sample for HPLC analysis was adjusted to 15 µL.

GABA content in the extracts was quantified in mg/100 g dry leaf using the following equation:

$$\text{GABA content} = \frac{A \times B}{C} \quad (2)$$

where $A = \frac{\text{Peak area of sample} - \text{Y intercept of calibration curve}}{\text{Slope of calibration curve}}$

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B = Weight of extract obtained per 100 g of dry leaf
C = Weight of extract diluted in distilled water before HPLC analysis

Determination of Related Properties

Total phenolic content

The total phenolic content of the extracts was performed according to the Folin-Ciocalteu assay [8]. Briefly, 50 µL of extract, 9.50 mL of distilled water and 500 µL of 10% Folin-Ciocalteu reagent were added to a screw-capped tube and shaken for 5 min. Then 2 mL of 10% Na_2CO_3 was added to the tube and the solution was stored in the dark for 2 hr. Absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 730 nm. The phenolic content was calculated from a gallic acid standard curve expressed as mg gallic acid equivalent (mg GAE/100 g dry leaf) [9].

Total flavonoid content

The total flavonoid content was evaluated following the method adapted from Hu et al [10]. First, 1 mL of extract was diluted to 10 mL with 55% ethanol in a screw-capped tube. Then 0.5 mL of the substance was added, along with 0.1 mL 5% $NaNO_2$ solution, mixed and allowed to stand for 5 min. Next, 1 mL of 10% $AlCl_3$ solution was added to the tubes, shaken and set aside for 5 min. Finally, 1.0 mL 1M NaOH solution was added to the tube, followed by 3 mL of distilled water and the mixture shaken and allowed to stand for 10 min. before analysis. A UV-Vis spectrophotometer was used to determine the flavonoids in the extract against the same mixed solution without utilising the sample as a blank, at a wavelength of 510 nm. Each experiment was conducted in triplicate. Results were reported as mg quercetin equivalent (QE)/100 g dry leaf; quercetin was used as standard substance.

DPPH scavenging activity

The DPPH radical scavenging activity of the extract was evaluated following Thabti et al. [11] with certain adjustments. Briefly, 2.0 mL of DPPH solution (0.2 mM DPPH in 95% ethanol) was thoroughly mixed with 1.0 mL of extract at a concentration of 300 µg/mL in a screw-capped tube. The mixture was forcefully shaken and stored at room temperature for 30 min. in the dark. A UV-Vis spectrophotometer was used to detect the absorbance at 517 nm. Lower absorbance of the reaction mixture suggests higher free radical scavenging activity. The blank control was 95% ethanol. The DPPH scavenging activity (%) was calculated according to the following equation:

$$\text{DPPH scavenging activity (\%)} = ((A_{control} - A_{sample}) / A_{control}) \times 100\% \quad (3)$$

$A_{control}$ = Light absorption value of the DPPH solution
A_{sample} = Light absorbance value of the extract mixed with DPPH solution

Statistical Analysis

Results from triplicate experiments were presented as mean ± standard deviation, with analysis of variance used to evaluate the statistical significance. If $P < 0.05$, the difference was judged to be statistically significant.

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RESULTS AND DISCUSSION

Chromatogram of GABA

The regression equation for GABA at various concentrations from 0.025 to 0.075 mg/L was Y = 62189.06X + 41.94, with good linearity (R^2 = 0.99976). Chromatograms of standard GABA and a sample mulberry leaf extract are shown in Figure 1. Peak detection was observed at retention time of 23.85 min. in both chromatograms. However, shortly before the GABA peak, other peaks were absorbed at the wavelength of the fluorescence at EX:330 and EM:440 nm. This was possibly the result of the derivatisation reaction [12].

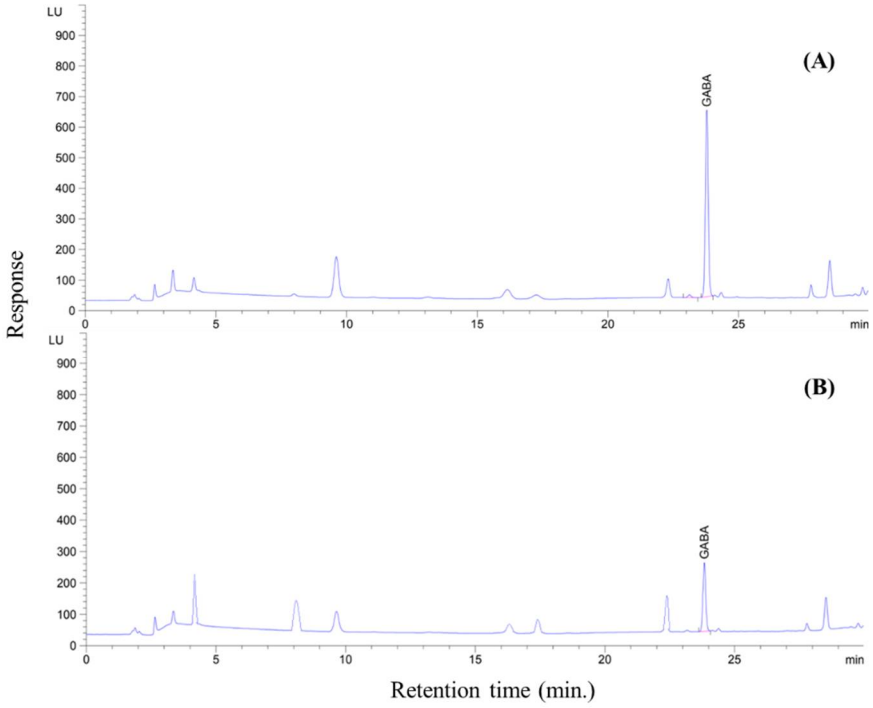

Figure 1. Chromatograms of standard GABA (A) and mulberry leaf extract (B)

Model Fitting, Statistical Analysis and Spectrophotometric Analysis

Twenty runs for GABA extraction from mulberry leaves were optimised using RSM based on three independent variables, viz extraction time, ethanol concentration and ratio of solvent to mulberry leaf powder, with results listed in Table 2. Effects of the three independent variables on GABA content of the extracts were studied and optimised using RSM with CCD. The GABA content of the extracts varied between 244.71 ± 1.02 - 390.53 ± 4.26 mg/100 g dry leaf. After performing multiple regression analysis on the experimental data, the response variable and test variables were displayed by the second-order polynomial equation, Eq (4).

$$Y = -64.9 - 0.414X_1 + 13.80X_2 - 5.32X_3 - 0.1080X_2^2 + 0.2022X_1X_3 \quad (4)$$

where Y, X_1, X_2 and X_3 are the GABA content in the extract (mg/100 g dry leaf), extraction time (min.), concentration of ethanol (%) and ratio of solvent to mulberry leaf powder (mL/g) respectively.

The experimental results were analysed using analysis of variance (ANOVA), with results reported in Table 3. The data match Equation (4) well, with the significance of the model

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statistically acceptable at $P < 0.05$. Furthermore, the lack of fit shows that the model equation is capable of predicting GABA content in the extract within the range of experimental variables, as evidenced by the P value of 0.793 (P value > 0.05). The coefficient of determination (R^2) of 0.9645 and adjusted coefficient of determination (adj-R^2) of 0.9326 show good accuracy and high correlation between the response and the independent variables.

The P value indicates the significance of the F value, which is determined by the number of degrees of freedom in the model (95% confidence level). As a result, the effects in P value column are less than 0.05 and considered significant [13]. When the F value is higher and the P value is lower, the corresponding variables become more significant [14]. The model has a high F value (F = 30.20) and a low P value ($P < 0.0001$), indicating that it is highly significant. The linear, square and 2-way interaction terms in this case all have high model F values of 47.37, 33.89 and 9.34 respectively and all P values ($P < 0.01$) are low, indicating that the model is highly significant.

Table 2. CCD and response values for GABA extraction from mulberry leaves

Run	Extraction time (X_1, min.)	Conc. of EtOH (X_2, %)	Ratio of solvent to mulberry leaf powder (X_3, mL/g)	GABA (mg/100 g dry leaf)
1	30.00	30.00	12.50	244.71 ± 1.02
2	48.00	69.86	16.96	364.50 ± 3.05
3	30.00	55.00	12.50	325.14 ± 1.16
4	30.00	55.00	12.50	345.47 ± 1.49
5	30.00	55.00	12.50	340.67 ± 2.61
6	30.00	80.00	12.50	293.12 ± 3.04
7	48.00	40.14	16.96	360.82 ± 2.65
8	30.00	55.00	5.00	332.16 ± 2.65
9	12.00	69.86	16.96	298.58 ± 2.72
10	30.00	55.00	12.50	339.60 ± 1.93
11	12.00	40.14	16.96	260.30 ± 1.88
12	30.00	55.00	12.50	320.10 ± 2.95
13	30.00	55.00	12.50	322.21 ± 3.00
14	48.00	69.86	8.04	320.54 ± 2.94
15	30.00	55.00	20.00	352.13 ± 6.57
16	0.00	55.00	12.50	301.11 ± 3.09
17	60.00	55.00	12.50	390.53 ± 4.26
18	12.00	69.86	8.04	310.13 ± 1.84
19	12.00	40.14	8.04	274.28 ± 2.25
20	48.00	40.14	8.04	300.44 ± 2.85

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Table 3. ANOVA for the regression model of GABA extraction

Source	Sum of squares	Degrees of freedom	Mean squares	F value	P value
Model	23759.15	9	2639.91	30.20	<0.0001
Linear	12422.40	3	4140.80	47.37	<0.0001
X_1	9142.80	1	9142.80	104.59	<0.0001
X_2	2354.88	1	2354.88	26.94	<0.0001
X_3	924.73	1	924.73	10.58	0.009
Square	8887.21	3	2962.40	33.89	<0.0001
X_2^2	8208.13	1	8208.13	93.90	<0.0001
2-way interaction	2449.54	3	816.51	9.34	0.003
X_1X_3	2108.24	1	2108.24	24.12	0.001
Residual	874.14	10	87.41		
Lack-of-fit	275.51	5	55.10	0.46	0.793
Pure error	598.62	5	119.72		
Total	24633.29	19			

Analysis of Response Surfaces

Three-dimensional (3D) response surface plots were created to visualise the effect of experimental level of each variable and the type of interaction between two independent variables and to determine the optimal level of each independent variable for maximum GABA content from mulberry leaves. The interaction between two independent variables was explored in each plot, while the other variable was kept constant. The 3D response surface plots are presented in Figures 2A-C.

The GABA content as a function of extraction time (0-60 min.) and concentration of ethanol (30-80%) at a fixed ratio of solvent to mulberry leaf powder (12.5 mL/g) is shown as a 3D response surface plot in Figure 2A. Maximum GABA content is predicted when the ethanol concentration and extraction time are 55% and 60 min. respectively. The amount of GABA increases as extraction time increases to a maximum of 60 min. The GABA content decreases when ethanol concentration is above 55%. This result concurs with that of Lin et al. [15] who found that 50% ethanol concentration was suitable for extracting GABA from tea leaves. Ethanol provides good solvent polarity and solubility for GABA extraction at a concentration of 50%.

The GABA content of extract at varying extraction times (0-60 min.) and different ratios of solvent to mulberry leaf powder (5-20 mL/g) at a fixed concentration of ethanol (55%) is shown as a 3D response surface plot in Figure 2B. Extraction time has a more significant effect on the GABA content than does the ratio of solvent to mulberry leaf powder. However, the GABA content from mulberry leaves also increases as the ratio of solvent to mulberry leaf powder increases from 5 to 20 mL/g.

Figure 2C shows a 3D response surface plot for different ethanol concentrations (30-80%) against ratios of solvent to mulberry leaf powder (5-20 mL/g) at a fixed extraction time (30 min.). Results show that GABA content increases as ethanol concentration increases from 30% to 55% but then decreases as ethanol concentration increases above this level. Furthermore, when the ratio of solvent to mulberry leaf powder increases from 5 to 20 mL/g with 55% ethanol concentration, the GABA content of mulberry leaves increases.

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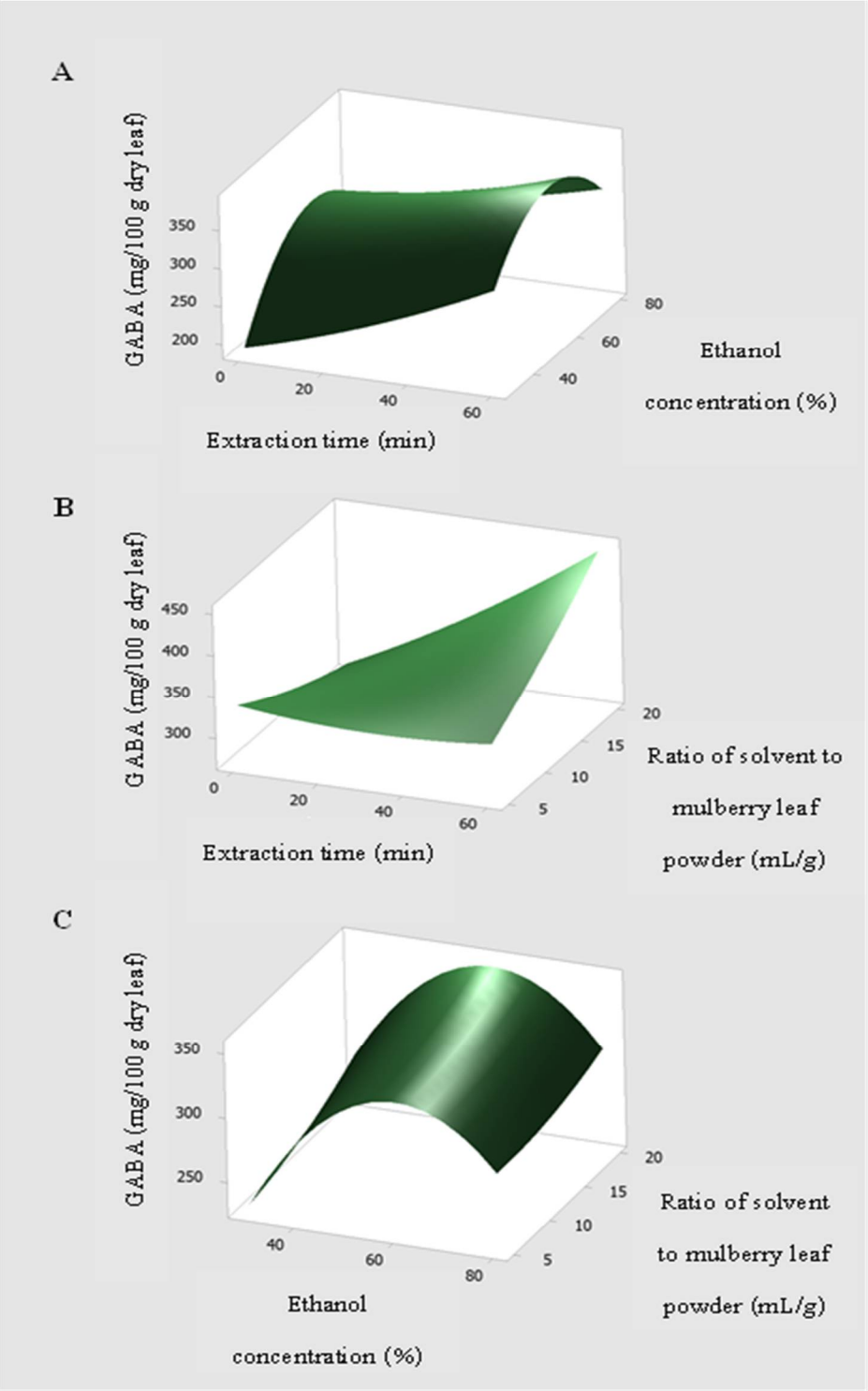

Figure 2. Response surface 3D plots showing effects of variables on GABA content: (A) extraction time vs concentration of ethanol; (B) extraction time vs ratio of solvent to mulberry leaf powder; (C) concentration of ethanol vs ratio of solvent to mulberry leaf powder

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Verification and Validation of Model

Extraction conditions were optimised for maximum GABA in the extract from mulberry leaves. Figure 3 shows the desirability function that was used to optimise the response variables. Because the values for desirability (D) and individual desirability (d) are equal to the optimal condition, this means that the condition is well optimised. The experimental results were obtained under optimal extraction conditions. The RSM method was used to compare the experimental and predicted values of the responses to ensure that the model was adequate [16]. Results in Figure 4 show no significant differences ($P > 0.05$) between the experimental and predicted values and the RSM models are deemed correct and valid.

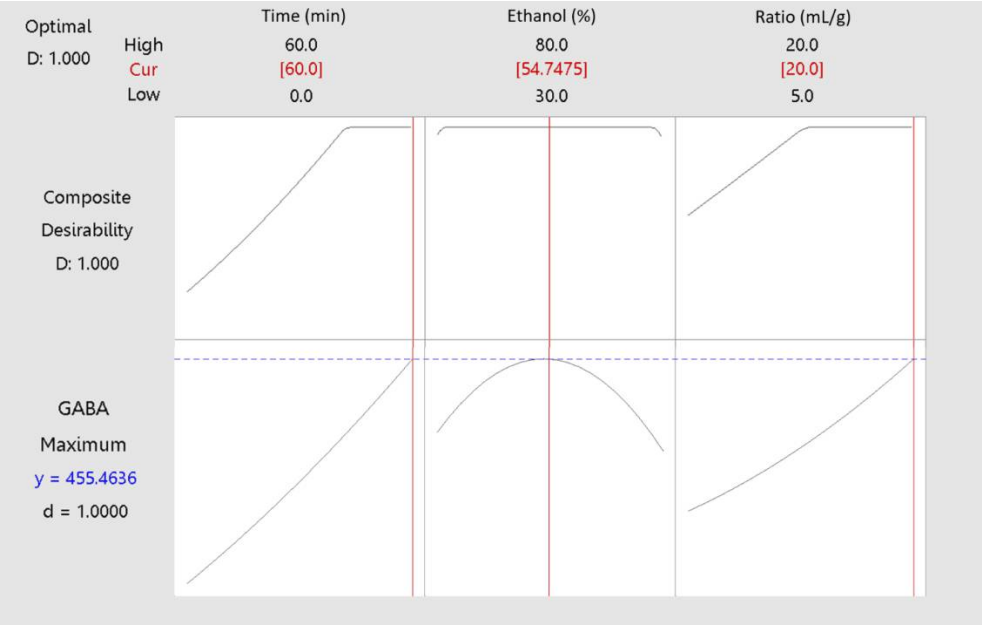

Figure 3. Desirability function applied in multiple responses

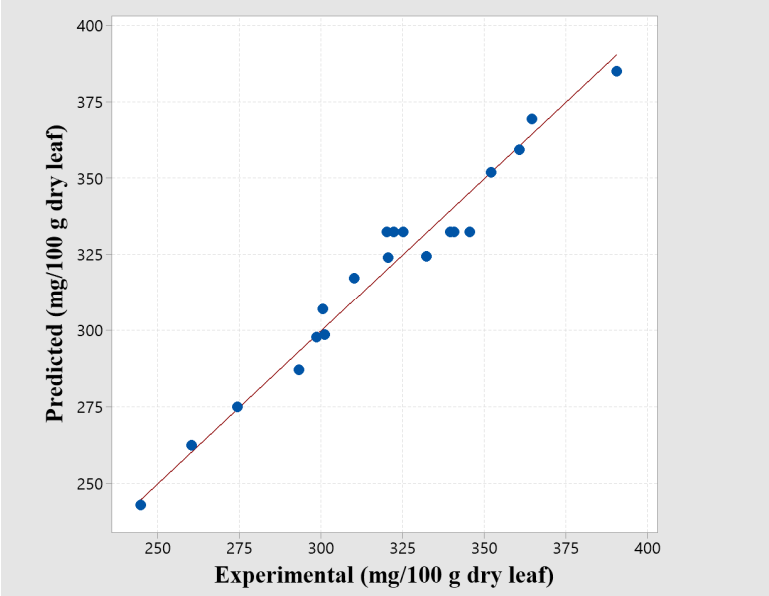

Figure 4. Comparison between experimental and predicted values of GABA content in extracts

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Optimisation of Extraction Parameters and Validation of Predictive Model

The experimental results were used to create an optimisation strategy to determine the optimal extraction condition to maximise the GABA content in mulberry leaf extract. The optimisation problem is presented in the following form:

Maximise:

$$Y = -64.9 - 0.414X_1 + 13.80X_2 - 5.32X_3 - 0.1080X_2^2 + 0.2022X_1X_3$$

Subject to:

$$0 \leq X_1 \leq 60$$
$$30 \leq X_2 \leq 80$$
$$5 \leq X_3 \leq 20$$

Numerical optimisation was used to find the condition that provided the highest GABA content using Minitab software (version 19.1, Pine Hall Road, State College, PA, USA). The optimised conditions are: extraction time of 60 min., 54.75% ethanol concentration and ratio of solvent to mulberry leaf powder of 20 mL/g. Under these conditions, the maximum GABA content of mulberry leaves predicted by the model is 455.46 mg/100 g dry leaf. To compare the predicted result with the experimental value, three duplicate experiments were carried out at the optimal extraction conditions. Under this condition, the experimental GABA content of mulberry leaf extract was 450.36 ± 3.50 mg/100 g dry leaf, which is close to the predicted value.

Comparison with Conventional Extraction and Other Extraction Methods

Extracts derived from optimal extraction conditions (60 min. extraction time, 54.75% ethanol concentration and 20 mL/g ratio of solvent to mulberry leaf powder) from both conventional and ultrasonic-assisted extractions were analysed for GABA, total phenolic content (TPC), total flavonoid content (TFC) and DPPH scavenging activity (%). The results are compared in Table 4. Ultrasonic waves moving through a medium create violent bubble collapse, thereby increasing cell tissue disruption and increasing extraction efficiency. Based on the experimental results in Table 4, ultrasonic-assisted extraction of active compounds from mulberry leaves provides greater extraction efficiency than does conventional extraction.

Table 4. Comparison of GABA content and related properties obtained from ultrasonic and conventional methods

Extraction method	GABA (mg/100 g dry leaf)	TPC (mg GAE/100 g dry leaf)	TFC (mg QE/100 g dry leaf)	DPPH scavenging activity (%)
Ultrasonic-assisted extraction	450.36 ± 3.50[a]	345.83 ± 4.53[a]	27.29 ± 1.23[a]	25.94 ± 2.12[a]
Conventional extraction	380.80 ± 1.72[b]	330.81 ± 4.69[b]	20.59 ± 2.11[b]	19.85 ± 0.61[b]

Note: Different superscripts in a column indicate significant differences ($P<0.05$).

The GABA content obtained in this study was lower than that reported by William et al. (505 mg/100 g dry leaf) [17]. However, they used water-to-mulberry leaf powder at a ratio of 40 mL/g, with extraction performed twice at 70°C for 40 min. In our study a single-cycle extraction was used and the extraction was performed at room temperature with a ratio of solvent to mulberry leaf powder at 20 mL/g. In the study of Yang-sheng et al. [18] they did the extraction at 50°C for

2.13 hr using the ratio of mulberry leaf powder to water of 1:30 to obtain GABA at 474.2 mg/100 g dry leaf. In the experiments on antioxidant activity, the TPC and TFC levels in our extracts were lower than those described by Kim et al. [19], who used an extrusion process to increase the amount of extractable flavonoids from mulberry leaves.

Another study reported that the optimisation of the extraction of flavonoids from mulberry leaves by RSM was achieved with an ethanol concentration of 61% at 72°C and solid-to-solvent ratio of 1:29 g/mL for 1.5 hr [20]. Still in another study [21], optimal conditions for extracting flavonoids from mulberry leaves were 70.85°C, 39.30% of ethyl acetate in water, 120 min. extraction period and solvent/solid ratio of 34.60:1. Our results have the advantage of being conducted at room temperature and using a safer and less toxic solvent, as well as saving solvent and reducing processing time.

CONCLUSIONS

Five-level CCD with RSM has been used to optimise the ultrasonic extraction of GABA from mulberry leaves. Optimal conditions are extraction time of 60 min., ethanol concentration of 54.75% and ratio of solvent to mulberry leaf powder of 20 mL/g. Under these conditions, GABA at 450.36 ± 3.50 mg/100 g dry leaf can be obtained in the extract. The experimental GABA content is close to the predicted value. The TPC, TFC and DPPH scavenging activity of 345.83 ± 4.53 mg GAE/100 g dry leaf, 27.29 ± 1.23 mg QE/100 g dry leaf and 25.94 ± 2.12% respectively are determined for the extract.

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