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**Isolation of Dihydroartemisinic Acid from the *Artemisia annua* L.
by-product by Combining Ultrasound-assisted Extraction with Response
Surface Methodology**

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Summary

Malaria is the most devastating parasitic disease worldwide. Artemisinin is the only drug that can cure malaria that is resistant to quinine-derived drugs. After the commercial extraction of artemisinin from *Artemisia annua*, the recovery of DHAA (dihydroartemisinic acid) from artemisinin extraction by-product has the potential to increase artemisinin commercial yield. Here we describe the development and optimization of an ultrasound-assisted alkaline procedure for the extraction of DHAA from artemisinin production waste using response surface methodology. Our results using this methodology established that NaOH at 0.36%, extraction time of 67.96 min, liquid:solid ratio of 5.89, and ultrasonic power of 83.9 W were the optimal conditions to extract DHAA from artemisinin production waste. Under these optimal conditions, we achieved a DHAA yield of 2.7%. Finally, we conducted a validation experiment, and the results confirmed the prediction generated by the regression model developed in this study. This work provides a novel way to increase the production of artemisinin per cultivated area and to reduce artemisinin production costs by recycling its commercial waste to obtain DHAA, an immediate precursor of artemisinin. The use of this technology may reduce the costs of artemisinin-based antimalarial medicines.

Keywords

dihydroartemisinic acid; ultrasound-assisted extraction; response surface methodology; *Artemisia annua* L.; alkaline extraction; acid precipitation

Introduction

Malaria is one of the world's most important parasitic diseases, affects approximately 300-500 million people worldwide, and causes more than one million deaths per year (WHO: World Malaria Report 2015, <http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/>).¹ *Artemisia annua*, L. is currently the only commercial source of artemisinin, the raw material for the production of ACTs (artemisinin combination therapies). ACTs are the frontline and life-saving medicine to treat malaria where *Plasmodium falciparum* is endemic and resistant to quinine-derived medicines. ACTs cost between US\$ 1.0 and 3.50 per treatment and can be required many times a year by people living in malaria-endemic areas. However, at the current cost ACTs are unaffordable for people living in economically-stricken countries, and who need it the most.² Thus, it is of paramount importance and urgency to reduce the production costs of artemisinin-derived antimalarial medicines. One way to achieve this goal is by increasing artemisinin yield per cultivated area and by improving the extraction efficiency of artemisinin and its related compounds from leaves of *A. annua*. Although the production of one of the artemisinin precursors (artemisinic acid) in genetically-engineered yeast was developed, no economically feasible artemisinin product is yet available from this technology, and its predicted cost is higher than current market prices for plant-based artemisinin. The production of artemisinin *in planta* surpasses what can be achieved by microorganisms engineered to produce the precursor artemisinic acid as an artemisinin precursor. *A. annua* uses widely-available resources such as soil, fertilizers, water, and solar energy to produce artemisinin as a natural result of the plant biosynthetic process controlled mostly by genetics, while the yeast needs an axenic environment, energy, nutrition, and oxygen to support its growth. Recent reports of low and variable artemisinin concentrations in *A. annua* are based on literature from the 90's with non-commercial cultivars.^{3,4} However,

crop-breeding programs have produced new varieties of *A. annua* with a consistently high yield of artemisinin ranging from 0.5 to 1.5 % (w/w).⁵⁻⁷ Considering that a plant can produce 350 g of dried leaves, and with 30,000 plants/ha each producing an average 1% (g/100g dw or w/w) artemisinin, the crop would produce 105 kg of artemisinin/ha, or 63kg/ha considering a 60% extraction efficiency with hexane (Malcolm Cutler, pers. comm.). Obviously, there is still room to increase the content of artemisinin in *A. annua* using classic breeding and selection or modern molecular techniques, such as genomic editing technology and genetic engineering that can successfully up-regulate biosynthetic genes and knock down negative regulators in the biosynthetic pathway of artemisinin. There are selections from Brazilian cultivars (3M) bred by CPQBA (Campinas, Brazil) that can produce 2% (w/w) artemisinin without reduction in plant biomass (Ferreira, unpublished). Therefore, production of artemisinin *in planta* continues to be the most competitive way to obtain affordable artemisinin, while empowering small farmers and communities willing to produce *A. annua* biomass for local artemisinin extraction plants. However, independently of its being present in higher concentrations in commercial cultivars, artemisinin commercial extraction still generates tons of waste that contains dihydroartemisinic acid, artemisinic acid, and some remaining artemisinin.

DHAA (dihydroartemisinic acid) is considered to be the main direct precursor of artemisinin,⁸⁻¹⁰ and it was reported to achieve levels twice as high as artemisinin in Chinese *A. annua*.¹⁰ Due to cost, commercial extraction uses hexane, which has a low extraction efficiency (60/70%) for artemisinin and an even poorer extraction efficiency for DHAA,^{11, 12} leaving most of the DHAA in the by-product of the artemisinin production waste (APW). Once recovered from the APW, DHAA can easily be transformed into artemisinin *in vitro*, without any enzyme, through the conversion of DHAA to artemisinin using photochemically-generated singlet oxygen in large scale.^{13,14} Thus, artemisinin yield and

costs can improve significantly if we use the DHAA already present in the extracted leaf biomass of *A. annua*. However, DHAA is discarded as a waste after artemisinin industrial extraction, leading to the loss of a valuable artemisinin raw material.

The isolation of DHAA from leaves of *A. annua* was reported at least 17 years ago¹⁵, but this isolation was time-consuming and only suited for laboratory scale. Previously, we have developed a simultaneous isolation of artemisinin and its precursors from *A. annua* by preparative reversed-phase HPLC, but it requires expensive instrument under laboratory settings.¹⁶ Others reported the extraction of artemisinin and artemisinic acid from *A. annua* using supercritical carbon dioxide,¹⁷ but they did not explore the technology to extract DHAA.

Because DHAA is a weak acid, we hypothesized that it would dissolve in water, after its conversion to salt by an alkaline solution. However, APW is normally a paste that consists of lipophilic pigments (chlorophylls) and leaf waxes that surrounds DHAA and blocks the alkaline solution from entering the paste to convert DHAA to its salt form. Ultrasound-assisted extraction is an inexpensive, simple, and efficient alternative that uses ultrasound waves to replace conventional extraction techniques.² When applied to a liquid, ultrasound waves consist of a cyclic succession of expansion (rarefaction) and compression phases caused by mechanic vibration. Rarefaction causes cavitation bubbles due to the radiated forces triggered by the ultrasound waves.² The collapse of the bubbles form fissions in plant cell walls that increase solvent penetration and the extraction of cell components.^{19,20} Recently, ultrasound waves have been widely applied to the extraction of active compounds from plant matrices showing advantages over conventional extraction techniques. These advantages include shorter time, less solvent, higher extraction yield, and lower cost.²¹⁻²³ However, to the best of our knowledge, there is yet no report on the ultrasound-assisted extraction of industrial waste, especially the ones that form a hydrophobic paste. We believe

that ultrasound waves could help to disrupt the film formed by oils and wax and disperse the paste into many small particles, increasing the surface of interaction between plant matrix and the solvent, thus improving DHAA extraction efficiency.

Once a new ultrasound-assisted extraction method is developed, optimization of extraction conditions, such as solvent concentration, extraction time, and solid-solvent ratio is very important to maximize extraction efficiency and time. RSM (Response surface methodology) uses mathematics and statistics for the generation of empirical models. Experiments that are carefully designed can optimize the response (output variable) that is influenced by several independent variables (input variables), improving the extraction process.²⁴ RSM is a statistical technique based on the fit of empirical models to the experimental data obtained in relation to experimental design.^{25,26} RSM generates a high-precision regression equation showing the relationship between responses and parameters through a series of tests that help to delineate an optimal response and to identify experimental parameters.²⁵ Response surface methodology has been widely used to optimize complex extraction procedures, thus reducing the number of experimental trials required.^{27,28} The present work aimed to develop optimal conditions for isolation and purification of DHAA from APW using RSM in order to maximize artemisinin production efficiency and reduce environmental contamination.

Experimental

Extraction Material

The APW used was acquired from Hunan Vigor Bio-Tech Company (Changsha, China). The APW was the byproduct of artemisinin commercial extraction from the same material.

Chemicals

NaOH, HCl, NaHCO₃, Na₂CO₃, anhydrous magnesium sulfate and chloroform were purchased from Changsha Chemical Reagent Company (Changsha, Hunan, China). DHAA

was purchased from National Institute for Control of Pharmaceutical and Biologic Products (Beijing, China).

Extraction of DHAA from artemisinin extraction waste

Samples of APW of about 0.1 g dry weight, added 1 mL of extraction solvent (different diluted bases to be tested), extracted 2 times for 1 h under sonication power of 100 W (SK3300LH, Kudos Ultrasonic Instrument, Shanghai, China) at 40 °C. Extraction mixtures were centrifuged for 5 min at 12000 rpm with the supernatant from two extractions being combined and transferred to a fresh Eppendorf tube. The DHAA in the alkaline extraction solution was determined using GC-FID (gas chromatography with flame ionization detection) according to the method described in section entitled “GC-FID chromatographic conditions”.

Separation of DHAA using acid precipitation

The NaOH extraction solution was concentrated 3-fold under vacuum at 40 °C, and then added of a suitable volume of 1 mol/L of HCl followed by vortexing and allowing the extract to stand for approximately 10 min until the formation of a precipitate. Then, mixture was centrifuged at 12000 rpm for 5 min. The precipitated samples were freeze dried and weighed, and then re-dissolved and diluted with chloroform to quantify the concentration of DHAA. The precipitates formed at different pHs were separately collected for assay.

Response surface methodology design

RSM was used to obtain the optimal conditions for extraction of DHAA by developing the relationships between the response and the variables. On the basis of the results from single factor experiments, a Box-Behnken design, with four-factor at three-level, was conducted to analyze the individual, interactive and quadratic effects of extraction parameters on the DHAA yield. The four independent variables were NaOH concentration (A), extraction time (B), liquid/solid ratio (C) and ultrasonic power (D), whose range and central point values were showed in Table 1. We carried out 29 experiments at random (Table 2) and the experimental data were analyzed using Design Expert 8.0.5 (Stat-Ease Inc, Minneapolis,

MN, USA) to establish the functional relationship between the content of DHAA and the extraction parameters based on a second-order polynomial model, which was widely accepted to describe the relationships between the responses and the independent variables,²⁶ expressed as below:

$$Y = \delta_0 + \sum_{i=1}^4 \delta_i X_i + \sum_{i=1}^4 \delta_{ii} X_i^2 + \sum_{i < j=2}^4 \delta_{ij} X_i X_j + \epsilon$$

where Y represents the value of response; δ_0 is the constant term; δ_i , δ_{ii} , and δ_{ij} represent the coefficients for linear, quadratic, and interactions, respectively; X_i and X_j are the values of the independent variables, and ϵ is the residue associated with the experiments.

Verification of optimized condition and regression model

Approximately 100 g of APW (DW) was weighed and broken to particle size of approximately 0.1 g. Then, DHAA was extracted from APW under the optimized conditions determined by RSM with two extractions. The extraction solutions were centrifuged for 10 min at 5000 rpm, the supernatants were combined, concentrated 3-fold under vacuum at 40 °C, and then adjusted the pH to 1 with 1 mol/L of HCl, let stand for approximately 10 min until a precipitate formed, and then the mixture was centrifuged at 5000 rpm for 10 min. The samples of these precipitates were freeze dried, weighed, and re-dissolved with chloroform to quantify the content of DHAA in the sample.

Quantification of DHAA in alkaline extraction solution

The alkaline extraction solution (2 mL) was added of 1 mol/L of HCl to adjust the pH to 7. The addition of HCl results in the precipitation of DHAA from the liquid medium. This solution was partitioned with 2.0 mL of chloroform. After shaking, the solution was allowed to rest for 30 min to form a by-layer. The chloroform layer was then transferred to a clean

Eppendorf tube and added anhydrous magnesium sulfate to remove residual water from chloroform, and centrifuged for 2 min at 12,000 rpm. An aliquot of 1 μ L of chloroform was used to perform GC-FID analysis.

GC-FID chromatographic conditions

The DHAA analysis was performed in a 6890N GC system (Agilent, Santa Clara, CA, USA) coupled with a flame ionization detector and a data collection system (ChemStation 32, Rev. A. 09.01) according to a previously reported method²⁹. Briefly, the capillary column was a HP-5 (Agilent, 30 m \times 0.32 mm ID, 0.25 μ m of 95% dimethyl polysiloxane). The flow rate of the carrier gas was 1 mL/min with a split ratio of 3:1. The temperatures of the injector and detector were set at 235 $^{\circ}$ C and 285 $^{\circ}$ C, respectively. The initial oven temperature was set at 180 $^{\circ}$ C, and then increased to 220 $^{\circ}$ C at 6 $^{\circ}$ C/min, holding for 3 min, then increasing to 280 $^{\circ}$ C at 30 $^{\circ}$ C/min, and holding at that temperature for 10 min.

Statistical analysis

Each test was repeated three times, and the data obtained from each experiment were described as the mean value \pm standard deviation, which was analyzed by RSM software Design Expert 8.05b or statistical software SPSS (Version 16.0; SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effect of various alkalis on the extraction of DHAA

Different alkaline solutions of different concentration (NaOH: 0.5%, 1%, 2%, 3%, 4%, and 5%; NaHCO₃: 2%, 3%, 4%, 5% ; Na₂CO₃ : 2%, 3%, 4%, and 5%) were tested on their

effectiveness to extract DHAA from APW. Our results (Fig. 1) clearly show that solutions of NaOH (0.5-4%) extracted the highest amounts of DHAA from APW, followed by NaHCO₃ (2-5%), then NaCO₃ solution. However, the high efficiency of DHAA extraction achieved with 0.5% NaOH solution decreased with increasing concentrations, indicating that DHAA (a weak acid) may degrade under increased pH of more concentrated NaOH solutions. Interestingly, the efficiency of extraction increased with higher concentrations of both NaHCO₃ and Na₂CO₃ (Fig. 1).

Effect of NaOH concentration on the extraction of DHAA

After establishing that NaOH was the best alkali for the extraction of DHAA from APW, we investigated the effect of its concentration on the extraction efficiency of DHAA by using of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% of NaOH. The results showed that when NaOH was below 0.5%, the extraction efficiency was improved in range from 0.2-0.3%, while concentrations of 0.4 and 0.5% had a negative impact on the extraction efficiency. Thus, the best extraction of DHAA from APW was achieved with 0.2-0.3% aqueous NaOH (Fig. 2).

The effect of ultrasonic power on the extraction of DHAA

In order to improve the extraction efficiency, ultrasound-assisted extraction was employed. The results showed that extraction efficiency of DHAA from APW increased consistently, although not always significantly, from 20 to 80 W, but no significant difference existed between 80 and 100 W (Fig. 3). Thus, we established that the power setting of 80 W was the most efficient in extracting DHAA, resulting in 20% of energy savings in the extraction process without loss of efficiency. This result might confirm our hypothesis that cavitation bubbles formed by ultrasound waves disrupt the film of hydrophobic pigments and wax surrounding DHAA. The dispersal of the APW paste into small particles results in a larger

contact area between DHAA and the extraction solvent.

The effect of time on ultrasonic extraction of DHAA

The ultrasonic extraction efficiency of DHAA from APW was evaluated from different ultrasonic times (30, 45, 60, 75, and 90 min) used with the alkali solution (0.3% NaOH). From 30 to 60 min, extraction time increased the efficiency of extraction but, after 60 min, prolonged extraction provided no advantage or reduced extraction efficiency (Fig. 4). Extended sonication extraction may lead to the degradation of DHAA in an alkaline solution, in our case 0.3% NaOH. Therefore, the extraction of DHAA from APW was more efficient when performed for 60 min.

The effect of the liquid:solid ratio on the extraction of DHAA

A study was conducted to determine the best liquid:solid ratio (v /w) for better extraction of DHAA from APW. Using the best extraction solvent, as determined in the first step, samples were extracted at liquid:solid ratio of 1:1, 3:1, 5:1, 7:1, and 10:1 (v/w), respectively. The results showed that the ratio of liquid:solid of 7:1 and 10:1 provided the best extraction efficiencies for DHAA, without significant difference between them (Fig. 5). Considering the extraction cost, the ratio of 7 was as efficient as that of 10 and would save 30% in solvent and alkali costs. Our results also show that extraction ratio of 5:1, usually followed as a rule of thumb for extractions of secondary metabolites from dry leaves, had only moderate extraction efficiency to extract DHAA from APW.

The effect of pH value on the precipitation of DHAA

The purification of DHAA from APW improved at low pH, conducted using acid precipitation. We used different volumes of HCl, resulting in pH values of 0.5, 1, 1.5, 2, 2.5

and 3 to precipitate DHAA. The pH=1 seemed to be appropriate and extraction efficiency tended to decrease above this pH value (Fig. 6). This was expected based on the acidic nature of DHAA.

Box-Behnken design and analysis

Response surface methodology is a statistical tool used to explore the relationship between the independent variables (input) and the response or output.³⁰ In the present work, the Box-Behnken design was employed to develop a polynomial model for the optimization of the extraction of DHAA from APW with 4 independent variables at 3 levels (Table 1). The results (Table 2) were fitted to a second-order polynomial model, which was described in the experimental section. A regression equation, expressing the relationship between the response and variables, was obtained as shown below:

$$Y = -37.476 + 9.714A + 0.225B + 2.049C + 0.558D - 0.043AB - 0.687AC + 0.204AD - 0.009BC - 0.00023BD - 0.006CD - 27.26A^2 - 0.001B^2 - 0.055C^2 - 0.0035D^2,$$

where Y was DHAA content in the extraction solution. While A, B, C and D were NaOH concentration, extraction time, liquid:solid ratio, and ultrasonic power, respectively.

The significance and suitability of the resulting model was evaluated using ANOVA. The results (Table 3) showed that the model *F*-value was 18.56 and the value of "Prob > *F*" (*P*-value) was less than 0.0001, which implied that the model was significant and there was only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise. Subsequently, the model fitting analysis was performed using R^2 (the coefficient of determination), which was the proportion of variability of the experimental data explained by the mathematic model.²⁵ The results (Table 4) showed that R^2 was 0.9489, suggesting that only 5.1% of the total variation could not be explained by the mathematical model in the extraction studies conducted. Additionally, the Pred- R^2 (predicted R-squared) of 0.7298 was

in reasonable agreement with the Adj-R² (adjusted R-squared) of 0.8977 (as shown in table 4). Adeq-Precision (the adequate precision used to measure the signal-to-noise ratio) of 12.771 was obtained (Table 4), indicating an adequate signal, which was greater than desirable ratio of 4. The “Lack of Fit *F*-value” of 3.13 implied that the “Lack of Fit” was not significantly relative to the pure error (Table 3). Moreover, there was a 14.12% chance that a "Lack of Fit *F*-value" this large could occur due to noise (See Table 3). Therefore, all of the above analyses confirmed the validity of the equation obtained.

Furthermore, data on Table 3 proves that the coefficients of linear terms (A, B, and D), the coefficients of interaction terms (AC, AD, BC, CD) and all the coefficients of quadratic terms (A², B², C², and D²) had significantly small *P*-values (*P* < 0.05), whereas the coefficients of other terms were not significant (*P* > 0.05). Moreover, from the data showed in Table 3, we could obtain the order of importance for the extraction conditions influencing the DHAA yield, as follows: NaOH concentration > extraction time > ultrasonic power > liquid:solid ratio.

Response surface analysis

The 3-dimensional response surface plots (Fig. 7) were given as a graphical representation of the regression model to illustrate the relationship between the response, the experimental levels of each independent variable, and the interaction of two investigated factors when the other variables were kept constant. Fig. 7A indicated the interaction of the extraction time and NaOH concentration on the DHAA yield when the liquid:solid ratio and ultrasonic power was 7 and 80 W, respectively. The DHAA extraction yield increased with the addition of the extraction time and NaOH concentration to a certain range, and then decreased slowly when the NaOH concentration and extraction time exceeded 0.35% and 69 min, respectively. Fig. 7B indicated the interaction of the NaOH concentration and liquid:solid ratio on the DHAA

yield when the extraction time and ultrasonic power was 60 min and 80 W, respectively. The DHAA yield increased sharply at first and then presented a smooth trend. Fig. 7C indicated that the interaction of the NaOH concentration and ultrasonic power improved the yield of DHAA from APW when the liquid:solid ratio and extraction time was 7 and 60, respectively. The amount of DHAA increased sharply when the ultrasonic power increased from 70 to 80 W but, beyond 80 W DHAA, extraction yield decreased rapidly as the ultrasonic power increased. The yield of DHAA also increased sharply with the concentration of NaOH below 0.35%, decreasing slowly after that value. Fig. 7D showed the interaction of the extraction time and liquid:solid ratio on the extraction yield of DHAA when NaOH concentration and ultrasonic power was 0.30% and 80 W, respectively. The DHAA extraction yield increased with both the extraction time and the liquid:solid ratio. From Fig. 7E, it was found that the extraction yield of DHAA increased sharply with the ultrasonic power increasing from 70 to 85 W, but beyond 85 W DHAA extraction yield decreased. The yield of DHAA also increased sharply with the extraction time before 63 min, and then plateaued after 60 min. When NaOH concentration and extraction time were unchanged, the yield of DHAA increased with higher ultrasonic power (Fig. 7). Moreover, when ultrasonic power was constant, the amount of DHAA increased with the liquid:solid ratio.

Optimization and Verification of predicted model

Based on the response surface analysis performed using the Design Expert 8.0.5 software, the optimum condition for extraction of DHAA from APW was established as a function of a NaOH concentration of 0.36%, extraction time of 67.96 min, liquid:solid ratio of 5.89, and ultrasonic power of 83.9 W. The estimated values for Y, 1.35 mg/mL (total volume was 2 mL), was obtained at those conditions, which means that 2.70 mg DHAA could be extracted from 100 mg of APW under the optimum condition.

In order to validate the optimized extraction condition calculated by Design-Expert 8.0.5b, a triplicate experiment was performed with 100 g of APW under the optimum condition. The results (Fig. 8) showed that 3.16 ± 0.53 g of the extracted product with a purity of $75.13 \pm 6.58\%$ (w/w) (Fig. 8B), representing 2.37 ± 0.4 g of DHAA, was obtained from 100 g of APW (containing 3.01% of DHAA) using the method developed in this work. Theoretically, 2.70 g of DHAA should be obtained based on the calculation of the established regression equation. There was only about 12% difference between the experimental value and the predicted value even though the data were scaled up to 1000-fold compared to the mathematic equation developed in this study, which indicated that the developed quadratic model was suitable to predict the extraction efficiency of DHAA from APW. Therefore, the validation experiments showed that the quadratic model developed was satisfactory within the specified range of extraction parameters and the extraction procedure developed in the present work was suitable to recycle APW.

Conclusion

In the present study, we developed and presented for the first time an ultrasound-assisted alkaline extraction of DHAA from byproduct of *A. annua*, artemisinin production waste. After investigating the effect of different conditions on the extraction efficiency provided by single-factor experiments, response surface methodology was employed to calculate the optimal extraction conditions. The results showed that NaOH concentration of 0.36%, extraction time of 67.96 min, liquid:solid ratio of 5.89, and ultrasonic power of 83.9 W were the optimal conditions for extraction of DHAA from APW. Under the optimal extraction conditions, 2.70 mg of DHAA could be extracted from 100 mg of APW. Finally, a verification experiment was conducted and the result confirmed the predicted results obtained by a regression model developed in this study. This work shows how to improve the

utilization of *A. annua* leaves as source of artemisinin by and providing a potential way to reduce the cost of artemisinin production and to recycle DHAA previously discarded with the APW.

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Conflict of Interest

The authors declare no conflict of interest. The APW was kindly provided by Hunan Vigor Bio-Tech Company without any conflict of interest.

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Figure legends

Figure 1. Effect of the type and concentration of alkali on the extraction of dihydroartemisinin acid from artemisinin production waste.

Figure 2. The effect of sodium hydroxide (NaOH) concentration on the extraction efficiency of dihydroartemisinin acid from artemisinin production waste.

Figure 3. The effect of ultrasonic power on the extraction of dihydroartemisinin acid from artemisinin production waste.

Figure 4. The effect of ultrasonic time on extraction of dihydroartemisinin acid from artemisinin production waste.

Figure 5. Effect of the liquid:solid ratio on the extraction efficiency of dihydroartemisinin acid from artemisinin production waste.

Figure 6. The effect of pH value on extraction of dihydroartemisinin acid from artemisinin production waste.

Figure 7. Response surface methodology graphs of DHAA: (A) extraction time versus NaOH concentration; (B) ultrasonic power versus NaOH concentration; methanol concentration versus liquid:solid ratio; (C) liquid:solid ratio versus NaOH concentration; (D) ultrasonic power versus liquid:solid ratio; (E) ultrasonic power versus extraction time; (F) extraction time versus liquid:solid ratio. The optimum extraction conditions were established through Response Surface Methodology using software Design-Expert 8.0.5b, as follows: an aqueous

solvent of 0.36% NaOH, an extraction time of 67.96 min and a liquid:solid ratio of 5.89 mL/g, with a ultrasonic power of 83.9 W.

Figure 8. Isolation of dihydroartemisinic acid (DHAA) from artemisinin production waste by combining alkali extraction with acid precipitation. The gas chromatograms of artemisinin production waste before isolation (A) and the dihydroartemisinic acid extract from artemisinin production waste (B) with ▼ indicating the peak for DHAA with an elution time of 6.5 minutes.

Figure 1.

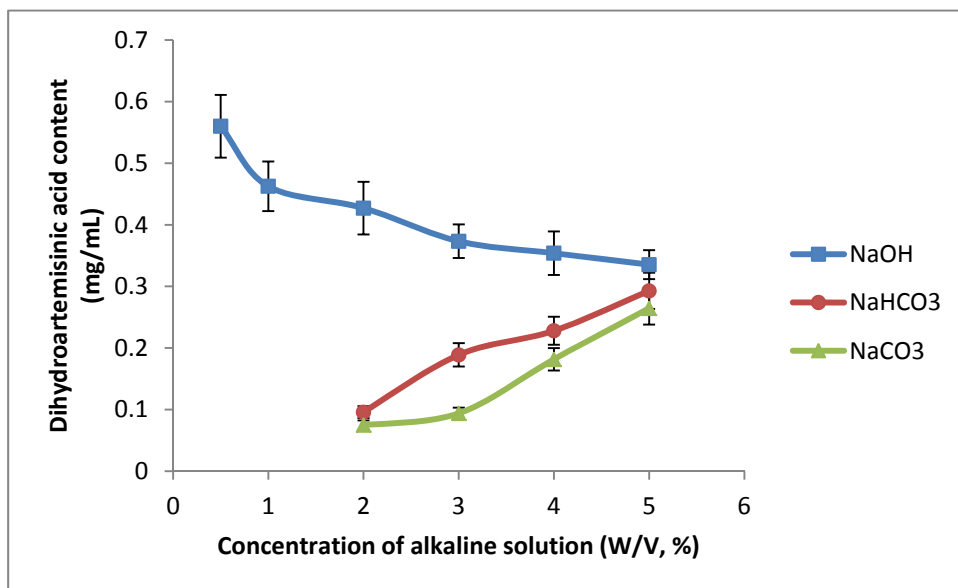


Figure 2.

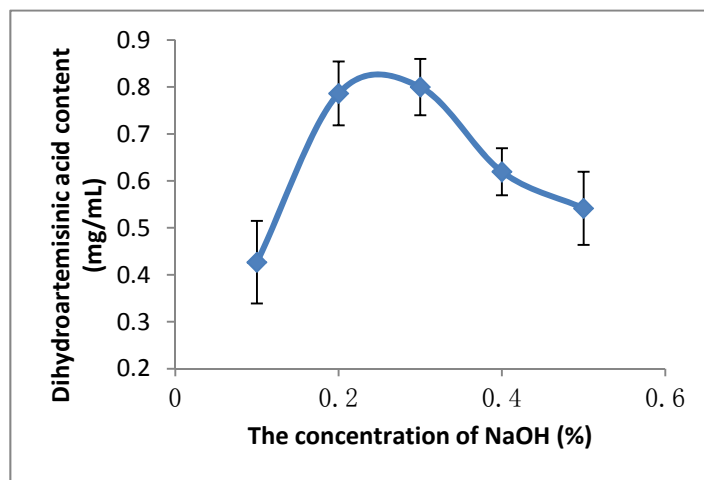


Figure 3.

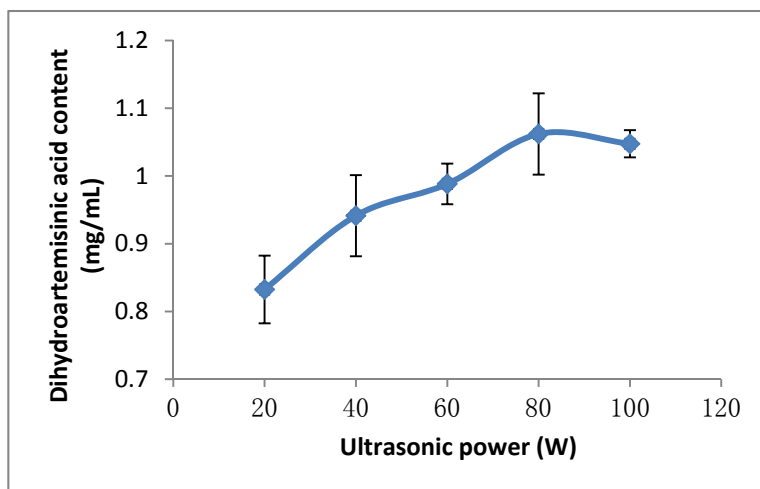


Figure 4.

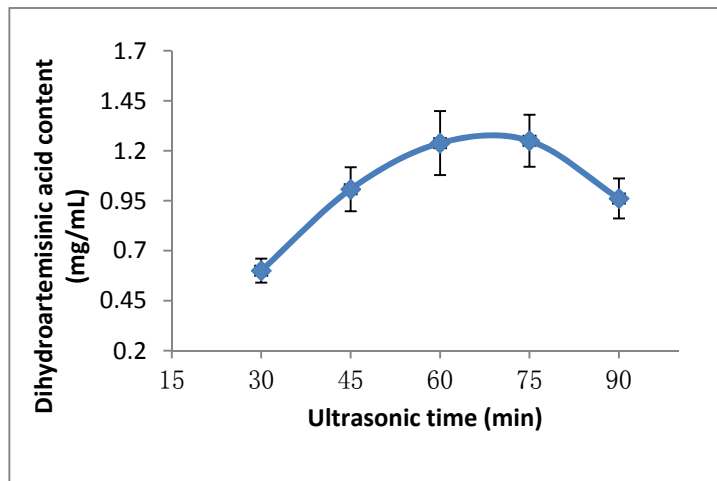


Figure 5.

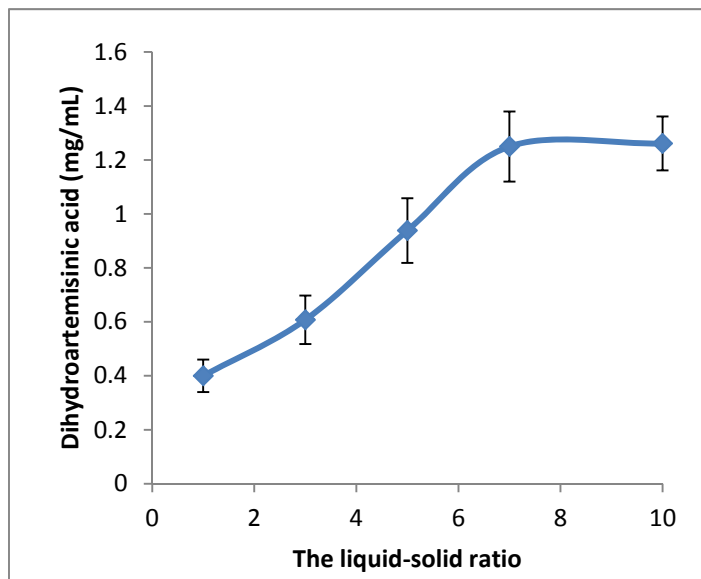


Figure 6.

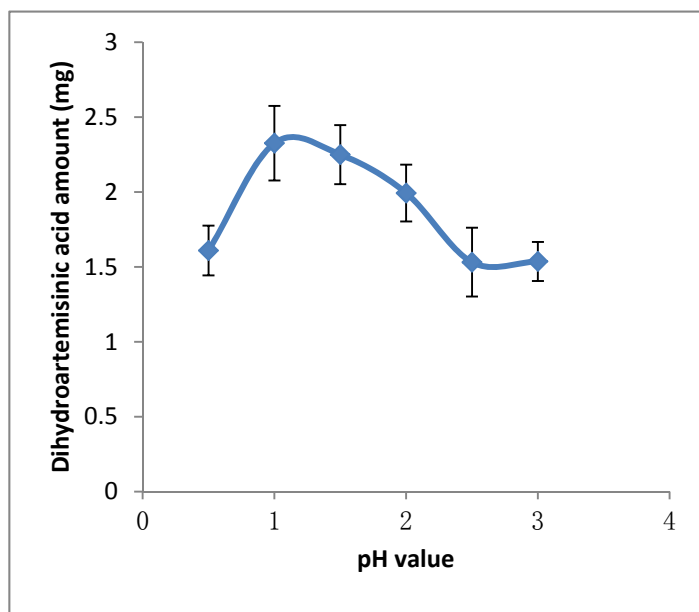


Figure 7

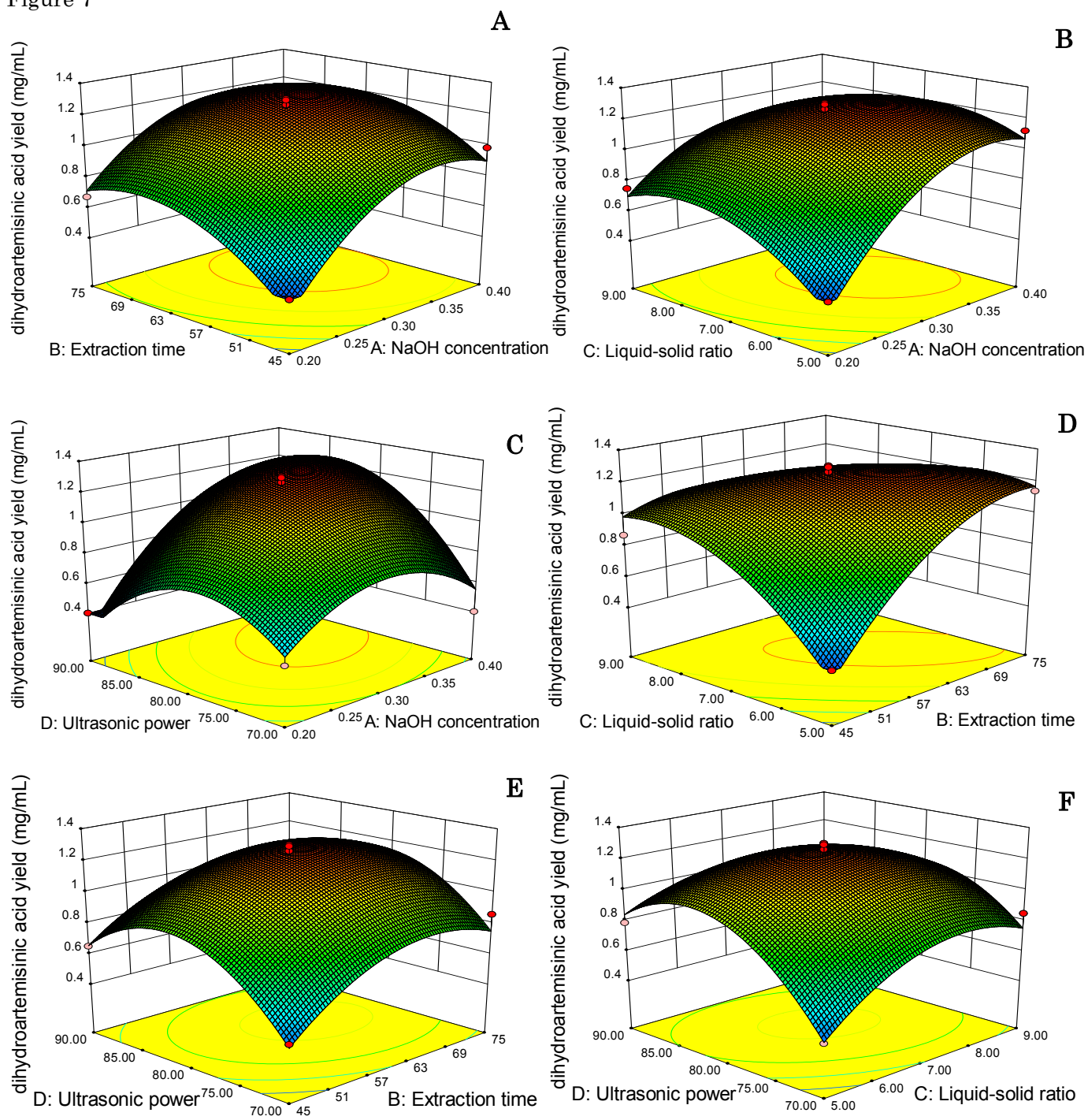


Figure 8.

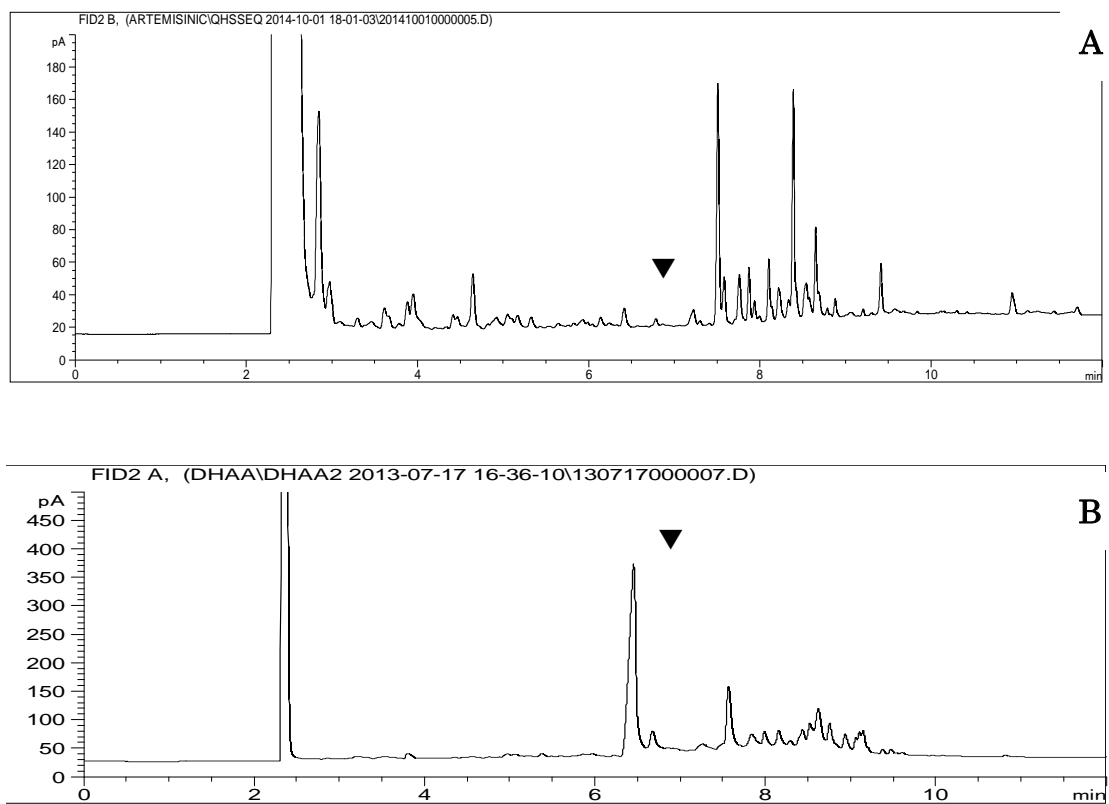


Table1. Factors and their levels for the Box-Behnken design

Factor	Symbol	Coded levels		
		-1	0	1
NaOH concentration (%)	A	0.2	0.3	0.4
Extraction time (min)	B	45	60	75
Liquid-solid ratio	C	5	7	9
Ultrasonic power (W)	D	70	80	90

Table 2.Box-Behnken experimental design and results for the yield of dihydroartemisinic acid in the ultrasound-assisted alkaline extraction

Run	A	B	C	D	Dihydroartemisinic acid content (mg/mL)
1	0.3	60	5	70	0.4093
2	0.3	60	7	80	1.2612
3	0.4	45	7	80	0.9842
4	0.3	75	9	80	0.5332
5	0.3	75	5	80	1.14
6	0.3	60	7	80	1.299
7	0.2	60	7	90	0.2913
8	0.4	60	7	70	0.391
9	0.2	75	7	80	0.6726
10	0.2	45	7	80	0.3159
11	0.3	75	7	90	0.9416
12	0.3	60	7	80	1.2908
13	0.3	60	7	80	1.1751
14	0.3	60	5	90	0.7874
15	0.3	60	9	70	0.8489
16	0.3	45	5	80	0.3487
17	0.4	60	5	80	1.1195
18	0.4	75	7	80	1.0826
19	0.3	60	9	90	0.7341
20	0.3	60	7	80	1.158
21	0.4	60	9	80	0.9596
22	0.2	60	9	80	0.7505
23	0.3	45	9	80	0.8694
24	0.2	60	7	70	0.4512
25	0.2	60	5	80	0.361
26	0.3	45	7	90	0.6512
27	0.4	60	7	90	1.047
28	0.3	75	7	70	0.8596
29	0.3	45	7	70	0.4307

Table 3 ANOVA for response surface quadratic model of DHAA

Sources of variation	Sum of squares	DOF	Mean square	F value	P value	Significance
Model	2.85	14	0.20	18.56	< 0.0001	**
A-A	0.63	1	0.63	57.08	< 0.0001	**
B-B	0.22	1	0.22	20.17	0.0005	**
C-C	0.023	1	0.023	2.13	0.1664	
D-D	0.094	1	0.094	8.56	0.0110	*
AB	0.017	1	0.017	1.52	0.2379	
AC	0.075	1	0.075	6.88	0.0201	*
AD	0.17	1	0.17	15.17	0.0016	**
BC	0.32	1	0.32	28.96	< 0.0001	**
BD	4.8E-003	1	4.8E-003	0.44	0.5193	
CD	0.061	1	0.061	5.54	0.0338	*
A ²	0.48	1	0.48	43.94	< 0.0001	**
B ²	0.32	1	0.32	29.19	< 0.0001	**
C ²	0.31	1	0.31	28.15	0.0001	**
D ²	0.77	1	0.77	70.57	< 0.0001	**
Residual	0.15	14	0.011			
Lack of Fit	0.14	10	0.014	3.13	0.1412	
Pure Error	0.017	4	4.4E-003			

Note:** means highly significant difference (P<0.01); * means significant difference (P<0.05)

Table 4 Model fitting analysis

Term	Value
R-Squared	0.9489
Adj R-Squared	0.8977
Pred R-Squared	0.7298
Adeq Precision	12.771