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*Full Length Research Paper*

# Optimization for ultrasonic-assisted extraction of salidroside from *Ligustrum lucidum* by central composite design-response surface methodology

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**As a new extraction method, ultrasonic-assisted extraction (UAE), whose procedure has higher extraction yield, lower extracting temperature and less power consumption, was explored to extract salidroside from *Ligustrum lucidum* firstly. The content of salidroside was determined by reverse phase-high performance liquid chromatography (RP-HPLC). The UAE process of salidroside from *L. lucidum* was optimized by using central composite design-response surface methodology (CCD-RSM). The results showed that the optimal extraction conditions were ethanol concentration of 74.14%, liquid-solid ratio of 30.31, extracting time of 68.14 min and the extraction efficiency of salidroside was 4.68 mg;g$^{-1}$. The results indicated that the UAE would be a novel method for extraction of salidroside from *L. lucidum.***

**Key words:** Ultrasonic-Assisted Extraction (UAE), central composite design-response surface methodology, *Ligustrum lucidum,* reverse phase-high performance liquid chromatography (RP-HPLC).

## INTRODUCTION

*Ligustrum lucidum* (Nvzhenzi in Chinese) has been known as tonic for kidney and liver in the traditional Chinese medicine prescription for a long time (Shi et al., 1998). It was reported that *L. lucidum* possess immunomodulatory, anti-inflammatory, hepatoprotective, anticancer, anti-aging, antioxidative, antiviral, anti-mutagenic and neuroprotective activities (He et al., 2001a, b; Kalsuhiko et al., 1989; Lau et al., 2002; Lin et al, 2007; Ma et al., 2011; Nagy et al., 2006; 2009; Shoemaker et al., 2005; Sung et al., 2006; Zhu et al., 2009). Salidroside (p-hydroxyphenethyl-b-D-glucoside, $C_{14}H_{20}O_7$, as shown in Figure 1) is one of the major active constituents in *L. lucidum.* It has been reported to possess various pharmacological properties including resisting anoxia, anti-aging, anti-cancer, anti-inflammation, antioxidative, antifatigue, antiviral, neuroprotective, hepatoprotective and cardioprotective *effects* (Diaz Lanza et al., 2001; Laremii and Grigor'eva, 2002; Kelly, 2001; Kucinskaite et al., 2004; Kanupriya et al., 2005; Ma et al., 2009; Nan et al., 2003; Wang et al., 2004; Wang et al., 2009; Zhang et al., 2007).

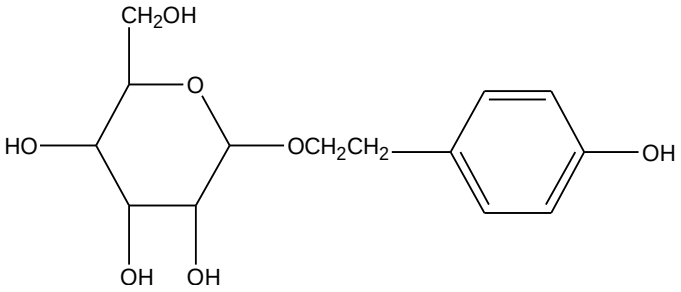


**Figure 1.** The chemical structure of salidroside.

The first step of analysis and utilization of the medicinal plant bioactive constituents is extraction, that is, the

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separation of components to be analyzed or used from the cellular matrix. The “ideal” extraction method should be quantitative, non-destructive and timesaving (Guo, 2003). In recent years, ultrasonic-assisted extraction (UAE) has been used for extraction of many components, such as flavonoids (Ji et al., 2005; Wang et al., 2011), ginsenosides (Ma et al., 2005), schisandrins (Yin et al., 2005) and polyphenols (Li et al., 2011). Therefore, this technique might be an ideal extraction method for salidroside from *L. lucidum.*

In the current study, UAE was employed for the extraction of salidroside from *L. lucidum*. Reverse phase-high performance liquid chromatography (RP-HPLC) was used to determine the salidroside in extract of *L. lucidum*. Moreover, the extraction parameters including ethanol concentration (%), liquid-solid ratio (ml : g) and extraction time (min) were optimized by the maximization of the extraction yield of salidroside. The relationship between the extraction parameters and the yield of salidroside was studied by running a central composite design (CCD), constructing a mathematical model, and investigating the relationship by response surface analysis methodology (RSM). The results indicated that UAE could be applied for the production of high quality salidroside extracts.

## MATERIALS AND METHODS

### Plant materials and chemicals

*L. lucidum* (Zhejiang Province, China) was obtained from a local pharmacy in China. Salidroside was purchased from China pharmaceutical and biological products inspection (Lot: 110818-201005). HPLC grade solvents (acetonitrile, methanol and ethanol) were purchased from Tianjin Kemiou chemical reagent Co. Ltd.

### Extraction of salidroside from *Ligustrum lucidum* by UAE

UAE was performed with an Ultrasonic Cleaners (KQ2200E, Kunshan, China). The output power is 100 W and the frequency is 40 kHz. Sample powder 2.00 g was transferred into a 100 ml conical flask and proportioned extraction solvent was added. Then the flask was sonicated in an ultrasonic water bath for a proposed time. After the extraction ended, the extract was then filtrated and transferred into a 50 ml volumetric flask, and the residues were then rinsed with the extraction solvent in triplicate. Then, pool the first extract and the later extracts. Finally, the volume was made up to the mark with the extraction solvent.

### Quantitative analysis method

The HPLC system HP 1100 series (Agilent Technologies, Waldbronn, Germany), equipped with the ChemStation software (Agilent Technologies) and comprised a binary high-pressure pump, an online vacuum degasser, an auto-sampler, a thermostated column compartment and a photodiode array detector using a maximum plot in the range of 190 to 800 nm, was used for chromatographic analysis. All separations were carried out on a Hypersil ODS C18 column (4.6 × 250 mm i.d., 5 μm particle size, Agilent). The mobile phase was acetonitrile (A): water (B) =13:87. The flow rate was kept at 1 $ml.min^{-1}$ and the column temperature was 30°C. Injection volume was 10 μl. The absorbance was measured at the wavelength of 275 nm for the detection of salidroside.

### Optimize the UAE extraction process of *Ligustrum lucidum* by CCD-RSM

A CCD with three variables was used to determine the response pattern and then to establish a model. Three variables used in this study were ethanol concentration ($X_1$), solid-liquid ratio ($X_2$) and extraction time ($X_3$) with five levels (−r, −1, 0, 1 and r) of each variable; the experimental factors and levels of coding are shown in Table 1, while the dependent variable was the yield of salidroside. All variables were taken at a central coded value considered as zero. In general, CCD is constructed in such a way that $2^k + 2k + 1$ experiments are required; where k represents the number of factors to be studied. Therefore, the twenty experiments shown in Table 2 were performed in triplicate. The experiments were randomly assigned to avoid unobserved error. The yield of salidroside was estimated by quadratic response surface model, which have the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_0 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Where $Y$ is the predicted response, $X_1$, $X_2$ and $X_3$ are the coded values of the factors; $\beta_0$, $\beta_i$ (i =1,2,3) and $\beta_{ij}$ (i =1,2,3; j =1,2,3, i≤j) are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

The statistical analysis of experimental results was performed by the software Design-Expert 8.0.5.

## RESULTS AND DISCUSSION

### Quantitative determination of salidroside

Salidroside are one of the kinds of the main effective ingredients in various medicinal plants including *L. lucidum*. The optimal mobile phase, consisting of acetonitrile and water, was described by Qi et al. (2010). Under the proposed condition, the high performance liquid chromatogram of reference substance and *L. lucidum* extracts are shown in Figure 2. Calibration curve of salidroside was linear in relatively wide of amounts (5.2 to 260 ng) and showed good linearity regressions ($Y$ = 5.6869$X$ + 6.0472) with high correlation coefficient value ($R^2$ = 0.9999) between peak area ($Y$) and the amount of salidroside ($X$, μg). Limit of detection (LOD) and limit of quantitation (LOQ) were defined as the signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ values of salidroside were 1.6 and 5.2 ng, respectively. The precision test was carried out by the intra-day variability for salidroside. The intra-day variability was

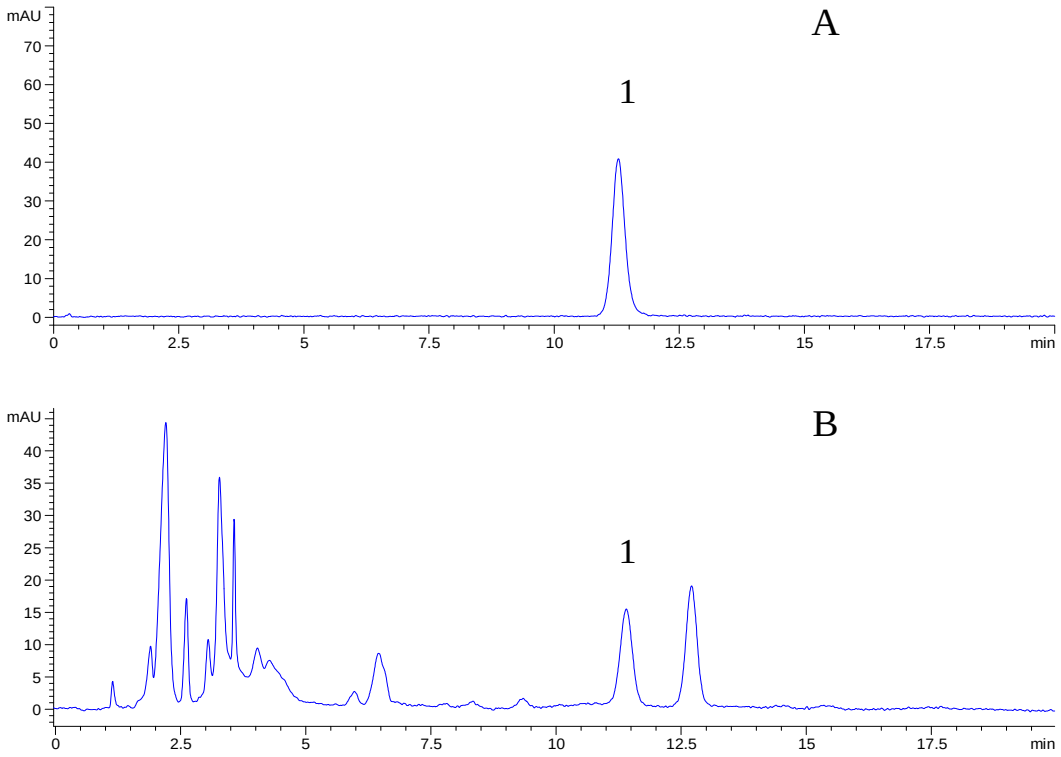


**Figure 2.** Chromatograms of the salidroside standard solution (A) and *Ligustrum lucidum* extraction (B); peak 1, salidroside.

**Table 1.** Factors and levels for CCD test.

| Factor | Level | | | | |
|---|---|---|---|---|---|
| | *-r* | -1 | 0 | 1 | *r* |
| Ethanol concentration ($X_1$)/% | 25 | 39.79 | 60 | 80.21 | 95 |
| Liquid-solid ratio ($X_2$) | 5.0 | 14.5 | 27.5 | 40.5 | 50 |
| Extraction time ($X_3$)/min | 30 | 42.7 | 60 | 77.3 | 90 |

*r* = 1.732.

assayed at one concentration on the same day. The RSD of intra-day was less than 0.02%, which demonstrated a good precision of this method. The accuracy was determined by the method of standard addition. The diluted sample solution was spiked with the standard solution of salidroside in different ratios. The resultant samples were analyzed using the proposed method. The mean recovery of salidroside was 99.36 to 99.67% (Table 3).

## Selection of variables

In UAE, solvent is a key factor affecting the yield of extraction. Mixed solvents of a lower alcohol and water have been generally used for the extraction of salidroside. Among the various alcohols, ethanol was chosen as it is suitable for pharmaceutical and food industry. Therefore, the effect of ethanol concentration in aqueous ethanol extractant on salidroside yield was evaluated. The ethanol concentration was varied in the range of 25 to 95% (v/v). Considering the needs of industry, the liquid-solid ratio was varied in the range of 5 to 50 and the extraction time of 30 to 90 min. The ranges

**Table 2.** The central composite design matrix of three test variables in coded and experimental results from the response variables.

| S/No. | $X_1$/% | $X_2$/mL:g | $X_3$/min | $Y$/mg·g$^{-1}$ |
|---|---|---|---|---|
| 1 | 39.79 (-1) | 14.5 (-1) | 42.7 (-1) | 4.11 |
| 2 | 80.21 (+1) | 14.5 (-1) | 42.7 (-1) | 4.28 |
| 3 | 39.79 (-1) | 40.5 (+1) | 42.7 (-1) | 4.38 |
| 4 | 80.21 (+1) | 40.5 (+1) | 42.7 (-1) | 4.48 |
| 5 | 39.79 (-1) | 14.5 (-1) | 77.3 (+1) | 4.14 |
| 6 | 80.21 (+1) | 14.5 (-1) | 77.3 (+1) | 4.53 |
| 7 | 39.79 (-1) | 40.5 (+1) | 77.3 (+1) | 4.45 |
| 8 | 80.21 (+1) | 40.5 (+1) | 77.3 (+1) | 4.50 |
| 9 | 25 (-1.732) | 27.5 (0) | 60 (0) | 3.88 |
| 10 | 95 (1.732) | 27.5 (0) | 60 (0) | 4.62 |
| 11 | 60 (0) | 5 (-1.732) | 60 (0) | 3.86 |
| 12 | 60 (0) | 50 (1.732) | 60 (0) | 4.08 |
| 13 | 60 (0) | 27.5 (0) | 30 (-1.732) | 4.52 |
| 14 | 60 (0) | 27.5 (0) | 90 (1.732) | 4.48 |
| 15-20 | 60 (0) | 27.5 (0) | 60 (0) | 4.65 |

$X_1$, Ethanol concentration; $X_2$, liquid-solid ratio; $X_3$, extraction time.

**Table 3.** Recovery of salidroside by standard addition method.

| Variable | Original amount (μg) | Added amount (μg) | Detected amount (μg) | Recovery[a] (%) | RSD[b] (%) |
|---|---|---|---|---|---|
| Salidroside | 56.29 | 26 | 82.20 | 99.67 | 0.71 |
| | 56.29 | 52 | 108.08 | 99.60 | 0.35 |
| | 56.29 | 78 | 133.79 | 99.36 | 0.77 |

n = 6.

**Table 4.** Regression results from the data of CCD experiments.

| Source | Sum of squares | df | Mean square | F-value | p-value Prob>F |
|---|---|---|---|---|---|
| $X_1$ | 1.238454 | 1 | 0.289359 | 20.77909 | 0.001 |
| $X_2$ | 0.289359 | 1 | 0.122963 | 8.830034 | 0.014 |
| $X_3$ | 0.122963 | 1 | 0.007393 | 0.530898 | 0.4829 |
| $X_1X_2$ | 0.007393 | 1 | 0.007875 | 0.565518 | 0.4694 |
| $X_1X_3$ | 0.007875 | 1 | 0.00357 | 0.256373 | 0.6236 |
| $X_2X_3$ | 0.00357 | 1 | 0.00012 | 0.008626 | 0.9278 |
| $X_1^2$ | 0.00012 | 1 | 0.206945 | 14.86083 | 0.0032 |
| $X_2^2$ | 0.206945 | 1 | 0.681148 | 48.91368 | 0.0001 |
| $X_3^2$ | 0.681148 | 1 | 0.015424 | 1.107597 | 0.3174 |
| Residual | 0.139255 | 10 | 0.013926 | | 0.0236 |
| Lack of fit | 0.122572 | 5 | 0.024514 | 7.34696 | |
| Pure error | 0.016683 | 5 | 0.003337 | | |
| Cor total | 0.139255 | 19 | | | |
| Model | 1.238454 | 9 | 0.137606 | 9.881575 | 0.0007 |

**Table 5.** Comparison between the predicted value and observed value for the response variables.

| Response | Predicted value ($mg \cdot g^{-1}$) | Observed value ($mg \cdot g^{-1}$) | Optimized condition | | |
|---|---|---|---|---|---|
| | | | $X_1$ | $X_2$ | $X_3$ |
| Y | 4.72 | 4.68 | 74.14 | 30.31 | 68.14 |

and the levels of the variables studied in this paper, and the consequent responses are shown in Table 2.

## Optimization of extraction by RSM

Proceeding with exploration and optimization of a fitted response, surface will likely give poor or misleading results unless the model is an adequate fit. Thus, it is always necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and verify that none of the least squares regression assumptions are violated (Myer et al., 1995). Analysis of variance showed that $R^2$ value of the fitted model for the yield of salidroside was determined as 0.8989, which indicated that the regression models defined well the true behavior of the system (Table 4). The test for lack of fit compares the variation around the model with pure variation within replicated observations, which measures the adequacy of the quadratic response surface model. The *P*-value for this test was large ($P$ = 0.0236), implying that the quadratic model is adequate (Table 4).

The optimum regression equation of salidroside is as follows:

$$Y = 4.1667 + 0.1438X_1 + 0.0937X_2 - 0.0230X_3 - 0.1146X_1^2 - 0.2080X_2^2 - 0.0313X_3^2 - 0.0314X_1X_2 + 0.0211X_1X_3 - 0.0039X_2X_3$$

Where $X_1$ is the ethanol conceSntration, $X_2$ is the liquid-solid ratio and $X_3$ is the extraction time.

The response of the experimental and value composite scores in the regression equation predicted that the correlation coefficient was 0.9993 ($R^2$ = 0.9993), showing that the model fitted well and could explain 99.93% of the change of the response value. Analysis of variance results showed that the F value of the overall model test showed a significant equation ($P < 0.05$).

The three main factors of interaction effects on the response value are shown in Figures 3 to 5 by the 3-D graphic, although the graphics directly reflected the

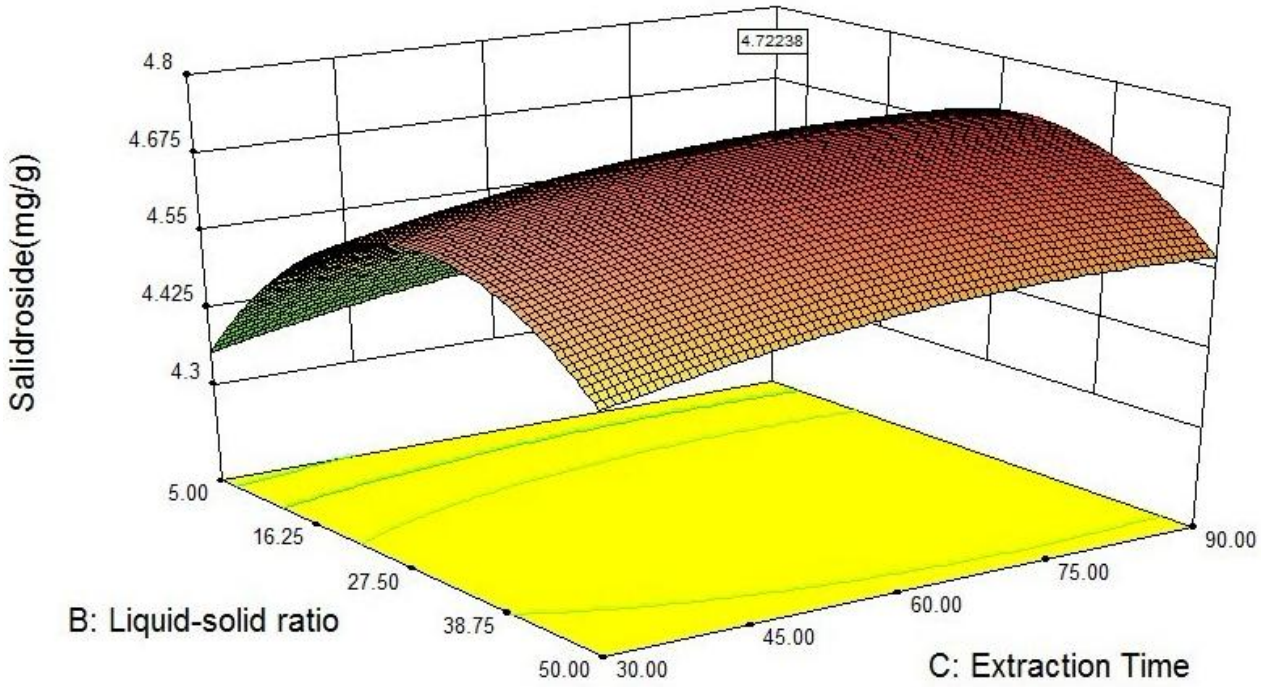


**Figure 3.** Effects of Solid-liquid ratio and extraction time on extraction yield.

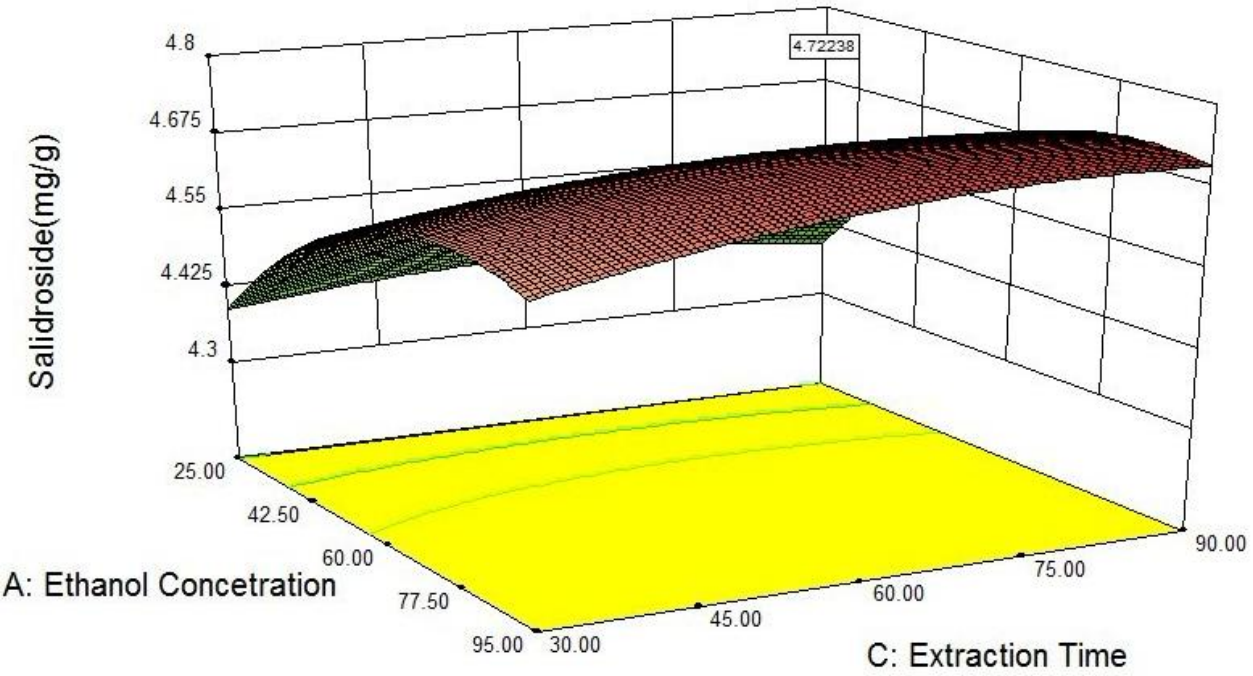


**Figure 4.** Effects of ethanol concentration and extraction time on extraction yield.

interaction of various factors on the response values. The graphics showed that the ethanol concentration and liquid-solid ratio had the highest significant extraction on the extraction yield of salidroside, showing a steep curve, while the lowest significant was the extraction time. However, the corresponding curves were shown to be relatively smooth, with the increase or decrease of the extraction time, while the response value did not change

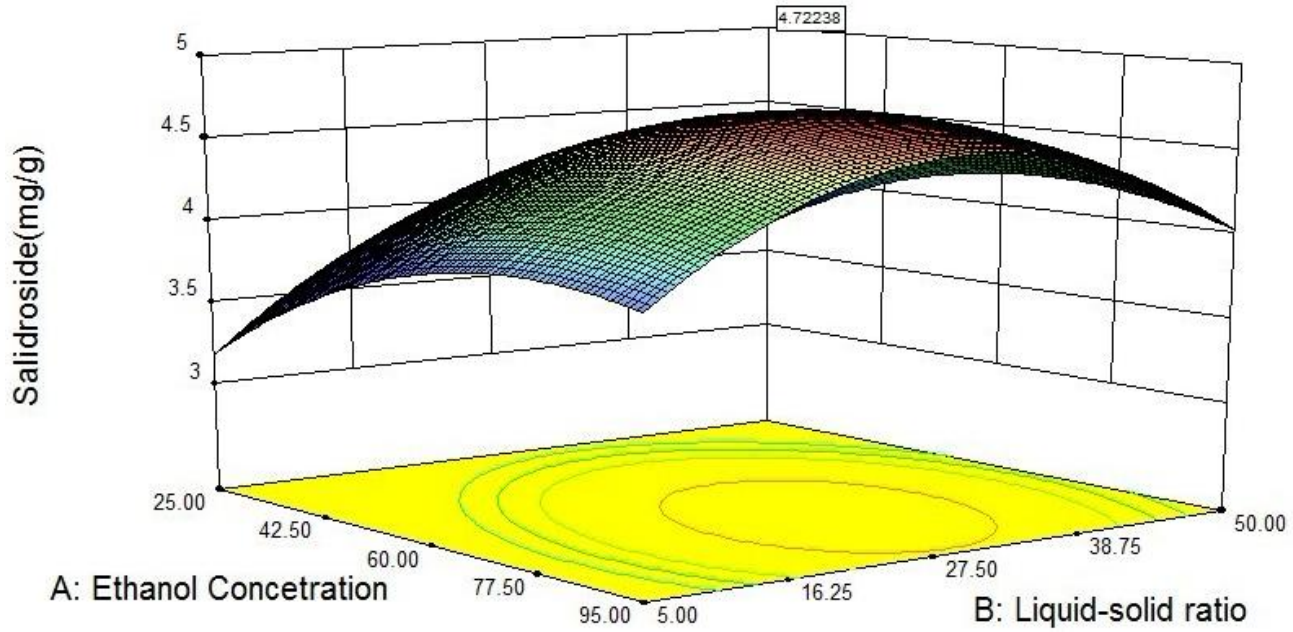


**Figure 5.** Effects of ethanol concentration and solid-liquid ratio on extraction yield.

significantly.

## Experimental validation of the optimal conditions

For solving equations, the Design-Expert 8.0.5 software was used to obtain the optimal extraction conditions, which were: ethanol concentration of 74.14%, liquid-solid ratio of 30.31 and extracting time of 68.14 min. In order to validate the predictive capacity of the model, the optimal extraction conditions to obtain the highest response variable were determined in the range of experimental conditions by prediction of computing program as shown in Table 5. It was validated after extraction of salidroside under this optimal condition. The result has no significantly different to the predicted value within 95% mean confidence interval. These results demonstrated the predictability of the model for the extraction of salidroside from *L. lucidum* in the experimental condition used.

## Conclusion

An adequate quadratic polynomial model for predicting the values of salidroside yield from *L. lucidum* by using UAE was determined according to the optimization designs. Student's *t*-test indicated ethanol concentration and liquid-solid ratio significantly influenced the extraction efficiency, while extraction time does not. The optimal condition derived from RSM was determined to be ethanol concentration of 74.14%, liquid-solid ratio of 30.31 and extraction time of 68.14 min. Under the optimal condition, the experimental value agreed with the predicted value by analysis of variance. For the quantitative determination of salidroside, an analytical method was developed by using RP-HPLC. The validation of this method showed good results of linearity, precision and accuracy.

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