



## Optimization of extraction process of hypericin from *St. John's Wort* by central composite design-response surface methodology

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### ABSTRACT

The aim of present work was to find out the optimum combination of extraction process variables of hypericin from *St. John's wort*, by revealing the mathematical functional relation of extraction temperature, ethanol concentration for the extraction yield, and to develop a validated HPLC method to determine hypericin in *St. John's wort* extracts prepared. The HPLC separation was performed on a Luna C<sub>18</sub> column (4.6 mm×250 mm, 5µm particle) using a mobile phase consisting of 2.5g•L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>- methanol (5:95 v/v) eluted in an isocratic mode, a flow rate of 1.0 ml/min, and a detection wavelength of 588 nm. The method is validated with respect to accuracy, precision, linearity, and limits of detection and quantitation. The experiments were carried out according to a five-level, two-factor Central Composite Design (CCD) with extraction temperature and ethanol concentration as variables while extraction yield as response. The optimum process was obtained through response surface methodology (RSM) based on the mathematical model established according to the experimental data, i.e. extraction temperature: 90 °C; ethanol concentration: 77%. Under the conditions above, the mean value of observed extraction yields(n=3) was 0.6767 mg/g of *St. John's wort* powder tested, with the deviation of 1.7% from the predicted value of 0.6654, which demonstrated that the mathematical model established in this study has satisfactory predictability. On the basis of result obtained, it was concluded that central composite design is a good approach to optimize the extraction of *St. John's wort*.

**Key words:** *St. John's wort*; Extraction process; HPLC; Central composite design; Response surface methodology

### INTRODUCTION

*St. John's wort* (*Hypericum perforatum* L.), a herbaceous perennial plant, belonging to Europe, Asia, and North Africa, naturalized to North and South America and Australia, has become one of the leading plant-based dietary supplements worldwide. The Greeks and the Romans documented its medicinal use in the treatment of nerve-related disorders in antiquity. Nowadays the use of *Hypericum* extracts is concerned mainly with anti-depressive applications (1-4).

As acting as an alternative herb medicine for the treatment of depression, *St. John's wort* has been found to be superior to placebo and equivalent to standard antidepressants for the treatment of mild to moderate depression. The randomized controlled studies have also provided evidence that *Hypericum perforatum* extracts are as effective as standard antidepressants in mild- to-moderate depression (4).

What's more, *St. John's wort* extracts are used not only as herbal medicinal products but also as a top-selling botanical dietary supplement by standardised using the naphthodianthrone of the hypericin group, calculated as 0.2–1 mg hypericin daily dose. Finally, *St. John's wort* preparations have recently been used as an ingredient in some food products sold as functional foods (5).

The main active components of *St. John's wort* are thought to be hypericin and hyperforin (6). The hypericin compounds exhibit photodynamic, anti-depressive and antiviral activities and produce a significant amount of

reactive oxygen species upon photo-activation, which can cause damage to areas of the body exposed to light (7-9). In addition, many of the more common plant constituents (e.g., flavonoids and flavonoid derivatives, xanthone derivatives, amentoflavone, biapigenin, volatile oil) present in *St. John's wort* have been found to possess antioxidant activity, and thus it may be possible that the oxidative damage produced by hypericin can be mediated through ROS scavenging by these constituents present in preparations made from *St. John's wort* extract (10-11). However, most available *St. John's wort* formulations are now standardized to include hypericin and hyperforin because these constituents have been researched the most extensively. The structure of hypericin is shown in Fig. 1.

Considering the upsurge interest and increasing market demand for *St. John's wort* extracts, this work aimed to develop a rapid HPLC assay method to quantify the hypericin in *St. John's wort* extracts, and then, to optimize the extraction process of hypericin from *St. John's wort* by HPLC, using response surface methodology.

## EXPERIMENTAL SECTION

### Materials

Hypericin (purity >98%) was purchased from Xi'an xiaocao botanical Co., Ltd. (Shanxi, China). The commercial dried aerial parts of *St. John's wort* (*Hypericum perforatum* L.) were obtained from Chengdu herb market (Chengdu, China) and authenticated by department of Pharmacognosy, School of Life Science and Engineering, Southwest Jiaotong University, pulverized and sieved through a 100-mesh sieve before use. Methanol and potassium dihydrogen phosphate were of HPLC grade and were purchased from Kemiou Co., Ltd. (Tianjin, China). All other reagents or solvent were of analytical grade. Water was purified by a Milli Q plus system from Millipore (Bedford, MA, USA).

### HPLC assay

#### *Chromatography conditions*

Hypericin was quantified using a Shimadzu HPLC system (Tokyo, Japan) consisting of a Shimadzu LC-20AT pump, a Shimadzu spectrophotometric detector SPD-20A, a 20- $\mu$ L sample loop, a manual injector, a column heater, and a Luna C18 column (4.6mm $\times$ 250 mm, 5  $\mu$ m particle) (Phenomenex, Guangzhou, China). Chromato solution Light software package was used for data analysis and processing.

For the preparation of the mobile phase, 2.5 g potassium dihydrogen phosphate was diluted and filled up to 1000 mL with ultrapure water. A 50-mL volume of this aqueous solution was combined with 950 mL methanol to constitute the mobile phase (12). The detection wavelength was 588nm. The flow-rate was 1.0 mL/min. The analytical column was kept at 30 °C. The substances were quantified using peak area.

#### *Preparation of standard stock solutions*

Stock solution of 50  $\mu$ g $\cdot$ mL<sup>-1</sup> of hypericin was prepared in a mixed solvent (methanol-pyridine, 9:1, v/v), into a 25-mL brown volumetric flask. The solution was stored at 4 °C.

#### *Preparation and assay of Sample solution*

About 25 g of the *St. John's wort* powder was accurately weighed, transferred into a 500-mL round bottom flask containing 250 mL of the ethanol-water mixture of corresponding concentration, and extracted for 120 min by heat-recirculation extraction at the corresponding temperature (the corresponding ethanol concentration and extraction temperature of each experimental run were shown in Table II). The extract solution was filtered through cellulose paper by vacuum filtration into a 250-mL volumetric flask. The round bottom flask was rinsed with extra ethanol and the washings were passed through the same filter to bring the filtrate to the graduation volume of the volumetric flask. The solution was passed through a 0.45  $\mu$ m millipore filter, and 10  $\mu$ L of the filtered sample solution was injected into the HPLC system. The whole procedure was performed under strict exclusion of light.

#### *Assay and validation*

The linearity of detector response to different concentrations of hypericin was evaluated by preparing standard solutions at six different levels ranging from 5 $\mu$ g $\cdot$ mL<sup>-1</sup> to 50  $\mu$ g $\cdot$ mL<sup>-1</sup>. The assays were processed in triplicate on a daily basis. Standard curve of peak area versus concentration was plotted. The linear regression equation and the linear range of six of hypericin were determined using the developed HPLC method.

To determine the Limits of detection (LOD) and quantification (LOQ), a part of the stock solution was diluted with solvent (methanol-pyridine, 9:1, v / v) to appropriate concentration, and an aliquot of the diluted solution was injected into the HPLC for analysis. Limits of detection (LOD) and quantification (LOQ) for hypericin were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

The sample solution was put in the dark at 4 °C and brought to room temperature for analysis on three consecutive days (24, 48 and 72 h) to observe the stability of sample solutions, the procedure was performed six times every day, and the intra- and inter-day RSD was calculated.

The intra- and inter-day variations were chosen to determine the precision (reproducibility) of the method, respectively. For intra-day variability tests, the standard solutions were analyzed six times at three different amounts within one day, while for inter-day variability tests, the samples were examined in triplicate on three consecutive days. Accuracy was defined as the percentage difference between the observed concentration and calculated amount of hypericin, and was always expressed as recovery. The recovery was determined by adding a known amount (low, medium and high level of 1, 2, 3 µL of stock solution) of hypericin standard to the sample solution prepared. The mixture was analyzed using the sample assay method mentioned above.

The hypericin content of each analyte was calculated from the calibration curve. Variations were expressed as the relative standard deviations (RSD, %) and relative errors (RE, %).

### Experimental design

A two factor three level Central Composite Design (CCD), which was a simplified representation in analytical form of a given reality, was used for the optimization procedure. As shown in Fig. 2, the design consisted of four factorial points, four axial points (two nearby axial points on the axis were at a distance of 1.414 from the design center) and six center points, leading to 14 sets of experiments (13, 14). This design could provide an empirical second order polynomial model used for prediction of the effect of extraction variables on the extraction yield using a number of experimental runs. In this mathematical approach the experimental response variable  $Y$  can be represented by quadratic equation of the response surface as shown in Eq. 1.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1^2 + b_4X_2^2 + b_5X_1X_2 \quad (1)$$

Where  $Y$  is the response variable,  $b_0, b_1, b_2, b_3, b_4, b_5$  are the regression coefficients of variables for corresponding terms, respectively.  $X_1$  and  $X_2$  are independent variables. The equation enables the analysis of the effects of each factor and their interactions over the response.

Due to its merits of non-toxicity and easy recovery by reduced pressure, ethanol is widely used as extraction solvent on large industrial scale, at the same time, hypericin shows good dissolubility in ethanol, thus ethanol of different concentrations were used as the extraction solvent in the following study. Generally, extraction temperature, extraction time, solvent volume, and solvent concentration are the effective factors of solvent extraction efficiency. However, the extraction yield always increases with the increase of extraction time and solvent volume, the extraction time and solvent volume seem not the critical factors. Therefore, the extraction temperature ( $X_1$ ) and ethanol concentration ( $X_2$ ) were chosen as factors in this experimental design. According to our preliminary experimental results and the principle of CCD, five levels of each factor were defined as shown in Table I. The experimental sets of runs are shown in Table II (each set was performed in triplicate, and the mean value was expressed as the final extraction yield), and the extraction yields of fourteen runs were determined by the HPLC method mentioned above.

Table I. The independent variables: factors and levels for CCD

Factors	Ranges and levels				
	-1.41421(- $\alpha$ )	-1	0	1	1.41421(+ $\alpha$ )
Extraction temperature $X_1$ / °C	50	55.86	70	84.14	90
Ethanol concentration $X_2$ / %	55	60.86	75	89.14	95

## RESULTS AND DISCUSSION

### HPLC assay validation

#### Separation and specificity

Hypericin was well separated under the developed HPLC conditions, the retention time was about 15 min for hypericin. Figs. 3–4 show the chromatograms of standard hypericin and aquatic-ethanol extracts of *St. John's wort*, respectively.

#### Limit of detection (LOD) and limit of quantitation (LOQ)

The limits of detection and quantification were less than  $1.8 \times 10^{-2} \mu\text{g} \cdot \text{mL}^{-1}$  and  $5.9 \times 10^{-2} \mu\text{g} \cdot \text{mL}^{-1}$  for hypericin, respectively.

**Linearity**

The regression equation, with hypericin concentration ( $\mu\text{g} \cdot \text{mL}^{-1}$ ) as independent and peak area(y) as dependent, calculated for the hypericin calibration curve by using linear regression analysis, was  $y = -4138.24 + 131658.44x$  ( $r = 0.99984$ ,  $n = 6$ ,  $0.072 - 0.72 \mu\text{g}$ ). The correlation coefficient value indicated satisfactory linear correlation between hypericin amount and its peak area within the test range.

**Stability**

The RSD of six determinations of the same sample solution within 12 hours was less than 1.73%, which indicated that the sample solution had good stability. Further study showed that sample solutions were stable for at least one week when stored out of light at 4 °C.

**Precision and accuracy**

The reproducibility (precision) of the proposed method was evaluated: the intra-day RSD ranged 2.0–3.6% ( $n = 6$ ). The inter-day RSD ranged 1.6–4.8% ( $n = 6$ ). The developed method had good accuracy with the mean recovery of 97.7% for hypericin.

The overall results above indicated that this HPLC method was precise, accurate, and sensitive for quantitative determination of hypericin.

**Optimization procedure***Fitting of experimental data to the model*

The experimental design was performed using Design-expert 7.1.3 software (Stat-Ease Co., USA), while the analysis of data was performed by Statistica 7.0 software (StatSoft Co., USA). The experimental results of fourteen runs arranged by the experimental design are shown in Table II. The data were fitted to the quadratic model for response Y using Statistica 7.0 software, and the fitting results are shown in Table III. With a confidence level of 95%, the quadratic polynomial model was calculated as Eq. 2, from it, the response factors at any regime within the interval of our experiment design can be calculated.

$$Y = -3.36786 - 0.01865X_1 + 0.1176X_2 + 1.13677 \times 10^{-4}X_1^2 - 8.0707 \times 10^{-4}X_2^2 + 7.53 \times 10^{-5}X_1X_2 \quad (2)$$

**Table II. Experimental matrix and results**

Std.	Run	Block	Coded factor value		Actual factor value		Extraction yield
			$X_1$	$X_2$	$X_1^a$	$X_2^b$	
2	1	Block1	1	-1	84.1	60.8	0.4417
5	2	Block1	-1	-1	70	75	0.5416
4	3	Block1	-1	1	84.1	89.1	0.5267
7	4	Block1	0	0	70	75	0.5397
6	5	Block1	0	0	70	75	0.5849
3	6	Block1	1	1	55.8	89.1	0.4001
1	7	Block1	0	0	55.8	60.8	0.3757
9	8	Block1	0	- $\alpha$	90	75	0.6341
10	9	Block1	0	0	70	55	0.1841
14	10	Block1	0	0	70	75	0.5684
8	11	Block1	+ $\alpha$	0	50	75	0.5376
13	12	Block1	- $\alpha$	0	70	75	0.5785
12	13	Block1	0	+ $\alpha$	70	75	0.5368
11	14	Block1	0	0	70	95	0.2507

<sup>a</sup> The extraction temperature (°C).

<sup>b</sup> The ethanol concentration (%).

<sup>c</sup> Weight hypericin / weight St. John's wort powder (mg/g).

**Table III. Regression equations obtained for Y (for actual factor value)**

Model Style	Regression Model	R-square	F-value	P-value
Quadratic	$Y = -3.36786 - 0.01865X_1 + 0.117589X_2 + 1.13677 \times 10^{-4}X_1^2 - 8.0707 \times 10^{-4}X_2^2 + 7.52985 \times 10^{-5}X_1X_2$	0.97664	861.5039	<0.0001

**Analysis of variance (ANOVA) and model validation**

The coefficients of the nonlinear polynomial model (Eq. 2), and their t-values and p-values are shown in Table IV, where the standard error of coefficient is a measure of the variation in estimating the coefficient and is the ratio of the coefficient to the standard error. Regarding to the analysis of variance for Y, various statistical data (sum of

squares, degree of freedom, mean squares, F-value, p-value of the regression) were examined as shown in Table V.

Experimental results and the predicted values obtained from model (Eq. 2) are given in Fig. 5. As can be seen, the predicted values match the experimental values well with R-square of 97.6% for response Y. Fig. 6 depicts the residual plots for Y in the model (Eq. 2), it shows that the distribution of the residuals for the response approximately follows the fitted normal distribution and the residuals of the response randomly scatter in the residual plots.

**Table IV. Estimated regression coefficients for Y (Level of confidence: 95.0%, alpha=0.05)**

Term	Coefficients	Standard error	t-value df=8	p-level	Lo. Conf Limit	Up. Conf Limit
Constant	-3.36786	0.495506	-6.7968	0.000138	-4.51050	-2.22523
$X_1$	-0.01865	0.008213	-2.2707	0.052829	-0.03759	0.00029
$X_2$	0.11759	0.008417	13.9703	0.000001	0.09818	0.13700
$X_1^2$	0.00011	0.000047	2.3998	0.043188	0.00000	0.00022
$X_2^2$	-0.00081	0.000047	-17.0661	0.000000	-0.00092	-0.00070
$X_1X_2$	0.00008	0.000064	1.1749	0.273832	-0.00007	0.00022

**Table V. Analysis of variance for Y**

Factor	Sum of Squares	Degree of freedom	Mean Squares	F-value	P-value
$X_1$	0.013466	1	0.013466	20.3000	0.001987
$X_1^2$	0.003820	1	0.003820	5.7592	0.043188
$X_2$	0.005172	1	0.005172	7.7959	0.023481
$X_2^2$	0.193207	1	0.193207	291.2533	0.000000
$X_1X_2$	0.000916	1	0.000916	1.3803	0.273832
Error	0.005307	8	0.000663		
Total	0.227201	13			

#### *Response surface (contour) plots*

In order to gain insight about the effect of each variable, the three-dimensional surface and contour showing the influence of independent variables  $X_1$  and  $X_2$  upon the response Y were respectively depicted in Fig. 7 and Fig. 8.

#### *Optimization of extraction condition*

As shown in Eq.2, coefficients with one factor represent the effect of that particular factor on response while the coefficients with more than one factor and those with second order terms represent the interaction between those factor and the quadratic nature of the phenomena, respectively. Positive sign in front of the terms indicates synergistic effect while negative sign indicates antagonistic effect upon the response. Therefore, from the Eq.2, it can be qualitatively concluded that  $X_2$ ,  $X_1^2$ , and the interaction between  $X_1$  and  $X_2$  had synergistic effects on the response of Y, on the contrary,  $X_1$  and the quadratic term of  $X_2^2$  had antagonistic effects.  $X_2$  was the most important parameter to affect the extraction yield, followed by  $X_1$ ,  $X_1^2$  and  $X_1X_2$ .

The response surface and contour plots shown in Fig. 7 and Fig. 8 demonstrate that, the extraction yield (Y) is increasing at first, then decreasing with the increase of ethanol concentration at the range of 55%–95%; the extraction yield (Y) is increasing slowly and continuously with the increase of extraction temperature at the range of 50 °C–90 °C. The response surface has the maximum point where  $X_1=90$ . Therefore, the optimum ethanol concentration( $X_2$ ) can be found depending on Eq.3.

$$\left[ \frac{\partial Y}{\partial X_2} \right]_{X_1=90} = 0 \quad (3)$$

Response optimization results of the nonlinear polynomial model are as follows: the response Y presents the predicted maximum value 0.6654 at the optimum parameters of  $X_1$  (90),  $X_2$  (77), i.e. extraction yield shows maximum value of 0.6654 mg/g under the conditions of extraction temperature 90 °C, ethanol concentration 77 %.

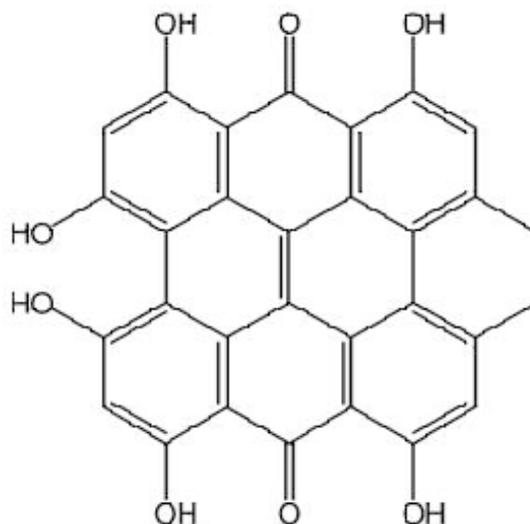
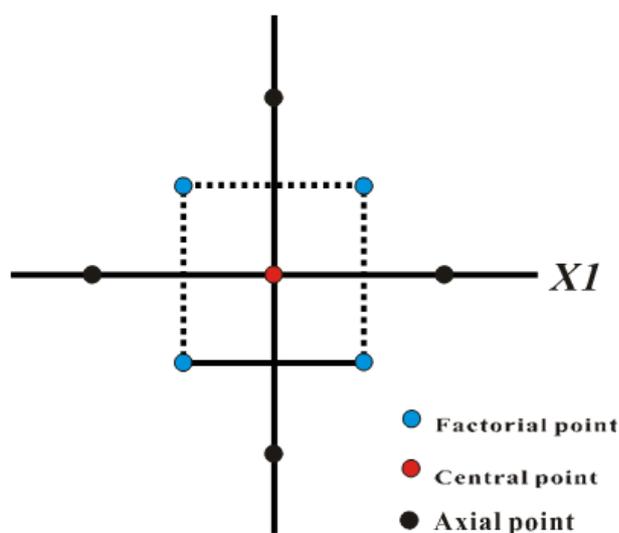
*Validation of the optimum condition*

To compare the observed extraction yield with the predicted one, experimental rechecking was performed under the deduced optimum extraction conditions shown in Table VI. A mean value of 0.6767 mg hypericin/g *St. John's wort* powder (n=3) obtained from actual experiments, with a low percentage of bias from the predicted one, showed that the observed value matched the predicted value reasonably well, and it could be concluded that the mathematical model was chosen correctly and that the model had satisfactory predictive power.

**Table VI.** Validation test results based on optimized extraction condition (n=3)

No.	Observed values (mg/g)	Predicted value (mg/g)	Deviation (%) <sup>a</sup>
1	0.6712	0.6654	0.87
2	0.6807		2.30
3	0.6783		1.94
Mean Value	0.6767		1.70

<sup>a</sup>Deviation = (Observed value - Predicted value) / Predicted value.

**Fig. 1.** Chemical structure of hypericin**Fig. 2.** Schematic diagram of central composite design (CCD) as a function of  $X_1$  and  $X_2$  according to the  $2^2$  factorial design with four axial points and six central points (k=2, replication)

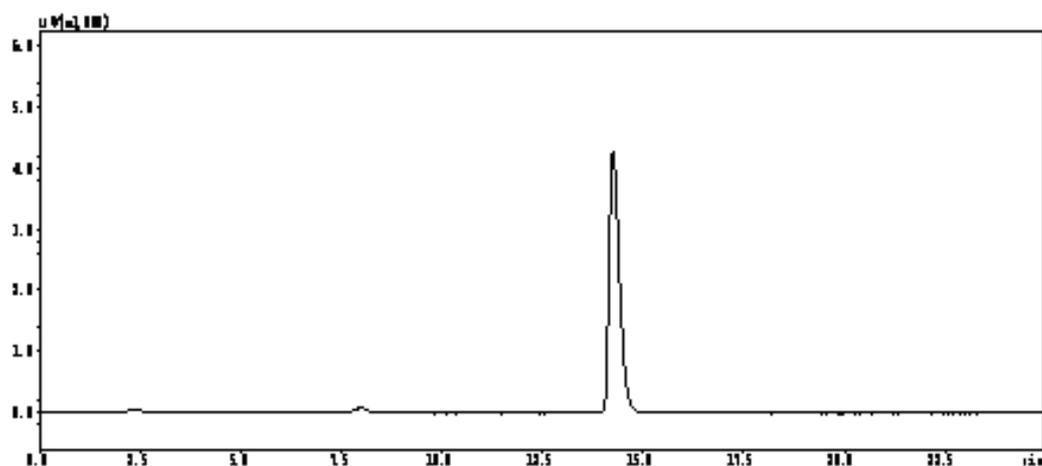


Fig. 3. Hypericin reference standard

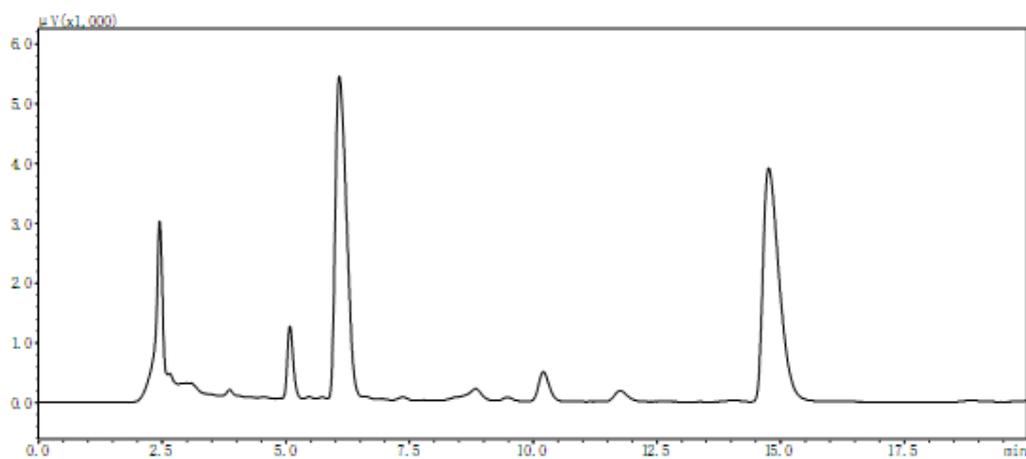


Fig. 4. A typical HPLC chromatogram of aquatic-ethanol extracts of St. John's wort

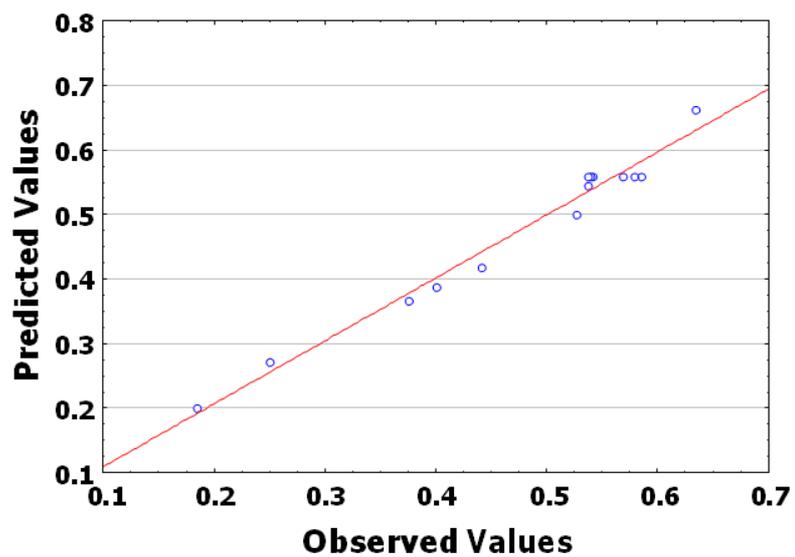


Fig. 5. Relation between experimental and predicted extraction yield basing on Eq. 2

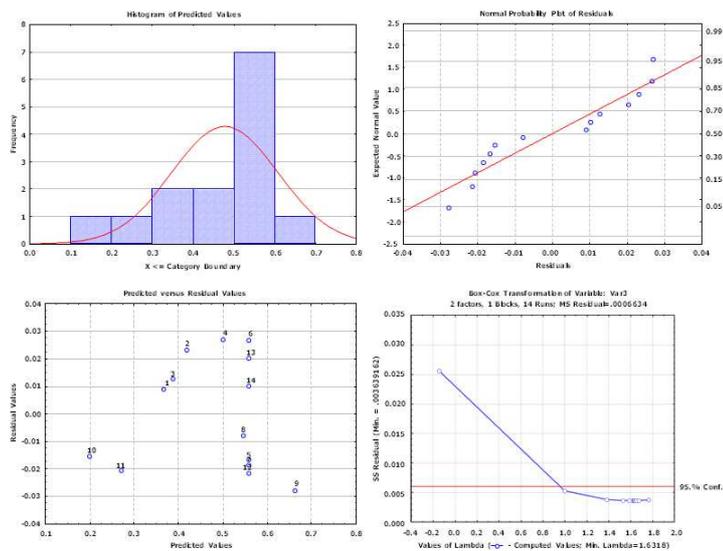


Fig. 6. Residual plots for  $Y$  (extraction) in the model (Eq.2)

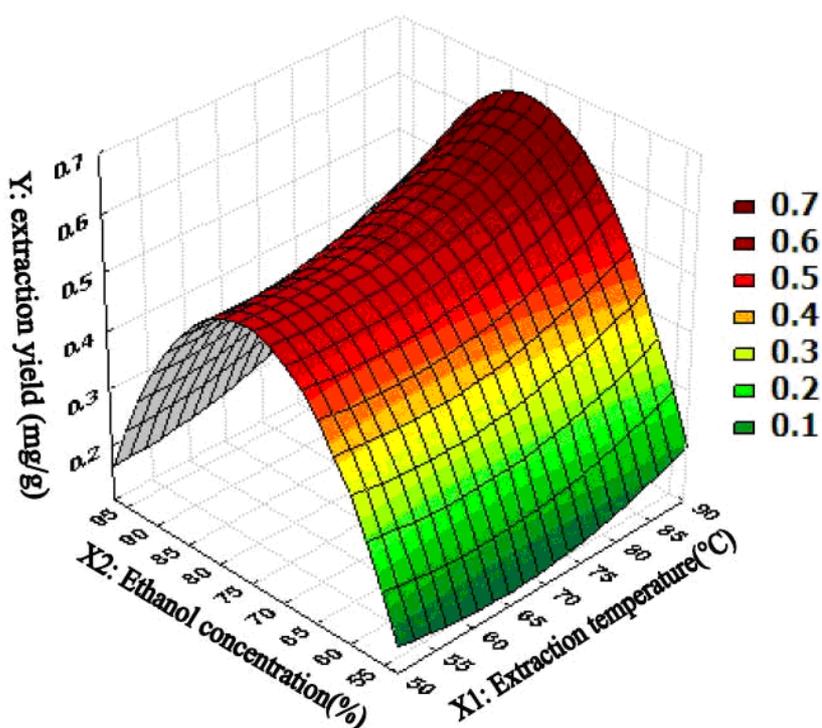


Fig. 7. The three-dimensional (3D) plots for extraction yield ( $Y$ ),  $X_1$ : Extraction temperature ( $^{\circ}\text{C}$ );  $X_2$ : Ethanol concentration(%)

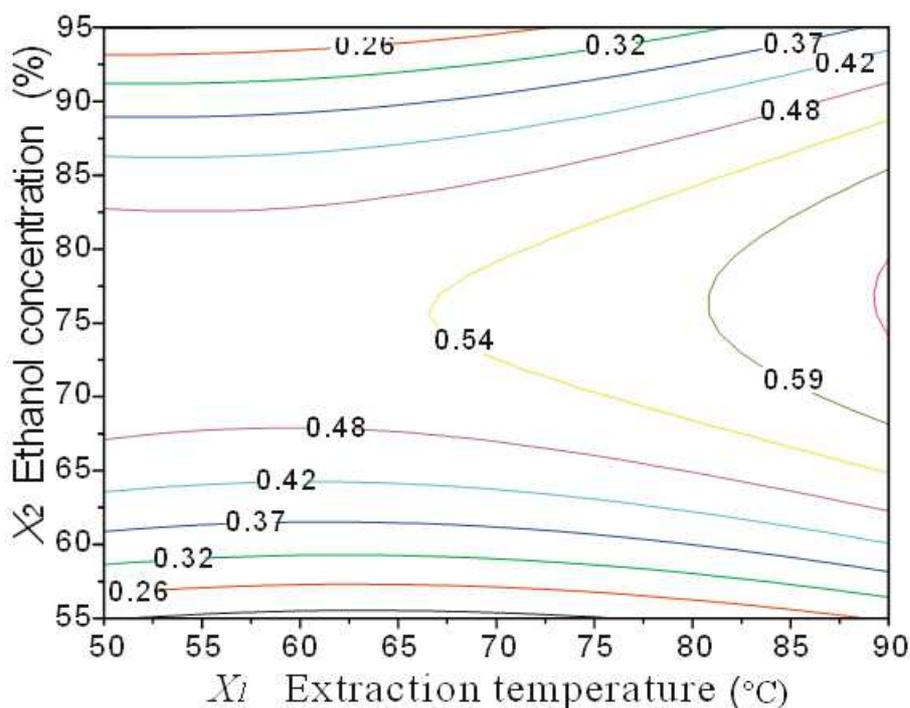


Fig. 8. The contour (2D) plots of Content (Y)  $X_1$ : Extraction temperature (°C);  $X_2$ : Ethanol concentration(%)

### CONCLUSION

This work used central composite to design the experiment, and it avoid the deficiency of uniform design and orthogonal test that can't predict the best value of the factor when continuous valued are from an extent range. A HPLC method established in this work was proved to be specific and suitable for routine analysis because of its simplicity and reproducibility. The mathematical model established in this work had satisfactory predictability on *St. John's wort* extraction yield.

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