Optimization of Conditions for Charantin Extraction in PEG/Salt Aqueous Two-Phase Systems Using Response Surface Methodology

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Abstract: *Momordica charantia*, commonly known as karela or bitter melon, is used as a vegetable in Chinese, Indian subcontinent. However, in Chinese traditional medicine, the plant is usually used as an antidiabetic agent. One of the active components is charantin. Aqueous two-phase systems (ATPS) were used for the purifying saponin because of their low cost. ATPS can reduce amount of organic solvent and the danger of explosion necessitates special equipment leading to increased costs. The levels of blood glucose decreased after administration of charantin purified from *Momordica charantia* with (ATPS *(p<0.05)*.

Key Words: *Momordica charantia*, charantin, ATPS, RSM, extraction.

1. INTRODUCTION

Momordica charantia (family, Cucurbitaceae), commonly known as karela or bitter melon (Japanese name "Tsurureishi"), is used as a vegetable in Chinese, Indian subcontinent, South America. The fruit of this plant has been used as a bitter stomachic, a laxative, an antidiabetic, and an anthelmintic for children.

In the orient, *Momordica charantia* is not only a popular vegetable crop, but also a source of traditional medicinal ingredient , having hypoglycemic , antiviral , antidiabetic , and antitumorigenic functions [1, 2]. The extracts of the fruit pulp and seed have been reported to have various medicinal properties, including antitumoir and antimutagenic activities. However, in Chinese traditional medicine, the plant is usually used as a hypoglycemic and antidiabetic agent and many components have been identified from *M. charantia,* which possess hypoglycemic properties. One of the active components responsible for this action is charantin. The methods for the separation and purification of charantin from *Momordica charantia* have been proposed. Generally, several solvents with different polarities should be used to separate it from other contaminants. The yield and/or purity of the charantin are relatively low. Furthermore, such processes in industrial applications inevitably consume a massive amount of organic solvent and the danger of explosion necessitates special equipment leading to increased costs.

Aqueous two-phase systems (ATPS), especially poly (ethyleneglycol) (PEG) /salt systems, have been widely used for the purifying biological materials in biochemistry and biotechnology because of their low cost and wide range of hydrophobicity differences between the two-phase systems [3, 4]. Employing these phase systems can enhance the partition selectivity of target products, especially for hydrophobic products [5]. Numerous reports dealing with macromolecule partition in an aqueous two-phase system have been published, mostly involving proteins or enzymes. Few cases of the partition of small molecules were studied. The present work studies the partition behavior of charantin in a small-scale aqueous two-phase extraction system. Before such a scheme can be developed, it is necessary to determine the distribution of these molecules in an aqueous two-phase system and how to manipulate the system to achieve the desirable partition. Thus, response surface methodology (RSM) had been considered as a useful method to optimize the extraction condition for production of charantin.

Response surface methodology (RSM), which has been extensively applied in optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing processes [6, 7], is a collection of statistical techniques for experiment designing, model developing, factors evaluating, and optimum conditions searching. But it has not yet been reported as a means to optimize conditions of charantin from *Momordica charantia*.

In this paper, we describe the direct extraction of charantin from *Momordica charantia* using the property that the PEG/salt aqueous two-phase systems can extract hydrophobic proteins. The objective of this study was to investigate and optimize the operating conditions of the charantin extraction in *Momordica charantia* using RSM.

2. METHODS AND MATERIALS

2.1. Chemicals and Reagents

PEG 4000 was obtained from Fluka. K_2HPO_4 was from Beijing Chemical Reagent Co. and were of analytical grade. The Ginsenoside Rg1 used as standard, was obtained from National Institute for the Control of Pharmaceutical and Biological Products. Xiaoke pills were purchased from Jilin Liuhe Pharmaceutic Factory, China. Xiaoke pill is a kind of Chinese medicine used in the treatment of diabetes.

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2.2. Plant Material

Momordica charantia were collected in Jinan, China, in June 2006, and identified by Dr. Z. Feng, Shandong University of Traditional Chinese Medicine.

2.3. Preparation for Samples

The fine powders of *Momordica charantia* for extraction study were prepared by milling dry *Momordica charantia* with a mechanical grinder and sieving through a 20-mesh metal sieve. Fifty gram of powders was mixed with 1000 ml water. After boiling for 30–40 min, the mixture was subject to filtration with a 100-mesh metal sieve. The 245ml filtrate (crude water extract) were collected and used directly for further experiment.

2.4. Aqueous Two-Phase Extraction

An aqueous two-phase system was prepared by adding various amounts (indicated in the text) of PEG, K_2HPO_4 and ethanol to various amounts of crude water extract. The system was vortexed to form a homogeneous phase. Then it was centrifuged at 3000 *g* for 5 min to facilitate the phase separation. The charantin-containing salt-rich layer was separated and subject to back-extraction three times with 95% ethanol. The ethanol extracts were collected and kept at 4^0C overnight to allow salt to precipitate. After removing the precipitant by centrifugation, the amounts of charantin were analyzed by ultraviolet spectrophotometry.

2.5. Estimation of Hypoglycemic Activity of Charantin Prepared by Aqueous Two-Phase (CPATP)

Thirty-six hyperglycemic mice were allocated equally into 3 groups: Alloxan-induced hyperglycemic group, alloxan and Xiaoke Pill-treated group, alloxan and *CPATP* -treated groups. The other 12 normal mice were injected (iv) with the normal saline and used as the control group. From then on, the 4 groups of mice were administered (ig) saline, Xiaoke Pill (200mg/kg/d), CPATP, and saline respectively. On the 20th day, after fasting the mice for12 h, blood samples were obtained from the tail veins to determine the blood glucose levels [8].

2.6. Experimental Design and Evaluation

RSM was used to investigate the influence of various amounts of PEG, K_2HPO_4, ethanol and crude water extract (sample loading) on the yield of charantin. A Box-Behnken factorial design with four factors and three levels, including three replicates at the center point, was used for fitting a second-order response surface. Tables **1** and **2** gave the factors, their values, and the experimental design, respectively. A mathematical model, describing the relationships between the process indices (charantin) and the factors in second-order equation, was developed as follows:

$$Y = B_0 + \sum_{i=1}^{n} B_i X_i + \sum_{i=j=1}^{n} B_{ij} X_i X_j \qquad (1)$$

Where Y is the predicted response variable; B_0, B_i, B_{ij} are constant regression coefficients of the model, and X_i, X_j represent the independent variables in the form of coded values. The accuracy and general ability of the above polynomial

Table 1. Variables and Experimental Design Levels for Response Surface

Independent Variables	Coded Symbols	Levels		
		-1	0	1
K_2HPO_4 (g)	X_1	1.0	1.2	1.4
PEG (g)	X_2	0.96	1.12	1.28
Ethanol (ml)	X_3	0	0.2	4
Sample loading (ml)	X_4	6	9	12

Table 2. Box-Behnken Design Matrix and the Responses of the Dependent Variables Charantin

Runs	Independent Variables				Dependent Variables	
	X_1	X_2	X_3	X_4	Y_1	(A)
1	1.0	0.96	0.2	9	0.007
2	1.0	1.28	0.2	9	0.005
3	1.4	0.96	0.2	9	0.008
4	1.4	1.28	0.2	9	0.006
5	1.2	1.12	0.0	6	0.004
6	1.2	1.12	0.0	12	0.013
7	1.2	1.12	0.4	6	0.005
8	1.2	1.12	0.4	12	0.011
9	1.0	1.12	0.2	6	0.002
10	1.0	1.12	0.2	12	0.007
11	1.4	1.12	0.2	6	0.004
12	1.4	1.12	0.2	12	0.008
13	1.2	0.96	0.0	9	0.011
14	1.2	0.96	0.4	9	0.010
15	1.2	1.28	0.0	9	0.006
16	1.2	1.28	0.4	9	0.005
17	1.0	1.12	0.0	9	0.004
18	1.0	1.12	0.4	9	0.003
19	1.4	1.12	0.0	9	0.003
20	1.4	1.12	0.4	9	0.004
21	1.2	0.96	0.2	6	0.012
22	1.2	0.96	0.2	12	0.014
23	1.2	1.28	0.2	6	0.005
24	1.2	1.28	0.2	12	0.014
25	1.2	1.12	0.2	9	0.013
26	1.2	1.12	0.2	9	0.013
27	1.2	1.12	0.2	9	0.014

model could be evaluated by the coefficient of determination R^2.

2.7. Data Analysis

Data from the Box–Behnken factorial design shown in Table **2** were used for determining the regression coefficients of the second-order multiple regression model. The analysis of regression and variance (ANOVA) was carried out using the RSREG procedure of the SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA). The 3D response surface and contour plot analysis were made by keeping two independent variables at constant level and changing the other two independent variables, and then calculating the response variables using RSREG analysis.

3. RESULTS

Table **3** showed the analysis of variance (F-test) for this experiment, and the coefficient of determination (R^2) was shown as 96.52%. This indicated that, the accuracy and general ability of the polynomial model was good, analysis of the response trends using the model was considered to be reasonable.

The regression coefficients, along with the corresponding P-values, for the model of the charantin production, were also presented in Table **3**. The P-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable.

Table **3** showed that, the regression coefficients of X_2, X_4, X_1^2, X_3^2 and X_2X_4 were significant at 1% level, and X_4^2 was also significant at 5% level. So the polynomial model for charantin yield Y was regressed by only considering the significant terms and was shown as below:

$$Y=0.01333-0.00175X_2+0.0029167X_4 +0.00175X_2X_4 - 0.006167X_1^2-0.003917X_3^2- 0.001417 X_4^2 \quad (2)$$

The model (2) indicated that sample loading (X_4) had a significant effect (P < 0.001) on Y as it had the largest coefficient followed by PEG (X_2). Positive coefficient indicated a linear effect to increase Y. However, negative effects indicated a linear effect to decreases Y. The quadratic terms (X_1, X_2, X_3) had negative effects while interaction term (X_2X_4) had positive effects on Y.

The 3D response surface based on independent variables PEG (X_2) and sample loading (X_4) was shown in Fig. (**1**),

Table 3.   Results of Regression Analysis of a Full Second-Order Polynomial Mode for Optimization of Production of Charantin

Source	DF	Estimate	Std Err	F	Pr > F
X1	1	0.000417	0.000317	1.724138	0.213716
X2	1	-0.00175	0.000317	30.41379	0.000133**
X3	1	-0.00025	0.000317	0.62069	0.446075
X4	1	0.0029167	0.000317	84.48276	0.0001**
X1*X1	1	-0.006167	0.000476	167.8467	0.0001**
X1*X2	1	0	0.0005	0	1
X1*X3	1	0.0005	0.00055	0.827586	0.380881
X1*X4	1	-0.00025	0.00055	0.206897	0.65733
X2*X2	1	-0.000917	0.000476	3.708812	0.078146
X2*X3	1	-2.17E-19	0.00055	1.56E-31	1
X2*X4	1	0.00175	0.00055	10.13793	0.007862**
X3*X3	1	-0.003917	0.000476	67.70881	0.0001**
X3*X4	1	-0.00075	0.00055	1.862069	0.197429
X4*X4	1	-0.001417	0.000476	8.858238	0.011565*
Model	14	0.000402	0.000029	23.73837	0.0001**
Error	12	0.000014	1.208E-6		
Total	26	0.000416			
R-square	96.52%		Adj.R-square	92.45%	

* Significant at 5% level.
** Significant at 1% level.

while the other independent variables, K_2HPO_4 (X_1) and ethanol (X_3) were kept at a constant level. The interaction relationship between the two independent variables (X_2, X_4) can be easily understood by examining the contour plots generated by keeping independent variables (X_1, X_3) as constant (Fig. **2**). Canonical analysis revealed a maximum production yield of charantin of 0.15, could be achieved at the point when K_2HPO_4, PEG, ethanol and sample loading were set at 1.2g, 1.12g, 0.2ml, 12ml, respectively.
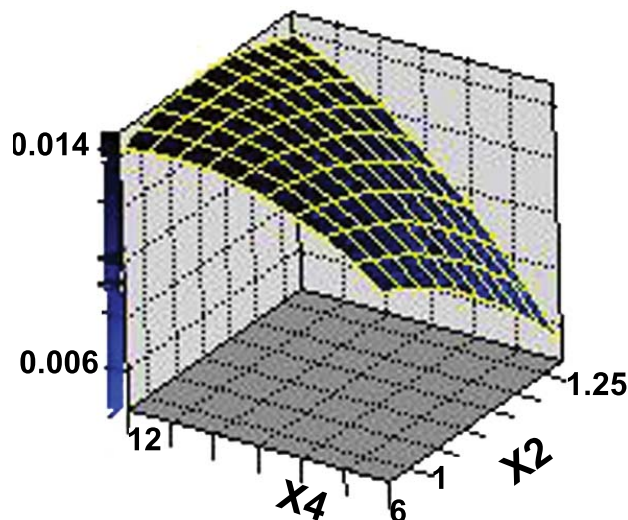
Fig. (1). Surface plot of the combined effects of PEG (X_2) and sample loading (X_4) on the yield of charantin (X_1=1.2g, X_3=0.2g).
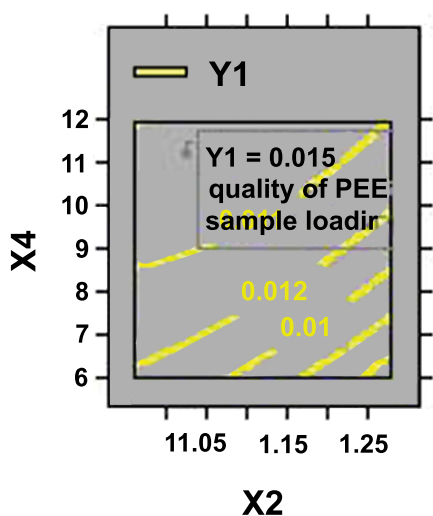
Fig. (2). Contour plot of the combined effects of PEG and sample loading on the yield of charantin, keeping independent variables (X_1, X_3) as constant (X_1=1.2g, X_3=0.2g).

The availability of the regression model (2) of the charantin production was tested using the calculated optimal conditions: K_2HPO_4 1.2g, PEG 1.12g, ethanol 0.2ml and sample loading 12ml with triplicate experiments. The mean value was 0.0148297, which agreed with the predicted value 0.015 well.

The results of blood glucose from hyperglycemic mice induced by alloxan are presented in Table **4**. The levels of blood glucose decreased after administration of *CPATP* and the Xiaoke Pill *(p<0.05)*.

Table 4.   Effect of *CPATP* on Blood Glucose Levels in Alloxan-Hyperglycemic Mice

Different Groups	Blood Glucose (mmol/L)
Alloxan- treated	21.9±2.9[a]
Alloxan and Xiaoke Pill-treated	12.8±3.0[b]
Alloxan and *CPATP*-treated	11.0±6.1[b]
Control group	6.0±1.8

The different letters in the same column indicate a statistical difference ($p<0.05$).

4. DISCUSSION

The quantitative partition of charantin derived from the crude extract of *Momordica charantia* in an aqueous two-phase system has been reported. Statistical experimental designs proved to be useful tools in optimizing charantin extraction in PEG/salt ATPS. RSM model, which could overcome the limitations of classic empirical methods, was proposed to study the combined effects of phase-forming components. The phase-forming components: PEG and sample loading, strongly affected yield of charantin. The optimal aqueous two-phase system for charantin extraction was K_2HPO_4 1.2g, PEG 1.12g, ethanol 0.2ml and sample loading 12ml.

In China, many farmers plant *Momordica charantia* as vegetable. If it is used to separate charantin, the farmers will benefit from it. Aqueous two-phase systems (ATPS) were used for the purifying saponin because of their low cost. ATPS can reduce amount of organic solvent and the danger of explosion necessitates special equipment leading to increased costs.

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REFERENCES

[1]   Ho WKK, Liu SC, Shaw PC, *et al*. Cloning of the cDNA of alpha-momorcharin: a ribosome inactivating protein. Biochem Biophys Acta 1991; 1088: 311-4.

[2]   Raza H, Ahmed I, Lakhani MS, *et al*. Effect of bitter melon (Momordica charantia) fruit juice on the hepatic cytochrome P450-dependent monooxygenases and glutathione S-transferases in streptozotocin-induced diabetic rats. Biochem Pharmacol 1996; 52: 1639-42.

[3]   Walter H, Brooks DE, Fisher D. Partitioning in aqueous two-phase systems: theory, methods, uses, and applications to biotechnology. Academic Press: Orlando, FL 1985.

[4]   Eiteman MA, Gainer JL. Peptide hydrophobicity and partitioning in poly(ethylene glycol)/magnesium sulfate aqueous two-phase systems. Biotechnol Prog 1990; 6: 479-84.

[5]   Li C, Ouyang F, Bai J. Extractive cultivation of *Lactococcus lactis* using a poly(ethylene glycol)/MgSO₄-7H₂O aqueous two-phase systims to produce nisin. Biotechnol Lett 2000; 22: 843-7.

[6]   Shi Y, Weimer PJ. Response surface analysis of the effects of pH and dilution rate on *Ruminococcus flavefaciens* FD-1in cellulose-fed continuous culture. Appl Environ Microbiol 1992; 58: 2583-91.

[7]   Oh S, Rheem S, Sim J, Kim S, Baek Y. Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-glucose medium by using response surface methodology. Appl Environ Microbiol 1995; 61: 3809-14.

[8]   Han C, Hui Q, Wang Y. Hypoglycaemic activity of saponin fraction extracted from Momordica charantia in PEG/salt aqueous two-phase systems. Nat Prod Res 2008; 22(13): 1112-9.