Evaluation and optimization of supercritical extraction of insulin from Otostegia persica

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Abstract

In this study, the extraction of insulin from otostegia persica was enquire by Soxhlet extraction and modified supercritical CO_2 with methanol as solvent and 30 min of static time. Design of experiment was executed with response surface methodology (RSM) using Mini Tab software 17. The operating temperature (40–60◦C), pressure (15–35 MPa),dynamic extraction time (60–120 min) and CO_2 flow rate (0.8–2 ml/min) were regarded as the range of operating variables UV test was applied to recognize and quantitatively determine the amount of extracted insulin. Response surface analysis proved that R_2 and modified R_2 of the model were 98.16%and 96.54%, respectively. The RSM modeling prophesy the optimal operating conditions to be the pressure of 28.1 MPa, temperature of 47◦C, CO_2 flow rate of 2 ml/min and dynamic extraction time of 109 min in which the maximum absorption coefficient of 0.7.

Keywords: Supercritical extraction, Insulin, Optimization, Response Surface Methodology, Otostegia Persica.

1. Introduction

Otostegia persica is native of Iran, Pakistan and Afghanistan that widely distributed in southern and southeastern Iran. In traditional Iranian medicine, this herb is used to treat diabetes, fever, malaria and toothache. Also, in clinical and experimental studies, there have been cases of relief, arthritis effects and anti-inflammatory peroxidation.

In addition, the hydro alcoholic extract improves the leave morphine syndrome. It has been reported that polyphenolic antioxidants in some plants are useful in the improvement of diabetes complications. Otostegia persica has polyphenols polar compounds such as Flavonoids and Tannins. Flavonoids usually do their antioxidant activity by combining with free radicals. It has been reported that methanolic extract of the o.persica has an antioxidant activity that has a beneficial role in the improvement of diabetes mellitus also o.persica has a hypoglycemic effect that is shown by improving the pancreatic islets and increasing insulin secretion [1,2].

According to the study, the methanolic extract of this plant has strong antioxidant activity due having of Morin and Quercetin flavonoids and prevents the destruction of DNA cells. Quercetin is able to collect xanthine superoxide and xanthine oxide radicals. This flavonoid is beneficial through reducing oxidative stress on improving the difficulty of the vessels and cataracts caused by diabetes. A series of studies show that this flavonoid is able to increase serum levels of insulin and calcium in the blood. In general, flavonoid Morin in plants of the family moraceae is non-toxic and has antioxidant, anti-allergic, anti-inflammatory, anti-mutagenic and anti-cancer effects. In addition to the activity of collecting free radicals, Quercetin in the o.persica also has the ability to inhibit pancreatic lipase. Flavonoids do from several procedures themselves antioxidant actions. For example, combining with free radicals and producing less active products, inhibiting metabolic enzymes such as lipoxygenase, cyclooxygenase, xanthine oxidase and nitric oxide synthase, which are involved in the production of free radicals. It has also been shown that flavonoids Morin and Quercetin oppose cholesterol oxidation. Generally, this plant probably reduces serum glucose by reducing free radicals and improving pancreatic islets and increasing insulin levels [3,4].

The main objective of this study was insulin extraction from o.persica by modified SC-CO_2 in periodic static-dynamic method for pharmaceutical application. The optimization was performed by response surface methodology (RSM). The RSM is beneficial for modeling, problem analysis and optimization when a response (i.e., extraction recovery) is influenced by several variables such as pressure, temperature, flow rate of SC-CO_2 and dynamic time. In this work the other impressive variable (static time) was fixed at optimum value of 30 min that was procure by experiment.

The methods used to extract this substance are all inclusive solvent-extracted, which, on the other hand, are not cost-effective due to the high consumption of energy and solvent and On the other hand, the provision and recovery of solvents that are very important in food and medicine is not entirely possible. Organic solvents, especially chlorinated solvents, are harmful for environment. This problem has been resolved by replacing CO_2 as the most common solvent in supercritical processes because carbon dioxide is cheap, it has a low critical temperature and pressure, non-explosive, non-toxic and non-reactive properties, and it is also safe for the environment [5-8].

2. Experimental

2.1. Materials

O.persica was prepared from Kerman co. The samples were screened with mesh size of 20–35 (0.841–0.507 mm). Methanol (purity ≥ 99.9%, Merck), deionized water and industrial grade carbon dioxide (purity > 99%) were utilized for Soxhlet and supercritical fluid extraction and UV test.

2.2. Soxhlet extraction

For Soxhlet extraction, 1 g of o.persica in a filter paper set in a Soxhlet device and then continued extraction for 8 hrs. and methanol was used as solvent. After this work, the solvent was vaporized by rotary vacuum evaporator (30◦C), and the extract was dried at 70◦C to delete remaining solvent to required amount. Then the amount of insulin was determined by UV test. The absorption coefficient obtained from the extraction of Soxhlet was 0.7 which is considered as a 100% extraction.

2.3. Supercritical fluid extraction: devices and procedure

To do this study, the supercritical extraction system shown in Fig. 1 was used.

2.3.1. CO_2 cylinder

The cylinder is intended to store carbon dioxide. There is a pipe to transfer carbon dioxide to the end of the tank. Its degree of purity is usually higher than 97%.

2.3.2. Molecular sieve

A molecular sieve is intended to dry out and increase the degree of purity of carbon dioxide gas. The sieve consists of a tube filled with molecular sieve K28751105148-sieve5A.

2.3.3. Cooler circulator

A cooler is used to convert carbon dioxide into a liquid before entering the pump. In these coolers, ethylene glycol is usually used as a cooling agent.

2.3.4. High pressure pump

High pressure liquid chromatography pumps are used to pump the solvent into the extraction vessel. The solvent present in the tank is pressurized by the pump about 3750 to10000 psi.

2.3.5. Needle valve

In order to control the input of carbon dioxide and to save carbon dioxide, one valve is placed at the pump outlet.

2.3.6. Oven

Ovens work with airflow and are commonly used to control and maintain the temperature of the chromatography column. The temperature range is from -15 ° C below ambient temperature to 80 ° C.

2.3.7. Extraction cell

The extraction cells in the SFE range from 10 to 200 milliliters and are mostly of stainless steel 316 or other similar resistant materials. The temperature control is usually done by placing the sample cells in the oven.

2.3.8. Back pressure regulator

The pressure of the system by the back pressure of the back pressure regulator not only ensures the back pressure, but also controls the flow rate of the fluid flowing through the system.

The Tescom Series 24-1762-26 was used in the project. The maximum and minimum pressure of this regulator is 414 and 34 bar, and the maximum operating temperature of this device is 204.

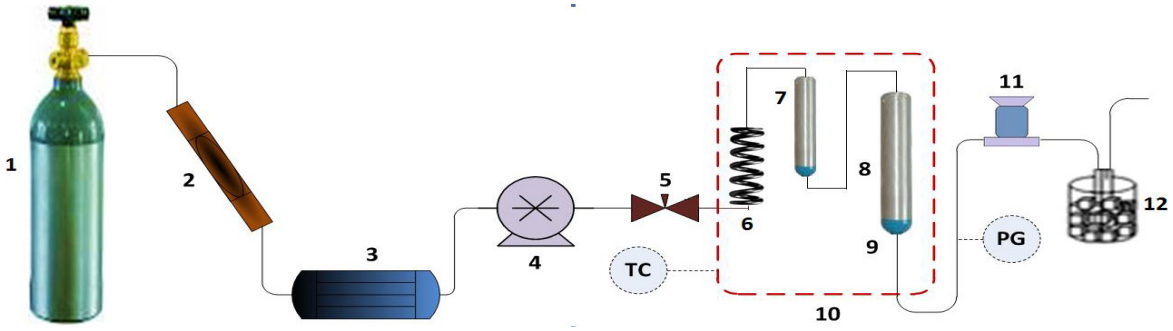

Figure 1. Schematic diagram of experimental setup for the supercritical extraction system; (1) CO2cylinder, (2) column consisting of molecular sieve and silica gel,(3) chiller, (4) pump head cooler, (5) HPLC pump, (6) needle valve, (13) BPR valve, (14) extracted sample collection vessel.

2.4. UV test

We first turn on the device and wait for it to start up. Then we set the wavelength of the device to 400 to 700 according to the standard method in order to determine the absorption factor to represent the required factor. The deionized water poured in the cell and added reactive and entered the device. Calibrate the device and then read the absorption coefficient of each sample.

3. Design of experiment

Traditionally, optimization has been executed by checking the impression of one factor at a time on response. In other words, just one parameter is investigated while others are saved at a constant level. The main disadvantages of this optimization method were (1) a large number of experiments that need to be done require time and cost and (2) the interactive effects between distinct operating variables are not considered. To solve these problems, the optimization was executed by using multi-variants statistic methods such as Response Surface Methodology (RSM). RSM method is combination of mathematical and statistical techniques that when a response is influenced by several variables are beneficial for solve modeling and analysis subjects. In order to obtain the best answer, simultaneously optimize the levels of these variables.

The steps in this procedure are as follows:

1. Select the variables that have the most impact on the response. Identify the variables that may affect the response. Therefore, it is necessary to pick out those variables with main effects.
2. Choose the experimental design and executed according to the selected experimental matrix and getting answers.

RSM model is shown in Eq (1):

$$Y = A_0 + \sum_{i=1}^{k} A_i Z_i + \sum_{i=1}^{k} A_{ii} Z_i^2 + \sum_{i} \sum_{j} A_{ij} Z_i Z_j + \varepsilon \qquad (1)$$

Where, A_0 is Regression coefficient for constant coefficient, A_i is Regression coefficient for linear terms, A_{ii} is Regression coefficient for square terms, A_{ij} is Regression coefficient for interactions terms, is A collection of statistical errors that are not covered by the main part of the function and k is the number of variables.

In RSM, the central composite design (CCRD) is usually used to determine how the tests are performed. In this case, prior to the design, the selected levels are encoded. The central value of the code is zero, smaller numbers get negative code and bigger numbers get positive code in which these codes are integers. To calculate the actual values of the parameters at the coded levels according to the following equation:

$$Z_i = \frac{X_i - X_{i,c,p}}{\Delta \xi} \qquad (2)$$

Where Z_i is code for each level, X_i is the value of the parameter in the maximum, minimum or center point, $X_{i,c,p}$ is the value of the parameters in the center point and ξ is the value of the distance between Xs in the maximum, minimum or central points.

Coded Levels	Temperature [ºC]	Dynamic time [min]	Pressure [Mpa]	Flow rate [ml/min]
-2	40	60	150	0.8
-1	45	75	200	1.1
0	50	90	250	1.4
1	55	105	300	1.7
2	60	120	350	2

Table 1. Uncoded and coded levels of independent variables used in the RSM design.

3) Detect a suitable model of the response variables according to the fit of a polynomial equation to the experimental data. After catching data linked to each experimental point, it is necessary to fit a mathematical equation to qualify the behavior of the response to the levels of studied values. In other words, the coefficients of Eq. (3) are acquired via least square method (LSM). LSM is a multiple regression technique with generating the lowest residual possible that used to fit a mathematical model to a collection of experimental data.

4) The evaluation of the model's validity. The attained mathematical model was appraised by the application of analysis of variance (ANOVA), coefficient of determination (R^2), and adjusted coefficient of determination (Adj. R^2).

5) Finding the optimum values of model for each variable that studied. Finally, the needful conditions were determined for optimum recovery of insulin extraction through the first derivative of the mathematical function, which depicts the response surface [9,10].

Between the several experimental design methods, RSM was chosen in this reading to specify the effects of pressure, temperature, flow rate and dynamic time in binary modifier on the extraction efficiency of insulin. Therefore, four levels central composite rotatable design (CCRD) was used with four independent variables.

The independent variables in this study were dynamic time (t), flow rate of SC-CO_2 (Q), temperature (T), pressure (P), in the range of 60–120 min, 0.8–2 ml/min, 40–60°C, 15–35 MPa, respectively. Recovery of insulin extraction was response variable (dependent variable). The Table 1 shows the coded and uncoded levels of independent variables and Table 2 also shows 31 experimental runs that are according to coded levels of the independent variables in the CCRD for performance of RSM used from Minitab17.

Number of experiments	(p)	(T)	(t)	(Q)	Absorb
1	-1	-1	-1	-1	0.4982
2	0	0	0	0	0.5435
3	1	1	-1	-1	0.54
4	0	0	0	0	0.5452
5	-1	-1	-1	1	0.5417
6	0	0	2	0	0.5640
7	1	1	1	1	0.5550
8	1	-1	-1	1	0.5375
9	0	0	0	-2	0.4773
10	0	0	0	0	0.5445
11	-1	-1	1	1	0.5648
12	1	1	1	-1	0.5042
13	0	0	0	0	0.5337
14	0	2	0	0	0.5032
15	1	-1	-1	-1	0.4880
16	0	0	0	0	0.5489
17	0	0	0	0	0.5487
18	0	0	0	0	0.5383
19	1	-1	1	1	0.5641
20	-1	1	-1	-1	0.4089
21	-1	1	1	1	0.5315
22	0	-2	0	0	0.5647
23	-2	0	0	0	0.4485
24	-1	-1	1	-1	0.5311
25	0	0	0	2	0.5676
26	2	0	0	0	0.5117
27	-1	1	1	-1	0.4669
28	1	1	-1	1	0.5185
29	-1	1	-1	1	0.4871
30	1	-1	1	-1	0.5268
31	0	0	-2	0	0.4919

Table 2. Results obtained from supercritical extraction of insulin.

4. Results and discussion

4.1. Fitting the model and analysis of experimental data

The experimental data available in Table 2 are used to obtain the coefficients of the second-order polynomial equation (Equation (5)) by the LSM. The regression anomalies are summarized in Table 3 and the value of each coefficient is determined based on the absolute value of t and the value of p in the table. For any of the terms in the model, a large absolute value of t and a small p-value would represent more notable effects on the corresponding response variables. In this project, the parameters with p <0.001 as parameters are very important and effective and Parameters with p <0.01 as important parameters and Parameters with a p value of more than 0.05 are known as parameters that do not have a significant effect. The second-order polynomial model was required for insulin recovery (R) as a function of independent variables in Eq. 3.

According to Table 3, the terms of pressure, temperature, dynamics time and flow rate were very important and highly significant from quadratic terms p^2 and t^2 were highly significant and other parameters were not important. The calculated coefficient of determination (R^2) and adjusted coefficient of determination (Adj. R^2) were 98.16% and 96.54%, respectively that which indicates the appropriate accuracy of the proposed model for the fitting of experimental data in a statement that about 98% of the changes are covered by the model.

$$R=29.506+2.501p+1.883T+1.840Q+1.022t-0.573p^2-0.457T^2+0.297Q2-1.649t^2 \\ -0.123Pt+0.402PQ-0.137pt+0.263TQ-0.078Tt+1.034Qt \quad (3)$$

4.2. Optimization of extraction operating conditions

Because there is no unique answer to designing a process, a design can include several designs, so choosing the best one from the available options is of particular importance. Optimization emphasizes the better the solution to a problem and tries to move the optimal solution by changing a basic idea. This path of motion may reach the optimal answer or be near optimum. Therefore, optimum conditions of the experiment were obtained at temperature of 47°C, pressure of 28.1 MPa, flow rate of 2 ml/min and dynamic time 109 min.

4.3. Response surface analysis

The interactions among different variables and optimum values for reaping the maximum recovery by a three-dimensional response surface model according to Eq. (5) were perused.

The best way to examine the effect of operating parameters on the rate of recovery of insulin is to plot the response surface diagrams in a three or two dimensional. In these plot, the axis A is designed for the response, and the axis B and P for the two independent variables and two other variables are zero (central point). In Fig. 2, the effect of pressure and temperature on insulin recovery is shown that in this figure Dynamic time and flow rate are 90 min and 2 ml/min, respectively.

The effect of the pressure parameter can be explained this way that the pressure and density of carbon dioxide are directly related to each other, with increasing pressure, the density of carbon dioxide increases. With increasing density, the molecular distance decreases and As a result, the molecular interactions between the solvent and the extract increase Which increases the supercritical fluid solubility And it has a positive effect on the recovery rate of the extract as it increases recovery, It should be noted that increasing pressure also has a negative effect on recovery And it decreases with increasing pressure, diffusivity and mass transfer coefficient [11, 12]. As shown in Figure 2, the increase in pressure initially increases the recovery rate and from a pressure of 28 up the recovery rate is reduced because repulsion increases between the solvent and the soluble component. In fact, it can be said that when pressure ranges from 15 to 28, the increase in carbon dioxide density is dominant and from the 28th onwards, the reduction of the mass transfer coefficient and the reduction of diffusivity are predominant. Flow rate and dynamic time are 1.4 ml/min and 90 min respectively, which are the same central points. As shown in Table 4, the pressure has a positive linear effect ($p < 0.001$) on the rate of recovery of insulin at low pressure levels that it's due to the increased carbon dioxide density because of the high pressure. At high pressures, the negative effect of the squared pressure parameter ($p < 0.001$) is also significant and with increasing pressure, as already said, the recovery rate is reduced which is due to increased repulsion between the solvent and the soluble component at high pressures and the reduction of diffusivity and mass transfer coefficient.

Regarding the effect of the temperature parameter, it can be said that this parameter can have both negative and positive effect. Increasing the temperature increases the density that this reduces the solvent of solubility power and thus reduces the recovery rate. The increase in temperature also increases the vapor pressure of the components which increases the solubility of the extract in supercritical carbon dioxide. What effect the temperature has on the recovery rate depends on which one has a dominant effect [13, 14].

term	coefficient	T-value	p-value
A_0	29.506	107.76	0.000
P	2.501	16.92	0.000
t	1.022	6.91	0.000
T	1.883	12.73	0.000
Q	1.84	-12.85	0.000
P^2	-0.573	-4.23	0.001
t^2	-1.649	-12.17	0.000
T^2	-0.457	-3.37	0.004
Q^2	0.297	-2.19	0.044
P×t	-0.137	-0.76	0.461
P×T	-0.123	0.68	0.506
P×Q	0.402	2.22	0.041
t×T	-0.078	-0.43	0.672
t×Q	1.034	5.71	0.000
T×Q	0.263	1.45	0.166
R^2=98.16%	, R^2(adj)= 96.54%		

Table 3. Regression coefficients of predicted second-order polynomial model for insulin recovery.

Source	Degree of freedom	Sum of squares	Mean square	f-value	p-value
Model	14	447.214	31.944	60.87	0.000
Linear	4	341.602	85.400	162.74	0.000
P	1	150.150	150.150	286.13	0.000
T	1	25.072	25.072	47.78	0.000
T	1	85.089	85.089	162.15	0.000
Q	1	81.291	81.291	154.91	0.000
Square	4	84.161	21.04	40.09	0.000
P^2	1	9.382	9.382	17.88	0.001
t^2	1	77.761	77.761	148.18	0.000
T^2	1	5.960	5.960	11.36	0.004
Q^2	1	2.514	2.514	4.79	0.044
Interactions	6	21.451	21.575	6.81	0.001
P×t	1	0.3	0.3	0.57	0.461
P×T	1	0.243	0.243	0.46	0.506
P×Q	1	2.584	2.584	4.92	0.041
t×T	1	0.098	0.098	0.19	0.672
t×Q	1	17.119	17.119	32.62	0.000
T×Q	1	1.108	1.108	2.11	0.166
Error	16	8.369	0.525	-	-
Lack of fit	10	6.375	0.638	1.89	0.225
Pure Error	6	2.021	0.337	-	-
Total	30	455.610	-	-	-

Table 4. ANOVA of RSM modeling for insulin recovery.

As shown in Fig. 2, the recovery rate is reduced by increasing the temperature. In fact, it can be said that when the temperature ranges from 40 to 47, the recovery rate is very good and decreases from 47 up. Table 4 also confirms these effects with a negative coefficient by having a positive effect for the linear term of the temperature and the negative effect for the temperature term ($p < 0.001$).The effect of this parameter is that the increase in flow rate causes reduction of the thickness of the film layer around solid particles and increases the film mass transfer coefficient which overcomes the mass transfer resistance and also the common surface resistance regarding the transfer of the soluble component from the sample matrix [15]. As shown in Fig. 3, with increasing flow rate the recovery rate increases at constant temperature which is due to the reduction of film thickness layer around solid particles and the mass transfer resistance. In these figure, the values of the pressure and temperature parameters are 25 MPa and 50 respectively. As shown in Table 3, this parameter has a positive effect on the recovery rate.

During the dynamic time, the new solvent passes through the samples. As long as there would have existed effective propulsion between the fresh fluid and the sample for mass transfer increasing the dynamics time increases insulin recovery. As shown in Fig. 5, the dynamic time increases, the insulin recovery rate also increases. As shown in Table 3, the extraction time has a positive linear effect on recovery rate of insulin ($p < 0.001$). By increasing the extraction time, the negative effect of square term time ($p < 0.001$) has also been important and this increase, after 120 minutes, will not have much effect on the recovery rate.

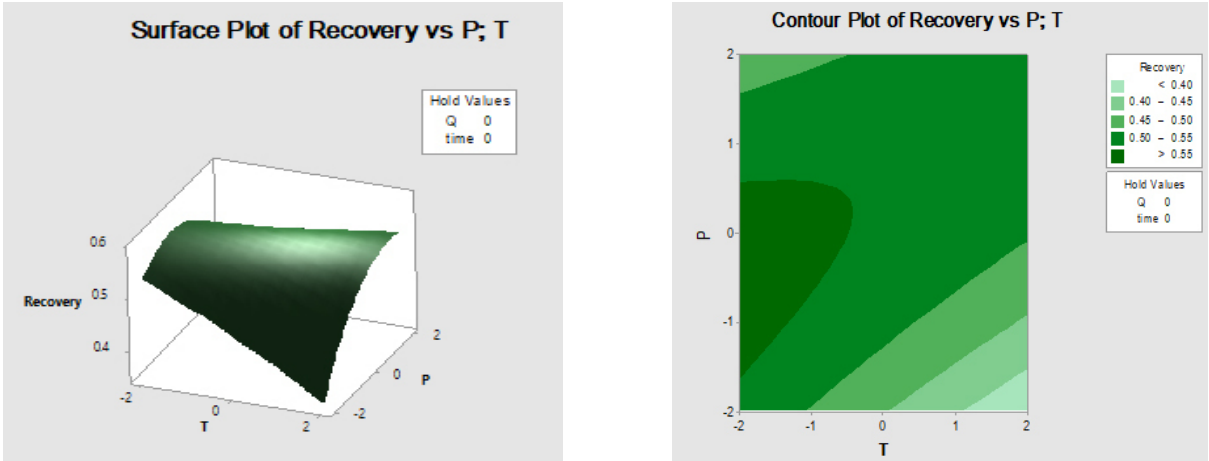

Figure 2. Surface and contour plots of recovery versus P and T.

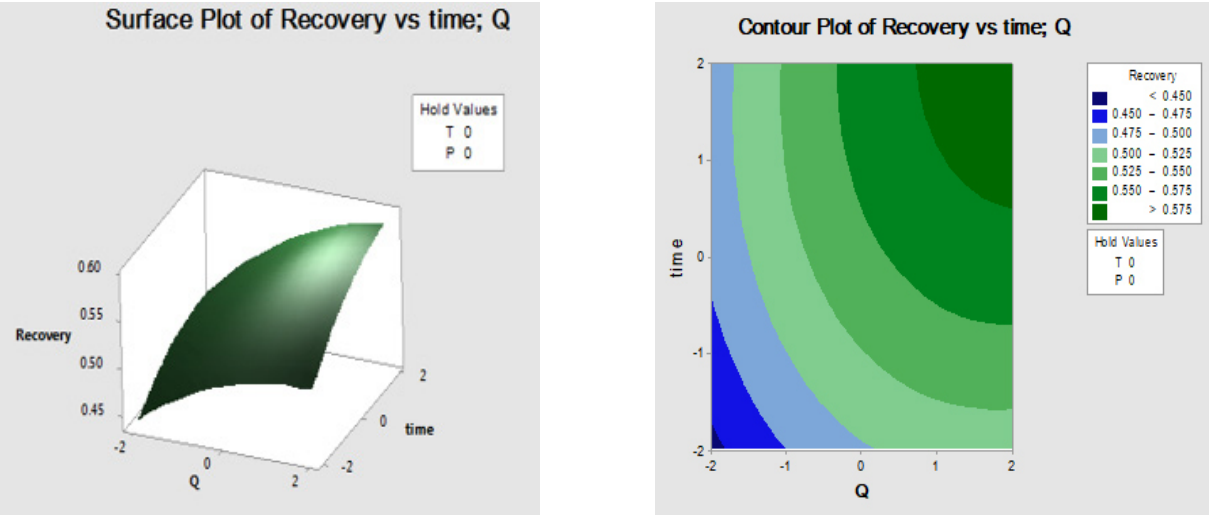

Figure 3. Surface and contour plots of recovery versus time and Q .

5. Conclusions

In this research, extraction of insulin from the plant was carried out using two different methods of extraction with supercritical carbon dioxide and extraction with Soxhlet. In extraction by Soxhlet with methanol solvent, the time of 8 hours was chosen. Also, the RSM to the most appropriate operating conditions for the extraction of insulin, pressure 28 Mpa, temperature 47, flow 2 ml/min, and dynamic time dynamics 109 min predicted. In general identified that the linear terms of pressure and time are very important this is while carbon dioxide flow rate is of relatively high importance. The square terms of all variables are also very important except carbon dioxide flow rate.

Acknowledgments

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