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Dispersive liquid-liquid microextraction for preconcentration and determination of phenytoin in real samples using response surface methodology-high performance liquid chromatography

Eslam Pourbasheer [a*], Samira Sadafi [a], Mohammad Reza Ganjali [b,c] Maryam Abbasghorbani [d]

[a]*Correspondence to:Department of Chemistry, Payame Noor University (PNU), P.O. Box, 19395-3697, Tehran, Iran, E-mail: pourbasheer@ut.ac.ir; Tel: +98 45 33515003*
[b]*Center of Excellence in Electrochemistry, Faculty of Chemistry, University of Tehran, P. O. Box 14155-6455, Tehran, Iran*
[c]*Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran*
[d]*Gas division, Research Institute of Petroleum Industry, P.O. Box 14665-137, Tehran, Iran*

In the present study, the dispersive liquid–liquid microextraction (DLLME) was developed for preconcentration and determination of phenytoin in real samples by high performance liquid chromatography (HPLC). Several experimental variables were investigated such as the extraction solvent, disperser solvent, salt effect, extraction time, centrifuge time, centrifuge speed and sample volume. Firstly, an orthogonal array design (OAD) was applied to choose the significant variables. Then, the significant factors were optimized using the central composite design (CCD). The variables were optimized with the aid of the response surface methodology. The chloroform and ethanol were selected as extraction and dispersive solvents, respectively. In this method, a linear range of 0.01-24 μg mL^{-1} and the relative standard deviation from 1.7 to 12.35% were obtained for water samples. Also, for urine samples, the linear range of 0.2-24 μg mL^{-1} and the relative standard deviation from 1.41 to 9.3% were obtained. The limit of detection (LOD) and limit of quantitative (LOQ) were 0.94 and 2.84 and also, 1.63 and 4.94 μg mL^{-1} for water and urine samples respectively.

1. Introduction

Phenytoin (PHT) is a phenylated hydantoin derivative used as an anti-epileptic totreat of seizure disorders.[1] Blitz synthesized, PHT in 1908.[2] PHT has a structure similar to phenobarbital. PHT became accessible in 1938 after the discovery of its antiseizure efficacy by Merritt and Putnam.[3] This drug is bound greatly to plasma proteins (about 90%) and metabolized in the liver.

Many analytical methods have been reported for the analysis of PHT and its metabolites [4,5] and PHT in combination with other antiepileptic drugs in plasma. Frequently published methods were included; high performance liquid chromatography, [6-8] thin layer chromatography, [9] gas chromatography, [10] fluorescence polarization immunoassay, [11] and spectrophotometry. [12] The extraction of PHT has been described by many publications which include solid-phase extraction (SPE) [7,5,8] and liquid–liquid extraction (LLE)[13] which LLE is one of the oldest methods of preconcentration and matrix isolation. The disadvantage of LLE is time-consuming, and it requires large amounts of organic solvent. The SPE uses much less solvent than LLE but this method is relatively expensive. Supercritical fluid extraction (SFE) also is expensive.[14] Solid- phase microextraction (SPME) is a solvent free process developed by Arthur and Pawliszyn,[15] that includes simultaneous extraction and preconcentration of analytes from aqueous samples. The disadvantages are: a) SPME is expensive b) its fiber is fragile c) has limited lifetime. [16] Liquid-phase microextraction (LPME) is method developed by He and Lee in 1997.[17,18] LPME was developed as a solvent-minimized sample pretreatment procedure and the benefits of this approach are inexpensive, little solvent is used. [19] The disadvantage are included: fast stirring would tend to format air bubble,[14] extraction is time-consuming, and equilibrium could not be attained after a long time in most cases.[20]

Dispersive liquid–liquid microextraction (DLLME) is an analytical technique among extraction, the method newly discovered by Assadi and co-workers. [13] In DLLME extracting solvent mixture and dispersive solvent quickly is injected by syringe to the water sample solution. Therefore it can be seen that the extraction solvent is distributed to form tiny droplets within the aqueous sample solution. After extraction, phase separation

is performed by centrifuges. In the final stage the enriched analyte in the sedimented phase is removed from the centrifugeand injected into the instrument.

The benefits of DLLME are its easily of operation, high enrichment factor, high extraction recovery. In this method minimal volume of extraction solvents is used. Large surface area of the droplets of solvent extracted and aqueous samples will lead to a rapid equilibrium.[21]

In the present study, the dispersive liquid–liquid microextraction was used for preconcentration and determination of phenytoin in real samples using response surface method and high performance liquid chromatography.

2. Experimental

2.1 Chemicals and reagents

Phenytoin was obtained from Merck. Carbon tetrachloride, chloroform, 1,2-dichlorobenzene, 1,2-dichloroethane, as extraction solvent and acetone, acetonitrile, methanol and ethanolas dispersive solvents were obtained from Merck. The stock solutions of phenytoin were prepared by dissolving in methanol. Working solutions were prepared by dilution of standard stock solution with methanol. High-purity deionized water was applied for all the experiments.

2.2. Instrumentation

Chromatographic measurements were performed using a HPLC system equipped with series UV detector model 2550 set at 254 nm, and model 3207 manual injector with a 20 μL sample loop. A centrifuge Hettich made in Germany with power of 6000 (rpm) was

used for centrifuging. The pH meter of Metrohom 744 model, made in Switzerland was applied to adjust the mobile phase. A 100.0 μL microsyringe (Hamilton) was used for injection.

2.3 Chromatographic conditions

Analytes were separated on NULEOSIL 100-5, C_{18} column (125 mm×4.6 mm internal diameter) under isocratic condition with a mobile phase consist of 30% acetonitrile and 70% acetate buffer solution (0.02 M, pH 4.6). The flow rate of mobile phase was 1.0 mL/min and the UV detection wavelength was set at 254 nm. The analytical column and precolumn were kept at 25±2°C.

2.4 Dispersive liquid–liquid microextraction procedure

The 5.66 mL of working standard solution was placed in a 10 mL of glass centrifuge tube with conical bottom. According to the optimized conditions, a mixture of 157 μL chloroform (extraction solvent) and 1.37 ml of ethanol (disperser solvent) was quickly injected into the sample solution using a 100 μL syringe. A cloudy solution (water, ethanol and chloroform) was formed in a test tube (the cloudy state was stable for a long time). Then the mixture was centrifuged for 3 min at 39000 rpm. Accordingly, the dispersed fine particles of extraction phase were sedimented in the bottom of conical test tube. The sedimented phase was withdrawn by a 100 μL microsyringe (Hamilton) and was transferred to conical bottom tube, and then the solvent evaporated and was dissolved in methanol (the methanol volume was equal to the evaporated solvent volume). Finally, it was injected into the HPLC instrument for analysis.

In this work, the urine samples were collected from healthy volunteer in our lab, who was not receiving any pharmaceutical treatment at the time of sampling and the samples were centrifuged for 3 min at 2000 rpm. Then, supernatants were decanted into a clean glass tube and filtered through a 0.2 µm filter. The 0.5 ml of filtration products was diluted to 10 mL. Also, the water sample was provided from our laboratory and filtered through a 0.2 µm filter and applied for extraction as same as the urine sample.

2.5 Calculation of enrichment factor and recovery

The enrichment factor (EF) was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) within the sample:

$$EF = \frac{C_{sed}}{C_0} \quad (1)$$

The C_{sed} was estimated from calibration graph of direct injection of PHT standard solution in the chloroform at the range of 1-55 µg mL^{-1}.

Also, for evaluation of the extraction, the extraction recovery ER(%) was calculated as the following:

$$ER(\%) = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100 \quad (2)$$

where ER (%), V_{sed} and V_{aq} are called the extraction recovery, volume of precipitated phase and volume of aqueous sample, respectively.

3. Results and discussion

3.1 Selection of extraction solvent

The selection of the solvent is important for the DLLME process. In DLLME, the extractor solvent should has the following properties: a) It should have more density than the water, b) immiscibility with aqueous phase, c) suitable chromatography behavior.[22] In this experiment, some solvents such as 1,2-dichlorobenzene, carbon tetrachloride, 1,2-dichloroethane and chloroform were studied as extraction solvent and acetone, acetonitrile, methanol and ethanol were considered as disperser solvents. The effect of these solvents on the extraction efficiency of DLLME was investigated using 1 ml of dispersive solvents and 100 µl of extraction solvents. Evaluation of carbon tetrachloride with ethanol showed a cloudy solution was formed, but the formed, organic phase after centrifugation was not clear. 1,2-dichlorobenzene with ethanol were examined that the result showed cloudy solution was not formed. 1,2-dichloroethane with ethanol was evaluated and cloudy solution was formed, however the organic phase was very little. Finally, the investigations of chloroform with ethanol showed that the very good cloudy solution was formed using the chloroform as extraction solvent. The results showed that the extraction response (peak area) of chloroform was excellent, and then was selected as extraction solvent for subsequent experiments. The results are shown in Figure 1.

Fig. 1

3.2 Selection of disperser solvent

Miscibility of dispersive solvent in both aqueous phase (sample solution) and organic phase (extracting solvent) is a significant factor to select a dispersive solvent. [23] Thereby, acetone, acetonitrile, methanol and ethanol are selected for this purpose and effect of these solvents on the proficiency of DLLME was evaluated using 1 mL of each above mentioned disperser solvents and 100 μL of extraction solvents. Results showed that the highest yield was obtained by ethanol. Finally, ethanol was chosen as the disperser solvent.

3.3. Optimization of DLLME

Experimental design

The experimental design techniques were used to reduce the number of experiments. The most important impact parameters on the performance of DLLME process, including the disperser solvent (A), extraction solvent (B), sample volume (C) and centrifuge speed (D), were chosen based on preliminary experiments. In order to select the most significant factors the primary testing was done by orthogonal array design (OAD). Then, in order to optimize the values of these factors and to achieve the best response, central composite design (CCD) was used.

Orthogonal array design (OAD)

In this work, a two-level orthogonal array design with an OA, 8 (2^{7-4}) matrix was used to select significant quantitative factors. In orthogonal array design, the two-variable interactions were considered to be ignored, so the attention could be intensive on the decreased significant variables. Then low and high values for variables were selected from the results of foregone experiments, which are shown in Table 1. The independent variables were considered such as extraction solvent (A), dispersive solvent (B), extraction time (C), centrifuge time (D), centrifuge speed (E) salt effect (F), and sample volume (G). Eight experiments were chosen and performed by the OAD design (Table 2).

Table 1

Table 2

In this study, analysis of variance (ANOVA) was used to identify significant factors. According to the ANOVA table, the model F-value of 60.75 implies that the model is significant. The "Prob> F" values less than 0.050 indicates model terms are significant and values greater than 0.1 indicate the model terms are not significant. In this case A, B, E, G were significant model terms. Also, the results were shown in Pareto chart (Fig .2). Pareto chart shows that the disperser solvent volume (chloroform), extraction solvent volume (Ethanol), sample volume and centrifuge speed are the most actuarial significant effects on the dependent variables at the $p < 0.05$ level. As can be seen in Figure 2, the salt effect (F), extraction time (C) and centrifuge time (D) were not significant.

Fig. 2

Central composite design

In the present work, after selecting the most important factor using OAD, to evaluate and for the optimization of variables, the central composite design (CCD) method was applied. CCD is one of the most frequently used response surface method (RSM). RSM plays principal role in designing, formulating, developing and analyzing new scientific research, as well as improving existing studies and products.[24]

A central composite design combines a two-level factorial design with plus points (star points) and at least wiseone point at the center of the experimental zone to achieve the properties such as: rotatability or orthogonality, in order to fit quadratic polynomials. In addition to describing the linear effects of factors on the response, CCD explains the interaction and quadratic effects of the variables.

In this work to reduce the number of experiments from central composite design, small central composite design was used. Small composite design is minimal point designs and they are very sensitive to outliers. With this method, alpha value 1.68 was obtained. Replicates of factorial points, center points in each factorial block replicates of axial (star) points and center points in each axial block was equal to 1, 4, 1, 3 respectively. In this study, four variables were investigated such as solvent extraction (A), solvent dispersive (B), centrifuged speed (C) and sample volume (D). Thus, 23 experiments were designed by CCD. The experiments were randomized and were divided into two blocks. The factor levels are shown in Table 3.

Table 3

The ANOVA data to evaluate the significance of the model equation and for response surface quadratic model are shown in Table 4. The model F-value of 4.94 implies the

model is significant. Values of "Prob> F" less than 0.050 indicate model terms are significant. In this case D, BD, A^2 are significant model terms. Values greater than 0.100 indicate the model terms are not significant. The second-order equation can quantitatively describe the relationship between the responses and independent variables. This model is shown in Eq (3) which includes: four main effects and six two-factor interaction effects and four curvature effects.

Table 4

Peak area = $+1.124\times10^6+1.196\times10^5$ (A)$+1.178\times10^5$ (B)+29475.17(C)$+2.517\times10^5$ (D)$+1.495\times10^5$ (AB) - 75952.5 (AC) + 1.517 $\times10^5$(AD)+98890.75(BC) + 2.942×10^5 (BD) + 1.138×10^5 (CD) $-1.848\times10^5(A^2)$ - 47454.90(B^2) +19994.96 (C^2) -38261.80 (D^2) (3)

The "lack of fit F-value" of 2.18 implies the lack of fit is not significant relative to the pure error. The quality of fit of the polynomial model equation was evaluated by the coefficient of determination (R^2, adjusted-R^2 and “adequate precision”). R^2 is a measure by the model and equal to 0.908. The adjusted-R^2 is regulated for the number of terms in the model. It decreases as the number of terms in the model increases, if those additional terms do not add value to the model and its value was equal to 0.724."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 8.367 indicates an adequate signal. This model can be used to navigate the design space.

Graphs obtained from the model are shown in figure 3. In figure 3a it is clear that, when the volume of extraction solvent (chloroform) increased from 100 to 200 µL, the peak area increased. In the other hand, by increasing the volume of extraction solvent, the

volume of sedimented phase increases and the recovery of extraction increases which cause to have high peak area value.

In figure 3b, by increasing the volume of disperser (ethanol) from 0.5 to 1.5 mL, dispersion of extraction solvent (chloroform) occurred properly and cloudy solution is formed completely, therefore the peak area is increased.

As can be seen in figure 3c, the sample volume is increased from 3 to 6 ml, and with increasing the sample volume at a fixed concentration of the phenytoin, the peak area increased. In fact, by increasing the sample volume at constant concentration of the drug, the amount of drug will increase and in high sample volume, the better cloudy solution will form. Therefore peak area will increase.

Fig. 3

Figure 4 shows the interaction diagrams. An interaction occurs when the response is different, depending on the settings of two factors. The plots make it easy to exegesis two factor interactions. If appear two non-parallel lines in the diagram, show that the effect of one factor depends on the level of the other. Figure 4(a) shows that there is not significant interaction between the volume of extractor and sample volume. Figure 4(b) and (c) shows the interaction of extractor- disperser and disperser-sample volume.

Fig. 4

Response surface methodology

The next step was to acquire the optimum value for each factor to attain the maximum response. An important goal of this method is to optimize the response surface which is

affected by diverse process parameters.[25] The response surface plots show the results of the extraction recovery modeling for some of the significant factors. The curvatures of the plots indicate the interaction between the factors. For the graphical interpretation of the interactions, the use of three-dimensional plots of the model is highly recommended. Three-dimensional graphs were used to evaluate the interactive effect of the two variables on the response using the central composite design obtained by plotting showed in figure 5. Figure 5a, shows that by increasing the volume of extraction solvent and sample volume, the peak area increases. By increasing the sample volume at a fixed concentration, the amount of phenytoin will increase and a stable cloudy solution will formed. With the increased volume of extraction solvent drops of extraction in water increasing, as a result, the peak area increases. Figure 5b, shows that in the range of 100-200 μL of extractor volume, the peak area was increased by increasing the volume of the disperser. With increasing dispersive solvent, extraction solvent interaction with analytes increased, as a result, the peak area increases. Figure 5c, shows that by increasing the dispersive solvent and sample volume the peak area increases.

Fig. 5

According to the overall results of optimization study, the following experimental conditions are chosen: volume of choloroform: 157 μl; volume of ethanol: 1.37 ml; sample volume: 5.66 mL; centrifugation speed: 39000 rpm; extraction time: 3 min. The observed experimental enrichment factor under the above conditions was 16.93.

Analysis of real sample

The different water samples and urine sample were tested under the optimum conditions. The obtained chromatograms of phenytoin (30 μg mL^{-1}), before and after DLLME method are shown in figure 6. Also, the statistical results of real samples are shown in Table 5. In this method, the linear ranges of 0.01-24 μg mL^{-1} and 0.2-24 μg mL^{-1} were obtained for phenytoin in water and urine samples respectively. The correlation coefficients (r) were calculated as 0.9991 and 0.9990 and also the relative standard deviations (RSD, n=3) were calculated from 1.7 to 12.35% and 1.41 to 9.3% for water and urine samples respectively. The LOD and LOQ in water samples and urine samples were 0.94, 2.84 and 1.63, 4.94 μg mL^{-1} respectively. As can be seen, the good statistical results are reported and the proposed method was successful in preconcentration and determination of phenytoin in real samples.

Fig. 6

Table 5

4. Conclusion

In the present study, the dispersive liquid-liquid microextraction was applied for preconcentration and determination of phenytoin in real samples using response surface method - high performance liquid chromatography. Firstly, an orthogonal array design was applied to choose the significant variables. Then, the significant factors were optimized by using a central composite design. The variables were optimized with the aid of the response surface methodology. Experiments showed that ethanol and chloroform are the most suitable as disperser and extractor solvents respectively. Using experimental

design reduced the number of tests, resulting in saving time and experimental costs. The proposed method was used for determination of phenytoin in urine and water samples and good statistical results were obtained.

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Table 1 Assignment of factors and levels of the orthogonal array design

Factor	Symbol	Low level	High level
Extraction solvent	A	100	200
Dispersive solvent	B	0.5	1.5
Extraction time	C	1	10
Centrifuge time	D	3	10
Centrifuge speed	E	2000	4000
Salt effect	F	0.5	2
Sample volume	G	3	6

Table 2 Design matrix and the response for the orthogonal array design.

Run	Block	A	B	C	D	E	F	G	Peak Area
1	Block 1	200	0.5	10	3	4000	0.5	6	707610
2	Block 1	100	0.5	1	3	2000	0.5	3	713026
3	Block 1	100	0.5	1	10	4000	2	6	995742
4	Block 1	100	1.5	10	10	4000	0.5	3	686692
5	Block 1	200	1.5	1	3	4000	2	3	337543
6	Block 1	200	1.5	1	10	2000	0.5	6	1.02×10^{6}
7	Block 1	200	0.5	10	10	2000	2	3	519863
8	Block 1	100	1.5	10	3	2000	2	6	1.39×10^{6}

Table 3 Factors and their levels for the central composite design.

Factor	Symbol	Level				
		-α	-1	0	+1	+α
Extraction solvent	A	66	100	150	200	234
Dispersive solvent	B	0.2	0.5	1	1.5	1.8
Centrifuged speed	C	1318	2000	3000	4000	4682
Sample volume	D	2	3	4.5	6	7

Table 4 Analysis of variance table (ANOVA) for response surface quadratic model.

Sourse[a]	Sum of squares[b]	d.f.[c]	Mean square[d]	F-Value[e]	p-value Prob>F[f]	Significance
Model	1.69×10^{12}	14	1.21×10^{11}	4.94	0.0205	Significant
A	8.10×10^{10}	1	8.10×10^{10}	3.31	0.1116	
B	7.85×10^{10}	1	7.85×10^{10}	3.21	0.1163	
C	1.19×10^{10}	1	1.19×10^{10}	0.49	0.5085	
D	3.58×10^{11}	1	3.58×10^{11}	14.66	0.0065	
AB	7.41×10^{10}	1	7.41×10^{10}	3.03	0.1253	
AC	4.62×10^{10}	1	4.62×10^{10}	1.89	0.2118	
AD	7.63×10^{10}	1	7.63×10^{10}	3.12	0.1206	
BC	7.82×10^{10}	1	7.82×10^{10}	3.2	0.1168	
BD	2.87×10^{11}	1	2.87×10^{11}	11.73	0.0111	
CD	1.04×10^{11}	1	1.04×10^{11}	4.24	0.0785	
A^2	5.38×10^{11}	1	5.38×10^{11}	21.99	0.0022	
B^2	3.55×10^{10}	1	3.55×10^{10}	1.45	0.2676	
C^2	6.30×10^{09}	1	6.30×10^{09}	0.26	0.6274	
D^2	2.31×10^{10}	1	2.31×10^{10}	0.94	0.3639	
Residual[g]	1.71×10^{11}	7	2.45×10^{10}			
Lack of Fit[h]	7.98×10^{10}	2	3.99×10^{10}	2.18	0.2082	Not significant
Pure Error[i]	9.14×10^{10}	5	1.83×10^{10}			
CorTotal[j]	1.86×10^{12}	22				

[a] Source of variation.
[b] Sum of the squared differences between the average values and the overall mean.
[c] Degrees of freedom.
[d] Sum of squares divided by d.f.
[e] Test for comparing term variance with residual (error) variance.
[f] Probability of seeing the observed F-value if the null hypothesis is true.
[g] Consists of terms used to estimate experimental error.
[h] Variation of the data around the fitted model.
[i] Variation in the response in replicated design points.
[j] Totals of all information corrected for the mean.

Table 5 The statistical results of real samples by DLLME method

Samples	Concentration ($\mu g\ mL^{-1}$)	Recovery (%)[a]	LOD[b] ($\mu g\ mL^{-1}$)	LOQ[c] ($\mu g\ mL^{-1}$)	r [d]	RSD [e]
Water sample	0.4	90.55	0.94	2.84	0.9991	12.35%
	3	99.00				5.48%
	21	98.33				1.7%
Urine Sample	0.4	73.87	1.63	4.94	0.9990	9.3%
	3	120.66				2.43%
	21	101.45				1.41%

[a] Mean recovery for three determination
[b] Limit of detection
[c] Limit of quantitative
[d] Correlation coefficients
[e] Relative standard deviation (n=3); calculated as [(standard deviation of analytical response/mean of analytical response) × 100]

Figure Captions

Fig. 1 Effect of extraction solvents on the peak area.

Fig.2 Pareto chart of the variables obtained from orthogonal array design.

Fig.3 Effect of each factor on the extraction efficiency: a) extraction solvent, b) dispersive solvent, c) sample volume from central composite design.

Fig.4 Two-factor interactions and their effects on the peak area: a) extractor-sample volume, b) extractor-disperser, c) disperser-sample volume.

Fig.5 Three-dimensional response surface for: a) extractor-sample volume, b) extractor-disperser, c) disperser-sample volume.

Fig.6 Obtained chromatograms of phenytoin spiked at 30 μg mL^{-1}: (a) before DLLME; (b) after DLLME.

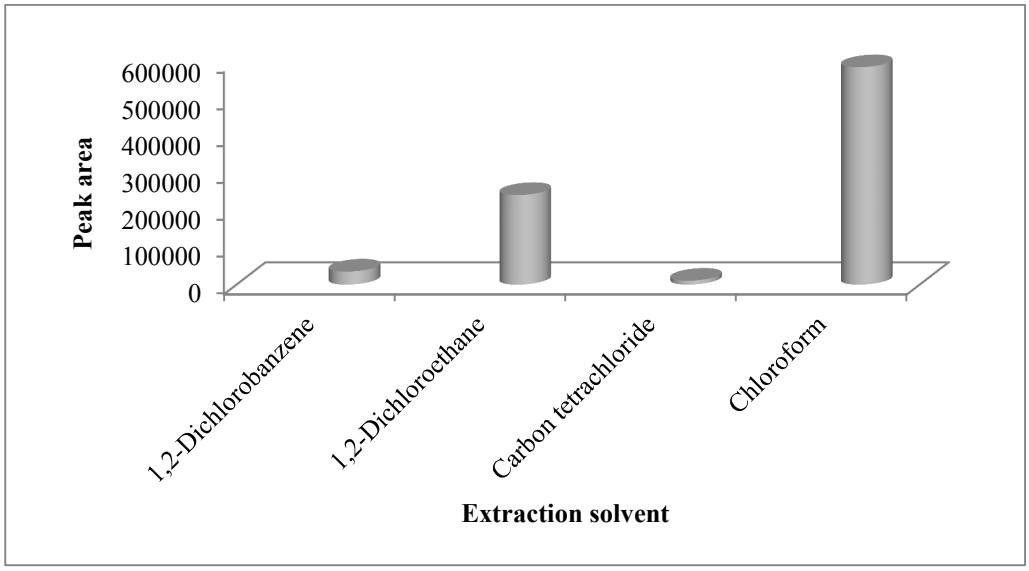


Fig. 1

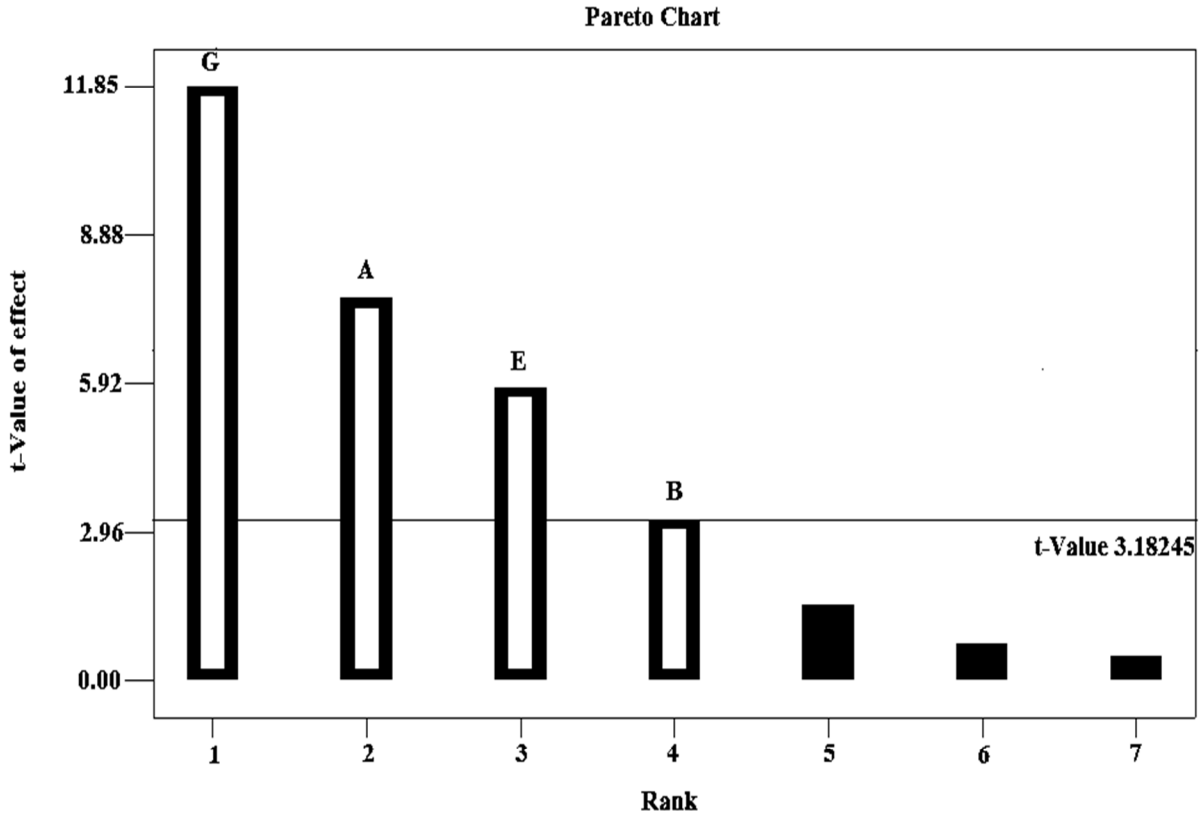
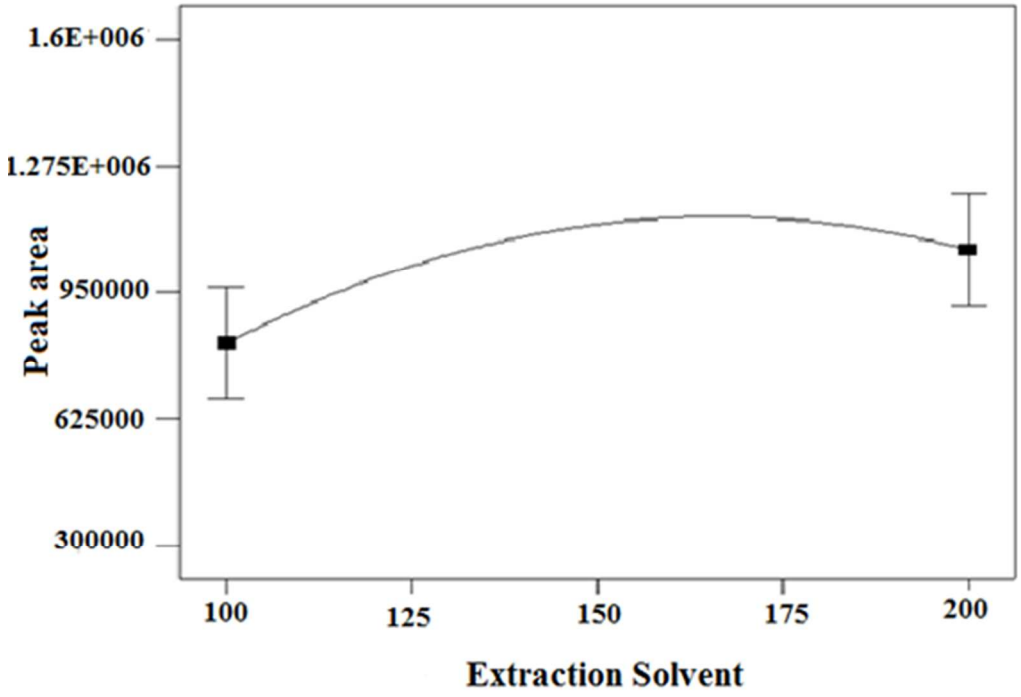
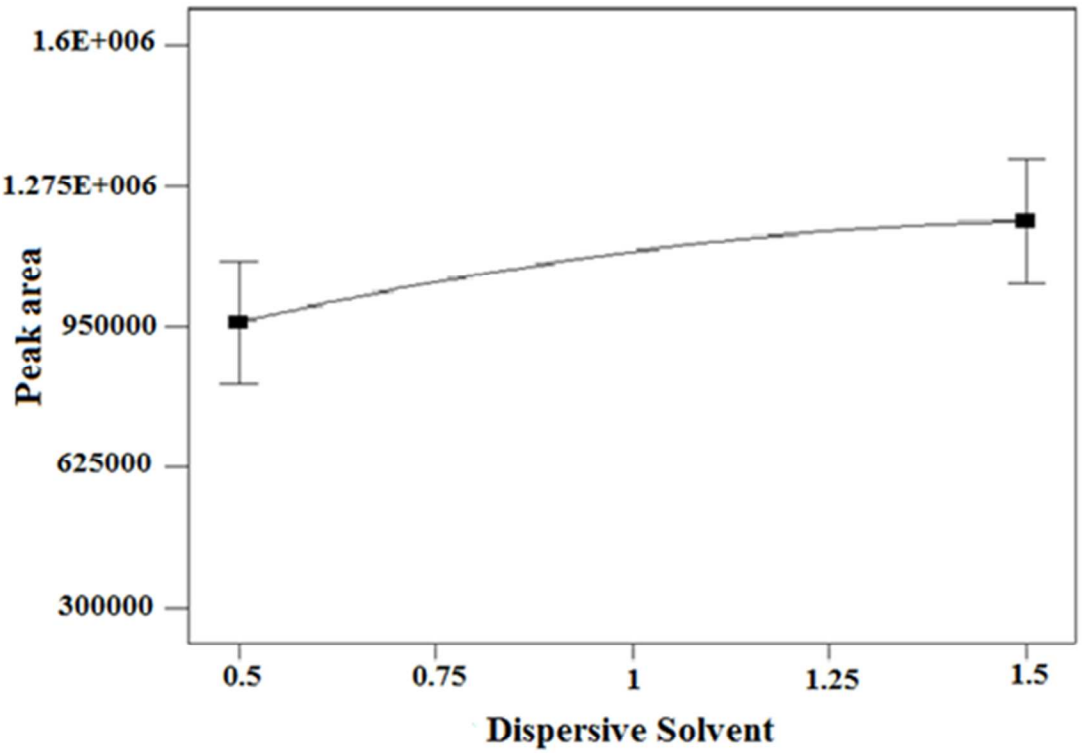


Fig. 2



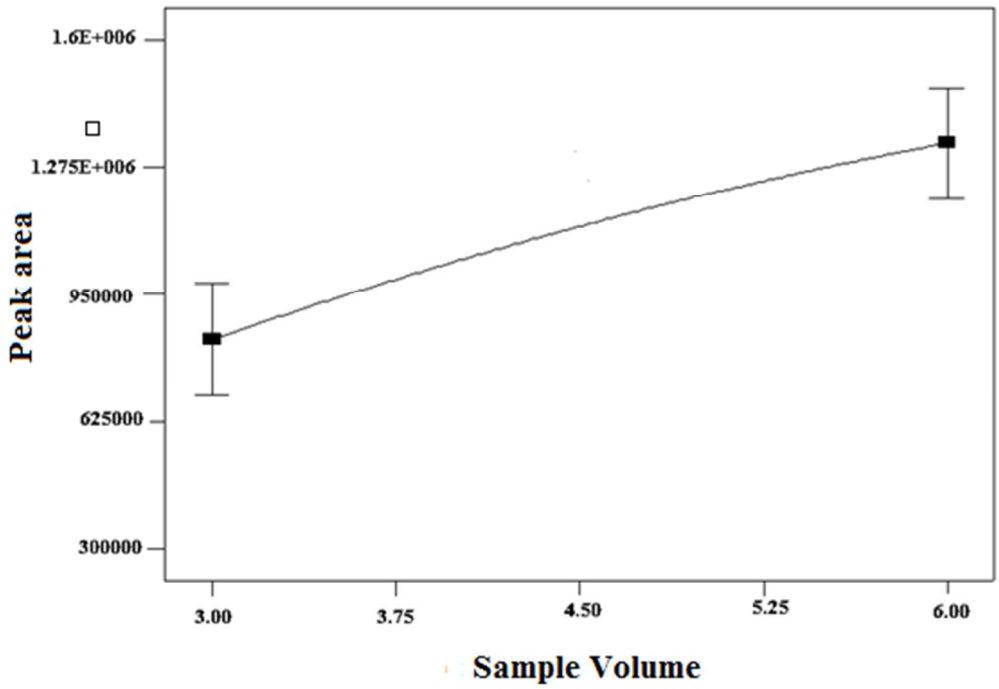
(a)

Fig. 3



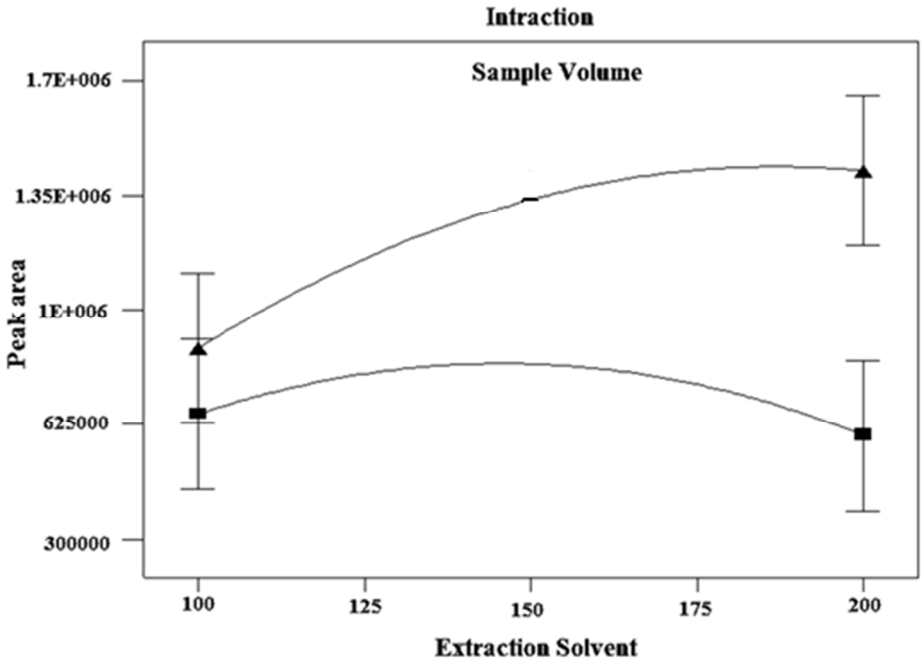
(b)

Fig. 3



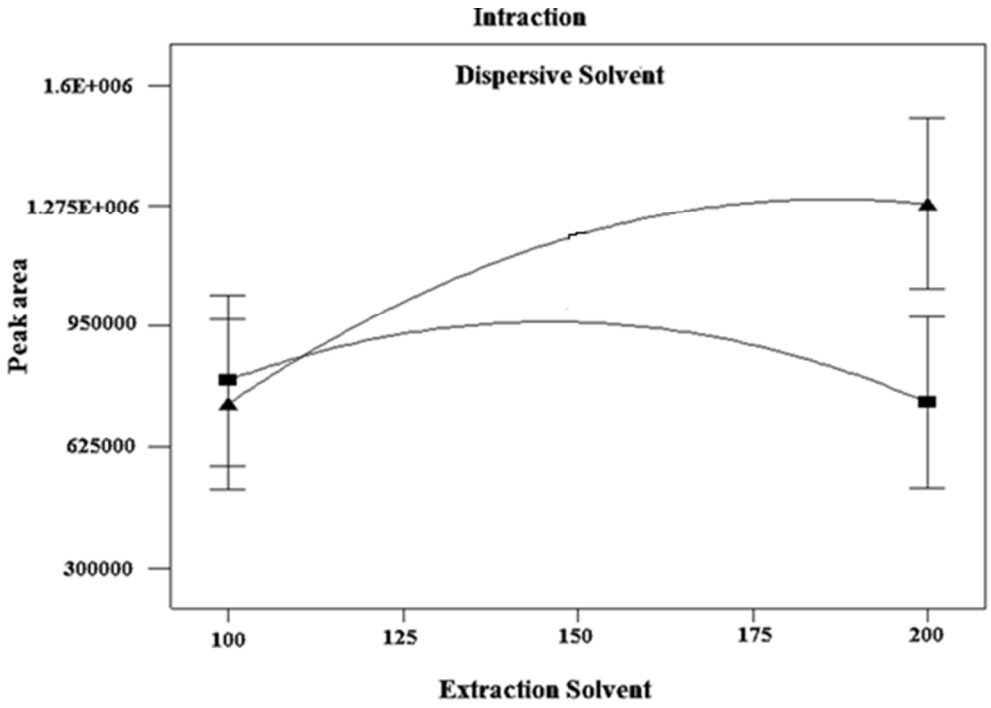
(c)

Fig. 3



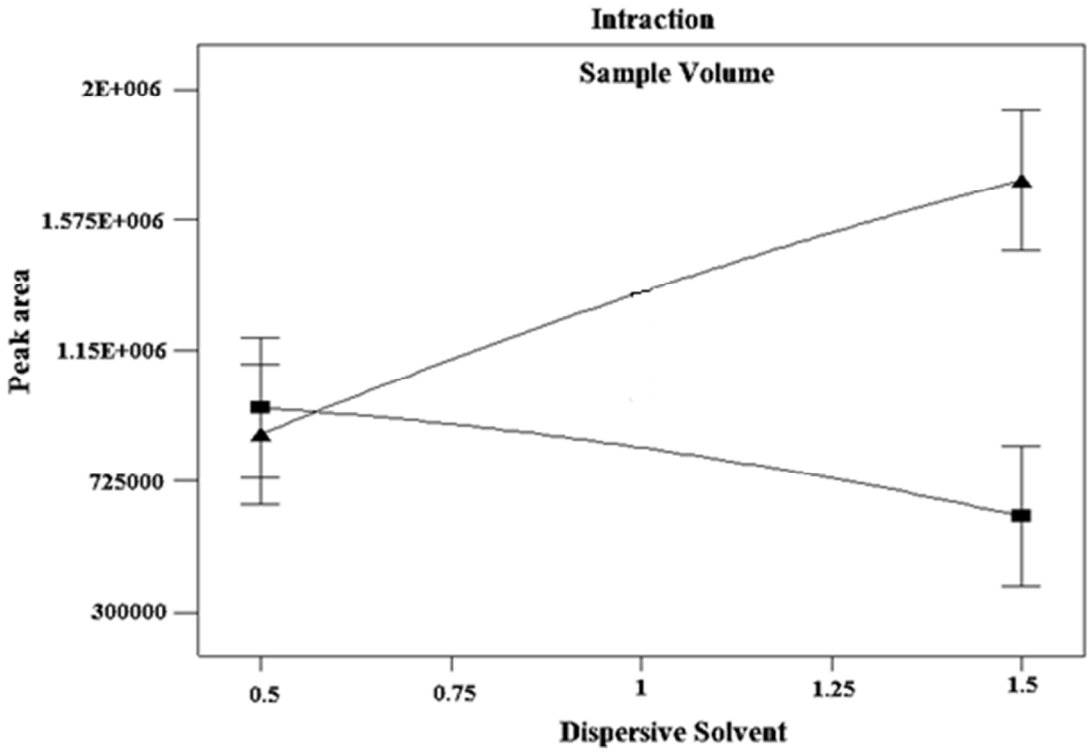
(a)

Fig. 4



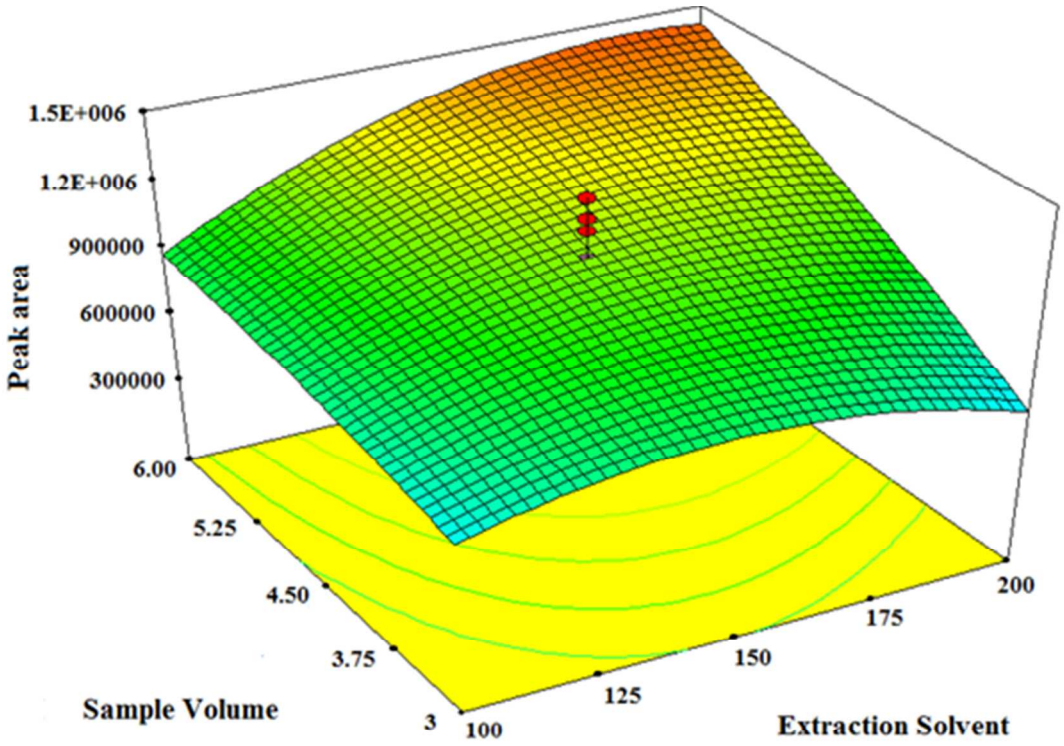
(b)

Fig. 4



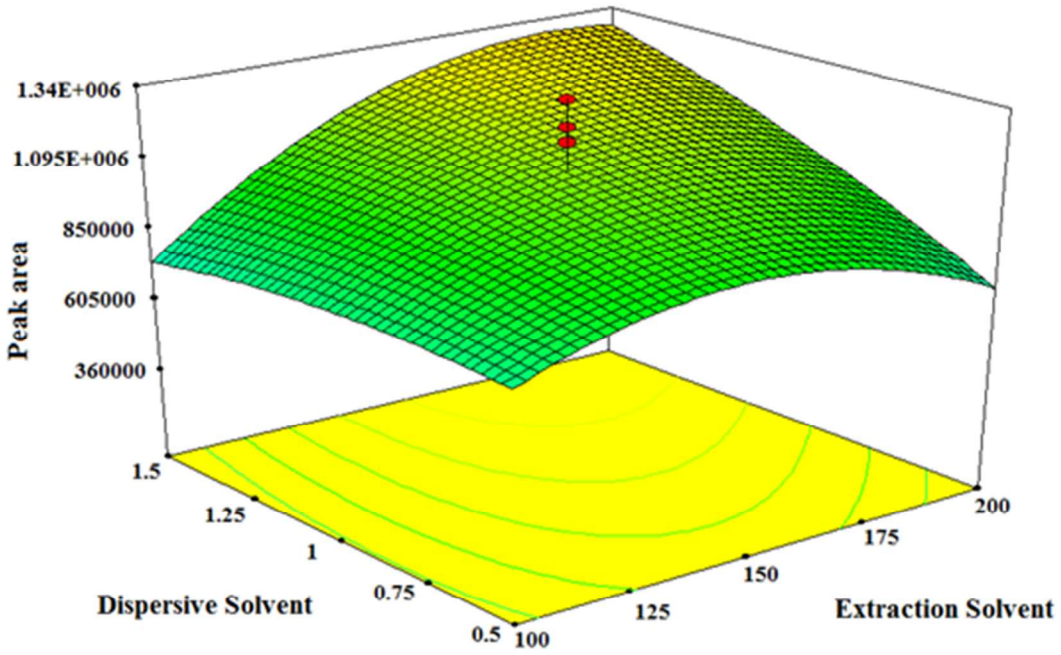
(c)

Fig. 4



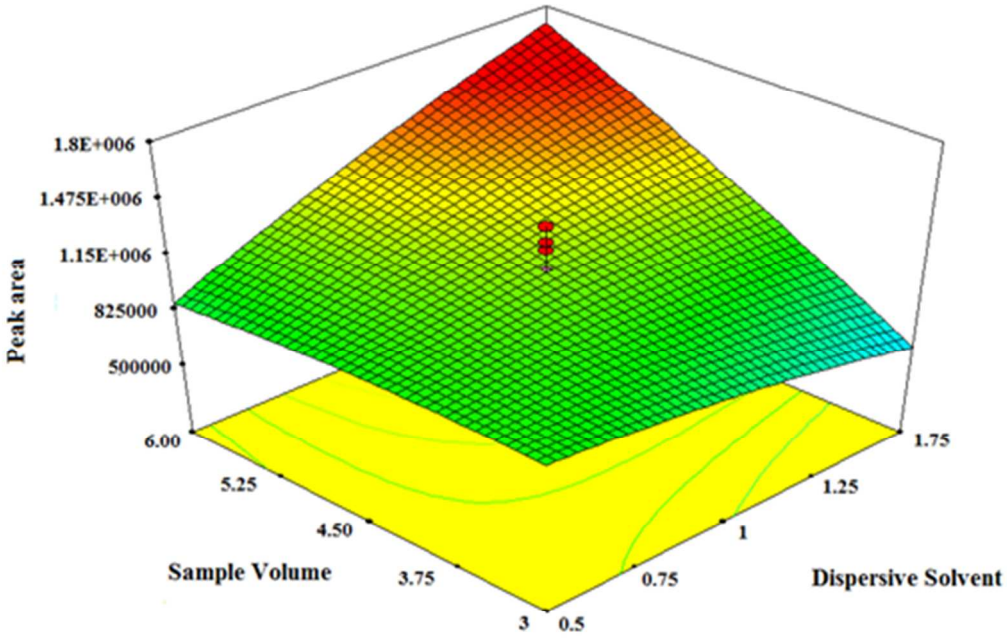
(a)

Fig.5



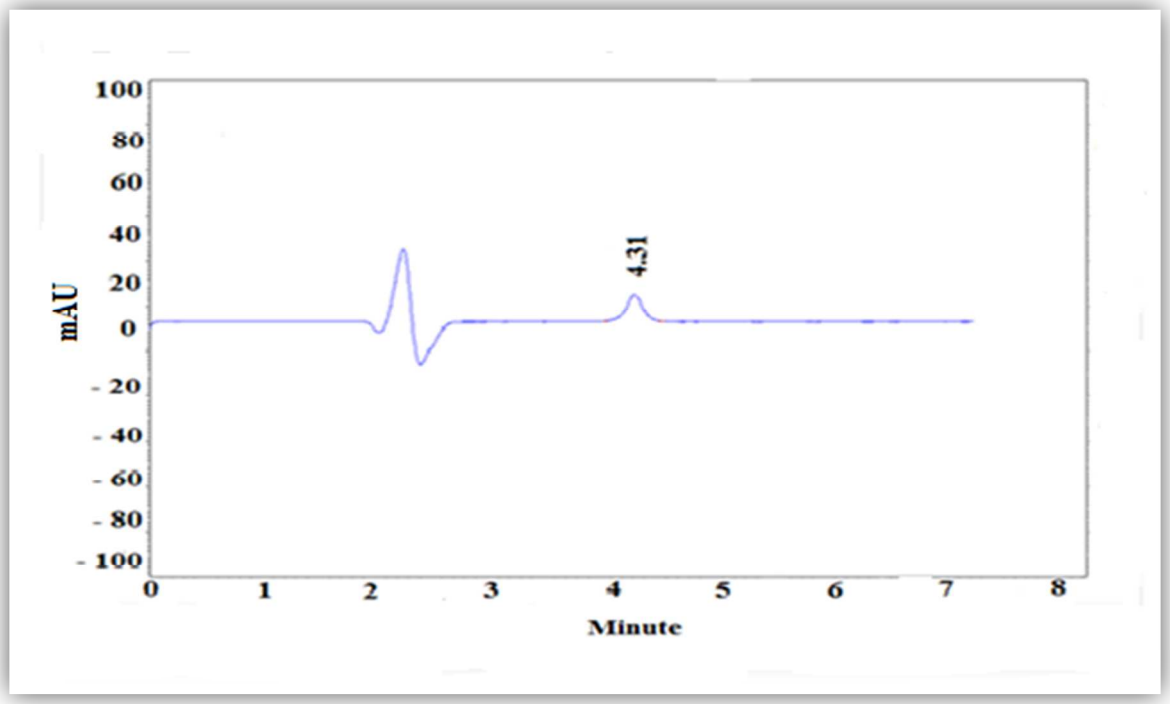
(b)

Fig.5



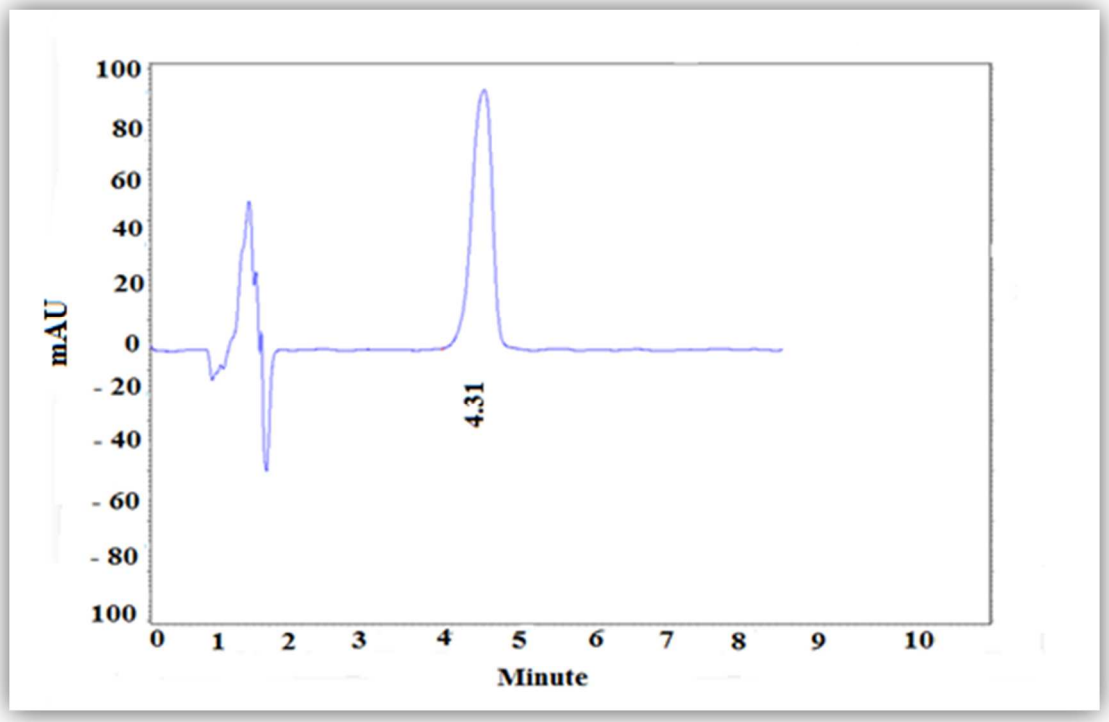
(c)

Fig.5



(a)

Fig.6



(b)

Fig.6