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Process optimisation, characterisation and evaluation of resveratrol-phospholipid complexes using Box-Behnken statistical design

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ABSTRACT

With the advent of 21st century, researchers worldwide have extensively reviewed herbs and botanicals for their marked clinical efficacy. It has been estimated that most of the newly discovered compounds offer poor bioavailability due to their low aqueous solubility. Phospholipid complexation of the drug often helps to improve its water solubility and enhance the bioavailability. This study includes optimization of resveratrol-phospholipid complexes using a 3-factor, 3-level box-behnken design (15 batches). Three independent variables *i.e.* phospholipid-resveratrol ratio, refluxing temperature and reflux time were optimized for two dependent variables, i.e. yield and entrapment efficiency (EE). Complexes were prepared by refluxing stoichiometric ratio of Phospholipon 90G and resveratrol in dichloromethane and retrieved by precipitation with n-hexane. Complexation was confirmed by Fourier Transform Infra-Red (FTIR) spectroscopy. The data was suitably used to explore quadratic response surfaces and construct second order polynomial models with Design Expert®. Formulation with highest desirability (D=0.994) was selected as optimum and prepared using 1.5:1 Phospholipon 90G-resveratrol ratio (X1) at 59.4°C temperature (X2) and 4 h time (X3) to give maximum yield and entrapment efficiency. Analysis of variance (ANOVA) was also found to be significant for both the responses. Complexes were optimised for good yield and EE. The partition coefficient was lowered to 2.25 hypothesizing good passive absorption.

***Key Words*:** Partition coefficient, phytosome, polyphenol, entrapment efficiency, yield, desirability.

INTRODUCTION

Resveratrol (trans-3,4',5-trihydroxystilbene), a polyphenolic phytoalexin, found in many plant species including grapes, peanuts, cranberries, Japanese knotweed (*Poligonum cusipdatum*) and others ("Resveratrol monograph," 2010). About 2000 years ago in ancient India, a well-known “Ayurvedic" medicine, "Darakchasava" was prescribed as a cardiotonic and for many other ailments whose active ingredient was resveratrol detected by high performance liquid chromatography analysis (Paul *et al.*, 1999). Resveratrol was first isolated in 1940 from the roots of white hellebore (*Veratrum grandiflorum*) (Aggarwal *et al.*, 2004). Later in 1963, resveratrol was extracted from the roots of *Polygonum cuspidatum*, a well-known plant used in Japanese and Chinese traditional medicine (Paul *et al.*, 1999). Resveratrol exhibits potent cardioprotective ("Resveratrol monograph," 2010) and chemotherapeutic activity (Das and Ka-Yun, 2010). It also offers antibacterial action against *Helicobacter pylori* and used for chronic inflammation developed by long-term use of aspirin and selective cycloxygenase-2 inhibitors (Baur and Sinclair, 2006). Resveratrol can also combat oxidative stress, improve health and survival by mimicking the effects of calorie restriction.

Resveratrol is a BCS class II drug with low solubility (3 mg/100 ml) and high permeability (log P 3.1) (Amri *et al.*, 2012). Poor aqueous solubility limits its *in vivo* bioavailability and thus it is a suitable candidate for phospholipid complexation which facilitates drug absorption by increased solubilization of drug in intestinal milieu by various ancillary mechanisms (Porter *et al.*, 2007). Phospholipids are important structural and functional component of cell membranes, which maintains cell membrane fluidity and useful in hepatic cirrhosis and acute liver disorders. Thus, phospholipids also offer distinct advantages in addition to solubilizing property when used as a carrier system (Schimitt, 2009; "Soy lecithin Fact Sheet, United Soyabean Board.," 2012).

Popularly known as Phytosomes, this technology has recently attracted the attention of many researchers due to its capacity of successful and effective delivery of potential therapeutics and dietary supplements (Agarwal *et al.*, 2012). It was developed by Indena S.p.A of Italy to augment the absorption and utilisation of poorly available phytomedicines. Indena is one of the world’s leading companies in lieu of exploring the nature’s riches and development of suitable dosage forms using the herbs & botanicals.

Objective of this study is to optimise resveratrol-phopsholipid complexes using a 3-factor, 3-level box-behnken design using desirability function. Independent variables (factors) were phospholipid-resveratrol ratio (X1), process temperature (X2) and reaction time (X3) for two dependent variables *i.e.* yield (Y1) and entrapment efficiency (Y2). Optimisation designs explore optimum conditions in a fewer runs rather than that of a full factorial design. A total of 15 batches were prepared and evaluated to find optimised conditions.

MATERIALS AND METHODS

Materials

Trans-resvertarol was purchased from Total Herbs Solution, Mumbai, India. Phospholipon 90G was a gift sample from Lipoid, Switzerland. Dichloromethane and n-hexane were procured from RFCL Ltd, New Delhi, India.

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Table 1: Formulation chart and evaluation data of prepared complexes.

Batch	X1	X2	X3	Y1, % Yield	Y2, % EE (Mean ± SD)
1	-1	-1	0	65.2	69.6 ± 0.65
2	1	-1	0	78.9	79.5 ± 2.10
3	-1	1	0	67.8	71.7 ± 0.60
4	1	1	0	80.5	81.6 ± 1.37
5	-1	0	-1	63.7	70.7 ± 0.51
6	1	0	-1	79.4	80.3 ± 0.90
7	-1	0	1	69.4	71.2 ± 0.86
8	1	0	1	81.9	83.6 ± 1.72
9	0	-1	-1	73.5	73.6 ± 1.62
10	0	1	-1	72.9	74.6 ± 1.37
11	0	-1	1	73.5	74.8 ± 1.22
12	0	1	1	76.3	75.6 ± 2.10
13[a]	0	0	0	74.7	74.2 ± 0.65
14[a]	0	0	0	73.8	75.0 ± 1.61
15[a]	0	0	0	75.7	74.4 ± 1.69

a: centre point replicates

Preparation of resveratrol-phospholipid complexes

Accurately weighed amounts of Phospholipon 90G and resveratrol in stoichiometric molar ratios (0.5:1/1:1/1.5:1) were dissolved in 20 ml dichloromethane (Bombardelli and Patri, 1988) and refluxed with condenser at constant temperature (40°/50°/60°C) for a defined period of time (2/3/4 h) according to box-behnken formulation design as specified in table 1. The mixture was concentrated to 2-3 ml by evaporating the solvent. Complexes were retrieved by precipitation by adding excess amount of n-hexane. Mixture was then allowed to stand, supernatant was decanted and residue was dried in dessicator (anhydrous calcium chloride) for 48 h to obtain resveratrol- phospholipid complexes.

Design of Experiments (DoE)

A 3-factor, 3-level box-behnken design was used to suitably explore the main, interaction and quadratic terms and construct second order polynomial equation using Design Expert® (Version 8.0.0, Stat-Ease Inc., Minneapolis, MN). This cubic design was characterised by a set of points lying at the midpoint of each edge of a multidimensional cube and centre point replicates (n = 3) (Yue *et al.*, 2010). A design matrix of 15 batches was constructed to generate a non-linear quadratic model equation as-

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

where Y is the measured response for each factor level combination; b_0 is an intercept; b_1 to b_{33} are regression coefficients figured from the observed experimental values of Y; and X_1, X_2 and X_3 are coded levels of independent variables. The terms X_1X_2 and X_i^2 (i = 1, 2 or 3) represent the interaction terms to study response on simultaneous change of three factors and quadratic terms to investigate the non-linearity, respectively. The dependent and independent variables selected are listed in table 2 along with their low, medium and high levels, selected on the basis of results from preliminary trials.

Evaluation of complexes

Yield

Complexes obtained were dried in dessicator and weighed to note the quantity. Yield (%) was calculated by given formula:

$$Yield\ (\%) = \frac{\text{Total amount of complexes}}{\text{Total amount of raw material}} \times 100$$

Entrapment efficiency (EE)

Accurately weighed quantity (10 mg) of complexes were dissolved in methanol and diluted. Absorbance was noted at 306 nm to record the concentration of resveratrol present in the solution. Accordingly, the amount of resveratrol in total yield was calculated and EE was determined by given formula:

$$EE\ (\%) = \frac{\text{Amount of resveratrol in complexes}}{\text{Total amount of resveratrol taken}} \times 100$$

Partition coefficient

Partition coefficient was calculated by adding excess amount (100 mg) of drug and complex in equal volume of n- octanol and water (each 10 ml). This dispersion was agitated for 24 h on mechanical rotary shaker model 24BL; Remi instruments Ltd. Vasai, India. The solutions were then separated using a separating funnel and were centrifuged at 4000 rpm for 15 min. Both layers were filtered and diluted to record the absorbance.

FTIR spectroscopy

Resveratrol, phospholipids and complexes are grounded separately with anhydrous potassium bromide into a fine powder using mortar and pestle and compressed into a disc (Das *et al.*, 2010). IR spectrum was recorded over a wave number region of 4000–400 cm^{-1} using FTIR spectrometer (model BX2, PerkinElmer, Norwalk, US).

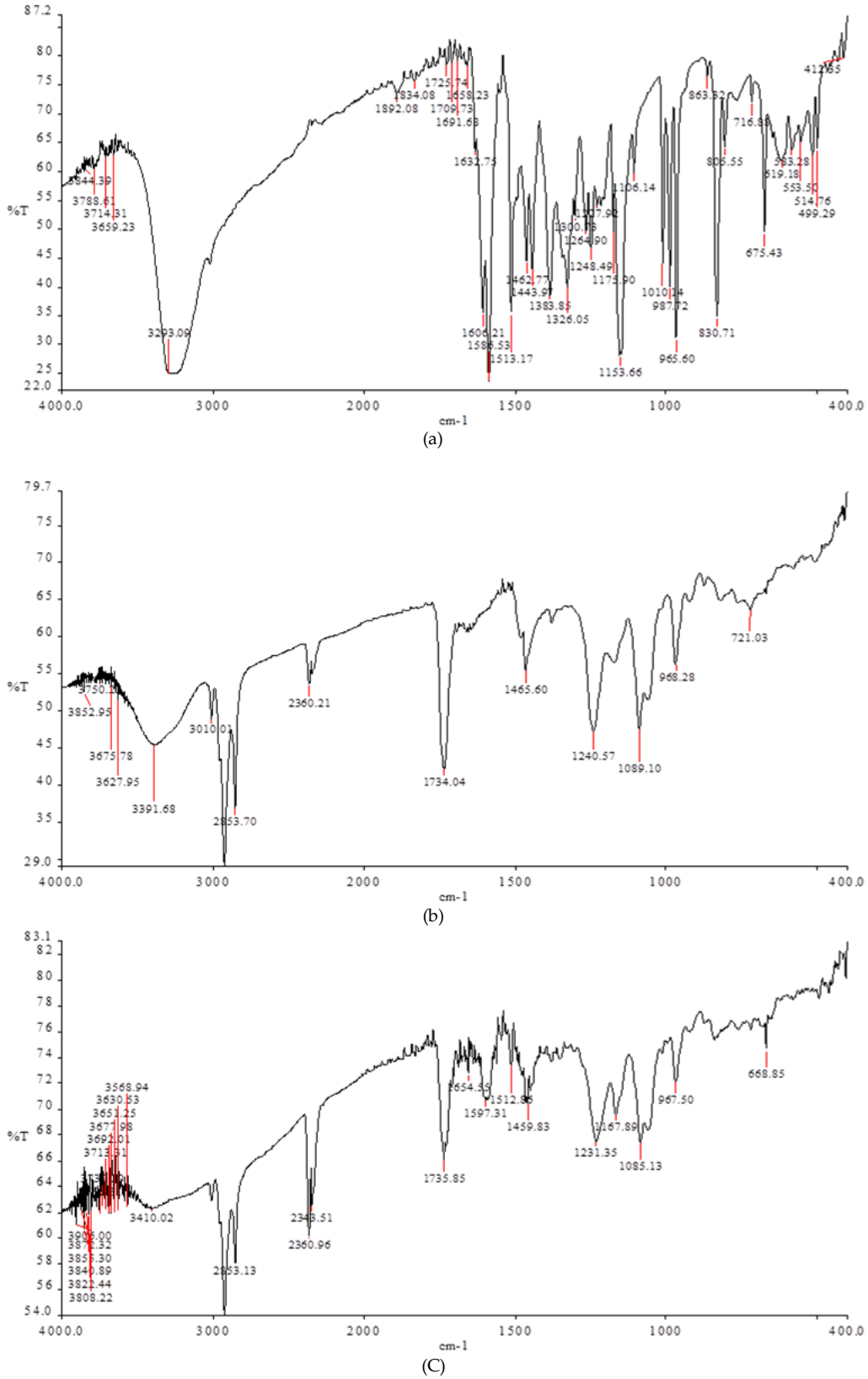
Optimisation using desirability and model validation

A total of 15 batches with triplicate centre points were prepared and evaluated for yield (%) and EE (%). This data was statistically analysed and validated by Design Expert® (Version 8.0.0, Stat-Ease Inc., Minneapolis, MN) on the basis of ANOVA and polynomial equations to find an optimised set of process parameters. The model was evaluated in terms of significant coefficients and R^2 values. The optimised formulation was selected making use of desirability functions. Each response is associated with its own partial desirability function. If the value of the response is optimum, its desirability equals 1, and if it is totally unacceptable, its value is zero. Thus the desirability for each response can be calculated at a given point in the experimental domain. The optimum is the point with the highest value for the desirability. Optimum formulation was prepared and evaluated for yield (%), EE (%) and partition coefficient. Various 3-D response surface graphs and contour plots were studied over the entire experimental region, to select six optimum checkpoint formulations for validation of the experimental domain and polynomial equation. The actual experimental values were quantitatively compared with the predicted values to compute the prediction error (%). Linear correlation plots between the actual and predicted values of each response were plotted using MS-Excel® along with the error bars (%) to obtain the R^2 values for optimum model validation.

RESULTS AND DISCUSSION

Evaluation of Complexes

Dried complexes were evaluated for yield (%) and entrapment efficiency (%) in triplicates and calculated for mean value with standard deviation (table 1). Results showed wide variation where yield (%) ranges between 63.7- 81.9% while EE lies between 69.6- 83.6%. This data was then subjected to optimisation and model validation.



(a)

(b)

(C)

Figure 1: FTIR spectra in infra-red region 4000-400 cm-1 (a) resveratrol (b) phospholipon (c) resveratrol- phospholipon complex.

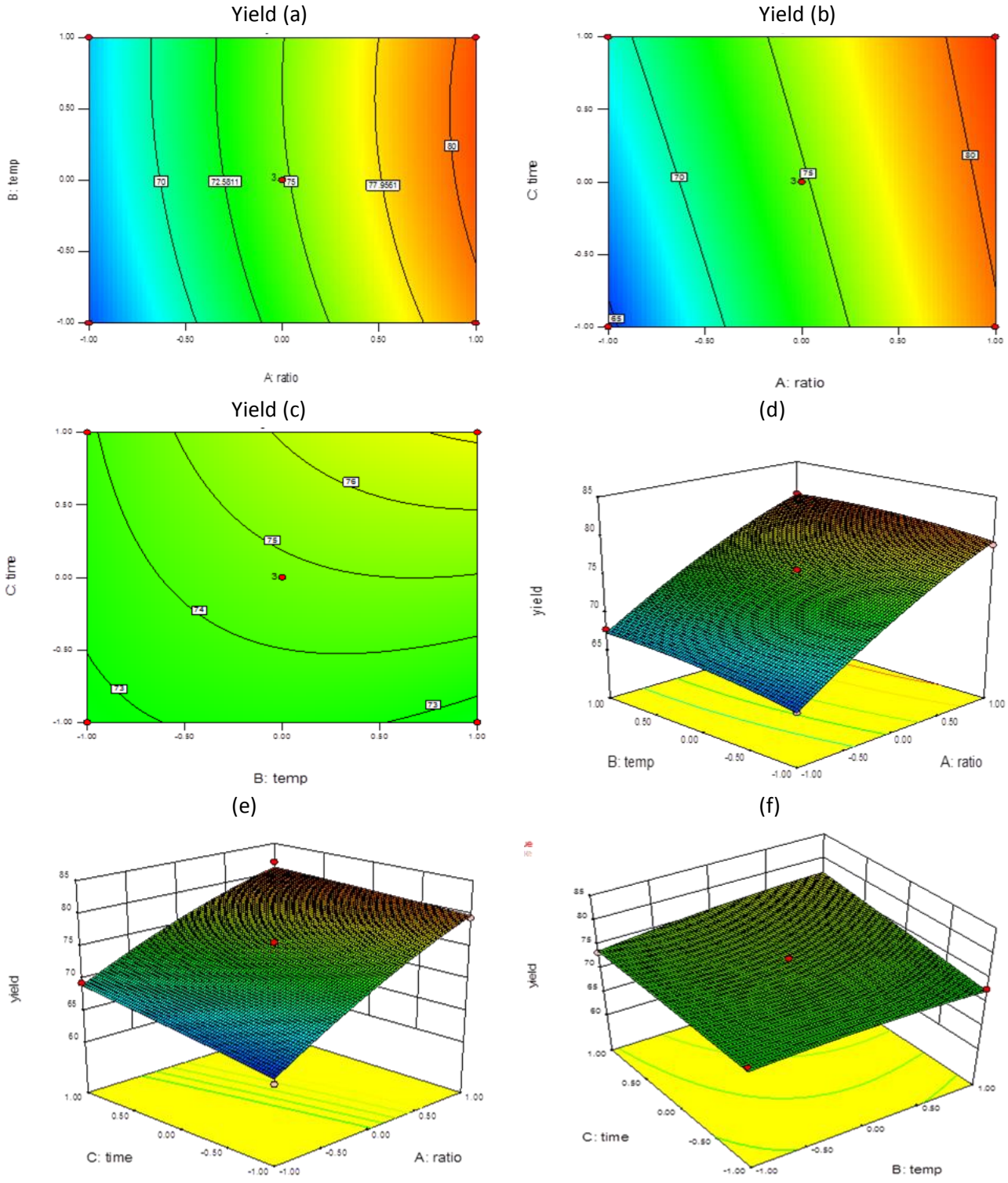


Figure 2: Contour plots for percent yield (a) phospholipon: resveratrol ratio and temperature, (b) phospholipon: resveratrol ratio and time, (c) temperature and time, and Response surface plots (d) phospholipon: resveratrol ratio and temperature, (e) phospholipon: resveratrol ratio and time, (f) temperature and time.

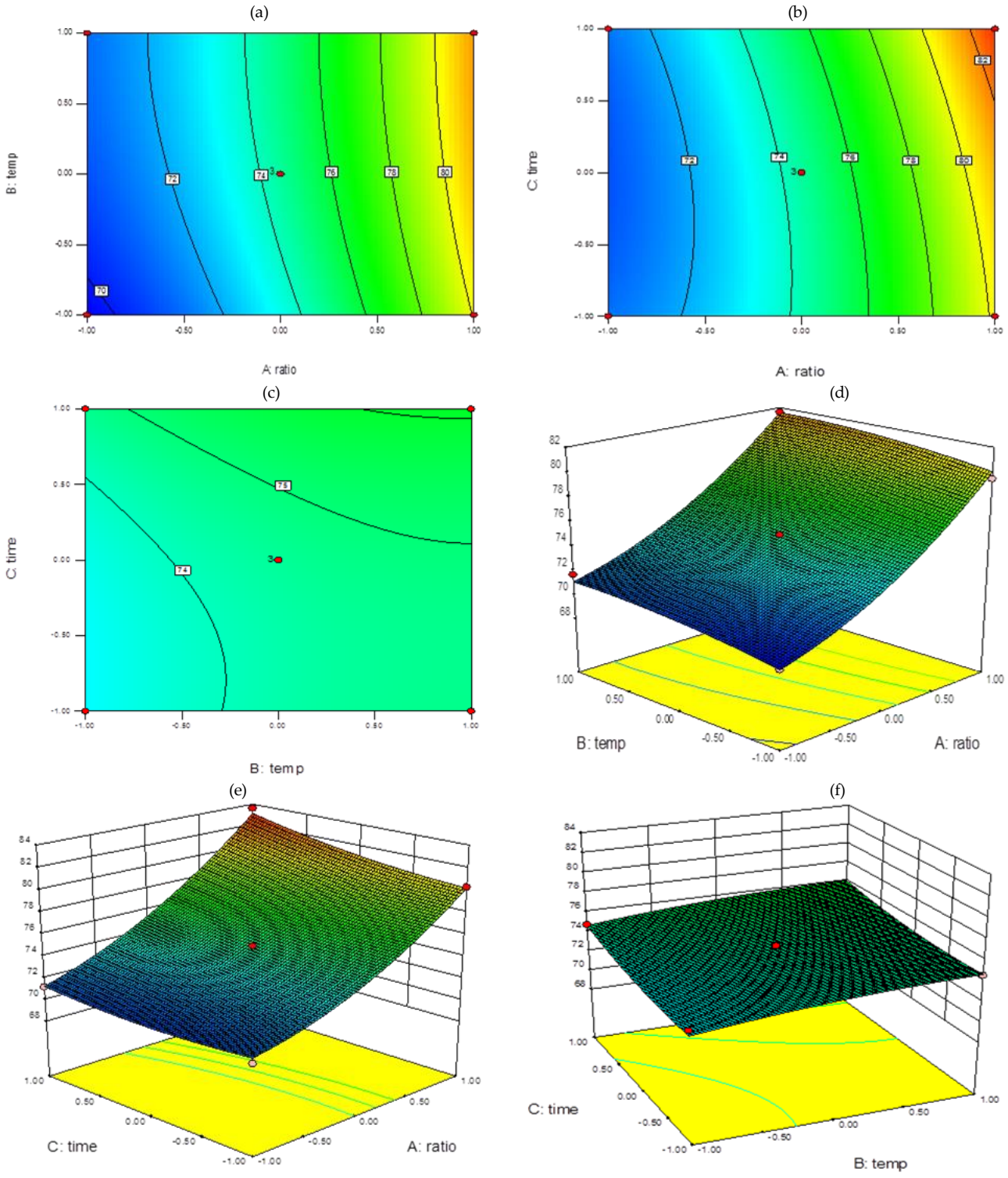


Figure 3: Contour plots for % EE (a) phospholipon: resveratrol ratio and temperature (b) phospholipon: resveratrol ratio and time (c) temperature and time, and response surface plots (d) phospholipon: resveratrol ratio and temperature (e) phospholipon: resveratrol ratio and time (f) temperature and time.

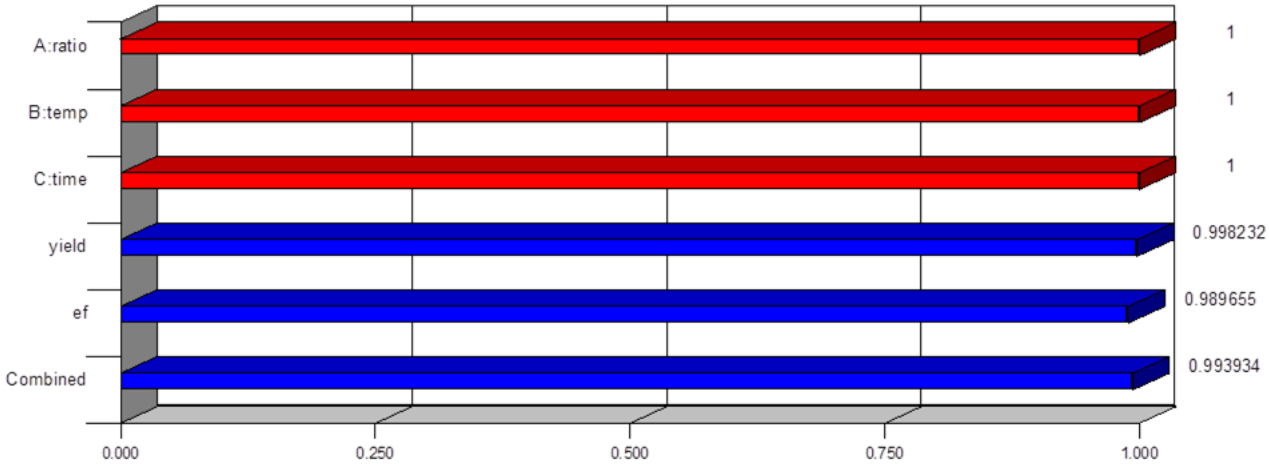


Figure 4: Individual and combined desirability for measured responses.

Table 2: Depdendant and independent variables in box-behnken design used for optimization studies.

Type of variable	Variable	Optimization Levels used: Actual (Coded)		
		Low (-1)	Medium (0)	High (+1)
Independent	X_1 [Phospholipon 90G: resveratrol ratio]	0.5:1	1:01	1.5:1
	X_2 [Temperature (°C)]	40	50	60
	X_3 [Time (h)]	2	3	4
Dependent	Y_1 [Yield (%)]		Maximise	
	Y_2 [Entrapment efficiency (%)]		Maximise	

Table 3: Summary of results of regression analysis for responses Y1 and Y2 for fitting to quadratic model.

Quadratic model	R^2	Adjusted R^2	Predicted R^2	SD	%CV
Response (Y1)	0.9864	0.9619	0.8427	1.06	1.43
Response (Y2)	0.9917	0.9768	0.8873	0.63	0.84

Regression equation of the fitted quadratic model[a]

$$Y1 = 74.73 + 6.825X_1 + 0.80X_2 + 1.45X_3 - 0.25X_1X_2 - 0.80X_1X_3 + 0.85X_2X_3 - 1.042X_1^2 - 0.592X_2^2 - 0.092X_3^2$$

$$Y2 = 74.53 + 5.23X_1 + 0.75X_2 + 0.75X_3 + 1.96X_1X_2 + 0.7X_1X_3 - 0.05X_2X_3 + 1.43X_1^2 - 0.367X_2^2 + 0.483X_3^2$$

[a]Only the terms with statistical significance are included

Table 4: Experimental levels and evaluation of optimised formulation.

Phospholipon: resveratrol ratio (X1)	Temperature (°C) (X2)	Time (hr) (X3)	% Yield	% EE	Partition coefficient, log P (mean ± SD)
1.5:1	59.4	4	81.7 ± 0.208	82.7 ± 0.96	2.25 ± 0.085

FTIR Spectroscopy

Infra-red spectrum of resveratrol showed a typical *trans* olefinic band at 965.6 cm^{-1} and narrow band of O-H stretching at 3293 cm^{-1} (figure 1a). Three characteristic intense bands at 1383.85, 1586.53 and 1606.21 cm^{-1} correspond to C–O stretching, C–C olefinic stretching and C–C aromatic double-bond stretching (13). On the other side, infra-red spectrum of phospholipid showed O-H stretching at 3391 cm^{-1} and characteristic P=O stretching band at 1218 cm^{-1} and P-O-C stretching band at 1089 cm^{-1} and $N(CH_3)_3$ stretching at 968 cm^{-1} (figure 1b).

Infra-red spectra of the complex formed showed remarkable shifting of P-O-C and P=O absorption band of phospholipon from 1089.10 cm^{-1} to 1085.13 cm^{-1} and from 1240.57 cm^{-1} to 1231.35 cm^{-1} respectively indicating interference at polar head of the Phospholipon 90G (figure 1c). Furthermore, O-H stretching at 3293 cm^{-1} show a narrow band in resveratrol IR spectra while IR spectra of the resveratrol- phospholipon complex showed broadening and shifting of this band (Silverstein *et al.*, 2005). Unlike intramolecular hydrogen bonding where peaks are sharp and well defined, spectrum of the complex gives broad bands indicating intermolecular hydrogen bonding between Phospholipon 90G and resveratrol thereby confirming the complexation (Sharma, 2007).

Data fitting to the model

A three-factor, three-level Box-Behnken statistical design was used. Measured responses for 15 runs were simultaneously fitted to first order, second order and quadratic models using Design Expert® software where, quadratic model was found best-fit and the comparative values of R^2, standard deviation and coefficient of variation (%) are given in table 3. ANOVA was found to be significant for

Table 5: Composition of optimised and checkpoint formulation, the predicted and experimental values of response variables and percentage prediction error.

Checkpoint formulations (X1:X2:X3)	Response variables	Experimental values	Predicted values	% prediction error
0.6:53.3:3.58	Y1	74.92	75.38	0.61
	Y2	74.39	74.71	0.428
0.8:43.9:2.10	Y1	70.05	69.61	-0.632
	Y2	72.35	71.99	-0.5
1.3:43.0:2.46	Y1	77.27	77.53	0.335
	Y2	76.53	76.88	0.455
1.1:55.8:2.40	Y1	75.86	75.61	-0.331
	Y2	76.89	76.34	-0.72
1.31:53.9:3.45	Y1	80.19	79.73	-0.577
	Y2	80.86	80.34	-0.647
1.4:55.5:3.06	Y1	79.13	79.95	1.025
	Y2	80.78	81.19	0.505
Optimized 1.5:59.4:4	Y1	81.71	81.86	0.183
	Y2	82.76	83.45	0.827

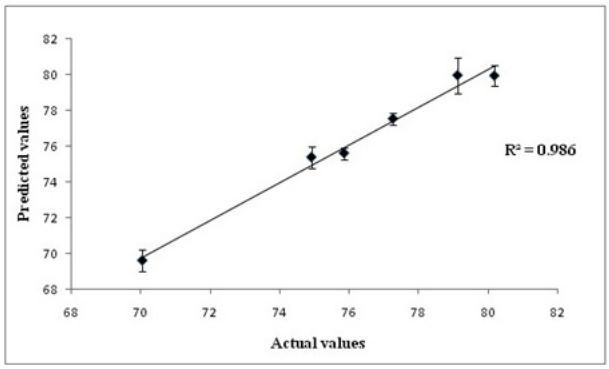
both responses and this model was used to navigate the design space to find the optimised conditions.

The quadratic equations for each response were generated as given in table 3, where only statistically significant ($p < 0.05$) coefficients are included and a positive coefficient indicates that response is favoured, while a negative value indicates an inverse relationship between the factor and the response. It is clear from the equations that all the three independent variables *i.e.* the phospholipon: resveratrol ratio (X1), temperature (X2) and time (X3) have positive effects on the two responses *i.e.* yield (Y1) and EE (Y2). The effect of phospholipon: resveratrol ratio (X1) on Y1 and Y2 was almost 7 and 5 fold greater than other two factors respectively which means that factor X1 is highly influencing the outcome or results of complexation process that are yield and EE. However, the effect of temperature and time on response Y1 and Y2 is not highly significant. This relationship between factors and responses is explained even more clearly by response surface graphs as presented in figure 2

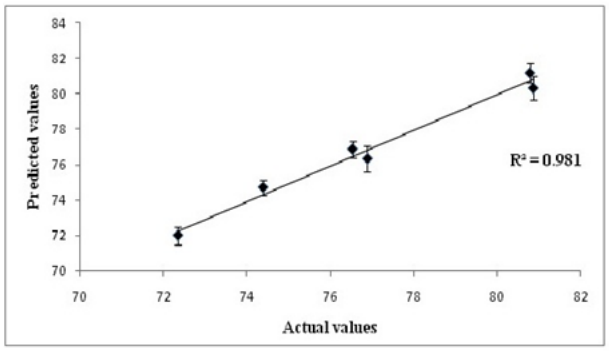
These are 2- dimensional graphs between two factors while the 3rd factor is constant. Figure 2d shows the effect of X1 and X2 on Y1 and the graph displays that there is sharp increase in yield with increase in phospholipon: resveratrol ratio (X1) while increase in temperature (X2) or time (X3) results in only slight increase or moderate enhancement in yield as revealed by Figure 2f . However, response Y1 is favoured more by X3 where yield approaches 70% unlike the mid-point of 65-70% in case of X2 clear from figure 2e.

Similar results were obtained with Y2. The figure 3 d, e and f display the more significant effect of X1 than X2 and X3 in achieving high entrapment efficiency (Y2). When the level of X1 was varied from low to high, EE increase was approaching 80%.

Desirability function was used to identify the optimum levels of factors and to get maximum desirable response. The individual desirability and the combined desirability of all the responses are reported in figure 4. The optimized batch was selected with maximum combined desirability value *i.e.* 0.994 using Design Expert® version 8.0.0 (Stat-Ease, Inc.). Thus, formulation composition with high levels of X1 (phospholipon: resveratrol ratio of 1.5:1), X2 (temperature 59.4°C) and X3 (time 4 h) was found to fulfil the maximum requisite of an optimum formulation by highly affecting the yield and EE



(a)



(b)

Figure 5: Linear correlation plots between actual and predicted values of checkpoint formulations (a) yield (%) (b) EE (%).

of complexes. The optimised formulation was prepared in triplicate and evaluated for the measured responses and also for partition coefficient, which are presented in table 4. Optimised formulation showed high yield (81.7%) and EE (82.7%).

Partition coefficient was decreased to 2.25 from 3.1 which suggest increase in aqueous solubility hypothesizing good passive absorption across the lipid membrane (Vemulapalli *et al.*, 2007).

Validation of Optimisation Model

To validate the optimisation model, six checkpoint formulations were selected from the experimental domain. Table 5 shows the factor levels for checkpoint formulations prepared and evaluated for yield and EE to find the experimental values. Predicted values for the same checkpoints were determined using the regression equations and compared with the experimental values to find the percentage prediction error or biasness (%), which was found to vary between -0.720% and 1.025%. Linear correlation plots between the actual and the predicted response variables were plotted to represent the scatter of the predicted versus actual values (figure 5a-b), in terms of R^2 (For Y1and Y2, R^2: 0.986, 0.981respectively). Thus, the low magnitude of error as well as the high values of R^2 in this study proves the significant analytical ability of the model.

CONCLUSION

Box-behnken design comprising of triplicate centre points was successfully applied on phospholipid complexes and had facilitated to investigate the effect of variables on responses *i.e.* yield and EE. Complexation between resveratrol and Phospholipon was confirmed by FTIR spectrum of complexes. Complete set of data was then subjected to quadratic model to generate polynomial regression equations, contour plots and response surface plots. Optimised formulation was selected on the basis of maximum desirability (D=0.994) and was found to be X1 (1.5:1), X2 (59.4°C) and X3 (4h). It was prepared and evaluated for yield and EE that was found to be 81.71% and 82.76% respectively and compared with predicted values to find prediction error to be 0.183% and 0.827%. Optimised formulation was also evaluated for partition coefficient which was found to be 2.25±0.085. A partition coefficient value between 1 and 3 hypothesizes good passive absorption and improved oral bioavailability. Additional six checkpoint formulations were randomly selected in the experimental domain and evaluated to find the actual values. These actual values were then compared with predicted values from the polynomial equation to determine the percent prediction error ranging between -0.720 and 1.025%. Linear correlation plots between actual and predicted values of checkpoints was plotted using Microsoft Excel and R^2 values was found close to be 0.986 and 0.981 for yield and EE respectively, indicating that model is validated.

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