Optimisation of the Oil Extraction from *Nigella sativa* Seeds Using Response Surface Methodology

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Nigella sativa, also known as black cumin, an annual herbaceous plant growing especially in Mediterranean countries, has recently gained considerable interest not only for its use as spice and condiment but also for its healthy properties of the fixed and essential oil and its potential as a biofuel. Nigella sativa seeds fixed oil, due to its high content in linoleic acid followed by oleic and palmitic acid, could be beneficial to human health. The objective of this study is to determine the optimum conditions for the solvent extraction of Nigella sativa seeds fixed oil using a three-level, three-factor Box-Behnken design (BBD) under response surface methodology (RSM). The obtained experimental data, fitted by a second-order polynomial equation were analysed by Pareto analysis of variance (ANOVA). From a total of 10 coefficients of the statistical model only 5 are important. The obtained experimental values agreed with the predicted ones.

Keywords: Nigella sativa seeds oil, solvent extraction, Response Surface Methodology, Box-Behnken design

In response to the needs of growing industrialization and food and pharmaceutical sector development, the interest in investigation of non-conventional plants as sources of sustaining oils received special attention in recent years [1-4].

Nigella sativa (Family Ranonculaceae) among numerous other plants recognized as containing bioactive components with beneficial properties has been extensively studied. It is considered a miracle herb with historical and religious background, world - wide used due to its proved pharmacological, phytochemical and nutritional properties [5-8].

The part of the plant which has the therapeutic effect is the seed. The seeds contain over one hundred chemical compounds: essential and fixed oils, proteins, alkaloids, saponins etc. [9].

Fixed oil, representing more than 30 % (w/w) of the seeds, is composed mainly of unsaturated fatty acids, including $C_{20:2}$ eicosadienoic acid [10, 11], glycolipids, phospholipids, and bioactive phytosterols [12]. Thanks to this valuable composition, *Nigella sativa* seed fixed oil is considered among newer sources of edible oils, with important function in nutrition and human health [10, 13].

The link between oil's chemical composition and its biological activity, although closely interconnected, is not yet completely defined. It is however a certitude that composition complexity and the yield of seed oil strongly depend on the employed extraction method. Numerous investigations refer to solvent extraction at different temperature levels, from cold extraction up to hot extraction conducted at 70°C. Researches indicated that higher extraction temperature may induce partial alteration, especially on active components, with negative impact on functional, anti-oxidative and pro-oxidative effects [10, 14]. Cold pressing was also applied as non-hazardous technique, involving no heat or solvents usage and no ulterior refining. Despite the resulted volatile oils contain numerous active constituents, with proved beneficial clinical effect, it were found as more susceptible to accelerated oxidation than solvent-extracted oils. Newer techniques, like microwave assisted extraction or supercritical (fractionated) CO_2 extraction were reported with good results [10, 15].

Disregarding the extraction method, most of the studies also emphasized the importance of plant chemotype and geographical conditions [16-18]. In all extraction studies the experimental results could be analyzed by means of semiempirical, phenomenological or statistical models [19-21].

This study uses classical solvent extraction method, as simple and effective method to investigate the potential usage of Romanian *Nigella sativa* for the production of oil proper for human consumption. The determination of optimal conditions for achieving valuable oils and higher yields involved response surface methodology (RSM) with a three-variable-three-level Box-Behnken design (BBD).

Experimental part

Materials and chemicals

Nigella sativa seeds were purchased from Research and Development Station for Agriculture, Secuieni, Neamt. Moisture content of the seeds (6.5 ± 0.2%) was determined using a thermo-balance OHAUS MB23.The seeds, after grinding using an electric grinder, were sieved to get particles with diameters in the range of 500-750 μm and stored in a refrigerator until extraction.

The solvents, n-hexane, petroleum ether 40-60°C fraction (p.a.) and CH_2Cl_2 (HPLC purity grade) have been purchased from Sigma-Aldrich. Methanol (HPLC isocratic grade) and BF_3-MeOH 10-14% complex have been purchased from J.T. Baker and Alpha Aesar.

The standard mixture of 37 fatty acid methyl esters (*Supelco*™ 37 Component FAME Mix) used for the gas-chromatographic analyses has been purchased from Supelco.

Extraction procedure

Laboratory Soxhlet extraction was used to determine the initial fixed oil content in *N. sativa* seeds. About 5 g of grinded seeds was weight and subjected to extraction with 150 mL n-hexane for 8 h. The average result of three

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experiments was expressed as the percentage of lipids in the dry matter of seed powder.The initial oil content was 40.02 ± 0.68%.

Batch extraction was used in all experiments, using a magnetic stirrer with heating plate to set up the working temperature. In a typical experiment 10 g of grinded *N. sativa* seeds was weighed and placed into a glass vessel foreseen with water condenser. After a predetermined time the mixture was filtered under vacuum and the solvent distilled at low pressure. The extracted oil was dried until constant weight. The relative oil extraction yield, Y (%), was computed with equation (1):

$$Y(\%) = \frac{m}{m_0 \cdot w_{oil}} \times 100 \qquad (1)$$

where m is the mass of extracted oil, m_0 the mass of seeds and w_{oil} represents the initial oil content of seeds established by Sohxlet extraction.

All extraction experiments were repeated three times and the average values are presented.

Analysis of fatty acid composition of the extracted oil

The extracted oil composition was established using chromatographic analysis. Fatty acid methyl esters (FAME) were prepared by transesterification using BF_3-MeOH complex as catalyst, according to a previously reported method [22].

The gas-chromatograms of FAME have been recorded in triplicate on an *Agilent Technologies* 7890 A instrument equipped with Agilent auto sampler and Triple Axis mass detector model 5975 C VL MSD (operating conditions: oven temperature 140°C for 5 min, then 4 °C/min to 240°C for 20 min – a total routine of 50 min., carrier gas He, split ratio 100:1). Separation into components has been done on a capillary column especially designed for FAME analysis (*Supelco* SP^{TM} 2560: 100 m length, 0.25 mm inner diameter, 0.2μm film thickness). The ready for injection solutions have been prepared in CH_2Cl_2 of HPLC purity grade. Fatty acids identification has been performed by comparing for each peak the retention time with those from a commercial standard mixture (*Supelco*^{TM} 37 Component FAME Mix). Fatty acids composition has been determined on the basis of the GC peak areas correlated with the response factors of the detector; these factors have been calculated for each FAME in the standard mixture by reporting the unit area of each peak to the unit area of the oleic acid methyl ester peak. The response factors of the detector have been calculated as an average of five determinations.

Experimental design and statistical analysis

A preliminary study was performed in order to establish the effect of the main process parameters (temperature, liquid/solid ratio and time) on the relative oil extraction yield. A single factor experimental method was used to determine the range for each independent variable. In all experiments grinded seeds with a particle size of 0.63±0.02 mm, humidity content 6.5% and n-hexane as solvent were used. The individual and interactive effect of the three independent variables: temperature (X_1), solid liquid ratio (X_2) and extraction time (X_3) on the response variable (Y – relative oil extraction yield) was evaluated using a three factors at three levels Box-Behnken experimental design (BBD).The uncoded and coded independent variables are listed in table 1.

A quadratic model was chosen for predicting the values of response variable, according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (2)$$

where Y is the response (relative extraction yield), β_0 a constant term, β_i are coefficients of linear terms, β_{ii} coefficients of quadratic terms and β_{ij} the coefficients of interactive terms. The important linear, quadratic, and interaction coefficients were determined by the least square regression followed by analysis of variance (ANOVA) with a confidence of p = 0.05. For experimental design analysis and data processing the statistical package software STATISTICA (Trial version Stat Soft Inc., Tulsa, USA) was used.

Results discussions

Composition of Nigella sativa seeds oil

The extracted oil using hexane as solvent after transesterification were GC analysed. A typical chromatograph is presented in figure 1.

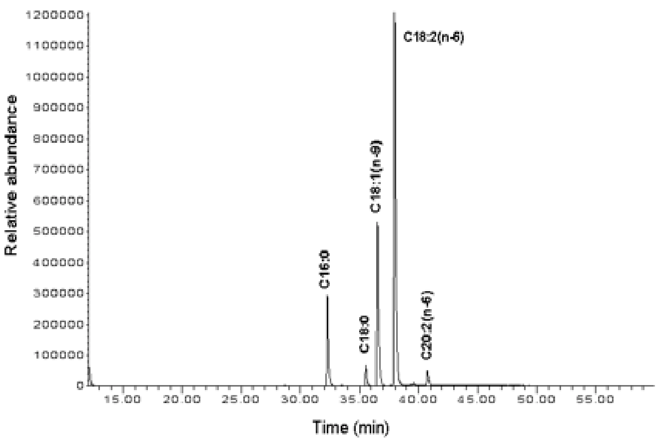

Fig.1. GC chromatogram of *N. sativa* seeds fixed oil obtained by Soxhlet extraction

Table 2 presents the main compounds of the extracted oil, revealing a high level of unsaturation.

Fitting the model

The three variables (temperature, liquid/solid ratio, time) and three factorial levels Box-Behnken design was applied for response function fitting. The whole design consisted of 17 experimental points carried out in random order. Five replicates (experiments 13-17) at the centre of the design were used for estimating of a pure error sum of squares. The uncoded and coded independent variables and the

Table 1
INDEPENDENT VARIABLES AND THEIR LEVELS USED IN EXPERIMENTS FOR FIXED OIL EXTRACTION FROM *NIGELLA SATIVA* SEEDS

Independent variables	Symbol coded	Factor level		
		-1	0	1
Temperature (°C)	X_1	20	40	60
Liquid/solid ratio (mL/g)	X_2	8	12	16
Time (h)	X_3	1	2	3

No.	Acid	Composition (molar % ± sd)
1.	Palmitic, $C_{16:0}$	12.80 (± 0.08)
2.	Stearic,$C_{18:0}$	2.38 (± 0.02)
3.	Oleic,$C_{18:1}$	22.02 (± 0.12)
4.	Linoleic, $C_{18:2}$	60.38 (± 0.35)
5.	Linolenic, $C_{18:3}$	0.24 (± 0.01)
6.	cis-11-Eicosenoic, $C_{20:1}$	0.12 (± 0.01)
7.	*cis*-11,14-Eicosadienoic, $C_{20:2}$	2.05 (± 0.04)

Table 2
COMPOSITION OF *NIGELLA SATIVA* SEEDS FIXED OIL

Exp	Coded level			Independent variables			Relative extraction efficiency, Y (%)	
	X_1	X_2	X_3	T (°C)	L/S ratio (v/wt.)	t (h)	Experimental	Predicted
1	-1	-1	0	20	8	2	73.16	73.28
2	1	-1	0	60	8	2	87.12	85.98
3	-1	1	0	20	16	2	79.04	80.18
4	1	1	0	60	16	2	89.74	89.62
5	-1	0	-1	20	12	1	72.4	71.96
6	1	0	-1	60	12	1	87.12	87.94
7	-1	0	1	20	12	3	76.65	75.83
8	1	0	1	60	12	3	81.56	82.00
9	0	-1	-1	40	8	1	76.75	77.08
10	0	1	-1	40	16	1	85.54	84.84
11	0	-1	1	40	8	3	77.84	78.54
12	0	1	1	40	16	3	81.65	81.32
13	0	0	0	40	12	2	82.32	82.95
14	0	0	0	40	12	2	84,12	82.95
15	0	0	0	40	12	2	81.91	82.95
16	0	0	0	40	12	2	82.87	82.95
17	0	0	0	40	12	2	83.52	82.95

Table 3
EXPERIMENTAL AND PREDICTED RESULTS FOR THREE LEVEL BOX-BEHNKEN DESIGN WITH THREE INDEPENDENT VARIABLES

experimental Box-Behnken design matrix are listed in table 3.

The regression coefficients, related to coded variables, obtained by fitting experimental data to the second order response model for the investigated response (relative extraction efficiency) are presented in table 4. It can be seen that the linear parameters corresponding to temperature and liquid/solid ratio, the quadratic parameter of time and the interaction parameter between temperature and time are the only significant parameters ($p < 0.05$).

These results indicate that the major contributing factor to the relative extraction efficiency is the temperature. This is confirmed also by the Pareto chart, presented in figure 2.

Fitting the model

The three variables (temperature, liquid/solid ratio, time) and three factorial levels Box-Behnken design was applied for response function fitting. The whole design consisted of 17 experimental points carried out in random order. Five replicates (experiments 13-17) at the centre of the design

Table 4
ESTIMATED COEFFICIENTS OF THE SECOND ORDER POLYNOMIAL EQUATION OF RESPONSE SURFACE OF OIL YIELD EXTRACTED FROM *NIGELLA SATIVA* SEEDS

Variables	Regression coefficients		Standard error	t - value	p - value*
Intercept	β_0	82.94800	0.499144	166.1806	0.000000
X_1	β_1	5.53625	0.394608	14.0298	0.000002
X_1^2	β_{11}	-0.84775	0.543929	-1.5586	0.163060
X_2	β_2	2.63750	0.394608	6.6839	0.000282
X_2^2	β_{22}	0.16475	0.543929	0.3029	0.770778
X_3	β_3	-0.51375	0.394608	-1.3019	0.234144
X_3^2	β_{33}	-2.66775	0.543929	-4.9046	0.001744
X_1X_2	β_{12}	-0.81500	0.558060	-1.4604	0.187554
X_1X_3	β_{13}	-2.45250	0.558060	-4.3947	0.003177
X_2X_3	β_{23}	-1.24500	0.558060	-2.2309	0.060886

* $p < 0.05$ indicates statistical significance

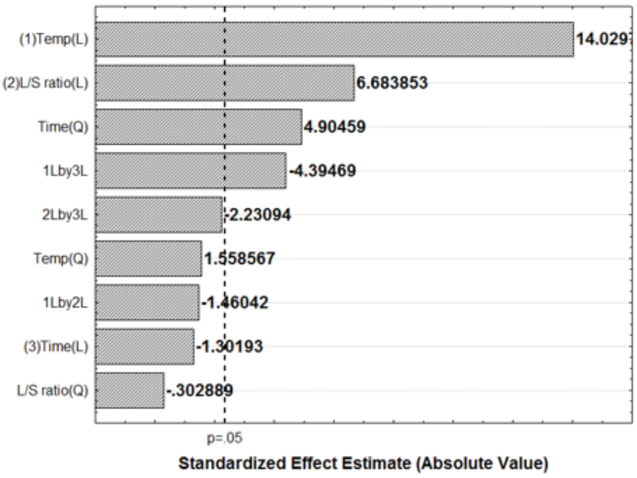

Fig. 2. Pareto chart for the effects of process parameters, temperature (Temp), liquid/solid ratio (L/S ratio), extraction time (Time) and of their interactions on the relative extraction efficiency of *Nigella sativa* seed oil

were used for estimating of a pure error sum of squares. The uncoded and coded independent variables and the experimental Box-Behnken design matrix are listed in table 3.

The regression coefficients, related to coded variables, obtained by fitting experimental data to the second order response model for the investigated response (relative extraction efficiency) are presented in table 4. It can be seen that the linear parameters corresponding to temperature and liquid/solid ratio, the quadratic parameter of time and the interaction parameter between temperature and time are the only significant parameters ($p < 0.05$).

These results indicate that the major contributing factor to the relative extraction efficiency is the temperature. This is confirmed also by the Pareto chart, presented in figure 2.

Multiple regression analysis on the experimental data was done and with the coefficients listed in table 4 an empirical relationship between the experimental variables and the response was obtained:

$$Y = 82.948 + 5.536X_1 - 0.848X_1^2 + 2.638X_2 + \\ +0.165X_2^2 - 0.514X_3 - 2.668X_3^2 - 0.815X_1X_2 - \\ -2.453X_1X_3 - 1.245X_2X_3 \quad (3)$$

where X_1, X_2, and X_3 are the coded factors of the test variables, extraction temperature, liquid/solid ratio and extraction time, respectively.

Eliminating the insignificant coefficients ($p > 0.05$) from equation (3), the following second-order polynomial equation was obtained for predicting the relative extraction efficiency(Y):

$$Y = 82.948 + 5.536X_1 + 2.638X_2 - \\ -2.668X_3^2 - 2.453X_1X_3 \quad (4)$$

Table 5 summarize the results for analysis of variance (ANOVA) followed by Fisher's statistical test (F-test) for the experimental data obtained according to the Box-Behnken Design. The validity of the quadratic model is sustained by the F-value (33.00). The values calculated for the coefficient of determination ($R^2 = 0.9770$) and adjusted-R^2 (0.9474) indicate a satisfactory adjustment of the quadratic model and a good correlation between the experimental and the predicted values. Also the value for predicted-R^2 of 0.7514 is in reasonable agreement with the adjusted-R^2.

The value computed for the adequate precision (20.434), measuring the signal to noise ratio, indicates an adequate signal (a ratio greater than 4 is desirable).

The coefficient of variation (CV) shows the scattering of the experimental points from the predicted values obtained from the quadratic model. The very low coefficient of variation value (1.32) sustains a high degree of precision and reliability of experiments. This result is also sustained by diagnostic plots such as the experimental data versus predicted ones, presented in figure 3. One can see that the data points are positioned closed to the straight line, signifying that there is a satisfactory agreement between experimental and model data.

All these data demonstrate that the quadratic polynomial model is significant and could be used to characterize the relationship between the model response and the process variables and adequate for predicting the relative extraction efficiency of *N. sativa* seeds oil.

The 3D response surface plots, presented in figure 4, were employed to point out graphically the effect of the independent process factors on the extraction of *N. sativa* seeds oil. They showed the influence of two factors on the process while the third was maintained as constant at the value characterizing the centre of experimental design (table 1).

Source	Sum of squares	Degree of freedom	Mean square	F - value	p - value
Model	369.97	9	41.11	33.00	< 0.0001
X_1 – temperature	245.20	1	245.20	196.83	<0.0001
X_1^2 –	3.03	1	3.03	2.43	0.1631
X_2–liquid/solid ratio	55.65	1	55.65	44.67	0.0003
X_2^2	0.11	1	0.11	0.092	0.7708
X_3 –time	2.11	1	2.11	1.70	0.2341
X_3^2	29.97	1	29.97	24.06	0.0017
X_1X_2	2.66	1	2.66	2.13	0.1876
X_1X_3	24.06	1	24.06	19.31	0.0032
X_2X_3	6.20	1	6.20	4.98	0.0609
Residual	8.72	7	1.25		
Total	378.69	16			
R-Squared 0.9770					
Adj R-Squared 0.9474					
Pred R-Squared 0.7528					
Adeq Precision 20.434					
C.V. 1.39					

Table 5
ANALYSIS OF VARIANCE (ANOVA) OF THE MODELED RESPONSE

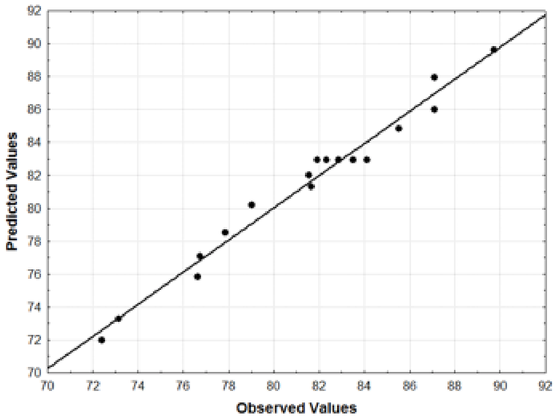

Fig.3. Comparison between predicted and experimental values for the relative extraction efficiency

Higher temperature softens seeds cells improving rate of diffusion, enhancing extraction efficiency and also increases the oil solubility.

Figure 4b shows the 3D surface plot of the effect of extraction temperature (X_1) and extraction time (X_3) upon the relative extraction efficiency, while the liquid/solid ratio is kept constant at zero level. It can be observed that the relative extraction efficiency increases with the increase of temperature, but extraction time has a favourable effect only until zero level, after that its influence is not significant. A similar influence of the liquid/solid ratio (X_2) and extraction time (X_3) on the relative extraction efficiency of *N. sativa* seed oil could be observed from the 3D surface plot presented in figure 4c.

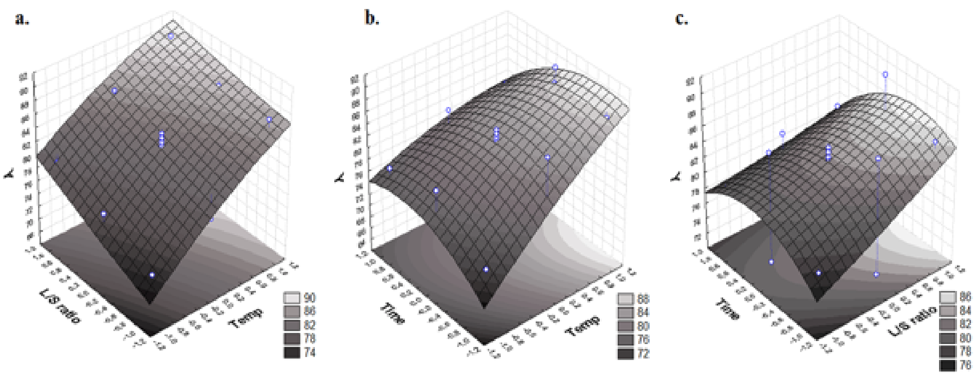

Fig. 4. 3D response surfaces of the relative extraction efficiency for *N. sativa* seed oil as a function of: (a) temperature and L/S ratio; (b) temperature and time and (c) L/S ratio and time

From figure 4a, where the effect of extraction temperature (X_1) and liquid/solid ratio (X_2) on the extraction efficiency is presented, the extraction time being constant at zero level, it could be observed that both process parameters have a strong effect on the extraction efficiency.

Optimization of the Nigella sativa seed oil extraction

The desirability profiles (fig. 5) indicates that the optimal extraction conditions can be achieved for the maximum level of temperature and liquid/sold ratio and at the central

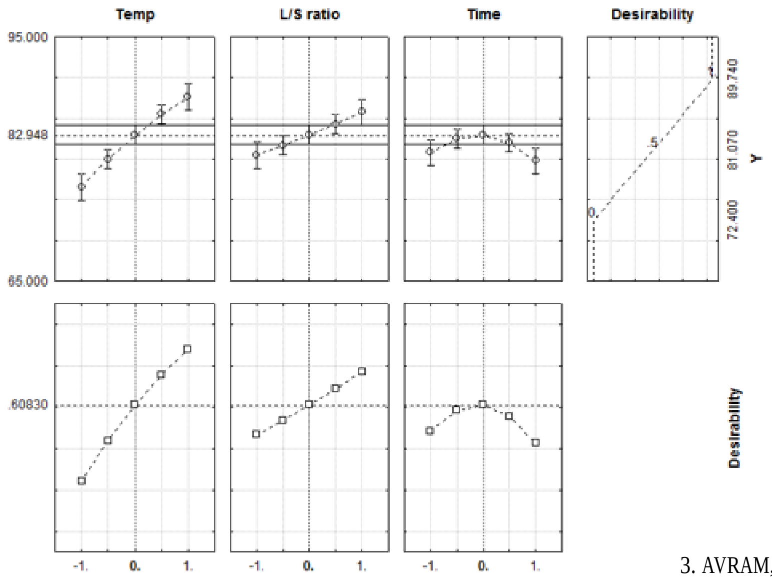

Fig. 5. Profiles for predicted relative extraction efficiency and the desirability level for different influencing factors for optimum oil extraction from *Nigella sativa* seeds

value for extraction time, which in uncoded variables it means an extraction temperature of 60°C, a liquid/solid ratio of 16/1 mL/g and an extraction time of 2 h. In order to validate the model prediction, experiments were performed under the above conditions obtaining for the relative extraction efficiency a value of 89.80 ± 0.25%, being in a good agreement with theoretical prediction.

Conclusions

In the last years there is a growing interest for new sources of vegetable oils with high content of unsaturated fatty acids. Due to the high content in unsaturated fatty acids, especially omega 3 and omega 6 of the fixed oil separated from seeds, *Nigella sativa* could be a good candidate. In the present paper, the oil extraction from *Nigella sativa* seeds was studied using a statistical method based on the response surface methodology (RSM) in order to identify and quantify the process parameters which could maximize the relative extraction efficiency. The Box-Behnken design (BBD) and RSM prove to be efficient in deducing the optimal conditions for the oil extraction. After a multiple regression analysis on the experimental data a quadratic polynomial model was chosen. The results for analysis of variance (ANOVA) followed by Fisher's statistical test (F-test) sustain the significance of the quadratic polynomial model, which could be used to characterize the relationship between the model response (oil extraction efficiency) and the process variables (extraction temperature, liquid/solid ratio, extraction time), and adequate for predicting the relative extraction efficiency of *N. sativa* seeds oil.

The most significant contribution to the relative oil extraction efficiency are the linear terms of temperature (X_1) and liquid/solid ratio (X_2), the quadratic terms of the extraction time (X_3^2) and the interaction term between the temperature and extraction time (X_1X_3).

For the optimum conditions (temperature 60°C, liquid/solid ratio 16/1v/wt and time 2h) for the extraction process a yield 89.80 ± 0.25% was obtained, which is in good agreement with the value predicted for optimized conditions generated by the RSM.

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