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Supercritical fluid extraction of pupae oil from mulberry silkworm (*Bombyx mori* L.)

Srinivas G, Udaykumar Nidoni, Ramachandra CT, Ramappa KT and Ashoka J

Abstract

Oil was extracted from the mulberry silkworm pupae using supercritical carbon dioxide (SC-CO_2). A Central Composite Design of Response Surface methodology (RSM) was employed to optimize the SC-CO_2 extraction parameters. The oil yield varied with interaction of pressure, temperature, extraction time and CO_2 flow rate. Optimum condition was observed within the variables of temperature from 30°C to 50°C, pressure from 125 bar to 225 bar, extraction time from 60 min to 180 min and CO_2 flow rate from 15 g/min to 27 g/min. However, the extraction parameters were found to be optimized at temperature 45°C, pressure 203bar, extraction time of 145 min and CO_2 flow rate of 24 g/min. At this optimized condition, the highest oil yield was found to be 30.10 g/100 g. The desirability at optimum operating condition was found to be 0.99. The production cost of oil from mulberry silkworm pupae using supercritical fluid extraction equipment was estimated and benefit cost ratio was found to be 1.57.

Keywords: Supercritical fluid extraction, silkworm pupae oil, extraction yield, optimization

Introduction

Like all other agro-industries, sericulture industries also generate a great deal of waste in the process of production. The by-product which is treated as the waste that originates at different stages of silk production in varying degrees but the overall waste-output ratio appears to be somewhat high here (Ismath, 1985) [6] which is around 80 to 90 per cent, This valued silkwaste is not effectively utilized because of lack of awareness, availability of waste utilization technology and market facility (Nelaballe *et al.*, 2014) [12].

During the silk reeling process, the spent pupae are produced in large quantities and are the major by-product of silk production (Datta *et al.,* 2007) [4]. Spent silkworm pupae is a by-product often discarded as waste in the open environment or used as fertilizer (Wei *et al.,* 2009) [24], and as feed in fresh water prawn culture (Ashoka *et al*., 1997) [3]. Spent silkworm pupae are a highly degradable product in silk production area and the disposal of large quantities of pupae can cause serious environmental problems (Jun *et al.,* 2010) [7]. There is accumulation of huge quantity of sericulture wastes but utilization of by-product has not kept pace with the strides the country has achieved in sericulture fronts over the years.

Silk worm pupae are known for their nutritional value, due to the presence of high protein and high fat content (about 30 per cent of the total dry pupae weight). The protein in silkworm pupae contains 18 known amino acids, which includes all of the essential amino acids and sulfur-containing amino acids, exhibiting high quality, according to the amino acid profile recommended by the Food and Agriculture Organization (FAO)/ World Health Organization (WHO). The silkworm pupae fat and oil was useful in soap/ cosmetic industries and was found to work in anti-ageing, darkening gray hair and body weight reduction (Velayudhan *et al.,* 2008) [23]. The silkworm pupae oil is abundant in unsaturated fatty acid, reaching 75 per cent of total fat, while polyunsaturated acid especially the alpha-linolenic acid (ALA) accounts for as high as of 34.27 per cent (Tao *et al.,* 2014) [21]. Silkworm pupae oil is also a highly nutritional and exploitable food resource that lowers cholesterol, improves memory and serves as an anti-oxidant by eliminating free radicals in the body (Wei *et al.,* 2009) [24]. The fatty acid composition of silkworm pupae oil varies according to its variety and origin, usually consisting of α-linolenic, oleic, linoleic, palmitic, palmitoleic and other fatty acids, with a total of more than 75 per cent unsaturated fatty acids (UFAs) and approximately 20 percent saturated fatty acids (Jun *et al.,* 2010) [7].

Extraction of silkworm pupae oil by conventional petroleum ether with soxhlet has been reported (Wen-Juan *et al.,* 2012 and Supanida *et al.,* 2008) [25, 19]. Compared with conventional technology for obtaining oils containing the solvent extraction (mainly petroleum ether), supercritical fluid extraction (SFE) has attracted during last decades.

The SC-$CO_2$is non-explosive, non-toxic, and available in high purity with low cost, non-solvent residues (Sapkale *et al.,* 2010 and Wen-Juan *et al.,* 2012) [18, 25]. Optimized conditions to enhance purity of α-linoleic acid from de-silked silkworm pupae oil can be easily and effectively done by Response Surface Methodology (Jun *et al.,* 2010) [7].

For one kg of raw silk, 8 kg of wet pupae (2 kg of dry pupae) is produced (Patil *et al*., 2013) [16]. However, at present pupae are not effectively utilized in the country. It is necessary to adopt suitable and improved methods of processing and handling of pupae to obtain pupa oil with desirable economic qualities (Halliyal, 2009) [5].

In recent times, many research investigations have has been carried out in application of silkworm pupae oil in bio-diesel production, cosmetic industries, soap industries, supplementing animal feed, synthesis of surfactants and food industries (Nelaballe *et al*., 2014; Gahlot and Suryanarayana, 2008) [12].

Keeping in view of the above facts, a research topic on “Supercritical fluid extraction of pupae oil from mulberry silkworm (*Bombxy mori* L.)” was undertaken with the following objectives.

Materials and Methods

Raw materials

Fresh Silkworm pupae were procured from Ramanagara town of Karnataka (India). The dried pupae were ground in a laboratory hammer mill to obtain fine powder (Nguyen *et al*., 2015) [13]. The solvents, chemicals and reagents (analytical grade) used throughout the experiment were procured from M/s. Sigma Aldrich Chemicals, Bangalore (Karnataka).

Soxhlet extraction of mulberry silkworm pupae oil

Oil extraction from mulberry silkworm pupae was carried out by soxhlet extraction method using SOCS- PLUS apparatus (Make: Pelican Equipments; Model: SCS-08) with hexane as solvent. Accurately, 100 g of the mulberry silkworm pupae powder was taken into the thimble and placed it in the sample compartment of the extractor. Sample compartment was attached to a 500 ml round bottom flask containing 300-350 ml hexane. SOCS- PLUS set-up was assembled and heated in a mantle. The SOCS- PLUS apparatus was run at 85 ºC for 90 min. Hexane in the oil extract was distilled out by using a rotary flash vacuum evaporator (Superfit, Rotavap; PBU-6D) (Malapit, 2010) [10].

Supercritical fluid (SC-CO2) extraction of mulberry silkworm pupae oil

The supercritical carbon dioxide extraction system (Thar; SFE 500 system) was used for extraction of mulberry silkworm pupae oil. Deionized water (at 5 °C) was used for cooling different zones in the SC-CO2 extraction system. The independent variables selected for the study were supercritical fluid pressure (125, 150, 175, 200 and 225 bar) temperature (30, 35, 40, 45 and 50 °C) at constant dynamic extraction time of (60, 90, 120, 150 and 180 min) and CO_2 flow rate of (15, 18, 21, 24 and 27 g/min) and using the experimental design the number of runs were minimized, resulting in a 31 simplified set of experiments (Wen-Juan *et al*., 2012) [25]. Static extraction process was performed for 30 min (Palafox *et al*., 2012) [15] which allowed the sample to soak in the CO_2 and co-solvent in order to equilibrate the mixture at desired pressure and temperature. After attaining desired pressure and temperature the dynamic extraction time (90 min) was started by opening the exit valve of the SC-CO_2 extraction system. During the dynamic extraction time, CO_2 carrying the crude extract flowed out of the extraction vessel and then into a collection vessel, where the CO_2 was separated through the vent connected to the fume hood.

Extraction yield

The silkworm pupae oil from the SC-CO_2 extraction was collected and the residual content of co-solvent was removed by using a rotary flash vacuum evaporator (Superfit, Rotavap; PBU-6D) under vacuum at 40 °C. The oil was then placed in the oven at 40 °C for 30 min for further removal of solvent traces. The extraction yield was computed by using the following equation.

$$\text{Extraction yield (g/100g)} = \frac{M_{extract}}{m_{feed}} \times 100 \qquad \text{... (1)}$$

Where,

$M_{extract}$ = Mass crude extract, g

m_{feed} = Feed mass, g

Optimization of process parameters

The numerical optimization technique of the Design-Expert software was used for the simultaneous optimization of the multiple responses. The desired goals for each variable and response were chosen. The desirability values of the minimum and maximum were configured as 0 and 1, respectively. All of the independent variables were kept within the range, while the responses were either maximized or minimized. Numerical optimization was applied for supercritical fluid extraction of silkworm pupae oil on the basis of extraction yield and extraction efficiency. The quadratic response surface analysis was based on multiple linear regressions taking into account of linear, quadratic and interaction effects according to the equation given below;

$$Y = b_0 + \sum a_i x_i + \sum a_{ij} x_i x_j + \sum a_{ii} x_i^2 \qquad \text{... (2)}$$

Where Y is the response value predicted by the model; b_0 is offset value, a_i, a_{ij} and a_{ii} are main (linear), interaction and quadratic coefficients, respectively. The adequacy of the models was determined using model analysis; lack-of fit test and coefficient of determination (R^2) analysis. For model to be suited, R^2 should be at least 0.80 for a good fitness of a response model (Mirhosseini *et al*., 2009).

Verification of predicted and actual responses

To verify the predicted and actual responses of SC-CO_2 mulberry silkworm pupae oil, the mean relative per cent deviation modulus was calculated by using the following relationship (Krishnaiah *et al.,* 2012) [8].

$$\text{Per cent relative deviation} = \frac{100}{N} \sum_{i=1}^{N} \frac{|e_i - p_i|}{p_i} \qquad \text{… (3)}$$

Where,

N = Total number of observations

e_i = Experimental value

p_i = Predicted value

Statistical design

Statistical analysis was carried out to study the effect of different parameters on the dependent variables by Composite Completely Randomized Design (CCRD) using the statistical software Design Expert (7.7.0 trial version).

A generalized factorial completely randomized design was employed to analyse the independent process variables. Statistical significance of the terms was examined by analysis of variance (ANOVA) for each response. ANOVA is important in determining the adequacy and significance of the model. The p-values were used as a tool to check the significance of each of the coefficients, which, in turn were necessary to understand the pattern of the mutual interactions between the test variables. The adequacy of regression model was checked by R^2 and Fisher's F-test (Montgomery, 2001) [11].

Cost of production of mulberry silkworm pupae oil using SC-CO_2 extraction process

The cost of production of mulberry silkworm pupae oil using supercritical fluid extraction equipment was estimated by considering the fixed and variable costs as well as other related costs. The standard procedure in accounting and cost calculation given by Ababa *et al.*, (2004) [1] was followed.

Results and Discussions

Extraction yield

The extraction yield of oil obtained from mulberry silkworm pupae powder at different treatment combinations are presented in Table 1. From the table, it is observed that the extraction yield varied in the range of 23.96 to 29.91 g/100 g. Among the treatment combinations, the extraction yield of 29.91 g/100 g was observed to be the highest at SC-CO_2 pressure of 225 bar, temperature of 40 °C, extraction time of 120 min and CO_2 flow rate of 21 g/min, whereas the lowest of 23.96g/100 g was recorded at SC-CO_2 pressure of 125 bar, temperature of 40 °C, extraction time of 120 min and CO_2 flow rate of 21 g/min. The interaction effect between treatments combination was found to be significantat one per cent level ($p<0.01$).

Analysis of variance (ANOVA) and estimated coefficient values of extraction yield of mulberry silkworm pupaeoil are depicted in Table 2. The Model F-value of 2.67 implies that the model is significant. The values of "Prob > F" are less than 0.05 indicating the model terms A, AD, A^2 are significant. The values greater than 0.1 indicate the model terms are not significant. The "Lack of Fit F-value" of 3.67 implies that the lack of fit is insignificant. Non-significant lack of fit is good. A negative "Pred R-Squared" implies that the overall mean is a better predictor of response than the current model. "Adeq Precision" value measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 6.83 indicates adequate signal. Hence, this model could be used to navigate the design space.

The optimized predictive model (in terms of actual factors) for extraction yield of mulberry silkworm pupae oil is presented in the following equation.

$$\text{Extraction yeild (g/100 g)} = 19.10+0.14 \times A+ 0.66 \times B+0.03 \times C-2.15 \times D-1.54\text{-}04 \times A \times B-2.73\text{-}04 \times A \times C+9.24\text{-}03 \times A \times D+7.95\text{-}04 \times B \times C-2.70\text{-}03 \times B \times D+4.49\text{-}003 \times C \times D-7.89\text{-}004 \times A^2-7.48\text{-}003 \times B^2-3.95\text{-}004 \times C^2+3.53\text{-}003 \times D^2 \quad \text{... (4)}$$

Effect of SC-CO_2 temperature, pressure, extraction time and CO_2 flow rate on extraction yield

Extraction yield of mulberry silkworm pupae oil extracted at different temperature, pressure, extraction time and CO_2 flow rate combinations are shown in Fig.1 The following illustrations fig. 1 to 6 fig. are discussed hereunder.

Fig. 1 shows the effect of SC-CO_2 temperature and pressure on the oil yield at the fixed extraction time of 125 min and CO_2 flow rate of 21 g/min. It is observed that the yield of oil was significantly increased with increasing pressure at a given temperature. If the given temperature is higher than a certain value (about 42 ^{o}C), while pressure was rising, the oil yield increased at low-pressure levels, but once the pressure reached the high levels, the oil yield slightly decreased. This can be explained by an increase in the supercritical CO_2 density due to increase in pressure which enhanced solubility of solutes. (Pourmortazavi and Hajimirsadeghi, 2007) [17].

The influence of temperature on extraction time was more difficult to predict than that of pressure because of its two counter effects on the yield of oil. First, the temperature elevation decreased the density of CO_2, leading to the reduction in the solvent power to dissolve the solute. Secondly, the temperature rise increased the vapour pressure of the solutes, bringing about the elevation in the solubility of oils in SC-CO_2. An increase in oil yield was observed with the enhancing of the temperature in early stage of extraction, because the change of solvent density was less effective than that of solute vapor pressure. Similar phenomena were also reported for the extraction of other lipids by SC-CO_2 (Wang *et al*., 2008; Wei *et al*., 2009) [24].

Fig.2 describes the effect of CO_2 flow rate and pressure on oil yield. The results indicated that the oil yield increased gradually with the increase of extraction time at a lower fixed extraction pressure, while the oil yield decreased gradually with the increase of extraction time at the higher extraction pressure.

Fig 3 describes the interaction between extraction pressure and CO_2 flow rate which revealed that the mulberry silkworm pupae oil yield increases slowly with an increase in CO_2 flow rate at a lower fixed extraction pressure; then, a decrease of CO_2 flow rate after the center point of pressure. Before the center extraction pressure (175 bar), the CO_2 flow rate only led to a gradual increase in oil yield, especially at 225 bar.

Fig. 4 illustrates the interaction between extraction time and extraction temperature on oil yield. It is observed that at a given temperature, especially at low or high temperatures, oil yield changed dramatically with extraction time. At the temperature center point, the oil yield rapidly increased with the extraction time.

Fig. 5 illustrates the interaction between extraction temperature and CO_2 flow rate on the oil yield, with a fixed extraction pressure and extraction time at 184.5 and 120 min, respectively. The CO_2 flow rate displayed a positive effect on the oil yield at low temperature. However, no obvious effect of CO_2 flow rate on the mulberry silkworm pupae oil was observed at a high extraction temperature.

Fig. 6 shows the interaction between time and CO_2 flow rate with a fixed extraction pressure of 184.5 bar and temperature of 40 °C. It is seen that no significant effect on oil yield was observed when the extraction time was fixed and the CO_2 flow rate increased. However, the obvious decreasing trends in oil yield with the extraction time were displayed when the CO_2 flow rate was fixed. The above phenomena were difficult to explain, however similar phenomena were also reported for the extraction of other oils by SF CO_2, e.g., *Passiflora* seed oil (Liu *et al.,* 2009).

The soxhlet extracted mulberry silkworm pupae oil was found to be lower in oil yield (25.82 g/100 g) compared to SC-CO_2

extraction. This might be due to higher temperature applied in soxhlet extraction compared to SC-CO_2extraction. High temperature might lead to thermal degradation of fatty acids, especially unsaturated fatty acids (Uddin and Chun, 2010) [22].

Optimization of process parameters for SC-CO_2extracted mulberry silkworm pupae oil

The optimized condition obtained was 45 °C temperature, pressure of 203, extraction time of 145.69 min and CO_2 flow rate of 24 g/min. In the present investigation, the lack of fit was found to be significant for all responses. The responses were predicted by Design-Expert 7.7.0 software for the optimum process conditions with desirability of 0.995. The desirability function of optimum operating parameters of SC-CO_2 extracted mulberry silkworm pupae oil at different treatments is depicted in Fig. 7.

Verification of predicted variables for SC-CO_2 extracted mulberry silkworm pupae oil

The suitability of the model equation for predicting the optimum response values was verified using the optimal condition. The experimental sample had the optimum process conditions of temperature (45 °C), pressure (203bar), extraction time (145 min) and CO_2 flow rate (24 g/min).This optimized parameter was carried out thrice and the average values of each response are presented. Verification of the predicted and actual responses of silkworm pupae oil is presented in Table 3. From the table, it is seen that, the % relative deviation modulus of 0.77 of extraction yield and with desirability of 0.99. This implies the process of extraction at optimum conditions is highly efficient.

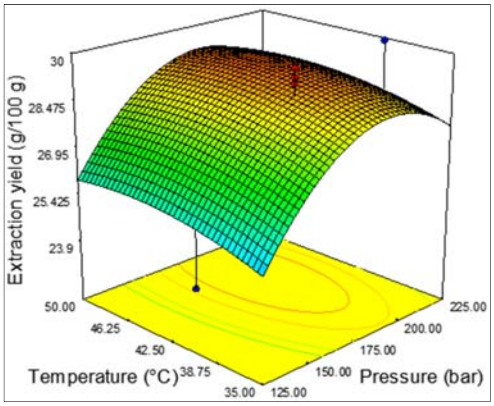

Fig 1: Effect of SC-CO_2 temperature and pressure on yield of mulberry silkworm pupae oil

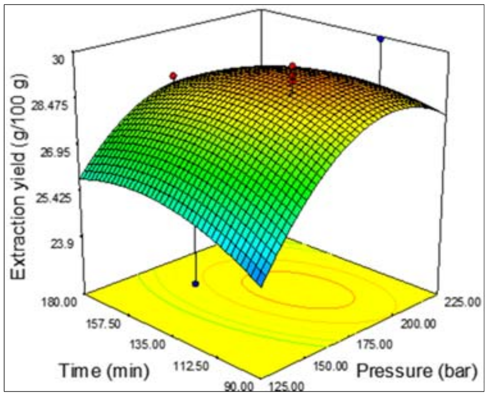

Fig 2: Effect of SC-CO_2 pressure and time on yield of mulberry silkworm pupae oil

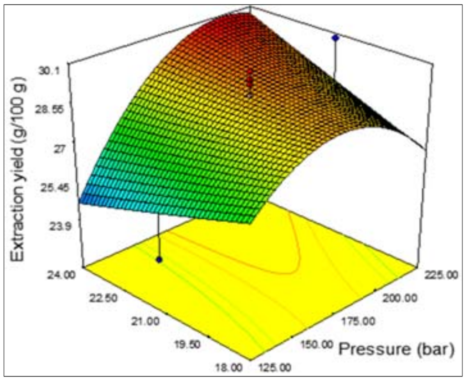

Fig 3: Effect of SC-CO_2 pressure and CO_2 flow rate on yield of mulberry silkworm pupae oil

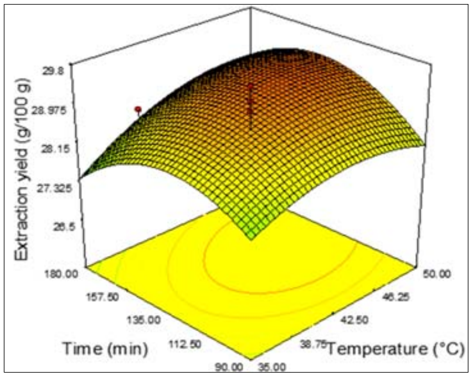

Fig 4: Effect of SC-CO_2 extraction time and temperature on yield of mulberry silkworm pupae oil

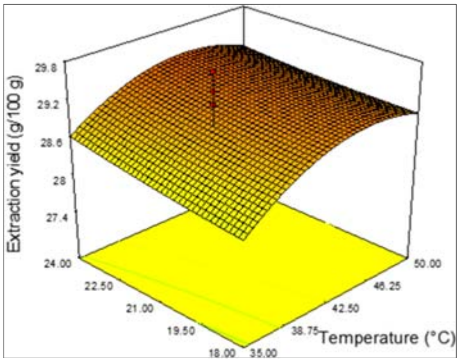

Fig 5: Effect of SC-CO_2 temperature and CO_2 flow rate on yield of mulberry silkworm pupae oil

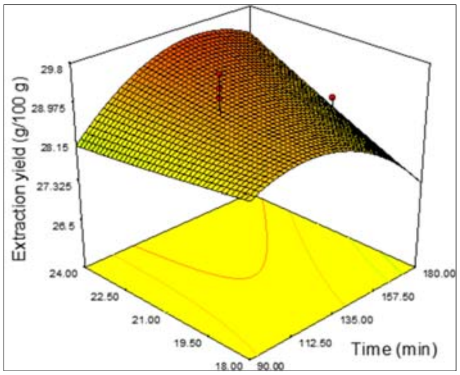

Fig 6: Effect of SC-CO_2 extraction time and CO_2 flow rate on yield of mulberry silkworm pupae oil

Fig 1-6: Effect of SC-CO_2 temperature, pressure, extraction time and CO_2 flow rate on extraction yield

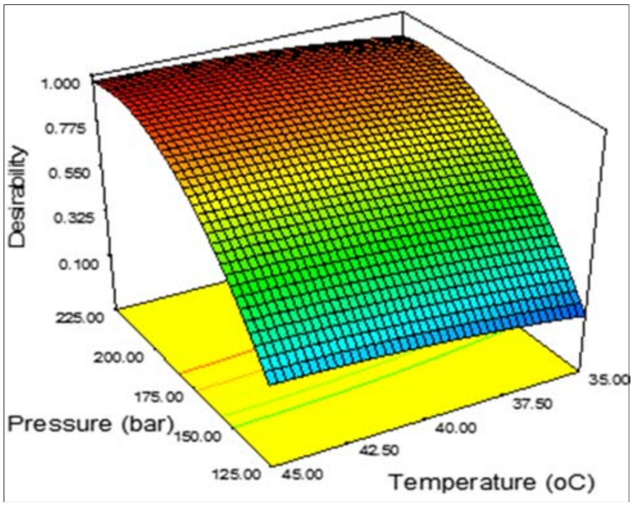

Fig 7: Desirability of optimum operating parameters of SC-CO_2 extracted mulberry silkworm pupae

Table 1: Effect of SC-CO2 temperature, pressure, extraction time and CO_2 flow rate on extraction yield for silkworm pupae oil

Treatments	(A) Temperature (°C)	(B) Pressure (bar)	(C) Extraction time (min)	(D) CO_2 flow rate (g/min)	Extraction yield (g/100g)
Tc	80	Normal	90	-	25.82
T_{1}	45	200	90	24	28.25
T_{2}	35	150	90	24	25.26
T_{3}	35	200	150	24	28.91
T_{4}	35	150	150	24	28.00
T_{5}	40	175	120	21	28.86
T_{6}	40	175	120	27	29.32
T_{7}	40	175	120	21	27.90
T_{8}	35	150	150	18	27.30
T_{9}	35	200	90	18	27.15
T_{10}	30	175	120	21	27.42
T_{11}	45	150	90	18	28.50
T_{12}	35	200	150	18	26.10
T_{13}	50	175	120	21	28.90
T_{14}	45	150	150	24	28.62
T_{15}	40	175	120	21	29.8
T_{16}	40	175	120	21	28.87
T_{17}	45	200	90	18	28.12
T_{18}	40	175	180	21	28.41
T_{19}	40	175	120	21	28.82
T_{20}	40	225	120	21	29.91
T_{21}	40	175	120	15	28.75
T_{22}	45	200	150	24	29.89
T_{23}	35	200	90	24	29.19
T_{24}	40	175	120	21	29.51
T_{25}	40	125	120	21	23.96
T_{26}	35	150	150	18	28.32
T_{27}	40	175	60	21	26.56
T_{28}	40	175	120	21	29.31
T_{29}	45	150	90	24	27.50
T_{30}	35	150	90	18	28.50
T_{31}	45	200	150	18	28.16

Tc = Control - Soxhlet extraction carried out at 80°C for 90 minutes

Table 2: ANOVA for extraction yield of SC-CO_2 extracted mulberry silkworm pupae oil

Source	Sum of squars	DF	Mean square	F Value	p-value Prob> F	Factor	Coeff. Est.	SE	95% CI Low	95% CI high
Model	37.38	14	2.67	2.67	0.0312	Intercept	29.01	0.38	28.21	29.81
A-A	8.32	1	8.32	8.33	0.0108	A-A	0.61	0.21	0.16	1.05
B-B	3.39	1	3.39	3.39	0.0841	B-B	0.39	0.21	-0.06	0.84
C-C	2.32	1	2.32	2.32	0.1470	C-C	0.32	0.21	-0.13	0.76
D-D	0.47	1	0.47	0.47	0.5008	D-D	0.14	0.21	-0.30	0.59
AB	5.26×10^{-3}	1	5.26×10^{-3}	5.26×10^{-3} $5.27E^{-3}$	0	AB	-0.02	0.27	-0.58	0.54
AC	0.62	1	0.62	0.62	0.4436	AC	-0.20	0.26	-0.76	0.35
AD	7.09	1	7.09	7.09	0.0170	AD	0.69	0.26	0.14	1.25
BC	0.20	1	0.20	0.20	0.6596	BC	0.12	0.27	-0.44	0.68

BD	0.02	1	0.02	0.02	0.8807	BD	-0.04	0.27	-0.60	0.52
CD	2.41	1	2.41	2.41	0.1400	CD	0.40	0.26	-0.15	0.96
A^2	6.93	1	6.93	6.93	0.0181	A^2	-0.49	0.19	-0.89	-0.10
B^2	1.00	1	1.00	1.00	0.3332	B^2	-0.19	0.19	-0.58	0.21
C^2	3.60	1	3.60	3.60	0.0758	C^2	-0.36	0.19	-0.75	0.04
D^2	0.03	1	0.03	0.03	0.8674	D^2	0.03	0.19	-0.37	0.43
Residual	15.99	16	1.00							
Lack of Fit	13.20	9	1.47	3.67	0.0501					
Pure Error	2.79	7	0.40							
Cor Total	53.37	30								
Std. Dev.	1.00		R-Squared		0.70		Significant			
Mean	28.20		Adj R-Squared		0.44					
CV%	3.55		Pred R-Squared		-0.60					
PRESS	85.34		Adeq Precision		6.83					

Table 3: Verification of predicted and Actual Process Parameters and Responses of SC-CO_2 Extraction of mulberry silkworm pupae oil

Process parameters	Predicated value	Experimental value	% Relative deviation
Extraction yield (g/100g)	30.43	30.1	0.77
Optimization desirability: 0.991			

Cost of production of oil from silkworm pupae powder using SFE equipment

The cost of production of mulberry silkworm pupae oil was computed as ₹. 8256 /- per kg of oil with benefit cost ratio of 1.57.

Conclusion

The optimized process parameters namely extraction pressure of 203, temperature of 45°C, extraction time of 120 min and CO_2 flow rate of 21 g/min recorded highest yield of 30.10 g/100g. Supercritical fluid extraction process yielded significantly higher yield compared to soxhlet extraction. The technical feasibility of pupae oil extraction by supercritical fluid extraction using CO_2 as solvent was clearly demonstrated in the present study which can effectively utilize generated waste in sericulture industry.

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