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Research Article

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Posted Date: June 12th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3014699/v1>

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Additional Declarations:

Tables 1 to 6 are available in the Supplementary Files section.

Version of Record: A version of this preprint was published at Chemical Papers on July 28th, 2023. See the published version at <https://doi.org/10.1007/s11696-023-02974-2>.

Extraction of capsaicin from *Capsicum chinense* (cv Bhut Jolokia) using supercritical fluid technology and degradation kinetics

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Abstract

Capsaicin (CC), the dominant pungent compound in hot chilies, is widely used as a flavouring agent, preservative, active compound in packaging film and functional foods. *Capsicum chinense* cv Bhut Jolokia is one of the richest sources of CC, yet scarcely studied. The present investigation aimed to optimize a clean and green method for extracting CC through supercritical fluid extraction (SFE) method. Low-grade, culled, and discarded fruits were used for the extraction, and process was optimized through central composite design of RSM. The optimized extraction condition, 68.31 °C/347.98 bars/102.50 min, resulted in maximum CC content (367.14 ± 1.12 mg/g) and oleoresin yield (7.23 ± 2.15 %) in a shorter extraction time (< 2 h). Accelerated thermal stability study suggested first-order degradation kinetics of CC at temperatures from 80 to 140 °C. The activation energy (kJ/mol) of the reaction was 71.86, while Q_{10} for 80 to 100 °C, 100 to 120, and 120 to 140 were 0.0548, 0.0574, and 0.1456, respectively. Valorisation of Bhut Jolokia for targeting an oleoresin with maximum CC opens a new window for its commercial exploitation in food industry. Furthermore, the study opens avenues for exploration of SFE-based extraction as clean, and sustainable method with reduced carbon footing.

Keywords:

Chili oleoresin; Supercritical carbon-dioxide extraction; Total phenolic content; Antioxidant activity; Thermogravimetric analysis; First-order kinetics

Statement of Novelty

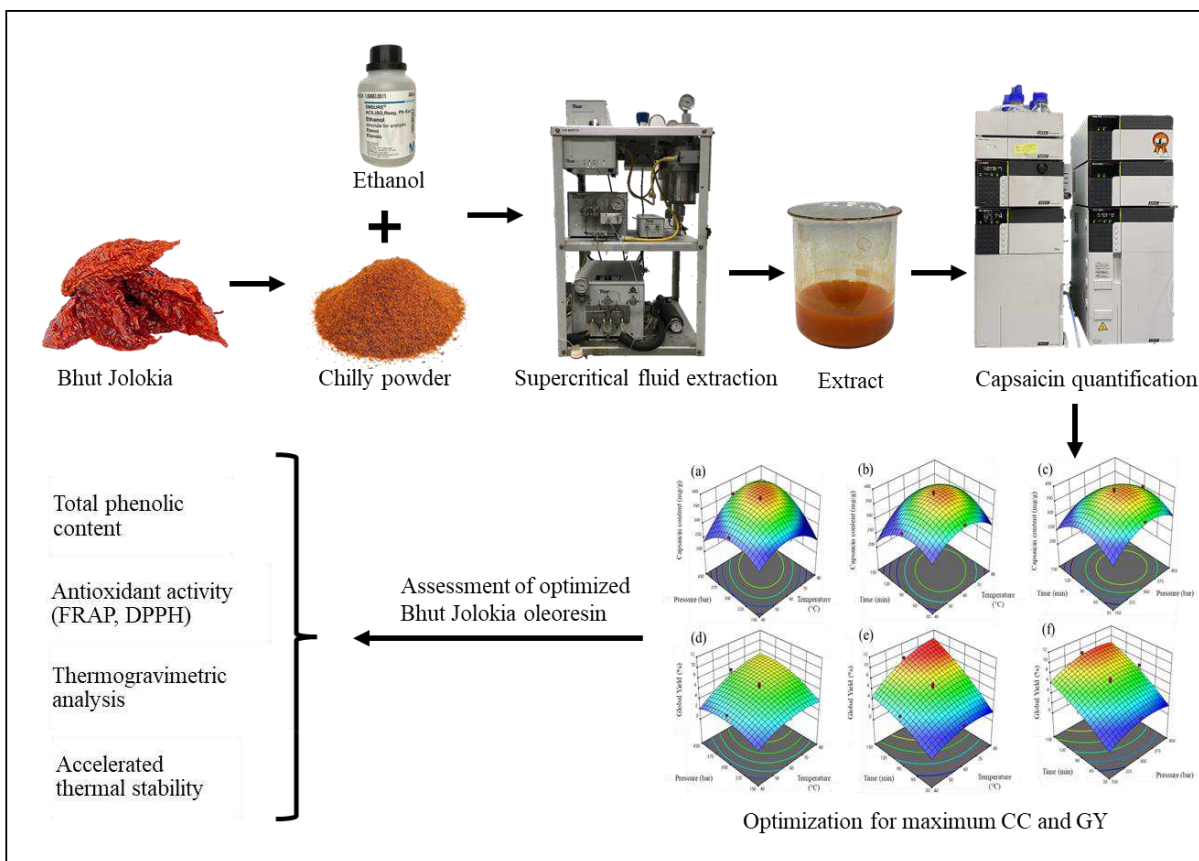
The study employs a green supercritical fluid method for extraction of capsaicin from *Capsicum chinense* (cv Bhut Jolokia). The technology envisaged an innovative non-hybrid mode which yielded the highest capsaicin content in the shortest time (<2 h). Bhut Jolokia represents a

34 promising candidate for commercial oleoresin extraction given its exceptionally high capsaicin
35 content (39.24 % of oleoresin).

36

37 **Highlights**

- 38 • Underutilized *Capsicum chinense* cv Bhut Jolokia was valorized.
- 39 • Green and clean process employed for extraction of capsaicin-rich oleoresin.
- 40 • Novel optimized process yielded maximum capsaicin in shortest time.



1. Introduction

Hot chili peppers are an important component of the staple diet of Asian and Mexican countries. They are indispensable in Indian curries, sauces, and pickles; and are valued for their characteristic aroma, taste, flavor, and pungency. Being the largest producer, India accounts for approximately 40 % (2.05 MT) of the world's total chili pepper production and is also the largest consumer, and exporter. India exported 0.56 MT of chilies to USA and China in the year 2021 (FAO, 2021).

Bhut jolokia (BJ), a cultivar belonging to the *Capsicum chinense* (Jacq.) species, is native to the northeastern region of India. It is primarily cultivated in the states of Manipur, Nagaland, and Assam and is commonly referred to as 'U-morok', 'Naga king chili', 'Raja mircha', 'Ghost pepper' etc. Traditionally, it is utilized as a flavoring agent in local cuisine and folk medicine for the treatment of asthma, gastrointestinal abnormalities, toothache, muscle pain, and arthritis (Bhagowati & Changkija, 2009). It was recognized as the 'world's hottest chili' (1001304 Scoville Heat Units) by the Guinness World Records in the year 2006 followed by a GI tag recognition in 2008 (Verma et al., 2013). The capsaicinoid content in BJ is approximately 338 and 18 times higher than those in Scotch Bonnet and Jalapeno, respectively (Liu & Nair, 2010). Capsaicin (CC) contributes to about 69 % of the total capsaicinoid (Popelka et al., 2017) and is the dominant pungency compound in BJ. The content of CC in BJ ranges from 21.87 to 59.87 mg/g powder (Islam et al., 2015; Moirangthem et al., 2014; Schmidt et al., 2017; Sweat et al., 2016); higher than that reported in 'Malagueta' (1.02 mg/g) the widely studied Brazilian pepper (de Aguiar et al., 2013, 2018; Santos et al., 2015).

Capsaicin is a potent nutraceutical compound and its selective impact on the peripheral portion of the sensory nervous system (specifically on the primary afferent neurons) is amongst its most

acknowledged attributes. Its ability to deplete neurotransmitter of painful impulses has established CC as a versatile therapeutic agent for alleviating rheumatoid arthritis, post-herpetic neuralgia, post-mastectomy pain syndrome, and diabetic neuropathy (Adhikari et al., 2023; Dlodla et al., 2022). Other proven health-promoting effect includes anti-cancerous, anti-inflammatory, anti-iron binding, anti-obesity, cardiovascular activity regulation, analgesic properties, and antimicrobial properties (Sanati et al., 2018; Xiang et al., 2022). Immunomodulatory response of CC on neurilemma cells due to the anti-inflammatory effect suggests its rational use for the treatment of Covid-19 (Grüter et al., 2020).

The extraction of CC is a challenging step governing the production and mass availability of this bioactive compound (Castro-Muñoz et al., 2022). Conventional extraction demands exhaustive solvent extraction for long periods, coupled with heat treatment for long durations. Although the yields are high, the overuse of harmful solvents violates environmental and health guidelines. Hence, the urgency for developing cost-effective and clean extraction processes capable of preserving the nutritional, functional, and biological properties of CC, while ensuring high extraction yields must be addressed.

Supercritical fluid extraction (SFE), particularly supercritical carbon dioxide (SC-CO₂) extraction, is used for the extraction of bio-actives and has attracted attention as its advantages include non-toxicity, affordability, non-inflammability, chemically inert, abundant availability, and ease of removal from the extract when exposed to room conditions (25 °C, 1 atm) (Al-Maqtari et al., 2021). Supercritical solvents diffuse into the solid matrix like a gas while simultaneously solubilizing certain compounds of interest from the solid matrix. The low polarity of CO₂ also makes it ideal for lipophilic compounds such as CC (de Aguiar et al., 2022). The use of co-solvents (ethanol) in conjunction with SC-CO₂ is reported to improve its extraction

efficiency (Ahangari et al., 2021; Yen et al., 2015). Katherine et al., (2008) investigated SFE of lycopene from watermelon and reported the highest lycopene yield (36 %) at 60 °C and 20.7 MPa, with 15 % ethanol (v/v) as co-solvent. Several SFE studies for the extraction of capsaicinoids or CC from different chili peppers are also available (de Aguiar et al., 2014; Pervazić et al., 2004; Santos et al., 2015; Shah et al., 2020; Silva & Martínez, 2014). However, the extraction period in reported studies is long (>5h); making the process uneconomical and detrimental to the stability of CC. Additionally, there are limited studies on CC extraction from Bhut Jolokia. For instance, Sarwa et al., (2017) attempted microwave and ultrasound-assisted extraction using acetone for the extraction of capsaicinoids. Bajer et al., (2015) applied pressurized hot extraction at extremely high temperatures (120-240 °C) but extracted only 0.91% of CC. Deka and Hulle (2021) extracted only 1.72 % of capsaicin despite using SFE. Considering the above said deficiencies related to low yield under different extraction conditions and vast potential of Bhut Jolokia as a promising source of CC, a systematic study was planned to target high yield and CC content.

The main objective envisaged was to optimize a non-hybrid SFE technique for maximizing CC extraction from discarded BJ fruits. A central composite design (CCD) of the response surface methodology (RSM) was employed for optimization. Ethanol was used as a co-solvent to improve the polarity of SC-CO₂. The optimized BJ oleoresin (BJO) was characterized for functional quality in terms of total phenolic content and antioxidant activity. Additionally, the oxidative stability and thermal stability of BJO were established through an accelerated thermal stability study and thermogravimetric analysis, respectively.

2. Materials and Methods

2.1 Chemicals

Capsaicin standard ($\geq 93\%$ assay) was purchased from M/s Wako Pure Chemical Industries Ltd., Japan while liquid CO₂ ($\geq 95\%$ purity) was acquired from M/s Amit Laboratory, New Delhi. All solvents and chemicals used in the analysis were of HPLC grades.

2.2 Material

Ten kg of sun-dried *Capsicum chinense* (cv Bhut Jolokia) was procured from two farm locations in Imphal, Manipur, India. Generally, after drying, top-quality graded fruits enter into spice mix whereas low culled fruits are discarded or collected for low-grade powder. These low-grade fruits were utilized for the extraction of oleoresin in this study.

2.2.1. Preparation of Chili Fruits

The fruits were cleaned and further dried using a recirculatory air dryer (AN CRYO RDAD-80, Ancryo Instruments Pvt. Ltd., India) at 60 °C with an airflow rate of 3 m/s for 2 h and 15 % relative humidity till moisture content of 10 % was achieved. The fruits were pulverized in a mixer-grinder (HL1605 Philips; 230 V, 50 Hz 500 W), sieved through BS 16 mesh (≤ 1 mm); loaded into airtight containers, and stored at -20 °C till further analysis. For homogeneity, two sets of powdered samples were considered for optimization by replicating some of the experimental runs (one from each sample) to assess the variability between samples (Section 2.5). Since the variability between the responses received from each sample was small (Table 1), further optimization was carried out by taking both the replicated points (both axial and center points). Trials were conducted at optimized levels of the input factors on a different sample prepared for the validation purpose and the result are presented in the paper to ensure the reproducibility of results.

2.3 Compositional Analysis of *Capsicum chinense* (cv Bhut Jolokia) Powder

The dried chili powder was analyzed for the carbohydrate, protein, crude fat, ash, and moisture contents in triplicates as per standard methods (AOAC, 2000; Shah et al., 2020).

2.3.1 Soxhlet Extraction

The oleoresin or the global yield (GY) of the BJ powder was determined by employing the Soxhlet extraction (SOX) method as described by Santos et al. (2015) with minor modifications. Hexane (100 mL) was used for extraction from Bhut Jolokia powder (3 g) for 6 h. The solution was evaporated in a rotary evaporator (Heidolph, Germany) at 40 °C vapor temperature, 335 mbar vacuum, and 50 °C water bath temperature to obtain a brownish-orange thick oleoresin which was weighed and stored at –20 °C until further analysis. The CC content was analyzed as per the protocol given in section 2.6.

2.3.2 Global Yield

After the removal of solvent, weight of BJO (X_{BJO}) was measured using an analytical balance. The oleoresin yield or global yield (GY) was determined using the following formula.

$$Global\ yield = \frac{X_{BJO}}{X_f} \times 100 \quad (1)$$

where X_{BJO} = weight of BJO (g), X_f = weight of feed (g).

2.4 Instrumentation for Supercritical Fluid Extraction

Extraction was performed out in a supercritical fluid extraction (SFE) system (Model No 7100, Thar Technologies Inc., USA) (Fig. 1). For each experiment, chili powder (100 g) was wrapped in a muslin cloth with the free ends fastened using threads to prevent the migration of particles

from the extraction vessel to other parts of the system and loaded into the extraction chamber (L). The extraction parameters were controlled using the system's software (Super Chrom SFC Suite v5.9, Thar Technologies Inc., USA).

The extraction parameter investigated were: pressure (150, 225, 300, 375, and 450 bar); temperature (40, 50, 60, 70, and 80 °C); and time (30, 60, 90, 120, and 150 min). Pressure levels were decided as per a previous report from our laboratory (Patel et al., 2019). Temperature range, on the other hand, was selected such that the lower limit was well above the critical temperature of CO₂ (31.1 °C) and the upper limit was capped at 80 °C to check the thermal degradation of CC. The maximum extraction period was restricted to 150 min in line with the objective to optimize an SFE technique with a shorter extraction period while CO₂ flow rate was set at 35 g/min as fixed for the SFE system in previous work (Patel et al., 2019). Further, to accentuate the extraction efficiency, ethanol at 5% (w/w) was used as a co-solvent. Post extraction, ethanol from the extract was vaporized in a rotary evaporator at 40 °C vapor temperature, 175 mbar vacuum, and 50 °C water bath temperature. Quantification of CC was carried out according to the protocol presented in section 2.6, and GY was calculated as per section 2.3.2.

2.5 Experimental Design and Statistical Analysis

Response surface methodology (RSM) was employed to optimize the extraction parameters (temperature, pressure, and time) for maximization of CC and GY in BJO. A five-level central composite design (CCD) was used for the experimentation. The experimental layout was obtained using the Design Expert software (ver. 13) (Licensed to ICAR-CMFRI, Kochi) for three input factors viz., temperature (°C), pressure (bar), and time (min). A total of 28 experiments

were conducted (Table 1) which include the replicated axial and center points to provide sufficient degrees of freedom for testing the lack of fit of the model. Second order polynomial model was fitted to establish a functional relationship between the responses (CC and GY) and the input factors (temperature, pressure, and time). Analysis of variance (ANOVA), R^2 , and lack of fit statistics were used for assessing the statistical significance of the fitted model. This model was employed for further optimization using Design Expert software by assigning suitable goals for each of the input factors and responses; factors were set to 'in range' while the responses were set to 'maximize.' Multi-response optimization was done to find out the optimum settings which maximize the responses, and optimum values for input factors were chosen based on the desirability function (Derringer & Suich, 1980). Optimized responses were further validated with significance assessed at $p < 0.05$. Three-dimensional surface plots were provided to depict the behavior of the response variables with varying levels of the input factors.

2.6 Quantification of Capsaicin

An Ultra-Fast Liquid Chromatography (UFLC) (Shimadzu Corporation, Nakagyo-ku, Japan) equipped with DGU-20 A5R (degassing unit), LC – 20 AD prominence chromatograph (pump), SIL 20 ACHT prominence autosampler, CBM20A communication bus module, SPD – M20A prominence diode array detector and CTO – 20 AC prominence column oven was employed for the quantification of CC from the extract. A shim-pack solar C_{18} reverse phase column (4.6 x 250 mm; 5 μ m particle size) was used with an isocratic elution of acetonitrile and water (70:30) having a flow rate of 0.8 mL/min, 15 min run time and column temperature was set at 40 °C. A volume of 20 μ L was injected and detection was observed at 280 nm. LabSolution software was used for the quantification of CC and data analysis. All samples for analysis were freshly

prepared by making a 1000 ppm solution of the BJO in acetonitrile. This was then filtered through a syringe filter (0.22µm PVDF hydrophilic membrane, Himedia).

For the calibration curve, a 1000 ppm (1 mg/mL) stock solution of CC standard was prepared in acetonitrile. From this, 50, 100, 200, 300, and 500 ppm working concentrations of the standard were prepared. The area obtained under each chromatogram was plotted against its respective concentration for constructing the calibration curve. Guidelines of the International Council on Harmonisation were employed for method validation.

2.7 Total Phenolic Content

The total phenolic content (TPC) of the optimized BJO was determined according to the method described by Ayob et al. (2021) with some modifications. Briefly, a 1000 ppm solution of BJO was initially prepared in acetonitrile; 100 µL of this solution containing 650 µg/mL of optimized BJO was added to 3 mL of distilled water; 500 µL of Folin-Ciocalteu reagent was added; and incubated for 2 min. Subsequently, 2 mL of 20 % sodium carbonate solution was added and absorbance at 750 nm was measured against a reagent blank after 30 min of incubation. The results were expressed as mg Gallic acid equivalents (GAE)/100 g dw.

2.8 Antioxidant Activity (AOX)

2.8.1 Ferric Ion Reducing Antioxidant Power (FRAP)

The reducing power of optimized BJO was ascertained with the FRAP assay as per the protocol reported by Visakh et al. (2022) with minor modifications. FRAP reagent was freshly prepared by mixing 10 mM TPTZ (2,4,6-Tripyridyl-S-triazine prepared in 40 Mm HCl), 20 mM FeCl₃, and 300 mM acetate buffer (pH 3.6) in the ratio 1:1:10. Thereafter, 100 µL of acetonitrile

218 solution containing 650 µg/mL of optimized BJO was added to 3 mL of FRAP reagent,
219 incubated at 37 °C for 10 min, and optical density was read at 593 nm.

220 **2.8.2 2,2-Diphenylpicrylhydrazyl (DPPH) Radical Scavenging Activity Assay**

221 The free radical scavenging capacity of optimized BJO was measured from the stable 2,2-
222 diphenyl-1-picrylhydrazil (DPPH) radicals scavenged by the optimized BJO (Visakh et al.,
223 2022). A volume of 100 µL of acetonitrile solution containing varying concentrations of BJO
224 (10–50 µg/mL) was taken in a series of test tubes, 3.9 mL of DPPH reagent was added, and after
225 30 min of incubation in the dark, absorbance was read at 517 nm. Results were expressed in
226 µmolTE/g. Additionally, the half maximal inhibitory concentration (IC₅₀) value was assessed
227 using probit linear regression analysis.

228 **2.9 Thermogravimetric Analysis of Optimized Bhut Jolokia Oleoresin**

229 Thermal stability of optimized BJO (≈4 mg) was determined using a thermogravimetric analyzer
230 (TGA 2 STAR^e system, METTLER TOLEDO, Switzerland), under nitrogen with a flow rate of
231 100 mL/min and heated from 25 to 700 °C at a heating rate of 10 °C/min.

232 **2.10 Accelerated Thermal Stability**

233 To understand how CC degrades during high-temperature processing, optimized BJO was
234 directly added to a carrier oil viz., rice bran oil (RBO) at a rate of 1 % (w/v) and homogenized (T
235 25 Ultra Turrax, IKA). This oil was heated to different temperatures (80, 100, 120, and 140 °C)
236 in a magnetic stirrer (Tarson Digital Spinot) for 90 min each with continuous stirring to ensure
237 uniform heating. An independent thermometer was utilized for simultaneous monitoring of
238 temperature throughout the experiment. Aliquots were drawn every 15 min for a total duration of
239 90 min, cooled immediately, and refrigerated at 4 °C until analysis.

240 The experimental data were fitted using first-order reaction (Eq. 2) and half-lives ($t_{1/2}$) of CC
 241 corresponding to each temperature were calculated as given in Eq. 3. The goodness of fit was
 242 established through the coefficient of determination (R^2) and the sum of squared residuals (SSR)
 243 (Eq. 4).

$$244 \quad C_t = C_o e^{(-kt)} \quad (2)$$

245 where C_o and C_t = CC content at the initial time and time t (mg/mL of oil), respectively, and k =
 246 degradation rate (h^{-1}).

$$247 \quad t_{1/2} = \ln(2)/k \quad (3)$$

248 where $t_{1/2}$ = half-life of CC (h).

$$249 \quad SSR = \sum \{ (C_t - C_{expt})^2 \} \quad (4)$$

250 where C_t = observed CC content at time t , and C_{expt} = expected CC content at time t .

251 Furthermore, the dependence of the thermal degradation of CC in RBO was ascertained by
 252 calculating the activation energy (E_a) and temperature quotient (Q_{10}) values using Eq. 5 and Eq.
 253 6.

$$254 \quad k = k_o e^{-E_a/RT} \quad (5)$$

255 where k = degradation rate (h), k_o = Arrhenius constant, E_a = activation energy J/mol, R =
 256 Universal gas constant (8.31 J/mol K), T = temperature (K).

$$257 \quad Q_{10} = \left(k_2/k_1 \right)^{10/T_2 - T_1} \quad (6)$$

where k_1 and k_2 = degradation rate corresponding to temperature T_1 and T_2 , respectively.

3. Results and Discussion

3.1. Compositional Analysis of *Capsicum chinense* (cv Bhut Jolokia)

The physicochemical composition of BJ powder in terms of carbohydrate, protein, crude fat, ash, and moisture was 64.79, 11.29, 4.98, 7.88, and 11.05 %, respectively (Table 2). The values obtained conform with the previous results (Deka & Swami Hulle, 2021; Kuna et al., 2018). The conventional SOX yielded 11.73 ± 1.15 % of oleoresin (referred to as GY i.e., global yield) and CC content of 485.83 ± 45.79 mg/g. The GY obtained was nearly twice the content reported in a previous study (6.23 %) (Deka & Swami Hulle, 2021). The differences may be attributed to the quality of raw materials, cultivation practices, and processing conditions (Das et al., 2016; de Aguiar et al., 2018).

3.2. Supercritical Extraction of Bhut Jolokia Oleoresin

The statistical significance of temperature, pressure, and time on the response factors was investigated through a second-order model using ANOVA (Tables 3 & 4). Second-order models with a non-significant lack of fit were developed for both CC and GY (Eq. 7 & 8).

3.2.1 Effect of Extraction Parameters on Capsaicin Content in Bhut Jolokia Oleoresin

The CC content in BJO ranged from 305.68 to 392.39 mg/g, with the highest and lowest levels observed in the 4th and 7th run, respectively (Table 1). ANOVA revealed that a quadratic model can significantly explain the impact of the extraction parameters on the CC content (Table 3). The test of significance (Table 4) indicated that the linear impact of temperature, pressure, and time; as well as the interaction effects of temperature and pressure were statistically significant

($p < 0.01$). The quadratic effects of all input factors were highly significant ($p < 0.0001$). The quadratic model (Eq. 7) so developed was significant and had a non-significant lack of fit, indicating that it can effectively depict the effect of the extraction parameters on the CC ($R^2 = 0.89$).

$$CC \text{ (mg/g)} = -220.70 + 12.34A + 0.82B + 1.59C + 0.01AB + 0.02AC - 0.0004BC - 0.13A^2 - 0.002B^2 - 0.01C^2 \quad (7)$$

where A = temperature ($^{\circ}\text{C}$), B = pressure (bar), C = time (min).

The interaction effect of temperature and pressure (Fig. 2 (a)) was found to be significant (Table 4). Increasing both these parameters increased CC content from 312.10 to 392.39 mg/g but at higher temperatures and pressure ($> 60^{\circ}\text{C}$ and > 300 bar), CC content reduced (349.93 mg/g at 450 bar and 338.62 mg/g at 80°C). At a given temperature, increasing the pressure is known to increase the solvent density of supercritical fluids which in turn improves its solvating power. On the other hand, the opposite holds true for temperature (Leila et al., 2022). This is the reason for the reduction of CC at temperatures beyond 60°C . At extreme extraction conditions, the combined effect of temperature and pressure may have reduced the solvating power of CO_2 or degraded CC resulting in lower CC yield. This shift in the effect of pressure from positive to negative on CC content was also observed at 75 bar by Deka and Hulle (2021) and 400 bar by Shah et al. (2020).

For all levels of temperature, increasing extraction duration up to 90 min promoted CC extractability, however, beyond 90 min, a reduction in CC content was observed (Fig. 2 (b)). A similar trend was also noted for all levels of pressure (Fig. 2 (c)). The highest CC content (392.39 mg/g) was achieved after 90 min (4th run) while after 150 min, (18th run), CC content

dropped to 310.09 mg/g. The initial increase in CC concentration for all levels of pressure and temperature elucidates the linear effect of time which was found to be statistically significant (Table 4). The reduction at longer extraction periods, particularly at higher pressure levels, was accounted to the compaction of the sample matrix due to extreme pressure which interfered with the flow path of SC-CO₂ (Lad & Kar, 2021). Another reason for such a phenomenon could be the lower solubility of CC as compared to triacylglycerols and carotenoids (de Aguiar et al., 2014).

The extraction efficiency of CC with SFE was found to be in the range of 62.93 to 80.77 % in comparison to conventional SOX. Despite low yields, our results are satisfactory considering two important facts: firstly, the short extraction period (<2 h) and, secondly, a clean process free of toxic solvents. Previous works have reported higher recovery after extended extraction periods. Perva-Uzunalic et al. (2004) reported that about 96 % of total capsaicinoids were extracted at 400 bar and 80 °C after 5.6 h of extraction. Likewise, de Aguiar et al. (2013) reported the highest CC yield (42 mg/g extract) at long extraction periods (5.3 h). A careful perusal of previous works reveals that though capsaicinoid yield in these cases is high, the process is uneconomical due to the long extraction duration.

It is noteworthy to observe that the maximum CC obtained in this study (4th run) was about 3 to 18 times higher than earlier reported results in extracts from ‘Malagueta’ and ‘Habanero’ pepper extract, respectively (de Aguiar et al., 2018; Martins et al., 2017).

3.2.2 Effect of Extraction Parameters on Oleoresin Yield

The yield of oleoresin in all SFE runs was lower than conventional SOX (11.73 %); quite expected as the boiling solvent permits easy access to the active sites within the substrate allowing easy solubilization of the target biomolecule into the solvent in conventional SOX. In

addition, a longer extraction period (6 h) ensured the complete extraction of CC along with other compounds resulting in a high GY. The lower GY as perceived for all runs of SFE could be due to the presence of some non-polar solutes that are soluble in hexane but insoluble in SC-CO₂ (Uquiche et al., 2005). Similar differences between conventional SOX and SFE were reported by de Aguiar et al. (2013) and Santos et al. (2015). The GY varying from 2.17 to 9.15 % in our SFE study corresponded to 18.50 to 78 % of GY obtained using SOX.

ANOVA table (Table 4) for GY obtained using SFE showed that the linear effects of all the extraction parameters were significant out of which temperature and time were highly significant ($p < 0.0001$). The quadratic effect of temperature and pressure was significant at $p < 0.05$. All other terms were found to be statistically non-significant (Table 4). A second-order polynomial equation (Eq. 8) with a good correlation ($R^2 = 0.90$) was found to be significant for GY (Table 3). An insignificant lack of fit indicated that this model can logically explain the effect of the input parameters (temperature, pressure, and time) on GY.

$$GY (\%) = -13.91 + 0.30A + 0.02B + 0.03C + 0.0003A + 0.0009AC - 0.00002BC - 0.003A^2 - 0.000059B^2 - 0.0002C^2 \quad (8)$$

where A = temperature (°C), B = pressure (bar), C = time (min).

For a given extraction period, at all levels of pressure, increasing the temperature from 40 to 80 °C positively impacted the GY (Fig. 2 (d)) but the effect of pressure on GY was not as prominent as that of temperature whose linear effect was found to be highly significant ($p < 0.0001$). Del Valle et al. (2003) reported an increment in extraction yield from pelletized Jalapeño peppers as the temperature was increased from 35 to 65 °C at 360 bar.

At all levels of temperature, for a given pressure level, increasing the extraction duration from 30 to 150 min increased the GY (Fig. 2 (e)). Likewise, GY increased as the temperature was elevated from 40 to 80 °C for all levels of extraction period. The surface plot predicts that maximum GY can be achieved at higher levels of temperature and extraction period; the linear effect of both these parameters was highly significant ($p < 0.0001$). Higher temperature increases solute volatility, enhances solubility, and improves extraction efficiency; however, this can also lower the density of SC-CO₂ and reduce its solvating power (Leila et al., 2022). Thus, it is important to arrive at a temperature that will increase the solute volatility without compromising the density of SC-CO₂. This may be the reason for the reduced yield at 80 °C in the 16th run (6.48%) with the maximum GY achieved at 60 °C in the 18th run (9.15%) after an extraction period of 150 min. Other SFE studies on chilies also support that longer extraction periods favor higher GY (Perva-Uzunalić et al., 2004; Uquiche et al., 2005). Longer extraction duration ensured enough time to achieve diffusion extraction as well as adequate contact time between the analyte and SC-CO₂ thereby, improving its extractability. During SFE of Biquinho pepper, diffusion extraction was reported to dominate the SFE process after 80 min at 150 bar and 60 °C (de Aguiar et al., 2014).

A similar trend, as discussed above, was observed for the interaction of pressure with time at constant temperature (Fig. 2 (f)). However, the positive impact of longer extraction periods on GY is less pronounced than that of temperature. The improvement in GY is justified as increasing pressure for a given temperature leads to an increase in SC-CO₂ density which in turn augments its solvating power and hence, promotes higher solubility of the target compound. Moreover, elevated pressure levels are known to encourage mass transfer between the analyte and the SC-CO₂ (Shah et al., 2020). The positive impact of pressure and time on the GY has also

been documented in other SFE studies (Deka & Swami Hulle, 2021; Perva-Uzunalić et al., 2004; Shah et al., 2020).

Interestingly, runs of SFE yielding maximum CC content did not necessarily coincide with high GY (Table 1). The 4th run, with the highest CC (392.39 mg/g), had a corresponding low yield (6.52%). Probably extraction variables (temperature, pressure, and time) were more favorable for extraction of CC than other related compounds (triacylglycerols, carotenoids, etc.) in oleoresin (de Aguiar et al., 2014; Deka & Swami Hulle, 2021; Del Valle et al., 2003). This highlights the potential of SFE to produce commercially valuable extracts of high purity such as BJO with a high concentration of CC.

3.3 Optimization and Model Validation

Appropriate goals were assigned to the input as well as response factors and optimized using Design Expert software. Since our main target was to maximize CC, the highest level of importance was assigned to CC. The optimum extraction condition was 68.31 °C/347.98 bar/102.50 min with a desirability value of 0.84. The values of CC and GY were predicted to be 380.65 mg/g and 7.82 %, respectively. For validation, experiments were conducted in duplicate at the optimized extraction condition and the CC and GY were determined to be 367.14 ± 1.21 mg/g and 7.23 ± 2.15 %, respectively. The relative deviation was found to be 3.55 % and 7.54 % for CC and GY, respectively, indicating a reasonably good agreement of the experimental values with that of the predicted values.

3.4 Total Phenolic Content and Antioxidant Activity

The antioxidant activity (AOX) of BJO ranged between 55.9 ± 1.94 to 64.66 ± 2.16 $\mu\text{molTE/g}$ in DPPH and FRAP assay, respectively, corresponding to TPC of 2.37 ± 0.01 g GAE/100. The IC₅₀

value in the DPPH assay was 37.57 ± 0.52 mg/mL. Our results agree with the findings of Meckelmann et al. (2013); where the TPC in the capsicum accessions ranged from 1.5 and 3.69 g GAE/100 g and AOX between 3 and 92 $\mu\text{molTE/g}$.

Generally, AOX of chili oleoresin is due to the synergistic action of different phytochemicals, ascorbic acid, carotenoids, Vitamin E, reducing sugar, and phenolic and flavonoid compounds, including CC (Dubey et al., 2015; Machado et al., 2021). A high concentration of CC is one of the major contributors to AOX in hot peppers; during maturation, the process of capsaicinoid synthesis supersedes that of the synthesis of flavonoids. During advanced stages of pepper maturation synthesis of flavonoids converges with the capsaicinoids pathway, resulting in the disappearance of flavonoids (Meckelmann et al., 2013). Kogure et al. (2002) reported CC to be a strong scavenger of several radicals and attributed its scavenging activity to the C7-benzyl carbon of the vanillin and 8-methyl-6-noneamide. Glycosides of ferulic, sinapic acid and quercetin, luteolin, and apigenin are the major phenolics and flavonoids reported in hot chili peppers (Bae et al., 2012).

3.5 Thermogravimetric Analysis of Optimized Bhut Jolokia Oleoresin

The thermograms (TG/dTG curves) showed three weight loss (WL) steps (Fig. 4). The BJO registered a residue of 40.51 % in the first WL step (97.83 to 308 °C) with maximum decomposition occurrence at peak temperature of 271 °C. Rezazadeh et al. (2022) reported a much lower residue of 26 % by 100 °C in red pepper extract. The difference is likely as the red pepper extract used in their study was locally procured while in our study, Bhut Jolokia oleoresin was extracted under controlled conditions in our laboratory. On the contrary, Huang et al., (2019)

reported a weight residue of nearly 10% of pure CC with its peak thermal decomposition at 325 °C.

A peak temperature of 371.67 °C was recorded for the second WL step (308 to 416.5 °C) with a residue weight of 9.70 %. Finally, third WL step (416.5 to 691.17 °C) corresponded to the terminus of the thermal degradation process with only 0.62 % of the initial weight remaining (Table 6). The decomposition at elevated temperatures is typical of chili oleoresin therefore, interventions such as encapsulation can be employed to protect against thermal degradation (Bai et al., 2023; de Aguiar et al., 2022; Rezazadeh et al., 2022).

3.6 Accelerated Thermal Stability

The incorporation of chili oleoresin in foods requires careful consideration of its thermal stability to preserve its flavor and nutritional value. Since CC is lipophilic, the CC-rich oleoresin (1% w/v) was dissolved in a carrier oil i.e., RBO, and subjected to accelerated storage study (80 to 140 °C for 90 min) to understand the degradation mechanism. Oil was chosen as a medium, to simulate the degradative heating effects of domestic/elevated processing on BJO. Degradation of CC was monitored over time, and as can be seen from Fig. 3 with increasing temperature and time duration, high degradation was observed at elevated temperatures. After 90 min, 78.99, 57.67, 38.56, and 7.84 % of CC were retained at 80, 100, 120, and 140 °C, respectively. As anticipated, a rise in temperature from 80 to 100 to 120 to 140 °C, resulted in a reduction in the half-life (h) corresponding to 4.13, 1.99, 1.27, and 0.38, respectively (Table 5). This suggests that the degradation of CC was temperature-dependent (Zhang et al., 2021). Further, the rate constant of the degradation reaction was higher for elevated temperatures (Table 5). There are scanty reports on degradation kinetics of CC although generating such information is essential

and a prerequisite for determining its effective incorporation in varied food matrixes, including bakery goods, packaging films, and pharmaceuticals.

The reaction kinetics of the thermal degradation of CC revealed a first-order reaction with an activation energy of 71.86 kJ/mol. Furthermore, the high R^2 and lower values of SSR also suggested that the first-order model was suitable for kinetics studies and half-life estimation (Song et al., 2017). Studies concerning the kinetics of chili oleoresins in carrier oils are not available for comparison. However, Bustamante, et al. (Bustamante et al., 2022) and Zhang et al. (Zhang et al., 2021) studied the thermal degradation of CC during cooking (100 to 210 °C) and in hotpot oil (120 to 180 °C) and found the degradation to follow first-order kinetics with activation energies corresponding to 84.0 kJ/mol and 52.77 kJ/mol, respectively. In another, drying of red chili pepper reportedly complied with second-order kinetics with an activation energy of 45.10 kJ/mol (Arifin et al., 2018). The lower temperatures (40 to 70 °C) employed in their study may have resulted in lower activation energy. Structural changes in CC like formation of vanillyl nonanoate through condensation of vanillylamin and fatty acid derivatives may occur during heating (Arifin et al., 2018). Additionally, 8-methyl-6-nonenoamide has been identified as the primary product while vanillin and 8-methyl-6-nonenoic acid as secondary products during decomposition (Zhang et al., 2021).

Temperature sensitivity of CC in terms of Q_{10} was in conformity with degradation rate constants. The Q_{10} value of 80 to 100, 100 to 120, and 120 to 140 °C were 0.0548, 0.0574, and 0.1456, respectively, demonstrating an increase in the degradation rate as the temperature was gradually raised. Further, Q_{10} was > 2.5 times higher between 120 to 140 °C than at lower temperatures indicating the susceptibility of CC at these temperatures. Thus, it seems that decomposition of CC is dependent on the initial concentration of reactant and reaction conditions i.e., time and

temperature of the reaction. The finding suggests that CC can be protected in an emulsion-based delivery system for improving its stability, strengthening a strong basis for future lines of research.

4. Conclusions

An innovative green supercritical fluid extraction process was optimized to target high capsaicin content and oleoresin yield from *Capsicum chinense* (cv Bhut Jolokia) in shortest extraction duration (<2h). The optimized extraction technique using CO₂ as the main solvent and ethanol as a co-solvent in a non-hybrid SFE setup yielded oleoresin with high CC content (367.14 mg/g) and functional quality. High AOX and oxidative stability of BJO as established by accelerated storage conditions, establishes CC from BJ as a functional ingredient for food applications. The study adds another dimension of research for encapsulating CC in polymer matrixes to expand its use in the food industry.

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664 **Acknowledgments**

665 The first author gratefully acknowledges Post Graduate School, ICAR-Indian Agricultural
666 Research Institute, New Delhi for providing financial assistance in the form of IARI Senior
667 Scholarship for Ph.D. and the Director, ICAR-Indian Agricultural Research Institute, for
668 providing all the necessary laboratory facilities.

669 **CRedit authorship contribution statement**

670 **Vikono Ksh:** Investigation, Validation, Data curation, Formal analysis, Writing – original draft,
671 & review. **Vishnu Anand:** Investigation, Formal analysis, Writing – original draft **Virendra**
672 **Singh Rana:** Resource, Writing – review & editing. **Jigni Mishra:** Resource. **Eldho**
673 **Varghese:** Formal analysis, Data curation, & Validation. **Neelam Upadhyay:** Data curation,
674 Writing – review & editing. **Charanjit Kaur:** Supervision, Data curation, Formal analysis,
675 Writing – review & editing.

676 **Funding** The inhouse funding received by the Division of Food Science & Post Harvest
677 Technology from ICAR – Indian Agriculture Research Institute, New Delhi was utilized.

678 **Data Availability** Data will be made available on request.

679 **Statements and Declarations**

680 **Competing Interest** The authors declare no competing interests.

Figures

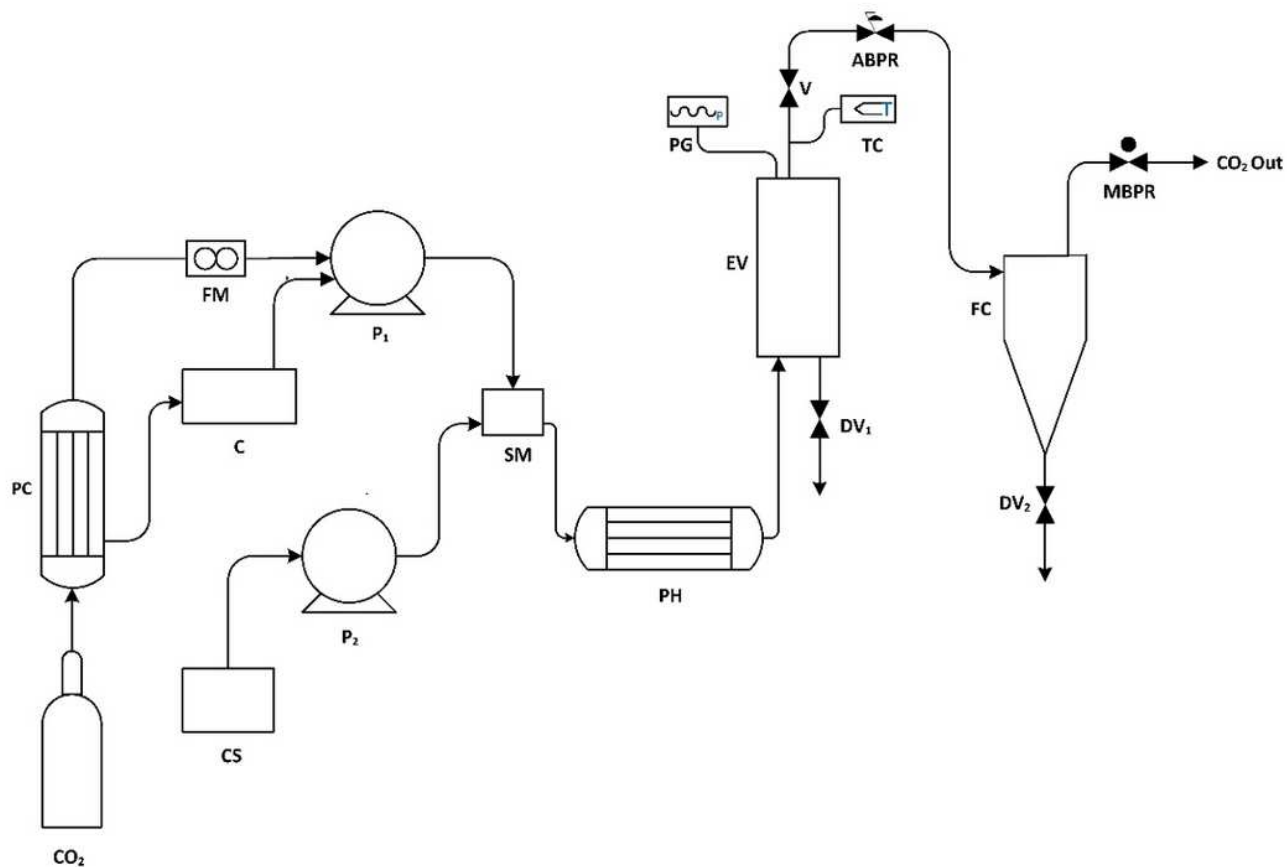


Figure 1

Diagram of the supercritical fluid extraction unit: PC – Pre-cooler; FM – Flowmeter; C – Chiller; CS – Co-solvent; P₁ – CO₂ pump; P₂ – Co-solvent pump; SM – Static mixer; PH – Pre-heater; EV – Extraction vessel (1 L); PG – Pressure gauge; TC – Thermocouple; V – on/off valve; ABPR – Automated back pressure regulator; FC – Fraction collector; MBPR – Manual back pressure regulator; DV₁ and DV₂ – Drain valve.

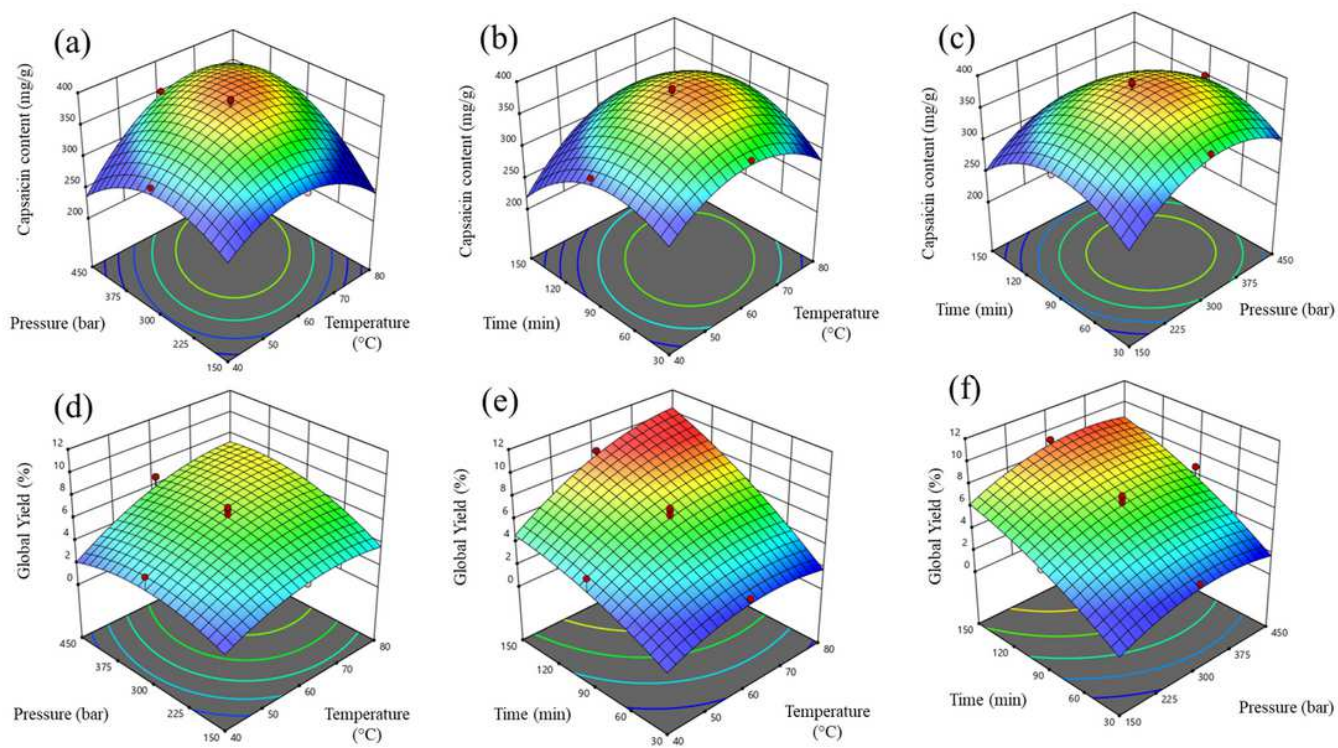


Figure 2

Response surface plots to visualize the effect of input variables (temperature, pressure, time) on the capsaicin content (a-c) and the global yield (d-f).

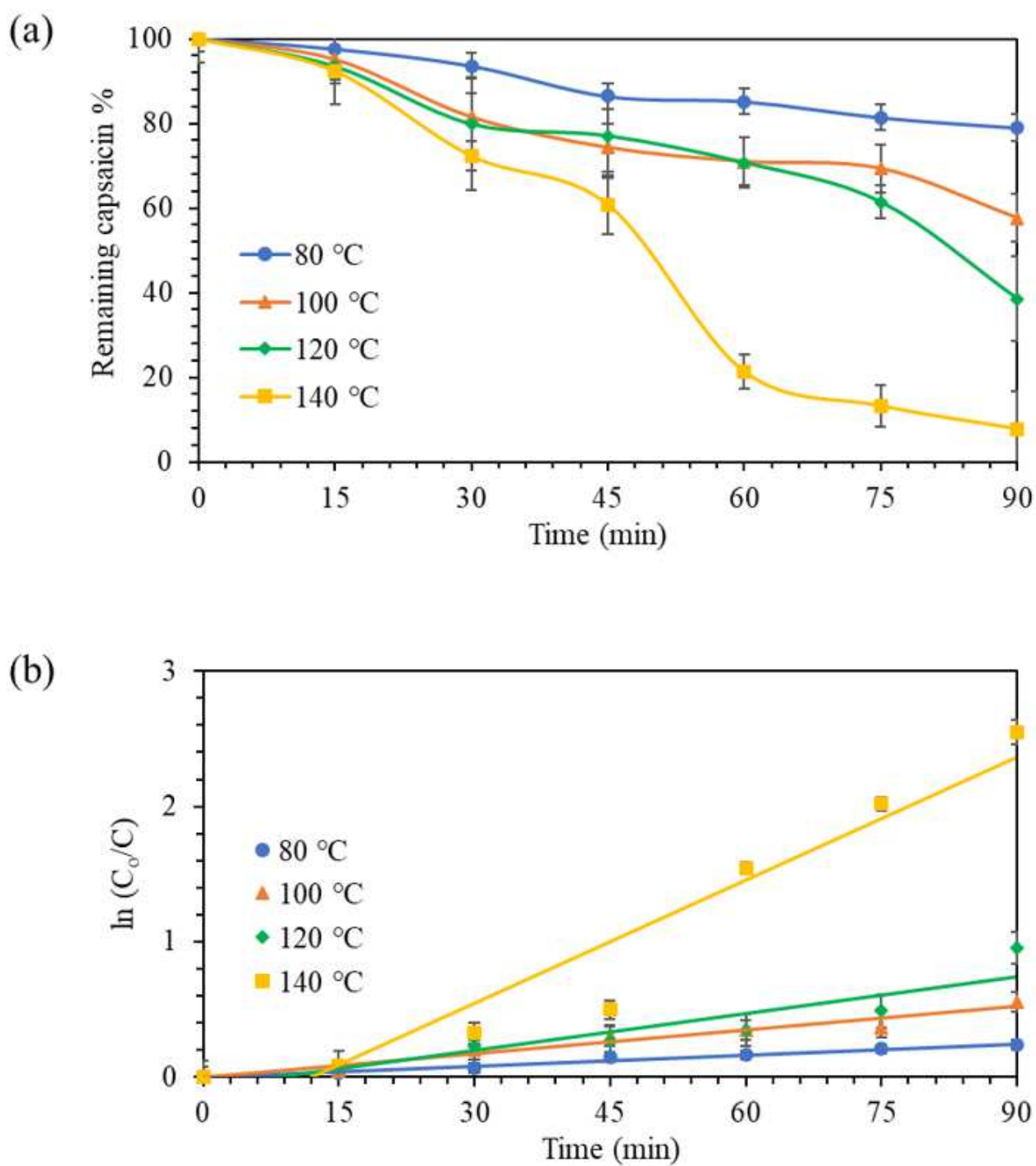


Figure 3

Degradation kinetics of capsaicin in rice bran oil (a) and degradation kinetics analysis of capsaicin using first-order model (b).

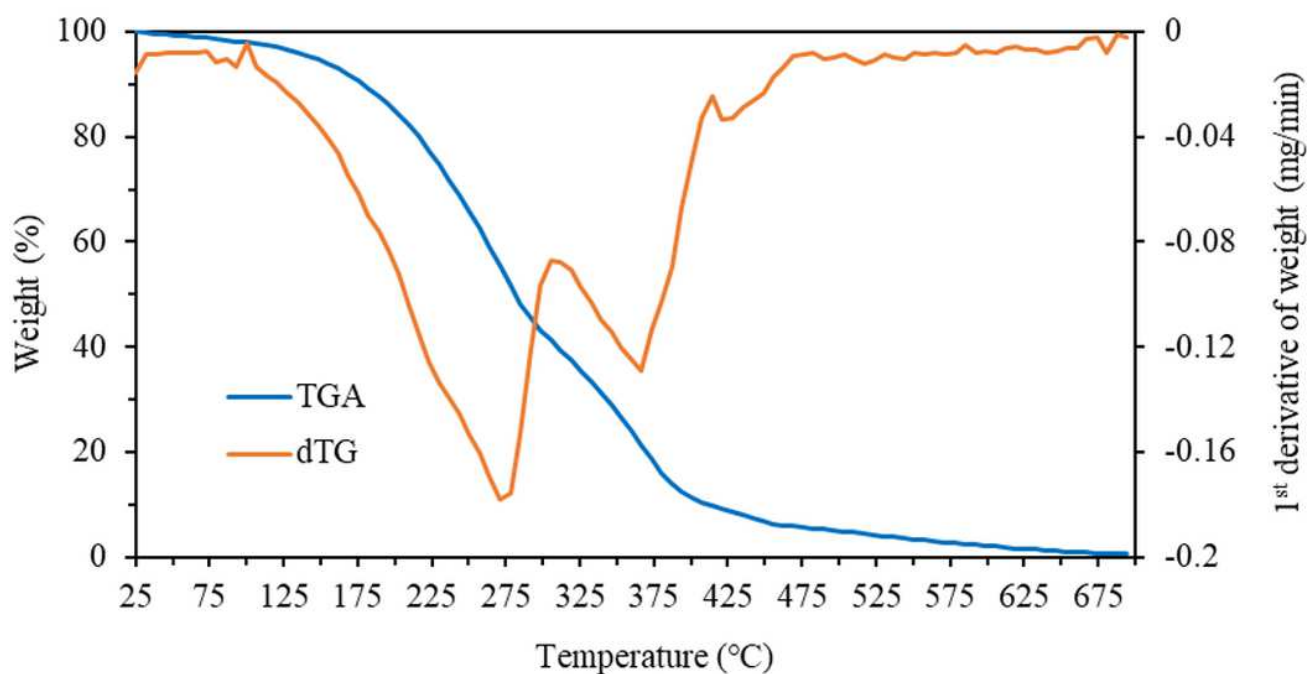


Figure 4

Thermogravimetric (TG) and differential thermogravimetric (dTG) curves of optimized Bhut Jolokia oleoresin.

Supplementary Files

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