https://doi.org/10.31925/farmacia.2023.5.8 **ORIGINAL ARTICLE**

A BOX-BEHNKEN DESIGN FOR OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF SINAPIC ACID FROM *FRAGARIA ANANASSA*

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Abstract

The present study focuses on optimizing the extraction process of sinapic acid from *Fragaria ananassa* using ultrasonic-assisted extraction (UAE). The extraction process study variables were: temperature in the range of 20 to 60°C, extraction time between 20 and 90 minutes and the solid-to-liquid ratio ranging from 10 to 30 mL/g. Response surface analysis determined that the most favourable conditions for sinapic acid extraction were at a temperature of 60°C, extraction time of 55 minutes and at a liquid-to-solid ratio of 30 mL/g. Under these conditions, the extraction yield of sinapic acid was measured at 23.56 mg/g. The experimental results closely aligned with the predicted values, affirming the reliability of the proposed model. Furthermore, UAE proved to be a highly effective method, exhibiting significantly reduced extraction time and producing higher levels of sinapic acid compared to traditional extraction techniques. HPLC analysis confirmed the presence of substantial amounts of sinapic acid in *Fragaria ananassa*. In conclusion, the optimized UAE method, in conjunction with HPLC-UV analysis, presents a valuable approach for the efficient extraction and estimation of sinapic acid from *Fragaria ananassa*. These results have the potential to greatly benefit industrial applications that need for extremely high extraction yields of these organic molecules.

Rezumat

Studiul urmărește optimizarea procesului de extracție a acidului sinapic din *Fragaria ananassa* folosind metoda cu ultrasunete (UAE). Variabilele de studiu ale procesului de extracție au fost: temperatura (20-60°C), timpul de extracție (20-90 de minute) și raportul solid-lichid (10-30 mL/g). Rezultatele au stabilit condițiile favorabile pentru extracția acidului sinapic: temperatura de 60°C, timpul de extracție de 55 minute și un raport lichid-solid de 30 mL/g. În aceste condiții, randamentul de extracție a acidului sinapic a fost de 23,56 mg/g. În plus, UAE s-a dovedit a fi o metodă foarte eficientă, cu un timp de extracție semnificativ redus și cu niveluri mari de acid sinapic în comparație cu tehnicile tradiționale de extracție. Analiza HPLC a confirmat prezența unor cantități substanțiale de acid sinapic în *Fragaria ananassa*. Rezultatele obținute au aplicabilitate industrială pentru obținerea moleculelor organice cu randament de extracție mare.

Keywords: *Fragaria ananassa*, sinapic acid, ultrasonic assisted extraction, Box-Behnken, optimization

Introduction

In recent years, there has been a growing interest in the development of novel extraction techniques that maximize the efficiency and yield of bioactive compounds from plant sources[1]. Ultrasonic-assisted extraction (UAE) has emerged as a promising method due to its ability to enhance extraction processes through the application of ultrasonic waves [2, 3]. The extraction of bioactive compounds from plant materials has been a subject of extensive research due to their potential applications in various industries, including pharmaceuticals, food and cosmetics [4]. Traditional extraction methods often involve the use of solvents and prolonged extraction times, which can be laborious and inefficient [5-7]. In recent years, UAE has gained significant attention as an alternative technique to enhance the extraction process and improve the recovery of phytoconstituents [3, 8-11]. UAE involves the application of ultrasonic waves to the extraction medium, resulting in the generation of cavitation bubbles. These bubbles collapse near the solid-liquid interface, leading to the release of shock-waves and the formation of microjets, which contribute to the disruption of plant cells and facilitate the release of target compounds into the extraction solvent. This phenomenon significantly increases mass transfer rates and promotes the extraction efficiency of bioactive compounds from plant matrices [12, 13]. Several studies have reported successful extraction of various classes of bioactive compounds, including phenolic acids, flavonoids, alkaloids and essential oils, using ultrasound-assisted techniques [14-18]. For instance, UAE has been utilized to extract phenolic compounds such as resveratrol, quercetin and curcumin from different plant sources [19-22]. The enhanced extraction efficiency provided by UAE can contribute to the development of high-value natural extracts and facilitate the utilization of plant-derived bioactive compounds in various industries [3].

Fragaria ananassa, commonly known as the garden strawberry, is a widely cultivated fruit that belongs to the Rosaceae family [23]. The garden strawberry is known for its sweet and succulent berries, which are consumed worldwide in various culinary applications [24]. Apart from its culinary appeal, *Fragaria ananassa* has also been recognized for its potential biological properties [25-27]. The plant contains several bioactive compounds, including vitamins, minerals, antioxidants and phytochemicals, which contribute to its human health benefits [27]. These biological properties have been explored in scientific research and traditional medicine practices [24]. One of the key applications of *Fragaria ananassa* is its potential anti-inflammatory properties [28]. Studies have shown that the plant contains bioactive compounds such as sinapic acid, anthocyanins and ellagitannins, which possess anti-inflammatory effects [29, 30]. These compounds have been found to inhibit the production of pro-inflammatory molecules in the body, thereby potentially reducing inflammation and related conditions [29, 30].

Sinapic acid, a phenolic compound found in various plant species, possesses significant antioxidant and antimicrobial properties, making it a subject of interest in the field of natural product research [30]. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is a derivative of cinnamic acid and belongs to the class of hydroxycinnamic acids [31, 32]. It is naturally present in various plant sources, including fruits, vegetables and grains. Several studies have reported the diverse pharmacological activities of sinapic acid, such as its antidiabetic, antioxidant, anti-inflammatory and anticancer properties [30, 33]. The multi therapeutic potential of sinapic acid has led to increased interest in its extraction and analysis from plant matrices [34-38]. In addition to its therapeutic effects, it is essential to consider the bioavailability of sinapic acid, as this factor significantly influences its potential for clinical application. Several preclinical studies have investigated the pharmacokinetics and interaction of sinapic acid, revealing insights into its drug disposition in animal models [39, 40]. These studies suggest that sinapic acid exhibits moderate bioavailability, with factors such as route of administration and dosage playing pivotal roles in its absorption and systemic availability [39-41]. This study aims to investigate the application of Box Behnken design for ultrasound assisted extraction of sinapic acid from *Fragaria ananassa*.

Materials and Methods

Plant material, apparatus and reagents

Fragaria ananassa (strawberries) were purchased from local market. The fruit were cut into smaller pieces dried in shades for 96 hours and then dehydrated by lyophilization (freeze drying) and coarsely ground for the extraction process. The ground powder was stored in a dark container free from moisture and light until used. Standard substance of sinapic acid (SA; 99% purity), was purchased from Merck Laboratories. Methanol and acetonitrile solvents of analytical grade were used in the analysis and were purchased from Panreac Chemicals, UK. Mili-Q water was produced from the Lab.

Extraction of sinapic acid

Powdered *Fragaria ananassa* (10 g *per* 250 mL) was added to a conical flask. Extraction aided by ultrasonic equipment (Raypa® Instrumentation, Barcelona, Spain) was used to extract sinapic acid from *Fragaria ananassa* in methanol at a frequency of 60 Hz and power of 550 W. Rota vapor was used to extract the liquid, and then it was cooled, filtered and dried to obtain the extract; the % yield was then determined. The content of sinapic acid in the extracted material was determined using the HPLC technique [42].

Another set of experiments involved vigorously shaking a mixture of 10 grams of dried *Fragaria ananassa* powder and 250 mL of methanol in a water bath at 60°C for an hour. The extracted material was collected at the end of the procedure, cooled and then dried using Rota vapor. Using a previously developed HPLC method [42], the sinapic acid concentration in the resulting extract was analysed. Briefly, the separation of the extract was conducted using a reversed-phase C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) supplied by Waters. The mobile phase employed in the HPLC system consisted of methanol (A) and a mixture of water/acetic acid (99:1 v/v) (B). A gradient elution was performed at a flow rate of 1.5 mL/min over a 20-minute period.

Box-Behnken design (BBD)

The extraction parameters were optimized at three different levels (Low, medium and high; -1, 0, +1) by using BBD 3-factorial design (3^3) and are presented in Table 1 (Design expert Version 8, Stat-Ease Inc., Minneapolis, MN, USA). Three parameters were selected to examine the impact of UAE variables (Temperature (A), time duration (B) and Solid-liquid ratio (C) for the maximum extraction of sinapic acid from *Fragaria ananassa*. The optimization was performed as performed as *per* the previously reported protocol [2, 43]. The model gives 17 runs of fitted with 5 central points to a second order polynomial equation to optimize the sinapic acid extraction. The significance of model terms and equations were carefully determined in terms of p-values using ANOVA and p values ≤ 0.05 were considered as significant. The experiments were conducted in 3-replicated (n = 3) and the experimental results were compared with the predicted values to validate the model. In order to maximize yield, we ran the experiment in the conditions recommended by the RSM's point prediction tool for the concentration of sinapic acid. The optimal conditions predicted by the model were valued in order to determine the model's reliability. These typical values were compared to the values (predicted) by the established model to establish its accuracy and applicability.

Statistical analysis

The data is presented as a mean standard deviation (± SD). Models were adjusted with the use of BBD, multiple regression and ANOVA. The statistical significance of the established quadratic mathematical model was simulated using the following statistics: p value, F value, R^2 (determination coefficient), R^{2a} (adjusted determination of coefficient), SS (sum of squares), DF (degrees of freedom), CV (coefficient of variation), MSS (mean sum of squares) and R (coefficient correlation). Microsoft Excel 2019 (Microsoft Inc., Seattle, WA, USA) was used for all of the calculations and analysis of the experiment data. The data was plotted and response surfaces were constructed. Significant results were defined as p-values below 0.05.

Results and Discussion

The optimized extract of *Fragaria ananassa* was analysed, and the chromatogram illustrating the separation of sinapic acid and other constituents is depicted in Figures 1A and 1B. Sinapic acid was detected and quantified at a UV wavelength of 320 nm. Incorporating a gradient system in the HPLC elution process enhances the performance, sensitivity and efficiency of the column. The implementation of a gradient system in HPLC analysis not only enhances the quality of separation and reduces analysis time and minimizes column degradation caused by strong analyte retention. In this particular setup, sinapic acid exhibited a retention time (Rt) of 4.10 min, which is comparable to the retention time (Rt = 4.058 min) of the constituent found in the optimized extract. The HPLC method demonstrated excellent linearity for sinapic acid, with an r^2 value of 0.999 within the linear range of 0.1 - 50 µg/mL.

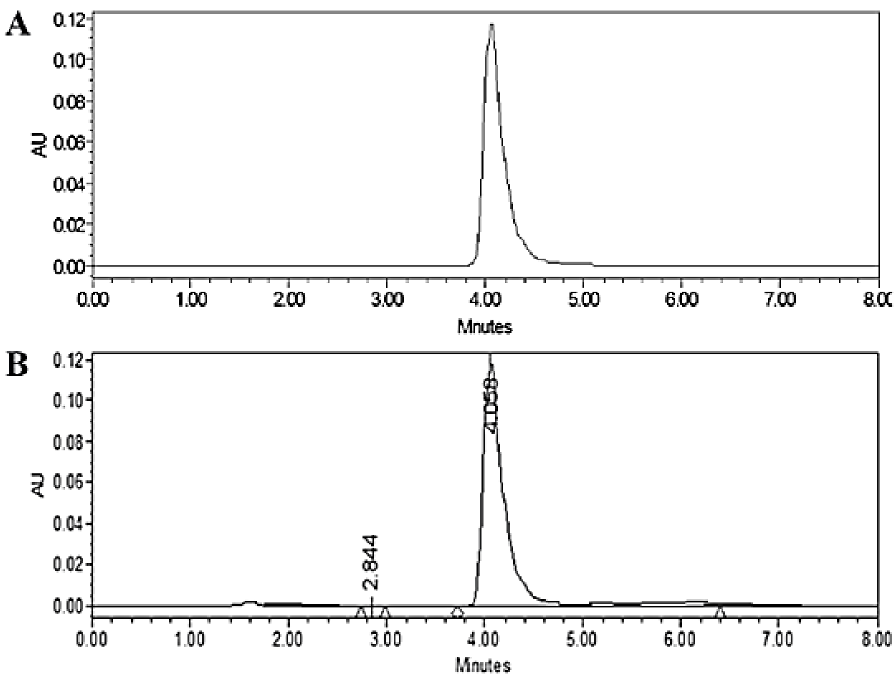

Figure 1.

Typical HPLC chromatograms of standard sinapic acid and extracted sinapic acid from *Fragaria ananassa*

BBD optimization and model fitting

A number of variables in the extraction process were held constant in an effort to enhance sinapic acid extraction from *Fragaria ananassa*. The BBD was then utilized to find the optimal values for these constants. Factor ranges of 20 - 60°C, 30 - 90 min and 10 - 30 mL/g were employed to determine the sinapic acid extraction (Table I). Sinapic acid extraction efficiency was determined by varying only one of three variables (temperature, time and pressure) in isolation from the other two. When looking at the impact of the other two variables, the same method was used. Sinapic acid extraction was shown to be most sensitive to temperature, followed by solid/liquid ratio and extraction duration, according to the BBD. Extraction at 60°C for 55 minutes at a solid-liquid ratio of 30 mL/g resulted in the best yield of sinapic acid (23.56 mg/g).

Table I

Box-Behnken response surface design summary

Design summary						
Study type	Response surface					
Initial design	Box-Behnken					
Design model	Quadratic					
Runs	17					
Factor name (Units)	**Low actual**	**High actual**	**Low coded**	**High coded**	**Mean**	**SD**
A. Extraction temperature (°C)	20	60	-1	1	40	13.72
B. Extraction time (Min)	20	90	-1	1	55	24.01
C. Solid/Liquid ratio (mg/g)	15	30	-1	1	22.50	5.14
Response name units	**Analysis**	**Minimum**	**Maximum**	**Mean**	**SD**	**Ratio**
Y1. Sinapic acid (mg/g)	Polynomial	8.23	23.56	16.87	4.82	2.86

A. Extraction temperature (°C); B. Extraction time (Min); C. Solid/LiquidrRatio (mg/g)

BBD was used to study the influence of UAE variables (A, B and C) on sinapic acid extraction in different modes like linear, quadratic and interacting. The 3-factorial BB experimental design and the obtained response (R) in terms of sinapic acid is presented in Table II. Sinapic acid concentration values varied from 8.23 to 23.56 mg/g of dry extract (Table II). The ANOVA showed that quadratic polynomial model was significant ($p < 0.001$) (Table III).

Table II

Experimental parameters of BBD and results of sinapic acid yield

Run	Temp. (°C)	Time (min)	Solid/Liquid (g/mL) ratio	Sinapic acid (mg/g)		Residual
				Actual	Predicted	
1	20	20	22.5	8.23	8.03	0.20
2	60	20	22.5	12.26	12.99	-0.73
3	20	90	22.5	9.12	8.39	0.73
4	60	90	22.5	21.27	21.46	-0.20
5	20	55	15	11.96	12.42	-0.46
6	60	55	15	18.26	17.78	0.48
7	20	55	30	9.96	10.44	-0.48
8	60	55	30	23.56	23.10	0.46
9	40	20	15	12.69	12.43	0.26
10	40	90	15	20.15	20.43	-0.28
11	40	20	30	17.96	17.68	0.28
12	40	90	30	18.26	18.52	-0.26
13	40	55	22.5	20.22	20.54	-0.32
14	40	55	22.5	20.95	20.54	0.41
15	40	55	22.5	20.99	20.54	0.45
16	40	55	22.5	21.32	20.54	0.78
17	40	55	22.5	19.23	20.54	-1.31

Influence of extraction parameters on sinapic acid

Fragaria ananassa methanolic extract contains anywhere from 8.23 mg/g to 23.56 mg/g of sinapic acid per gram of dry extract. At 20°C and 20 minutes of extraction time, the yield was 8.03 mg/g, which was the lowest. The best yield, 23.56 mg/g, was achieved with an extraction temperature of 60°C and a duration of 55 minutes. A statistical evaluation of the data revealed that the yield of sinapic acid was significantly influenced by the extraction temperature, extraction duration and liquid-to-solid ratio. *Fragaria ananassa* sinapic acid yield was modelled using a second-order polynomial equation. The equation is as follows:

$$\text{Sinapic Acid} = 20.54 + 4.51 * A + 2.21 * B + 0.83 * C + 2.03 * A * B + 1.82 * A * C - 1.79 * B * C - 4.58 * A^2 - 3.25 * B^2 - 0.030 * C^2,$$

where Y is the yield of sinapic acid in mg/g, A is the extraction temperature in °C, B is the extraction time in minutes and C is the liquid-to-solid ratio in mL/g. This equation can be used to optimize the extraction conditions for the isolation of sinapic acid.

Method validation

Sinapic acid yield was compared between experimental and predicted values to verify the BBD method. The BBD model and the resulting polynomial equation were tested using a minimal percentage point prediction tool to ensure their accuracy. Table III shows a linear relationship between observed and predicted amounts of sinapic acid, and the high R^2 values that approach 0.999 indicate an unusual degree of correlation ($p \leq 0.001$). Figure 2 displays diagnostic graphs comparing experimental data with those of the models used to evaluate their accuracy. The Box-Cox figure is shown in Figure 2A for power transformations of variables. Figure 3B shows a normal distribution and a linear relationship between the expected and actual response values for each run. The closeness of the residual plot to the straight line and the lack of variance deviation in Figure 3C exemplify the response variable's normal distribution. The current model significantly enhances the connection between process variables and the outcome. Figure 2D shows the excellent concordance between calculated and measured values. All data points were found to be within the allowed range after inspecting the internally studentized residuals *vs.* experimental runs (Figure 2E). Figure 2F shows the perturbation of variables within the allowed range, further illuminating the model's efficacy and precision.

Table III

The analysis of variance and regression coefficients employed to assess the calculated surface quadratic model's impact on sinapic acid yield

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	390.30	9.00	43.37	59.51	< 0.0001
A-Extraction temperature	162.68	1.00	162.68	223.24	< 0.0001
B-Extraction time	38.98	1.00	38.98	53.49	0.0002
C-Solid/Liquid ratio	5.57	1.00	5.57	7.65	0.0279
AB	16.46	1.00	16.46	22.59	0.0021
AC	13.32	1.00	13.32	18.28	0.0037
BC	12.83	1.00	12.83	17.60	0.0041
A^2	88.21	1.00	88.21	121.05	< 0.0001
B^2	44.37	1.00	44.37	60.89	0.0001
C^2	0.004	1.00	0.00	0.01	0.9445
Residual	5.10	7.00	0.73		
Lack of fit	2.30	3.00	0.77	1.10	0.0469
Pure error	2.80	4	0.70		
Cor total	395.40	16			
Regression analysis and response equation					
Std. Dev.	0.85	R-Squared		0.99	
Mean	16.85	Adj R-Squared		0.97	
C.V. %	5.07	Pred R-Squared		0.90	
PRESS	41.23	Adeq Precision		23.02	

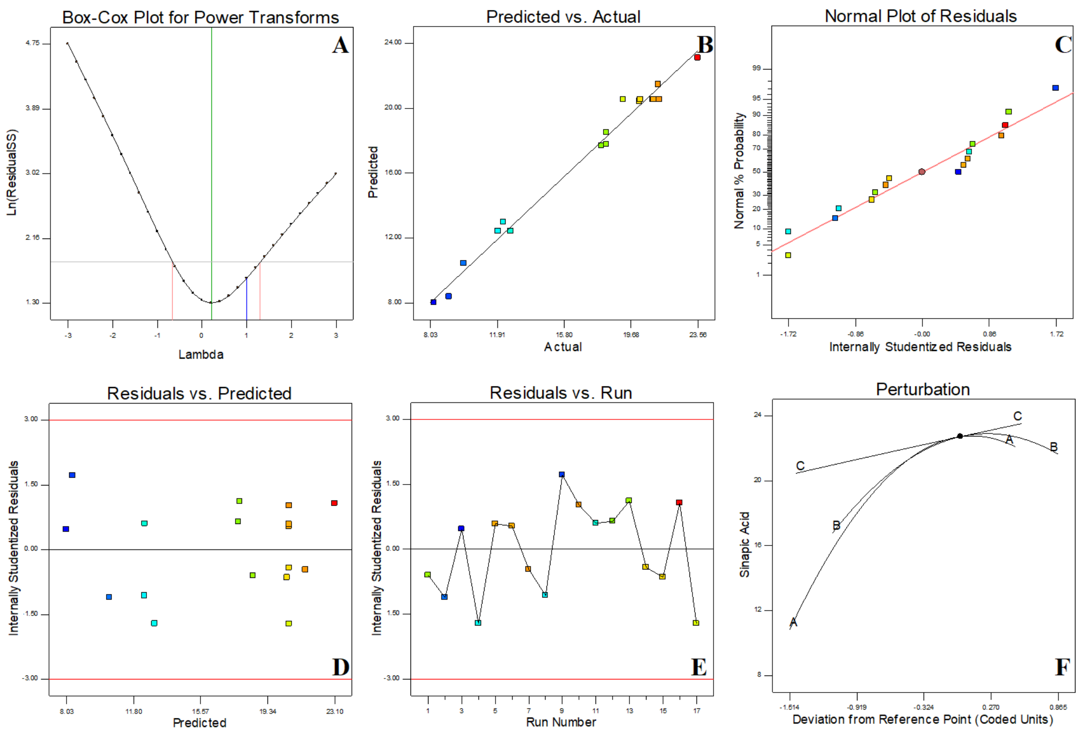

Figure 2.

The diagnostic plots evaluating the adequacy of the Box-Behnken model for the tested variable, specifically the relationship between predicted and actual sinapic acid yield

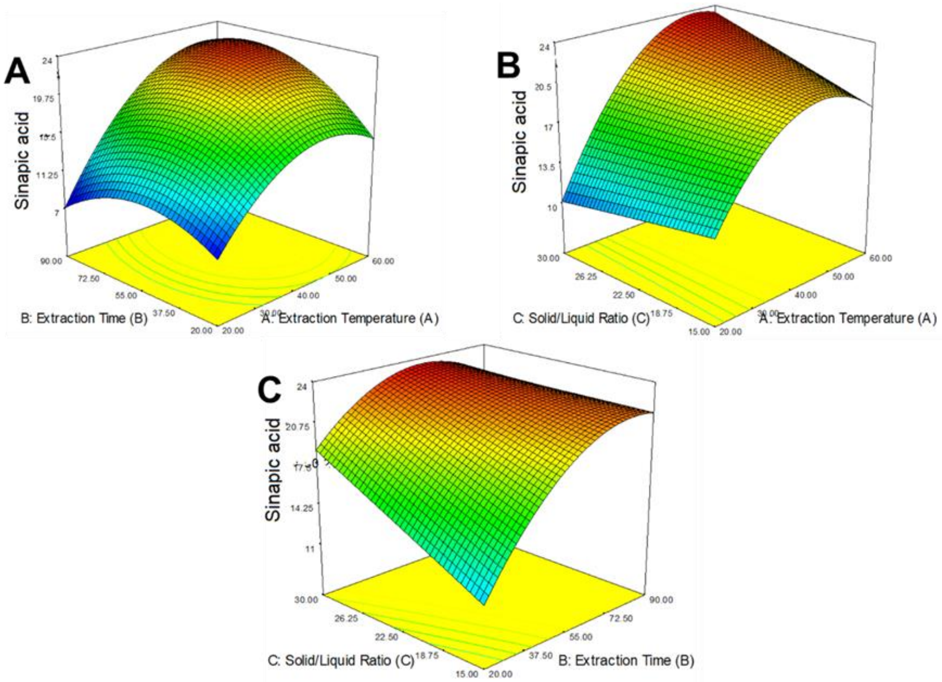

Figure 3.

The response surface relation between variables was visualized using 3D plots. Figure A illustrates the impact of extraction time and extraction temperature on sinapic acid yield, while Figure B demonstrates the relationship between solid-liquid ratio and extraction temperature. Furthermore, Figure C displays the influence of solid-liquid ratio and extraction time on sinapic acid yield. In each figure, the intensity of the dark red colour represents the concentration of sinapic acid. Additionally, the UAE method yielded a significantly higher amount of sinapic acid (23.56 ± 0.69 mg/g) compared to the CSE method (sinapic acid yield: 15.26 ± 0.46 mg/g) at a significance level of $p < 0.05$. In addition to the improved extraction efficiency, the UAE method also demonstrated a notable reduction in solvent usage and extraction time compared to the CSE method.

The BBD approach was employed to determine the optimal extraction conditions for achieving maximum sinapic acid extraction from the methanol extract of *Fragaria ananassa*. By maximizing the desirability of the response, the most favourable extraction parameters were predicted (Table IV). The desirability function assigns a value between 0 and 1 to each response, with 0 indicating an undesirable outcome and 1 representing the most desirable result. Design Expert 8 software was utilized to calculate the highest desirability value, considering the intended objectives of the responses. The maximum desirability value for sinapic acid was determined to be 0.956. Numeric optimization of the UAE extraction parameters (extraction temperature of 50.435°C, extraction time of 60.68 min and liquid-to-solid ratio of 25.64 mL/g) resulted in the highest predicted sinapic acid yield of 24.64 ± 0.39 mg/g of dried extract (Figures 3A, 3B and 3C). Subsequently, experiments were conducted under the optimized UAE conditions, and the experimental sinapic acid yield (22.96 ± 0.69 mg/g of dried extract) aligned with the predicted value with 93.18% validity, confirming the reliability of the BBD model in forecasting sinapic acid extraction using UAE.

Table IV

Point prediction tool model validation: prediction *vs*. observed

Factor	Name	Level	Low level	High level	Coding
A	Extraction Temperature (°C)	50.43	20	60	Actual
B	Extraction Time (min)	60.68	20	90	Actual
C	Solid/Liquid Ratio (mg/g)	25.14	15	30	Actual
Response	**Prediction**	**Observed (validation)**			
Sinapic acid	24.64 ± 0.39	22.96 ± 0.69			

Phytoconstituents are a class of polyphenols that are abundant in nature [44, 45]. To address the rising market demand and environmental laws, modern techniques are replacing conventional methods of flavonoid extraction. These methods are more efficient, selective and use less energy and solvent than their predecessors. Modern techniques include microwaves, ultrasound, pressured liquids, supercritical and electric fields [44, 46, 47]. When compared to traditional methods, these new approaches are typically faster, friendlier to the environment, economical and more automated [44, 49]. During the experiment, it was observed that the maximum content of sinapic acid increased with the extraction temperature up to 60°C, at 60 minutes with liquid-to-solid ratio of 30 mL/g reaching a maximum value of 23.56 mg/g of dried extract. Further alterations in these variables did not result in a significant increase in sinapic acid content. However, increasing the extraction time and solvent-to-drug ratio did not significantly increase sinapic acid yield. These findings helped establish the range for various UAE parameters which were further used in the optimization using BBD to maximize sinapic acid extraction from *Fragaria ananassa*.

Sinapic acid yield values from multiple BBD runs showed a significant dependence on various extraction settings, indicating the need for process adjustment. The quadratic polynomial model for sinapic acid extraction, developed to improve the extraction technique, was found to be highly significant ($p < 0.0001$) compared to other models such as linear, 2FI and cubic models. The model's F-value of 59.51 confirmed its significance [49]. Greater significance indicates a higher degree of agreement between observed and predicted values [50]. The lack of fit was found to be non-significant ($p > 0.05$), indicating the model's capacity to predict sinapic acid extraction. The experimental data for sinapic acid demonstrated low coefficient of variation (CV) of 5.07%, indicating high reliability. The model's precision was adequate with an adequate precision ratio of 23.02, indicated sufficient signal for exploring the design space. The residual and pure error of the suggested model were determined to be 3.10 and 2.30, respectively. The linear and quadratic effects of all three UAE parameters on sinapic acid yield were evident from the data. The P-value is used to assess the significance of each individual factor and the power of the interactions between variables [51].

The interaction of extraction parameters (A, B and C) on sinapic acid yield was visualized through 3D response surface plots. Sinapic acid extraction from *Fragaria ananassa* increased with temperature up to 60°C, after which it decreased. The highest yield of 23.56 mg/g was obtained at an extraction temperature of 60°C and extraction time of 55 min. This proved that the optimum yield may be obtained with an extraction period of only 60 minutes. Increasing the amount of time spent extracting has a similar beneficial effect [52-54]. Cavitation nuclei proliferation and the cavitation margin where it begins are both known to be temperature-dependent. At first, rising temperatures led to a greater extraction yield, but eventually, acoustic cavitation in UAE produced hydroxyl radicals, which led to chemical breakdown [55, 56]. The effect of A and C on sinapic acid yield was observed at a constant B of 55 minutes, resulting in a maximum yield. The influence of B and C on sinapic acid yield, with a fixed A of 60°C, showed a decrease in yield with increasing B and C values. The maximum sinapic acid yield of approximately 23.56 mg/g was achieved at T2 of 60 minutes and T3 of 30 mL/g. More fast diffusion of compounds was seen at greater water-to-raw-material ratios, suggesting an extra concentration differential between the compounds and water [57]. The diffusion zone was pushed further into the solid compounds' interior tissues as the water-to-raw-material ratio was increased [44, 57]. A greater yield of sinapic acid was achieved by increasing the water to raw material ratio from 10 to 30 mL/g. Extracting sinapic acid using UAE at its most efficient required the following settings, as established by BBD analysis: 50.43°C extraction temperature, 60.68 minutes' extraction duration and 25.64 mL/g liquid-to-solid ratio. These optimum UAE conditions were used in the experiments. The proposed HPLC-UV technique was used to determine the concentration of sinapic acid in the extract of *Fragaria ananassa* that had been produced using the BBD-optimized UAE parameters. The 23.96 ± 0.69 mg/g of dry extract discovered experimentally in the BBD-optimized *Fragaria ananassa* was in line with the projected value. This illustrates that the proposed model is capable of accurately estimating sinapic acid yield. Compared to the CSE approach, UAE not only speeds up the extraction of sinapic acid but also requires fewer solvents and less time.

Conclusions

Response Surface Methodology (RSM) in combination with Box-Behnken Design (BBD) was employed to optimize the extraction parameters for sinapic acid from *Fragaria ananassa*. The optimal conditions for maximizing sinapic acid extraction using UAE were determined as follows: an extraction temperature of 50.43°C, an extraction time of 60.68 minutes and a liquid-to-solid ratio of 25.64 mL/g. UAE proved to be highly effective, significantly reducing the extraction time and yielding higher sinapic acid contents compared to traditional or conventional methods. HPLC analysis confirmed that strawberries contained a substantial amount of sinapic acid. Therefore, this modified UAE technique presents a promising alternative for the extraction of sinapic acid from dried *Fragaria ananassa* extracts, which serve as a rich source of natural antioxidant sinapic acid. Moreover, the UAE method also led to a significant reduction in solvent use and extraction time compared to the conventional solvent extraction (CSE) method. Overall, the improved UAE method, coupled with the developed HPLC-UV method, holds great potential for efficient extraction and quantification of sinapic acid from *Fragaria ananassa*, catering to industrial applications.

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Conflict of interest

The authors declare no conflict of interest.

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