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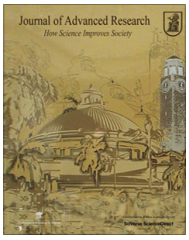

ORIGINAL ARTICLE

Optimization of supercritical fluid extraction and HPLC identification of wedelolactone from *Wedelia calendulacea* by orthogonal array design

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ABSTRACT

The purpose of this work is to provide a complete study of the influence of operational parameters of the supercritical carbon dioxide assisted extraction (SC CO_2E) on yield of wedelolactone from *Wedelia calendulacea* Less., and to find an optimal combination of factors that maximize the wedelolactone yield. In order to determine the optimal combination of the four factors *viz.* operating pressure, temperature, modifier concentration and extraction time, a Taguchi experimental design approach was used: four variables (three levels) in L_9 orthogonal array. Wedelolactone content was determined using validated HPLC methodology. Optimum extraction conditions were found to be as follows: extraction pressure, 25 MPa; temperature, 40 °C; modifier concentration, 10% and extraction time, 90 min. Optimum extraction conditions demonstrated wedelolactone yield of 8.01 ± 0.34 mg/100 g *W. calendulacea* Less. Pressure, temperature and time showed significant ($p < 0.05$) effect on the wedelolactone yield. The supercritical carbon dioxide extraction showed higher selectivity than the conventional Soxhlet assisted extraction method.

Abbreviations: SC CO_2E, supercritical carbon dioxide assisted extraction; SAE, Soxhlet assisted extraction; CAL STDs, calibration standards; QC STDs, quality control standards; LQC, low quality control; MQC, medium quality control; HQC, high quality control; Diff%, % difference.

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Introduction

The genus *Wedelia* comprises over 60 species of which nearly two dozen species are reported to be medicinally active. Among these is *Wedelia calendulacea* (Less.) or *W. chinensis* [1], commonly called 'pila bhangra' and used as a cure for several ailments [2]. The plant has been extensively studied for its hepatoprotective activity, and a number of herbal preparations comprising of *W. calendulacea* are available for treatment for jaundice and viral hepatitis [3]. The alcoholic extract of whole plant of *W. calendulacea* exhibited protective activity against

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carbon tetrachloride-induced liver injury *in vivo* [4]. The herb *W. calendulacea* is said to possess properties and main active constituent coumestans i.e., wedelolactone similar to *Eclipta alba* Hassk [3,5,6]. Wedelolactone exerts diverse biological activities including antivenom, anti-inflammatory, antitumor, antiosteoporotic and hepatoprotective effects [3,7–13].

Very few methods *viz*. homogenization [14] and Soxhlet extraction (SAE) [13,15] were reported for extracting wedelolactone from *W. calendulacea*. It is well known fact that conventional solvent extraction methods are tedious and time consuming. Moreover, these processes may lead to thermal, oxidative and photo-decomposition of active phyto-constituents. Supercritical carbon dioxide assisted extraction (SC CO_2E) has immediate advantages over traditional extraction techniques *viz*. it is a flexible process due to the possibility of continuous modulation of the solvent power/selectivity of the supercritical CO_2, it allows the elimination of polluting organic solvents and the expensive post-extraction processing of the extracts for solvent elimination [16].

Until now, there has been no literature reporting the use of SC CO_2E of wedelolactone from *W. calendulacea*. In present work, we have utilized SC CO_2E technique for the extraction of wedelolactone from *W. calendulacea*. The main objectives of the present study were (a) to analyze the influence of sample preparation conditions such as pressure, temperature, modifier concentration and extraction time on the wedelolactone yield; (b) to investigate the effects of various parameters on the SC CO_2E performance using Taguchi L_9 orthogonal array design.

Material and methods

Plant material and reagents

The authenticated dried plant material of *W. calendulacea* was ground to a powder using a pulveriser (K.C. Engineers, Ambala, HR, India). To select uniform particle size, plant powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, GJ, India) with sieves of different sizes (12, 24, 65, 85 and 120 meshes, Swastika electric and scientific works, Ambala, HR, India) for a period of 15 min. The plant powder passed through 65 mesh sieve and retained on 85 mesh sieve was collected and used for further extraction experiments. The standard wedelolactone (purity 98% by HPLC) was obtained from Natural Remedies Pvt. Ltd. (Bangalore, KA, India). All solvents used for the extraction and the chromatographic purpose were of analytical grade (Finar Chemicals Ltd., Ahmedabad, GJ, India) and HPLC grade (Merck, Darmstadt, Germany), respectively. CO_2 gas (99% purity) was procured from M/s Jain Cylinders (Aurangabad, MH, India).

Bench top SC CO_2E unit (Model: SFE 2000 series, Jasco International Co. Ltd., Hachioji, Tokyo, Japan) was used for the extraction purposes. The extracts were prepared freshly and stored temporarily in desiccators (Riviera glass Pvt. Ltd., Mumbai, MH, India) under vacuum until the analysis.

HPLC analysis

The HPLC analysis of wedelolactone was performed using in-house HPLC method as described below.

HPLC instrumentation and operating conditions

The HPLC system consisted of a Waters e2695 Separation Module with auto-sampler and Waters 2489 ultraviolet spectrophotometric detector (Waters, Milford, MA, USA) equipped with MassLynx data acquisition software, version 4.1. All samples and standards were filtered through 0.45 μm syringe filters (Millipore, Bangalore, KA, India). Separation was achieved on Waters XTerra C-18 column (250 mm × 4.6 mm, 5 μm particle sizes) (Waters, Milford, MA, USA) at 40 °C with mobile phase consisting of methanol and 0.5% acetic acid buffer (pH 5.0, 55:45 v/v) in isocratic elution with 0.5 mL/min flow rate. The UV detection of analytes was carried out at 351 nm.

Preparation of calibration standards and quality control samples

Reference stock solution (1 mg mL^{-1}) of wedelolactone was prepared by accurately weighing 5 mg of wedelolactone which was transferred to 5 mL volumetric flasks, dissolved and diluted up to 5 mL with HPLC grade methanol. Stock solution was diluted suitably with HPLC grade methanol to achieve 6 calibration standards (CAL stds) containing wedelolactone. CAL STD-1: 2.5 μg mL^{-1}; CAL STD-2: 5 μg mL^{-1}; CAL STD-3: 7.5 μg mL^{-1}; CAL STD-4: 10 μg mL^{-1}; CAL STD-5: 12.5 μg mL^{-1}; CAL STD-6: 25 μg mL^{-1}. Three quality control standards (QC stds) containing wedelolactone (LQC: 3.5 μg mL^{-1}; MQC: 8.5 μg mL^{-1} and HQC: 24 μg mL^{-1}) were prepared from stock solution.

Method validation

The analytical method was validated to meet the acceptance criteria as per International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. Recovery studies were performed using standard addition method. The linearity and range was established using six CAL STDs. The peak area vs concentration plots were subjected to linear least square regression analysis. Intra- and inter-day accuracy was established from QC STDs by evaluating nominal and mean measured concentrations of QC STDs which were compared and expressed as % difference (Diff%). The Diff% between mean measured and nominal concentrations was calculated as follows:

$$[(\text{Mean measured concentration} - \text{nominal concentration})/\text{nominal concentration}] \times 100 \quad (1)$$

The intra- and inter-day precision (% RSD) was established by analyzing five replicates each of 3 QC STDs on day 1 and again on each of three consecutive days. The lowest concentration with acceptable accuracy and precision was reported as limit of quantification (LOQ) for wedelolactone. Robustness of the method was assessed by multiple ratio adjustments in mobile phase composition, pH of the aqueous buffer and column oven temperature. For the study, methanol composition was changed over the range of 53–57%, pH range was modified in between 4.5 and 5.5 and column oven temperature was varied in the range of 38–42 °C. Less than 2% change in the final results was defined as the acceptance criteria.

Soxhlet assisted extraction (SAE) of W. calendulacea

SAE was used for the extraction of wedelolactone from the *W. calendulacea*. Twenty grams of powdered drug was placed in thimble (Borosil, Mumbai, MH, India), which was inserted into a Soxhlet apparatus and extracted with 600 mL methanol. The extraction was performed for 24 h. After extraction, methanol was removed from extract at 40 °C using rotary vacuum evaporator, and analyzed for wedelolactone content by HPLC. The SAE of *W. calendulacea* was performed in triplicates.

Experimental design and data analysis

The Taguchi experimental design approach has been used for optimization of extraction variables. It is a robust methodology against uncontrollable environmental changes (also known as noise factors), as is the case for raw material variability. A four-factor, three-level orthogonal array design (OAD), L_9 $(3)^4$ was employed as a chemometric method for investigating the effects of the following factors on the extraction efficiency of wedelolactone from *W. calendulacea*: extraction pressure (A), temperature (B), modifier concentration (C), and extraction time (D). From all the different orthogonal arrays available, an L_9 array fitted perfectly. Nine experiments were performed in order to estimate the best conditions for the extraction of wedelolactone. Factors and levels tested are reported in Table 1. All the experiments were carried out in triplicate, leading to a total of 9 experiments for the experimental design. The analysis of variance tables was generated, and the *p*-values of less than 0.05 were considered to be statistically significant. Design-Expert software (version 8.0.6.1, Stat-Ease, Inc., Minneapolis, USA) was used for the ANOVA analysis of the obtained experimental data.

Supercritical carbon dioxide extraction (SC CO_2E) of wedelolactone from W. calendulacea

The extractor column was densely packed with 5 g of *W. calendulacea* powder. The column was carefully fixed in a column oven. The CO_2 from the cylinder was passed through chiller unit (~277 K) via a siphon tube, delivered and compressed to the desired working pressure by CO_2 delivery pump (PU 2080-CO_2 Plus, Jasco International Co. Ltd., Hachioji, Tokyo, Japan) mounted with a pressure regulator (BP-2080 Plus, Jasco International Co. Ltd., Hachioji, Tokyo, Japan), respectively. Methanol was introduced into system as an organic modifier using a solvent pump (PU 2080 Plus, Jasco International Co. Ltd., Hachioji, Tokyo, Japan). The temperature and pressure of CO_2 was manipulated with a pressure regulator. The SC CO_2 was passed through an extraction column (150 mm length × 15 mm i.d.) which was placed in a thermostatically controlled oven (CO-2060 Plus, Jasco International Co. Ltd., Hachioji, Tokyo, Japan). After the pressure and the fluid flow rate reached the desired values, the six-port valve was opened so as to pass SC CO_2 through the extractor; this was counted as the start of the extraction cycle. In the first operating mode, SC CO_2 was introduced into the extractor for 10 min static conditioning so as to achieve sufficient contact with *W. calendulacea* powder. The second operating mode consisted of a steady flow of SC CO_2 under the dynamic extraction. The exit fluid from the extractor was expanded to ambient pressure by a pressure regulator. The extract was collected in a glass vial and analyzed for wedelolactone content by HPLC.

Table 1 Variables and experimental design levels of the OAD.

Independent variables	Coded symbols	Levels		
		1	2	3
Extraction pressure (MPa)	*A*	25	30	35
Extraction temperature (°C)	*B*	40	60	80
Modifier concentration (%)	*C*	5	10	15
Extraction time (min)	*D*	30	60	90

Statistical analysis

Each experiment was performed in triplicates and the data were subjected to calculations of mean ± S.E. The mean values were used for drawing the graphs.

Results and discussion

HPLC analysis and validation

Wedelolactone content was determined by referring to the calibration curve established by running wedelolactone standard at varying concentrations through the HPLC system under the same conditions. The calibration curve of wedelolactone was linear over the concentration range of 2.5–25 μg mL^{-1} ($y = 262.18x - 27.164$; r^2, 0.999) (Fig. 1). The recovery of wedelolactone was 98.12 ± 1.97% as calculated by addition of known amounts of wedelolactone to the *W. calendulacea* extract. The intra-day accuracy in terms of Diff% was in the range of −3.11 to +2.04 whereas inter-day accuracy was in the range of −4.17 to +3.31. Intra-day precision (% RSD) was in the range of 2.11–3.24 whereas inter-day precision was in the range of 2.92–4.51. Limit of quantification for wedelolactone was 2.5 μg mL^{-1}. The slight, intentional change in mobile phase composition, pH of aqueous buffer and column oven temperature did not affect the final results viz. peak area and retention time.

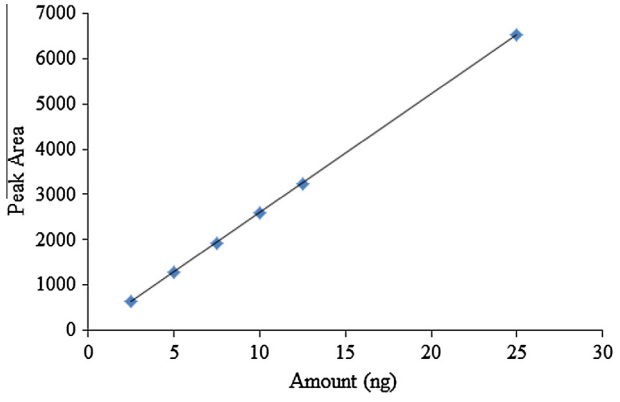

Fig. 1 Representative calibration curve for wedelolactone.

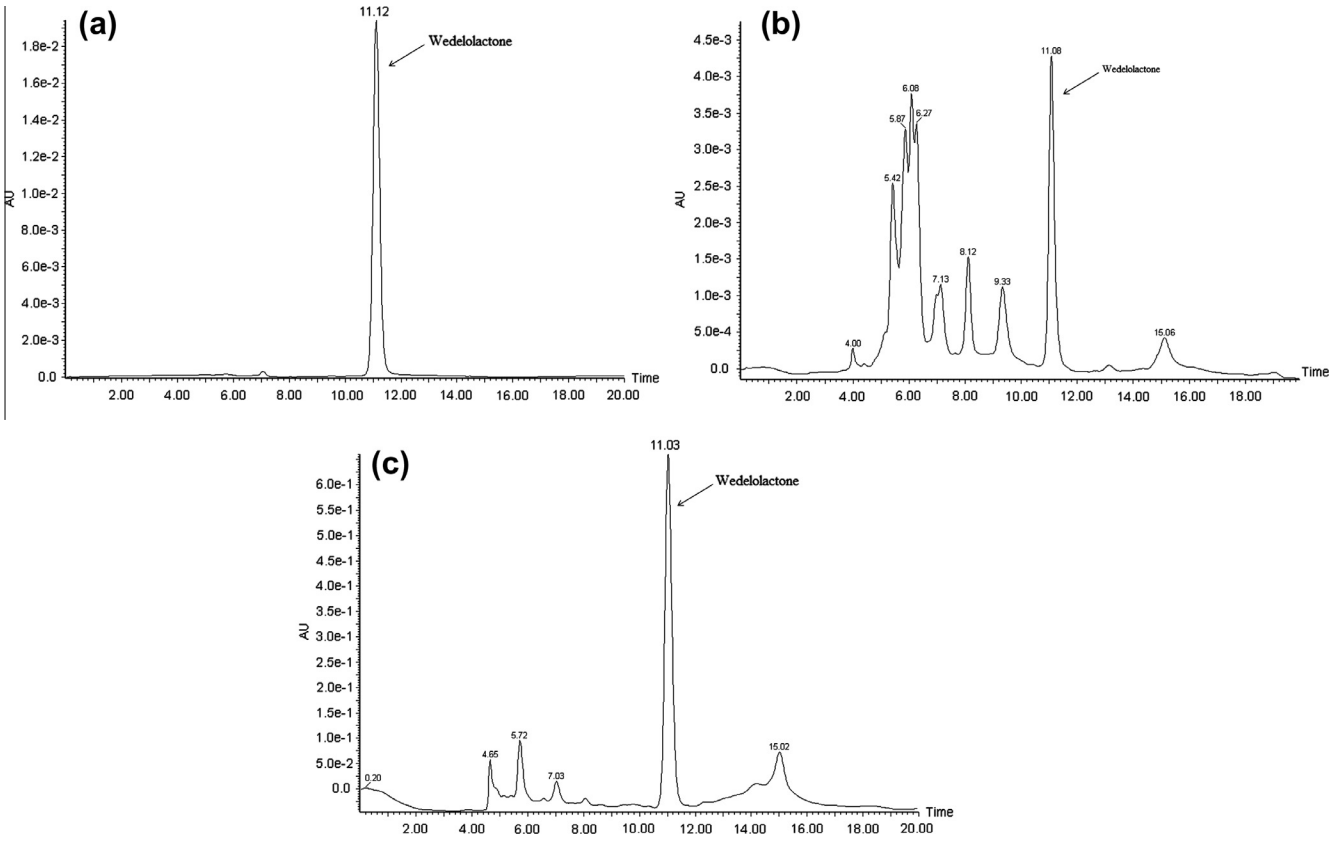

Fig. 2 HPLC Chromatograms showing (a) standard wedelolactone, (b) extracts obtained by SAE and (c) extracts obtained by SC CO_2E at optimize conditions.

Typical HPLC chromatograms of the standard wedelolactone and sample extracts obtained by SAE and SC CO_2E are shown in Fig. 2a–c.

Soxhlet assisted extraction (SAE)

The conventional SAE of *W. calendulacea* was carried out to recover the maximum extractable amount of wedelolactone. After SAE, 7.08 ± 0.29 mg wedelolactone/100 g *W. calendulacea* was obtained.

Analysis of experimental design

The first step in the SC CO_2E is to optimize the operating conditions to obtain an efficient extraction of the target compounds and avoid the coextraction of undesired compounds. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in developing an SC CO_2E method. Based on the previous knowledge of SC CO_2E, the four different process variables *viz*. extraction pressure (A), extraction temperature (B), modifier concentration (C) and dynamic extraction time (D) are considered as the most important factors of SC CO_2E [17]. These factors were investigated at first during this study using a three-level OAD. Focus was on the main effects of the factors and not the interactions among different variables in the matrix. The extract obtained from each test was quantitatively analyzed by HPLC for wedelolactone content and extraction yield was calculated. The experimental results are listed in Table 2.

Table 2 Experimental results of the orthogonal test.

Run no.	*A*	*B*	*C*	*D*	Yield[a] (mg/100 g)
1	25	40	5	30	5.2 ± 0.22
2	30	40	10	90	7.3 ± 0.34
3	30	80	5	60	2.4 ± 0.12
4	30	60	15	30	3 ± 0.11
5	25	60	10	60	5.9 ± 0.22
6	25	80	15	90	4.9 ± 0.2
7	35	60	5	90	4.5 ± 0.16
8	35	40	15	60	3.9 ± 0.19
9	35	80	10	30	0.3 ± 0.02

[a] Yield values are averages of three determinations.

The maximum extraction yield of the wedelolactone was 7.3 ± 0.34 mg/100 g (Table 2). It was noticed that each process variable imparted different influence upon the yield of wedelolactone. Therefore, if the analysis is only made based on the statistics listed in Table 2, it was difficult to select the best extraction conditions. So further analysis was subsequently performed and listed in Table 3. From Table 3, it could be inferred that the Factor B is the most significant factor according to the *R* values, while the Factor C is the insignificant one compared with the others. Fig. 3 was also helpful to obtain the optimized SC CO_2E conditions. It shows the relationship between the extraction yield and the four process variables, *viz*. extraction pressure (25–35 MPa), extraction temperature

Table 3 Analysis of L_9 $(3)^4$ test results.

	Variables			
	A	*B*	*C*	*D*
M_1	16 ± 0.64	16.4 ± 0.75	12.1 ± 0.5	8.5 ± 0.35
M_2	12.7 ± 0.57	13.4 ± 0.49	13.5 ± 0.58	12.2 ± 0.53
M_3	8.7 ± 0.37	7.6 ± 0.34	11.8 ± 0.5	16.7 ± 0.7
m_1	5.33 ± 0.21	5.47 ± 0.25	4.03 ± 0.17	2.83 ± 0.12
m_2	4.23 ± 0.19	4.47 ± 0.16	4.5 ± 0.19	4.07 ± 0.18
m_3	2.9 ± 0.12	2.53 ± 0.11	3.93 ± 0.17	5.57 ± 0.23
R	2.43 ± 0.09	2.93 ± 0.14	0.47 ± 0.03	2.73 ± 0.12
Optimal level	A_1	B_1	C_2	D_3

M: Sum of yield for the factors at each level.
m: The mean values of yield for the factors at each level.
$R = m$, max $- m$, min.

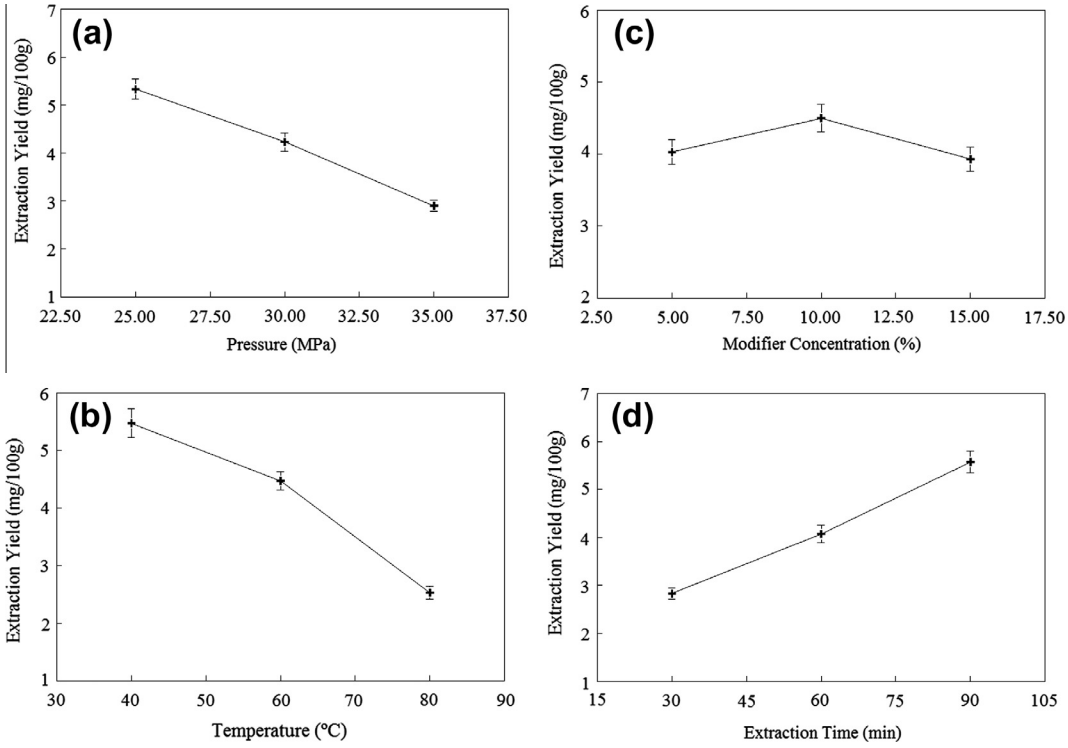

Fig. 3 Effects of (a) pressure, (b) temperature, (c) modifier concentration, and (d) extraction time on SC CO_2E yield of wedelolactone from *W. calendulacea*.

(40–80 °C), modifier concentration (5–15%), and dynamic extraction time (30–90 min).

The significance of each coefficient was determined using *p*-value. When a process variable has a *p*-value smaller than 0.05, it influences the process in a significant way for a confidence level of 95% [18]. In general, the effects lower than 0.05 are significant. Table 4 shows the analysis of variance (ANOVA) of the experimental results, wherein pressure, temperature and dynamic extraction time contributes as a significant factor for yield of wedelolactone with $p < 0.05$, while modifier concentration have no significant effect on the yield of wedelolactone with $p > 0.05$.

Effect of SC CO_2E condition on wedelolactone yield

Effect of extraction pressure

It is usually considered that the yield of target compounds with SC CO_2E is influenced by the extraction pressure, temperature, modifier concentration and time. The fluid density can be increased by elevating pressure. In addition, the solubility of solid compounds in supercritical fluid could be influenced by the repulsive solute–fluid interaction [19]. It is well known fact that the solubility of supercritical CO_2 is affected by density and vapor pressure. When the solubility of the solutes in supercritical CO_2 is controlled predominantly by density rather than by

Table 4 Analysis of variance (ANOVA) for SC CO_2E of wedelolactone from *W. calendulacea* using L_9 $(3)^4$ design.

	Sum of squares	DF[a]	Mean square	*F*-value	*p*-Value	Significant
A	8.882	1	8.882	34.42	0.0042	**
B	12.9027	1	12.9027	50.02	0.0021	**
C	0.015	1	0.015	0.06	0.8213	NS
D	11.2027	1	11.2027	43.43	0.0027	**
Pure error	1.0322	4	0.2582			
Cor total	34.0422	8				

[a] Degrees of freedom.
** Significant at $p < 0.01$.
NS: Not significant.

vapor pressure, the solubility of the solutes increases in response to increase in supercritical CO_2 density at higher pressures under constant temperature; however, the dissolving power decreases as the temperature increases at constant pressure due to decreased density of supercritical CO_2 [20]. As the pressure continues to increase, however, the repulsive solute–fluid interaction becomes more and more. When pressure reaches a certain value for some compounds, the repulsive solute–fluid interaction may become greater than the increase in the solubility obtained from the increased solvent density. In this situation, the solubility of the compounds decreases. A lower solubility leads to a decrease in extraction yield. The solubility of solute in supercritical fluid depends on a complex balance among fluid density, solute vapor pressure and the repulsive solute–fluid interaction, which are controlled by temperature and pressure.

In this study, the influence of pressure on the extraction efficiency of wedelolactone was studied under different conditions by changing the pressure from 25 to 35 MPa. As shown in Fig. 3a, the extraction efficiency of the wedelolactone was decreased markedly when pressure was increased from 25 to 35 MPa. Unfavorable effect on extraction efficiency was may be due to increase in the repulsive solute–fluid interactions at high extraction pressure. Taking all of the results into consideration, within the ranges of the parameters studied, 25 MPa was selected as the optimal extraction.

Effect of extraction temperature

While considering the effect of temperature on solubility of solid compounds, two different effects can appear. One is the increase in solid volatility with temperature rise, causing an increase in vapor pressure, and another is the decrease in solvent density with temperature rise. The improvement of solubility by temperature is dependent on which effect is more important [21]. The effect of the extraction temperature is demonstrated in Fig. 3b. In this work, effect of temperature on extraction yield at three different values (40, 60, and 80 °C) was evaluated to optimize the extraction process. As shown in Fig. 3b, the wedelolactone yield was decreased when extraction temperature was increased from 40 to 80 °C. The highest extraction yield was obtained when temperature was at 40 °C. This effect of temperature may be resulted from decrease in solvent density leads to decrease in solubility of solutes in the supercritical fluid.

Effect of modifier concentration

Although CO_2 is the most common medium in supercritical fluid extraction, in certain instances supercritical CO_2 cannot quantitatively extract target analytes under conventional SC CO_2E conditions because of its weak solvating power. Modifier is added to an extraction process mainly for two reasons: (i) to increase the polarity of the SC CO_2 in order to improve the solubility of the analytes; and (ii) to facilitate desorption of analytes from the plant matrix. The polar modifier molecules accelerate desorption processes by competing with the analytes for the active binding sites; as well as by disrupting matrix structures. Various polar co-solvents have been tried over the years for the supercritical CO_2 extraction of polar constituents, but methanol remains the most popular [22]. The wedelolactone yield increased with increasing methanol concentration from 5% to 10%, but when the concentration increased from 10% to 15%, the extraction efficiency decreased (Fig. 3c). Besides, the methanol concentration is found to be insignificant to influence the extraction yield rather than the other three factors according to the *R* values in Table 3. Therefore, 10% could be selected as the optimal methanol concentration.

Effect of dynamic extraction time

Time is one of the main factors for exhausted extraction and is an important index for evaluation of extraction efficiency. Shorter extraction time could cause incomplete extraction and longer extraction time could be time and solvent wasting. In order to obtain high yield of wedelolactone, an important extraction step of static extraction (10 min) was performed. This step could make a better penetration of the fluid into the matrix compared with the only dynamic extraction mode. This step was followed by a dynamic extraction to enhance solubility of wedelolactone in the supercritical fluid. To evaluate the effect of dynamic extraction time on SC CO_2E of wedelolactone, extraction was performed for 30, 60, and 90 min separately. Fig. 3d shows that the extraction yield of wedelolactone increases significantly in the extension of extraction time.

Optimized SC CO_2E conditions and validation of the model

As shown in Table 3, the combination of factors found after the calculation as optimal (A1–B1–C2–D3) had not been tested previously. As a consequence it was necessary to perform a confirmatory experiment to probe the reliability of the results obtained, for extraction of wedelolactone. The extraction yield obtained at optimal conditions was 8.01 ± 0.34 mg/100 g *W. calendulacea* Less., slightly higher than the maximum observed in trial number 2 of the experimental design, proving the reliability of the statistical analysis.

Comparison of SAE and SC CO_2E on the basis of yield and extraction time

The conventional SAE of *W. calendulacea* powder resulted in 7.08 ± 0.29 mg/100 g wedelolactone yield after an extraction period of 24 h. Optimized SC CO_2E showed 8.01 ± 0.34 mg/100 g wedelolactone recovery after an extraction period of 90 min. The comparison of wedelolactone yield and its required extraction time demonstrated that SC CO_2E technique is more efficient than SAE technique. This could be attributed to action of SC CO_2, which produces cell disruption leading to a greater contact area between solid and liquid phase and better access of solvent to valuable components.

Conclusions

In this study, the effects of pressure, temperature, modifier concentration and extraction time were evaluated in order to develop an optimized SC CO_2E method. Taguchi L_9 orthogonal array design was successfully applied for optimization of total wedelolactone yield. The extent of the impact of variables on extraction yield followed the order: variable B (extraction temperature) > D (extraction time) > A (extraction pressure) > C (modifier concentration). We also concluded that extraction temperature and time were the two major factors affecting extraction yield. An efficient HPLC method was developed for determination of wedelolactone from the product of SAE and SC CO_2E with good sensitivity, precision, and repeatability. It can be used as an improved quality control analysis method for wedelolactone in near future.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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