Ultrasonic-Assisted Extraction Process and Method Validation for Deoxypodophyllotoxin from the Roots of *Anthriscus sylvestris*: Application of Response Surface Methodology and UPLC–PDA–QDa

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Deoxypodophyllotoxin (DPT), or anthricin, is a lignan isolated from the roots of *Anthriscus sylvestris* and is reported to exhibit anti-inflammatory, anti-oxidant, and anti-asthmatic effects. Herein, the conditions for the extraction of DPT from *A. sylvestris* are optimized using a Box–Behnken design (BBD) method based on response surface methodology (RSM). DPT was detected by ultra-performance liquid chromatography coupled with photodiode array and quadrupole detector (UPLC–PDA–QDa) and analytical validation methods based on International Conference on Harmonization (ICH) guidelines. In preliminary experiments, the experimental conditions of extraction time, solvent percentage, and temperature were selected for optimization. The adequacy of the experimental model was statistically evaluated, and the regression coefficient (R^2), adjusted regression coefficient (R^2_{adjust}), and p -value of the lack-of-fit were determined as 97.86%, 94.02%, and 0.124, respectively. The maximum yield of DPT was estimated to be 2.341 mg/g for 30 min in 100% methanol at 60 °C, and the actual yield was measured as 2.295 mg/g (± 0.023) under the same conditions.

Keywords: *Anthriscus sylvestris*, deoxypodophyllotoxin, optimization, response surface methodology, UPLC–PDA–QDa

Introduction

Anthriscus sylvestris (L.) Hoffm., commonly known as wild chervil or cow parsley, belongs to the Apiaceae family and is traditionally used as an antitussive, antipyretic, analgesic, diuretic, and cough remedy in many countries [1]. The roots of *A. sylvestris* contain monoterpenes [2], lignans [3], coumarins, phenylpropene, and polyacetylene derivatives [1]. The most effective compound found in *A. sylvestris* roots is the lignan deoxypodophyllotoxin (DPT), which is reported to elicit anti-lung inflammation [4], anti-tumor [1], anti-proliferative, and anti-viral activities [5].

The distinct advantages of DPT as a therapeutic agent, as well as other active ingredients in *A. sylvestris*, warrant its extraction in high yields so that such compounds can be effectively used in the food industry. For this purpose, various extraction factors and methods have been studied such as Soxhlet extraction, solvent extraction, microwave-assisted extraction, supercritical fluid extraction, and ultrasonic-assisted extraction [6]. Among them, ultrasonic-assisted extraction has been used in the food industry and to extract phytochemical ingredients [7, 8] because, relative to other methods, it is eco-friendly, cost-effective, and requires relatively less time, energy, and solvent; it also has a low physical risk and enhanced extraction quality [8].

Some of the extraction factors that must be considered include time, temperature, pH, particle size, pressure, solvent type, and concentration [9]. The traditional approach of “one-variable-at-a-time” is well accepted, but it is time-consuming and requires significant effort [9]. Moreover, this approach does not consider the effect of interactions among different factors [10]. To overcome these shortcomings, Box and Wilson suggested the use of response surface methodology (RSM) [11]. RSM is an effective and efficient mathematical statistics method to construct models, evaluate the effects of multiple variables, and determine the optimal conditions to obtain desirable

responses; collectively, this approach helps to overcome the limitations of conventional methods [12]. The main advantage of RSM is the reduced number of experimental trials required to evaluate multiple parameters and their interactions [13].

In this study, components from the roots of *A. sylvestris* are extracted using RSM with a Box–Behnken design (BBD). The experimental factors selected for investigation were extraction time, temperature, and percentage of methanol in distilled water, with preliminary tests based on the one-variable-at-a-time approach. Notably, the active component DPT was detected by ultra-performance liquid chromatography (UPLC) within 5.0 min and validated according to the International Conference on Harmonization (ICH) guidelines.

Experimental

Plant Materials and Chemicals. Dried roots of *A. sylvestris* were purchased from an oriental pharmacy in Jeollanamdo, South Korea. Their morphology and genetic identification were determined by Drs. Goya Choi and Byeong Cheol Moon, respectively, at the Korea Institute of Oriental Medicine (KIOM). A voucher specimen was deposited in the Korean Herbarium of Standard Herbal Resources at KIOM (Index herbariorum code KIOM, Specimen No. KIOM 2-15-0480). A fine powder of the material was obtained after grinding and passing it through a 600 μm sieve.

DPT (>95.0%) was obtained from the National Development Institute of Korean Medicine (Gyeongsangbuk-do, South Korea), and podophyllotoxin (PD; $\geq 98\%$) and yatein ($\geq 98\%$) were obtained from ChemFace (Hubei, China). The structures of these chemicals are represented in Figure 1. High-performance liquid chromatography (HPLC) grade solvents were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA).

Genetic Authentication of Plant Materials. The genomic DNA of authentic control plants and samples were extracted

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Figure 1. Chemical structure of deoxypodophyllotoxin (A), podophyllotoxin (B), and yatein (C)

using the DNeasy Plant Mini Kit (GIAGEN, USA) following the manufacturer's protocol. To verify the plant species, multiplex polymerase chain reaction (PCR) was carried out using six combinations of species-specific Sequence Characterized Amplified Region (SCAR) primers and the total genomic DNA, as described in a previous report [14]. After gel electrophoresis of the PCR products, we compared the size and specificity of the resultant DNA bands with those of control DNA extracted from authentic plant materials (Figure 2) [14].

Extraction Procedure. An aliquot of the powdered sample (0.2 g) to be extracted was placed in 10 mL of the appropriate solvent ($n = 5$), capped in a vial, and subjected to an ultrasonic bath (44 kHz, 8510 Ultrasonic; Branson Co., Danbury, CT, USA). The extract was filtered through a 0.2 μm membrane syringe filter before injection into the UPLC system.

UPLC Analysis. Identification of the chemical patterns and the analysis of the optimal experimental conditions was performed using a UPLC–photodiode array (PDA)–quadrupole detector (QDa) instrument. The UPLC system (Acquity™ UPLC system; Waters Corporation, Milford, MA, USA) comprised a binary solvent pump, sample manager, column oven, online degasser, PDA detector, and QDa detector. The UPLC data were processed with Empower™ 3 software (Waters Corporation). The analytical column

comprised an Acquity UPLC® ethylene-bridged hybrid (BEH) C_{18} column (2.1×150 mm, 1.7 μm ; Waters Corporation). The mobile phase was 0.05% formic acid containing distilled water (A) and methanol (B). The linear gradient conditions of the mobile phase for pattern analysis began with 40% methanol and changed to 100% methanol over 30 min. The optimization conditions included an isocratic mobile phase comprising 62% methanol for 5 min at a flow rate of 0.2 mL/min and an injection volume of 2 μL . The wavelengths were monitored at 200–400 nm, and the sample peak was detected at 290 nm. The sample peaks were confirmed with a standard peak in terms of retention time and consistency with ultraviolet (UV) wavelength and MS spectrum analyses. Nitrogen was used as the carrier gas for the QDa detector under the following conditions: mass condition, m/z range of 350–450; capillary voltage, 0.8 V; probe temperature, 600 °C; sampling frequency, 8 Hz; cone voltage, 15 V; source temperature, 120 °C; and turbo temperature, 45 °C. The selective ion recording (SIR) of DPT was monitored at m/z 421 in positive mode.

Method Validation. The DPT standard compound was dissolved in methanol. The linearity of DPT (6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$) was established via a correlation coefficient-derived calibration curve. The relative standard deviation (RSD) was calculated as $\text{SD}/\text{mean} \times 100$. The limit of detection (LOD) and limit of quantitation (LOQ) of DPT were computed as $3.3\sigma/s$ and $10\sigma/s$, respectively, where σ is the SD of the response and s is the slope of the calibration curve. Both intra-day ($n = 6$) and inter-day precision and accuracy of DPT were determined over three consecutive days. The recovery tests were conducted at high, medium, and low spiking concentrations using the relation $\{(\text{detected concentration} - \text{initial concentration})/\text{spiked concentration}\} \times 100$.

Preliminary Conditions. To select the optimal experimental conditions, the sample was extracted using the one-variable-at-a-time method by considering the following factors: solvent type, extraction time, temperature, and solvent percentage. Water, ethanol, and methanol were selected as the solvents because they have low toxicity and are eco-friendly. The extraction time and temperature were set to 10–70 min and $30\text{--}60 \pm 2$ °C, respectively. Finally, 40–100% (v/v) methanol containing distilled water was chosen as the solvent. Statistical analyses (i.e., t -test, one-way ANOVA, and Kruskal-Wallis test [15]) were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and Minitab 16.0 (Minitab Inc., State College, PA, USA).

RSM Design. The most widely used RSM designs comprise a central composite design (CCD) and a BBD. These designs

Figure 2. Authentication of plant material based on the multiplex-SCAR marker. Line 1–2, line 3–4, line 5–6, and line 7 indicate the PCR product form authentic *A. decursiva*, *P. praeruptorum*, *A. sylvestris*, and root of *A. sylvestris* used in this study, respectively. The size of precise PCR products and DNA ladders is represented by the arrowheads at the right and left side of gel image, respectively. M represents 100 bp DNA ladder

Figure 3. Chemical profiling for root of *A. sylvestris*; standard components of chromatogram (A), sample chromatogram (B), mass spectra of podophyllotoxin (C), deoxypodophyllotoxin (D), and yatein (E)

were used to identify the relationship between the independent factors and the dependent variables [16]. BBD has been widely used in the optimization of bioactive compounds [17] and to determine the best combination of extraction variables for determining DPT yields [13]. The Minitab BBD includes variables with codes -1 , 0 , and 1 . The three variables considered in this study were extraction time (X_1 ; 30, 45, and 60 min), methanol percentage (X_2 ; 50%, 75%, and 100%), and extraction temperature (X_3 ; 40, 55, and 70 °C), and the range of these factors was determined by a preliminary experiment. This

experimental design consisted of a total of 15 variable configuration points, with each point repeated in 5 times. The data were fitted to a quadratic polynomial model and the regression coefficients were obtained [13]. The polynomial equation was as follows:

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

Figure 4. Chromatogram of DPT standard (A), methanol extraction of *A. sylvestris* (B) at 290 nm, mass spectrum (C), and UV spectrum of DPT (D)

Table 1. Intra-day and inter-day precision for DPT

	Concentration ($\mu\text{g/mL}$)	Intra-day ($n = 6$)		Inter-day ($n = 6$)	
		Found ($\mu\text{g/mL}$)	RSD (%)	Found ($\mu\text{g/mL}$)	RSD (%)
DPT	50	50.7	0.45	51.4	1.72
	25	25.6	0.74	25.9	1.19
	12.5	12.3	1.17	12.4	0.92

where Y is the dependent or response variable; ε is the residual term; β_0 , β_b , β_{ii} , and β_{ij} are the intercept, linear, quadratic, and interaction coefficients, respectively; and X_i and X_j are the independent variables [15].

Results and Discussion

UPLC Analysis and Method Validation. The chromatograms of *A. sylvestris* containing DPT, PD, and yatein are shown in Figure 3. As seen, PD, DPT, and yatein were detected at approximately 10.1, 12.4, and 23.8 min, respectively, and their corresponding values of selective ion recording (SIR) in positive mode were 437.2 m/z $[\text{M}+\text{Na}]^+$, 421.2 m/z $[\text{M}+\text{Na}]^+$, and 400.5 m/z $[\text{M}]^+$, respectively. The contents of PD, DPT, and yatein were 0.011 ($\pm 2.51 \times 10^5$), 2.235 (± 0.007), and 0.014 ($\pm 2.86 \times 10^5$) mg/g, respectively. The optimization procedure of the method was performed for DPT only because of the trace contents of PD and yatein (Figure 3).

Chromatograms of DPT in *A. sylvestris* are represented in Figure 4. As seen, DPT was detected at 2.951 (± 0.002) min with a wavelength of 292.7 nm. The total ion chromatogram (TIC) in positive mode at 350–450 m/z and selective ion recording (SIR)

mode identified $[\text{M}+\text{Na}]^+$ at 421 m/z . The standard curve indicated good linearity, and the standard range, linear equation, and correlation coefficient were 6.25–100 $\mu\text{g}/\text{mg}$, $Y = 6414.3784X + 11343.9028$, and $R^2 = 0.9997$, respectively. The LOD and LOQ of DPT were 0.0479 and 0.1451 $\mu\text{g}/\text{mL}$, respectively.

The values of the inter-day (0.92–1.72%) and intra-day (0.45–1.17%) precision of DPT were acceptable, with $\text{RSD} \leq 2.0\%$ (Table 1). Inter- and intra-day recovery tests had values of 101.202–105.040% and 101.693–105.729%, respectively, and all precision and recovery RSD values were less than 2.0% (Table 2).

Preliminary Extraction Conditions. *A. sylvestris* was extracted in distilled water, methanol, and ethanol. Distilled water was chosen because of its widespread availability and non-toxicity compared with methanol and ethanol [18, 19]. DPT did not dissolve in distilled water. The yields of DPT in methanol and ethanol were 2.366 ± 0.078 and 2.105 ± 0.025 mg/g, respectively (t -test, $p = 0.031$). Given the higher yield of DPT in methanol, this solvent was selected as the optimal solvent (Figure 5A). Although DPT was not detected in either 0% or 20% methanol, its yields in 40%, 60%, 80%, and 100% methanol were detected as 2.100 ± 0.102 , 2.386 ± 0.039 , 2.404 ± 0.017 , and 2.407 ± 0.038 mg/g, respectively (Kruskal-Wallis). Based on these results, a methanol range of 50–100% was selected for the subsequent studies (Figure 5B).

The DPT contents after extraction for 10, 20, 30, 40, 50, 60, and 70 min were 2.346 ± 0.086 , 2.366 ± 0.047 , 2.206 ± 0.043 , 2.354 ± 0.020 , 2.513 ± 0.056 , 2.520 ± 0.018 , and 2.523 ± 0.014 mg/g, respectively (one-way ANOVA, post-hoc by Tukey's test). The range of the extraction time was

Table 2. Intra-day and inter-day recovery test of DPT

	Spike concentration ($\mu\text{g/mL}$)	Intra-day ($n = 6$)				Inter-day ($n = 6$)			
		Initial concentration ($\mu\text{g/mL}$)	Detected concentration ($\mu\text{g/mL}$)	Recovery (%)	RSD (%)	Initial concentration ($\mu\text{g/mL}$)	Detected concentration ($\mu\text{g/mL}$)	Recovery (%)	RSD (%)
DPT	25	10.931	36.363	101.202	0.267	10.946	36.555	101.693	0.459
	12.5	10.931	24.451	104.353	0.439	10.946	24.599	104.915	1.011
	6.25	10.931	18.047	105.040	0.208	10.946	18.182	105.729	0.986

Figure 5. The content of DPT for solvents type, times, temperatures, and solvent percentage (ratio of methanol and distilled water). * $p < 0.05$ by t -test (without water). ^{a,b,c}Post-hoc by Tukey

delimited to 30–60 min because 60 and 70 min were not statistically significant (Figure 5C).

The yields of DPT at extraction temperatures of 30, 40, 50, and 60 °C were 2.348 ± 0.088 , 2.469 ± 0.050 , 2.478 ± 0.092 , and 2.521 ± 0.032 mg/g, respectively (Kruskal-Wallis). Clearly, the DPT content increased as the temperature increased (Figure 5D). The temperature was maintained between 40 and 70 °C. Note that this temperature range includes the boiling point of methanol (64.7 °C).

Fitted Model. The contents of DPT based on the BBD factors are listed in Table 3. The DPT yield ranged from 2.108 ± 0.049 to 2.344 ± 0.031 mg/g. The DPT content under various conditions was calculated by one-way ANOVA, which includes the regression coefficient (R^2) of the second-order polynomial equation (Table 4). The fitness of the quadratic polynomial model was verified by R^2 values [20]. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 , and the significance of the regression coefficient was checked by the corresponding F -test and p -value estimates [13]. As is well known, the value of R^2 always ranges between 0 and 1, and a model is more predictive when its R^2 value is closer to 1 [21]. In this model, the R^2 and R^2_{adjust} values of DPT were 97.86% and 94.02%, respectively. In a valid statistical model, the R^2_{adjust} and R^2 values should be close [20]. This model could be used to show why R^2 should be close to R^2_{adjust} in a good model. The p -value of the lack-of-fit was 0.124, and the F -test suggests that the model had a high F -value and low p -value [13]. Overall, these results ($p > 0.05$) demonstrate that the quadratic model was valid within the spatial influence of the variables on the response [15].

Table 3. Box–Behnken design matrix and experimental results from the response variables ($n = 5$)

No.	X_1 (min)	X_2 (v/v)	X_3 (°C)	DPT (mg/g)
1	30 (−1)	50 (−1)	55 (0)	2.164 (±0.034)
2	60 (1)	50 (−1)	55 (0)	2.211 (±0.028)
3	30 (−1)	100 (1)	55 (0)	2.297 (±0.043)
4	60 (1)	100 (1)	55 (0)	2.344 (±0.031)
5	30 (−1)	75 (0)	40 (−1)	2.230 (±0.033)
6	60 (1)	75 (0)	40 (−1)	2.237 (±0.017)
7	30 (−1)	75 (0)	70 (1)	2.241 (±0.024)
8	60 (1)	75 (0)	70 (1)	2.263 (±0.053)
9	45 (0)	50 (−1)	40 (−1)	2.108 (±0.049)
10	45 (0)	100 (1)	40 (−1)	2.236 (±0.027)
11	45 (0)	50 (−1)	70 (1)	2.148 (±0.047)
12	45 (0)	100 (1)	70 (1)	2.299 (±0.030)
13	45 (0)	75 (0)	55 (0)	2.274 (±0.014)
14	45 (0)	75 (0)	55 (0)	2.287 (±0.048)
15	45 (0)	75 (0)	55 (0)	2.276 (±0.041)

Table 4. Analyses of the regression model with a dependent Y variable for the extraction conditions

Variable	DPT ($R^2 = 97.86\%$, $R^2_{\text{adjust}} = 94.02\%$)			
	DF	Sum of squares	F -value	P -value
Regression	9	0.054411	25.44	0.001
Linear	3	0.041648	58.41	0.000
X_1	1	0.001889	7.95	0.037
X_2	1	0.037324	157.03	0.000
X_3	1	0.002434	10.24	0.024
Square	3	0.012585	17.65	0.004
X_1X_1	1	0.000894	1.44	0.283
X_2X_2	1	0.003717	19.26	0.007
X_3X_3	1	0.007974	33.55	0.002
Interaction	3	0.000179	0.25	0.858
X_1X_2	1	0.000000	0.00	0.995
X_1X_3	1	0.000048	0.20	0.671
X_2X_3	1	0.000131	0.55	0.492
Residual error	5	0.001188		
Lack of fit	3	0.001088	7.22	0.124
Pure error	2	0.000100		
Total	14	0.055600		

The linear and square terms of DPT included large F -values (58.41, 17.65) and small p -values (0.000, 0.004); however, the interaction terms included a small F -value (0.25) and a large p -value (0.858). This result suggests that the yield of DPT was influenced by the linear and square terms more than the interaction term.

RSM. As the main active compound of *A. sylvestris*, the amount of DPT extracted and detected was optimized in this study. In the food and pharmaceutical industries, ultrasonic-assisted extraction and RSM are powerful approaches with many advantages [15]. In this study, BBD was used in conjunction with CCD, which is a common approach. The quadratic model was computed as a second-order polynomial equation, with extraction time (X_1 , min), solvent percentage (X_2 , %), and extraction temperature (X_3 , °C) as variables:

$$Y = -3.692 \times 10^{-3}X_1 + 1.034 \times 10^{-2}X_2 + 0.220 \times 10^{-1}X_3 + 4.286 \times 10^{-5}X_1^2 - 5.633 \times 10^{-5}X_2^2 - 2.065 \times 10^{-4}X_3^2 + 1.360 \times 10^{-7}X_1X_2 + 1.543 \times 10^{-5}X_1X_3 + 2.524 \times 10^{-5}X_2X_3 + 1.211 \quad (2)$$

The regression coefficients for DPT are tabulated in Table 5. All linear terms (time, percentage, and temperature) and two square terms (percentage $\times$ percentage and temperature $\times$ temperature) were significant for DPT, with p -values of 0.037, 0.000, 0.024, 0.007, and 0.002, respectively. However, not all of the interaction terms were significant. The p -value was used as a measure of the significance of each coefficient and indicates correlated interactions between variables [22]. Small p -values indicate that the DPT extraction yield is influenced by the associated variables [7]. In this work, time, percentage, temperature, percentage $\times$ percentage, and temperature $\times$ temperature were found to affect the DPT extraction efficiency.

The quadratic model of BBD can be shown as 3D surface plots and 2D contour images for interactions among the three variables (i.e., X_1 , extraction time; X_2 , methanol percentage; and X_3 , extraction temperature). The response curves were plotted to understand the interactions among the variables and to determine the optimum level of each variable for maximum response [23]. Figure 6 shows the 2D contour and 3D surface plots of DPT for the response variables. The three surface plots exhibited the highest yield at 55–65 °C, 55–60 min, and 80–100% methanol. These data indicate that the DPT content increased as the methanol content increased. Additionally, the DPT content tends to decrease after 55–65 °C. This may occur because increasing the extraction temperature accelerates the chemical decomposition of DPT owing to the durative ultrasonic effect, which causes lower extraction yields [6, 24]. Figure 7 shows the optimal conditions for DPT; at 60 min, 59.4 °C, and 100% methanol, the DPT content was

Table 5. Regression coefficients result from the data of BBD experiments

Variable	DPT			
	Coefficient	Standard error coefficient	T -value	P -value
Constant	2.27940	0.008901	256.084	0.000
X_1	0.01537	0.005451	2.819	0.037
X_2	0.06830	0.005451	12.531	0.000
X_3	0.01744	0.005451	3.200	0.024
X_1X_1	0.00964	0.008023	1.202	0.283
X_2X_2	−0.03521	0.008023	−4.388	0.007
X_3X_3	−0.04647	0.008023	−5.792	0.002
X_1X_2	0.00005	0.007708	0.007	0.995
X_1X_3	0.00347	0.007708	0.450	0.671
X_2X_3	0.00571	0.007708	0.741	0.492

Figure 6. 2D-contour plots (A–C) and 3D surface plots (D–F) of DPT; extraction time (X_1), solvent percentage (X_2), and extraction temperature (X_3)

determined to be 2.341 mg/g. Under the same conditions, the actual content of DPT was detected as 2.295 (± 0.023) mg/g.

Conclusions

This work shows that UPLC–PDA–QDa is a very effective method for detecting DPT. The optimum extraction conditions (i.e., extraction time, solvent percentage, and extraction temperature) for ultrasonic-assisted extraction with response surface methodology using a Box–Behnken design were determined. The ranges of these factors were selected during preliminary tests. As DPT is the main active compound in *A. sylvestris* roots, its optimal extraction conditions were determined. Under these optimal conditions (of 60 min, 60 °C, and 100%

methanol), the DPT yield was calculated as 2.341 mg/g. Under the same conditions, the actual yield of DPT was determined as 2.295 (± 0.023) mg/g. Thus, the predicted yield was close to the actual yield under the same optimized extraction conditions.

Conflict of Interest

The authors declare no conflicts of interest.

Compliance with Ethical Standards

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

Figure 7. Optimal conditions of DPT

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