Research Article

Interprof. J. Health Sci. 2023, 21(1-2): IJHS-0239

Impact of Solvent-to-Solid Ratio and Infusion Duration on Extraction Yield and Nitrate Content of *Cyanthillium cinereum* (L.) H.Rob. and *Clausena anisata* (Willd.) Hook.f. ex Benth.: An Optimization Approach

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Received 6 October 2023; Revised 31 October 2023; Accepted 5 November 2023

Abstract: *Cyanthillium cinereum* (L.) H.Rob. and *Clausena anisata* (Willd.) Hook.f. ex Benth. are herbal plants used as smoking cessation aids. These are nitrate-rich plants that can induce tongue numbness and alter cigarette flavor. This study aimed to optimize the infusion conditions of the whole plant of *C. cinereum* and the leaves of *C. anisata*. The circumscribed central composite design was applied to investigate the two infusion factors, including solvent-to-solid ratio and infusion duration. The extracts were analyzed for nitrate content using reversed-phase ion-interaction high-performance liquid chromatography. Results showed that both the extraction yield and nitrate content of *C. anisata* were higher than those of *C. cinereum*. The simultaneous achievement of high extraction yield and nitrate content was observed when a high solvent-to-solid ratio and a long infusion duration were used. Analysis of variance revealed that the solvent-to-solid ratio was the only factor significantly affecting extraction yield and nitrate content. The optimal condition for achieving the highest extraction yield and nitrate content simultaneously was found to be a solvent-to-solid ratio of 27:1 and an infusion duration of 13.5 min. For *C. cinereum*, the obtained extraction yield was 4.67%, with a nitrate content of 0.16%. For *C. anisata*, the extraction yield was 20.86%, with a nitrate content of 0.83%. The computer program's predictions were reliable and precise, as indicated by the low percentage error. In conclusion, this study successfully clarified the impact of solvent-to-solid ratio and infusion duration on the extraction yield and nitrate content of *C. cinereum* and *C. anisata* using an optimization approach.

Keywords: Central composite design, Design of Experiments, Smoking cessation, *Cyanthillium cinereum*, *Clausena anisata*, Herbal tea

INTRODUCTION

Smoking poses a significant global health challenge, causing more than eight million deaths annually. The World Health Organization reports that eight million of these deaths result from direct tobacco use, while 1.3 million are attributed to second-hand smoke exposure among nonsmokers (1). Smoking is linked to various health issues, including cancer, chronic obstructive pulmonary disease, lung and heart diseases, stroke, and diabetes. Additionally, it heightens the risk of tuberculosis, eye diseases, and compromises the immune system (2). There are two approaches to smoking cessation: non-pharmacologic and pharmacologic treatments. Pharmacologic treatments encompass various products such as nicotine-replacement therapy, bupropion, varenicline, nortriptyline, and clonidine (3). Traditional and folk medicines also incorporate herbal remedies for smoking cessation. One such plant is *Cyanthillium cinereum* (L.) H.Rob., officially recognized in the Thai National List of Essential Medicines for smoking cessation. Extracts from this plant are utilized in smoking cessation aids (4-7)

Wongwiwatthananukit et al. investigated the effectiveness of *C. cinereum* tea in 64 subjects over 24 weeks. Primary outcomes, including continuous abstinence rate (CAR) and 7-day point prevalence abstinence rate (PAR), were evaluated. While CAR and PAR were higher than placebo, the differences were not statistically significant. The study suggested that the daily cost of *C. cinereum* was lower than other pharmacologic options (8). Furthermore, *C. cinereum* pastilles demonstrated efficacy and safety, with CAR at week 12 being more effective than placebo for low to moderate nicotine dependence smokers (9). Another study by Srisoi et al. reported significant differences in CAR and PAR compared to the placebo group (10). A systematic review and meta-analysis found that *C. cinereum* showed better smoking cessation benefits than a placebo after 8-week treatment, with low adverse events, cost-effectiveness, and accessibility being notable advantages (11).

Clausena anisata (Willd.) Hook.f.ex. Benth. is another plant used in Thai traditional medicines for smoking cessation, although it lacks clinical trial support. Reports indicate that the plant contains nitrate salts, which may induce tongue numbness and alter the smell and taste of cigarettes (4, 5). Previous research suggests that *C. anisata,* like *C. cinereum*, holds the potential for smoking cessation based on its nitrate content (12). The easy way to consume these two plants is in herbal tea form, prepared using the infusion method.

The traditional method of experimentation often relies on trial and error, which is unstructured, inefficient, and particularly challenging when lacking expertise in the subject matter. Another frequently used approach is the one factor at a time (OFAT) method. In OFAT, one factor is altered, the response is measured, and the process is then repeated with a different factor. However, this approach seldom reveals the optimal set of conditions, especially when multiple factors are at play. This is where the Design of Experiments (DOE) proves advantageous (13). By utilizing DOE, substantial time, money, and resources can be saved compared to traditional trial-and-error or OFAT methods. Additionally, DOE allows for the identification of factor interactions and the characterization of the response surface (14, 15). Furthermore, a statistical model can be employed to predict the simultaneous impact of multiple factors (13).

This work aimed to evaluate the impact of the solvent-to-solid ratio and infusion duration on the extraction yield and nitrate content of two plants, *C. cinereum* and *C. anisata*, using a circumscribed central composite design. The authors expected that the data obtained from this work could be used as a guide for the infusion of these two plants to achieve the highest extraction yield and nitrate content for smoking cessation purposes.

MATERIALS AND METHODS

Materials

Sodium nitrate (purity 99%) and 85% orthophosphoric acid were purchased from Carlo Erba Reagents, France. Methanol (HPLC grade) was purchased from Duksan Pure Chemicals, Korea. Octylamine was purchased from Sigma-Aldrich, USA. *C. cinereum* (whole plant) was obtained from Pathum Thani Province, Thailand, and *C. anisata* (leaf) was obtained from Sing Buri Province, Thailand.

Experimental design, optimization, and verification

Each dried plant underwent grinding using a grinder. The different solvent-to-solid ratios were varied. The range of each factor was based on the preliminary study. A specific quantity of powdered plant material was then added to a 100-mL beaker. Subsequently, 50 mL of boiling water was added, and the mixture was stirred. The infusion process took place for a specific duration, as outlined in Table 1, designed based on a circumscribed central composite design. Following infusion, the filtrate was promptly collected using the vacuum filtration technique with Whatman® filter paper no. 1.

The collected filtrate was subjected to lyophilization for a period of 20-24 h. The resulting extract powder was collected, and the extraction yield was determined. The nitrate content of the extracts was analyzed using a validated high-performance liquid chromatography (HPLC) method.

The extraction yield and nitrate content of plant extracts obtained under various infusion conditions were analyzed using Design-Expert® version 11. Contour plots for each response were generated. Additionally, analysis of variance (ANOVA) was performed. The optimal condition, providing the highest extraction yield and nitrate content, were selected based on the desirability function (16). The accuracy of Design-Expert®'s prediction of the optimal condition was confirmed by re-extracting *C. cinereum* and *C. anisata*. Experimental values were compared to predicted values, and the percent error was calculated using Equation 1.

$$Error\ (\%) = \frac{(Experimental\ value - Predicted\ value)}{Experimental\ value} \times 100 \quad \textbf{(1)}$$

Table 1. The circumscribed central composite design presented as coded and actual values of solvent-to-solid ratio and infusion duration.

Condition	Solvent-to-solid ratio		Infusion duration	
	Coded	Actual value	Coded	Actual value (min)
1	-1	12.9:1 (50 mL: 3.87 g)	-1	6.5
2	1	27.1:1 (50 mL: 1.85 g)	-1	6.5
3	-1	12.9:1 (50 mL: 3.87 g)	1	13.5
4	1	27.1:1 (50 mL: 1.85 g)	1	13.5
5	$-\sqrt{2}$	10:1 (50 mL: 5 g)	0	10.0
6	$\sqrt{2}$	30:1 (50 mL: 1.67 g)	0	10.0
7	0	20:1 (50 mL: 2.5 g)	$-\sqrt{2}$	5.0
8	0	20:1 (50 mL: 2.5 g)	$\sqrt{2}$	15.0
9	0	20:1 (50 mL: 2.5 g)	0	10.0
10	0	20:1 (50 mL: 2.5 g)	0	10.0

Sample preparation

A standard nitrate solution at a concentration of 1 mg/mL was prepared by dissolving 13.7 mg of sodium nitrate in a 10-mL volumetric flask, using water as the solvent. Subsequently, the solution was diluted to concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL. Each dilution underwent filtration through a nylon syringe filter with a pore size of 0.45 µm and was subjected to triplicate analysis for each concentration. A calibration curve was then established. For plant extracts, they were dissolved in water at a concentration of 2.5 mg/mL (n = 3). Similar to the standard solution, the plant extract solutions were filtered through a nylon syringe filter. Subsequently, HPLC analysis was performed, and the nitrate content was determined based on the calibration curve.

HPLC condition

HPLC condition was performed as the previous works (12, 17, 18). The examination was carried out using the HPLC instrument (Agilent 1260 Infinity, Agilent Technologies, USA). Utilizing the ACE-GENERIX column (150×4.6 mm, i.d., 5 µm), the process was regulated at 25°C. A 0.01 M octylammonium orthophosphate isocratic system (pH 7.0) was employed as the mobile phase. The mobile phase was prepared by dissolving 1.3 g of octylamine in a 30%v/v methanol solution in a 1,000-mL volumetric flask. The pH was adjusted to 7.0 with 10% orthophosphoric acid, and the volume was then adjusted to 1,000 mL with 30%v/v methanol. The flow rate of the mobile phase was set at 0.8 mL/min, with an injection volume of 10 µL. Detection was performed at a wavelength of 213 nm. The sample was initially assessed once the signal displayed a consistently reproducible retention time and peak area.

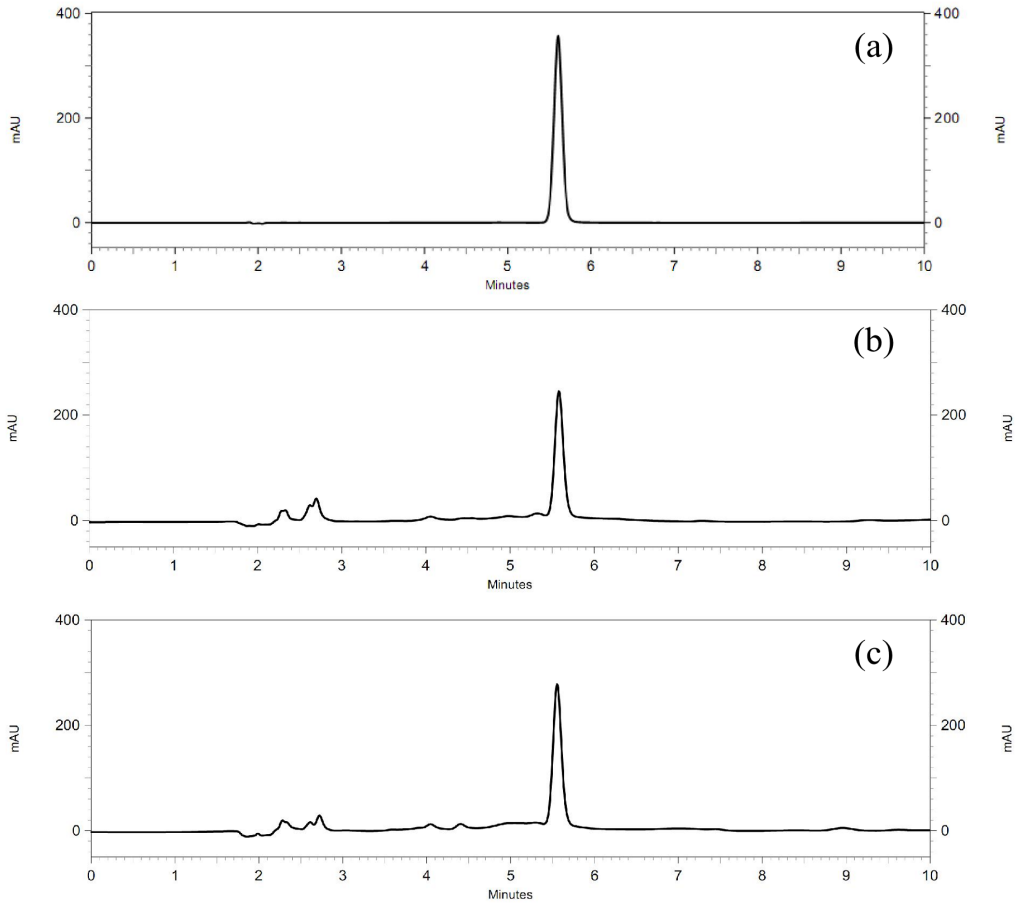

Figure 1. HPLC chromatograms of (a) standard nitrate (50 µg/mL) (b) *C. cinereum* extract (2.5 mg/mL) (c) *C. anisata* extract (2.5 mg/mL)

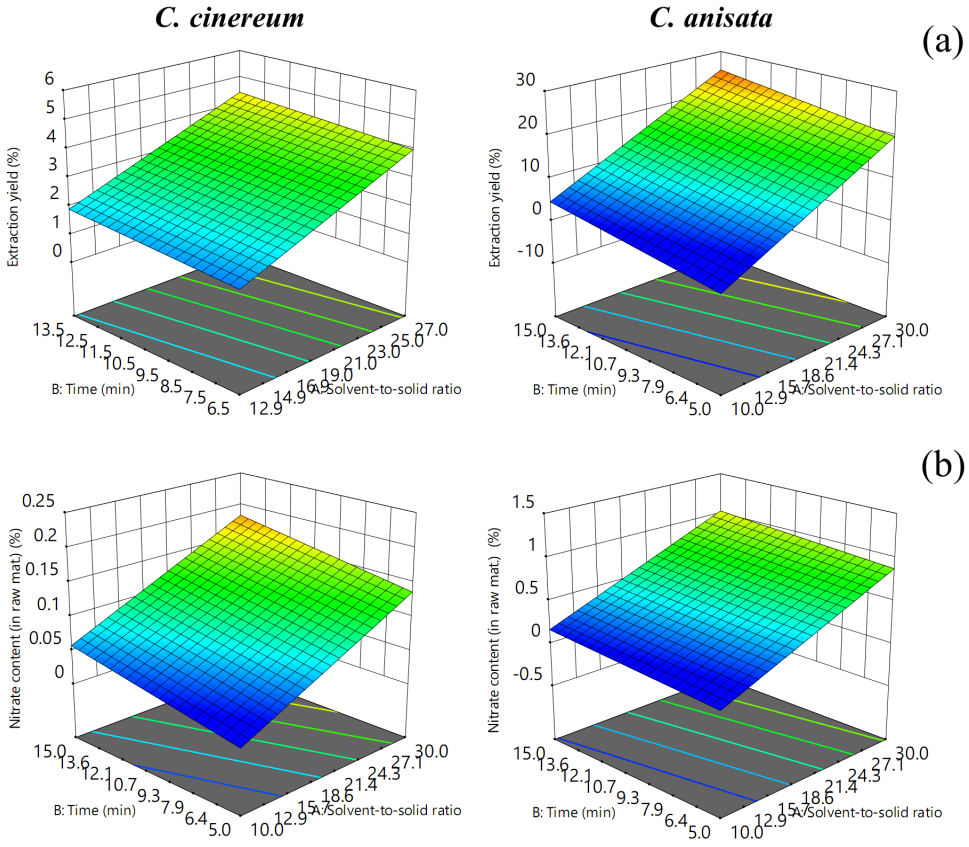

Figure 2. Response surfaces of (a) extraction yield and (b) nitrate content of *C. cinereum* (left) and *C. anisata* (right)

RESULTS AND DISCUSSION

The HPLC chromatograms of *C. cinereum* and *C. anisata* extracts are shown in Figure 1. The nitrate peak was eluted at a retention time of approximately 5.6 min. These data support the concept that both plants contain nitrate and can be used as smoking cessation aids.

Contour plots of the extraction yield and nitrate content of *C. cinereum* and *C. anisata* are presented in Figure 2. The results indicate that an increase in the solvent-to-solid ratio led to higher extraction yield and nitrate content. An increase in infusion duration resulted in slightly higher extraction yield and nitrate content. ANOVA data showed that the model was significant, and the lack of fit was not significant, meeting the desired criteria. The solvent-to-solid ratio was identified as the only factor significantly affecting the extraction yield and nitrate content of both *C. cinereum* and *C. anisata*, as demonstrated in Table 2.

The infusion conditions of *Thunbergia laurifolia* leaves were optimized by varying infusion time, temperature, and water volume to maximize the contents of caffeic acid and rosmarinic acid. It was observed that increasing infusion temperature and time led to an increase in caffeic acid content. At a low infusion time (5 min), raising the water volume from 150 mL to 250 mL resulted in an increased caffeic acid content. However, at a longer infusion time (15 min), increasing the water volume from 150 mL to 250 mL decreased caffeic acid content. Furthermore, maintaining the water volume at 200 mL while increasing the infusion temperature resulted in increased contents of both caffeic acid and rosmarinic acid. Although increasing infusion time enhanced caffeic acid content, it did not affect rosmarinic acid content (19). The obtained data were consistent with the findings of the current study. Both water volume (solvent-to-solid ratio) and infusion time were identified as

Table 2. Analysis of variance for extraction yield and nitrate content of *C. cinereum* and *C. anisata*

Source	Sum of Squares	df	Mean Square	F-value	p-value
Extraction yield (C. cinereum)					
Model	13.11	2	6.55	4.94	0.0459*
A-Solvent-to-solid ratio	12.72	1	12.72	9.59	0.0174*
B-Time	0.38	1	0.38	0.29	0.6080
Residual	9.28	7	1.33		
Lack of Fit	7.14	6	1.19	0.56	0.7713
Pure Error	2.14	1	2.14		
Cor Total	22.39	9			
Nitrate content (C. cinereum)					
Model	0.0179	2	0.0090	4.97	0.0454*
A-Solvent-to-solid ratio	0.0158	1	0.0158	8.75	0.0212*
B-Time	0.0021	1	0.0021	1.19	0.3112
Residual	0.0126	7	0.0018		
Lack of Fit	0.0109	6	0.0018	1.05	0.6333
Pure Error	0.0017	1	0.0017		
Cor Total	0.0305	9			
Extraction yield (C. anisata)					
Model	460.03	2	230.01	23.13	0.0008*
A-Solvent-to-solid ratio	428.91	1	428.91	43.13	0.0003*
B-Time	31.12	1	31.12	3.13	0.1202
Residual	69.61	7	9.94		
Lack of Fit	59.08	6	9.85	0.94	0.6589
Pure Error	10.52	1	10.52		
Cor Total	529.64	9			
Nitrate content (C. anisata)					
Model	0.7669	2	0.3835	8.24	0.0145*
A-Solvent-to-solid ratio	0.7434	1	0.7434	15.98	0.0052*
B-Time	0.0236	1	0.0236	0.51	0.4998
Residual	0.3257	7	0.0465		
Lack of Fit	0.3103	6	0.0517	3.37	0.3946
Pure Error	0.0154	1	0.0154		
Cor Total	1.0900	9			

An asterisk (*) denotes a significant value.

factors positively influencing the content of bioactive compounds.

In contrast to the nitrate content observed in the microwave-assisted extraction of *C. cinereum* and *C. anisata*, the results showed a conflicting trend. An augmentation in microwave time led to an increase in extraction yield but a decrease in nitrate content (18). The authors explained that elevating microwave power, time, and

irradiation cycle resulted in greater extraction of other chemical components, causing a dilution effect and consequently reducing the nitrate content (18). According to the solvent-to-solid ratio, it differed from the findings in other studies. Increasing the solvent-to-solid ratio of *Vernonia amygdala* leaf increased extraction yield and total phenolic content to their maximum levels. However, further increases in the solvent-to-solid ratio led to a decrease in both extraction yield and total phenolic content (20). Sometimes, a single factor like the solvent-to-solid ratio did not seem to affect the extraction yield. Instead, interactions among factors, such as the solvent-to-solid ratio interacting with extraction time or extraction temperature, played a significant role (21).

The optimal condition, selected based on the desirability function to achieve the highest simultaneous extraction yield and nitrate content in the raw material, consisting of a solvent-to-solid ratio of 27:1 and an infusion duration of 13.5 min for both *C. cinereum* and *C. anisata* (Table 3). Verification was conducted by re-extracting *C. cinereum* and *C. anisata* using these optimal conditions. The results indicated that the percent errors for all parameters were less than 10% (Table 4), confirming the accuracy and reliability of the Design-Expert® program.

CONCLUSION

This study has provided valuable insights into the optimization of infusion conditions for *C. cinereum* and *C. anisata*, both recognized herbal plants used in smoking cessation. The investigation, employing a circumscribed central composite design, focused on two critical infusion factors: solvent-to-solid ratio and infusion duration. Through the application of reversed-phase ion-interaction HPLC, the nitrate content of the extracts was analyzed. The results revealed notable disparities between *C. cinereum* and *C. anisata*, with the latter exhibiting higher extraction yield and nitrate content. The simultaneous optimization of extraction yield and nitrate content was achieved by employing a high solvent-to-solid ratio and an extended infusion duration. ANOVA revealed the significant influence of the solvent-to-solid ratio on both extraction yield and nitrate content. The identified optimal condition for achieving the highest extraction yield and nitrate content simultaneously were determined as a solvent-to-solid ratio of 27:1 and an infusion duration of 13.5 min. Specifically, for *C. cinereum*, an extraction yield of 4.67% and a nitrate content of 0.16% were obtained. In the case of *C. anisata*, the extraction yield reached 20.86%, accompanied by a nitrate content of 0.83%. The reliability and precision

Table 3. The optimal condition predicted by Design-Expert® with their predicted values and desirability values.

Samples	Optimal condition (uncoded)		Predicted values		Desirability
	Solvent-to-solid ratio	Infusion duration (min)	Extraction yield (%)	Nitrate content (%)	
C. cinereum	27.0	13.5	4.432	0.158	0.745
C. anisata	27.0	13.5	21.493	0.883	0.683

Table 4. Verification data presented as predicted values, experimental values, and percent error.

Samples	Responses	Predicted values	Experimental values (n = 3)	Error (%)
C. cinereum	Extraction yield	4.432	4.674 ± 0.394	5.18
	Nitrate content	0.158	0.160 ± 0.016	1.25
C. anisata	Extraction yield	21.493	20.862 ± 2.407	-3.02
	Nitrate content	0.883	0.834 ± 0.052	-5.88

of the computer program's predictions were confirmed through the low percentage error. In summary, this study successfully elucidates the impact of solvent-to-solid ratio and infusion duration on the extraction yield and nitrate content of *C. cinereum* and *C. anisata*. These findings contribute to the broader understanding of the optimization approach for utilizing these herbal plants in smoking cessation, providing valuable insights for further exploration and application in related fields.

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