Optimization of Ultrasonic-Assisted Extraction of Antioxidant Compounds in Black Shallots *(Allium ascalonicum)* from Vietnam using Response Surface Methodology

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Response surface methodology (RSM) was used to optimize the extraction conditions for total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity (DPPH) in black shallots *(Allium ascalonicum)* using ultrasonic-assisted extraction (UAE). The variables used in the process were extraction temperature (40-60ºC), extraction time (60–80 min), ethanol concentration (30–70%) and power level (60–100%/600W). The results indicated that extraction temperature, extraction time, ethanol concentration and ultrasonic power level had an impact on TPC, TFC and DPPH radical scavenging activity. The optimum extraction conditions obtained from RSM were: extraction temperature of 53 ºC, extraction time of 72 min, ethanol concentration of 48 % and ultrasonic power level of 84 %. The experimental values of TPC, TFC and DPPH radical scavenging activity were 17.28 ± 0.12 mg GAE/g, 5.42 ± 0.08 mg QE/g and 75.35 ± 0.15%, respectively.

Key words: *Allium ascalonicum*; black shallot; total phenolic content (TPC); total flavonoid content (TFC); DPPH radical scavenging activity

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Shallots (*Allium ascalonicum* L.) are used as a spice in food around the world. They have also been used as a traditional medicine with health benefits. The chemical constituents of shallots include mainly flavonoid glycosides, sulfur-containing compounds and saponins. Flavonol glucosides include quercetin and isorhamnetin mono- and diglucosides found in French and Italian shallots [1]. Previous studies have shown that many plants of the *Allium* family (except *A. sativum* and *A. cepa*) play an important role in the composition of fragrances, with their antioxidant, aromatic and therapeutic properties [2]. The bulbs of the *Allium* species are used as a food flavouring and have been prized over the years not only for their characteristic taste and odour, but also as a significant source of bioactive compounds such as allicin and their derivatives [3, 4, 5] or flavonoid glycosides [6, 7, 8].

The antioxidant activity of the *Allium* species is derived from sulfur-containing compounds and their precursors. Other compounds such as polyphenols, fibre and trace elements are also claimed to contribute to this activity [9]. Polyphenols are compounds that are generously distributed in natural products. They have been shown to have a variety of biological properties, such as antioxidant, antiviral, anti-inflammatory, antimicrobial and anti-epithelial activities [10, 11]. Polyphenol-rich medicinal plants can slow the oxidation of lipids, and improve the nutritional value and quality of food [12]. In addition to being a good source of antioxidants, purple onions also have important benefits such as improving heart health and preventing cancer and diabetes. They also possess anti-inflammatory, antibacterial and anti-obesity properties, and help prevent or treat allergies [13].

Shallots contains secondary metabolites such as flavonoids, especially flavonols, anthocyanins, plant sterols and saponins [13]. *β*-Sitosterol, campesterol and stigmasterol are among the most common plant sterols in natural products. Sterols may lessen the risk of atherosclerosis and protect against cardiovascular diseases [14]. They also have the potential to reduce the risk of breast, prostate and colon cancers [15, 16]. Furthermore, plant sterols have anti-inflammatory and immunomodulatory properties [17]. All plant sterols cannot be synthesized in the human body and must be obtained from the diet. *β*-Sitosterol, stigmasterol and campesterol account for more than 95% of total dietary plant sterols [14]. The *Allium* genus embraces more than 500 species found all over the world. Antibacterial and antifungal properties have been demonstrated in *A.*

sativum, *A. Porrum* [18], *A. cepa* [19], *A. ascalonicum* [20], and *A. fistulosum* [21].

Shallots (*Allium ascalonicum* L.) offer many health benefits but are still limited by their pungent odour and spicy taste. However, the unpleasant odour and taste of shallots can be eliminated through heat treatment as the less stable compounds and unpleasant odors are converted into durable and odourless compounds. On this basis, black shallots are processed and produced by controlled aging at an appropriate temperature and humidity to improve the their taste and quality.

In recent years, there have been many reports on the use of ultrasonic-assisted extraction (UAE) for biologically active compounds. As a result, we know that ultrasound is a well-established method in the processing of plant materials, particularly for the extraction of low molecular weight substances as it can accelerate extraction and thus improve bioactive compound extraction [22, 23, 24, 25, 26]. UAE breaks plant cell walls and allows all soluble substances into the solvent [27]. The recovery of various compounds from other sources by ultrasonic extraction has received considerable interest [26, 28]. Factors such as solvent composition, extraction time, extraction temperature, solvent to solid ratio and extraction method influence the extraction efficacy [29, 30]. Optimization of the process can be achieved by empirical or statistical methods and is essential for commercial applications.

The optimization of UAE during the extraction of bioactive compounds, effectively applied the response surface methodology (RSM) with a suitable experimental design, the Box-Behnken design (BBD) [31]. Currently, there is no research applying ultrasonic-assisted extraction on raw black shallots. This study aimed to identify the total phenolic and total flavonoid contents and the antioxidant activity of black shallots *(Allium ascalonicum)*, employing RSM to optimize its UAE and determine the antioxidant activity of the samples.

EXPERIMENTAL

1. Materials

Shallots were collected from the Vinh Chau District of Soc Trang Province, Vietnam. The shallots were classified and the specimens selected were whole, round and firm, free of pests, and not physically damaged. Shallots were cleaned with water and the outside husks and damaged parts were removed. The whole shallots subsequently were put in an aging chamber (Shellab, USA) based on the procedure reported by Tran et al. [32]. In addition, the selected onions were uniform in colour and size (about 20-30 mm in diameter). These were washed to remove the sandy soil, the stems and roots were cut off, and they were then peeled and dried outside. After preliminary treatment, the shallots were wrapped in foil (with small holes 1.5 - 2 mm in diameter for steam release) and incubated (75°C, 90% RH) for 20 days. The product of the heat treatment process was dark brown, dry, and flexible in texture, with a slight sweet taste.

2. Methods

2.1. Total Phenolic Content (TPC)

The TPC of the black shallot extracts was obtained using a slightly modified version of the method reported by Singleton et al. 1999 [33]. This method is based on the measurement of the colour change induced in the reagent by phenolate in the presence of sodium carbonate. The black shallot extract (1 ml) was introduced into the Folin-Ciocalteu reagent (5 ml) for 3 min. Then, Na_2CO_3 (4 mL, 7.5%) and deionized water (10 mL) were added, and the solution was incubated in the dark for 60 min at room temperature. The absorbance of the solution was measured at 765 nm with an Agilent 8453 UV-Visible spectrophotometer. The TPC of the black shallot extract was determined from the linear equation of a standard curve at different concentrations prepared with gallic acid. The total content of phenolic compounds was expressed as mg of gallic acid equivalent/g dry weight (mg GAE/g DW).

2.2. Total Flavonoid Content (TFC)

The TFC of the black shallot extracts was determined following the method of Chang et al. (2002) with some modifications [34]. The black shallot extract (0.5 mL) was reacted with ethanol (1.5 mL, 75%), aluminum chloride (0.1 mL, 10%), potassium acetate (0.1 mL, 0.1 M), and distilled water (2.8 mL) at room temperature for 30 minutes. The absorbance of the reaction mixtures was measured at 415 nm with an Agilent 8453 UV-Visible spectrophotometer. The standard calibration curve was constructed with a quercetin standard and TFC was described as mg quercetin equivalent/g dry weight (mg QE/g DW).

2.3. DPPH Radical Scavenging Activity

The free radical scavenging activity of the black shallot extract, using DPPH radicals, was performed as described by the method of Xu and Chang (2007) with minor modifications [35]. The black shallot extract (0.2 mL) was mixed with a DPPH ethanol solution (3.8 ml, 0.15mM), which was shaken vigorously for 1 min. Then, it was incubated in the dark for 30 min at room temperature. The absorbances of the sample (Abs_{sample}) and the control ($Abs_{control}$) were measured at 517 nm (Agilent 8453 UV-Visible Spectrophotometer). Each

experiment was performed in triplicate and expressed as mean ± SD. The radical scavenging activity was calculated according to Eq. (1) below:

$$\text{Radical scavenging activity (\%)} = [1 - (Abs_{sample}/Abs_{control})] \times 100 \quad (1)$$

2.4. Ultrasonic Extraction

The ultrasonic extraction was conducted using an ultrasonic probe with a maximum power of 600 watts. A mixture of 10 g of black shallot powder and 300 mL of suitable solvent was contained in a stainless steel vessel and extracted with an ultrasonic probe. The resulting suspension was centrifuged and filtered with a 0.45µm syringe filter before further analysis.

2.5. Box-Behnken Design

In this optimization study, a three-level, four-variable Box-Behnken design (BBD) consisting of 27 experimental runs was used based on the results of the preliminary experiments. The independent variables were extraction temperature (X_1), extraction time (X_2), ethanol concentration (X_3) and power level (X_4), while the dependent variables were total phenolic content (Y_1), total flavonoid content (Y_2) and DPPH radical scavenging activity (Y_3). Experiments were repeated and mean values were used as responses. The Design Expert software, version 7.0 was used to perform ANOVAs on the means of the triplicate experiments. Each analysis was performed in triplicate and all experimental results were expressed as mean ± SD. *P* values < 0.05 were considered significant and *P* values < 0.01 very significant.

RESULTS AND DISCUSSION

1. Fitting the Models

The TPC, TFC and DPPH results for the black shallot extract were obtained by employing optimized ultrasonic extraction based on the BBD. The four independent variables of BBD were as follows: extraction temperature and time, ethanol concentration and ultrasonic power level. The preliminary experiments presented in Table 1 show that the input range of the selected variables were determined. After extraction under optimal conditions, three dependent variables, total phenolic and total flavonoid contents and DPPH radical scavenging activity, were determined.

The Design-Expert® program, version 7.0.0 was used to evaluate the effects of the extraction process. The response variables were fitted with a second-order polynomial model as follows:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j$$

where Y is the predicted response; β_o is the intercept coefficient; β_i is the linear coefficient; β_{ii} is the squared coefficient; β_{ij} is the interaction coefficient; X_i and X_j are the coded independent variables; X_iX_j and X_i^2 are the interaction and quadratic terms respectively.

Table 2 shows the values of the three evaluation indices for each extraction condition. The maximal TPC was 17.55 mg GAE/g, the maximal TFC was 5.67 mg QE/g and the maximal DPPH was 76.21%. From the multiple linear regression analysis of the 27 data entries, empirical second-order polynomial models for TPC, TFC and DPPH radical scavenging activity scavenging capacity were created (Table 3).

Table 1. Coded level of independent variables used in the RSM design

Independent variables	Units	Coded symbols	Coded variable levels		
			-1	0	+1
Extraction temperature	°C	X_1	40	50	60
Extraction time	min	X_2	60	70	80
Ethanol concentration	%	X_3	30	50	70
Ultrasonic power level	%	X_4	60	80	100

Table 2. Experimental Design and Response Values

Run No.	Coded and Processed Variable level				Response		
	X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
	Extraction temperature (°C)	Extraction time (min)	Ethanol concentration (%)	Power level (%)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	DPPH radical scavenging activity (%)
1	50(0)	70(0)	70(+1)	60(-1)	12.81	5.67	67.83
2	60(+1)	60(-1)	50(0)	80(0)	14.39	4.85	71.19
3	50(0)	80(+1)	30(-1)	80(0)	16.95	3.87	72.05
4	40(-1)	70(0)	30(-1)	80(0)	13.78	3.48	70.24
5	40(-1)	70(0)	50(0)	100(+1)	13.72	4.13	71.13
6	50(0)	70(0)	70(+1)	100(+1)	15.39	4.04	72.68
7	50(0)	80(+1)	50(0)	60(-1)	15.45	5.10	72.14
8	50(0)	70(0)	50(0)	80(0)	16.84	5.42	74.56
9	60(+1)	70(0)	70(+1)	80(0)	13.36	4.89	70.11
10	40(-1)	80(+1)	50(0)	80(0)	14.45	4.69	71.56
11	50(0)	80(+1)	50(0)	100(+1)	16.42	4.77	74.12
12	50(0)	60(-1)	50(0)	100(+1)	15.73	3.73	76.21
13	50(0)	70(0)	50(0)	80(0)	17.55	5.51	75.71
14	60(+1)	70(0)	50(0)	100(+1)	17.05	4.39	73.81
15	60(+1)	70(0)	30(-1)	80(0)	16.57	4.36	73.53
16	50(0)	60(-1)	70(+1)	80(0)	13.11	4.30	70.08
17	50(0)	70(0)	50(0)	80(0)	16.35	5.73	74.43
18	60(+1)	70(0)	50(0)	60(-1)	12.79	5.41	70.34
19	40(-1)	70(0)	70(+1)	80(0)	12.89	4.71	68.78
20	40(-1)	60(-1)	50(0)	80(0)	12.20	3.97	69.53
21	40(-1)	70(0)	50(0)	60(-1)	13.57	4.54	70.44
22	50(0)	70(0)	30(-1)	60(-1)	16.22	4.17	71.73
23	60(+1)	80(+1)	50(0)	80(0)	14.99	5.05	73.03
24	50(0)	60(-1)	50(0)	60(-1)	12.95	4.85	67.25
25	50(0)	70(0)	30(-1)	100(+1)	16.78	3.77	73.61
26	50(0)	60(-1)	30(-1)	80(0)	14.12	4.07	70.53
27	50(0)	80(+1)	70(+1)	80(0)	13.89	5.41	69.83

Table 3. Empirical second-order polynomial models for TPC, TFC and DPPH

Response	Model equations	R^2	p- value
Y_1 – TPC	$Y_1 = 16.91 + 0.71X_1 + 0.80X_2 – 1.08X_3 + 0.94X_4 – 0.41X_1X_2 – 0.58X_1X_3 + 1.03X_1X_4 – 0.51X_2X_3 – 0.45X_2X_4 + 0.5X_3X_4 – 1.8X_1^2 – 1.19X_2^2 – 1.04X_3^2 – 0.66X_4^2$	0.9785	<0.0001
Y_2 – TFC	$Y_2 = 5.55 + 0.29X_1 + 0.26X_2 + 0.44X_3 – 0.41X_4 – 0.13X_1X_2 – 0.18X_1X_3 – 0.15X_1X_4 + 0.33X_2X_3 + 0.2X_2X_4 - 0.31X_3X_4 – 0.48X_1^2 – 0.45X_2^2 – 0.69X_3^2 – 0.46X_4^2$	0.9867	<0.0001
Y_3 – DPPH	$Y_3 = 74.9+ 0.86X_1 + 0.66X_2 – 1.03X_3 + 1.82X_4 - 0.047X_1X_2 – 0.49X_1X_3 + 0.69X_1X_4 - 0.44X_2X_3 – 1.74X_2X_4 + 0.74X_3X_4 – 2.06X_1^2 – 1.58X_2^2 – 2.4X_3^2 – 1.11X_4^2$	0.9253	0.0001

The corresponding p and R^2 values in Table 3 were used to evaluate ANOVA results for multiple regression analysis and response surface quadratic modeling of Y_1, Y_2 and Y_3. F values of Y_1, Y_2 and Y_3 were calculated to be 39.07; 63.45 and 10.61, both leading to a p value <0.05, which indicated that both models were extremely statistically significant. The coefficient of determination of the model (R^2) was 0.9785, 0.9867 and 0.9253, showing that more than 97.85%, 98.67% and 92.53% of the response variations were explained, and showing that the established model had good accuracy within the range limits used. The F-values of lack of fit for Y_1, Y_2 and Y_3 were 0.21, 0.36 and 1.86, respectively, implying that the lack of fit was insignificant compared to the pure error. This indicated that the accuracy of the polynomial model was sufficient.

2. Response Surface Analysis

Variables such as extraction temperature and time, ethanol concentration, and power level affected the extraction conditions for the total phenolic and flavonoids content and DPPH. Three-dimensional model graphs were plotted as shown in the corresponding figures. The response surface plot of the model was taken with two different variables within the experimental range being investigated while keeping the other variables at the central level.

Table 4. Regression coefficients of the predicted second-order polynomial models for total phenolic content, total flavonoid content and DPPH.

Source	Y_1 – TPC		Y_2 – TFC		Y_3 – DPPH	
	F- value	p- value	F- value	p-value	F- value	p- value
Model	39.07	< 0.0001***	63.45	< 0.0001***	10.61	0.0001***
X_1	49.08	< 0.0001***	82.09	< 0.0001***	10.45	0.0072**
X_2	62.66	< 0.0001***	67.92	< 0.0001***	6.18	0.0290*
X_3	113.20	< 0.0001***	195.99	< 0.0001***	15.02	0.0022**
X_4	85.92	< 0.0001***	168.21	< 0.0001***	46.69	< 0.0001***
X_1X_2	5.50	0.0414*	5.66	0.0371*	0.011	0.9197^{NS}
X_1X_3	10.87	0.0071**	10.26	0.0075**	1.13	0.3089^{NS}
X_1X_4	34.10	0.0001***	7.79	0.0170*	2.27	0.1576^{NS}
X_2X_3	8.48	0.0162*	35.92	< 0.0001***	0.92	0.3562^{NS}
X_2X_4	6.61	0.0268*	13.06	0.0036**	14.32	0.0026**
X_3X_4	8.24	0.0156*	31.67	0.0001***	2.59	0.1333^{NS}
X_1^2	139.92	< 0.0001***	101.64	< 0.0001***	26.65	0.0002**
X_2^2	61.11	< 0.0001***	91.77	< 0.0001***	15.71	0.0019**
X_3^2	46.47	< 0.0001***	214.66	< 0.0001***	36.05	< 0.0001***
X_4^2	18.90	0.0010**	96.38	< 0.0001***	7.75	0.0165*
Lack of Fit	0.21	0.9578^{NS}	0.36	0.8741^{NS}	1.86	0.4011^{NS}
R^2	0.9786		0.9867		0.9253	

*p< 0.05; **p< 0.01; ***p< 0.001; NS: non-significant.

2.1. Response Surface Analysis of Total Phenolic Content

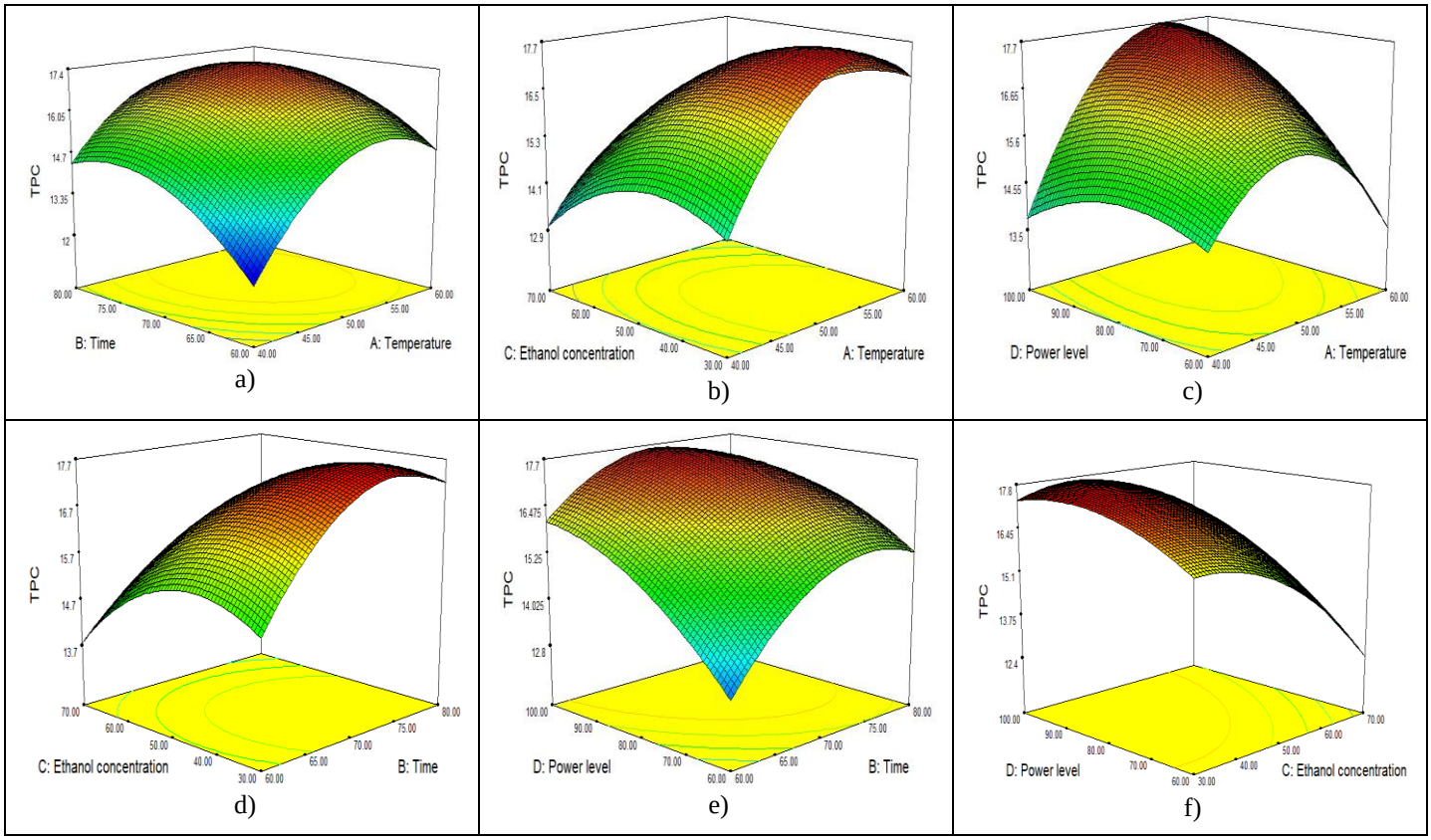

Figure 1. The response surface plots of TPC

TPC is influenced by temperature, extraction time, concentration of ethanol and power level. The three-dimensional plots were used for TPC in black shallots because of the functional response of the two factors keeping the other fixed at the middle level as shown in Fig. 1. It can be observed that TPC increased with increasing extraction temperature at 50% ethanol concentration and 80% power level. TPC also increased with increasing extraction temperature from 30°C to 55°C but then decreased. When the extraction time was increased from 60 to 75 min, TPC increased but from 75 to 80 min, TPC decreased. It was observed that TPC increased as the concentration of ethanol increased from 30%v/v to 50%v/v, and then decreased from 50% v/v to 70%v/v at a constant extraction time of 50 min and ultrasonic power level of 80%. This result was the same as that reported by Jayaprakasha [36]. It was found that the degree of cell membrane rupture of the raw materials and the solubility of phenolic substances were improved when using ethanol in water [37]. The yield of TPC increased when the ultrasonic power level increased from 60% to 100%. TFC is directly proportional to ultrasonic power at any temperature as higher ultrasonic power produces a higher cavitation effect, which effectively breaks down plant tissues and cell walls [38].

Figure 2 presents the combined effects of four variables on TFC. Fig. 2a displays the effect of 3D coalescence of extraction temperature and time on TFC when the remaining factors were maintained at constant level. At any extraction time, TFC is proportional to extraction temperature because of the positive effect of temperature on extraction [39]. At a constant temperature, extraction time also had a significant effect on TFC yield. At any extraction temperature, an increase in extraction time resulted in a significant increase in TFC. The interaction of power level and ethanol concentration at a fixed extraction time (70 min) and extraction temperature (50 °C) are shown in Fig. 2f. The 3D image shows that total flavonoid content was significantly affected by ultrasonic power and ethanol concentration. It can be observed that the TFC value increased when ultrasonic power was increased from 60% to 80%, but then decreased with the increase of power from 80% to 100% of 600W at a fixed temperature of extraction, 50°C. When the ethanol concentration was increased, the extraction resulted in an enhancement in the TFC value at any power level [38].

2.2. Response Surface Analysis of Total Flavonoid Content

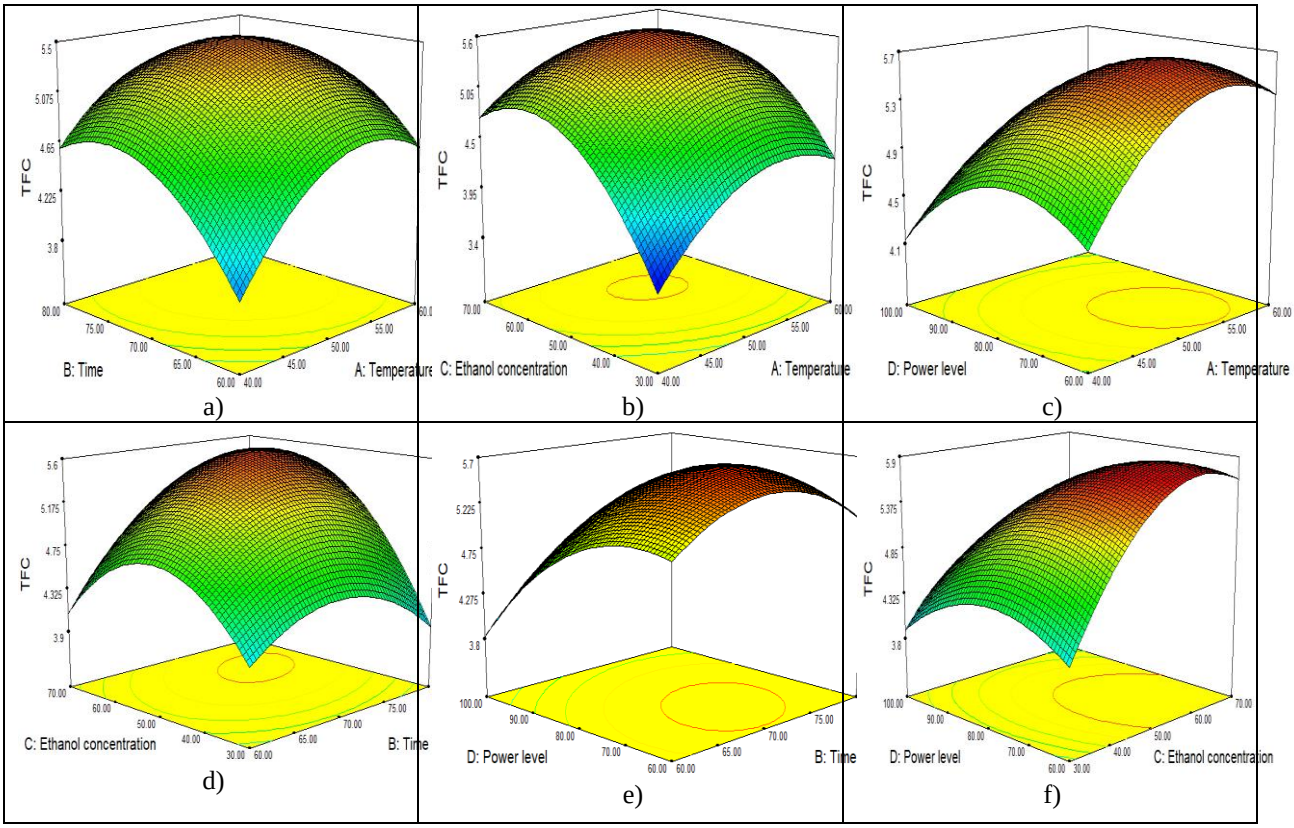

Figure 2. The response surface plots of TFC

The 3D images in Fig. 3 show the effects of extraction temperature, extraction time, ethanol concentration and power level on DPPH radical scavenging activity. The linear effects of these four factors on the DPPH value were significant. The 3D image in Fig. 3b also shows the effect of temperature and ethanol concentration on DPPH with the remaining factors maintained at a constant level. It can be observed that DPPH increased with the increase in extraction temperature from 40oC to 55oC, but then decreased when the extraction temperature increased from 55oC to 60oC at a constant extraction time of 70 min and ultrasonic power level of 80%. The effects of ethanol concentration and extraction temperature were similar. DPPH increased with increasing ethanol concentration from 30% to 50% then decreased when ethanol concentration increased from 50% to 70%. The 3D image in Fig. 3e shows the influence of time and ultrasonic power level on DPPH when ethanol concentration and extraction temperature were kept constant. The DPPH value increased with increasing extraction time and ultrasonic power level.

3. Optimization and Model Verification

From the second-order polynomial equation, the optimum conditions for total phenolic and total flavonoid content and antioxidant activity in black shallots were as follows: extraction temperature of 52.51oC, extraction time of 72.19 min, ethanol concentration of 47.99 % and ultrasonic power level of 84.08 %. The predicted maximum values of TPC, TFC and DPPH were 17.39 mg GAE/g, 5.47mgQE/g and 75.42%, respectively. Based on the operability in actual production, we modified the optimum conditions slightly as follows: extraction temperature of 53oC, extraction time of 72 min, ethanol concentration of 48 % and ultrasonic power level of 84%. Under these conditions, the mean values of TPC, TFC and DPPH were 17.28 ± 0.12 mg GAE/g, 5.42 ± 0.08 mg QE/g, and 75.35 ± 0.15% (n = 3). The results suggest that the experimental values are in agreement with the predicted values (Table 5).

2.3. Response Surface Analysis of DPPH

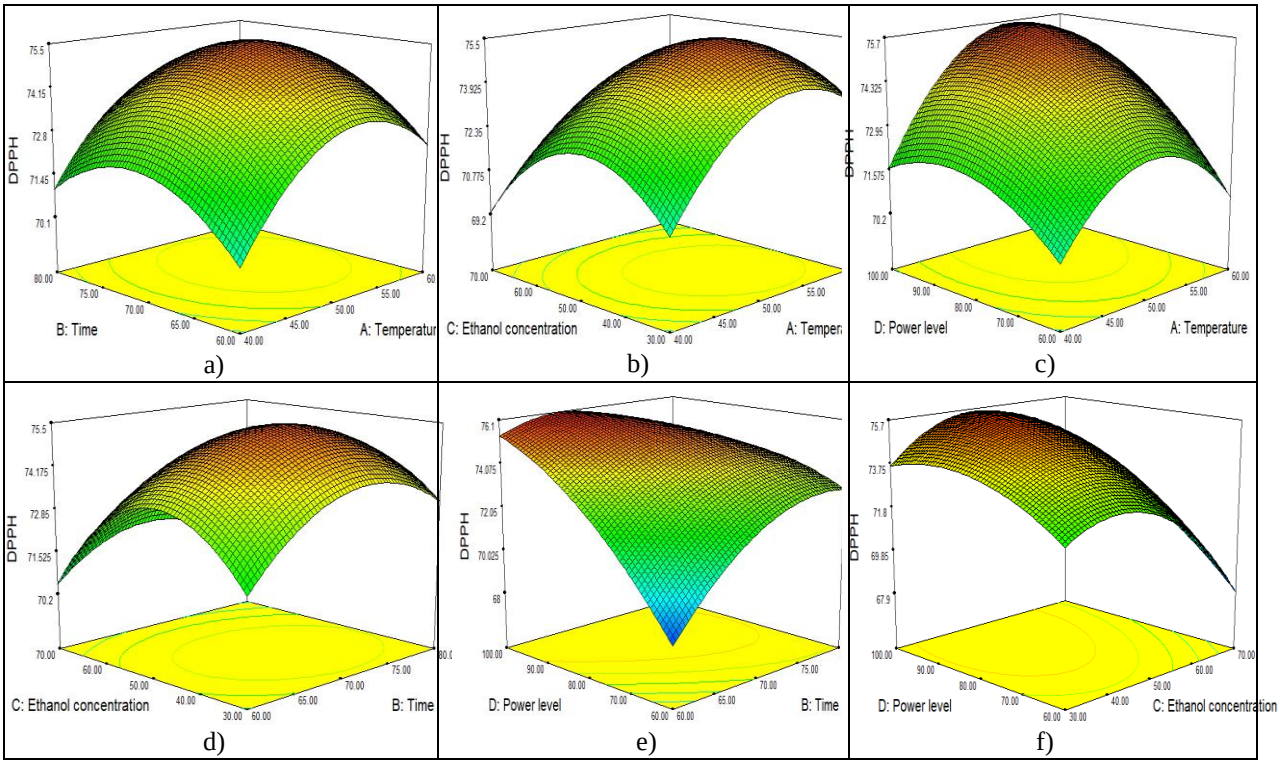

Figure 3. Response surface plot of DPPH

Table 5: Predicted and experimental values of responses under optimal conditions

Responses	Optimum extraction conditions				Maximum value	
	X_1	X_2	X_3	X_4	Experimental[a]	Predicted
TPC (mg GAE/g DW)	53°C	72min	48%	84%	17.28 ± 0.12	17.39
TFC (mg QE/g DW)					5.42 ± 0.08	5.47
DPPH radical scavenging activity (%)					75.35 ± 0.15	75.42

X_1, extraction temperature (°C); X_2, extraction time (min); X3, ethanol concentration (%); X_4, ultrasonic power level (%); Y_1, TPC (mg GAE/g); Y_2, TFC (mg QE/g); Y_3, DPPH (%). [a]Responses are the means ± SD (n = 3).

CONCLUSION

In this study, for the first time, UAE was employed to study the properties of black shallots. The optimal conditions for UAE and the antioxidant activity of black shallot extracts were investigated by response surface methodology using the Box-Behnken design. Under optimal extraction conditions (extraction temperature of 53°C, extraction time of 72min, ethanol concentration of 48% v/v and ultrasonic power level of 84% (504W)), the experimental values for TPC, TFC and DPPH were found to be 17.28 ± 0.12 mgGAE/g, 5.42 ± 0.08 mg QE/g and 75.35 ± 0.15%, respectively.

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