

Comparison of Deep Eutectic Solvent-based Ultrasound- and Heat-assisted Extraction of Bioactive Compounds from *Withania somnifera* and Process Optimization Using Response Surface Methodology

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Abstract

Extraction of bioactive compounds from *Withania somnifera* roots was studied using sodium acetate-glycerol deep eutectic solvent (DES) and two techniques of extraction: ultrasound-assisted extraction (UAE) and heat-assisted extraction (HAE) under response surface methodology (RSM). For UAE and HAE, total phenolic content (TPC, mg gallic acid equivalents per g dry weight (mg GAE g⁻¹ DW)), total flavonoid content (TFC, mg rutin equivalents g⁻¹ DW (mg RE g⁻¹ DW)), radical scavenging activity (RSA, mg AAE (ascorbic acid equivalents) g⁻¹ DW), and iron chelating activity (ICA, mg EDTAE (ethylenediaminetetraacetate equivalents) g⁻¹ DW) were 6.51, 6.08, 12.56, and 3.57, respectively, and 3.33, 3.98, 6.57, and 2.48, respectively. For UAE, the optimal conditions were a DES concentration of 50%, temperature of 60 °C, and time of 20 min, and for HAE, a DES concentration of 60%, temperature of 60 °C, and time of 75 min. The discovered models were strongly supported by the validation experiments. UAE was more efficient and less time-consuming for extracting phytoconstituents of the *W. somnifera* than HAE.

Keywords: *Withania somnifera*, phenols and flavonoids, antioxidant activity, deep eutectic solvent, ultrasound-assisted extraction, response surface methodology

1. Introduction

Withania somnifera is a shrub belonging to the family Solanaceae. It is locally known as “Ashwagandha” (lit., horse smell) in South Asia due to the horse-like smell of its root powder.¹ Other names include “Asghand” in Urdu and “Winter Cherry” in English.² It is found in drier parts of Pakistan, the Middle East, South Asia, and Europe.³ *W. somnifera* is said to promote male fertility, reduce stress levels, and increase overall health.⁴ *W. somnifera* is rich in natural products which include phenolics, flavonoids, and steroidal lactones.⁵ It has many therapeutic properties including antioxidant and anti-inflammatory effects.⁶ Commercially, *W. somnifera* is also used as a dietary supplement and is available on the market as tablets, capsules, and syrups. Due to its high demand, there is a need to find a green extraction technique that can reduce manufacturing costs and waste and give a high yield.^{7,8}

Commonly, natural products are extracted using organic solvents including methanol, ethanol, and acetone.⁹ However, their use has several drawbacks such as high tox-

icity, volatility, and poor biodegradability. It is, therefore, important to find safer and more sustainable extractants.¹⁰ One possible solution is the use of deep eutectic solvents (DESs) for the extraction of bioactive natural products from plants. The efficacy of the DESs for this purpose is demonstrated by a rapidly growing number of studies. For instance, recently, tartaric acid-glycerol and tartaric acid-ethylene glycol have been shown very effective solvents to extract antioxidant compounds from *Rosa canina* L.¹¹ In several studies, glycerol-based DES exhibited higher efficiency in extracting phenolics and antioxidants as compared to organic solvents.¹² DESs can be easily tailored for extracting the compound of interest from the plant material. Sodium acetate-glycerol DES proved to be a promising solvent for the extraction of polyphenols from the plant matrices.¹³ It is prepared by the interaction of sodium acetate as hydrogen bond acceptor (HBA) and glycerol as hydrogen bond donor (HBD).¹⁴ Recently, the sodium acetate-glycerol DES at a molar ratio of 1:3 has been proven to be more efficient to extract polyphenols from raw

mango peels than any other DESs.¹⁵ It is also effective in extracting antioxidants from the agri-food waste biomass.¹⁶

Extraction techniques are generally classified into two broad categories, namely, conventional techniques and modern techniques. Conventional techniques include maceration, percolation, infusion, and refluxing which are solvent specific and require prolonged extraction time. On the other hand, modern techniques include supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction which are less time consuming and, generally, require lesser amounts of solvents.¹⁷ Heat-assisted extraction (HAE) is commonly employed due to the ease of its use and availability. It, however, also has certain disadvantages including a long extraction period and high energy consumption. Prolonged extraction at a certain temperature can also cause thermal degradation of bioactive compounds.¹⁸ On the other hand, ultrasound-assisted extraction (UAE), can show a better extraction efficiency as compared to conventional extraction techniques (maceration, stirring-assisted extraction, refluxing).¹⁹ The mechanism behind ultrasound-assisted extraction involves acoustic cavitation. When ultrasound waves pass through a solvent, the compression and rarefaction in the solvent medium form a vacuum that produces cavitation bubbles. When the cavitation bubbles collide with the plant surface, produce the shear effect and break the plant cell wall.²⁰ The interaction between the two phases increases and bioactive constituents are transferred into the extracting medium. The phenomenon is known as mass transfer. In UAE, several parameters influence the extraction process which includes ultrasound frequency, power, treatment time, temperature, solvent-to-solid ratio, and type of solvent used.²¹

The current research aimed to find the efficiency of UAE to recover bioactive compounds from *W. somnifera* dried roots in comparison to HAE. For this purpose, preliminary single-factor extractions were carried out to find out the most effective levels of the independent factors both for UAE and HAE. Based on the results of the preliminary study, extraction optimization of both techniques was done according to the Box–Behnken design (BBD) of response surface methodology. The results of this study will contribute to the advancement of extraction technology and provide valuable insights for the nutraceutical industries, leading to the development of standardized extracts from *W. somnifera* for therapeutic applications. To the best of our knowledge, optimization of the extraction of bioactive compounds from *W. somnifera* using DES has not been performed so far. With the growing realization of environmental safety, exploring green industrial processes is highly desirable. The industrial process must be environmentally sustainable. In this context, the research embodied in the article is an important contribution to the field.

2. Materials and Methods

2. 1. Plant Material

A sample of *Withania somnifera* roots was collected from the Akbari market, Lahore. The roots were converted into fine powder in a high-speed multi-function comminutor (RRH-250A). The pulverized powder went through an 80-sized mesh sieve. The plant powder was then placed in a polyethylene zip-locked bag and then refrigerated at 5 °C until further use.

2. 2. Chemicals

Sodium acetate trihydrate and glycerol were acquired from Duksan (Seoul, Korea). Folin–Ciocâlteu reagent was from Scharlab (Spain). Sodium carbonate and aluminum chloride were obtained from Merck (Darmstadt, Germany). Sodium nitrite was from Honeywell (Charlotte, USA). Sodium hydroxide, ferrozine, methanol, DPPH, gallic acid, rutin, ascorbic acid, and EDTA were obtained from Sigma-Aldrich (Steinheim, Germany).

2. 3. Extraction Procedure

HAE was carried out in a shaking incubator (Vision Scientific-VS-8480SN, Korea) at the constant shaking speed of 200 rpm and the solvent-to-solid ratio was also kept constant (30 mL g⁻¹). A measured amount (1 g) of dried plant material was mixed with 30 mL of solvent in a 100 mL Erlenmeyer flask. The temperature was varied from 40–60 °C, DES concentration varied from 30–70 (%v/v), and extraction time varied from 30–150 min. The extract was filtered through Whatman filter paper no. 42 and stored in a refrigerator in a glass vial at 5 °C.

UAE was conducted in a sonication bath (Fischer Scientific-FS60, Mexico) at the frequency of 42 kHz and power of 110 W. One gram (1 g) of dried plant material was mixed with 30 mL of solvent in a 100 mL Erlenmeyer flask. The temperature was varied from 30–70 °C, DES concentration varied from 30–70 (%v/v), and extraction time varied from 10–50 min. The extract was filtered through Whatman filter paper no. 42 and stored in the refrigerator in a glass vial at 5 °C.

2. 4. Single-factor Experiments

The preliminary single-factor experiments were carried out before the HAE and UAE- optimization study to find the factor levels. The effect of DES concentration, temperature, and extraction time on total phenolic content (TPC) from the *W. somnifera* roots was investigated. The single-factor results are shown in Figure 1 and 2.

2. 5. Total Phenolic Content (TPC)

TPC was assessed using a previously stated method with some slight modifications.²² The assay was based on

Folin–Ciocâlteu reagent (FC reagent). Briefly, test tubes were covered from the sides with aluminum foil, 100 μL extract of *W. somnifera* roots was taken and diluted with 8 mL of DI water. Afterward, 300 μL of FC reagent was added and incubated for 8 min. Afterward, 1.5 mL of 20% Na_2CO_3 solution was added. The mixture was heated in the dark at 40 $^\circ\text{C}$ for 1 hour in an oven. The absorbance was recorded at 765 nm. A calibration curve of gallic acid was drawn at different concentrations (50–400 mg L^{-1} , $R^2 = 0.9982$) and TPC was estimated in terms of its equivalents.

2. 6. Total Flavonoid Content (TFC)

A reported method was used to estimate the TFC with some slight modifications.²³ The assay is based on the complexation of flavonoids with aluminum. In a test tube, 300 mL extract of *W. somnifera* roots was pipetted out. Then, 3 mL of aqueous methanol (70% DI water: 30% methanol) was added. Afterward, 150 μL of NaNO_2 solution and then 150 μL of AlCl_3 solution were added to the solution, which was then left to rest for 5 min. Then, 1 mL of NaOH solution was added. The absorption was recorded at 506 nm wavelength. A calibration curve of rutin was obtained with different concentrations (50–400 mg L^{-1} , $R^2 = 0.9987$) and TFC was calculated as its equivalents.

2. 7. Radical Scavenging Activity (RSA)

RSA was estimated as per a previously reported method based on DPPH radical assay.²⁴ Test tubes were covered with aluminium foil and 500 μL of the root extract

was put in them. Then, 1 mL of DPPH solution which was prepared earlier was added and then 5 mL of DI water was added. The test tubes were incubated at 37 $^\circ\text{C}$ for the completion of the reaction in the oven. After incubation, absorbance was taken at 517 nm wavelength. A calibration curve of ascorbic acid was obtained with different concentrations (10–50 mg L^{-1} , $R^2 = 0.9975$) and antioxidant activity was measured in terms of ascorbic acid equivalents.

2. 8. Iron Chelating Activity (ICA)

With some slight modifications, the ICA was estimated as per a reported protocol.²⁵ In an aluminum foil-wrapped test tube, 100 μL plant extract was taken in test tubes. 3 mL of DI water was added, then, 100 μL of FeSO_4 solution was added. After that, 50 μL of ferrozine was added and incubated for 15 min in the dark. Then, absorbance was taken at 562 nm wavelength. A calibration curve of EDTA was obtained with different concentrations (10–50 mg L^{-1} , $R^2 = 0.9839$) and ICA was expressed as EDTA equivalents.

2. 9. Experimental Design

The optimization parameters for both HAE and UAE were kept the same to the sake of comparison of the two techniques. Three-factor-three-level BBD was used for modelling and optimization. The coded levels of each factor were -1 , 0 , $+1$ (lower, middle, high). The designs of experiments for HAE and UAE are shown in Table 1 along with the experimental results. Each design had 15 runs including 3 central points.

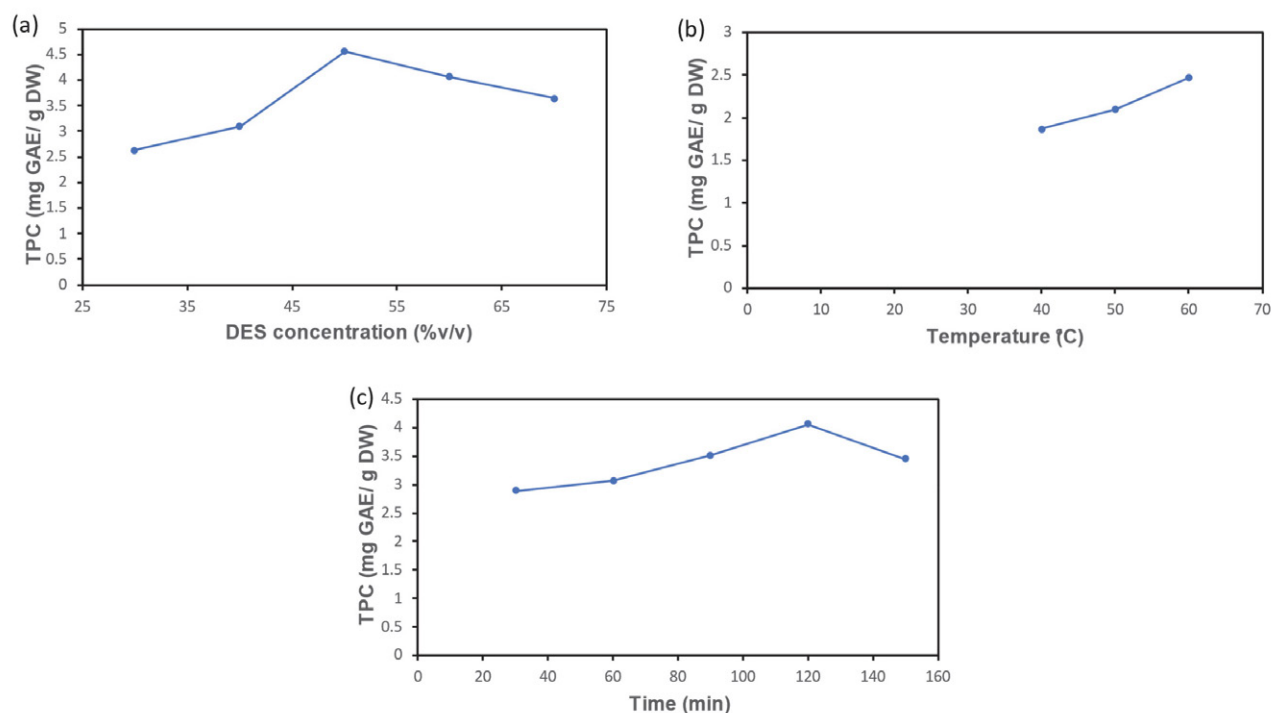


Figure 1. Single-factor experiments showing the effect of HAE parameters on TPC.

The analysis of variance (ANOVA) was performed to determine the interaction between the independent variables and their influence on the observed responses. Co-efficient of determination (R^2) was used to determine the adequacy of the model, and p -values determined the significance of the model. The p -values < 0.05 were considered significant statistically. Lack of fit represents the failure of the model to describe the relationship between variables and the responses.

3. Results and Discussion

3.1. HAE Single-factor Experiments

The single-factor experiments were conducted to discover the effective factors and their levels on the extraction of phenolics. The outcomes of these experiments are shown in Figure 1. Shaking speed (200 rpm) and solvent-to-solid ratio (30 mg L⁻¹) were kept constant.

Figure 1a shows that as the concentration of the DES increased from 30% to 50%, there was a corresponding increase in TPC. A further increase in DES concentration, however, resulted in a decrease of TPC. Figure 1b displays the effect of temperature on TPC while keeping the other factors (DES concentration and time) constant. There was an increase in TPC as the temperature increased from 40 °C to 60 °C. Figure 1c exhibits the effect of time on TPC while keeping all other factors constant. As the time increased to 120 min, there was a corresponding increase in TPC.

The single-factor experiments were very useful for designing the optimization experiments for HAE as well as UAE. Figure 1a shows the increase in TPC was due to the decrease in polarity with the increasing DES concentration, which enabled moderately polar polyphenols to be extracted into the solvent.²⁶ However, as the DES concentration increased beyond 50%, TPC started to decrease. This may be because of the increased viscosity of the solvent, which made the solvent less able to penetrate into the plant biomass.²⁷ Figure 1b shows an increase in TPC can be attributed to the mass transfer. Kinetic energy of the system increases with the increase in temperature resulting in a stronger interaction between the solvent and the plant biomass. Moreover, increase in temperature also results in a decrease in the solvent viscosity. As a result, the solvent penetrates more effectively into the plant biomass extracting a higher amount of phenolics.²⁸ Figure 1c shows an increase in TPC with solvent, it can be attributed to an effective exposure of the plant biomass to the solvent, allowing the release of phenolic compounds from the biomass. However, an extended exposure of the plant material to the solvent can cause a breakdown of the phenolic compounds. That may lead to a decrease in TPC.²⁹

3.2. UAE Single-factor Experiments

The results of the UAE single-factor experiments are shown in Figure 2. Power (110 W), frequency (42 kHz), and solvent-to-solid ratio (30 mg L⁻¹) were kept constant.

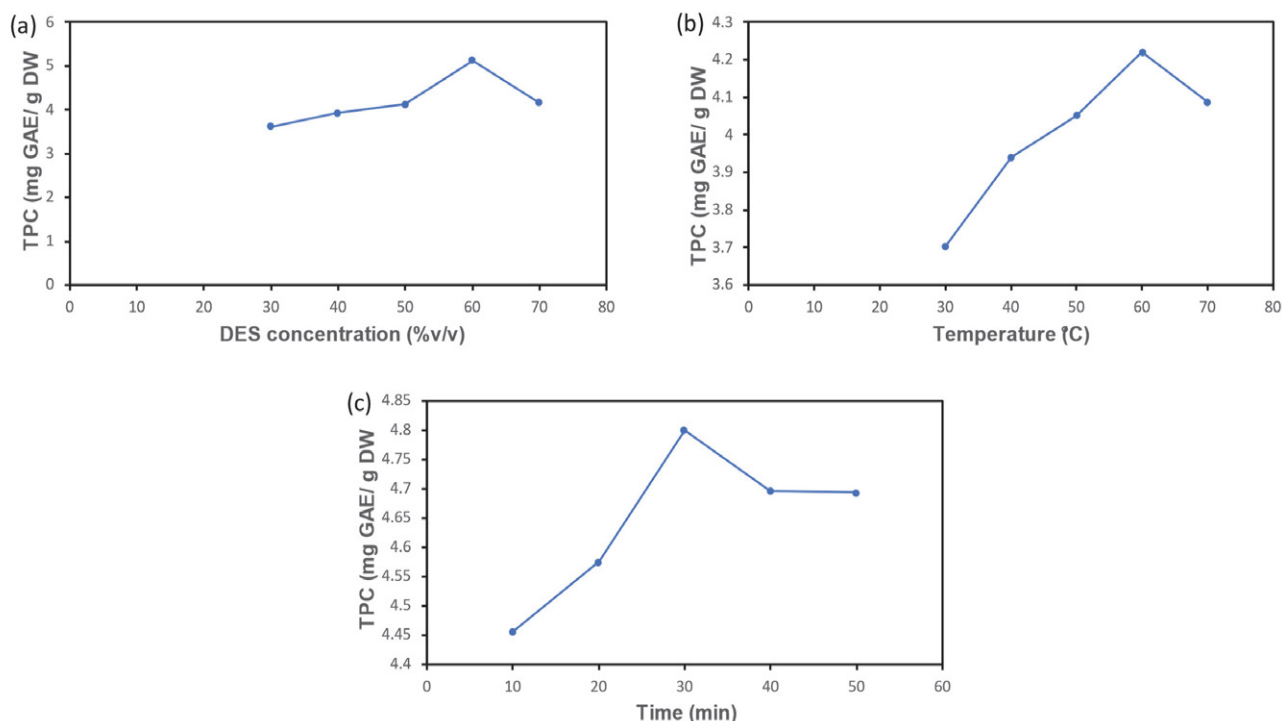


Figure 2. Single-factor experiments showing the effect of UAE parameters on TPC.

Figure 2a shows that there is a considerable effect of DES concentration on TPC. It was noted that as the DES concentration increased from 30% to 60%, TPC also increased. However, after reaching 60% concentration, the TPC started decreasing with any further increase in the DES concentration. Figure 2b demonstrates that TPC increases with the temperature until 60 °C, however, beyond that it starts decreasing. Figure 2c displays the effect of ultrasound treatment time on TPC. With time TPC shows an increase and reaches a maximum at 30 min after which TPC decreases.

Figure 2a shows that, due to the DES being more viscous than water, increased DES concentration led to a corresponding increase in the viscosity of the solution. With the high viscosity of the solvent, it was difficult for it to penetrate the plant biomass. This effect might be responsi-

ble for the decrease in TPC at higher DES concentration.²⁶ Figure 2b shows that the high temperature might be damaging heat-sensitive phenolics that undergo chemical degradation at elevated temperatures.³⁰ Figure 2c shows that cavitation effect produced through various mechanisms causes ultrasound waves to promote release of chemical compounds from the cell matrix. However, ultrasound treatment for a certain threshold duration of time may cause breakdown of the chemicals and thus show a lower TPC. Many studies have shown this trend.³¹

3. 3. HAE Optimization

The results of HAE optimization experiments as per the Box–Behnken design of experiment are shown in Table 1.

Table 1. Box–Behnken designs of experiments for HAE and UAE and results.

Heat-assisted extraction (HAE)							
Independent variables				Responses			
Run order	A: DES concentration (%v/v)	B: Temperature (°C)	C: Time (min)	TFC mg RE g ⁻¹ DW	TPC mg GAE g ⁻¹ DW	RSA mg AAE g ⁻¹ DW	ICA mg EDTAE g ⁻¹ DW
1	40	50	75	3.10	2.81	5.38	2.20
2	50	50	120	3.75	3.16	5.89	2.47
3	30	50	120	2.96	2.71	5.32	1.33
4	50	40	75	2.88	2.76	5.56	2.66
5	40	60	120	3.81	3.23	6.35	1.83
6	40	50	75	3.55	2.87	5.88	1.78
7	30	60	75	3.72	2.64	6.45	1.51
8	40	40	30	2.65	2.79	5.15	2.25
9	50	50	30	3.19	3.22	5.95	2.36
10	50	60	75	3.81	3.23	6.72	2.70
11	30	50	30	2.58	2.55	5.52	1.63
12	40	60	30	3.50	3.13	6.36	1.95
13	40	50	75	3.66	2.77	5.64	1.99
14	40	40	120	3.04	2.74	5.26	2.04
15	30	40	75	2.25	2.32	5.16	1.53
Ultrasound-assisted extraction (UAE)							
1	30	30	20	3.38	4.86	10.18	2.16
2	45	45	20	4.05	5.72	11.81	2.88
3	45	30	30	4.95	5.42	10.63	2.78
4	60	45	10	5.20	6.34	12.35	3.35
5	45	60	30	5.53	5.61	10.95	3.04
6	45	45	20	4.39	5.33	11.25	3.19
7	30	45	30	5.02	5.48	10.24	2.35
8	45	60	10	4.03	6.32	11.50	3.06
9	45	45	20	4.57	5.67	11.83	2.87
10	45	30	10	3.73	5.35	10.97	2.98
11	60	45	30	6.57	6.27	11.87	3.45
12	60	30	20	4.75	5.68	12.15	3.53
13	60	60	20	6.66	6.70	13.10	3.47
14	30	60	20	4.70	5.30	10.56	2.55
15	30	45	10	3.61	5.41	10.97	2.21

Table 2. Predicted models and their regression equations based on significant terms.

Heat-assisted extraction (HAE)			
Response	Model	Model equation	Eq. No.
TPC	Linear	HAE-TPC = $2.86 + 0.2687A + 0.2025B$	Eq. 1
TFC	Linear	HAE-TFC = $3.23 + 0.2650A + 0.5025B + 0.2050C$	Eq. 2
RSA	Linear	HAE-RSA = $5.77 + 0.2087A + 0.5937B$	Eq. 3
ICA	Linear	HAE-ICA = $2.92 + 0.5238A$	Eq. 4
Ultrasound-assisted extraction (UAE)			
TPC	Linear	UAE-TPC = $5.70 + 0.3275A + 0.4925B$	Eq. 5
TFC	Linear	UAE-TFC = $4.74 + 0.5137A + 0.8087B + 0.6875C$	Eq. 6
RSA	Linear	UAE-RSA = $11.36 + 0.2725A + 0.9400B$	Eq. 7
ICA	Linear	UAE-ICA = $2.92 + 0.5662B$	Eq. 8

The data was fitted in the 2nd order polynomial equation to obtain mathematical models for the responses. ANOVA was carried out to determine the significance of the predicted model and the terms. The model equations including only the significant terms are shown in Table 2.

For each response, a linear model was predicted. Based on the *p*-values and lack of fit *p*-values of the models, the significance of the predicted models was determined. The models were regarded significant if their *p*-values were less than 0.050 and lack of fit *p*-values were higher than 0.050. Similarly, the terms of a model were considered as significant if their *p*-values were less than 0.050. The ANOVA details are given in Table 3 while the coefficients are shown in Table 4. The predicted models were further supported by *R*², adjusted *R*² and predicted *R*² values (Tables 3 and 4).

3. 3. 1. Effects of HAE Parameters on Responses

In HAE, the term A (DES concentration) has a significant positive effect on all the responses. Term B (temperature) also significantly affected all the responses, except ICA. Term C (time) has significant effect only on TFC.

All the factors affected the responses positively. It means that within the experimental ranges of the factors, an increase in them resulted in an increase in the responses. Figures 3a and 3b show that for HAE, DES concentration affects the responses positively. The DES as such is a viscous liquid. Water as a diluent lowers the DES viscosity and, therefore, increases its ability to diffuse into the plant biomass and extract its chemical constituents more effectively.³²

Figure 3a shows that the temperature has been demonstrated in studies to facilitate the extraction of phenolics from plant roots. It increases the kinetic energy of the system creating strong interaction between the solvent and the plant biomass being extracted. Temperature also decreases the viscosity of the solvent enabling it to penetrate the plant biomass more effectively. Both these effects result in an enhanced extraction of chemical constituents of the biomass.

Figures 3c and 3d show that temperature is more significant as compared to the other factors. An elevated temperature

lowered the viscosity of the extracting solvent, resulting in increased movement of phytochemicals from the plant cell wall into the solvent. As a result, more flavonoids were extracted from the plant material.

Interestingly, time was not a significant factor in TPC, RSA, and ICA. This may be because the extraction rate is initially high and becomes gradually slower as time passes, resulting in little change in the overall extraction efficiency over time.³³ However, time was a significant factor in TFC indicating that a change in time significantly affects the extraction of TFC.

Furthermore, the dilution of DES also played a role in TFC extraction. As the ratio of the DES concentration increases, the extracting solvent becomes less polar. This change in polarity allowed flavonoids with moderate polarity to be extracted more efficiently into the extracting solvent.³⁴

Figures 3e and 3f show a drastic increase in RSA with increasing temperature demonstrating that temperature-tolerant phytochemicals are extracted into the solvent which is responsible for the radical scavenging activity. A slight increase of RSA with an increase in DES concentration shows to reduce the polarity of DES which makes it possible for moderately polar phytochemicals to transfer into the extracting solvent. Longer exposure may adversely affect the antioxidant activity of the extracted polyphenols. This may be due to the degradation of the extracted antioxidants over time.³⁵

In the current study, temperature and time did not have significant effect on ICA as shown in Figures 3f and 3g. Elevated temperatures for longer extraction time can cause the degradation of the phytochemicals which shows the iron chelating activity. On the other hand, the DES concentration had a significant effect on the ICA. As the DES concentration increases the extracting medium becomes less polar, resulting in better extraction of natural products having similar polarity, such as vitamins, proteins, and carbohydrates that can influence the metal chelating activity. Polyphenols are not the only bioactive compounds that show ICA. Other compounds, such as vitamins and proteins, can also contribute to the overall metal-chelating activity of the extracted compounds.³⁶

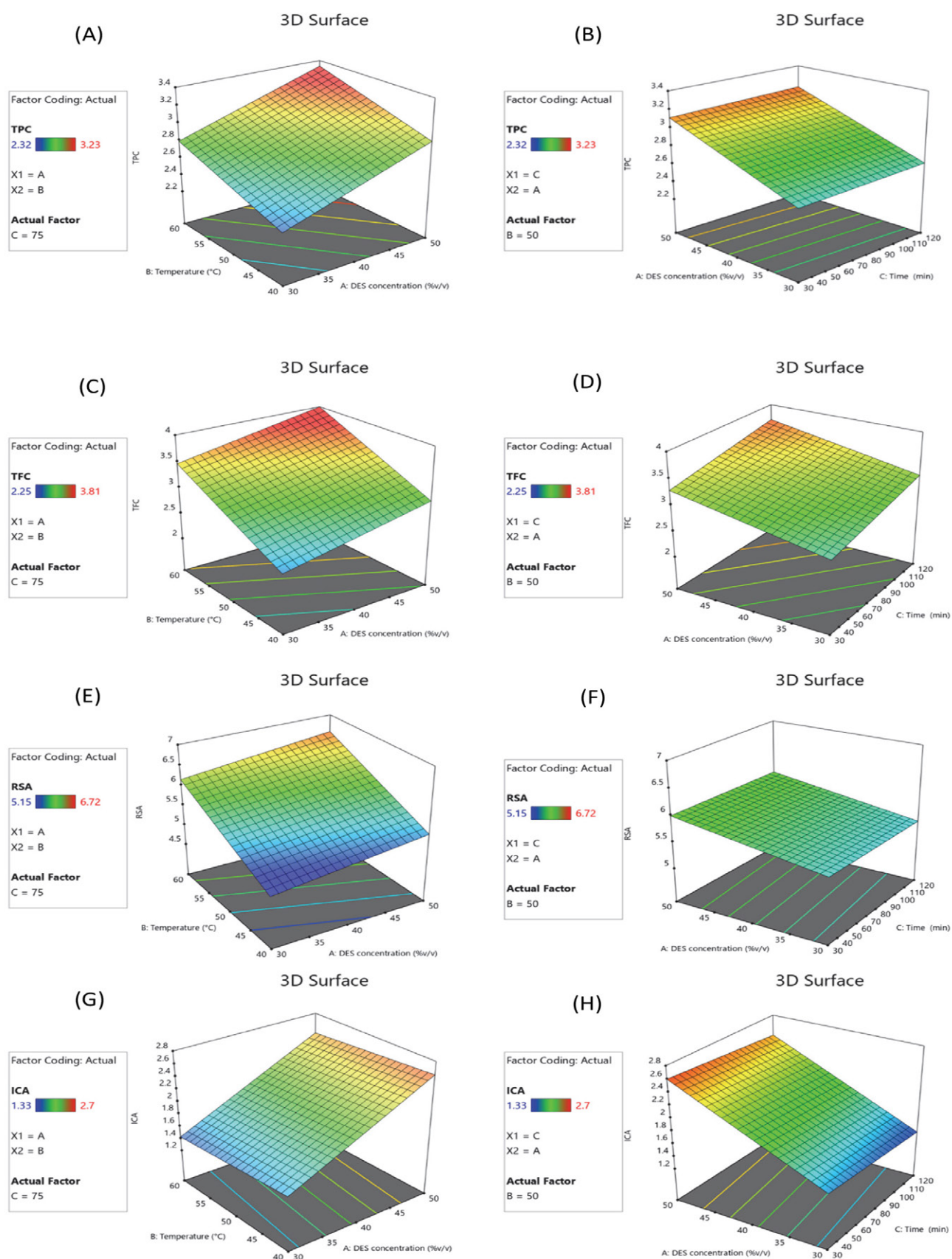


Figure 3. HAE-3D surface plots show combined effect of any two factors on the responses.

Table 3. HAE ANOVA table for all responses.

Source	TPC (mg GAE g ⁻¹ DW)		TFC (mg RE g ⁻¹ DW)		RSA (mg AAE g ⁻¹ DW)		ICA (mg EDTAE g ⁻¹ DW)	
	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value
Model	<0.0001	21.34	0.0001	17.99	<0.0001	27	<0.0001	33.79
A	<0.0001	40.7	0.0081	10.39	0.0124	8.9	<0.0001	98.51
B	0.0005	23.11	<0.0001	37.36	<0.0001	72.02	0.2703	1.35
C	0.6649	0.1981	0.0298	6.22	0.7803	0.0817	0.2473	1.52
Lack of fit	0.1379	6.63	0.7939	0.5284	0.7863	0.5432	0.8657	0.3952
<i>R</i> ²	0.8533		0.8307		0.8804		0.9021	
Adjusted <i>R</i> ²	0.8133		0.7845		0.8478		0.8761	
Predicted <i>R</i> ²	0.6964		0.7235		0.7945		0.8261	

Table 4. Coefficient table for HAE and UAE.

Heat-assisted extraction (HAE)				
Model	TPC Linear	TFC Linear	DPPH Linear	ICA Linear
Intercept	2.86	3.23	5.75	2.02
A	0.2687	0.2650	0.2085	0.5238
B	0.2025	0.5025	0.5937	-0.0613
C	0.0188	0.2050	-0.0200	-0.0650
<i>p</i> -value	<0.0001	0.0001	<0.0001	<0.0001
Lack of fit <i>p</i> -value	0.1379	0.7939	0.7863	0.8657
<i>R</i> ²	0.8533	0.8307	0.8804	0.9021
Adjusted <i>R</i> ²	0.8133	0.7845	0.8478	0.8754
Predicted <i>R</i> ²	0.6964	0.7235	0.7945	0.8261

Ultrasound-assisted extraction (UAE)				
Model	Linear	Linear	Linear	Linear
Intercept	5.70	4.74	11.36	2.92
A	0.3275	0.5137	0.2725	0.0837
B	0.4925	0.8087	0.9400	0.5662
C	-0.0800	0.6975	-0.2625	0.0025
<i>p</i> -value	0.0002	0.0001	<0.0001	<0.0001
Lack of fit <i>p</i> -value	0.5097	0.2607	0.5242	0.8426
<i>R</i> ²	0.8225	0.8382	0.8522	0.9303
Adjusted <i>R</i> ²	0.7741	0.7940	0.8119	0.9112
Predicted <i>R</i> ²	0.6590	0.7009	0.7288	0.8772

3. 4. UAE Optimization

The UAE results are shown in Table 1 and regression equations based on only significant terms are given in Table 2. The coefficients are given in Table 3 while ANOVA details are given in Table 5.

For all the responses, linear models were predicted which were well fitted based on the significant *p*-values and nonsignificant lack of fit *p*-values. The models were further supported by *R*², adjusted *R*² and predicted *R*² values.

3. 4. 1. Effects of UAE Parameters on Responses

Like in HAE, TPC and RSA in UAE were affected by the terms A and B, and TFC was affected by A, B and C.

However, in UAE, ICA was not affected by A or C, but only by B. All the factors affected the responses positively. It means that within the experimental ranges of the factors, an increase in them resulted in an increase in the responses.

For UAE, temperature imparts a crucial role in extracting phenolics from plant biomass. The effect can be seen in Figures 4a and 4b. This is because higher temperatures lower the viscosity of the liquids, which in turn speeds up the transfer of the bioactive molecules into the solvent. Thus, the polyphenols can be extracted efficiently by increasing the temperature. DES was diluted with water to lower the viscosity of the DES, making it easier for the extracting medium to penetrate plant tissues and extract the desired phenolic compounds. This method is beneficial in increasing the phenolic content extracted. However, it is important to note that an increase in water content in DES increases the solvent polarity resulting in poor phenolics. Therefore, a balance must be maintained between the water content and the DES for optimal extraction. Prolonged exposure to elevated temperatures can cause phenolic compounds to decompose, reducing their concentration and bioactivity. Therefore, optimization helps to achieve maximum extraction efficiency while preserving the integrity of the polyphenols extracted.³⁷

Interestingly, both TPC and TFC have similar *R*² values. Since the *R*² value represents a goodness of fit of a regression model, it indicates how well the data points fit the regression line. A similar *R*² value for TPC and TFC suggests that the relationship between the two variables is similar in strength and direction.

Figures 4c and 4d show that temperature, DES concentration, and time have a considerable impact on TFC. However, when comparing these three factors, it becomes obvious that temperature imparts a less crucial role in the TFC. The probable reason behind this is that as the temperature increases, the vapor pressure difference between the inside and outside of the collapsing bubbles decreases, leading to a decrease in the intensity of the collapsing bubbles. As we know, the collapse of bubbles produced by cavitation is responsible for the extraction process. The force created by these collapsing bubbles damages the plant cell,

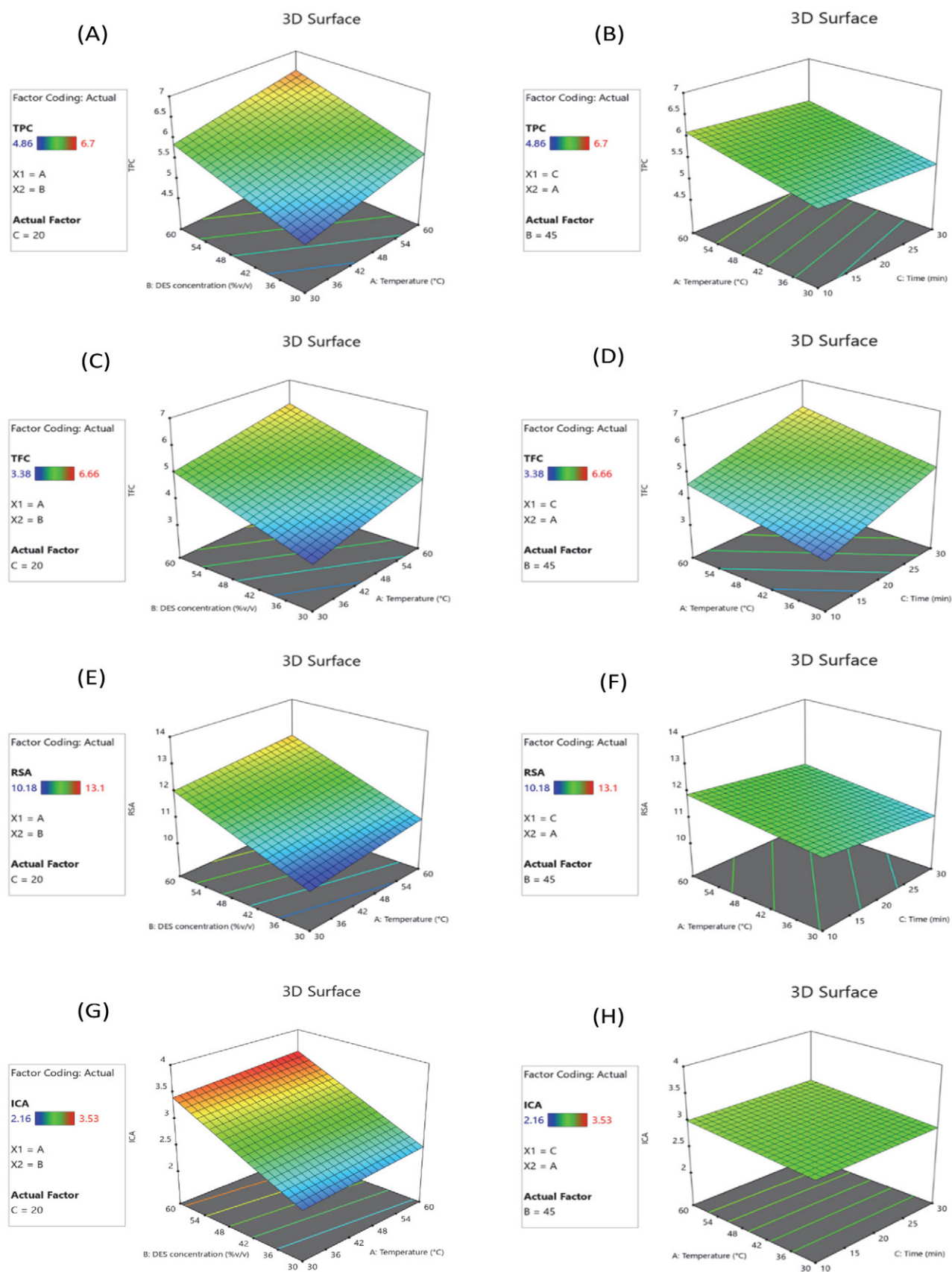


Figure 4. UAE-3D surface plots show combined effect of any two factors on the responses.

Table 5. UAE ANOVA for all responses.

Source	TPC (mg GAE g ⁻¹ DW)		TFC (mg RE g ⁻¹ DW)		RSA (mg AAE g ⁻¹ DW)		ICA (mg EDTAE g ⁻¹ DW)	
	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value
Model	0.0002	17	0.0001	18.99	<0.0001	21.14	<0.0001	48.91
A	0.0024	15.35	0.0072	10.81	0.0555	4.59	0.1040	3.14
B	0.0001	34.72	0.0003	26.8	<0.0001	54.57	<0.0001	143.6
C	0.3591	0.9161	0.0011	19.36	0.0635	4.26	0.9588	0.0028
Lack of fit	0.5097	1.29	0.2607	3.20	0.5242	1.24	0.8426	0.4374
R ²	0.8225		0.8382		0.8522		0.9303	
Adjusted R ²	0.7741		0.7940		0.8119		0.9112	
Predicted R ²	0.6590		0.7009		0.7288		0.8772	

thereby releasing the phytochemicals into the extracting solvent. On the other hand, time plays a crucial part in the extraction process. A longer extraction period can increase the chance of the collapsing bubbles produced by cavitation. These collapsing bubbles can then disrupt the plant cell wall, causing the phytochemicals to diffuse into the extracting solvent more efficiently.³⁸

Figures 4e and 4f show the slight increases in RSA observed with increasing temperature suggesting that antioxidants are more easily hydrolysed at elevated temperatures. As the concentration of DES increases, more antioxidants are solubilized in the solvent, leading to an increase in radical scavenging activity. This finding highlights the advantages of utilizing DESs as solvents in the extraction of antioxidants. Prolonged exposure to high temperature does not impact the extraction of antioxidants, likely due to the decomposition over time.³⁹

Figures 4f and 4g show that the ICA is favoured by an increase in DES concentration, but not by the tempera-

ture or longer extraction process. Specifically, the results suggest that the solvent's polarity decreases as DES concentration is increased, which facilitates the extraction of moderately polar bioactive substances responsible for the iron chelating activity. However, elevated temperatures and prolonged exposure to solvents do not have a significant effect on iron chelating activity which can lead to the degradation of heat-sensitive iron chelating agents.

3. 5. Process Optimization and Experimental Verification

Numerical optimization was conducted to discover a single model of all the responses. For HAE and UAE, numerical optimization was done by keeping the independent factors at 'in range' option while the responses at 'maximize'. Under these constraints, the desirability factors for HAE and UAE were 0.935 and 0.882, respectively, which were close to 1 and, thus, a strong indication of the signif-

Table 6. HAE and UAE predicted and experimental values of the responses obtained at optimal conditions.

Heat-assisted extraction (HAE)				
Input and output parameters	Goal values	Predicted	Experimental values	Percentage error %
DES concentration (%v/v)	in range			
Temperature (°C)	in range			
Time (min)	in range			
TPC (mg GAE g ⁻¹ DW)	Maximize	3.33	3.24 ± 0.14	-2.70
TFC (mg RE g ⁻¹ DW)	Maximize	3.98	3.81 ± 0.10	-4.27
RSA (mg AAE g ⁻¹ DW)	Maximize	6.57	6.38 ± 0.19	-2.89
ICA (mg EDTAE g ⁻¹ DW)	Maximize	2.48	2.61 ± 0.08	5.24
Ultrasound-assisted extraction (UAE)				
DES concentration (%v/v)	in range			
Temperature (°C)	in range			
Time (min)	in range			
TPC (mg GAE g ⁻¹ DW)	Maximize	6.51	6.34 ± 0.17	-2.61
TFC (mg RE g ⁻¹ DW)	Maximize	6.08	5.78 ± 0.17	-4.93
RSA (mg AAE g ⁻¹ DW)	Maximize	12.56	12.78 ± 0.16	1.75
ICA (mg EDTAE g ⁻¹ DW)	Maximize	3.57	3.64 ± 0.04	-2.24

icance of the models. For HAE, the optimal conditions were DES concentration 50%, temperature 60 °C, and time 75 min, and for UAE, DES concentration 60%, temperature 60 °C, and time 20 min. Validation experiments were performed under these conditions and the predicted and experimental values of the responses are given in Table 6.

The minimal percentage errors ranging from 1.75 to 5.24% given in Table 6 indicate a good correlation between the predicted and experimental values of the given responses and fitted well. This leads to the conclusion that within the experimental domain under study, polynomial equations are valid, and they may be employed for point prediction.

The efficacy of both HAE and UAE were tested in terms of response TPC, TFC, RSA, and ICA for both techniques. As Table 6 shows, UAE was more effective than HAE in all the responses at the optimum conditions. UAE also required much less time (only 20 min) as compared to 75 min of HAE. This is an important advantage of UAE over HAE. Many studies have shown similar results when compared to conventional technologies for extraction. Extraction of antioxidants from *Limonium sinuatum* was carried out by UAE at the optimal extraction time 9.8 min showing higher antioxidant activity as compared to maceration and Soxhlet extraction. UAE remarkably reduces the extraction period while enhancing the extraction yield and antioxidant activity.⁴⁰ In another study, polyphenolics extraction was carried out using UAE from *Thymus serpyllum*. L. herb compared to HAE and maceration was found to be more effective in all responses, while HAE and maceration do not have a significant difference among responses.³³ Finally, UAE has also been shown to be very efficient in extracting polyphenolics from *Adansonia digitata* which proved to be significant in terms of TPC, TFC, and antioxidant activity, when compared to HAE and maceration at the optimal time of 20 min.⁴¹

4. Conclusions

Extraction optimization of phenolics including flavonoids, radical scavengers, and iron-chelators from *W. somnifera* roots was successfully done using UAE and HAE and glycerol-sodium acetate DES. Well-fitted linear models were obtained for all the responses in both techniques. DES concentration and temperature were the most influential factors in both of the techniques. Optimum conditions suggested by numerical optimization for UAE and HAE were almost the same, except time which was much less in the case of UAE as compared to HAE. Response values were also much higher in UAE than in HAE. TPC, TFC, RSA and ICA of UAE were 6.51, 6.08, 12.56, and 3.57, respectively, which were much higher than for HAE being 3.33, 3.98, 6.57, and 2.48, respectively.

Thus, UAE was not only more efficient but also less time demanding. The optimized models were strongly

supported by the validation study with minimal % errors. The current study can be used for the development of processes that can be applied on an industrial scale for the extraction of bioactive compounds from *W. somnifera*.

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Competing interest statement

The authors declare no competing interest of any type.

5. References

1. P. K. Mukherjee, S. Banerjee, S. Biswas, B. Das, A. Kar, C. Katiyar, *J. Ethnopharmacol.* **2021**, 264, 113157. DOI:10.1016/j.jep.2020.113157
2. S. Paul, S. Chakraborty, U. Anand, S. Dey, S. Nandy, M. Ghorai, S. C. Saha, M. T. Patil, R. Kandimalla, J. Proćkó, A. Dey, *Biomed. Pharmacother.* **2021**, 143, 112175. DOI:10.1016/j.biopha.2021.112175
3. S. Aslam, N. I. Raja, M. Hussain, M. Iqbal, M. Ejaz, D. Ashfaq, H. Fatima, M. A. Shah, M. Ehsan, *Am. J. Plant Sci.* **2017**, 8, 1159–1169. DOI:10.4236/ajps.2017.85076
4. S. Rayees, F. Malik: *Withania somnifera*: From Traditional Use to Evidence Based Medicinal Prominence. In: S. Kaul, R. Wadhwa (Eds.) *Science of Ashwagandha: Preventive and Therapeutic Potentials*. **2017**, Springer, Cham.. DOI:10.1007/978-3-319-59192-6_4
5. N. Alam, M. Hossain, M. I. Khalil, M. Moniruzzaman, S. A. Sulaiman, S. H. Gan, *BMC Complement. Altern. Med.* **2011**, 11, 65. DOI:10.1186/1472-6882-11-65
6. L. Cornara, M. Biagi, J. Xiao, B. Burlando, *Front. Pharmacol.* **2017**, 8, 412. DOI:10.3389/fphar.2017.00412
7. F. Chemat, M. Abert-Vian, A. S. Fabiano-Tixier, J. Strube, L. Uhlenbrock, V. Gunjevic, G. Cravotto, *Trends Anal. Chem.* **2019**, 118, 248–263. DOI:10.1016/j.trac.2019.05.037
8. N. Rombaut, A. S. Tixier, A. Bily, F. Chemat, *Biofuels, Bioprod. Biorefin.* **2014**, 8, 530–544. DOI:10.1002/bbb.1486
9. K. Papoutsis, P. Pristijono, J. B. Golding, C. E. Stathopoulos, M. C. Bowyer, C. J. Scarlett, Q. V. Vuong, *Eur. Food Res. Technol.* **2018**, 244, 1353–1365. DOI:10.1007/s00217-018-3049-9
10. L. Panzella, F. Moccia, R. Nasti, S. Marzorati, L. Verotta, A. Napolitano, *Front. Nutr.* **2020**, 7, 60. DOI:10.3389/fnut.2020.00060
11. H. Koraqi, B. Qazimi, C. Česko, A. T. Petkoska, *Acta Chim. Slov.* **2022**, 69, 665–673. DOI:10.17344/acsi.2022.7559
12. A. S. Dheyab, M. F. Abu Bakar, M. AlOmar, S. F. Sabran, A. F. Muhamad Hanafi, A. Mohamad, *Separations*. **2021**, 8, 176. DOI:10.3390/separations8100176

13. C. B. T. Pal, G. C. Jadeja, *Food Sci. Technol.* **2020**, *26*, 78–92. DOI:10.1177/1082013219870010
14. E. Mouratoglou, V. Malliou, D. P. Makris, *Waste Biomass Valor.* **2016**, *7*, 1377–1387. DOI:10.1007/s12649-016-9539-8
15. C. B. T. Pal, G. C. Jadeja, *Biomass Convers. Biorefin.* **2022**, *1–13*. DOI:10.1007/s13399-022-02550-w
16. P. Gullón, B. Gullón, A. Romani, G. Rocchetti, J. M. Lorenzo, *Trends Food Sci. Technol.* **2020**, *101*, 182–197. DOI:10.1016/j.tifs.2020.05.007
17. D. Danjoll-Hashani, Ş. Selen-Işbilir, *Acta Chim. Slov.* **2022**, *69*, 430–436. DOI:10.17344/acsi.2021.7332
18. S. Rodríguez-Rojo, A. Visentin, D. Maestri, M. J. Cocero, *J. Food Eng.* **2012**, *109*, 98–103. DOI:10.1016/j.jfoodeng.2011.09.029
19. H. Guo, S. Liu, S. Li, Q. Feng, C. Ma, J. Zhao, Z. Xiong, *J. Pharm. Biomed. Anal.* **2020**, *185*, 113228. DOI:10.1016/j.jpba.2020.113228
20. C. Wen, J. Zhang, H. Zhang, C. S. Dzah, M. Zandile, Y. Duan, H. Ma, X. Luo, *Ultrason. Sonochem.* **2018**, *48*, 538–549. DOI:10.1016/j.ultsonch.2018.07.018
21. C. S. Dzah, Y. Duan, H. Zhang, C. Wen, J. Zhang, G. Chen, H. Ma, *Food Biosci.* **2020**, *35*, 100547. DOI:10.1016/j.fbio.2020.100547
22. S. Iftikhar, M. T. Qamar, A. Y. Aydar, D. Ahmed, *Folia Hort.* **2022**, *34*, 163–171. DOI:10.2478/fhort-2022-0013
23. S. Ihsan, D. Ahmed, H. Khalid, *J. Medicinal Plants By-products* **2022**, *11*, 119–127. DOI: 10.22092/JMPB.2021.355098.1374.
24. D. Ahmed, M. M. Khan, R. Saeed, *Antioxidants* **2015**, *4*, 394–409. DOI:10.3390/antiox4020394
25. R. Sudan, M. Bhagat, S. Gupta, J. Singh, A. Koul, *BioMed. Res. Int.* **2014**, *2014*, 179865. DOI:10.1155/2014/179865
26. J. B. Barbieri, C. Goltz, F. Batistão Cavalheiro, A. Theodoro Toci, L. Igarashi-Mafra, M. R. Mafra, *Ind. Crops Prod.* **2020**, *144*, 112049. DOI:10.1016/j.indcrop.2019.112049
27. B. Ozturk, C. Parkinson, M. Gonzalez-Miquel, *Sep. Purif. Technol.* **2018**, *206*, 1–13. DOI:10.1016/j.seppur.2018.05.052
28. R.-E. Ghitescu, I. Volf, C. Carausu, A.-M. Bühlmann, I. A. Gilca, V. I. Popa, *Ultrason. Sonochem.* **2015**, *22*, 535–541. DOI:10.1016/j.ultsonch.2014.07.013
29. H. Hosseini, S. Bolourian, E. Yaghoubi Hamgini, E. Ghanuni Mahababadi, *J. Food Process. Preserv.* **2018**, *42*, e13778. DOI:10.1111/jfpp.13778
30. A. Antony, M. Farid, *Appl. Sci.* **2022**, *12*, 2107. DOI:10.3390/app12042107
31. B. Sik, Z. Ajtony, E. Lakatos, R. Szekelyhidi, *Heliyon* **2022**, *8*, e12048. DOI:10.1016/j.heliyon.2022.e12048
32. S. K. Saha, S. Dey, R. Chakraborty, *J. Mol. Liq.* **2019**, *287*, 110956. DOI:10.1016/j.molliq.2019.110956
33. A. A. Jovanović, V. B. Đorđević, G. M. Zdunić, D. S. Pljevljakušić, K. P. Šavikin, D. M. Godevac, B. M. Bugarski, *Sep. Purif. Technol.* **2017**, *179*, 369–380. DOI:10.1016/j.seppur.2017.01.055
34. Z.-L. Sheng, P.-F. Wan, C.-L. Dong, Y.-H. Li, *Ind. Crops Prod.* **2013**, *43*, 778–786. DOI:10.1016/j.indcrop.2012.08.020
35. G. A. Nayik, B. N. Dar, V. Nanda, *Int. J. Food Prop.* **2016**, *19*, 1738–1748. DOI:10.1080/10942912.2015.1107733
36. İ. Gulcin, S. H. Alwasel, *Processes* **2022**, *10*, 132. DOI:10.3390/pr10010132
37. B. Zheng, Y. Yuan, J. Xiang, W. Jin, J. B. Johnson, Z. Li, C. Wang, D. Luo, *LWT* **2022**, *154*, 112740. DOI:10.1016/j.lwt.2021.112740
38. T. P. Vo, L. N. H. Nguyen, N. P. T. Le, T. P. Mai, D. Q. Nguyen, *Curr. Res. Food Sci.* **2022**, *5*, 2013–2021. DOI:10.1016/j.crfs.2022.09.021
39. I. A. Almusallam, I. A. M. Ahmed, E. E. Babiker, F. Y. Al-Juhaimi, G. J. Fadimu, M. A. Osman, S. A. Al Maiman, K. Ghafoor, H. A. Alqah, *LWT* **2021**, *140*, 110816. DOI:10.1016/j.lwt.2020.110816
40. D.-P. Xu, J. Zheng, Y. Zhou, Y. Li, S. Li, H. B. Li, *Food Chem.* **2017**, *217*, 552–559. DOI:10.1016/j.foodchem.2016.09.013
41. B. B. Ismail, M. Guo, Y. Pu, W. Wang, X. Ye, D. Liu, *Ultrason. Sonochem.* **2019**, *52*, 257–267. DOI:10.1016/j.ultsonch.2018.11.023

Povzetek

Raziskovali smo ekstrakcijo bioaktivnih spojin iz korenin rastline *Withania somnifera* pod vplivom ultrazvoka (UAE) ali pa ob uporabi segrevanja (HAE), kjer smo kot topilo uporabili evtektično zmes (DES) natrijevega acetata in glicerola. Ekstrakcije smo študirali s pomočjo metodologije površin odgovora (RSM). Določali smo celokupno vsebnost fenolov (TPC) v mg galne kisline (in njej ekvivalentnih snovi) na g suhe snovi (mg GAE g⁻¹ DW), celokupno vsebnost flavonoidov (TFC) v mg rutina (in njemu ekvivalentnih snovi) na g suhe snovi (mg RE g⁻¹ DW), aktivnost lovljenja radikalov (RSA) v mg askorbinske kisline (in njej ekvivalentnih snovi) na g suhe snovi (mg AAE g⁻¹ DW) ter aktivnost keliranja železovih ionov (ICA) v mg etilendiamintetraacetatnih ekvivalentov na g suhe snovi (mg EDTAE g⁻¹ DW). Če smo ekstrakcijo izvedli ob uporabi ultrazvoka, smo dobili naslednje vrednosti: 6,51 za TPC, 6,08 za TFC, 12,56 za RSA in 3,57 za ICA; v primeru termične ekstrakcije pa so bile vrednosti sledeče: 3,33 za TPC, 3,98 za TFC, 6,57 za RSA in 2,48 za ICA. Za izvedbo ekstrakcije pod vplivom ultrazvoka so bili optimalni naslednji parametri: koncentracija DES 50 %, temperatura 60 °C in čas 20 min; za termično ekstrakcijo pa se je najbolje izkazala koncentracija DES 60 %, temperatura 60 °C in čas 75 min. Razviti modeli so bili temeljito potrjeni z validacijskimi eksperimenti. Izkazalo se je, da je ekstrakcija rastlinskih snovi iz *W. somnifera* pod vplivom ultrazvoka bolj učinkovita in časovno hitrejša kot pa pri uporabi termične ekstrakcije.



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