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Modeling and optimizing microwave-assisted extraction of antioxidant compounds from marigold (*Calendula officinalis* L.) using response surface methodology

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Abstract: *Calendula officinalis* L. is a commercially important plant that finds application in the treatment of various diseases in traditional medicine. The total antioxidant capacity, radical scavenging activity, and total phenolic content of marigold extracts were investigated by Folin, CUPRAC, and DPPH assays, respectively. The optimum operating conditions of microwave-assisted extraction (MAE) including temperature, extraction time, solvent-to-solid ratio, and solvent concentration were ascertained by employing response surface methodology (RSM). The solvent (ethanol) concentration was the most significant operating factor among all responses of MAE. At the optimum extraction conditions, the maximum yield of total phenolic content, total antioxidant capacity, and radical scavenging activity obtained experimentally were very close to their predicted values, thus showing the suitability of the model used and the success of RSM in optimizing the extraction conditions. Chromatographic analysis of marigold extract was performed by UPLC–PDA–ESI–MS/MS system and chlorogenic acid was the main component (1742.50 ± 42.23 μg/g DS).

Key words: Microwave-assisted extraction, response surface methodology, antioxidants, marigold, UPLC–DAD–ESI–MS/MS analysis

1. Introduction

Marigold (*Calendula officinalis* L.), a member of the family Asteraceae, is commonly used as a traditional medicinal plant for skin disorders, burns, and menstrual irregularities. Moreover, marigold, with yellow or golden-orange flowers, is used as an ointment, tea, or spice [1]. Marigold is therefore a commercially important plant that can be easily grown in Mediterranean regions [2]. It has been reported that marigold contains carotenoids, steroids, terpenoids, and phenolic compounds such as gallic acid, caffeic acid, isoquercitrin, rutin, and various quercetin glucosides [3,4]. It has antiinflammatory and antitumor-promoting properties and strong antioxidant activity [5].

In the literature, many studies have investigated the total flavonoid content (TFC), free radical scavenging activity (RSA), and total phenolic content (TPC) of marigold species using various extraction techniques [1,6,7]. Fatima et al. [8] determined the antioxidant capacity, RSA, UV-hydrogen peroxide-induced DNA damage protection activity, and antimicrobial properties of the ethanolic extract of flowers of *Calendula officinalis*. Zeinsteger et al. [9] reported that *Calendula officinalis* L. protects rat brain mitochondria from peroxidative damage due to its high content of phenolic acids and flavonoids. Erçetin et al. [6] evaluated the antioxidant and cholinesterase inhibitory properties of extracts obtained using different solvents from *Calendula arvensis* L. and

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Calendula officinalis L. The highest free radical scavenging activity was observed in the methanolic extracts of *Calendula officinalis*. In another similar study, the content of polyphenols of marigold extract was determined by TLC and HPLC–UV–DAD and the antioxidant efficiency was estimated from Fe^{3+}/Fe^{2+} reducing power, Cu^{2+} and Fe^{2+} chelation, $\bullet$OH radical scavenging, ABTS [2,2'-azino bis (3-ethylbenzothiazoline-6-sulfonic acid)], and DPPH (2,2'-diphenyl-1-picrylhydrazyl) assays by Hernández-Rosas et al. [10]. Zheng et al. [11] determined the TPC, total flavonoid content, ferric reducing antioxidant capacity, and RSA of samples of 65 flowers commonly consumed in China. Their study showed that marigold has an important place in terms of antioxidant capacity among other samples.

Ćetković et al. reported that the polyphenol content of marigold extract correlated with its antioxidant features [1]. Therefore, the most important step in accurately measuring the antioxidant activity of extract is effective extraction of polyphenols. Solid–liquid extraction is the most widely used technique for the extraction of antioxidants from marigold species. The classical solid–liquid extraction procedure has many disadvantages such as long extraction time, large quantities of toxic organic solvents required, and low extraction yields [12]. Moreover, the optimization of conditions that affect the extraction efficiency such as temperature, solvent-to-solid ratio, and solvent concentration in classical optimization studies is expensive and time-consuming [13]. Response surface methodology (RSM), an effective method that involves statistical and mathematical analysis of data used for optimizing the extraction processes [14], can overcome these limitations. Gong et al. [15] used RSM to investigate solid–liquid extraction parameters of bioactive components from marigold (*Tagetes erecta* L.). Göktaş et al. [16] produced sterilizing agents from *Calendula officinalis* extracts optimized by RSM. To the best of our knowledge, optimization of microwave-assisted extraction of antioxidant compounds from marigold (*Calendula officinalis* L.) using RSM has not been reported yet. In a recent study the ultrasound-assisted extraction (UAE) of flavonoids from pot marigold flowers was optimized by employing a Box–Behnken design. The optimum operating conditions of UAE including temperature, extraction time, solvent-to-solid ratio, and ethanol concentration were ascertained and the total flavonoid content of pot marigold was investigated with aluminum(III) chloride [17].

Marigold is known to have a rich composition including many bioactive compounds such as carotenoids [18], phenolic acids (protocatechuic, vanillic, syringic acids), hydroxycinnamic acids (*p*-coumaric, caffeic, chlorogenic acids), and flavonoids and their glycosides (quercetin, isorhamnetin, isoquercitrin, rutin) [19,20]. Moreover, there are studies in the literature on the determination of the polyphenol content of *Calendula officinalis* L. by chromatographic analysis. Kotb et al. [19] reported that cinnamic acid, chrysin, rutin, caffeic acid, and syringic acid were identified in *Calendula officinalis* L. In another study, Frum [21] found gallic acid, cinnamic acid, syringic acid, ferulic acid, and rutin in the HPLC analysis of *Calendula officinalis* L. extracted through maceration. Augšpole et al. [20] performed the characterization of phenolic contents present in five herbal tea infusions, including *Calendula officinalis* L., by HPLC. Phenolic compounds such as chlorogenic acid, 4-hydroxybenzoic acid, gallic acid, and vanillin were detected in a *Calendula officinalis* L. infusion. Alsaraf et al. [22] performed HPLC analysis on marigold flower extract and detected nine different active compounds including rutin, quercetin derivatives, quercitrin, myricetin, and apigenin, which act as antioxidants to restore skin health.

In the present study, marigold (*Calendula officinalis* L.) extracts for analysis were prepared using microwave-assisted extraction (MAE), which has distinct advantages over conventional solid–liquid extraction methods such as reduced cost and time and increased reproducibility. Many parameters such as solvent con-

centration, temperature, solvent-to-solid ratio, and extraction time influence the yield of the extraction. The optimization of these parameters using RSM presents the maximum extraction yield. Moreover, the phenolic profile of *Calendula officinalis* L. was reported using UPLC–PDA–ESI–MS/MS for the first time.

2. Materials and methods

2.1. Reagent and chemicals

Chemical substances including neocuproine, methanol, ethanol, protocatechuic acid, vanillic acid, syringic acid, isoquercitrin (quercetin 3-β-D-glucoside), ferulic acid, chlorogenic acid, 4-hydroxybenzoic acid, caffeic acid, rosmarinic acid, quercetin, hesperidin, rutin, formic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin–Ciocalteu reagent, copper(II) chloride, ammonium acetate, sodium potassium tartrate ($NaKC_4H_4O_6$), copper(II) sulfate, sodium hydroxide, and sodium carbonate were of analytical reagent grade and sourced from Merck (Darmstadt, Germany). Marigold (*Calendula officinalis* L.) was purchased from Arifoğlu, a commercial herb seller (İstanbul, Turkey). The purchased herbal plant was milled into uniform dry powder by electric chopper and stored at 4 °C.

2.2. Instrumentation

A UV-Vis spectrophotometer (Varian CARY 100) was used to record the UV-visible spectra and absorption measurements. An Eppendorf automatic micropipette, 10–100-μL volume range, was used in the experiments. MAE was performed using an ETHOS - One (Milestone) closed vessel oven system. A Waters Ultra Performance Liquid Chromatography system coupled with a diode array detector (DAD) and Xevo TQD (double quadrupole) mass spectrometer was used for chromatographic analysis.

2.3. Preparation of solutions

Ammonium acetate solution (1 M, pH 7) and $CuCl_2$ solution (10 mM) were prepared in water; neocuproine (Nc) solution (7.5 mM) was prepared in ethanol. All antioxidant standards were freshly prepared in methanol. The reagents used in the Folin assay were prepared as follows: 2% Na_2CO_3 in 0.1 M NaOH is a Lowry A solution; 0.5% $CuSO_4$ in 1% $NaKC_4H_4O_6$ is a Lowry B solution; Lowry C reagent was freshly prepared as a mixture of 50 mL of Lowry A solution + 1 mL of Lowry B solution; Folin–Ciocalteu reagent was prepared from the commercial reagent by diluting with water. The DPPH radical ($DPPH^{\cdot}$) solution (0.2 mM) was prepared with methanol.

2.4. Microwave-assisted extraction of marigold

MAE was performed in a closed vessel oven system equipped with an automatic fiber optic temperature control system.

Thirty experimental MAE runs were carried out with variations in temperature (50–100 °C), solvent concentration (ethanol % in water), extraction time (1–10 min), and solvent-to-solid ratio (mL/0.2 g). In each experimental run, 0.2 g of sample was mixed with varying quantities (5 mL, 12.5 mL, and 20 mL) of ethanol solution (0%, 50%, and 100% (v/v)) in an inner vessel, and a magnetic stirring rod was added to each vessel. The microwave power (500–1500 W) was automatically controlled by a system in the oven according to the temperature of the oven. The marigold sample in solvent was irradiated with microwaves for 3 min to heat it to the desired temperature and for varying extraction times (1 min, 5.5 min, and 10 min) for balance at

the desired temperature with 5 min for cooling. The extracts were successively filtered through filter paper and 0.45-μm PTFE syringe filters. Three replicate extractions were obtained from the marigold sample and spectrophotometric measurements of each marigold extract were repeated three times.

2.5. Determination of total phenolic content

The TPC of the marigold extract was determined using the Folin–Ciocalteu assay as described by Singleton et al. [23]. To a test tube were added $x\mu$L of the marigold extract, 2.5 mL of Lowry C solution, and (1 – x) mL of H_2O in this order. Ten minutes later, 0.25 mL of Folin–Ciocalteu reagent was added, and the blue color formed was measured at 750 nm against a reagent blank after 30 min. TPC was calculated with the following equation using the molar absorptivity of Trolox ($\varepsilon_{TR} = 1.33 \times 10^3$ L mol^{-1} cm^{-1}) [24]: TPC (mmol TR/g dried sample) = (A_{750}/ε_{TR}) (total volume (3.75 mL)/volume of marigold extract taken) (extract volume (mL)/0.2 g dry weight of marigold).

2.6. Total antioxidant capacity assay

The TAC of marigold extract was determined using the CUPRAC assay developed by Apak et al. [25]. A cupric neocuproine complex (Cu(II)-Nc) that comprised 1 mL each of copper(II) chloride, neocuproine, and ammonium acetate buffer solution was reduced to the cuprous chelate (Cu(I)-Nc) in the presence of marigold extract. The final volume, 4.1 mL, was made with the addition of water. The absorbance at 450 nm (A_{450}) was recorded against a reagent blank after 30 min. The TAC was calculated with the following equation using the molar absorptivity of Trolox ($\varepsilon_{TR} = 1.67 \times 10^4$ L mol^{-1} cm^{-1}) [24]: TAC (mmol TR/g dried sample) = (A_{450}/ε_{TR}) (total volume (4.1 mL)/volume of marigold extract taken) (extract volume (mL)/0.2 g dry weight of marigold).

2.7. Free radical scavenging activity assay

The free RSA of the marigold extract was determined using the DPPH assay as described by Sánchez-Moreno et al. [26] with minor modifications. First 2 mL of methanolic DPPH solution (0.2 mM) was added to the mixture solution of x mL of extract and (2 – x) mL of methanol. The mixture was incubated for 30 min at room temperature. At the end of this period, the absorbance was recorded at 525 nm. The radical scavenging activity was calculated with the following equation using the molar absorptivity of Trolox (ε_{TR} being 1.67×10^4 L mol^{-1} cm^{-1} under the specified conditions) [24]: RSA (mmol TR/g dried sample) = (A_{525}/ε_{TR}) (total volume (4.0 mL)/volume of marigold extract taken) (extract volume (mL)/0.2 g dry weight of marigold).

2.8. Chromatographic analysis of antioxidant compounds in marigold extract

The chromatographic analysis of antioxidant compounds in marigold extracts was performed by UPLC–PDA–ESI–MS/MS system (Waters) as described by Çelik et al. [27]; 0.1% HCOOH in bidistilled H_2O (A) and 0.1% HCOOH in MeOH (B) were used as the mobile phase. The antioxidant compounds were analyzed using the following experimental conditions: C18 analytical column (100 × 2.1 mm, 1.7 μm); flow rate = 0.45 mL min^{-1}; $V_{sample} = 10$ μL; temperature of column = 30 °C; λ = 190 to 400 nm; and gradient program: 0 min 90% (A) - 10% (B); 2 min 85% (A) - 15% (B); 3 min 80% (A) - 20% (B); 4 min 70% (A) - 30% (B); 6 min 60% (A) - 40% (B); 8–10 min isocratic elution 50% (A) - 50% (B); 12 min 90% (A) - 10% (B). The ionization conditions for

double quadrupole analyzer mass spectrometry were cone gas flow rate, 50 L/h; desolvation gas flow rate, 650 L/h; collision energy, 20 V; desolvation temperature, 400 °C; capillary voltage, 3 kV. The phenolic antioxidant profile of marigold was characterized according to the mass-to-charge (m/z) ratios of precursor and product ions in multiple reaction monitoring (MRM) mode.

2.9. Experimental design

Using Design-Expert Software Version 11 Trial, a face-centered composite design (FCCD) was applied to the independent parameters (temperature, extraction time, solvent-to-solid ratio, and solvent (ethanol) concentration in water) to optimize the extraction parameters for the antioxidant compounds from marigold. The values of the independent variables and their coded forms with their symbols are given in Table 1. In the present study, TPC (Y_1), TAC (Y_2), and RSA (Y_3) were selected as the response (also known as the dependent) variables. The functional relationship between independent variables and response (dependent variables) can be described as [28]:

$$Y = f(X1, X2, \ldots Xk) + \quad \varepsilon. \tag{1}$$

Table 1. Values of the independent variables and their coded forms with their symbols employed in RSM for optimization of MAE.

Factors	Units	Symbols of the variables	Levels		
			–1	0	1
Temperatures	°C	X_1	50	75	100
Extraction time	min	X_2	1	5.5	10
Ethanol concentration	%, v/v	X_3	0	50	100
Solvent-to-solid ratio	mL/0.2 g	X_4	5	12.5	20

The experimental data conformed to second-order polynomial models, which define the values of TPC, TAC, and RSA as a function of independent variables as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \gamma \tag{2}$$

where Y is the response (dependent variable); X is the independent variable; β_0 is an intercept term; β_i , β_{ii}, and β_{ij} are the interaction coefficients; k is the factor number; and ε is experimental error. Analysis of variance (ANOVA) was conducted to identify the relation between the independent and dependent variables using Design-Expert.

3. Results and discussion

According to the proposed design, 30 experiments were performed in triplicate and the experimental values of TPC, TAC, and RSA are shown in Table 2. The values of TPC, TAC, and RSA were 0.12–1.34 mmol TR/g DS, 0.07–0.68 mmol TR/g DS, and 0.09–0.41 mmol TR/g DS, respectively.

Table 2. FCCD layout and experimental data.

Run no.	Factors					Response	
	X_1	X_2	X_3	X_4	TPC (mmol TR/g DS)	TAC (mmol TR/g DS)	RSA (mmol TR/g DS)
1	50	1	0	5	0.52	0.29	0.17
2	100	1	0	5	1.22	0.57	0.34
3	50	10	0	5	0.59	0.33	0.18
4	100	10	0	5	1.32	0.59	0.35
5	50	1	100	5	0.12	0.09	0.08
6	100	1	100	5	0.15	0.12	0.09
7	50	10	100	5	0.14	0.11	0.10
8	100	10	100	20	0.16	0.13	0.12
9	50	1	0	20	0.75	0.42	0.28
10	100	1	0	20	1.16	0.60	0.36
11	50	10	0	20	0.77	0.43	0.30
12	100	10	0	20	1.09	0.60	0.35
13	50	1	100	20	0.17	0.07	0.10
14	100	1	100	20	0.19	0.15	0.16
15	50	10	100	20	0.20	0.13	0.10
16	100	10	100	20	0.25	0.20	0.18
17	50	5.5	50	12.5	1.00	0.56	0.33
18	100	5.5	50	12.5	1.31	0.52	0.35
19	75	1	50	12.5	0.82	0.45	0.34
20	75	10	50	12.5	1.17	0.65	0.37
21	75	5.5	0	12.5	1.07	0.59	0.34
22	75	5.5	100	12.5	0.35	0.25	0.16
23	75	5.5	50	5	1.21	0.59	0.35
24	75	5.5	50	20	1.33	0.63	0.38
25	75	5.5	50	12.5	1.33	0.66	0.36
26	75	5.5	50	12.5	1.32	0.64	0.38
27	75	5.5	50	12.5	1.34	0.68	0.41
28	75	5.5	50	12.5	1.34	0.65	0.39
29	75	5.5	50	12.5	1.31	0.66	0.38
30	75	5.5	50	12.5	1.31	0.65	0.39

3.1. Modeling and optimization of MAE using RSM

To predict the yield of TPC, TAC, and RSA, a second-order polynomial model was generated using multiple nonlinear regression analysis of the experimental data. The adapted quadratic models for TPC, TAC, and RSA in coded variables are given in Eqs. (3)–(5), respectively.

$$TPC = -0.670985 + 0.027268X_1 + 0.132452X_2 + 0.020142X_3 - 0.003291X_4 - 0.000010X_1X_2 - 0.000103X_1X_3 - 0.0000230X_1X_4 + 0.00000389X_2X_3 - 0.000304X_2X_4 + 0.000020X_3X_4 - 0.000090X_1^2 - 0.010991X_2^2 - 0.000202X_3^2 + 0.000994X_4^2 \quad (3)$$

$$TAC = -0.608758 + 0.023174X_1 + 0.040519X_2 - 0.007091X_3 + 0.0008549X_4 - -0.000031X_1X_2 - 0.000035X_1X_3 - 0.000029X_1X_4 + 0.000020X_2X_3 + 0.000046X_2X_4 - -0.000028X_3X_4 - 0.000124X_1^2 - 0.003148X_2^2 - 0.000077X_3^2 - 0.000085X_4^2 \quad (4)$$

$$RSA = -0.221073 + 0.009808X_1 + 0.010227X_2 + 0.004429X_3 + 0.0009292X_4 + 0.000005X_1X_2 - 0.000015X_1X_3 - 0.000035X_1X_4 + 0.000012X_2X_3 - -0.000065X_2X_4 - 0.000016X_3X_4 - 0.000048X_1^2 - 0.000792X_2^2 - 0.000050X_3^2 - 0.000098X_4^2 \quad (5)$$

Solvent concentration was the most significant operating factor for all MAE responses, while extraction time had the least significant effect on extraction efficiency. Table 3 shows the results of the ANOVA, which demonstrated the significance and adequacy of the developed quadratic models for TPC, TAC, and RSA. All of the models were meaningful and adequate to show the correlation between the response and parameters with low P-values (<0.0001 for TPC, TAC, and RSA) from the ANOVA. The determination coefficient (R^2) showing the model's compatibility with the experimental data and the adjusted determination coefficient (Adj. R^2) were compatible with each other (the difference was less than 0.2). The predicted R^2 values of 0.8996, 0.8687, and 0.8996 agree reasonably well with the adjusted R^2 values of 0.9613, 0.9441, and 0.9613 for TPC, TAC, and RSA, respectively. The adequate precision, which evaluates the signal-to-noise ratio and is desired to be greater than 4, was 20.798, 16.217, and 19.798 for TPC, TAC, and RSA, respectively.

Table 3. ANOVA for the effect of temperature, extraction time, solvent concentration, and solvent-to-solid ratio on the yield of TPC, TAC, and RSA using a quadratic response surface model.

Source	TPC			TAC			RSA		
	Model	Lack of fit	Pure error	Model	Lack of fit	Pure error	Model	Lack of fit	Pure error
df	14	10	5	14	10	5	14	10	5
SS	6.60	0.1340	0.0009	1.32	0.0460	0.0015	0.3736	0.0096	0.0011
MS	0.4714	0.0134	0.0002	0.0944	0.0046	0.0003	0.0267	0.0010	0.0002
F value	52.40	74.8		29.83	15.55		37.49	4.32	
P value	< 0.0001	< 0.0001		< 0.0001	0.0037		< 0.0001	0.0599	

df: Degrees of freedom; SS: sum of squares; MS: mean square.

3.2. Effects of process parameters on MAE

The 3D response surface plots as a function of solvent concentration shown in Figures 1–3 demonstrate different changes in TAC, TPC, and RSA values.

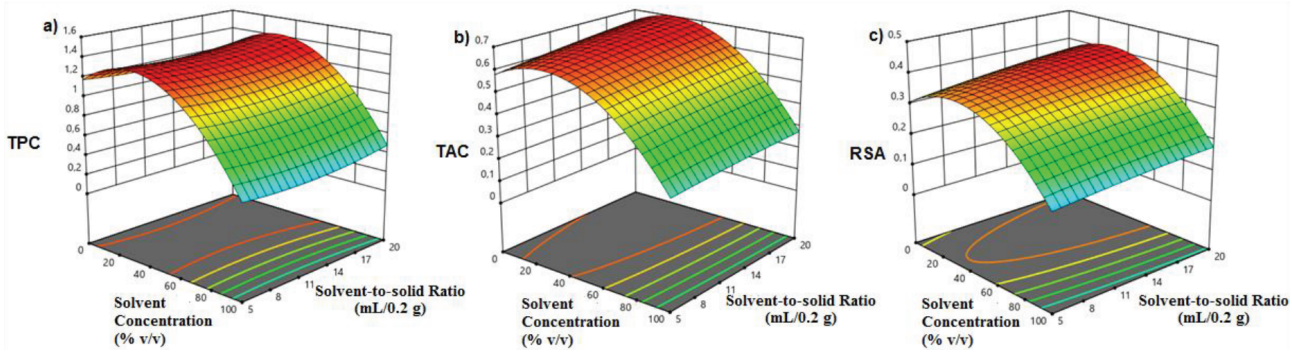

Figure 1. The 3D surface plot for the (a) TAC, (b) TPC, and (c) RSA of *Calendula officinalis* L. extract as a function of solvent concentration to solvent-to-solid ratio (extraction temperature = 75 °C and extraction time = 5.5 min).

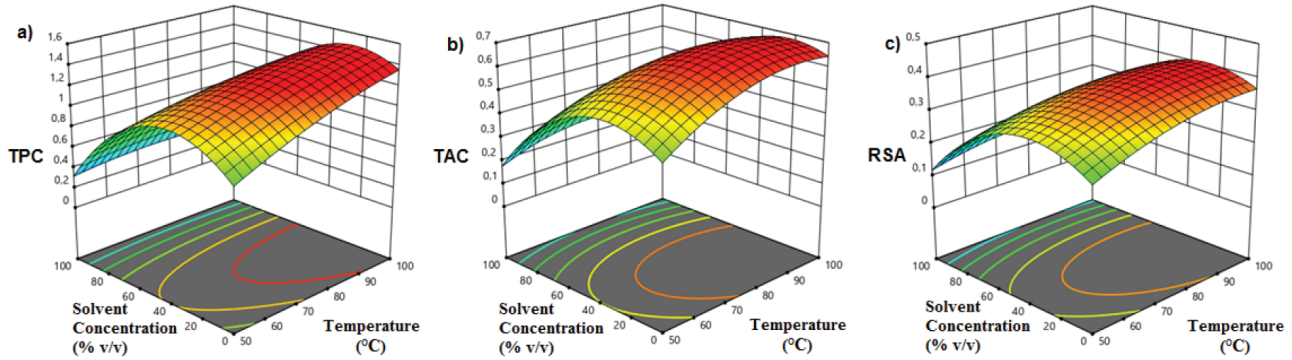

Figure 2. The 3D surface plot for the (a) TAC, (b) TPC, and (c) RSA of *Calendula officinalis* L. extract as a function of solvent concentration to temperature (solvent-to-solid ratio = 12.5 mL/0.2 g and extraction time = 5.5 min).

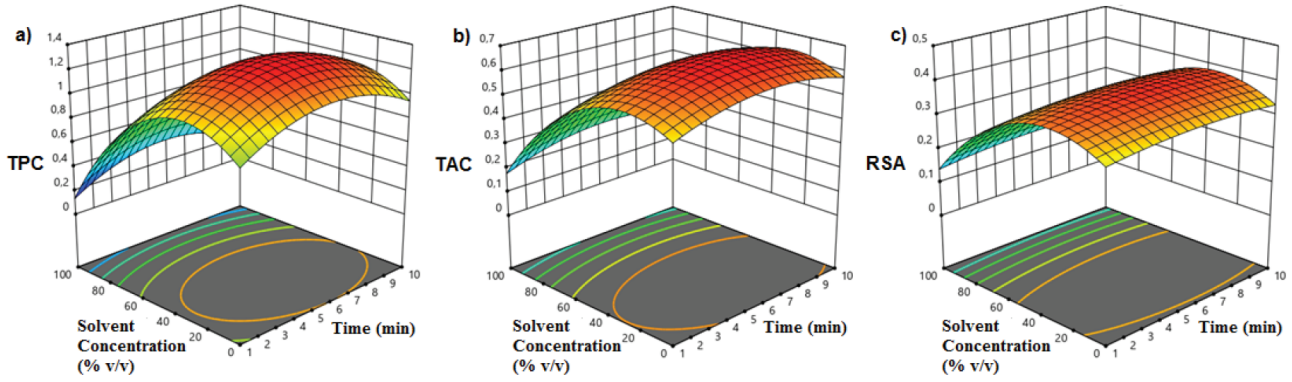

Figure 3. The 3D surface plot for the (a) TAC, (b) TPC, and (c) RSA of *Calendula officinalis* L. extract as a function of solvent concentration to extraction time (extraction temperature = 75 °C and solvent-to-solid ratio = 12.5 mL/0.2 g).

Compared to the other analyzed factors, solvent concentration had the highest impact on the yields of TAC, TPC and RSA. Methanol, ethanol, and water are the most commonly used solvents in extraction processes. In the present study, the ethanol–water mixture was chosen as a suitable solvent mixture for the extraction process due to its health compatibility, environmental suitability (green solvent), and low cost [29].

Zerajić et al. [17] used ethanol as the extraction solvent for UAE of flavonoids from pot marigold flowers.

They reported that the use of ethanol in extraction provides acceptability for pharmaceutical applications. The use of aqueous mixtures of alcohols as solvents for the extraction of antioxidants is also known to be more effective than the corresponding single component solvent system and improves the extraction rate [30,31]. While ethanol increases the solubility of the solute, water aids its desorption from the plant matrix [32]. In addition, as shown in Figures 1–3, the yields of TAC, TPC, and RSA increased when the ethanol percentage in water increased to a certain value and then decreased moving towards pure water and pure ethanol. Moreover, 23%–33% ethanol concentration showed the greatest efficiency in the extraction of antioxidants due to their low viscosity and also allowed the solvent to penetrate the plants via swelling of the matrix.

Compared to the other analyzed factors, the solvent-to-solid ratio and time had the lowest impact on the yields of TAC, TPC, and RSA. Figure 1 shows that there was no significant change in the yield of TAC or TPC with increasing solvent-to-solid ratio. Rajha et al. [33] emphasized that keeping the volume of the solvent to a minimum with optimization processes helps to reduce the total cost of the extraction process. The solvent-to-solid ratio of 11 mL/0.2 g was sufficient to achieve high extraction efficiency. Pinelo et al. [34] explained that the mass transfer phenomenon accelerates when the taking of lower sample amounts permits one to obtain a higher phenolic concentration gradient between sample and solvent. The decrease in extraction efficiency after a given solid ratio in solvent is consistent with the principles of mass transfer because when a lower solid ratio in solvent is used the pushing force is expected to be higher [35].

Spingo et al. [30] reported that temperature and extraction time are the most significant parameters that need to be optimized in the process to reduce the energy cost. The continuous increase in the TAC, TPC, and RSA values of the extracts as a function of temperature can be seen from Figures 2a–2c. This can be clarified by the enhancing of the solubility of solute and the diffusion coefficient with an increase in temperature as mentioned previously [36], but at high temperatures the yield can be reduced by structural decomposition of phenolics accounting for decreased antioxidant capacity [30,37,38]. In the present study, 80–84 °C showed the greatest efficiency for the extraction of antioxidants from marigold. This temperature is extremely convenient to prevent degradation of antioxidant compounds and is economically practical. On the other hand, the yields of TAC, TPC, and RSA increased slightly with extraction time (Figure 3). In general, prolonged extraction times increase the extraction efficiency of antioxidants from plants. This may be due to the time required for the solvent to penetrate the solid sample, dissolve the soluble components in the sample, and release the solvent from the sample with the dissolved components [39–41]. However, long extraction times are economically not feasible. Zerajić et al. evaluated the optimum extraction time of 29 min as shorter than that of other conventional extraction techniques [17]. In the present study, the optimum extraction time was 6 min with regard to the TAC, TPC, and RSA values obtained.

The MAE operation conditions were optimized for maximum responses using suitable software. The aim was to obtain high antioxidant yields within the extraction parameters; here, yield, the feasibility of the experiment, and suitability in terms of green chemistry were considered. The optimum MAE conditions for the TPC, TAC, and RSA from marigold are given in Table 4. The highest yields of TPC (1.34 mmol TR/g DS), TAC (0.68 mmol TR/g DS), and RSA (0.41 mmol TR/g DS) from marigold were obtained in the following ranges: 23%–33% ethanol aqueous solution (v/v), 80–84 °C, 5.85–9.0 min, and 10.88–19.0 mL/0.2 g. The predicted values are compatible with experimental values obtained at optimum extraction conditions, which were confirmed by good correlation. As a result, the design model was considered to be reliable and accurate for determining the TPC, TAC, and RSA of marigold extracts obtained using MAE.

Table 4. Predicted and experimental values under optimum conditions based on individual responses.

Responses	Process factors				Predicted values (mmol TR/g DS)	Experimental values (mmol TR/g DS)
	X_1 (°C)	X_2 (min)	X_3 (%)	X_4 (mL/0.2 g)		
TPC	88	5	23	10	1.43	1.38
TAC	81	6	28	13	0.70	0.68
RSA	84	9	33	19	0.41	0.43

3.3. Chromatographic analysis of antioxidant compounds in marigold extract

The phenolic antioxidant profile of the marigold extract was characterized by UPLC–DAD–ESI–MS/MS system. The chromatographic method was validated with good precision (RSD% 0.75–1.76 for intraday, 1.89–4.20 for interday) and reproducibility (REC% 91.0–105.0). The negative ionization mode was used for obtained MS data. The working conditions (dwell time: 0.02, cone and collision voltage), precursor ions $(M - H^-)$, and their retention times are shown in Table 5. As a result of fragmentation, rutin and hesperidin lost their glycoside group to form their aglycone, quercetin, and hesperetin, respectively. Some of the antioxidant compounds such as protocatechuic acid 179 > 135, 4-hydroxybenzoic acid 137 > 93, and caffeic acid 163 > 119 lost their carboxylic acid group (M – H – 44) as a result of fragmentation. Figure 4 presents the total and extracted ion chromatograms of phenolic antioxidants identified in marigold extract. Peak identification was done by matching the retention times and mass and UV-Vis spectra of individual phenolic antioxidants with those of standards. In the total ion chromatogram, the peaks of rutin and isoquercitrin overlap due to their proximity to retention times. However, in the extracted ion chromatograms rutin and isoquercitrin can be identified through different precursor ions.

Table 5. Optimal conditions for MRM transitions of antioxidants compounds in marigold extracts.

Compound	t_R (min)	MRM (m/z)	Fragments	Cone (V)	Collision (V)
Protocatechuic acid	1.83	$152.95^a > 108.94^b$		35	14
4-Hydroxybenzoic acid	2.94	136.95 > 93.25	64.96	30	36
Chlorogenic acid	3.40	353.31 > 191.01	85.02	54	18
Vanillic acid	3.83	166.99 > 107.98		32	18
Caffeic acid	3.90	178.97 > 135.00		32	18
Syringic acid	4.37	197.03 > 181.99	122.94	30	16
Ferulic acid	5.55	193.03 > 134.01	178.05	32	15
Rutin	6.59	609.27 > 300.19	271.06	65	38
Isoquercitrin	6.62	463.17 > 300.20	271.07	62	26
Hesperidin	6.98	609.28 > 301.10	164.02	56	26
Rosmarinic acid	7.21	359.09 > 160.97	197.00	46	22
Quercetin	9.69	301.23 > 150.95		30	26

[a] Precursor ion $(M - H)^-$. [b] Product ion.

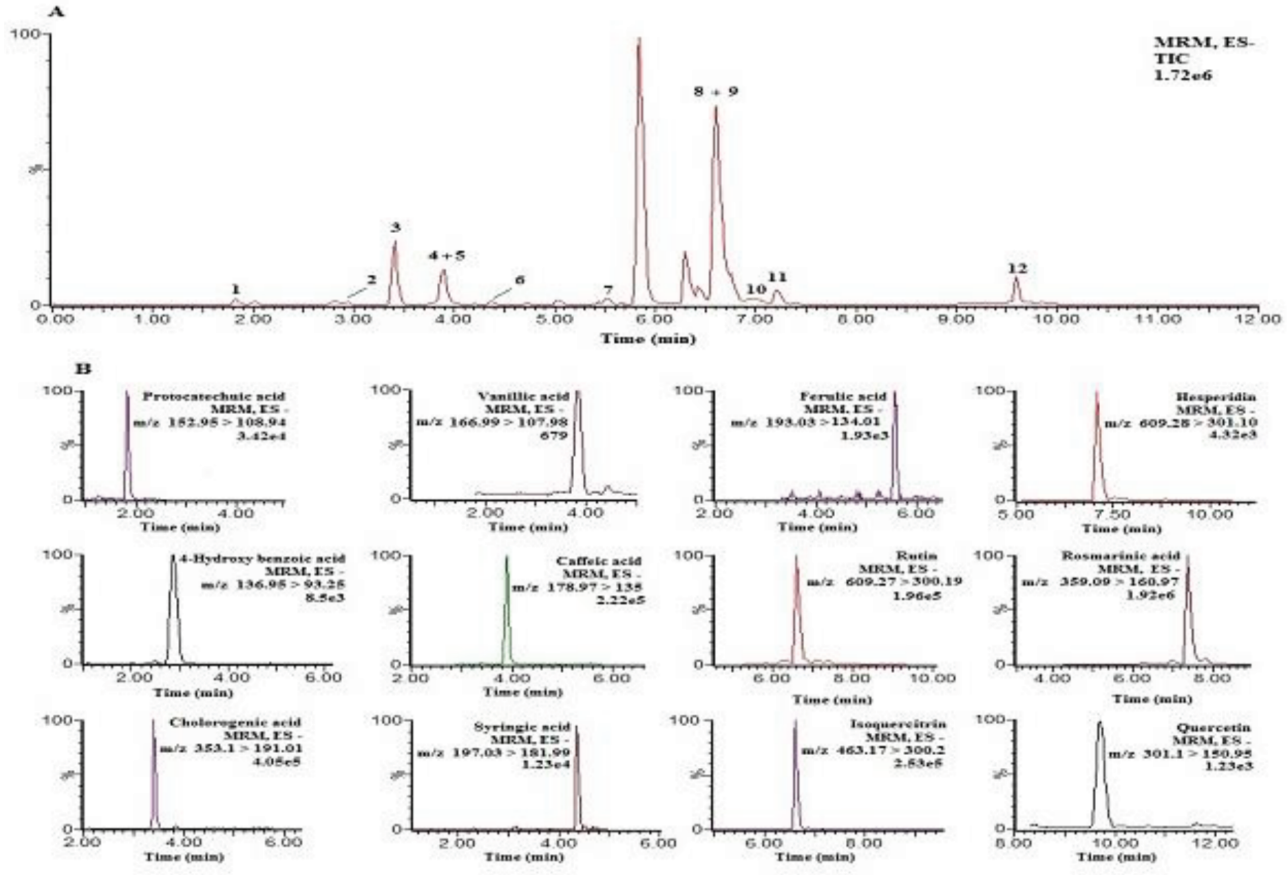

Figure 4. Total (A: ES-) and extracted (B) ion chromatograms of antioxidants in marigold: (1) protocatechuic acid; (2) 4-hydroxybenzoic acid; (3) chlorogenic acid; (4) vanillic acid; (5) caffeic acid; (6) syringic acid; (7) ferulic acid; (8) rutin; (9) isoquercitrin; (10) hesperidin; (11) rosmarinic acid; (12) quercetin.

The amounts of the different antioxidant compounds present in marigold extract quantified using their linear calibration curves (chromatographic peak area vs. concentration) are summarized in Table 6. Chlorogenic acid (1742.50 $\pm$ 42.23 μg/g DS) and quercetin (7.86 $\pm$ 0.24 μg/g DS) were the major and minor constituents, respectively, compared to other antioxidant compounds. Hydroxycinnamic acids (chlorogenic acid and caffeic acid) and rutin were detected in the marigold extracts by LC–MS/MS detection according to the results given by Lungu et al. [42]. These findings are highly consistent with the phenolic antioxidant profile characterized by UPLC–DAD–ESI–MS/MS system in the present study.

4. Conclusion

In our study, MAE of antioxidants from *Calendula officinalis* L. plant was used, optimized, and modeled for the first time in the literature. Microwave extraction was used, having distinct advantages of reduced cost and time and increased reproducibility over classical extraction methods (Soxhlet extraction, liquid–liquid extraction, etc.). RSM was successfully used for the design of experiments, modeling, and optimization of the extraction of antioxidant compounds, which is the most important step in the determination of the antioxidant capacity/activity of marigold. The optimum extraction conditions for obtaining the maximum yield of TPC,

Table 6. Amounts of phenolic antioxidants present in marigold extract (number of measurements = 3).

Antioxidant	Content (μg/g DS)
Protocatechuic acid	52.70 ± 2.04
4-Hydroxybenzoic acid	333.70 ± 9.58
Chlorogenic acid	1742.50 ± 42.23
Vanillic acid	25.22 ± 1.15
Caffeic acid	237.81 ± 10.54
Syringic acid	70.95 ± 3.25
Ferulic acid	12.43 ± 0.54
Rutin	639.82 ± 28.45
Isoquercitrin	375.22 ± 15.29
Hesperidin	186.83 ± 9.34
Rosmarinic acid	452.56 ± 21.74
Quercetin	7.86 ± 0.24

TAC, and RSA were determined using a face-centered composite design. The basis of the study was investigation of the effects of important parameters such as extraction temperature, extraction time, solvent concentration (ethanol in water), and solvent-to-solid ratio on extraction of marigold. The ethanol concentration was the most significant operating factor in the MAE of antioxidants from marigold. A second-order polynomial model was employed and all models calculated for the three responses (TPC, TAC, and RSA) were significant ($P < 0.0001$). The results of the RSM suggest that 28% ethanol concentration, 81 °C temperature, 6 min time, and 13 mL/0.2 g solvent-to-solid ratio should be employed as the optimum extraction conditions to obtain the greatest TPC (1.40 mmol TR/g DS) and TAC (0.70 mmol TR/g DS) from marigold through MAE. These experimental conditions allow fast, quantitative, and maximum extraction of antioxidants from marigold. The MAE method optimized in the present study, which is simple, fast, and highly efficient, will be an important alternative method for the extraction of antioxidants from marigold.

In the chromatographic studies performed by UPLC–PDA–ESI–MS/MS system, 12 important phenolic antioxidant compounds were identified in marigold at optimum MAE conditions. This is why marigold is a commercially important plant. Among the phenolic compounds identified, chlorogenic acid is the major phenolic compound in amounts of 1742.50 ± 42.23 μg/g DS.

In the future, the proposed extraction method can be applied for effective, economical, and environmentally friendly extraction of antioxidants from marigold in the food, cosmetic, and pharmaceutical industries, where marigold is frequently used.

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