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AUTOMATIC SOLVENT EXTRACTION OF BIOACTIVE MOLECULES RICH IN PHENOLICS AND FLAVONOIDS FROM *HYPERICUM PERFORATUM* L.: OPTIMIZATION AND MULTIVARIATE ANALYSIS

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ABSTRACT
This study employed automatic solvent extraction (ASE) to obtain extracts rich in phenolic and flavonoid compounds from *Hypericum perforatum L.* (St. John's Wort). Response surface methodology (RSM) based on Box-Behnken Design was used to optimize three factors (solid mass, immersion time and ethanol concentration). Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (DPPH and ABTS assays) were evaluated as response variables. Ethanol concentration had the most significant effect on TPC and TFC, while antioxidant activity was significantly influenced by the *H. perforatum* amount. The optimal conditions (0.5 g solid mass, 30 min immersion time and ~30-33% ethanol) yielded the highest TPC (95.1093 mg-GAE/g-DM), TFC (66.9977 mg-CE/g-DM), antioxidant activity value of DPPH (38.4081 mg-TEAC/g-DM) and antioxidant activity value of ABTS (42.3328 mg-TEAC/g-DM) with a desirability index close to 1. Principal component analysis (PCA) supported correlations among the responses, showing that ASE is a sustainable and effective extraction method.
Keywords: *Hypericum perforatum* L., antioxidant activity, green extraction, RSM, PCA.

HYPERICUM PERFORATUM L.'DEN FENOLİK VE FLAVONOİDLER AÇISINDAN ZENGİN BİYOAKTİF MOLEKÜLLERİN OTOMATİK ÇÖZÜCÜ EKSTRAKSİYONU: OPTİMİZASYON VE ÇOK DEĞİŞKENLİ ANALİZ

ÖZ
Bu çalışmada, *Hypericum perforatum* L. (sarı kantaron) bitkisinden fenolik ve flavonoid bileşikler açısından zengin özütler elde etmek için otomatik çözücü ekstraksiyonu (ASE) kullanılmıştır. Box-Behnken Tasarımına dayalı tepki yüzey metodolojisi (RSM), üç faktörü (katı kütle, daldırma süresi ve etanol konsantrasyonu) optimize etmek için kullanılmıştır. Toplam fenolik içerik (TPC), toplam flavonoid içerik (TFC) ve antioksidan aktivite (DPPH ve ABTS analizleri) tepki değişkenleri olarak değerlendirilmiştir. Etanol konsantrasyonu TPC ve TFC üzerinde en önemli etkiye sahipken, antioksidan aktivite *H. perforatum* miktarından önemli ölçüde etkilenmiştir. Optimum koşullar (0.5 g

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katı kütle, 30 dakikalık daldırma süresi ve ~%30-33 etanol) en yüksek TPC'yi (95.109 mg-GAE/g-DM), TFC'yi (66.998 mg-CE/g-DM), DPPH'nin (38.408 mg-TEAC/g-DM) antioksidan aktivite değerini ve ABTS'nin (42.333 mg-TEAC/g-DM) antioksidan aktivite değerini, 1'e yakın bir arzu edilirlik indeksiyle vermiştir. Temel bileşen analizi (PCA), yanıtlar arasındaki korelasyonları desteklemiş ve ASE'nin sürdürülebilir ve etkili bir ekstraksiyon yöntemi olduğunu göstermiştir.
Anahtar kelimeler: *Hypericum perforatum* L., antioksidan aktivite, yeşil ekstraksiyon, RSM, PCA.

INTRODUCTION

Hypericum perforatum L. also known as St. John's Wort is a medicinal plant due to its bioactive properties such as antioxidant, antimicrobial and anti-inflammatory effects (Tumbarski et al., 2024). The antioxidant activity of *H. perforatum* is generally attributable to its phenolic and flavonoid contents (Cossuta et al., 2012). Considering the growing interest in natural antioxidants, *H. perforatum* extracts have been attracting attention as potential ingredients in functional food formulations (Jakubczyk et al., 2021; Lim et al., 2025; Mišina et al., 2025; Sánchez-Muniz et al., 2012; Kaloteraki, C., et al., 2021). Therefore, recovery of the active materials from *H. perforatum* with maximum yield and quality is of great significance. Ultrasound-assisted extraction (Kakouri et al., 2023; Seyrekoglu et al., 2022; Seyrekoğlu and Temiz, 2020) and microwave-assisted extraction (Milutinović et al., 2024) were used for obtaining bioactive molecules from *H. perforatum* in earlier studies. Unlike these conventional assisted methods, the recovery of phenolic- and flavonoid-rich extracts from *H. perforatum* was performed using automatic solvent extraction (ASE) in the present study. ASE is considered as a promising green extraction technique due to its properties such as efficiency (high recovery), speed (shorter extraction time), environmentally friendly (low solvent consumption) and reproducibility (automation and control possibilities) (Chemat et al., 2019). Since the extraction efficiency of ASE is influenced by multiple factors (time, solid/solvent ratio, concentration of the solvent), response surface method (RSM) was employed as a statistical optimization tool to determine the best conditions for maximizing bioactive compound recovery. Box-Behnken Design (BBD) was chosen as one of the most common designs used in RSM due to the fact that it requires less experimental runs than central composite designs (Szpisják-Gulyás et al., 2023). Moreover, BBD avoids extreme factor combinations that might cause degradation of thermolabile compounds (Ahmad et al., 2020). Therefore, it is unavoidable to adopt for improving the recovery of phenolic and flavonoid compounds from *H. perforatum*.

To the best of our knowledge, this is the first study to investigate the application of ASE coupled with statistical optimization for the extraction of bioactive compounds from *H. perforatum*. In addition, the present study integrates principal component analysis (PCA) with RSM to provide a comprehensive evaluation of the extraction parameters and the responses. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity (measured by ABTS and DPPH assays) were accepted as response variables. This comprehensive approach will present ASE as a sustainable method for producing high-quality extracts from *H. perforatum*.

MATERIALS AND METHODS

Materials

Dried plant material was purchased from a local herbal store in Istanbul, Turkey. The samples were stored in sealed containers at ambient conditions until the extraction step. Chemicals and reagents (ethanol, methanol, Folin-Ciocalteu reagent, gallic acid, sodium carbonate, aluminum chloride, catechin, trolox, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl)) were of analytical grade (≥98% purity) and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Automatic solvent extraction was performed using an autoextractor (VELP Scientifica, Usmate, Italy). Spectrophotometric measurements were performed by UV-Vis spectrophotometer (PG Instruments, T60/Leicestershire, England).

Extraction

Active ingredients were extracted from *H. perforatum* using the automatic solvent extractor. This system owns five times the speed of a Soxhlet. Glass cup containing was filled with 80 mL solvent (aqueous ethanol). The glass cups were then filled with the cellulose extraction thimbles (single thickness, 33 mm x 80 mm, Whatman, Maidstone, UK) that held the solid samples.

The overall workflow of the experimental procedure, including the steps from sample preparation to data analysis, is summarized in Figure 1.

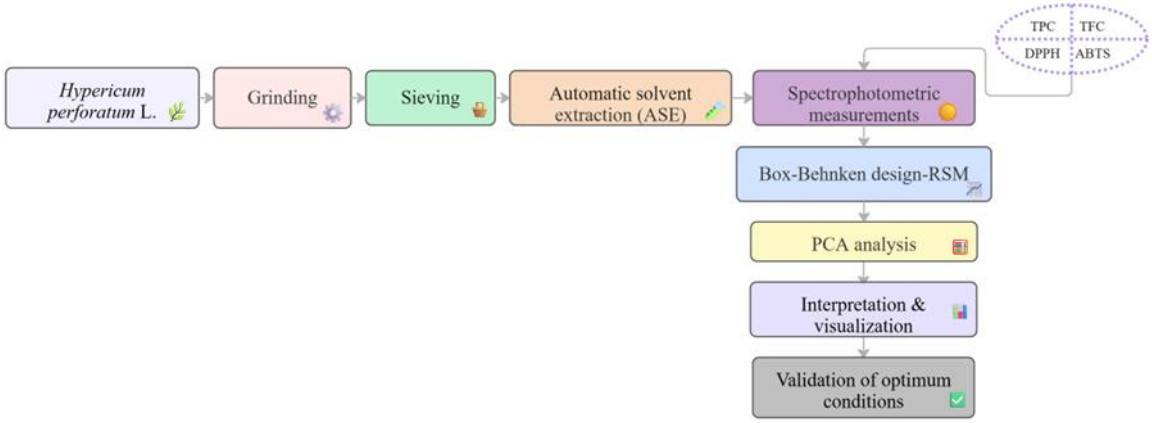


Figure 1. Schematic representation of the experimental workflow, illustrating the steps from sample preparation to data analysis.

The ASE system was programmed to run through the following steps: immersion time (variable according to the design), removing time (0 min), washing time (20 min), recovery time (30 min), and cooling time (5 min). For each extraction run, the solvent was freshly prepared to ensure consistency and to avoid any possible contamination or solvent degradation between experiments.

Table 1 represents the process parameters and their levels determined by the preliminary experiments.

Table 1. Process parameters, units, symbols, and coded levels.

Process Parameter	Units	Symbol	Coded levels		
			−1	0	1
Solid mass	g	A	0.5	1	1.5
Immersion time	min	B	10	20	30
Solvent concentration	%, v/v	C	30	60	90

Spectrophotometric measurements

TPC, TFC and antioxidant activity (DPPH and ABTS) determinations were measured spectrophotometrically. The Folin-Ciocalteu colorimetric method was used to determine the TPC amount in the extracts (Şahin, 2015). The sample of the extract was mixed with pure water. Folin-Ciocalteu solution that had been diluted was added to the extract that had been diluted and mixed. The sodium carbonate solution was added and left for 30 min. The absorption was found at a wavelength of 760 nm. The amount of TPC was given as mg of gallic acid equivalent per gram of dried material (mg-GAE/g-DM).

TFC was determined by aluminum chloride colorimetric assay as described in (Sakanaka et al., 2005). A complex between flavonoids and aluminum chloride is formed, causing to color change in the solution. Then, this color change is

measured under 510 nm of wavelength. The absorbance values measured were compared to a reference flavonoid solution (catechin) to determine the TFC. The amount of TFC was given as mg of catechin equivalent per gram of dried material (mg-CE/g-DM).

Antioxidant activity values of the extracts were determined by DPPH and ABTS assays, respectively. These two methods represent the ability of the extract to scavenge free radicals. The color changes of the solution in DPPH and ABTS assays were measured at 517 nm and 734 nm, respectively (Şahin, 2015). The antioxidant activity values were given in trolox equivalent antioxidant capacity per gram of dried matter (mg-TEAC/g-DM).

Box-Behnken design

BBD with three factors and three levels was applied. The independent variables selected were solid mass (A, g), extraction time (B, min) and ethanol concentration (C, % v/v). Each variable was studied at three coded levels (-1, 0, +1) as given in Table 1. The experimental design consisted of 17 runs including replicates at the center point to estimate the pure error. The responses (dependent variables) were TPC and TFC. Minitab statistical software 22 (Minitab Inc., State College, PA, USA) was used for experimental design, regression analysis and response surface modeling.

Multivariate data analysis

Principal Component Analysis (PCA) was applied to reduce the dimensionality of the dataset and to visualize correlations among total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities (DPPH and ABTS). Prior to analysis, data were standardized, and PCA was carried out on the correlation matrix to eliminate the effect of differences in measurement scales. PCA was performed using Minitab® Statistical Software, version 22.2.1 (64-bit) (Minitab LLC, State College, PA, USA). Score plots and biplots were generated to assess sample clustering and to reveal the association between measured variables.

RESULTS and DISCUSSION

Effect of process parameters on the bioactive substances of the *Hypericum perforatum* extract

Table 2 summarizes the design matrix of the experimental study with the measured variables (TPC, TFC and antioxidant acitivity values measured by 2 assays) for the recovery of *H. perforatum* extract through the ASE system.

TPC values ranged from 33.592±0.003 mg-GAE/g-DM to 94.840±0.001 mg-GAE/g-DM, while TFC values in the *H. perforatum* extract were between 30.714±0.004 mg-CE/g-DM and 66.920±0.001 mg-CE/g-DM. When the process conditions are evalauted, it is seen that the highest TPC and TFC values are observed under 30% ethanol conditions (Table 2). On the other hand, 90% ethanol gave rise to produce poor yields of TPC and TFC as seen in Table 2. Consequently, the same tendency was observed for both TPC and TFC towards the extraction conditions. This can be also supported by the positive strong correlation between the TPC and TFC values of the extracts ($r=0.9034$).

Actually, analysis of variance (ANOVA) test (Table 3) also supports that solvent concentration was the most significant factor influencing both TPC and TFC values with the highest F-values ($P<0.001$). This finding is also supported by the derived quadratic models (Eq.1 and Eq.2), where there are strong negative coefficients for the ethanol concentration. This shows that relatively low ethanol concentration favours the extraction of phenolic and flavonoid compounds. This can be explained by some factors such as polarity, hydrogen bond interactions and matrix swelling properties. First of all, water is more polar than ethanol. Therefore, the solvent gets more polar when the water concentration is higher than ethanol concentration in the solution (Özbek et al., 2020). So, 30% ethanol (v/v) causes the diffusion of much more polar phenolics and flavonoids from the solid matrix comparing to 90% ethanol. Another explanation might be the role of water as swelling agent for the plant matrix, which enhances the mass transfer of the solute into the solvent (Rodríguez De Luna et al., 2020).

Table 2. Process parameters and experimental results for the total phenolic content, total flavonoid content and antioxidant activity (DPPH and ABTS).

Run	Solid mass (g)	Immersion time (min)	Solvent concentration (%,v/v)	TPC (mg-GAE/g-DM)	TFC (mg-CE/g-DM)	DPPH (mg-TEAC/g-DM)	ABTS (mg-TEAC/g-DM)
1	0.5	30	60	76.115±0.003	53.878±0.002	38.808±0.000	42.456± 0.000
2	1	20	60	65.174±0.006	47.869±0.001	19.285±0.001	21.295±0.000
3	1	10	90	33.849±0.004	30.714±0.004	19.387±0.004	21.243±0.001
4	1.5	30	60	71.227±0.003	49.720±0.006	12.403±0.001	14.123±0.001
5	0.5	10	60	62.982±0.003	50.809±0.002	38.625±0.001	42.126±0.001
6	1.5	20	90	33.592±0.003	32.242±0.001	12.940±0.002	14.254±0.000
7	1	30	30	94.840±0.001	64.146±0.004	19.000±0.001	21.188±0.000
8	1.5	20	30	89.622±0.004	65.069±0.001	11.896±0.002	14.200±0.001
9	1	20	60	69.608±0.001	47.019±0.006	18.797±0.003	21.061±0.001
10	1	10	30	81.476±0.002	52.494±0.001	18.865±0.000	21.086±0.000
11	1	20	60	64.807±0.004	47.023±0.001	18.960±0.002	21.245±0.001
12	1	30	90	49.698±0.003	32.142±0.006	19.364±0.002	21.344±0.001
13	0.5	20	30	93.022±0.006	66.920±0.001	37.896±0.001	42.155±0.000
14	1	20	60	65.060±0.004	48.973±0.001	19.374±0.001	21.328±0.000
15	1.5	10	60	55.085±0.003	42.774±0.001	12.507±0.004	14.158±0.000
16	0.5	20	90	45.692±0.001	43.218±0.001	38.935±0.000	42.394±0.000
17	1	20	60	66.510±0.004	46.147±0.001	19.016±0.002	21.031± 0.001

Abbreviations: DM, dried matter; GE, equivalents of gallic acid; TPC, total phenolic content; CE, equivalents of catechin; TFC, total flavonoid content; TEAC, Trolox equivalent antioxidant capacity; DPPH, DPPH radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl); ABTS, ABTS radical scavenging activity (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)).
* Data are given as the mean (n=3)±standard deviation.

Table 3. Analysis of variance for the responses.

	Source	DF	Adj SS	Adj MS	F-Value	P-Value
TPC	Model	9	5364.00	596.00	134.84	0.000
	Linear	3	5335.93	1778.64	402.39	0.000
	Solid mass (g)	1	100.01	100.01	22.62	0.002
	Immersion time (min)	1	427.61	427.61	96.74	0.000
	Solvent concentration (%,v/v)	1	4808.32	4808.32	1087.81	0.000
	Square	3	5.34	1.78	0.40	0.756
	Solid mass (g)*Solid mass (g)	1	0.43	0.43	0.10	0.765
	Immersion time (min)*Immersion time (min)	1	0.16	0.16	0.04	0.852
	Solvent concentration(%,v/v)*Solvent concentration (%,v/v)	1	4.80	4.80	1.09	0.332
	2-Way Interaction	3	22.73	7.58	1.71	0.251
	Solid mass (g)*Immersion time (min)	1	2.26	2.26	0.51	0.497
	Solid mass (g)*Solvent concentration (%,v/v)	1	18.92	18.92	4.28	0.077
	Immersion time (min)*Solvent concentration (%,v/v)	1	1.54	1.54	0.35	0.573
	Error	7	30.94	4.42		
	Lack-of-Fit	3	14.94	4.98	1.25	0.404
	Pure Error	4	16.00	4.00		
	Total	16	5394.94			
Model summary	R^2= 99.43%, R^2 (adjusted)=98.69%, R^2 (predicted)=95.10%					

	Source	DF	Adj SS	Adj MS	F-Value	P-Value
TFC	Model	9	1822.41	202.49	211.00	0.000
	Linear	3	1666.04	555.35	578.68	0.000
	Solid mass (g)	1	78.25	78.25	81.54	0.000
	Immersion time (min)	1	66.67	66.67	69.47	0.000
	Solvent concentration (%,v/v)	1	1521.12	1521.12	1585.02	0.000
	Square	3	105.66	35.22	36.70	0.000
	Solid mass (g)*Solid mass (g)	1	82.95	82.95	86.44	0.000
	Immersion time (min)*Immersion time (min)	1	27.37	27.37	28.52	0.001
	Solvent concentration(%,v/v)*Solvent concentration (%,v/v)	1	0.00	0.00	0.00	0.972
	2-Way Interaction	3	50.71	16.90	17.61	0.001
	Solid mass (g)*Immersion time (min)	1	3.76	3.76	3.92	0.088
	Solid mass (g)*Solvent concentration (%,v/v)	1	20.82	20.82	21.69	0.002
	Immersion time (min)*Solvent concentration (%,v/v)	1	26.13	26.13	27.23	0.001
	Error	7	6.72	0.96		
	Lack-of-Fit	3	2.17	0.72	0.63	0.631
	Pure Error	4	4.55	1.14		
	Total	16	1829.13			
Model summary	R^2= 99.63%, R^2 (adjusted)=99.16%, R^2 (predicted)=97.72%					
	Source	DF	Adj SS	Adj MS	F-Value	P-Value
DPPH	Model	9	1539.36	171.04	2715.37	0.000
	Linear	3	1366.61	455.54	7231.94	0.000
	Solid mass (g)	1	1365.50	1365.50	21678.24	0.000
	Immersion time (min)	1	0.00	0.00	0.07	0.796
	Solvent concentration (%,v/v)	1	1.10	1.10	17.49	0.004
	Square	3	172.72	57.57	914.03	0.000
	Solid mass (g)*Solid mass (g)	1	171.44	171.44	2721.78	0.000
	Immersion time (min)*Immersion time (min)	1	0.06	0.06	0.94	0.366
	Solvent concentration(%,v/v)*Solvent concentration (%,v/v)	1	0.01	0.01	0.17	0.691
	2-Way Interaction	3	0.03	0.01	0.14	0.932
	Solid mass (g)*Immersion time (min)	1	0.02	0.02	0.33	0.585
	Solid mass (g)*Solvent concentration (%,v/v)	1	0.00	0.00	0.00	0.992
	Immersion time (min)*Solvent concentration (%,v/v)	1	0.01	0.01	0.10	0.762
	Error	7	0.44	0.06		
	Lack-of-Fit	3	0.21	0.07	1.26	0.401
	Pure Error	4	0.23	0.06		
	Total	16	1539.80			
Model summary	R^2= 99.97%, R^2 (adjusted)=99.93%, R^2 (predicted)=97.75%					
	Source	DF	Adj SS	Adj MS	F-Value	P-Value
ABTS	Model	9	1788.60	198.73	17088.27	0.000
	Linear	3	1579.18	526.39	45262.64	0.000
	Solid mass (g)	1	1579.11	1579.11	135781.32	0.000
	Immersion time (min)	1	0.03	0.03	2.67	0.147
	Solvent concentration (%,v/v)	1	0.05	0.05	3.95	0.087
	Square	3	209.37	69.79	6000.98	0.000
	Solid mass (g)*Solid mass (g)	1	208.07	208.07	17890.77	0.000
	Immersion time (min)*Immersion time (min)	1	0.00	0.00	0.01	0.914
	Solvent concentration(%,v/v)*Solvent concentration (%,v/v)	1	0.00	0.00	0.31	0.597
	2-Way Interaction	3	0.04	0.01	1.20	0.377
	Solid mass (g)*Immersion time (min)	1	0.03	0.03	2.86	0.134

	Solid mass (g)*Solvent concentration (%,v/v)	1	0.01	0.01	0.74	0.419
	Immersion time (min)*Solvent concentration (%,v/v)	1	0.00	0.00	0.00	0.996
	Error	7	0.08	0.01		
	Lack-of-Fit	3	0.01	0.00	0.11	0.947
	Pure Error	4	0.07	0.02		
	Total	16	1788.68			
Model summary	R^2= 100.00%, R^2 (adjusted)=99.99%, R^2 (predicted)=99.99 %					

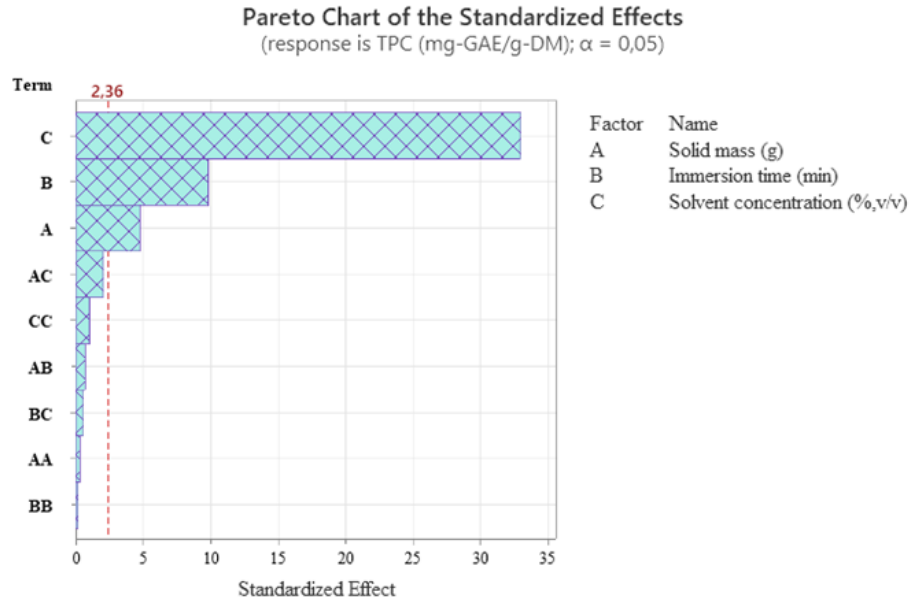
$$\mathbf{TPC} = 100.72 - 3.9\,\text{Solid mass (g)} + 0.536\,\text{Immersion time (min)} - 0.571\,\text{Solvent concentration (\%, v/v)} + 1.27\,\text{Solid mass (g)} * \text{Solid mass (g)} - 0.0020\,\text{Immersion time (min)} * \text{Immersion time (min)} - 0.00119\,\text{Solvent concentration (\%, v/v)} * \text{Solvent concentration (\%, v/v)} + 0.150\,\text{Solid mass (g)} * \text{Immersion time (min)} - 0.1450\,\text{Solid mass (g)} * \text{Solvent concentration (\%, v/v)} + 0.00207\,\text{Immersion time (min)} * \text{Solvent concentration (\%, v/v)} \quad (1)$$

$$\mathbf{TFC} = 67.62 - 36.52\,\text{Solid mass (g)} + 1.626\,\text{Immersion time (min)} - 0.1395\,\text{Solvent concentration (\%, v/v)} + 17.75\,\text{Solid mass (g)} * \text{Solid mass (g)} - 0.02550\,\text{Immersion time (min)} * \text{Immersion time (min)} + 0.000019\,\text{Solvent concentration (\%, v/v)} * \text{Solvent concentration (\%, v/v)} + 0.1938\,\text{Solid mass (g)} * \text{Immersion time (min)} - 0.1521\,\text{Solid mass (g)} * \text{Solvent concentration (\%, v/v)} - 0.00852\,\text{Immersion time (min)} * \text{Solvent concentration (\%, v/v)} \quad (2)$$

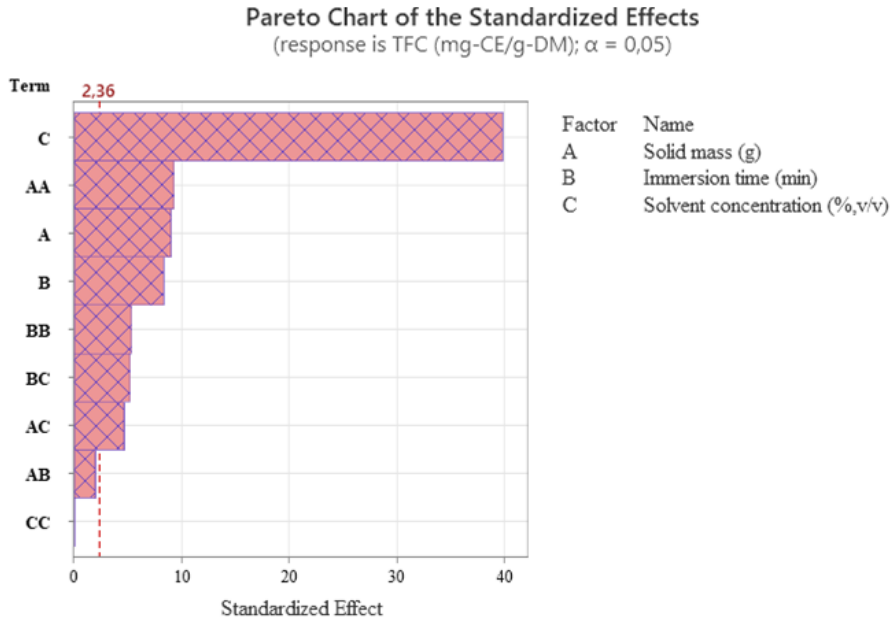
The adequacy of the derived models was confirmed by high coefficients of determination (R^2) as seen in Table 3. R^2, R^2 (adjusted) and R^2 (predicted) are all higher than 0.95 for TPC, whereas the R^2 values of TFC were also in good agreement with each other by extremely satisfactory results (>0.95). Additionally, the models had non-significant lack-of-fit value (P>0.05), showing that the derived models by Box-Behnken design describe the data without systematic bias (Chen and Chen, 2025).

Additionally, other parameters were also found statistically significant for both TPC and TFC as given in Pareto charts (Figure 2). Figures 2a and 2b display the superior effect of the ethanol concentration over the other process terms on the phenolic and flavonoid yields.

The interaction effects between the variables can be observed in 3D response surface plots (Figures 3 and 4). Figure 3 indicates that the solvent concentration had the strongest negative effect, followed by immersion time and solid mass for the TPC yield. Figure 3a shows that solid mass decreases the yield under the constant solvent volume, which is expectable (Wong et al., 2013). Because increasing the amount of solids restricts the penetration of the solvent into the plant matrix. So, the solute cannot be released from the matrix. On the other hand, TPC increases slightly as time increases as seen in Figure 3a. This extraction interval (30 min) is also in agreement with the report of Pan et al., where the risk of phenolics breaking down beyond 30 min was observed (Pan et al., 2010). However, the main determining factor is the solvent concentration as shown in Figures 3b and 3c. Increasing the ethanol concentration decreases the yield sharply. This result is consistent with that of Lohvina et al. (Lohvina et al., 2021). They observed the same ethanol effect on the extraction of total phenolics from the seeds of fenugreek.

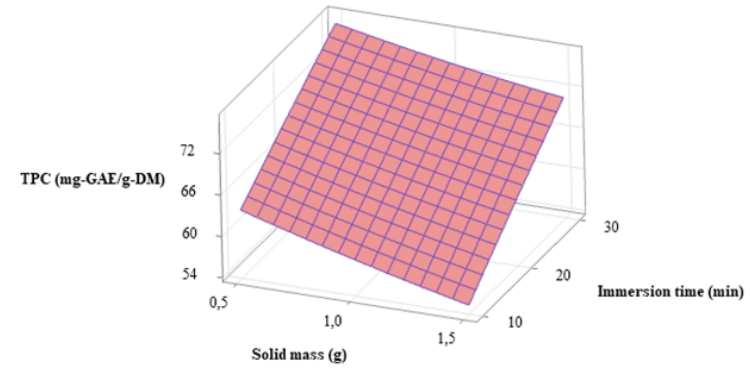


a)

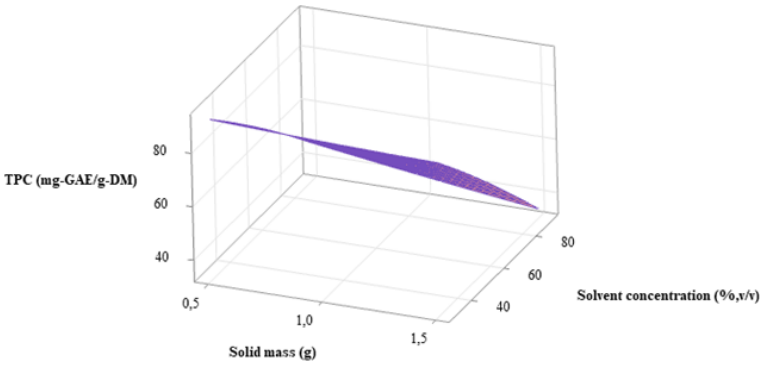


b)

Figure 2. Pareto chart of standardized effects for TPC **(a)** and TFC **(b)** obtained by the Box-Behnken design.



a)



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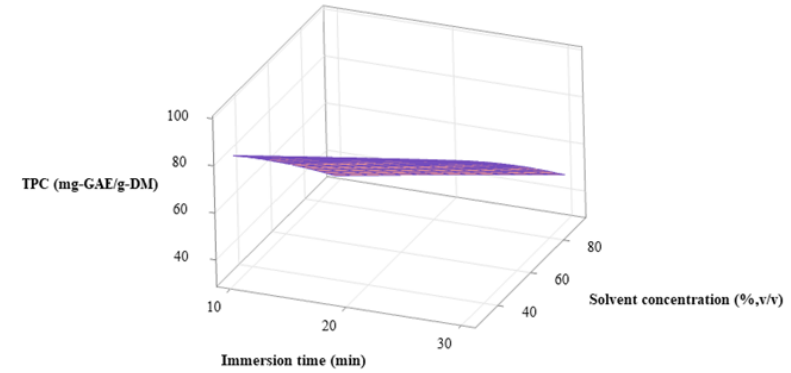


c)

Figure 3. Response surface plot showing the combined effect of solid mass and immersion time (a), solid mass and solvent concentration (b) and immersion time and solvent concentration (c) on total phenolic content of the *H. perforatum* extract.

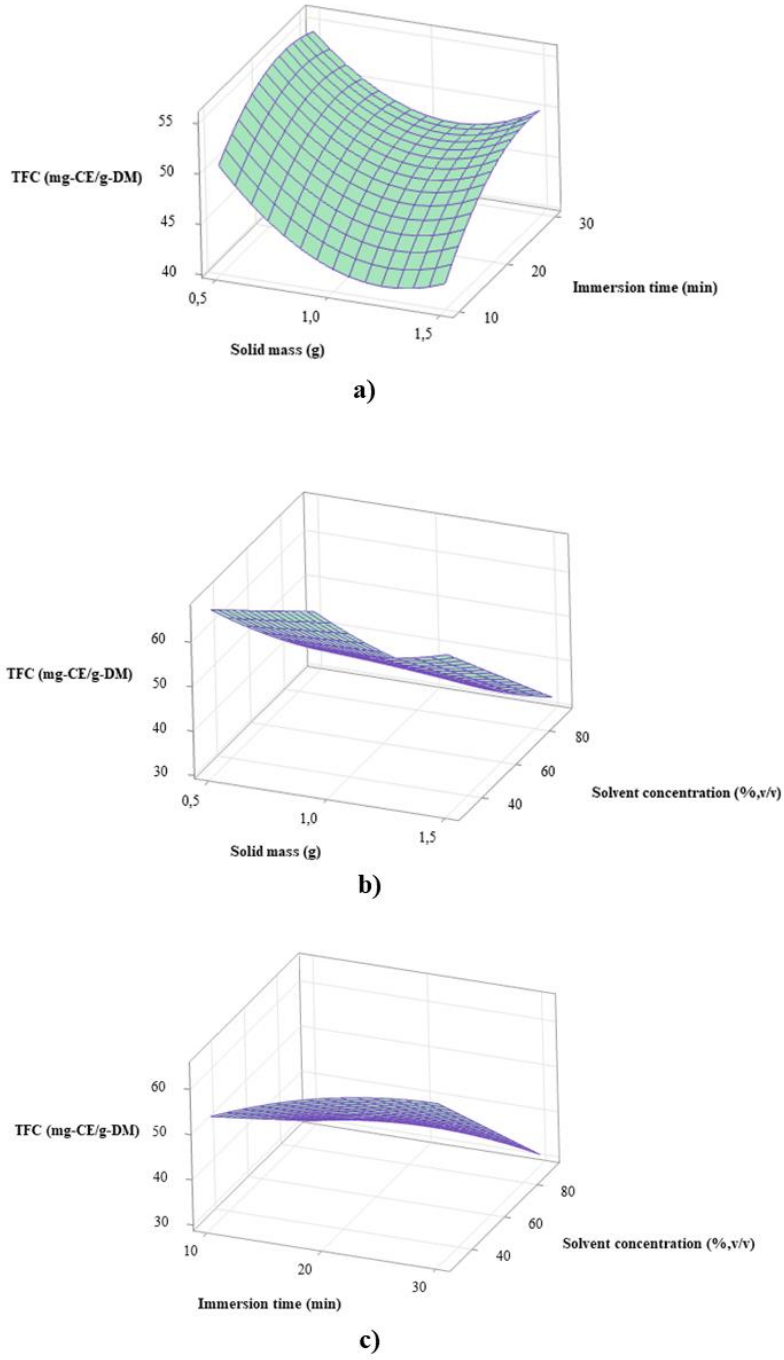


Figure 4. Response surface plot showing the combined effect of solid mass and immersion time (a), solid mass and solvent concentration (b) and immersion time and solvent concentration (c) on total flavonoid content of the *H. perforatum* extract.

The quadratic effects of solid mass and its interaction with time were also significant for the TFC yield as seen in the curved surface (Figure 4a). These trends are also supported with the ANOVA and Pareto analyses as described earlier (Table 3 and Figure 2). Since there is a strong correlation between TPC and TFC ($r>0.90$), the tendency of the responses towards the parameters are generally similar (Figures 3b, 3c, 4b and 4c).

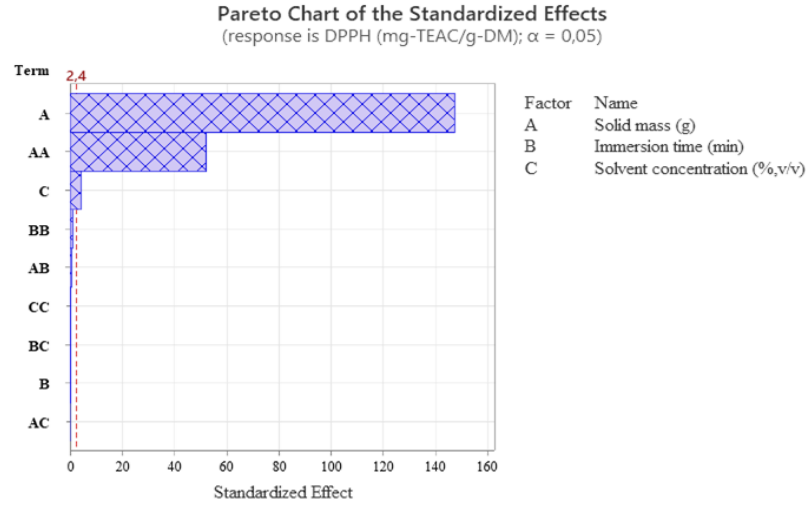
Effect of process parameters on the antioxidant capacity of the *Hypericum perforatum* extract

As already presented in Table 2, antioxidant activity values of DPPH test were between 11.896±0.002 mg-TEAC/g-DM and 38.935±0.000 mg-TEAC/g-DM in the *H. perforatum* extracts obtained by the ASE system. In case of ABTS assay, the antioxidant activity values in the extract changed from 14.123±0.001 mg-TEAC/g-DM to 42.456±0.000 mg-TEAC/g-DM. Furthermore, there was a perfect correlation between the results of 2 antioxidant activity assays ($r=0.9992$). When the effects of the parameters are evaluated, it can be seen that low solid mass (0.5 g) gave rise to the highest antioxidant capacities for both method, while 1.5 g of plant material (high mass) produced the lowest antioxidant activity values (Table 2). This effect was also recorded during the solid-liquid extraction of antioxidants from grape by-products (Pinelo et al., 2006). It was observed that high solid concentration reduced the extraction efficiency, decreasing the antioxidant capacity. The highest linear and second power effects of plant material amount can be also observed in ANOVA results with the highest F-values at $P<0.0001$ (Table 3) and the Pareto charts (Figure 5). The linear and second power effects of time and ethanol concentration show non-significant effects (Table 3, Figures 5a and 5b). Even though solvent concentration and extraction time significantly influenced TPC and TFC (Figure 2 and Table 3), they did not significantly affect the antioxidant capacities (Figure 5). This means that other components such as lipophilic or other antioxidant substances might be responsible of antioxidant capacity independent of phenolic and flavonoid concentrations. This is also evident from the lack of correlation between antioxidant activity results and TPC/TFC ($r<0.06$).

Eq. 3 and Eq. 4 also support the observed trends, where there are strong negative influences of increased solid mass on the DPPH and ABTS radical scavenging activities. Antioxidant activity models derived by Box-Behnken design showed even higher predictive performance with R^2 values exceeding 0.999 (Table 3). The lack-of-fit test was not also significant ($P>0.05$). To conclude, it has been confirmed that these models based on the Box-Behnken design can reliably predict extraction performance.

$$\begin{aligned}\textbf{DPPH} = {} & 69.78 - 76.90\ \text{Solid mass (g)} \\ & - 0.0227\ \text{Immersion time (min)} + 0.0217\ \text{Solvent concentration (\%, v/v)} \\ & + 25.524\ \text{Solid mass (g)} * \text{Solid mass (g)} + 0.00118\ \text{Immersion time (min)} \\ & * \text{Immersion time (min)} \\ & - 0.000056\ \text{Solvent concentration (\%, v/v)} * \text{Solvent concentration (\%, v/v)} \\ & - 0.0144\ \text{Solid mass (g)} * \text{Immersion time (min)} \\ & + 0.00008\ \text{Solid mass (g)} * \text{Solvent concentration (\%, v/v)} \\ & - 0.000132\ \text{Immersion time (min)} * \text{Solvent concentration (\%, v/v)}\end{aligned} \quad (3)$$

$$\begin{aligned}\textbf{ABTS} = {} & 76.675 - 83.786\ \text{Solid mass (g)} \\ & + 0.0269\ \text{Immersion time (min)} + 0.00174\ \text{Solvent concentration (\%, v/v)} \\ & + 28.118\ \text{Solid mass (g)} * \text{Solid mass (g)} - 0.000059\ \text{Immersion time (min)} \\ & * \text{Immersion time (min)} \\ & + 0.000032\ \text{Solvent concentration (\%, v/v)} * \text{Solvent concentration (\%, v/v)} \\ & - 0.0183\ \text{Solid mass (g)} * \text{Immersion time (min)} \\ & - 0.00308\ \text{Solid mass (g)} * \text{Solvent concentration (\%, v/v)} \\ & - 0.000001\ \text{Immersion time (min)} * \text{Solvent concentration (\%, v/v)}\end{aligned} \quad (4)$$



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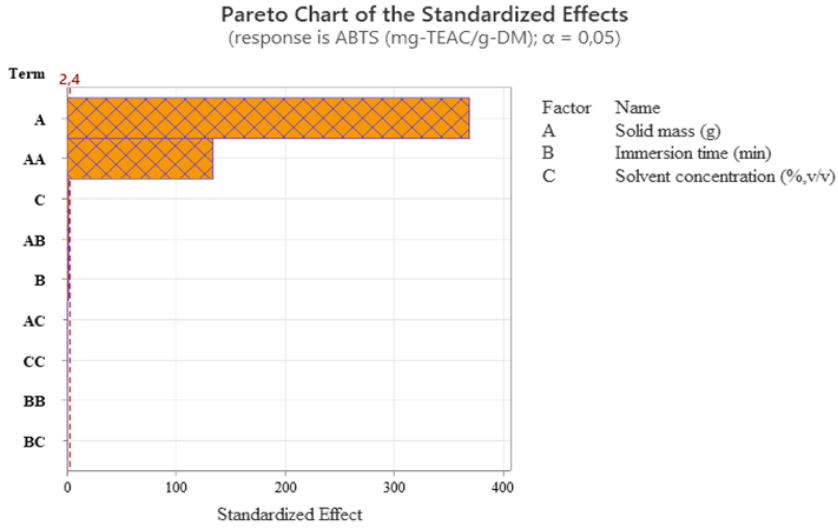


b)

Figure 5. Pareto chart of standardized effects for DPPH (a) and ABTS (b) obtained by the Box-Behnken design.

As shown in the response surface plots (Figures 6 and 7), both antioxidant assays (DPPH and ABTS) responded in a highly consistent manner to the process variables. This can be explained by the fact that both assays measure radical scavenging activity based on single electron transfer reactions, leading to strong correlation (Knez et al., 2025). Therefore, similar trends were observed in response to the ASE conditions. Imenšek et al. also recorded a high correlation between the DPPH and ABTS in the antioxidant activity values of elderberry fruits (Imenšek et al., 2021).

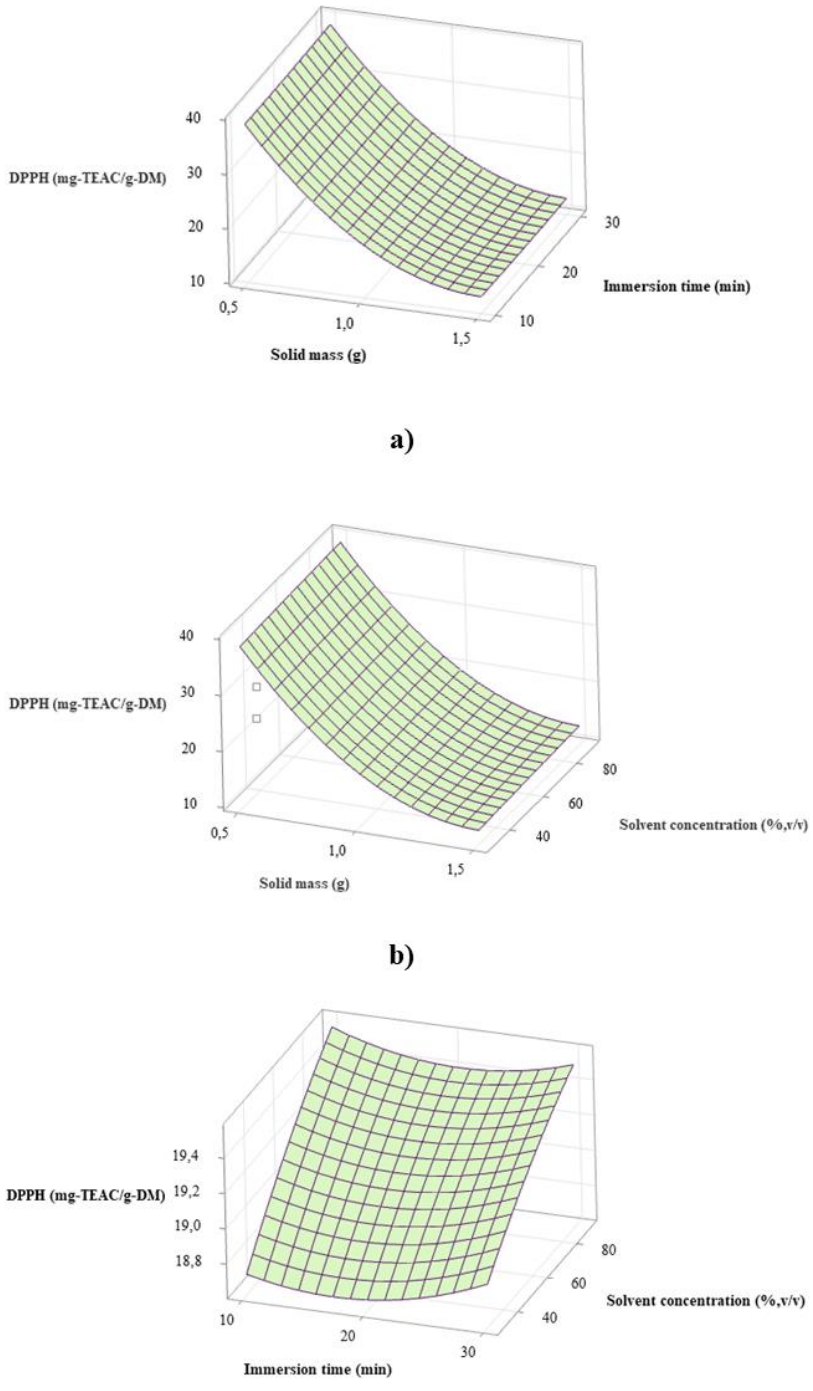


Figure 6. Response surface plot showing the combined effect of solid mass and immersion time (a), solid mass and solvent concentration (b) and immersion time and solvent concentration (c) on DPPH antioxidant activity values of the *H. perforatum* extract.

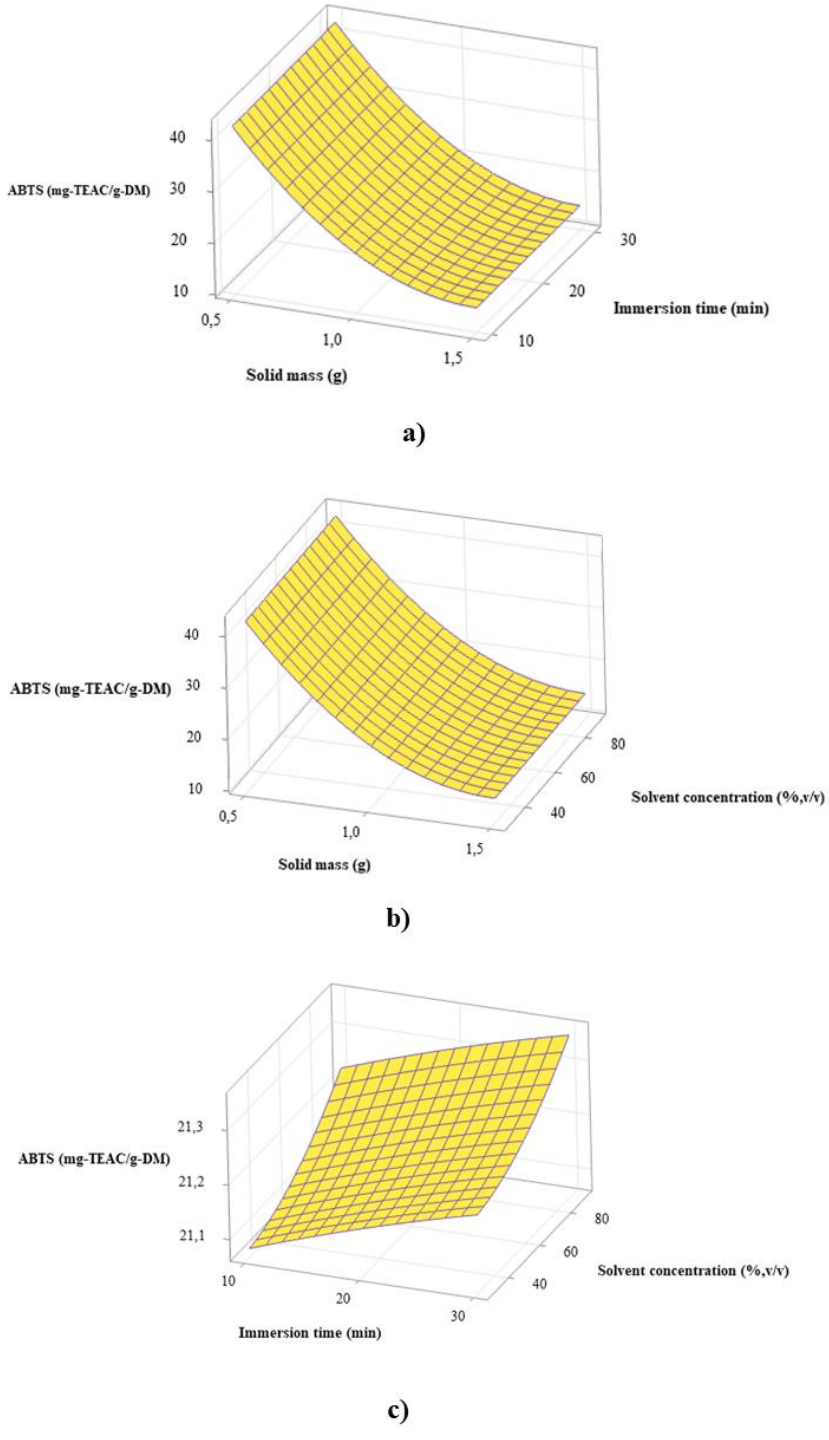


Figure 7. Response surface plot showing the combined effect of solid mass and immersion time (a), solid mass and solvent concentration (b) and immersion time and solvent concentration (c) on ABTS antioxidant activity values of the *H. perforatum* extract.

The solid mass exerted the most significant influence, where increasing solid loadings caused a sharp decline in antioxidant activity as observed in Figures 6a, 6b, 7a and 7b. High solids loading limits mass transfer and diffusion at constant solvent volume, while reducing the solvent penetration into the matrix. This causes to saturate the medium and reduce the antioxidant transfer. It has been clearly demonstrated that high solids loading reduces phenolic/tannin yields in various biomasses (Ozdemir et al., 2024; Song et al., 2025).

However, solvent concentration and immersion time were not significant (Figures 6c and 7c). Similarly, the maximum is reached around 10 min under the optimized extraction conditions of bioactive molecules from brown macroalga sargassum horneri (Song et al., 2025). After 10 min, there was no recovery of bioactives. This is in agreement with the poor time effect at 10-30 min in the current design (Figures 6a, 6c, 7a and 7c).

The congruent responses of DPPH and ABTS (Figures 6 and 7) verify the ANOVA results (Table 3, Figures 5a and 5b), where only solid mass was statistically significant. The previous reports also support that high solid-to-solvent ratios decrease the efficiency of free radical scavenging due to mass transfer limitations (Bhadange et al., 2024; Vo et al., 2023; Wu et al., 2024).

Multi-response optimization

Table 4 summarizes the numerical optimization outcomes. The extraction conducted with 0.5 g of dried *H. perforatum*, an immersion time of 30 minutes, and an ethanol concentration of approximately 30–33 % (v/v) yielded the highest responses. The overall desirability value of 0.994 confirms that the selected conditions are very close to the global optimum (Harkat-Madouri et al., 2025; Karabegoić et al., 2023). The strong agreement between the measured and predicted data demonstrates that the optimization model provides a reliable representation of the experimental system.

Table 4. Optimization results.

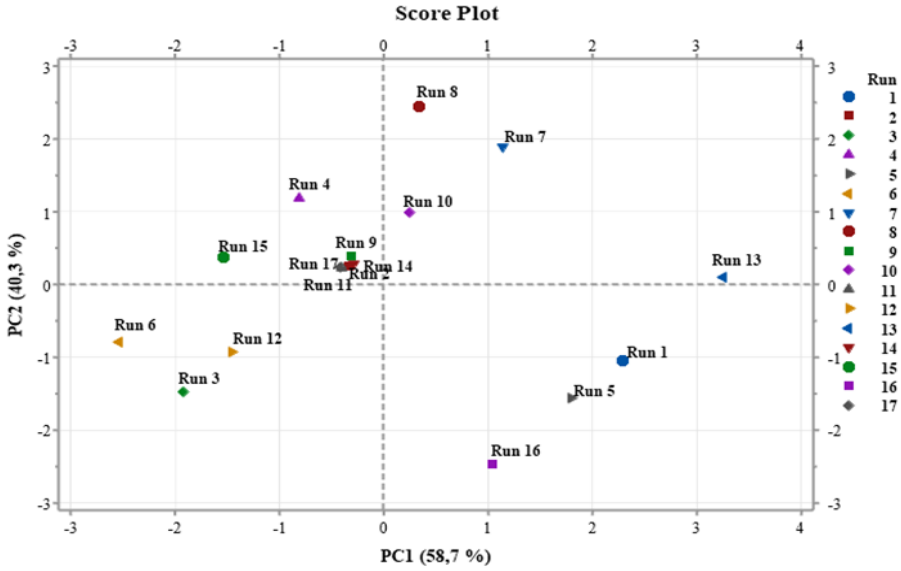
Parameter	Value	Response	Experimental value	Fitted value	Desirability
Solid mass (g)	0.5	TPC (mg-GAE/g- DM)	94.504	95.109	0.994
Immersion time (min)	30	TFC (mg-CE/g-DM)	67.103	66.998	
Solvent concentration (%, v/v)	33.030	DPPH (mg-TEAC/g-DM)	37.805	38.408	
		ABTS (mg-TEAC/g-DM)	43.104	42.333	

On the other hand, these measured variables in the *H. perforatum* extract is close to those of the earlier studies (Alahmad et al., 2022; Kaplan et al., 2021). Alahmad et al. extracted *H. perforatum* with different solvents, where they found the TPC value to be ≈93.2 Mg-GAE/g in methanol and ≈64.4 Mg-GAE/g in ethanol (Alahmad et al., 2022). Kaplan et al. found that increasing the liquid/solid ratio in 45% ethanol increased the TPC, where 50.7 Mg-GAE/g was measured at a 1/20 ratio and 60.5 Mg-GAE/g at a 1/40 ratio (Kaplan et al., 2021).

Principal component analysis

PCA accounted for 99.0% of the total variance, signifying that almost all variations in the dataset were well represented. The first principle component (PC1) possessed an eigenvalue of 2.35, accounting for 58.7% of the variance, and the second principal component (PC2) had an eigenvalue of 1.61, elucidating an additional 40.3%. Together, PC1 and PC2 captured nearly the entire variation, providing a clear distinction among the experimental runs based on their bioactive properties.

As seen in the score plot (Figure 8a), the runs were grouped according to their bioactive properties. Runs 7 and 13 were associated with PC1 and showed the highest TPC and TFC values. Runs 1, 5 and 16, on the other hand, were associated with PC2 and showed stronger antioxidant activity (DPPH and ABTS). In contrast, Runs 3 and 15 appeared in the opposite quadrant, reflecting generally low values for all responses.



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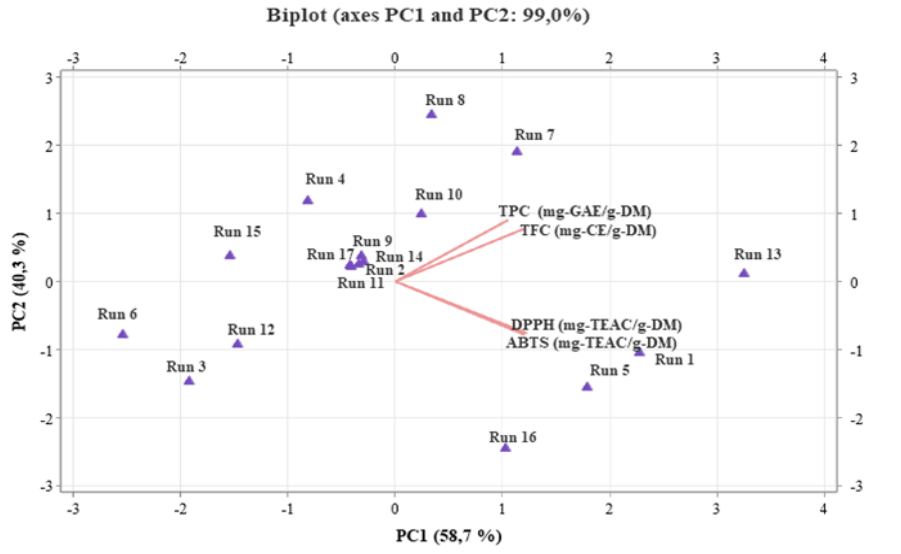

b)

Figure 8. (a) PCA score plot of extraction runs based on TPC, TFC, DPPH, and ABTS values, with each sample labelled by run number. (b) PCA biplot showing the relationships between the variables (TPC, TFC, DPPH, ABTS) and sample distribution along the principal components (PC1, PC2).

The biplot (Figure 8b) supported these findings. TPC and TFC were grouped along PC1, confirming their close relationship (R^2>0.90), while DPPH and ABTS clustered on PC2, showing their strong correlation (R^2>0.99), as antioxidant assays. Overall, the results show that the extraction conditions affected both the amount and the composition of the phenolic compounds. Less concentrated solvent systems (around 30% ethanol) encouraged phenolic and flavonoid enrichment (Runs 7 and 13), while higher ethanol levels (60–90%) enhanced radical scavenging capacity (Runs 1, 5 and 16). In summary, PCA successfully separated the extracts enriched in phenolic and flavonoid compounds from those showing greater antioxidant activity, emphasizing the role of solvent concentration in shaping the bioactive profile.

CONCLUSION

Bioactive compounds from *Hypericum perforatum* L. were recovered by automatic solvent with RSM optimization effectively. The concentration of ethanol and solid mass were determined to be statistically significant factors affecting the yields of phenolic compounds, flavonoids, and antioxidant activity at P<0.0001. Optimum extraction conditions (0.5 g of plant, 30 min of extraction and ~30% ethanol) gave the highest yields with a satisfying desirability function (0.994). The observed phenolic and flavonoid yields were similar or superior to the earlier studies on the *H. perforatum* extract. Furthermore, the results of the DPPH and ABTS tests for antioxidant activity were very similar, which proved the reliability of the current findings. The last but not the least, PCA distinguished the *H. perforatum* extracts high in phenolics/flavonoids from those with better radical scavenging capacity. This result also supports the affect of extraction parameters on the bioactive profiles. To conclude, the proposed extraction system can be recommended as a fast and environmentally friendly technique for producing high quality of *H. perforatum* extracts with potential applications in functional food formulations.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest in writing upon submission of the manuscript.

AUTHORS' CONTRIBUTIONS

İrem Toprakçı Yüksel: Formal analysis, Investigation, Software, Validation, Conceptualization, Writing-review & editing.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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