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Modeling and Optimizing Homogenizator-Assisted Extraction of Antioxidant Compounds from Linden and Characterization by HPLC-PDA

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Ihlamurdan Antioksidan Bileşiklerin Homojenizatör Destekli Ekstraksiyonunun Modellenmesi ve Optimize Edilmesi ve HPLC-PDA ile Karakterizasyonu

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Abstract

In this study, the optimization of homogenizer-assisted extraction (HAE) of antioxidants from linden were carried out using response surface methodology (RSM). Experimental factors included homogenization speed, extraction time, solvent-to-solid ratio, and solvent concentration. The HAE process was optimized to maximize the total antioxidant capacity (TAC) which determined by using CUPric Reducing Antioxidant Capacity (CUPRAC) of the linden extract. The models created for the TAC responses demonstrated a significant relationship ($p < 0.0001$) between the dependent response and the independent parameters. Water ratio was identified as the most significant operational factor in the HAE process, while solvent-to-solid ratio was determined to be the least significant parameter. According to the experimental data obtained from the model, the optimum conditions were found to be 14980 rpm homogenizer speed, 15 min extraction time, 98 mL solvent for 0.1 g solid and 41% water in EtOH solvent ratio and showed strong agreement with the results predicted by the model. Under the optimum operational conditions of HAE, 0.912 mmol TR/g-dried sample was achieved as TAC. Under the same experimental operating conditions, when looking at the TAC values, it was determined that the HAE method was much more efficient than the traditional heat removal method. The individual antioxidants of the linden extract were identified using HPLC on a C18 column with a gradient elution method. Using HPLC–PDA analyses, 12 antioxidants were identified in the linden extract. In conclusion, the modeled methodology is proposed as a feasible method for the extraction of antioxidants from linden in the product industry.

Keywords: *Extraction; Bioactive compounds; Optimization; Chromatography; Natural product.*

Öz

Bu çalışmada, ıhlamurdan elde edilen antioksidanların homojenizatör destekli ekstraksiyonunun (HAE) optimizasyonu, yanıt yüzey metodolojisi (RSM) kullanılarak gerçekleştirilmiştir. Deneysel faktörler homojenizasyon hızı, ekstraksiyon süresi, çözücü-katı oranı ve çözücü konsantrasyonudur. HAE işlemi, ıhlamur ekstraktının CUPric İndirgeyici Antioksidan Kapasitesi (CUPRAC) kullanılarak belirlenen toplam antioksidan kapasitesini (TAC) maksimize etmek için optimize edilmiştir. TAC yanıtları için oluşturulan modeller, bağımlı yanıt ile bağımsız parametreler arasında anlamlı bir ilişki ($p < 0,0001$) göstermiştir. Su oranı, HAE işleminde en anlamlı operasyonel faktör olarak tanımlanırken, çözücü-katı oranı en az anlamlı parametre olarak belirlenmiştir. Modelden elde edilen deneysel verilere göre optimum koşullar, 14980 rpm homojenizatör hızı, 15 dk ekstraksiyon süresi, 0,1 g katı için 98 mL çözücü ve çözücünün oranı da EtOH içinde %41 su olarak bulunmuştur ve model tarafından öngörülen sonuçlarla güçlü bir uyum göstermiştir. HAE'nin optimum operasyonel koşulları altında, 0,912 mmol TR/g-kuru örnek TAC olarak elde edilmiştir. Aynı deneysel çalışma koşulları altında, TAC değerlerine bakıldığında, HAE yönteminin geleneksel ısı giderme yönteminden çok daha verimli olduğu belirlenmiştir. Ihlamur ekstraktının standart antioksidanları, bir C18 kolonunda gradyan elüsyon yöntemi ile HPLC kullanılarak tanımlanmıştır. HPLC-PDA analizleri kullanılarak, ıhlamur ekstraktında 12 antioksidan tanımlandı. Sonuç olarak, modellenen metodoloji, ürün endüstrisinde ıhlamurdan antioksidanların ekstraksiyonu için uygulanabilir bir yöntem olarak önerilmektedir.

Anahtar Kelimeler: *Ekstraksiyon; Biyoaktif bileşikler; Optimizasyon; Kromatografi; Doğal ürün.*

1. Introduction

Linden, commonly known as Tilia spp., refers to a group of deciduous trees and shrubs widely distributed across Europe, North America, and Asia. These species are highly valued for their medicinal and aromatic properties, particularly the flowers, which have been used in traditional medicine for centuries. Linden flowers are rich in bioactive compounds, including flavonoids, phenolic acids, and essential oils, which contribute to their antioxidant, anti-inflammatory, and sedative properties (Pourmorad *et al.* 2006). The antioxidant activity of linden is primarily attributed to its high phenolic content. Flavonoids such as quercetin, kaempferol, and their derivatives are the major compounds responsible for

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scavenging free radicals and preventing oxidative stress (Dorman *et al.* 2003). These bioactive compounds are of significant interest in pharmacology, nutraceuticals, and food industries due to their potential health benefits, including reducing the risk of chronic diseases such as cardiovascular disorders, diabetes, and cancer (Demir *et al.* 2024; Nabavi *et al.* 2020). Extracting antioxidants from linden flowers involves optimizing techniques to preserve their bioactivity while maximizing yield. Common extraction methods include solvent-based techniques, such as maceration, Soxhlet extraction, and ultrasonic-assisted extraction (UAE), using solvents like ethanol, methanol, or water. Advanced techniques such as supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) are gaining attention for their efficiency and eco-friendliness. Parameters such as solvent type, extraction time, temperature, and pH play critical roles in determining the extraction yield and antioxidant activity of the final extract (Chemat *et al.* 2017). Homogenizer-assisted extraction (HAE) offers significant advantages over classical, microwave-assisted, and ultrasonic-assisted extraction methods, particularly in the recovery of bioactive compounds from plant materials. By generating high shear forces, HAE disrupts cellular structures effectively, enhancing the contact surface between the solvent and the target compounds, which results in higher extraction yields within shorter times (Rombaut *et al.* 2014). Unlike MAE, which can cause thermal degradation of heat-sensitive compounds due to localized overheating, HAE operates under controlled temperatures, minimizing compound loss (Chemat *et al.* 2017). Moreover, HAE is more energy-efficient and requires less solvent compared to ultrasonic methods, while avoiding issues like foaming and energy dissipation often observed in ultrasonication. Its simplicity, lower operational risks, and adaptability to various sample types make HAE a robust and eco-friendly alternative for routine extraction processes, especially in applications requiring the preservation of thermally sensitive bioactive compounds. Given the rising demand for natural antioxidants, linden flowers serve as a promising source of bioactive compounds. Selecting the appropriate extraction method for the target is the first step. Understanding and optimizing the extraction methods not only ensure the effective utilization of these natural resources but also contribute to developing sustainable approaches in functional food and pharmaceutical industries (Gök *et al.* 2024). Optimizing experimental parameters in one go is time-consuming, expensive, and difficult. Therefore, it is important to develop an experimental design. Response surface methodology (RSM) involves a series of experimental studies in which the independent variables are methodologically varied (Behaiyn *et al.* 2023). HAE method was used for antioxidant extraction from linden and the most important parameters for extraction were obtained in the most appropriate way using response surface methodology (RSM). The best points of the parameters aimed to maximize the total antioxidant capacity (TAC) evaluated by the CUPric Reducing Antioxidant Capacity (CUPRAC) method. The optimized parameters included homogenization speed, extraction time, solvent-to-solid ratio, and solvent concentration. Furthermore, the effects of these operational factors on TAC were analyzed and discussed. The antioxidant capacity of the HAE sample obtained under optimal conditions was then compared with that of the sample extracted using conventional heat extraction. For this comparison, CUPRAC, ABTS, DPPH, and Folin methods were employed. The novelty of this study lies in the application of homogenizer-assisted extraction (HAE) as a green, efficient, and thermally gentle technique for recovering antioxidants from linden. By combining this advanced method with statistical optimization, the study offers a sustainable and rapid alternative to conventional extraction techniques, contributing significantly to the development of eco-friendly extraction strategies for thermolabile bioactive compounds. As a result of the study, the HAE method, in which the most suitable conditions for extraction are determined, is presented to the literature as a simple, fast and low-cost method for antioxidant extraction from linden.

2. Materials and Methods

2.1 Chemicals and instrumentations

In this study, analytical grade chemicals were used and sourced from the following suppliers:

Copper(II) chloride ($CuCl_2$) was from Merck, ammonium acetate (NH_4Ac) and copper(II) sulfate from Riedel–de Haën (Seelze, Germany); Folin–Ciocalteu reagent, gallic acid (GA), potassium sodium tartrate tetrahydrate, copper(II) chloride dihydrate, potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), methanol (MeOH), ethanol (EtOH) from Sigma-Aldrich (St. Louis, MO, USA); neocuproine (Nc), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin (QR), chlorogenic acid (CLA), chlorogenic acid (CLA) from Aldrich (Taufkirchen, Germany); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), catechin (CT), kaempferol-3-*O*-glucoside (Kaem-3-*O*–G), from Fluka (Buchs, Switzerland); epicatechin (EC), rutin (RT), p-coumaric acid (COU), from Sigma (Taufkirchen, Germany); protocatechuic acid (3,4-

dihydroxybenzoic acid, 3,4-DHBA), vanillic acid (VA), sinapic acid (SPA) from Ambeed (IL, USA). Linden (Tilia cordata L.) was supplied by a local market (Şah Sultan, 2022) in Istanbul, Turkey.

Shimadzu UV-1900i spectrophotometer (Kyoto, Japan) was used for the absorption measurements. HAE of the antioxidants from Tilia L. was done with using DAIHAN homogenizator extraction system (Gangwondo, South Korean). Shimadzu high-performance liquid chromatography (HPLC) system coupled with photodiode array detector (PDA) (Kyoto, Japan) was used for characterization and quantification of linden phenolic antioxidants. InertSustain-C18 coloumn (250 mm, 4.6 mm, 5 µm) was used for the HPLC analysis.

2.2 Homogenizator-assisted extraction (HAE)

Before starting the study, the moisture content of the freeze-dried linden samples was determined (6.67%). Then, they were ground using a mortar (300-600 µM) and stored in the refrigerator. The HAE treatment of linden was conducted under varying operational conditions, including homogenization speeds (5000, 10000, and 15000 rpm), durations (5, 10, and 15 minutes), solvent concentrations (0, 25, and 50% water in ethanol), and solvent-to-solid ratios (20, 60, and 100 mL/0.1 g). The resulting extracts from each treatment were filtered and kept at 4 ℃ for subsequent analysis.

2.3 Total antioxidant capacity (TAC) assay

The cupric reducing antioxidant capacity (CUPRAC) method was used to determine the antioxidant capacity of linden extract (Apak *et al*. 2004). In this method, while antioxidants are oxidized, the Cu(II)-Nc complex is reduced to Cu(I)-Nc. 450 nm is the wavelength at which the absorbance change is measured. The analysis procedure was carried out as follows:

A mixture consisting of 1 mL of $CuCl_2$ solution, 1 mL of Nc solution, and 1 mL of NH_4Ac solution was prepared. Then, x mL sample (linden extract) was added and the total was completed to 4.1 mL with distilled water. After an incubation period of 30 minutes, absorbance values were read at 450 nm. A blank solution without sample was used as a reference. The molar absorptivity of the TR standard was used as the slope of the line obtained from the concentration-absorbance graph. TAC results for linden samples are given as mmol TR/g-dried sample.

$$TAC\ (\frac{mmol\ TR}{g-dried\ sample}) = \frac{As}{\varepsilon}\ x \frac{Vt}{V\ddot{o}}\ x\ df\ x \frac{Ve}{m}$$

A_s: Absorbance value recorded for sample
ε: Molar absorptivity coefficient for TR (ε_{TR} =16700 L $mol^{-1}.cm^{-1}$)
V_t: Total method volume (mL)
V_s: Sample volume in method (mL)
df: Dilution factor
V_e: Volume of extract (mL)
m: Total amount of sample during extraction (g)

2.4 ABTS radical ($ABTS^{\cdot+}$) scavenging capacity (ARC) assay

This assay evaluates antioxidant activity based on the decolorization of the $ABTS^{\cdot+}$ radical cation by antioxidants (Re *et al*. 1999). The procedure was carried out as follows:

1 mL of diluted $ABTS^{\cdot+}$ solution, (x) mL of sample, and (4 − x) mL of ethanol were added to a test tube. The mixture was incubated for 6 minutes, and absorbance was then measured at 734 nm against ethanol as the reference for both the sample and blank solutions. To calculate the absorbance difference (ΔA), the absorbance of the blank radical solution (containing the solvent instead of the sample) was subtracted from the absorbance of the sample solution. Using a calibration curve constructed by plotting TR standard concentrations against absorbance, TAC of the extracts was expressed in units of mmol TR/g-dried sample.

$$TAC\ (\frac{mmol\ TR}{g-dried\ sample}) = \frac{\Delta A}{\varepsilon}\ x \frac{Vt}{V\ddot{o}}\ x\ df\ x \frac{Ve}{m}$$

$\Delta A = A_{ABTS} - (A_E - A_0)$
A_{ABTS}: Absorbance of $ABTS^{\cdot+}$ reagent without the sample
A_E: Absorbance of the extract
A_0: Absorbance of the extract without $ABTS^{\cdot+}$ reagent.
ΔA: Absorbance value recorded for sample
ε: Molar absorptivity coefficient for TR (ε_{TR} =26000 L $mol^{-1}.cm^{-1}$)
V_t: Total method volume (mL)
V_s: Sample volume in method (mL)
df: Dilution factor
V_e: Volume of extract (mL)
m: Total amount of sample during extraction (g)

2.5 Free radical scavenging capacity (FRC) assay

This assay measures the ability of antioxidants to scavenge the DPPH radical, a stable organic nitrogen radical. The DPPH radical exhibits a purple color with maximum absorbance at 517 nm (Sanchez-Moreno *et al*. 1998). The procedure was conducted as follows: 1 mL of diluted DPPH (0.2 mM) radical solution, (x) mL of sample, and (4 − x) mL of ethanol were added to a test tube. The mixture was stirred and incubated at room temperature for 30 minutes. After the incubation period, absorbance was measured at 515 nm with ethanol as the reference for both the sample and blank solutions. The absorbance difference (ΔA) was calculated by subtracting the absorbance of the blank radical solution (containing the solvent instead of the sample) from the absorbance of the

sample solution. Using the calibration curve, created by plotting TR standard concentrations against absorbance, TAC of the extracts was expressed in units of mmol TR/g-dried sample.

$$TAC\ (\frac{mmol\ TR}{g-dried\ sample}) = \frac{\Delta A}{\varepsilon}\ x \frac{Vt}{V\ddot{o}}\ x\ df\ x \frac{Ve}{m}$$

$\Delta A = A_{DPPH} - (A_E - A_0)$
A_{DPPH}: Absorbance of DPPH reagent without the sample
A_E: Absorbance of the extract
A_0: Absorbance of the extract without DPPH reagent.
ΔA: Absorbance value recorded for sample
ε: Molar absorptivity coefficient for TR (ε_{TR} =24592 L mol^{-1}.cm^{-1})
V_t: Total method volume (mL)
V_s: Sample volume in method (mL)
df: Dilution factor
V_e: Volume of extract (mL)
m: Total amount of sample during extraction (g)

2.6 Total phenolic content (TPC) assay

Total phenolic content (TPC) of the linden extract was determined using the Folin-Ciocalteu assay (Singleton *et al*. 1999). The reagents were prepared as follows:

Lowry A solution: 2% Na_2CO_3 in 0.1 M NaOH,
Lowry B solution: 0.5% $CuSO_4$ in 1% $NaKC_4H_4O_6$,
Lowry C solution: A mixture of 50 mL of Lowry A and 1 mL of Lowry B.

The analysis procedure was conducted as follows: A test tube was prepared by adding x mL of the extract, 2.5 mL of Lowry C, and (1 − x) mL of distilled water. The mixture was allowed to sit for 10 minutes, followed by the addition of 0.25 mL of Folin reagent, which had been diluted three times. After a 30 min incubation period at room temperature, the absorbance change was read at 750 nm against a non-antioxidant blank solution. The molar absorptivity of the TR standard was used as the slope of the line obtained from the concentration-absorbance graph. TAC results for linden samples are given as mmol TR/g-dried sample.

$$TAC\ (\frac{mmol\ TR}{g-dried\ sample}) = \frac{As}{\varepsilon}\ x \frac{Vt}{V\ddot{o}}\ x\ df\ x \frac{Ve}{m}$$

A_s: Absorbance value recorded for sample
ε: Molar absorptivity coefficient for TR (ε_{TR}= 4650 L mol^{-1}.cm^{-1})
V_t: Total method volume (mL)
V_s: Sample volume in method (mL)
df: Dilution factor
V_e: Volume of extract (mL)
m: Total amount of sample during extraction (g)

2.7 Chromatographic Analysis with HPLC-PDA

Separation of antioxidants was carried out using a InertSustain-C18 (250 mm, 4.6 mm, 5 µm) analytical column. The injection volume was 20-µL and the flow rate was 1 mL min−1. Two analytical detection wavelengths, 270 and 320 nm, were used to observe all antioxidants in the linden sample. A gradient elution program was applied using two different mobile phases: solvent A (2% (v/v) acetic acid in water) and solvent B (MeOH). Applied gradient elution program:

initially 95% A–5% B (0. min)
90% A–10% B (5. min) (slope 1.0);
80% A–20% B (20. min) (slope 1.0);
70% A–30% B (30. min) (slope 1.0);
60% A–40% B (35. min) (slope 1.0);
50% A–50% B (45. min) (slope 2.0);
95% A–5% B (65. min) (slope 1.0).

2.8 Statistical Analysis

The program used for optimization is Design-Expert® Software Version 11. Analyses were guided by RSM including face-centered composite design (FCCD). In this study, four independent variables were selected: X_1 (homogenization speed, ranging from 5000 to 15000 rpm), X_2 (extraction time, measured in minutes), X_3 (the solvent-to-solid ratio, specified as 20-100 mL/0.1 g), and X_4 (solvent concentration, defined as the percentage of water in ethanol). The study identified TAC as the sole dependent variable. Experimental data were used to determine the independent variables and their respective levels, which are detailed in Table 1. The results are given in Table 2 along with the experimental data. A quadratic equation, as shown in Eq. 1 (Stadler *et al*., 2002), was applied to model the experimental findings.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta\, x_i + \sum_{i=1}^{k} \beta_{ii}\, x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij}\, x_i x_j + \varepsilon \quad \text{(Eq. 1)}$$

In Eq. 1:

β_0: The constant regression coefficient,
β_i, β_{ii}, β_{ij}: The interaction coefficients,
k: The factor number,
Ɛ: is experimental error.

Table 1. The independent variables, their levels, units, and symbols.

Independent variables	Symbol of the variables	Units	Levels		
			-1	0	1
Homogenizator speed	A	rpm	5000	10000	15000
Time	B	min	5	10	15
Solvent-to-solid ratio	C	mL/0.1 g	20	60	100
Solvent concentration	D	%, v/v	0	25	50

3. Results and Discussions

Table 2 provides an overview of how the independent variables influenced the TAC of linden extract produced via HAE in the experimental study. The TAC levels in the extracts were determined to vary between 0.141 and 0.911 mmol TR/g-DS.

3.1. Modeling and Optimization of HAE Using RSM

It was determined that the model used to explain the relationship between the obtained results and the independent variables was significant (p<0.0001). Among the parameters, solvent concentration had the most substantial effect on TAC outcomes. Table 3 presents the outcomes of applying quadratic models to the data. The ANOVA results indicate that the quadratic model made a statistically significant contribution

The quadratic models for TAC are presented in Eqs. (2). The significance of each coefficient was assessed through the F-test and p-values provided in Table 3.

$$CUPRAC = +0.5265 + 0.0794A + 0.0411B - 0.0080C + 0.2341D + 0.0111AB + 0.0232AC + 0.0107AD - 0.0032BC - 0.0272BD + 0.0312CD + 0.0753A^2 + 0.0880B^2 - 0.0027C^2 - 0.1828D^2$$

Table 2. FCDD of independent factors for the HAE and experimental results for the CUPRAC.

Run No	Factors				TAC (mmol TR/ g-DS)
	A	B	C	D	
1	5000	5	20	0	0.176
2	15000	5	20	0	0.282
3	5000	15	20	0	0.275
4	15000	15	20	0	0.470
5	5000	5	100	0	0.141
6	15000	5	100	0	0.221
7	5000	15	100	0	0.185
8	15000	15	100	0	0.363
9	5000	5	20	50	0.666
10	15000	5	20	50	0.749
11	5000	15	20	50	0.667
12	15000	15	20	50	0.742
13	5000	5	100	50	0.604
14	15000	5	100	50	0.890
15	5000	15	100	50	0.626
16	15000	15	100	50	0.911
17	5000	10	60	25	0.581
18	15000	10	60	25	0.722
19	10000	5	60	25	0.549
20	10000	15	60	25	0.780
21	10000	10	20	25	0.602
22	10000	10	100	25	0.546
23	10000	10	60	0	0.159
24	10000	10	60	50	0.629
25	10000	10	60	25	0.478
26	10000	10	60	25	0.473
27	10000	10	60	25	0.475
28	10000	10	60	25	0.486
29	10000	10	60	25	0.471
30	10000	10	60	25	0.473

Table 3. The ANOVA for quadratic equations for the CUPRAC.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.27	14	0.0905	20.48	< 0.0001	significant
A-Speed	0.1135	1	0.1135	25.67	0.0001	
B-Time	0.0305	1	0.0305	6.89	0.0191	
C-Solvent-to-solid ratio	0.0012	11	0.0012	0.2620	0.6162	
D-Water ratio	0.9862	1	0.9862	223.11	< 0.0001	
AB	0.0020	1	0.0020	0.4427	0.5159	
AC	0.0086	1	0.0086	1.94	0.1836	
AD	0.0018	1	0.0018	0.4111	0.5311	
BC	0.0002	1	0.0002	0.0363	0.8515	
BD	0.0118	1	0.0118	2.68	0.1226	
CD	0.0156	1	0.0156	3.52	0.0803	
A^2	0.0147	1	0.0147	3.32	0.0885	
B^2	0.0201	1	0.0201	4.54	0.0500	
C^2	0.0000	1	0.0000	0.0043	0.9486	
D^2	0.0866	1	0.0866	19.58	0.0005	
Residual	0.0663	15	0.0044			
Lack of fit	0.0662	10	0.0066	237.80	< 0.0001	significant
Pure error	0.0001	5	0.0000			
Cor Total	1.33	29				

The quadratic models for TAC are presented in Eqs. (2). The significance of each coefficient was assessed through the F-test and p-values provided in Table 3.

$$CUPRAC = +0.5265 + 0.0794A + 0.0411B - 0.0080C + 0.2341D + 0.0111AB + 0.0232AC + 0.0107AD - 0.0032BC - 0.0272BD + 0.0312CD + 0.0753A^2 + 0.0880B^2 - 0.0027C^2 - 0.1828D^2$$

The models developed for TAC were shown to be significant (p > 0.05). Additionally, the predicted R^2 value of 0.7731 was found to be in good agreement with the adjusted R^2 value of 0.9039, as the difference between them was less than 0.2. For TAC, an adequate precision value, which evaluates the signal-to-noise ratio, exceeded the desired threshold of 4 and was calculated as 17.263. As a result of the experiments, it was seen that the

solvent-to-solid ratio variable had no significant effect on the extraction. If the solvent-to-solid ratio was ignored for modeling, the lack-of-fit value was found not significant. The Lack of Fit F-value of 1.98 implies the Lack of Fit is not significant relative to the pure error. There is a 13.98% chance that a Lack of Fit F-value this large could occur due to noise. In conclusion, using the Design-Expert software, the HAE process variables were optimized to achieve the highest CUPRAC value. The maximum CUPRAC (0.912 mmol TR/g-DS) was obtained under the following conditions: A = 14980 rpm, B = 15 minutes, C = 98 mL/0.1 g-DS, and D = 41% water in ethanol.

3.1. Effects of Selected Parameters on HAE

The 3D response surface plots in Figure 1 were generated based on Eqs. 2 to visualize the interactions among the various factors and their respective effects on the TAC responses. Figure 1 shows the 3D representation of the CUPRAC results for time, homogenizer speed, and solvent-to-solid ratio against water ratio. When the results are examined, the solvent concentration is the most critical parameter for this study. The second most important parameter is homogenizer speed. In general, as the water content increases, TAC increases up to a certain level, but after a certain point the water content begins to balance. When Figure 1c is examined, it is seen that the change of solvent-to-solid ratio does not affect the extraction efficiency much. Pereira *et al.* (2017) studied the optimization of the extraction of phenolics from banana peel by HAE process and reported that the solvent-to-solid ratio did not affect the extraction efficiency, similar to our findings. It was also reported that 50% water ratios gave the best results, which is consistent with the results of our study. While the polarity of water is quite high, ethanol has low polarity, but both are mixed to form effective combinations in the extraction of phenolics (Roby *et al.* 2013). The mixture of organic solvent and water at a ratio of approximately 50% (v/v) is generally effective in the extraction of phenolics from solid samples (Turkmen *et al.* 2007). The use of aqueous solvents promotes the hydration of particles, facilitates the penetration of the organic solvent into the matrix and consequently enhances mass transfer by diffusion (Ghitescu *et al.* 2015).

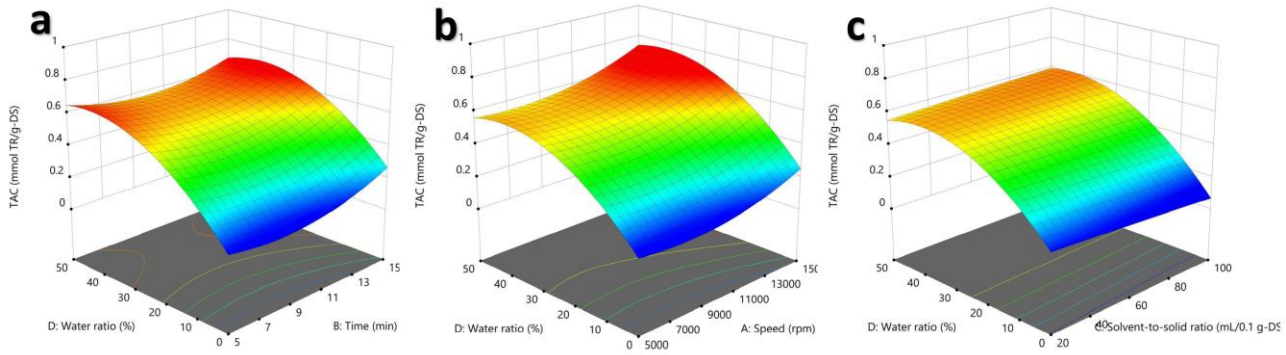

Figure 1. The 3D surface plot for the CUPRAC of the linden extract as a function of extraction: a) water ratio to time, b) water ratio to speed, and c) water ratio to solvent-to-solid ratio.

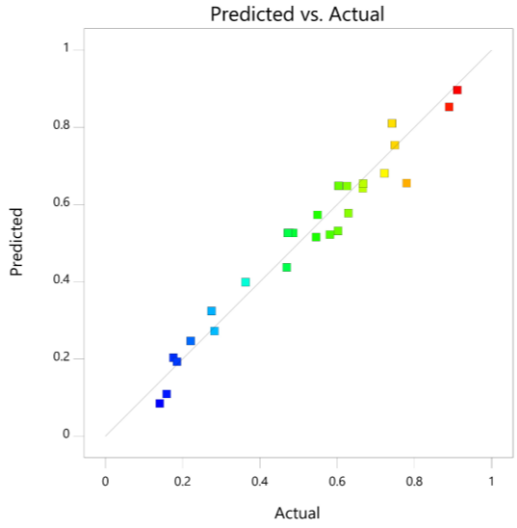

Figure 2. The correlation between the predicted and experimental CUPRAC values of the linden extracts.

3.2. Verification of Predictive Models

Figure 2 illustrates the correlation between the TAC values of linden extract, experimentally determined and predicted using quadratic models, across various combinations of HAE operational parameters. The strong agreement between the predicted and experimental results confirms that the developed models successfully achieved their objective.

3.3. Comparison of the CUPRAC, ABTS, DPPH, and TPC Values of Linden Extracts Obtained via HAE and Classical Heat Extraction (CHE)

Linden extract was produced using classical heat extraction (CHE) under the optimized conditions of the HAE method. The TAC values for linden extracts prepared

via HAE and CHE were measured as 0.71 and 0.27 mmol TR/g-sample, respectively.

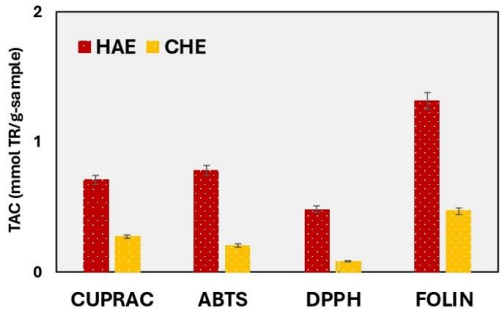

Figure 3. Comparison of TAC, ABTS, DPPH and Folin results using linden extract HAE and CHE.

Similarly, the ABTS values were determined to be 0.78 mmol TR/g-sample for HAE and 0.20 mmol TR/g-sample for CHE. For DPPH, the extracts showed values of 0.48 mmol TR/g-sample with HAE and 0.08 mmol TR/g-sample with CHE. The TPC values were recorded as 1.31 mmol TR/g-sample for HAE and 0.47 mmol TR/g-sample for CHE. These results indicate that the TAC, ABTS, DPPH, and TPC values of linden extract produced using the HAE method were approximately twice as high as those obtained with the CHE method.

3.4. Chromatographic Analysis with HPLC–PDA

The identification of 12 antioxidants (GA, 3,4-DHBA, CT, 4-HBA, CLA, VA, EC, COU, SPA, RT, Kaem-3-*O*-G, QR) in linden was made by the developed HPLC method. The retention time, calibration equation, correlation coefficient, linear range and the results of the content of the standards in linden obtained from the PDA system, where a specific wavelength of 270 nm and 320 nm was used for each analyte, are given in Table 4.

Table 4. The standard antioxidants identified in linden by HPLC–PDA analyses.

Identified compounds	λ_{max} (nm)	t_R (min)	Calibration equation and correlation coefficient (r^2)	Linear range (mg L^{-1})	Content (mg L^{-1})
GA	270	7,40	y = 64974x - 1243.8 r^2=1	0.094-9.407	0.213
3,4-DHBA	270	11,80	y = 53255x + 162.9 r^2=1	0.015-7.706	0.759
CT	270	15,67	y = 9840.8x + 295.12 r^2=1	0.145-14.514	6.951
4-HBA	270	17,20	y = 91940x + 308.9 r^2=1	0.014-6.906	0.071
CLA	320	19,36	y = 9840.8x + 295.1 r^2=1	0.177-17.716	0.393
VA	270	21,73	y = 73705x + 1062.7 r^2=1	0.017-8.408	0.175
EC	270	24,53	y = 9986.7x - 1225.7 r^2=1	0.290-14.514	0.987
COU	320	32,64	y = 183725x + 961.8 r^2=1	0.016-8.208	0.104
SPA	320	36,53	y = 100661x + 523.2 r^2=1	0.022-11.211	0.001
RT	320	42,26	y = 20020x + 271.5 r^2=1	0.061-30.526	6.742
Kaem-3-*O*-G	320	46,17	y = 36531x + 267.5 r^2=1	0.045-22.429	6.211
QR	320	51,61	y = 34238x - 680.42 r^2=1	0.030-15.112	0.298

Figures 4A and B show the HPLC chromatogram of a standard mixture of 12 antioxidant compounds at 270 and 320 nm, respectively, while Figures 4C and D show the HPLC chromatogram of the linden sample obtained as a result of homogenizer-supported extraction under optimum conditions at 270 and 320 nm, respectively. In order to calculate the amounts of antioxidants in the linden extract, separate calibration lines were created for each compound by HPLC method. As a result, CT, RT, and Kaem-3-*O*-G were determined as the main components of linden. The amounts of CT, RT, and Kaem-3-*O*-G compounds were determined as 6.951, 6.742, and 6.211 mg L^{-1}, respectively.

4. Conclusions

In this study, the extraction of antioxidants from linden was optimized and modeled using RSM with four key factors of HAE: homogenizer speed, extraction time, solvent concentration, and solvent-to-solid ratio. Statistical analysis revealed that the models developed for TAC was significant in describing the relationship between the responses and the independent variables ($p < 0.0001$). Among the operational factors, solvent concentration was identified as the most effective parameter affecting the TAC values of the linden extract. Furthermore, the predicted values closely matched the

experimental results, demonstrating the reliability of the model. As a result of RSM, the optimum conditions for the extraction of antioxidants from linden were determined. Homogenizer speed, time solvent-to-solid ratio, and solvent concentration were determined as 14980 rpm, 15 min, 98 mL/0.1 g-DS, and 41% water in EtOH, respectively for optimal extraction. CUPRAC, ABTS, DPPH and Folin methods were applied to the samples obtained under optimum conditions and the results were compared with those obtained by the classical thermal method.

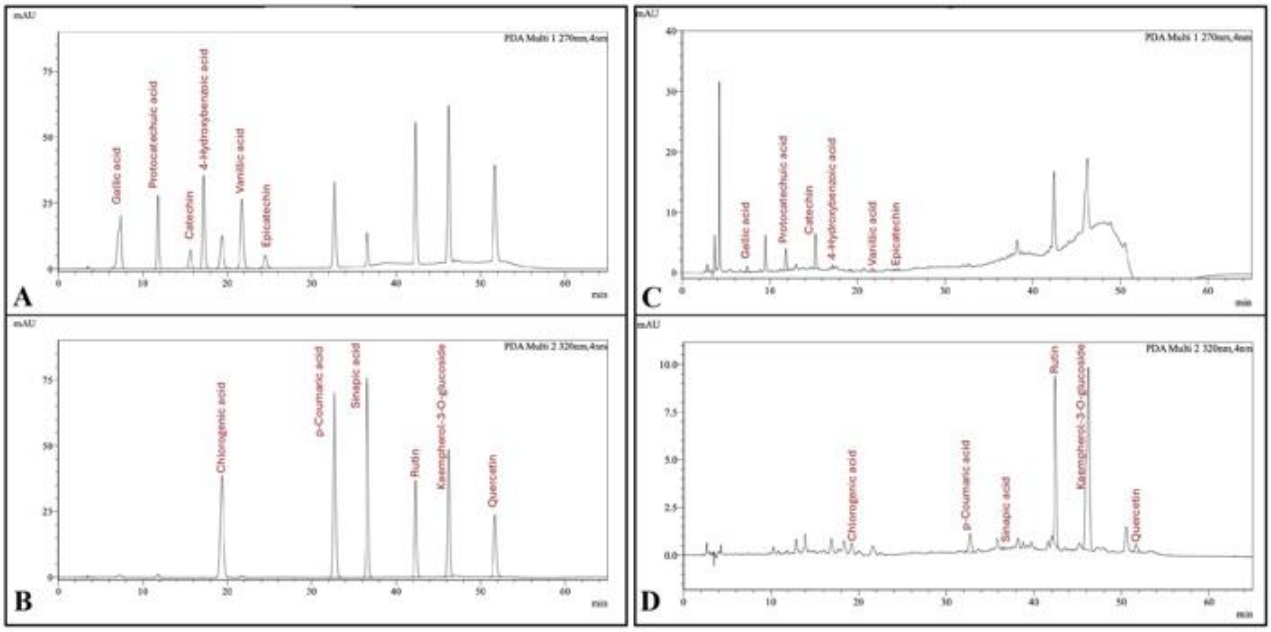

Figure 4. HPLC chromatogram of the standard antioxidant mixture (A) at 270 nm and (B) at 320 nm, and the HPLC chromatogram of the linden extract (C) at 270 nm and (D) at 320 nm (1: GA, 2: 3,4-DHBA, 3: CT, 4: 4-HBA, 5: CLA, 6: VA, 7: EC, 8: COU, 9: SPA, 10: RT, 11: Kaem-3-O-G, and 12: QR).

A fast, effective and low-cost method was proposed for the extraction of antioxidants from the linden sample with the HAE method, which gave quite good results compared to the traditional method. In addition, 12 antioxidant compounds were identified using the HPLC-DAD method and their amounts were calculated in the extract obtained under optimum conditions. This developed method can be an alternative for the extraction of antioxidants from linden in many sectors such as cosmetics, medicine and food.

Declaration of Ethical Standards

The author declares that she complies with all ethical standards.

Credit Authorship Contribution Statement

Author: Methodology/study design, Experiment and data analysis, Writing – original draft, Writing-review and editing

Declaration of Competing Interest

The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The author declares that the main data supporting the findings of this work are available within the article.

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