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Optimization of Three Extraction Factors for Flavonoid Contents in *Morus alba* var. *shalun* using Box–Behnken Response Surface Methodology

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Abstract. This study aimed to optimize the extraction conditions of total flavonoid content (TFC) using Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD). Three extraction variables were evaluated, namely ethanol concentration (40–80%), extraction temperature (40–70 °C), and extraction time (30–90 min). The experimental results showed that all extraction parameters and their interactions influenced flavonoid recovery. Contour plots, desirability analysis, and cube plot visualization demonstrated that moderate extraction conditions produced better responses compared to extreme conditions. The optimum extraction region was obtained at approximately 60% ethanol concentration, 55 °C extraction temperature, and 60 min extraction time, resulting in relatively high total flavonoid content with a good desirability value. The interaction among extraction variables indicated that balanced extraction conditions were important for improving extraction efficiency and maintaining compound stability. Overall, the developed RSM model provided useful information for optimizing flavonoid extraction from natural materials.

Keywords: Flavonoid extraction, Response surface methodology (RSM), Box-behnken design, Optimization, Total flavonoid content

Abstrak. Penelitian ini bertujuan untuk mengoptimasi kondisi ekstraksi total flavonoid content (TFC) menggunakan Response Surface Methodology (RSM) berbasis Box–Behnken Design (BBD). Tiga variabel ekstraksi yang dievaluasi meliputi konsentrasi etanol (40–80%), suhu ekstraksi (40–70 °C), dan waktu ekstraksi (30–90 menit). Hasil penelitian menunjukkan bahwa seluruh parameter ekstraksi beserta interaksinya memengaruhi perolehan flavonoid. Analisis plot kontur, fungsi desirability, dan visualisasi cube plot menunjukkan bahwa kondisi ekstraksi sedang memberikan respon yang lebih baik dibandingkan kondisi yang terlalu ekstrem. Daerah optimum diperoleh pada konsentrasi etanol sekitar 60%, suhu ekstraksi sekitar 55 °C, dan waktu ekstraksi sekitar 60 menit, yang menghasilkan kandungan flavonoid total relatif tinggi dengan nilai desirability yang baik. Interaksi antar variabel ekstraksi menunjukkan bahwa kondisi yang seimbang penting untuk meningkatkan efisiensi ekstraksi dan menjaga stabilitas senyawa. Secara keseluruhan, model RSM yang dikembangkan memberikan informasi yang bermanfaat untuk optimasi ekstraksi flavonoid dari bahan alam.

Kata kunci: Ekstraksi flavonoid, Response surface methodology, Box–Behnken design, Optimasi, Total flavonoid content

1. Introduction

Morus alba commonly known as white mulberry, is a medicinal plant widely distributed throughout Asia and traditionally utilized for various therapeutic purposes. Different parts of the plant, including leaves, fruits, and roots, have been reported to contain various bioactive compounds such as flavonoids, phenolics, alkaloids, and anthocyanins that contribute to antioxidant, anti-inflammatory, antimicrobial, and antidiabetic activities [1], [2]. Among these compounds, flavonoids are considered one of the major secondary metabolites responsible for many of the biological activities of *Morus alba* [3], [4]. Consequently, the total flavonoid content (TFC) has become an important quality parameter for evaluating the therapeutic potential and functional value of this plant [5].

The extraction process plays an essential role in determining the yield and composition of flavonoid compounds obtained from plant materials. Several extraction parameters, including solvent concentration, extraction temperature, and extraction time, are known to significantly influence extraction efficiency and flavonoid recovery [6]. Ethanol is widely used as an extraction solvent because of its relatively low toxicity and effectiveness in dissolving semi-polar compounds [7]. In addition, extraction temperature and extraction time affect solvent penetration, mass transfer, and compound stability during the extraction process [8]. However, excessively high temperatures or prolonged extraction times may lead to degradation of thermolabile compounds, resulting in reduced extraction

efficiency [9]. Therefore, optimization of extraction conditions is necessary to obtain high flavonoid recovery while maintaining compound stability.

Conventional optimization approaches based on varying one factor at a time are often inefficient and unable to adequately explain the interaction effects among extraction variables [10]. To overcome these limitations, response surface methodology (RSM) has been widely applied as an effective statistical and mathematical approach for optimizing phytochemical extraction processes [11], [12]. Among various RSM designs, the box–behnken design (BBD) is frequently preferred because it requires fewer experimental runs while still allowing evaluation of linear, interaction, and quadratic effects among variables [13], [14]. In addition, contour plots generated from RSM models provide visual interpretation of variable interactions and facilitate identification of optimum extraction regions [15]. Several studies have reported the application of RSM for flavonoid extraction from medicinal plants; however, most studies primarily focus on optimization results without providing detailed interpretation of interaction patterns through contour plot analysis [16].

Furthermore, studies specifically investigating the interaction effects of ethanol concentration, extraction temperature, and extraction time on total flavonoid content in *Morus alba* var. *shalun* remain limited. In particular, comprehensive interpretation of contour plots and cube plot visualization for understanding the synergistic interactions among extraction variables has not been extensively discussed in previous studies. Therefore, this study aimed to investigate the effects of ethanol concentration, extraction temperature, and extraction time on the total flavonoid content of *Morus alba* var. *shalun* using a box–behnken design. In addition, contour plot and cube plot analyses were employed to provide a clearer visualization and interpretation of the interaction effects among extraction variables. The objective of this study was to determine the optimum extraction conditions for maximizing total flavonoid content and to provide a better understanding of the interaction behavior among extraction parameters in *Morus alba* var. *shalun* extraction.

2. Method and Experimental

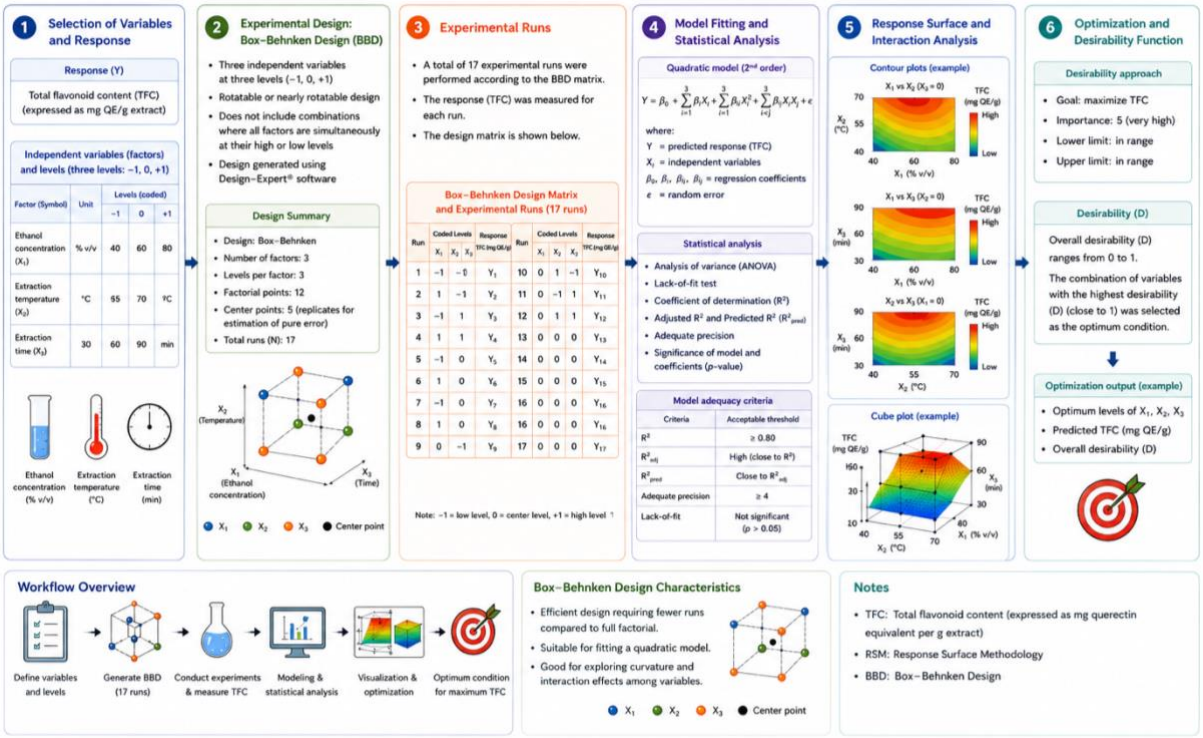


Fig 1. Workflow of Box–Behnken Design (BBD) and Response Surface Methodology (RSM) for optimization of total flavonoid content extraction

Figure 1 illustrates the overall workflow used in this study for optimizing total flavonoid content (TFC) extraction from Morus alba var. Shalun using Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD). Three independent variables were evaluated, namely ethanol concentration (40–80%), extraction temperature (40–70 °C), and extraction time (30–90 min), while TFC was used as the response variable. The experimental design consisted of 17 experimental runs generated using the BBD matrix, including factorial and center points. Experimental data obtained from each run were fitted into a second-order polynomial model and analyzed using analysis of variance (ANOVA) to evaluate the significance and adequacy of the model. Contour plots and cube plots were subsequently generated to visualize the interaction effects among extraction variables. Finally, desirability function analysis was employed to determine the optimum extraction conditions for maximizing total flavonoid content.

The raw experimental data presented in Table 1 were generated using the Box–Behnken Design (BBD) consisting of 17 experimental runs with three independent variables, namely ethanol concentration (%), extraction temperature (°C), and extraction time (min). Each factor was evaluated at three levels to investigate its effect on total phenolic content (TPC) expressed as mg GAE/g. The experimental TPC values ranged from 35.8 to 49.4 mg GAE/g, indicating variations in extraction efficiency under different experimental conditions. The obtained data were subsequently analyzed using Response Surface Methodology (RSM), ANOVA, and regression modeling in Design-Expert software version 12 to evaluate model adequacy and determine the optimum extraction conditions.

Table 1. Raw data factor and response

Run	Factor 1 Ethanol concentration	Factor 2 Temperature	Factor 3 Time	Response Total Flavonoid content (mg QE/g)
1	60	70	30	23
2	60	40	30	21
3	60	55	60	25.3
4	40	70	60	22.7
5	80	70	60	27.3
6	80	40	60	25.8
7	40	40	60	21.2
8	40	55	30	19.9
9	80	55	30	23.4
10	60	40	90	24.3
11	80	55	90	26.1
12	40	55	90	22.4
13	60	55	60	25.3
14	60	55	60	25.3
15	60	70	90	27.7
16	60	55	60	25.3
17	60	55	60	25.3

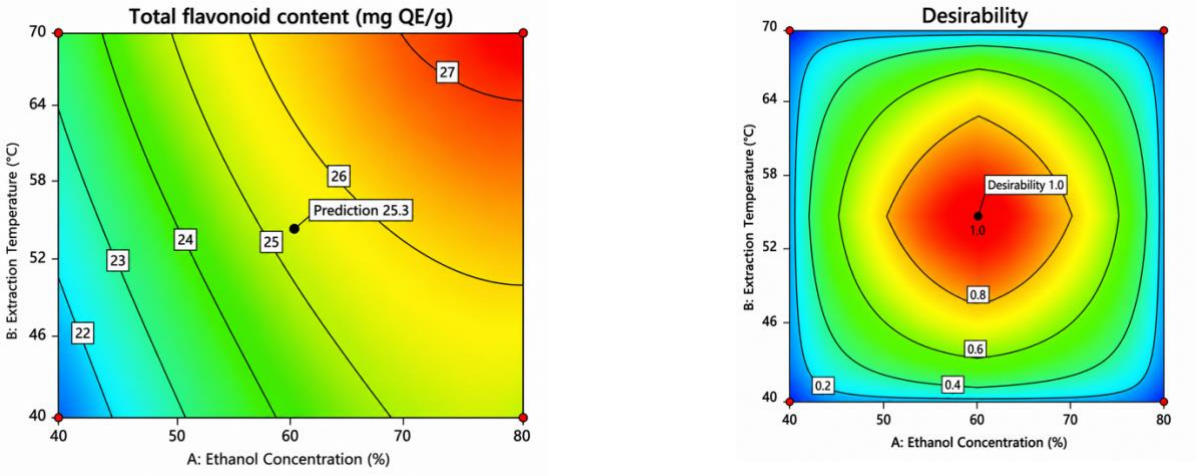
3. Results and Discussion

The optimization table presents the parameters used in the desirability function analysis within the Response Surface Methodology (RSM) approach to determine the optimum extraction conditions for maximizing the total flavonoid content (TFC). In this study, three independent variables were employed, namely ethanol concentration (A), extraction temperature (B), and extraction time (C). Each factor was assigned lower and upper limits based on the experimental ranges established in the Box–Behnken Design (BBD).

Table 2. Optimization criteria and desirability function parameters for total flavonoid content extraction using Box–Behnken Design

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:Ethanol concentration	target = 60	40	80	1	1	3
B:Extraction temperature	target = 55	40	70	1	1	3
C:Extraction time	target = 60	30	90	1	1	3
Total flavonoid content	none	19.9	27.7	1	1	3

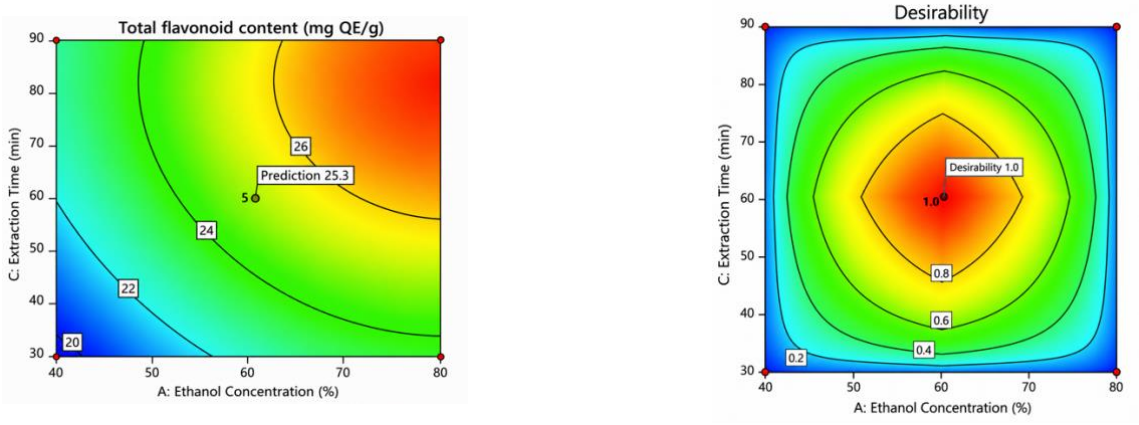
Factor A, ethanol concentration, was optimized within the range of 40–80% with a target value of 60%. This target indicates that a moderate ethanol concentration was considered optimal for providing a suitable solvent polarity balance to effectively dissolve flavonoid compounds. Excessively low ethanol concentrations may reduce the extraction efficiency of bioactive compounds, whereas excessively high concentrations may decrease extraction performance due to polarity incompatibility with semi-polar flavonoid compounds. As shown in Fig. 2, the interaction between ethanol concentration and extraction temperature demonstrated that increasing ethanol concentration from 40% to approximately 60–70% gradually increased the TFC value, particularly when combined with moderate extraction temperatures. Similarly, Fig. 2 indicated that ethanol concentrations around 60% combined with longer extraction times produced higher flavonoid recovery.



(a) (b)

Fig 2. Response surface contour plots of desirability and total flavonoid content (TFC) as a function of factor A (ethanol concentration) and factor B (temperature)

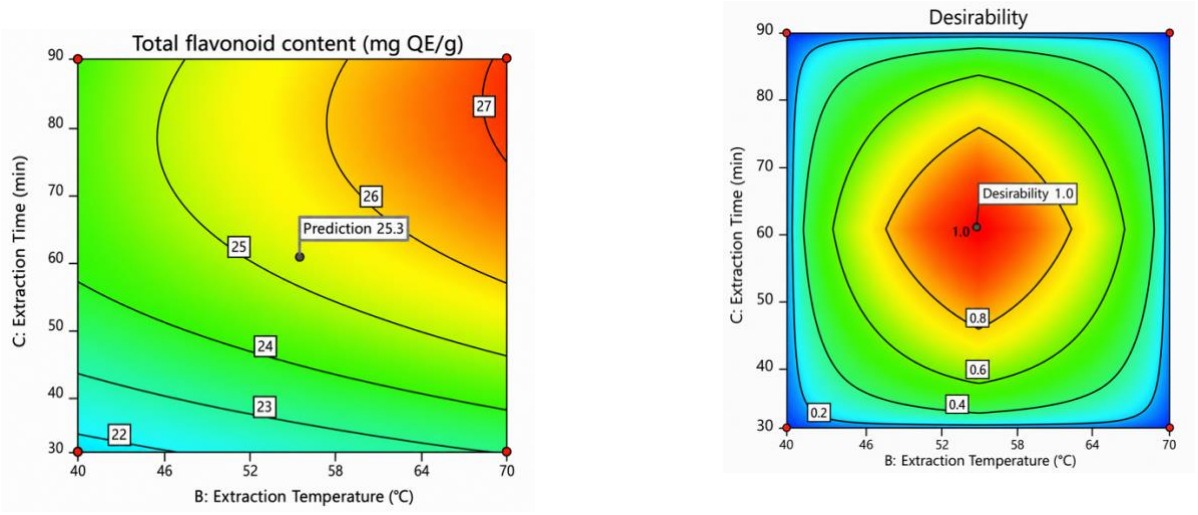
Factor B, extraction temperature, was optimized within the range of 40–70 °C with a target value of 55 °C. Extraction temperature plays an important role in enhancing flavonoid solubility and accelerating diffusion and mass transfer processes between the sample matrix and the solvent. However, excessively high temperatures may cause degradation of heat-sensitive flavonoid compounds. Therefore, a moderate extraction temperature was selected as the optimum condition to achieve high extraction efficiency while maintaining compound stability. The interaction between ethanol concentration and extraction temperature shown in Fig. 2 revealed that moderate-to-high temperatures positively affected TFC values. In addition, Fig. 4 demonstrated that increasing extraction temperature together with extraction time enhanced flavonoid extraction efficiency, as indicated by the higher TFC region located at elevated temperatures and longer extraction durations.



(a) (b)

Fig 3. Response surface contour plots of desirability and total flavonoid content (TFC) as a function of factor A (ethanol concentration) and factor C (extraction time)

Factor C, extraction time, was optimized within the range of 30–90 min with a target value of 60 min. Adequate extraction time is required to ensure sufficient interaction between the solvent and the sample matrix, allowing flavonoid compounds to diffuse efficiently into the solvent system. Nevertheless, excessively prolonged extraction may reduce process efficiency and increase the possibility of compound degradation due to extended heat exposure. As illustrated in Fig. 3, the interaction between ethanol concentration and extraction time showed that increasing extraction time improved flavonoid recovery, particularly at ethanol concentrations above 60%. Likewise, Fig. 4 showed that longer extraction times combined with higher extraction temperatures significantly increased TFC values due to enhanced solvent penetration and mass transfer. The response variable, total flavonoid content, exhibited a response range of 19.9–27.7 mg QE/g. This range indicates that variations in extraction conditions significantly affected flavonoid recovery. Higher TFC values demonstrate improved extraction efficiency and enhanced release of flavonoid compounds from the sample matrix. The contour plots in Fig. 2–4 clearly demonstrated the interaction effects among the extraction variables, where the optimum response region was generally located near the center of the experimental design space.



(a) (b)

Fig 4. Response surface contour plots of desirability and total flavonoid content (TFC) as a function of factor B (Extraction temperature) and factor C (extraction time)

Furthermore, the desirability contour plots in Fig. 2(b), Fig. 3(b), and Fig. 4(b) showed that the optimum desirability region was consistently concentrated around moderate extraction conditions, particularly near 60% ethanol concentration, 55 °C extraction temperature, and 60 min extraction time. The relatively symmetrical contour patterns indicated good agreement between the experimental results and the developed RSM model. These findings suggest that balanced extraction conditions were more favorable than extreme operating conditions for maximizing flavonoid recovery while maintaining process efficiency and compound stability. In addition, all factors and the response variable were assigned equal lower weight and upper weight values of 1, along with an importance value of 3. These results indicate that all factors were considered equally important during the optimization process. Consequently, the optimization model did not prioritize a single parameter but instead aimed to identify the best overall combination of extraction conditions for maximizing total flavonoid content. Overall, the desirability analysis demonstrated that extraction conditions near the target values produced the optimum response with a high desirability value. The optimum conditions were obtained at approximately 60% ethanol concentration, 55 °C extraction temperature, and 60 min extraction time. These conditions represent the best balance between extraction efficiency, bioactive compound stability, and overall process effectiveness. Overall, the developed RSM model successfully identified efficient extraction conditions for enhancing total flavonoid content.

The cube plot presented in Fig. 5 illustrates the combined effects of ethanol concentration (A), extraction temperature (B), and extraction time (C) on total flavonoid content (TFC). The cube plot provides a three-dimensional visualization of the response distribution at the lower and upper levels of each extraction factor. The predicted TFC values obtained from the model ranged from approximately 18.6 to 28.1 mg QE/g, indicating that the extraction variables significantly influenced flavonoid recovery. The lowest predicted TFC value (18.6 mg QE/g) was observed at the lower levels of ethanol concentration (40%), extraction temperature (40 °C), and extraction time (30 min). This result suggests that insufficient solvent concentration, low extraction temperature, and short extraction time reduced the extraction efficiency of flavonoid compounds. Under these conditions, solvent penetration and mass transfer between the sample matrix and the solvent were likely limited, resulting in lower flavonoid recovery.

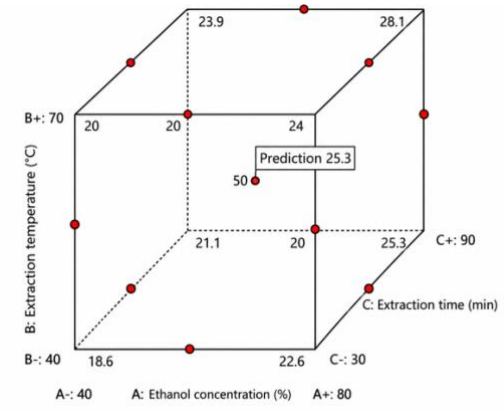


Fig 5. Cube plot illustrating the interaction effects of ethanol concentration (A), extraction temperature (B), and extraction time (C) on total flavonoid content (TFC)

In contrast, the highest predicted TFC value (28.1 mg QE/g) was obtained at higher extraction conditions, particularly at elevated ethanol concentration, extraction temperature, and extraction time. This finding demonstrates that increasing the extraction parameters positively affected flavonoid extraction efficiency. Higher ethanol concentration improved the solubility of semi-polar flavonoid compounds, while elevated temperature accelerated diffusion and mass transfer processes. Additionally, longer extraction time enhanced solvent interaction with the sample matrix, allowing greater release of flavonoid compounds into the solvent system. The cube plot in Fig. 4 also revealed the interaction effects among the three extraction variables. The increase in TFC values was not influenced by a single factor alone, but rather by the simultaneous combination of ethanol concentration, extraction temperature, and extraction time. The interaction between extraction temperature and extraction time appeared particularly

important, as higher temperatures combined with prolonged extraction duration produced significantly higher TFC values. Similarly, moderate-to-high ethanol concentration further enhanced extraction performance under these conditions. The central predicted value of approximately 25.3 mg QE/g shown in Fig. 4 indicates that the selected optimum region provided balanced extraction conditions capable of producing high flavonoid content without requiring excessively extreme operating conditions. This result is consistent with the contour plots presented in Fig. 1–3, where the optimum region was concentrated around moderate extraction conditions, namely approximately 60% ethanol concentration, 55 °C extraction temperature, and 60 min extraction time. The agreement between the cube plot and contour plots confirms the reliability of the developed RSM model in predicting the optimum extraction conditions. Overall, Fig. 4 confirms that the developed RSM model successfully described the interaction effects among extraction variables and effectively predicted the optimum conditions for maximizing total flavonoid content.

4. Conclusion

This study demonstrated the potential of response surface methodology (RSM) combined with a Box–Behnken Design (BBD) to evaluate the effects of ethanol concentration, extraction temperature, and extraction time on total flavonoid content (TFC). The experimental results indicated that the extraction variables and their interactions influenced the flavonoid yield to varying degrees. Based on the contour plots, desirability analysis, and cube plot visualization, moderate extraction conditions generally provided more favorable results compared to extreme operating conditions. The optimum region was observed at approximately 60% ethanol concentration, 55 °C extraction temperature, and 60 min extraction time, which produced a relatively high flavonoid response with good desirability value.

The interaction among extraction variables suggested that balanced extraction conditions were important for improving flavonoid recovery while maintaining process efficiency and minimizing the possibility of compound degradation. In particular, ethanol concentration, extraction temperature, and extraction time worked synergistically in influencing the extraction performance. Overall, the developed RSM model provided useful information for understanding the extraction behavior of flavonoid compounds under different processing conditions. These findings may serve as a reference for future studies related to the optimization of bioactive compound extraction from natural materials.

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