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Optimization of the ultrasonic-assisted extraction of the total methoxyflavones content from black ginger *Kaempferia parviflora* rhizomes

Le Hong Luyen*, Dong Thi Thu Hien, Vu Cam Tu

University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do Ward, Ha Noi, Viet Nam

*Email: *le-hong.luyen@usth.edu.vn*

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Abstract. The well-known black ginger *Kaempferia parviflora* (KP) has been proven to possess many health benefits. However, the optimization of bioactive compounds extraction from this medicinal plant was limited, despite its various applications in the functional food industry. In the current study, response surface methodology (RSM) using Box-Behnken Design (BBD) was employed to optimize both the extraction efficiency and the total content of six methoxyflavones in KP rhizomes harvested in Viet Nam. The extraction yield was maximized at 229.13 mg/g dried sample after 23 min extraction at 75 °C with an ultrasonic power of 46 %, a solid-liquid ratio of 1:60 g/mL, and an ethanol concentration of 42 % (v/v). Meanwhile, the highest amount of methoxyflavones (58.38 mg/g) was achieved with 40 % ultrasonic power, 1:40 g/mL solid-liquid ratio, 23 minutes extraction, and 99 % ethanol (v/v). These extraction conditions were applied to KP samples collected from five different cultivation regions, including Dak Lak, Gia Lai, Nghe An, Hung Yen, and Yen Bai. The results indicated that KP rhizomes in Yen Bai province yielded the highest efficiency (236.78 mg/g) and also contained the highest total methoxyflavone content (76.29 mg/g) compared to other locations ($p < 0.05$). In addition, 5,7,4'-trimethoxyflavone was consistently found to be the most abundant methoxyflavonein all five ginger samples. This is the first time the optimal extraction for maximizing the total amount of six major methoxyflavones from KP was assessed. The findings in this study can support the exploration of *K.parviflora* as a great source for further applications in the production of methoxyflavones-rich food supplements.

Keywords: *Kaempferia parviflora,* methoxyflavones, ultrasonic-assisted extraction, response surface methodology, Box-Behnken design.

Classification numbers: 1.1.3, 1.2.1, 1.3.1.

1. INTRODUCTION

Kaempferia parviflora (KP) is a tropical herbaceous plant belonging to the Zingiberaceae family. This botanical species is indigenous to Southeast Asia, particularly Thailand [1]. In the last two decades, *K. parviflora* has been of much scientific interest due to its phytochemical composition, especially methoxyflavone compounds. These flavonoids are characterized by one or several methoxy groups (-OCH_3) attached to a flavone skeleton (Figure 1). Several

methoxyflavones contained in KP rhizomes, such as 5,7,4'-trimethoxyflavone,5,7-dimethoxyflavone or 5-hydroxy-3,7- dimethoxyflavone exhibited various pharmacological effects including antioxidant properties [2], improving sexual health [3], anti-inflammatory [4, 5], anti-allergenic [6] or antithrombotic activity [7, 8], etc. Therefore, many dietary supplement products containing methoxyflavones from KP as the main bioactive components were delivered in the market. For instance, Black ginger extract distributed by the Swanson brand (USA) contains 4 % of 5,7-dimethoxyflavone in its composition. This product is introduced as potentially supporting cardiovascular and brain health. In addition, Black ginger capsules (Vitabowl, USA) are marketed as testosterone boosters to improve male vitality. According to their claims, methoxyflavone compounds found in KP can relax muscles and increase blood flow throughout the body without impacting heart rate and blood pressure. Another example is the FAME's Black ginger – an Energy booster from Myanmar. The producers assert that via stimulating AMP-activated protein kinase in myoblasts, methoxyflavones in KP can improve energy generation, muscular endurance, fat metabolism, and physical fitness performance. With such many health-beneficial properties, the extraction of methoxyflavones from this black ginger should be improved for many potential applications in functional food production. Recently, an investigation was undertaken to optimize the extraction yield and total content of three methoxyflavone, including 3,5,7,3,4-pentamethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4'-trimethoxyflavone [9]. In that study, the optimizing process only comprised of 4 extraction factors including solid-liquid ratio, extraction time, extraction temperature, and ethanol concentration. However, this paper did not consider ultrasound power as one of the critical factors that could influence the expected results.

In Viet Nam, research on KP has been growing, focusing on its potential health benefits and quantification of its bioactive compounds. In 2016, Nguyen and her colleagues demonstrated remarkable anti-osteoporotic and antioxidant effects of several methoxyflavones isolated from KP rhizomes [10]. Total flavonoid content in KP rhizomes harvested in several provinces such as Nghe An, Thanh Hoa, Lai Chau was 4.10 %, 5.68 %, and 5.91 % respectively [11]. Nguyen and Pham reported the antioxidant activity of the KP's ethanolic extract [12] while Ha *et al.* showed the androgenic activity of this extract at 0.156 ml/kg in hypogonadal mouse models by increasing levels of plasma testosterone and seminal vesicles' weight in the prostate [13]. In 2021, five well-known components were isolated from the n-hexane extract of KP rhizomes [14]. In a recent study of our research group, six major methoxyflavones from KP rhizomes, including 5-hydroxy-3,7- dimethoxyflavone (**1**), 5,7,4′- trimethoxyflavone (**2**), 5-hydroxy-3,7,4'-trimethoxyflavone (**3**), 5-hydroxy-7-methoxyflavone (**4**), 3,5,7-trimethoxyflavone (**5**) and 3,5,7,4′- tetramethoxyflavone (**6**), have been demonstrated as potential antiaggregatory and anticoagulant agents [7]. Moreover, in that study, we also established a powerful UPLC-MS/MS method for simultaneously quantifying those six methoxyflavones in KP rhizomes. Hence, optimizing the factors that influence the KP's extraction process to achieve the highest total content of these compounds is essential. It can aid in the production of safer and more potent antithrombotic drugs (or functional foods) compared to existing medications which can currently cause several adverse effects [15].

Taking into consideration of the above facts, the current study aimed to optimize theultrasonic-assisted extraction process of *K. parviflora* rhizome on both the extraction yield and the total amount of six methoxyflavone compounds (**1** - **6**). The optimization process was investigated through five factors including ultrasonic power, solid-liquid ratio, extraction time, extraction temperature, and ethanol concentration. Then, the optimal extraction factors were

used to evaluate the cultivation efficiency of KP ginger harvested from different locations in Viet Nam such as Dak Lak, Gia Lai, Hung Yen, Nghe An, and Yen Bai province.

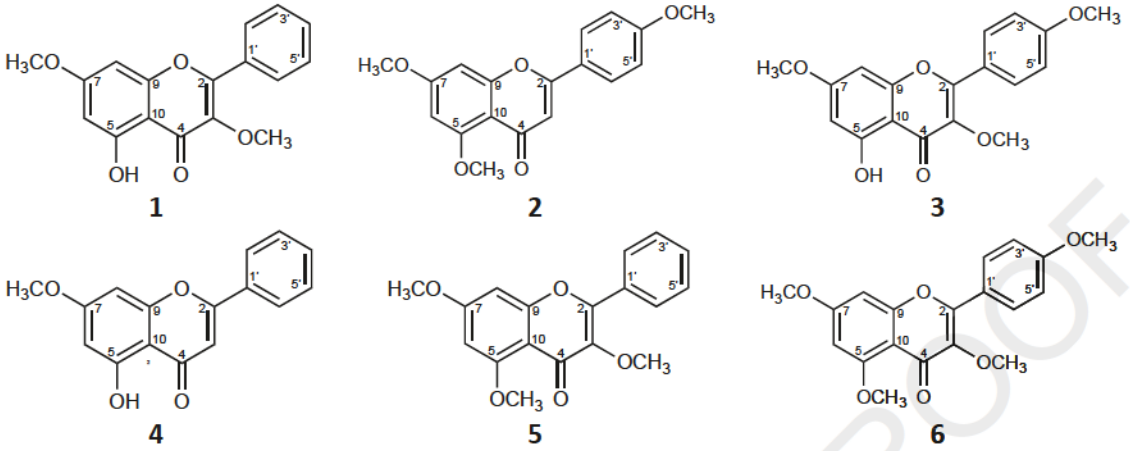

Figure 1. Structures of 6 major methoxyflavones **1** – **6** isolated from KP rhizomes.

2. MATERIALS AND METHODS

2.1. Materials

Methanol and pure water for UPLC-MS analysis, EtOH 99.99 %, and dimethyl sulfoxide (DMSO) for plant extraction were obtained from Fisher Scientific, UK. Six reference compounds **1** – **6** isolated from *K. parviflora* rhizomes were provided by Dr. Le Hong Luyen, University of Science and Technology of Hanoi (USTH).

2.2. Plant materials

Kaempferia parviflora (KP) rhizomes collected from Hung Yen province in February 2023 were used for the optimization experiments. The plant's identity was determined by Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature. A plant specimen was stored at the Department of Life Science, University of Science and Technology of Hanoi. Four other ginger rhizome samples were obtained from Gia Lai, Dak Lak, Nghe An, and Yen Bai provinces in February 2023.

2.3. Quantification of methoxyflavoneusing UPLC-MS/MS

Six standard compounds were dissolved in DMSO to form a 1 mg/mL stock solution. The working solution at 1000 µg/L was injected at 0.1, 0.2, 0.4, 0.8, and 1.6 µL into the UPLC-MS system. The calibration curves were constructed by plotting the peak areas obtained versus the corresponding concentrations.

KP extracts at 1 mg/mL were prepared in DMSO and centrifuged at 3700 rpm. The supernatant was filtered through 0.22 µm membranes before being transferred into glass vials for analysis. The 6 methoxyflavones in KP extracts were quantified using the previously described method [7] with the following equations: y = 585.53x +1779.4 for compound **1**, y = 876.52x + 2450.9 for compound **2**, y = 1041.6x + 1236.9 for compound **3**, y = 236.63x + 312.01 for compound **4**, y = 265.44x + 1187.5 for compound **5** and y = 1068.9x + 3051.5 for compound **6**.

2.4. Single-factor experimental analysis

This study employed a single experimental design to assess the influence of five factors including ultrasonic power, solid-liquid ratio, extraction time, extraction temperature, and ethanol concentration, on the extraction yield of KP. However, only four factors were assessed to maximize the total content of 6 methoxyflavone, excluding the extraction temperature. The experiment involved varying the levels of the factor under evaluation while maintaining the other factors unchanged.

Ultrasonic power

The effect of ultrasonic power using a Digital Ultrasonic Cleaner (Daihan, Korea, 990W) was investigated at 30, 40, 50, 60, 80, and 100 % with a solid-liquid ratio of 1:20 g/mL, for 20 min extraction, at 40 °C, with 70 % v/v ethanol.

Solid-liquid ratio

The effect of the solid-liquid ratio was assessed from 1:10 to 1:70 g/mL. The other extraction factors were kept constant at 30 %, 20 min, 40 °C, and 70 % v/v for ultrasonic power, extraction time, extraction temperature, and ethanol concentration, respectively.

Extraction time

The influence of extraction time was determined from 20 to 90 min with ultrasonic power of 30 %, solid-liquid ratio of 1:20 g/mL, extraction temperature of 40 °C, and 70 % v/v ethanol.

Extraction temperature

The extraction temperature ranged from 30 °C to 80 °C. The ultrasonic power, solid-liquid ratio, extraction time, and ethanol concentration were respectively kept constant at 30 %, 1:20 g/mL, 20 min, and 70 % v/v.

Ethanol concentration

Finally, the ethanol concentration varied from 40, 50, 60, 70, 80, 90, to 100 % v/v. Other factors were kept constant at 30 % ultrasonic power, 1:20 g/mL solid-liquid ratio, 40 °C, and a 20-minute extraction time.

Two grams of powdered black ginger were extracted with ethanol following specified conditions. The resulting supernatants were filtered and evaporated using a rotary vacuum evaporator until the solvent was removed entirely. Finally, the extraction yield of KP was determined as milligrams of extract per gram of dry matter (DM). The total methoxyflavones in KP were quantified as the total peak area of 6 compounds **1** – **6** using UPLC-MS/MS. Each trial was done twice.

$$Extraction\ yield\ \left(\frac{mg}{g}\right) = \frac{Mass\ of\ dried\ extract\ (mg)}{Mass\ of\ dried\ sample\ (g)}$$

2.5. Box-Behnken design (BBD)

The BBD is a widely used statistical experimental design technique, particularly in optimization and response surface methodology (RSM). It is beneficial for determining the optimal conditions for a process when multiple factors or variables need to be considered. BBD

employs a specific set of experimental runs that allows for assessing both linear and quadratic effects of factors, making it suitable for modeling complex processes [16]. The general quadratic polynomial formula for a BBD can be expressed as:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} X_i X_j + e_i$$

in which Y represents the response variable, X_i andX_j are variables (i and j range from 1 to k), β_0 is the model intercept coefficient, β_i, β_{jj}, and β_{ij} are interaction coefficients of linear, quadratic, and the interactive two variables, respectively, and k is the number of independent variables.

For optimizing the extraction yield, a BBD was employed involving five factors as described in Table 1, each factor with three levels of influence (-1, 0, +1), to assess their combined impact on the extraction yield of KP rhizome. This BBD comprised 46 experiments (See Supplement Table S1), with six central points.

Table 1. BBD with 5 factors for optimizing extraction yields.

Independent variable	Symbol	Factor level			Dependent variable
		-1	0	+1	
Ultrasonic power (%)	X_1	30	40	50	
Solid-liquid ratio (g/mL)	X_2	40	50	60	
Extraction time (min)	X_3	20	30	40	Y_1: Extraction yield
Ethanol concentration (% v/v)	X_4	40	50	60	
Extraction temperature (°C)	X_5	60	70	80	

For optimizing the total content of 6 methoxyflavones, only four factors were chosen according to the screening results (Table 2). This BBD encompassed 29 experiments (See Supplement Table S2), with five central points included.

Table 2. BBD with 4 factors for optimizing the total content of methoxyflavone.

Independent variable	Symbol	Factor level			Dependent variable
		-1	0	+1	
Ultrasonic power (%)	X_1	40	50	60	
Solid-liquid ratio (g/mL)	X_2	40	50	60	Y_2: Total
Extraction time (min)	X_3	20	30	40	methoxyflavone content
Ethanol concentration (% v/v)	X_4	80	90	100	

2.6. Statistical analysis

Results (extraction yield – Y_1 and total methoxyflavones – Y_2) were presented as mean ± standard deviation. Analysis of variance (ANOVA) of the results was conducted using Design-Expert software (12.0.3.0, Stat-Ease Inc., Minneapolis, MN, USA).

3. RESULTS AND DISCUSSION

3.1. Effect of a single factor on the extraction yield and the total content of methoxyflavones

3.1.1. Effect of the ultrasonic power

The impact of ultrasonic power on the yield of extraction and the total content of methoxyflavone was illustrated in Figure 2a and Figure 3a, respectively. The extraction yield peaked at 156.11 mg/g when the rhizome sample was treated under 40 % ultrasound power, whereas the total content of six methoxyflavones peaked at 50% ultrasound power. Ultrasound power can influence the extraction yield and the content of 6 methoxyflavones in KP. Usually, it generates localized pressure changes and microstreaming, effectively enhancing mass transfer by breaking down the plant cell membranes and promoting endogenous substances to be released into the solvent [17]. Ultrasonic waves aid in better solvent penetration into the plant matrix, ensuring efficient contact between the solvent and the target compounds [18]. This generally results in an increased mass of extracted compounds. However, excessive ultrasound power can lead to the breakdown of substances that are sensitive to heat and the formation of undesirable emulsions, which may decrease the overall extraction yield and affect the content of specific compounds like methoxyflavones [19]. Consequently, the ultrasound power levels selected for RSM experiments to optimize extraction yield were set at 30 %, 40 %, and 50 % for the levels -1, 0, and +1. In comparison, the ultrasound power levels were chosen as 40 %, 50 %, and 60 % to optimize the total content of methoxyflavone.

3.1.2. Effect of the solid-liquid ratio

Figures 2b and 3b show that the extraction yield and total methoxyflavone content peaked at a 1:50 g/mL solid-liquid ratio. Increasing this ratio led to a slight decrease in both responses. A higher solid-liquid ratio may increase extraction yield by providing more solvent for extraction and facilitating the release of compounds into the solvent. However, beyond the optimal point, excessive solid-liquid ratios may lead to reduced yields due to limitations in solvent penetration and the saturation of available binding sites [20]. Therefore, three solid-liquid ratios were chosen for the RSM experiments with values of 1:40, 1:50, and 1:60 g/mL.

3.1.3. Effect of the extraction time

The highest yield of extraction, at 152.71 mg/g, was attained after a 30-minute extraction process. Interestingly, extending the duration from 40 to 90 minutes consistently resulted in a proportional decrease in the yield of extraction (Figure 2c). This pattern was also obtained for the total methoxyflavone content, which declined when the extraction duration exceeded 30 minutes (Figure 3c). Initially, a prolonged extraction time can result in higher extraction yields and more remarkable contents of methoxyflavones as it allows more time for the solvent to interact with the plant material. However, there is a limit to this effect, and prolonged extraction times may lead to a decline in both yield and content due to potential thermal degradation or other adverse reactions [21]. Therefore, for the RSM experiments, the extraction duration varied between 20 and 40 minutes.

3.1.4. Effect of the extraction temperature

The extraction yield rose from its lowest point of 128.88 mg/g at 30 °C to its peak of 153.49 mg/g at 70 °C but slightly declined to 140.50 mg/g when the temperature further increased to 80 °C (Figure 2d). It can be explained that higher temperatures can enhance mass

transfer by promoting the diffusion of substances of interest from the plant matrix into the solvent used. However, the extraction yield can also be reduced when thermally sensitive compounds are degraded at elevated temperatures [22]. As a result, extraction temperature values of 60 °C (-1), 70 °C (0), and 80 °C (+1) were chosen for the RSM process. In contrast, the total content of methoxyflavone did not significantly change when the temperature grew from 30 °C to 80 °C (Figure 3d). Therefore, the extraction time was not involved in the further optimization process for the total content of methoxyflavone.

3.1.5. Effect of the ethanol concentration

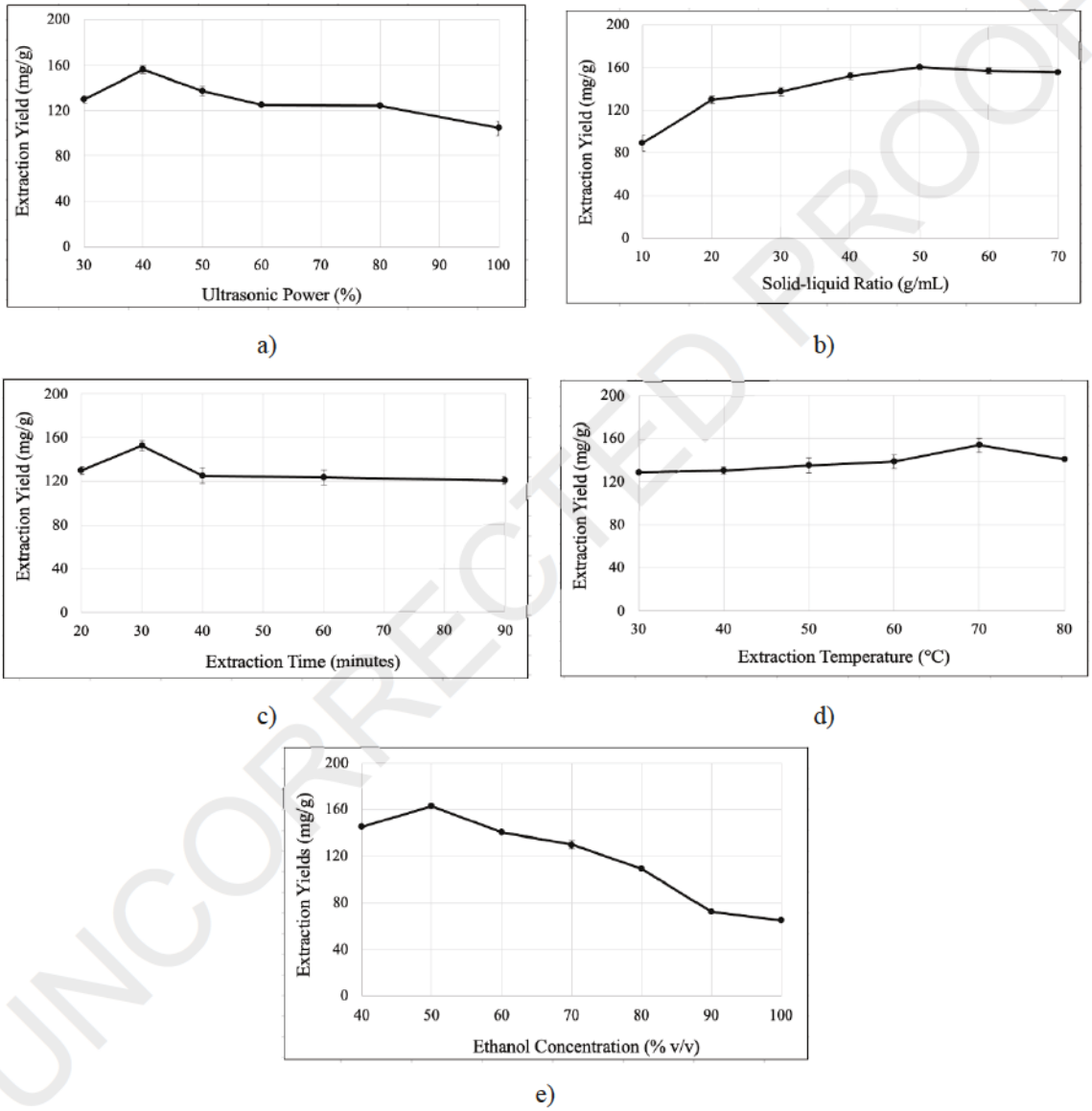

Figure 2. The effect of the single factor on the extraction yields: a) Ultrasonic power, b) Solid-liquid ratio, c) Extraction time, d) Extraction temperature, and e) Ethanol concentration.

The highest extraction yield at 153.61 mg/g was achieved when the rhizomes were extracted with 50 % ethanol. Then, this yield significantly decreased with increasing ethanol

concentrations (Figure 2e). Conversely, the highest content of six methoxyflavones was obtained with a much higher concentration of ethanol (90 % v/v) (Figure 3e). A similar trend was also observed in recent research by Karongrawa and colleagues [9]. This inverse relationship can be attributed to the solvent's polarity and solubility characteristics. Lower alcohol concentrations create a more polar environment, favoring the dissolution of a broader range of compounds and leading to a higher extraction yield. Conversely, a higher alcohol concentration decreases the polarity of the solvent, which is beneficial for extracting specific bioactive compounds like methoxyflavones, resulting in an increased total content of these compounds in the extract [23]. Therefore, in the RSM experiments, ethanol concentration variations spanned from 40 % to 60 % for optimizing extraction yields and from 80 % to 100 % for optimizing the total methoxyflavone content.

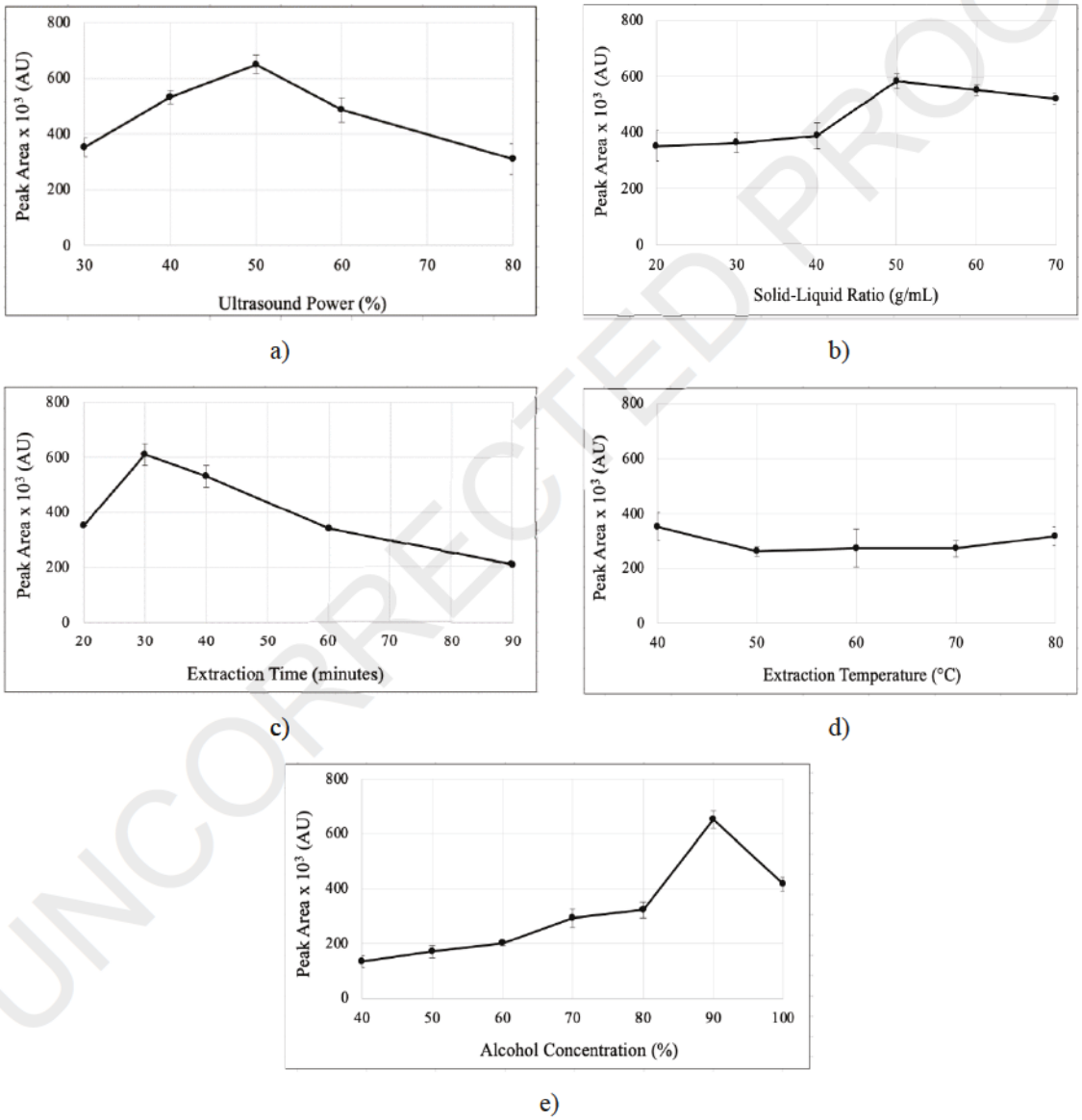

Figure 3. The effect of the single factor on the total content of methoxyflavone: a) Ultrasonic power, b) Solid-liquid ratio, c) Extraction time, d) Extraction temperature, and e) Ethanol concentration.

3.2. Optimization of variables by Box-Behnken design (BBD)

3.2.1. Statistical analysis and model fitting

The association between the variables and the responses (X_1 to X_5 for Y_1 and X_1 to X_4 for Y_2) was assessed through multiple regression analysis, and the resulting second-order polynomial equations are presented below:

$$Y_1 = 213.429 + 4.212X_1 + 7.594X_2 + 13.380X_3 - 5.461X_4 + 0.449X_5 + 11.747X_1X_2 - 2.537X_1X_3 - 0.208X_1X_5 - 6.826X_1X_5 - 6.675X_2X_3 + 3.070X_2X_5 - 5.529X_2X_4 - 12.575X_3X_5 + 8.180X_3X_4 - 6.976X_4X_5 - 11.387{X_1}^2 + 1.303{X_2}^2 - 9.340{X_3}^2 - 11.253{X_4}^2 - 23.709{X_5}^2$$

$$Y_2 = 1282147.998 - 176405.571X_1 - 159067.458X_2 + 73762.646X_3 + 243570.12X_4 - 4567.029X_1X_2 + 22123.625X_1X_3 + 5232.488X_1X_4 - 21543.10X_2X_3 - 13598.692X_2X_4 + 15414.183X_3X_4 - 92.593\,{X_1}^2 - 5177.513\,{X_2}^2 + 29072.293\,{X_3}^2 - 20558.987\,{X_4}^2$$

An ANOVA was used to assess the significance and appropriateness of the model, and the corresponding statistical data is provided in Table 3. The models optimizing extraction yield and the total content of methoxyflavone display significant F-values of 154.14 and 353.15, respectively. The low p-value ($p < 0.0001$) emphasizes the models' high significance. Regarding extraction yields, the R^2 value of 0.992 is notably favorable, indicating that the model can elucidate approximately 99.2 % of the fluctuations in the response variable. Furthermore, for total methoxyflavone content, the R^2 value is even higher at 0.9972. This underscores the model's commendable accuracy in fitting the data. The adjusted R^2 reflects the proportion of variance explained by the model, adjusted for the number of predictors while the predicted R^2: Measures how well the model predicts new data. A high predicted R^2 indicates good model generalizability. In Table 3, the adjusted coefficient closely aligns with the R^2 value, suggesting a strong concordance between the expected and experimental values. As can be seen, both coefficients of variation (C.V. %) are notably low at 1.06 % for Y_1 and 1.34 % for Y_2, signifying a high level of repeatability of the model's performance. The lack of fit indicates how well the model explains variation in the data that is not due to pure error. In both cases, a non-significant lack of fit value suggests no significant discrepancy between the predicted values generated by the model and the actual experimental results within the range of the design space.

Table 3. The quadratic polynomial mode's analysis of variance (ANOVA).

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significant
Y_1 (Extraction yield)						
Model	13007.25	20	650.36	154.14	<0.0001	Yes
X_1- Ultrasonic power	283.83	1	283.83	67.27	< 0.0001	Yes
X_2- Solid/liquid ratio	922.76	1	922.76	218.70	< 0.0001	Yes
X_3- Extraction time	2864.45	1	2864.45	678.88	< 0.0001	Yes
X_4- Ethanol concentration	477.24	1	477.24	113.11	< 0.0001	Yes
X_5- Extraction temperature	3.22	1	3.22	0.7635	0.3906	No
X_1X_2	551.94	1	551.94	130.81	< 0.0001	Yes

X_1X_3	25.75	1	25.75	6.10	0.0207	Yes
X_1X_4	186.35	1	186.35	44.17	< 0.0001	Yes
X_1X_5	0.1737	1	0.1737	0.0412	0.8408	No
X_2X_3	178.21	1	178.21	42.24	< 0.0001	Yes
X_2X_4	122.28	1	122.28	28.98	< 0.0001	Yes
X_2X_5	37.69	1	37.69	8.93	0.0062	Yes
X_3X_4	267.65	1	267.65	63.43	< 0.0001	Yes
X_3X_5	632.51	1	632.51	149.90	< 0.0001	Yes
X_4X_5	194.64	1	194.64	46.13	< 0.0001	Yes
Residual	105.48	25	4.22			
Lack of Fit	93.74	20	4.69	1.99	0.2284	No
Pure Error	11.75	5	2.35			
Cor Total	13112.73	45				
R^2	0.9920					
Adjusted R^2	0.9855					
Predicted R^2	0.9701					
CV %	1.06					
Y_2 (Total methoxyflavone content)						
Model	1.470E+12	14	1.050E+11	353.15	< 0.0001	Yes
X_1- Ultrasound power	3.734E+11	1	3.734E+11	1255.69	< 0.0001	Yes
X_2- Solid/liquid ratio	3.036E+11	1	3.036E+11	1020.99	< 0.0001	Yes
X_3- Extraction time	6.529E+10	1	6.529E+10	219.55	< 0.0001	Yes
X_4- Ethanol concentration	7.119E+11	1	7.119E+11	2393.89	< 0.0001	Yes
X_1X_2	8.343E+07	1	8.343E+07	0.2805	0.6046	No
X_1X_3	1.958E+09	1	1.958E+09	6.58	0.0224	Yes
X_1X_4	1.095E+08	1	1.095E+08	0.3683	0.5537	No
X_2X_3	1.856E+09	1	1.856E+09	6.24	0.0255	Yes
X_2X_4	7.397E+08	1	7.397E+08	2.49	0.1371	No
X_3X_4	9.504E+08	1	9.504E+08	3.20	0.0955	
Residual	4.163E+09	14	2.974E+08			
Lack of Fit	3.655E+09	10	3.655E+08	2.88	0.1600	No
Pure Error	5.081E+08	4	1.270E+08			
Cor Total	1.474E+12	28				
R^2	0.9972					
Adjusted R^2	0.9944					
Predicted R^2	0.9852					
CV %	1.34					

Statistical analysis reveals that linear variables (X_1, X_2, X_3, X_4) exhibited remarkable effects (p-value < 0.05) on the extraction yield. In contrast, linear variable X_5 does not affect the response variable significantly (p-value > 0.1). From an examination of the linear and quadratic coefficients, the selected factors significantly influenced the extraction yield in the following descending order: extraction time > solid-liquid ratio > ethanol concentration > ultrasonic power according to the reduction in the F-values (Figure 4). It is also confirmed that all linear variables

(X_1, X_2, X_3, and X_4) significantly affected the total content of 6 methoxyflavones. Examining the linear andquadraticcoefficients reveals that these selected factors influenced the response Y_2 in this order: ethanol concentration > ultrasonic power > solid-liquid ratio > extraction time. It is worth noting that the effect order of tested factors on two responses of interest is completely different. For instance, the ethanol concentration was the most important factor for the total methoxyflavone content, but its effect was remarkably weaker than other factors for the extraction yield. Meanwhile, the extraction time expressed the most potent effect on the extraction yield, but it was the weakest factor for the total methoxyflavone. Therefore, the selection of predominant parameters is strongly dependent on the purpose of the extraction, whether the crude extract or the amount of methoxyflavone is more important. Moreover, in the current study, the ultrasonic power displayed a significant impact on the extraction yield and the total methoxyflavone content. Meanwhile, this factor was not investigated in the study of Krongrawa *et al.* [9], for the same optimization purposes. This might explain the different findings between the two reports which were discussed in the next section.

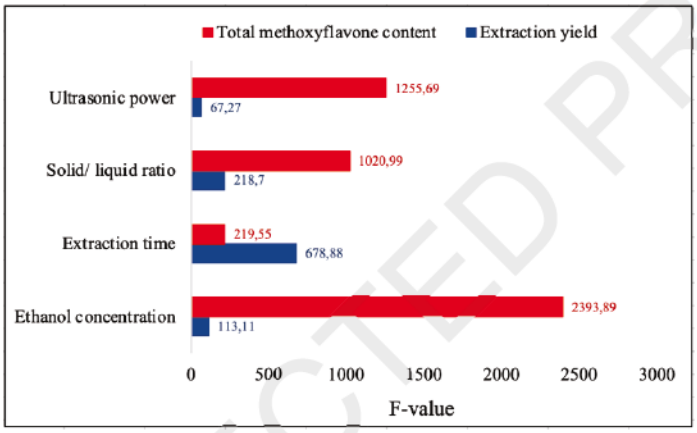

Figure 4. Significant factors on the extraction yield and total methoxyflavone content.

3.2.2. Optimization of UAE procedure

A 3D contour plot in a BBD is a valuable graphical representation that illustrates how changes in two independent variables simultaneously influence the response variable. In the context of 3D plots, a circular contour plot typically signifies a more symmetrical and balanced interaction between the two independent variables, where changes in both factors have a similar or proportional impact on the response. However, a flat contour plot suggests that the relationship between the two variables is less balanced and more asymmetric. This means changes in one factor have a more important impact on the response than changes in the others [24]. In addition, red and blue colors visually represent the variations in the response. Typically, the blue color represents lower response values, while the red color signifies higher values. The transition between these colors indicates the changing magnitude of the response variable values [25]. Figures 5 and 6 show that the 3D plots representing the interaction between two variables concerning extraction yields tend to exhibit circular shapes. In contrast, those representing the total content of methoxyflavone tend to be flat. Specifically, as seen in Figures 5d, 5g, and 5i, the ethanol concentration factor appears to exert a more pronounced effect on the response than the other variable, indicating its greater significance to the response.

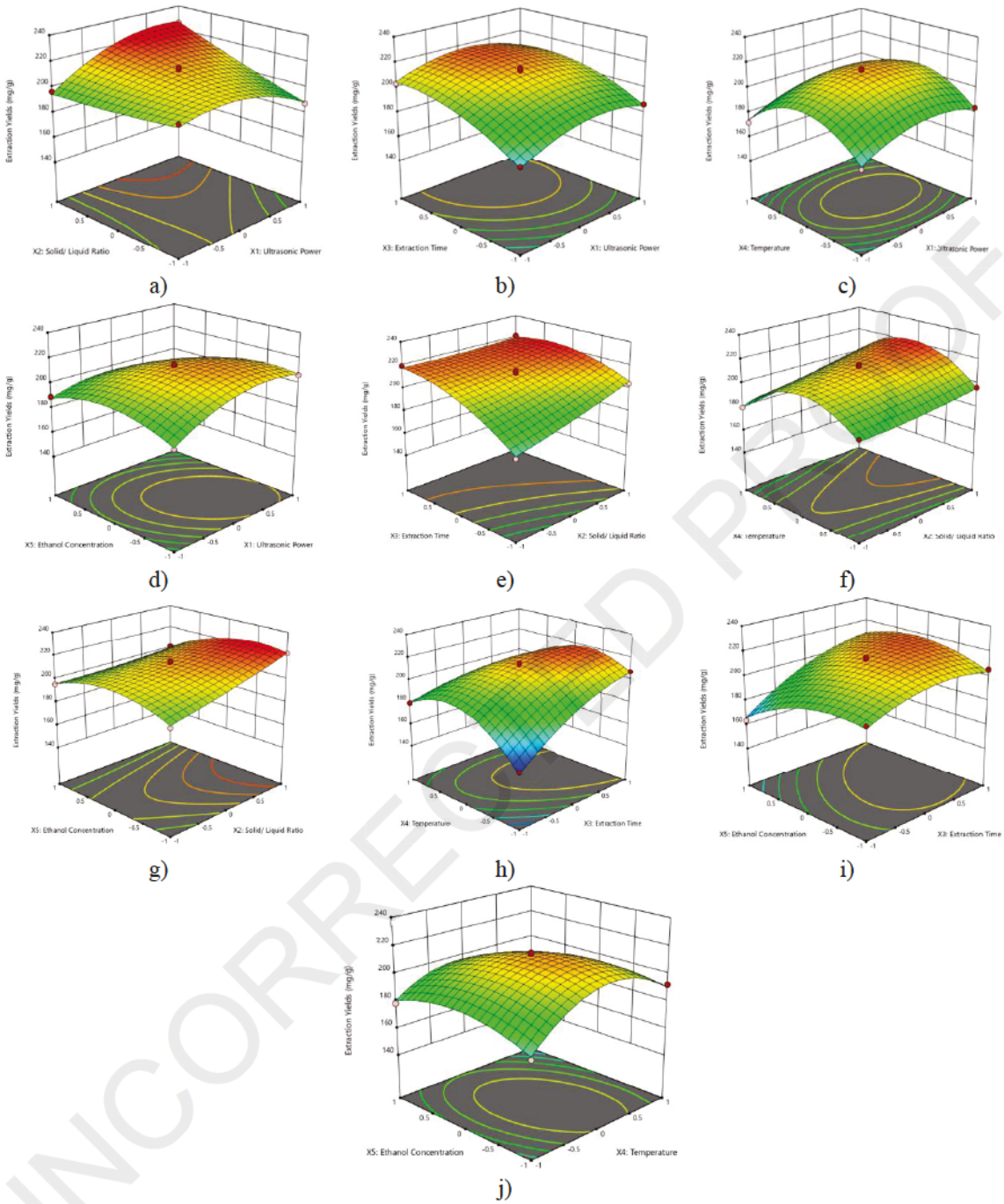

Figure 5. Three-dimensional response surface graphs present process conditions' significant effect on the extraction yields (mg/g): a) Solid-liquid ratio & ultrasound power, b) Extraction time & ultrasonic power, c) Temperature & ultrasonic power, d) Ethanol concentration & ultrasonic power, e) Extraction time & solid-liquid ratio, f) Temperature & solid-liquid ratio, g) Ethanol concentration & solid-liquid ratio h) Temperature & extraction time i) Ethanol concentration & extraction time j) Ethanol concentration & temperature.

The optimal conditions for the highest extraction yield were determined and confirmed using Design Expert version 12.0 software, resulting in the following recommended settings: 45.73 % ultrasonic power, a solid-liquid ratio of 1:59.57 g/mL, an extraction time of 22.87 minutes, an extraction temperature of 75.44 °C, and an ethanol concentration of 42.25 % v/v. Under these conditions, the estimated value of Y_1 is approximately 229.51 mg/g. The KP rhizome was extracted using the predicted optimal UAE conditions to validate the RSM models further. For operational ease, the ideal parameters in the verification experiment were marginally modified as follows: 46 % ultrasonic power, a solid-liquid ratio of 1:60 g/mL, an extraction time of 23 minutes, an extraction temperature of 75 °C, and an ethanol concentration of 42 % v/v. The result shows that the experimental value (229.13 ± 0.70 mg/g) was close to the predicted value (C.V % = 0.31 %). Consequently, the RSM experimental parameters provide precision and reliability and offer values that accurately depict the predicted optimization. Given that this model accounts for the interaction of multiple independent factors, it becomes evident that optimizing key extraction variables can significantly enhance the extraction of KP extract in ethanol.

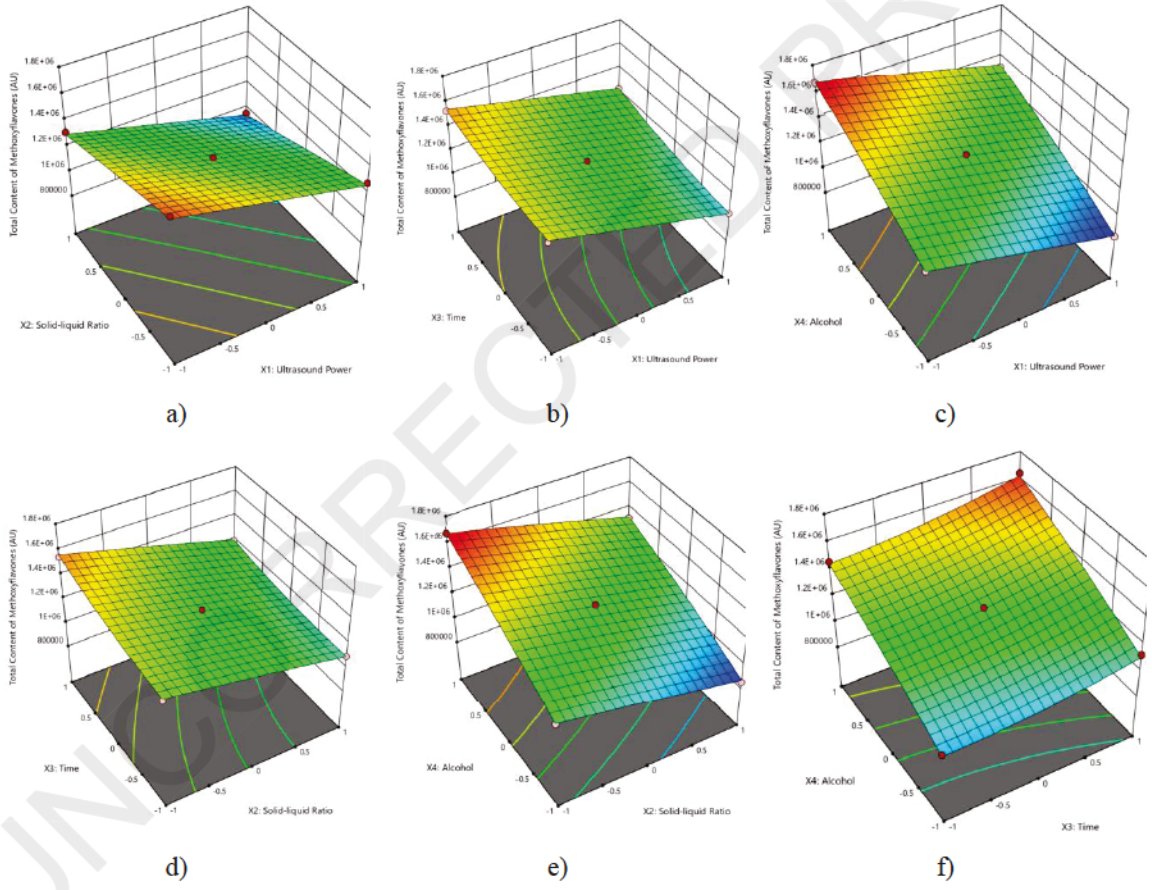

Figure 6. Three-dimensional response surface graphs present process conditions' significant effect on the total methoxyflavone content expressed as total peak area (AU): a) Solid-liquid ratio & ultrasound power, b) Extraction time & ultrasonic power, c) Ethanol concentration & ultrasonic power, d) Extraction time & solid-liquid ratio, e) Ethanol concentration & solid-liquid ratio, f) Ethanol concentration & extraction time.

The ideal extraction factors for maximizing the total mass of 6 methoxyflavones were determined to be an ultrasonic power of 40.24 %, a solid-liquid ratio of 1:40.22 g/mL, an extraction time of 22.81 minutes, and an ethanol concentration of 99.34 % v/v. The expected Y_2 value was approximately 1746259.66. Some minor adjustments were also made for the verification experiment, resulting in 40 % ultrasonic power, 1:40 g/mL solid-liquid ratio, 23 minutes extraction, and 99 % ethanol (v/v). These extraction conditions resulted in an experimental value of 1771180 ± 45344, which closely agrees with the predicted value (C.V % = 2.56 %).

3.3. Extraction yield and total methoxyflavone content in KP collected in different provinces

The ideal factors obtained from the optimization process were used to evaluate the extraction efficiency and total methoxyflavones of KP samples harvested in different regions (Table 4). This experiment determined the methoxyflavone content (mg/g DW) when each compound's peak area was plotted according to the calibration curve which is the function between the peak areas and the corresponding concentrations.

Table 4. Extraction yield and total methoxyflavone content of KP rhizomes harvested from five different cultivation places.

Province	Extraction yields (mg/g DW)	Total methoxyflavone contents (mg/g DW)	Estimated total methoxyflavone contents (mg/g extract)
Yen Bai	236.78 ± 2.21^a	76.29 ± 3.14^a	322.20
Hung Yen	229.13 ± 0.70^b	58.38 ± 1.28^b	254.79
Dak Lak	227.41 ± 1.55^b	49.61 ± 2.05^c	218.15
Gia Lai	199.20 + 0.85^c	37.78 ± 2.12^d	189.66
Nghe An	183.19 ± 0.66^d	30.32 ± 1.73^e	165.51

DW: dried weight of the sample, different superscript letters indicate significant differences among samples ($p < 0.05$)

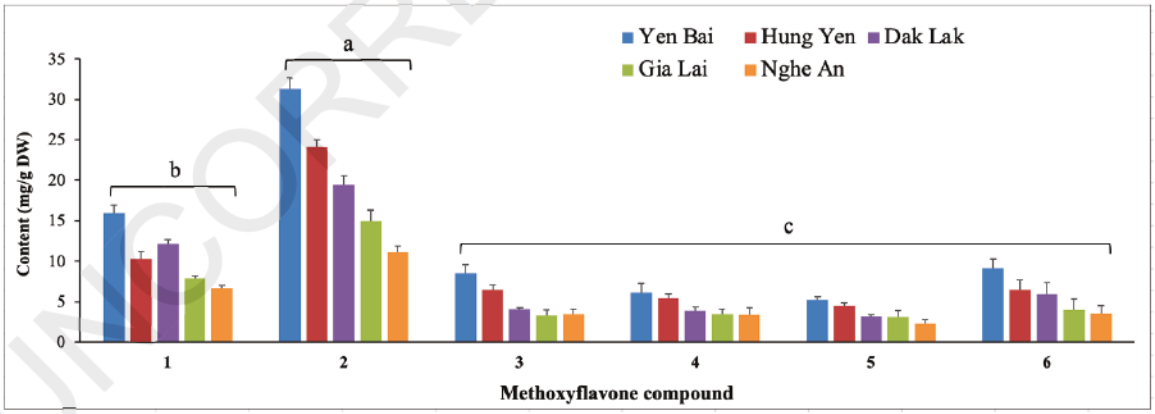

Figure 7. Methoxyflavones composition in ginger samples harvested in different provinces (**1**: 5-hydroxy-3,7- dimethoxyflavone; **2**: 5,7,4′- trimethoxyflavone; **3**: 5-hydroxy-3,7,4'-trimethoxyflavone; **4**: 5-hydroxy-7-methoxyflavone; **5**: 3,5,7- trimethoxyflavone; **6**: 3,5,7,4′- tetramethoxyflavone).

Methoxyflavones in KP rhizome can vary based on the plant's cultivation conditions and geographical location. Hence, it is crucial to assess and compare the concentration of

methoxyflavones in samples harvested from different places. As shown in Table 4, the black ginger sample from Yen Bai boasted the highest methoxyflavone content after extraction under optimized conditions, followed by samples from Hung Yen, Dak Lak, Gia Lai, and Nghe An. The Yen Bai sample also exhibited the highest extraction yield compared to other provinces. Regarding the composition of each methoxyflavone, compound **2** (5,7,4'-trimethoxyflavone) is the most abundant in all samples tested, with its mass exceeding that of other compounds by 2 to 10 times. Previous research has highlighted the potential health-promoting effects of this compound, including its antioxidant, anti-inflammatory, and anti–osteoporoticproperties [10]. In a recent study investigating Thai black ginger, the highest recorded extraction yield was 169.50 mg/g DW, and the maximal amount of the three methoxyflavones (3,5,7,3',4'-pentamethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4' -trimethoxyflavone) was around 322 mg/g extract [9]. Compared to the optimized results in the current study, all black ginger samples from Viet Nam exhibited higher extraction yields but only Yen Bai's sample contained a similar total methoxyflavone content. This finding suggests that besides 5,7,4' -trimethoxyflavone, other major compounds isolated from different KP samples might vary largely depending on the ginger origins.

4. CONCLUSIONS

In this study, RSM combined with BBD effectively assessed multiple extraction parameters for optimal KP responses. The highest extraction yield reached 229.13 mg/g while methoxyflavone content was 58.38 mg/g. Optimal conditions for maximum extraction yield involved 46 % ultrasonic power, a 1:60 g/mL solid-liquid ratio, 23 minutes of extraction time, 75 °C extraction temperature, and 42 % v/v ethanol concentration. A different set of optimal conditions was identified for the highest total methoxyflavone content as follows: 40 % ultrasonic power, a 1:40 g/mL solid-liquid ratio, 23 minutes of extraction time, and 99 % v/v ethanol concentration. Experimental results closely matched predicted values, confirming the model's reliability. In comparative experiments, Yen Bai's KP rhizomes showed the highest extraction yield (236.78 mg/g) and total methoxyflavone content (76.29 mg/g), with 5,7,4'-trimethoxyflavone consistently as the most abundant compound. Further research on the KP is needed to explore its potential in the functional food industry due to its various health benefits.

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CRediT authorship contribution statement. Le Hong Luyen: Methodology, Investigation, Supervision, Writing – review & editing. Dong Thi Thu Hien: Investigation, Formal analysis. Vu Cam Tu: Investigation, Formal analysis, Writing – original draft.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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