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Highly Efficient Recovery of Bioactive Puerarin from Roots of *Pueraria lobata* Using Generally Recognized as Safe Solvents

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Abstract: Puerarin (daidzein-8-C-glucoside), one of the bioactive isoflavones, has attracted attention in various industries due to its excellent pharmacological effects such as antioxidant effect, estrogen-like activity, reduction of blood sugar, and neuroprotective effect. Puerarin is most abundantly found in the roots of *Pueraria lobata* (RPL) among various biomass sources. To improve the utilization feasibility of puerarin, a high-yield extraction process should be designed for RPL. This study aimed to optimize the extraction process to more efficiently recover puerarin from RPL while using generally recognized as safe solvents as extraction solvents, considering the potential industrial applications of puerarin. The extraction variables were optimized by the one-factor-at-a-time method, response surface methodology, and time profiling study. As a result, puerarin yield was achieved at 60.56 mg/g biomass under optimal conditions (ethanol concentration of 46.06%, extraction temperature of 65.02 °C, ratio of extraction solvent to biomass of 11.50 mL/g, and extraction time of 22 min). High puerarin yield achieved in this study contributed to improving the industrial applicability of puerarin.

Keywords: *Pueraria lobata*; flavonoid; puerarin; bioactive compound; extraction; response surface methodology

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1. Introduction

Pueraria lobata, a legume widely distributed in Korea, Japan, and China, contains flavonoids and other bioactive compounds. Due to these bioactive compounds, it is used as a medicinal herb for treating diseases, such as cold, headache, diarrhea, and hypertension, and as a health supplement because of its health-promoting effects, including antipyretic, slaking thirst, and detoxifying alcohol [1–3]. Among the various bioactive compounds in *P. lobata*, puerarin (daidzein-8-C-glucoside) is the component with the most outstanding pharmacological effect (Figure 1). Puerarin is the major isoflavone in *P. lobata* and is most abundantly found in the roots of *P. lobata* (RPL) among various parts of biomass feedstocks [4]. Flavonoids are polyphenol compounds synthesized by plants and exhibit various biological properties, including antioxidant, anti-inflammatory, anticancer, antibacterial, and antiviral properties [5]. Owing to these properties, flavonoids are utilized

in numerous industries, such as food, cosmetics, and pharmaceutical industries [5], and research on flavonoids is attracting attention [6]. Puerarin has not only antioxidant, anti-inflammatory, anticancer, antiviral, and antibacterial properties, which are the properties of flavonoids, but also estrogenic activities, controlling blood pressure and lowering blood sugar. Additionally, puerarin has attracted worldwide attention because of its extensive neuroprotective functions in various central nervous system diseases, such as Alzheimer's disease, Parkinson's disease, and depression [7].

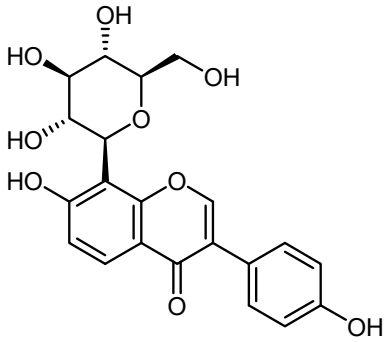

Figure 1. Chemical structure of puerarin.

Recently, with the increasing demand for natural bioactive compounds in several industrial fields, research on recovering puerarin from RPL has been actively conducted. Furthermore, natural antioxidants recovered from medicinal plants, such as puerarin, are preferred in the antioxidant market due to their safety and low toxicity [8–10]. As most of these natural bioactive compounds are present at low levels in plants and are recovered through extraction and purification processes, the design of high-yield processes is required. Therefore, to utilize the natural bioactive compounds recovered from biomass on an industrial scale, designing more efficient extraction processes is necessary. This can be achieved by optimizing the extraction variables, such as the type of extraction solvent, solvent concentration, solvent-to-biomass ratio, and temperature.

Typically, organic solvents are used to recover bioactive compounds, including flavonoids from biomass. Nevertheless, the use of organic solvents can exert harmful effects on the environment and human health due to the flammability, volatility, and toxicity of these solvents [11]. For the application of bioactive compounds in food and cosmetic products, extraction with generally recognized as safe (GRAS) solvents is recommended. This concept has been successfully applied to the extraction of bioactive substances, such as anthocyanins and phenolic compounds from biomass feedstocks such as elderberries and algae [12,13]. In particular, puerarin is mainly utilized in the food, pharmaceutical, and cosmetic industries; thus, the extraction process should be designed based on GRAS solvents.

This study aimed to design an extraction process that could more efficiently recover puerarin from RPL by optimizing the extraction variables. Additionally, considering the potential industrial applications of puerarin, GRAS solvents were used as extraction solvents. To optimize the extraction variables, the one-factor-at-a-time (OFAT) method was first used to identify the effects of these variables, including the type of extraction solvent, solvent concentration, extraction solvent-to-biomass ratio, and extraction temperature, on the puerarin yield. Subsequently, based on the influences of these variables determined via the OFAT method, the optimal conditions for effectively extracting puerarin from RPL were derived by analyzing the interactions among solvent concentration, solvent-to-biomass ratio, and extraction temperature via response surface methodology (RSM).

2. Materials and Methods

2.1. Materials

The RPL was purchased from Bomin herbal agricultural corporation (Yeongcheon, Republic of Korea). RPL was ground in a blender followed by sieving to obtain a powder with a particle size of 600–850 μm. The powder was dried in a freeze-dryer (Ilshin biobase, TFD8501: Dongducheon, Republic of Korea) for 24 h followed by sealing and storage at −20 °C until use. Acetone (99.5%) was purchased from Daejung (Siheung, Republic of Korea), and ethyl alcohol (94.5%) was purchased from Samchun (Seoul, Republic of Korea). Ethyl acetate (99.8%) was obtained from Sigma–Aldrich (St. Louis, MO, USA), and n-hexane was purchased from Junsei (Tokyo, Japan). Puerarin was purchased from Alibaba Co., Ltd. (Hangzhou, China). HPLC-grade acetic acid, methanol, and water as mobile phases for high-performance liquid chromatography (HPLC) were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Extraction of Puerarin from the Roots of Pueraria lobata via One-Factor-at-a-Time Method

Puerarin extraction variables (type of extraction solvent, concentration of extraction solvent, extraction temperature, and extraction solvent-to-biomass ratio) affecting the extraction yield were optimized stepwise using the OFAT method. The OFAT method is a method of deriving optimal conditions by changing only one variable at a time while maintaining all other variables under default conditions [14,15]. Optimization was performed under the following default extraction conditions: 10 mL/g extraction solvent-to-biomass ratio, 30 °C extraction temperature, and 3 h extraction time. Extraction was conducted in a water bath, and the extract was centrifuged at 13,000 rpm for 5 min. The supernatant acquired via centrifugation was used for quantitative analysis of recovered puerarin, and quantitative analysis was performed using HPLC. At this time, all experiments were repeated three times.

Initially, to identify the effect of the type of extraction solvent on the extraction yield, an experiment was performed using four GRAS solvents (acetone, ethanol, ethyl acetate, and n-hexane). 1 g of RPL and 10 mL of extraction solvent were added to a 50 mL conical tube, and extraction was conducted in a water bath at 30 °C for 3 h.

After selecting the optimal extraction solvent (ethanol), concentrations of the extraction solvent were set to 0, 25, 50, 75, and 100% to determine the effect of solvent concentration on the extraction yield. 10 mL of extraction solvent was added to 1 g of RPL, and extraction was performed at 30 °C for 3 h.

Next, to identify the effect of the extraction temperature on the extraction yield, experiments were conducted at the temperatures of 30, 40, 50, and 60 °C. 10 mL of the optimal extraction solvent (50% ethanol) determined in the previous experiment was added to 1 g of RPL followed by extraction for 3 h.

Finally, to identify the effect of the extraction solvent-to-biomass ratio on the extraction yield, experiments were performed by adjusting the extraction solvent-to-biomass ratio to 5, 7.5, 10, 12.5, and 15 mL/g. 10 mL of the optimal extraction solvent (50% ethanol) selected in the previous experiment was used, and extraction was conducted at the optimal extraction temperature (50 °C) for 3 h.

2.3. Enhancement of Puerarin Extraction from the Roots of Pueraria lobata via Response Surface Methodology

According to the optimal conditions derived from the OFAT method, the extraction conditions were optimized again via RSM using Design-Expert (Stat-Ease, Inc.: Minneapolis, MN, USA) to further investigate the interactions among variables (Figure 2). RSM is a statistical optimization method that derives optimal conditions by analyzing the results of

the experiments designed based on statistics to identify the interactions among variables. Experimental design was performed using a 5-level-3-factor central composite rotatable design (CCRD) of RSM, and a regression model was developed via statistical analysis of the experimental data. As the variables for the RSM experimental design require continuity with the response values for statistical analysis and regression model development, the extraction solvent concentration, extraction temperature, and extraction solvent-to-biomass ratio were selected as variables, and the center point of each variable was chosen as the optimal condition determined from the OFAT method (Table 1). Twenty experiments were designed using CCRD, and extraction was conducted in a water bath for 30 min under each extraction condition. Puerarin yield (mg/g biomass) was set as the response value (i.e., the dependent variable), and finally, an empirical regression model was established to predict the puerarin yield. The corresponding model equation was developed according to the following equation (Equation (1)).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where Y is the response value; β_0 represents the constant coefficient; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients, respectively; and X_i and X_j denote the coded values of the variables. Statistical significance and reliability of the established model were evaluated using analysis of variance (ANOVA).

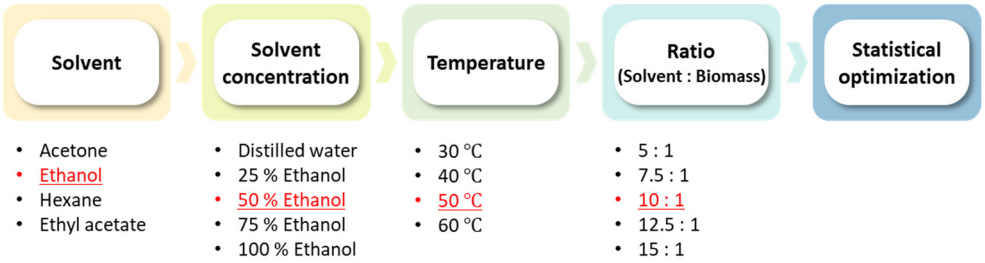

Figure 2. Optimization procedure for puerarin extraction from RPL. Red highlights with underlines indicate optimal conditions for puerarin extraction.

Table 1. Central composite rotatable design (CCRD) with three parameters affecting the extraction of puerarin from RPL.

Parameter	Unit	Symbol	Coded Level				
			−1.682	**−1**	**0**	**1**	**1.682**
Ethanol concentration	vol%	X_1	7.955	25	50	75	92.045
Extraction temperature	°C	X_2	33.182	40	50	60	66.818
Solvent-to-biomass ratio	mL/g	X_3	5.796	7.5	10	12.5	14.204

2.4. HPLC Analysis

Puerarin was quantitatively analyzed using HPLC according to a previously reported study [5,16]. HPLC vials for analysis were prepared as follows: First, 100 µL of reaction solution was obtained and diluted 10-fold with methanol. Then, impurities present in the diluted solution were removed using a 0.2 µm syringe filter (Advantec, DISMIC 13HP020AN, Tokyo, Japan). Finally, the filtered solution was placed in a 2 mL HPLC vial. Analysis was performed using Agilent 1260 infinity II (Agilent, Santa Clara, CA, USA) equipped with an

INNO Column C18 (120 Å, 5 µm, and 4.6 × 250 mm^2) and a variable wavelength detector under the following conditions: injection volume: 5 µL, column temperature: 50 °C, and wavelength: 250 nm. (A) 1% acetic acid in water and (B) methanol were used as mobile phases, and the flow rate of the mobile phase was maintained at 1 mL/min. The gradient system was configured as follows: 0 min (70% A, 30% B), 5 min (0% A, 100% B), 10 min (0% A, 100% B), 15 min (70% A, 30% B), and 20 min (70% A, 30% B).

The amounts of puerarin in the extract were evaluated using the standard curve of puerarin, which was acquired using a standard solution of puerarin dissolved in methanol. The linear regression equations and correlation coefficients for puerarin are $Y = 0.0000517X - 0.0053$ $(R^2 = 0.9999)$, where Y is the concentration of puerarin (mg/mL), and X is the peak area of puerarin (mAU × s). The linear range of puerarin concentration is 0.1–0.8 mg/mL. The results demonstrated that the HPLC method is reliable for the quantitative analysis of puerarin. The chromatograms for the puerarin standard and RPL extracts are shown in Figure 3. Puerarin yield was calculated using Equation (2).

$$\text{Puerarin yield (mg/g biomass)} = \frac{[C]_{\text{Puerarin}} \times N \times V}{W} \quad (2)$$

where $[C]_{\text{Puerarin}}$ is the puerarin concentration (mg/mL) calculated from the standard curve, N denotes the dilution factor, V is the volume of the extraction solvent used for extraction (mL), and W represents the dry weight of RPL (g).

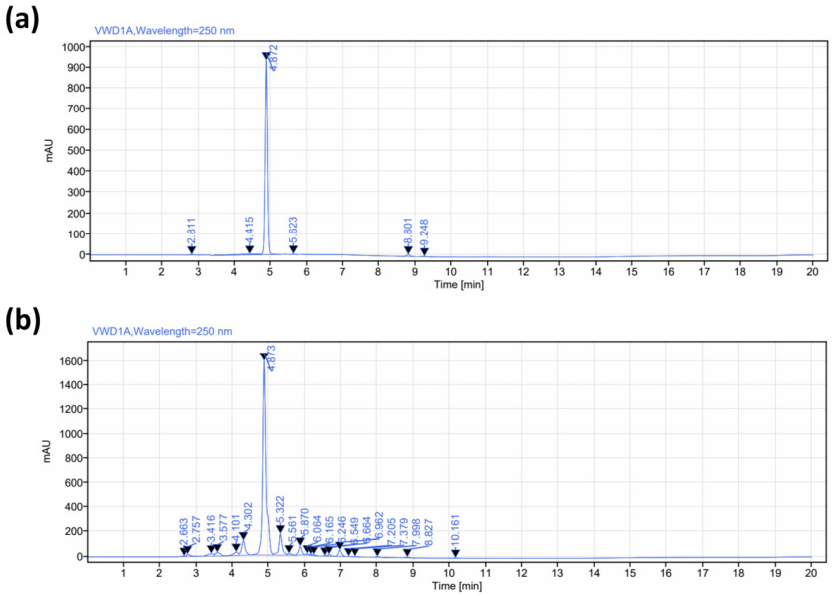

Figure 3. HPLC chromatograms of puerarin standard (**a**) and RPL extract (**b**). The puerarin peaks were observed at a retention time (RT) of 4.87 min in both the standard and the extract.

2.5. Statistical Analysis

All extractions were performed in triplicate, and the results were represented as mean values. The results were analyzed with Tukey's test for paired comparison using SigmaPlot (Systat Software, Inc.: San Jose, CA, USA). Data with different letters are significantly different ($p < 0.05$).

3. Results and Discussion

3.1. Extraction of Puerarin via One-Factor-at-a-Time Method

3.1.1. Effect of Type of Extraction Solvent on the Puerarin Yield

The type of extraction solvent is an important factor affecting puerarin recovery [12,13,17]. To determine the influence of solvent type on puerarin recovery, extraction experiments were conducted using different types of solvents. As extraction solvents, we selected GRAS solvents among the industrial organic solvents commonly used to recover phenolic compounds from biomass. Four solvents (acetone, ethanol, ethyl acetate, and n-hexane) with different polarities were tested to extract puerarin from RPL [9,12,13,18]. In the experiments, when acetone, ethanol, ethyl acetate, and n-hexane were used as extraction solvents, 0.64, 13.14, 0.83, and 0.45 mg/g biomass yields were obtained, respectively. Relatively high yield was acquired in the case of ethanol (Figure 4).

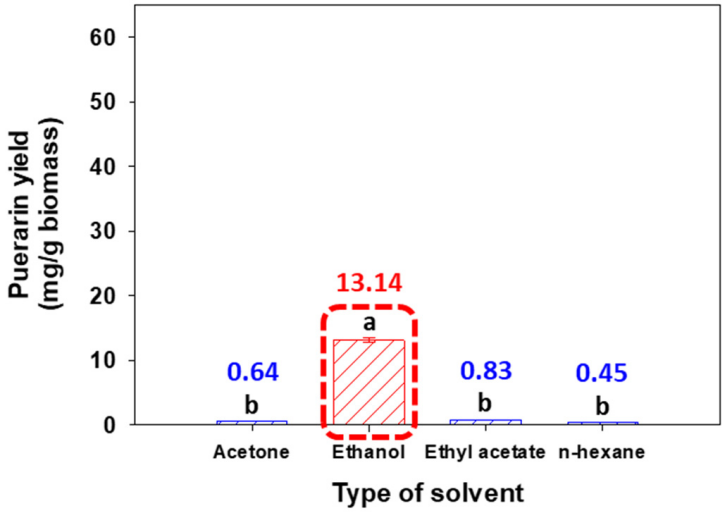

Figure 4. Effect of solvent type on the puerarin yield from RPL. (solvent-to-biomass ratio of 10 mL/g, extraction temperature of 30 °C, and extraction time of 3 h). Data with different letters (i.e., a and b) are significantly different ($p < 0.05$).

Amounts of phenolic compounds that can be obtained via solvent extraction are determined by the solubilities of these compounds in the extraction solvent, which depend on the polarity of the extraction solvent [12,13]. Generally, a polar organic solvent is the most effective solvent for extracting polar phenolic compounds [12,13,19]. Puerarin is a polar phenolic compound with high polarity owing to its many hydroxyl groups. The polarities of the solvents used for extraction are in the following order: ethanol > acetone > ethyl acetate > n-hexane [12]. As ethanol exhibits the highest polarity among those of the four solvents, the largest amount of puerarin, which has high polarity, was recovered from the biomass when ethanol was used as the extraction solvent. Therefore, ethanol was selected as the optimal solvent for extraction. Ethanol increases the extraction efficiency of polar phenolic compounds and exhibits low toxicity and cost, rendering it the most preferred solvent for extracting bioactive compounds [9,13,20,21]. Due to these advantages, ethanol has been utilized as an extraction solvent in several studies on the extraction of polar phenolic compounds from plant resources [22–26].

3.1.2. Effect of Solvent Concentration on the Puerarin Yield

The polarity of the extraction solvent changes with respect to the concentration of the extraction solvent, and the solubility of the compound in the extraction solvent accordingly varies [9,12,13]. Therefore, identifying the appropriate concentration of the extraction solvent is crucial. Additionally, as the amount of the extraction solvent consumed is related

to the process cost, reducing the consumption of the extraction solvent by selecting a suitable concentration of the extraction solvent is necessary for an economical process [13,27]. Therefore, the effects of the concentrations of the extraction solvent in the range of 0–100% on the puerarin yield were examined. With an increase in the concentration of the extraction solvent from 0 to 25 and 50%, the puerarin yield increased from 39.97 to 49.34 and 52.36 mg/g biomass, respectively. However, at higher concentrations of 75 and 100%, the yield decreased to 50.14 and 13.14 mg/g biomass, respectively (Figure 5).

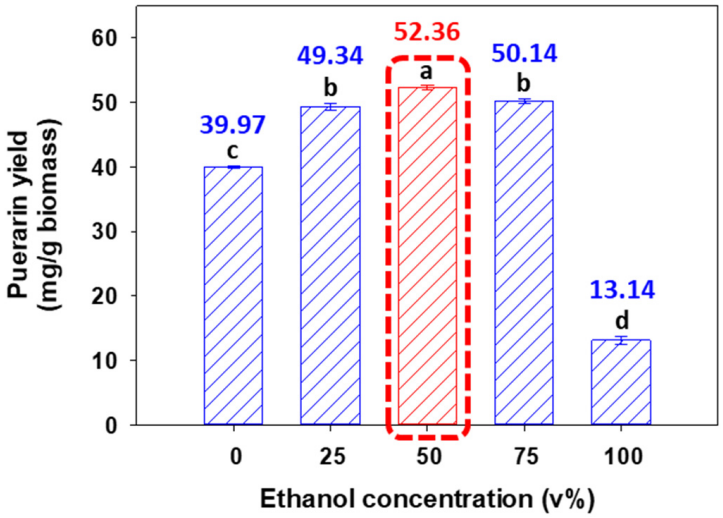

Figure 5. Effect of solvent concentration on the puerarin yield from RPL. (Ethanol as extraction solvent, solvent-to-biomass ratio of 10 mL/g, extraction temperature of 30 °C, and extraction time of 3 h). Data with different letters (i.e., a, b, c, and d) are significantly different ($p < 0.05$).

High-purity organic solvents, such as 100% ethanol, cause dehydration and disruption of plant cells and denaturation of cell wall proteins and increase the viscosity of the plant matrix, inhibiting the release of polyphenols into the extraction solvent [12,23,25]. Mixtures of ethanol and water are more efficient in recovering phenolic compounds than the cases of single-component solvents [13,18]. The polarity of the extraction solvent changes with respect to the mixing ratio of ethanol and water, which alters the solubilities of the phenol compounds in the extraction solvent. Thus, determining an appropriate mixing ratio that can increase the yield of the target compound to be extracted is important, and in this study, 50% ethanol, which demonstrated the highest yield, was chosen as the optimal extraction solvent. Similar to this study, Liao et al. have extracted flavonoids from peanut skins using ethanol as an extraction solvent and investigated the influence of ethanol concentration on the extraction [22]. They analyzed ethanol concentrations in the range of 50–80% and confirmed that the flavonoid yield increased with an increase in the ethanol concentration from 50 to 70% and rapidly decreased at concentrations higher than 70%. Moreover, this trend has been reported by Yu et al. [24]. They extracted flavonoids from *Crinum asiaticum* and investigated the flavonoid yield with respect to the ethanol concentration in the range of 30–80%. Results indicated that the flavonoid yield increased with an increase in the ethanol concentration and decreased at ethanol concentrations above 60%.

3.1.3. Effect of Temperature on the Puerarin Yield

Extraction temperature is an important factor that directly affects the puerarin yield; therefore, selecting a suitable extraction temperature is necessary [17]. Herein, the influences of the extraction temperatures of 30, 40, 50, and 60 °C on the puerarin yield were examined, and corresponding puerarin yields of 52.36, 54.39, 57.71, and 55.26 mg/g biomass were achieved (Figure 6). With an increase in the extraction temperature from

30 to 50 °C, the puerarin yield increased; nevertheless, the puerarin yield decreased at temperatures higher than 50 °C. Therefore, 50 °C was chosen as the optimal extraction temperature, which exhibited the highest puerarin yield.

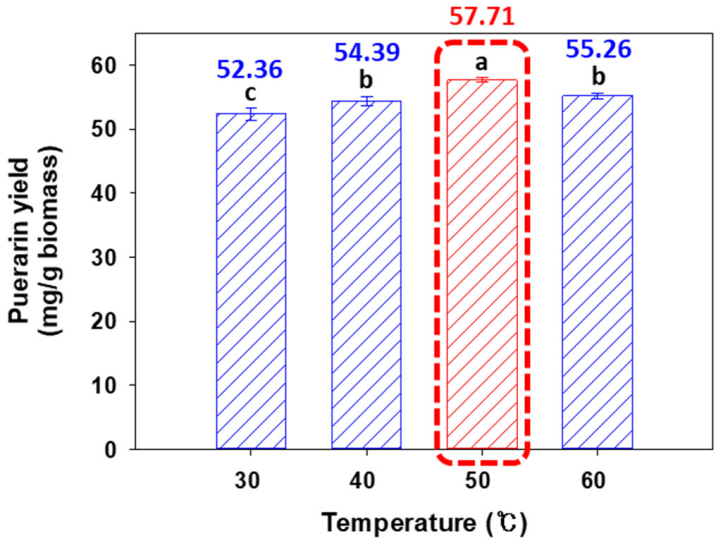

Figure 6. Effect of extraction temperature on the puerarin yield from RPL. (50% ethanol as extraction solvent, solvent-to-biomass ratio of 10 mL/g, and extraction time of 3 h). Data with different letters (i.e., a, b, and c) are significantly different ($p < 0.05$).

Generally, as the extraction temperature increases, the viscosity of the solvent decreases, which can improve the extraction rate, while both solubility and mass transfer rate increase, enhancing the solubility of the analyte [28]. Additionally, at high temperatures, the interactions between phenolic compounds and proteins and between phenolic compounds and polysaccharides in biomass are weakened, which increases the diffusion rate of phenolic compounds into the solvent [13,29,30]. However, the increase in extraction yield above a certain temperature is limited. Extraction at extremely high temperatures may cause decomposition of phenolic compounds or increase solvent loss via evaporation [13,29,31]. Similar to this study, a related study focusing on the extraction of flavonoids from *Cyclocarya paliurus* (Batal.) Iljinskaja leaves investigated the effect of temperature in the range of 30–70 °C on the flavonoid yield [32]. Results revealed that the flavonoid yield increased with an increase in the extraction temperature from 30 to 60 °C; in contrast, it decreased at temperatures higher than 60 °C. Furthermore, in studies on the optimization of the conditions for the extraction of flavonoids from biomass, 50–60 °C was mainly selected as the optimal extraction temperature [13,29,33–37].

3.1.4. Effect of Ratio of Solvent to Biomass on the Puerarin Yield

The extraction solvent-to-biomass ratio is an important factor affecting the extraction yield [9,38]. Therefore, the influence of the extraction solvent-to-biomass ratio in the range of 5:1–15:1 on the puerarin yield was analyzed. With an increase in the extraction solvent-to-biomass ratio from 5 to 7.5 and 10, the puerarin yields substantially improved from 40.77 to 52.25 and 57.71 mg/g biomass, respectively. Nevertheless, at higher ratios of 12.5 and 15, the puerarin yields decreased to 53.27 and 52.05 mg/g biomass, respectively (Figure 7).

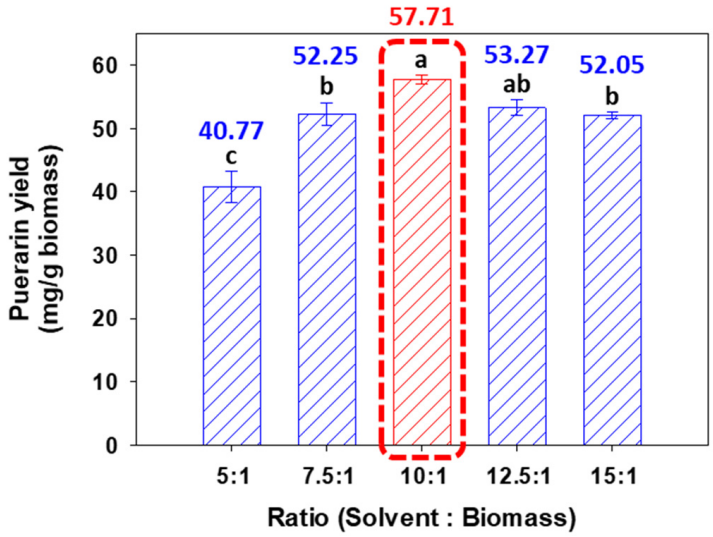

Figure 7. Effect of solvent-to-biomass ratio on the puerarin yield from RPL. (50% ethanol as extraction solvent, extraction temperature of 50 °C, and extraction time of 3 h). Data with different letters (i.e., a, b, and c) are significantly different ($p < 0.05$).

The extraction solvent amount should be sufficient to completely soak the biomass. Typically, with an increase in the extraction solvent amount, the target compound can be entirely dissolved, thereby increasing the extraction yield. However, if the solvent amount exceeds a certain value, more impurities are dissolved, which interferes with the dissolution of the target compound, and consequently, the extraction yield decreases [9,26,29]. Additionally, if the extraction solvent-to-biomass ratio is considerable, the extraction solvent is wasted and the process cost increases; therefore, choosing an appropriate extraction solvent-to-biomass ratio is crucial [9,29,39]. Results of these experiments indicated that the highest yield was obtained at an extraction solvent-to-biomass ratio of 10:1, and thus, 10:1 was selected as the optimal extraction solvent-to-biomass ratio.

3.2. Enhancement of Puerarin Extraction via Response Surface Methodology

3.2.1. Model Fitting

In Section 3.1, the effects of four variables (solvent type, solvent concentration, extraction temperature, and extraction solvent-to-biomass ratio) on the puerarin yield were determined using the OFAT method. Based on these experiments, herein, the interaction among variables was analyzed using RSM, and an empirical model was developed to predict the puerarin yield from RPL. The designed extraction conditions and experimental results are presented in Table 2. Extraction was conducted for 30 min. Identifying the suitable extraction time is important because an extraction time longer than necessary reduces process efficiency. Therefore, puerarin yield with respect to the extraction time was investigated under the optimal extraction conditions derived via the OFAT method. Extraction was completed in 30 min, and no significant increase in the puerarin yield was observed after 30 min. Based on this result, extraction was performed for 30 min under the designed conditions, and the model equation for the actual factors achieved via regression analysis based on the experimental data is as follows (Equation (3)):

$$Y = -14.9548 + 0.3508X_1 + 0.4797X_2 + 8.7267X_3 + 0.0028X_1X_2 - 0.0033X_1X_3 + 0.0207X_2X_3 - 0.0056{X_1}^2 - 0.0063{X_2}^2 - 0.4204{X_3}^2 \quad (3)$$

where Y is the predicted puerarin yield (mg/g biomass) and X_1, X_2, and X_3 represent the ethanol concentration (vol%), extraction temperature (°C), and extraction solvent-to-biomass ratio (mL/g), respectively. Moreover, model terms with positive signs in the model

equation indicate a synergistic effect that increases the puerarin yield, whereas model terms with negative signs imply a hostile effect that reduces the puerarin yield [40]. Y values estimated by Equation (3) are provided in Table 2.

Table 2. Experimental and predicted puerarin yields acquired using the RSM central composite rotatable design (CCRD).

No.	Point Type	Coded Level			Y: Puerarin Yield (mg/g Biomass)	
		X_1	X_2	X_3	Experimental	Predicted by Model Equation (3)
1	Factorial	−1	−1	−1	44.87	45.38
2	Factorial	1	−1	−1	39.49	38.06
3	Factorial	−1	1	−1	48.30	47.88
4	Factorial	1	1	−1	45.24	46.08
5	Factorial	−1	−1	1	51.26	50.53
6	Factorial	1	−1	1	41.69	42.22
7	Factorial	−1	1	1	56.46	58.01
8	Factorial	1	1	1	55.62	55.22
9	Axial	−1.682	0	0	52.6	52.11
10	Axial	1.682	0	0	43.29	43.62
11	Axial	0	−1.682	0	43.33	44.05
12	Axial	0	1.682	0	57.98	57.09
13	Axial	0	0	−1.682	40.66	41.00
14	Axial	0	0	1.682	53.53	53.02
15	Center	0	0	0	56.43	57.71
16	Center	0	0	0	58.73	57.71
17	Center	0	0	0	57.92	57.71
18	Center	0	0	0	56.86	57.71
19	Center	0	0	0	57.38	57.71
20	Center	0	0	0	58.93	57.71

To evaluate the significance and validity of the model derived from the experimental data, ANOVA was conducted, and the results are presented in Table 3. A high F-value (>1) and low p-value (<0.05) of the model reveal the statistical significance of the model [13,29,41]. The F-value indicates the probability that the differences between groups are statistically significant, and a higher F-value means that the variation between groups is greater relative to the variation within groups. The p-value is a tool that assesses the significance of each coefficient, and the smaller the p-value, the more important the coefficient [4,9,12]. The F-value and p-value of the derived model are 72.56 and less than 0.0001, respectively, which indicates that the probability of the origin of the F-value from noise is only 0.01% and the model is meaningful [13]. Generally, if the p-value of a model term is less than 0.05, the term demonstrates a statistically significant effect [42]. Model terms that exhibit significant effects on the puerarin yield prediction model are as follows: linear terms of ethanol concentration (X_1), temperature (X_2), and solvent-to-biomass ratio (X_3); quadratic terms of ethanol concentration ($X_1{}^2$), temperature ($X_2{}^2$), and solvent-to-

biomass ratio (X_3^2); and interaction terms of ethanol concentration–temperature (X_1X_2) and temperature–solvent-to-biomass ratio (X_2X_3).

Table 3. Analysis of variance (ANOVA) results for the quadratic model of predicting puerarin yield.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	*p*-Value Prob > F	Remarks
Model	892.65	9	99.18	72.56	<0.0001	Significant
X_1	87.18	1	87.18	63.78	<0.0001	
X_2	205.17	1	205.17	150.09	<0.0001	
X_3	174.29	1	174.29	127.50	<0.0001	
X_1X_2	15.24	1	15.24	11.15	0.0075	
X_1X_3	0.48	1	0.48	0.35	0.5654	
X_2X_3	12.36	1	12.36	9.04	0.0132	
X_1^2	174.71	1	174.71	127.81	<0.0001	
X_2^2	91.83	1	91.83	67.18	<0.0001	
X_3^2	206.30	1	206.30	150.92	<0.0001	
Residual	13.67	10	1.37			
Lack of fit	8.62	5	1.72	1.71	0.2855	Not significant
Pure error	5.05	5	1.01			
Cor total	906.32	19				

Coefficient of determination (R^2): 0.98. Adjusted R^2: 0.97. Predicted R^2: 0.91. Coefficient of variation (CV): 2.29%. Adequate precision (AP): 24.13.

According to the ANOVA results, R^2, adjusted R^2, and predicted R^2 of the puerarin yield prediction model are 0.98, 0.97, and 0.91, respectively. Herein, R^2 is the square of the statistical deviation between the experimental and predicted values. The closer the R^2 to 1, the better the correlation between the experimental and predicted values, indicating that the response can be better predicted [43]. Even if the terms are not statistically significant, R^2 can increase if the terms are continuously added to the model. Therefore, typically, adjusted and predicted R^2 are used to determine the model accuracy, and the difference between adjusted and predicted R^2 should be less than 0.2. The difference between adjusted and predicted R^2 of the puerarin yield prediction model is 0.06 (<0.2), which implies sufficiently high accuracy of the model for puerarin yield prediction. Lack-of-fit reveals the extent to which the model fails to fit data from experimental areas that are not included in the regression model, and a non-significant lack-of-fit indicates excellent predictability of the model [44]. The coefficient of variation is the ratio of the standard deviation of the predicted value to the mean of the observed response value and is an indicator of the reproducibility of the model. Generally, when the coefficient of variation of a model is less than 10%, the accuracy and reliability of the performed experiment are high; thus, the model is considered reproducible [44,45]. Also, Adequate Precision is a signal-to-noise ratio measured by comparing the predicted value of design points with the average prediction error [46], and a value of 4 or higher reveals that the model is suitable for exploring the designed space [45,47]. The lack-of-fit F-value of the puerarin yield prediction model is 1.71, which indicates that the lack-of-fit is not considerable compared to the pure error. Additionally, the coefficient of variation and adequate precision are 2.29% (<10%) and 24.13 (>4), respectively, implying that the model is reliable and appropriate for estimating the puerarin yield from RPL.

Distributions of residuals were examined to evaluate the suitability of the model. Residual represents the error between the predicted and actual values at each point. As the standard deviation of the residual significantly varies at each design point, comparing the residuals at different design points is irrelevant. When the experimental errors are random, the residuals follow a normal distribution [47]. Then, the residuals are normalized based on the standard deviation (studentized) [46,47]. The distributions between the predicted and experimental studentized residuals appear as a straight line (Figure 8a), revealing that the studentized residuals follow a normal distribution [48]. Moreover, if an incorrect model is used or additional transformation of the response is necessary, the distribution between the predicted and experimental studentized residuals appears as an S-shaped curve [47]. Figure 8b shows a plot of the studentized residuals and predicted puerarin yield; random distributions of the residuals indicate that the proposed model is appropriate. Figure 8c depicts a plot of the predicted and actual puerarin yields, and the data points are evenly distributed along the diagonal line, which verifies that the model adequately fits the experimental data [44]. Herein, the predicted values were calculated using Equation (3), and the actual values were the experimentally obtained data. Figure 8d shows an outlier t plot for all runs. The outlier t plot reveals the sizes of the residuals for each experiment, which confirms the existence of experiments with particularly large residuals. Typically, the standard residuals must be within a standard deviation interval of 3, and outliers outside this interval indicate potential errors in the model or manipulation errors in the experimental data [46]. No out-of-interval data are present in Figure 8d, implying that the model is consistent with all the data [48].

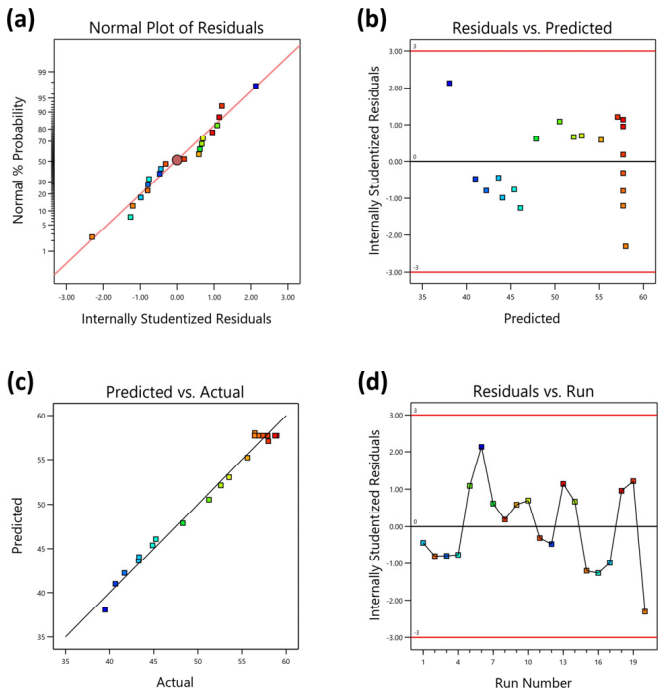

Figure 8. Statistical analysis of the response surface model for predicting puerarin yield. Plots of (**a**) normal% probability and studentized residuals, (**b**) studentized residuals and predicted response, (**c**) predicted and actual responses, and (**d**) outlier t plot. Points near the red line in (**a**) imply that the residuals are normally distributed. Red lines in (**b**,**d**) represent the boundaries of ±3 studentized residuals.

3.2.2. Mutual Effect of Parameters

To understand the influences of the interactions among variables on puerarin yield, one variable was fixed at level 0 and the response surface and contour lines were plotted for

the other two variables (Figure 9). Figure 9a depicts the interaction between the extraction temperature and ethanol concentration and the effect of this interaction on the puerarin yield at an extraction solvent-to-biomass ratio of 10:1. The puerarin yield increased with an increase in the ethanol concentration from 25 to approximately 45%; however, it rapidly decreased at concentrations exceeding 45%. The highest yield of puerarin was achieved at an extraction temperature of approximately 65 °C. Figure 9b shows a graph depicting the influence of the interaction between the extraction solvent-to-biomass ratio and ethanol concentration on the puerarin yield at an extraction temperature of 50 °C. Figure 9c shows the effect of the interaction between the extraction solvent-to-biomass ratio and extraction temperature on the puerarin yield when 50% ethanol was used as the extraction solvent. In both graphs, the puerarin yield was the highest when the extraction solvent-to-biomass ratio was approximately 11.5.

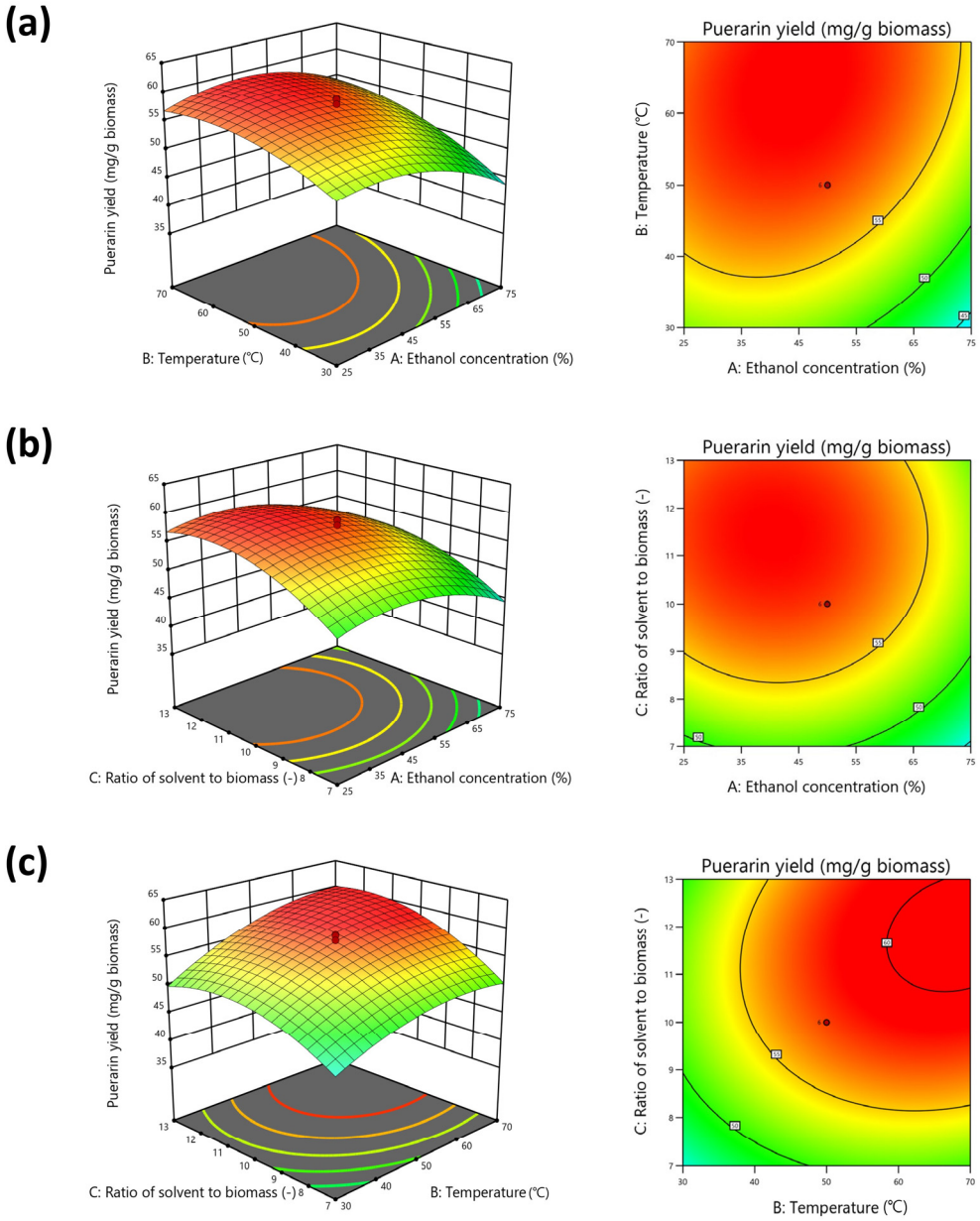

Figure 9. Response surface and contour plots for the influences of independent parameters on puerarin yield: (**a**) temperature and ethanol concentration, (**b**) solvent-to-biomass ratio and ethanol concentration, and (**c**) solvent-to-biomass ratio and temperature.

3.2.3. Obtaining Optimal Extraction Conditions and Model Validation

Numerical optimization was conducted to determine the optimal puerarin extraction conditions using the puerarin yield prediction model. Goals were set as ethanol concentration, "in range"; temperature, "in range"; solvent-to-biomass ratio, "in range"; and puerarin yield, "maximize." The optimal conditions derived via numerical optimization were as follows: ethanol concentration: 46.06%, extraction temperature: 65.02 °C, and extraction solvent-to-biomass ratio: 11.50. The predicted puerarin yield under the optimal conditions was 60.68 mg/g biomass (Table 4). To verify the model, an experiment was performed according to the derived optimal conditions, and the experimental puerarin yield acquired under the optimal conditions was 60.75 mg/g biomass. These results indicate that the model successfully estimated the puerarin yield, demonstrating the reliability of the model.

Table 4. Predicted and experimental puerarin yields obtained under the optimal extraction conditions.

Ethanol Concentration (vol%)	Temperature (°C)	Solvent-to-Biomass Ratio (mL/g)	Predicted *Y* (mg/g Biomass)	Experimental *Y* (mg/g Biomass)
46.06	65.02	11.50	60.68	60.75 ± 0.27

3.3. Extraction of Puerarin Under the Optimal Conditions

Puerarin yields with respect to the extraction time under the optimal conditions derived by the OFAT method and RSM were confirmed (Figure 10). After 22 min of extraction, a puerarin yield of 60.56 mg/g biomass was obtained, and no considerable increase in the puerarin yield was observed thereafter. Therefore, 22 min was determined to be the optimal extraction time. Finally, the optimal puerarin extraction conditions established via this study were as follows: solvent: 46.06% ethanol, extraction temperature: 65.02 °C, solvent-to-biomass ratio: 11.50 mL/g, and extraction time: 22 min, and the puerarin yield acquired under these optimal conditions was 60.56 mg/g biomass, approximately 4.6 times the initial puerarin yield of 13.14 mg/g biomass. Herein, a high yield of puerarin was achieved in a short time by optimizing the puerarin extraction conditions using the OFAT method and RSM and measuring the time profile.

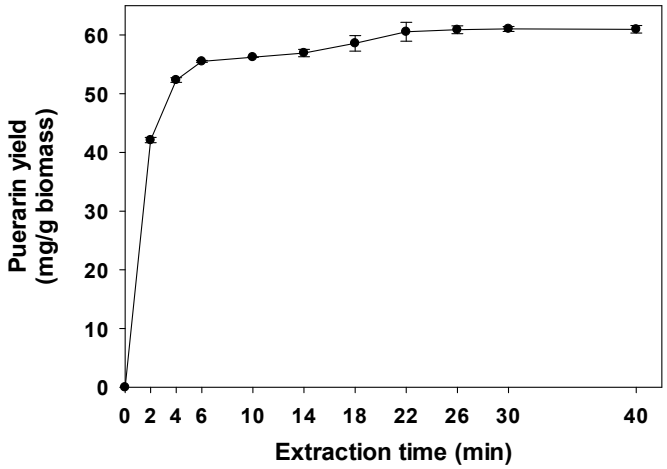

Figure 10. Puerarin yield as a function of extraction time. (46.06% ethanol as extraction solvent, extraction temperature of 65.02 °C, and solvent-to-biomass ratio of 11.50 mL/g).

Table 5 presents a summary of research on the recovery of puerarin from RPL. Puerarin has been recovered by various extractions such as ultrasound-assisted extraction, supercritical CO_2 fluid extraction, pressurized solvent extraction, and solvent extraction.

Ultrasound-assisted extraction is an extraction technology that can obtain high yields using a minimal amount of solvent in a short time using ultrasound to rupture plant cell walls and cell membranes, thereby promoting the diffusion of intracellular substances into the solvent [26]. This method decomposes puerarin by locally generating high temperatures and pressures in the extract [49]. Supercritical CO_2 fluid extraction is an environmentally friendly technology that requires short extraction times and is suitable for heat-sensitive compounds [50]. However, non-polar CO_2 is not effective in extracting polar compounds including puerarin, and an appropriate polar co-solvent must be used to recover puerarin [51]. Yang et al. achieved a puerarin yield of approximately 0.14 mg/g biomass from RPL via supercritical CO_2 extraction using 36.7% ethanol as a co-solvent [51]. Pressurized solvent extraction, also known as accelerated solvent extraction, is an extraction technology that can selectively extract polar and non-polar compounds using solvent polarity, which changes depending on the pressure and temperature [52]. Nevertheless, as this method is conducted at high temperatures and pressures, puerarin may be destroyed and the process operating costs are high [53,54]. Solvent extraction, a traditional extraction, has been widely used for a long time because it does not require specific equipment and can produce large amounts of products [55]. This extraction is low-cost and necessitates low installation and maintenance costs, resulting in lower operating costs for the entire process than the cases of other new extractions [56]. GRAS solvents are recognized as safe for use in food, pharmaceutical, and cosmetic applications, which aligns well with the industrial applicability of puerarin. Additionally, the GRAS solvents used in this study are easy to handle and cost-effective, making solvent extraction with these solvents a more sustainable extraction process without compromising the extraction efficiency of bioactive compounds such as puerarin. Yields of the bioactive compounds recovered from biomass vary depending on the type of extraction [57]. Therefore, choosing an appropriate extraction method and optimizing the process variables are significantly important to efficiently obtain bioactive components in high yields [58]. In this study, puerarin was extracted from RPL via solvent extraction, which does not need particular equipment, and the extraction variables were optimized based on the OFAT method and RSM. Consequently, puerarin was recovered in high yield using a small amount of solvent in a shorter extraction time than the cases of other puerarin extractions. Thus, this study will contribute to the development and commercialization of cost-effective puerarin recovery in the future. The main challenges for the practical industrial application of the developed process are scale-up studies and the continuous development of the downstream process, including the formulation of RPL extract or puerarin. In our follow-up study, (1) economic analysis through process simulation and (2) application or formulation of the extracts will be carried out.

Table 5. Summary of extraction conditions for puerarin recovery.

Biomass	Type of Extraction	Conditions				Puerarin Yield (mg/g Biomass)	Ref.
		Solvent	Temperature (°C)	Time (min)	Solvent-to-Biomass Ratio (mL/g)		
Pueraria lobata roots	Ultrasound-assisted extraction	100% ethanol (with 0.05% HCl)	57.82	39.79	10	43.04	[59]
Pueraria lobata roots	Ultrasound-assisted extraction	71.35% ethanol	-	49.08	21.72	41.00	[4]

Table 5. *Cont.*

Biomass	Type of Extraction	Conditions				Puerarin Yield (mg/g Biomass)	Ref.
		Solvent	Temperature (°C)	Time (min)	Solvent-to-Biomass Ratio (mL/g)		
Pueraria lobata roots	Ultrasound-assisted extraction	Natural deep eutectic solvent	80	120	20	12.13	[60]
Pueraria lobata roots	Ultrasound-assisted extraction	1.06 M 1-butyl-3-methylimidazolium bromide	35	27.43	23.26	37.71	[11]
Pueraria lobata roots	Supercritical CO_2 fluid extraction	36.7% ethanol	51.5	30	66.67	0.14	[51]
Pueraria lobata roots	Solvent extraction	Water	99	60	25	22.00	[61]
Pueraria lobata roots	Pressurized solvent extraction	Water	141	58	23	48.00	[61]
Pueraria lobata roots	Pressurized solvent extraction	95% ethanol	100	10	10	2.74	[54]
Pueraria lobata roots	Solvent extraction	95% ethanol	-	120	10	1.19	[54]
Pueraria lobata roots	Solvent extraction	46.06% ethanol	65.02	22	11.50	60.56	This study

4. Conclusions

In this study, a puerarin extraction process was designed using a statistical method to efficiently recover puerarin from RPL. Industrial GRAS solvents were used as extraction solvents, and a puerarin yield prediction model was proposed by analyzing the correlations among the variables, such as solvent concentration, temperature, and extraction solvent-to-biomass ratio, influencing the extraction yield. The following optimal conditions were determined via the model: ethanol concentration: 46.06%, temperature: 65.02 °C, extraction solvent-to-biomass ratio: 11.50 mL/g, and extraction time: 22 min. A puerarin yield of 60.56 mg/g biomass was acquired under these optimal conditions, which was approximately 4.6 times that achieved before extraction variable optimization. The novelty of this study lies in the design of cost-effective puerarin recovery that can obtain a high yield of puerarin via extraction using a small amount of solvent in a short extraction time without stirring or specific equipment. This study provides useful information on the recovery of puerarin from RPL, which will contribute to the development of a biorefinery strategy for biomass-based flavonoid ester production to be explored in subsequent research. In order to produce and apply puerarin-rich RPL extracts on an industrial scale, further research should focus on scale-up studies of the optimal process, application to practical products, and life-cycle assessment.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: Author Soo Kweon Lee is employed by the Lotte R&D center. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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