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Optimization of ultrasonic–microwave synergistic extraction of flavonoids from sweet potato leaves by response surface methodology

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Abstract

Ultrasonic–microwave synergistic extraction was used to extract flavonoids from sweet potato leaves (SPL) by response surface methodology. The optimal conditions for flavonoids extraction were 1:40 (g/ml) of solid–liquid ratio, 57°C of extraction temperature, 76 s of extraction time, and 72% (v/v) ethanol for 2 times, the highest extraction efficiency was 91.65 ± 3.37%. After purification, the flavonoids purity reached to 76.10 ± 3.11 (%, DW). The result of high-performance liquid chromatography revealed 11 compounds including astragalin (473.8 ± 7.3 mg/g, DW), quercetrin (86.5 ± 0.7 mg/g, DW), 4,5-chlorogenic acid (76.4 ± 0.5 mg/g, DW), isoquercitrin (62.4 ± 0.4 mg/g, DW), tiliroside (18.8 ± 0.3 mg/g, DW), quercetin (12.5 ± 0.2 mg/g, DW), 3,4,5-chlorogenic acid (6.5 ± 0.2 mg/g, DW), caffeic acid (6.1 ± 0.2 mg/g, DW), kaempferol (6.0 ± 0.2 mg/g, DW), myricetrin (5.9 ± 0.1 mg/g, DW), and rhamnetin (4.3 ± 0.1 mg/g, DW) in sweet potato leaf flavonoids, which possessed good antioxidant activity compared to soy isoflavones, ginkgo biloba extract, and propolis flavone. The IC_{50} value of sweet potato leaf flavonoids was 13.26 ± 0.09 μg/ml in ferric reducing antioxidant power and 5.41 ± 0.21 in 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) scavenging capacity, respectively.

Practical applications

China is the leading country of sweet potato production in the world, but sweet potato leaves (SPL) have been neglected except for a partial use as livestock feed in most parts of China, and detailed reports on the effective components in the leaves are scarce. Ultrasonic–microwave synergistic extraction is an efficient way to select flavonoids from SPL which has a potential to be extended in natural flavonoids industry.

1 | INTRODUCTION

Flavonoids are a large class of secondary metabolites widely existing in plants. Its basic structure is flavane nuclear parent (2-benzyl ketone), which means two benzene rings (A ring and B ring) are connected by pyran ring (C ring), often containing hydroxyl, methoxy, methyl, isoamyl group, and other substituents, widely exists in the fruit, leaf, wood, and bark of plant. Flavonoids possess biological activities such as antioxidant activity, improving blood circulation, reducing cholesterol, preventing ultraviolet, inhibiting angiogenesis, antibacterial, and antiinflammatory (Chen et al., 2011).

Some studies found that sweet potato leaves (SPL) are rich in flavonoids and the content ranges from 18 to 73 mg quercetin equivalent/100 g (DW) in four cultivars in Taiwan (Liao, Lai, Yuan, Hsu, & Chan, 2011). At present, the flavonoids including quercetin, myricetin, luteolin, and apigenin have been found in SPL (Ojong et al., 2008). Peonidin has been found in purple sweet potato cultivar Eshu No. 8 (Zhang, Luo, Zhou, & Zhang, 2018). Meanwhile, it has been reported

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that SPL flavonoids possess antioxidant activity (Huang et al., 2013), anti-LDL oxidation activity (Taira, Taira, Ohmine, & Nagata, 2013), anticytotoxic activity (Liao et al., 2011), and antiproliferation activity (Taira et al., 2013).

It is well known that flavonoids are soluble in polar solvents and are usually extracted by aqueous mixtures of organic solvent such as methanol, ethanol and acetone, and 70% ethanol extract had the highest yield of total flavonoid (3.4 mg QE/g DW) (Fu et al., 2016), meanwhile methanol and acetone are toxic and ethanol and its aqueous mixture are the safest solutions for extraction. The extraction of flavonoids from SPL included conventional stirring extraction (Miu et al., 2011), reflux extraction (Liao et al., 2011), sonication extraction (Isabelle et al., 2010), and dynamic high pressure microfluidization-assisted extraction (Huang et al., 2013), which were time-consuming, activity destroying, solvent wasting, and not eco-friendly.

Ultrasonic–microwave synergistic extraction (UMSE) is the extraction method combined with ultrasonic and microwave which is economic and time-saving comparing with other new extraction technologies such as pressurized liquid extraction, supercritical fluid extraction, enzyme-assisted extraction, and pulsed electric field extraction (Mandal, Mohan, & Hemalatha, 2007). Ultrasound-assisted extraction (UAE) uses ultrasound to produce “cavitation” in liquid, destroying plant cell and cell membrane structure, thus enhancing the penetration of cell contents through cell membrane, which is beneficial to the release and dissolution of flavonoids (Chemat, Zill-e-Huma, & Khan, 2011). The biggest advantage of UAE is to increase the yield significantly such as the yield of chlorogenic acid from artichoke leaves extracted by 80% methanol for 15 min increased 50% toward to maceration at ambient temperature, which was close to the yield by boiling extraction (Saleh et al., 2016). Microwave-assisted extraction (MAE) is based on the principle of its penetrating heating, in the microwave field, the absorption difference of various materials makes certain components of the matrix material or some components in the extraction system be selectively heated and get enough energy to escape (Mustapa, Martin, Gallego, Mato, & Cocero, 2015). The biggest advantage of MAE is time-saving, compared to 2 hr of conventional reflux extraction of total polyphenols from the leaves of *Pistacia lentiscus*, the MAE could only take 60 s to attain the highest yield of total polyphenols, saving more than 99% of the time (Dahmoune et al., 2014). Meanwhile, the highest antioxidant activity could be obtained in 70% ethanol extracts from elecampane (*Inula helenium*) roots collected by UAE which was 86.0 mM TE/g DW in ABTS and 67.0 mM TE/g DW in ferric reducing antioxidant power (FRAP) (Petkova, Ivanov, Vrancheva, Denev, & Pavlov, 2016).

Response surface methodology (RSM) is an experimental design which utilize minimum trials to optimize the multiple variables experiments, at the same time detecting the interrelationship between the variables comparing to the traditional orthogonal design (Ferreira et al., 2007). Especially, for the bioactive compounds extraction optimization experiments, RSM shows its superiority which could flexibly optimize the dependent variables more than one according to the same variables (Derrien, Badr, Gosselin, Desjardins, & Angers, 2017).

In the present study, flavonoids were extracted from SPL by UMSE. The optimal extraction parameters were assessed with RSM. The individual flavone composition was analyzed by high-performance liquid chromatography (HPLC). In addition, the antioxidant activity was investigated and compared with some common commercial flavonoids (soy isoflavones, ginko biloba extract, and propolis flavone). The aim of this study was to exploit the potentialities of UMSE application in the industrial extraction of flavonoids and the application of SPL as a good source in commercial flavonoids production.

2 | MATERIALS AND METHODS

2.1 | Materials

According to the previous study in our lab (Sun, Mu, Xi, Zhang, & Chen, 2014), the SPL (leaf and petiole) from sweet potato cultivar Yuzi No. 7 was selected in the present study, which was heart type leaf, contained the highest polyphenols content and highest antioxidant activity among 40 cultivars around China. Fresh SPL were provided by Chongqing Sweet Potato Engineering and Technology Research Center, Chongqing, China. Tubers were sowed in the late period of June and SPL were harvested in the early period of September, 2017, approximate 70 days of growth period at Baiyun Village, Muer Town, Yubei District, Chongqing. Air transported to the lab immediately, washed gently, and dried by vacuum freezing and then grounded by ultra-micro pulverizer, sieved through 100-mesh screens, and then sealed in aluminum foil bags and stored in the refrigerator at 4°C for further use.

Quercetin, kaempferol, myricetrin, astragalin, tiliroside, quercitrin, isoquercitrin, rhamnetin, caffeic acid (CA), 4, 5-chlorogenic acid (4, 5-CQA), and 3, 4, 5-chlorogenic acid (3, 4, 5-CQA) were purchased from An Apoptosis and Epigenetics Company (Houston, USA), the purity of the standards was more than 98%. Soy isoflavones, ginkgo biloba extract, and propolis flavone were purchased from Shanghai Yuanye Biotech Co., Ltd. (Shanghai, China). 2, 4, 6-Tri (2-pyridyl)-1, 3, 5-triazine (TPTZ) and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Solarbio Life Sciences (Beijing, China). Other analytical grade chemicals were obtained from Sinopharm Chemical Reagent (Beijing, China). HPLC grade solvents were purchased from Fisher Chemical (Beijing, China). HPLC water was prepared by the Molgene water purification system from Molecular (Shanghai, China).

2.2 | Basic components of SPL

The SPL powder was analyzed for moisture, ash, crude protein, crude fat, crude fiber, carbohydrate, and flavonoids content. The moisture content was determined by AOAC method 930.15, ash content was determined by AOAC method 923.03, crude protein was analyzed according to Kjeldahl procedure by AOAC method 955.04, crude fat was determined by AOAC method 960.39, and crude fiber was determined by AOAC method 991.43. Carbohydrate content was

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calculated by subtracting the sum of ash, crude protein, crude fat, and crude fiber from 100. The flavonoids content in SPL powder was measured by colorimetric aluminum method described by Shi et al. (2016) with some improvements: 0.1 ml of crude extract was absorbed and added into 10-ml volumetric flask, 4.9 ml of 30% ethanol was complemented, shook the solution for even, and 0.3 ml of 5% $NaNO_2$ was added later, mixing uniformity, and stood still for another 5 min, then 0.3 ml of 10%$Al(NO_3)_3$ was added to the mixture, mixing evenly, and stood still for another 6min, finally 4.0 ml of 4%NaOH was added and constant volume to 10 ml with 30% ethanol, lasting for 10 min and measured the absorbance by spectrophotometer at 320 nm (maximum absorbance), quercetin was taken as the standard.

2.3 | Single factor for extraction efficiency of SPL flavonoids

Five grams SPL powder was extracted by Ultrasonic-Microwave Assisted Extractor CW-2000 (ultrasonic frequency was 40 kHz, ultrasonic power was 50 W, microwave frequency was 2450 MHz, microwave power was 100 W, microwave wavelength was 0.12 m) (Shanghai Xintuo Analytical Instruments Co., Ltd, China) for 1, 2, 3 times, immersed in ethanol solution of 10%, 30%, 50%, 70%, 90%, 100% by solid–liquid ratio (g/ml) of 1:10, 1:20, 1:30, 1:40, 1:50 at the temperature of 35, 45, 55, 65, 75°C continued for 25, 50, 75, 100, 200, 600, 999 s, centrifuged at 7,000 rpm for 30 min, the supernatant was combined and constant the volume to 500 ml with 30% ethanol. The extraction efficiency (%) was calculated by the equation:

$$\text{Extraction efficiency (\%)} = \frac{\text{Content of SPL flavonoids extracted by certain parmeters}}{\text{Actual content of SPL flavonoids}} \times 100\% \quad (1)$$

2.4 | RSM optimization for extraction efficiency of SPL flavonoids

Since 2 times and 3 times of extraction had no significant difference and 1:40 (g/ml) of solid–liquid ratio had the highest extraction efficiency of SPL flavonoids according to result of single-factor experiments, meanwhile considering the solvent saving, extraction times, and solid–liquid ratio were settled down as 2 and 1:40. Three factors and three levels model consisted of 17 experiments with five replicates at central point were shown in Table 1. Three experimental factors included extraction temperature (X_1: 45, 55, 65°C), extraction time (X_2: 50, 75, 100 s), and ethanol concentration (X_3: 50%, 70%, 90%), which were optimized by Box–Behnken design, extraction efficiency of SPL flavonoids (Y) was chosen as the dependent variable. The experimental data were fitted into the equation:

$$Y = Ao + \sum_{i=1}^{3} AiXi + \sum_{i=1}^{3} AiiXi2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} AijXiXj, \quad (2)$$

where Y was the response variable, A_0 was the intercept constant, A_i, A_{ii}, A_{ij} were the regression coefficients for linear, quadratic, cross effect of X_1, X_2, X_3, X_i, X_j were coded values of independent variables.

TABLE 1 Experimental and predicted values of extraction efficiency in Box–Behnken design

Factor	Coded symbol	Level		
		−1	0	1
Extraction temperature	X_1	45	55	65
Extraction time	X_2	50	75	100
Ethanol concentration	X_3	50	70	90

Run	Independent variables			Measured	Predicted
	X_1	X_2	X_3	Y	Y
1	−1	−1	0	71.05	69.45
2	1	−1	0	82.95	82.06
3	−1	1	0	76.02	76.91
4	1	1	0	74.07	75.49
5	−1	0	−1	71.76	69.63
6	1	0	−1	84.19	82.95
7	−1	0	1	79.93	77.26
8	1	0	1	85.97	87.74
9	0	−1	−1	79.57	77.80
10	0	1	−1	73.71	76.38
11	0	−1	1	82.59	84.72
12	0	1	1	66.61	66.25
13	0	0	0	89.88	89.17
14	0	0	0	91.65	89.17
15	0	0	0	87.57	89.17
16	0	0	0	88.63	89.17
17	0	0	0	88.10	89.17

Note. X_1: extraction temperature (°C); X_2: extraction time (s); X_3: ethanol concentration (%); Y: Extraction efficiency (%) of SPL flavonoids.

The optimal extraction parameters were calculated by the "desirability" algorithm (Wong, Li, Li, Razmovski-Naumovski, & Chan, 2017). To determine the optimization of extraction, the experimental variables in the Design Expert 8.0 were set as "in the range," meanwhile the response variable was set as "maximize." The combination of independent variables which made the highest desirability was chosen as the optimal extraction parameters.

2.5 | SPL flavonoids purification

Liquid–liquid extraction is the method to separate materials with different polarity using different organic solvent. Phenolic acids in SPL were mainly chlorogenic acid with different position substituted (Xi, Mu, & Sun, 2015), which hardly dissolved in ethyl acetate while flavonoids could dissolved easily (Lu, Wang, Xie, & Ding, 2013), so crude extract obtained by optimum process was vacuum concentrated to half volume and extracted by the same volume of petroleum ether in order to remove chlorophyll and other lipid soluble impurities, water phase was extracted by ethyl acetate with the same volume, the mixture was shaken thoroughly and stood for 5 min to make

the ester phase and water phase separate completely, ethyl acetate phase was vacuum concentrated and evaporated to dryness, which was SPL flavonoids.

2.6 | Qualitative and quantitative analysis of SPL flavonoids

The SPL flavonoids standards (quercetin, myricetrin, astragalin, tiliroside, quercitrin, isoquercitrin, kaempferol, rhamnetin, CA, 4,5-CQA, 3,4,5-CQA) were qualified and quantified by HPLC (Shimadzu), which was equipped by the system control unit (CBM-20A), the UV detector (SPD-20A), the degaser (DGU-20A$_3$), the liquid infusion unit (LC-20AB), the automatic sampler (SIL-20AC), and the column oven (CTO-20AC), C_{18} was the column (4.6 × 150 mm, 5 μm, Inertsil ODS-SP, Shimadzu). The mobile phase was consisted of A and B, A: ultrapure water with 0.5% (v/v) phosphoric acid, B: 100% acetonitrile, the elution procedure was performed as follows: 0–15.0 min, 20%–65% B; 15.0–15.1 min, 65%–80% B; 15.1–20.0 min, static 80% B; 20.0–20.1 min, 80%–20% B; 20.1–25.0 min, static 20% B, the elution rate was 1.0 ml/min and the injection volume was 20 μl, the oven temperature was kept at 30°C, and detection wavelength was set at 326 nm according to the method of Xi et al. (2015) with some modification.

The SPL flavonoids and standards were precisely weighed and dissolved in the HPLC grade methanol to prepare the stock solution at the concentration of 1 mg/ml, kept at 4°C until use. Standard stock solutions were diluted to 50 μg/mL with methanol and 1 mg/ml of SPL flavonoids were filtered through 0.22 μm membrane and injected into HPLC to detect and compare the retention time and response of each peak with standards. The concentration of standards was adjusted to 25, 50, 100, 150, 200 μg/ml for quercetin, myricetrin, tiliroside, quercitrin, isoquercitrin, kaempferol, rhamnetin, CA, 4, 5-CQA, 3, 4, 5-CQA and 100, 200, 300, 400, 500 μg/ml for astragalin according to the response of the SPL flavonoids.

2.7 | Antioxidant activity of SPL flavonoids

2.7.1 | Ferric reducing antioxidant power

FRAP was carried out according to the method of Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Byrne (2006) with some modification, the specific procedure was as follows: 10 mmol/L TPTZ (40 mmol/L HCl was the solvent), 20 mmol/L $FeCl_3$ (0.3 mol/L, pH 3.6 PBS was the solvent). One portion of TPTZ, 1 portion of $FeCl_3$ and 10 portion of PBS were mixed together and incubated in the water bath at 37°C away from light, that was how to prepare the FRAP working solution.

The sample solutions with different flavonoid concentration (5, 10, 20 μg/ml) were prepared by diluting with distilled water. An aliquot of 0.15-ml flavonoid sample solution was mixed with 2.85 ml of FRAP working solution, incubated for 30 min from light at room temperature, and measured the absorbance by spectrophotometer at 593 nm immediately; the blank control was the mixture while flavonoid sample solution was replaced by distilled water. The scavenging rate was calculated according to the equation:

$$\text{Scavenging rate (\%)} = \frac{A_0 - A}{A_0} \times 100\% \tag{3}$$

A_0 was the absorbance of the mixture using distilled water to substitute the flavonoids sample solution at 593 nm and A was the absorbance of the mixture with flavonoids sample solution at 593 nm. The IC_{50} value was calculated by Graph Pad Prism 6.

2.7.2 | ABTS$\cdot^+$ scavenging activity

According to the method of Li, Lin, Gao, Han, and Chen (2012) with some modification: 2.5 ml of ABTS (7.4 mM) was mixed with 2.5 ml of $K_2S_2O_8$ (2.6 mM), the mixture was kept quite away from light at 4°C for 24 hr to produce ABTS$^{\cdot+}$, then diluted with ethanol for about 50 times to make the absorbance reach to 0.70 ± 0.02 at 734 nm which was ABTS$^{\cdot+}$ working solution.

Two milliliter ABTS$^{\cdot+}$ working solution reacted with 1.0 ml of flavonoids sample solution of different flavonoids concentration (5, 10, 20 μg/ml), incubated for 6 min, and detected their absorbance at 734 nm. The scavenging rate was calculated according to the equation:

$$\text{Scavenging rate (\%)} = \frac{A_0 - A}{A_0} \times 100\% \tag{4}$$

where A_0 is the absorbance of the mixture using ethanol to substitute the flavonoids sample solution at 734 nm and A is the absorbance of the mixture with flavonoids sample solution at 734 nm. The IC_{50} value was calculated by Graph Pad Prism 6.

2.8 | Data analysis

The data of RSM was processed by Design Expert 8.0, other were analyzed by SAS 8.0. Experiments were carried out in triplicate and data were expressed as mean ± *SD*, p < 0.05 was considered as there was no significant difference.

3 | RESULTS AND DISCUSSION

3.1 | Basic components of SPL

The ash, crude protein, crude fat, and crude fiber content of SPL were 10.47 ± 0.12, 28.79 ± 0.04, 3.28 ± 0.23, and 18.49 ± 0.20 g/100 g DW and the carbohydrate content was 38.97 ± 0.28 g/100 g DW. The flavonoids content of SPL was 5.63 ± 0.21 g/100 g DW.

3.2 | Analysis of single-factor experiments

The effects of UMSE variables, including extraction times, solid–liquid ratio, ethanol concentration, extraction temperature, and extraction time on the extraction efficiency (%) of SPL flavonoids were evaluated by single-factor experiments (Supplement

Figure 1). For the effect of extraction times on the extraction efficiency of SPL flavonoids, there was no significant difference between 2 and 3 times. Considering the solvent-saving and follow-up concentration operation simplifying, extracted 2 times would be better. For the effect of varying solid–liquid ratio on the extraction efficiency of SPL flavonoids, there was a significant increase when solid–liquid ratio increased from 1:10 to 1:40 and then decreased. This might due to the reason that huge solution volume made complete stirring impossible. In addition, the large volume of solution needed to absorb more energy to heat itself up, thus led to inadequate energy diffusion in ultrasonic and microwave field which would slow down the cell wall breaking and flavonoids leaching (Alara, Abdurahman, & Olalere, 2018). For the effect of ethanol concentration on the extraction efficiency of SPL flavonoids, the maximum extraction efficiency was obtained at the concentration of 70%, following with the flavonoids decreasing when the ethanol concentration kept increasing. Relative high ethanol concentration made the alcohol-soluble substances dissolved into the solvent easily. For the effect of extraction temperature on the extraction efficiency of SPL flavonoids, there was a significant rising when the temperature reached to 55°C compared to 45°C, which increased 23.10% then slightly decreased 2.51% when the temperature kept increasing to 65°C. Relative high temperature could lower the viscosity of solvent and made bioactive components transferring through cell membrane more easily from plant matrix (Chew et al., 2011), but high-temperature environment could accelerate the bioactive compounds degradation (Alara et al., 2018). The extraction efficiency of SPL flavonoids achieved to maximum when the extraction time lasting to 75s compared to 50s and 100s, which were 9.09% and 2.79% higher, respectively, ultrasonic and microwave power needed time to transfer the energy to the matrix.

3.3 | Statistical analysis and model fitting of RSM

The advantage of Box–Behnken design was trying minimum times to obtain the optimal model of the experiment. The experimental and predicted values were shown in Table 1. The analysis of variance was summarized in Table 2. *p* value of the model was below 0.0001 indicated the model was approximate to the reality ($p < 0.05$). X_1, X_2, X_3, X_1X_2, $X_1^{\ 2}$, X_2^2, and X_3^2 were below 0.05 showed these factors were quite significant to the extraction efficiency of SPL flavonoids.

The lack of fit was insignificant ($p = 0.6854 > 0.05$) represented the model was significant compared to the pure error, which might due to the noise, the model could predict the extraction process quite well. The $R^2 = 0.9793$ indicated the model could explain the 97.93% of real extraction process, there was only 2.07% of the total variation could not be explained (Wai, Alkarkhi, & Easa, 2010). Moreover, the adjusted $R^2 = 0.9526$ showed the high significance of the model, after deleting the insignificant parameters, there was still 95.26% of the data could be explained by this model.

Three-dimensional plots were chosen to represent the predicted model and the interaction between different parameters. The plots show the interaction between two factors while another was kept at medium level. The response surface plots were shown in Figure 1A–C.

3.4 | Interaction of variables on extraction efficiency of SPL flavonoids

The extraction efficiency of SPL flavonoids, which ranged from 66.61 ± 1.42% to 91.65 ± 3.37% (Table 1), depended on the extraction temperature, extraction time, and ethanol concentration and their interaction (Figure 1A–C). Extraction temperature was the

TABLE 2 Analysis of variance (ANOVA) for response surface model

Source	Sum of squares	*df*	Mean square	*F* Value	*p*-value Prob > *F*	
Model	1.82	9	0.2	36.73	<0.0001	Significant
A-temperature	0.87	1	0.87	158.51	<0.0001	
B-time	0.22	1	0.22	39.32	0.0004	
C-concentration	0.061	1	0.061	10.98	0.0129	
AB	0.12	1	0.12	22.23	0.0022	
AC	4.00E−04	1	4.00E−04	0.073	0.7954	
BC	0.018	1	0.018	3.31	0.1118	
A^2	0.83	1	0.83	150.45	<0.0001	
B^2	0.22	1	0.22	39.11	0.0004	
C^2	0.12	1	0.12	22.41	0.0021	
Residual	0.039	7	5.51E−03			
Lack of fit	0.011	3	3.66E−03	0.53	0.6854	Not significant
Pure error	0.028	4	6.90E−03			
Cor total	1.86	16				
R^2			0.9793			
Adj R^2			0.9526			
Pred R^2			0.8824			

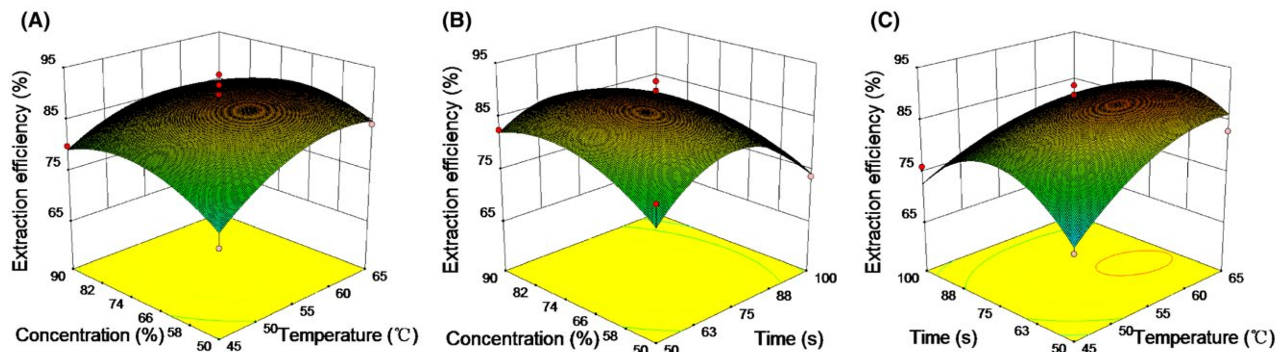

FIGURE 1 Three-dimensional diagrams of extraction temperature, extraction time, and ethanol concentration on the extraction efficiency of SPL flavonoids (A: Interaction of extraction temperature and ethanol concentration on the extraction efficiency of SPL flavonoids; B: Interaction of extraction time and ethanol concentration on the extraction efficiency of SPL flavonoids; C: Interaction of extraction temperature and extraction time on the extraction efficiency of SPL flavonoids)

most important factor (p-value < 0.0001) for the extraction which affected the extraction efficiency of SPL flavonoids effectively. The result showed that for the extraction temperature rising from 45 to 65°C, the extraction efficiency of SPL flavonoids was shown first rising then falling tendency. The increase of extraction temperature could definitely enhance the flavonoids dissolving, but high temperature would also affect the stability of flavonoids and induced them degradating rapidly.

Extraction time was the second critical factor for the extraction. We could see from Figure 1B,C that increased time from 50 to 100 s could enhance the microwave and ultrasonic reacted with the sample and accelerated the solvent and energy penetrating into the matrix, but too much time of severe reaction would definitely destroy the chemical bonds of flavonoids and induced them dissociating (Wong et al., 2017).

Ethanol concentration was also vital for the extraction because it decided the polarity of the whole system. When the ethanol concentration changed from 50% to 90%, the extraction efficiency showed first rising then falling tendency as we saw from Figure 1A,B. Flavonoids were a class of weak polarity organic compounds which could dissolved in high percentage of ethanol easily. If the ethanol concentration was not high enough there were a large number of water-soluble impurities escaped into the solution, and if the ethanol concentration was too high that meant the polarity of the solution was not high enough to dissolve the flavonoids completely.

3.5 | Optimization of extraction condition and method validation

According to the results and discussion, the optimum extraction was required to find the desire condition for maximizing the extraction efficiency of SPL flavonoids, the extraction temperature was 57°C, the extraction time was 76 s, the ethanol concentration was 72%, solid–liquid ratio was 1:40, and extracted 2 times according to the above condition, the maximum extraction efficiency of SPL flavonoids was 91.65 ± 3.37%, the predicted maximum extraction efficiency fitted by the software was 89.17% which correlated quite well with the actual data, demonstrated the model could simulate the reality and the optimum condition was quite valid for this experiment.

3.6 | Purification of SPL flavonoids

The crude solution extracted by optimum parameters then went through liquid–liquid extraction to get petroleum ether phase, ethyl acetate phase, and water phase. The purity of SPL flavonoids in ethanol extract was 16.81 ± 0.76 (%, DW) and in ethyl acetate phase it was rising up to 76.10 ± 3.11 (%, DW).

3.7 | Qualitative and quantitative analysis of SPL flavonoids

Qualitative and quantitative analyses were shown in Figure 2, Supplement Figure 2 and Table 3. There were 11 flavonoids detected in SPL flavonoids which were CA, 4,5-CQA, myricetrin, 3,4,5-CQA, isoquercitrin, astragalin, quercitrin, tiliroside, quercetin, kaempferol, and rhamnetin according to the references and comparing to the retention time of standards (Anastácio & Carvalho, 2013; Ojong et al., 2008; Xi et al., 2015). Astragalin was the highest amount of flavonoids in SPL flavonoids, which was 473.8 ± 7.3 mg/g DW, followed by quercitrin (86.5 ± 0.7 mg/g DW), 4,5-CQA (76.4 ± 0.5 mg/g DW), isoquercitrin (62.4 ± 0.4 mg/g DW), tiliroside (18.8 ± 0.3 mg/g DW), quercetin (12.5 ± 0.2 mg/g DW), 3,4,5-CQA (6.5 ± 0.2 mg/g DW), CA (6.1 ± 0.2 mg/g DW), kaempferol (6.0 ± 0.2 mg/g), myricetrin (5.9 ± 0.1 mg/g DW), and rhamnetin (4.3 ± 0.1 mg/g DW). Ojong et al. (2008) found out that apigenin, kaempferol, luteolin, quercetin, and myricetin existed in SPL usually grown in Southern United States. Anastácio and Carvalho (2013) also took the research on purple SPL flavonoids and luteolin, myricetin, and quercetin were detected. There were some differences between our results which might due to the different cultivar contained different flavonoid monomer, the difference of physiological stage of the plant or cultural practices.

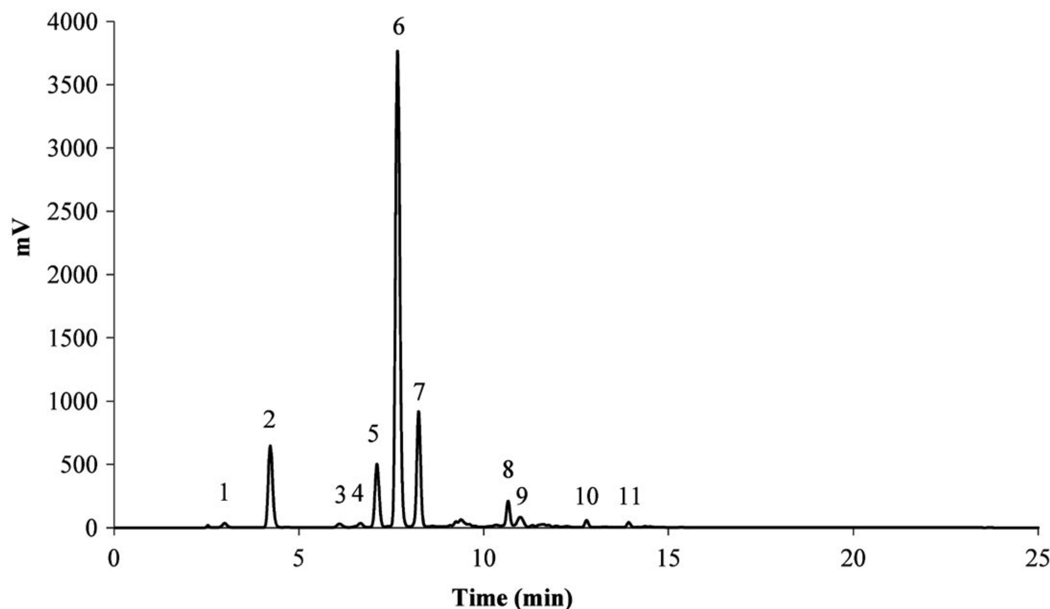

FIGURE 2 The HPLC chromatography of SPL flavonoids. Peak 1: CA, peak 2:4, 5-CQA, peak 3: myricetrin, peak 4:3, 4, 5-CQA, peak 5: isoquercitrin, peak 6: astragalin, peak 7: quercitrin, peak 8: tiliroside, peak 9: quercetin; peak 10: kaempferol, peak 11: rhamnetin

TABLE 3 Qualitative and quantitative analysis of SPL flavonoids by HPLC

No.	Ret. time (min)	Identification	Standard curve	R^2	Peak area	Content (mg/g DW)
1	2.98	CA	y = 114133x−292197	0.9993	323,630 ± 10,611	6.1 ± 0.2g
2	4.198	4,5-CQA	y = 72434x−293019	0.9984	3,611,795 ± 23,637	76.4 ± 0.5c
3	6.099	Myricetrin	y = 29502x−120219	0.9979	347,722 ± 5,894	5.9 ± 0.1g
4	6.662	3,4,5-CQA	y = 74873x−468134	0.9958	321,973 ± 9,907	6.5 ± 0.2g
5	7.095	Isoquercitrin	y = 34321x−101233	0.9996	3,293,152 ± 21,110	62.4 ± 0.4d
6	7.642	Astragalin	y = 40295x−219384	0.9995	25,006,624 ± 385,286	473.8 ± 7.3a
7	8.207	Quercitrin	y = 35135x−132682	0.9991	5,064,650 ± 40,986	86.5 ± 0.7b
8	10.613	Tiliroside	y = 61425x−236252	0.9923	992,268 ± 15,834	18.8 ± 0.3e
9	10.965	Quercetin	y = 33083x−92209	0.9983	664,719 ± 10,636	12.5 ± 0.2f
10	12.735	Kaempferol	y = 44840x−122699	0.9990	316,534 ± 10,551	6.0 ± 0.2g
11	13.879	Rhamnetin	y = 42177x−71175	0.9979	229,180 ± 5,330	4.3 ± 0.1h
Sum						759.2 ± 7.3

Note. Different letters (a–h) mean values are significantly different ($p < 0.05$).

3.8 | Antioxidant activity of SPL flavonoids

Antioxidant activity of SPL flavonoids were evaluated by FRAP and ABTS method. The results were shown in Figure 3A,B. The flavonoids purity of SPL flavonoids, soy isoflavones, ginkgo biloba extract, and propolis flavone were 76.10 ± 3.11a, 59.32 ± 3.20b, 45.77 ± 3.34c, and 56.85 ± 2.78b (%, DW), respectively.

Figure 3A showed the Fe^{3+} scavenging capacities of SPL flavonoids and soy isoflavones, ginkgo biloba extract, and propolis flavone at the concentration of 5, 10, and 20 μg/ml, SPL flavonoids had the significant advantage than the other three positive controls. The Fe^{3+} scavenging capacities of SPL flavonoids was 17.74 ± 1.95%, 37.36 ± 0.98, and 76.22 ± 0.49% at the concentration of 5, 10, and 20 μg/ml, which was approximately 0.31 times and 3.08 times higher than ginkgo biloba extract and propolis flavone at the concentration of 5 μg/ml, 0.18 times and 2.25 times higher than ginkgo biloba extract and propolis flavone at the concentration of 10 μg/ml and 0.23 times and 1.80 times higher than ginkgo biloba extract and propolis flavone at the concentration of 20 μg/ml. Soy isoflavones showed poor activity in this experiment. IC_{50} values of SPL flavonoids, soy isoflavones, ginkgo biloba extract, and propolis flavone were 13.26 ± 0.09a, 143.71 ± 1.33d, 15.99 ± 0.11b, and 34.01 ± 0.68c μg/ml, respectively.

Figure 3**B** showed $ABTS^{\cdot+}$ scavenging capacity at the flavonoids concentration of 5, 10, and 20 μg/ml. The $ABTS^{\cdot+}$ scavenging rate of

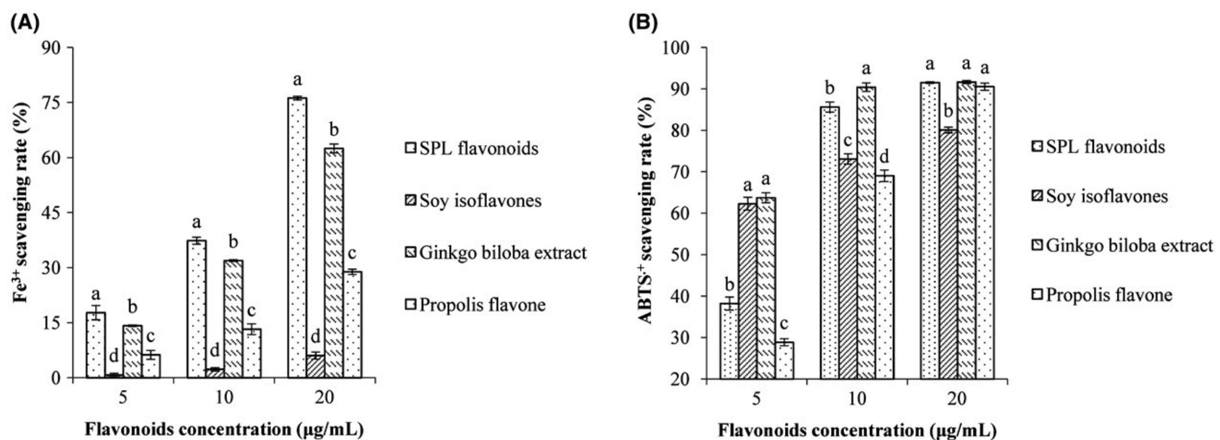

FIGURE 3 Antioxidant activities of SPL flavonoids. (A) Fe^{3+} scavenging capacity. (B) $ABTS^{\cdot+}$ scavenging capacity *Note*. Different letters (a–d) mean values are significantly different ($p < 0.05$).

SPL flavonoids at the concentration of 5 μg/ml was 38.21 ± 1.56%, which was 61.34%, 59.93%, and 132.35% of scavenging capacity comparing to the corresponding concentration of soy isoflavones, ginkgo biloba extract, and propolis flavone. When the concentration reached to 10 μg/ml, the $ABTS^{\cdot+}$ scavenging rate of SPL flavonoids achieved to 85.63 ± 1.22%, which was 17.14% and 24.03% higher than soy isoflavones and propolis flavone and 5.30% a little lower than ginkgo biloba extract. When the concentration reached up to 20μg/mL, the $ABTS^{\cdot+}$ scavenging rate of SPL flavonoids achieved to 91.52 ± 0.17%, which was 14.26% higher than soy isoflavones and had no significant difference between ginkgo biloba extract and propolis flavone. IC_{50} values of SPL flavonoids, soy isoflavones, ginkgo biloba extract, and propolis flavone were 5.41 ± 0.21c, 2.11 ± 0.06a, 3.20 ± 0.09b, and 6.99 ± 0.11d μg/ml, respectively.

The antioxidant activity is related to many factors, including the structure of the flavonoids from different sources, other ingredients in the flavonoid sample (Chen et al., 2017). In addition, it has been reported that daidzin and genistin were the dominant flavonoids in soy isoflavones (Szymczak et al., 2017), quercetin, kaempferol, and isorhamnetin were the dominant flavonoids in ginkgo biloba extract (Sati, Dhyani, Bhatt, & Pandey, 2017), rutin, isorhamnetin, kaempferol, luteolin, naringenin, and quercetin-3-glucoside were the main flavonoids detected in propolis flavone (Andrade et al., 2018), which meant the difference of antioxidant activity among SPL flavonoids, soy isoflavones, ginkgo biloba extract, and propolis flavone might also due to the differences of flavonoids composition.

4 | CONCLUSION

The results showed that the highest extraction efficiency of SPL flavonoids at the optimum condition (extraction temperature was 57°C, extraction time was 76 s, ethanol concentration was 72%, solid–liquid ratio (w/v) was 1:40, and extracted 2 times) was 91.65 ± 3.37%, which was confirmed through the validation experiment 89.17%. The crude extract was selected by petroleum ether and ethyl acetate, the latter phase was collected to obtain the SPL flavonoids with the purity of 76.10 ± 3.11 (%, DW). HPLC analysis results showed that the SPL flavonoids mainly consisted of astragalin, quercetrin, 4, 5-CQA, isoquercitrin, tiliroside, quercetin, 3, 4, 5-CQA, CA, kaempferol, myricetrin, and rhamnetin, which possessed high antioxidant capacity. Meanwhile, UMSE was an economic method to obtain SPL flavonoids which was time-saving and easy to scale up at the pilot test and industrial scale, providing a potential possibility for industrial extraction of flavonoids from SPL, enriching the flavonoids health products market.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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