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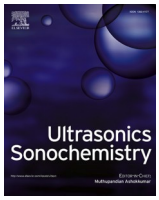

Enzyme-assisted ultrasonic extraction of total flavonoids and extraction polysaccharides in residue from *Abelmoschus manihot* (L)

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ABSTRACT

Abelmoschus manihot (L) is a traditional chinese herb and the present study focused on its comprehensive development and utilization. Enzyme-assisted ultrasonic extraction (EUAE) was investigated for the extraction and qualitative and quantitative analysis of flavonoids from *Abelmoschus manihot* (L) using a combination of ultra-performance liquid chromatography-photodiode array (UPLC-PDA), polysaccharides was extracted from residues and compared with directly extracted from raw materials. The optimal yield of 3.46±0.012 % (w/w) was obtained when the weight ratio of cellulase to pectinase was 1:1, the enzyme concentration was 3 %, the pH was 6.0, the solvent was a mixture of 70 % ethanol (v/v) and 0.1 mol/L NaH2PO4 buffer solution, the ultrasonic power was 500 W, the extraction time was 40 min, and the temperature of the extraction was 50 °C. The individual concentrations of interested flavonoids (rutin, neochlorogenic acid, nochlorogenic acid, lsoquercitrin, quercitrin, gossypin, quercetin) were effectively increased with the using of EUAE, compared with ultrasonic extraction (UE) method. Polysaccharides were extracted from each residue, respectively, the Polysaccharides yield in residue from EUAE was higher than that from UE, and closed to the yield from direct extraction in raw materials. The above results shown that the experimental process had the potential to be environmentall, friendly, straightforward and efficient.

1. Introduction

Plant natural active components has recently attracted more and more attention because of their abundant sources, safety, reliability and remarkable activity. Such as polysaccharides, saponins, flavonoids, resveratrol, polyphenols, curcumin, proteins, and so on [1–3]. Due to their distinct physical properties and biological activities, these particular natural active components have found extensive application in numerous fields including medicine, food, and cosmetics [4–7]. The corresponding extraction and separation are also developing. With people's attention to environmental protection, the extraction of natural bioactive ingredients is seeking green environmental protection methods [8], especially in the cosmetic, food and pharmaceutical industries, increasing request for the natural products of sustainable and environmentally responsible manner [9]. Some plants, herbs and fruits are often extracted with unique active ingredients, there are still a variety of valuable bioactive components in the residues, which are neglected and abandoned, resulting in a large waste of resources [10], and recycling of valuable bioactive components in the residues is very much in line with the requirements of green and sustainable development [11]. Table 1.

Abelmoschus manihot (L), an edible hibiscus in the Mallow family, is a traditional chinese herb. Research has shown that *Abelmoschus manihot* (L) is rich in polysaccharides, flavonoids and other bioactive components, and has antibacterial, anti-inflammatory [12], antioxidant, liver protection [13], heart protection [14] and other biological activities. Traditional extraction methods of total flavonoids in *Abelmoschus manihot* (L) (TFAM) include soxhlet extraction method, heat reflux extraction method, etc [15], but these traditional extraction methods have obvious advantages and disadvantages, such as long time and high

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Table 1
Independent variables of Box-Behnken design.

Variables	Unit	Symbol	levels		
			−1	0	1
Extraction pH		A	5.5	6	6.5
Ethanol concentration	%	B	60	70	80
Extraction temperature	°C	C	45	50	55
Extraction time	min	D	30	40	50

temperature, which lead to the destruction of some flavonoid structures [16]. In recent years, advanced extraction methods such as ultrasound-assisted extraction, microwave-assisted extraction, and enzyme-assisted extraction have also been widely used in the extraction of total flavonoids [17–19]. Compared with traditional methods, these methods are gentler, more efficient, energy-saving and environmentally friendly. In pursuit of higher extraction efficiency, later research has found that by synergistic combination of two or more extraction methods, the advantages of each method are enlarged, the disadvantages are reduced, and the extraction efficiency is improved [20,21]. The polysaccharides of *Abelmoschus manihot* (L) has good antioxidant activity [22] and potential medicinal value [23], and usually after extracting the total flavonoids of *Abelmoschus manihot* (L), the residue is rich in polysaccharides [24], the extracting the residual polysaccharides have a lot of potential value. The present study aimed to improve the resource utilization efficiency of *Abelmoschus manihot* (L). Enzyme-assisted ultrasonic extraction (EUAE) process was established to improve the efficiency of TFAM extraction, the process was optimized using Box-Behnken experimental design and the optimized extraction method was validated and compared with other extraction methods. In addition, polysaccharide was extracted from the corresponding residues and compared with those extracted directly in order to fully utilize the material, protect the environment and comply with green development. To provide some help for its wider and efficient application in food and medical services.

2. Mateial and methods

2.1. Sample preparation

Abelmoschus manihot (L) flower were harvested from Henan Province, China. The flowers were dried in a hot air oven at 55 °C for 24 h. After the buds were ground and sieved for 80 mesh screens, the flower powder needed for the experiment was obtained.

2.2. The content of total flavonoids

The content of total flavonoids was determinated by modified sodium nitrite-aluminum nitrate colorimetry [25]. Briefly, each extract solution (1.0 mL) and 5 % Sodium nitrite (0.6 mL) was absolutely mixed in a 10 mL tube, followed by 10 % aluminum nitrate (0.6 mL), 4 % sodium hydroxide (3 mL), and 70 % ethanol solution to 10 mL. The absorbance value at 510 nm after 15 min of reaction was recorded. Calculation of total flavonoid content based on rutin concentration. The formula was as follows:

$$X\% = \left(\frac{cVN}{1000M}\right) \times 100\%$$

Where N is the multiple of dilution, c is the standard solution concentration of rutin (mg/mL), M the flower powder weight (g), V is the sample solution volume (mL).

2.3. Determination of polysaccharides content

Phenol-sulfuric acid method was used for determination [26]. Briefly, 1.0 mL extract solution, 6 % phenol standard solution and concentrated suulfuric acid (5 mL) was absolutely mixed in a 10 mL. The absorbance value at 490 nm after 30 min of reaction was recorded. The polysaccharides content was computed based on glucose concentration as given below:

$$X\% = \left(\frac{cVN}{M}\right) \times 100\%$$

Where N is the multiple of dilution, c is the standard solution concentration of glucose (mg/mL), M the crude polysaccharides weight (mg), V is the sample solution volume (mL).

2.4. Ultrasonic assisted extraction method

The different ethanol concentrations and 0.2 g *Abelmoschus manihot* (L) flower powder were added to a 10.0 mL tube. Using an ultrasonicator (KQ-500DB 500 W, 40 kHz, Shumei Ultrasonic Instruments Co. Ltd, Kunshan, China) extractio.

2.5. Enzyme-assisted and ultrasound extraction method

After the different concentrations of ethanol (50 %, 60 %, 70 %, 80 %, and 90 %) were prepared in a 100 mL conical flask, Using PBS (phosphate buffered solution) adjusted the pH values of the solution. The different concentrations of ethanol and 0.2 g *Abelmoschus manihot* (L) flower were added to a 10.0 mL tube containing 3 % mixed enzymes (1: 1 = cellulase: pectinase). Using an ultrasonicator extraction.

2.6. Extraction of polysaccharides from residue by water and alcohol precipitation

Abelmoschus manihot (L) flower powder was added ethanol (1: 10 g/mL) and heated to reflux at 90 °C for 4 h to remove pigment molecules and lipid. After heating to reflux, the flower powder was filtered and naturally dry. 2 g treated *Abelmoschus manihot* (L) flower powder and dried residue (after extraction flavonoids) was respectively added to distilled water with a material-liquid ratio of 1:50 and extracted by heating and refluxing, respectively. The filtrate was then centrifuged, concentrated under reduced pressure, deproteinized, and the supernatant was collected. Add 3 times anhydrous ethanol to stand overnight, then centrifuge and collect precipitation and freeze drying to obtain the polysaccharides.

2.7. Single-factor experiment

The effect of extraction temperature, pH, time and ethanol concentration on the extraction efficiency were preliminarily determined by single-factor experiments. During the optimization process, each extraction condition was changed to 5 different points in an interval as follows: time, 10 min, 20 min, 30 min, 40 min, 50 min; ethanol concentration, 50 %, 60 %, 70 %, 80 %, and 90 %; temperature, 35 to 55 ∘C; and pH, 4.5 to 6.5.

2.8. Response surface experimental design

Design-Expert v.8.0 software was used to investigate the effects of four independent variables (pH (A), ethanol concentration (B), temperature (C) and time (D)) on the yield of flavonoids, based on the results obtained from the single-factor experiment. The independent variables were set to 3 levels, and the whole design had 29 experimental points, of which the center point was repeated three times.

2.9. Antioxidant activity analysis

The antioxidant activities of TFAM and polysaccharides in the residue have been studied by ABTS free radical and DPPH free radical

scavenging activity. V_C was used as a positive control. Experiments were carried out using a UV–visible spectrophotometer (UV-2600, Mepida Instrument Co. LTD, Shanghai, China) and repeated 3 times.

2.9.1. DPPH free radical scavenging activity

DPPH free radical scavenging activity experiments were performed using a slightly modified version of previously reported protocols [27]. 2 mL of different concentrations of extracts were mixed with 2 mL of DPPH working solution (0.1 mmol/mL). Thereafter, the mixture of liquid was incubated at room temperature away from light for 30 min, and then the absorbances at 517 nm was measured. Vc (Vitamin C) as a positive control. DPPH clearance was calculated as follow:

$$X/\% = \left(1 - \frac{A_1 - A_2}{A}\right) \times 100\%$$

where A is the negative control (DPPH + ethanol solution), A_1 is the absorbances of the reaction solution (sample + DPPH) and A_2 is the blank control (ethanol solution + sample).

2.9.2. ABTS free radical scavenging activity

Determination of free radical scavenging activity of ABTS by a slightly modified method [28]. 140 mmol/L potassium persulfate was blended with ABTS working solution (7 mmol/L) in the dark at room temperature and then stored overnight. The working solution was diluted with anhydrous ethanol to an absorbance of 0.7±0.02 at 734 nm before use. Subsequently, this working solution (3.5 mL) was blended with the sample (0.5 mL). After 10 min, the absorbance at 734 nm was recorded. Vc as a positive control. ABTS clearance were calculated as given below:

$$X/\% = \left(1 - \frac{A_1 - A_2}{A}\right) \times 100\%$$

where A_1 is the absorbances of the sample reaction solution (sample + ABTS), A_2 is the blank control (sample + ethanol solution) and A is the negative control (ABTS + ethanol solution).

2.10. IR analysis of residual polysaccharides

10 mg of polysaccharides was added to 500 mg of dried potassium bromide, thoroughly ground and pressed into a transparent sheet and placed in a FTIR spectrometer for spectral measurement in the frequency range: 4000–500 cm^{-1}.

2.11. HPLC analysis of flavonoids

Quantitative analysis of 7 flavonoids in *Abelmoschus manihot* (L) extracts by high performance liquid chromatography (HPLC) using Agilent 1260 system (USA).

Detection conditions: Eclipse Plus C18 column (4.6 mm × 250 mm, 5 μm), Agilent 1260 liquid chromatograph (with DAD detector). The mobile phase B and A were acetonitrile and 1 % formic acid aqueous solution, and the gradient elution was performed by varying the ratio of acetonitrile from 12%→22%→24%→32%→33%→12% at the time points of 0 min→8 min→10 min→25 min→28 min→30 min. The injection volume was 5 μL, the column temperature was 20 °C, the detection wavelength was 360 nm, and the flow rate was 1.0 mL/min. The separation chromatogram of flavonoids detected at 360 nm after comparison with the corresponding standards is shown in Fig. 3.

2.12. Analysis of monosaccharide composition

The monosaccharide composition of polysaccharides was determined according to a modified method [29]. Lyophilized polysaccharides powder (5 mg) and 2 mol/L trifluoroacetic acid (5 mL) was mixed in ampoules with nitrogen to seal and hydrolyzed at 110 °C for 240 min. After cooling, hydrolysate (2 mL) and methanol (1 mL) were intensive mixed, and then blow-dry with nitrogen at 50 °C was repeated twice. Add 0.2 mol/L NaOH (2 mL) solution to the blow-dried residue and dissolve it fully.

Monosaccharide derivatization: 1 mL the mixed monosaccharide standard solution and the hydrolysis solution of polysaccharides (1 mL) added 1 mL of PMP (1-pheny-3-methyl-5-pyrazolone, 0.5 mol/L) and heated at 70 °C for 2.5 h in a water bath, respectively. After cooling to room temperature, added 0.2 mol/L HCl (1 mL) to mixed well, and then added equal volume chloroform extraction, discard the chloroform layer, collected the hydrolytic layer, and repeated extraction three times. The aqueous phase was filtered through 0.45 μm microporous membrane and the filtrate detected by HPLC.

Detection conditions: Agilent 1260 liquid chromatograph (with DAD detector), Curosil-PFP column (4.6 mm × 250 mm, 5 μm). The mobile phase A was sodium phosphate buffer (100 mmol/L, pH 6.4), and the mobile phase B was acetonitrile, and the gradient elution was performed by varying the ratio of acetonitrile from 15%→28%→40%→15% at the time points of 0 min→30 min→35 min→40 min. The flow rate was 1.0 mL/min, the column temperature was 30 °C, the injection volume was 5 μL, and the detection wavelength was 250 nm.

2.13. Statistical analysis

The analysis result of the mean values was compared using 95 % confidence interval tukey test and one-way variance analysis in the IBM SPSS statistical 25.0 software. Regression analysis and response surface optimization were performed using design-expert 8.0.6 software. All measurements were repeated three times and expressed as mean ± standard deviation.

3. Results and discussion

3.1. Analysis of one-way experiment

3.1.1. Effect of enzymatic pH on TAFM yield

Fig. 1(a) shown that the yield of flavonoids extracted from *Abelmoschus manihot (L)* increased significantly whan enzymatic pH values increased from 4.5 to 6, whereas it decreased significantly at pH >6. This might be due to the fact that at different pH values, the spatial structure of the enzyme might change, thus altering the enzyme's conformation and enzyme activity, while the pH value of about 6, the enzyme's activity was the best, which could effectively hydrolyze the cell wall, accelerating the destruction of the structure of the cell wall, so that the flavonoids in the cells can be extracted fully.

3.1.2. Effect of ethanol concentration on TAFM yield

As shown in Fig. 1(b), when the ethanol concentration increased from 50 to70%, the yield of flavonoids extracted from *Abelmoschus manihot* (L) increased gradually. However, the yield gradually decreased when the ethanol concentration was further increased. This indicated that the yield could be increased when the system contains enough water. This might be due to the fact that some flavonoids had higher solubility in polar solvents and the presence of sufficient amount of water in the system to increase the polarity of the solvent improved to promote the release of flavonoids. Relatively too much water might also result in low flavonoid extraction. Optimal conditions for flavonoid extraction with 70 % ethanol were determined.

3.1.3. Effect of extraction temperature on TAFM yield

The effect of extraction temperature on TAFM yield was shown in Fig. 1 (c). When the extraction temperature was 35–45 °C, the yield of TAFM was positively correlated with the extraction temperature, while the yield was basically stable when the extraction time was increased from 45 °C to 50 °C, and the yield of TAFM reached the maximum value at 50 °C. As is well-known, all enzymes have the maximum hydrolytic

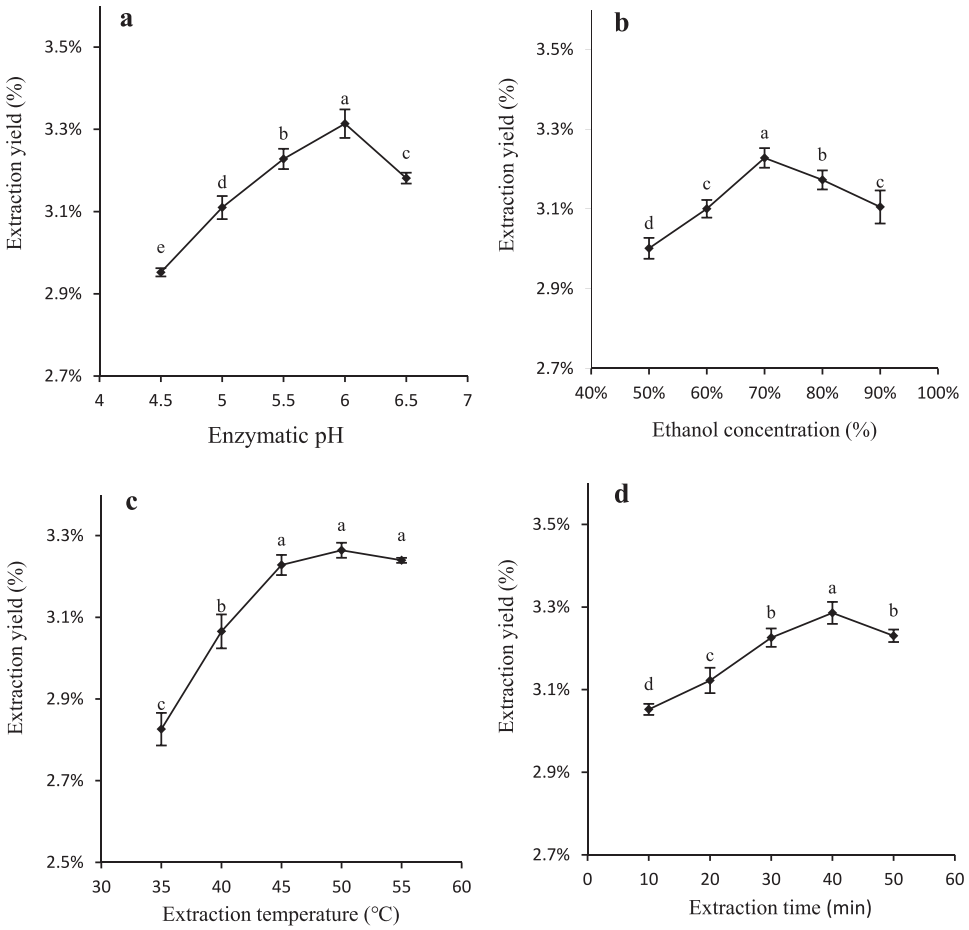

Fig. 1. Effect of different enzymatic pH (a), ethanol concentration (b), extraction temperature (c), extraction time (d) on the extraction yield. According to ANOVA, the different letters (a, b, c, d) mean the difference is not significant and the same letter are not significantly different at ($P < 0.05$).

activity at their optimum temperature, and if the enzyme is at a temperature other than the optimal temperature, the activity of enzyme will be greatly reduced [30]. So 50 °C was the optimum temperature for mixing enzymes in this study.

3.1.4. Effect of extraction time on TAFM yield

The results were shown in Fig. 1(d), when the extraction time was 40 min, the yield of TAFM obtained a maximum value. The extraction yield of flavonoids decreased when the extraction time exceeded 40 min. A possible reason for this situation was that the extraction time was too long, in the limited solvent, the dissolution of other substances inhibited the dissolution of flavonoids. The optimal extraction time for flavonoid extraction with 40 min were determined.

3.2. Response surface experimental design analysis

3.2.1. Model of BBD-RSM

The extraction conditions were further optimized using a

Table 2
ANOVA for reponse surface quadratic model.

Parameter	Sum of Squares	df	Mean Squares	F-Value	P-value	Significant
Model	0.66	14	0.047	50.29	< 0.0001	***
A-PH	4.80E-03	1	4.80E-03	5.1	0.0404	*
B-Ethanol concentration	0.024	1	0.024	25.83	0.0002	***
C-Temperature	0.011	1	0.011	11.48	0.0044	**
D-Time	3.33E-05	1	3.33E-05	0.035	0.8534	
AB	6.25E-04	1	6.25E-04	0.66	0.4287	
AC	6.40E-03	1	6.40E-03	6.8	0.0206	*
AD	2.50E-05	1	2.50E-05	0.027	0.8728	
BC	1.00E-04	1	1.00E-04	0.11	0.7492	
BD	2.50E-05	1	2.50E-05	0.027	0.8728	
CD	9.00E-04	1	9.00E-04	0.96	0.3446	
A^2	0.55	1	0.55	579.56	< 0.0001	***
B^2	0.15	1	0.15	160.18	< 0.0001	***
C^2	0.062	1	0.062	65.44	< 0.0001	***
D^2	0.039	1	0.039	41.33	< 0.0001	***
Residual	0.013	14	9.41E-04			
Lack of Fit	9.65E-03	10	9.65E-04	1.1	0.5065	

$R^2 = 0.9805$, $R^2_{Adj} = 0.9610$, C.V.%=0.9. [a] ***($p < 0.001$), **($p < 0.01$), *($p < 0.05$).

combination of BBD and RSM, and the results of 29 experiments were illustrated in Table 2. The experimental data were analyzed by multiple regression, and the regression equations were as follows:

$$Y = 3.40 + 0.020A + 0.045B - 0.030C - 0.013AB - 0.040AC + 0.015CD - 0.29A^2 - 0.15B^2 - 0.097C^2 - 0.077D^2 \quad D2$$

where Y, A, B, C and D are the TFAM yield (%), pH, ethanol concentration (%), extraction temperature (°C) and extraction time (min).

The ANOVA result of the regression model was shown in Table 3. The p-value (<0.0001) and F-value (50.29) of the model were highly remarkable. The coefficient of determination (R^2) was 0.9805 manifesting a good model fit. The p-value for underfitting was 0.5065 ($p > 0.05$), which confirmed that underfitting was indistinctive relative to the pure error, which was more indicative of the dependability of the models. From the validity analysis of the variables, the independent variables (A, B and C), the term of interaction (AC) and all the quadratic terms (A^2, B^2, C^2, D^2) ($p < 0.05$) had a remarkable effect on the extraction yield of TFAM. Whereas, other terms coefficients (D, AB, AD, BC and CD) had insignificant ($p > 0.05$) effects. All the results effectively verified that the regression model had sufficient exactitude for TFAM extraction.

3.3. Comparisons of diffierent TFAM extraction methods

Comparison of TFAM extraction rates of different methods was shown in Table 4. It could be found that the highest extraction rate of flavonoids extracted by the EURE method with an extraction rate of 3.46 ±0.012 %, which was 1.13 times higher than those of UE (3.06±0.006 %). In addition, the results of Fig. 2 (2) shown that EURE had higher content of seven flavonoid compounds than UE. This might because the enzyme-assisted ultrasonic extraction method could combine the strong cavitation effect, perturbation effect, high acceleration, fragmentation and agitation effects produced by ultrasonic radiation pressure [31] with the ability of enzymes to degrade or disrupt the cell wall, which could accelerate the disruption of the plant cell wall and the dissolution of solutes in the cells [32]. And the synergistic action of enzymes and ultrasound increased the extraction rate. Greatly shortened the extraction time and resultful increased the extraction efficiency [33]. The results showed that EUAE is an efficient and environmentally friendly method to extract TFAM.

Table 3
Experimental design and results of response surface analysis.

Run	pH (A)	Ethanol concentration (B)	Temperature (C)	Time (D)	Response value (yield, %)
1	6	70	45	30	3.26±0.006
2	6.5	60	50	40	2.93±0.045
3	6.5	70	45	40	3.16±0.008
4	6	70	50	40	3.44±0.008
5	6	80	50	50	3.21±0.014
6	6	70	55	30	3.19±0.002
7	6	60	45	40	3.11±0.007
8	5.5	60	50	40	2.90±0.051
9	6	70	50	40	3.43±0.006
10	6.5	70	50	50	3.07±0.042
11	5.5	70	50	30	3.01±0.035
12	6	60	50	30	3.14±0.004
13	6	80	55	40	3.19±0.004
14	6	80	45	40	3.22±0.008
15	6	70	55	50	3.22±0.012
16	5.5	70	55	40	2.96±0.027
17	6	60	50	50	3.13±0.003
18	6	70	50	40	3.39±0.009
19	5.5	70	50	50	3.03±0.033
20	6	70	45	50	3.23±0.010
21	5.5	80	50	40	3.01±0.031
22	5.5	70	45	40	3.00±0.042
23	6.5	70	55	40	2.96±0.043
24	6.5	70	50	30	3.04±0.036
25	6	70	50	40	3.39±0.006
26	6	80	50	30	3.23±0.002
27	6	60	55	40	3.10±0.004
28	6	70	50	40	3.37±0.008
29	6.5	80	50	40	2.99±0.034

3.4. HPLC analysis of TFAM by different extraction methods

The composition of TFAM was analyzed by HPLC. Qualitative and quantitative analysis were performed on seven major flavonoids. As shown in F ig. 7, seven flavonoids in EUAE (nochlorogenic acid: 43.7 ±2.9 mg/L, neochlorogenic acid: 45.8±5.0 mg/L, rutin: 101.9±9.3 mg/L, lsoquercitrin: 237.4±19.7 mg/L, quercitrin: 545.3±15.4 mg/L, gossypin: 148.6±6.0 mg/L and quercetin: 74.4±5.3 mg/L) were significantly higher than UE (nochlorogenic acid: 28.2±1.0 mg/L, neochlorogenic acid: 28.4±1.8 mg/L, rutin: 78.8±0.7 mg/L, lsoquercitrin: 205.7±13.2 mg/L, quercitrin: 452.4±11.8 mg/L, gossypin: 120.8 ±4.6 mg/L and quercetin: 62.0±5.2 mg/L). It could be seen the compositions of TFAM in different extraction methods exhibits significant difference, which also indicated that different extraction methods will have a certain effect on the product.

3.5. Comparisons of polysaccharides from diffierent residue

Extract polysaccharides from the residue after extracting flavonoids, makes full use of plant resources, green environmental protection, and greatly protects the ecological environment. As shown in Table 4, the extraction yield of polysaccharides directly extracted from the material was a little higher than that from the residue. The extraction yield was 17.4±0.21, which was 1.43 times of UE and 1.22 times of EURE. This might be due to the extraction of polysaccharides from the residue, eliminating the first step of conventional polysaccharides extraction to remove pigment and lipid molecules, the residue after the extraction of flavonoids had more oil residues, resulting in a slightly lower polysaccharides extraction yield, which could explain why EUAE had a higher polysaccharides extraction yield than UE. In short, from the perspective of environmental protection and the difference of polysaccharides extraction rate was not large, residue reextraction of polysaccharides, make full use of plant resources, greatly protect the ecological environment, was an environmentally friendly and efficient method.

3.6. FTIR spectroscopy of polysaccharides

FT-IR spectroscopy is an efficient preliminary qualitative analysis of organic functional group, and the basic functional groups of extraction polysaccharides of different methods were analyzed by infrared spectroscopy. As shown in Fig. 3 (2), a strong and wide absorption peak appeared at about 3300 cm^{-1}, which could be judged to be the O-H stretching vibration caused by the hydrogen bond in its molecules, while the absorption peakes at 2900–2950 cm^{-1} indicated the existence of C-H stretching vibration, and both absorption peaks were the main characteristic absorption peaks of polysaccharides [34]. A strong absorption peak appeared at about 1600 cm^{-1}, suggesting that there was an asymmetric C = O stretching vibration of –COOH [35], and a weak absorption peak at about 1400 cm^{-1} reflected the C-O stretching vibration of –COOH, indicating that it contains uronic acid [36]. The absorption peaks at 1000–1200 cm^{-1} indicated the presence of C-O-C and C-O-H stretching vibrations of pyranose rings [37]. The above results showed that polysaccharides extracted from different residues and raw materials have typical polysaccharide absorption peaks, and whether or not the polysaccharides were extracted from raw materials or different residues had indistinctive influence on the type of glycosidic bonding and sugar rings of the polysaccharides.

Table 4
Comparison of polysaccharides and flavonoids extracted by different extraction methods.

Methods	Ethanol content (%)	Soild-liquid ratio (g/mL)	Time (min)	pH	Temperature (°C)	Flavonoids extraction yield (%)	Polysaccharides extraction yield (%)	Polysaccharides content (%)
EUAE	70	1:25	40	6	50	3.46±0.012[a]	14.3±0.37[b]	58.2±0.74
UE	70	1:25	50	–	50	3.06±0.006[b]	12.2±0.36[c]	58.4±0.16
DE	–	1:50	180	–	100	–	17.4±0.21[a]	58.1±0.42

Labeling: different letters (a, b, c) indicate significance of differences between the same columns.

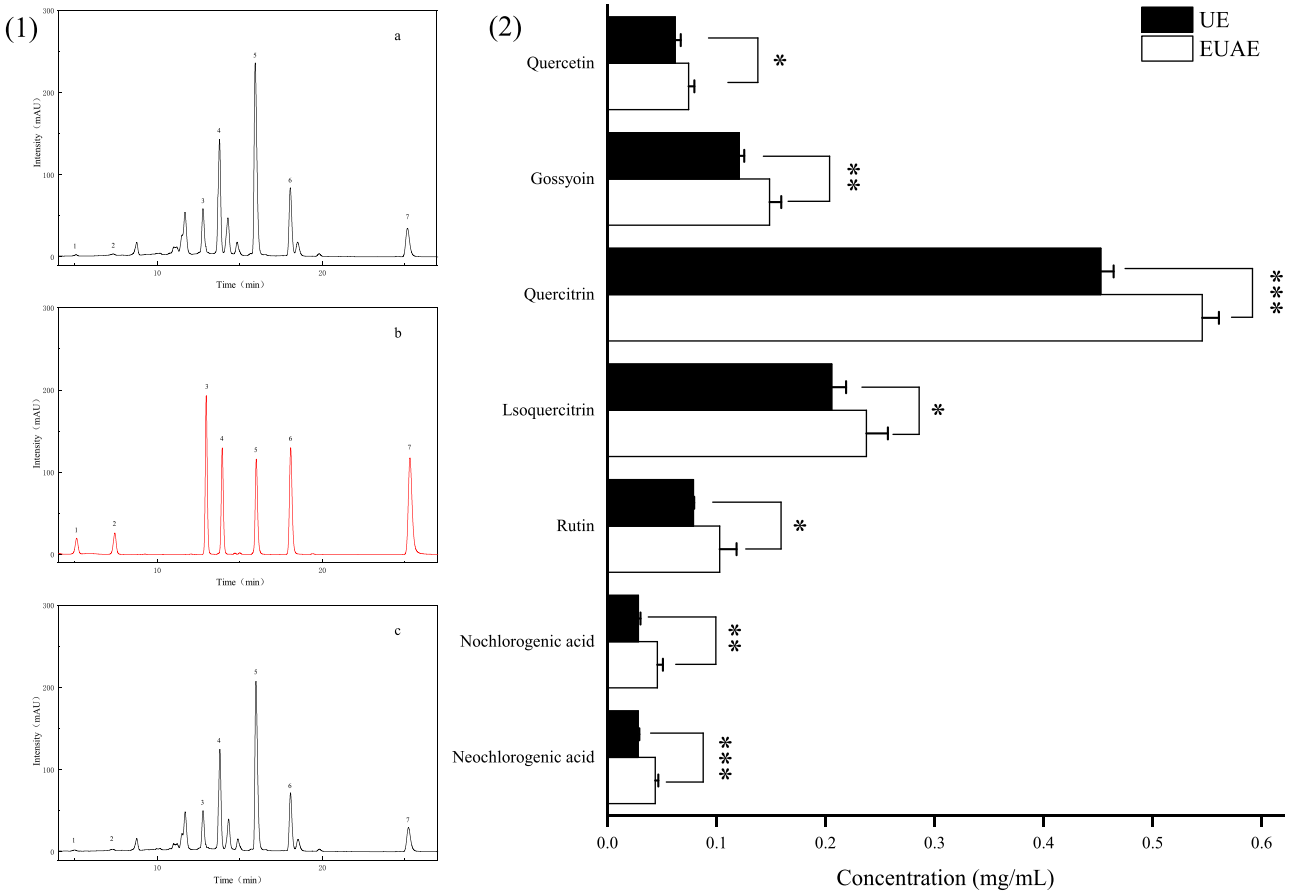

Fig. 2. Analysis on the difference of seven flavonoids in UE and EUAE. (1a) HPLC analysis of the flavonoids extracted by EUAE, (1b) HPLC analysis of mixed standards, (1c) HPLC analysis of the flavonoids extracted by UE. 1: Neochlorogenic acid, 2: Nochlorogenic acid, 3: Rutin, 4: Lsoquercitrin, 5: Quercitrin, 6: Gossypin, 7: Quercetin. (2) Significant difference analysis. ***($p < 0.001$), **($p < 0.01$), *($p < 0.05$).

3.7. Composition analysis of monosaccharides

Research has shown that the monosaccharide component of polysaccharides does not alter observably depending on the extraction method, only the content is affected [38]. It showed that the polysaccharides of the same species extracted by the same extraction method had little effect on the monosaccharide composition of their polysaccharides. Therefore, the polysaccharides extracted from the raw materials were selected and analyzed for the compositional components of the monosaccharides in the polysaccharides by HPLC as demonstrated in Fig. 3 (1). *Abelmoschus manihot* (L) polysaccharides consisted of rhamnose, glucose, galactose, arabinose, galacturonic acid and mannose, and its molar ratio is 0.19: 1: 2.52: 2.33: 3.96: 0.02.

3.8. Antioxidant activity of analysis

Oxidative stress, caused by reactive oxygen species and free radicals, is extremely harmful to human health and can lead to various diseases such as cardiovascular disease, liver damage, cytotoxicity, and aging. However, some studies have shown that antioxidants can mitigate the damage caused by oxidative stress and the scavenging activities of DPPH• radical and $ABTS^+$ radical are widely used to evaluate antioxidant activities [39].

3.8.1. Analysis antioxidant activity of TFAM analysis

The effect of the $ABTS^+$ free radical and DPPH• free radical clearance yields of TFAM were demonstrated in Fig. 4(A and B). It could be shown that both the total flavonoid by different extraction methods shown significant antiradical activities, and the scavening rates increased with the total flavonoids concentration. The Fig. 4(A) shown that the clearance activity of $ABTS^+$ radical of flavonoids extracted by different extraction methods. In the range of 1–2.5 mg/mL, the $ABTS^+$ free radical clearance yields of the total flavonoids extracted by the two extraction methods increased with increasing concentration of the total flavonoids. The $ABTS^+$ radical cation scavenging yields of TFAM extracted by the two extraction methods was about 95–98 % when the TFAM concentration was 2.5 mg/mL. The DPPH• clearance activity of the total flavonoids extracted by the two extraction methods was shown in the Fig. 4

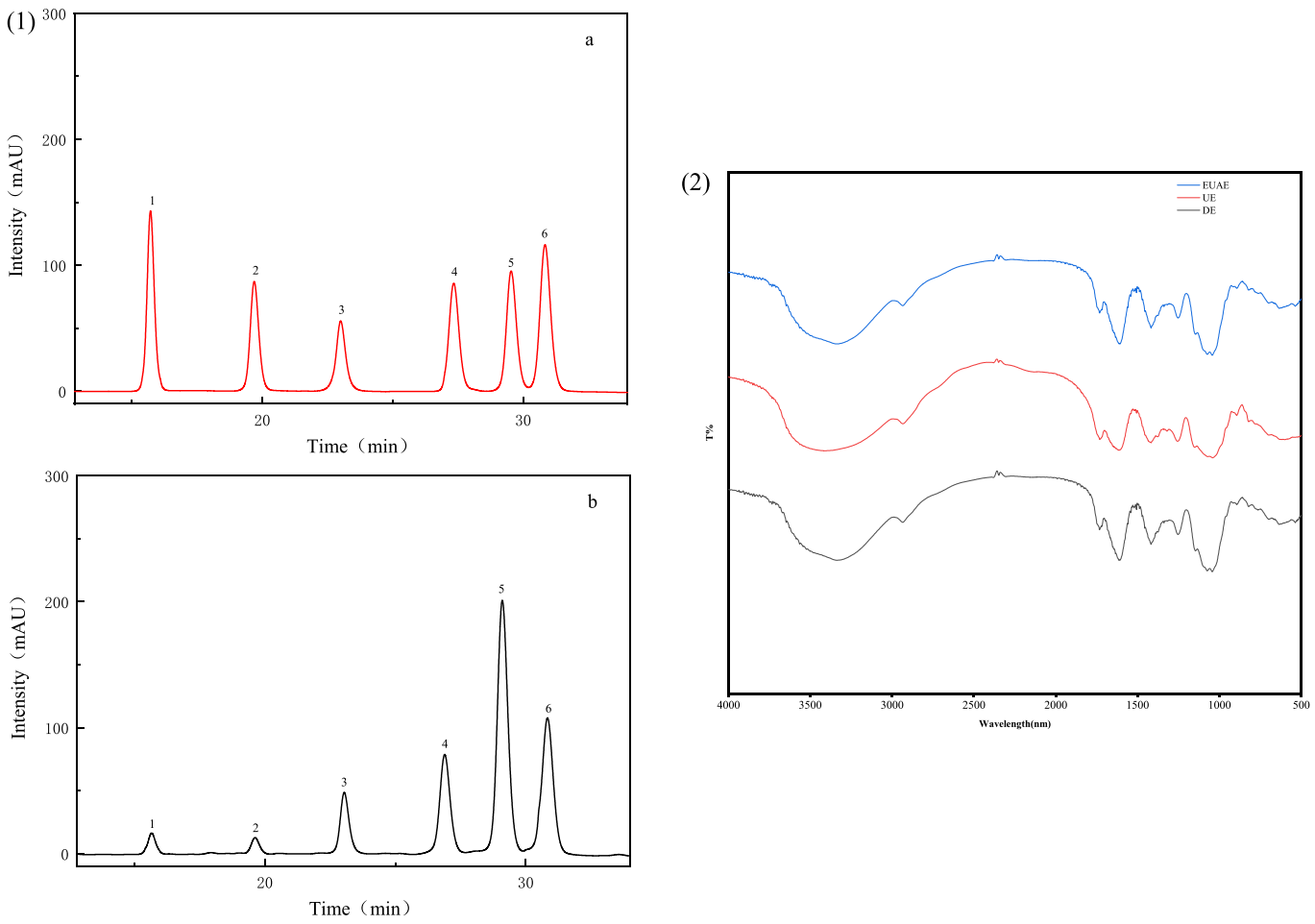

Fig. 3. FTIR spectra of *Abelmoschus manihot L* polysaccharides extracted and HPLC of standard monosaccharides and polysaccharides derivatives. (1a) HPLC analysis of mixed standard monosaccharides, (1b) HPLC analysis of *Abelmoschus manihot (L)* polysaccharides derivatives. 1: D-mannose, 2: rhamnose, 3: galacturonic acid, 4: D-glucose, 5: D-galactose, 6: DL-arabinose. (2) FTIR analysis.

(B). The DPPH• radical clearance yields of flavonoid extracted by UE and EUAE increased with increasing concentration of flavonoid in the range of 0–0.25 mg/mL in a significant dose-dependent manner. When the total flavonoids concentration was 0.25 mg/ml, the DPPH• radical clearance rate of total flavonoids extracted by different extraction methods was close to that under the same concentration of Vc. Compared with the total flavonoids by UE, the total flavonoids by EUAE have higher scavenging rates of $ABTS^+$ radical and DPPH• radical. This might because the synergistic action of ultrasound and mixed enzymes to advance extraction yeild of polyphenols, this was consistent with the HPLC analysis of total flavonoids by different extraction methods.

3.8.2. *Analysis antioxidant activity of polysaccharides*

As shown in Fig. 4(C and D), it was obvious that at the concentrations of 0.3–7 mg/mL ($ABTS^+$) and 0.2–2 mg/mL (DPPH•), the $ABTS^+$ radical and DPPH• free radical clearance yields of the three polysaccharides was dose dependent manner. The $ABTS^+$ radical cation scavenging rates of the three polysaccharides was essentially flat in the range of 7–10 mg/mL, and the $ABTS^+$ radical clearance rates of the three polysaccharides approached those of Vc at 7 mg/mL. The Fig. 4(D) shown the DPPH• radical clearance rate of the three polysaccharides was about 85–87 % at a concentration of 2–3 mg/mL. According to ABTS + and DPPH• free radical scavenging activity assays, the polysaccharides of Abelmoschus manihot (L) showed good free radical scavenging activity. In fact, most natural polysaccharide compounds possess antioxidant activity, which is related to their electron- or hydrogen-donating capacities [40]. And the antioxidant capacity of polysaccharides can be affected by factors such as their glyoxylate content and monosaccharide composition. Under acidic conditions, the ketone or aldehyde groups (electrophilic groups) of polysaccharides contribute to the release of hydrogen from the O-H single bond, and thus polysaccharides with a high content of galacturonic acid have a strong antioxidant capacity [41,42]. Related studies have shown that the higher the content of rhamnose, galactose and arabinose in polysaccharides, the stronger their antioxidant activity [43]. The high content of galacturonic acid, rhamnose, galactose and arabinose in Abelmoschus manihot (L) polysaccharides might contribute to the good antioxidant properties of the polysaccharides.

All of two antioxident activity shown that TFAM and the polysaccharides of *Abelmoschus manihot* (L) had the free radicals scavenging potential antioxidant power. Therefore, TFAM and the polysaccharides of *Abelmoschus manihot* (L) would be good sources of natural antioxiodants for application in the functional food, pharmaceutical and cosmetics industries. Furthermore, compared with the other extrction methods, the antioxidant activity of the total flavonoids by EUAE was stronger. The results showed that EUAE was a good extraction method for TFAM extraction.

4. Conclusion

In this work, the flavonoid of *Abelmoschus manihot* (L) was extracted by enzyme-assisted ultrasound extraction method, and response surface design was used to optimize the main extraction conditions based on the results of single factor experimen. At ultrasonic temperature of 50°C, ultrasonic time of 40 min, 70 % ethanol content, pH of 6, the optimal

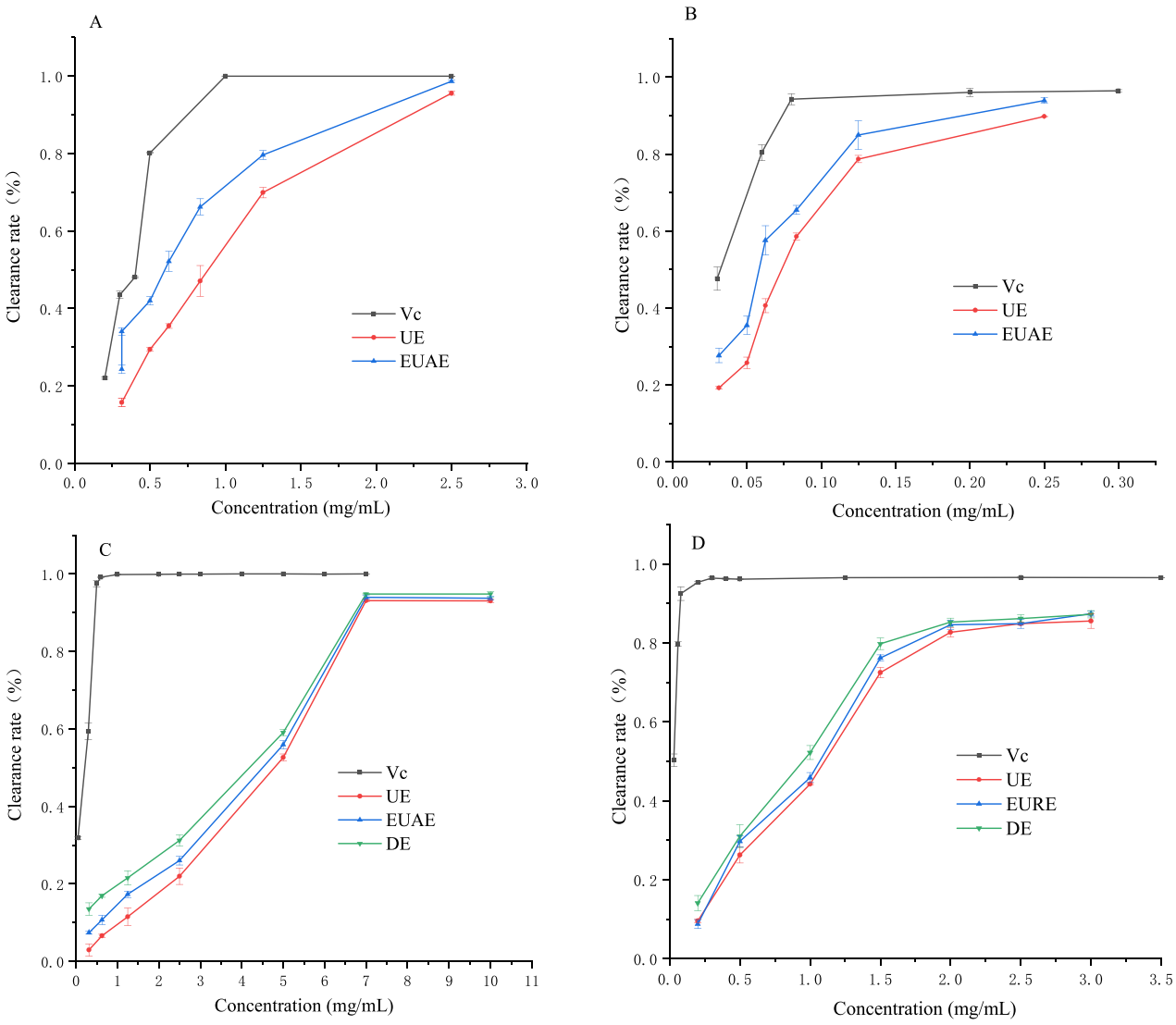

Fig. 4. *In vitro* antioxidant activities of total flavonoids and polysaccharides from *Abelmoschus manihot L.* (A) Scavenging ability of total flavonoids on ABTS + free radical, (B) scavenging ability of total flavonoids on DPPH• free radical, (C) scavenging ability of polysaccharides on $ABTS^{+}$ free radical, (D) scavenging ability of polysaccharides on DPPH• free radical.

yield of flavonoid was 3.46±0.012. Compared with ultrasonic extraction, the extraction rate of flavonoids was significantly increased. The 7 flavonoids content in the extracts was also significantly increased by HPLC. In order to fully utilize plant resources, polysaccharides were extracted from waste residue and compared with those extracted from raw materials. The activity and structure of polysaccharides were analyzed by liquid chromatography and infrared spectroscopy. The results shown that it consisted of glucose, galactose, mannose, rhamnose, arabinose and galacturonic acid, with pyranose cyclofunctional group. The above results shown that the experimental process had the potential of green, simple and efficient utilization of *Abelmoschus manihot* (L).

CRediT authorship contribution statement

Qiming Chu: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis. **Shengnan Xie:** Writing – original draft. **Hongling Wei:** Investigation. **Xuchen Tian:** Data curation. **Zhonghua Tang:** Resources. **Dewen Li:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Ying Liu:** Supervision, Resources, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2024.106815.

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