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Study on the Ultrasonic Extraction Technology of Total Flavonoids in Alfalfa

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Abstract **Original Research Article**

The best extraction process of total flavonoids from salinity tolerant alfalfa in Yellow River Delta region was studied. In this study, ultrasonic-assisted ethanol extraction method was used to design orthogonal experiments based on one-factor experiments, and then the optimal conditions for total flavonoids extraction were determined by analysis of variance. The influence of ultrasonic power, liquid-solid ratio, ethanol concentration, extraction time and extraction temperature on the extraction rate of total flavonoids in alfalfa were explored. The experimental results show that the optimal conditions for extraction are temperature is 60℃, liquid-solid ratio is 50:1 (mL/g), ultrasound time is 60 min, and ethanol concentration is 70%. This experiment can provide some theoretical basis for the comprehensive utilization of alfalfa total flavonoids, and also provide a reference direction for the extraction of total flavonoids, and finally improve the economic benefits of alfalfa planting industry.

Keywords: Alfalfa, Total Flavonoids, Ultrasound-Assisted Extraction, Method, Orthogonal Experiment

INTRODUCTION

Alfalfa (Alfalfa), as a perennial herbaceous plant of the legume family, Rosaceae, lives in areas with abundant sunlight and moist soil. Alfalfa has a deep root system with a well-developed primary root and abundant lateral roots, which facilitates the absorption of water and nutrients from the soil; the stems are erect with numerous branches; the alfalfa's leaf blades consist of three ovate leaflets with serrated edges, long-stalked petals, and a butterfly-shaped corolla that is purple or blue. Alfalfa has a well-developed root system, making it highly drought-tolerant and able to grow well even in areas with low precipitation. Developed alfalfa roots can produce rhizobacteria, and alfalfa is able to convert atmospheric nitrogen into ammonia-nitrogen in the soil through the nitrogen fixation of the root system, which increases the fertility of the soil[1].In addition, alfalfa is grown to improve soil structure, which helps to conserve water and improve the environment. Alfalfa contains many valuable nutrients, including essential amino acids, minerals, vitamins, and dietary fiber, and alfalfa has unique properties as an antioxidant, making it useful in limiting a number of diseases, including conditions such as diabetes mellitus, atherosclerosis, and hypercholesterolemia [2].

Flavonoids are a class of organic compounds found mainly in plants and their products that have high nutritional and medicinal value. Many flavonoids have antioxidant activity, which can scavenge free radicals in the body and reduce oxidative stress damage, helping to prevent chronic diseases such as cardiovascular disease and cancer. Flavonoids also have anti-inflammatory and antimicrobial activities, and play a role in the prevention of atherosclerosis and the value-added of cancer cells, as well as anti-aging and antioxidant [3-4], and in recent years have become a hotspot in the study of natural antioxidants. Studies have shown that there are abundant flavonoid resources in alfalfa, which can be used as raw materials for extracting flavonoids and play an important medical value, for example, the total flavonoids in alfalfa have a strong inhibitory effect on experimental nephropathy with the involvement of down-regulation of anti-inflammatory genes [5].

There are many methods for extracting total flavonoids, such as solvent extraction, Soxhlet extraction, heated reflux extraction, and supercritical fluid extraction. One of the other commonly used methods is the microwave-assisted extraction method [6], which utilizes microwave penetrating heating to heat the material in three dimensions, resulting in energy differences between the various parts of the substance and sufficient energy to separate from the substance; The second is the ultrasonic-assisted extraction method [7], which utilizes the shocking effect of ultrasound to rupture the substance and thus extract the active ingredients. Although the traditional organic solvent extraction method is cheap in terms of extraction cost, the extraction rate is not high enough to reach the expected vision; supercritical fluid extraction method has high extraction efficiency and no solvent residue, but the equipment cost is high. Ultrasonic extraction method has been applied to flavonoid extraction due to its easy-to-achieve extraction conditions and short time-consumption, while supplemented with ethanol extraction can achieve better results [8]. Therefore, this paper will combine ultrasonic and solvent extraction technology to extract flavonoids, and apply orthogonal method to optimize the extraction conditions, in order to find a lower cost, high extraction rate, with high economic benefits of the extraction technology for the comprehensive utilization of alfalfa and flavonoids extraction provides a theoretical reference.

In this experiment, the effects of extraction time, liquid-solid ratio, ultrasonic power, ethanol concentration, and extraction temperature on the yield of flavonoids were explored using one-way experiments with reference to the studies on the extraction of plant flavonoids, and then combined with an orthogonal test to determine the most suitable conditions for the extraction of total flavonoids from alfalfa, with a view to providing a basis for the further development of alfalfa.

1. MATERIALS AND METHODS

1.1 Experimental Materials

1.1.1 Experimental Raw Materials

Collection of salinity-tolerant alfalfa in the Yellow River Delta:

- Place: Yellow River Delta Region, Shandong Aviation Academy (37°23′1 “N, 117°58′57” E)
- Time: March 26, 2024, 16:00 hrs.
- Weather: Cloudy, 17°C

1.1.2 Experimental Equipment

Equipment	Manufacturers
Infrared drying oven	Shanghai Jingmai Instrument and equipment Co., LTD
Refrigerator	Shandong Hisense Company
JJ-2 tissue masher	Shanghai Zuule Instrument Co., LTD
ES520 Precision Electronic Balance	Shanghai Jinghai Instrument Co., LTD
T6 ultraviolet spectrophotometer	Beijing Pujie General Instrument Co., LTD
ST 16R High-speed Freezing Centrifuge	Thermo Fisher Scientific China Co., Ltd
KQ-A1000DE CNC ultrasonic cleaner	Kunshan Ultrasound Instrument Co., LTD

1.1.3 Experimental Instruments

Tray, scissors, sieve (pore size 60 mesh), volumetric flask (100 mL), centrifuge tube (50 mL), beaker, volumetric cylinder, pipette gun (maximum range 1000 μL).

1.1.2 Experimental reagents

Reagents	Manufacturers
ethanol	Yantai Far East Fine Chemical Co., Ltd
Sodium nitrite	Tianjin Fuchen Chemical Reagent Factory
Aluminum nitrate	Tianjin Fuchen Chemical Reagent Factory
Sodium hydroxide	Tianjin Fuchen Chemical Reagent Factory
Rutin Standard	Hefei Pomomei Biotechnology Co., LTD

1.2 Experimental Methodology

1.2.1 Sample Handling

The collected alfalfa grass was sifted to remove impurities, laid flat on a tray lined with newspaper for uniform heating, placed in an infrared drying oven for drying, and the temperature was set at 45°C. After the drying operation, the alfalfa grass was pulverized so that it was processed into a powder, passed through a 60-purpose mesh sieve, and was placed in a self-sealing bag, and then preserved for spare use in a refrigerator.

1.2.2 Preparation of a Standard Curve for Rutin

Firstly, a 0.2 mg/mL standard solution of rutin was equipped, which was also used as a control. The procedure was as follows: 20.00 mg of rutin standard sample was accurately weighed out with an electronic balance, ethanol solution (concentration of 70%) was added in a water bath at a constant temperature of 40 ℃ to make the rutin completely dissolved, and then poured into a 100 ml volumetric flask to be fixed, and then shaken evenly.

Then, 1.0, 2.0, 3.0, 4.0 and 5.0 mL of the prepared rutin standard solutions were pipetted into each test tube, while a set of blank tests were set up for control. Add 1.5 mL of sodium nitrite solution with concentration of 5% in each test tube, shake well and leave it for 6 minutes, then add 1.5 ml of aluminum nitrate solution with concentration of 10%, shake well and leave it for the same time, and finally add 7.5 ml of sodium hydroxide solution with concentration of 4%, mix well and leave it for 15 minutes. The absorbance of each sample was measured at 510 nm using a UV spectrophotometer with the blank group as a comparison, and the average value was taken after three parallel measurements.

Finally, the curve was plotted to make a standard curve of rutin using the calculated concentration of rutin standard solution (x) as the horizontal coordinate and the absorbance value (y) as the vertical coordinate.

1.2.3 Flavonoid Extraction

Ultrasonic-assisted ethanol extraction method, accurately weigh 0.5 g of treated alfalfa leaf powder, placed in a 50 ml centrifuge tube, add the required volume and concentration of ethanol solution, mix thoroughly, extract the total flavonoids under the corresponding ultrasonic conditions, and then centrifuged under the conditions of 4200 r/min for 20 min, take up the clarified supernatant, and then add the corresponding concentration of ethanol to make the volume of the extract the same. The total flavonoids yield was determined by adding the corresponding concentration of ethanol to make the volume of the extract the same.

1.2.4 Determination of Flavonoid Contents

The total flavonoid content was determined using $NaNO_2$-Al (NO_3)-NaOH photometric method. Using a pipette, 1 mL of flavonoid extract was tested in a tube, and then $NaNO_2$ solution, Al $(NO_3)_3$ solution and NaOH solution were added sequentially with a pipette gun, mixed well, and left to stand. Afterwards, the absorbance value was measured at 510 nm, and the total amount of flavonoids was deduced in accordance with the standard curve of rutin and the formula, which is as follows (a cross-reference of the letters in the formula is shown in Table 1.1 below):

$$\boldsymbol{w} = \frac{\mathbf{c} \times \mathbf{n} \times \mathbf{v}}{\mathbf{m}} \times \mathbf{100\%}$$

Table 1.1 Letters represent the meaning

Letters	Represents the meaning
c	Concentration of total flavonoids calculated from the standard curve (mg/mL)
n	Dilution ratio
v	Total sample volume (mL)
m	Quality of alfalfa powder (mg)

1.2.5 Single-Factor Tests

Taking the extraction rate of total flavonoids of alfalfa as an index, the power, temperature, time, liquid-solid ratio, and ethanol concentration during ultrasonication were set as the experimental investigation factors respectively, and the better levels that had a greater effect on the extraction of total flavonoids were screened out. The specific levels are listed in the table below.

Table 1.2 Single-factor test level

Level	Ultrasound power/W	Ethanol concentration/%	Ultrasound time/min	Liquid solid ratio / (mL/g)	Ultrasound temperature/℃
1	400	30	30	20:1	40
2	500	40	40	30:1	50
3	600	50	50	40:1	60
4	700	60	60	50:1	70
5	800	70	70	60:1	80

1.2.6 Orthogonal Tests

After completing the single-factor experimental operation, four factors with relatively significant effects on the yield of total flavonoids were selected to design orthogonal experiments, and the levels of each factor are shown in the table below. Orthogonal experiments were carried out and analyzed to obtain the optimum extraction process of total flavonoids.

Table 1.3 Orthogonal test factor level table

Level	Element			
	Ultrasound time/min	Ethanol concentration/%	Ultrasound temperature/℃	Liquid solid ratio/(mL/g)
1	55	65	55	45:1
2	60	70	60	50:1
3	65	75	65	55:1

1.2.7 Data Handling

The data obtained from the orthogonal test were analyzed using the statistical software SPSS.

2. EXPERIMENTAL RESULTS AND ANALYSIS

2.1 Plotting of Standard Curves

Linear regression was performed using the mass concentration of rutin as the horizontal coordinate and absorbance as the vertical coordinate, yielding a linear regression equation of y=12.665x-0.0051 (R^2=0.9987), the results of which are shown in Figure 2.1 below. The coefficient of determination was close to 1, indicating that the model fit was relatively good for rutin concentrations in the range of 0.000-0.080 mg/mL.

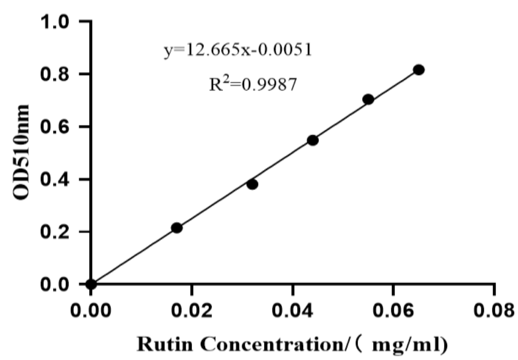

Fig2.1 Standard curve for rutin

2.2 Results of the one-factor test

2.2.1 Results of the Effect of Ultrasonic Power on the Yield of Total Flavonoids in Alfalfa

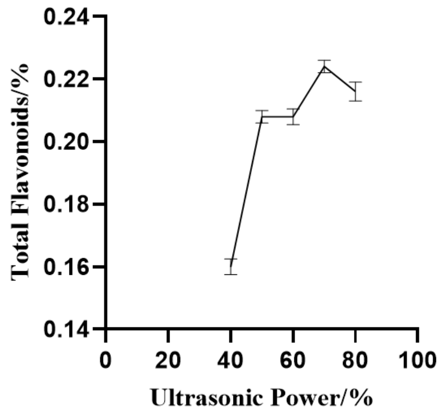

Fig 2.2 Effect of ultrasonic power on the yield of total flavonoids

From Figure 2.2, it can be seen that in the ultrasonic time, extraction temperature, ethanol concentration, liquid-solid ratio and other alfalfa total flavonoids extraction conditions are the same, the ultrasonic power is different, alfalfa yield is different, ultrasonic power in the range of 400W-700W, the higher the power, the higher the rate of total flavonoids, when the ultrasonic power reaches 700W, the total flavonoids yield is the highest, when the ultrasonic power of 800W, total flavonoids yield declined. When the ultrasonic power was 800W, the total flavonoid yield decreased.

2.2.2 The Results of Liquid-Solid Ratio on the Extraction Rate of Rotal Flavonoids from Alfalfa

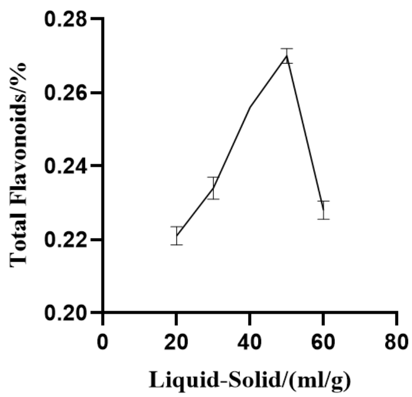

Fig 2.3 Effect of liquid-solid ratio on the yield of total flavonoids

As can be seen from Fig. 2.3, under the same conditions of total flavonoid extraction such as ultrasonic power, ultrasonic time, extraction temperature and ethanol concentration, different volumes of ethanol added resulted in different flavonoid yields, and the extraction rate of total flavonoids was maximized when the liquid-solid ratio was 50.

2.2.3 Results of the Effect of Ethanol Concentration on the Extraction Rate of Total Flavonoids from Alfalfa

Ethanol is a green solvent, because of its cheap and easy to get so as a common extraction solvent, according to the principle of similar solubility can meet the needs of different kinds of components of the extraction, strong penetration.

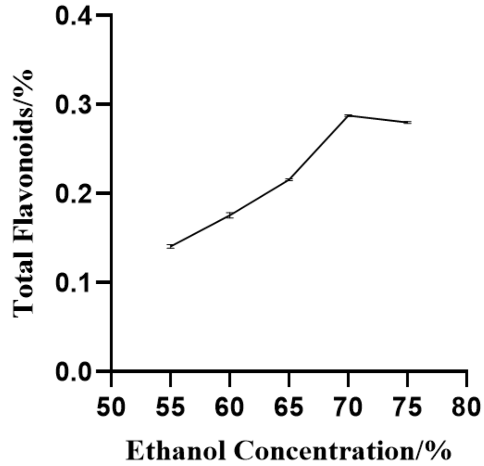

Fig 2.4 Effect of ethanol concentration on the yield of total flavonoids

As can be seen in Figure 2.4, the total flavonoid yield was increasing at ethanol concentrations from 55% to 70%, reaching a peak at an ethanol volume fraction of 70%, the second half of the fold may be the result of the increase in the volume fraction of ethanol causing many non-targeted extracts to be dissolved into the extract, and flavonoids extraction was impeded so the total flavonoids yield was reduced.

2.2.4 Results of the effect of extraction temperature on the extraction rate of total flavonoids from alfalfa

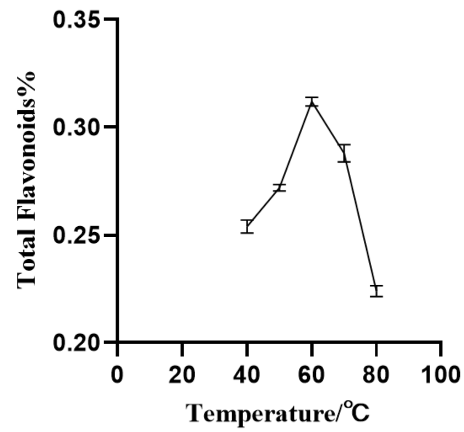

Fig 2.5 Effect of extraction temperature on the yield of total flavonoids

From Figure 2.5, the total flavonoid yield increases when the temperature is in the range of the interval from 40°C to 60°C, and the total flavonoid yield is maximum at a temperature of 60°C. When the temperature is in the range of the interval from 60°C to 80°C, the total flavonoid yield decreases slowly and then decreases sharply, and the increase in the temperature beyond 70°C leads to the degradation or even inactivation of the active ingredient so that the total flavonoid yield will appear to decrease sharply.

2.2.5 Results of the effect of extraction time on the extraction rate of total flavonoids in navel orange peel

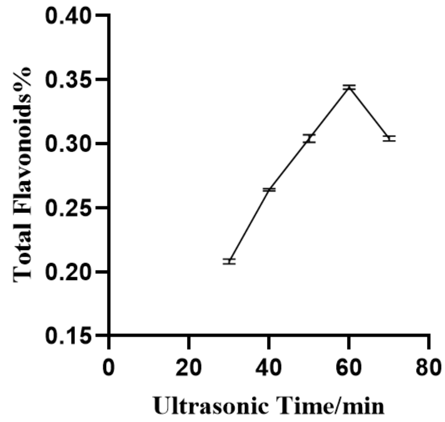

Fig2.6 Effect of ultrasound time on the yield of total flavonoids

From Figure 2.6, when the extraction time consumed increased (30-60 min), the increase of extraction time consumed increased the total flavonoid yield, and the maximum total flavonoid yield was obtained when the ultrasonic time was 60 min, and when the ultrasonic extraction time consumed more time (70 min), the total flavonoid yield showed a decreasing tendency due to the increase of time consumed instead. This may be due to the long extraction time, which increased the dissolution of non-flavonoid components and impurities, and may even lead to the degradation and inactivation of the active ingredients, so the total flavonoid yield decreased.

2. 3 Orthogonal Test Results

Experiment number	Factor				Total flavonoid yield/%
	A Ultrasound time/min	B ethanol concentration /%	C Ultrasonic temperature/°C	D Liquid-solid ratio/mL/g	
1	1	1	1	1	0.225
2	1	2	3	2	0.270
3	1	3	2	3	0.220
4	2	1	2	3	0.231
5	2	2	3	1	0.252
6	2	3	1	2	0.280
7	3	1	2	2	0.180
8	3	2	1	3	0.187
9	3	3	3	1	0.261
K_{1j}	0.715	0.636	0.692	0.738	
K_{2j}	0.763	0.709	0.631	0.730	
K_{3j}	0.628	0.761	0.783	0.638	
$\overline{K}_{1j}$	0.238	0.212	0.231	0.246	
$\overline{K}_{2j}$	0.254	0.236	0.210	0.243	
$\overline{K}_{3j}$	0.209	0.254	0.261	0.213	
R	0.054	0.042	0.051	0.033	
Priority	A>C>B>D				
Optimal Mix	$A_2B_3C_1D_2$				

As can be seen from the table, the influencing factors were A, C, B and D in descending order, and the optimal combination was A2B3C1D2, i.e., the ultrasonic time was 60 min, the ethanol concentration was 70%, the ultrasonic temperature was 60°C, and the liquid-to-solid ratio was 50:1 (mL/g), at which time the total flavonoid yield reached 0.280%.

3. CONCLUSION

In this paper, the optimal extraction process of total flavonoids from saline-tolerant alfalfa in the Yellow River Delta region was investigated. By using the data from the results of the one-factor test of the ultrasonic-assisted ethanol extraction method and the results of the orthogonal test among the several influencing factors, the extraction temperature plays a relatively large role in the total flavonoids yield of umbilical cord saline-tolerant alfalfa, and the one that has the least role is the ultrasonic extraction time. The final optimum extraction process for flavonoids in saline-tolerant alfalfa was obtained as the optimum conditions for extraction were temperature of 60°C, liquid-solid ratio of 50:1 (mL/g), ultrasound time of 60 min and ethanol concentration of 70%. This experiment provides a research method to study the extraction of total flavonoids.

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