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Optimization of supercritical carbon dioxide extraction process for total flavonoids in *Suaeda salsa* by orthogonal experiment

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Abstract: To determine the optimal method for extracting flavonoids from *Suaeda salsa*, this study investigated the impact of various factors, including extraction time, temperature, pressure, and ethanol concentration, on the yield of total flavonoids obtained through the utilization of supercritical carbon dioxide (SFE-CO2) as the extraction medium. The study employed both single-factor experiments and orthogonal experiments to accomplish this objective. The single-factor experiment adopted the control variable method, with fixed parameters for each factor: temperature of 40℃, pressure of 30 MPa, time of 1.5 hours, and ethanol concentration of 90%. The study found that the optimal extraction conditions were as follows: a temperature of 45℃, a pressure of 30 MPa, a duration of 2 hours, and an ethanol concentration of 95%. Under these conditions, the total extraction yield of total flavonoids from *Suaeda salsa* achieved 10.25%, which is higher than the existing reports (6.57%). This study not only promotes the development and utilization of *Suaeda salsa* but also serves as a significant avenue for future research on the development of nutritional and healthy foods and pharmaceutical products derived from the natural resource *Suaeda salsa*.

Keywords: Supercritical CO2 extraction; *Suaeda salsa*; Total flavonoids; Orthogonal experiment

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INTRODUCTION

Suaeda salsa is an annual herbaceous wild plant,that grows widely in coastal mudflat and saline alkali lands in regions such as Jiangsu, Hebei, Liaoning, Shandong, and Northeast China (Wang *et al*., 2022). Renowned for its strong and rapid growth capacity, *Suaeda salsa* also contains various valuable components. In addition to being rich in fats, proteins, polysaccharides, and alkaloids, it is abundant in flavonoids and vitamins, which give it a high edible and medicinal value. Recent medical research has found that this plant has various functions, including blood sugar reduction, blood pressure reduction, vasodilation, heart disease prevention and treatment, and strengthening human immunity (Mohammed, 2020, Fu *et al*., 2019, Wang *et al*., 2022). They may be suitable for preventing cardiovascular system diseases and have health benefits for elderly and hypertensive patients. At present, due to the small planting area and the need for manual harvesting, most *Suaeda salsas* are in a state of self-extinction, resulting in serious resource waste (Sun *et al*., 2018). The extraction of flavonoids from plants has always been a hot topic in academic research. Currently, many of the plants being studied are widely used, including ginkgo biloba, honeysuckle, agarwood leaves, sweet vine, etc. The extraction methods mainly include ultrasonic-assisted extraction, enzyme impregnation extraction, microwave heating extraction, supercritical fluid extraction technology, etc. (Zhao *et al*., 2021) However, research on the extraction of flavonoids from *Suaeda salsa* by supercritical dioxide carbon (SFE-CO2) extraction has not yet been reported.

Compared with traditional extraction methods, SFE-CO2 extraction has a wider scope of action and a faster rate of action, making it a relatively advanced physical extraction method. Many flavonoids in plants have been extracted using this method (Song *et al*., 2019). There are currently reports on the extraction processes of total flavonoids from *Suaeda salsa* (TFSS), including ultrasonic- assisted extraction, alcohol extraction, alkali extraction, and acid precipitation. The alcohol extraction has been found to have a lengthy extraction time and low efficiency, while ultrasound-assisted extraction has been observed to potentially worsen solvent evaporation (Elferjane *et al.,* 2023). On the other hand, the alkali acid extraction method is susceptible to external pollution and interference (Waseem *et al.,* 2023). Furthermore, a study reported a relatively low extraction yield of 6.57% and noted that the purity of the extracted compounds was not high (Guo *et al*., 2018). To date, the SFE-CO2 extraction method has not been used to optimize the extraction of TFSS. This study aims to determine the optimal process conditions for SFE-CO2 extraction of TFSS through orthogonal experiments to provide a scientific foundation as guidance for its development and utilization.

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MATERIAL AND METHODS

Pretreatment of Suaeda salsa stems and leaves in saline soil

Stems and leaves of *Suaeda salsa* (The mature plant which is fiery red and highly ornamental) was collected from the coast of Dafeng in Yancheng, Jiangsu, and verified by Professor Bian Qingya, a botanist from Jiangsu Vocational College of Medicine. They were freeze-dried in a low-temperature drying oven (LABCONCO, Beijing, China) at -20°C to -30°C, crushed and sieved, and then stored in a sealed and dry environment at room temperature for no more than six months for later use (Jiang *et al.,* 2022).

Standard curve preparation

Rutin standard with a purity of 92.2% (SINOPHARM, China) was precisely weighed for 5.4 mg on the analytical balance (Mettler Toledo Technology (China) Co. Ltd.), dissolved in 70% ethanol (SINOPHARM, China), transferred to a 25-mL volumetric flask, and thoroughly shaken. Then, a 0.2 g/mL Rutin standard was prepared. A range of Rutin reference solution (i. e. 0 mL, 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, and 6 mL) were precisely measured and placed in 25 mL volumetric flasks, and added with 1 mL of 5% sodium nitrite solution (SINOPHARM, China), thoroughly shaken, and incubated at room temperature for 6 min. Then, 1 mL of 10% aluminum trichloride solution (SINOPHARM, China) was added, thoroughly shaken, and incubated for 6 min. Next, 10 mL of 4% sodium hydroxide solution (SINOPHARM, China) were added and diluted to 25 mL with a 70% ethanol solution before being shaken and incubated for 15 minutes. The absorbance was measured at wavelength of 510 nm with UV spectrophotometry (UV-1800PC-DS2 UV-vis, Shanghai Yuan Xi Instrument Co., Ltd.). Subsequently, a standard curve was developed, whereby the concentration of Rutin (mg/mL) was represented on the x-axis and the absorbance was represented on the y-axis (Wang *et al.*, 2020).

Selection of entrainer

CO_2 belongs to the category of non-polar solvents, and according to the principle of similar solubility, it has only good solubility for low-polarity lipophilic solutes. Flavonoids belong to a group of compounds with strong polarity; therefore, a polar solvent is needed as a co-solvent or entrainer to improve and maintain extraction selectivity and increase the solubility of volatile and polar solutes such as ethanol, methanol, and acetone (Zhao *et al.*, 2021). Anhydrous ethanol was used as an entrainer to improve the overall polarity of the extraction solvent.

Extraction of total flavonoids from Suaeda salsa

As shown in Fig. 1, a total of 20 g of *Suaeda salsa* powder was weighed and added to the supercritical extraction instrument SFE-2 (Applied Separations (ASI), USA). The required extraction temperature, time, pressure, and ethanol concentration were set on the fluid extraction equipment according to different experimental requirements, and the fixed flow rate was 2 mL/min. When the temperature of the equipment dropped to around 3°C to 4°C, the system began to add anhydrous ethanol and pressurize according to demand. CO_2 (Zheng Da, Yan Cheng, China) with a purity higher than 99.9% was converted into a liquid state after passing through the gas bottle into the condenser, and then metered and injected into the extraction kettle by a high-pressure pump. The temperature gradually rose to a supercritical condition, and extraction began. After reaching the set pressure value, the equipment began to cycle and maintain a constant temperature and pressure. The CO_2 fluid (carrying the extract of *Suaeda salsa*) entered the separation vessels I and II in sequence, and the depressurization valve began to depressurize and control the temperature. The extract of *Suaeda salsa* was separated from the CO_2. After reaching the set time, the pressure of the extraction vessel was released to normal pressure, and the discharge port was opened to obtain a TFSS extraction solution sample (Zhao *et al.,* 2021, Chu *et al.,* 2021).

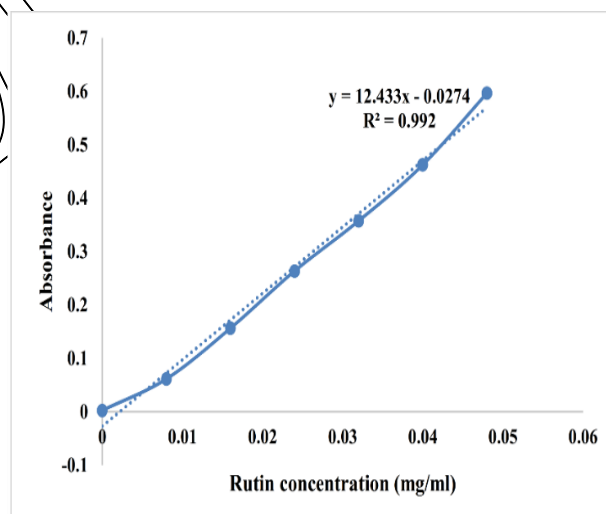

Fig.1: Rutin Standard Curve

The petroleum ether was added to the same volume as the extraction solution, extracted 3~4 times, and fat-soluble impurities were removed before transferring the alcohol solution layer to a 27-microporous adsorption resin column (D101, Lebiochem, Shanxi) (Dias *et al.*, 2021). The ratio of the original solution to the amount of resin is 1: 1.5. It was eluted with water and 70% ethanol until the solution changed from light yellow to colorless.

The eluent of the ethanol solution was collected and concentrated on a rotary evaporator (RE-6000AT) to obtain *Suaeda salsa* flavonoid extract (Zhou *et al.*, 2018). Then, the powder was obtained by freeze-drying the solution in a SCIENTZ-10N/A freezer dryer (Ningbo, Xinzhi Biotechnology Co. Ltd.) and stored in a sealed place away from light at 4□.

Table 1: Single-factor experimental factors and levels for *Suaeda salsa* extraction

Factors	Levels				
Extraction time (h)	1	1.5	2	2.5	3
Extraction temperature (□)	35	40	45	50	55
Extraction pressure (MPa)	20	25	30	35	40
Ethanol concentration (%)	80	85	90	95	100

Table 2: Orthogonal experimental design

Level	Factors			
	A Temperature (□)	B Pressure (MPa)	C Time (h)	D Ethanol concentration (%)
1	40	25	1.5	85
2	45	30	2	90
3	50	35	2.5	95

Table 3: Orthogonal experimental results of extracting *TFSS* using supercritical CO_2 fluid extraction method

Serial Number	A Temperature (□)	B Pressure (MPa)	C Time (h)	D Ethanol concentration (%)	*TFSS* Yield (%)
1	1	1	1	1	9.07
2	1	2	3	2	9.35
3	1	3	2	3	9.85
4	2	1	3	3	9.52
5	2	2	2	1	9.63
6	2	3	1	2	9.3
7	3	1	2	2	9.71
8	3	2	1	3	9.59
9	3	3	3	1	9.03

Table 4: Range analysis results of orthogonal experiments

Term	Level	A Temperature (□)	B Pressure (MPa)	C Time (h)	D Ethanol concentration (%)
K value	1	28.27	28.30	27.96	27.73
	2	28.45	28.57	29.19	28.36
	3	28.33	28.18	27.90	28.96
K average value	1	9.42	9.43	9.32	9.24
	2	9.48	9.52	9.73	9.45
	3	9.44	9.39	9.30	9.65
Optimal level		2	2	2	3

Determination of total flavonoid extraction yield

1 mL of sample was prepared according to the standard curve preparation conditions. Absorbance was measured at a wavelength of 510 nm to obtain the total flavonoid concentration in the sample. The formula is as follows:

Total yield of flavonoid extraction (%) = [(C × V)/M] × 100%

where C: total flavonoid concentration; V: volume of extraction solution; M: the weight of *Suaeda salsa* powder.

Single-factor experiment

The single-factor control variates method (Xue *et al.,* 2023) was employed to set the parameters for each factor: temperature at 40 °C, pressure at 30 MPa, duration of 1.5 hours, and ethanol concentration at 90%. Specific factor levels are detailed in Table.1.

Orthogonal design for optimization

Based on the principles of orthogonal experimental design and single-factor experiments (Jia *et al.,* 2021), four main factors that significantly affect the extraction yield of flavonoids were selected to explore the TFSS yield and determine the optimal extraction process for TFSS. The factor level design is shown in table. 2, where A represents temperature, B represents pressure, C represents time, and D represents ethanol concentration.

DATA STATISTICS AND ANALYIS

All results were expressed as the mean ± standard deviaton (SD). Differences were analyzed by one-way analysi of variance (ANOVA) and multiple comparison tests (SPSS software 26.0). Values of $p < 0.05$, were considered statistically significant.

RESULTS

Rutin standard curve

As shown in fig.2, the regression equation obtained for flavonoid concentration determination was: y =12.433x-0.0274, R^2 = 0.992. This curve indicates a good linear relationship from 0 mg/mL to 0.048 mg/mL. Single-factor result analysis of the influence of pressure, temperature, time, and ethanol concentration on TFSS yield.

As shown in fig. 3a, as the pressure increased, the yield of TFSS first increased and then decreased. At an extraction pressure of 30 MPa, the yield of extraction reached 9.75%, at its highest value. When the pressure exceeded 30 MPa, its diffusion rate further decreased, resulting in a decrease in the contact between total flavonoids and CO_2, resulting in a gradual decrease in its yield.

Based on Fig. 3b, the extraction yield of TFSS gradually rose with the increase in temperature, reaching its maximum value at 50 ℃. Therefore, this experiment selected an extraction temperature of 50 ℃ for the next optimization experiment.

Within 1~2 hours, the extraction yield of TFSS increased correspondingly with the prolongation of time (Fig. 3c). At 2 hours, the extraction rate of TFSS was the highest. But after 2 hours, with the extension of time, the TFSS in the sample was almost completely extracted, so the yield change was not significant.

As shown in fig. 3d, ethanol as the entrainer has a significant impact on the extraction yield of TFSS. As the concentration increases, the yield of TFSS shows a significant upward trend. When the concentration exceeds 90%, the extraction yield begins to decrease.

Thus, this indicates that the optimal ethanol extraction concentration for this experiment is 90%.

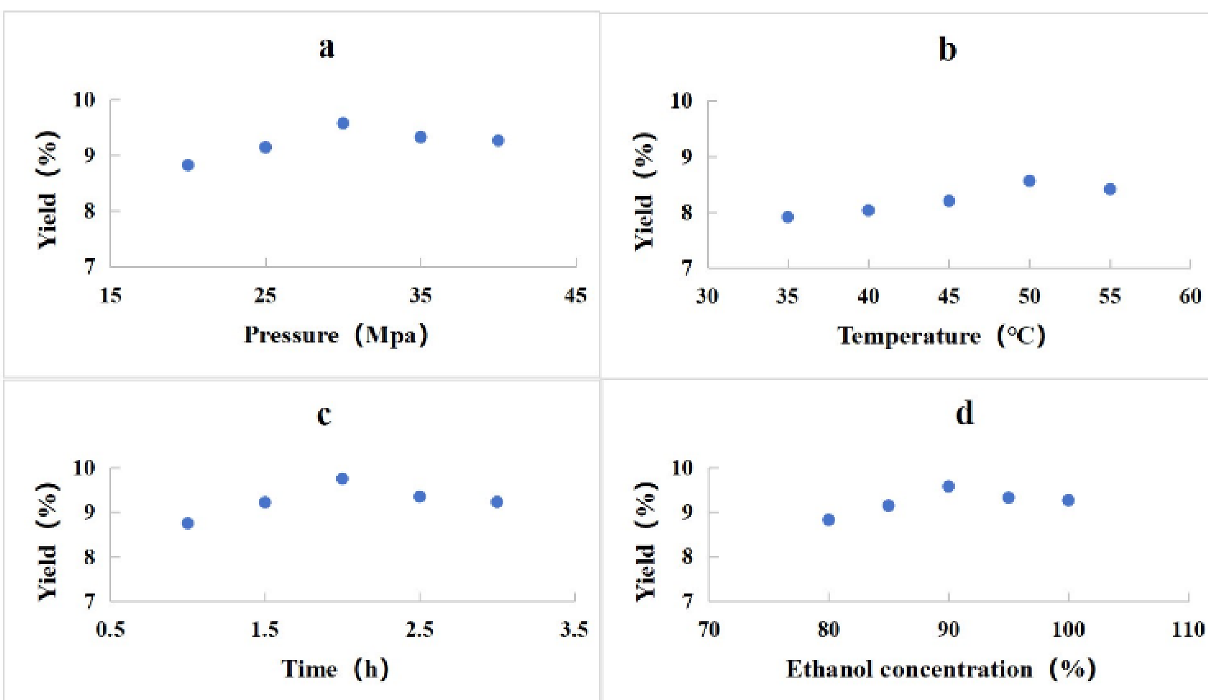

Fig.2: Effect of pressure, temperature, time, and ethanol concentration on TFSS yield
a: the influence of pressure; b: the influence of temperature; c: the influence of time; d: the influence of ethanol concentration.

Fig.3. Effect of pressure, temperature, time, and ethanol concentration on TFSS yield a: the influence of pressure; b: the influence of temperature; c: the influence of time; d: the influence of ethanol concentration.

Visual analysis of orthogonal experiments

The experiment used L9 (34) to design an orthogonal experiment to learn more about the four factors listed above (Table. 3) to improve the extraction process based on single factor analysis. As shown in Table. 4, the order of influence factors on TFSS extraction yield is: C> D> B> A. It means that time has the greatest impact on TFSS extraction yield, and temperature has the smallest impact. Based on the comprehensive orthogonal experimental results, the optimal extraction process combination is A2B2C2D3, which means the optimal extraction conditions are extraction temperature 45 □, extraction pressure 30 MPa, extraction time 2 hours, and ethanol concentration 95%.

Validation experiment

According to the result obtained from the orthogonal experiment, three parallel experiments were conducted, and the yield of TFSS was (10.25 ± 0.23) %, the purity was higher than 60%, which were significantly better than the extraction rates of single factor experiments and orthogonal experiments in each group ($P = 0.01$). The result indicates that the SFE-CO2 extraction process parameters of TFSS optimized by the orthogonal experimental method have high accuracy and repeatability. This yield is higher than that reported by the traditional methods, including ethanol extraction, water extraction, ultrasonic-assisted extraction, and microwave-assisted extraction, which were reported previously (Gao, 2005, Zhang, 2008, Wan and Li, 2012, Lin *et al*., 2013).

DISCUSSION

The extraction of flavonoids from plant materials involves several critical steps and considerations to ensure efficient recovery of these bioactive compounds. Various factors significantly impact the extraction process, including the selection of plant material (Sun and Shahrajabian, 2023), pre-treatment of plant material (Shi *et al*., 2003), choice of solvent (Rostagno and Prado, 2013), extraction method (Chemat *et al*., 2012), optimization of extraction conditions, filtration and concentration (Goufo and Trindade, 2014), storage (Justesen, 2000, Karacabey and Mazza, 2010). Based on the above factors, this study selected the stems and leaves of *Suaeda salsa* at flowering season for their high flavonoid content as reported in the literature (Fu *et al*., 2019). The plants were subjected to low-temperature drying to minimize flavonoid degradation, and the extracted raw materials were obtained after crushing. Ethanol was chosen as the extraction solvent due to its moderate polarity, low toxicity, safety, good biocompatibility, volatility, and recyclability, as well as its broad application foundation and cost-effectiveness (Teixeira *et al*., 2023). This solvent is particularly effective at extracting flavonoids while ensuring the quality and safety of the extract, making it an ideal choice for the extraction process (Suran *et al*., 2021). Through this optimized approach, the process can effectively maximize flavonoid yield and maintain the integrity of the extracted compounds.

Some studies reported on the extraction processes of TFSS, including ultrasonic-assisted extraction, alcohol extraction, alkali extraction, and acid precipitation (Guo *et al.,* 2018). Alcohol extraction has been found to have a lengthy extraction time and low efficiency, while ultrasound-assisted extraction has been observed to be effective, yet potentially worsen solvent evaporation (Chemat *et al.*, 2017). On the other hand, the alkali acid extraction method poses risks related to external pollution and interference (Waseem *et al.*, 2023). Furthermore, a previous study reported a relatively low extraction yield of 6.57% and indicated that the purity of the compounds extracted remains suboptimal (Guo *et al.*, 2018). To date, the SFE-CO_2 extraction method has not been explored for optimization of TFSS extraction. This study aims to determine the optimal process conditions for SFE-CO_2 extraction of TFSS through orthogonal experiments to provide a scientific foundation that can guide the development and utilization of more efficient and pure extraction methods.

The extraction of flavonoids using SFE-CO_2 is influenced by various factors that can affect the efficiency, yield, and quality of the extracted compounds. These factors include pressure (Reverchon and De Marco, 2006), temperature (Hossain *et al.*, 2011), co-solvents (Rostagno and Prado, 2013), extraction time (Pourmortazavi and Hajimirsadeghi, 2007), flow Rate of CO_2 (Reverchon and De Marco, 2006), particle size of plant material (Li *et al.*, 2005), moisture content of plant material (Khaw *et al.*, 2017). Among these factors, pressure, temperature, co-solvents and extraction time are paramount because they directly influence the solubility and stability of flavonoids in the supercritical CO_2 environment, in turn, determine the overall efficiency and success of the extraction process (Chen, 2020). Optimization of these critical variables is essential to maximize the yield and quality of the extracted flavonoids.

The result in Figure. 3a indicates that the density of supercritical carbon dioxide increased with increasing pressure, thereby improving its solubility for solutes. But, at the same time, the diffusion coefficient of carbon dioxide fluid decreased with increasing extraction pressure, which was not conducive to the dissolution of flavonoids. There may be a complex synergistic system involved in this process (Dong, 2017). Therefore, in practical applications, it is crucial to strike a balance by selecting an appropriate extraction pressure. This involves optimizing the pressure to maximize solubility and extraction efficiency while minimizing costs and preventing damage to the raw materials. Achieving this balance ensures a more efficient, cost-effective, and high-quality extraction process.

As shown in figure. 3b, temperature affected the diffusion rate of supercritical carbon dioxide within the extraction materials. An appropriate temperature was conducive to increasing the volatility of the solute, and the diffusion coefficient of the material also increased accordingly. As a result, flavonoids in *Suaeda salsa* were more easily extracted. Due to the continuous increase in temperature, leading to a decrease in the concentration and density of carbon dioxide, which affected the solubility of carbon dioxide and obstructing the continuation of extraction, the extraction rates no longer increased and showed a downward trend when the temperature exceeded 50 ℃. In addition, exceeding a certain temperature range could also reduce the physiological function of flavonoids (Sun, 2016).）Therefore, in practical operation, it is necessary to choose an appropriate extraction temperature. This involves selecting a temperature that is high enough to enhance the diffusion rate and improve extraction efficiency but not so high that it significantly reduces the density of SFE-CO_2 or causes thermal degradation of the sensitive compounds. By optimizing the extraction temperature, one can achieve a balance that maximizes yield and maintains the integrity and quality of the extracted substances.

Extraction time is one of the important factors affecting the extraction effect. if the extraction time was too short, the supercritical CO_2 fluid cannot have good contact with the solute, and the extraction amount naturally decreased. After the extraction time reached a certain level, the CO_2 fluid and the flavonoid compounds of *Suaeda salsa* reached a near dissolution equilibrium state. If the extraction time continued to increase, it can only increase the energy consumption of supercritical extraction and may reduce the extraction rate actually (Zhao *et al.*, 2021). Therefore, in practical applications, it is necessary to choose an appropriate extraction time based on the characteristics of the raw materials and the experimental purpose. This is congruent with our findings where the extraction yield of TFSS increased correspondingly with the prolongation of time and the yield remained stagnant after 2 hours of extraction (figure. 3c).

As a polar solvent, ethanol can enhance the solubility of polar compounds in supercritical CO_2. With the increase of ethanol concentration, the polarity of the supercritical CO_2 system increases, which can more effectively dissolve and extract compounds with stronger polarity. Therefore, in practical applications, it is necessary to choose an appropriate ethanol concentration (figure. 3d).

However, it was worth noting that although the influence of extraction temperature ranked last in this experiment, the results of the previous single factor experiment indicated that only an appropriate temperature could enhance the volatility of solutes and increase the diffusion coefficient of materials, making the extraction of flavonoid compounds from *suaeda salsa*. Therefore, extraction temperature was still a relatively important part of the flavonoid extraction process, and "R_A= 0.006" also confirms this.

The result of the orthogonal experiment indicates that the SFE-CO_2 process parameters of TFSS optimized by the orthogonal experimental method have high accuracy and reproducibility. This yield is higher than that reported by the traditional methods (6.57%), including ethanol extraction, water extraction, ultrasonic-assisted extraction, and microwave-assisted extraction, which were reported previously (Gao, 2005, Zhang, 2008, Wan and Li, 2012, Lin *et al.*, 2013, Gong, 2016).

CONCLUSION

The experimental results revealed that the optimal process conditions for extracting TFSS using the supercritical CO_2 extraction method are: 45 ℃, 30 MPa, 2 hours of extraction time, and 95% ethanol concentration. Under these optimal conditions, parallel experiments were conducted, yielding a TFSS extraction of 10.25%, which is higher than the yields obtained from of single factor experiments and orthogonal experiments. This study provides a solid foundation for future research on the creation of nutrient-dense, healthful meals and medicinal products made from the natural resource *Suaeda salsa*. It also encourages the development and use of *Suaeda salsa*.

Several benefits were observed when comparing this optimized process to traditional approach, including significantly improved recovery and purity, as well as decreased time and organic solvent use (Wang 2016, Chu *et al.*, 2021). The findings from UV spectrophotometry analysis confirmed the effectiveness of supercritical carbon dioxide extraction as a method for obtaining TFSS.

While the orthogonal experimental optimization method successfully determined the optimal combination of various factors for TFSS extraction, conditions beyond this optimal combination were not explored. Therefore, further research is needed to fully understand the influence of different extraction parameters. By using the response surface optimization method to construct a quadratic regression model, the magnitude and significance of the influence of various factors on the extraction rate of flavonoids as well as the optimal extraction process beyond the current experimental setting can be determined (Zhou *et al.*, 2018). This approach may offer valuable insights for optimizing the supercritical CO_2 extraction TFSS process in the future studies.

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