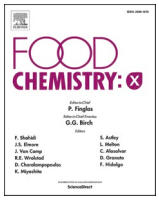
Food Chemistry: X 14 (2022) 100287

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Effects of five extraction methods on total content, composition, and stability of flavonoids in jujube

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ARTICLE INFO

Keywords:
Jujube
Rutin
Deep eutectic solvent
Extraction methods
Stability

ABSTRACT

The present study investigated the effects of different extraction methods including water-water bath (W-WB), ethanol-water bath (E-WB), deep eutectic solvent (DES) combined with ultrasound-assisted extraction (DES-UAE), microwave-assisted extraction (DES-MAE), and enzyme-assisted extraction (DES-EAE) on flavonoids (total flavonoid content, flavonoid composition, and stability) in jujube. The highest total flavonoid content of 8.03 mg/g was obtained by the DES-MAE extraction. Fifteen types of flavonoids were identified from jujube. The amount of rutin produced by the E-WB and DES-UAE methods was 66.88 ± 1.58 μg/g and 45.23 ± 3.22 μg/g, respectively. The retention of flavonoids in DES-UAE extracts were 98.15 ± 0.51%, 64.25 ± 2.21% after 2 h of high temperature treatment at 90 °C and 21 days of dark storage, respectively. The flavonoids extracted by different methods were suitable for dark storage under different light contrasts, where the retention of flavonoids extracted by DES-UAE method was 86.44 ± 2.45%. In conclusion, DES-UAE would be an efficient method for flavonoid extraction from jujube.

Introduction

Jujube, a plant belongs to the Rhamnaceae genus, and is commonly used as a traditional medicine and food. This native Chinese plant has been cultivated for more than 3,000 years. Globally, China is the largest producer of jujube, with cultivation areas and annual production accounting for more than 95% of the global output (Wang, Liu, Huang, & Luo, 2020). Xinjiang Province, as one of the world's six major fruit production belts, has suitable climatic conditions for the growth of jujube. Thus, jujube is widely cultivated in Xinjiang and admired for its good taste and high nutritional value.

Jujube fruits from Xinjiang province are rich in carbohydrates, dietary fibers, vitamins, minerals, and biologically active ingredients such as flavonoids, polyphenols, polysaccharides, and saponins (Wang et al., 2020). However, the capacity to process and utilize jujube in Xinjiang province is low. Jujube is mainly sold in dried form, juice form, and other processed products. Flavonoids are natural products with significant biological activities, including antiviral, anti-allergic, lipid-lowering, anti-bacterial and anti-inflammatory effects as well as prevention of cardiovascular and cerebrovascular diseases (Hamed et al., 2019). Flavonoids are found in various plants and have been used as natural pigment and antioxidants in food industry. In plants, natural flavonoids occur in form of aglycones (oxyglycosides and carbon glycosides). However, the instability of aglycones is correlated with low bioavailability (Cao, Wang, Liu, Ren, Han, & Deng, 2020), which limits their application in food and drug-related fields (Huang et al., 2017; Liu, Sun, You, Liu, Ren, & Wang, 2020). This calls for development of extraction methods that do not alter the stability of flavonoid.

Due to the complex structure of flavonoids, there is no general extraction method that is suitable for extracting flavonoids from all plants. Several organic solvents have been used for flavonoids extraction including methanol, ethanol, and ethyl acetate. However, the use of these solvents involves many processing steps and requires high temperature conditions. Moreover, the current extraction methods require large amounts of organic solvents and are time consuming. Furthermore, many organic solvents cause ionization, hydrolysis, oxidation and deactivation of flavonoids (Song et al., 2019). It should be noted that some organic solvents cause serious environmental problems and are hazardous to human health (Xu, Ran, Chen, Fan, Ren, & Yi, 2019).

At present, there is a growing research interest to develop green

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https://doi.org/10.1016/j.fochx.2022.100287
Received 16 October 2021; Received in revised form 13 March 2022; Accepted 14 March 2022
Available online 16 March 2022

extraction methods for flavonoids that are environmentally friendly, adjustable solvents, and require low amount of energy (Bosiljkov et al., 2017). Deep eutectic solvents (DESs), originally proposed by Abbott and co-workers, are promising types of green and sustainable solvents (Abbott, Capper, Davies, Rasheed, & Tambyrajah, 2003). They have similar physical properties such as feasible structural designs and excellent solubility with ionic liquids, compared to traditional organic solvents. (Cvjetko Bubalo et al., 2016; Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013; Jeong, Lee, et al., 2015; Mansur, Song, Jang, Lim, Yoo, & Nam, 2019). To date, DESs has been successfully used for extraction of several biologically active compounds in plants, including flavonoids (Yin, Zhong, Bian, Cheng, & Li, 2020), phenolic acids (Zhang et al., 2020), anthocyanins (Bosiljkov et al., 2017), polyphenols, and polysaccharides (Makris, 2018). Compared to traditional organic solvents, DES has excellent solubility. The solubility of DESs in different properties compounds vary depending on the combination of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA). This means that solubility and extraction efficiencies of target compounds can be improved by selecting appropriate components. Compounds with significant differences in properties can be extracted and efficiently separated simultaneously, implying that DESs can be applied as green solvents in the extraction of bioactive components (Oomen, Begines, Mustafa, Wilson, Verpoorte, & Choi, 2020; Wang et al., 2017). In recent years, advances in the development of extraction techniques have resulted in design of methods that utilize small amounts of solvents and energy, which has enhanced the extraction rates of various compounds. Such innovative technologies are currently being used in different fields, including pharmaceuticals, food, and cosmetic production. These technologies include ultrasound extraction (Milani et al., 2020), supercritical fluid extraction (Song et al., 2019), subcritical water extraction (Wang et al., 2017), ultra-high pressure extraction (Zhang et al., 2018), enzymolysis (Nadar, Rao, & Rathod, 2018; Wei, Sun, & Fang, 2019), and microwave extraction (Niu, Gao, & Liu, 2020).

The study identified a suitable method for extracting flavonoids from plants through the study of DESs combined with different extraction techniques.

Materials and methods

Chemicals and materials

Methanol, acetonitrile, and acetic acid were purchased from ANPEL. All solvents were of HPLC gradient grade. Ultra-pure water in-house was prepared using the Milli-Q water purification system (Millipore, Bedford, MA, USA). Choline chloride was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Malic acid, acetic acid, citric acid, fructose, glucose, urea, ethylene glycol, sorbitol, xylitol, and glycerol were obtained from YongSheng Fine Chemical Co. Ltd. (Tianjin, China). Jujube (Fig. 1a) was purchased from the local market (Shihezi, China). Jujube fruits were refrigerated at -18 °C for 24 h and thereafter crushed to powder. Samples were refrigerated at -18 °C to prevent moisture absorption and agglomeration.

Preparation of DESs

DESs were prepared by mixing Choline chloride with various HBDs, including malic acid, lactic acid, citric acid, fructose, glucose, urea, ethylene glycol, sorbitol, xylitol, and glycerol (Table 1). The mixture was stirred and heated continuously for 2 h (until a homogeneous, transparent liquid was formed) with magnetic Stirrer and subsequently cooled at room temperature (Abbott et al., 2003). All prepared DESs were stored in sealed glass bottles to avoid moisture or any other contaminations that can adversely affected DESs.

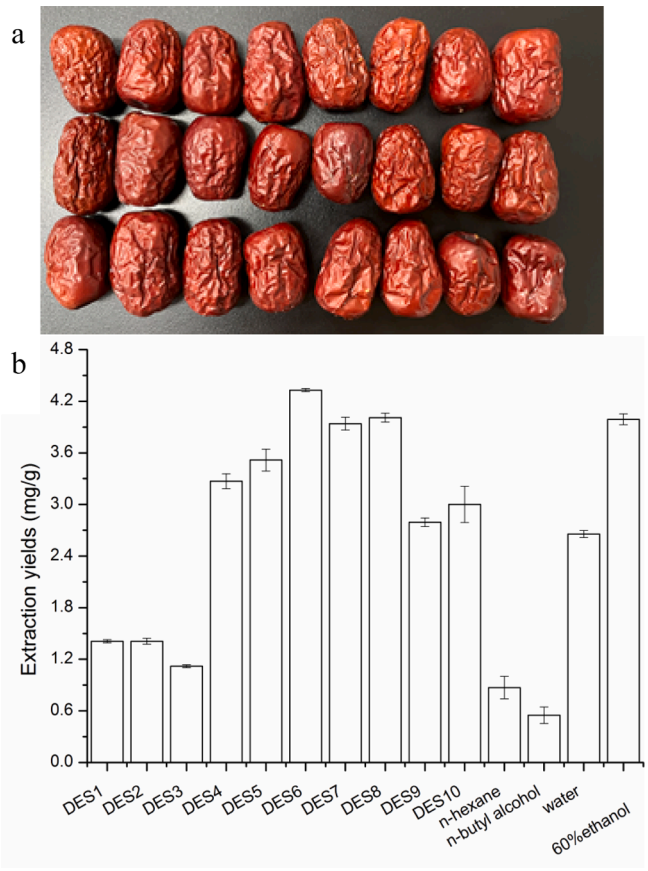

Fig. 1. Xinjiang jujube were used as the experimental materials (a). Extraction yields of different DESs and conventional solvents for flavonoids (b).

Table 1
List of the studied DESs.

Abbreviation	Component 1	Component 2	Mole ratio
DES1	Choline chloride	malic acid	1:2
DES2	Choline chloride	lactic acid	1:2
DES3	Choline chloride	citric acid	1:2
DES4	Choline chloride	fructose	1:2
DES5	Choline chloride	glucose	1:2
DES6	Choline chloride	urea	1:2
DES7	Choline chloride	ethylene glycol	1:2
DES8	Choline chloride	sorbitol	1:2
DES9	Choline chloride	xylitol	1:2
DES10	Choline chloride	glycerol	1:2

Selection of optimal solvents

About 0.5 g of jujube powder was extracted in 15 mL of 10 DESs of interest (solidliquid ratios, 1:30 g/mL) in a water bath for 0.5 h at 60 °C. Then, extracts were centrifuged at 4,400×*g* (6000 rpm in a High Conic™ || rotor, Teraeus Multifuge X1, Thermoscientific, New York, USA) for 10 min at 4 °C. The supernatant was collected for further analyses. To compare the extraction efficiencies of DESs to those of other extraction solvents, extraction was also done using conventional solvents (*N*-hexane, *N*-butanol, water, 60% ethanol), under similar conditions. All extraction procedures were performed in triplicate.

Preliminary evaluation of DES-based extraction method

Five different methods were used to extract flavonoids from jujube. The optimal extraction conditions of each method were determined by single factor and response surface optimization experiments. The effects

of the same factors on different methods were explored.

Traditional extraction

Traditional extractions included water-water bath (W-WB) and ethanol-water bath (E-WB). About 15 mL of water and ethanol concentrations (0, 20, 40, 60, 80, and 100%) were added to a 20.0 mL tube with 0.5 g jujube powder, respectively. The solvents and samples were evenly stirred and heated in a water bath. The effects of solid-liquid ratios (1:15, 1:20, 1:25, 1:30, 1:35, 1:40, and 1:45 g/mL), temperature (50, 60, 70, 80, and 90 °C), and time (30, 50, 70, 90, and 110 min) were investigated using the same procedure. After extraction, supernatants were collected for further analysis.

Ultrasound-assisted DES extraction (UAE-DES)

The extraction solvent (optimal DES) was added to a 20.0 mL tube containing 0.5 g jujube powder. The effects of water content (10, 20, 30, 50, 70, and 90%) in selected DESs, in mole ratios of 1:1, 1:2, 1:3, 2:1, and 3:1, and solid-liquid ratios of 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, and 1:45 g/mL were explored using the procedure described earlier. Extraction was performed using an ultrasonicator (SK5200BT 200 W, 35 kHz, Kedao Ultrasonic Instruments Co. Ltd, Shanghai, China): temperature (45, 50, 55, 60, 65, and 70 °C), time (20, 30, 40, 50, and 60 min).

Microwave-assisted extraction (DES-MAE)

The DES and sample were put in a glass beaker, evenly stirred and heated in a microwave (M1-L213C, Midea Kitchen Appliance Co., Ltd, Guangdong, China): power (70, 210, 350, 560, and 700 w), time (60, 80, 100, 120, 140, and 160 s). The molar ratio, water content of selected DES, and solid–liquid ratios were studied using the procedure described above.

Enzyme-assisted extraction (DES-EAE)

After mixing DES with the sample in a 50 mL clean conical flask, the pH values of the extraction solution was adjusted to 5 using a phosphate buffered solution (PBS). Different types of enzymes were weighed and mixed with 2 mL of 40 °C warm water to activate them. The EP tube was constantly shaken to ensure complete dissolution of the enzymes. The mixture was heated in a constant temperature water bath at 50 °C for 1 h, and at 90 °C for 40 s to inactivate the enzymes. The mixture was cooled to room temperature and the extracted liquid used to supplement the amounts of extracted solvent that had evaporated. The effect of enzymolysis factors: species of enzymes (cellulose, pectinase, cellulose: pectinase = 2:1, cellulose: pectinase = 1:1, cellulose: pectinase = 1:2), enzyme addition (5, 10, 15, 20, 25, and 30 mg/g), pH (4, 4.5 5, 5.5, 6, and 6.5), temperature (30, 40, 50, 60, and 70 °C), and time (1, 1.5, 2, 2.5, and 3 h) were evaluated. Follow-up operations were the same as the preceding steps.

Total flavonoid content

Sodium nitrite-aluminum nitrate colorimetry described previously was used to quantify total flavonoid content with minor modifications (Masci, Coccia, Lendaro, Mosca, Paolicelli, & Cesa, 2016). Briefly, each extract solution (1.0 mL) was mixed with 0.3 mL of 5% Sodium nitrite, followed by 0.3 mL of 10% Aluminum nitrate, 4.0 mL of 4% Sodium hydroxide, and 4.4 mL of distilled water. After incubation at room temperature for 15 min, absorbance was recorded using a multiwell plate reader (Eon, Biotek, US) at 510 nm. The total flavonoid content was calculated based on rutin concentration.

UHPLC analysis

The Waters UHPLC system was used to identify the different flavonoids in the jujube extracts. Chromatographic separation was performed on a HSS T3 column (1.8 μm, 2.1 × 50 mm, Waters, USA). A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) were used as mobile phases in a gradient elution mode: 90:10 V/V at 0 min, 90:10 V/V at 1.0 min, 90:10 V/V at 7.0 min, 90:10 V/V at 7.1 min, and 90:10 V/V at 12.0 min. The flow rate was 0.3 mL/min while the injection volume was 2.0 μL. The column temperature was maintained at 40 °C.

High-resolution mass spectrometry was used to identify the flavonoids in jujube. This was done using the Q Exactive hybrid Q-Orbitrap mass spectrometry detection system (Thermo, USA), equipped with a heated electrospray ionization (ESI) ion source and XCalibur workstation. Using the electrospray ion source (ESI), the analyte was analyzed under a single ion detection (SIM) mode and simultaneous scanning of negative ions, which improved sensitivity. Optimized conditions for mass spectrometry were: Shepherd-gas pressure 40 arb, auxiliary gas pressure 10 arb, ion spray voltage +3000 V, capillary temperature, 320 °C; And aux gas heater temperature of 350 °C. Prior to injection, each sample solution was filtered through a 0.22 μm PTFE filter.

Evaluation of the flavonoids stabilizing effects of the five extraction methods

Several factors, including storage conditions (temperature, light source) and time were investigated to assess the stability of extracted flavonoids. Flavonoids extracted by the five different methods were stored at 4 °C and in a water bath with temperature of 30, 40, 50, 60, 70, 80, and 90 °C for 120 min, and then cooled to room temperature. The extracts were stored in the dark and sampled every day for 21 consecutive days. The five samples were then exposed to different light sources (UV radiation, indoor natural light, and dark light). Sample positions were fixed to ensure the same height from the light source. Samples were obtained after every 2 h in the first 12 h and after every 12 h in the 44 consecutive hours.

Statistical analysis

Experimental data were analyzed using the Origin8.5 software. The design-expert 8.0.6 software was used for regression analysis and response surface optimization. Measurement data are expressed as mean ± S.D. Extraction yields of flavonoids for different extraction methods were compared using ANOVA in the IBM SPSS statistical 25.0 software. $p \leq 0.05$ was set as the threshold for statistical significance.

Results and discussion

Initial screening of DESs

Ten DESs and four conventional solvents were investigated to identify the effective DES in the present study. Interactions between HBD and HBA in DES were the main factors influencing extraction efficiencies of target products. The DESs exhibit different surface tension, polarity, viscosity, and solubility properties depending on the different combinations of HBD and HBA. Therefore, it is evident that different DESs can affect the extraction efficiencies of target compounds from the plant matrix (Wang et al., 2020). Compared to traditional solvents, pure DES exhibited a higher viscosity at room temperature which affected mass transfer efficiency. Water was added to reduce viscosity and increase the extraction efficiency. In a decreasing order, the extraction yields included amides > alcohols > sugars > carboxylic acids (Fig. 1b). Notably, the DES6 effectively extracted flavonoids, as compared with the conventional solvents including *N*-hexane, *N*-butanol, water, and 60% ethanol among other DESs. Further, it was noted that DES6-8 enhanced the extraction yield of flavonoids. Therefore, DES6 was chosen as the extraction solvent in this study.

Comparisons of extraction methods

The currently used flavonoid extraction method is hot water extraction, which is based on ethanol and water as solvents. When water

is used as the extraction solvent, a large number of inorganic salts and polysaccharide impurities dissolve and this inconveniences the subsequent flavonoid separation and purification. Other extraction methods, including ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and enzyme-assisted extraction (EAE) have been applied in flavonoid extraction. However, the methods are limited by high temperatures, labor, and low efficiencies, resulting in unstable rates of flavonoid extraction (Liang, Zeng, Wang, & Lou, 2019). The current study evaluated the extraction efficiencies of DES-UAE, DES-MAE and DES-EAE and compared them to those of the traditional water-water bath (W-WB) and ethanol-water bath (E-WB) methods. In addition, optimal conditions for the five jujube flavonoid extraction methods were determined using single-factor and response surface optimization experiments (Table 2).

The water content and mole ratio of the same DES solvents varied across different extraction methods. Moreover, the DES-MAE and DES-EAE extraction methods required the same molar ratio of DES, whereas DES-UAE required more choline chloride to improve efficiency of the extraction. Different molar ratios of DES led to different pH values, which can affect electrostatic bond and hydrogen bond interactions between DES and solute molecules, and play an important role in the extraction process. Thus, the molar ratio of the components affected the physical and chemical properties of DESs, which also significantly affected the extraction efficiency (Guo, Zou, Li, Kou, Liu, & Fu, 2020).

Compared to other solvents, viscosity of the obtained pure DES6 was higher at room temperature. The flavonoid yield was higher when the water content was 30-60% compared with other water contents (Table 2). This was because addition of water rapidly reduced viscosity of the DESs system and altered its polarity. The DESs with low viscosity are considerably effective extractants. This is because low viscosity promotes the mass transfer of target compounds to the DES solution, although this has not been absolutely established (Xu et al., 2019). For the DES system, water content of 20% has been reported to be the best for extractions of echinacoside and oleuropein in DES-UAE for the DES system. Moreover, it has been shown that water contents $>$ 50% or $<$20% are unfavorable as they weaken the interactions between DESs and the desired components (Wang et al., 2020). Huang et al. (2017) reported that excess water in the DES solution reduces the extraction efficiency, which is attributed to reduced solubility of the target compounds in the extraction medium and interactions between the sample and DES. Excessive dilution of DES using water is associated with destruction of hydrogen bonds among DES components, resulting in the loss of the supramolecular structure. Therefore, structural changes of DESs that are caused by dilution, affects its physical and chemical properties as well as applications (Huang et al., 2017). In summary, water contents of DESs between 20 and 50% are conducive for target compound extraction.

This work evaluated the effects of different solidliquid ratios (1:15, 1:20, 1:25, 1:30, 1:35, 1:40, and 1:45 g/mL) on flavonoid extraction. The extraction yields of flavonoids gradually increased with increasing the solid-liquid ratios. These findings were consistent with a previous explanation that as more DES entered plant cells, the more flavonoids were released into the extraction system. The increase in extraction solvent content increased the contact area with the sample, which accelerated the mass transfer of the target compound to the extraction solvent, thereby improving the extraction efficiency. Elsewhere, when DES-UAE was used to extract flavonoids from citrus peel wastes, the best condition was established to be a solid-liquid ratio of 1:50 g/mL (Xu et al., 2019). In this study, using the same extraction method, the solid-liquid ratio of flavonoids from jujube was 1:40 g/mL. Therefore, citrus peel wastes required more solvents to dissolve and obtain better yields. Furthermore, different raw materials affect the solid-liquid ratio in the same extraction method. Apart from the alcohol extraction method, other extraction methods require almost similar solid-liquid ratios (between 1:40 and 1:45 g/mL), which is suitable for extraction.

Findings of the current study indicated that the optimal extraction temperature for DES-UAE and DES-EAE was 70 °C, which was mild compared to that of W-WB and E-WB (their extraction temperatures are 90 °C). DES-MAE used the microwave maximum power of 700 w to obtain higher yields. Microwave-assisted extraction of flavonoids from leaves of *Alpinia oxyphylla* miq was more suitable at an extraction temperature between 70 and 80 °C and maintained high activity (Niu, Gao, & Liu, 2020). Thus, flavonoid extraction does require higher temperatures. In addition, temperature elevation reduces the viscosity of the DES system, increases the diffusion coefficient, accelerates molecular movements, and facilitates the precipitation of effective components. Temperature elevation can break cell walls and is beneficial for the extraction of flavonoids. However, high temperatures are not conducive for extraction, as they can destroy flavonoid activities. Therefore, the release of flavonoids increases at optimum temperatures.

The extraction time is a crucial factor in the extraction process of target compounds from plants samples (Xu et al., 2019). Prolonged extraction time increases total flavonoid content yield. As the extraction time increased, the extraction rate decreased significantly. This could be because after a long extraction time, other substances in the sample tissue diffused and attached to the sample surface possibly inhibiting the contact between sample and solvent and extraction efficiency. Among the five extraction methods, the DES-EAE method took the longest time of 3 h, whereas DES-MAE took only 160 s using the maximum microwave power. This finding contrasts with that obtained from other previous studies. The DES-MAE extraction of bioactive phenolic compounds from onion peel wastes showed that the microwave time was 15 min, with a smaller power of 100 w to obtain higher yields (Pal & Jadeja, 2019). The biggest advantage of microwave heating is that the internal temperature of cells increases sharply to quickly reach the required temperature, avoids the thermal gradient caused by traditional heating, and a large number of flavonoids are dissolved and released from cells (Makris, 2018). However, Biesaga (2011) showed that when the microwave extraction temperature was too high, the other substances instantly dissolved and large quantities of non-flavonoids were extracted, which caused degradation of the target compound. In the present study, apart from DES-MAE, DES-UAE was also the most time-saving extraction method. Moreover, the high total flavonoid content of 5.84 mg/g was obtained by the DES-MAE extraction with the extraction temperature of 70 °C and extraction time of 44 min. However, evaluation of the total flavonoids content is only a rough measurement. Therefore, there is a need to further analyze the differences in flavonoid contents to find a suitable method for extracting flavonoids from jujube.

Table 2
Comparison of different extraction methods and optimum process parameter.

Methods	Mole ratio	Water content (%)	Solid-liquid ratio (g/mL)	Extraction Temperature/ power	ExtractionTime (min)	Total flavonoid content (mg/g)
W-WB	–	–	1:45	90 °C	70	3.61
E-WB	–	60	1:24.5	90 °C	48	5.14
DES-UAE	2:1	45	1:40	70 °C	44	5.84
DES-MAE	1:3	34	1:42	700 w	2.67	8.03
DES-EAE	1:3	50	1:45	70 °C	180	6.85

Annotation: Other extraction conditions of DES-EAE: The amount of enzyme 14.5 mg/g, cellulose/pectinase ratio2:1 (mg/mg), enzyme solution pH 5.10.

UHPLC analysis

Flavonoids are important secondary plant metabolites present in several foodborne plants and most medicinal plants, such as fruits, vegetables, and tea. Several flavonoids have been identified, including subclasses of flavonols, flavones, flavanols, flavanones, isoflavones, anthocyanidins, and proanthocyanidins (George, Dellaire, & Rupasinghe, 2017). The biggest flavonoid-associated challenge is poor stability and solubility that mainly depends on their structure, subclass, molecular weight, glycosylation, and esterification resulting in low bioavailabilities.

Fifteen flavonoids were identified in jujube and quantified, most of which belong to subclass flavonols. Flavonols are major flavonoid compounds with a 3-hydroxyl flavonoid backbone structure and different phenolic hydroxyl positions (Seleem, Pardi, & Murata, 2017). Some differences between extracts obtained from different extraction methods are presented in Table 3 ($p \leq 0.05$). Apart from DES-EAE, which did not extract catechins and L-epicatechin, other 15 flavonoids were extracted through all the extraction methods. In addition, the amounts of compounds extracted through DES-MAE were almost the lowest as compared with other methods. Notably, the W-WB and E-WB methods were superior to the other methods of assisted DES in the extraction of catechins and L-epicatechin (belong to the flavanols). Rutin (quercetin 3-O-rutin), a common dietary flavonoid in most plants, has high bioactivities and multiple bioefficacy including anticancer, anti-inflammatory, antimicrobial, and antidiabetic activities (Song et al., 2019). The current study established that among the identified compounds, rutin concentration was the highest for all the five extraction methods. In particular, it was found that the contents of rutin in E-WB, DES-UVE, DES-MAE, DES-EAE, and W-WB extracts were 66.88 ± 1.58, 45.23 ± 3.22, 34.16 ± 0.75, 20.94 ± 0.56, and 7.47 ± 0.09 μg/g, respectively. Catechin exhibited the second highest concentration for the five extraction methods, with its content being significantly higher in E-WB (5.86 μg/g) compared with W-WB (5.46 μg/g) and DES-UAE (1.75 μg/g). In addition, the limits of detection (LOD) and quantitation (LOQ) were determined by gradually decreasing the concentrations of analytes until signals could still be recorded at a signal: noise ratio of 3 (S/N = 3) and 10 (S/N = 10). The LODs of rutin, catechin, and L-epicatechin ranged from 0.5 to 1 ng/mL, whereas their LOQs ranged from 1 to 10 ng/mL. Intra-day RSDs for catechin and L-epicatechin were in the range between 0.06 and 0.15%. Relatively small amounts of flavonoids extracted from jujube included dihydromyricetin, vitexin, (+)-dihydrokaempferol, and naringenin chalcone. The amounts of different types of flavonoids extracted by different extraction methods were completely different.

Table 3
Flavonoid compositions in the extracts identified and quantified by HPLC and UPLC-ESI-MS.

Variables (μg/g)	W-WB	E-WB	DES-MAE	DES-UAE	DES-EAE
Catechin	4.73 ± 0.065^{b}	5.64 ± 0.034^{a}	0.132 ± 0.011^{d}	2.06 ± 0.081^{c}	N.D
Dihydromyricetin	0.0253 ± 0.05^{a}	0.02 ± 0.002^{ab}	0.0153 ± 0.005^{bc}	0.0156 ± 0.005^{bc}	0.01 ± 0.003^{c}
L-Epicatechin	5.55 ± 0.127^{b}	5.83 ± 0.102^{a}	0.05 ± 0.007^{d}	1.71 ± 0.38^{c}	N.D
Rutin	7.47 ± 0.09^{e}	66.88 ± 1.58^{a}	34.16 ± 0.745^{c}	45.2 ± 3.22^{b}	21.0 ± 0.562^{d}
Vitexin	0.067 ± 0.003^{a}	0.021 ± 0.002^{bc}	0.03 ± 0.02^{b}	0.008 ± 0.004^{c}	0.008 ± 0.003^{c}
Quercetin 3-β-D-glucoside	4.058 ± 0.045^{a}	0.94 ± 0.04^{b}	0.286 ± 0.003^{d}	0.352 ± 0.007^{c}	0.171 ± 0.006^{e}
(+)-taxifolin	0.212 ± 0.006^{a}	0.196 ± 0.004^{b}	0.144 ± 0.005^{c}	0.191 ± 0.006^{b}	0.051 ± 0.005^{d}
Quercitrin	1.630 ± 0.02^{a}	1.50 ± 0.047^{b}	0.585 ± 0.018^{c}	0.581 ± 0.031^{c}	0.545 ± 0.047^{c}
(+)-dihydrokaempferol	0.074 ± 0.003^{a}	0.023 ± 0.003^{b}	0.028 ± 0.006^{b}	0.0133 ± 0.005^{c}	0.027 ± 0.003^{b}
Luteolin	1.66 ± 0.035^{a}	0.362 ± 0.161^{d}	0.562 ± 0.027^{b}	0.393 ± 0.025^{c}	0.296 ± 0.016^{e}
Quercetin	2.21 ± 0.081^{a}	0.761 ± 0.035^{b}	0.694 ± 0.034^{b}	0.461 ± 0.034^{c}	0.373 ± 0.015^{d}
Naringenin chalcone	0.195 ± 0.003^{a}	0.061 ± 0.009^{a}	0.203 ± 0.27^{a}	0.02 ± 0.01^{a}	0.035 ± 0.023^{a}
Apigenin	0.779 ± 0.026^{a}	0.149 ± 0.004^{c}	0.187 ± 0.014^{b}	0.126 ± 0.008^{cd}	0.119 ± 0.013^{d}
Kaempferol	1.94 ± 0.018^{a}	0.457 ± 0.06^{b}	0.481 ± 0.043^{b}	0.297 ± 0.004^{c}	0.245 ± 0.011^{c}
Isorhamnetin	2.51 ± 0.032^{a}	0.528 ± 0.032^{c}	0.583 ± 0.009^{b}	0.38 ± 0.017^{d}	0.324 ± 0.021^{e}

ND = not detected. Different letters in the same row indicate significant differences ($p < 0.05$) between the samples.

In this study, the DES-UVE method had higher extraction efficiency for 15 flavonoids. Notably, it was noted that E-WB and DES-UAE methods were suitable for extracting rutin. Globally, DES-UAE has been used to extract bioactive substances from plants. In addition, DES had higher extraction efficiency, as compared to other solvents, especially water. In addition, it has been documented that the yield of anthocyanins from grape skin was double or even higher from DES-UAE method than from of the other conventional methods of extraction (Jeong, Zhao, et al., 2015). Further, the DES-UVE method is remarkably more efficient as compared with other the traditional extraction methods that are time-consuming and use volatile organic solvents. In the extraction of phenolic compounds from grape skin using DES, the best extraction method was UAE, which had a higher extraction efficiency than MAE and the conventional extraction methods (Cvjetko Bubalo et al., 2016). In the present study, the ultrasonic treatment temperature was lower than that of other methods, which prevented the degradation and oxidation of tested flavonoids. In addition, the presence of many hydroxyl groups in flavonoid promotes its degradation, whereas the presence of sugars and methoxy groups prevent flavanoid degradation (Biesaga, 2011). Higher content of rutin was obtained by DES-UAE method, indicating that the sugar groupo of rutin and mild ultrasound extraction conditions played a certain protective role on the structure of rutin. The extraction of plant natural compounds using the DES-EAE method has been extensively studied in recent years. Enzymes degrade cellulose and pectin in cell walls and membranes. Thus, they are ideal catalysts that facilitate extraction while reducing mass transfer resistance of naturally active compounds (Chiang & Lai, 2019; Wei et al., 2019; Zhang et al., 2020). However, enzymatic hydrolysis conditions are relatively strict and require suitable temperature, pH, and other factors. Results showed that DES-MAE extraction was less effective and time consuming compared with other extraction methods. High temperatures and long extraction times may lead to degradation of the target compound due to unwanted reactions in the extraction solvent. Therefore, the stability of extracted compounds should be verified to identify the optimal extraction method for flavonoids.

Stability of the flavonoids extracted by different methods

Storage conditions influence the quality flavonoids and their by-products. This is because flavonoids are unstable and easily undergo changes during storage (West & Mauer, 2011). Various degradation pathways of flavonoid are affected by oxygen, light, and temperature

(Biesaga, 2011). Storage conditions, including temperature, time, or techniques affect the synthesis, retention, and decomposition of flavonoids. The degradation rate varies with the complexity of processing (Ren, Nian, & Perussello, 2020). These processes affect the structures of the flavonoids and their antioxidant activities (Chaaban et al., 2017). In the current study, the levels of total flavonoids content were taken as indices to determine stability of different extraction methods.

Flavonoid stability and bioactivity were particularly affected by temperature. For instance, flavonoids were more or less sensitive to heat treatment depending on their structure. Compared to aglycone flavonoids, glycosylated flavonoids are more resistant to heat treatment. Results of the current study established that the flavonoids extracted by different methods showed significant differences in temperature sensitivity (Fig. 2a). Flavonoids extracted using the DES-UAE method in the temperature range of 4-90 °C were more stable, as compared with those extracted using other extraction methods. DES-UAE extraction at a high temperature of 90 °C for 2 h resulted in a flavonoid retention of 98.15 ± 0.51%. The stability of flavonoids decreased in the following order: DES-UAE > E-WB > DES-MAE > DES-EAE > W-WB. In a previous study, the stability of sweet potato leaf flavonoids after heat treatment at 75 °C for 90 min or HHP treatment at 600 MPa for 30 min was high, which did not cause any significant destruction (J. Liu, Mu, Sun, & Fauconnier, 2020). Under milder operating conditions, DES-UAE was protective against the decomposition of certain flavonoids (Makris, 2018). In addition, it has been reported that heat treatment reduces flavonoid contents due to thermal degradation. However, some heat treatment methods including short time frying have been shown to improve flavonoid retention by inactivating plant microbes and/or enzymes (Ren et al., 2020). In this study, stability of the flavonoids extracted using DES-UAE, DES-EAE, and DES-MAE methods increased in the temperature range of 80-90 °C (Fig. 2a). This observation may be explained by the possibility that DES preserved the structure of rutin. It has been reported that there is a correlation between rutin structure, antioxidant activities and heat treatment. Therefore, high temperatures can reduce the degradation rate of some flavonoids.

Fig. 2b shows changes in stability of flavonoids extracted using the five extraction methods after 21 days. The rate of degradation of flavonoids extracted using the DES-UAE method was lower compared with that of other extraction methods implies that the method maintained a good flavonoid stability (flavonoid retention rate was 64.25 ± 2.21%) after 21 days. On the other hand, flavonoids extracted using the E-WB method exhibited the most gentle downward trend and the stability was second, only to DES-UAE method. Flavonoids extracted using the DES-EAE method were the least stable with retention of only 30% residue. It is worth noting that the stability of flavonoids extracted using several methods degraded quickly during the early stages, but slowed down in the latter stages. The current study speculated that flavonoid degradation was associated with flavonoid accumulation. Notably, W-WB and E-WB extraction methods showed turbidity during storage, especially in water, whereas methods-assisted DES did not. This indicated that W-WB and E-WB are unsuitable for extracting flavonoids in terms of storage stability.

Extracts from different extraction methods were placed under different light sources for 44 h. It was noted that light avoidance was beneficial for flavonoid storage, maintaining the high retention rate of flavonoids (Table 4). Further, ultraviolet radiation in between 250 and 260 nm range was lethal to most microorganisms, including bacteria and viruses, and can be used to reduce incidences of putrefying mold. In a separate study, Ren et al. (2020) showed that the levels of flavonoid in some plants increased at low (1.2 kJ/m^2) and medium (6.0 kJ/m^2) UV doses, whereas the levels of quercetin decreased at high (12 kJ/m^2) UV doses. The effects of various doses of UV on different types of flavonoids are dissimilar. The five extraction methods showed fastest flavonoid decline rates in UV radiation. The retention rate of flavonoids extracted using W-WB was comparatively the high, which may be attributed to the degree of accumulation as speculated earlier (W-WB yield was the lowest). In addition, the retention rate of flavonoids was maintained at about 80% when water and alcohol were used as solvents, under the three light sources. In the 44 h period, the highest retention rate of flavonoids was 86.44 ± 2.45% by DES-UAE extraction method, whereas that of DES-EAE was lowest. Currently, the DES-UAE method is widely used for flavonoid extraction and it is therefore suitable for extraction of flavonoids from jujube. Compared to the other methods, this method showed better extraction rates and maintained good flavonoid stability under different light and temperature conditions as well as storage time.

Conclusions

The current study evaluated the effects of five different extraction methods on flavonoids in Jujube (total flavonoid contents, flavonoid composition, and stability). Fifteen kinds of flavonoids were identified from jujube extracts, among which rutin was the most abundant, especially when E-WB and DES-UAE methods were used for extraction. High temperatures are associated with dissolution of non-flavonoids, resulting in high yields of total flavonoids when using the DES-MAE approach. Using the DES-UAE method, at a high temperature of 90 °C for 2 h and

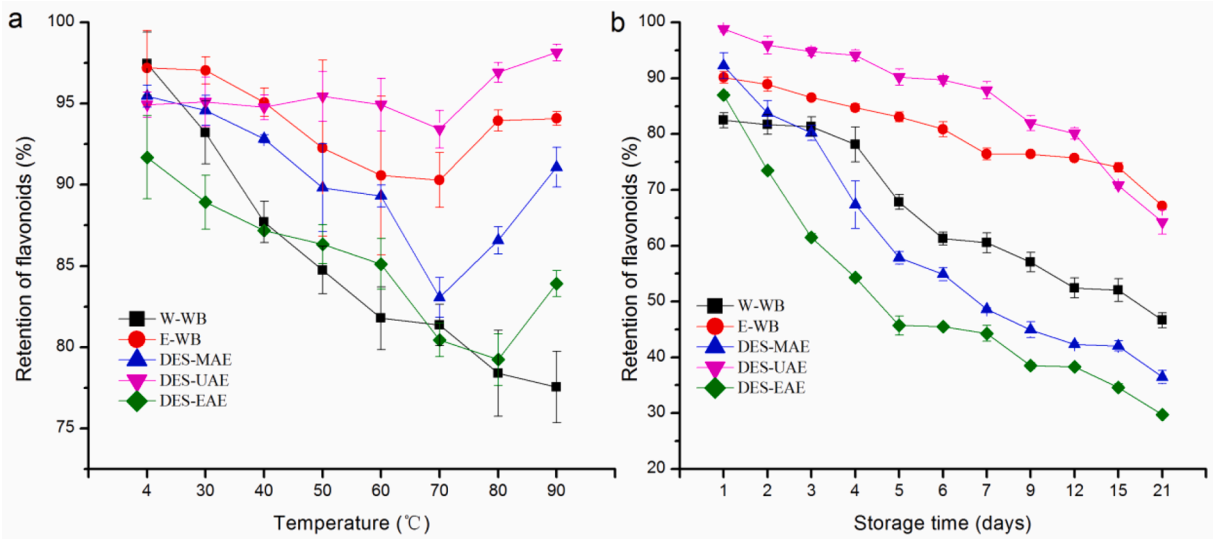

Fig. 2. Effect of temperature (a) and Storage time (b) on stability of flavonoids in jujube.

Table 4
Stability of flavonoids in extracts under different light sources.

Variables	Time (hour)	Water-Water bath	Ethanol-Water bath	DES-MAE	DES-UAE	DES-EAE
UV radiation	2	98.35 ± 0.71%	89.41 ± 0.63%	89.87 ± 0.84%	95.22 ± 0.27%	97.58 ± 1.56%
	8	93.00 ± 2.57%	79.92 ± 0.63%	91.84 ± 1.36%	88.75 ± 1.60%	84.95 ± 0.30%
	20	90.95 ± 1.89%	81.57 ± 2.27%	83.10 ± 1.27%	81.50 ± 1.62%	73.18 ± 1.56%
	32	81.89 ± 2.57%	78.40 ± 0.83%	74.26 ± 1.12%	71.96 ± 2.82%	66.09 ± 1.97%
	44	80.66 ± 3.11%	77.79 ± 1.04%	66.81 ± 1.71%	65.79 ± 2.33%	55.54 ± 0.52%
Indoor natural light	2	93.8 ± 1.23%	87.0 ± 2.51%	92.50 ± 0.88%	99.30 ± 2.12%	93.2 ± 1.08%
	8	95.88 ± 1.43%	80.19 ± 0.86%	89.45 ± 0.42%	90.60 ± 1.67%	92.56 ± 0.30%
	20	93.83 ± 1.23%	83.36 ± 1.72%	87.34 ± 1.52%	89.21 ± 0.92%	82.35 ± 1.59%
	32	77.37 ± 0.71%	81.91 ± 1.09%	80.73 ± 0.24%	80.43 ± 1.67%	71.11 ± 1.87%
	44	81.48 ± 2.14%	80.06 ± 0.95%	74.96 ± 0.64%	83.51 ± 1.16%	65.22 ± 1.31%
Under dark light	2	95.47 ± 2.57%	89.41 ± 1.95%	92.69 ± 1.36%	98.00 ± 3.03%	92.39 ± 0.52%
	8	97.53 ± 3.27%	84.46 ± 4.56%	90.44 ± 0.64%	93.84 ± 1.67%	92.73 ± 1.2%
	20	87.65 ± 0.71%	85.28 ± 2.75%	90.72 ± 1.12%	90.29 ± 0.71%	82.18 ± 1.59%
	32	82.30 ± 0.71%	85.14 ± 0.63%	85.09 ± 0.49%	89.68 ± 1.22%	76.30 ± 2.08%
	44	81.07 ± 1.43%	81.29 ± 0.48%	78.90 ± 0.73%	86.44 ± 2.45%	70.07 ± 1.04%

21 days of dark storage, retention of flavonoids was 98.15 ± 0.51% and 64.25 ± 2.21%, respectively. On the other hand, the flavonoid retention was 86.44 ± 2.45% under dark light conditions. Therefore, compared to the other methods, the DES-UAE method can efficiently reduce the degradation of some flavonoids under different conditions. Based on findings of the present study, the DES-UAE approach is efficient and environmentally friendly for extracting flavonoids from jujube because it maintains their bioavailability and stability. However, there is need for Follow-up studies should explore the specific mechanisms for the effects of storage conditions on individual flavonoids and generation of new by-products.

CRediT authorship contribution statement

Xiu-min Liu: Conceptualization, Methodology, Formal analysis, Writing – original draft, Data curation, Writing – review & editing. **Ya Liu:** Conceptualization, Methodology, Formal analysis, Writing – original draft. **Chun-hui Shan:** Conceptualization, Methodology. **Xin-quan Yang:** Conceptualization, Methodology. **Qin Zhang:** Software. **Na Xu:** Software. **Li-ying Xu:** Software. **Wen Song:** Software.

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number: 32060527); Xinjiang Production and Construction Corps Innovation Team Building Projects in Key Areas (grant number: 2017CB012); Projects of Innovation and Development Pillar Program for Key Industries in Southern Xinjiang of Xinjiang Production and Construction Corps (grant number: 2018DB002).

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