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A Rapid and Non-Thermal Process of Five Flavonoids from Safflower using Ultrahigh Pressure-Assisted Extraction

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A rapid and non-thermal process using ultrahigh pressure extraction has been applied in extracting five flavonoids from safflower. The optimized ultrahigh pressure extraction conditions of the target compounds was 40 % ethanolic solution (v/v) as extraction solvent, 100 MPa of extraction pressure, 1 min of extraction time, 1:30 (g/mL) of solid/liquid ratio and only 1 cycle. Compared with HRE and ultrahigh pressure extraction, the ultrahigh pressure extraction technique showed highest efficiency in extracting target components. The yields of hydroxysafflor yellow A, rutin, safflor yellow A, astragalin and kaempferol were 31.59, 0.23, 11.86, 1.31 and 0.080 mg/g, respectively. The contents of five flavonoids in seven safflowers from different regions were analyzed by the ultrahigh pressure extraction-HPLC method. The samples with highest total content of five flavonoids originated from Xinjiang, China. In addition, SEM micrographs revealed that the florets tissues were disrupted after ultrahigh pressure extraction process in the optimized conditions.

Keywords: Safflower, Flavonoids, Ultrahigh pressure extraction.

INTRODUCTION

Carthamus tinctorius L., commonly known as safflower in Asian, is the only species of this genus in China, which has been used as natural pigment additive for food and traditional Chinese medicinal herb[1]. The florets of safflower are commonly used for promoting blood circulation[2], treating heart disease, cerebral thrombosis and male sterility[3]. Flavonoids are main biological active compounds in the florets of safflower, which share many pharmacological effects including antidiabetic[4], antioxidant[5], antiproliferation of cancer[6] and antimelanogenesis activity[7].

In previous studies, bioactive compounds in safflower have been extracted by heat reflux extraction[8,9] and ultrasonic-assisted extraction[10]. These methods are time consuming with high solvent consumption. Microwave-assisted extraction[11] was also used for extraction of safflower yellow at much reduced extraction time. However, it is a heat process that may destroy or degrade natural products with low thermal stability. Therefore, new extraction techniques involve both short extraction time and non-thermal process are demanded.

The ultrahigh pressure technique, ranging from 100 MPa to 800 MPa, has been widely used in pharmaceutics and food industry[12]. Ultrahigh pressure extraction is a novel technology to enhance solvent permeability in cells and mass transport phenomena[13]. Ultrahigh pressure extraction technique can reduce the processing time and obtain higher extraction yield[14-17]. Thus, it could be used to extract bioactive compounds from plants or herbal materials in short time involving non-thermal process. Recently, ultrahigh pressure extraction was used for extraction of salidroside from *Rhodiola sachalinensis*[18], polyphenols from green tea leaves[19], anthocyanins from grape[20], ginsenosides from ginseng[21] and lignans from *Dysosma versipellis*[22]. All of these applications have demonstrated that ultrahigh pressure extraction results in high product yields with fast processing time. However, there is no report about using ultrahigh pressure extraction techniques for extraction of active compounds from safflower until now.

In this study, we developed a rapid and non-thermal ultrahigh pressure extraction method for five flavonoids from safflower. After ultrahigh pressure extraction processing, five flavonoids were simultaneously analyzed using an HPLC/DAD. The peaks were assigned based on retention time and UV spectrum. The chemical structures of the five flavonoids, including hydroxysafflor yellow A, rutin, safflor yellow A, astragalin and kaempferol are shown in Fig. 1. Then, the extraction results by ultrahigh pressure extraction were compared with conventional extraction methods. Seven batches of safflower samples collec-

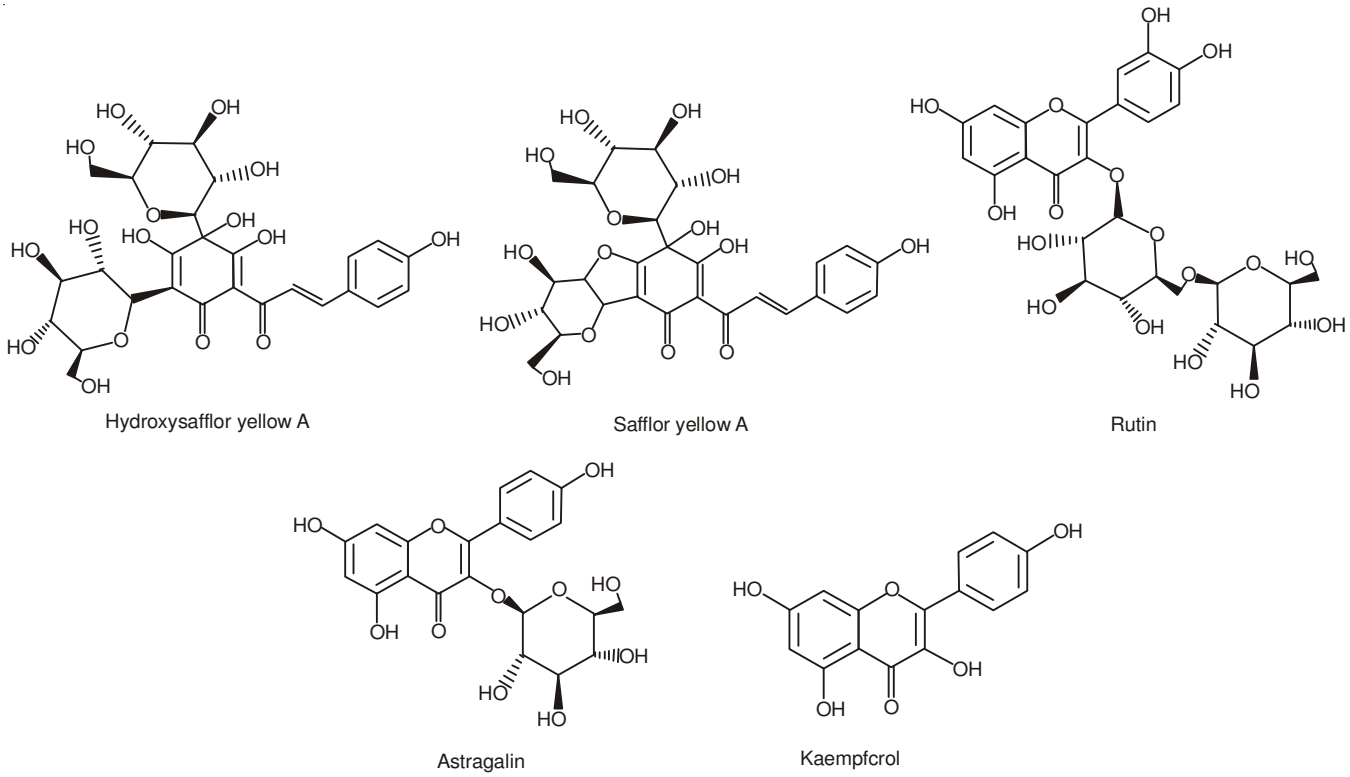

Fig. 1. Chemical structures of the five flavonoids extracted from safflower by ultrahigh pressure extraction

ted from different sources were extracted under optimized ultrahigh pressure extraction condition. The microstructure of the untreated and ultrahigh pressure extraction treated samples were examined by scanning electron microscopy (SEM).

EXPERIMENTAL

The *Carthamus tinctorius* L. samples were purchased from herbal origin markets in seven provinces of China and were identified by Dr. Jia Li (Shandong University of Traditional Chinese Medicine, Jinan, Shandong, China). The sources of the seven tested samples are listed in Table-3. All safflower samples were harvested in 2012. The herbs were dried in an oven (50 °C for 24 h), then powdered and sieved through a 40 mesh screen.

Standard hydroxysafflor yellow A, rutin, safflor yellow A, astragalin and kaempferol were purchased from National Institute of the Control of Pharmaceutical and Biological Products (China). Ethanol and methanol were all of analytical grades (Guangcheng Chemical, Tianjin, China). Chromatographic grade methanol and phosphoric acid (Kemiou Chemical, Tianjin, China) were used for High Performance Liquid Chromatography (HPLC) analysis and the water used was distilled water.

Ultrahigh pressure-assisted extraction was conducted with a High Hydrostatic Pressure Processor (HPP. L3-600, Huataisenmiao Biology Engineering Technology, Tianjin, China). The pressure ranged from 0 to 900 MPa and the pressure precision was controlled at ± 5 MPa.

HPLC analysis was carried out on an Agilent 1120 HPLC equipped with G4290A system, an autosampler and a DAD detector (Agilent, California, USA).

Ultrahigh pressure extraction: In each test 0.5 g sample powder was extracted with solvent and the mixture was poured into a polyethylene bag. Extractions were performed at different solvents composition of water, methanol solution (20, 40, 60 and 80 % v/v) and ethanol solution (20, 40, 60 and 80 % v/v); extraction pressure (100, 200, 300, 400 and 500 MPa); time (1, 2, 3, 4 and 5 min); Solid/liquid ratio (1:10, 1:20, 1:30, 1:40 and 1:50, g/mL) and number of cycles (1, 2 and 3). After ultrahigh pressure extraction, the extraction solution was centrifuged at 4000 rpm for 10 min. Then the supernatant were evaporated and redissolved to 50 mL with ethanol and filtered through 0.22 mm membrane. The filtrate was injected into the HPLC for further analysis. All experiments were carried out in triplicate.

Traditional extraction methods: Heat reflux extraction and ultrasonic extraction were chosen as the conventional extraction methods for comparison. The dried safflower (1 g) sample was weighed in a 100 mL round bottom flask and 30 mL extraction solvent (40 % ethanol solution) was added. Extraction was carried out at boiling state for a given time.

The dried plant sample (1.0 g) was weighed in a 100 mL flask and 30 mL of 40 % ethanol solution was added. The flask was sonicated for 0.5 h, 50 kHz and 300 W by an ultrasonicator (SB-3200DT, Xinzhi Biochemical, Ningbo, China).

The extraction solution was centrifuged at 4000 rpm for 10 min. Then the supernatant were evaporated and redissolved to 50 mL with ethanol and filtered through 0.22 mm membrane. Then, the filtrate was injected into the HPLC for further analysis.

HPLC analysis: The crude extracts were analyzed by HPLC. Chromatographic separations were accomplished with

an Inertsil ODS-SP C18 column (250 × 4.6 mm, 5 μm) at room temperature. The mobile phase consisted of A (methanol) and B (0.4 % phosphoric acid solution, v/v), used in gradient elution: 0-10 min, 20-40 % A; 10-25 min, 40-50 % A; 25-32 min, 50-70 % A; 32-38 min, 70-100 % A; 38-45 min, 100 % A. The flow rate was 1 mL/min, the injection volume was 10 mL and the effluent was monitored at 350 nm. Hydroxysafflor yellow A, rutin, safflor yellow A, astragalin and kaempferol in each extract were identified by comparing their retention time and the UV absorption with standard solutions. All experiments were carried out in triplicate.

Statistical analysis: The one way ANOVA test was used to calculate the significance of the differences of extraction efficiency for the flavonoids. The results of HPLC analysis were expressed as means of extraction efficiency ± SD.

Examination of florets microstructure: After ultrahigh pressure extraction processed in optimum conditions, the mixture was centrifuged at a speed of 4000 rpm for 10 min. The supernatants were discarded and the residues were dried at 50 °C for 12 h in an oven. The remaining samples were fixed on a specimen holder with aluminum tape and then sputtered with gold in a sputter-coater. All the specimens were examined with a Zeiss Supra-55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) under high vacuum condition and at an accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Optimization of ultrahigh pressure extraction procedure: Four major parameters of ultrahigh pressure extraction were optimized: extraction solvents water, methanol solution (20, 40, 60 and 80 % v/v), ethanolic solution (20, 40, 60 and 80 % v/v); pressure at 100, 200, 300, 400 and 500 MPa; time of 1, 2, 3, 4 and 5 min; solid/liquid ratio (1:10, 1:20, 1:30, 1:40 and 1:50 g/mL); and number of cycles (1, 2 and 3). The concentration of target compounds was used as the marker for evaluation of extraction efficiency.

Nine different composition of ethanol and methanol of different composition in water (0, 20, 40, 60 and 80 % v/v) as solvents were performed in condition of 200 MPa, 2 min and 1:20 (g/mL) of solid/liquid ratio at room temperature. Different polarities of the extraction solvent led to different extraction yields for the five flavonoids. As shown in Fig. 2a, when ethanol percentage from 0 to 60 %, the extraction yield of hydroxysafflor yellow A are obvious higher than in 80 % ethanol solution. The maximum extraction yield was achieved using 40 % ethanol solution for other four flavonoids. Fig. 2b demonstrated a similar trend as Fig. 2a showing that the sum of target compounds contents was increased with the increase of methanol concentration from 0 to 60 %. The extraction yield of flavonoids decreased strongly with the methanol concentration higher than 60 %, except for astragalin. Extraction with 40 % ethanolic solution resulted in the best extraction yield and the sum of five flavonoids contents was 35.59 mg/g.

The effect of extraction pressure was investigated in the range of 100-500 MPa. The extraction solvent was 40 % ethanolic solution, the extraction time was 2 min and the solid/liquid ratio was 1:20 (g/mL) and the result is plotted in Fig. 2c. It shows that the extraction yield did not significant change in the experiment pressure ranges. During the pressure promoting period, the extracting solvent comes into cells to integrate with bioactive compounds. When the ultrahigh pressure is released the cell wall is disrupted to release the solvent with target compounds. According to a previous report pressure of 100 MPa is enough to cause rupture of intracellular vacuoles and plant cell walls in onions[23]. Therefore, when extraction pressure reached 100 MPa, the safflower cell wall might be broken and the extraction yield reached the maximum value rapidly.

The effects of extraction time were also investigated in condition of 100 MPa, 40 % ethanol solution and a solid/liquid ratio of 1:20 (g/mL). As shown in Fig. 2d, the extraction efficiency of target compounds had no significant change when increasing the extraction time from one minute. The different pressure between the inner and the exterior of the cell is very large under ultrahigh pressure extraction conditions. Under this large differential pressure, the solvent permeates very fast through the broken cell membranes and the mass transfer rate or dissolution are very quick. This leads to a very short extracting time with ultrahigh pressure extraction. Therefore, 1 min was sufficient for the process of ultrahigh pressure extraction.

The influence of raw material to solvent ratio and number of cycles on the extraction yield was evaluated at 40 % ethanol, pressure 100 MPa and extraction time of 1 min, as is shown in Fig. 2e and f. The extraction yield increases with the increasing of the solvent volume. Fig. 2f shows the yields obtained for extraction cycles from 1 to 3. The extract contents of the five flavonoids were similar in different number of extraction cycle. Taking the extraction yield, the solvent and processing costs into consideration and the best choice of the ratio is 1:30 (g/mL) at 1 cycle.

The optimal conditions for extraction of the five flavonoids by ultrahigh pressure extraction were 40 % ethanolic solution, 100 MPa of pressure, 1 min, 1:30 (g/mL) of solid/liquid ratio at 1 cycle. Under the optimum ultrahigh pressure extraction conditions, the extraction yields of hydroxysafflor yellow A, rutin, safflor yellow A, astragalin and kaempferol were 31.59, 0.23, 11.86, 1.31 and 0.080 mg/g, respectively. HPLC chromatograms of the standard compound solutions and compounds extracted from safflower under optimal ultrahigh pressure extraction conditions are shown in Fig. 3.

Validation of ultrahigh pressure extraction quantitative method: Table-1 shows some of the parameters used to investigate the analytical performance of the optimized ultrahigh pressure extraction method. These parameters include calibration curves, correlation coefficients (R^2), calibration range, recovery, limits of detection (LOD) and limits of quantification (LOQ). Good linearity was observed and all the correlation coefficients were ≥ 0.9990. The LOD and the LOQ of the analytes were within 0.019-0.033 and 0.058-0.100 ng/mL, respectively. The range of recovery was 92.3-103.8 %. All of these above showed that the present extraction method was credible.

Comparison of ultrahigh pressure extraction with conventional methods: To compare ultrahigh pressure extraction with other extraction methods, parallel experiments were carried out using heat reflux extraction and ultrasonic-assisted extraction. The extraction yields of five flavonoids obtained

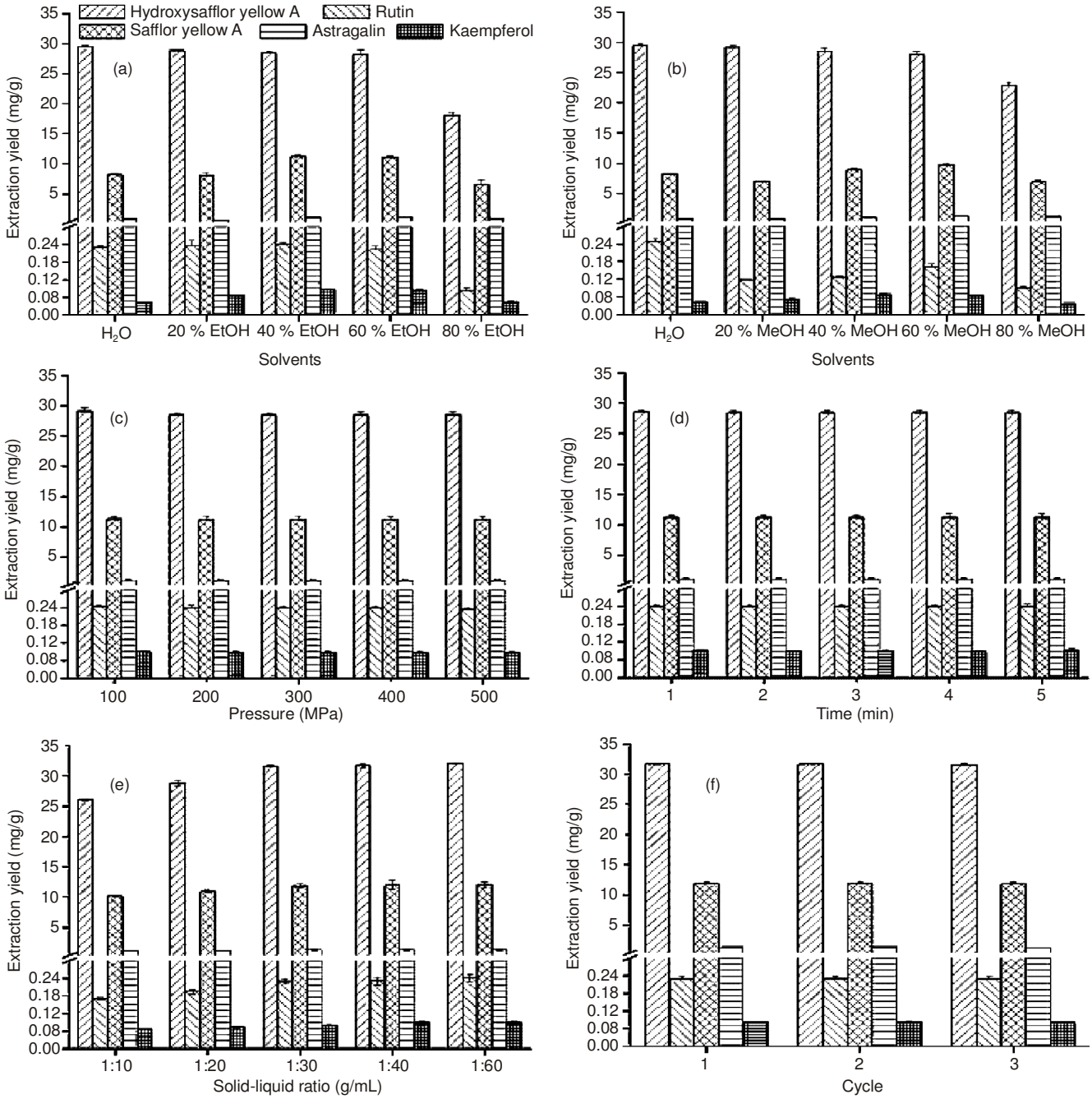

Fig. 2. Effects of ethanol concentration; (a) methanol concentration; (b) extraction pressure; (c) time; (d) solid/liquid ratio (e) and cycle (f) on the extraction yield of five flavonoids from safflower by ultrahigh pressure extraction

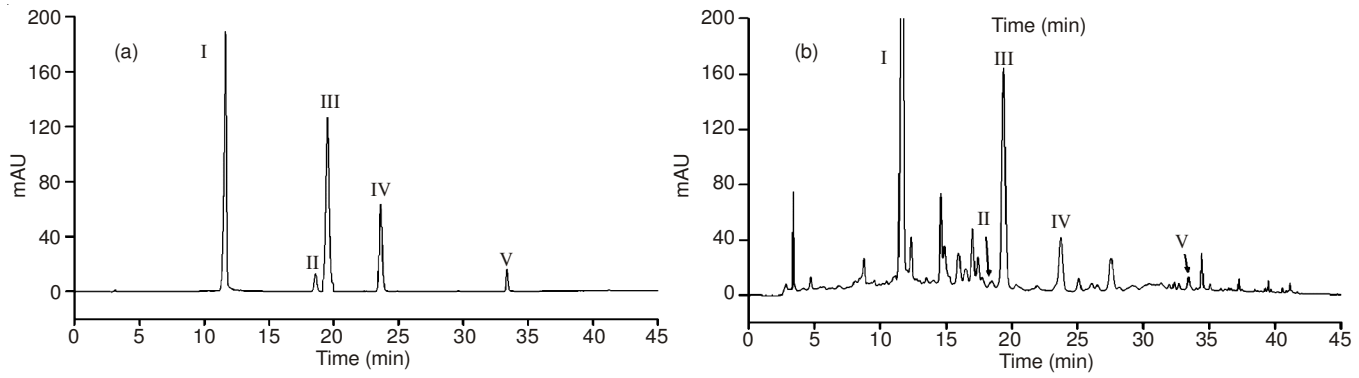

Fig. 3. HPLC chromatogram of (a) the standards mixture of five flavonoids; (b) the safflower extracts by ultrahigh pressure extraction at optimum parameters: 100 MPa of pressure, 1 min and 1:30 (g/mL) of solid/liquid ratio in 40 % ethanol (v/v). Peaks of the target analytes: (I) hydroxysafflor yellow A; (II) rutin; (III) safflor yellow A; (IV) astragalin and (V) kaempferol

using ultrahigh pressure extraction, ultrasonic-assisted extraction and heat reflux extraction are shown in Table-2. The extraction yields for ultrahigh pressure extraction were higher than ultrasonic-assisted extraction in 10 min and heat reflux extraction in 0.5 h. In same solvent and solid/liquid ratio system, ultrahigh pressure extraction had similar extraction yield compared with ultrasonic-assisted extraction in 0.h and heat reflux extraction in 60 min. Ultrahigh pressure extraction required only 1 min, which is 1/60 of the time required for heat reflux extraction and 1/30 of the time required for ultrasonic-assisted extraction. Furthermore, the ultrahigh pressure extraction was processed in room temperature that heat consumption is remarkable less than that required by other extraction methods. Therefore, ultrahigh pressure extraction method obviously reduced the extraction time and heat consumption for extraction of flavonoids from safflower.

Quantification of flavonoids content in safflower: Seven samples collected from different sources were extracted under optimized ultrahigh pressure extraction condition. Significant variations were found in the contents of these compounds in the samples and the results are listed in Table-3. Hydroxysafflor yellow A and safflor yellow A contribute in the total yield. And the contents were 15.48-41.17 and 8.72-15.67 mg/g. The highest total content of five flavonoids was 55.91 mg/g, found in Xinjiang. And lowest content was 26.01 mg/g, found in Henan, China.

Structural changes after ultrahigh pressure extraction: In order to elucidate the microstructure and to understand the extraction mechanism, the untreated florets and ultrahigh pressure extraction samples were examined by SEM. Fig. 4a and b show the micrographs of the untreated sample in different magnifications, respectively. In the untreated sample, some granules attached on the florets surface and the stripes of florets tissues kept intact. While after ultrahigh pressure extraction treatment, as shown in Fig. 4c, burr-like projection were generated on the florets surface. Further magnification micrograph shown that the florets tissues were found completely cracked which shown in Fig. 4d. This result demonstrated that when pressure reached 100 MPa florets tissues and cellulose were ruptured, which enhanced the mass transfer of the solvents into the materials and the soluble constituents into the solvents.

Conclusion

In this paper, a rapid and non-thermal extracting system for five flavonoids from safflower by ultrahigh pressure extraction was established. Compared with the other techniques the ultrahigh pressure extraction was found to be better in terms of time consuming, the extraction time was only 1 min. It greatly reduced the extraction time. This method is an alternative extraction technique for the fast extraction of flavonoids from safflower. In addition, processing using ultrahigh pressure

TABLE-1
LINEAR REGRESSION DATA, RECOVERY, LOD AND LOQ OF INVESTIGATED COMPOUNDS

Analytes	Linearity	Correlation coefficient (R^2)	Calibration range (μg/mL)	Recovery (%) ± S.D.[b] (%)	LOD (ng/mL)	LOQ (ng/mL)
Hydroxysafflor yellow A	Y = 802.52x+75.33[a]	0.9991	0.61-50.33	92.3 ± 2.81	0.028	0.085
Rutin	Y = 1406.83x-4.56	0.9998	0.029-0.72	95.1 ± 3.69	0.031	0.094
Safflor yellow A	Y = 1762.38x+46.27	0.9991	0.89-20.39	97.2 ± 5.07	0.019	0.058
Astragalin	Y = 2174.85x+32.58	0.9995	0.092-2.30	103.8 ± 3.67	0.024	0.073
Kaempferol	Y = 3551.01x-1.46	0.9990	0.0088-0.22	96.3 ± 2.58	0.033	0.100

[a]x, y are sample concentration and the absorption peak area, respectively; [b] S.D. is standard deviation

TABLE-2
COMPARISON OF EXTRACTION YIELDS UNDER DIFFERENT EXTRACTION METHODS (n = 3) AT 1:30 (g/mL) OF SOLID/LIQUID RATIO

Methods	Time (min)	Extraction yields (mg/g) ± S.D. (%)				
		Hydroxysafflor yellow A	Rutin	Safflor yellow A	Astragalin	Kaempferol
Ultrahigh pressure extraction	1	31.59 ± 5.06[b]	0.23 ± 0.83[ab]	11.86 ± 3.75[b]	1.31 ± 1.66[a]	0.080 ± 0.27[b]
Ultrasonic-assisted extraction	10	10.22 ± 3.51[f]	0.06 ± 0.42[e]	3.97 ± 1.25[e]	0.32 ± 0.81[c]	0.019 ± 0.20[c]
Ultrasonic-assisted extraction	30	29.34 ± 4.47[d]	0.20 ± 0.75[c]	11.04 ± 3.16[d]	1.09 ± 1.37[b]	0.062 ± 0.41[d]
Heat reflux extraction	30	12.32 ± 3.51[e]	0.08 ± 0.32[d]	4.18 ± 1.34[e]	0.36 ± 0.71[c]	0.027 ± 0.31[e]
Heat reflux extraction	60	30.82 ± 5.27[c]	0.22 ± 0.91[b]	11.50 ± 3.28[c]	1.30 ± 1.21[a]	0.071 ± 0.30[f]
Heat reflux extraction	120	32.01 ± 5.92[a]	0.24 ± 0.94[a]	12.32 ± 3.19[a]	1.36 ± 1.62[a]	0.087 ± 0.35[a]

a, b, c, d, e, f represent significance level 5 %

TABLE-3
CONTENTS (mg/g) OF INVESTIGATED COMPOUNDS IN *Carthamus tinctorius* L. FROM DIFFERENT REGIONS IN CHINA (n = 3)

Samples	Source	Extraction yields (mg/g) ± S.D. (%)					Total
		Hydroxysafflor yellow A	Rutin	Safflor yellow A	Astragalin	Kaempferol	
1	Gansu	19.88 ± 4.23	0.26 ± 0.96	12.53 ± 3.19	0.39 ± 0.98	0.09 ± 0.35	33.15
2	Xinjiang	41.17 ± 7.35	0.22 ± 0.82	12.90 ± 4.52	1.55 ± 1.89	0.08 ± 0.31	55.92
3	Shandong	24.32 ± 5.21	0.17 ± 0.76	8.72 ± 2.17	1.24 ± 1.22	0.05 ± 0.21	34.5
4	Hubei	28.25 ± 4.66	0.16 ± 0.69	15.67 ± 3.96	0.77 ± 1.01	0.12 ± 0.18	44.97
5	Henan	15.48 ± 3.81	0.15 ± 0.72	9.50 ± 2.87	0.80 ± 1.09	0.09 ± 0.49	26.02
6	Hebei	22.74 ± 4.57	0.19 ± 0.92	10.76 ± 3.69	1.25 ± 1.28	0.09 ± 0.52	35.03
7	Sichuan	31.59 ± 5.06	0.23 ± 0.83	11.86 ± 3.75	1.31 ± 1.66	0.08 ± 0.27	45.07

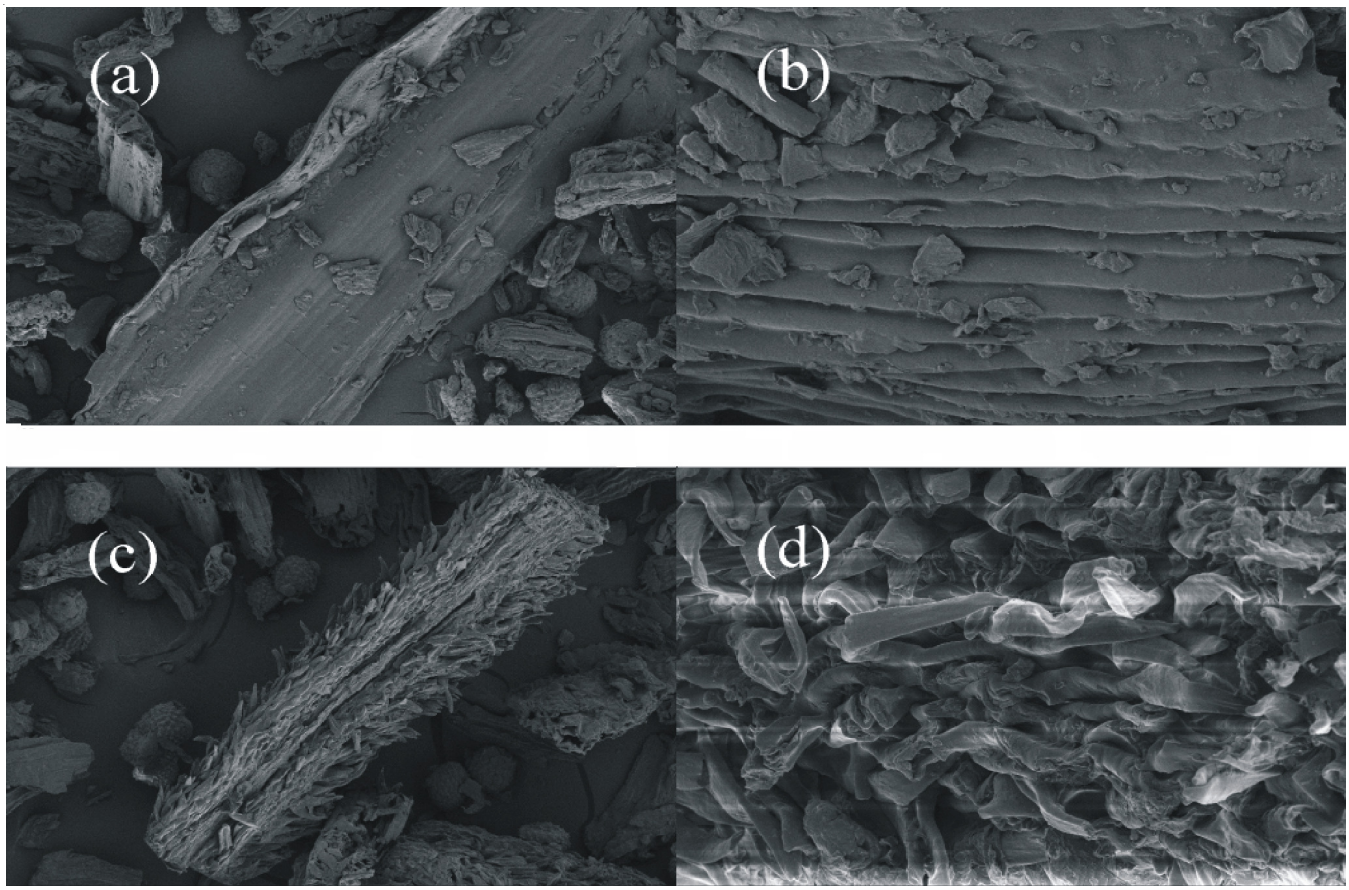

Fig. 4. Scanning electron micrographs of (a and b) untreated; and (c and d) after ultrahigh pressure extraction

extraction can be carried out at room temperature which is favorable for the thermally unstable compounds. It could be a very useful tool for the extraction of natural products.

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