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Characterization of Fatty Oil of *Zizyphi spinosi semen* Obtained by Supercritical Fluid Extraction

Feng Wang · Xuesong Liu · Yong Chen · Longhu Wang

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Abstract *Zizyphi spinosi semen* (ZSS) has been widely used for treatment of insomnia in oriental countries. The aim of this study is to characterize the fatty oil of ZSS obtained by supercritical fluid extraction in terms of chemical composition and physicochemical properties. The chemical composition, including fatty acids and unsaponifiable constituents, was analyzed by gas chromatography–mass spectrometer (GC–MS). The results revealed that 9-octadecenoic acid (43.38 ± 0.03%) and 9,12-octadecadienoic acid (40.58 ± 0.03%) were the main fatty acids, and β-sitosterol (37.39 ± 0.02%) and squalene (30.79 ± 0.01%) were the key unsaponifiables. Furthermore, four indexes were assayed according to Chinese Pharmacopeia (2005) to reflect the physicochemical properties of ZSS oil, their values being determined as follows: acid value (10.3 ± 0.1 mg KOH/g), peroxide value (0.05 ± 0.01 g/100 g), saponification value (194.4 ± 0.5 mg KOH/g) and iodine value (109.7 ± 0.8 g I/100 g). The basic information obtained provides data support for quality evaluation and efficacy research of ZSS oil, and suggests its prospects for development in pharmaceutical and food industries.

Keywords *Zizyphi spinosi semen* · Supercritical fluid extraction · Fatty oil · Chemical composition · Physicochemical properties · GC–MS

F. Wang · X. Liu · Y. Chen · L. Wang (✉)
Department of Chinese Medicine Sciences and Engineering, Zhejiang University, Hangzhou 310058, China
e-mail: wang2000@zju.edu.cn

Introduction

Zizyphi spinosi semen (ZSS), an important traditional Chinese medicine, has been widely applied in oriental countries such as China and Korea for centuries. Its remarkable curative effects, such as hypnotic-sedative, anticonvulsant and analgesic action [1–3], have also been confirmed by a large amount of clinical research. Besides application in pharmaceutical industry, ZSS has been developed as an additive for supplementary health food [4–7] and attracted increasing attention from food industry. The curative effect and commercial value of ZSS are mostly attributed to the fatty oil [8], which has been proven to regulate the level of lipoproteins [9] and improve sleep [10].

Generally, fatty oil can be obtained by conventional extraction methods such as Soxhlet extraction and expelling. However, these techniques usually cause degradation of thermolabile compounds and consequently influence the extraction efficiency of fatty oil as well as the safety of products [11, 12]. Meanwhile, it is hard to remove residual solvent by practical manufacturing techniques to meet product specifications, good manufacturing practices or other quality-based requirements. In this study, to avoid the defects of conventional extraction methods and obtain the original fatty oil, supercritical fluid extraction (SFE) is introduced as an environmentally responsible and efficient extraction technique. Being performed at relatively low temperature without or with only a little organic solvent, SFE has been widely reported for collection of fatty oil from various natural sources in the literature [13–15].

In view of the integral utilization of ZSS oil in pharmaceutical and food industries, it is necessary to collect basic information on characterization of ZSS oil to evaluate its quality and understand its mechanism of action. Hence,

the objective of the present study is to analyze the chemical composition of ZSS oil by GC–MS, and determine its main physicochemical properties according to Chinese Pharmacopeia (2005), so that ZSS oil can be characterized systematically.

Methods

Materials and Reagents

Zizyphi spinosi semen, supplied by Yan'an Changtai Pharmaceutical Co., Ltd., was cultivated in Shanxi Province, China. The materials were chosen with care, and only those at the same stage of maturity were used. The sample was stored in hemp bags at ambient temperature protected from light and then ground in a sample mill immediately prior to extraction.

CO_2 (purity 99.9%) was purchased from Hangzhou Electrochemical-Gas Factory (Zhejiang, China). Iodine and potassium iodide were purchased from Xilong Chemical Plant (Guangdong, China). Bromine was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The other chemicals such as ethanol were used as received.

Preparation of ZSS Oil

To separate fatty oil from ZSS, different extraction methods including SFE and petroleum ether extraction (PEE) were investigated and compared to determine the optimum method and conditions. The fatty oil obtained by the most suitable method which gave the highest yield of ZSS oil was characterized in terms of chemical composition and physicochemical properties.

SFE

SFE was carried out using an extraction system (model HA220-50-06; Hua An Supercritical Co., Ltd., Jiangsu, China) consisting of a 1-L extractor, two 1-L separators, a syringe pump, and a condensing unit. In each experiment, 250 g ZSS powder was loaded into the extractor and the SFE process was employed at various pressures (15–30 MPa), temperatures (35–50 °C), extraction times (1–2.5 h) and particle sizes (0.25–1.45 mm) so that the effect of each parameter could be investigated. After SFE, two layers were formed in the extract: fatty oil at the top and the watery layer underneath. The oil was separated from the water phase using a separatory funnel and its weight was measured for calculating the SFE extraction ratio.

PEE

A conventional solvent extraction method, PEE, was also performed to obtain fatty oil from ZSS. ZSS powder (100 g) was placed in a 2,000-mL round-bottomed flask, and 1,000 mL petroleum ether was added. The flask was connected to a condenser pipe, and the suspensions were boiled for 2 h. Thereafter, the extract was removed, and the extraction process was repeated with fresh extractant once more. Finally the extracts were combined, filtered, and evaporated to get rid of solid foreign matter and petroleum ether. The fatty oil obtained was measured to calculate the PEE extraction ratio.

Chemical Composition

The chemical composition of fatty oil obtained from ZSS by SFE was analyzed using a GC–MS system consisting of an Agilent 6890N GC equipped with a split-splitless injector and a HP-5-MS fused-silica column (5% phenylmethylpolysiloxane, 30 m × 0.25 mm × 0.25 μm; Agilent, USA) and an Agilent 5975I MS equipped with an EI ion source and a quadrupole array detector. Individual components were identified by matching mass spectra to those of reference compounds in the National Institute of Standards and Technology (NIST) Mass Spectra Library, and the relative amount of each component was calculated by dividing each peak area by the total peak area.

Fatty Acids

The fatty acid analysis of ZSS oil was divided into two steps: preparation of fatty acid methyl esters, and GC–MS analysis. The procedure for preparing fatty acid methyl esters was carried out according to the guideline published by Kou et al. [16]. Firstly, 50 mg ZSS oil was weighed accurately and dissolved with 2 mL petroleum ether. Then 2 mL 0.4 mol/L potassium hydroxide methanol solution was added, blended, and set aside for 30 min at room temperature. Finally, distilled water was added and the organic phase was injected into the GC–MS system for analysis after filtering through a 0.45-μm microporous membrane.

GC–MS conditions were confirmed as follows: GC column temperature was programmed from 150 to 215 °C at 5 °C/min, holding for 5 min, then heating at the same rate to 240 °C (1 min) and finally heating at 10 °C/min to 270 °C (5 min). Quadrode, ion source, injector and additional channel temperatures were 150, 230, 250 and 280 °C, respectively. The carrier gas (helium) was adjusted to linear flow rate of 1.0 mL/min. The sample (0.2 μL) was injected into the system in split mode with split ratio of

20:1. Mass spectra were recorded over 50–550 amu range with ionisation energy of 70 eV.

Unsaponifiable Constituents

For analysis of unsaponifiable constituents, ZSS oil also needed to be pre-treated. Firstly 0.5 g ZSS oil was weighed accurately, and 20 mL 2 mol/L sodium hydroxide ethanol solution was added. After circumfluence extraction for 1 h, 15 mL saturated sodium chloride solution was added and the mixture was transferred into a 125-mL separating funnel. Thereafter, 20 mL petroleum ether was added to extract unsaponifiables. Finally, the extract was transferred into a 25-mL volumetric flask, brought up to volume with petroleum ether, and filtered through a 0.45-μm microporous membrane.

GC–MS conditions were confirmed as follows: Quadrode, ion source, injector, GC column and additional channel temperatures were 150, 230, 280, 280 and 290 °C, respectively. The carrier gas was adjusted to linear flow rate of 1.5 mL/min. The sample (1.0 μL) was injected into the system by split mode with split ratio of 20:1. Mass spectra were recorded over 50–550 amu range with ionisation energy of 70 eV.

Physicochemical Properties

As an essential part of the characterization of fatty oil, physicochemical indexes, including acid value, saponification value, peroxide value and iodine value, were assayed according to Chinese Pharmacopeia (2005).

Statistical Analysis

Statistical analysis to determine significant differences among the different treatments was performed using one-way analysis of variance (ANOVA). Least significant differences were calculated at $p = 0.05$. All treatments were carried out in triplicate, and results are expressed as mean ± standard deviation (SD).

Results and Discussion

Preparation of ZSS Oil

In the current study, SFE and PEE were employed and compared to select the favourable method for ZSS oil extraction. For SFE, four important parameters, including extraction time, pressure, temperature and particle size, were investigated to explore the suitable extraction conditions, and all the results are listed in Table 1. The effect of extraction time was investigated first, under otherwise constant conditions (25 MPa, 45 °C and 0.4 mm), and the results showed that extended extraction time increased recovery up to 2 h ($p > 0.05$, ANOVA). After this period, the extraction ratio remained constant. Thus, it is not necessary to use a longer time for efficient extraction, and 2 h was chosen for all subsequent experiments. Then, pressure was studied in the range from 15 to 30 MPa while the other parameters were fixed (2 h, 45 °C and 0.4 mm), presenting a similar impact trend. The extraction ratio increased significantly with increasing pressure up to 25 MPa ($p < 0.05$, ANOVA), so 25 MPa was selected for subsequent experiments. Next, four different temperatures between 35 and 50 °C were tested using fixed extraction time of 2 h, pressure of 25 MPa and particle size of 0.4 mm. The results showed that temperature of 45 °C gave the highest amount of ZSS oil when compared with the other three extraction temperatures ($p < 0.05$, ANOVA). Therefore, subsequent experiments were conducted at 45 °C. Finally, particle size was investigated under fixed conditions in which other parameters were chosen according to the previous investigations as follows: extraction time 2 h, pressure 25 MPa and temperature 45 °C. Similar to the effect trend of temperature, the highest extraction ratio occurred for particle size of 0.32 mm. Hence, 0.32 mm was chosen as an adequate

Table 1 Effect of parameters on SFE of ZSS oil

Parameter	Range	Extraction ratio (%)
Extraction time (h)[a]	1	25.9 ± 1.0
	1.5	28.8 ± 0.9
	2	29.6 ± 0.8
	2.5	29.6 ± 0.8
Pressure (MPa)[b]	15	14.7 ± 1.0
	20	23.0 ± 0.9
	25	29.6 ± 0.8
	30	29.6 ± 0.6
Temperature (°C)[c]	35	22.3 ± 1.7
	40	27.5 ± 0.6
	45	29.6 ± 0.8
	50	26.0 ± 1.7
Particle size (mm)[d]	0.25	28.1 ± 1.5
	0.32	31.1 ± 0.8
	0.4	29.6 ± 0.8
	0.52	24.1 ± 1.3
	0.75	18.5 ± 0.9
	1.45	16.5 ± 1.0

[a] The other conditions were fixed as follows: 25 MPa, 45 °C and 0.4 mm

[b] The other conditions were fixed as follows: 2 h, 45 °C and 0.4 mm

[c] The other conditions were fixed as follows: 2 h, 25 MPa and 0.4 mm

[d] The other conditions were fixed as follows: 2 h, 25 MPa and 45 °C

Table 2 Comparison of SFE with PEE for extraction of ZSS oil

Extraction method	Extraction time (h)	Extraction ratio (%)
SFE	2	31.1 ± 0.8
PEE	4	20.8 ± 0.6

particle size for extraction of ZSS oil. In summary, the optimized SFE conditions were established to be: extraction time 2 h, pressure 25 MPa, temperature 45 °C and particle size 0.32 mm, under which the extraction ratio of ZSS oil reached about 31.1%. Compared with PEE (about 20.8%), as shown in Table 2, SFE extracted much more ZSS oil in half the extraction time, indicating the excellent extraction efficiency of SFE. Thus, SFE was regarded as the most suitable extraction method, and the ZSS oil obtained was further characterized.

Fatty Acid Composition

Detailed identification and quantization of fatty acids found in ZSS oil, produced by SFE under the optimum conditions described above, were performed by GC–MS, and the results are listed in Table 3. As displayed in the table, 20 compounds were detected in ZSS oil, among which 17 were identified as fatty acids. The total relative amount of fatty acids reached about 99.81%. 9-Octadecenoic acid (43.38 ± 0.03%) was the principal unsaturated fatty acid, followed by 9,12-octadecadienoic acid (40.58 ± 0.03%) and 11-eicosenoic acid (3.69 ± 0.07%). On the other hand, hexadecanoic acid (5.45 ± 0.02%) was the predominant saturated fatty acid, followed by octadecanoic acid (3.31 ± 0.01%). Among all the fatty acids, these five fatty acids contributed about 96.41%, while the other 12 minor fatty acids made up only about 3.40%.

Due to their notable physiological activities and nutritional value, unsaturated fatty acids have been regarded as higher quality than saturated ones. Clinical research has proved that unsaturated fatty acids can regulate the level of plasma lipids and prevent cardiovascular disease [17, 18]. On the contrary, assimilating too much saturated fatty acids can increase the risk of atherosclerosis. In ZSS oil, nine unsaturated fatty acids were identified, and their total relative amount reached about 89.25%, while eight saturated fatty acids only contributed about 10.56%. The ratio of unsaturated to saturated fatty acids (about 8.45) suggests that ZSS oil possesses high pharmacological effect and nutritional value, and consequently may be very appropriate for application in pharmaceutical and food industries.

Unsaponifiable Constituents

A relatively simple GC–MS chromatographic pattern was found on analysis of the unsaponifiable constituents in ZSS oil. The results (Table 4) showed that ten compounds were detected, of which seven were identified accurately. Among these, β-sitosterol (37.39 ± 0.02%) and squalene (30.79 ± 0.01%) were the main components. β-Sitosterol is one of the most important phytosterols, and has been proven to lower plasma total cholesterol and lipoproteins [19, 20]. Squalene, as a biologically active substance, can enhance the activity of superoxide dismutase [21], prevent coronary heart disease [22] and inhibit cancer cell proliferation [23]. In addition, the natural antioxidant β-tocopherol, which protects against oxidative deterioration and prolongs quality guarantee period, was found in the ZSS oil (3.73 ± 0.01%). The presence of these bioactive components gives ZSS oil greater medicinal value, as well as opening more extensive applications.

Physicochemical Properties

Another main aspect of the characterization of ZSS oil is determination of its physicochemical properties, since they are required for quality control of technological processes [24]. According to Chinese Pharmacopeia (2005), four significant physicochemical properties of ZSS oil, including acid value, peroxide value, saponification value and iodine value, were determined, and the results are presented in Table 5.

Acid value and peroxide value, reflecting the content of free fatty acids and peroxide, respectively, have frequently been used as two important parameters to monitor the quality of fatty oil. The acid value of ZSS oil was determined as 10.3 ± 0.1 mg KOH/g, higher than the value (4.0 mg KOH/g) in GB 2716-2005. This may be blamed on the long preservation time, but the high acid value can be reduced by neutralization with alkali. By contrast, the peroxide value of ZSS oil (0.05 ± 0.01 g/100 g) meets the provisions of GB 2716-2005 (peroxide value ≤0.25 g/100 g). In addition, saponification value is regulated to reflect the total level of free fatty acids and glycerides in fatty oil, but it should be emphasized that, in fact, it is frequently used to evaluate the average molecular weight of fatty acids in fatty oil. The saponification value of ZSS oil was found to be 194.4 ± 0.5 mg KOH/g, within the range of 185–200 mg KOH/g, satisfying the requirement for oil for injection in Chinese Pharmacopeia (2005). Finally, iodine value, as a quantitative expression of unsaturated level, is defined as the amount of iodine which is absorbed by 100 g fatty oil. The iodine value of ZSS oil (109.7 ± 0.8 g I/100 g) also meets the provisions for oil for injection in Chinese Pharmacopeia (2005) (78–128 g I/100 g). The determination of these physicochemical indexes shows the relative high quality and feasibility as oil for injection of ZSS oil.

Table 3 Fatty acid composition of ZSS oil obtained by SFE

No.	t_R (min)	Composition	Molecular formula	Chain	Relative amount (%)
1	8.60	Tetradecanoic acid	$C_{14}H_{28}O_2$	C14:0	0.02 ± 0.00
2	10.37	Unidentified			0.01 ± 0.00
3	11.72	7-Hexadecenoic acid	$C_{16}H_{30}O_2$	C16:1	0.02 ± 0.00
4	11.79	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	C16:1	0.03 ± 0.00
5	12.16	Hexadecanoic acid	$C_{16}H_{32}O_2$	C16:0	5.45 ± 0.02
6	13.56	2-Hexyl-cyclopropaneoctanoic acid	$C_{17}H_{32}O_2$	C17:1	0.03 ± 0.01
7	13.99	Heptadecanoic acid	$C_{17}H_{34}O_2$	C17:0	0.03 ± 0.00
8	15.32	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	C18:2	40.58 ± 0.03
9	15.48	9-Octadecenoic acid	$C_{18}H_{34}O_2$	C18:1	43.38 ± 0.03
10	15.55	11-Octadecenoic acid	$C_{18}H_{34}O_2$	C18:1	1.43 ± 0.01
11	16.00	Octadecanoic acid	$C_{18}H_{36}O_2$	C18:0	3.31 ± 0.01
12	20.62	11,14-Eicosadienoic acid	$C_{20}H_{36}O_2$	C20:2	0.05 ± 0.01
13	20.78	11-Eicosenoic acid	$C_{20}H_{38}O_2$	C20:1	3.69 ± 0.07
14	21.51	Eicosanoic acid	$C_{20}H_{40}O_2$	C20:0	1.03 ± 0.01
15	23.96	Heneicosanoic acid	$C_{21}H_{42}O_2$	C21:0	0.02 ± 0.00
16	25.65	13-Docosenoic acid	$C_{22}H_{40}O_2$	C22:1	0.05 ± 0.01
17	26.20	Docosanoic acid	$C_{22}H_{44}O_2$	C22:0	0.50 ± 0.02
18	27.85	Unidentified			0.03 ± 0.00
19	29.33	Tetracosanoic acid	$C_{24}H_{48}O_2$	C24:0	0.21 ± 0.01
20	30.61	Unidentified			0.15 ± 0.01
		Total fatty acids			99.81 ± 0.20
		Main fatty acids			96.41 ± 0.14
		Unsaturated fatty acids			89.25 ± 0.14
		Saturated fatty acids			10.56 ± 0.06
		Additional components			0.19 ± 0.01

Table 4 Unsponifiable constituents of ZSS oil obtained by SFE

No.	t_R (min)	Composition	Relative amount (%)
1	2.72	Di-*n*-octyl phthalate	1.32 ± 0.01
2	4.20	Squalene	30.79 ± 0.01
3	5.94	Unidentified	2.95 ± 0.01
4	6.44	*β*-Tocopherol	3.73 ± 0.01
5	9.23	4-Hydroxyphenyl pyrrolidinyl thione	2.86 ± 0.01
6	9.80	Dodeca-hydropyrido(1,2-*b*)isoquinolin-6-one	4.44 ± 0.02
7	10.93	*β*-Sitosterol	37.39 ± 0.02
8	12.77	2-Ethyl-acridine	2.91 ± 0.01
9	17.67	Unidentified	5.12 ± 0.01
10	18.49	Unidentified	8.50 ± 0.03

Conclusions

In this study, ZSS oil was extracted by SFE and PEE. Optimizing the extraction processes and comparing the two extraction methods, SFE was selected as the suitable method, and the ZSS oil obtained was analyzed in terms of chemical composition and physicochemical properties. The GC–MS results showed that the main fatty acids were 9-octadecenoic acid (43.38 ± 0.03%) and 9,12-octadecadienoic acid (40.58 ± 0.03%) and that the key unsaponifiables were *β*-sitosterol (37.39 ± 0.02%) and squalene (30.79 ± 0.01%). The ratio of unsaturated to saturated fatty acids was approximately 8.45, illustrating the high pharmacological effect and nutritional value of ZSS oil.

Table 5 Physicochemical characteristics of ZSS oil obtained by SFE

Characteristic	Value
Acid value (mg KOH/g)	10.3 ± 0.1
Peroxide value (g/100 g)	0.05 ± 0.01
Saponification value (mg KOH/g)	194.4 ± 0.5
Iodine value (g I/100 g)	109.7 ± 0.8

Moreover, four main physicochemical properties of ZSS oil were assayed as well. The determination of this basic information not only provides data support for quality evaluation and efficacy research of ZSS oil, but also suggests its extensive prospects for development in pharmaceutical and food industries.

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