

Simultaneous determination of saponins and fatty acids in *Ziziphus jujuba* (Suanzaoren) by high performance liquid chromatography–evaporative light scattering detection and pressurized liquid extraction

J. Zhao^a, S.P. Li^{a,*}, F.Q. Yang^a, P. Li^b, Y.T. Wang^a

^a Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau

^b Key Laboratory of Modern Chinese Medicines and Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China

Received 1 November 2005; received in revised form 14 December 2005; accepted 20 December 2005

Available online 3 February 2006

Abstract

The seed of *Ziziphus jujube* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou, Suanzaoren in Chinese, is one of commonly used Chinese medicines. Saponins and fatty oil contains several fatty acids in Suanzaoren are responsible for its therapeutic activities. In this study, a new HPLC coupled with evaporative light scattering detection (ELSD) and pressurized liquid extraction (PLE) method was developed for the simultaneous quantitative determination of 11 major components of 2 saponins and 9 fatty acids, namely jujuboside A, jujuboside B, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid and docosanoic acid in Suanzaoren. Simultaneous separation of these eleven compounds was achieved on a C18 analytical column. The mobile phase consisted of (A) 0.1% aqueous acetic acid and (B) methanol with 0.1% acetic acid using a gradient elution. The drift tube temperature of ELSD was set at 75 °C, and nitrogen flow-rate was 1.8 l/min. All calibration curves showed good linearity ($r^2 > 0.9955$) within test ranges. This method showed good reproducibility for the quantification of these eleven components in Suanzaoren with intra- and inter-day variations of less than 3.41 and 4.37%, respectively. The validated method was successfully applied to quantify 11 investigated components in nine commercial samples of Suanzaoren.

© 2006 Elsevier B.V. All rights reserved.

Keywords: *Semen Ziziphi Spinosa* (Suanzaoren); *Ziziphus jujube* Mill. var. *spinosa*; HPLC–ELSD; Pressurized liquid extraction (PLE); Saponin; Fatty acid

1. Introduction

Semen Ziziphi Spinosa, Suanzaoren in Chinese, is the seed of *Ziziphus jujube* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou (Rhamnaceae), which has been used for its action on insomnia and anxiety [1]. Modern pharmacological studies showed that Suanzaoren possesses multiple activities such as hypnotic-sedative, hypotensive, antihypoxia, antihyperlipidemia, and hypothermic effects [2]. Generally, the curative effect of traditional Chinese medicine is an integrative result of a number of bioactive compounds. Both saponins [3,4] and fatty oil [5,6] with abundance of fatty acids were considered as major active fractions for sedative and hypnotic effects in Suanzaoren. Actually, free fatty acids have been shown to modulate many intracellular processes [7]. Also, oleic acid (OA) directly interferes with the inflammatory response characterizing early

atherogenesis due to the endothelial expression of adhesion molecules for circulating monocytes [8,9]. In addition, conjugated linoleic acid has multiple pharmacological activities, such as the role in the prevention of cancer [10], increasing endurance exercise capacity [11], and positively benefiting bone mineral density (BMD) in postmenopausal women [12]. Therefore, quantitative determination of saponins and fatty acids is helpful to control the quality of Suanzaoren. For most cases, HPLC with UV–vis detection is the prevailing technique. However, due to the low UV absorptivity of the molecules, low-wavelength UV [13] is required for detection of saponins, which suffers low sensitivity and baseline drift when using steep gradients. On the other hand, fatty acids are commonly determined by GC–MS as the esters after derivatization because they have no UV absorptivity [14,15]. In the context of routine work, the drawbacks of derivatization techniques are widely recognized (dependence of various experimental parameters, incompleteness of derivatization reactions, analyte degradation, prolonged analysis time, additional cost for derivatization system and reagents).

* Corresponding author. Tel.: +853 397 4692; fax: +853 841 358.
E-mail address: SpLi@umac.mo (S.P. Li).

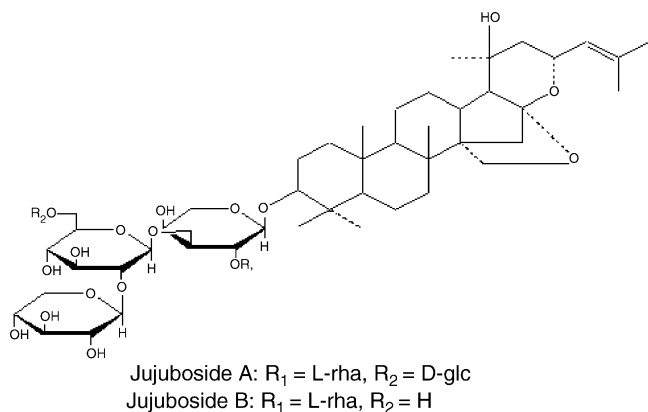


Fig. 1. The structures of investigated saponins.

The evaporative light scattering detector (ELSD) response does not depend on the samples' optical characteristics, which eliminates the common problems associated with other HPLC detectors. It can achieve stable baseline with multi-solvent gradients for improved resolution and faster separations. Therefore, ELSD is increasingly being used in liquid chromatography (LC) as a quasi-universal detector, which has been successfully applied to the analysis of non-volatile compounds such as saponins [16–18] and lipids [19,20]. However, there is no report for simultaneous determination of saponins and fatty acids using HPLC-ELSD.

In present study, by using HPLC-ELSD and pressurized liquid extraction (PLE), a simple method for simultaneous determination of eleven compounds of saponins and fatty acids in Suanzaoren was developed. The validated method was also applied to quantify the investigated components in Suanzaoren material.

2. Experimental

2.1. Chemicals, reagents and materials

Methanol and acetic acid used for HPLC was purchased from Merck (Darmstadt, Germany). The water was purified with a Milli-Q system. Methanol and ethyl acetate of analytical grade used for extraction were purchased from DaMao Chemical Reagent Factory, TianJin, China. Fatty acids such as lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid and docosanoic acid were purchase from Sigma-Aldrich (St. Louis, USA). Jujuboside A and jujuboside B (Fig. 1) were gifts from Professor Li Ping, China Pharmaceutical University.

Nine commercial samples of Suanzaoren were confirmed by Dr. S. P. Li, University of Macau. All corresponding voucher specimens were deposited at the Institute of Chinese Medical Sciences, Macau University, Macau, China.

2.2. Pressurized liquid extraction (PLE)

Pressurized liquid extractions were performed on a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA, USA) system. Powder

of Suanzaoren (sample No. 6, 0.5 g) were mixed with diatomaceous earth in a proportion (1:1) and placed into an 11-ml stainless steel extraction cell, respectively. The extraction cell was extracted under the optimized conditions. Then, the extract was transferred to a 25 ml volumetric flask, which was brought up to its volume with ethyl acetate and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC-ELSD system.

2.3. HPLC analysis

All separations were performed on an Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and an evaporative light scattering detector (ELSD 2000ES, Alltech, USA). Data were acquired and processed using Agilent ChemStation software. A ZORBAX SB-C18 column (2.1 mm \times 150 mm I.D., 5 μm) with a ZORBAX ODS C18 guard column (3.9 mm \times 20 mm I.D., 5 μm) was used. Solvents that constituted the mobile phase were A (0.1% aqueous acetic acid) and B (methanol with 0.1% acetic acid). The elution conditions applied were: 0–5 min, isocratic 20% B; 5–20 min, linear gradient 20–70% B; 20–25 min, isocratic 70% B; 25–30 min, linear gradient 70–90% B; 30–45 min, isocratic 90% B; 45–60 min, linear gradient 90–100% B; and finally, reconditioning steps of the column was 20% B isocratic for 15 min. The flow-rate was 0.4 ml/min and the injection volume was 10 μl . The column operated at 30 $^{\circ}\text{C}$. The analytes were monitored with ELSD. The impactor position of ELSD was set off. The other parameters including nebulizing gas flow rate and drift tube temperature were optimized based on the ratio of signal to noise (S/N).

3. Results and discussion

3.1. Optimization of PLE procedure

PLE procedure was optimized. And the parameters include the type of solvent [methanol, ethyl acetate, ethyl acetate-methanol (through 5:95 to 50:50) with interval of 5], particle size (20–40 mesh, 40–60 mesh and 60–80 mesh), pressure (500, 1000, 1200 and 1500 psi), temperature (60, 100, 140 and 180 $^{\circ}\text{C}$), time (5, 10, 15 and 20 min), flush volume (20, 40, 60 and 80%) and cycles of extraction (1, 2 and 3) were studied by using univariate approach. The amount of 11 investigated components was used as the marker for evaluation of extraction efficiency. Influences of solvent, particle size, pressure, temperature, static extraction time, flush volume and cycles of extraction on PLE was shown in Fig. 2. The recovery efficiency for the PLE procedure was determined by performing consecutive pressurized liquid extractions on the same sample under the optimized PLE conditions, until no investigated compounds were detected by the analysis. The recovery was calculated based on the total amount of individual investigated components. Taking into account the results of optimization and recovery experiment, the conditions of the PLE method proposed were: solvent, methanol–ethyl acetate (95:5); temperature, 140 $^{\circ}\text{C}$; particle size, 40–60 mesh; static extraction time, 15 min; pres-

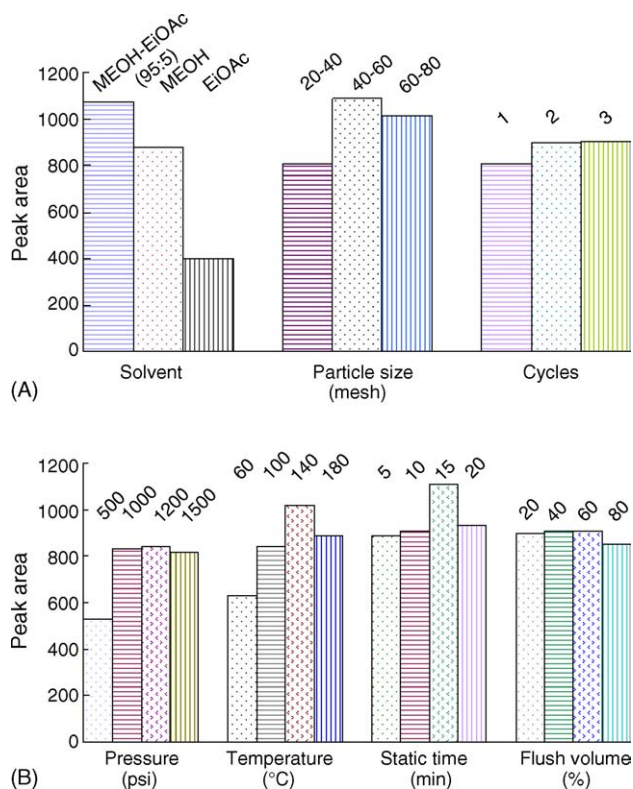


Fig. 2. Influences of (A) solvent, particle size and cycles, as well as (B) pressure, temperature, static extraction time and flush volume on PLE.

sure, 1200 psi; static cycle, 2 and flush volume, 40%. Generally, solvent is a key factor for PLE, and methanol is a desired solvent for extraction of most compounds from herbs [21–23]. Here, methanol, ethyl acetate and their mixture with different ratios were chosen for test because the polarity of saponins and fatty acids were significantly different. The experiments were performed at the default conditions (temperature, 100 °C; pressure, 1500 psi; static extraction time, 5 min; flush volume, 60% and one extraction cycle). As shown in Fig. 2A, the extraction efficiency of methanol-ethyl acetate (95:5) was the highest.

3.2. Optimization of ELSD parameters

The detection of investigated compounds was achieved by using an ELSD 2000ES (Alltech, USA). The parameters of ELSD such as nebulizing gas flow rate and evaporator tube temperature were optimized to obtain the best signals. The ratio of signal to noise (S/N) was taken as a measurement. For optimization of evaporator tube temperature, nebulizing gas flow rate was kept at the constant of 1.8 l/min. Fig. 3A showed that the effect of evaporator tube temperature of ELSD on S/N of major components investigated in Suanzaoren, which showed that the optimum temperature was 75 °C. In general, nebulizer gas flow rate determines the size of the droplets formed during nebulization. Ideally, the highest signal should be obtained at the lowest gas flow rate. Fig. 3B showed that the effect of nebulizing gas flow rate of ELSD on S/N of the analytes. The baseline was unstable when gas flow rate decreased to 1.6 l/min, as well as 1.7 l/min. Therefore, the optimum gas flow rate was 1.8 l/min.

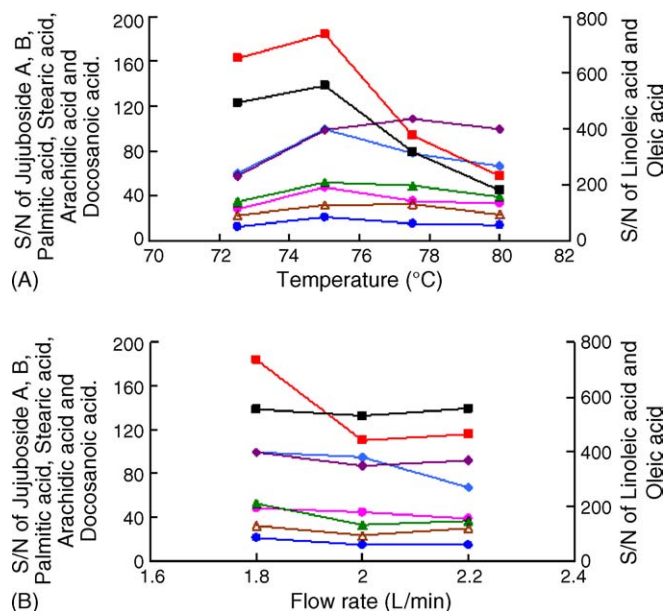


Fig. 3. Effects of evaporator tube temperature (A) and nebulizing gas flow rate (B) on signal-to-noise ratio (S/N). Jujuboside A (●), jujuboside B (●), palmitic acid (◆), stearic acid (▲), arachidic acid (△), docosanoic acid (◆), linoleic acid (■), oleic acid (■).

Considering the results mentioned above, the optimized parameters of ELSD were as follows: evaporator tube temperature, 75 °C; nebulizing gas flow rate, 1.8 l/min.

3.3. Calibration curves

Methanol–ethyl acetate (95:5) stock solutions containing eleven analytes were prepared and diluted to appropriate concentration for the construction of calibration curves. Six concentrations of the eleven analytes' solution were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the amount (μg) of each analyte. The results were shown in Table 1.

3.4. Limits of detection and quantification

Methanol–ethyl acetate (95:5) stock solution containing eleven reference compounds were diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions were injected into HPLC for analysis. According to the literature [24], the limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at S/N (the ratio of signal to noise) of 3 and 10, respectively. The ratio of signal to noise was calculated using Agilent ChemStation software. Table 1 showed the data of LOD and LOQ for each investigated compound.

3.5. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. A certain concentration solution of eleven reference compounds was tested. For intra-day variability, the samples were analyzed in triplicate for three times

Table 1
Linear regression data, LOD and LOQ of investigated compounds from Suanzaoren

Analytes	Linear regression data			LOD (ng)	LOQ (ng)
	Regressive equation	r^2	Linear range (μg)		
Jujuboside A	$y = 3011.2x - 37.802$	0.9983	0.0229–0.1375	9.2	18.3
Jujuboside B	$y = 2776x - 29.449$	0.9993	0.0208–0.1249	8.3	16.7
Lauric acid	$y = 101.46x + 33.374$	0.9955	0.1380–1.1043	30.0	110.0
Myristic acid	$y = 459.4x + 19.964$	0.9985	0.0458–0.2750	6.9	22.9
Palmitoleic acid	$y = 81.196x + 71.434$	0.9989	0.2104–1.2615	21.0	84.2
Linoleic acid	$y = 1341.1x - 1.791$	0.9984	0.0609–1.4625	7.6	30.5
Palmitic acid	$y = 1507.9x - 1.068$	0.9981	0.0158–0.1900	2.9	12.7
Oleic acid	$y = 720.24x + 40.166$	0.9990	0.0440–0.8792	13.7	41.6
Stearic acid	$y = 1749.2x + 5.987$	0.9983	0.0271–0.3250	6.8	21.7
Arachidic acid	$y = 1485.6x - 43.281$	0.9976	0.0406–0.4875	27.1	54.2
Docosanoic acid	$y = 3722.3x - 60.212$	0.9973	0.0313–0.2000	10.4	20.8

Table 2
Repeatability ($n=3$) of investigated compounds from Suanzaoren

Peak	Analytes	Concentration ($\mu\text{g/ml}$)	Inter-day (RSD %)	Intra-day (RSD %)
1	Jujuboside A	13.75	2.11	4.37
2	Jujuboside B	12.49	2.54	2.44
3	Lauric acid	110.43	0.83	0.93
4	Myristic acid	27.50	2.11	3.85
5	Palmitoleic acid	126.15	3.17	3.68
6	Linoleic acid	146.25	3.41	1.08
7	Palmitic acid	19.00	1.36	2.92
8	Oleic acid	87.92	2.84	3.33
9	Stearic acid	32.50	2.78	1.24
10	Arachidic acid	48.75	2.15	3.04
11	Docosanoic acid	20.00	2.99	3.15

Concentrations correspond to the amount injected 10 μl .

within 1 day, while for inter-day variability, the samples were examined in triplicate for consecutive 3 days. Variations were expressed by the relative standard deviations (RSD), which were less than 3.41 and 4.37%, respectively (Table 2).

Recovery test was used to evaluate the accuracy of this method. A known amount of eleven investigated compounds were added to approximate 0.3 g of Suanzaoren, and then extracted and analyzed as described above. The results were shown in Table 3.

Table 3
Recoveries of investigated compounds from Suanzaoren

Peak	Analytes	Contained ^a (mg)	Added (mg)	Found (mean, mg)	Recovery (mean, %)	RSD (% , $n=3$)
1	Jujuboside A	0.120	0.118	0.234	98.46	0.65
2	Jujuboside B	0.088	0.086	0.168	96.74	2.47
3	Lauric acid	0.000	0.142	0.141	98.59	1.42
4	Myristic acid	0.000	0.012	0.012	102.78	4.68
5	Palmitoleic acid	0.000	0.079	0.081	103.80	1.89
6	Linoleic acid	2.845	2.872	5.936	103.83	1.63
7	Palmitic acid	0.380	0.385	0.774	101.22	2.33
8	Oleic acid	2.115	2.110	4.254	100.69	1.92
9	Stearic acid	0.211	0.220	0.416	96.52	1.68
10	Arachidic acid	0.179	0.185	0.365	97.59	3.16
11	Docosanoic acid	0.690	0.699	1.353	97.43	3.23

^a Results are expressed as mg/0.3 g.

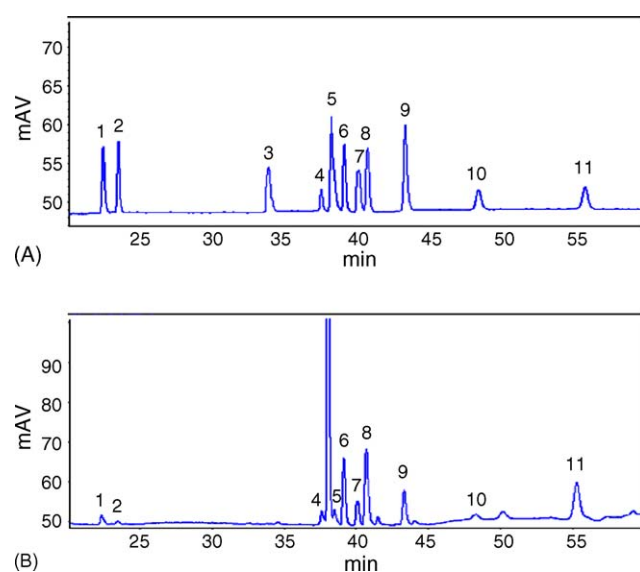


Fig. 4. HPLC-ELSD chromatograms of (A) mixed standards and (B) typical sample of *Ziziphus jujuba*. (1) jujuboside A; (2) jujuboside B; (3) lauric acid; (4) myristic acid; (5) palmitoleic acid; (6) linoleic acid; (7) palmitic acid; (8) oleic acid; (9) stearic acid; (10) arachidic acid; (11) docosanoic acid.

3.6. Quantification of investigated components in *Suanzaoren*

The chromatograms of PLE extract for *Suanzaoren* materials were shown in Fig. 4. The identification of investigated components was carried out by comparison of their retention time with those obtained injecting standards in the same conditions

or by spiking *Suanzaoren* samples with stock standard solutions, which was confirmed by LC–MS. The MS and MS₂ of the investigated components were shown in Fig. 5.

By using the calibration curve of each investigated compound, nine *Suanzaoren* samples were analyzed. Table 4 showed the summary results. In general, the components were similar in all samples of *Suanzaoren* though the contents were various.

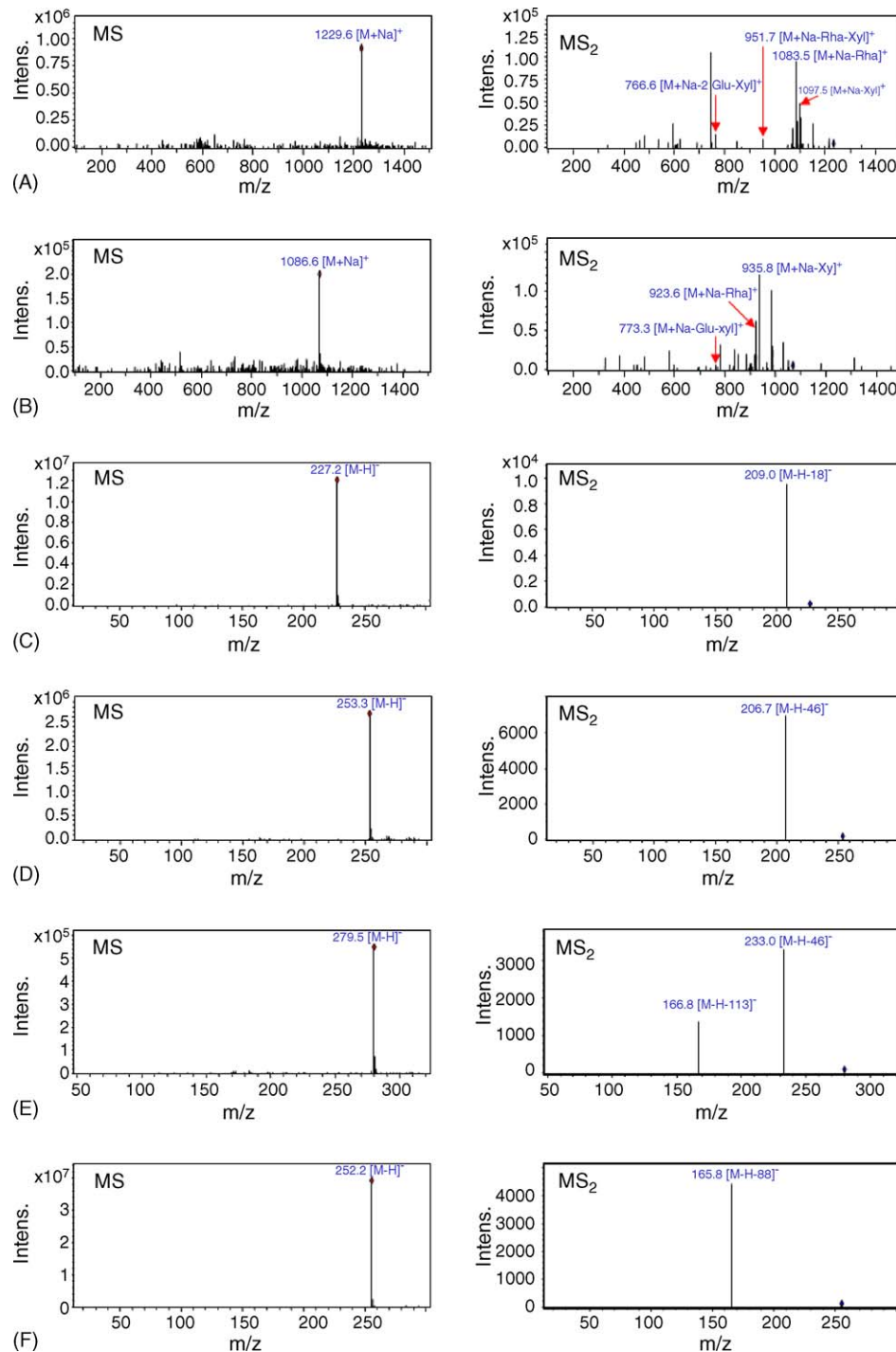


Fig. 5. MS and MS₂ of investigated components in *Ziziphus jujuba*. (A) jujuboside A; (B) jujuboside B; (C) myristic acid; (D) palmitoleic acid; (E) linoleic acid; (F) palmitic acid; (G) oleic acid; (H) stearic acid; (I) arachidic acid; (J) docosanoic acid.

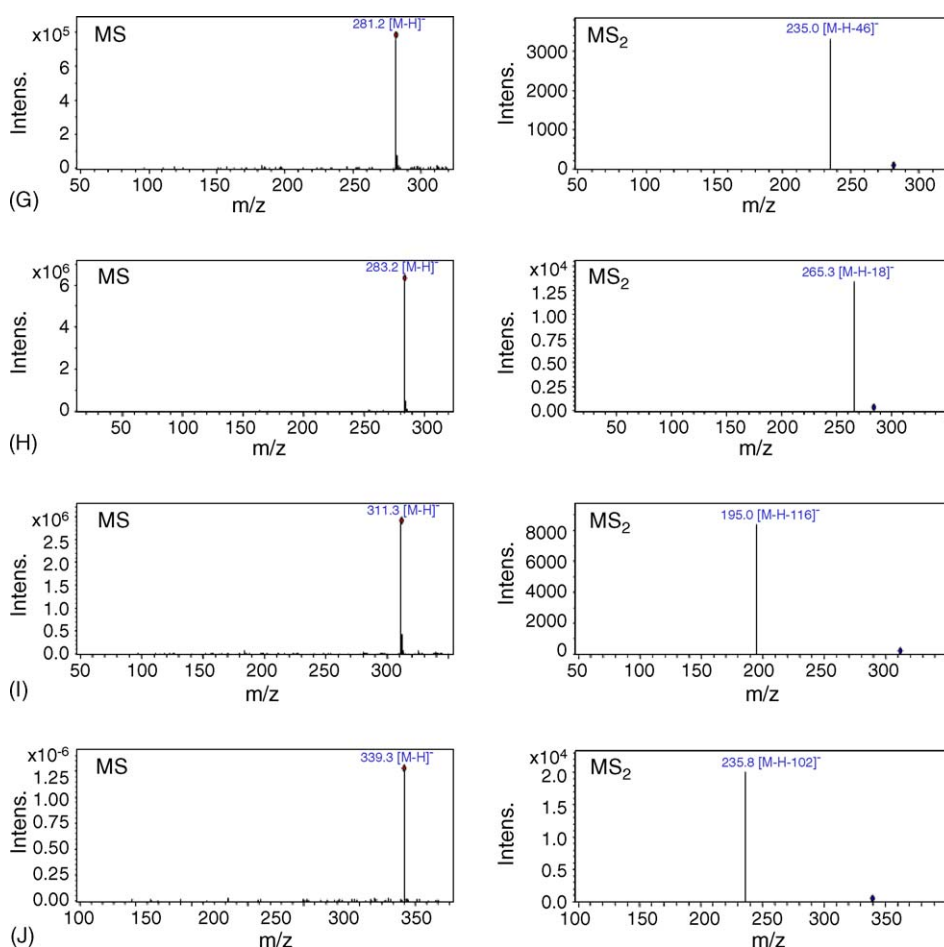


Fig. 5. (Continued).

Table 4
The contents (mg/g) of eleven investigated components in Suanzaoren materials

Analytes	Sample No.								
	1	2	3	4	5	6	7	8	9
Jujuboside A	0.476 ^a	± ^b	0.267	0.358	0.126	0.384	0.424	0.357	0.277
Jujuboside B	0.348	±	0.157	0.290	0.106	0.409	0.339	0.405	0.516
Lauric acid	– ^c	–	–	–	–	–	–	–	–
Myristic acid	0.154	0.140	0.132	0.342	0.145	–	0.325	0.722	0.784
Palmitoleic acid	–	1.765	0.374	0.545	1.786	–	–	–	–
Linoleic acid	11.434	6.244	14.340	21.156	8.850	28.554	16.502	26.983	51.349
Palmitic acid	1.530	2.238	2.226	1.820	2.270	2.024	1.622	2.178	2.460
Oleic acid	8.450	13.959	11.781	25.679	11.964	26.395	12.504	26.483	50.195
Stearic acid	0.861	1.432	1.316	0.980	1.354	1.522	0.618	1.262	2.892
Arachidic acid	0.193	0.583	0.578	0.748	0.542	0.939	0.577	0.561	0.370
Docosanoic acid	2.778	4.652	4.775	7.158	5.814	5.242	7.725	7.370	6.882

^a The data was presented as average of three replicates (RSDs < 3%).

^b Under limit of quantification.

^c Not detected.

Lauric acid and palmitoleic acid were not detected in all and partial samples of Suanzaoren, respectively. The amount of fatty acids especially linoleic acid and oleic acid were much higher than that of saponins in Suanzaoren. The data were corresponding with those previous studies [25–27].

4. Conclusions

Generally, fatty acids are determined by GC–MS as the esters after derivatization, which had the drawbacks such as prolonged analysis time, additional cost for derivatization and so on. The

developed method in this paper could be used for simultaneous determination of saponins and fatty acids in Suanzaoren without derivatization, which simplified the sample preparation. It is helpful to control the quality of Suanzaoren and other Chinese medicines.

Acknowledgements

We are grateful to Wan J. B., Leon Lai and Sandy Lao from our institute for their expert technical assistances.

References

- [1] Pharmacopoeia Commission of PRC (Eds.), Pharmacopoeia of the People's Republic of China (English edition), Chemical Industry Press, Beijing, PR China, 2000, p. 254.
- [2] Q.R. Shi, Y. Zhou, P. Zhou, W.D. Zhang, *J. Pharm. Practice* 22 (2004) 94.
- [3] Z.Y. Feng, D.W. Guo, S. Su, H. Zhao, X.X. Zheng, *J. Zhejiang Univ. (Med. Sci.)* 31 (2002) 103.
- [4] S.Y. Huang, S.R. Xie, C.Y. Huang, Z.M. Zhang, *J. Dalian Univ.* 23 (2002) 90.
- [5] Q.X. Zhao, Q.L. Wang, J.H. Huang, *J. Xi'an, Med. Univ.* 16 (1995) 432.
- [6] S.L. Wu, B.X. Yuan, Z.Y. Ma, *Northwest Pharm. J.* 16 (2001) 114.
- [7] M.J. Jackson, *Proc. Nutr. Soc.* 49 (1990) 77.
- [8] M. Massaro, R. De Caterina, *Nutr. Metab. Cardiovasc. Dis.* 12 (2002) 42.
- [9] M. Massaro, M.A. Carluccio, R. De Caterina, *Cardiologia* 44 (1999) 507.
- [10] K.W. Lee, H.J. Lee, H.Y. Cho, Y.J. Kim, *Crit. Rev. Food Sci. Nutr.* 45 (2005) 135.
- [11] W. Mizunoya, S. Haramizu, T. Shibakusa, Y. Okabe, T. Fushiki, *Lipids* 40 (2005) 265.
- [12] R.A. Brownbill, M. Petrosian, J.Z. Ilich, *J. Am. Coll. Nutr.* 24 (2005) 177.
- [13] Y.J. Li, Z.T. Che, K.S. Bi, Z.M. Xu, M. Li, *Chin. J. Chin. Mat. Med.* 26 (2001) 309.
- [14] J. Gamazo-Vázquez, M.S. García-Falcón, J. Simal-Gándara, *Food Control* 14 (2003) 463.
- [15] L. Akoto, R. Pel, H. Irth, U.A.T. Brinkman, R.J.J. Vreuls, *J. Anal. Appl. Pyrolysis* 73 (2005) 69.
- [16] M.K. Park, J.H. Park, S.B. Han, Y.G. Shin, I.H. Park, *J. Chromatogr. A* 736 (1996) 77.
- [17] X.Y. Chai, S.L. Li, P. Li, *J. Chromatogr. A* 1070 (2005) 43.
- [18] H.J. Li, P. Li, *Chin. J. Pharm. Anal.* 20 (2000) 82.
- [19] J. Nordbäck, E. Lundberg, *J. High Resol. Chromatogr.* 22 (1999) 483.
- [20] A. Avalli, G. Contarini, *J. Chromatogr. A* 1071 (2005) 185.
- [21] S.P. Li, P. Li, C.M. Lai, Y.X. Gong, Kelvin.K.W. Kan, Tina.T.X. Dong, Karl.W.K. Tsim, Y.T. Wang, *J. Chromatogr. A* 1036 (2004) 239.
- [22] S.C. Lao, S.P. Li, K.K.W. Kan, P. Li, J.B. Wan, Y.T. Wang, T.T.X. Dong, K.W.K. Tsim, *Anal. Chim. Acta* 526 (2004) 131.
- [23] Y.X. Gong, S.P. Li, P. Li, J.J. Liu, Y.T. Wang, *Electrophoresis* 1055 (2005) 215.
- [24] N. Kaul, H. Agrawal, B. Patil, A. Kakad, S.R. Dhaneshwar, *Il Farmaco* 60 (2005) 351.
- [25] H.J. Li, P. Li, *Chin. J. Pharm. Anal.* 20 (2000) 82.
- [26] J.Y. Gong, Q.Z. Zhao, L. Shi, *J. Changchun, Coll. Trad. Chin. Med.* 19 (2003) 87.
- [27] Z.D. Chen, C.Y. Xu, L. Xie, *Chin. Trad. Herbal Drugs* 32 (2001) 976.