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Short communication

High-performance liquid chromatography—Two wavelength detection of triterpenoid acids from the fruits of *Ziziphus jujuba* containing various cultivars in different regions and classification using chemometric analysis

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ABSTRACT

A simple and sensitive HPLC–DAD method has been developed for the first time to simultaneously determine 10 triterpenoid acids (ceanothic acid, alphitolic acid, zizyberanal acid, zizyberanalic acid, epiceanothic acid, ceanothenic acid, betulinic acid, oleanolic acid, ursonic acid and zizyberenalic acid) in the dried fruit of *Ziziphus jujuba* (called Dazao) which has been widely used as one of the traditional Chinese medicines (TCMs). This HPLC assay was performed on a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm) with the column temperature at 35 °C. The mobile phase was composed of A (acetonitrile) and B (0.05% aqueous phosphoric acid, v/v). The flow rate was 1.0 ml/min and the detection wave length was set at 205 nm for reference compounds **1**–**9** and 238 nm for reference compound **10**. All calibration curves showed good linear regression ($r^2 > 0.9999$) within the test range. The established method showed good precision and accuracy with overall intra-day and inter-day variations of 0.43–1.72% and 0.53–2.45%, respectively, and overall recoveries of 94.98–104.09% for the 10 compounds analyzed. The validated method was successfully applied for the simultaneous determination of the 10 triterpenoid acids in 42 batches of Dazao which contained 36 cultivars from 22 cultivation regions, and were investigated and authenticated as *Z. jujuba*. Hierarchical clustering analysis (HCA) and principal components analysis (PCA) were performed to differentiate and classify the samples based on the contents of the 10 triterpenoid acids. The presented HPLC–DAD method conjugated with chemometrics approach was demonstrated to be very helpful in using Dazao resources, and was possibly useful in chemotaxonomic characterization.

1. Introduction

Ziziphus jujuba Mill. is a thorny Rhamnaceous plant, and more than 700 cultivars have been found in China, especially in Henan, Shandong, Hebei, Shanxi, Shaanxi, Ningxia, Xinjiang, and Gansu provinces. Its dried fruit called Dazao in China has been used as a traditional Chinese medicine (TCM) for immunity stimulant, antitumor activities, etc. [1–3]. As for the chemical constituents of this plant, several triterpenoid acids, cyclopeptide alkaloids, saponins, and flavonoids have been isolated from its fruit, bark and leaf [3–9].

Triterpenoid acids are one of the major active constituents in the fruit of *Z. jujuba*, which exhibited many biological activities such as cytotoxic [10], anti-complementary [5], anti-microbial [11], anti-plasmodial, anti-mycobacterial [12], anti-HIV [13], anti-inflammatory [14,15] and cyclooxygenase-2 inhibitory activities [3]. Especially, their selective inhibitory effect on human melanoma *in vitro* and *in vivo* test systems without any signs of acute or chronic toxicity, even at repeated doses of up to 500 mg/kg weight [16,17]. Until now, more than 15 triterpenoid acids were found in the fruit of *Z. jujuba*. It is known that the therapeutic effect of TCM is due to the synergic effect of its multiple chemical bioactive compounds [18,19]. So, it is essential that a few markers or pharmacologically active compounds should be used as standards in the quality control of TCM. High-performance liquid chromatography (HPLC) have been applied for determination of one or two triterpenoid acids in the fruit of *Z. jujuba* [20,21].

It is well known that herbs collected from various cultivation regions or different cultivars are discrepant in the types and quantities of chemical constituents, which influence their therapeutic effects [22]. So 42 batches of *Z. jujuba* fruits were collected from 22 cultivation regions containing 36 cultivars, and the contents of the 10 markers were simultaneously determined for evaluate the quality in this study. Furthermore, hierarchical clustering analysis (HCA) and principal components analysis (PCA) were performed to evaluate and classify the samples according to the contents of the 10 chemical constituents.

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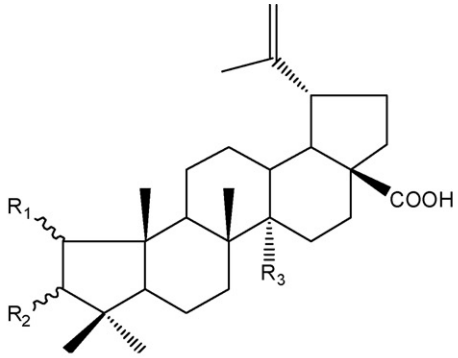
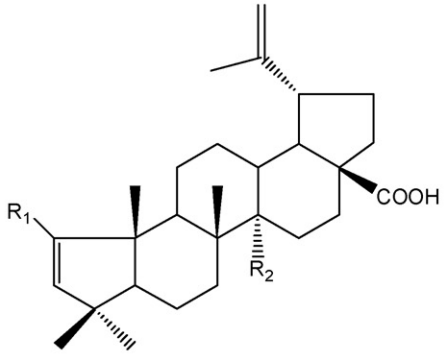
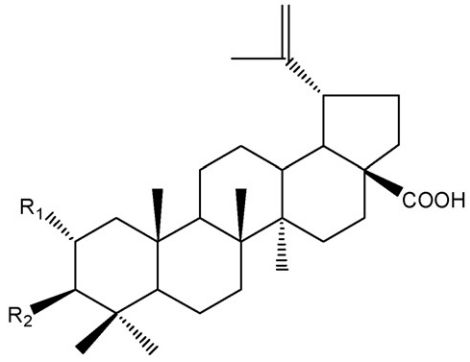


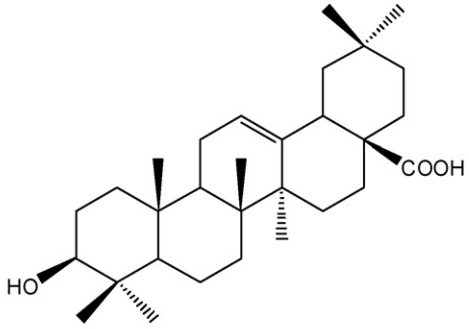
Ceanothic acid (1) R_1=αCOOH, R_2=βOH, R_3=CH_3
Zizyberanal acid (3) R_1=αCHO, R_2= H, R_3=COOH
Zizyberanalic acid (4) R_1=βCHO, R_2=αOH, R_3=CH_3
Epiceanothic acid (5) R_1=βCOOH, R_2=βOH, R_3=CH_3



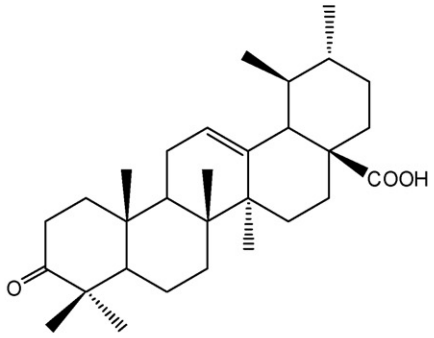
Ceanothenic acid (6) R_1=H, R_2=COOH
Zizyberenalic acid (10) R_1=CHO, R_2=H



Alphitolic acid (2) R_1=OH, R_2=OH
Betulinic acid (7) R_1=H, R_2=OH



Oleanolic acid (8)



Ursonic acid (9)

Fig. 1. Chemical structures of 10 investigated compounds in *Ziziphus jujuba*.

2. Experimental

2.1. Chemicals and reagents

The 10 reference compounds (ceanothic acid, alphitolic acid, zizyberanal acid, zizyberanalic acid, epiceanothic acid, ceanothenic acid, betulinic acid, oleanolic acid, ursonic acid and zizyberenalic acid) were isolated from fruits of *Z. jujuba* by our laboratory and their structures were confirmed based on spectroscopic analysis (^{1}H NMR, ^{13}C NMR and ESI-MS), and the purity of each compound was more than 98% determined by HPLC analysis. The chemical structures of these reference compounds were shown in Fig. 1. Acetonitrile was HPLC-grade from Merck (Darmstadt, Germany) and deionized water was purified by a EPED superpurification system (Eped, Nanjing, China). Other reagent solutions were of analytical grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

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

Analytes	Slope ($A \pm$ S.E.)	Intercept ($B \pm$ S.E.)	r^2	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
1 Ceanothic acid	8176.6 ± 12.6	+1461.2 ± 1140.1	1.0000	4.80–192.00	0.46	1.86
2 Alphitolic acid	9325.8 ± 36.1	−6901.0 ± 3377.4	0.9999	3.65–146.00	0.51	1.54
3 Zizyberanal acid	14776 ± 101.8	−3910.6 ± 6903.0	0.9999	3.60–144.00	0.35	1.06
4 Zizyberanalic acid	15582 ± 66.5	−1326.7 ± 3945.6	0.9999	3.15–126.00	0.42	1.66
5 Epiceanothic acid	6696.6 ± 20.4	−5095.3 ± 1020.3	0.9999	2.65–106.00	0.68	2.65
6 Ceanothenic acid	12574 ± 32.3	−884.17 ± 3106.2	0.9999	5.10–204.00	0.29	1.15
7 Betulinic acid	12469 ± 25.5	−7295.2 ± 7198.9	0.9999	15.00–600.00	0.49	1.48
8 Oleanolic acid	17743 ± 49.7	−8726.6 ± 7258.8	0.9999	7.75–310.00	0.41	1.63
9 Ursonic acid	12796 ± 28.7	2579.2 ± 5358.2	0.9999	9.90–396.00	0.50	2.00
10 Zizyberenalic acid	10284 ± 32.0	−5056.2 ± 3426.2	0.9999	3.15–126.00	0.36	1.08

$Y = Ax + B$, Y is peak area; x is concentration of the reference compounds (μg/ml); S.E. is the standard error and S.D. is the standard deviations; r^2 is the correlation coefficient of the equation; LOD refers to limit of detection and LOQ refers to limit of quantification.

2.2. Plant materials

Forty-two batches of samples (samples 1–42) of *Z. jujuba* were collected from Henan, Shandong, Hebei, Shanxi, Ningxia, Xinjiang and Gansu provinces, PR China. After collection, the fruits were allowed to dry at 45 °C for 6 days. The botanical origin of materials was identified by the corresponding author, and the voucher specimens were deposited at the Herbarium in Nanjing University of Chinese Medicine, China.

2.3. Preparation of standard solutions

A mixed standard stock solution containing ceanothic acid (1), alphitolic acid (2), zizyberanal acid (3), zizyberanalic acid (4), epiceanothic acid (5), ceanothenic acid (6), betulinic acid (7), oleanolic acid (8), ursonic acid (9) and zizyberenalic acid (10) was prepared in methanol. Working standard solutions were prepared by diluting the mixed standard stock solution with methanol to give six different concentrations within the ranges: **1**, 4.80–192.00 μg/ml; **2**, 3.65–146.00 μg/ml; **3**, 3.60–144.00 μg/ml; **4**, 3.15–126.00 μg/ml; **5**, 2.65–106 μg/ml; **6**, 5.10–204.00 μg/ml; **7**, 15.00–600.00 μg/ml; **8**, 7.75–310.00 μg/ml; **9**, 9.90–396.00 μg/ml and **10**, 3.15–126.00 μg/ml for calibration curves. The standard solutions were filtered through a 0.45 μm membrane prior to injection. All solutions were stored in a refrigerator at 4 °C before analysis.

2.4. Preparation of sample solutions

The dried powder of the fruits *of Z. jujuba* (5.0 g, 40 mesh) was weighed accurately into a 100 ml conical flask with stopper and then extracted two times (30 min each) by ultrasonic with 100 ml chloroform. The extracts were combined and filtered through analytical filter paper. After evaporating chloroform of the extracts to dryness by a rotary evaporator, the residue was dissolved in methanol in a 10 ml volumetric flask, and then filtrated through a 0.45 μm membrane filter before injected into the HPLC system for analysis.

2.5. Apparatus and chromatographic conditions

Analysis was performed using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), consisting of a quaternary pump solvent management system, an on-line degasser, and an autosampler. The raw data were detected by 2998 PDA, acquired, and processed with Empower™ Software. An Waters SunFire™ C_{18} column (250 mm × 4.6 mm, 5 μm) preceded by a Waters Symmetry Shield RP C_{18} guard column (20 mm × 3.9 mm, 5 μm) was applied for all analyses. Detection wavelength was set at 205 nm for reference compounds **1**–**9** and 238 nm for reference compound **10**. The mobile phase was composed of A (acetonitrile) and B (0.05% aqueous phosphoric acid, v/v) with a linear gradient elution: 0–16 min, 55% A; 16–36 min, 55–65% A; 36–46 min, 65–77% A; 46–56 min, 77–80% A; 56–70 min, 80–95% A. Re-equilibration duration was 20 min between individual runs. The flow rate of the mobile phase was 1.0 ml/min, and the column temperature was maintained at 35 °C.

2.6. Method for HCA of samples

The hierarchical clustering was done by SPSS 15.0 software. Ward's method was applied, and squared Euclidean distance was selected as a measurement. Dendrogram resulting from the 10 investigated compounds' contents derived from HPLC profiles of the tested samples.

2.7. Method for PCA of samples

The principal components analysis was done by SPSS 15.0 software. In this study, the contents of the 10 markers analyzed from the 42 samples composed a data matrix with 42 rows and 10 columns, which was used for PCA analysis after normalization. The first two principal components were extracted, and the scatter plot obtained by plotting the scores of PC 1 versus PC 2.

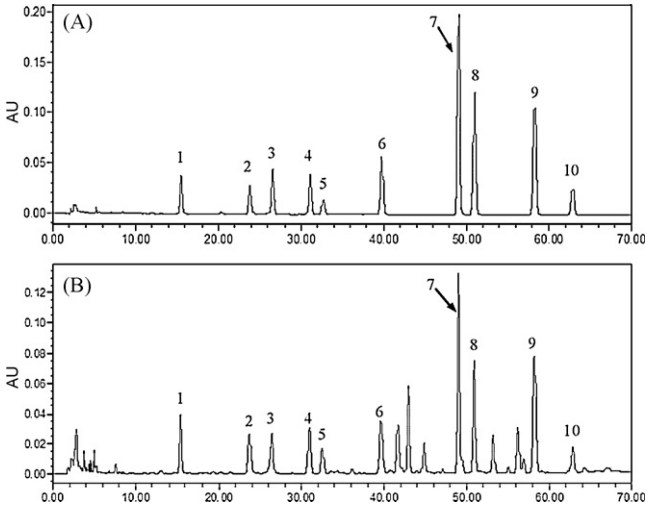


Fig. 2. Typical HPLC chromatograms of (A) mixed standards and (B) *Z. jujuba*. Ceanothic acid (**1**), alphitolic acid (**2**), zizyberanal acid (**3**), zizyberanalic acid (**4**), epiceanothic acid (**5**), ceanothenic acid (**6**), betulinic acid (**7**), oleanolic acid (**8**), ursonic acid (**9**) and zizyberenalic acid (**10**).

Table 2
Precision, repeatability, stability and recovery of 10 analytes.

Analytes	Precision (R.S.D., %)		Repeatability (R.S.D., %, $n = 6$)	Stability (R.S.D., %, $n = 6$)	Recovery (%, $n = 3$)	
	Intra-day ($n = 6$)	Inter-day ($n = 3$)			Mean	R.S.D. (%)
Ceanothic acid	1.20	2.45	2.40	1.72	98.52	1.40
Alphitolic acid	0.91	1.60	2.03	1.42	104.09	2.73
Zizyberanal acid	1.72	2.22	1.96	2.79	94.98	2.59
Zizyberanalic acid	0.43	1.06	1.95	1.37	101.90	1.73
Epiceanothic acid	0.48	1.37	1.78	1.31	97.66	0.93
ceanothenic acid	0.95	1.54	2.77	1.72	99.24	2.35
Betulinic acid	1.46	0.53	2.23	2.06	102.65	2.18
Oleanolic acid	0.49	1.85	2.41	2.05	95.59	0.63
Ursonic acid	1.72	1.59	2.64	2.61	99.38	0.30
Zizyberenalic acid	0.30	1.33	2.33	1.95	97.50	0.42

3. Results and discussion

3.1. Optimization of extraction procedure

Several extraction methods, solvents and times were investigated to obtain the best extraction efficiency. The results revealed that ultrasonic bath extraction was more effective than refluxing for the 10 triterpenoid acids analyzed, so the further experiments were carried out with ultrasonic bath extraction. Various solvents including methanol, ethanol, ethyl acetate, and chloroform were screened, and the best solvent was found to be chloroform, which enabled less interfering peaks and provided the highest values in the contents of the 10 markers. The volume of chloroform, times of ultrasonic extraction and duration of extraction were also investigated to optimize the extraction procedure. The results demonstrated that the established extraction method (each sample was extracted two

Table 3
The contents (mg/g) of 10 investigated compounds in samples of *Z. jujube*.

Sample no.	Cultivar (*Z. jujuba* cv.)	Analytes										Total
		1	2	3	4	5	6	7	8	9	10	
1	Junzao	0.0827	0.2411	0.1270	0.0134	0.0498	0.0476	0.2898	0.1917	0.3893	0.0564	1.4888
2	Junzao	0.0260	0.2090	0.0402	nd	nd	0.0325	0.2387	0.0924	0.3093	0.0187	0.9669
3	Hupingzao	0.1820	0.3432	0.1716	0.0321	0.1026	0.1161	0.3713	0.2674	0.4731	0.1381	2.1974
4	Lizao	0.1987	0.1044	0.0733	0.0159	0.1729	0.1002	0.2112	0.2156	0.7498	0.0916	1.9336
5	Lizao	0.1778	0.0721	0.0795	0.0138	0.1012	0.2744	0.1973	0.2541	0.9009	0.0842	2.1554
6	Lizao	0.0345	0.1129	0.0970	0.0030	nd	0.0631	0.2028	0.1497	0.1917	0.0206	0.8752
7	Banzao	0.0592	0.0843	0.1073	0.0092	nd	0.1129	0.2104	0.1739	0.7000	0.0273	1.4846
8	Chipingyuanzao	0.0531	0.1988	0.1075	0.0263	0.0158	0.1772	0.4311	0.1176	0.4570	0.0865	1.6708
9	Yuanlingzao	0.1470	0.1853	0.0601	0.0372	0.0463	0.1073	0.2906	0.0566	0.2217	0.0480	1.2002
10	Yuanlingzao	0.1389	0.1954	0.0871	0.0525	0.0502	0.1491	0.2607	0.0678	0.2816	0.0686	1.3518
11	Changhongzao	0.1300	0.2875	0.1161	0.0232	0.0183	0.2003	0.2822	0.1424	0.5738	0.0634	1.8371
12	Mingshenzao	0.0962	0.1497	0.1034	0.0360	0.0291	0.1433	0.2285	0.1349	0.4507	0.0725	1.4444
13	Lingbaoyuanzao	0.0600	0.1211	0.0953	0.0174	0.0107	0.1832	0.1897	0.1177	0.4505	0.0461	1.2920
14	Bianhesuan	0.0935	0.2549	0.1375	0.0528	0.0213	0.1652	0.5073	0.1311	0.5539	0.0903	2.0077
15	Xingtangdazao	0.1120	0.2501	0.1031	0.0264	0.0407	0.1307	0.3407	0.1860	0.3794	0.0867	1.6557
16	Dongzao	0.2860	1.6498	2.2386	0.1403	0.0227	1.0049	0.4797	0.6272	1.5124	0.2376	8.1991
17	Lingwuchangzao	0.0404	0.3349	0.0732	0.0132	0.0319	0.1343	0.3500	0.1782	0.6252	0.0531	1.8344
18	Huizao	0.0283	0.7057	0.2541	0.0095	0.0147	0.0557	0.4129	0.1524	0.3340	0.0293	1.9966
19	Huizao	0.0063	0.1218	0.0658	nd	nd	0.0659	0.2128	0.1053	0.4456	0.0203	1.0437
20	Huizao	0.2016	0.4730	0.1264	0.0177	nd	0.0861	0.4284	0.2628	0.9289	0.0447	2.5696
21	Jixinzao	0.1093	0.1893	0.0692	0.0215	0.0184	0.0818	0.4633	0.2617	1.1984	0.0468	2.4597
22	Lingzao	0.0334	0.1964	0.1750	0.0138	0.0224	0.1631	0.4110	0.2098	0.9071	0.0259	2.1580
23	Zanhuangdazao	0.2339	0.7069	0.1777	0.1445	0.0751	0.2029	0.4022	0.1332	0.5403	0.1416	2.7582
24	Ruoqiangdazao	0.0307	0.0713	0.0647	0.0037	0.0100	0.0456	0.1621	0.1166	0.1333	0.0202	0.6580
25	Hamidazao	0.2772	0.0535	0.0273	0.0034	nd	0.1060	0.1738	0.1642	0.3347	0.0106	1.1507
26	Pingliangyuanzao	0.0118	0.0273	0.0064	nd	nd	0.0219	0.0496	0.0285	0.0616	0.0071	0.2143
27	Ningxiayuanzao	0.0718	0.5071	0.0586	0.0203	0.0280	0.2311	0.4255	0.1014	0.3796	0.0815	1.9050
28	Shouguangdazao	0.2035	0.2357	0.0969	0.0378	0.0722	0.0926	0.2800	0.0724	0.3266	0.0488	1.4665
29	Sihongdazao	0.3323	0.1200	0.0963	0.0273	0.1757	0.1909	0.1291	0.2480	1.1380	0.1104	2.5679
30	Mangguozao	0.0400	0.1968	0.1805	0.0191	0.0216	0.1967	0.3594	0.1773	0.9519	0.0156	2.1590
31	Hetaowen	0.0878	0.3260	0.1054	0.0515	0.0352	0.0474	0.6240	0.1456	0.6699	0.1230	2.2159
32	Dalilongzao	0.3014	0.2271	0.1179	0.0386	0.1271	0.3191	0.5372	0.4581	1.2425	0.1556	3.5246
33	Guantanzao	0.2962	0.1667	0.0674	0.0374	0.0235	0.2289	0.4177	0.2012	1.0107	0.0944	2.5440
34	Jidanzao	0.3239	0.1937	0.1311	0.0383	0.1468	0.1379	0.2786	0.2142	0.8824	0.1283	2.4751
35	Chahuzao	0.1102	0.2690	0.1613	0.0446	0.0430	0.1348	0.5284	0.1512	0.6343	0.1185	2.1953
36	Jinsixiaozao	0.2039	0.3711	0.1367	0.0698	0.0370	0.3350	0.3121	0.2135	1.0898	0.0983	2.8670
37	Mianzao	0.1772	0.2059	0.1281	0.0349	0.0197	0.1682	0.1935	0.2339	0.6774	0.0614	1.9003
38	Damuzao	0.4135	0.1023	0.1900	0.0576	0.1223	0.1985	0.1282	0.2069	1.0520	0.0937	2.5651
39	Wuhezao	0.1694	0.2680	0.1368	0.0476	0.0187	0.1695	0.2729	0.1832	0.9984	0.0675	2.3320
40	Yiwudazao	0.3860	0.5278	0.0297	0.0942	0.1062	0.3318	0.3323	0.0544	0.1409	0.1948	2.1981
41	Xiangzao	0.2937	0.0808	0.0959	0.0273	0.0132	0.1905	0.1815	0.2791	0.8514	0.0352	2.0486
42	Chuanlingzao	0.1035	0.2718	0.1993	0.0451	0.0394	0.1462	0.5401	0.1652	0.6615	0.0913	2.2634

nd = not detected; Samples 1, 3–5, 7 from Shanxi province; samples 2, 6, 19, 24–25 from Xinjiang province; samples 8–11, 16, 28, 30–31 from Shandong province; samples 12–14, 20–22 from Henan province; samples 15, 23, 32–42 from Hebei province; samples 17–18, 27 from Ningxia province; sample 26 from Gansu province and 29 from Jiangsu province.

times by ultrasonic with 100 ml chloroform for 30 min per time) was adequate and appropriate for the analysis.

3.2. Optimization of the chromatographic conditions

In order to obtain chromatograms with better resolution of adjacent peaks within shorter time, the chromatographic conditions were optimized. The resolutions of these compounds were tested and compared with different reversed phase conditions using a variety of analytical columns such as Hanbon Lichrospher C_{18} (250 mm × 4.6 mm, 5 μm), Diamonsil™ C_{18} (250 mm × 4.6 mm, 5 μm) and Waters SunFire™ C_{18} (250 mm × 4.6 mm, 5 μm). Considering most of the compounds under study have wavelength maximum at 205 nm, in order to avoid blank interference, acetonitrile was chosen as organic solvent because of its low wavelength maximum compared to methanol. Since, all the 10 reference compounds are the derivates of triterpenoid acids, the investigation is commenced with a buffer at acidic pH to improve the resolution and eliminate the tailing of the target peak. Hence, 0.1% formic acid, 0.4% acetic acid and 0.05% phosphoric acid were compared in the experiment. As a result, an Waters SunFire C_{18} (250 mm × 4.6 mm, 5 μm) column with acetonitrile and 0.05% aqueous phosphoric acid (v/v) as the mobile phase was chosen as the preferred chromatographic conditions, and gradient elution was applied. It was also suggested that separation was better when column temperature was kept at 35 °C rather than 30 and 40 °C. According to the absorption maxima of 10 reference compounds on the UV spectra with three-dimensional chromatograms of HPLC–DAD detection, the wavelength was set at 205 nm for compounds **1**–**9** and 238 nm for compound **10**. Representative chromatograms for the standard analytes and for a sample were shown in Fig. 2. The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with the optimized mobile phases. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the analytes' peaks.

3.3. Validation of the HPLC method

3.3.1. Calibration curves, limits of detection and quantification

The stock solution containing the 10 markers was prepared and diluted to six appropriate concentrations for the establishment of calibration curves. The calibration graphs were plotted after linear regression of the peak areas versus the corresponding concentrations. The lowest concentration of working solution was diluted with methanol to a series of appropriate concentrations, and aliquots of diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) for each analyte under the chromatographic conditions were determined at the signal-to-noise ratio (S/N) of 3 and 10, respectively. The results of *F* test for comparison of the variance of residuals and the variance of the regression indicated that all 10 reference compounds showed good linearity ($P < 0.01$, $r^2 > 0.9999$) in a relatively wide concentration range. The LOD and LOQ of the 10 analytes were 0.29–0.68 and 1.06–2.72 μg/ml, respectively (Table 1).

3.3.2. Precision, repeatability and stability

The intra-day and inter-day precisions were investigated by determining the 10 analytes in six replicates during a single day and by duplicating the experiments on three consecutive days. Repeatability was confirmed with six different working solutions prepared from sample 10 and one of them was injected into the apparatus at 0, 4, 8, 12, 24 and 48 h within 2 days, respectively, to evaluate the stability of the solution. Variations were expressed by relative standard deviations (R.S.D.). All the results were shown in Table 2, indicating that the intra-day, inter-day, repeatability and stability R.S.D. values of the 10 compounds were all less than 3.0%.

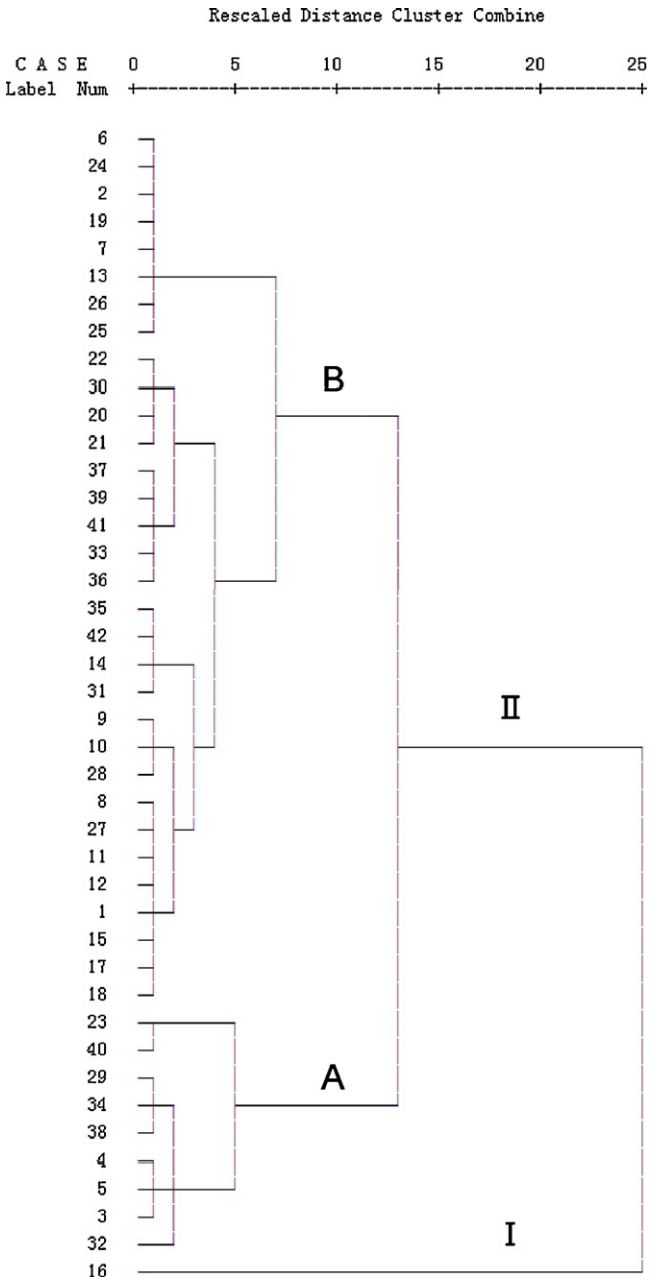


Fig. 3. Dendrograms of hierarchical cluster analysis for the 42 tested samples of *Z. jujuba*. The 42 samples are the same as in Table 3.

3.3.3. Recovery

The recovery was performed by adding known amounts of the 10 standards at low (80% of the known amounts), medium (same as the known amounts) and high (120% of the known amounts) levels. The spiked samples were then extracted, processed, and quantified in accordance with the methods mentioned above. The results were shown in Table 2. The overall recoveries lay between 94.98 and 104.09% for all reference compounds, with R.S.D. less than 3.0%, and the recoveries are comparable to 100% at 95% confidence level ($P > 0.05$), which indicating that the established method was accurate enough for the determination of the 10 triterpenoid acids in *Z. jujuba*.

Table 4
Component loading matrix for PCA.

Components (PCs)	Analytes									
	1	2	3	4	5	6	7	8	9	10
1	0.584	0.797	0.812	0.795	0.329	0.900	0.438	0.785	0.667	0.856
2	0.689	−0.454	−0.407	0.015	0.836	−0.154	−0.375	−0.002	0.175	0.224

3.4. Sample analysis

The established analytical method was then subsequently applied to a simultaneous determination of the 10 markers in 42 batches of Dazao, which contained 36 cultivars from 22 cultivation regions. The results (Table 3) showed that there were remarkable differences among the contents of the 10 triterpenoid acids in different Dazao. For example, although ursonic acid was a highest content constituent in almost all Dazao, its contents varied from 0.48 to 1.65 mg/g. The same variation could also be found in other constituents. Furthermore, the total content of the 10 triterpenoid acids in the sample 16 (which was called Dongzao in China) was triple of other samples, which suggested that Dongzao is a potential cultivar of *Z. jujuba* considering the content of triterpenoid acids. In fact, the differences in the content of triterpenoid acids among different Dazao may attribute to the many factors, including genetic variation, plant origin, and climate or geography (soil or minerals).

3.5. HCA of the samples

In order to evaluate the variation of *Z. jujuba*, hierarchical cluster analysis (HCA) was performed based on the contents of 10 triterpenoid acids from HPLC profiles. The results (Fig. 3) showed that 42 tested samples of *Z. jujuba* were divided into two main clusters (I and II) according to their contents. Sample 16 was in cluster I and the other samples were in cluster II, which was divided into two subgroups again (A and B). The results indicated that the samples collected from same cultivation region were mostly classified in one cluster, such as samples 2, 6 and 19 (from Hetian, Xinjiang province, China) were classified in same cluster, and samples 20, 21 and 22 (from Xinzheng, Henan province, China) were classified in another cluster, and the same examples could also be found in other samples, which implies that the influence of Dazao cultivation regions on the contents of the 10 triterpenoid acids is very obvious.

3.6. PCA of the samples

The determination results of 42 samples were further analyzed and classified by principal components analysis (PCA). The first two principal components (PC 1 and PC 2) with more than 69% of the whole variances were extracted for analysis. PC 1 accounted for 51.7% variances and PC 2 accounted for 17.9%. The other principal components which had a minor effect on the model were discarded. The components loading matrix was shown in Table 4. According their loadings, PC 1 had good correlation with compounds **2**–**4**, **6**, **8**–**10** and PC 2 had good correlation with compounds **1** and **5**, which indicated that almost all of the 10 compounds may contribute to the classification of the samples. The scatter plot was shown in Fig. 4, where each sample was represented as a marker. It was noticeable that the samples were clearly clustered into four domains. Sample 16 was in domain I, samples 32 was in domain II, samples 4, 29, 34 and 38 were in domain III and the others were in domain IV. Actually, these results were consistent with their natural properties. For example, sample 16, named Dongzao, whose ripping season was the latest in the 42 tested samples, and was usually consumed as fresh table fruit in China with their succulent and sweet taste characters. Sample 32 (Called Dalilongzao in China) was mainly used as ornamental plant with their unique character of tortuous branch. And these results furtherly supported the viewpoints of Yu et al. [23] and Gao and Zhang [24] that the genetic distance of Dongzao was remote to the other cultivars of *Z. jujuba*, which suggested that the presented method may be helpful for

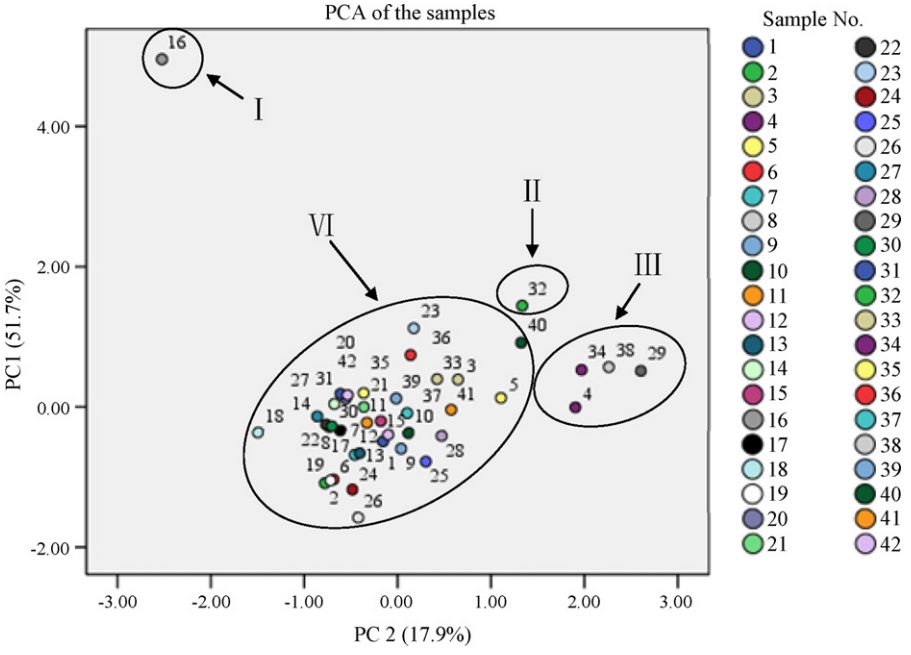


Fig. 4. The scatter plot obtained by PCA of the 42 samples of *Z. jujuba*.

discrimination Dongzao from the other cultivars in chemotaxonomy.

4. Conclusions

In the present study, the 10 triterpenoid acids were simultaneously determined in fruits of *Z. jujuba* by the developed HPLC–DAD method, and the method was successfully applied to 42 samples which contained 36 cultivars from 22 cultivation regions. These 10 triterpenoid acids were analyzed simultaneously with acceptable performance of linearity, precision, repeatability and accuracy in an analysis time of 70 min. From the results, the cultivar of Dongzao should be the best choice in the 42 tested cultivars of *Z. jujuba* when the tertenpoid acids were utilized as major active constituents in TCM. HCA and PCA approaches applied on chromatographic data obtained using HPLC–UV techniques allow to cluster the cultivars of *Z. jujuba* and distinguish the cultivar of Dongzao from the others. Furthermore, the results of HCA also implied that besides the factor of cultivar, cultivation region may be a major factor to influence the contents of the triterpenoid acids of Dazao. The presented HPLC–DAD method conjugated with HCA and PCA was proved to be very helpful in utilizing Dazao better and may be useful for classification the cultivars of *Z. jujuba*. Based on the results that almost all of analyzed Dazao contained several triterpenoid acids, the presence or absence of triterpenoid acids may have the chemotaxonomic significance.

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References

[1] J.W. Li, L.P. Fan, S.D. Ding, X.L. Ding, Food Chem. 103 (2007) 454–460.
[2] S.M. Lee, B.S. Min, C.G. Lee, K.S. Kim, Y.H. Kho, Planta Med. 69 (2003) 1051–1054.
[3] B.N. Su, M. Cuendet, N.R. Farnsworth, H.H.S. Fong, J.M. Pezzuto, A.D. Kinghorn, Planta Med. 68 (2002) 1125–1128.
[4] A.B. Kundu, B.R. Barik, D.N. Mondal, A.K. Dey, A. Banerji, Phytochemistry 28 (1989) 3155–3158.
[5] S.M. Lee, J.G. Park, Y.H. Lee, C.G. Lee, B.S. Min, J.H. Kim, Biol. Pharm. Bull. 27 (2004) 1883–1886.
[6] K. Yoshidawa, N. Shimono, S. Arihara, Tetrahedron Lett. 32 (1991) 7059–7062.
[7] K. Yoshidawa, N. Shimono, S. Arihara, Chem. Pharm. Bull. 40 (1992) 2275–2278.
[8] J.R. Lewis, J. Nat. Rep. 9 (1992) 81–101.
[9] B.H. Han, M.H. Park, S.T. Wah, Tetrahedron Lett. 28 (1987) 3957–3958.
[10] R.L. Huang, W.Y. Wang, Y.H. Kuo, Y.L. Lin, Chin. Pharm. J. 53 (2001) 179–184.
[11] X.C. Li, L. Cai, C.D. Wu, Phytochemistry 46 (1997) 97–101.
[12] S. Suksamrarn, P. Panseeta, S. Kunchanawatta, T. Distaporn, S. Ruktasing, A. Suksamrarn, Chem. Pharm. Bull. 54 (2006) 535–537.
[13] T. Fujioka, Y. Kashiwada, R.E. Kilkuskie, L.M. Cosentino, L.M. Ballus, J.B. Jiang, W.P. Janzen, I.S. Hen, K.H. Lee, J. Nat. Prod. 57 (1994) 243–247.
[14] M.C. Recio, R.M. Giner, S. Manez, Planta Med. 61 (1995) 9–12.
[15] S. Manez, M.C. Recio, R.M. Giner, J.L. Rios, Eur. J. Pharmacol. 334 (1997) 103–105.
[16] E. Pisha, H. Chai, I.-S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, Nat. Med. 1 (1995) 1046–1051.
[17] V. Zuco, R. Supino, S.C. Righetti, L. Cleris, E. Marchesi, C.G. Passerini, F. Formelli, Cancer Lett. 175 (2002) 17–25.
[18] B. Shao, H.Z. Guo, Y.J. Cui, A.H. Liu, H.L. Yu, H. Guo, M. Xu, D.A. Guo, J. Pharm. Biomed. Anal. 44 (2007) 737–742.
[19] X.J. Tan, Q. Li, X.H. Chen, Z.W. Wang, Z.Y. Shi, K.S. Bi, Y. Jia, J. Pharm. Biomed. Anal. 47 (2008) 847–853.
[20] X.H. Wang, T. Cui, X.J. Qi, G.S. Du, J. Zhao, Food Sci. 23 (2002) 137–138.
[21] J. Chen, R. Tang, Pract. Clin. J. Integr. Trad. Chin. Western Med. 4 (2004) 68–69.
[22] Y. Zhao, Z. Li, X. Zhou, W. Cai, X. Gong, C. Zhou, J. Pharm. Biomed. Anal. 48 (2008) 1230–1236.
[23] C.G. Yu, J.Z. Chen, Y.L. Yin, J. Jiangsu Forest Sci. Technol. 32 (2005) 15–18.
[24] X.M. Gao, J.H. Zhang, J. Northwest Sci.-Technol. Univ. Agric. For. 32 (2004) 109–110.