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Simultaneous quantification of major bioactive constituents from Zhuyeqing Liquor by HPLC-PDA

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Abstract

Background: Zhuyeqing Liquor (ZYQL) is a famous traditional Chinese functional liquor. For quality control of ZYQL products, quantitative analysis using high-performance liquid chromatography coupled with photodiode array detector (HPLC-PDA) was undertaken.

Methods: Eighteen compounds from ZYQL were simultaneously detected and used as chemical markers in the quantitative analysis, including 3-hydroxy-4,5(*R*)-dimethyl-2(5*H*)-furanone (**M1**), isobiflorin (**M2**), vanillic acid (**M3**), biflorin (**M4**), genipin 1-O-*β*-D-gentiobioside (**M5**), 1-sinapoyl-*β*-D-glucopyranoside (**M6**), geniposide (**M7**), epijasmnoside A (**M8**), ferulic acid (**M9**), luteolin 8-C-*β*-glucopyranoside (**M10**), isoorientin (**M11**), narirutin (**M12**), hesperidin (**M13**), 6′-O-sinapoylgeniposide (**M14**), 3,5-dihydroxy-3′,4′,7,8-tetramethoxyl flavones (**M15**), 3′,4′,3,5,6,8-hexamethoxyl flavone (**M16**), kaempferide (**M17**), and tangeretin (**M18**).

Results: The separation by gradient elution was achieved on SHIMADZU VP-ODS column (4.6 × 150 mm, 5 μm) at 30°C with methanol (A)/0.1% phosphoric acid (B) as the mobile phase. The detection wavelengths were 254, 278, and 335 nm. The optimized HPLC method provided a good linear relation ($r \geq 0.9991$ for all the target compounds), satisfactory precision (RSD values less than 1.47%) and good recovery (97.40% to 103.44%). The limits of detection ranged between 0.20×10^{-4} and 64.90×10^{-4} μg/μL for the different analytes. Furthermore, the optimum sample preparation was obtained from HPD_{100} column eluted with water and 95% ethanol, respectively.

Conclusions: Quality control of ZYQL products, in total seven samples and twelve parent plants, was examined by this method, and results confirmed its feasibility and reliability in practice.

Keywords: Zhuyeqing Liquor; Bioactive constituent; Quantitative analysis; HPLC-PDA

Background

Zhuyeqing Liquor (ZYQL), authorized as a functional health liquor in 1998 by the Ministry of Public Health in China, is a famous traditional Chinese functional liquor. The history of ZYQL could be traced back to the Warring States Period and became popular among people in the South and North Dynasties. In the Tang Dyansty and Song Dynasty, it had reached its climax (Yang 2007). ZYQL was designed based on the principles of traditional Chinese medicine (TCM) and comprises 12 herbs: *Lophatherum gracile* Brongn. (Zhuye), *Gardenia jasminoides* Ellis (Zhizi), *Lysimachia capillipes* Hemsl. (Paicao), *Angelica sinensis* (Oliv.) Diels (Danggui), *Kaempferia galanga* L. (Shannai), *Citrus reticulata* Blanco (Chenpi), *Chrysanthemum morifolium* Ramat. (Juhua), *Amomum villosum* Lour. (Sharen), *Santalum album* L. (Tanxiang), *Eugenia caryophyllata* Thunb. (Gongdingxiang), *Aucklandia lappa* Decne. (Guangmuxiang), and *Lysimachia foenum-graecum* Hance (Linglingxiang). According to its long-term history use, ZYQL has various biological properties such as anti-oxidant, anti-fatigue, and immunoenhancement (Han 2007).

Up to now, many studies show solicitude for the color, smell, and taste of the health functional liquor; few studies pay close attention to its chemical constituents and quality control. Currently, chemical analytical methods for the quality control of ZYQL have not been established. Therefore, it is necessary to establish a rapid and effective method

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for the quantitative analysis of the health functional liquor. In this study, the system of high-performance liquid chromatography coupled with photodiode array detector (HPLC-PDA) was used for analyzing the chemical profile of ZYQL. This method includes many advantages like high speed detection, excellent peak shapes, less solvent usage, well-defined chemical constituents, and simultaneous detection of multi-constituents, which is better than finger-printing. Thus, simultaneous determination by RP-HPLC method is suitable for quantitative analysis and can be used as an effective tool to evaluate herbal medicine products.

Methods

Chemicals and materials

Methanol (HPLC-grade) was purchased from Fisher Scientific Co. (Franklin, MA, USA). Water for HPLC analysis was purified by a Milli-Q water purification system (Millipore, Billerica, MA, USA). Phosphoric acid (analytical grade) was purchased from Tianjin Guangfu Chemical Reagent Co. Ltd. (Tianjin, China). Other solvents from Tianjin Guangfu Chemical Reagent Co. Ltd. (Tianjin, China) were all of analytical grade.

Reference compounds of **M1** to **M18** (Figure 1) were isolated previously from ZYQL by author, structures of which were elucidated by comparison of spectral data (UV, MS, ^{1}H NMR, and ^{13}C NMR) with the literature data (Lin et al. 2006; Okamuraα et al. 1998; Huang et al. 2012; Zhang and Chen 1997; Ma et al. 2009; Miyake et al. 2007; Liu et al. 2011; Chen et al. 2008; Rayyan et al. 2005; Kumarasamy et al. 2004; Ke et al. 1999; Yoo et al. 2002; Dinda et al. 2011; Esteban et al. 1986; Ballester et al. 2013; Wang et al. 2010; Hòrie et al. 1998). The purity of each reference standard

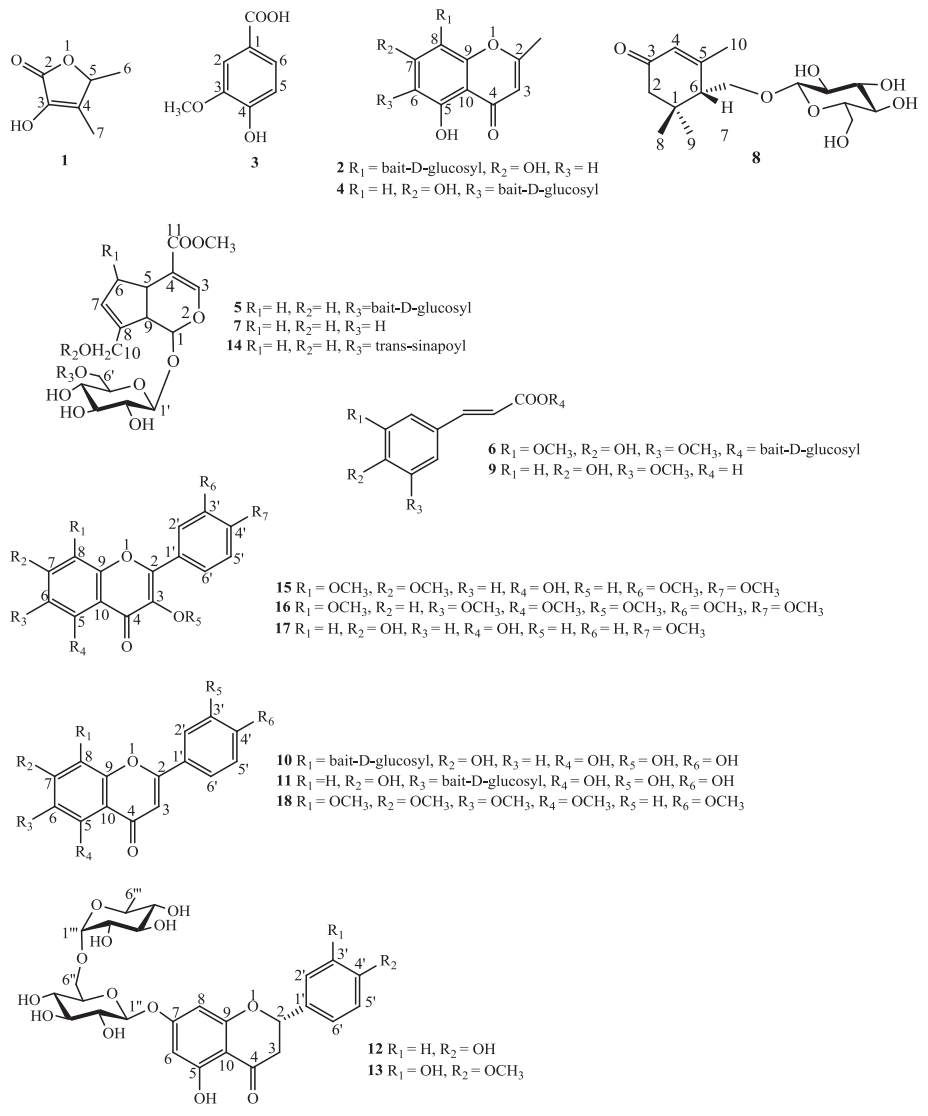

Figure 1 Structures of compounds M1 to M18.

was determined to be above 98% by HPLC analysis based on a peak area normalization method, detected by HPLC-PDA and confirmed by HR-ESI-TOF-MS and NMR spectroscopy.

The samples of different batch and different alcoholicity of ZYQL and the 12 parent plants were provided by Shanxi XinghuaCun Fen Jiu Group Co., Ltd. (Shanxi, China). The 12 parent plants were identified by Professor Jincai Lu (Shenyang Pharmaceutical University, Shenyang, China). The voucher specimen was deposited at Shenyang Pharmaceutical University (Shenyang, China) and registered under the number ZYQL 2011050101.

Instrumentation and chromatographic conditions

Chromatographic analysis was performed on Waters 2695 Alliance HPLC system (Waters Co., Milford, MA, USA) with Waters 2998 PDA detector. Chromatographic separation was carried on a SHIMADZU VP-ODS column (4.6 mm × 150 mm, 5 μm; Shimadzu, Kyoto, Japan) at a column temperature of 30°C using methanol (A) and 0.1% phosphoric acid (B) as mobile phase with the gradient elution procedure show in Table 1. The flow rate was set at 1.0 ml/min and the detection wavelengths were 254 nm (for compounds **M1** to **M5**, **M7**, **M8**, and **M17**), 278 nm (for compounds **M12** and **M13**), and 335 nm (for compounds **M6**, **M9** to **M11**, **M14** to **M16**, and **M18**), which were chosen based on the maximum absorption of all the tested compounds. The injection volume was 10 μL, and the analytes were well separated in chromatographic conditions above.

Standard solution preparation

Individual stock solutions were prepared by dissolving the standards in methanol to obtain 3-hydroxy-4,5(*R*)-dimethyl-2(5*H*)-furanone (**M1**) 19.920 mg mL^{-1}, isobiflorin (**M2**) 8.330 mg mL^{-1}, vanillic acid (**M3**) 5.802 mg mL^{-1}, biflorin (**M4**) 3.911 mg mL^{-1}, genipin 1-O-*β*-D-gentiobioside (**M5**) 4.405 mg mL^{-1}, 1-sinapoyl-*β*-D-glucopyranoside (**M6**) 1.115 mg mL^{-1}, geniposide (**M7**) 23.804 mg mL^{-1}, epijasmnoside A (**M8**) 12.060 mg mL^{-1}, ferulic acid (**M9**) 2.515 mg mL^{-1}, luteolin 8-C-*β*-glucopyranoside (**M10**) 1.510 mg mL^{-1}, isoorientin (**M11**) 2.203 mg mL^{-1}, nairutin (**M12**) 1.032 mg mL^{-1}, hesperidin (**M13**) 4.801 mg mL^{-1}, 6′-O-sinapoylgeniposide (**M14**) 5.312 mg mL^{-1}, 3,5-dihydroxy-3′,4′,7,8-tetramethoxyl flavones (**M15**) 5.021 mg mL^{-1}, 3′,4′,3,5,6,8-hexamethoxyl flavone (**M16**) 15.005 mg mL^{-1}, kaempferide (**M17**) 6.408 mg mL^{-1}, and tangeretin (**M18**) 17.155 mg mL^{-1}. A mixed solution containing all the 18 standards was prepared as accurately as 108 μL **M1**, 6.8 μL **M2**, 2.4 μL **M3**, 8.0 μL **M4**, 165 μL **M5**, 96 μL **M6**, 106 μL **M7**, 3.4 μL **M8**, 8.2 μL **M9**, 9.5 μL **M10**, 7.9 μL **M11**, 35 μL **M12**, 40 μL **M13**, 80 μL **M14**, 8.2 μL **M15**, 11 μL **M16**, 12 μL **M17**, and 4.6 μL **M18** and were placed in a 2-mL flask with stopper, diluted with methanol to make sure the volume reached 2 mL. All prepared solutions were respectively stored in a refrigerator at 4°C when not in use.

Table 1 Time program of the gradient elution

Time (min)	Flow (mL/min)	Methanol (%)	0.1% Phosphatic acid (%)
0	1	5	95
70	1	55	45
75	1	60	40
110	1	80	20
120	1	98	2
125	1	98	2

Treatment for samples

For the analysis, 40 mL of ZYQL were evaporated in vacuum at 50°C to dryness. The dry residue was processed as follows in order to obtain better analytical results: The residue was dissolved with water (10 mL) and applied to an HPD_{100} column eluted with water (150 mL); the water eluent was discarded and then eluted with 95% ethanol (150 mL). The 95% ethanol eluent was condensed and dissolved with methanol and then placed in a 2-mL flask with stopper, with a methanol-metered volume. Prior to HPLC analysis, the sample solution was passed through a 0.22-μm millipore filter.

The 12 crude dried parent plants were pulverized and sifted through 40 mesh sieve, respectively. One gram of the powder from the parent plant was placed in a 50-mL flask with stopper, then weighed again correctly, and extracted by ultrasonic method with 20 mL methanol for 30 min. Then standing, it was cooled down to room temperature (22°C) and the weight was mended to the incipient weight with methanol. Prior to HPLC analysis, the sample solution was passed through a 0.22-μm millipore filter.

Validation of the method

Calibration curves

Linearity was established by the injection of 1, 2, 4, 8, 12, 16, and 20 μL of the mixed reference standard solution prepared, respectively. Calibration graphs were plotted subsequently based on linear regression analysis of the integrated peak (Y) versus content (X, μg).

Limits of detection and quantitation

In order to evaluate the limits of detection (LODs) and the limits of quantification (LOQs) of the compounds, mixed standard stock solution was further diluted serially to provide a series of appropriate concentrations, and an aliquot of the diluted solutions was injected into HPLC for analysis. The LOD and LOQ for each analyte was calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

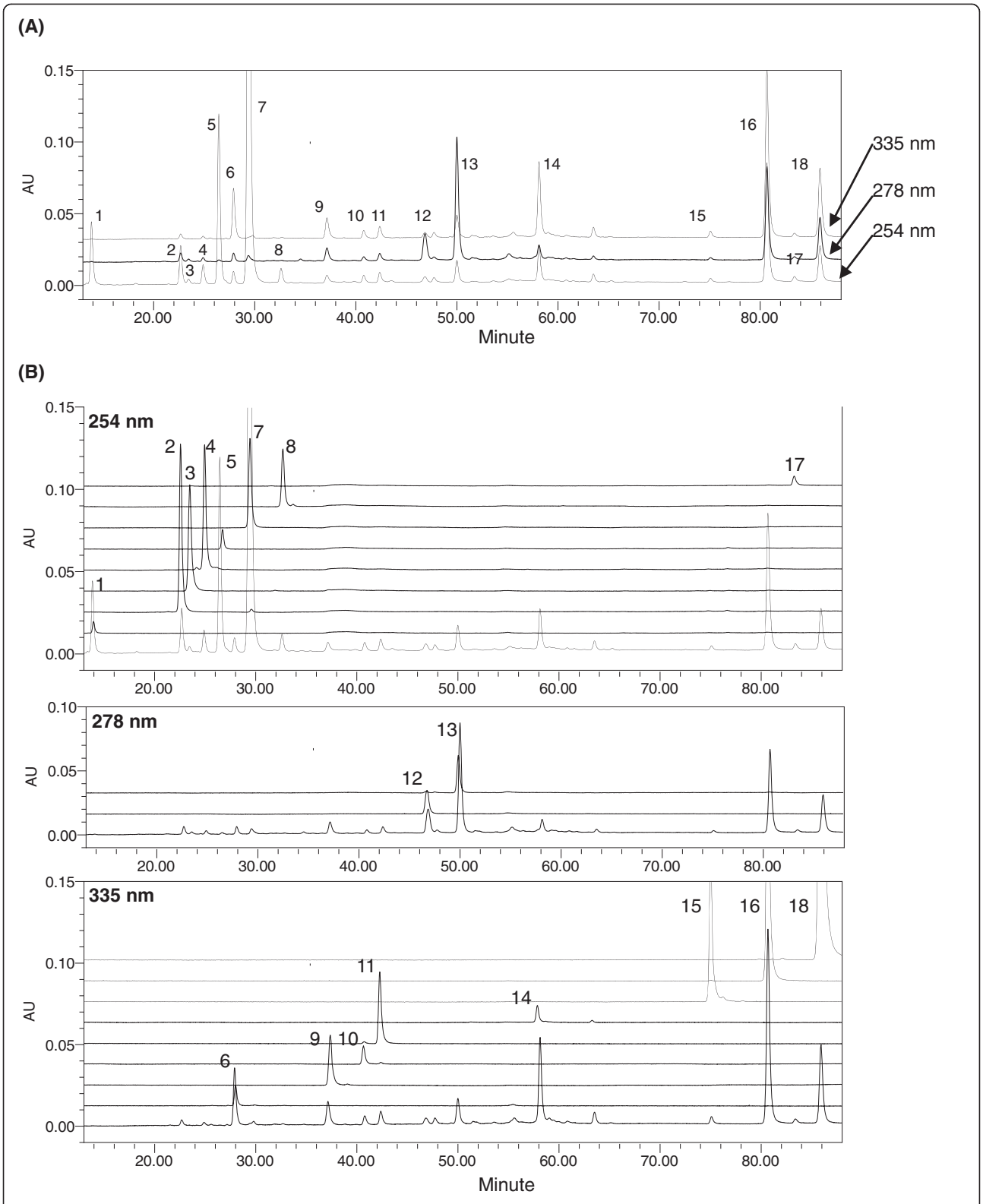

Figure 2 Stack views. (A) Different detector-wavelength HPLC chromatograms of mixed reference standards (from up to down: 335, 278, 254 nm). Column: SHIMADZU VP-ODS column (4.6 mm × 150 mm, 5 μm), temperature of 30°C. **(B)** HPLC chromatograms of **M1** to **M18** and mixed reference standards (from up to down: 254 nm **M1**, **M2**, **M3**, **M4**, **M5**, **M7**, **M8**, **M17**, mixed reference standards; 278 nm **M12**, **M13**, mixed reference standards; 335 nm **M6**, **M9**, **M10**, **M11**, **M14**, **M15**, **M16**, **M18**, mixed reference standards).

Table 2 Optimization of the treatment method of Zhuyeqing Liquor (μg/mL)

Compound[a]	Treatment method				
	Method 1	Method 2	Method 3	Method 4	Method 5
M1	ND	5.2494	3.1331	9.5943	16.4270
M2	0.0888	0.5371	0.5078	0.5818	0.5942
M3	0.0922	0.0906	0.0388	0.0939	0.1063
M4	0.0263	0.4980	0.5098	0.5069	0.5153
M5	ND	44.0545	101.6907	100.6372	101.7888
M6	0.1479	0.1880	0.1902	0.1927	0.1936
M7	45.2421	563.2436	570.3556	566.0022	574.2514
M8	20.8481	24.6279	25.6049	24.5513	25.7319
M9	0.1931	0.1782	0.1906	0.1918	0.1968
M10	0.0324	0.0526	0.0600	0.0705	0.0736
M11	0.2009	0.4601	0.4871	0.4915	0.4954
M12	0.7071	1.0544	1.0683	1.0699	1.0877
M13	1.1371	2.5392	2.8461	2.8029	2.9909
M14	ND	3.4939	4.0839	4.0563	4.3756
M15	ND	0.0641	0.0678	0.0688	0.0689
M16	0.5189	0.6113	0.5842	0.3282	0.6623
M17	2.9599	2.9776	2.1520	1.6446	2.9957
M18	0.4448	0.5307	0.4278	0.4673	0.5413
Sum[b]	72.6396	650.4512	713.9987	713.3521	733.0967

Sample in optimization of the treatment method was 45° Zhuyeqing Liquor (20130207). Method 1, acetoacetate extract; method 2, *n*-butanol extract; method 3, 70% ethanol treatment; method 4, SPE column eluted with methanol; method 5, HPD_{100} column eluted with ethanol. [a]'ND' in the 'Compound' column expressed under LOQ. [b]Total content of the 18 investigated compounds.

Precision and stability

The precision of the chromatographic system was validated by injecting 10 μL of the mixed reference solution six times during 1 day. Stability study was performed with sample solution in 48 h (the time points are 0, 5, 10, 15, 25, 35, and 48 h, respectively). Variations were expressed by relative standard deviations (RSD) of peak area.

Repeatability and recovery

The repeatability test was analyzed by injecting six independently prepared samples (45° ZYQL (20130207), the concentration, and prepared method as the 'Treatment for samples'). The RSD value of concentration was adopted to evaluate repeatability. The recovery tests were studied by adding the proper amount of mixed-reference standard

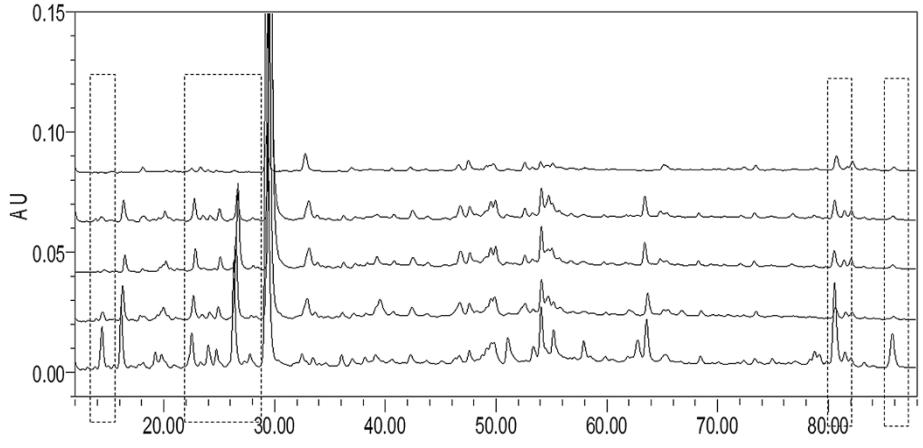

Figure 3 Stack views of 45° Zhuyeqing Liquor preparation method HPLC chromatograms (254 nm, from up to down: method 1, method 2, method 3, method 4 and method 5).

Table 3 Regression equations, correlation coefficients, and linear range for 18 analytes in Zhuyeqing Liquor

Analyte	Time (t_R)	Linear regression				
		Regression equation ($n = 3$)	Correlation coefficients r	Linear range (μg)	LOD (10^{-4} μg/μL)	LOQ (10^{-4} μg/μL)
M1	14.004	$Y = 5.48e+003X - 1.45e+003$	0.9998	1.08~21.63	64.90	216.34
M2	22.819	$Y = 2.86e+006X - 8.84e+002$	0.9999	2.80×10^{-2}~5.60×10^{-1}	1.68	5.60
M3	23.573	$Y = 4.73e+006X - 5.52e+003$	0.9991	1.70×10^{-3}~3.40×10^{-2}	0.20	0.67
M4	25.069	$Y = 1.62e+006X - 3.23e+003$	0.9997	1.55×10^{-2}~3.10×10^{-1}	3.10	10.34
M5	26.671	$Y = 5.41e+005X - 3.44e+004$	0.9994	3.63×10^{-1}~7.25	2.42	8.08
M6	28.087	$Y = 1.31e+006X - 4.70e+003$	0.9998	5.25×10^{-2}~1.05	8.40	28.0
M7	29.646	$Y = 7.08e+005X + 4.19e+003$	0.9998	1.26~25.21	25.46	84.87
M8	32.875	$Y = 9.81e+005$ X - 5.87 e + 003	0.9998	2.04×10^{-2}~4.08×10^{-1}	4.08	13.60
M9	37.264	$Y = 3.17e+006X - 1.51e+004$	0.9992	1.03×10^{-2}~2.06×10^{-1}	2.06	6.87
M10	40.883	$Y = 1.65e+006X - 1.26e+002$	0.9993	7.10×10^{-3}~1.42×10^{-1}	2.13	7.11
M11	42.396	$Y = 2.38e+006X - 6.23e+003$	0.9991	8.70×10^{-3}~1.74×10^{-1}	1.74	5.83
M12	46.878	$Y = 2.67e+006X - 3.22e+002$	0.9998	1.75×10^{-2}~3.50×10^{-1}	5.25	17.51
M13	50.008	$Y = 1.69e+006\ X + 3.71e+003$	0.9998	9.56×10^{-2}~1.91	5.74	19.12
M14	58.096	$Y = 4.55e+005X - 2.11e+003$	0.9998	2.13×10^{-1}~4.25	12.75	42.50
M15	74.987	$Y = 2.15e+006X - 7.62e+002$	0.9991	4.10×10^{-3} ~ 8.20×10^{-2}	3.28	10.95
M16	80.609	$Y = 3.59e+006X - 2.08e+004$	0.9999	8.23×10^{-2}~1.65	0.55	1.84
M17	83.248	$Y = 1.97e+005X - 3.36e+003$	0.9991	3.85×10^{-2}~7.70×10^{-1}	15.40	51.32
M18	85.890	$Y = 2.69e+006X - 1.38e+004$	0.9996	3.93×10^{-2} ~ 7.86×10^{-1}	0.79	2.64

Y is the peak area and X is the content of standard solutions; LOD refers to the limits of detection, S/N = 3; LOQ refers to the limits of quantity, S/N = 10.

Table 4 Precision, stability, recovery, and repeatability data of 18 analytes in Zhuyeqing Liquor

Analyte	Precision ($n = 6$)		Stability RSD (%)	Recovery (n = 6)					Repeatability ($n = 6$)	
	Concentrations (mg/mL)	RSD (%)		Original (μg)	Spiked (μg)	Detected (μg)	Recovery (%)	RSD (%)	Average concentration (μg/mL)	RSD (%)
M1	1.08	0.92	1.70	164.16	162.26	326.57	100.17	3.10	16.0862 ± 0.2722	1.50
M2	2.80×10^{-2}	0.82	1.52	7.11	4.20	11.41	102.44	1.84	0.5580 ± 0.0045	1.39
M3	1.70×10^{-3}	1.30	1.66	0.27	0.26	0.52	97.40	2.52	0.1072 ± 0.0020	1.96
M4	1.55×10^{-2}	0.78	1.08	5.88	2.33	8.23	100.88	2.27	0.5154 ± 0.0032	0.64
M5	3.63×10^{-1}	0.87	1.62	1217.80	54.39	1273.19	101.83	3.30	101.1963 ± 0.7206	0.72
M6	5.25×10^{-2}	0.86	1.58	6.00	7.88	14.15	103.44	1.68	0.1940 ± 0.0019	1.00
M7	1.26	0.49	1.18	1270.60	1260.30	2543.54	101.00	0.78	568.4991 ± 2.7722	0.98
M8	2.04×10^{-2}	1.04	1.76	211.50	102.00	316.34	102.79	1.46	25.1942 ± 0.2548	1.45
M9	1.03×10^{-2}	0.30	1.49	2.82	1.55	4.37	100.76	2.63	0.1947 ± 0.0018	1.00
M10	7.10×10^{-3}	1.10	1.67	3.22	1.07	4.28	99.54	3.26	0.0728 ± 0.0011	1.61
M11	8.70×10^{-3}	1.15	1.62	4.65	1.31	5.96	100.66	3.09	0.5101 ± 0.0057	1.28
M12	1.75×10^{-2}	1.09	1.39	2.43	2.63	5.14	103.13	1.48	1.0800 ± 0.0155	1.44
M13	9.56×10^{-2}	1.02	1.36	37.69	14.34	52.20	101.14	1.99	2.8911 ± 0.0351	1.21
M14	2.13×10^{-1}	0.74	1.59	943.73	31.88	975.00	98.10	2.25	4.2790 ± 0.0314	1.30
M15	4.10×10^{-3}	1.47	1.75	1.99	1.23	3.22	100.30	2.66	0.0645 ± 0.0009	1.53
M16	8.23×10^{-2}	0.91	1.58	21.52	12.35	33.77	99.20	2.44	0.6444 ± 0.0052	0.81
M17	3.85×10^{-2}	0.93	1.75	133.12	115.50	249.54	100.79	2.63	2.9457 ± 0.0498	1.36
M18	3.93×10^{-2}	1.11	1.49	10.02	11.79	21.89	100.65	1.58	0.5368 ± 0.0060	1.13

RSD refers to relative standard deviation. Samples in stability, recovery, and repeatability methods were taken from 45°Zhuyeqing Liquor (20130207).

solution to the sample (45° ZYQL (20130207)), and then processed by the method described in the 'Treatment for samples' section to yield the final concentration. The experiment was repeated six times.

Results and discussion

Optimization of chromatographic conditions

To improve resolution and sensitivity of analysis but reduce analytical time, the following chromatographic conditions were optimized (Gao et al. 2013), including different mobile phase compositions (methanol, acetonitrile, and aqueous phosphatic acid of different concentrations), column temperature, and wavelength: To inhibit ionization of the acidic ingredients in the ZYQL sample, phosphatic acid was added in mobile phase. Two mobile phase systems, methanol-phosphatic acid aqueous solution and acetonitrile-phosphatic acid aqueous solution, were examined, and then column temperatures at 25°C, 30°C, 40°C, and 50°C were compared. A sensitive wavelength was determined by PDA with reference compounds. Present researches indicated that better separation and results were obtained using a mobile phase of water and methanol rather than water and acetonitrile. Therefore, in this work, the optimum resolution was achieved using methanol (A) and 0.1% phosphatic acid (B) as mobile phase, with a column temperature of 30°C at different detection wavelengths, which were described in 'Instrumentation and chromatographic conditions' section, with gradient elution (Table 1). All 18 standard analytes could be eluted with baseline separation in 90 min. Representative chromatograms for the mixed reference standard and 18 standard compounds were shown in Figure 2A,B.

Optimization of sample preparation

In order to eliminate the water-soluble constituents and obtain the liposoluble constituents, the optimization of sample preparation was performed using 45° ZYQL (20130207). Forty milliliters of ZYQL was evaporated in vacuum at 50°C to dryness. And the following five methods were choosen to select the best method for sample preparation. First, the dry residue was suspended with water (10 mL) and extracted with acetoacetate (10 mL). The acetoacetate extract was condensed and then methanol was used to meter the volume

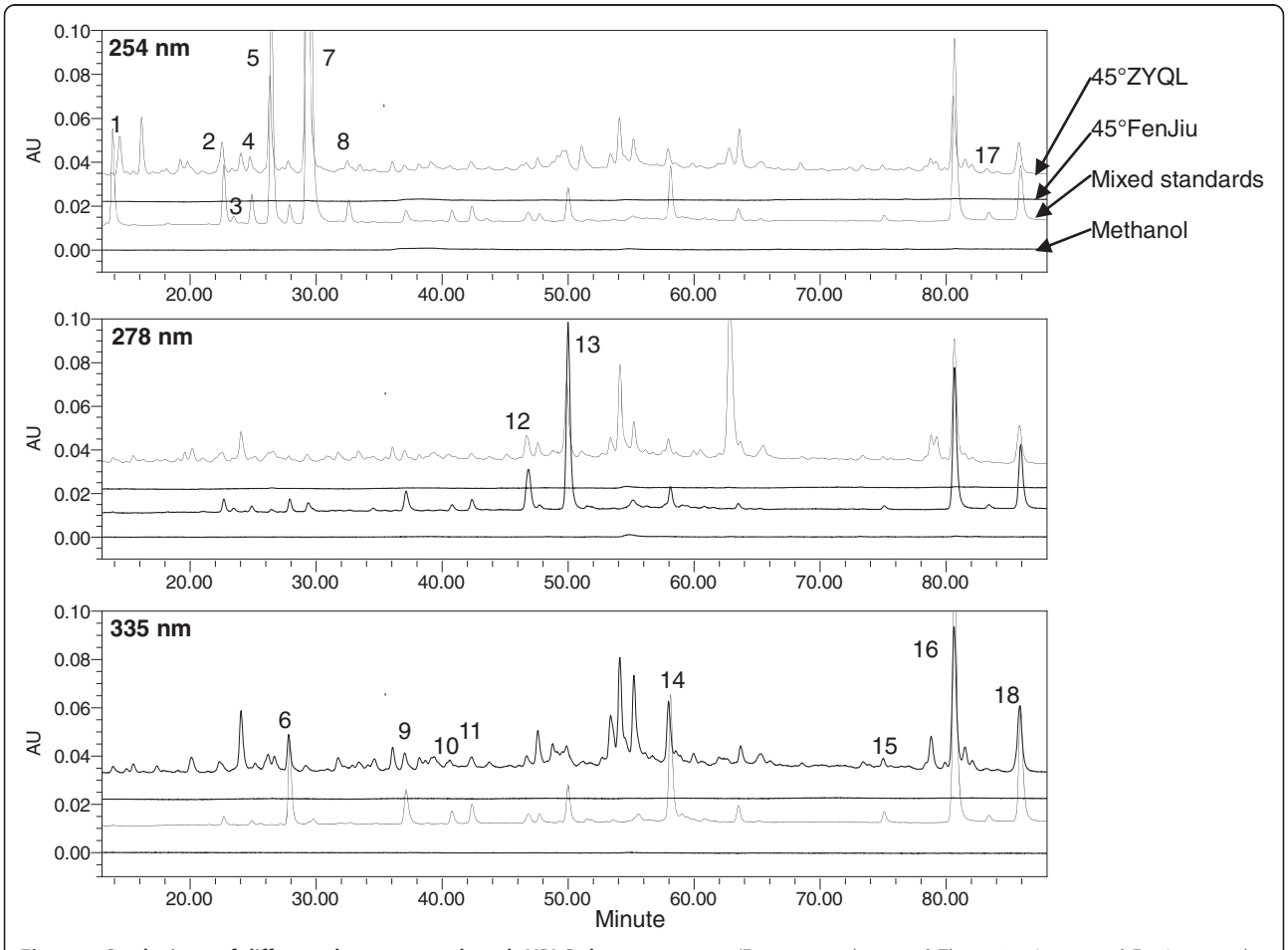

Figure 4 Stack views of different detector-wavelength HPLC chromatograms. (From up to down: 45° Zhuyeqing Liquor, 45° FenJiu, mixed reference standards, blank solvent: methanol, respectively).

(2 mL). Second, the dry residue was suspended with water (10 mL) and extracted with *n*-butanol (10 mL) The *n*-butanol extract was condensed and then methanol was used to meter the volume (2 mL). Third, the dry residues was dissolved with 70% ethanol (20 mL) to precipitate the polysaccharide and then condensed the supernate, use methanol to metered volume (2 mL). Fourth, the dry residues was dissolved with water (10 mL) as fraction A, then the remanent residues was dissolved with methanol (10 mL) as fraction B. Fraction A was applied to an SPE column eluted with water (150 mL); the water eluent was discarded; fraction B was applied to the same SPE column eluted with methanol (150 mL); the methanol eluent was condensed and methanol was used to meter the volume (2 mL). Fifth, the dry residue was dissolved with water (10 mL) and applied to an HPD_{100} column eluted with water (150 mL). The water eluent was discarded and then eluted with 95% ethanol (150 mL). The 95% ethanol eluent was condensed and then methanol was used to meter the volume (2 mL). Comparing the analytical results of the target constituents, though the former three methods proved to be more simple than the other, they could not obtain all the tested constituents and some content too lower to accurately reflect the real content. So, these three methods were deserted. The fourth one although could obtain all the tested constituents but at a lower content. Therefore, the optimized condition was selected, the fifth one (Table 2, Figure 3).

Validation of the method

The method was validated in terms of linearity, LOD and LOQ, precision, repeatability, stability, and recovery test. All calibration curves exhibited good linearity ($r \geq 0.9991$) in a relatively wide linear range as shown in Table 3. For the quantified compounds, the LOD and LOQ were 0.20×10^{-4}~64.90×10^{-4} μg/μL and 0.67×10^{-4}~216.34×10^{-4} μg/μL, respectively (Table 3), which were calculated with corresponding standard solution on the basic of a signal-to-noise ratio (S/N) of 3 and 10, respectively. Table 4 showed the results of precision, stability, recovery and repeatability of the 18 analytes. It was indicated that the RSD of the precision variations were less than 1.47% for all 18 analytes. The RSD of repeatability was less than 1.96% for all the analysis, which proved that this assay had good reproducibility. Stability test results, with RSD less than 1.76%, indicated that the sample solution was stable at room temperature for at least 48 h. The mean recovery rates, which ranged from 97.40% to 103.44% with RSD values less than 3.30% for the analytes concerned, showed that the developed analytical method had good accuracy. All these values fall within acceptable limits, which indicates this HPLC method is reliable with significant repeatability, recovery rate, and precision. The results proved that HPLC is appropriate for analyzing and assessing the quality of ZYQL.

Table 5 Contents of 18 analytes in different batches and different alcoholicity of Zhuyeqing Liquor (μg/mL)

Compound[a]	45° FenJiu	38°	42°	45°				
	20130207	20130207	20130207	20130207	20120601	20110507	20100417	20090302
M1	ND	15.1395	15.1395	16.7150	16.6674	16.6558	16.6543	16.6239
M2	ND	0.3689	0.3945	0.5851	0.5854	0.5846	0.5839	0.5840
M3	ND	0.0792	0.0830	0.1063	0.1064	0.1058	0.1032	0.1060
M4	ND	0.3028	0.3742	0.5180	0.5139	0.5106	0.5081	0.5111
M5	ND	65.7206	71.8348	101.7175	101.3777	101.0265	101.1293	100.9297
M6	ND	ND	ND	0.1930	0.1904	0.1901	0.1893	0.1934
M7	ND	273.1958	309.9846	574.4770	574.1508	574.1103	573.6367	573.2887
M8	ND	12.6644	19.9230	25.8595	25.4397	25.2009	25.4234	25.1453
M9	ND	0.1395	0.1659	0.1956	0.1934	0.1909	0.1933	0.1938
M10	ND	0.0536	0.0612	0.0715	0.0714	0.0715	0.0715	0.0721
M11	ND	0.2612	0.2963	0.4993	0.4972	0.4983	0.4919	0.5000
M12	ND	0.5690	0.6861	1.0884	1.0835	1.0822	1.0806	1.0830
M13	ND	2.0079	2.0709	2.9740	2.9731	2.9587	2.8982	2.9883
M14	ND	2.5058	2.7162	4.3718	4.3672	4.3615	4.3586	4.3699
M15	ND	0.0549	0.0585	0.0689	0.0686	0.0684	0.0685	0.0686
M16	ND	0.4758	0.5602	0.6434	0.6435	0.6422	0.6427	0.6383
M17	ND	2.6999	2.8338	3.0047	2.9943	2.9927	2.9836	2.9735
M18	ND	0.3534	0.4045	0.5495	0.5432	0.5427	0.5389	0.5433
Sum[b]	ND	376.5922	427.5872	733.6385	732.4671	731.7937	731.5560	730.8129

[a]'ND' in the 'Compound' column expressed under LOQ. [b]Total content of the 18 investigated compounds.

Table 6 Contents of 18 analytes in 12 parent plants (mg/g)

Compound[a]	Zhuye	Zhizi	Paicao	Danggui	Shannai	Chenpi	Juhua	Sharen	Tanxiang	Gongdingxiang	Guangmuxiang	Linglingxiang
M1	ND	2.1057	ND	ND	ND	ND	ND	ND	ND	ND	ND	19.8802
M2	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.7841	ND	0.0790
M3	ND	ND	ND	ND	ND	ND	ND	ND	0.0038	ND	ND	ND
M4	ND	ND	ND	ND	ND	ND	ND	ND	ND	5.4547	ND	0.0498
M5	ND	19.9996	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M6	ND	1.6513	ND	ND	ND	0.2580	ND	ND	ND	ND	ND	ND
M7	ND	43.3886	ND	2.5174	ND	ND	ND	ND	ND	ND	ND	ND
M8	ND	5.0078	ND	ND	ND	1.0468	ND	ND	ND	ND	ND	ND
M9	ND	ND	ND	0.6170	ND	0.0805	ND	ND	ND	ND	ND	ND
M10	0.3268	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M11	0.4161	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
M12	ND	0.0111	ND	ND	ND	3.6198	0.2104	ND	ND	ND	0.0217	ND
M13	ND	ND	ND	ND	ND	4.6719	0.3139	ND	ND	ND	ND	ND
M14	ND	8.6950	0.0302	ND	ND	ND	ND	0.0292	0.0025	ND	ND	ND
M15	ND	ND	ND	ND	ND	0.1017	0.0472	ND	ND	ND	ND	ND
M16	0.0638	0.0750	ND	0.2479	17.7933	0.6813	0.4755	ND	ND	ND	ND	0.3214
M17	ND	1.9330	ND	ND	ND	0.8970	1.4942	ND	ND	ND	ND	ND
M18	ND	0.0648	ND	ND	ND	0.5263	0.1625	ND	ND	ND	ND	ND
Sum[b]	0.8067	82.9319	0.0302	3.3823	17.7933	11.8833	2.7037	0.0292	0.0063	10.2388	0.0217	20.3304

[a]'ND' in the 'Compound' column expressed under LOQ. [b]Total content of the 18 investigated compounds.

Sample analysis

The HPLC analytical method described above was subsequently used to simultaneously quantify 18 compounds in seven commercial products and 12 parent plants supplied by Shanxi XinghuaCun Fen Wine Group Co., Ltd. (Shanxi, China). Generally, the 18 compounds were authenticated by comparison of their retention times and MS spectra with those of reference standards. The representative HPLC chromatograms of mixed standard solution and sample solutions are shown in Figure 4. The analytical results are summarized in Tables 5 and 6. According to the chromatographic results shown in Table 5, there was no any constituents to be detected in 45° FenJiu (solvent of ZYQL). Moreover, the concentration of compounds **M1** to **M18** in 45° ZYQL were higher than those in 42° and 38°, which showed that with the increase of alcoholicity, the content of bioactive constituents increased as well. In addition, there was no content difference between the successive 5 years of 45° ZYQL. This indicated that the quality of 45° ZYQL was stable for at least 5 years.

Table 6 showed the content of compounds in 12 parent plants, which exhibited that the major bioactive constituents were mainly from *Gardenia jasminoides* Ellis (Zhizi), *Kaempferia galanga* L. (Shannai), *Citrus reticulata* Blanco (Chenpi), and *Lysimachia foenum-graecum* Hance (Linglingxiang). And this result was greatly useful and helpful for the quality control and further formula optimization of the technical study of Zhuyeqing Liquor.

Conclusions

An HPLC-PDA method has been developed for the simultaneous determination of 18 major compounds extracted from ZYQL for the first time. The validation data indicated that this method is reliable and can be applied to determine the contents of the 18 compounds in different ZYQL products. This valuable information concerning the concentration of these bioactive constituents in ZYQL could be of great importance for the quality assessment and should therefore be useful for the guidance of development of the new health care products. Furthermore, this HPLC-PDA assay supplies a rapidness and effectiveness method for the simultaneous determination of multiple constituents in ZYQL.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HYG carried out the whole experiment, SYW participated in the sample preparation, HYG and JHW performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

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