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Simultaneous quantification of 17 constituents from Yuanhu Zhitong tablet using rapid resolution liquid chromatography coupled with a triple quadrupole electrospray tandem mass spectrometry

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ABSTRACT

A rapid resolution liquid chromatography coupled with a triple quadrupole mass spectrometry (RRLC-QQQ) was employed to identify and quantify the major bioactive constituents in Yuanhu Zhitong tablet (YZT), a traditional Chinese medicine. Seventeen constituents were simultaneously determined and quantified by reference standards in 9 min, namely scopoletin, protopine, α-allocryptopine, tetrahydropalmatine, coptisine, tetrahydroberberine, corydaline, berberine, byakangelicin, byakangelicol, xanthotoxin, bergapten, pimpinellin, oxypeucedanin, imperatorin, osthole and isoimperatorin. All of them were performed on an Agilent XDB C_{18} column (4.6 mm × 50 mm, 1.8 μm) with linear gradient elution of acetonitrile–0.3% formic acid water (pH 2.7). The proposed method was applied to analyze 15 batches of samples with acceptable linearity (r^2, 0.9938–0.9999), precisions (RSD, 1.01–4.92%), repeatability (RSD, 1.33–4.91%), stability (RSD, 1.46–4.86%), recovery (RSD, 1.11–4.81%) of the seventeen compounds. Furthermore, the Hierarchical Cluster Analysis was applied to classify 15 samples based on characteristics of the 17 compound markers. As a result, the analytical method possessing high sensitivity and speed is suitable for the quality control of YZT.

1. Introduction

Traditional Chinese medicine (TCM) is getting more and more attention all over the world due to its exactly clinical practice. However, quality control is one of the most important problems for the application and development of TCM, and manufacturers have paid particular consideration on its efficacy and safety [1,2]. Since the effect of an herb medicine always results from the synergy of multiple components, and the conventional quality control mode, which cannot represent the real quality of herb medicine, is the simply quantitative analysis of one or only a few components, quantitative analysis of multiple active components is becoming the most direct and indispensable method for quality control of TCM, and increasing number of researchers have begun to apply this technology in the TCM, especially for prescription [3–7].

Yuanhu Zhitong tablet (YZT, commercial product) is a classical herb medication composed of 223 g of *Radix Angelicae dahuricae* and 445 g of *Rhizoma Corydalis* (processing with vinegar). *Radix Angelicae Dahuricae* includes two origins of *Angelica Dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. and *Angelica Dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. var. *formosana* (Boiss.) Shan et Yuan in *China Pharmacopoeia* (ChP.), which are both used in commercial markets. YZT is widely used in the treatment for gastralgia, costalgia, headache and dysmenorrhea caused by qi stagnancy and blood stasis [8]. Chemically, alkaloids in *Rhizoma Corydalis* and coumarins in *Radix Angelicae Dahuricae* have been considered as the active components [9–16], and of which the DL-tetrahydropalmatine (DL-THP) is the most important compound. It has been officially chosen as the quality control marker for this preparation in ChP. Due to the different origins, sources, harvest time, pretreatments and manufacturing processes of medicinal materials, the quality of YZTs varies significantly. Thus, quality control of YZT products is very critical to ensure their efficacy and safety.

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To date, only a few bioactive components such as DL-THP, protopine, berberine, imperatorin and isoimperatorin have been chosen as markers for quality assessment [17–19]. A variety of chromatographic techniques including thin layer chromatography (TLC) [20], high performance liquid chromatography (HPLC) [21], rapid resolution liquid chromatography (RRLC) [22] and capillary electrophoresis (CE) [23] have been utilized to do the qualitative or quantitative analysis in YZT. The digitized HPLC fingerprints could reflect overall properties of YZT at present; however, the most common peaks and exact compounds could not be identified and quantified [24]. Additionally, there are a lot of micro-compounds (scopoletin, tetrahydroberberine, osthole, oxypeucedanin and so on) below LOD of UV or DAD detector and longer analytical time in above-mentioned methods. Therefore, it is necessary to develop a new detecting method that is more sensitive and faster. Rapid resolution liquid chromatography coupled with a triple quadrupole electrospray tandem mass spectrometry (RRLC-QQQ) is a powerful tool to solve the above problems because of its high sensitivity and rapid resolution [25–27]. In this paper, the established RRLC-QQQ method has a great advantage for simultaneous identification and quantitative analysis of 17 components including scopoletin, protopine, α-allocryptopine, DL-THP, coptisine, tetrahydroberberine, corydaline, berberine, byakangelicin, byakangelicol, xanthotoxin, bergapten, pimpinellin, oxypeucedanin, imperatorin, osthole and isoimperatorin in YZT within 9 min (Fig. 1). The results have indicated that this advanced method is fast, sensitive and convenient to show the real quality of the formula. Therefore, this proposed method could be reliable and feasible for quality assessment of YZT and other herb medications.

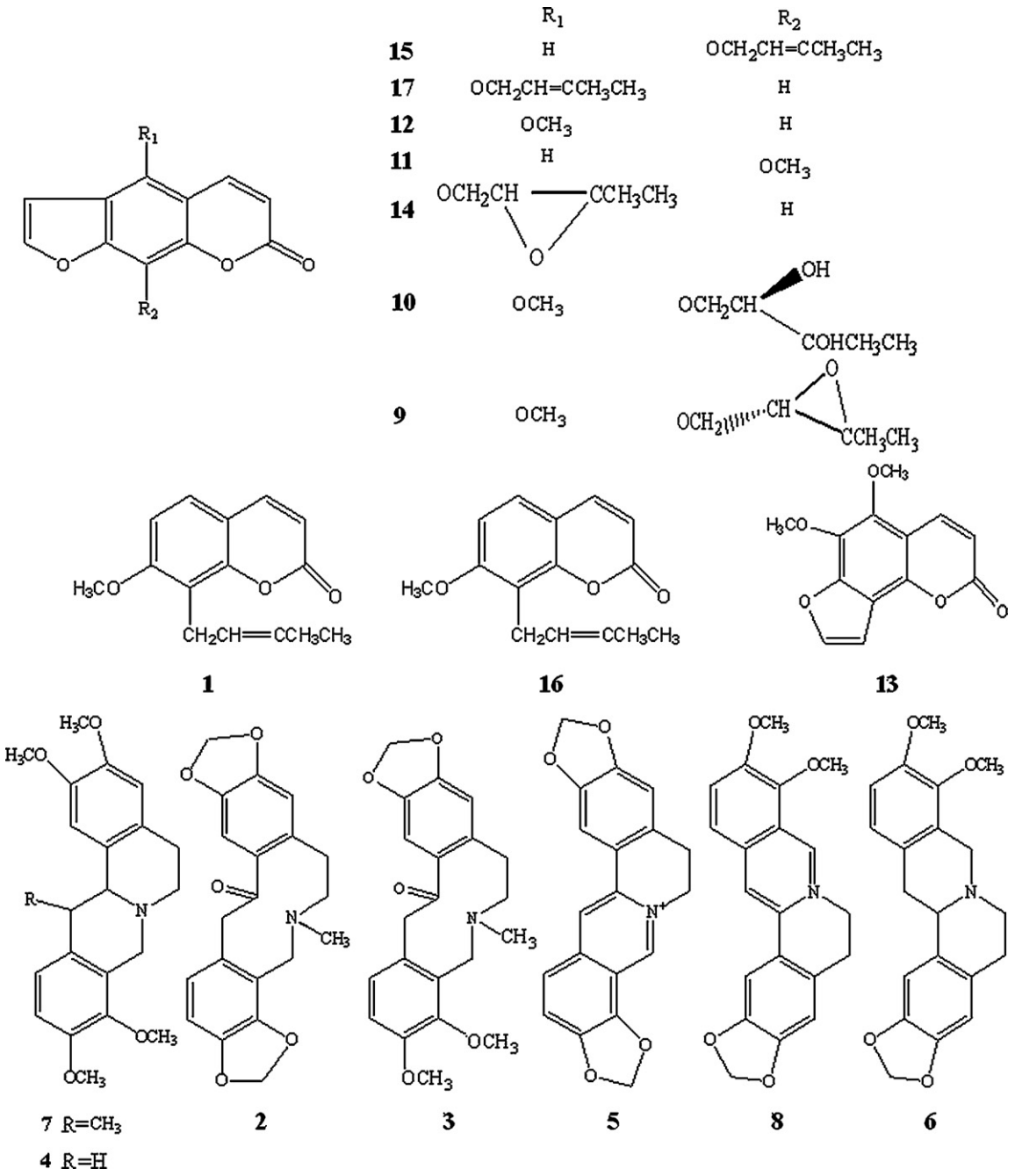

Fig. 1. Chemical structures of 17 markers: **1** scopoletin; **2** protopine; **3** α-allocryptopine; **4** DL-THP; **5** coptisine; **6** tetrahydroberberine; **7** corydaline; **8** berberine; **9** byakangelicol; **10** byakangelicin; **11** xanthotoxin; **12** bergapten; **13** pimpinellin; **14** oxypeucedanin; **15** imperatorin; **16** osthole; **17** isoimperatorin.

Table 1
A summary of the tested samples of YZT.

Sample no.	Pharmaceutical factory	Batch no.	Production date
S1	Shuzhong, Sichuan	100502	May 14, 2010
S2	Shuzhong, Sichuan	100403	April 30, 2010
S3	Shuzhong, Sichuan	100601	June 3, 2010
S4	Shuzhong, Sichuan	091203	December 17, 2009
S5	Shuzhong, Sichuan	100803	August 19, 2010
S6	Shuzhong, Sichuan	091004	October 24, 2009
S7	Banzhou Tianlong, Guangxi	100501	May 8, 2010
S8	Banzhou Tianlong, Guangxi	090501	May 3, 2009
S9	Banzhou Tianlong, Guangxi	091201	December 30, 2009
S10	Shibiao, Guangxi	100401	April 2, 2010
S11	Shibiao, Guangxi	100105	January 8, 2010
S12	Foshan Dezhong, Guangdong	10004	April 29, 2010
S13	Foshan Dezhong, Guangdong	10008	September 14, 2010
S14	Wanxi, Henan	100401	April 20, 2010
S15	Geruilin, Chongqing	091201	December 4, 2009

2. Experiments

2.1. Reagents and materials

HPLC grade acetonitrile was obtained from Tedia (Fairfield, OH, USA). Formic acid and ammonia water was of analytical grade obtained from Shanghai Chemical Regent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Billerica, MA, USA). Fifteen batches of YZT samples produced by different manufacturers were purchased from local drug stores (Table 1).

The standards of imperatorin, isoimperatorin, osthole, protopine, berberine, DL-THP and scopoletin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Byakangelicin, byakangelicol and coptisine were purchased from Chengdu Herbpurify Co. (Chengdu, China). Xanthotoxin, oxypeucedanin and tetrahydroberberine were obtained from Shanghai Winherb Co. (Shanghai, China). Corydaline was supplied by Sigma Co. (Sigma, USA). Pimpinellin was from Shanghai Touto Biotech. (Shanghai, China). Bergapten was acquired from Shanghai Hundred Million Biotech. (Shanghai, China). α-Allocryptopine was purchased from Shenzhen Meihe Biotech. (Shenzhen, China). The purities of all standards were no less than 98% and suitable for RRLC-QQQ analysis.

2.2. Samples and standard solution preparations

The coatings of 15 samples were removed completely, and the remains were smashed into powder with 60 mesh. Pulverized sample (0.5 g) was weighed precisely, and all samples were ultrasonically extracted using 50 ml 75% methanol at room temperature for 30 min, settled to the volume of 50 ml, then filtered through 0.22 μm nylon membrane filters. The filtrates were analyzed directly by RRLC-QQQ.

A stock solution containing 17 standards (scopoletin, protopine, α-allocryptopine, DL-THP, coptisine, tetrahydroberberine, corydaline, berberine, byakangelicin, byakangelicol, xanthotoxin, bergapten, pimpinellin, oxypeucedanin, imperatorin, osthole and isoimperatorin) was weighed accurately, dissolved in 75% methanol, and diluted to provide a series of standard solutions with gradient concentrations which were used to establish the calibration curves. Meanwhile, each standard was also prepared respectively. All solutions were stored at 4 °C for analysis.

2.3. Instrument and chromatographic conditions

RRLC analysis was performed on an Agilent 1200 Rapid Resolution Liquid Chromatography (Agilent, MA, USA) equipped with

Table 2
RT, related MS data of the 17 target markers detected on the RRLC-MS.

Marker	RT (min)	M+H/M (*m/z*)	MS^n (*m/z*)	Lost ions	Quantitative ion (*m/z*)	FV (V)	CE (V)
1	3.21	193	132.9, 177.9, 149.9	193−177.9=CH_3 177.9−149.9=CO	132.9	110	25
2	3.99	354	149.0, 189.0, 206.0	354+H−149.0=$C_{11}H_{13}O_3N$(206.0) 206.0−189.0=OH	189.0	130	28
3	4.37	370	187.9, 290, 352.0	370−352.0=H_2O	187.9	120	30
4	4.37	356	165.0, 192.0	356.1−192.0=165.0 (RDA)	192.0	120	22
5	4.59	320	292.0, 262.0	320−292.0=CO 292.0−262.0=CH_2O	292.0	120	35
6	4.89	340	149.0, 176.0	340.1−149.0=$C_{11}H_{13}O_2N$(191.0) 191.0−176.0=CH_3	176.0	115	25
7	4.97	370	165.0, 192.0	370.1+H−192.0=$C_{11}H_{15}O_2$(179.0) 179.0+H−165.0=CH_3	192.0	130	27
8	5.48	336	292.0, 320.0	336−320.0=CH_3+H 320.0−292.0=CO	320.0	110	35
9	5.57	317	175.0, 203.0, 231.0	317.0−231.0=$C_5H_{10}O$ 231.0−203.0=CO 203.0−175.0=CO	231.0	115	25
10	5.57	335	175.0, 203.0, 231.0	335.0−231.0=$C_5H_{12}O_2$ 231.0−203.0=CO 203.0−175.0=CO	231.0	100	25
11	6.94	217	161.1, 202.1	217.1−202.1=CH_3 217.1−161.1=2CO	202.1	130	28
12	7.36	217	174.0, 202.1	217.1−202.1=CH_3 202.1−174.0=CO	202.1	130	20
13	7.60	247	202.8, 216.8, 231.8	247−231.8=CH_3 231.8−216.8=CH_3 231.8−202.8=CH_2O	231.8	120	20
14	7.79	287	203.0, 287.0	287.0−203.0=C_5H_9O	203.0	80	15
15	8.38	271	203.0, 271.0,	271.0−203.0=C_5H_8	203.0	130	10
16	8.65	245	130.9, 161.0, 189.0	245.0−189.0=C_4H_8 189.0−161.0=CO 161.0−130.9=CH_2O	130.9	110	20
17	8.77	271	147.0, 159.0, 203.0	271.0−203.0=C_5H_8 203.0−159.0=CO_2 203.0−147.0=2CO	203.0	80	25

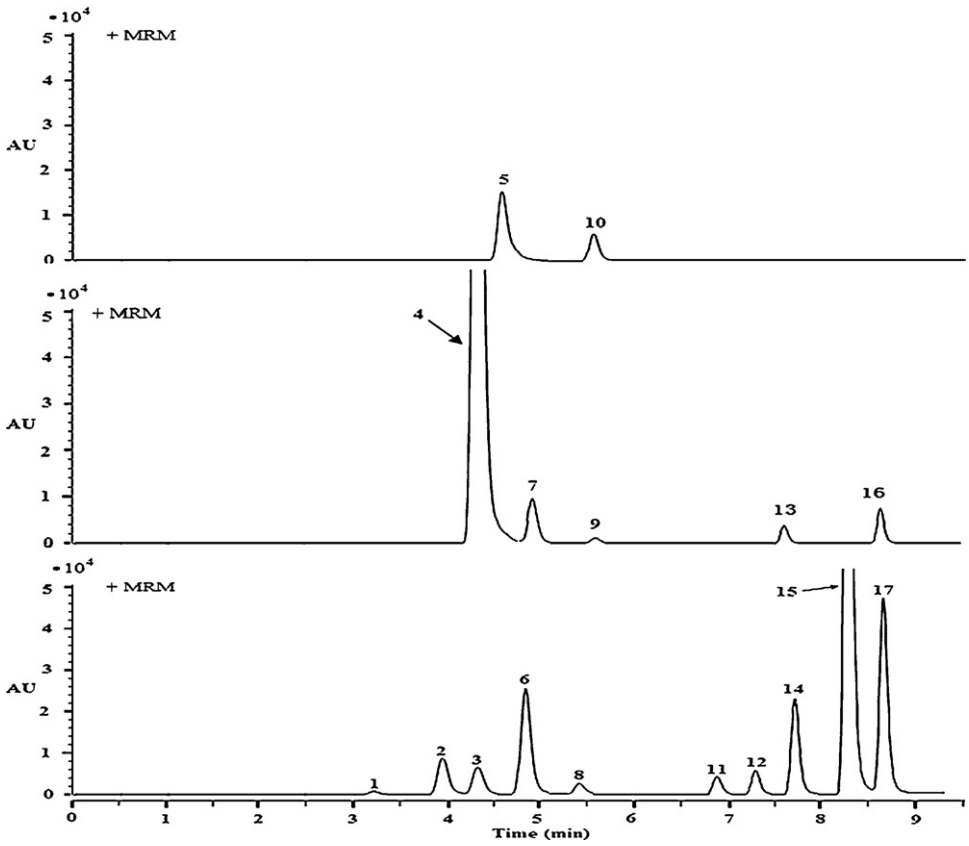

Fig. 2. The RRLC–MS/MS analysis MRM chromatogram of 17 markers.

an online vacuum degasser, a binary pump, an autosampler and a thermostated column compartment. The analytes were isolated on an Agilent XDB C_{18} column (4.6 mm × 50 mm, 1.8 μm). The linear gradient conditions were solvent A of 0.3% aqueous formic acid (pH 2.7, adjusted by ammonia water) and solvent B of acetonitrile with gradient elution as follows: 0–4 min, 20–40% B; 4–6 min, 40–80% B; 6–9 min, 80% B. Elution was performed with a solvent at the flow rate of 0.60 ml/min. The column temperature was set to 30 °C, and the injection volume was 5 μl for analysis.

The Agilent G6410 Triple Quadrupole mass spectrometer equipped with an electrospray ion source (ESI) (Agilent, MA, USA) was applied to quantitative analysis of 17 components. ESI-MS spectra were acquired in positive ion multiple reaction monitoring (MRM) mode. The conditions of MS analysis were designed as follows: the electrospray capillary voltage at 4000 V; nebulizer pressure at 38 psi; nitrogen was used as a drying gas at the flow rate of 10 L/min with a temperature of 345 °C and high-purity nitrogen was used as the collision gas. The full-scan range was from 100 to 500 *m/z*. The fragmentor voltage (FV) and collision energy (CE) were different according to the detection of different markers.

2.4. Hierarchical clustering analysis

Hierarchical cluster analysis (HCA) is a tool to identify relatively homogeneous groups of cases based on selected characteristics, using an algorithm that starts with each case in a separate cluster until only one is left. The method has been widely applied on the quality assessment of traditional Chinese medicine [28,29]. In the

Table 3
Calibration curves, linear ranges, LOD and LOQ of 17 markers.

Compound	Regression equation	R^2	Linear range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Scopoletin	$y = 66.3447x + 46.3638$	0.9994	0.88–22.00	0.0658	0.2193
Protopine	$y = 211.2781x - 1393.9063$	0.9998	67.60–3380.00	0.0202	0.0675
α-Allocryptopine	$y = 317.4065x - 1690.9943$	0.9996	13.60–1700.00	0.0129	0.0430
DL-THP	$y = 334.6602x + 113734.2021$	0.9938	140.50–7025.00	0.6276	2.0921
Coptisine	$y = 199.7884x - 771.7549$	0.9996	28.48–3560.00	0.0423	0.1409
Tetrahydroberberine	$y = 4984.1583x + 1712.7853$	0.9999	1.07–400.00	0.0118	0.0393
Corydaline	$y = 218.2076x + 11283.9356$	0.9996	21.76–5440.00	0.0106	0.0352
Berberine	$y = 179.3332x + 1518.4637$	0.9999	8.16–2040.00	0.0239	0.0798
Byakangelicol	$y = 142.0539x + 919.0076$	0.9988	13.28–332.00	0.0366	0.1219
Byakangelicin	$y = 11.7909x + 397.9368$	0.9978	30.00–1500.00	0.1327	0.4425
Xanthotoxin	$y = 502.5395x + 127.7538$	0.9999	6.40–240.00	0.0688	0.2293
Bergapten	$y = 587.1250x + 3278.8302$	0.9992	15.04–376.00	0.0270	0.0901
Pimpinellin	$y = 35.8175x + 686.4594$	0.9991	18.13–1360.00	1.2697	4.2323
Oxypeucedanin	$y = 220.9350x + 582.1600$	0.9992	7.40–184.50	0.5873	1.9577
Imperatorin	$y = 913.2649x + 135415.9686$	0.9966	32.53–2440.00	1.2882	4.2939
Osthole	$y = 380.6752x + 105.0663$	0.9995	1.66–41.50	0.0888	0.2959
Isoimperatorin	$y = 467.3272x + 37903.2183$	0.9962	68.32–1708.00	0.0714	0.2381

experiment, HCA of 15 samples were performed by SPSS software (SPSS 13.0 for Windows, SPSS Inc., USA), in which a method called average linkage between groups was employed and 17 markers were selected as the measurement.

3. Results and discussion

3.1. Optimization of the extraction conditions

In order to optimize the extraction conditions, the extraction methods, different solvents and varieties of time on efficiency were investigated. The results showed that ultrasonic extraction was more convenient and effective than refluxing extraction. It was also found that 75% methanol was the most efficient extraction solvent among the tested different concentrations of ethanol and methanol. In addition, the efficiencies of ultrasonic extraction were measured in different time—20, 30, 40, 50 and 60 min. It was demonstrated that the components could be extracted completely within 30 min. Finally, the sample solutions were prepared by ultrasonic extraction with 50 ml 75% methanol for 30 min.

3.2. Optimization of the chromatographic and mass spectrometric conditions

Mobile phases (methanol–water, acetonitrile–water, methanol–acid aqueous solution, and acetonitrile–acid aqueous solution) were examined and compared in order to obtain good chromatographic behavior and appropriate ionization. It was showed that acetonitrile–acid aqueous solution was better than others. Furthermore, formic acid (ammonia water used to adjust pH value) was added into the mobile phase to improve the peak shape and restrain the peak tailing, which was demonstrated to be helpful to ion response and resolution of these components. We also compared different concentrations of aqueous formic acid (0.1%, 0.2%, 0.3%, and 0.4%) with acetonitrile. The data indicated that the optimal condition was acetonitrile–0.3% aqueous formic acid (pH 2.7).

MS spectra were studied in both positive and negative modes. The positive mode was employed in our paper as it had better sensitivity of ion response than the negative mode. The chemical structures of 17 components were characterized based on their retention behavior and MS information, such as quasi-molecular ions $[M+H]^+$, fragment ions $[M+H-CH_3]$, $[M+H-C_5H_8]$, and [M+H−CO], compared to related standards and literatures [16,30,31]. For the optimization of MS conditions, FV and CE were chosen as the major optimal factors because they played important roles in parent and product ion responses. Each compound was investigated to respectively achieve optimal FV and CE. Under the optimized RRLC and MS/MS conditions, all 17 compounds in YZT were identified. The markers were further quantified mainly based on their MS fragmentations and highly sensitive and stabile daughter ions. Retention time (RT) and MS information for each analyte including [M+H] or [M], MS^n fragmentation ions, possible lost ions, quantitative ions, FV and CE are shown in Table 2, and RRLC–MS/MS chromatography of 17 markers in Fig. 2.

Table 4
Precision, repeatability and stability of 17 markers.

Compound	Precision intra-day	(RSD, $n=6$) inter-day	Repeatability (RSD, $n=6$)	Stability (24 h) (RSD, $n=5$)
Scopoletin	1.85	3.53	3.54	3.88
Protopine	2.16	1.35	4.91	4.86
α-Allocryptopine	2.16	2.54	4.84	3.70
DL-THP	1.01	1.36	4.71	4.51
Coptisine	4.92	2.63	1.33	1.46
Tetrahydroberberine	1.32	3.01	4.14	4.29
Corydaline	1.57	3.65	3.37	3.45
Berberine	4.33	2.40	4.33	3.91
Byakangelicol	2.18	2.10	3.11	3.22
Byakangelicin	1.79	4.79	3.74	4.14
Xanthotoxin	2.03	2.73	1.15	1.28
Bergapten	2.43	3.28	1.05	1.07
Pimpinellin	2.05	1.61	2.10	2.24
Oxypeucedanin	2.41	3.20	4.61	4.74
Imperatorin	3.01	3.39	4.50	4.72
Osthole	2.79	2.27	3.95	4.33
Isoimperatorin	2.64	2.81	3.56	3.94

Table 5
Recoveries of 17 markers with $n=3$.

Compound	Original (ng)	Addition (ng)	Detection (ng)	Recovery (%)	RSD (%)
Scopoletin	177.10	140.0	319.67	101.84	2.05
		175.0	357.57	103.13	4.66
		215.0	384.81	96.61	4.36
Protopine	5243.38	4200.0	9533.33	102.14	2.43
		5250.0	10625.29	102.51	4.36
		6300.0	11266.67	95.61	2.09
α-Allocryptopine	2023.23	1600.0	3603.83	98.79	3.11
		2100.0	4217.09	104.47	2.91
		2400.0	4309.84	95.28	3.14
DL-THP	94249.49	75400.0	171516.67	102.48	3.40
		94250.0	189092.36	100.63	1.11
		113100.0	204413.55	97.40	3.35
Coptisine	3743.89	3000.0	6599.80	95.20	4.30
		3750.0	7573.33	102.12	2.20
		4500.0	8312.61	101.53	4.21
Tetrahydroberberine	235.88	185.0	417.57	98.21	1.70
		235.0	486.87	106.80	1.50
		285.0	510.92	96.50	4.81
Corydaline	6358.18	5085.0	11688.73	104.83	2.88
		6360.0	12682.38	99.44	3.21
		7625.0	13727.17	96.64	1.42
Berberine	1291.41	1025.0	2361.67	104.42	2.75
		1275.0	2603.93	102.94	2.98
		1525.0	2772.4	97.12	3.78
Byakangelicol	3530.82	2825.0	6392.57	101.30	1.77
		3530.0	7059.43	99.96	3.82
		4235.0	7724.92	99.03	4.71
Byakangelicin	14647.03	12100.0	27509.64	106.30	4.55
		14650.0	28692.67	95.87	3.61
		17550.0	31485.59	95.95	1.83
Xanthotoxin	1755.28	1400.0	3102.52	96.23	3.01
		1750.0	3595.25	105.14	1.71
		2100.0	3838.13	99.18	1.42
Bergapten	5737.77	4600.0	10261.54	98.34	3.10
		5750.0	11707.63	103.82	2.86
		6900.0	12448.99	97.26	2.38
Pimpinellin	2477.11	1980.0	4463.76	100.34	1.94
		2475.0	4975.23	100.93	4.75
		2975.0	5420.43	98.94	2.29
Oxypeucedanin	8562.20	6850.0	15179.32	96.60	2.54
		8550.0	17500.52	104.54	1.67
		10250.0	18707.48	98.98	4.02
Imperatorin	64322.02	51450.0	117173.87	102.72	2.32
		64300.0	126083.33	96.05	3.16
		77200.0	142621.14	101.42	1.78
Osthole	228.92	185.0	416.39	101.34	2.88
		230.0	453.41	97.60	2.24
		275.0	503.20	99.74	4.60
Isoimperatorin	19630.79	15700.0	35480.98	100.96	3.38
		19600.0	40228.96	105.09	1.45
		23550.0	41945.69	94.76	3.52

Table 6
Contents of 17 compounds in 15 samples YZT.

S	Content of each compound in 15 batches of YZT sample ($\mu g\,g^{-1}$)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
S1	0.34	53.08	22.87	131.40	75.00	3.39	184.95	24.55	11.05	51.76	10.92	22.02	24.23	3.50	214.34	0.47	126.00
S2	0.27	60.80	27.12	200.34	69.07	4.98	291.99	21.47	11.92	50.07	9.93	22.75	25.42	4.01	219.70	0.41	124.89
S3	0.36	53.08	24.70	132.50	75.82	3.46	191.89	48.15	13.14	59.79	12.69	25.43	26.06	3.80	226.61	0.46	114.31
S4	0.35	72.24	31.68	246.42	78.92	6.11	405.83	19.57	10.64	44.95	8.96	20.40	22.05	3.08	207.52	0.37	109.48
S5	0.27	60.03	25.98	143.96	79.37	3.60	199.12	30.88	15.78	69.34	9.00	27.17	25.19	3.10	220.27	0.46	104.74
S6	0.22	74.59	32.42	254.23	79.57	6.45	393.92	19.13	7.17	30.81	8.33	15.41	14.52	2.34	183.66	0.37	119.26
S7	0.26	12.90	5.89	268.58	4.10	0.86	29.69	3.45	6.22	28.37	2.70	11.11	12.30	0.98	79.23	0.22	42.44
S8	0.29	15.80	5.86	280.58	12.10	0.61	23.84	5.15	7.02	30.48	3.92	13.63	14.48	1.13	97.87	0.27	54.16
S9	0.29	13.72	5.51	260.87	8.50	0.53	18.11	4.57	6.65	28.77	3.31	11.96	13.46	–	93.26	0.25	54.32
S10	0.11	18.23	8.15	264.11	23.74	0.88	55.37	12.93	2.15	8.81	2.14	6.22	3.37	0.12	52.98	0.15	23.68
S11	0.28	22.22	9.01	198.07	21.85	0.99	83.65	5.44	2.35	10.54	2.92	5.90	6.38	0.23	46.17	0.13	29.02
S12	0.43	258.25	99.88	562.03	311.08	12.77	810.27	193.42	10.03	44.72	10.51	28.83	38.46	3.44	299.83	0.71	122.41
S13	0.49	244.54	101.81	629.38	224.46	14.90	902.79	127.81	16.38	72.71	10.07	29.81	36.29	3.71	217.10	0.73	102.95
S14	1.07	169.24	69.91	444.07	123.55	10.44	508.73	75.86	17.51	75.87	5.99	29.78	25.18	2.08	48.57	0.39	10.17
S15	0.31	15.53	7.19	679.28	6.17	0.86	33.96	3.31	12.11	50.74	3.34	10.41	11.86	–	31.70	0.30	17.26
$\bar{M}$	0.36	76.28	31.86	313.06	79.55	4.72	254.32	39.71	10.01	43.85	6.98	18.72	19.95	2.10	149.25	0.38	77.01

Reducing sampling amount to half overcame the problem that the DL-THP content of S15, the corydaline content of S12, S13 were beyond the established linear ranges, and then preparing the new samples as the same method to analyze.
"–" means not being detected.

3.3. Analytical method validation

3.3.1. Linearity, limits of detection (LOD) and limits of quantification (LOQ)

The calibration curves were plotted with a series of concentrations of standard solutions. Each marker curve was made at least 6 levels. Acceptable linear correlation and high sensitivity at these conditions were confirmed by the correlation coefficients (r^2, 0.9938–0.9999). LOD and LOQ expressed by 3- and 10-fold of the ratio of the signal-to-noise (S/N) were also acquired, respectively. Detailed information regarding calibration curves, linear ranges, LOD and LOQ is displayed in Table 3.

3.3.2. Precision, repeatability and stability

The precision of the method was validated by the determination of intra- and inter-day variances. The intra-day precisions were evaluated by measuring a standard mixture solution composed of 17 markers six times a day under the optimized conditions, while inter-day precisions were investigated twice a day over three consecutive days. The concentration of each solution was determined by a calibration curve formed at the same day. The intra- or inter-day precisions calculated as relative standard deviation (RSD) were within the range of 1.01–4.92% or 1.35–4.79%.

Six independent samples of YZT (S12 was randomly selected) were extracted and analyzed in parallel by the above-established method for the evaluation of repeatability. The RSD values of 17 compounds were within the range from 1.05% to 4.91%, which revealed high repeatability of the method.

Stability of sample solution was tested at room temperature. The RSD values of 17 compounds were all within 5%, which demonstrated a good stability in 75% methanol solution within the tested period. The data of precision, repeatability and stability are listed in Table 4.

3.3.3. Recovery

Recovery was used to further evaluate the accuracy of the method. Known amounts of each standard solution at different concentration levels (high, middle and low) were mixed with known amounts of YZT samples (S12). Then the samples were extracted and analyzed by the above-established method, and triplicate experiments were repeated at each level. The average recoveries were estimated by the formula (1):

$$\text{recovery}\,(\%) = \left[\frac{\text{detection} - \text{original amount}}{\text{addition}}\right] \times 100 \quad (1)$$

As shown in Table 5, the mean recovery rates of 17 markers varied from 94.7% to 106.8% (RSD ≤ 4.81%).

3.4. Quantitative analysis of samples

3.4.1. Sample analysis

The developed analytical method was subsequently applied to analysis of 17 marker compounds in 15 batches of YZTs from different manufacturers or different batches of the same manufacturer. The contents, summarized in Table 6, were calculated with external standard methods based on the respective calibration curves.

The proposed RRLC-QQQ assay was a successful application in quantifications of major alkaloids and coumarins in different YZT samples. All 17 compounds were eluted within 9 min, which was suitable for RRLC-QQQ. The results showed that protopine, DL-THP, coptisine, corydaline, byakangelicin, imperatorin, isoimperatorin and so on were the major dominant compounds according to the content. Among them, the content of DL-THP was highest, and its mean value was 313.06 $\mu g\,g^{-1}$. On the other hand, the contents of scopoletin, tetrahydroberberine, osthole, oxypeucedanin, etc., were relatively low, for our previous data proved that they could not be detected by UV or DAD detector in the preparation. However, these micro-constituents play an important role in relieving pain from different pathways in low dose due to their various pharmacological effects [32–39], especially related to analgesia such as antinociceptive effect, anticonvulsant, neuroprotective effect, and anti-inflammatory. As a whole, multiple active components, including macro and micro components, are frequently considered to be responsible for the therapeutic effects, which may work 'synergistically' and reflect the characteristic of TCM [1]. The present work will be benefit for quality control of the preparation roundly.

In addition, it suggested that the variations of the contents of 17 markers were obvious in the samples from different companies or even different batches from the same company. These results showed that it was a reliable and efficient method as a simultaneous determination of multiple components in YZT.

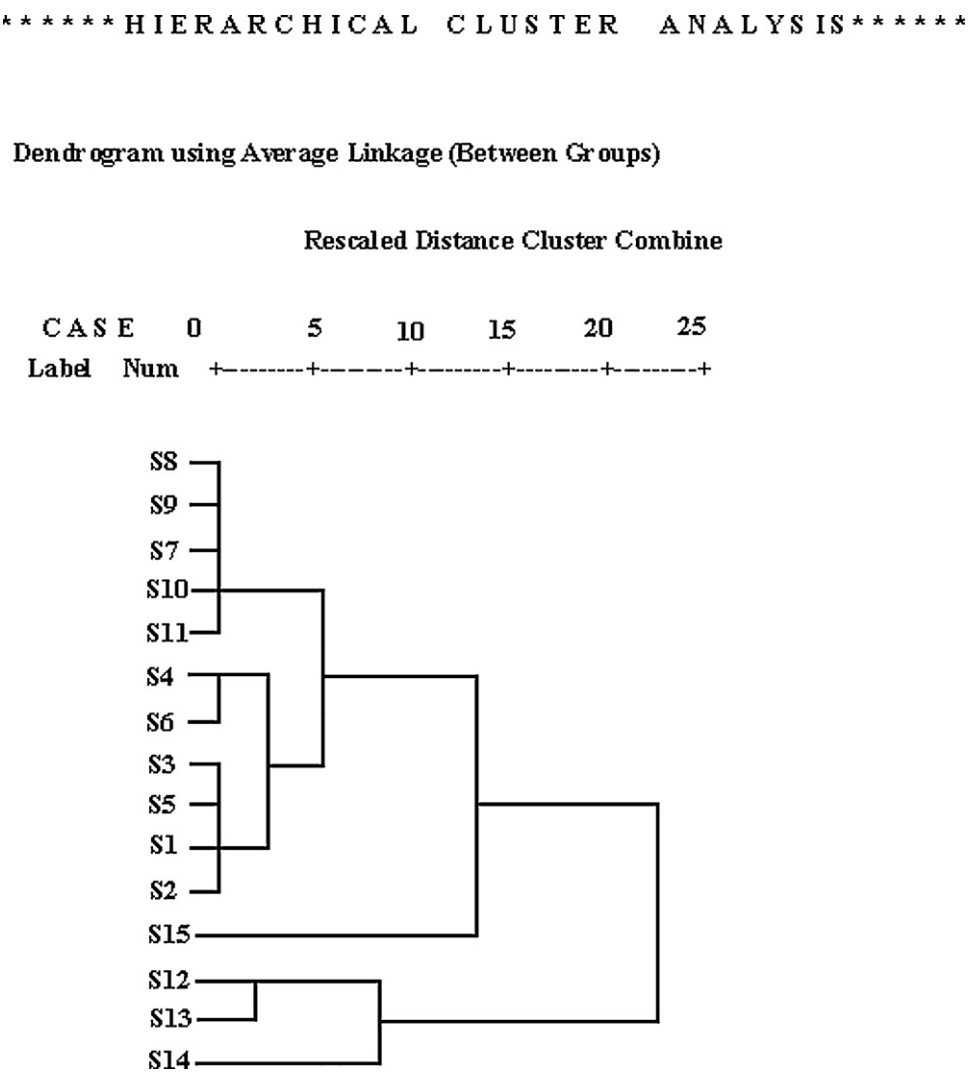

Fig. 3. Dendrograms of the hierarchical cluster analysis for 15 batches of YZT.

3.4.2. Quality assessment of YZT by hierarchical cluster analysis

To evaluate the YZT variation, HCA was performed based on the characteristics of the contents of 17 marker compounds. The marker contents in 15 YZT samples formed a 15×17 matrix. Distances between the 15 samples were calculated using the SPSS software.

As shown in Fig. 3, sample nos. 1–6 from Shuzhong Pharmaceutical Factory, sample nos. 7–11 from two pharmaceutical factories of Guangxi (nos. 7–9 from Banzhou Tianlong and nos. 10–11 from Shibiao), and sample nos. 12–13 from Foshan Dezhong could be included in the same group, respectively. HCA clearly indicated that chemical profiles from the same factory were similar, whereas chemical profiles from different factories produced significant difference.

4. Conclusion

In this paper, the RRLC-QQQ method was proved as a reliable and powerful technique for simultaneous conformation and quantification of 17 active components in YZT within 9 min. This method was applied to evaluate 15 commercial samples of YZT. The results showed that the contents of 17 marker compounds were obviously discrepant from different companies or even different batches from the same company, however, chemical profiles were similar in the same factory by HCA. The developed method was demonstrated to be simple, sensitive and reproducible, and have significant importance and comprehensive evaluation for quality control of YZT and related preparations. The established methodology will apply to or widely adapted to other sources to speed up the internationalization of Chinese medicine.

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