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Article

Simultaneous quantification of 53 flavonoids, iridoid glycosides, phenolic acids, free amino acids and nucleosides in Zhi-zi-chi decoction using UFLC/QTRAP-MS

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Abstract: To evaluate the flavonoids, iridoid glycosides, phenolic acids, free amino acids and nucleosides contents in Zhi-zi-chi decoction (ZZCD), a sensitive and rapid method was developed using UFLC/QTRAP-MS for the simultaneous quantification. The results showed that all known ingredients were determined in ZZCD and the method was suitable for the simultaneous determination of 53 target components in ZZCD. Of which three isoflavones, nine free amino acids and eight iridoid glycosides were the main constituents. This study simultaneously determined 53 target components of 5 major categories in ZZCD for the first time and made up the blank on the target components contents of ZZCD, and promoted the construction of the ZZCD fingerprint at the same time, which have great significance for the comprehensive guarantee of the clinical therapeutic effect of ZZCD. The present study also offered an experimental foundation for more in-depth research on the pharmacochemistry analysis of ZZCD and effective fractions selection. The determined iridoid glycosides have been reported to possess antidepressant effects, which also provides a material basis for the subsequent research on the anti-depressant effects of ZZCD.

Keywords: Zhi-zi-chi decoction (ZZCD); simultaneous quantitative analysis; UFLC/QTRAP-MS; multiple reaction monitoring (MRM)

1. Introduction

Zhi-zi-chi decoction (ZZCD), a TCM formula originally described in "Shanghan Lun" (Treatise on Cold Pathogenic Diseases), is composed of *Gardenia jasminoides* J.Ellis and *Sojae semen praeparatum*, whose Chinese herbal name is dandouchi, a product of Chinese fermented preparation obtained from the ripe seed of soybean [*Glycine max* (L.) Merr.] [1,2]. ZZCD has been widely used in Chinese clinical application to treat depression for centuries [3–5].

Our previous qualitative analysis study of ZZCD (accepted by Analytical Science Advances) and its individual herbs have detected iridoid glycosides, isoflavones, flavonoids and amino acids. Previous studied also shown that the effective anti-depressant parts of ZZCD and their individual herbs might be iridoid glycosides, isoflavones, flavonoids, amino acids, and volatile oils [6–8]. Iridoid glycosides are characteristic components of *Gardenia jasminoides* J.Ellis [9], while isoflavones are characteristic components of *Sojae semen praeparatum* [10]. At present, research has been conducted on the composition identification and extraction process of a certain kind of effective parts such as iridoid glycosides, sterols, flavonoids, phenolic acids, polysaccharides, nucleosides, and amino acids in

ZZCD and their individual herbs [11–15]. There were also relevant reports on the analysis of migrating components and metabolites of ZZCD in rat blood, urine, bile, and brain tissues [16,17]. There were few reports in simultaneous quantification of multiple types of constituents in ZZCD.

LC-MS/MS method in multiple reaction monitoring (MRM) mode was widely used in drug and food safety supervision, quantification of chemical composition, quality control and pharmacokinetics of traditional Chinese medicine for its high sensitivity and accuracy, less interference and good reproducibility and selectivity [18–20]. MRM is a multiple reaction monitoring technique commonly used in triple quadrupole mass spectrometry. Compared to liquid chromatography, it is suitable for simultaneous quantification of multiple target compounds. Compared with single quadrupole mass spectrometry, it has higher selectivity by monitoring the reactions of parent and daughter ion pairs [21–23].

Therefore, the present study was aimed to simultaneously determined 53 target flavonoids, iridoid glycosides, phenolic acids, free amino acids and nucleosides contents in ZZCD by UFLC/QTRAP-MSfor the first time, thereby making up the blank on the target components contents of ZZCD, and promoting the construction of the ZZCD fingerprint at the same time.

2. Experimental

2.1. Materials and methods

Gardenia jasminoides J.Ellis was obtained from KANGTAI TCM Co. (Jurong, China), *Sojae semen praeparatum* was obtained from YiFeng TCM shop (Nanjing, China).

The reference standards of daidzin, genistin, glycitin, daidzein, genistein, glycitein, ornithine (Orn), glutamic acid (Glu), arginine (Arg), γ-aminobutyric acid (GABA), serine (Ser), alanine (Ala), citrulline (Cit), threonine (Thr), lysine (Lys), proline (Pro), valine (Val), glutamine (Gln), aspartic acid (Asp), methionine (Met), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Try), uridine, cytosine, hypoxanthine, xanthine, 2′-deoxycytidine, 2′-deoxyinosine, 2′-deoxyadenosine, deacetyl asperulosidic acid, shanzhiside, geniposidic acid, deacetyl asperulosidic acid methyl, scandoside methyl ester, shanzhiside methyl ester, genipin1-β-gentiobioside, gardenoside, geniposide, genipin, crocin-I, crocin-II, rutin, isoquercetin, quercetin, malic acid, gallic acid, protocatechuic acid, procatechin, chlorogenic acid, p-coumaric acid and ferulic acid were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

LC/MS-grade acetonitrile, formic acid, methanol, and water were purchased from Merck Co. (Darmstadt, Germany).

2.2. Instruments

An ultra-fast liquid chromatography (Shimadzu Corporation UFLC XR; Kyoto, Japan) system was coupled with a triple quadruple and linear-ion trap mass spectrometer (AB SCIEX 5500 QTRAP; Foster City, CA) with an electrospray ionization (ESI) source.

2.3. Preparation of ZZCD solution

ZZCD is comprised of *Gardenia jasminoides* J.Ellis and *Sojae semen praeparatum* at the ratio of 105:248. The decoction was prepared in laboratory following the stpes described in detail by Chai [24] and run in triplicate for method validation for consistency.

The steps and parameters were as follows: *Gardenia jasminoides* J.Ellis (21 g) was boiled with water (800 mL) until mixture reduced to 500 mL in about 30 min, then *Sojae semen praeparatum* (49.6 g) was added and decocted later until mixture reduced to 300 mL in about 20 min, 750 μL filtered decoction was rediluted with 750 μL methanol and then centrifuged at 12000 rpm for 10 min. The supernatant was transferred to another tube and filtered through a filter membrane (0.22 μm).

2.4. Preparation of standard solutions

Mix standard solution was prepared by accurately weighing the standard substances and mixing them in methanol and water until completely being dissolved. This standard mixture was filtered through a filter membrane (0.22 μm).

The mixture standard solution was prepared by accurately weighing all the standard substances and mixing them in methanol (flavonoids, iridoid glycosides and phenolic acids) or water (free amino acids and nucleosides). This standards mixture was further diluted with 70% methanol to make a series standard solutions of different concentrations. The standard solutions were filtered through 0.22 μm membrane before injection.

2.5. Chromatography and mass spectrometry

An ultra-fast liquid chromatography (Shimadzu Corporation UFLC XR; Kyoto, Japan) system was coupled with a triple quadruple and linear-ion trap mass spectrometer (AB SCIEX 5500 QTRAP; Foster City, CA) with an electrospray ionization (ESI) source.

A Waters XBridge C_{18} column (4.6 mm × 100 mm, 3.5 μm) was used throughout this study. The mobile phase was a binary solvent system consisting of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The UFLC gradient was: 0~5 min, 98% A; 5~9 min, 98%~60% A; 9~11 min, 60%~5% A, 11~12 min, 5% A; 12~13 min, 5%~98% A in 1 min; 98% A hold for 3 min, thus, the total analysis time was 16 min, including column washing and requilibration. The flow rate was 800 μL/min and the injection volume was 2 μL. The column temperature was set at 30 ℃.

This hybrid system was used for the determination of targeted analytes in high sensitivity and selectivity performance. The capillary and voltage of ion source were maintained at 500 ℃ and 5500 V/−4500 V, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulization, 55 psi; heater gas pressure, 55 psi; curtain gas, 35 psi; collision gas, high.

In both positive and negative ion modes, the standard solutions of the target compounds were diluted and subjected to full scan of the parent ion using a flow injection method to identify the molecular ion peaks. Subsequently, the molecular ions of the target compounds were used as parent ions for full scanning of their

daughter ions. The mass spectrometry parameters de-clustering voltage (DP), collision energy (CE), and collision chamber exit voltage (CXP) were optimized using multiple reaction monitoring (MRM). And the mass spectrometry detection parameters of the 53 target components are listed in **Table 1**.

Table 1. The mass spectrometry detection parameters for the 53 target components.

Ionization mode	Ascription	No.	Analytes	Molecular formula	MW	MRM Transitions (m/z)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Positive	Isoflavones	1	Daidzin	$C_{21}H_{20}O_9$	416.38	417.2/255.1	66	10	21	20
		2	Genistin	$C_{22}H_{22}O_{10}$	446.39	447.2/285.1	66	10	19	24
		3	Glycitin	$C_{21}H_{20}O_{10}$	432.38	433.1/271.1	131	10	17	20
		4	Daidzein	$C_{15}H_{10}O_4$	254.24	254.9/152.2	106	10	55	18
		5	Genistein	$C_{16}H_{12}O_5$	284.26	284.7/270.1	146	10	35	18
		6	Glycitein	$C_{15}H_{10}O_5$	270.24	270.8/153.2	151	10	39	12
	Free amino acids	7	Orn	$C_5H_{12}N_2O_2$	132.19	133.02/70.03	51	10	21	10
		8	Glu	$C_5H_9NO_4$	147.13	148.06/83.91	58	10	14	14
		9	Arg	$C_6H_{14}N_4O_2$	174.20	175.12/70.02	88	10	18	10
		10	GABA	$C_4H_9NO_2$	103.12	104.07/87.00	83	10	10	11
		11	Ser	$C_3H_7NO_3$	105.09	106.05/59.99	67	10	8	19
		12	Ala	$C_3H_7NO_2$	89.09	90.06/44.02	79	10	10	20
		13	Cit	$C_6H_{13}N_3O_3$	175.19	176.10/69.89	60	10	20	17
		14	Thr	$C_4H_9NO_3$	119.12	120.07/74.00	93	10	20	28
		15	Lys	$C_6H_{14}N_2O_2$	146.19	147.06/83.91	66	10	14	18
		16	Pro	$C_5H_9NO_2$	115.13	116.07/70.02	68	10	10	16
		17	Val	$C_5H_{11}NO_2$	117.15	118.09/72.06	54	10	10	24
		18	Gln	$C_5H_{10}N_2O_3$	146.15	147.06/83.91	105	10	19	12
		19	Asp	$C_4H_7NO_4$	133.10	134.05/87.96	59	10	10	20
		20	Met	$C_5H_{11}O_2NS$	149.21	150.06/104.03	91	10	10	19
		21	Leu	$C_6H_{13}NO_2$	131.18	132.10/86.05	98	10	10	14
		22	Ile	$C_6H_{13}NO_2$	131.18	132.10/86.05	64	10	10	17
		23	Phe	$C_9H_{11}NO_2$	165.19	166.10/120.05	56	10	14	14
		24	Try	$C_{11}H_{12}N_2O_2$	204.23	205.10/146.03	67	10	18	20
	Nucleosides	25	Uridine	$C_4H_4N_2O_2$	112.09	113.01/96.04	86	10	21	12
		26	Cytosine	$C_4H_5N_3O$	111.10	112.00/95.10	106	10	23	14
		27	Hypoxanthine	$C_5H_4N_4O$	136.11	137.05/137.05	51	10	24	17
		28	Xanthine	$C_5H_4N_4O_2$	152.11	153.09/110.02	106	10	25	18
		29	2′-deoxycytidine	$C_9H_{15}N_3O_4$	227.24	228.20/112.05	76	10	13	19
		30	2′-deoxyinosine	$C_{10}H_{12}N_4O_4$	252.23	253.10/137.07	66	10	13	12
		31	2′-deoxyadenosine	$C_{10}H_{13}N_5O_3$	251.25	252.00/135.90	51	10	24	24

Table 1. (*Continued*).

Ionization mode	Ascription	No.	Analytes	Molecular formula	MW	MRM Transitions (m/z)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
Negative	Iridoid glycosides	32	Deacetyl asperulosidic acid	$C_{16}H_{22}O_{11}$	390.34	389.2/59.13	−5	−10	−62	−7
		33	Shanzhiside	$C_{16}H_{24}O_{11}$	392.35	391.15/59.21	−135	−10	−64	−7
		34	Geniposidic acid	$C_{16}H_{22}O_{10}$	374.34	373.14/123	−130	−10	−26	−7
		35	Deacetyl asperulosidic acid methyl	$C_{17}H_{24}O_{11}$	404.36	403.30/45.02	−40	−10	−36	−7
		36	Scandoside methyl ester	$C_{17}H_{24}O_{11}$	404.36	403.30/45.02	−40	−10	−36	−7
		37	Shanzhiside methyl ester	$C_{17}H_{26}O_{11}$	406.38	405.33/243.08	−75	−10	−18	−13
		38	Genipin1-β-gentiobioside	$C_{23}H_{34}O_{15}$	550.51	549.21/225.14	−200	−10	−20	−19
		39	Gardenoside	$C_{17}H_{24}O_{11}$	404.36	403.30/45.02	−40	−10	−36	−7
		40	Geniposide	$C_{17}H_{24}O_{10}$	388.37	387.12/225.03	−180	−10	−12	−11
		41	Genipin	$C_{11}H_{14}O_5$	226.23	225.07/101.14	−30	−10	−16	−13
		42	Crocin-I	$C_{44}H_{64}O_{24}$	976.96	975.43/651.21	−10	−10	−30	−25
		43	Crocin-II	$C_{38}H_{54}O_{19}$	814.82	813.35/327.21	−165	−10	−36	−33
	Other flavonoids	44	Rutin	$C_{27}H_{30}O_{16}$	610.51	609.07/300.03	−245	−10	−46	−17
		45	Isoquercetin	$C_{21}H_{20}O_{12}$	464.38	463.01/299.90	−160	−10	−36	−17
		46	Quercetin	$C_{15}H_{10}O_7$	302.24	301.10/151.02	−140	−10	−28	−11
	Phenolic acids	47	Malic acid	$C_4H_6O_5$	134.09	132.90/114.91	−80	−10	−14	−13
		48	Gallic acid	$C_7H_6O_5$	170.12	168.95/125.03	−120	−10	−18	−7
		49	Protocatechuic acid	$C_7H_6O_4$	154.12	153.11/109.11	−35	−10	−20	−13
		50	Procatechin	$C_7H_6O_3$	138.12	136.94/108.10	−90	−10	−30	−7
		51	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	353.01/191.05	−95	−10	−20	−15
		52	p-Coumaric acid	$C_9H_8O_3$	164.16	162.95/119.03	−75	−10	−18	−9
		53	Ferulic acid	$C_{10}H_{10}O_4$	194.19	192.99/133.91	−65	−10	−20	−15

2.6. Method validation

The method was fully validated in accordance with guidelines on linearity, precision, recovery, detection limit, quantification limit, and stability. Calibration curves were generated by plotting peak area against the concentration of standard solutions. The repeatability was analyzed on six sample solutions from the same sample in parallel. The recovery was used to evaluate the accuracy at different spiking concentration levels (80%, 100% and 120% as compared to the nominal concentration) of standard solutions. The precisions were examined for six replicates on the mixed standard solutions and expressed using the relative standard deviation (RSD). The limits of detection (LOD) and quantification (LOQ) were calculated based on the peak-to-noise ratio of 3:1 and 10:1, respectively. Sample stability was tested by periodically analyzing at room temperature for various periods (0, 2, 4, 8, 12, 16, 20 and 24 h).

2.7. Data processing

The retention time between samples, the ion response of chromatographic peaks and the acquisition of secondary mass spectra were observed and processed using Analyst 1.5.2 software (Applied Biosystems/ MDS Sciex). The content of each component was calculated by external standard curve method.

3. Results and discussion

3.1. Optimization of UFLC conditions

Chromatographic conditions, including composition of mobile phase and column temperature were optimized to obtain high resolution and sensitivity chromatograms. For the mobile phase, eight solvent systems were selected: acetonitrile-water, methanol-water, acetonitrile-0.1% formic acid water, methanol-0.1% formic acid water, 0.1% formic acid acetonitrile-0.1% formic acid water, 0.1% formic acid methanol-0.1% formic acid water, 0.2% formic acid acetonitrile-0.2% formic acid water, and 0.2% formic acid methanol-0.2% formic acid water. The results showed that acetonitrile as the mobile phase had a significantly better separation effect than methanol. By adding a certain amount of formic acid to both phases, it was possible to improve sensitivity and stabilize the shape of the chromatographic peaks. However, the addition of excessive formic acid did not optimize sensitivity or the chromatographic peaks. Therefore, this experiment chose 0.1% formic acid acetonitrile-0.1% formic acid water as the mobile phase. Additionally, column temperatures at 25, 30, 35, and 40 ℃ were examined, the retention time got shorter with rising of the column temperature, but the separation of Leu and Ile was unsatisfactory at the temperature up to 35 ℃. Therefore, 30 ℃ was chosen as the appropriate column temperature.

3.2. Optimization of MS conditions

Through repeated experiments, each target component exhibited high response values, good stability, and well-defined peak shapes under the detection conditions. The MRM extracted ion chromatograms for the 53 target components are shown in **Figures 1** and **2**.

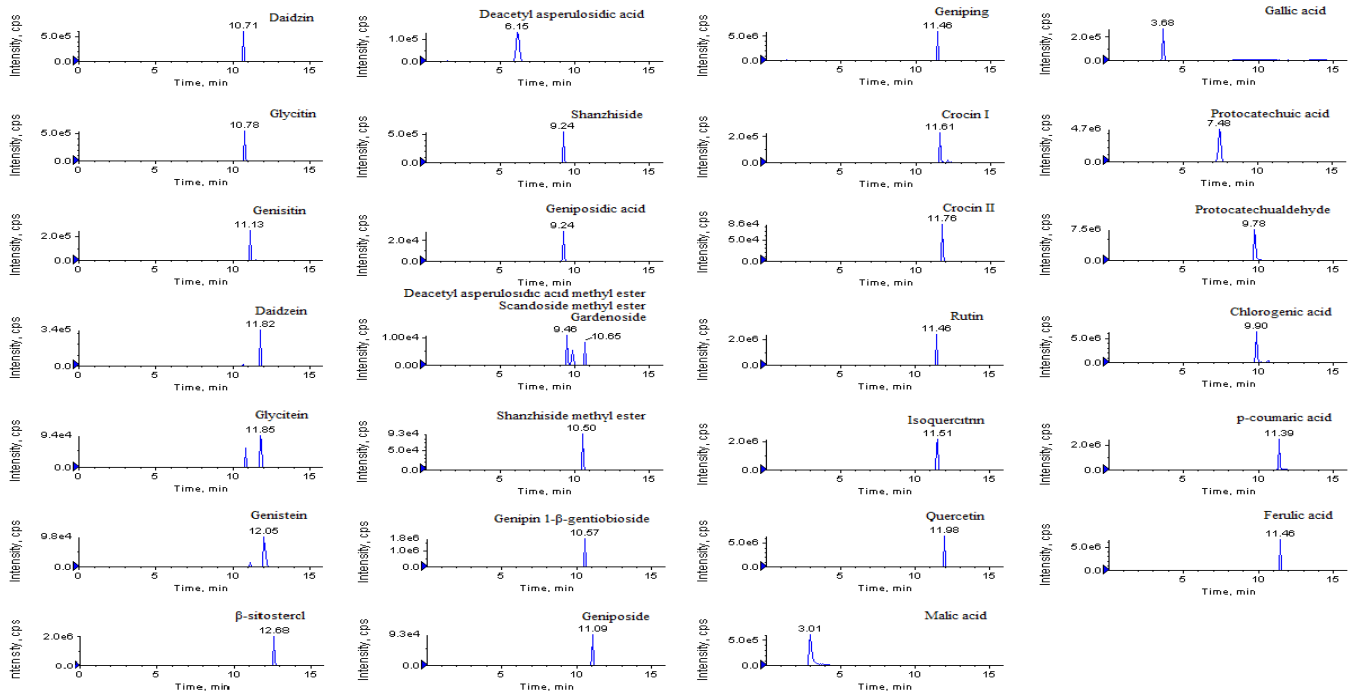

Figure 1. MRM extracted ion chromatogram of flavonoids, iridoid glycosides and phenolic acids in ZZCD.

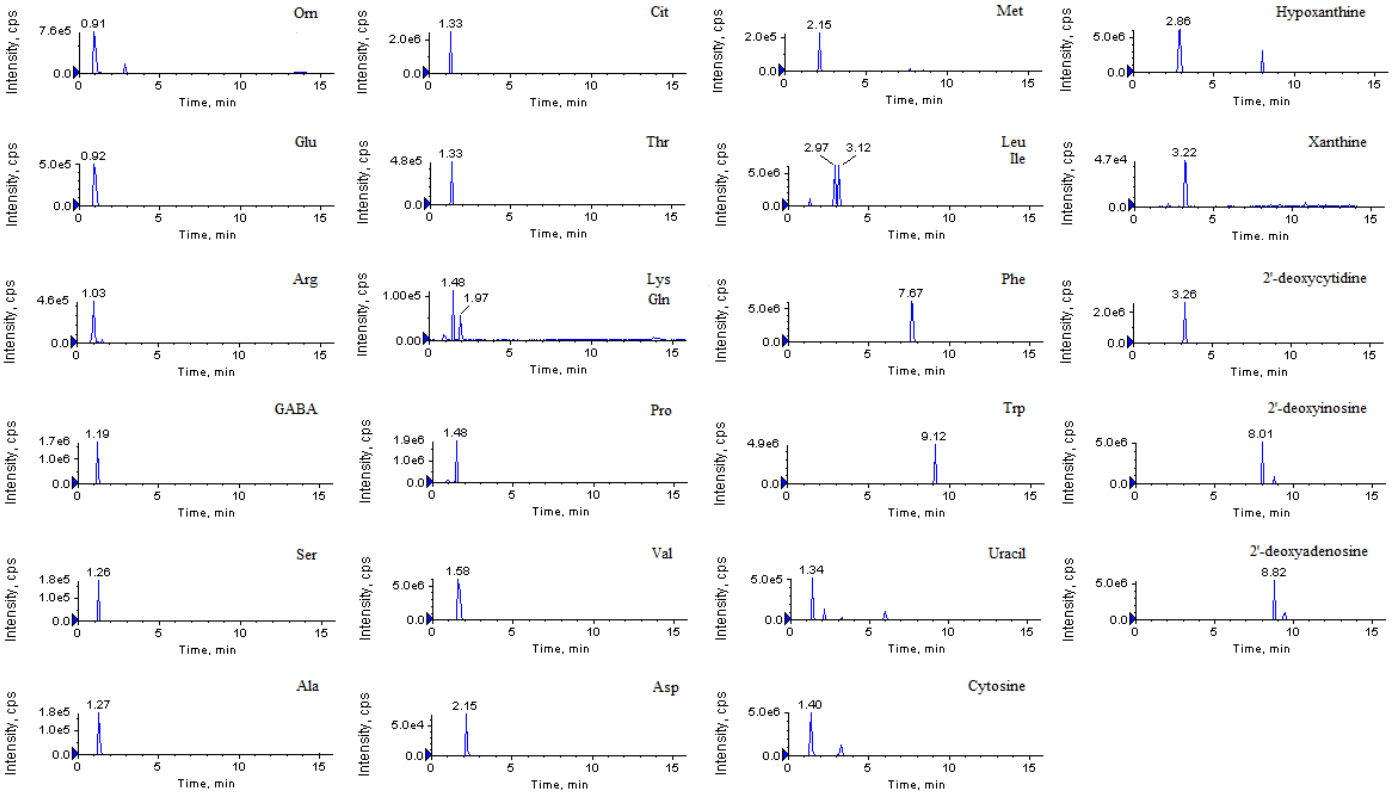

Figure 2. MRM extracted ion chromatogram of free amino acids and nucleosides in ZZCD.

3.3. Validation study

The method was fully validated in accordance with some related guidelines with respect to specificity, linearity, precision, recovery, detection limit, quantification limit and stability [25]. All data were summarized in **Tables 2** and **3**.

Table 2. Regression equation, linearity ranges, correlation coefficients and limit of detection (LOD) and quantitation (LOQ) of 53 targeted analytes.

Ionization mode	Ascription	No.	Analytes	tR (min)	Calibration curves	*r*	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Positive	Isoflavones	1	Daidzin	10.71	$Y = 89{,}792.21\ X + 30{,}154.34$	0.9991	1−10,000	0.5	1
		2	Genistin	10.78	$Y = 109{,}750.22\ X + 20{,}400.93$	0.9971	25−10,000	2.5	10
		3	Glycitin	11.13	$Y = 58{,}894.81\ X + 11{,}045.57$	0.9991	2.5−500	0.5	1
		4	Daidzein	11.82	$Y = 159{,}762.29\ X + 282{,}469.39$	0.9988	100−20,000	10	50
		5	Genistein	11.85	$Y = 221{,}212.24\ X + 273{,}869.66$	0.9987	2.5−500	0.5	1
		6	Glycitein	12.05	$Y = 226{,}385.63\ X + 89{,}508.03$	0.9959	100−20,000	10	50
	Free amino acids	7	Orn	0.91	$Y = 70.77\ X + 296{,}274.63$	0.9972	50−500,000	10	25
		8	Glu	0.92	$Y = 13.66\ X - 16{,}649.69$	0.9983	1000−400,000	10	50
		9	Arg	1.03	$Y = 47.99\ X + 243{,}564.27$	0.9977	50−300,000	10	25
		10	GABA	1.19	$Y = 111.85\ X - 17{,}529.34$	0.9987	250−200,000	25	100
		11	Ser	1.26	$Y = 8.99\ X + 4295.57$	0.9993	100−200,000	2.5	10
		12	Ala	1.27	$Y = 24.73\ X + 1{,}224{,}477.60$	0.9986	2500−500,000	25	100
		13	Cit	1.33	$Y = 164.67\ X + 243{,}174.49$	0.9976	2.5−200,000	0.5	1
		14	Thr	1.33	$Y = 44.11\ X + 35{,}027.58$	0.9992	25−200,000	2.5	10
		15	Lys	1.48	$Y = 1091.37\ X + 4253.88$	0.9996	10−10,000	1	5
		16	Pro	1.48	$Y = 192.21\ X + 188{,}322.36$	0.9994	1000−300,000	25	100
		17	Val	1.58	$Y = 2526.29\ X + 723{,}470.91$	0.9997	2.5−100,000	0.5	1
		18	Gln	1.97	$Y = 58.24\ X + 18{,}141.84$	0.9990	50−300,000	2.5	25
		19	Asp	2.15	$Y = 31.61\ X + 173{,}743.88$	0.9983	250−400,000	25	100
		20	Met	2.15	$Y = 26.27\ X + 56{,}871.24$	0.9978	100−100,000	2.5	10
		21	Leu	2.97	$Y = 1329.27\ X + 791{,}965.52$	0.9989	25−50,000	2.5	10
		22	Ile	3.12	$Y = 1419.35\ X + 939{,}467.85$	0.9986	25−50,000	2.5	10
		23	Phe	7.67	$Y = 2310.62\ X + 1{,}313{,}260.72$	0.9976	100−20,000	2.5	10
		24	Try	9.12	$Y = 442.15\ X + 1{,}174{,}687.34$	0.9999	10−200,000	0.5	1
	Nucleosides	25	Uridine	1.34	$Y = 103.91\ X - 407.72$	0.9984	50−20,000	2.5	10
		26	Cytosine	1.4	$Y = 2842.92\ X - 6102.24$	0.9993	1−5000	0.5	1
		27	Hypoxanthine	2.86	$Y = 2941.76\ X + 823{,}607.37$	0.9998	2.5−100,000	0.5	1
		28	Xanthine	3.22	$Y = 42.02\ X - 463.26$	0.9999	50−50,000	2.5	5
		29	2′-deoxycytidine	3.26	$Y = 455.86\ X - 14{,}570.08$	0.9998	50−10,000	2.5	10
		30	2′-deoxyinosine	8.01	$Y = 997.77\ X - 31{,}227.69$	0.9998	50−10,000	2.5	10
		31	2′-deoxyadenosine	8.82	$Y = 773.27\ X + 2{,}265{,}736.02$	0.9991	10−300,000	0.5	1

Table 2. (*Continued*).

Ionization mode	Ascription	No.	Analytes	tR (min)	Calibration curves	*r*	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Negative	Iridoid glycosides	32	Deacetyl asperulosidic acid	6.15	$Y = 26.05\ X + 1621.04$	0.9999	50−20,000	1	2.5
		33	Shanzhiside	9.24	$Y = 68.02\ X + 23{,}704.47$	0.9996	2.5−300,000	0.5	1
		34	Geniposidic acid	9.24	$Y = 15.893\ X + 5558.34$	0.9984	100−100,000	0.5	10
		35	Deacetyl asperulosidic acid methyl	9.46	$Y = 373\ X - 5633.7373$	0.9990	20−10,000	1	10
		36	Scandoside methyl ester	9.89	$Y = 5.13\ X + 546.19$	0.9993	50−10,000	2.5	5
		37	Shanzhiside methyl ester	10.5	$Y = 5.89\ X - 1683.6416$	0.9992	500−300,000	10	50
		38	Genipin1-β-gentiobioside	10.57	$Y = 13.11\ X + 4742{,}833.33$	0.9943	100−500,000	2.5	10
		39	Gardenoside	10.65	$Y = 1.33\ X + 3597.15$	0.9979	50−100,000	2.5	5
		40	Geniposide	11.09	$Y = 6.93\ X + 38470.04$	0.9980	25−400,000	2.5	10
		41	Genipin	11.46	$Y = 105.19\ X + 437{,}621.92$	0.9977	25−200,000	2.5	10
		42	Crocin-I	11.61	$Y = 1167.33\ X - 22{,}012.37$	0.9982	25−50,000	2.5	10
		43	Crocin-II	11.76	$Y = 270.12\ X - 31{,}648.28$	0.9984	250−20,000	2.5	100
	Other flavonoids	44	Rutin	11.46	$Y = 1531.42\ X + 176{,}446.24$	0.9970	2.5−100,000	0.5	1
		45	Isoquercetin	11.51	$Y = 279.52\ X + 14{,}205.63$	0.9994	1−5000	0.5	1
		46	Quercetin	11.98	$Y = 182.86\ X + 34{,}963.04$	0.9967	1−5000	0.5	1
	Phenolic acids	47	Malic acid	3.01	$Y = 2730.68\ X - 33{,}304.67$	0.9999	25−300,000	2.5	10
		48	Gallic acid	3.68	$Y = 437.32\ X + 217{,}327.78$	0.9981	50−100,000	2.5	10
		49	Protocatechuic acid	7.48	$Y = 1631.84\ X - 23{,}993.12$	0.9977	25−400,000	2.5	10
		50	Procatechin	9.78	$Y = 362.22\ X + 19{,}663.11$	0.9984	2.5−500,000	0.5	1
		51	Chlorogenic acid	9.9	$Y = 422.78\ X + 1{,}996{,}556.30$	0.9978	2.5−500,000	0.5	1
		52	p-Coumaric acid	11.39	$Y = 418.91\ X + 183{,}692.12$	0.9997	5−5000	0.5	1
		53	Ferulic acid	11.46	$Y = 189.11\ X + 35{,}393$	0.9982	5−200,000	0.5	1

The precisions were examined for six replicates on the mixed standard solutions and expressed using the relative standard deviation (RSD), whose values were less than 3.92%; the repeat, recovery and stability were tested on the analytes in S6, the repeatabilities analyzed on six sample solutions from the same sample in parallel were within 0.76%–2.80%; the recoveries were between 93.74% and 101.65% at different spiking concentration levels (80%, 100% and 120% as compared to the nominal concentration) of standards stock solutions; the short-term stability analyzed at 0, 2, 4, 8, 12, 16, 20 and 24 h were less than 4.00%, which indicated that the method was reproducible.

Linear equations, detection limits, and quantitative limits: A series of concentrations of control stock solutions were used for LC-MS analysis, with peak area on the vertical axis *Y* and control stock concentration (ng/ml) on the horizontal axis *X*. Linear regression was performed to draw a standard curve, and the correlation coefficient was calculated. The signal-to-noise ratio (S/N) of approximately 3 was used to calculate the lowest detection limit (LOD) for each component, and the S/N of approximately 10 was used to calculate the lowest quantitative limit (LOQ). The calibration curves showed satisfactory linearity, the correlation coefficient (r) ranged from 0.9943 to 0.9999 for all the analytes. The limits of detection (LOD) and quantity (LOQ) values were between 0.5-25 ng/mL and 1–100 ng/mL.

Table 3. Methodological examination results for 53 target analytes.

Ionization mode	Ascription	No.	Analytes	Precision (RSD/%)	Repeat (RSD/%)	Stability (RSD/%)	Recovery	
							Mean/%	RSD/%
Positive	Isoflavones	1	Daidzin	3.06	1.58	0.75	95.85	2.74
		2	Genistin	1.64	1.82	0.69	98.29	1.99
		3	Glycitin	0.49	2.48	2.22	108.92	3.14
		4	Daidzein	2.06	1.11	2.52	100.44	2.72
		5	Genistein	1.89	1.76	0.91	91.98	4.78
		6	Glycitein	0.8	1.41	3.12	95.82	4.49
	Free amino acids	7	Orn	0.62	0.63	3.18	99.66	3.96
		8	Glu	1.22	0.48	1.77	108.26	1.32
		9	Arg	0.66	1.74	1.04	107.11	1.19
		10	GABA	0.70	0.55	3.34	91.50	2.36
		11	Ser	0.45	2.31	1.98	107.50	3.31
		12	Ala	1.34	1.37	1.28	102.59	1.69
		13	Cit	1.47	2.42	2.18	104.8	3.18
		14	Thr	0.83	1.71	2.07	92.23	1.42
		15	Lys	0.66	1.04	2.19	105.02	3.95
		16	Pro	1.08	0.21	3.96	98.17	1.26
		17	Val	0.67	0.22	1.55	97.69	1.26
		18	Gln	0.45	1.95	2.17	97.26	3.81
		19	Asp	0.09	0.26	3.84	101.35	4.28
		20	Met	1.70	1.37	1.5	106.72	1.17
		21	Leu	1.02	0.13	1.01	105.62	1.82
		22	Ile	2.22	0.45	1.1	102.86	3.67
		23	Phe	2.07	2.89	1.03	103.91	3.79
		24	Try	2.32	0.78	3.68	101.91	3.99
	Nucleosides	25	Uridine	0.59	1.64	1.63	109.51	1.09
		26	Cytosine	0.89	0.85	0.53	103.68	2.87
		27	Hypoxanthine	1.87	2.06	1.40	108.57	1.76
		28	Xanthine	2.55	1.39	2.46	99.46	3.92
		29	2′-deoxycytidine	3.29	0.85	0.63	96.94	2.94
		30	2′-deoxyinosine	0.41	0.41	0.86	106.21	2.48
		31	2′-deoxyadenosine	0.54	2.64	1.35	99.11	3.74

Table 3. (*Continued*).

Ionization mode	Ascription	No.	Analytes	Precision (RSD/%)	Repeat (RSD/%)	Stability (RSD/%)	Recovery	
							Mean/%	RSD/%
Negative	Iridoid glycosides	32	Deacetyl asperulosidic acid	2.33	0.73	0.29	93.97	0.68
		33	Shanzhiside	0.85	3.83	1.45	93.88	3.93
		34	Geniposidic acid	0.59	1.57	0.84	99.26	3.54
		35	Deacetyl asperulosidic acid methyl	1.53	3.71	3.51	98.25	4.32
		36	Scandoside methyl ester	2.62	2.03	1.42	93.19	2.34
		37	Shanzhiside methyl ester	1.07	2.85	2.02	97.33	4.67
		38	Genipin1-β-gentiobioside	3.12	1.85	0.7	100.88	2.01
		39	Gardenoside	0.58	2.84	1.32	103.84	3.48
		40	Geniposide	0.14	0.64	0.57	102.42	3.67
		41	Genipin	1.75	2.76	0.95	93.29	3.15
		42	Crocin-I	1.75	1.83	2.57	97.81	3.77
		43	Crocin-II	0.41	1.59	3.74	106.95	3.21
	Other flavonoids	44	Rutin	2.98	2.84	3.16	102.43	3.7
		45	Isoquercetin	1.94	2.42	1.15	97.27	4.3
		46	Quercetin	0.24	3.03	2.18	103.89	2.06
	Phenolic acids	47	Malic acid	3.07	1.01	3.62	99.17	2.01
		48	Gallic acid	1.84	3.29	1.67	95.95	2.68
		49	Protocatechuic acid	3.6	3.74	1.58	107.46	1.87
		50	Procatechin	1.8	1.77	2.4	99.97	1.8
		51	Chlorogenic acid	3.08	3.33	0.93	104.8	3.99
		52	p-Coumaric acid	2.87	4.53	2.43	99.96	3.52
		53	Ferulic acid	2.77	4.21	3.42	98.79	3.55

Precision, reproducibility, and stability experiments: a mixture of control stock solutions was used, and six injections were made consecutively. The RSD values of the peak areas of the 53 target components were calculated. An equal amount of the test sample was weighed and prepared into six test samples in parallel, and the contents of each target component were determined and the RSD values were calculated. One of the test sample solutions was taken and the peak areas of the target components were measured and the RSD values were calculated at 0, 2, 4, 8, 12, 16, 20, and 24 hours. The results showed that the RSD values of the precision of the 53 target components were all less than 3.60%, indicating good instrument precision. The RSD values of the reproducibility were all less than 4.53%, indicating good reproducibility of the measurement method. The RSD values of the stability were all less than 3.84%, indicating good stability of the test sample solution at room temperature for 24 hours.

The addition recovery experiment: 10.5 g of Gardenia jasminoides J.Ellis was taken, 800 mL of water was added, and an equal amount of mixed reference

substances was added for decoction. When the liquid was 500 mL, 24.5 g of *Sojae semen praeparatum* was added later, and the decoction was boiled until the liquid was 300 mL. The parallel preparation and determination of each target component's addition recovery rate and RSD value were calculated. The detailed results are shown in **Table 3**, with the addition recovery rates of the 53 flavonoid, iridoid glycosides, phenolic acids, amino acid, and nucleotide components ranging from 91.80% to 109.51%. Their RSD values are all less than 4.78%.

The above results show that the LC-MS/MS method established in this experiment can be used for the simultaneous determination of the contents of 53 flavonoid, iridoid glycosides, phenolic acids, amino acid, and nucleotide components in the sample.

3.4. Sample analysis

The results showed that all the 53 target components were determined in ZZCD in **Table 4**, which shows that the method is well suited for simultaneous determination. Compared with previous studies, more components and categories were detected in this study. All target components were detected in the test sample, with the total isoflavones content being 2.270 mg/g, the total iridoid glycosides content being 35.573 mg/g, the total other flavonoid content being 0.294 mg/g, the total phenolic acids content being 0.959 mg/g, the total amino acid content being 14.19 mg/g crude herb weight, the total nucleic acid content being 0.554 mg/g crude herb weight. The iridoid glycosides, isoflavones, and free amino acids are the main component classes of ZZCD. Daidzin, glycitin, daidzein, Glu, GABA, Ala, Pro, Gln, Asp, Leu, Ile, Phe, deacetyl asperulosidic acid, shanzhiside, deacetyl asperulosidic acid methyl, scandoside methyl ester, genipin1-β-gentiobioside, gardenoside, geniposide, crocin-I are the main components of ZZCD. The contents of deacetylcycloartanoside, deacetylcycloartanol glycoside methyl ester, kenipin 1-β-gentiobioside, kenipin glycoside, glutamic acid, alanine, glutamino acid, and aspartic acid are much higher than those of other components. This study can provide methodological reference for the comprehensive evaluation of ZZCD and is of great significance for ensuring the clinical efficacy of ZZCD.

Table 4. Contents of 53 target analytes in ZZCD (mg/g, n = 3).

Ionization mode	Ascription	No.	Analytes	Contents (mg/g)
Positive	Isoflavones	1	Daidzin	0.526
		2	Genistin	0.089
		3	Glycitin	0.755
		4	Daidzein	0.448
		5	Genistein	0.089
		6	Glycitein	0.363
			Total isoflavones	2.270
	Free amino acids	7	Orn	0.066
		8	Glu	3.234
		9	Arg	0.295
		10	GABA	0.651
		11	Ser	0.278
		12	Ala	1.676
		13	Cit	0.055
		14	Thr	0.322
		15	Lys	0.104
		16	Pro	0.603
		17	Val	0.187
		18	Gln	3.062
		19	Asp	1.086
		20	Met	0.152
		21	Leu	0.531
		22	Ile	0.601
		23	Phe	0.879
		24	Try	0.407
			Total free amino acids	14.19
	Nucleosides	25	Uridine	0.035
		26	Cytosine	0.006
		27	Hypoxanthine	0.032
		28	Xanthine	0.226
		29	2’-deoxycytidine	0.003
		30	2’-deoxyinosine	0.059
		31	2’-deoxyadenosine	0.198
			Total nucleosides	0.554
	Iridoid glycosides	32	Deacetyl asperulosidic acid	2.714
		33	Shanzhiside	1.895
		34	Geniposidic acid	0.452
		35	Deacetyl asperulosidic acid methyl	7.244
		36	Scandoside methyl ester	0.565
		37	Shanzhiside methyl ester	0.147
		38	Genipin1-β-gentiobioside	6.956
		39	Gardenoside	1.980
		40	Geniposide	11.910
		41	Genipin	0.045
		42	Crocin-I	1.413
		43	Crocin-II	0.219
			Total iridoid glycosides	35.537

Table 4. (*Continued*).

Ionization mode	Ascription	No.	Analytes	Contents (mg/g)
Negative	Other flavonoids	44	Rutin	0.131
		45	Isoquercetin	0.061
		46	Quercetin	0.102
			Total flavonoids	0.294
	Phenolic acids	47	Malic acid	0.197
		48	Gallic acid	0.021
		49	Protocatechuic acid	0.059
		50	Procatechin	0.028
		51	Chlorogenic acid	0.419
		52	p-Coumaric acid	0.136
		53	Ferulic acid	0.099
			Total phenolic acids	0.959

4. Conclusion

In this study, a method based on UFLC/QTRAP-MS technique was established for the simultaneous determination of 53 flavonoids, iridoid glycosides, phenolic acids, free amino acids and nucleosides in ZZCD firstly. In view of the physiological activities of these components, they were taken as the basis of the efficacy substances of ZZCD. The results showed that all the 53 target components were determined in ZZCD and the method was suitable for the simultaneous determination of 53 target components in ZZCD, with iridoid glycosides, isoflavones and free amino acids as the main constituent categories of ZZCD. Daidzin, glycitin, daidzein, Glu, GABA, Ala, Pro, Gln, Asp, Leu, Ile, Phe, deacetyl asperulosidic acid, shanzhiside, deacetyl asperulosidic acid methyl, scandoside methyl ester, genipin1-β-gentiobioside, gardenoside, geniposide, crocin-I were the main constituents in ZZCD. In conclusion, the quantitative method of the active ingredients is stable and has good repeatability, which can serve as an indicator for the quality control of ZZCD. Compared with previous studies, more components and categories were detected in this study, which made up the blank on the target components contents of ZZCD, and promoted the construction of the ZZCD fingerprint at the same time, which have great significance for the comprehensive guarantee of the clinical therapeutic effect of ZZCD. The present study also offers an experimental foundation for more in-depth research on the pharmacochemistry analysis of ZZCD and effective fractions selection.

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