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Original Article

Simultaneous quantification combined with multivariate statistical analysis of multiple chemical markers of Wu Ji Bai Feng Pill by UHPLC–MS/MS

*Sheng-Nan Duan, Wen Qi, Si-Wen Zhang, Kun-Kun Huang, Dan Yuan**

Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang 110016, PR China

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ABSTRACT

Wu Ji Bai Feng Pill (WJBFP) is a traditional Chinese medicine (TCM) complex formula, which has been widely used in the treatment of various gynecological disorders. However, the quality control of multiple components in WJBFP is challengeable by using the methods applicable to analysis of several phytochemicals in single herbs or simple herbal preparations. The purpose of this study is to establish an ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC–MS/MS) method for the quantitative determination of 20 bioactive compounds in WJBFP. The modified chromatographic conditions were achieved on an Agilent Poroshell 120 EC-C_{18} column with a gradient elution consisted of 0.1% formic acid in acetonitrile and 0.1% aqueous formic acid (v/v). All analytes were determined using a triple quadrupole mass spectrometry in positive or negative ionization modes with multiple reaction monitoring (MRM) mode. An UHPLC–MS/MS method was optimized and validated for linearity, limits of detection and quantification, precision, repeatability, stability and recovery. The proposed method was applied for the analysis of 20 compounds in 19 batches of commercial WJBFP products. principal component analysis and hierarchical cluster analysis were applied to evaluate intrinsic quality and to identify chemical markers most responsible for quality evaluation. In conclusion, the established method offered speedy and sensitive determination for 20 compounds and is helpful for chemical standardization of commercial WJBFP products.

* *Corresponding author*. Department of Traditional Chinese Medicine, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, PR China. Fax: +86 024 23986502.
E-mail address: yuandan_kampo@163.com (D. Yuan).

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1. Introduction

Traditional Chinese medicine is a therapeutic system with its unique tradition, over thousands of years of continual practice and improvement through observation, investigation and critical thinking [1]. It aims to reinstate the whole-body balance of patients by using herbal formula, which is usually comprised of multiple herbal materials and has the capacity of systematically treating disease. However, it has always been questionable and unacceptable for modern medicinal science that the approaches successfully used in the analysis of one, two or three phytochemicals in a single herb or a TCM preparation were applied to the quality evaluation of most herbal products.

WJBFP (Wu Ji Bai Feng Pill) is a grand TCM complex formula, which has been popularly used to treat various gynecological disorders, such as dysmenorrhea, amenorrhea and infertility for hundreds of years. The pills are originally made of fourteen herbal materials and six animal crude materials, namely *Angelica sinensis* (Oliv.) Diels (Angelicae Sinensis Radix), *Ligusticum chuanxiong* Hort. (Chuanxiong Rhizoma), *Paeonia lactiflora* Pal. Paeoniae Radix Alba, *Rehmannia glutinosa* Libosch. (Rehmanniae Radix), *R. glutinosa* (Gaert.) *Libosch.* ex Fisch. et Mey. (Rehmanniae Radix Praeparata), *Salvia miltiorrhiza* Bge. (Salviae Miltiorrhizae Radix et Rhizoma), *Glycyrrhiza uralensis* Fisch. (Glycyrrhizae Radix et Rhizoma), *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao. (Astragali Radix), *Panax ginseng* C. A. Mey. (Ginseng Radix et Rhizoma), *Cyperus rotundus* L. (Cyperi Rhizoma), *Dioscorea opposita* Thunb. (Dioscoreae Rhizoma), *Asparagus cochinchinensis* (Lour.) Merr. (Asparagi Radix), *Stellaria dichotoma* L. var. *lanceolata* Bge. (Stellariae Radix), *Euryale ferox* Salisb. (Euryales Semen), *Gallus domesticlus* Brisson (Silky fowl), Cervi Cornus Colla, Cervi Cornu Degelatinatum, *Ostrea gigas* Thimberg. (Ostreae Concha), *Trionyx sinensis* Wiegmann (Trionycis Carapax), and *Tenodera sinesis* Saussure (Mantidis Oötheca) [2]. Modern pharmacological studies showed that WJBFP was reasonably combined and could reduce the androgen level, promote the follicular development, as well as improve the ovulation disorders [3].

According to our previous study [4], the main constituents in WJBFP extracts were structurally divided into more than ten phytochemical groups. Among them, monoterpene glycosides, flavonoids, triterpenoid saponins, tanshinones, phenolic compounds and phthalides were characteristic as far as both their contents and biological activities are concerned. Monoterpene glycosides are closely related to the efficacy of Paeoniae Radix Alba. A case in point is albiflorin (AF) and paeoniflorin (PF), which exhibited analgesia, anti-inflammation and anticoagulation activities [5–7]. Flavonoids mainly from Glycyrrhizae Radix et Rhizoma and Astragali Radix also showed antioxidant [8,9], anti-inflammatory [10,11], vascular protective [12], analgesic and uterine relaxant effects [13]. Estrogen-like, anticancer and neuroprotective effects of triterpenoid saponins in Glycyrrhizae Radix and Ginseng Radix, such as glycyrrhizic acid [14], and ginsenoside Rg_3 and Rb_1 [15–17], were also documented. Tanshinones and phenolic compounds from Salviae Miltiorrhizae Radix played an important role in the treatment of cardiovascular diseases due to their antioxidative stress, antiplatelet aggregation, anti-inflammation and antithrombosis activities [18,19]. In addition, phthalides in Angelicae Sinensis Radix and Chuanxiong Rhizoma also demonstrated anti-myocardial ischemia, blood vessel protection, anti-thrombotic and muscle relaxant effects [20,21]. Thus, the above constituents with various biological activities maybe speculated to be the biomarker components of WJBFP.

Although some studies on the quantitative analysis of WJBFP have been developed using TLC, HPLC, UHPLC and near infrared spectroscopy [22–24], the available methods of comprehensive and systematic quality evaluation for WJBFP have not been reported. Accordingly, it is indispensable to develop a rapid and sensitive method to simultaneously quantify the multiple compounds in WJBFP, which is instrumental to investigate the effectiveness and assess the quality of WJBFP.

Liquid chromatography coupled with tandem mass spectrometry has been increasingly popular in quantitative analysis of herbs and TCM formulas [25–27]. While single MS filtering offers advantages over non-mass selective techniques, the use of tandem mass spectrometry offers an extensive range and fast method development, as well as significant improvements in the accuracy of composite chemical system. As a result, the MRM experiment performed on the triple quadrupole mass spectrometers has become a practical choice for highly sensitive and selective quantification in complex matrices.

In the present study, an UHPLC–MS/MS method was developed to simultaneously determine the contents of 20 bioactive compounds (the chemical structures are shown in Supplementary Fig. S1) in WJBFP for the first time. The newly developed method was validated and applied for the simultaneous quantification of 20 components in 19 batches of WJBFP commercial products. Moreover, principal component analysis and hierarchical cluster analysis were used to evaluate intrinsic quality and to identify chemical markers most responsible for quality control.

2. Methods

2.1. Chemicals

Twenty authentic compounds were used in the present study. Gallic acid (GA, 1), ferulic acid (FA, 5), liquiritin (LQ, 6), isoliquiritin (ILQ, 8), ginsenoside Re (Re, 10), Rg_1 (11) and Rb_1 (12), cryptotanshinone (CTS, 18) and tanshinone II_A (TS2A, 20) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), calycosin-7-O-β-D-glucoside (CG, 4), acteoside (AT, 7), salvianolic acid B (SAB, 9) and Z-ligustilide (ZLG, 15) from Shanghai ANPEL Scientific Instrument Co., Ltd. (Shanghai, China), ginsenoside 20(S)-Rg_3 (Rg3, 14), Rk_1 (17), Rg_5 (16), and α-cyperone (CP, 19) from Beijing Anyp Remit Secco Biological Technology Co., Ltd. (Beijing, China), albiflorin (AF, 2) from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China), paeoniflorin (PF, 3) from Yoneyama Yakuhin Kogyo Co,. Ltd. (Osaka, Japan), and glycyrrhizic acid (GCA, 13) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The purity of each reference was determined to be above 98% by HPLC-DAD method.

2.2. Reagents and materials

Acetonitrile (ACN, LC–MS grade) and formic acid (spectroscopy grade) were purchased from Fisher Scientific UK (Loughborough, UK). Ultra-pure water (18.2 MΩ) was daily prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Analytical grade methanol (Yuwang Chemical Reagent Co. Ltd., Shandong, China) and purified water (Hangzhou Wahaha Group Co. Ltd., Hangzhou, China) were used for the extraction of samples. Polytetrafluoroethylene (PTFE) membranes of 0.22 μm used for processing samples were from ANPEL (Shanghai, China), and Nylon membranes from JinLong (Tianjin, China). Nineteen batches of WJBFP commercial products (Sample #1–#19, see Supplementary Table S1 for details) were acquired from different manufactures in China. And the voucher specimens are kept in the reference library for the medical herbs in Shenyang Pharmaceutical University.

2.3. Liquid chromatography

An Agilent 1290 Infinity II UHPLC system was employed (Agilent, California, USA), equipped with a binary solvent delivery system, an autosampler and a column compartment. Samples were separated on an Agilent Poroshell 120 EC-C_{18} column (2.1 × 100 mm, 1.9 μm, Agilent, California, USA). The mobile phase, consisting of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), was applied with the optimized gradient program as follows: 0–2.0 min, 5–10% (B); 2.0–3.0 min, 10–17% (B); 3.0–4.0 min, 17–20% (B); 4.0–4.5 min, 20–25% (B); 4.5–5.5 min, 25–30% (B); 5.5–7.1 min, 30–40% (B); 7.1–9.1 min, 40–50% (B); 9.1–10.0 min, 50–60% (B); 10–12.0 min, 60–70% (B); 12.0–14.0 min, 70–75% (B); 14.0–15.0 min, 75–99% (B); 15.0–15.9 min, 99–99% (B); 15.9–16.0 min, 99–5% (B); post-run time, 1 min. The column and autosampler temperatures were maintained at 40 °C and 4 °C, respectively. The flow rate was maintained at 0.4 mL/min and the injection volume was 2.0 μL.

2.4. Mass spectrometry

UHPLC–MS/MS was carried out on an AB SCIEX API 4000™ LC–MS\MS system (AB SCIEX, California, USA) equipped with an electrospray ionization (ESI) interface. The ESI source was operated in both positive and negative ionization modes, and quantification was performed in MRM mode. The operation conditions were as follows: ion spray voltage, 5500/−4500 V; turbo spray temperature, 450 °C; curtain gas (CUR), 25 psi and with interface heater on; collision gas, medium; nebulizer gas (Gas 1) and heater gas (Gas 2), 50 and 50 psi, respectively; entrance potential (EP), 10/−10 V. Nitrogen was used in all cases. The results of the precursor ion, product ion, corresponding declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) are listed in Table 1. The dwell time of each ion pair was 50 ms in the positive mode and 35 ms in the negative mode.

2.5. Standard solution preparation

Each reference compound (1–20) was accurately weighed and dissolved in 75% aqueous methanol to make its stock solution (200 μg/mL). Then, 20 stocks were mixed and diluted and with 75% aqueous methanol to prepare a final mixed standard solution (PF, SAB, AF, Rb_1, Re, GA and Rg_5 in 50 μg/mL, LQ, FA, AT, ILQ, ZLG, Rg_1, Rg_3, GCA, Rk_1 and CTS in 10 μg/mL, CG, CP and TS2A in 1.5 μg/mL). A series of five calibration solutions was prepared by appropriate dilution of the final mixed standard solution with 75% aqueous methanol for construction of the regression equations. All solutions were stored at 4 °C before determination. The concentration range is given in Table 1.

2.6. Sample solution preparation

Nineteen batches of WJBFP commercial products were ground and well mixed respectively. The powdered WJBFP (0.5 g) was dispersed in 25 mL of 75% aqueous methanol (v/v) and ultrasonically extracted (100W, 40 KHz) for 30 min at room temperature [4]. The extracted solution was adjusted to the original weight by adding 75% aqueous methanol, and then the mixtures were filtered. The filtrate was centrifuged at 13,000 rpm for 10 min. An aliquot (2.0 μL) of each sample filtered through a 0.22 μm PTFE membrane was injected into the UHPLC instrument for analysis.

2.7. Method validation

2.7.1. Linearity, LOQs and LODs

Calibration curves were constructed for at least five different concentrations by plotting the peak areas of the standard compounds (Y) versus the corresponding concentration of the injected standard solutions (X). Linear regression analysis was used to calculate the slope, intercept and correlation coefficient of each calibration line. Typically, LODs and LOQs are the lowest mass of a compound that can be detected or accurately and precisely quantified. For each target constituent, the LODs and LOQs were determined at a signal-noise ratio (S/N) of about 3 and 10 by serial dilution of standard solution.

2.7.2. Precision, repeatability and stability

Intra- and inter-day variations, which were chosen as indicators of the precision of the developed method, were evaluated by determining the 20 analytes in six replicates during a single day and by duplicating the experiments for three consecutive days. Variation in peak area was expressed as percent RSD. The repeatability of the developed method was described by analyzing six samples of WJBFP (S4) prepared using the same method. The RSD was used to evaluate the method repeatability. Meanwhile, the stability of the samples was also investigated at 0, 2, 4, 8, 12, and 24 h after initial storage at room temperature.

2.7.3. Accuracy

To further evaluate the accuracy of the developed method, a recovery test was validated by spiking the reference solutions to known amounts of WJBFP samples (S4) at the middle concentration level (100%). The mixture was extracted and analyzed as described above and six replicates were performed. The recovery percentages were calculated by the formula: Recovery (%) = (Detected amount − Original amount)/Spiked amount × 100%, RSD (%) = (S.D./mean) × 100%.

Table 1 – The retention time, precursor ions (MS1), product ions (MS2), DP, CE, CXP, linear regression data, LOD and LOQ of the 20 targeted components.

Compounds	t_R (min)	MS1 (*m*/*z*)	MS2 (*m*/*z*)	DP (V)	CE (eV)	CXP (V)	Regression equation	R^2	Linear range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
1 GA	1.08	169.0	125.0	−62	−20	−10	$y = 4.56e^5x - 2.58e^5$	0.9997	5.00–20.0	150	450
2 AF	4.02	525.2	121.2	−74	−31	−5	$y = 2.70e^5x + 4.70e^5$	0.9997	1.00–30.0	2.18	6.54
3 PF	4.25	525.1	449.4	−74	−18	−10	$y = 8.94e^5x + 1.83e^5$	0.9990	2.00–40.0	2.38	7.14
4 CG	4.75	447.2	285.3	90	22	8	$y = 1.55e^6x + 7.46e^3$	0.9994	5.00×10^{-3} to 0.500	0.28	0.74
5 FA	4.75	193.0	134.0	−62	−20	−12	$y = 3.16e^5x + 5.75e^3$	0.9992	2.50×10^{-2} to 2.50	1.17	3.52
6 LQ	4.82	417.1	255.0	−90	−27	−15	$y = 7.87e^5x + 1.66e^5$	0.9993	0.250–10.0	0.42	1.25
7 AT	5.07	623.2	161.1	−135	−53	−8	$y = 4.42e^5x - 3.62e^3$	0.9998	0.100–5.00	3.73	11.2
8 ILQ	5.98	417.1	255.0	−90	−27	−15	$y = 6.31e^5x - 6.89e^3$	0.9998	0.100–5.00	0.44	1.33
9 SAB	5.99	717.8	339.5	−46	−23	−7	$y = 1.54e^5x + 1.78e^5$	0.9989	1.00–40.0	3.17	9.50
10 Re	6.08	945.6	161.2	−220	−60	−8	$y = 1.22e^3x + 3.70$	0.9999	0.500–25.0	98.0	294
11 Rg_1	6.11	799.6	637.5	−160	−32	−15	$y = 1.76e^4x - 3.80e^2$	0.9996	5.00×10^{-2} to 5.00	12.4	37.0
12 Rb_1	7.62	1107.7	179.2	−173	−74	−12	$y = 3.66e^3x + 1.37e^3$	0.9990	0.500–20.0	133	400
13 GCA	8.43	821.6	351.3	−170	−55	−6	$y = 9.10e^4x + 1.87e^4$	0.9996	0.500–10.0	3.03	9.09
14 Rg_3	10.46	783.6	161.2	−190	−48	−8	$y = 3.15e^5x + 4.90e^3$	0.9991	0.250–5.00	6.35	19.1
15 ZLG	10.93	197.0	169.0	120	30	15	$y = 5.28e^5x + 1.95e^5$	0.9987	0.250–10.0	5.31	15.9
16 Rg_5	11.73	765.7	161.3	−175	−48	−12	$y = 1.08e^3x + 1.58e^3$	0.9999	2.50–50.0	290	870
17 Rk_1	11.87	765.6	161.3	−176	−48	−18	$y = 5.72e^3x + 7.84e^2$	0.9992	0.250–5.00	70.2	211
18 CTS	12.05	297.2	251.2	120	32	8	$y = 1.96e^6x + 6.87e^3$	0.9990	2.00×10^{-2} to 1.50	0.28	0.83
19 CP	12.47	219.2	111.2	83	22	10	$y = 6.54e^6x + 9.75e^3$	0.9999	3.00×10^{-3} to 0.100	0.53	1.59
							$y = 4.73e^6x + 1.12e^5$	0.9981	2.50×10^{-2} to 0.500		
20 $TSII_A$	13.45	295.1	249.2	128	30	8	$y = 1.86e^6x + 5.34e^4$	0.9980	5.00×10^{-2} to 1.50	0.44	1.33

2.8. Data analysis

AB SCIEX Analyst 1.6.1 software and MutiQuant™ 3.0.2 software (AB SCIEX, California, USA) were used for MS data acquisition and processing. Principal component analysis (PCA) was analyzed by SIMCA-P 11.5 software (Umetrics, Umeå, Sweden) and hierarchical cluster analysis (HCA) was analyzed using Heatmap Illustrator 1.0 software [28].

3. Results and discussion

3.1. Modification of LC conditions

Several UHPLC parameters were modified to obtain a better separation, a higher sensitivity, and a reduced analysis time on the base of our previous study [4]. Firstly, mobile phases, such as acetonitrile and methanol with formic acid, were tested comparatively. It was found that the elution with acetonitrile with 0.1% formic acid (B) and water with 0.1% formic acid (A) yielded the best peak shape and baseline resolution. Meanwhile, formic acid can not only improve the chromatographic separation, but also enhance the abundance of $[M-H + HCOOH]^-$ in the negative mode, such as compounds 2 and 3. On chromatographic columns, the best resolution of the isomers (compounds 2/3, 6/8, 16/17) was achieved using an Agilent Poroshell 120 EC-C_{18} column (2.1 × 100 mm, 1.9 μm) in the adequate elution gradient, with symmetric peaks and better separation.

3.2. Optimization of MS parameters

At first, the twenty analytes were characterized according to their mass spectra, which were obtained from syringe pump infusion analysis of the reference compounds, to ascertain their precursor ions and product ions in Q1 (MS1) and product ion (MS2) mode. The mass spectra showed that compounds 4, 15 and 18–20 only responded in positive mode, whereas compounds 1, 5, 7, 9, 13, 14, 16 and 17 only responded in negative mode. The ionization of the other seven compounds 2, 3, 6, 8 and 10–12 was more abundant in negative mode than in positive mode. So individual positive and negative mode runs were carried out. Furthermore, full-scan and collision-induced dissociation tests were operated to optimize the appropriate MRM method. In the full-scan mass spectra, most analytes formed predominant deprotonated molecular ions $[M-H]^-$ and protonated molecules ions $[M + H]^+$, except that the most abundant ions of albiflorin and paeoniflorin were $[M-H + HCOOH]^-$. Therefore, these ions were selected as the precursor ions for MS/MS fragmentation analysis. In tandem mass analysis, only the precursor ions of known mass were filtered by Q1 quadrupole and then fragmented in the middle (Q2) quadrupole which was employed as a collision cell. Subsequent fragments were passed through to Q3 where they may be filtered or fully scanned. At last, the most suitable DP, CE and CXP values were optimized to obtain the maximum sensitivity of the detected ion pairs. The optimum conditions are shown in Table 1 and the Extract Ion Chromatograms (XICs) of 20 channels in sample 4 are displayed in Fig. 1.

3.3. Selection of microfiltration membranes

It is a standard practice in laboratory analysis to use 0.22 μm polymeric membranes to filter samples prior to UHPLC chromatographic analysis, among which, Nylon 66 (also named polyamide 66, PA 66) and PTFE membranes are commonly used. As an aliphatic polyamide, the structure of PA 66 was characterized by cross-linked amide groups (–CO–NH–) separated by methylene sequences ($-CH_2-$) [29]. Most of the amide groups in PA 66 were associated by hydrogen bonds which were formed by the carbonyl oxygen atom binding to an amine proton on an adjacent amide group. When interacting with foreign molecules with strong proton donor moieties, the existing hydrogen bonds in PA could undergo changes to form preferential hydrogen bonds with the molecules in contact [30]. Whereas the structure of PTFE was formed by cross-linked tetrafluoroethylene (–CF2=CF2–), the construction of hydrogen bonds with strong proton donor moieties was likely to be very limited.

In this study, four representative compounds 2, 7, 9, and 13 mixed in a solution (1.0 μg/mL) were selected to investigate the hydrogen bond driven sorption in microfiltration membranes. Supplementary Fig. S2 showed the XICs of two sample solutions separately filtered with Nylon and PTFE membranes. It was clear that compared with compounds 2 and 7, compounds 9 and 13 displayed significant removal after filtered by Nylon membranes, indicating the sorption of compound 9 and 13 in Nylon membranes. On the contrary, there was marginal removal of all the four compounds in PTFE membranes. This phenomenon may be explained by the strong binding affinity originated from the hydrogen bonds between polyamide amide groups and phenol or carboxyl groups in compound 9 and 13. Thus, we finally chose PTFE membranes as the syringe filters, rather than Nylon membranes. And it was notable that Nylon membranes should be avoided when applied to compounds possessing multi hydroxyl groups.

3.4. Method validation

3.4.1. Linearity, LOQs and LODs

The calibration curves with the R^2, linear range and regression equation, LOD and LOQ of 20 targeted components are listed in Table 1. All the calibration curves indicated satisfactory linearity with correlation coefficients (R^2) from 0.9980 to 0.9999. The LOD and LOQ for the 20 analytes were in the range of 0.25–289.83 and 0.74–869.50 ng/mL, respectively, indicating that the developed method exhibited high sensitivity.

3.4.2. Precision, repeatability, stability and recovery

As shown in Table 2, results of precision, repeatability, stability and recovery test are listed. The RSD values for intra- and inter-day precisions ranged from 0.4% to 4.8% and 1.4% to 4.7%, respectively, indicating acceptable precision of the quantitative method. Repeatability with RSD less than 5.1% suggested that the developed method was repeatable enough for the quantitative evaluation of the analytes in WJBFP. And RSD value of the stability test varied from 1.5% to 4.8% in 24 h at room temperature. It was proved that all the analytes in the sample solution exhibited good stability. As listed in Table 2,

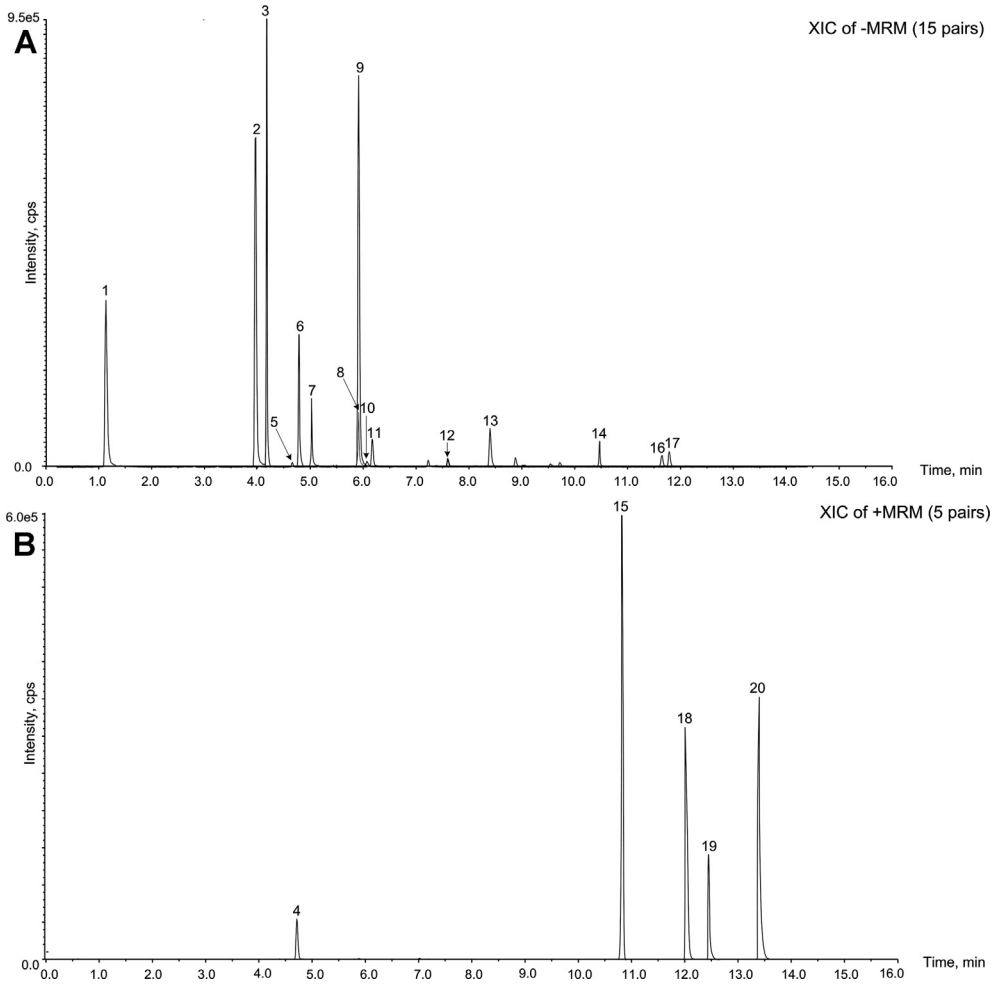

Fig. 1 – The XICs of the 20 analytes in sample 4 by UHPLC–MS/MS, 15 pairs in negative ion (A) and 5 pairs in positive method (B), respectively.

Table 2 – Precision, stability, repeatability and recovery for 20 compounds in WJBFP.

Compounds	Precision (RSD%, $n = 6$)		Stability (RSD%, $n = 6$)	Repeatability (RSD%, $n = 6$)	Recovery ($n = 6$)				
	Intra-day	Inter-day			Original (μg)	Spiked (μg)	Detected (μg)	Recovery (%)	RSD (%)
1 GA	2.0	3.8	4.4	3.6	585.5	530.0	1113.7	99.7	1.8
2 AF	3.7	3.9	2.7	3.1	101.6	100.0	200.3	98.8	4.1
3 PF	0.4	4.7	3.7	3.7	456.2	370.0	832.3	101.6	1.9
4 CG	2.7	2.5	3.2	4.7	2.2	1.25	3.5	101.5	3.9
5 FA	3.4	3.4	3.5	4.7	3.5	3.7	7.1	99.6	1.0
6 LQ	2.4	2.9	3.7	3.1	34.7	37.5	73.1	102.4	3.3
7 AT	2.4	3.3	2.5	3.8	37.9	40.0	77.2	98.1	2.5
8 ILQ	3.1	2.4	1.5	3.9	13.6	11.0	24.6	99.4	4.8
9 SAB	3.2	3.8	4.5	3.1	585.5	530.0	1113.7	99.7	1.8
10 Re	3.8	3.6	3.6	5.1	50.9	44.0	95.0	100.1	4.4
11 Rg_1	3.0	4.6	2.9	2.7	18.1	18.0	35.8	98.5	3.0
12 Rb_1	4.8	2.2	4.5	4.7	84.9	96.0	180.5	99.6	1.5
13 GCA	2.4	4.2	4.8	1.2	101.6	100.0	200.3	98.8	4.1
14 Rg_3	3.4	3.7	4.0	3.1	49.0	45.0	94.4	100.9	4.0
15 ZLG	2.7	2.6	3.9	4.2	66.2	65.0	131.5	100.4	1.1
16 Rg_5	4.5	1.4	4.0	4.3	605.1	570.0	1176.1	100.2	3.0
17 Rk_1	4.1	4.4	4.3	2.7	28.4	25.0	53.6	101.0	2.7
18 CTS	1.7	2.5	1.7	2.3	8.1	6.5	14.6	101.1	0.5
19 CP	2.2	3.3	3.6	3.1	1.5	1.4	2.9	99.3	0.7
20 $TSII_A$	1.5	1.9	1.9	2.6	7.5	7.0	14.5	100.4	2.6

the average recoveries of 20 standards were in the range of 98.1–102.4%, with RSD values from 0.5% to 4.8%. The results revealed that the method showed good reliability and accuracy.

3.5. *Sample analysis*

The validated UHPLC–MS/MS method was subsequently applied to determine 20 representative constituents in 19 batches of commercial WJBFP products. The quantitative results are presented in Supplementary Table S2. It was noticeable that the content of each constituent differed greatly among three dosage forms, water-honeyed pills, big honeyed pills and tablets, and among the batches made by different manufacturers.

PF that is a marker compound in the chemical assay for WJBFP according to Chinese Pharmacopeia [2], and AF with analgesia, spasmolysis, anti-inflammation and anti-coagulation activities [6,31], were detected to be the most abundant constituents in WJBFP (0.32–2.1 mg g^{-1} for PF, 0.21–1.8 mg g^{-1} for AF). All the products fulfilled the standards of PF content in Chinese Pharmacopeia, i.e., 0.35 mg g^{-1} for water-honeyed pills, 0.22 mg g^{-1} for big honeyed pills, and 1.4 mg g^{-1} for tablets. There was a relatively higher abundance of SAB (0.086–1.5 mg g^{-1}), Rg_5 (trace–1.1 mg g^{-1}) and Rb_1 (trace–0.76 mg g^{-1}) in the 19 batches of WJBFP. However, CP, an essential oil, was detected at a low amount (0.0013–0.0091 mg g^{-1}), which is easily volatilize during the manufacturing process.

According to Supplementary Table S2, the contents of the marker compounds in samples S1, S10 and S14, three of which were made in the same dosage form, but from different manufactures, were various due to the discrepancy in raw materials and processing procedures. On the other hand, samples S1 and S2, S4 and S5, and S6 and S7 from the same manufactures had a similar ratio in the content of the components, due to using the same intermediate. In addition, majority of marker compounds in the sample S15 in tablet dosage form displayed relatively high content because the preparation of a tablet is the process of the extraction and concentration of multiple phytochemicals from the formulated herbal materials. Moreover, it was interesting to find that Rg_1, Re and Rb_1 were not detectable in samples S1, S2, S16, and S17, instead, Rg_3, Rk_1, and Rg_5 were relatively abundant. The opposite result was shown in samples S11 and S14. It was elucidated in the previous reports that such chemical discrimination could be attributed to transformation of ginsenosides during process of high temperature treatment [32,33].

Our results indicated that the contents of the 20 analytes differed significantly among different commercial WJBFP products, which might lead to variances in the pharmacologic actions, even their therapeutic effects. Thus, determination of multiple components is essential for the quality evaluation of WJBFP products. The observed variations in the content of the target compounds might depend on the raw materials, dosage forms as well as processing procedures. Therefore, Good Manufacturing Practice guidelines and quality criterion for commercial products of WJBFP should be standardized to ensure the stability, safety and efficacy for clinical use.

3.6. *Principal components analysis*

To discriminate the samples from various manufactures or different dosage forms, the principal components analysis (PCA) was carried out by using the contents of 20 analytes as input data. The 3D matrix was composed of 19 observations and 20 variables, which indicated samples and the various markers measured by UHPLC–MS/MS, respectively. Based on eigenvalues higher than 1, the total variance explained of PC1 and PC2 accounted for 69.5% accumulation contribution rate.

The PCA score plot (Fig. 2A) showed the 19 batches of WJBFP samples were divided into three groups, Group 1 consisted of samples S1–S14 in the dosage of water-honeyed pills, with Group 2, namely sample S15 in that of tablets and Group 3 consisting of samples S16–S19 in that of big honeyed pills. The PCA analysis results indicated that the 20 components could be used as markers for evaluating the WJBFP samples of different dosage forms.

In general, 2D loading plots (e.g. the first principal component (PC1)/the second principal component (PC2) loading plot) provide useful information to identify important features in the first and second PC dimensions. In Fig. 2B, it was clear that the PF, AF, SAB, Rg_5, and Re located at the two ends of "S", demonstrating the greater correlativity with PC1 and PC2, representatively. Accordingly, the five compounds with various bioactivities such as analgesia [31], spasmolysis

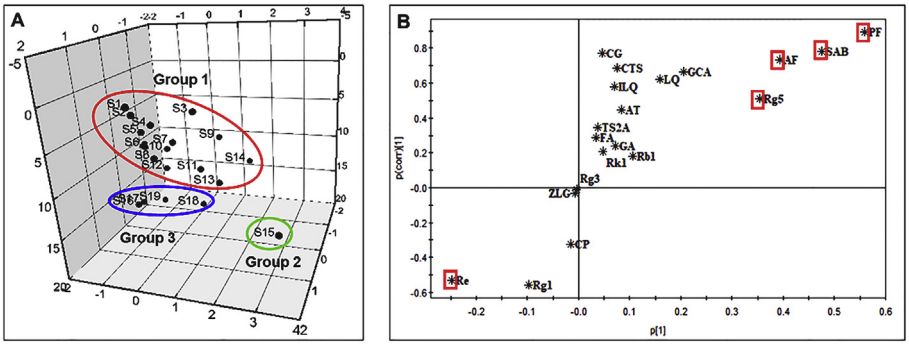

Fig. 2 – Scores (A) and loadings (B) plots of PCA. (A) refer to the sample number, S1~S19 represent 19 batches of WJBFP. The 20 compounds are shown in (B).

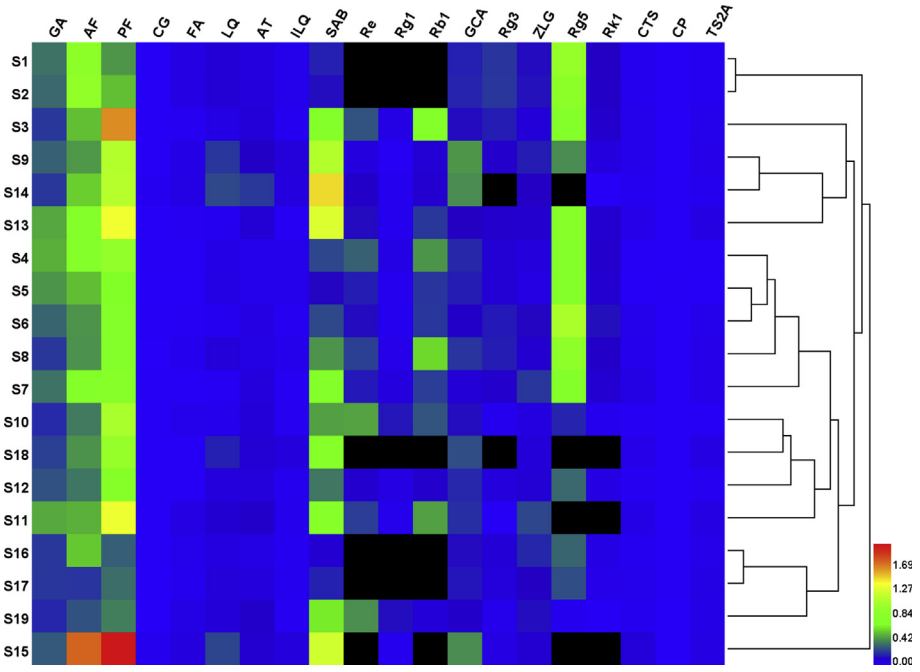

Fig. 3 – Dendrogram and heatmap of HCA of 19 WJBFP samples, S1~S19 represent 19 batches of WJBFP. Red color corresponds to higher concentration, while blue color corresponds to lower concentration. And the black color represents trace concentration.

[6], regulation of vascular [34] and vasoprotective effects [35] might be the potential chemical markers for the quality evaluation of WJBFP products. Therefore, besides PF according to China Pharmacopeia [2], AF, SAB, Rg_5, and Re also should be taken into consideration for chemical markers for the quality of WJBPP commercial products.

3.7. *Hierarchical cluster analysis*

Using the relative the contents of 20 constituents as input data matrix with a method called "average linkage between groups" of HCA, a dendrogram is constructed to reveal the relationships among the samples as shown in Fig. 3. In general, it was evident that 19 WJBFP samples were clearly clustered into two main groups as follows: S1–S14 and S16–S17 in cluster 1, and S15 in cluster 2, which indicated that tablets (S15) could be properly separated from honeyed pills using this method. Correspondingly, Group 2 (S15) was far away from the other two groups in the PCA score plot. Additionally, samples S4, S5, S6, S8 and S7 could better cluster with each other in HCA dendrogram, consistent with the samples S4, S5, S6, S8 and S7 which clustering tightly in the left part of Group 1.

On the other hand, the variation trend of content of compounds in each group can be seen intuitively from the HCA heatmap. It was noticed that S1 and S2, S5 and S6, S16 and S17 displayed the similar color row in the heatmap, as well as smallest distance between their subgroups in the dendrogram, demonstrating how insignificant the differences were. Furthermore, the colors varied dramatically among the "AF" column, "PF" column, "SAB" column, "Rg5" column, respectively. This may mean that the content of these fours changed greatly among different batches of products, which is consist with the chemical markers found in PCA loading plots.

4. Conclusions

In this study, a selective and rapid UHPLC–MS/MS method was established to evaluate the quality of commercial WJBFP products. The method was fully validated and used to simultaneously determine 20 compounds in WJBFP products in 16 min. The results revealed significant chemical variation in the contents of maker compounds among 19 batches of WJBFP. Furthermore, the results of multivariate statistical analysis illustrated that samples from different dosage forms could be classified, and PF, AF, SAB, Rg_5, and Re were highlighted as potential chemical markers for quality control of WJBFP. This study is helpful in establishing a scientific and rational method for chemical standardization of commercial WJBFP products.

Conflicts of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary material

Supplementary data related to this article can be found online at https://doi.org/10.1016/j.jfda.2018.10.004.

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