



Chemical Analysis and Multi-Component Determination in Chinese Medicine Preparation Bupi Yishen Formula Using Ultra-High Performance Liquid Chromatography With Linear Ion Trap-Orbitrap Mass Spectrometry and Triple-Quadrupole Tandem Mass Spectrometry

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Bupi Yishen Formula (BYF), a Chinese medicine preparation, has been clinically applied for the recovery of chronic kidney disease and for delaying its progress. Nevertheless, the chemical components in BYF have yet to be fully clarified. Ultra-high performance liquid chromatography with linear ion trap-Orbitrap mass spectrometry (UHPLC-LTQ-Orbitrap-MSⁿ) and triple-quadrupole tandem mass spectrometry (UHPLC-TQ-MS/MS) methods were developed for qualitative chemical profiling and multi-components quantitative analysis in BYF. The chromatographic separation was performed on a Phenomenex Kinetex C₁₈ column (2.1 × 100 mm i.d., 1.7 μm) using gradient elution of water (A) and acetonitrile (B) both containing 0.1% formic acid. Eighty-six compounds, including flavones, saponins, phenolic acids, and other compounds were authenticated or temporarily deduced according to their retention behaviors, mass mensuration, and characteristic fragment ions with those elucidated reference substances or literatures. Among the herbal medicinal materials of the formula, Astragali Radix, Codonopsis Radix, Salviae Miltiorrhizae Radix Rhizoma, and Polygoni Multiflori Radix Praeparata contributed to the bulk of the dissolved metabolites of the formula extraction. In addition, seven analytes were simultaneously determined by UHPLC-TQ-MS/MS, which was validated and has managed to determine major components in BYF. The study indicated that the established qualitative and quantitative methods would be potent and dependable analytical tools for characterizing multi-constituent in complex prescriptions decoction and provided a basis for the evaluation of bioactive components in BYF.

Keywords: Chinese medicine preparation, chemical analysis, multi-component determination, linear ion trap-orbitrap, quality control

INTRODUCTION

Chinese herbal medicine is the main form of clinical prevention and treatment of Traditional Chinese Medicine (TCM), the composition of which is composed of many different ingredients, and the organic combination of these different ingredients is different from adding individual ingredients simply. The material basis of Chinese herbal medicine is to coordinate and interact with each other so as to achieve the integrate function. Different from western medicine research, studies on Chinese herbal compound emphasize the integrity of the complex prescription, which should not split off from intrinsic characteristics of TCM and pursuit monomer compound (Wang et al., 2005). The material base of single herb or prescription is active substance groups. These groups of active substances are compatibly combined according to certain requirements, which act on multiple targets and thus has pleiotropic effects by multiple pathways (Xiong et al., 2015). Therefore, it is imperative to use modern advanced techniques to intrinsically explain the material basis of Chinese herbal medicine and to elaborate the connotation of compatibility and its curative effect.

Bupi Yishen Formula (BYF) is a non-herbal combination preparation of TCM which possesses the basic characterization of formula compatibility of TCM. BYF is prepared from the extract mixture of nine herbs, namely Astragali Radix, Codonopsis Radix, Atractylodis Macrocephalae Rhizoma, Poria, Dioscoreae Rhizoma, Polygoni Multiflori Radix Praeparata, Cuscutae Semen, Coicis Semen, and Salviae Miltiorrhizae Radix Rhizoma (Liu et al., 2012). The clinical application of BYF is treating and delaying the progression of chronic kidney disease, including postponing chronic renal failure symptoms, deferring early and mid-renal dysfunction, delaying entering the dialysis time, and protection of residual renal function (Mao et al., 2015). Modern pharmacological studies revealed that the decoction could effectively delay glomerular filtration rate (GFR) of patients on the fourth stage of chronic kidney disease. Unambiguously, detecting and identifying the major components in BYF is a prerequisite and the hinge to disclose the active constituents and how they produce the effectiveness.

In recent years, reports on global characterizations of complicated ingredients in TCM prescriptions continues to grow steadily due to the recently rapid development of multifarious hyphenated and hybrid mass spectrometry (MS). Analytical methods have exhibited good performance in analysis of unknown targets from TCM prescriptions, containing LC-ESI/MS (Dou et al., 2009; Shaw et al., 2012), LC-TOF/MS (Sun et al., 2013), LC/MS-IT-TOF, etc. (Hao et al., 2008; Liu et al., 2016).

Ultra-high performance liquid chromatography (UHPLC) has been utilized in many bioanalytical fields in recent years due to its rapid analysis and excellent separation (Simons et al., 2009; Ha et al., 2013). Equipped with a relatively short column with a low flow rate, UHPLC usually cost a remarkably shorter analysis time to achieve the same separation efficiency as HPLC. The hybrid LTQ-Orbitrap analytical platform, being composed of an ion trap coupled with an Orbitrap mass analyzer, enables two

scan types obtained at the same time. The Orbitrap provides relatively higher mass accuracy (<3 ppm) and mass resolution than a number of other mass spectrometers, which is available for determining exact molecular formulas (Dunn et al., 2008; Tchoumtchoua et al., 2013). Moreover, multi-stage MSⁿ mass spectra can be detected using ion trap by data-dependent scan and also minimize total analysis time, owing to its trigger for fragment spectra of target ions, and avoiding duplication by dynamic exclusion settings (Qiu et al., 2013). Thus, the LTQ-Orbitrap platform provides elemental compositions as well as multiple-stage mass data, which allow fast, sensitive, and reliable detecting, thus facilitating the identification of unknown compounds. Constituents of BYF could be structurally classified based on similar carbon skeletons, which should share a similar fragmentation pathway of each type and hence generate common characteristic product ions. Thus, mass spectra analysis for structural identifications could be facilitated by proposed strategies. In our previous study, the combination of UHPLC and LTQ-Orbitrap-MSⁿ has been successfully used in analyzing multiple components in single herbal extracts (Xu et al., 2014; Wang et al., 2015; Zhang et al., 2015). In this study, we attempt to exploit it to detect and identify the TCM prescription, which contain hundreds of different chemical constituents.

The present work attempted to establish an expeditious UHPLC-LTQ-Orbitrap-MSⁿ applicable approach for rapid separation and reliable identification of major constituents in BYF extract. Several strategies were used during the process, such as diagnostic fragment ions screening and fragment monitoring. In the decoction, eighty-six components altogether were identified or tentatively identified according to retention time and MS spectra data. Besides, a quantitative analysis approach has been constructed by Ultra-high performance liquid chromatography with triple quadrupole mass spectrometry (UHPLC-TQ-MS/MS). Seven representative compounds of relatively high contents unequivocally identified, were selected as marker components to evaluate the quality of BYF. The UHPLC-LTQ-Orbitrap MSⁿ, and UHPLC-TQ-MS/MS platforms were proved as potent tools for both rapid qualitative and quantitative detection and analysis of complicated constituents from natural resources and the study facilitated the comprehensive quality control of BYF.

EXPERIMENTAL

Chemicals, Reagents, and Materials

Chemical references including calycosin-7-O-β-D-glucopyranoside, calycosin, formononetin, astragaloside, salvianolic acid B, (*E*)-2,3,5,4'-Tetrahydroxystilbene-2-O-glucopyranoside ((*E*)-THSG) Astragaloside I, Astragaloside II, Astragaloside III, Astragaloside IV, soyasaponin I, lobetyolin, emodin were bought from Must (Chengdu, China). Rosmarinic acid, lithospermic acid, formononetin-7-O-glucopyranoside were from Yuanye (Shanghai, China). Salvianolic acid A was purchased from Feiyu (Jiangsu, China). Isomucronulatol-7-O-β-D-glucopyranoside were isolated from *Astragalus membranaceus* and provided by Prof. Zhu Dayuan from Shanghai Institute of Materia Medica. (*S*)-THSG,

emodin-8-O- β -D-glucoside and physcion-8-O- β -D-glucoside were isolated from *Polygonum multiflorum* in our lab. The purity of each standard was determined by HPLC ($\geq 95\%$) and their structures were confirmed by MS, $^1\text{H-NMR}$, and $^{13}\text{C-NMR}$. All references were deliquated with methanol for at a concentration of 50.0 $\mu\text{g/mL}$.

HPLC-grade Acetonitrile, methanol, and formic acid were from Sigma Aldrich (MO, USA). Ultra-pure water was prepared by a Milli-Q water system (Millipore, MA, USA). Other reagents and chemicals were of analytical grade.

Astragali Radix (No. 11050419, Neimenggu), Codonopsis Radix (No. 110653271, Gansu), Atractylodis Macrocephalae Rhizoma (No. 110600711, zhejiang), Poria (No. 110506341, Hunan), Dioscoreae Rhizoma (No. 121001014, Henan), Polygoni Multiflori Radix Praeparata (No. 110400831, Henan), Cuscutae Semen (No. 110502581, Shandong), Coicis Semen (No. 110600371, Guizhou), and Salviae Miltiorrhizae Radix Rhizoma (No. 110601741, Anhui) were from Kangmei(Guangdong, China). They were authenticated by Dr. Huang Zhihai and the specimens were preserved in Guangdong Provincial Hospital of TCM. Two batches of BYF concentrated granule was produced in the pilot-scale by Peili Pharmaceutical Co., Ltd. (NanNing, China).

Preparation of Calibration Standard Solutions

Standard of seven compounds was accurately weighed and dissolved in methanol separately to prepare the stock solution of each. A mixed stock solution was obtained, containing seven stock solutions, giving a concentration of 15.30 $\mu\text{g/mL}$ for calycosin-7-O-Glc, 6.45 $\mu\text{g/mL}$ for calycosin, 644.80 $\mu\text{g/mL}$ for (E)-THSG, 6.03 $\mu\text{g/mL}$ for astragalin, 15.60 $\mu\text{g/mL}$ for rosmarinic acid, 8.10 $\mu\text{g/mL}$ for salvianolic acid A, 0.812 $\mu\text{g/mL}$ for salvianolic acid B, respectively. Daidzein (50.0 $\mu\text{g/mL}$) was also prepared with methanol to obtain the internal standard (IS) stock solution. To construct calibration curves, the mixed stock solution was continuously diluted for series concentrations at 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128 of the original one. In a 2 mL volumetric flask, 0.2 mL of each concentration solution above, as well as 100 μL IS solutions were added, and all concentrations were finally diluted to 2 mL with 18% aqueous methanol. The acquired solutions were conserved at 4°C in refrigerator until use. All the solutions were filtered through 0.22 μm membranes before analysis.

Sample Preparation

(i) Extraction of crude drugs: A total 125 g dry pieces of nine medicinal materials were mixed by prescription ratio and extracted with boiling water (1:10) for three times (45, 30, and 30 min, respectively), filtered through gauze. Then three filtrates were combined and vacuum evaporated to recover the solvent at 56°C, and then BYF extract could be obtained. Extract was transferred into 250 mL volumetric flask, then adjusted to desired level with 10% methanol solution (final crude drug concentration was 0.5 g/mL). Solid-phase extraction (SPE) with C-18 column (ProElut, 200 mg, 3 mL column volume) was used for the pretreatment procedure, which had been conditioned with

methanol (2 mL) and water (2 mL). After 1.0 mL of BYF extract was loaded, the column was washed by 10% methanol (2 mL), and eluted with 1.0 mL 100% methanol slowly. The dry pieces of each herb were disposed through the same procedure, thus individual decoction was obtained. All the sample solutions were passed through 0.22 μm membranes prior to analysis.

(ii) Pretreatment of decoction for quantitative study: The lyophilized powder of BYF decoction was produced by freeze-drying. 11.20 g lyophilized powder was acquired from 100 mL of BYF decoction. Five hundred and sixty milligrams of BYF was filtered, a 0.1 mL portion of which was added with 10 μL of the IS solution, and then was diluted with methanol to 5 mL. The sample solutions were passed through 0.22 μm membranes.

(iii) Pretreatment of BYF concentrated granule: Concentrated granule (2.0 g) was accurately weighed and precisely dissolved in 100 mL of 80% aqueous methanol, and then refluxed for 1 h. The extract was cooled down to room temperature, weighed and made up a deficiency by 80% methanol, which was then treated in the same way as (ii).

UHPLC-LTQ-Orbitrap-MSⁿ Conditions

Chromatographic separation was conducted by a Thermo Accela UHPLC system (San Joes, USA) comprising an autosampler, a quaternary pump, a diode-array detector (DAD), and a column compartment settled to room temperature. A Phenomenex Kinetex C₁₈ column (2.1 \times 100 mm i.d., 1.7 μm) was utilized for sample separating. The mobile phase was mixture of water (A) and acetonitrile (B), both containing 0.1% formic acid. The elution gradient was set as follows: 0–12 min (10–25% B), 12–25 min (25–32% B), 15–42 min (32–56% B), 42–51 min (56–95% B). The injection volume of samples was 2 μL with a flow rate of mobile phase at 200 $\mu\text{L/min}$.

For qualitative experiments, a Thermo Fisher Scientific LTQ-Orbitrap XL hybrid mass spectrometer (Bremen, Germany) was hyphenated to the LC instrument via an electron spray ionization (ESI) interface. The samples were determined in negative mode. The ESI parameters were set (spray voltage was –3.5 KV; capillary temperature was 325°C; tube lens voltage was –76 V; Sheath gas and auxiliary gases were 45 and 6 units, respectively). The Orbitrap mass analyzer was set up the full scan mass range at m/z 120–1,200 of 30,000 resolution in centroided-type mass mode. In data-dependent MSⁿ acquisition, the most intense ions were always selected for online MS²-MS³ analysis by FT and MS⁴-MS⁵ analysis by LTQ, and dynamic exclusion detection was also conducted during the process for repetition prevention. Dynamic exclusion parameters was set as follows: Repeat count, 2; Repeat duration, 0.35 min; Exclusion duration, 1.0 min; Exclusion mass width, 3 amu. The collision energy for collision-induced dissociation (CID) was set as 30 % of maximum.

The number and types of expected atoms were fixed as follows for possible elemental composition of components: carbons ≤ 50 , hydrogens ≤ 80 , oxygens ≤ 30 , nitrogens ≤ 2 . The accuracy error threshold was set at 3 ppm. The software of Thermo Fisher Scientific Xcalibur 2.1 was applied for data analysis.

UHPLC-TQ-MS/MS Analysis

An AccelaTM UPLC system and a Thermo Scientific TSQ Quantum Ultra triple-quadrupole spectrometer (San Jose, USA) fitted with an ESI probe were employed for quantitative analysis. The separation column, column temperature, and the mobile phase were identical with those of qualitative conditions, with a gradient elution of 18–39% B at 0–5 min, 39–65% B at 5–7 min, 65–95% B at 7–9 min at 10–12 min with a flow rate at 250 μ L/min. The injection volume was set at 5 μ L.

Multiple reaction monitoring (MRM) was used for MS data acquisition and the conditions were designed as below: capillary temperature was 400°C; capillary voltage was 2.5 kV for negative mode and 3.0 kV for positive mode; sheath gas (N_2) was pressure 40 psi; auxiliary gas was 8 psi; the dwell time was 100 ms. The detection parameters of target compounds were summarized in **Table 1**. Peak areas of each analyte and IS acquired in MRM mode were employed for calibration curve establishing. Data were collected and analyzed by Thermo Xcalibur 2.1.0 Software.

Validation of Quantitative Method

The linear calibration curves were established by the analyte/IS ratio of each analyte (peak area ratio between each analyte and IS). Diluted standard solutions were successively analyzed until a signal-to-noise ratio (S/N) 3:1 and 10:1 were reached, respectively, to measure the limit of detection (LOD) and limit of quantification (LOQ) of each target compound. The intra-day precision was evaluated by detecting six times during 1 day, while the inter-day precision was assessed for 3 days in a row. Repeatability was obtained by six independent sample solutions using identical procedure in section Sample Preparation and variations were displayed by the relative standard deviation (RSD). One sample solution was tested at room temperature at different times within 24 h for stability evaluation. The recovery test was validated by adding known amounts of mixed reference solution to sample solutions at three concentration levels.

RESULTS AND DISCUSSION

Optimization of Extraction Procedure and Analysis Conditions

Variable factors during extraction procedures of BYF granule, including extraction solvent (water, 50, 80, and 100% methanol),

method (reflux and sonication), solvent volume (30, 60, and 100 mL), and time (15, 30, 45, and 60 min) were optimized so as to extract the compounds efficiently. The optimized method was finally determined to extract the BYF granule with 100 ml of 80% methanol by refluxed for 1 h.

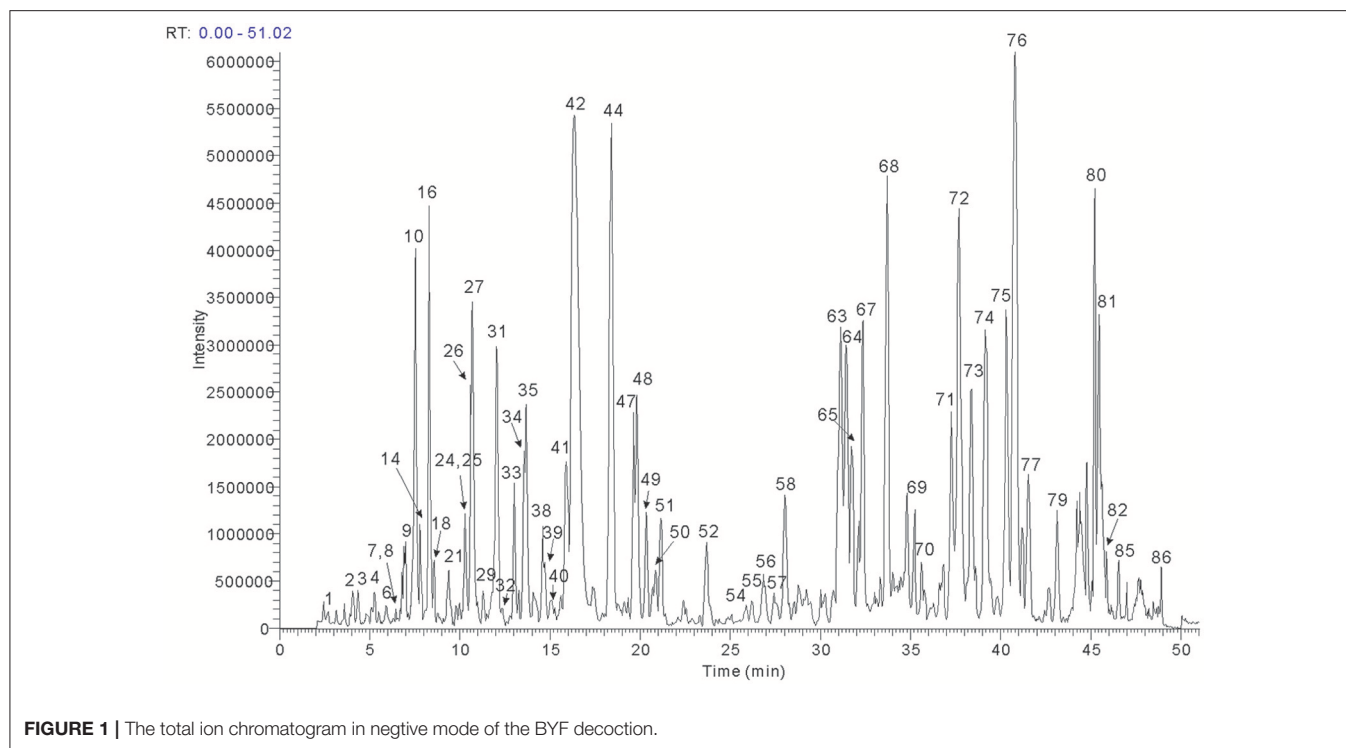
The UHPLC conditions were optimized, containing type of column, column temperature, mobile phase system, and flow rate. The Phenomenex Kinetex C_{18} column was selected based upon our previous multi-constituents analysis. Besides, different kinds of mobile phases were tested (acetonitrile and methanol with added modifiers, including formic acid, acetic acid, and ammonium acetate). A combination of acetonitrile and water both containing 0.1% (v/v) formic acid was found not only compatible to MS analysis, but also suitable for compounds separation for qualitative analysis. Comparing the TIC of the negative and positive modes, signal response was found more sensitive to the majority of components in negative mode, thus the MSⁿ data were detected in negative mode. The total ion chromatogram of BYF was acquired for structure confirmation (shown in **Figure 1**).

Characterization of Constituents in BYF Extract

BYF extract was analyzed using the optimized UHPLC-LTQ-Orbitrap-MSⁿ method. To elucidate the chemical components, known compounds were identified by comparing with the data of reference standards. Based on the MSⁿ analysis of the authentic compounds, the characteristic fragmentation behaviors of each type with the same carbon skeleton were conducted, and thus applied the obtained rules to structure characterization of their derivatives. For other unknown compounds, the structures were tentatively identified according to MSⁿ spectra and previous data in literatures. Eighty-six compounds in all were identified or tentatively identified (**Table 2**), including 15 flavones, 10 saponins, 12 phenolic acids, and other compounds. Nine herbs made markedly different chemical contributions to BYF. Specifically, the major constituents in BYF extract came from *Astragali Radix* (25 compounds), *Codonopsis Radix* (18 compounds), *Salviae Miltiorrhizae* (11 compounds), *Cuscutae Semen* (16 compounds), and *Polygoni Multiflori Radix Praeparata* (6 compounds).

TABLE 1 | Chromatographic retention time, MRM parameters, and collision energy for the seven investigated compounds.

Analytes	t_R (min)	Ionization mode	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Calycosin-7-O-Glc	2.33	ESI(+)	447.13	285.10	27
(E)-THSG	2.65	ESI(-)	405.02	243.04	21
Astragulin	3.18	ESI(-)	447.02	255.04	35
Rosmarinic acid	3.71	ESI(-)	359.99	161.03	20
Salvianolic acid A	4.73	ESI(-)	492.99	295.03	18
Salvianolic acid B	4.21	ESI(-)	717.00	321.03	33
Calycosin	4.88	ESI(-)	283.36	268.04	24
IS daidzein	4.47	ESI(+)	254.90	199.01	23



Compounds From Astragali Radix

Isoflavones and saponins, the major bioactive compounds in Astragali Radix, have various effects such as tonic, immunostimulant, cardioprotective diuretic, and hepatoprotective properties (Xu et al., 2006; Auyeung et al., 2009). In our work, 20 compounds from RA were totally characterized in BYF, including 12 isoflavones and 8 saponins. By comparing with information of reference standards, calycosin-7-O- β -D-glycoside, ononin, calycosin, formononetin, isomucronulatol-7-O- β -D-glucoside, 9,10-diMP-3-O-glucoside, 9,10-di-methoxypterocarpan-3-O- β -D-glucopyranoside, Astragaloside I, Astragaloside II, Astragaloside III, Astragaloside IV, and soyasaponin I were identified. Based on the MSⁿ analysis of these authentic compounds, the characteristic fragmentation behaviors of isoflavones and saponins were proposed in our previous study (Zhang et al., 2015), which were applied for the structure elucidation of their derivatives.

The MS² spectra of Compound 39 and Compound 55 exhibited characteristic product ions $[M-C_2H_2O]^-$ (m/z 475.1 and 429.1) and $[M-glu-C_2H_2O]^-$ (m/z 283.1 and 267.1), and their characteristic product ions yielded from the aglycone ion coincided with those of calycosin and formononetin. Based on the cleavage rules of loss of acetyl (42 Da) and acetylglucosyl (204 Da) groups, the two compounds were deduced as acetylglucoside of calycosin and formononetin.

Astragalosides from BYF decoction were mainly constituted by cycloastragenol aglycone, while aglycone ions or ions originated from the neutral loss of different glycosyl moiety in their MS² spectra. Take Compound 69 as an example, the $[M-H]^-$ ion was m/z 783.450 20 ($C_{41}H_{67}O_{14}^-$), which easily lose the

sugar units in its MS² spectra and gained typical product ions at m/z 651, 621, 489 from the loss of one xylose ($[M-132]^-$), one glucose ($[M-164 (glu)]^-$), one xylose and glucose ($[M-132 (xyl)-164 (glu)]^-$), respectively. In addition, one soyasaponin (Compound 78) of lower content from Astragali Radix was found in BYF decoction.

Compounds From Codonopsis Radix

The identified compounds of Codonopsis Radix in BYF can be classified into four main classes, namely, phenylpropanoid glycosides, acetylene glycosides, hexyl (hexenol) glycosides (Lin et al., 2013). In (-)-ESI-MS spectra of BYF decoction, apart from phenylpropanoid glycosides (including compounds 8, 12, 23, 37, and 40) existing in $[M-H]^-$ ion forms, others displayed as both $[M+HCOO]^-$ and $[M-H]^-$ ions.

By comparing the retention time values and mass data with those of the references, Compound 8 and 30 were unambiguously identified as tangshenoside I and lobetyolin, which are the representative compounds of phenylpropanoid glycosides and acetylene glycosides in Radix Codonopsis. Their MSⁿ spectra and proposed fragmentation pathways were summarized in **Figures 2A–C, 3, 4**, respectively. The $[M-H]^-$ ion and the typical ions in the MS² spectra of Compound 19 ($C_{26}H_{37}O_{13}^-$), namely, m/z 557.2 $[M-H]^-$, 467.2 $[M-C_7H_6]^-$, 341.1 $[M-C_{14}H_{17}O_2]^-$, were all 162 Da less compared to those of Compound 30, demonstrating that they have identical site cleavage. Compound 19 was therefore characterized as lobetyolinin by comparison with the literature (Kanji et al., 2003).

Compounds 37 and 40 were identified as diastereomers by the same deprotonated ions at m/z 823.265 8 ($C_{38}H_{47}O_{20}^-$) and the

TABLE 2 | Compounds detected and identified in BYF decoction.

No.	t_R /min	$[M-H]^-$ (Mass error, ppm)	Characteristic fragment ions	Molecular formula	Compounds	Resources
1	2.67	353.086 82 (-0.44)	MS ² [353]: 191.1, 179.0	C ₁₆ H ₁₈ O ₉	3-Caffeoyl-quinic acid	Cs
2	4.01	353.086 76 (-0.49)	MS ² [353]: 191.1, 179.0	C ₁₆ H ₁₈ O ₉	5-Caffeoyl-quinic acid	Cs
3	4.35	353.086 98 (-0.28)	MS ² [191]: 173.0, 127.0 MS ² [353]: 191.1, 179.0, 173.0	C ₁₆ H ₁₈ O ₉	4-Caffeoyl-quinic acid	Cs
4	5.30	469.191 35 ^a (-0.22)	MS ² [469]: 423.2	C ₁₈ H ₃₂ O ₁₁	(S)-3-Hexenyl-β-D-sophoraside	Cr
5	5.48	423.185 76 (-0.32)	MS ³ [423]: 261.1, 221.1, 179.1, 161.1			
5	5.48	350.19608 ^b (-0.12)	MS ² [350]: 250.1, 205.1, 161.1	C ₁₉ H ₂₈ NO ₅ ⁺	Codonopyrrolidium A ^c	Cr
6	5.84	469.19125 ^a (-0.32)	MS ² [469]: 423.2	C ₁₈ H ₃₂ O ₁₁	(E)-2-Hexenyl-β-D-sophoraside	Cr
7	6.44	423.185 91 (-0.60) 405.118 07 (0.06)	MS ³ [423]: 261.1, 221.1, 179.1, 161.0 MS ² [405]: 243.1	C ₂₀ H ₂₂ O ₉	(Z)-THSG	Pm
8	6.52	677.228 09 (-0.65)	MS ² [243]: 225.1, 215.1, 201.0, 173.0, 149.0, 137.0 MS ² [677]: 497.2, 453.2, 261.1, MS ³ [261]: 99.1, 161.1	C ₂₉ H ₄₂ O ₁₈	Tangshenoside I	Cr
9	6.96	471.207 40 ^a (0.18)	MS ² [471]: 425.2	C ₁₈ H ₃₄ O ₁₁	Hexyl β-sophoraside	Cr
10	7.50	425.201 57 (-0.17) 491.118 65 ^a (0.25)	MS ³ [425]: 263.1, 161.0 MS ² [417]: 283.1	C ₂₂ H ₂₂ O ₁₀	Calycosin-7-O-β-D-glup	Ar
11	7.45	595.129 33 (-0.03)	MS ³ [283]: 268.0 MS ⁴ [268]: 240.0, 224.0, 211.0, 184.0 MS ² [595]: 463.1, 301.0, 300.0	C ₂₆ H ₂₈ O ₁₆	Quercetin 3-O-(2-O-apisyl)-galactoside	Cs
12	7.70	469.133 51 (-0.54)	MS ³ [300]: 271.0, 255.0 MS ⁴ [271]: 243.0, 227.01, 199.0 MS ² [469]: 325.1, 265.1, 235.1	C ₂₁ H ₂₆ O ₁₂	Tangshenoside IV isomer	Cr
13	7.74	441.196 66 ^a (0.01) 395.191 10 (-0.21)	MS ³ [325]: 265.1, 235.1 MS ² [395]: 263.1, 161.0	C ₁₇ H ₃₂ O ₁₀	Hexyl-(pen)-glup	Cr
14	7.79	633.180 91 (-0.49)	MS ² [633]: 471.1, 307.1	C ₃₀ H ₃₄ O ₁₅	Unknown	Cs
15	7.97	521.128 72 ^a (0.25)	MS ³ [307]: 247.1, 205, 187, 163.1, 145.0 MS ² [521]: 475.1, 359.1, 313.1, 298.1, 207.0	C ₂₃ H ₂₄ O ₁₁	Odoratin-glup	Ar
16	8.32	405.118 47 (-0.09)	MS ³ [313]: 298.0, 283.0, 270.0 MS ² [405]: 243.1	C ₂₀ H ₂₂ O ₉	(E)-THSG	Pm
17	8.40	309.154 85 ^a (0.46)	MS ² [243]: 225.1, 215.1, 201.0, 173.0, 149.0, 137.0 MS ² [309]: 263.1	C ₁₂ H ₂₄ O ₆	Hexyl β-D-glup	Cr
18	8.54	463.087 52 (0.42)	MS ³ [263]: 161.1 MS ² [595]: 463.1, 301.0, 300.0	C ₂₁ H ₂₀ O ₁₂	Quercetin 3-O-galactoside (hyperoside)	Cs
19	8.88	603.227 84 ^a (-0.51)	MS ³ [301]: 271.0, 255.0, 179.0, 151.0 MS ² [603]: 557.2, 467.2, 341.1, 323.1	C ₂₆ H ₃₈ O ₁₃	Lobetyolinin	Cr
20	9.10	557.128 11 (-0.86)	MS ² [557]: 405.1, 313.1, 243.1	C ₂₇ H ₂₅ O ₁₃	2,3,5,4'-Tetrahydroxystilbene-2-(galloyl)-O-glc	Pm
21	9.32	261.133 79 (0.53)	MS ³ [313]: 169.0, 295.0 MS ² [261]: 187.1, 125.1	C ₁₂ H ₂₂ O ₆	Hexenyl-β-D-glup	Cr
22	9.40	565.191 10 ^a (-1.01) 519.185 85 (-0.79)	MS ² [519]: 357.1 MS ² [357]: 151.0, 136.0	C ₂₆ H ₃₂ O ₁₁	Unknown	

(Continued)

TABLE 2 | Continued

No.	t_R /min	$[M-H]^-$ (Mass error, ppm)	Characteristic fragment ions	Molecular formula	Compounds	Resources
23	9.72	469.133 82 (-0.23)	MS ² [469]: 407.1, 367.1, 325.1 MS ³ [325]: 163.0, 119.1	C ₂₁ H ₂₆ O ₁₂	Tangshenoside V	Cr
24	10.21	417.081 63[M-H] ⁻ (0.01)	MS ² [417]: 373.1, 175.0 MS ² [307]: 175.0	C ₂₀ H ₁₇ O ₁₀	Salvianolic acid D	Sm
25	10.30	537.102 29 (-1.9)	MS ² [537]: 493.1, 339.0, 295.1 MS ³ [339]: 321.0, 295.1, 185.0	C ₂₇ H ₂₂ O ₁₂	Salvianolic acid H or I	Sm
26	10.60	187.097 35 (0.89)	MS ² [187]: 125.1, 97.1	C ₉ H ₁₆ O ₄	Azelaic acid	Co / Cr
27	10.69	447.092 41 (-0.33)	MS ² [447]: 327.0, 285.0, 284.0 MS ³ [284]: 255.0, 227.0	C ₂₁ H ₂₀ O ₁₁	Astragalin	Cs
28	10.83	507.113 01 ^a (-0.31)	MS ² [507]: 461.1, 299.1 MS ³ [299]: 284.0 MS ⁴ [284]: 256.0, 227.0, 212.0	C ₂₂ H ₂₂ O ₁₁	Pratensein-7-O-Glu	Ar
29	11.30	477.102 60 (-1.5)	MS ² [477]: 314.0 MS ³ [314]: 285.0, 271.0, 243.0	C ₂₂ H ₂₂ O ₁₂	Unknown	/
30	11.86	441.175 29 ^a (-0.23) 395.169 65 (-0.40)	MS ² [441]: 395.1, 305.1, 215.1, 185.1, 179.1	C ₂₀ H ₂₈ O ₈	Lobetyolin	Cr
31	12.03	359.076 51 (0.36)	MS ² [359]: 223.0, 197.1, 179.0, 161.0	C ₁₈ H ₆ O ₈	Rosmarimic acid	Sm
32	12.33	537.102 48 (-0.27)	MS ² [537]: 493.1, 339.0, 295.1 MS ² [493]: 321.0, 295.1	C ₂₇ H ₂₂ O ₁₂	Salvianolic acid H or I	Sm
33	13.03	723.499 08 ^a (0.78) 677.493 90 (1.08)	MS ² [677]: 451.3 MS ² [677]: 433.4, 225.1	C ₄₈ H ₆₈ O ₅	Unknown	Cr/Am/Dr/ Ar
34	13.56	537.102 97 (0.22)	MS ² [537]: 493.1 MS ³ [493]: 295.1 MS ⁴ [295]: 277.1, 159.0, 109.0	C ₂₇ H ₂₂ O ₁₂	Lithospermic acid	Sm
35	13.69	475.123 66 ^a (0.17)	MS ² [475]: 429.1, 267.1, MS ³ [267]: 252.0, 22.9, 208.1	C ₂₂ H ₂₂ O ₉	Formononetin-7-O-Glc	Ar
36	14.43	505.133 64 ^a (-0.42)	MS ² [475]: 297.1, MS ³ [297]: 282.1, MS ⁴ [282]: 267.1, 254.1, 239.1	C ₂₃ H ₂₄ O ₁₀	6,4'-Dimethoxyisoflavone-7-O-Glc	Ar
37	14.48	823.265 87 (0.35)	MS ² [823]: 497.2, 453.2, 261.1	C ₃₈ H ₄₈ O ₂₀	6'''-Trans-p-coumaroyl-tangshenoside I	Cr
38	14.60	471.128 48 (-1.4)	MS ² [471]: 307.1 MS ³ [307]: 247.1, 187.0, 163.1, 145.0	C ₂₄ H ₂₄ O ₁₀	Unknown	Cs
39	14.74	533.128 66 ^a (-1.6)	MS ² [533]: 487.1, 445.1, 283.1 MS ³ [283]: 268.0 MS ⁴ [268]: 240.0, 224.0, 211.0	C ₂₄ H ₂₄ O ₁₁	Calycosin-7-O-glc-6''-O-acetate	Ar
40	15.13	823.265 81 (0.34)	MS ² [823]: 497.2, 453.2, 261.1	C ₃₈ H ₄₈ O ₂₀	6'''-Cis-p-coumaroyl-tangshenoside I	Cr
41	15.92	269.092 59 (-0.03)	MS ² [269]: 225.1 MS ³ [225]: 207.1, 183.1, 166.1, 156.1	C ₁₅ H ₁₄ O ₃ N ₂	Cuscutamine	Cs
42	16.38	717.144 65 (-0.36)	MS ² [717]: 519.1, 321.0 MS ³ [519]: 339.0, 321.0 MS ⁴ [321]: 293.0, 279.1, 277.1 MS ⁵ [279]: 251.1	C ₃₆ H ₃₀ O ₁₆	Salvianolic acid B	Sm
43	16.43	507.150 36 ^a (0.66)	MS ² [507]: 461.1, 299.1, 284.1	C ₂₃ H ₂₆ O ₁₀	9,10-Di-methoxypterocarpan-3-O-β-D-glucopyranoside	Ar

(Continued)

TABLE 2 | Continued

No.	t_R /min	$[M-H]^-$ (Mass error, ppm)	Characteristic fragment ions	Molecular formula	Compounds	Resources
44	18.40	283.060 73 (0.63)	MS ³ [299]: 284.1, 269.0, 241.0	C ₁₆ H ₁₂ O ₅	Calycosin	Ar
45	18.54	509.165 44 ^a (0.08)	MS ² [283]: 268.0, 240.0, 224.0, 211.0 MS ² [509]: 463.1, 445.1, 346.1, 301.1, 286.0 MS ³ [301]: 286.1, 135.0, 109.0	C ₂₃ H ₂₈ O ₁₀	Isomucronulatol-7-O-β-D-glucopyranoside	Ar
46	19.37	523.123 23 (-0.27)	MS ² [523]: 491.1, 343.1, 325.1, 293.0	–	–	–
47	19.63	493.113 16 (-0.6)	MS ² [493]: 295.1 MS ³ [295]: 277.1, 267, 185, 159.0, 109.0	C ₂₆ H ₂₂ O ₁₀	Salvianolic acid A	Sm
48	19.81	717.143 92 (-1.09)	MS ² [717]: 519.1 MS ³ [519]: 339.0, 321.0 MS ⁴ [321]: 279.1, 251.1	C ₃₆ H ₃₀ O ₁₆	Salvianolic acid E	Sm
49	20.34	551.118 35 (-1.0)	MS ² [551]: 519.1 MS ³ [519]: 353.1, 321.0	C ₂₈ H ₂₄ O ₁₂	Monomethyl lithospermat	Sm
50	20.81	431.097 08(-0.19)	MS ² [431]: 269.0 MS ³ [269]: 225.0, 241.0 MS ⁴ [225]: 181.0, 210.0	C ₂₁ H ₂₀ O ₁₀	Emodin-8-O-glc	Pm
51	21.14	493.112 70 (-0.22)	MS ² [493]: 295.1 MS ³ [295]: 277.1, 267.1, 185.1	C ₂₆ H ₂₂ O ₁₀ C ₃₆ H ₃₀ O ₁₅	Isomer of Salvianolic acid A	Sm
52	23.68	491.097 23 (-0.04)	MS ² [491]: 293.0, 311.1 MS ³ [293]: 276.0, 265.0, 249.1	C ₂₆ H ₂₀ O ₁₀	Salvianolic acid C	Sm
53	24.23	577.154 30 (-0.88)	–	C ₂₇ H ₃₀ O ₁₄	Unknown	
54	25.32	297.076 02 (0.27)	MS ² [297]: 282.1, 267.1, 254.1, 239.1	C ₁₇ H ₁₄ O ₅	7-Hydroxy-6,4'-dimethoxyisoflavone	Ar
55	26.18	517.133 67 ^a (-0.38)	MS ² [517]: 429.1, 267.1 MS ³ [267]: 252.0 MS ⁴ [252]: 223.1, 208.1	C ₂₄ H ₂₄ O ₁₀	Formononetin-7-O-β-D-glycoside-6''-O-acetate	Ar
56	26.85	285.039 64 (-0.27)	MS ² [285]: 257.0, 229.1, 213.1, 169.1, 199.1, 151.0	C ₁₅ H ₁₀ O ₆	Kaempferol or luteolin	Cs
57	27.86	299.055 33 (0.32)	MS ² [299]: 284.1, 271.1, 255.1, 240.1, 227.0	C ₁₆ H ₁₂ O ₆	Rhamnocitrin	Ar
58	28.03	327.217 10 (0.50)	MS ² [327]: 291.2, 229.1, 211.1, 171.1	C ₁₈ H ₃₂ O ₅	9,12,13-Trihydroxy-octadec-10,15-dienoic acid	Sm / Cr
59	28.28	549.159 85 ^a (-0.42)	MS ³ [229]: 211.1 MS ² [549]: 485.1, 459.1, 415.1, 299.1	C ₂₅ H ₂₈ O ₁₁	9,10-Di-methoxyptero-carpan-3-O-β-D-glucopyranoside-acetate	Ar
60	29.56	991.509 34 ^a (-1.50)	MS ³ [299]: 284.1, 269.0, 241.0 MS ² [991]: 783.5, 765.5, 489.4	C ₄₇ H ₇₈ O ₁₉	Astragaloside VI or VII	Ar
61	29.99	671.138 73 (-0.80)	MS ² [671]: 473.1 MS ³ [473]: 339.1, 321.0	C ₃₅ H ₂₉ O ₁₄	Unknown	-
62	30.51	491.118 59 ^a (0.19) 445.112 92 (-0.01)	MS ² [445]: 283.1 MS ² [283]: 268.0, 240.0 MS ² [240]: 212.0, 184.1	C ₂₂ H ₂₂ O ₁₀	Physcion-8-O-glc	Pm
63	31.13	267.065 92 (0.7)	MS ² [267]: 252.0 MS ³ [252]: 223.0, 208.0, 132.0	C ₁₆ H ₁₂ O ₄	Formononetin	Ar
64	31.42	329.232 70 (0.45)	MS ² [329]: 311.2, 293.2, 229.1, 211.1 MS ³ [171]: 153.0, 127.1, 125.1	C ₁₈ H ₃₄ O ₅	9,12,13-Trihydroxy-octadec-10-enoic acid	Cr

(Continued)

TABLE 2 | Continued

No.	t_R /min	$[M-H]^-$ (Mass error, ppm)	Characteristic fragment ions	Molecular formula	Compounds	Resources
65	31.72	329.232 64 (-0.16)	MS ² [329]: 311.2, 293.2, 229.1, 211.1 MS ³ [229]: 211.1, 209.1	C ₁₈ H ₃₄ O ₅	5,6,9-Trihydroxy-octadec-7-enoic acid	Cr / Co
66	33.03	991.508 61 ^a (-2.22)	MS ² [991]: 783.5, 765.5, 621.4, 489.4	C ₄₇ H ₇₈ O ₁₉	Astragaloside V	Ar
67	32.35	843.421 45 (-0.57)	MS ² [843]: 797.4, 779.4, 633.5	C ₃₈ H ₆₈ O ₂₀	Cuscutic acid C/Isomer	Cs
68	33.70	843.421 08 (-0.94)	MS ² [843]: 797.4, 779.4, 633.5	C ₃₈ H ₆₈ O ₂₀	Cuscutic acid C/Isomer	Cs
69	35.23	829.456 18 ^a (-1.83) 783.450 20 (-2.33)	MS ² [783]: 651.4, 621.4, 489.4	C ₄₁ H ₆₈ O ₁₄	Astragaloside IV	Ar
70	35.62	829.455 26 ^a (-2.75) 783.450 26 (-2.27)	MS ² [783]: 621.4, 489.4	C ₄₁ H ₆₈ O ₁₄	Astragaloside III	Ar
71	37.26	885.431 03 (-2.10)	MS ² [885]: 839.4, 821.4, 633.3	C ₄₀ H ₇₀ O ₂₁	Unknown	Cs
72	37.70	885.431 27 (-1.31)	MS ² [885]: 839.4, 821.4, 633.3	C ₄₀ H ₇₀ O ₂₁	Unknown	Cs
73	38.35	885.431 09 (-1.49)	MS ² [885]: 839.4, 821.4, 633.3	C ₄₀ H ₇₀ O ₂₁	Unknown	Cs
74	39.13	885.431 27 (-1.31)	MS ² [885]: 839.4, 821.4, 635.3	C ₄₀ H ₇₀ O ₂₁	Unknown	Cs
75	40.30	871.467 29 ^a (-1.29) 825.460 75 (-2.35)	MS ² [825]: 783.5, 765.4, 489.4	C ₄₃ H ₇₀ O ₁₅	Astragaloside II	Ar
76	40.78	885.431 40 (-1.18)	MS ² [885]: 839.4, 821.4, 633.5	C ₄₀ H ₇₀ O ₂₁	Acely-cuscutic acid C	Cs
77	41.54	681.367 55 (-1.6)	-	C ₃₂ H ₅₈ O ₁₅	Unknown	-
78	42.40	987.513 61 ^a (-2.31) 941.508 18 (-2.26)	MS ² [941]: 923.5, 795.5, 615.4, 457.4, 437.4	C ₄₈ H ₇₈ O ₁₈	Soyasaponin I	Ar
79	43.14	871.466 67 ^a (-1.91) 825.46057 (-2.52)	MS ² [871]: 783.5, 765.5, 717.4, 633.4, 603.4, 489.4	C ₄₃ H ₇₀ O ₁₅	Isoastragaloside II	Ar
80	45.19	913.477 66 ^a (-1.48) 867.471 07 (-2.59)	MS ² [867]: 825.5, 807.5, 765.5, 747.5, 729.5, 717.4, 705.5, 699.4, 633.4, 567.4, 489.4	C ₄₅ H ₇₂ O ₁₆	Astragaloside I	Ar
81	45.45	295.227 36 (0.59)	MS ² [295]: 277.2, 195.1, 171.1 MS ³ [277]: 233.2	C ₁₈ H ₃₂ O ₃	Coronaric acid	Co
82	45.49	913.476 5 ^a (-2.58)	MS ³ [867]: 825.5, 807.5, 765.5, 747.5, 729.5, 717.4, 705.4, 699.4, 633.4, 567.4, 489.4	C ₄₅ H ₇₂ O ₁₆	Isoastragaloside I	Ar
83	45.80	269.045 04 (0.59)	MS ² [269]: 225.0 MS ³ [225]: 181.0, 210.0	C ₁₅ H ₁₀ O ₅	Emodin	Pm
84	45.86	915.493 23 ^a (-1.56)	-	C ₄₅ H ₇₂ O ₁₆	Dioscin	Dr
85	46.55	955.487 79 (-1.92)	MS ² [955]: 891.5, 763.5, 701.4, 613.4, 523.4	C ₄₇ H ₇₄ O ₁₇	Acetylastragaloside I	Ar
86	48.91	339.231 75 (-0.3)	MS ² [339]: 163.1	C ₂₃ H ₃₂ O ₂	Unknown	-

^a[M+HCOO]⁻; ^b[M+H]⁺; ^conly detected in positive mode.

Cs, *Cuscutae Semen*; Cr, *Codonopsis Radix*; Pm, *Polygoni Multiflori Radix Praeparata*; Ar, *Astragali Radix*; Sm, *Salviae Miltiorrhizae Radix Rhizoma*; Co, *Coicis Semen*; Am, *Atractylodis Macrocephalae Rhizoma*; Dr, *Dioscoreae Rhizoma*; P, *Poria*; pen, pentoside; glup, glucopyranoside.

same productions at m/z 497.2, m/z 453.2, and m/z 261.1, and they could be differentiated by their elution order. Their log P calculated by Discovery Studio were 0.47 and 0.59. As cis-isomers with lower polarity could be eluted relatively later than trans-isomers, compounds 37 and 40 were identified as 6'''-trans- and 6'''-cis-p-coumaroyl-tangshenoside I.

Compounds 9, 13, and 17 exhibited the $[M+HCOO]^-$ precursor ion at m/z 471.207 40, 441.196 66, and 309.154 85, and their MS² and MS³ spectra all yielded ions at m/z

263.1 (C₁₂H₂₃O₆⁻) and 161.1 (C₆H₉O₅⁻) as the base peak, respectively. It is inferred that Compounds 9 and 13 were, respectively, substituted by an additional glucopyranoside and pentoside compared to Compound 17. Compounds 9, 13, and 17 were tentatively characterized as hexyl β -sophoroside, hexyl-(pen)-glucopyranoside, and hexyl β -D-glucopyranoside.

Isomers (Compounds 4 and 6) were obtained by the EIC of m/z 423. Both of Them displayed $[M-H]^-$ ion at m/z 423.185 76 (C₁₈H₃₁O₁₁⁻) and their MS² spectra all exhibited

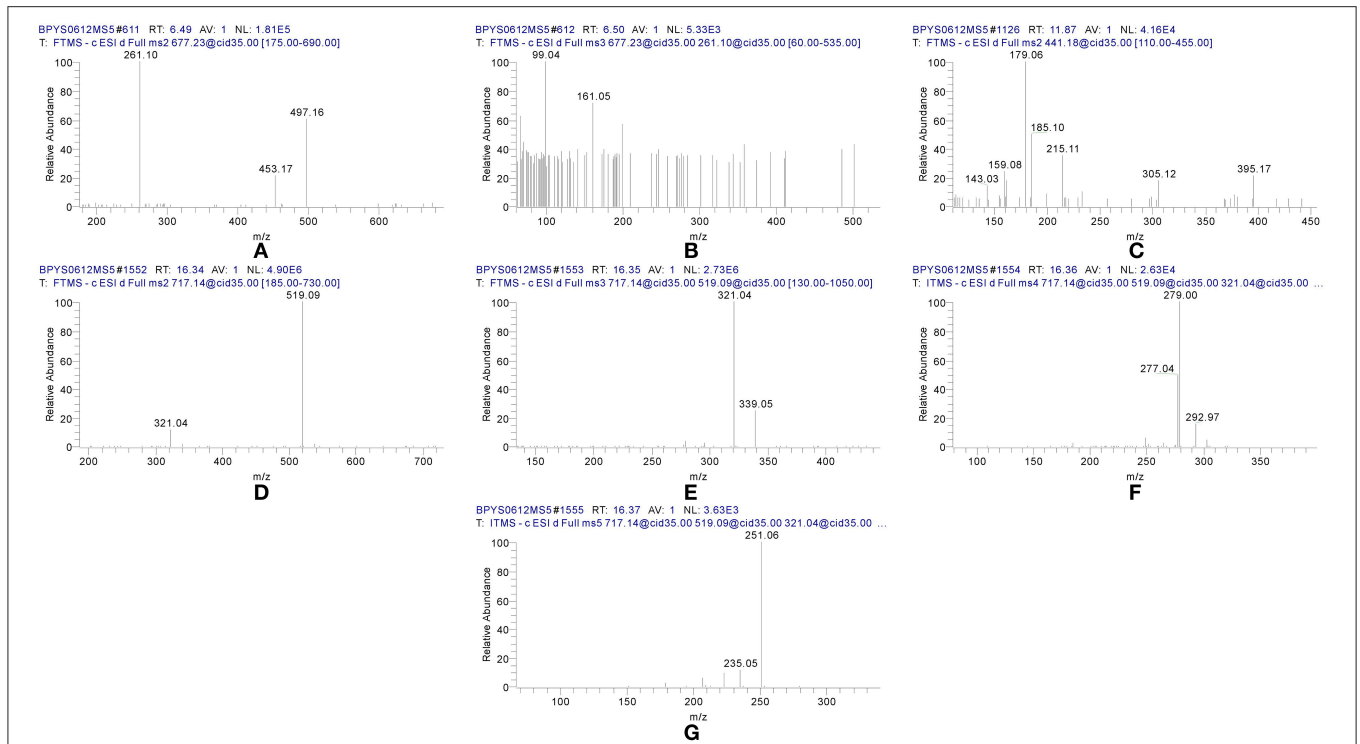


FIGURE 2 | MSⁿ spectra of tangshenoside I, lobetyolin, and salviaric acid B. (A) MS² of 667; (B) MS³ of 261; (C) MS² of 441; (D) MS² of 717; (E) MS³ of 519; (F) MS⁴ of 321; (G) MS⁵ of 279.

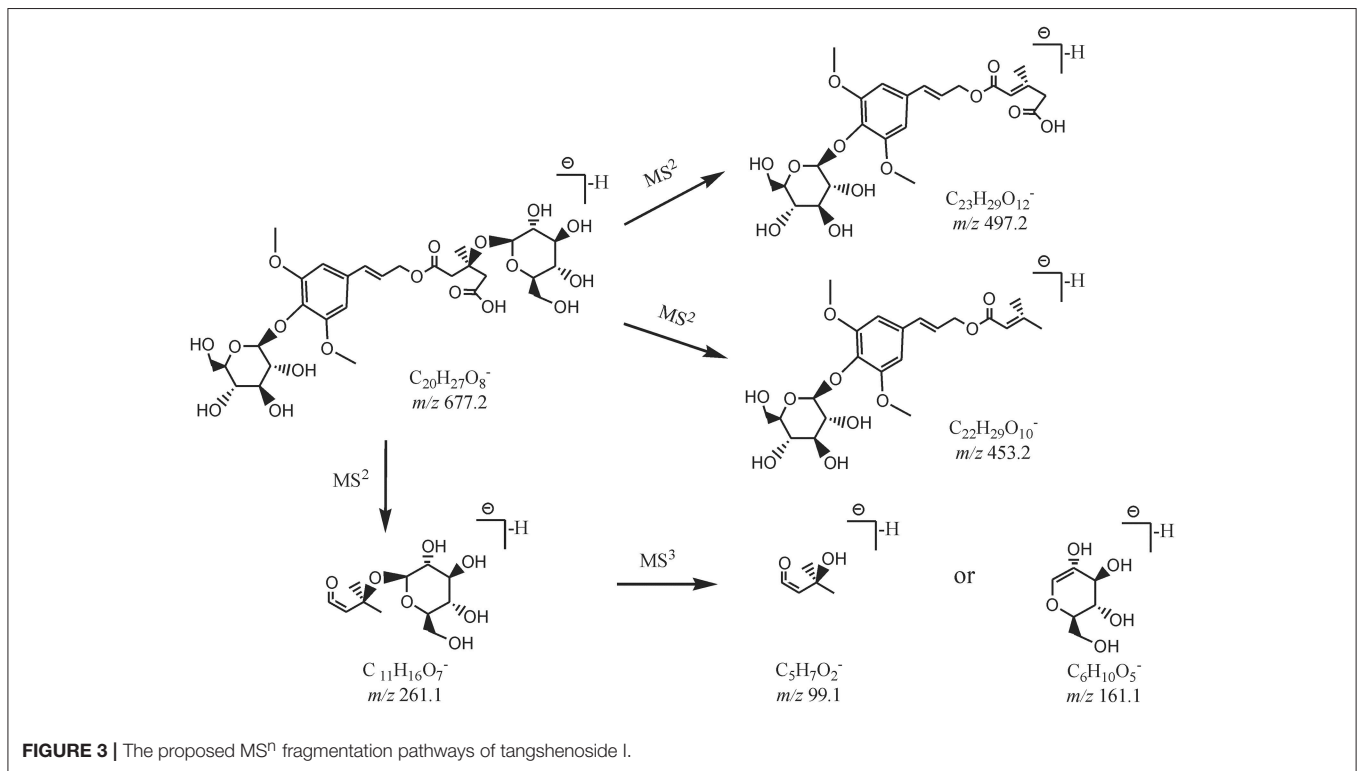
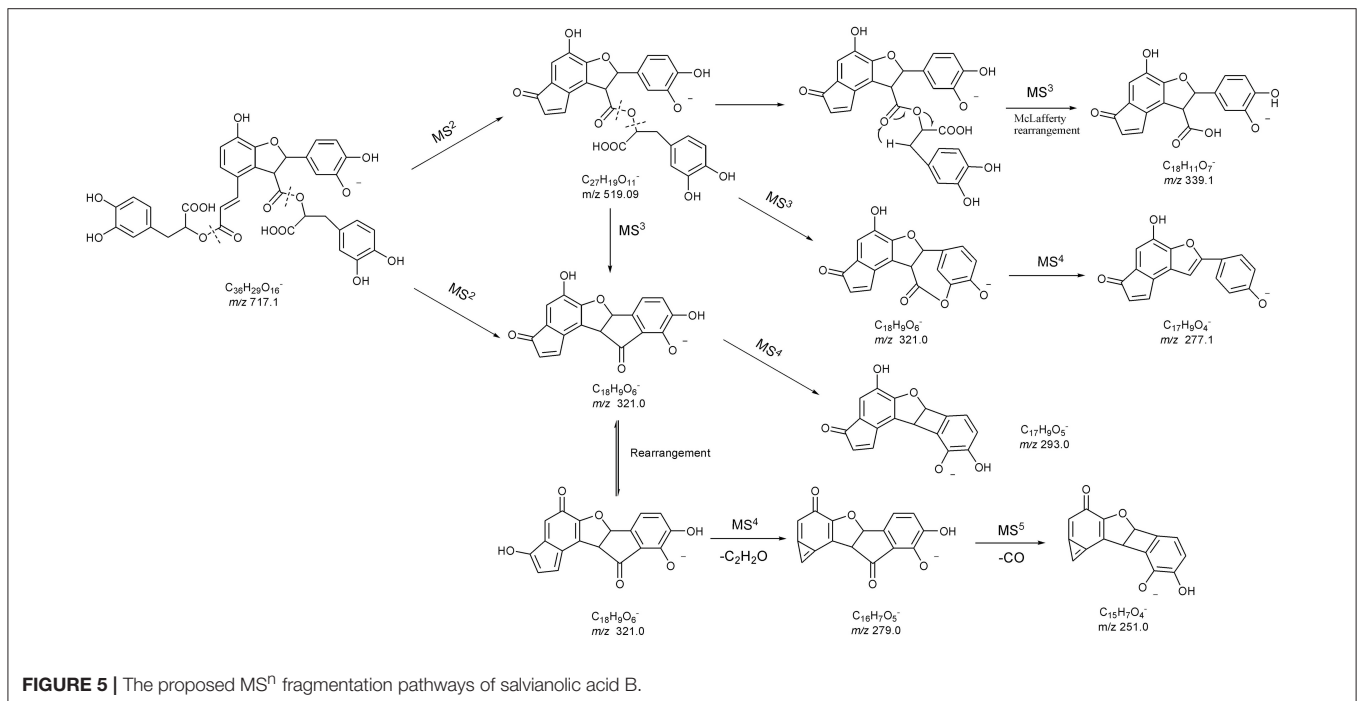
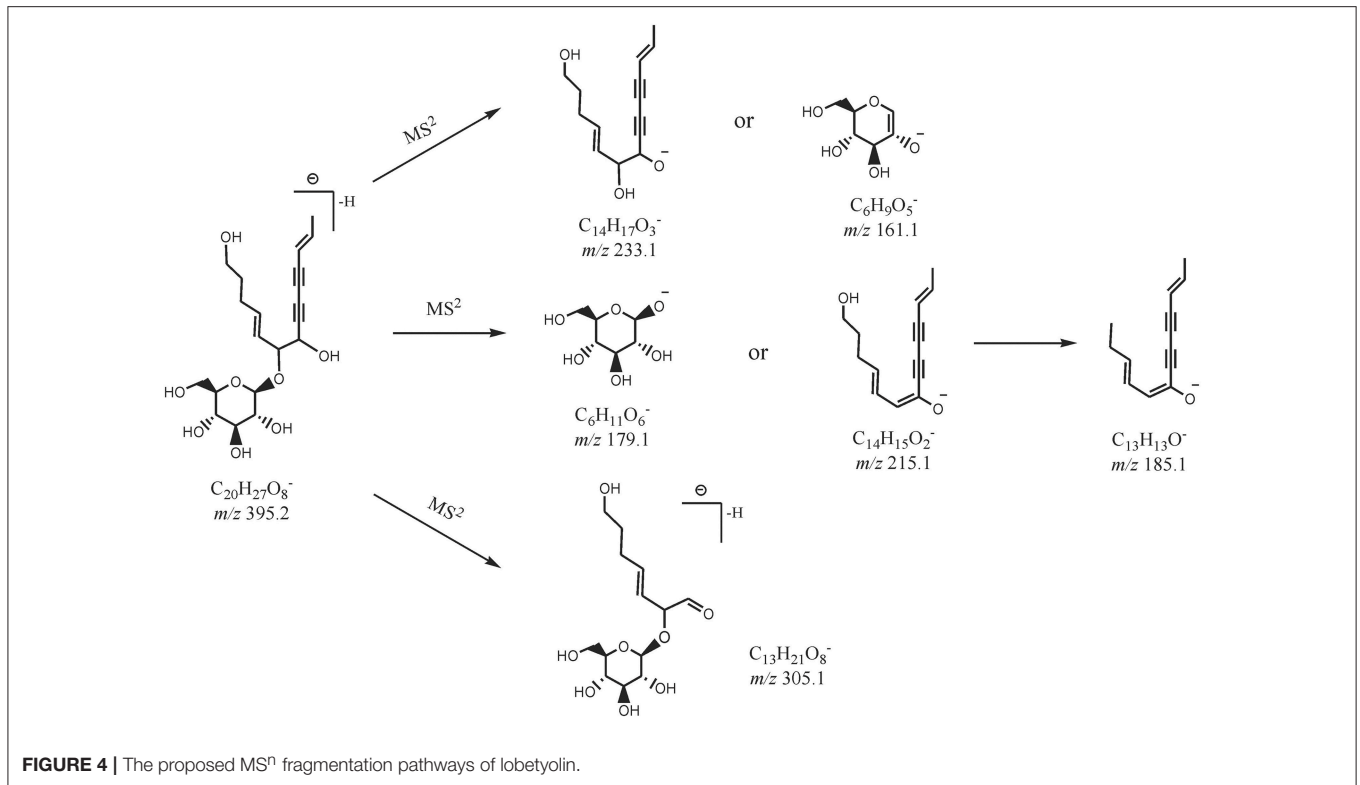


FIGURE 3 | The proposed MSⁿ fragmentation pathways of tangshenoside I.



ion at m/z 261.1 $[M-H-C_6H_{10}O_5]^-$ and 161.1 ($C_6H_9O_5^-$). Based on the fragmentation information and related literature (Tsai and Lin, 2010), they were primarily identified as (*S*)-3-hexenyl- β -D-sophoridase and (*E*)-2-hexenyl- β -D-sophoridase.

Their log P calculated by Discovery Studio were -1.9 and -2.0 and *cis*-isomers was eluted relatively later, therefore, compounds 4 and 6 were assigned as (*S*)-3-hexenyl- β -D-sophoridase and (*E*)-2-hexenyl- β -D-sophoridase.

TABLE 3 | Regression equations, linearity ranges, correlation coefficients, LOD, and LOQ data of the seven analytes.

Analytes	Regression equations	Linear range (ng/mL)	R ²	LOD ^a (ng/mL)	LOQ ^b (ng/mL)
Calycosin-7-O-Glc	Y = 0.00591514X + 0.00256763	11.48~765.0	0.9999	0.03	0.08
(E)-THSG	Y = 0.000746494X - 0.0040087	48.36~3,224.0	1.0000	0.02	0.04
Astragulin	Y = 0.000933611X - 0.00599332	4.523~301.5	0.9996	0.02	0.75
Rosmarinic acid	Y = 0.000437025X - 0.00906707	11.70~780.0	0.9994	0.98	3.91
Salvianolic acid A	Y = 0.000390993X - 0.0022037	6.075~405.0	0.9995	0.51	2.53
Salvianolic acid B	Y = 0.000286894X + 0.004386	609.0~40,600.0	0.9999	1.27	5.08
Calycosin	Y = 0.00265233X + 0.00139572	5.038~322.4	0.9995	0.04	0.15

^aLOD, limit of detection.^bLOQ, limit of quantification.**TABLE 4** | Precision, repeatability, and stability of the seven investigated compounds.

Analytes	Precision (RSD %)				Repeatability (RSD%; n = 6)	Stability (RSD%; n = 6)
	Standard solution		BYF extract			
	Intra-day (n = 6)	Inter-day (n = 3)	Intra-day (n = 6)	Inter-day (n = 3)		
Calycosin-7-O-Glc	2.99	2.02	0.84	2.16	1.06	1.95
(E)-THSG	1.48	0.64	0.77	2.03	0.45	0.36
Astragulin	2.27	2.80	0.53	3.72	1.18	2.38
Rosmarinic acid	1.07	2.74	0.73	2.90	1.24	1.11
Salvianolic acid A	0.97	2.15	2.32	4.51	3.00	1.70
Salvianolic acid B	0.24	2.74	1.02	2.83	0.74	1.42
Calycosin	2.09	3.04	0.77	3.04	0.78	3.38

Compounds From *Salviae Miltiorrhizae Radix Rhizoma*

Salviae Miltiorrhizae Radix Rhizoma was mainly composed of hydrophilic salvianolic acids and lipophilic diterpenoid quinines (Wu et al., 2007). This research adopted the water extraction method so that the major ingredients of *Salviae Miltiorrhizae Radix Rhizoma* in the BYF are primarily salvianolic acids. This type of compounds has high molecular weight and a lot of homologs, which display similar ESI-MSⁿ behaviors for their differentiations.

Compound 42 displayed the [M-H]⁻ ions at *m/z* 717.144 65 with the elemental composition of C₃₆H₃₀O₁₆. Its MS² spectrum gave diagnostic fragment ions at *m/z* 519.1 and 321.0, caused by the loss of one and two molecular unit of Danshensu, respectively. In the MS³ spectrum, two distinctive ions at *m/z* 339.1 and 321.0, resulted from neutral loss of one danshensu and the McLafferty rearrangement, respectively, were observed. The CID of ion *m/z* 321.0 could furtherly produced MS⁴ and MS⁵ spectra. Its MSⁿ spectra, as well as fragmentation pattern were shown in **Figures 2D–G, 5**. As its retention time and fragmentation ions were identical with those of the reference compound, Compound 42 was identified as salvianolic acid B.

As for the fragmentation pathway of Salvianolic acids, with Danshensu as their parent nucleus, their loss of H₂O and CO, as well as successive losses of Danshensu, occurred based on their MS spectra. Based on molecular weight and multi-stage information provided by MSⁿ, combining with literatures (Hu et al., 2005; Liu et al., 2007; Zhu et al., 2010), 11 phenolic acids in the prescription were identified accurately.

TABLE 5 | Recovery of the analytes.

Analytes	Initial (mg)	Added (mg)	Detected (mg)	Recovery (%)	RSD (%)
Calycosin-7-O-Glc	36.452	36.117	73.102	100.69	2.36
	45.465	45.147	90.417	100.76	
	54.716	54.176	110.166	103.44	
(E)-THSG	130.131	129.649	257.833	98.50	4.02
	161.804	161.901	328.248	102.80	
	194.186	194.367	386.662	99.03	
	12.832	12.884	25.643	99.43	
Astragulin	16.183	16.100	32.122	99.00	3.96
	19.397	19.316	37.913	95.86	
	35.905	35.932	71.485	99.02	
Rosmarinic acid	45.219	44.928	89.772	99.16	1.24
	53.911	53.924	107.457	99.30	
	13.590	13.694	27.325	100.75	
Salvianolic acid A	17.258	17.107	34.256	100.74	1.12
	20.036	20.542	40.565	101.07	
	930.601	928.765	1,869.590	101.10	
Salvianolic acid B	1,162.726	1,160.186	2,309.808	98.87	2.11
	1,395.901	1,393.148	2,806.526	101.25	
	15.531	15.505	31.663	104.04	
Calycosin	19.362	19.374	39.527	104.08	2.58
	23.197	23.243	46.378	99.73	

Compounds From Other Component Herbs

Phenolic constituents, including stilbenes and anthraquinones, were regarded as the main active components in *Polygoni Multiflori Radix Praeparata* (Chen et al., 2012). Most of these

stilbenes previously reported were mainly 2,3,5,4'-tetrahydroxy substituted type (Liu et al., 2011). Compounds 7, 16, 50, 62, and 82 were indubitably distinguished as (*E*)-THSG, (*S*)-THSG, emodin-8-O- β -D-glucoside, physcion-8-O- β -D-glucoside, and emodin, by comparing their t_R -values and mass information with those of the standards. The MS and MS² spectra of Compounds 7, 16, and 20 showed characteristic ions at m/z 405.118 29 and 243.065 55, representing the corresponding elemental composition of C₂₀H₂₁O₉⁻ and C₁₄H₁₁O₄⁻, which was consistent with our previous studies (Qiu et al., 2013). Fragmentation behaviors of anthraquinones were also accordance with previous results.

The components in Cuscutae Semen are mainly phenolic acids and flavonoids. For example, The MS² and MS³ spectra of Compounds 1, 2, and 3 were in accordance with those of 3-CQA (caffeoyl-quinic acid), 5-CQA, and 4-CQA available from the literature (Zhang et al., 2007). Although the fragmentation data of 5-CQA and 4-CQA were the same, retention time of 5-CQA was shorter in reversed-phase chromatography. Elution orders of the three isomers were consistent with the reported.

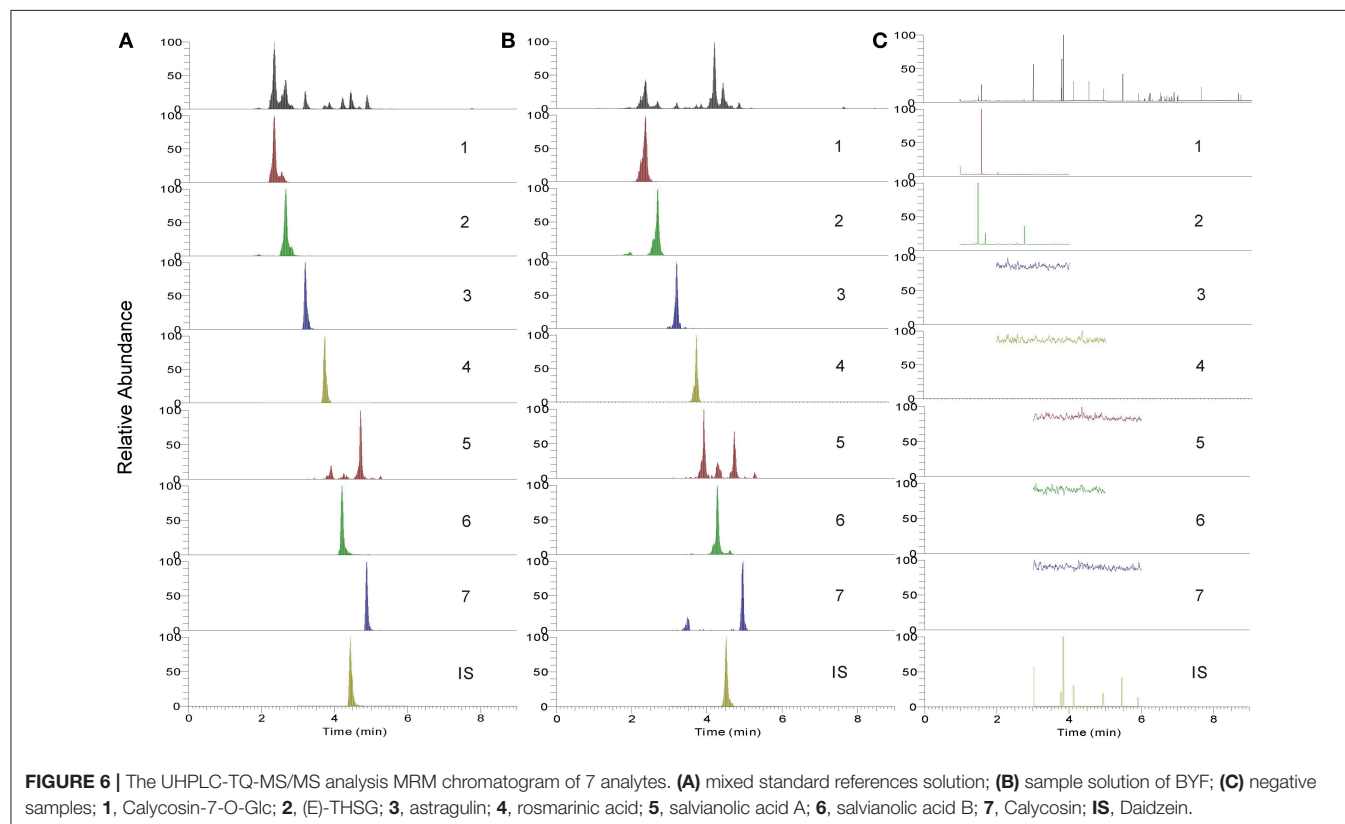
Compounds 67 and 68 both exhibited [M-H]⁻ ion at m/z 843.421 75 (C₃₈H₆₇O₂₀⁻) and their MS² spectra all identical ions, they were tentatively identified as cuscutic acid C and its isomers. Five isomers of acely-Cuscutic acid C (Compounds 71, 72, 73, 74, and 76) were presented by ion extraction at m/z 885 from TIC. They showed identical precursor ion and MS² spectrum, while substituted position of their acetyl group was remained to be further studied.

Validation of Quantitative Method

Seven compounds, unequivocally identified with relatively high content in both the decoction and the granule, were selected as marker components to evaluate the quality of BYF. As the extraction process that we applied was through traditional method, which is extracted by water, the major components of high content were mainly water-soluble and highly polar compounds. These compounds have been observed at the early 25 min of the UHPLC-LTQ-Orbitrap-MS spectra. Meanwhile, those less polar compounds of much lower content emerged between 25 and 50 min. However, the relatively high peak area of such compounds in mass spectra has no direct relationship to their actual content in samples. Seven compounds for quantitative analysis were mainly phenolic and flavonoid compounds. Thus daidzein, a flavonoid, was chosen as the IS due

TABLE 6 | Contents ($\mu\text{g/g}$, $n = 3$) of the seven investigated compounds in the samples of BYF extract powder and BYF granule.

Analytes	Contents of BYF extract powder			Contents of BYF granule	
	1	2	3	1	2
Calycosin-7-O-Glc	244.124	246.061	251.827	122.741	137.846
(<i>E</i>)-THSG	382.673	466.849	438.201	270.693	305.022
Astragulin	52.899	61.430	57.947	27.192	39.693
Rosmarinic acid	10.447	29.943	23.690	63.700	103.399
Salvianolic acid A	11.303	18.126	19.853	97.021	108.978
Salvianolic acid B	5,395.621	5,769.462	5,507.373	2,059.143	2,671.567
Calycosin	38.498	43.058	40.046	16.167	11.441



to its structural and polar similarity with the analytes, and no daidzein exist nor be detected in BYF.

Nice linearity with coefficients of determination ($R^2 > 0.9994$) were gained for the seven compounds. LOD and LOQ tests were carried out and listed in **Table 3**. The intra- or inter-day variations (RSD) were within the range of 0.24–2.99, 0.64–3.04, and 0.53–2.32%, 2.16–3.72% for mixed standard solution and sample solution, respectively (**Table 4**). Analytes in the sample solution were found stable for 24 h with a RSD $< 3.38\%$. Recoveries of the fourteen compounds ranged from 95.86 to 104.04% with RSD from 1.12 to 4.02% (shown in **Table 5**). As a result, the developed UHPLC-TQ-MS/MS method was considered as a sensitive, repeatable and accurate tool for the quantitative analysis of main compounds in BYF.

Application to Analysis of BYF Samples

The established UHPLC-TQ-MS/MS method was subsequently applied for quantitative analysis of both BYF decoction and its preparations. Two different batches of BYF extract powder and three different batches of BYF granule were detected using the developed method. MRM chromatograms of seven main compounds in BYF were displayed in **Figure 6**. The contents of the investigated compounds were determined and the outcomes were shown in **Table 6**.

As shown in **Table 6**, salvianolic acid B was found as the most abundant compound, and compared with BYF decoction, the contents of most investigated compounds are relative low in concentrated granule. This difference might result from manufacturing procedures, namely, concentration, mixing, granulation, and drying processes. Meanwhile, the contents of rosmarinic acid and salvianolic acid A were much higher in concentrated granule than in BYF decoction. The variability could be explained because salvianolic acid B could be degraded and oxidized in the manufacturing procedures thus transform to other phenolic acids, such as rosmarinic acid, salvianolic acid A, lithospermic acid, etc. (Zheng and Qu, 2012).

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CONCLUSION

In this paper, chemical constituents of BYF were systematically investigated by UHPLC-LTQ-Orbitrap-MSⁿ and UHPLC-TQ-MS/MS methods, which provided comprehensively both qualitative and quantitative information for analysis of major components in BYF. Eighty-six compounds including flavones, saponins, phenolic acids, and other compounds were identified. The quantitative method was proved to have nice linearity, good accuracy, sensitivity, and repeatability. Although the bioactive components have not be determined, the present method will be helpful for providing the chemical basis for the further pharmacokinetic studies and effective quality evaluation of BYF, which would be of great importance for its safety use and mechanisms of action.

AUTHOR CONTRIBUTIONS

JZ and WX performed the experiments, analyzed the data, and wrote the paper. XL, ZH, and XQ conceived and designed the experiment, contributed reagent, materials, analysis tools, and revised the manuscript. PW, JH, and JB provided constructive suggestions for this research. All authors gave approval to the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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