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Qualitative and Quantitative Analysis of Banhasasim-Tang Using UHPLC-Q-Orbitrap-MS and UHPLC-TQ-MS/MS

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Abstract: In this study, we analyzed the components of Banhasasim-tang (BHSST), a traditional prescription used to treat gastrointestinal disorders. Qualitative analysis was performed using ultra-high-performance liquid chromatography/quadrupole Orbitrap mass spectrometry (UHPLC-Q-Orbitrap-MS), and a total of 42 compounds were accurately and unambiguously identified by comparison with the corresponding reference standards and mass spectrometry (MS) results. A simultaneous quantitative method for analysis of the 22 identified compounds was established using UHPLC coupled with a triple-quadrupole tandem mass spectrometer (UHPLC-TQ-MS/MS) in multiple reaction monitoring mode. The established method was validated in terms of linearity (R^2 , 0.9990–0.9996), recovery (RSD, 0.37–3.60%), and intraday/interday precision (RSD, 0.35–8.90%). This method was applied to three batches of BHSST samples and revealed that two flavonoids from *S. baicalensis*, baicalin and wagonoside, were the most abundant components. This study presents the first comprehensive method for simultaneously identifying and quantifying the components of BHSST. Our method offers a valuable tool for the quality control of BHSST and serves as a foundational reference for further research on similar preparations.

Keywords: Banhasasim-tang; UHPLC-Q-Orbitrap-MS; UHPLC-TQ-MS/MS; quality control



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1. Introduction

Traditional oriental medicine has long been used for the prevention and treatment of various diseases, exhibiting promising anti-inflammatory, antioxidant, and anti-cancer effects [1–3]. Banhasasim-tang (BHSST, the Banxia Xiexin decoction in traditional Chinese medicine) is a typical prescription that originated from the Treatise on Febrile Diseases, commonly used to treat gastrointestinal disorders [4]. BHSST consists of seven herbal extracts (*Pinellia ternata* Breitenbach, *Zizyphus jujuba* Miller var. *inermis* Rehder, *Scutellaria baicalensis* Georgi, *Zingiber officinale* Roscoe, *Panax ginseng* C. A. Meyer, *Glycyrrhiza uralensis* Fischer, and *Coptis chinensis* Franchet) and has antioxidant and anti-inflammatory properties [5]. BHSST is effective in the treatment of ulcerative colitis and has been shown to reduce the symptoms of chronic gastritis [1,6]. Additionally, BHSST is potentially effective against gastric cancer, as it may inhibit the invasive behavior and metastasis of gastric cancer cells [7]. Some studies have examined the efficacy and mechanism of action of BHSST in treating depression [8], while clinical trials have revealed its efficacy against ulcerative colitis and irritable bowel syndrome without exhibiting side effects [9,10]. A study on gastric ulcers and functional dyspepsia revealed that such symptoms were reduced via BHSST treatment, with BHSST being effective in clinical trials involving patients with hepatocellular carcinoma [11–13].

Previous studies have shown that BHSST contains several active components, including flavonoids, alkaloids, and saponins [14]. According to the composition of each herbal extract, it contains flavonoids such as baicalin, baicalein, and wogonin from *S. baicalensis* and liquiritin and isoliquiritin from *G. uralensis* [15,16]. In addition, alkaloids such as

berberine and coptisine, which are the main components of *C. japonica*, and ginsenosides Rg1 and Rb1, which are saponin compounds of *P. ginseng*, are included in BHSST [17,18].

Prescriptions such as BHSST which combine extracts from several herbs exhibit complex chemical properties that can affect each other; therefore, each component must be comprehensively identified and quantified [10,19]. Previously, representative components of BHSST have been analyzed via high-performance liquid chromatography (HPLC) analysis, with ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) methods being developed for the simultaneous analysis of multiple components for the quality control of BHSST [20–22]. However, such studies have only included the representative components of BHSST, and few studies have reported the analysis of the chemical profile of BHSST and the simultaneous quantification of the identified components. Therefore, an efficient method must be developed to analyze the components of BHSST qualitatively and quantitatively.

UHPLC-MS/MS is a useful method offering efficient component separation and accurate analysis of their structural characteristics for the identification of unknown components [23]. Analytical techniques using Orbitrap mass spectrometry are employed for screening unknown components and for comprehensively characterizing the components [19]. Additionally, UHPLC coupled with a triple-quadrupole tandem mass spectrometer (UHPLC-TQ-MS/MS) is a fast and sensitive quantitative method widely used to provide precise and accurate detection and analysis of complex compounds [24]. In particular, multiple reaction monitoring (MRM) mode serves as a highly sensitive tool to selectively detect and quantify compounds based on ion transition [25].

In this study, we established an analytical method to characterize and identify the components of Banhasasim-tang (BHSST) using ultra-high-performance liquid chromatography/quadrupole Orbitrap mass spectrometry (UHPLC-Q-Orbitrap-MS). A total of 42 components were identified via comparison with retention times and MS results of reference standards. Subsequently, a simultaneous quantitative analytical method using UHPLC-TQ-MS/MS and MRM mode was established to quantify the contents of these identified compounds in BHSST. This established method was then applied to investigate the content distribution of 22 components in actual BHSST extracts.

2. Materials and Methods

2.1. Materials and Reagents

BHSST comprises seven herbal extracts, all of which were purchased from Kwangmyungdang Pharmaceutical (Ulsan, Republic of Korea). Each raw herbal extract was deposited in the KM Convergence Research Division of the Korea Institute of Oriental Medicine; the herbal composition and specimen numbers are as follows: *P. ternate* (TDC-09); *Z. jujuba* (TDC-11); *S. baicalensis* (TDC-10); *Z. officinale* (TDC-03); *P. ginseng* (TDC-13); *G. uralensis* (TDC-12); and *C. chinensis* (TDC-14). Twenty-two analytical standards were purchased from ChemFaces (Wuhan, China), exhibiting a purity of 98% or higher. Warfarin (IS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade methanol, acetonitrile, formic acid, and water were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. BHSST Sample Preparation

Seven herbal medicines, *P. ternate* (60 g), *Z. jujuba* (60 g), *S. baicalensis* (30 g), *Z. officinale* (30 g), *P. ginseng* (30 g), *G. uralensis* (30 g), and *C. chinensis* (10 g) were mixed, followed by the addition of a 10-fold mass amount of distilled water. The mixture was refluxed at 100 °C for 3 h, followed by filtration and concentration under reduced pressure using a rotary evaporator. The concentrated water extract was freeze-dried, yielding a powder (28.6%). To prepare the sample for analysis, the freeze-dried BHSST powder was dissolved in methanol (20 mg/mL) and then extracted in an ultrasonic bath for 30 min. The extracted solution was centrifuged at 12,500 rpm for 15 min before analysis.

2.3. Preparation of Standard Solutions and Control Samples

Precisely measured reference standards were dissolved in methanol to prepare stock solutions of target compounds. A mixed solution containing the 22 analytes was prepared and progressively diluted with methanol to obtain a mixed standard working solution with an appropriate concentration range. The prepared solution was used to create a calibration curve for each compound. Calibration samples of the 22 analytes of BHSST were prepared at concentrations within the range of the standard curve. Warfarin was added as IS in all samples at a consistent concentration, after dissolution in methanol. Quality control (QC) samples were prepared at three concentration levels (high, medium, and low) for method validation following the same preparation procedure as the calibration samples.

2.4. Analytical Conditions of UHPLC-Q-Orbitrap-MS and UHPLC-TQ-MS/MS

A Dionex UltiMate 3000 system equipped with a Thermo Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for qualitative analysis. The 22 compounds identified in BHSST were quantitatively analyzed via an UHPLC-TQ-MS/MS method performed on an Agilent 1290 Infinity II system interfaced with an Agilent 6495C triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The detailed operational conditions for the LC analysis methods used in qualitative and quantitative analyses, as well as for each piece of respective equipment, are summarized in Table 1. Additionally, quantitative analysis of the 22 compounds was performed in the dynamic MRM mode using the positive or negative modes. For each analyte, the MS/MS detection parameters, such as MRM transitions and collision energies, were optimized by setting them to the most appropriate values, and the results are shown in Table 2.

Table 1. Analysis conditions of UHPLC-Q-Orbitrap-MS and UHPLC-TQ-MS/MS.

UHPLC		Q-Orbitrap-MS		TQ-MS/MS	
Parameter	Condition	Parameter	Condition	Parameter	Condition
Column	Acquity BEH C18 column (100 × 2.1 mm, 1.7 µm)	Ion mode	Positive/ Negative	Ion mode	Positive/ Negative
Column temp.	40 °C	Ion source	ESI	Ion source	ESI
Injection volume	3.0 µL	Scan mode	Full MS-ddMS2	Capillary voltage	3500 V (pos) 3000 V (neg)
Sample temp.	4 °C	Scan range	100–1500 <i>m/z</i>	Gas temp.	130 °C
Mobile phase A	0.1% Formic acid in water	Capillary temp.	320 °C	Nebulizer gas	25 psi
Mobile phase B	Acetonitrile	Spray voltage	3.8 kV	Drying gas flow	11 L/min
Flow rate	0.25 mL/min	AUX gas	10 au	Nozzle voltage	500 V (pos) 1500 V (neg)
	Time (min)	Sheath gas	40 au	Software	MassHunter v.10.1
	0	Collision energy	25 eV		
	1	MS resolution	70,000		
	2	MS/MS resolution	17,500		
Gradient program	13	Software	Xcalibur v.3.0 Tracefinder v. 3.2		
	20				
	23				
	23.5				
	27.5				

Table 2. Optimized parameters for MRM of analytes and internal standards for UHPLC-TQ-MS/MS analysis.

No.	Compound Name	Rt (min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	Polarity
9	Rutin	5.23	609.1	300.1	166	40	4	Negative
10	Liquiritin apioside	5.48	549.2	255.1	166	34	4	Negative
11	Scutellarin	5.58	463.0	287.0	166	22	4	Positive
12	Liquiritin	5.59	417.2	255.1	166	18	4	Negative
13	Acteoside	5.64	623.2	161.0	166	40	4	Negative
16	Jatrorrhizine	6.74	338.1	322.1	166	30	4	Positive
19	Coptisine	6.82	320.0	292.1	166	30	4	Positive
20	Isoliquiritin apioside	7.05	549.1	255.1	166	30	4	Negative
21	Isoliquiritin	7.37	417.0	255.1	166	18	4	Negative
22	Baicalin	7.58	447.1	271.0	166	22	4	Positive
23	Ginsenoside Re	7.52	991.6	945.6	166	26	4	Negative
24	Ginsenoside Rg1	7.52	845.5	799.5	166	26	4	Negative
26	Palmitine	7.69	352.1	336.1	166	34	4	Positive
27	Berberine	7.81	336.1	320.1	166	34	4	Positive
29	Wogonoside	9.08	461.1	285.1	166	22	4	Positive
31	Ginsenoside Rb1	10.32	599.3	553.3	166	14	4	Negative
32	Baicalin	10.60	271.0	123.0	166	34	4	Positive
35	Glycyrrhizic acid	11.86	821.4	351.0	166	40	4	Negative
36	Wogonin	12.67	285.1	270.0	166	26	4	Positive
38	Oroxylin A	13.22	285.0	270.0	166	26	4	Positive
39	6-Gingerol	13.24	277.1	177.1	166	10	4	Positive
41	6-Shogaol	16.50	277.1	137.0	166	22	4	Positive
IS1	Warfarin	13.64	309.0	163.0	166	14	4	Positive
IS2	Warfarin	13.64	307.0	250.0	166	22	4	Negative

2.5. Validation of the Quantitative Method

A mixed-standard solution containing 22 analytes and IS was serially diluted to different concentrations for linearity evaluation: 0.02–6.25 ng/mL (rutin, scutellarin, jatrorrhizine, isoliquiritin, wogonin, oroxylin A, and 6-shogaol), 0.05–12.50 ng/mL (acteoside, coptisine, isoliquiritin apioside, ginsenoside Re, baicalin, and 6-gingerol), 0.10–25.00 ng/mL (liquiritin, ginsenoside Rg1, palmitine, and ginsenoside Rb1), 0.20–50.00 ng/mL (liquiritin apioside and berberine), 0.39–100.00 ng/mL (glycyrrhizic acid), and 1.56–400.00 ng/mL (baicalin and wogonoside). Calibration curves were constructed for each analyte using the ratio of the peak area of the analyte to the IS versus the analyte concentration. The lower limit of quantification, which represents the sensitivity of the analytical method, was determined as the lowest measured concentration at which accuracy and precision were exhibited. To recover the analyte, various concentrations (high, middle, and low levels) of each standard were added to a sample solution. The recovery was evaluated by comparing the difference between the known added content and the detected concentration for each analyte. The precision of the analytical method was assessed by analyzing intra- and interday variations: Intraday precision was measured by preparing six QC samples mixed with standard solutions of analytes in one day. Interday precision was measured by preparing one sample and analyzing it for three consecutive days following the same method. Both analyses were performed using QC samples at three concentrations, and the precision of all analytes was assessed using the relative standard deviation of the repeatedly measured values.

3. Results

3.1. Characterization of Chemical Compounds in BHSST via UHPLC-Q-Orbitrap-MS

The BHSST extract was analyzed using UHPLC-Q-Orbitrap-MS based on a qualitative analysis method in which 42 chemical compounds constituting BHSST were identified and

characterized. The base peak chromatograms of BHSST were obtained in both positive and negative ion modes, as shown in Figure 1. Among the 42 detected compounds, 39 compounds were unambiguously identified by comparing the retention times and MS results of the corresponding reference standards. The other three compounds were tentatively characterized by comparing their respective MS data with previous reports, and the retention times and MS results of the identified compounds are summarized in Table 3 and Figure S1.

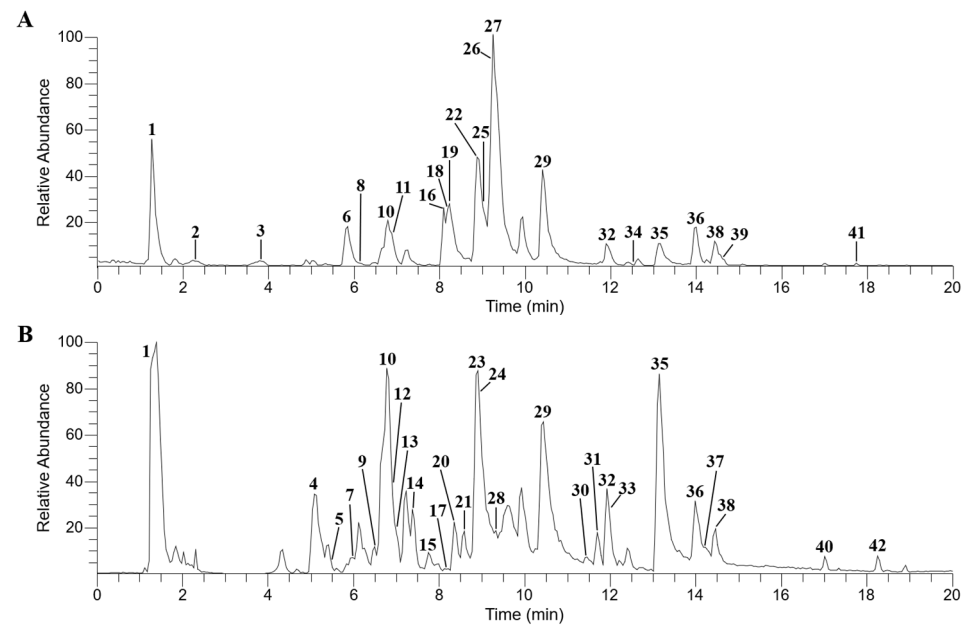


Figure 1. Base peak ion chromatograms of the BHSST extract in positive (A) and negative (B) ion modes obtained via UHPLC-Q-Orbitrap-MS. The ID numbers of the various types of phytochemicals are listed in Table 1.

Table 3. Characterization of chemical compounds in BHSST via UHPLC-Q-Orbitrap-MS.

No.	Rt (min)	Identification	Formula	Adduct	Measured Mass (<i>m/z</i>)	Error (ppm)	MS ² Fragment (<i>m/z</i>)
1	1.26	Maltose *	C ₁₂ H ₂₂ O ₁₁	[M − H] [−]	341.1088	−0.3058	89.0228, 71.0122, 59.0122
2	2.24	Leucine *	C ₆ H ₁₃ NO ₂	[M + H] ⁺	132.1021	1.1812	86.0972
3	3.80	Phenylalanine	C ₉ H ₁₁ NO ₂	[M + H] ⁺	166.0863	0.3551	120.0811
4	5.09	Darendoside A	C ₁₉ H ₂₈ O ₁₁	[M − H] [−]	431.1556	−0.5575	125.0229, 101.0228, 57.0329
5	5.51	Vicenin-2 *	C ₂₇ H ₃₀ O ₁₅	[M − H] [−]	593.1514	0.4016	473.1086, 353.0664
6	5.86	Magnoflorine *	C ₂₀ H ₂₄ NO ₄	[M] ⁺	342.1698	−0.4374	342.1700, 297.1122
7	5.98	Schaftoside *	C ₂₆ H ₂₈ O ₁₄	[M − H] [−]	563.1406	−0.0089	443.0982, 383.0774, 353.0665
8	6.09	Daidzin *	C ₂₁ H ₂₀ O ₉	[M + H] ⁺	417.1177	−0.6549	255.0659
9	6.54	Rutin *	C ₂₇ H ₃₀ O ₁₆	[M − H] [−]	609.1462	0.2138	300.0272, 223.0607
10	6.77	Liquiritin apioside *	C ₂₆ H ₃₀ O ₁₃	[M − H] [−]	549.1613	−0.0803	255.0660
11	6.89	Scutellarin *	C ₂₁ H ₁₈ O ₁₂	[M + H] ⁺	463.0869	−0.4015	287.0553
12	6.91	Liquiritin *	C ₂₁ H ₂₂ O ₉	[M − H] [−]	417.1191	0.0734	255.0660
13	7.00	Acteoside *	C ₂₉ H ₃₆ O ₁₅	[M − H] [−]	623.1981	−0.1298	161.0233
14	7.37	Isoacteoside *	C ₂₉ H ₃₆ O ₁₅	[M − H] [−]	623.1981	−0.0319	161.0233
15	7.70	Acanthoside B *	C ₂₈ H ₃₆ O ₁₃	[M − H] [−]	579.2084	0.1105	417.1552, 181.0496
16	8.10	Jatrorrhizine *	C ₂₀ H ₂₀ NO ₄	[M] ⁺	338.1384	−0.9265	338.1385, 336.1230
17	8.17	Scutellarin methylester *	C ₂₂ H ₂₀ O ₁₂	[M − H] [−]	475.0880	−0.3300	299.0559, 113.0228
18	8.19	Epiberberine *	C ₂₀ H ₁₈ NO ₄	[M] ⁺	336.1228	−0.5853	336.1230

Table 3. Cont.

No.	Rt (min)	Identification	Formula	Adduct	Measured Mass (<i>m/z</i>)	Error (ppm)	MS ² Fragment (<i>m/z</i>)
19	8.24	Coptisine *	C ₁₉ H ₁₄ NO ₄	[M] ⁺	320.0916	−0.3631	320.0917
20	8.36	Isoliquiritin apioside	C ₂₆ H ₃₀ O ₁₃	[M − H] [−]	549.1613	−0.0803	255.0660
21	8.68	Isoliquiritin *	C ₂₁ H ₂₂ O ₉	[M − H] [−]	417.1190	−0.2924	255.0659
22	8.89	Baicalin *	C ₂₁ H ₁₈ O ₁₁	[M + H] ⁺	447.0919	−0.6063	271.0600
23	8.91	Ginsenoside Re *	C ₄₈ H ₈₂ O ₁₈	[M + HCO ₂] [−]	991.5478	−0.534	945.5434, 637.4317, 113.0229, 101.0228
24	8.96	Ginsenoside Rg1 *	C ₄₂ H ₇₂ O ₁₄	[M + HCO ₂] [−]	845.4902	−0.2799	113.0229, 101.0228, 71.0121
25	9.02	Ononin *	C ₂₂ H ₂₂ O ₉	[M + H] ⁺	431.1336	−0.0546	269.0807
26	9.21	Palmatine *	C ₂₁ H ₂₂ NO ₄	[M] ⁺	352.1541	−0.7873	352.1543
27	9.26	Berberine *	C ₂₀ H ₁₈ NO ₄	[M] ⁺	336.1229	−0.4038	336.1230
28	9.32	Liquiritigenin *	C ₁₅ H ₁₂ O ₄	[M − H] [−]	255.0662	−0.2229	255.0652, 135.0073, 119.0487
29	10.41	Wogonoside *	C ₂₂ H ₂₀ O ₁₁	[M + H] ⁺	461.1076	−0.4435	285.0757
30	11.46	Ginsenoside Rf *	C ₄₂ H ₇₂ O ₁₄	[M + HCO ₂] [−]	845.4905	0.0810	113.0229, 101.0228, 71.0121
31	11.69	Ginsenoside Rb1 *	C ₅₄ H ₉₂ O ₂₃	[M + HCO ₂] [−]	1153.6008	−0.2687	1107.5956, 179.0551, 101.0228
32	11.90	Baicalein *	C ₁₅ H ₁₀ O ₅	[M + H] ⁺	271.0603	0.6353	271.0599
33	12.02	Ginsenoside Rc *	C ₅₃ H ₉₀ O ₂₂	[M + HCO ₂] [−]	1123.5902	−0.3293	1077.5838, 149.0442, 89.0227
34	12.56	Formononetin *	C ₁₆ H ₁₂ O ₄	[M + H] ⁺	269.0809	0.3442	269.0808
35	13.15	Glycyrrhizic acid *	C ₄₂ H ₆₂ O ₁₆	[M − H] [−]	821.3965	0.0431	351.0574, 193.0341
36	14.01	Wogonin *	C ₁₆ H ₁₂ O ₅	[M + H] ⁺	285.0757	−0.1259	285.0757
37	14.13	Chrysin *	C ₁₅ H ₁₀ O ₄	[M − H] [−]	253.0505	−0.4274	253.0504
38	14.53	Oroxylin A *	C ₁₆ H ₁₂ O ₅	[M + H] ⁺	285.0757	−0.2329	285.0757
39	14.62	6-Gingerol *	C ₁₇ H ₂₆ O ₄	[M] ⁺	294.1830	1.3355	150.0677, 137.0598
40	17.01	Glabridin *	C ₂₀ H ₂₀ O ₄	[M − H] [−]	323.1290	0.2701	323.1287, 135.0438
41	17.75	6-Shogaol *	C ₁₇ H ₂₄ O ₃	[M + H] ⁺	277.1798	−0.1477	137.0598
42	18.24	Hederagenin *	C ₃₀ H ₄₈ O ₄	[M − H] [−]	471.3478	−0.3631	471.3475

* Compared with a reference standard.

3.2. Quantitation of Chemical Compounds in BHSST via UHPLC-TQ-MS/MS

Based on the results of the compounds identified through qualitative analysis of BHSST, quantitative analysis was performed on the 22 major components of each herbal medicine. These chemical compounds are distributed in the constituent herbal medicines of BHSST as follows: 6-shogaol from *P. ternate*; rutin from *Z. jujuba*; scutellarin, acteoside, baicalin, wogonoside, baicalein, wogonin, and oroxylin A from *S. baicalensis*; 6-gingerol from *Z. officinale*; ginsenoside Re, ginsenoside Rg1, and ginsenoside Rb1 from *P. ginseng*; liquiritin apioside, liquiritin, isoliquiritin apioside, isoliquiritin, and glycyrrhizic acid from *G. uralensis*; and jatrorrhizine, coptisine, palmatine, and berberine from *C. chinensis*. The 22 compounds detected in BHSST were quantified via UHPLC-TQ-MS/MS analysis, with all compounds being detected simultaneously within 20 min. All compounds were analyzed individually in both positive and negative ion modes to establish the optimal MS/MS conditions. Consequently, scutellarin, jatrorrhizine, coptisine, baicalin, palmatine, berberine, wogonoside, baicalein, wogonin, oroxylin A, 6-gingerol, and 6-shogaol exhibited higher sensitivity in the positive ion mode. Conversely, the remaining compounds exhibited higher sensitivity in the negative ion mode.

In this study, the dynamic MRM mode was applied to perform sensitive and accurate quantitative analysis. The best MRM transition for quantitative analysis was determined by identifying the appropriate precursor ion for each analyte and selecting the most specific product ion for MRM analysis. In addition, to optimize the quantitative analysis method, the fragmentor was set to 166 V, and the collision energy was determined to be the optimal value for each compound in the range from 10 to 40 V. The optimized analytical conditions of

the MRM parameters for all analytes, including the internal standard (IS), are summarized in Table 2. The obtained chromatogram is presented in Figure 2.

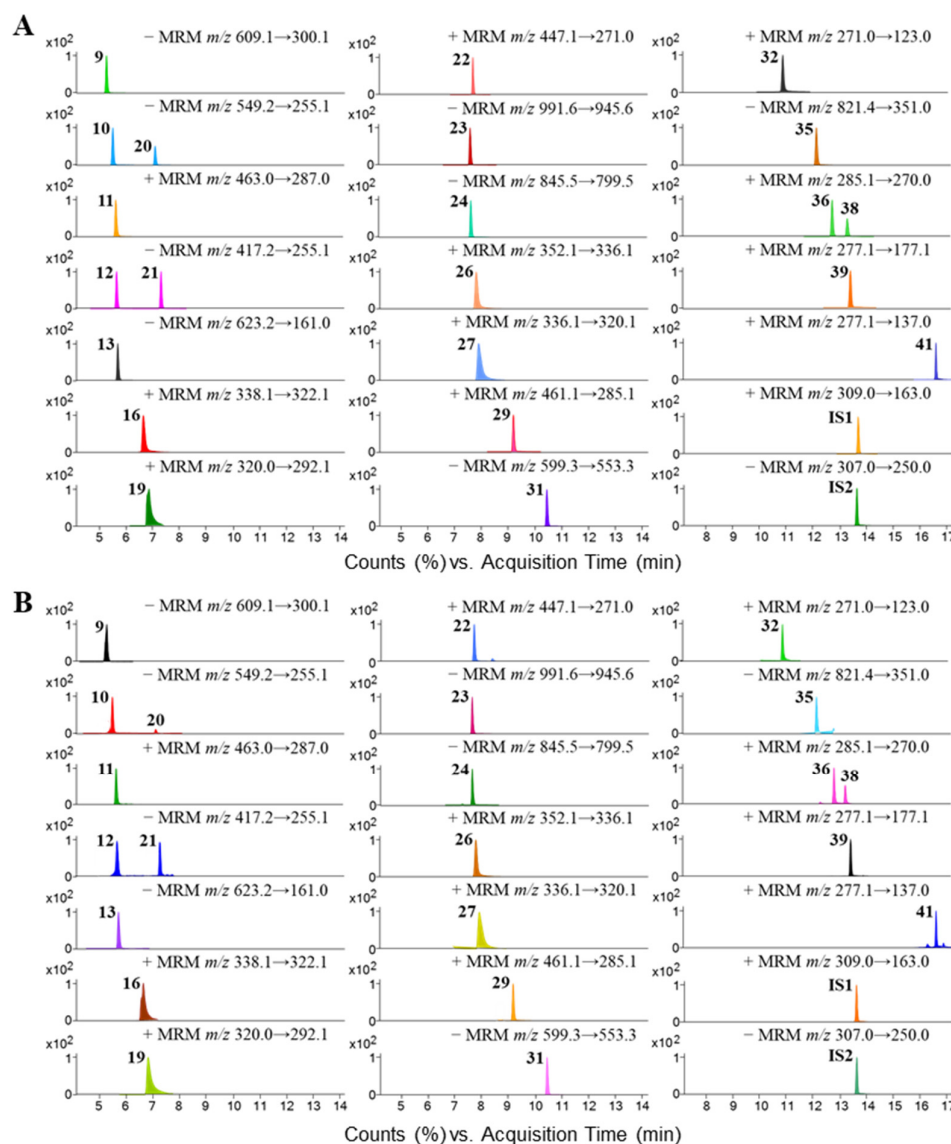


Figure 2. UHPLC-TQ-MS/MS chromatograms of the mixed standard solution (A) and BHSST extract (B) with their MRM modes. The ID numbers of the chemical compounds are presented in Table 2.

3.3. Method Validation

The BHSST quantitative analytical method was validated for its linearity, recovery, precision, and accuracy. The detailed data of the linear relationship and the lower limit of quantitation (LLOQ) for the 22 analytes are presented in Table 4. Calibration curves for all compounds showed good linearity with correlation coefficients (R^2) greater than 0.9990 within specific concentration ranges, indicating an excellent coefficient of determination. Additionally, the LLOQ obtained from UHPLC-TQ-MS/MS analysis was <1.56 ng/mL. Therefore, this method is sufficiently suitable for the quantitative analysis of the 22 target compounds.

The recovery of the method was determined by adding a mixed standard solution containing three different concentrations of the target compound to a sample of known concentration. The average recovery of the 22 analytes ranged from 91.32% to 110.54%, and the relative standard deviation (RSD) values were within 3.60%. The obtained results are summarized in Table 5.

Table 4. Linear regression data and LLOQ of the 22 analytes in BHSST for quantitative analysis method validation.

No.	Compound Name	Regression Equation	R ²	Linear Range (ng/mL)	LLOQ (ng/mL)
9	Rutin	$y = 0.0891x - 0.000393$	0.9992	0.02–6.25	0.02
10	Liquiritin apioside	$y = 0.1956x - 0.007461$	0.9994	0.20–50.00	0.20
11	Scutellarin	$y = 0.0107x + 0.000035$	0.9993	0.02–6.25	0.02
12	Liquiritin	$y = 0.3732x - 0.006084$	0.9994	0.10–25.00	0.10
13	Acteoside	$y = 0.1284x - 0.002549$	0.9992	0.05–12.50	0.05
16	Jatrorrhizine	$y = 0.5496x - 0.004375$	0.9993	0.02–6.25	0.02
19	Coptisine	$y = 0.2028x - 0.004268$	0.9991	0.05–12.50	0.05
20	Isoliquiritin apioside	$y = 0.2073x - 0.001545$	0.9994	0.05–12.50	0.05
21	Isoliquiritin	$y = 0.3112x - 0.001585$	0.9994	0.02–6.25	0.02
22	Baicalin	$y = 0.0106x + 0.001108$	0.9993	1.56–400.00	1.56
23	Ginsenoside Re	$y = 0.2309x - 0.002048$	0.9992	0.05–12.50	0.05
24	Ginsenoside Rg1	$y = 0.0690x - 0.001711$	0.9992	0.10–25.00	0.10
26	Palmitine	$y = 0.5096x - 0.021645$	0.9991	0.10–25.00	0.10
27	Berberine	$y = 0.3658x - 0.037572$	0.9990	0.20–50.00	0.20
29	Wogonoside	$y = 0.0123x - 0.005180$	0.9990	1.56–400.00	1.56
31	Ginsenoside Rb1	$y = 0.2998x - 0.006713$	0.9993	0.10–25.00	0.10
32	Baicalein	$y = 0.0120x - 0.000040$	0.9990	0.05–12.50	0.05
35	Glycyrrhizic acid	$y = 0.0805x - 0.007355$	0.9991	0.39–100.00	0.39
36	Wogonin	$y = 0.6316x - 0.001552$	0.9992	0.02–6.25	0.02
38	Oroxylin A	$y = 0.1985x - 0.001333$	0.9996	0.02–6.25	0.02
39	6-Gingerol	$y = 0.0621x - 0.000717$	0.9993	0.05–12.50	0.05
41	6-Shogaol	$y = 0.1483x - 0.000504$	0.9992	0.02–6.25	0.02

Table 5. Recovery test of the 22 analytes in BHSST for quantitative analysis method validation.

No.	Compound Name	Recovery (%)								
		Low Level			Medium Level			High Level		
		Added (ng/mL)	Mean	RSD (%)	Added (ng/mL)	Mean	RSD (%)	Added (ng/mL)	Mean	RSD (%)
9	Rutin	0.81	94.88	3.43	1.33	103.13	2.34	2.37	99.93	1.73
10	Liquiritin apioside	17.08	94.91	0.77	21.25	91.81	1.19	29.58	96.20	1.03
11	Scutellarin	1.01	93.39	2.80	1.53	106.49	1.07	2.57	102.51	3.40
12	Liquiritin	4.65	97.38	1.43	6.73	98.46	0.91	10.90	103.77	0.53
13	Acteoside	2.24	92.36	2.21	3.28	99.12	2.25	5.36	103.52	3.60
16	Jatrorrhizine	1.42	100.08	1.25	1.94	100.27	0.90	2.98	100.13	2.18
19	Coptisine	2.66	96.04	1.17	3.70	95.69	0.84	5.79	92.26	0.37
20	Isoliquiritin apioside	2.68	95.28	1.33	3.72	96.94	2.04	5.81	107.33	2.18
21	Isoliquiritin	0.96	96.12	1.28	1.48	104.95	2.30	2.52	106.23	1.59
22	Baicalin	97.13	94.94	1.00	130.46	102.59	0.80	197.13	101.22	0.72
23	Ginsenoside Re	2.66	95.33	1.94	3.70	100.32	2.05	5.78	103.62	0.74
24	Ginsenoside Rg1	5.17	100.63	1.11	7.25	100.86	0.86	11.42	101.43	1.46
26	Palmitine	4.55	97.66	3.02	6.63	97.31	0.62	10.80	97.19	2.77
27	Berberine	14.56	94.83	1.13	18.72	97.63	0.93	27.06	105.44	0.95
29	Wogonoside	114.86	92.54	0.64	148.19	96.34	1.28	214.86	99.21	1.28
31	Ginsenoside Rb1	4.99	98.38	1.40	7.07	104.24	1.27	11.24	110.54	0.65
32	Baicalein	2.50	97.19	3.56	3.55	108.86	0.44	5.63	102.40	1.43
35	Glycyrrhizic acid	23.07	93.11	1.46	31.41	99.03	1.89	48.07	101.14	0.52
36	Wogonin	1.02	92.33	0.96	1.54	101.54	1.56	2.58	105.80	0.78
38	Oroxylin A	1.12	93.05	1.70	1.64	95.02	1.10	2.68	91.32	1.00
39	6-Gingerol	2.79	94.45	1.06	3.83	91.90	1.43	5.91	97.50	1.17
41	6-Shogaol	0.82	94.58	1.38	1.34	100.26	2.12	2.38	99.37	0.62

The precision of the method was evaluated via the intraday and interday relative standard deviations (RSD). Intraday precision was evaluated by analyzing six replicates

of samples containing the target compounds at three concentrations. Interday precision was evaluated following the same method; however, analyses were performed on three consecutive days. The results of the analyses are presented in Table 6; the RSD for precision was 0.35–6.45% for intraday and 0.46–8.90% for interday evaluation. Additionally, the accuracy range for these analytes was 90.74–112.25% for intraday and 91.33–107.36% for interday analyses.

Table 6. Precision and accuracy of the 22 analytes in BHSST for quantitative analysis method validation.

No.	Compound Name	Concentration (ng/mL)	Precision (RSD, %)		Accuracy (%)	
			Intraday	Interday	Intraday	Interday
9	Rutin	4.17	1.80	5.53	110.26	104.14
		1.04	1.50	2.64	102.93	99.99
		0.26	1.20	2.71	94.01	94.63
		33.33	1.58	5.97	110.77	103.67
10	Liquiritin apioside	8.33	0.82	4.24	105.55	100.93
		2.08	1.29	1.07	102.83	103.91
		4.17	1.53	8.00	112.25	104.66
		1.04	2.82	1.32	101.37	99.85
11	Scutellarin	0.26	3.19	4.08	91.35	95.04
		16.67	1.12	5.27	109.75	103.53
		4.17	0.50	3.64	104.67	100.47
12	Liquiritin	1.04	1.39	3.73	100.93	103.91
		8.33	1.07	2.51	108.43	107.36
		2.08	1.04	1.45	103.12	104.74
13	Acteoside	0.52	1.04	8.08	93.47	101.33
		4.17	0.82	3.59	102.85	99.29
		1.04	2.13	4.77	102.28	96.94
16	Jatrorrhizine	0.26	1.84	5.11	98.13	94.13
		8.33	0.42	1.15	99.75	98.73
		2.08	1.42	2.04	97.36	95.12
19	Coptisine	0.52	0.73	2.88	93.17	91.33
		8.33	0.63	7.21	111.90	103.33
		2.08	1.13	5.26	108.42	102.21
20	Isoliquiritin apioside	0.52	1.88	1.39	100.09	101.48
		4.17	0.75	5.47	110.60	104.10
		1.04	1.96	2.06	103.08	100.77
21	Isoliquiritin	0.26	6.45	4.83	99.19	105.02
		266.67	0.60	8.90	110.47	100.78
		66.67	0.35	5.35	104.33	99.05
22	Baicalin	16.67	0.75	3.54	99.13	97.18
		8.33	0.98	5.06	110.27	104.22
		2.08	2.72	4.11	107.39	102.54
23	Ginsenoside Re	0.52	4.97	2.77	109.41	106.12
		16.67	1.06	5.75	111.14	104.22
		4.17	1.74	2.66	104.24	101.20
24	Ginsenoside Rg1	1.04	3.48	1.67	100.17	101.18
		16.67	1.11	5.02	105.18	100.01
		4.17	0.86	1.07	95.02	94.77
26	Palmatine	1.04	0.61	1.75	95.26	93.52
		33.33	0.50	6.84	106.78	98.97
		8.33	0.44	1.12	94.20	93.79
27	Berberine	2.08	1.37	3.44	97.08	93.64
		266.67	0.64	4.22	102.30	97.56
		66.67	1.98	3.52	99.72	95.83
29	Wogonoside	16.67	1.38	3.42	92.78	94.75
		4.17	0.89	3.35	110.83	106.99
		1.04	1.19	2.82	106.66	103.42
31	Ginsenoside Rb1	8.33	2.12	2.76	103.24	105.03
		2.08	0.42	8.38	109.11	101.09
		0.52	1.44	4.58	101.74	96.64
32	Baicalein	2.08	1.44	4.58	101.74	96.64
		0.52	0.90	3.16	102.34	105.00

Table 6. Cont.

No.	Compound Name	Concentration (ng/mL)	Precision (RSD, %)		Accuracy (%)	
			Intraday	Interday	Intraday	Interday
35	Glycyrrhizic acid	66.67	1.24	6.10	111.73	104.39
		16.67	0.74	5.97	108.92	102.22
		4.17	3.15	7.98	93.12	102.56
36	Wogonin	4.17	2.11	6.70	108.71	100.95
		1.04	2.16	2.67	98.57	95.64
		0.26	2.07	2.43	90.74	92.42
38	Oroxylin A	4.17	0.74	7.92	106.92	98.68
		1.04	1.08	1.85	98.16	97.18
		0.26	1.36	2.82	93.98	95.55
39	6-Gingerol	8.33	0.66	6.13	104.57	97.87
		2.08	0.50	1.11	92.29	93.43
		0.52	1.24	2.33	97.30	94.78
41	6-Shogaol	4.17	0.72	4.52	107.11	102.34
		1.04	2.37	0.46	98.98	98.71
		0.26	2.02	4.36	98.58	100.19

The above results demonstrate that the dynamic MRM mode method of UHPLC-TQ-MS/MS established for quantitative analysis is accurate and precise, suggesting that it can be applied effectively and efficiently to quantify the compounds present in BHSST.

3.4. Quantitative Application to BHSST Samples

The content of BHSST was analyzed using the validated UHPLC-TQ-MS/MS MRM analytical method, with all compounds being detected simultaneously. Each sample was spiked with IS and injected six times to obtain the mean and standard deviation values. The contents of the three batches of BHSST samples were not significantly different from each other; the detailed data are presented in Table 7.

Table 7. Contents of the 22 analytes in 3 batches of BHSST.

No.	Compound	BHSST-1			BHSST-2			BHSST-3		
		Mean (mg/g)	SD	CV (%)	Mean (mg/g)	SD	CV (%)	Mean (mg/g)	SD	CV (%)
9	Rutin	0.125	0.005	3.60	0.127	0.007	5.26	0.126	0.005	3.93
10	Liquiritin apioside	5.306	0.033	0.61	5.300	0.044	0.84	5.257	0.051	0.96
11	Scutellarin	0.256	0.012	4.57	0.263	0.011	4.31	0.263	0.004	1.58
12	Liquiritin	1.002	0.004	0.41	1.003	0.010	0.99	0.999	0.011	1.15
13	Acteoside	0.595	0.013	2.10	0.601	0.011	1.89	0.596	0.015	2.55
16	Jatrorrhizine	0.459	0.012	2.65	0.432	0.006	1.45	0.437	0.005	1.21
19	Coptisine	0.583	0.015	2.65	0.568	0.007	1.19	0.571	0.006	1.01
20	Isoliquiritin apioside	0.655	0.011	1.62	0.624	0.013	2.07	0.613	0.008	1.25
21	Isoliquiritin	0.171	0.004	2.45	0.170	0.006	3.60	0.167	0.005	2.99
22	Baicalin	35.157	0.521	1.48	34.848	0.388	1.11	34.661	0.401	1.16
23	Ginsenoside Re	0.558	0.008	1.52	0.554	0.006	1.16	0.551	0.004	0.69
24	Ginsenoside Rg1	1.080	0.006	0.58	1.132	0.006	0.56	1.110	0.006	0.54
26	Palmitine	0.831	0.007	0.84	0.814	0.008	1.03	0.813	0.010	1.21
27	Berberine	2.749	0.065	2.35	2.654	0.041	1.55	2.678	0.042	1.58
29	Wogonoside	28.919	0.657	2.27	28.818	0.598	2.07	28.893	0.510	1.76
31	Ginsenoside Rb1	1.120	0.008	0.75	1.112	0.008	0.68	1.091	0.007	0.61
32	Baicalin	0.626	0.011	1.80	0.630	0.007	1.10	0.617	0.012	1.97
35	Glycyrrhizic acid	4.869	0.059	1.20	4.898	0.058	1.19	4.894	0.050	1.01
36	Wogonin	0.186	0.004	2.14	0.183	0.001	0.81	0.183	0.001	0.78
38	Oroxylin A	0.204	0.012	5.77	0.202	0.009	4.40	0.205	0.006	2.69
39	6-Gingerol	0.624	0.009	1.50	0.610	0.007	1.15	0.606	0.004	0.64
41	6-Shogaol	0.101	0.001	0.82	0.098	0.001	1.47	0.100	0.001	1.32

4. Discussion

This study performed a qualitative analysis to identify the components of BHSST, a representative prescription used to treat gastrointestinal diseases, and a total of 42 compounds were identified. The detected compounds were reported to be constituents of 6-shogaol from *P. ternate* [26]; rutin from *Z. jujuba* [27]; scutellarin, acteoside, baicalin, wogonoside, baicalein, wogonin, and oroxylin A from *S. baicalensis* [15]; 6-gingerol from *Z. officinale* [28]; ginsenosides Re, Rg, and Rb1 from *P. ginseng* [18]; liquiritin, isoliquiritin, and glycyrrhizic acid from *G. uralensis* [16]; and coptisine, jatrorrhizine, palmatine, and berberine from *C. chinensis* [17]. Among these identified compounds, 24 components were in agreement with compound information reported in the literature related to BHSST [22,29,30].

Previously reported studies were either qualitative analyses that identified constituents of BHSST or quantitative analyses of representative constituents [22,29]. Accordingly, in this study, in order to efficiently analyze the components of BHSST, we established not only a chemical profile analysis but also a quantitative analysis method for some of the identified components. A total of 22 compounds were simultaneously quantitatively analyzed through MRM analysis using UPLC-TQ-MS/MS, and the optimal precursor and product ion for quantitative analysis were set for each component. Rutin exhibited a precursor ion at m/z 609.1, whereas its product ion was detected at m/z 300.1, owing to the loss of the rhamnosyl-glucoside moiety [31]. The two isomeric compounds, liquiritin and isoliquiritin, lost their glucose residue (162 Da) from the precursor ion to form a product ion at m/z 255. The two isomeric compounds, liquiritin apioside and isoliquiritin apioside, also exhibited a product ion of $[M - H - \text{xyl} - \text{glc}]^-$ at m/z 255 [32,33]. Regarding glycyrrhizic acid, its product ion was formed at m/z 351.0 in the negative ion mode, which resulted from the loss of the triterpenic aglycone [34]. Baicalin, wogonoside, and scutellarin generated product ions at m/z 271.0, 285.1, and 287.0, respectively, by neutral loss of the glucuronic acid moiety from the precursor ion $[M + H]^+$ [35,36]. In the case of baicalein, the trihydroxyphenyl moiety at m/z 123.0 was the product ion formed from the precursor ion $[M + H]^+$. Regarding wogonin and oroxylin A, the same product ion was observed at m/z 270.0 via the loss of CH_3 [37]. The product ion of acteoside detected at m/z 161.0 was generated via the successive loss of H_2O from the CA moiety ($\text{C}_{20}\text{H}_{26}\text{O}_{11}$) lost from the precursor ion [38,39]. Jatrorrhizine, palmatine, and berberine exhibited precursor ions $[M]^+$ at 338.1, 352.1, and 336.1, respectively. Furthermore, the product ions for all three compounds showed characteristic m/z values of 322.1, 336.1, and 320.1, respectively, owing to $[M - \text{CH}_3 - \text{H}]^+$ [40]. Coptisine exhibited a precursor ion at m/z 320.0 and a product ion at m/z 292.1, which resulted in the observed loss of the CO group [41]. Ginsenoside Re, Rg₁, and Rb₁ formed product ions via the loss of HCOOH from the precursor ion. Therefore, ginsenoside Re and Rg₁ exhibited $[M - \text{H}]^-$ ions at m/z 945.6 and 799.5, respectively, whereas Rb₁ showed $[M - 2\text{H}]^{2-}$ ions at m/z 553.3 [42,43]. 6-Gingerol was formed as a product ion of $[M + \text{H} - \text{H}_2\text{O} - \text{C}_6\text{H}_{12}\text{O}]^+$ at m/z 177.1 owing to the loss and rearrangement of the neutral alkyl moiety from the precursor ion [44]. Additionally, 6-shogaol cleaved the bond between the carbonyl moiety and the aromatic ring from the precursor ion $[M + \text{H}]^+$ formed at m/z 277.1, resulting in the formation of a product ion at m/z 137.0 [45].

The contents of 22 compounds in BHSST samples were measured using this established quantitative analysis method. Among the measured compounds, two flavonoids, baicalin and wogonoside, exhibited the highest content. These two flavonoids are the main components of *S. baicalensis*, and they have been previously reported as the most abundant components in BHSST [21]. Additionally, liquiritin apioside and glycyrrhizic acid, derived from *G. uralensis*, were detected in larger amounts compared to other components. Our results indicate that the components of BHSST were accurately quantified in actual samples analyzed via the validated UHPLC-TQ-MS/MS method. The established quantitative analytical method can therefore be applied in BHSST component analysis and quality control.

5. Conclusions

In this study, an UHPLC-Q-Orbitrap-MS method was applied for the qualitative evaluation of BHSST, and 42 compounds were identified. The characterized components were quantified using the MRM mode of UHPLC-TQ-MS/MS, and a rapid method capable of simultaneously quantifying the 22 compounds was established. The established method was validated for its linearity, precision, and accuracy and was proven effective for the analysis of real samples. Our method can provide comprehensive information on the composition of BHSST by revealing its chemical profile, while enabling simultaneous quantitative evaluation. In addition, it can provide reliable data for quality control of BHSST and related preparations. These results can contribute to evaluating the pharmaceutical effects of each component of BHSST in in vivo research or identifying the mechanisms of interaction. In the future, based on this study, there is a need to evaluate the actual clinical application potential of BHSST in various disease models, and further research is needed to systematically analyze the interaction network between each compound to better understand various pharmaceutical effects and mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr12081563/s1>, Figure S1: The MS² spectrum of 42 chemical compounds in BHSST via UHPLC-Q-Orbitrap-MS.

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