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Research Article

Quality Evaluation of Bioactive Ingredients in Lianhua Qingwen Capsule Based on Quantitative Analysis of Multicomponent by the Single Marker Method and the Chemical Recognition Patterns Method

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Based on high performance liquid chromatography with the diode array detector (HPLC-DAD), a new strategy for simultaneous determination of ten bioactive ingredients in Lianhua Qingwen capsule (LHQW) was developed for comprehensive quality assessment of LHQW. In this work, with rhein regarded as the internal reference substance (IRS), the relative correction factors (RCFs) of neochlorogenic acid, amygdalin, chlorogenic acid, forsythoside A, quercitrin, phillyin, glycyrrhizic acid, isoforsythiaside, and (+) pinoresinol-β-D-glucoside were calculated for simultaneous determination of ten bioactive ingredients. More importantly, compared to previous work, the simultaneous determination of the content of ten pharmacologically important active ingredients at one detection wavelength with only one reference substance has been achieved. Based on the contents of ten bioactive ingredients, the quality of the 20 batches of LHQW samples was further analyzed by chemical recognition patterns method. Ten bioactive ingredients showed a good linear relationship in their respective concentration ranges ($r \geq 0.999$). The relative standard deviations (RSDs) of precision (≤4.62%), stability (≤4.04%), repeatability (≤3.87%), and the average recovery of ten bioactive components (99.8%~104.1%) demonstrated the QAMS developed for LHQW which had good durability. The correlation coefficient ($P > 0.05$) showed that no significant difference existed in the results of QAMS and external standard method (ESM). Hierarchical clustering analysis (HCA) divided samples into three main groups. Radar plot analysis and principal component analysis (PCA) found some quality differences existed between the three groups of samples. Orthogonal partial least-squares discrimination analysis (OPLS-DA) showed that forsythoside A could be used as the primary marker responsible for the quality differences. In conclusion, the established QAMS method combined with chemometric analysis can simultaneously determine the content of 10 active components and comprehensively evaluate the quality of different batches of LHQW. It can provide scientific basis and reference of quality consistency evaluation for the formulation manufacturers and drug regulatory authorities.

1. Introduction

Lianhua Qingwen capsule (LHQW), which originated from classic ancient recipes of three dynasties, played an irreplaceable role in evidently alleviating the symptoms caused by coronavirus disease 2019 (COVID-19) [1]. A study collected clinical data from 284 patients with COVID-19, founding the recovery rate of the treatment group taking LHQW was higher than that of the control group (91.5% vs. 82.4%) [2]. Also, it has been confirmed that LHQW has an

excellent therapeutic effect on pulmonary infection and influenza [2, 3]. As the safety and efficacy of LHQW were gradually verified, the treatment of COVID-19 of LHQW was incorporated into the drug indications of LHQW by National Medical Products Administration. In daily life, LHQW is extensively used to treat influenza with relatively high sales [4]. Combined with the prevention and treatment of COVID-19, the sales of LHQW will further increase.

LHQW is comprised of *Forsythiae Fructus*, *Lonicerae japonicae* Flos, and other Chinese herbal medicines [5]. Due to the holistic treatment concept of traditional Chinese medicine, the therapeutic effect of LHQW is a comprehensive efficacy of each medicinal herb. One of the studies applied the human exposure-based approach to identify pharmaceutically active components in LHQW. Also, after screening data by comprehensive two-dimensional angiotensin-converting enzyme 2 (ACE2) biochromatography, results showed that RHE, FA, NA, and its isomers exhibited high inhibitory effect on ACE2 [6]. Also, through network pharmacology and molecular docking technology, other studies found that AMY, CA, GA, and ISF could be the candidate compounds for COVID-19 treatment [7–10]. Another study has demonstrated through network pharmacology that QUE and other components have strong antiviral potential against SARS-CoV-2 [11]. A study has revealed the therapeutic potential of phillyrin in COVID-19 and influenza coinfection through a series of bioinformatics network pharmacology methods [12]. In addition, PIN may have more important pharmacological activity as a strong cyclic phosphodiesterase (PDE) inhibitor [13]. Inspired by the abovementioned work, these ten more meaningful active ingredients were selected for QAMS to better evaluate the quality of LHQW in our study. In fact, the pharmacological effects of LHQW result from the interaction of various active components. Also, the source of the Chinese herbal medicines contained in LHQW will directly affect the content of each active ingredient, which will ultimately have a specific impact on the quality of LHQW. However, in the Chinese pharmacopoeia, only phillyrin is involved in the determination of LHQW's quality evaluation method [5]. Only a single quality control index may not adequately reflect the overall quality level of LHQW containing multiple active ingredients. Therefore, it is necessary to establish a method for the simultaneous determination of the contents of multiple active ingredients in LHQW.

Based on advanced techniques such as HPLC-Q Exactive-Orbitrap-MS in combination with GC-MS, the chemical components contained in LHQW were revealed in a more comprehensive manner [14]. More importantly, this provides an important prerequisite for the selection of suitable active ingredients in our work and lays the foundation for the establishment of the QAMS method for LHQW. However, quantitative analysis is not involved in their work. In another study, the characterization of components contained in LHQW and the quantification of 12 representative compounds, such as salidroside and chlorogenic acid, were achieved by UPLC-DAD-QTOF-MS [15]. Interestingly, in order to achieve quantitative analysis of these components, different detection wavelengths and 12 reference substances for the 12 components were adopted in that study. Due to the different maximum absorption values of different compounds, three detection wavelengths, 210 nm, 225 nm, and 254 nm, were chosen in that study to achieve the determination of the 12 components with high detection sensitivity and low interference reduction. Compared to previous work, we have achieved the simultaneous determination of the content of 10 pharmacologically important active ingredients at one detection wavelength with only one reference substance. Meanwhile, compared to the mass spectrometry detectors mentioned in the two articles, the HPLC-DAD instrument we used, which has a lower operating cost, is a common quantitative analysis instrument used by many drug manufacturers and drug testing organizations. Thus, the established QAMS method for LHQW could improve the analytical efficiency of LHQW quality evaluation with certain advantages. In addition, previous research for LHQW mainly focused on the determination of single or multiple components by the external standard method (ESM), which seemed to be exceedingly costly and time-consuming due to the need to purchase reference substances for all analytes [16–18]. More importantly, the efficacy of LHQW often relies on the synergistic effect of many active ingredients, so it seems unreasonable to evaluate the overall quality of LHQW by only one ingredient. Compared with ESM, the method of quantitative analysis of multicomponent by single marker (QAMS) has the potential to improve LHQW's quality evaluation system with its unique advantage. To determine several compositions simultaneously by a single component, the QAMS method explores the inherent functional connection in the contents of multiple ingredients. Also, the inherent connection of content could be expressed by relative correction factors (RCFs) to calculate the content of each component.

QAMS method is simple, efficient, and economical, and it could be an alternative method to complement imperfection caused by determining a single ingredient [19]. With the wide application of chemometrics in the quality control and evaluation of traditional Chinese medicine, chemical recognition patterns have become a prominent and effective method for screening traditional Chinese medicine Q-markers [20]. Accordingly, crucial components in the quality difference will be screened out of the various active ingredients after analyzing LHQW samples' information in depth by hierarchical clustering analysis (HCA), radar plot analysis, principal component analysis (PCA), and orthogonal partial least-squares discrimination analysis (OPLS-DA). Although QAMS method could provide a more rational indicator of LHQW's quality control and evaluation in combination with the chemical recognition patterns method, there is currently starved research on this. Therefore, a strategy integrating QAMS method with chemical recognition patterns could improve the quality evaluation level of LHQW to ensure the safety and effectiveness of clinical use and the economic benefits of the pharmaceutical company.

2. Materials and Methods

2.1. Chemicals and Reagents. The reference substance of chlorogenic acid (batch no. 110325, purity >98%, and CAS: 327-97-9) was purchased from Chengdu Chroma Biotechnology Co., Ltd. (Chengdu, China). Neochlorogenic acid (batch no. DSTDX001503, purity>98%, and CAS: 906-33-2), (+) pinoresinol-β-D-glucoside (batch no. DSTDS002401, purity >98%, and CAS: 69251-96-3), and isoforsythiaside (batch no. DST200315-411, purity >97%, and CAS: 1357910-26-9) were purchased from Desite Bio-Chem Technology Co., Ltd. (Chengdu, China). Amygdalin (batch no. 21110935, purity >98%, and CAS: 29883-15-6) and phillyrin (batch no. 21041221, purity >98%, and CAS: 487-41-2) were purchased from Tauto Biotech Co., Ltd. (Shanghai, China). Forsythoside A (batch no. 10059, purity >98%, and CAS: 79916-77-1) was purchased from Shanghai Standard Technology Co., Ltd. (Shanghai, China). Quercitrin (batch no. MUST-21111917, purity >98.68%, and CAS: 522-12-3) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). Glycyrrhizic acid (batch no. AMU488, purity >95%, and CAS: 1405-86-3) was purchased from Shanghai Bidepharm Co., Ltd. (Shanghai, China), and rhein (batch no. SR8100, purity >98%, and CAS: 478-43-3) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Also, the chemical structures of ten bioactive compositions are listed in Figure 1. All 20 batches of LHQW samples labeled S1–S20 were collected from Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, China), with the batch numbers of B2102150, B2102154, B2102009, B2102186, B2101158, B2102166, B2102148, B2102078, B2101161, B2102162, B2101315, B2102175, B2101162, B2101153, B2011041, B2009007, B2012044, B2010217, B2101064, and B2012099. In the prescription of LHQW, the composition of the herbs for 1000 capsules is recorded as follows: 255 g of *Forsythiae Fructus*, 255 g of *Lonicerae japonicae* Flos, 85 g of roasted *Ephedrae Herba*, 85 g of fried *Armeniacae Semen Amarum*, 255 g of *Gypsum Fibrosum*, 255 g of *Isatidis Radix*, 255 g of *Dryopteridis Crassirhizomatis Rhizoma*, 255 g of *Houttuyniae Herba*, 85 g of *Pogostemonis Herba*, 51 g of *Rhei Radix Et Rhizoma*, 85 g of *Rhodiolae Crenulatae Radix Et Rhizoma*, 7.5 g of L-menthol, and 85 g of *Glycyrrhizae Radix Et Rhizoma*.

The acetonitrile of HPLC grade was provided by Chengdu Chron Chemicals Co., Ltd. (Chengdu, China). The phosphoric acid of analytical grade was supplied by Shanghai Titan Scientific Co., Ltd. (Shanghai, China). Also, the ultrapure water was produced by a Milli-Q water purification system (Millipore, USA).

2.2. Instruments and Conditions. The analyses were performed on the Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a double solvent delivery system, a low-pressure mix quaternary pump (LC-20AT), an autosampler (SIL-20A), a column temperature controller, and a diode array detector (DAD, SPD-M20A). For the satisfactory separation of chromatographic peaks, a Zorbax reverse phase C18 column (4.6 × 250 mm, 5 μm, Agilent) was employed with the injection volume of 10 μL at 30°C. Moreover, another HPLC system (Waters Alliance e2659 system, Waters Corp., USA) and two additional C18 columns, InertSustain C18 (4.6 × 250 mm, 5 μm, GL Sciences) and Hypersil BDS C18 (4.6 × 250 mm, 5 μm, Thermo Fisher), were also applied for exploring durableness of the method in this study. The LS-220, an analytical balance with a resolution of 0.1 mg, was purchased from Shanghai Precisa Gravimetrics Co., Ltd. Also, the SQP electronic analysis balance (0.01 mg) was purchased from Sartorius Co., Ltd. (Germany).

The mobile phase, comprised of the 0.1% phosphoric acid aqueous solution (A) and acetonitrile solution (B), was programmed with the following gradient elution: 0–5 min, 5%–6% B; 5–35 min, 6%–7% B; 35–40 min, 7%–8% B; 40–50 min, 8%–15% B; 50–85 min, 15% B; 85–110 min, 15%–17% B; 110–115 min, 17%–20% B; 115–140 min, 20%–23% B; 140–155 min, 23%–35% B; 155–165 min, 35%–40% B; 165–180 min, 40%–55% B; 180-181 min, 55%–90% B; and 181–185 min, 90% B. Considering that the chromatographic peak time of the bioactive ingredients to be analyzed under the experimental conditions was scattered, it is necessary to apply a long analysis time to ensure that each ingredient could be well separated as far as possible. Different chromatographic conditions such as 60, 90, 120, 175, and 200 min were applied to achieve the separation of analytes in LHQW. However, it was found that shorter analysis times often resulted in less satisfactory separations. Therefore, the abovementioned chromatographic condition with better separation was finally selected to establish the QAMS method for LHQW to make the quantitative analysis results more accurate and reliable. By applying the full-wavelength scan of DAD detector, different detection wavelengths such as 205, 207, 210, 225, 230, 238, 254, 277, and 327 nm have been tried in the previous work for the analysis of the ten bioactive ingredients in LHQW. Also, the chromatograms at different wavelengths are shown in Supplementary Figure S1. In this study, the contents of ten active ingredients were determined simultaneously, and the selected detection wavelength could not satisfy the maximum absorption of different compounds at the same time, which is more common in the determination of Chinese pharmaceutical preparations. However, in order to improve the analysis efficiency and simplify the experimental process, the detection at the same wavelength seems to have certain advantages. Therefore, the experimental conditions need to adopt a wavelength that can take into account the ten bioactive ingredients, that is to say, let all the ten bioactive ingredients have good response signals at the same wavelength. Eventually, the detection wavelength was set at 207 nm. Also, the flow rate was set at 1.0 mL/min for the best separation.

2.3. Standard and Sample Solution Preparations. 12.52 mg of neochlorogenic acid (NA), 12.41 mg of amygdalin (AMY), 10.00 mg of phlorogenic acid (CA), 15.96 mg of forsythoside A (FA), 19.49 mg of quercitrin (QUE), 10.32 mg of phillyin

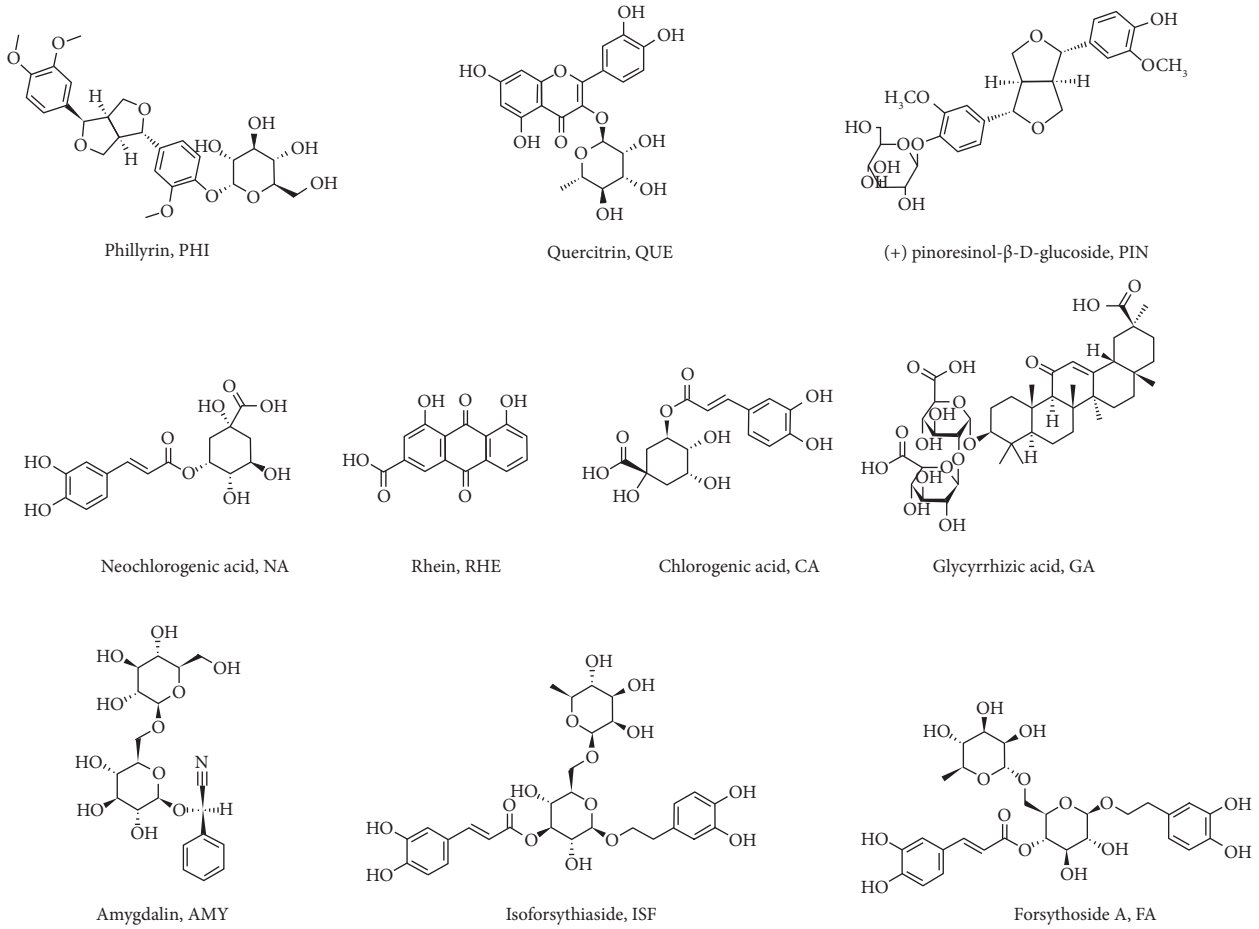

Figure 1: Chemical structures of ten bioactive compounds in LHQW.

(PHI), 18.72 mg of glycyrrhizic acid (GA), 14.50 mg of isoforsythiaside (ISF), 12.71 mg of (+) pinoresinol-β-D-glucoside (PIN), and 11.75 mg of rhein (RHE) were weighed precisely and dissolved in methanol to prepare the respective reference stock solutions. The final concentrations of each reference stock solution in order are 2.504 mg/mL, 1.241 mg/mL, 1.999 mg/mL, 3.191 mg/mL, 3.898 mg/mL, 1.032 mg/mL, 3.743 mg/mL, 2.900 mg/mL, 2.542 mg/mL, and 0.047 mg/mL.

Chromatograms of the ten active ingredients were obtained by injecting each reference stock solution separately (Supplementary Figure S2). Certain volumes of the above-mentioned individual reference stock solutions were measured accurately to obtain the following mixed standard solutions, consisting 0.1947 mg/mL of NA, 0.1053 mg/mL of AMY, 0.1321 mg/mL of CA, 1.452 mg/mL of FA, 0.1350 mg/mL of QUE, 0.3020 mg/mL of PHI, 0.1879 mg/mL of GA, 0.4688 mg/mL of ISF, 0.3319 mg/mL of PIN, and 0.0218 mg/mL of RHE.

After grinding and mixing the sample powder, approximately 1.0 g of powder of LHQW under the item of weight variation was accurately weighed and extracted with methanol by ultrasonication (200 W, 40 kHz) for 30 min. The exact weighing mass of each sample is shown in SupplementaryTable S1. After cooling to room temperature, additional methanol was added to compensate for the weight loss of extraction. Subsequently, 0.22 μm filter membranes were utilized for the final sample solution. In fact, the accuracy of determination results is of great significance to the method of QAMS and ESM; so it is necessary to assure the bioactive compositions in the sample could be fully extracted by solvent. In this study, the influences on extraction efficiency of different solvents, extraction methods, and time were investigated successively. In this study, different solvents (methanol, 50% methanol, 70% methanol, ethanol, and ethyl acetate), different extraction methods (ultrasonic extraction and reflux extraction), and different extraction times (15 min, 30 min, 45 min, and 60 min) were compared to obtain the better extraction efficiency. Finally, efficiency of 30 min ultrasonic extraction with methanol proved to have higher extraction efficiency. Also, all the standard substances were weighed accurately and dissolved in methanol. Also, all the sample solutions and standard solutions were stored at 4°C protected from light.

2.4. Calculation of Relative Correction Factors. Based on the fixed dosage of each medicinal material contained in LHQW from the immutability of prescription, there should be a certain internal proportional relationship between the contents of each component within a specific linear range. With a suitable component selected as the internal reference

substance (IRS), this intrinsic relationship between the other components to be tested relative to ISR, also known as relative correction factors (RCFs), is obtained. Accordingly, the simultaneous determination of multiple components with merely a single standard substance could be accomplished by RCFs. Also, the IRS is expected to be pharmacologically active, stable, cheap, widely available, and responding well [20]. In view of this, rhein could be regarded as the IRS because it is inexpensive and readily available while also showing by good resolution, responsivity, and stability under the chromatographic conditions of this study. More importantly, by comprehensive 2D angiotensin-converting enzyme 2 (ACE2) biochromatography, rhein was screened and identified as one of the bioactive compounds with potential ACE2 targeting ability in LHQW [21]. For the abovementioned reasons, rhein was regarded as the IRS and RCFs of the other nine ingredients were calculated by the following equation (formula (1)). Consequently, the contents of other analytes could be calculated by the following equations (formulas (2) and (3)):

$$\begin{aligned} \mathrm{RCF} &= \frac{f_i}{f_s} \\ &= f_{(i/s)} \\ &= \frac{(C_i/A_i)}{(C_S/A_S)}, \end{aligned} \tag{1}$$

$$C_i = f_{(i/s)} \times \frac{C_s}{A_S} \times A_i, \tag{2}$$

$$\omega_i = \frac{C_i \times V_i}{m_i}, \tag{3}$$

where $f_{i/s}$ represents the relative correction factor of the bioactive composition to be measured by the IRS. A_i and C_i are the peak area and concentration of the bioactive components to be measured, and A_s and C_s represent the peak area and concentration of IRS. ω_i represents the mass concentration of the analyte. V_i represents the extraction volume and m_i is the mass of the LHQW sample.

2.5. Statistical Analysis. SPSS 26.0 statistical software was employed to perform Hierarchical clustering analysis (HCA). Origin 2018 was applied for principal component analysis (PCA). Also, orthogonal partial least-squares discrimination analysis (OPLS-DA) was conducted by SIMCA 13.0. Radar plot analysis was performed by Microsoft Excel 2016.

3. Results

3.1. System Adaptability. Due to the complexity of the ingredients of LHQW and the complexity of Chinese patent medicine matrix, a gradient elution method has been developed to separate ten bioactive components utterly (in Section 2.2). In Figure 2, chromatograms of the blank solution, mixed standard solution, and sample solution illustrated that the bioactive ingredients were fully separated within 185 min. In addition, the resolution, theoretical plate number, and tailing factor of NA were 6.06, 17424, and 0.99, which met the requirements of system suitability. Also, the resolution is calculated by the following equation (formula (4)). Then, DAD detection was utilized for scanning the mixed reference standard from 200 to 400 nm. In the absorption spectrums of ten bioactive components, the maximum absorption wavelength of all compositions focused in the range of 200 nm–230 nm (Figure 3). For achieving satisfactory responses in all analytes, 207 nm was set as the final detection wavelength.

$$R = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2}, \tag{4}$$

where t_{R2} and t_{R1} represent the retention times of the component 1 and component 2, respectively. W_1 and W_2 represent the widths of the corresponding components.

3.2. Calibration Curves. The calibration curves of ten bioactive ingredients were constructed by the peak area (y) and concentration (x) with the gradient content of the mixed standard solution. The limits of quantity and detection were determined at the signal-to-noise ratios of 10 : 1 and 3 : 1. LOQs of the 10 active ingredients in this study were obtained by using the minimum concentration of the mixed standard solution in the linear range series, diluting it 10 times and injecting 20 μL for analysis. The final S/N of NA, AMY, CA, FA, QUE, PHI, GA, ISF, PIN, and RHE were obtained in the order of 36.14, 9.21,15.50, 40.15, 273.84, 119.51, 43.30, 201.21, 7.69, and 21.69, respectively. LOQs for each active ingredient were then calculated according to the limit of quantification requirement of 10 for S/N. LODs of the 10 active ingredients in this study were obtained by using the minimum concentration of the mixed standard solution in the linear range series, diluting it 25 times and injecting 20 μL for analysis. The final S/N of NA, AMY, CA, FA, QUE, PHI, GA, ISF, PIN, and RHE were obtained in the order of 16.26, 3.59, 9.30, 19.45, 109.50, 43.04, 17.31, 80.54, 4.61, and 13.04, respectively. LODs for each active ingredient were then calculated according to the detection limit requirement of S/N of 3. In Table 1, the correlation coefficients (r) of ten bioactive components ($r \geq 0.999$) manifested that the calibration curves built for the QAMS method of LHQW were acceptable and appropriate.

3.3. Method Validation. Method validation ought to be thoroughly investigated to promote the use value of the method. Intraday and interday precision were verified by six consecutive injections and two injections per day for ten consecutive days of the mixed standard solution, respectively. Also, the stability was investigated by injecting the sample solution (S19) after 0, 12, 24, 48, and 72 h to record the peak areas of ten bioactive components. The method's repeatability was examined by preparing six sample solutions (S19) in parallel. The recovery experiment was conducted by adding standard substances to the

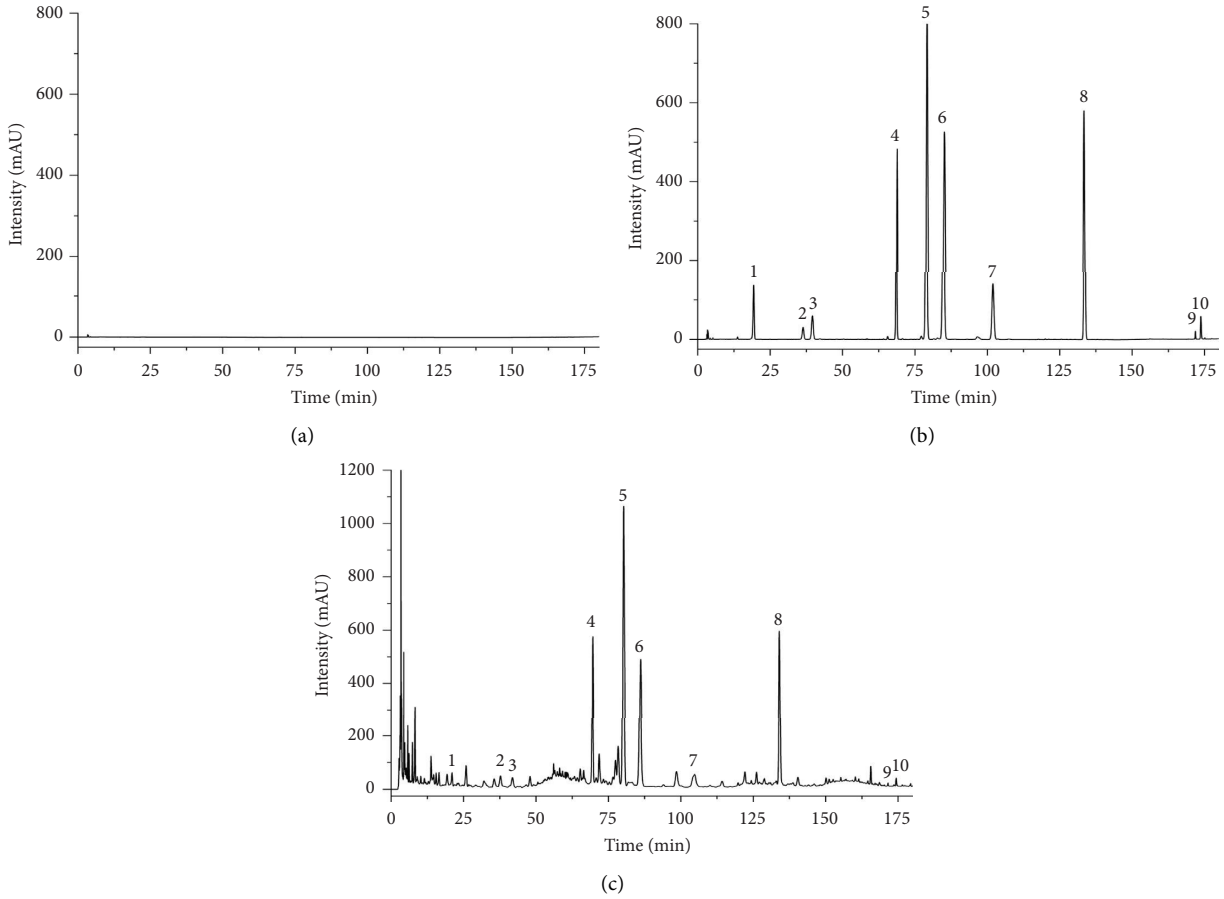

Figure 2: HPLC chromatograms of blank solution (a), mixed standard solution (b), and sample solution of LHQW (c). (1) Neochlorogenic acid; (2) amygdalin; (3) chlorogenic acid; (4) isoforsythiaside; (5) forsythoside A; (6) (+) pinoresinol-β-d-glucoside; (7) quercitrin; (8) phillyin; (9) glycyrrhizic acid; (10) rhein.

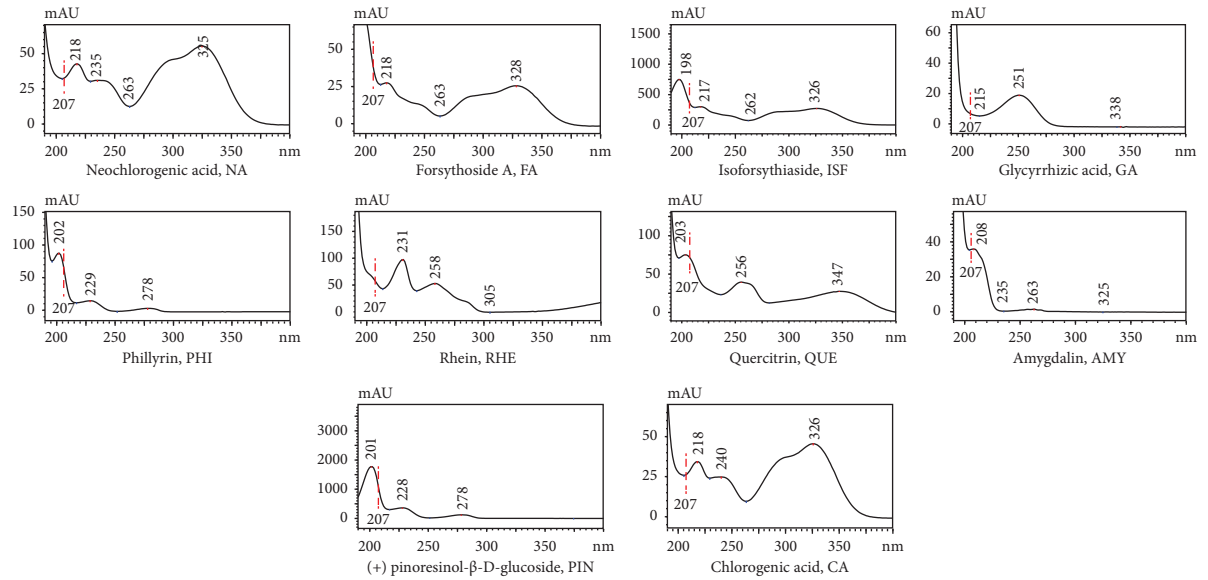

Figure 3: The UV spectrograms of ten bioactive components in LHQW.

sample solutions (S19) with a ratio of 1 : 1 for preparing six mixed solutions (R1–R6). Then, the recoveries were calculated by the analytes' peak areas to determine the method's accuracy. As shown in Table 2, the relative standard deviations (RSDs) of precision, stability, repeatability (all<5%), and the average recovery of ten

Table 1: Results of the investigation of the linear relationship, limit of detection (LOD), and limit of quantitation (LOQ).

Analytes	Regression equations	r	Linearity (mg/mL)	LOD (ng/mL)	LOQ (ng/mL)
NA	$y = 20{,}000{,}000x - 105566$	0.9997	0.019–0.487	140.2	525.8
AMY	$y = 10{,}000{,}000x + 16528$	0.9995	0.011–0.263	367.4	1194
CA	$y = 20{,}000{,}000x - 22586$	0.9995	0.013–0.330	167.8	838.8
ISF	$y = 30{,}000{,}000x + 191895$	0.9999	0.047–1.172	290.0	1160
FA	$y = 20{,}000{,}000x + 509547$	0.9996	0.145–4.355	158.9	529.5
PIN	$y = 60{,}000{,}000x - 91210$	0.9995	0.034–0.847	94.8	284.5
QUE	$y = 60{,}000{,}000x - 153093$	0.9999	0.0135–0.338	93.6	311.8
PHI	$y = 70{,}000{,}000x - 178729$	0.9999	0.030–0.754	44.7	149.1
GA	$y = 2{,}000{,}000x - 7122.5$	0.9997	0.019–0.470	494.1	2470
RHE	$y = 60{,}000{,}000x - 21663$	0.9999	0.002–0.054	18.4	92.20

Table 2: Precision, stability, repeatability, and recovery results of ten bioactive compositions in LHQW.

		NA	AMY	CA	ISF	FA	PIN	QUE	PHI	GA	RHE
Intraday precision (RSD, %, $n = 6$)		0.63	0.75	2.83	0.28	1.45	0.20	0.51	1.37	1.54	1.35
Interday precision (RSD, %, $n = 10$)		3.07	3.42	2.79	2.55	4.24	3.71	2.76	4.62	3.11	3.79
Stability (RSD, %, $n = 6$)		3.10	2.22	2.21	1.92	1.12	1.33	1.09	1.56	2.05	4.04
Repeatability (RSD, %, $n = 6$)		3.14	3.87	1.18	0.38	1.89	0.14	1.99	2.16	1.55	3.04
Recovery (%)	Mean	101.1	100.7	100.2	107.1	99.80	104.1	100.4	100.6	100.0	100.0
	RSD	0.82	1.07	1.02	2.90	1.07	2.17	0.72	1.03	1.11	0.96

bioactive components (99.8%~104.1%) demonstrated the QAMS developed for LHQW was credible, effective, and applicable.

3.4. Quantitative Analysis of Multicomponent by the Single Marker

3.4.1. The Calculations of the RCFs. Injecting mixed standard solutions at a range of injection volumes under chromatographic conditions of Section 2.2, RCFs were calculated by the equation in Section 2.4. As shown in Table 3, the average relative correction factor, corrected by six different concentrations, was ultimately utilized in the QAMS of LHQW. The RSDs of results (all<4%) showed that the relative correction factors had certain robustness to different concentrations under the same experimental conditions.

3.5. Assessment of QAMS Method for LHQW

3.5.1. Durability and Validation of RCFs. The durability of the QAMS method, verified under different experimental conditions, could demonstrate the reproducibility and stability of the resulting relative correction factors [22]. Consequently, the influence factors of the RCFs, mainly covering flow rates, column temperatures, columns, instruments, and acidity of mobile phase, were comprehensively examined with the Waters e2659 system and other conditions. As the results are shown in Table 4 and Table 5, the RSD values of RCFs under different experimental conditions were less than 5%, indicating that the QAMS method could remain durable and stable when experimental conditions change.

3.5.2. Location of the Peaks of Ten Bioactive Ingredients in LHQW. With the QAMS method extended to different laboratories, the changes in experimental conditions may influence the location of peaks. Therefore, the relative retention time (RRT) and retention time difference of ten bioactive ingredients under different conditions were recorded to choose the suitable method for the peaks' location. Results showed that only the RSD values of RRTs were less than 5% under different experimental conditions. The RRTs were more suitable for the peak location of the method (Table 6). Moreover, the existence of other compositions and isomers in LHQW would tend to inhibit the analytes' peaks from being located precisely. As a consequence, the UV absorption spectrums of the DAD detector were introduced to this study for the further precise location of peaks. However, in order to simplify the analysis steps, 207 nm was finally chosen as the detection wavelength of QAMS, which bioactive components were well separated and the response values were relatively high.

3.6. Similarity Evaluation of the Results between QAMS and ESM. As a commonly used quantitative method, ESM is applied to the determination of phillyrin in the current quality evaluation index of LHQW [5]. Therefore, the consistency of determination results by the QAMS method and ESM could evaluate the accuracy of QAMS. In order to more visually represent the differences between the results of the two methods, the content determination results of the two methods were plotted as bar charts. As shown in Figure 4, a high degree of symmetry emerged between the results of the QAMS method and the EMS method, indicating a high correlation between the results of the two methods. With applying SPSS 26.0 statistical software, no conspicuous difference ($P > 0.05$)

Table 3: Results of the calculations of the RCFs.

Injection volume/μL	RCFs								
	$f_{NA/RHE}$	$f_{AMY/RHE}$	$f_{CA/RHE}$	$f_{ISF/RHE}$	$f_{FA/RHE}$	$f_{PIN/RHE}$	$f_{QUE/RHE}$	$f_{PHI/RHE}$	$f_{GA/RHE}$
1	2.472	4.399	2.538	1.830	2.419	0.8707	0.9300	0.7779	36.07
3	2.535	4.465	2.588	2.034	2.464	0.8838	0.9486	0.7916	36.15
5	2.504	4.467	2.561	1.967	2.450	0.8935	0.9402	0.7904	36.09
10	2.496	4.389	2.609	1.984	2.541	0.9590	0.9380	0.7952	35.19
15	2.522	4.645	2.762	2.038	2.520	0.9304	0.9541	0.8152	35.77
20	2.542	4.503	2.609	2.009	2.623	0.9561	0.9516	0.8198	35.07
25	2.452	4.370	2.665	2.066	2.627	0.9321	0.9342	0.8042	35.99
Mean	2.503	4.463	2.619	1.990	2.521	0.9179	0.9424	0.7992	35.76
RSD (%)	1.32	2.10	2.86	3.93	3.28	3.85	0.97	1.85	1.26

Table 4: Results of RCFs on different instruments and columns.

Instruments	Columns	RCFs								
		$f_{NA/RHE}$	$f_{AMY/RHE}$	$f_{CA/RHE}$	$f_{ISF/RHE}$	$f_{FA/RHE}$	$f_{PIN/RHE}$	$f_{QUE/RHE}$	$f_{PHI/RHE}$	$f_{GA/RHE}$
Shimadzu LC-20AT	Zorbax reverse phase C18	2.675	4.590	2.663	1.975	2.440	0.8012	0.9738	0.8086	36.05
	InertSustain C18	2.674	4.696	2.806	2.033	2.597	0.8737	0.9902	0.8434	38.13
	Hypersil BDS C18	2.554	4.514	2.599	2.048	2.460	0.9215	0.9506	0.8474	34.44
Waters e2659	Zorbax reverse phase C18	2.496	4.389	2.609	2.238	2.541	0.8275	0.9380	0.7952	35.19
	InertSustain C18	2.639	4.649	2.701	2.114	2.623	0.8593	0.9920	0.8229	36.72
	Hypersil BDS C18	2.414	4.266	2.486	2.199	2.387	0.8554	0.9068	0.7702	35.86
	Mean	2.575	4.517	2.644	2.101	2.508	0.8564	0.9585	0.8146	36.07
	RSD (%)	4.13	3.63	4.08	4.84	3.74	4.79	3.47	3.63	3.54

Table 5: Results of RCFs of different flow rates, column temperatures, and acidity of the mobile phase.

Influence factors		$f_{NA/RHE}$	$f_{AMY/RHE}$	$f_{CA/RHE}$	$f_{ISF/RHE}$	$f_{FA/RHE}$	$f_{PIN/RHE}$	$f_{QUE/RHE}$	$f_{PHI/RHE}$	$f_{GA/RHE}$
Flow rate (mL/min)	0.8	2.425	4.285	2.507	2.281	2.376	1.076	0.9005	0.7666	36.18
	1	2.496	4.389	2.609	2.195	2.541	1.015	0.9380	0.7952	35.19
	1.2	2.419	4.264	2.501	2.308	2.413	1.065	0.9133	0.7728	37.84
	Mean	2.446	4.313	2.539	2.261	2.443	1.052	0.9173	0.7782	36.41
	RSD (%)	1.75	1.56	2.40	2.61	3.55	3.09	2.08	1.93	3.67
Column temperature (°C)	29	2.604	4.591	2.669	2.041	2.572	0.8321	0.9817	0.8320	34.63
	30	2.639	4.649	2.701	2.195	2.623	0.8012	0.9920	0.8229	36.28
	31	2.541	4.456	2.601	2.220	2.509	0.8777	0.9578	0.8114	34.34
	Mean	2.595	4.565	2.657	2.152	2.568	0.8370	0.9772	0.8221	35.08
	RSD (%)	1.91	2.16	1.91	4.51	2.21	4.60	1.80	1.25	2.98
Phosphoric acid aqueous solution	0.05%	2.549	4.486	2.614	2.135	2.541	0.8808	0.9559	0.8161	34.79
	0.10%	2.496	4.389	2.609	1.975	2.523	0.8012	0.9380	0.7952	35.19
	0.15%	2.415	4.244	2.472	2.077	2.388	0.8285	0.9050	0.7736	36.94
	Mean	2.486	4.373	2.565	2.063	2.484	0.8369	0.9330	0.7950	35.64
	RSD (%)	2.70	2.78	3.15	3.92	3.37	4.83	2.77	2.67	3.21

occurred in the results of the QAMS method and ESM. Therefore, the QAMS method established for LHQW was reliable and feasible for the determination of multicompound. In addition, the content levels of ten active ingredients showed that FA, ISF, PIN, GA, PHI, and AMY had relatively high mean contents (≥0.51 mg/g). Also, the abovementioned bioactive components are the main components of the herbs from which they are derived. ISF, PIN, FA, and PHI are derived from *Forsythiae Fructus*, GA from *Glycyrrhizae Radix Et Rhizoma*, and AMY from stir-fried *Armeniacae Semen Amarum*. The high levels of content reflected in these bioactive ingredients may be related to the prescribed dosage of each herb, such as the higher dosage of *Forsythiae Fructus*, or may also be associated with the choice of detection wavelength. Meanwhile, the RE values of the content results of the ten bioactive ingredients in 20 batches of samples were all less than 5%, indicating the accuracy of the QAMS method for multicomponent determination.

3.7. Chemical Recognition Patterns Method

3.7.1. Hierarchical Clustering Analysis (HCA). Based on the proximity of properties between samples, hierarchical clustering analysis (HCA) clusters samples with similar properties into one class first and samples with far different properties into classes later. Also, the procedure proceeds sequentially until the clustering ends when the expected number of classes or classes are merged [23]. With the contents of ten active ingredients of

Table 6: Relative retention times (RRTs) of analytes by different instruments and columns.

Instruments	Columns	RRT								
		NA/RHE	AMY/RHE	CA/RHE	ISF/RHE	FA/RHE	PIN/RHE	QUE/RHE	PHI/RHE	GA/RHE
Shimadzu	Zorbax reverse phase C18	0.121	0.247	0.262	0.395	0.505	0.488	0.655	0.812	0.982
	InertSustain C18	0.114	0.246	0.253	0.399	0.506	0.496	0.643	0.810	0.982
	Hypersil BDS C18	0.110	0.238	0.256	0.393	0.490	0.553	0.657	0.802	0.975
Waters	Zorbax reverse phase C18	0.120	0.225	0.249	0.377	0.465	0.495	0.615	0.769	0.980
	InertSustain C18	0.120	0.234	0.244	0.408	0.472	0.505	0.608	0.778	0.985
	Hypersil BDS C18	0.116	0.224	0.249	0.402	0.459	0.502	0.634	0.771	0.986
	Mean	0.117	0.236	0.252	0.396	0.483	0.507	0.635	0.790	0.982
	RSD (%)	3.55	4.13	2.47	2.63	4.20	4.65	3.18	2.49	0.39

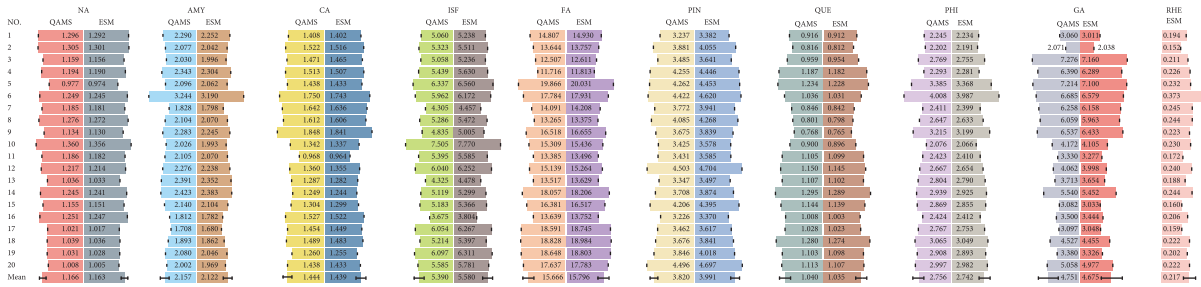

Figure 4: The bar charts of determination results by QAMS and ESM (mg/g).

20 batches of LHQW samples imported into the SPSS 26.0 software for performing HCA, the original data matrix was established by taking the square Euclidean distance as the measure with intergroup connection method to evaluate the quality differences between different batches of samples. The choice of the Euclidean distance usually needs to be based on the characteristics of the samples under different clusters. As shown in Figure 5, when the Euclidean distance was chosen 10, 20 batches of LHQW samples were divided into three main groups, and the production dates were closer between the samples under each group. Therefore, it seems that the Euclidean distance selection of 10 is more meaningful. As shown in Figure 5, a dendrogram drawn by the contents of ten bioactive components clearly showed that 20 batches of LHQW samples were divided into three main groups. Group one was composed of eight batches of LHQW samples (S5-6, S9, S14, and S17–20) and were marked in red in Figure 5. Group two marked in blue contained four batches of LHQW samples (S3-4 and S7-8). The rest samples were classified into the third group marked in green (S1-2, S10–13, and S15–16). The classification result showed that samples with similar production dates were grouped within different groups (e.g., S2 and S14). Therefore, the correlation between the classification results and the production date period was low. Since it is impossible to guarantee the production conditions are exactly the same in every production, it is reasonable to have some quality differences between samples from batch to batch. In addition, differences in the quality of the samples may be influenced by the source of the herbs, the production process, and the conditions of transport and storage. As a result, there were likely differences in sample quality between different production batches. However, no further information on specific quality differences from G1 to G3 could be provided by HCA. Consequently, radar plot analysis was introduced for further quality analysis of LHQW samples.

3.7.2. Radar Plot Analysis. With the content of ten active ingredients used as indicator values and expressed on the corresponding indicator axes of the radar chart in turn, a radar diagram with a certain shape was formed by connecting the numerical points of the indicators on different axes with a straight line. Also, it is used to reflect visually and graphically the difference and variations in the quality of different groups of samples on a two-dimensional plane [24]. The average contents of the bioactive ingredients of G1, G2, and G3 and the total batch of samples were imported into Origin 2018 software in turn for radar plot analysis. The results are shown in Figure 6, and there was a certain similarity in the content distribution of the three groups and the whole. Regarding radar chart shape, the shape of Figure 6(b) was highly consistent with Figure 6(a), while Figure 6(d) and Figure 6(a) had some differences. It confirmed that the sample quality of G1 was more similar to the overall quality and there were some differences in the quality of G3 and the overall. The more similar the quality to the overall sample, the better the quality consistency is likely to be. Therefore, the G1 seemed to have better quality consistency in comparison. Figure 6(e) shows a superimposition of the three radar plots of G1, G2, and G3, indicating some differences between the three radar plots of G1–G3. The results showed that the distribution of active ingredients' contents in the three groups was inconsistent, thus validating the accuracy and reliability of HCA classification

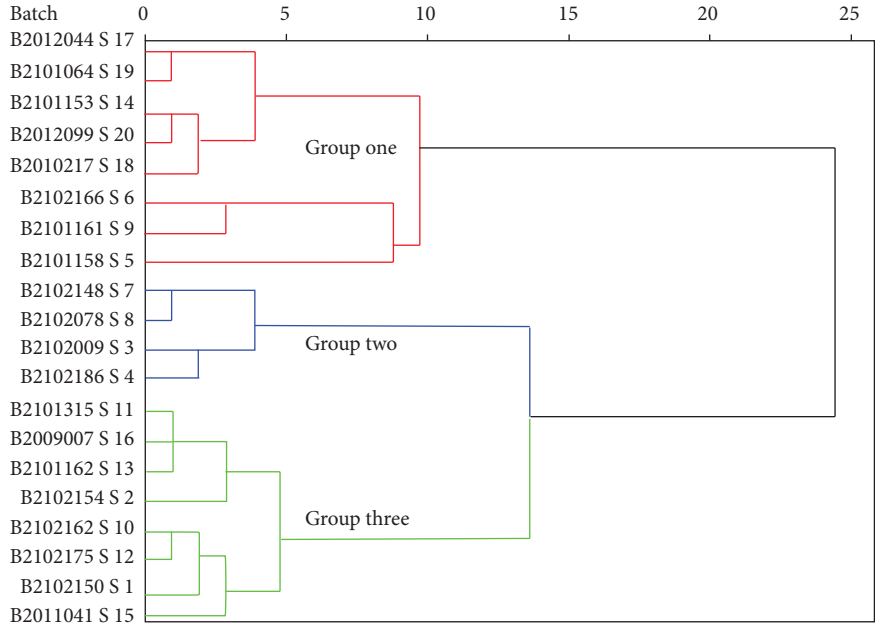

Figure 5: Dendrograms of hierarchical cluster analysis of 20 batches of samples of LHQW.

results. Further comparative analysis revealed that the differences in the radar plots of the three groups were mainly caused by differences in the content of GA, FA, ISF, and PHI. Meanwhile, the content levels of FA and ISF decreased in G1, G3, and G2, GA decreased in G2, G1, and G3, and the content levels of PHI were higher in G1 than in G2 and G3. The content of the other components did not show significant differences between the three groups. Results suggested that these four bioactive ingredients, which varied considerably in content among the three groups, may have the potential to become primary quality markers.

3.7.3. Principal Component Analysis (PCA). As a widely used multivariate statistical analysis method, principal component analysis (PCA) can be utilized to extract the main component features from multiple indicators that have some correlation by dimensionality reduction of the data [25]. For further analysis of sample quality, PCA was applied to observe the quality of the samples within each group and to verify the accuracy of HCA results. The contents of ten active ingredients of the 20 batches of LHQW samples were used as variables and imported into Origin 2018 software for PCA. As shown in Figure 7(a), sample points were all within the 95% confidence interval, indicating that the overall quality of the 20 batches of LHQW samples was stable. In addition, G2 and G3 had a more concentrated distribution of sample points than G1. In PCA, the closer the distance between sample points, the higher the quality similarity between samples. Therefore, the quality of samples within G2 and G3 was more consistent, while there were some differences between samples within G1. Therefore, to better ensure uniformity of drug quality, the pharmaceutical manufacturers need to focus on samples that show large quality differences and trace the causes of these differences. Furthermore, supervised statistical methods of discriminant analysis can be applied for further analysis to screen the components that cause differences in quality.

3.7.4. Orthogonal Partial Least-Squares Discrimination Analysis (OPLS-DA). With active ingredient content data imported into SIMCA 13.0 software, orthogonal partial least-squares discrimination analysis (OPLS-DA) was employed to find the main markers that caused quality differences. With the separation between groups improved, OPLS-DA model could be used for supervised chemical pattern recognition analysis [25]. As shown in Figure 7(b), all 20 batches of samples were within the 95% confidence interval in the score scatter plot of OPLS-DA, proving the stability of all samples. Also, all samples were divided into three main groups, in general agreement with the results of HCA, which verified the reliability and accuracy of the classification result of HCA. In Figure 7(c), since its VIP value > 1 and the error bar ranged at $X > 0$, FA could be identified as the component that contributed significantly to the quality difference. Also, in Figure 7(d), FA was the farthest from the origin, so it is proved that FA, with definite pharmacological effects, was most likely to be the main marker that caused quality differences. In addition, in a study on the active ingredients screening of LHQW, it was found that components such as FA could bind to angiotensin-converting enzyme 2 (ACE2), which is closely related to the mechanism of action of coronavirus drug treatment and significantly inhibits its activity [19]. Therefore, FA could be considered as a potential bioactive component in LHQW. More importantly, the result was consistent with the conclusion in the radar chart analysis (in Section 3.7.2). It is suggested that the manufacturer of LHQW should pay more attention to the source of *Forsythiae Fructus*, the main ingredient of which is FA, to better ensure the stability and homogeneity of drug quality.

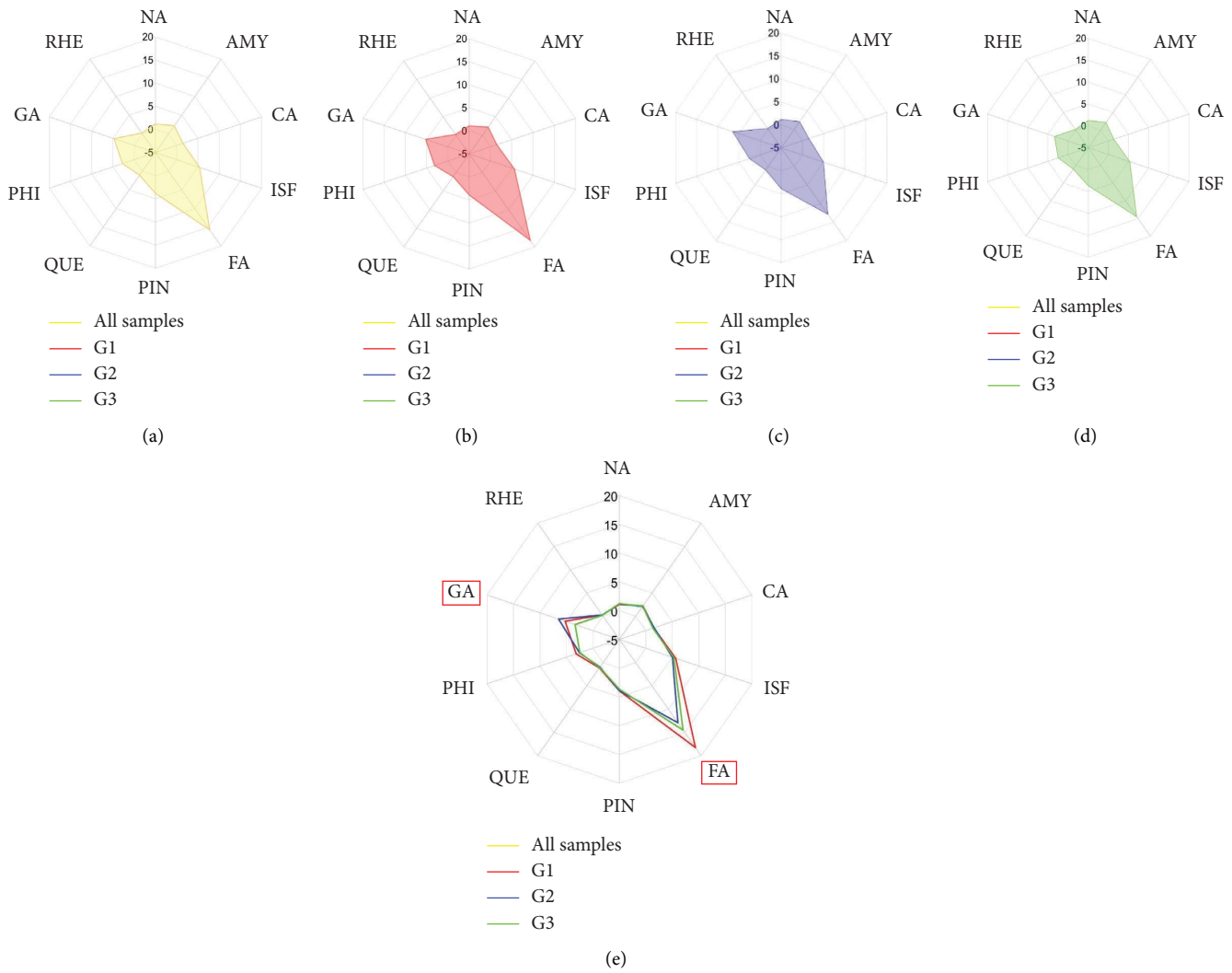

Figure 6: Radar plots showing the difference in LHQW samples within G1–G3: (a–d) the distribution of all samples, G1, G2, and G3, respectively; (e) the distribution of ten bioactive component patterns in three groups.

4. Discussion

QAMS enables the simultaneous determination of multiple components by a mere single standard substance in TCMs, which is highly suitable for the quality evaluation of TCMs. Compared to general multicomponent measurement methods that require the simultaneous purchase of 10 components, the QAMS method established for LHQW requires only one component reference substance as the IRS. In this way, QAMS method can largely reduce the cost of study generated by the purchase of reference substances and has the advantage of low cost. Due to its low-cost, apparent pharmacological activity, and chemical stability, rhein was selected as the internal standard substance to calculate the RCFs of the other nine bioactive ingredients. The QAMS method established for LHQW can largely reduce the cost generated by the purchase of reference substances. Therefore, with the advantages of feasibility, lower cost, and higher effectiveness, QAMS could optimize the quality evaluation system of LHQW.

The DAD detector offers unique advantages in the simultaneous determination of the content of multiple active ingredients in TCMs. Considering that DAD detector provides UV spectrogram of each component with a certain degree of specificity, it can assist the reference standards in qualitative analysis of the components in the sample. In addition, DAD detector can be applied to select a wavelength suitable for multicomponent content determination to facilitate the establishment of the QAMS method. More importantly, once the QAMS method has been established, the quality evaluation can be carried out by HPLC-UV with single wavelength detection, which could be used as a method reference for pharmaceutical companies, medical product administration, and researchers interested in the quality assessment of LHQW for quality monitoring, evaluation, and control.

Furthermore, the application of chemical recognition patterns method could better evaluate and classify the quality of different batches of LHQW samples. To analyze

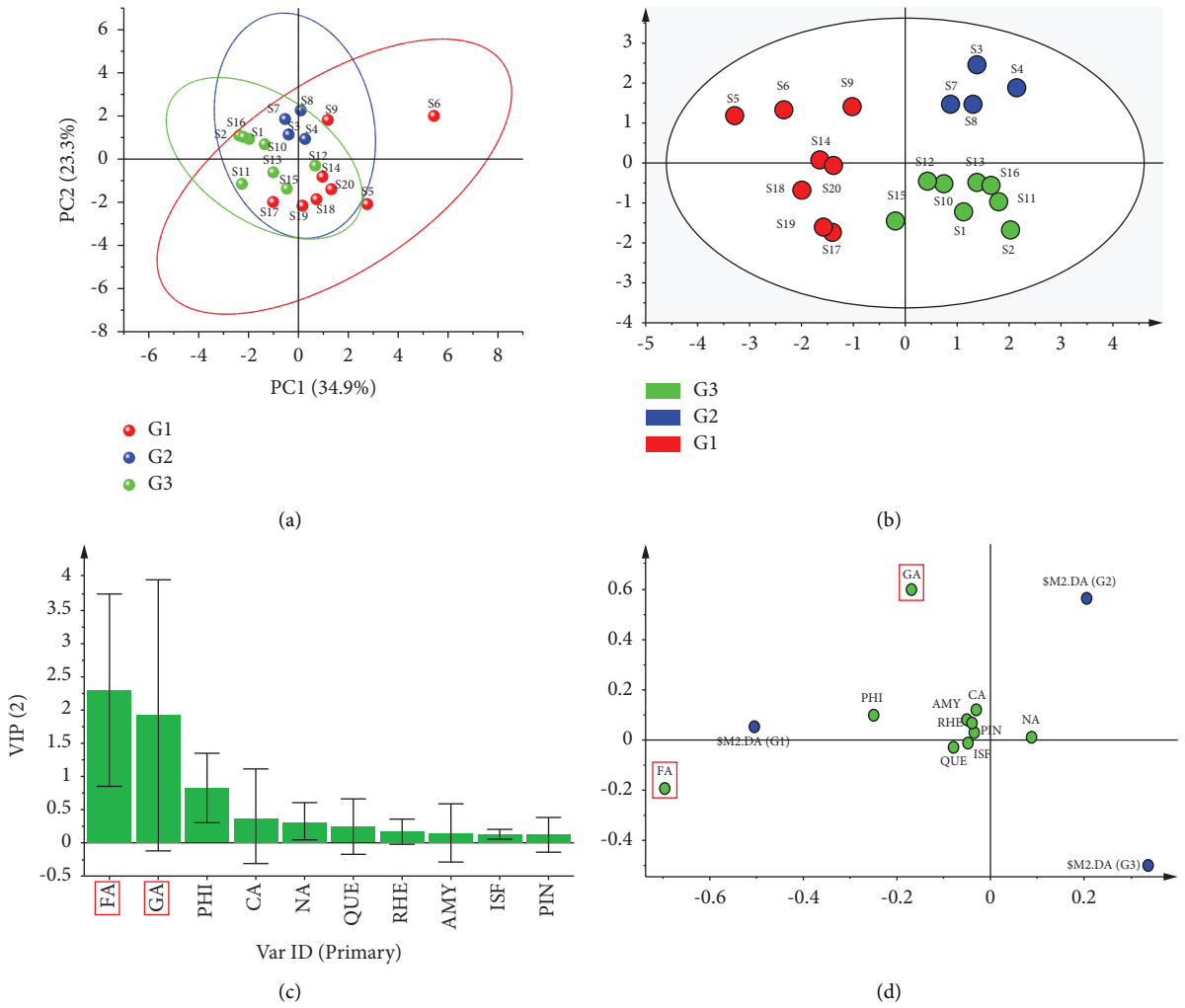

Figure 7: Technical analysis in 20 batches of Lianhua Qingwen capsule (LHQW): (a) score plot of principal component analysis (PCA), (b) score scatter plot of orthogonal partial least-squares discrimination analysis (OPLS-DA), (c) variable importance plot (VIP) of OPLS-DA, and (d) loading scatter plot of OPLS-DA.

the quality differences among the 20 batches of samples and classify them by the appropriate classification method, HCA, radar plot analysis, PCA, and OPLS-DA were applied to further analysis of experimental data. The results of HCA and PCA showed that 20 batches of LHQW samples were divided into three main groups. It indicated that samples showed some quality differences, which were related to the source of the herbs, the production process, and the conditions of transport and storage. Also, OPLS-DA and radar plot analysis revealed that the quality differences between three groups of samples were mainly caused by FA, which is derived from the medicinal herb *Forsythiae Fructus*. It suggested that pharmaceutical companies need to focus on controlling the quality of herbs, such as *Forsythiae Fructus*, so as to further reduce the possibility of quality difference and ultimately guarantee the effectiveness and quality consistency of LHQW.

5. Conclusion

In this study, a strategy of QAMS was developed to determine the contents of NA, AMY, CA, FA, QUE, PHI, GA, ISF, PIN, and RHE simultaneously in 20 batches of LHQW samples by HPLC. The accuracy of qualitative analysis of the components can be improved as HPLC-DAD provides UV spectrograms with a certain degree of specificity. In addition, HPLC-DAD simplified experimental operations and improved analytical efficiency by selecting a suitable detection wavelength for QAMS. The simultaneous determination of ten important pharmacological active ingredients by a single detection wavelength and a single component reference substance could simplify experimental operations, reduce research costs, and improve analytical efficiency. QAMS enables the simultaneous determination of multiple components by a mere single standard

substance. With RHE being used as the IRS, the RCFs of other ingredients relative to RHE were calculated to obtain the contents of nine active ingredients. Therefore, the QAMS of LHQW was established to achieve the simultaneous determination of the contents of ten bioactive ingredients. Under the comparison of the determination results of QAMS and ESM, there was no evident difference between the two results ($P > 0.05$). However, compared to ESM method, the QAMS method allows for the determination of multiple bioactive ingredients without the need to purchase reference substances of all analytes, thus greatly reducing the cost of the study. Therefore, QAMS method of LHQW could offer the advantages of being economical, accurate, durable, and efficient. In addition, HCA, radar plot analysis, PCA, and OPLS-DA in the chemical recognition patterns method were applied to further analyze the quality differences of different batches of LHQW samples. The results showed that the quality difference of herbs such as *Forsythiae Fructus* may be the main source of quality difference among different batches of LHQW samples. Therefore, the established QAMS method can be comprehensive and scientifically evaluate the quality consistency of LHQW.

Abbreviations

LHQW: Lianhua Qingwen capsule
TCMs: Traditional Chinese medicines
RA: Radix Astragali
COVID-19: Coronavirus disease 2019
QAMS: Quantitative analysis of multicomponents by single marker
HPLC-DAD: High performance liquid chromatography with the diode array detector
IRS: Internal reference substance
RCFs: Relative correction factors
ESM: External standard method
HCA: Hierarchical clustering analysis
PCA: Principal component analysis
OPLS-DA: Orthogonal partial least-squares discrimination analysis
NA: Neochlorogenic acid
AMY: Amygdalin
CA: Chlorogenic acid
FA: Forsythoside A
QUE: Quercitrin
PHI: Phillyin
GA: Glycyrrhizic acid
ISF: Isoforsythiaside
PIN: (+)-Pinoresinol-β-D-glucoside
RHE: Rhein
RRT: Relative retention time.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Qianqian Zhou and Lin Yang contributed equally to this work.

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Supplementary Materials

Table S1: Detailed information of 20 batches of LHQW samples. (*Supplementary Materials*)

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