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Development of a Quantitative Chromatographic Fingerprint Analysis Method for Sugar Components of Xiaochaihu Capsules Based on Quality by Design Concept

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Abstract: Background: Xiaochaihu capsule is composed of seven traditional Chinese medicines. The pharmacopoeia only focuses on the quantitative detection of baicalin, which cannot fully reflect the quality of the preparation. Some medium polar components were used to establish the fingerprint of Xiaochaihu capsule, but there was no report on the strong polar components. Methods: A high performance liquid chromatography-corona charged aerosol detection technology was used to establish a fingerprint analysis method for Xiaochaihu capsules following an analytical quality by design approach. Definitive screening designed experiments were used to optimize the method parameters. A stepwise regression method was used to build quantitative models. The method operable design region was calculated using the experimental error simulation method. Plackett–Burman designed experiments were carried out to test robustness. Results: The contents of four components were simultaneously determined. There were seven common peaks in the fingerprint. The common peak area accounted for 91.72%. Both fingerprint and quantitative analysis methods were validated as applicable in the methodology study. The quantitative fingerprint analysis method for sugar components can fill the gap in the detection of strong polar components in the existing methods. It provides a new technology for the comprehensive overall evaluation of Xiaochaihu capsule.

Keywords: quality by design; Xiaochaihu capsule; quantitative chromatographic fingerprint; design space; definitive screening design; Plackett–Burman

Citation: Lan, J.; Wu, G.; Wu, L.; Qu, H.; Gong, P.; Xie, Y.; Zhou, P.; Gong, X. Development of a Quantitative Chromatographic Fingerprint Analysis Method for Sugar Components of Xiaochaihu Capsules Based on Quality by Design Concept. *Separations* **2023**, *10*, 13. https://doi.org/10.3390/separations10010013

Academic Editors: Marcello Locatelli and Aleksandra Mišan

Received: 11 November 2022
Revised: 10 December 2022
Accepted: 23 December 2022
Published: 26 December 2022

1. Introduction

In recent years, analytical quality by design (AQbD) has been successfully used in the development of analytical methods for drug discovery [1–7]. AQbD allows analytical methods to be adapted within the method operable design region (MODR). The changes in analytical parameters within MODR do not affect method validity, which meets the method objectives [8–10]. AQbD facilitates the development of robust, effective, and economical analytical methods for the entire product life cycle. This promotes flexibility in the regulatory process of analytical methods [11–13]. AQbD implementation steps include determining the analytical method objective profile, identifying critical method attributes (CMAs) and critical method parameters, establishing mathematical models and MODR, conducting method validation, implementing control strategies, etc. [14–16].

Xiaochaihu capsule is composed of *Bupleurum* root, *Scutellaria* root, *Glycorrhiza* root and rhizome, *Codonopsis* root, jujube fruit, fresh ginger rhizome, and *Pinellia* rhizome prepared with ginger and aluminum. It is prepared by decoction, percolation, concentration, drying,

mixing, granulation, and capsule filling. It can be used for the treatment of symptoms of exogenous diseases, such as bitter fullness in the chest, loss of appetite, irritability and vomiting, bitter mouth, and dry throat [17–19]. High performance liquid chromatography (HPLC) is used to determine the content of baicalin in Xiaochaihu capsules for quality control in the 2020 edition of the Chinese Pharmacopoeia Vol. 1. The qualitative identification of *Bupleurum* root, liquorice root, and *Glycyrrhiza* root and rhizome is also used as reference herbs [20]. Xiaochaihu capsules are made from seven medicinal materials; therefore, it is obvious that the quantitative determination of baicalin cannot fully reflect Xiaochaihu capsule quality.

Simultaneous quantitative determination of multi-indicator components is more often used in the 2020 edition of the Chinese Pharmacopoeia Vol. 1. It is a widely accepted and feasible method for testing the quality of traditional Chinese medicine (TCM) [21,22]. Fingerprinting is another effective method for the detection of TCM preparations. Similarity and other indicators are used to reflect the overall spectral or peak information of fingerprints [23]. Recently, fingerprint technology has been greatly developed [24–26]. Zhang Xue et al. established a quantitative fingerprint analysis method of the moderately polar components such as glycyrrhizin, baicalin, and chaihu saponin B1 in Xiaochaihu granules [27]. Liu Aoxue et al. also determined the moderately polar components of saponins in Xiaochaihu granules using quantitative analysis of multi-components by a single marker [28]. However, there is no quantitative fingerprint analysis of the sugar components of Xiaochaihu capsules [29].

Because sugar components from Chinese herbs can be easily extracted in the process of decocting with water, they are often the main components of Chinese patent medicines. The chemical composition of Xiaochaihu capsules can be reflected more comprehensively by the detection of sugar components. Stachyose is a functional oligosaccharide [30]. It is naturally found in the Lamiaceae herbs [31]. It can significantly promote the value of beneficial intestinal flora in humans [32]. *Scutellaria* root belongs to the Lamiaceae. *Scutellaria* root is regarded as the minister drug of Xiaochaihu capsules. This may explain the effects of Xiaochaihu capsules in treating loss of appetite, irritability, and vomiting. Ribitol is a reduction product of D-ribose. It is present in the *Bupleurum* root in its free state. It is a characteristic component of *Bupleurum* root. The detection of ribitol can reflect the presence of *Bupleurum* root. This can be used as a supplement to the existing quality control methods of the Pharmacopoeia.

As strongly polar components, sugar components are difficult to analyze with conventional reversed-phase columns. They usually show weak UV absorption. These reasons result in more difficulties in the analysis of sugar components than that of moderately polar components. Therefore, Amino columns and hydrophilic columns are often used to separate sugar components, such as the Prevail Carbohydrate ES (250 mm × 4.6 mm, 5 µm) of Garce Alltech [33], Asahipak NH2P-50 4E column (4.6 × 250 mm, 5 µm) of Shodex [34], and XBridge BEH Amide XP column (3 × 150 mm, 2.5 µm) of Waters [35].

Recently, several researchers have used charged aerosol detection (CAD) to detect sugar components [36–38]. The detection principle of CAD is as follows. The eluent is formed into particles by atomization. Compared to evaporative light-scattering detector, CAD has higher sensitivity, better reproducibility, and a wider linearity range [39]. Thus, CAD is expected to achieve a better separation with a lower detection limit in the quantitative analysis of sugar components of the Xiaochaihu capsule.

In this work, AQbD was used to establish a fingerprint analysis method for Xiaochaihu capsules. Parameters were determined. Definitive screening design (DSD) was used to investigate the relationships between CMAs and method parameters. A stepwise regression method was used to build quantitative models between CMAs and method parameters. The experimental error simulation method was used to calculate and verify the probability-based MODR. After optimizing the analysis conditions, the content determination components were identified, and the quantitative fingerprint was established. Finally, the durability of the analytical method was investigated.

2. Materials and Reagents

Acetonitrile was purchased from Merck (chromatographic purity, Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q purification system (Millipore, Billerica, MA, USA). Ribitol (Lot No. 210916, HPLC > 99%), fructose (Lot No. 210519, HPLC > 99%), sucrose (Lot No. 210620, HPLC > 99%), stachyose (Lot No. 211026, HPLC > 99%), glucose (Lot No. 210917, HPLC > 99%), maltose (Lot No. 2110522, HPLC > 99%), and raffinose (Lot No. 210528, HPLC > 99%) were purchased from Shanghai Ronghe Pharmaceutical Technology Development Co., Ltd. (Shanghai, China).

There were 12 batches of Xiaochaihu capsule samples. The specific source merchant and lot number information are shown in Table S1.

3. Methods

3.1. Sample Preparation

3.1.1. Preparation of the Chemical Reference Solution

The four chemical reference substances were weighed precisely (AB204-N, Mettler Toledo, Zurich, Switzerland) and dissolved in 10 mL 60% acetonitrile solution. Four kinds of single-standard solution were pipetted into the same 50 mL volumetric flask. 60% acetonitrile solution was used for constant volume. The mixed reserve standard solution was diluted 5 times to obtain the mixed standard solution. The mixed standard solution was composed of 0.6512 mg/mL ribitol, 2.189 mg/mL fructose, 1.636 mg/mL sucrose, and 0.6767 mg/mL stachyose.

3.1.2. Preparation of the Sample Solution

The contents of Xiaochaihu capsules were weighed precisely and dissolved in 25 mL 60% acetonitrile solution. After being ultrasonically heated (LMTD15, Lumiere Tech, Beijing, China) and centrifuged (Minispin, Eppendorf, Hamburg, Germany), the supernatant was separated from the solution to obtain the sample solution.

3.2. HPLC Analysis

All HPLC analyses were performed on a Dionex Ultimate 3000 system (Thermo Fisher, Waltham, MA, USA) equipped with an SRD-3600 degasser, an HPG-3400RS pump, a WPS-3000TRS autosampler, a TCC-3000RScolumn thermostat, a photodiode array detector, and a Corona VeoRS CAD. The evaporation temperature was set at 35 °C. Chromatographic separation was carried out on an Asahipak NH2P-50 4E column (4.6 × 250 mm, 5 μm). The column temperature was set at 30 °C. The injection volume was set at 10 μL. The mobile phase consisted of solvent A (water) and solvent B (acetonitrile). The gradient elution program was as follows: 0–10 min, 78–74% B; 10–28 min, 74–50% B; 28–33 min, 50% B. The flow rate was set at 0.6 mL/min.

3.3. Experimental Design

3.3.1. DSD Experiment

Potential critical method parameters were identified with a fishbone diagram, as shown in Figure S1. In Figure 1, an improved AObD process was proposed based on the characteristics of traditional Chinese medicine. Based on the preliminary experiment results, the gradient, column temperature (X_5), and flow rate (X_6) were selected as potential critical method parameters for the experimental design. The other elution conditions are described in Section 3.2.

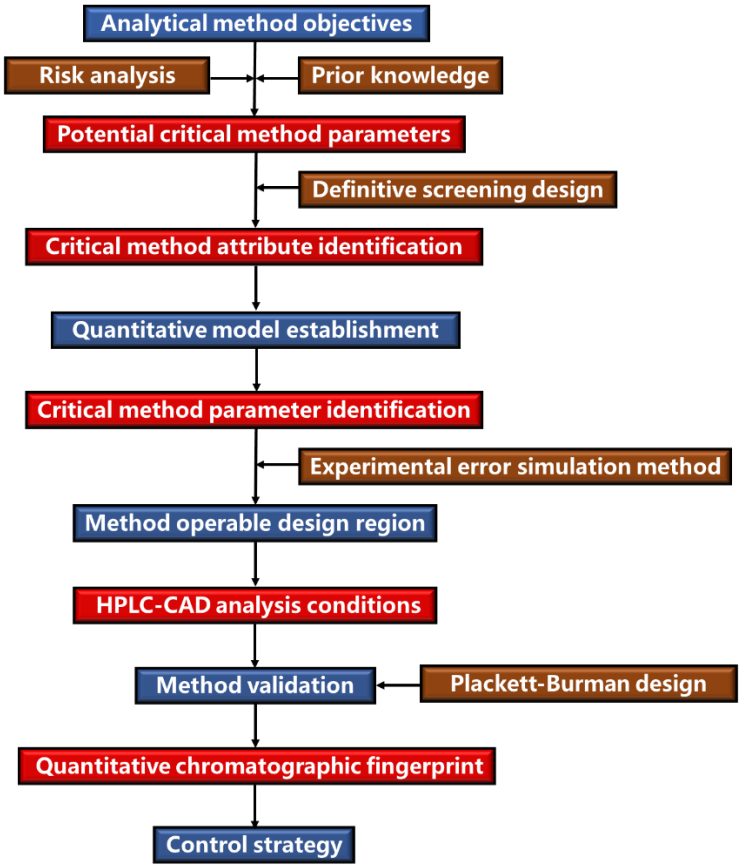


Figure 1. The AQbD process of this work.

As shown in Table 1, the mobile phase gradient was designed as 3 gradients involving 4 parameters (X_1–X_4).

Table 1. HPLC gradient conditions.

t/min	B%
0	X_1
X_2	X_3
X_4	50
$X_4 + 5$	50

A DSD method was employed to determine the relationships between the factors (X_1–X_6) and the response variables. After preliminary experiments, the levels of the factors were defined. The coded and uncoded values of each factor are summarized in Table 2. The CMAs were the peak number (Y_1), percentage of common peak (Y_2), and retention time of the last peak (Y_3). The center point was repeated 3 times. There were 2 additional dummy factors. The total number of experiments was 20. The specific experimental conditions are shown in Table 3.

Table 2. Factors and levels of DSD.

Level	Phase B Content in Mobile Phase at 0 min X_1/%	Closing Time of the First Gradient X_2/min	Phase B Content in Mobile Phase at the Beginning of the Second Gradient X_3/%	Closing Time of the Second Gradient X_4/min	Column Temperature X_5/°C	Flow Rate X_6 /(mL/min)
−1	78.0	8.0	71.0	28.0	26.0	0.60
0	80.0	10.0	73.0	30.0	28.0	0.70
1	82.0	12.0	75.0	32.0	30.0	0.80

Table 3. Experimental conditions and results of DSD.

Run	X_1/%	X_2/min	X_3/%	X_4/min	X_5/°C	X_6/(mL/min)	Y_1	Y_2/%	Y_3/min
1	78.0	8.0	71.0	30.0	30.0	0.60	15	88.86	24.43
2	82.0	10.0	75.0	28.0	30.0	0.60	15	85.26	27.18
3	78.0	10.0	71.0	32.0	26.0	0.80	12	89.63	22.92
4	80.0	10.0	73.0	30.0	28.0	0.70	11	91.21	25.27
5	82.0	8.0	71.0	32.0	28.0	0.80	12	90.45	21.88
6	80.0	12.0	75.0	32.0	30.0	0.80	9	93.88	26.83
7	80.0	8.0	71.0	28.0	26.0	0.60	12	92.43	24.03
8	82.0	12.0	71.0	28.0	26.0	0.70	11	92.16	24.95
9	82.0	12.0	75.0	30.0	26.0	0.80	11	91.93	26.29
10	78.0	8.0	75.0	28.0	26.0	0.80	10	92.51	22.27
11	82.0	12.0	71.0	32.0	30.0	0.60	14	88.22	27.41
12	78.0	12.0	75.0	28.0	28.0	0.60	14	89.58	27.98
13	78.0	8.0	75.0	32.0	30.0	0.70	12	90.51	25.73
14	82.0	8.0	73.0	28.0	30.0	0.80	14	88.44	22.09
15	78.0	12.0	71.0	28.0	30.0	0.80	12	90.4	23.19
16	78.0	12.0	73.0	32.0	26.0	0.60	13	89.47	28.68
17	82.0	8.0	75.0	32.0	26.0	0.60	17	86.24	27.78
18	80.0	10.0	73.0	30.0	28.0	0.70	11	91.59	25.25
19	80.0	10.0	73.0	30.0	28.0	0.70	11	91.32	25.25
20	80.0	10.0	73.0	30.0	28.0	0.70	11	91.63	25.24

3.3.2. Data Processing and Model Validation

The quantitative model between each CMA and the method parameters was developed using Equation (1). The model was simplified with the stepwise backward method (α = 0.1) using Minitab software (v19, Minitab, State College, PA, USA).

$$Y = a_0 + \sum_{i=1}^{6} a_i X_i + \sum_{i=1}^{6} a_{ii} X_i^2 + \sum_{i=1}^{5} \sum_{j=i+1}^{6} a_{ij} X_i X_j \quad (1)$$

where a_0 is the constant; a_i, a_{ii} and a_{ij} are the regression coefficients of the primary, secondary and interaction terms, respectively; X refers to a method parameter; and Y is a CMA.

The experimental error simulation method was used to calculate MODR [40]. MATLAB software (R2017b, MathWorks, Natick, MA, USA) was used for program calculations. All parameters were calculated in the form of coded values. The calculation steps of X_1 to X_5 were set at 1. The calculation step of X_6 was set at 0.1. The number of simulations was 500. After obtaining the prediction results with different combinations of method parameters, the corresponding probability values were calculated statistically. The MODR was obtained with the lowest acceptable probability of 0.8.

3.3.3. Plackett–Burman Designed Experiment

In the robustness test, Plackett–Burman designed experiments were carried out to investigate the variation in the response variables with slight changes in analytical conditions to ensure the results had good reproducibility. The parameters and levels were as follows: 78.5 ± 0.5% of phase B content in mobile phase at 0 min (X_1), 8.5 ± 0.5 min of the closing time of the first gradient (X_2), 73.5 ± 0.5% of phase B content in mobile phase at the beginning of the second gradient (X_3), 30.5 ± 0.5 min of the closing time of the second gradient (X_4), 30.0 ± 1.0 °C of column temperature (X_5), 0.60 ± 0.01 mL/min of flow rate (X_6). The experimental design is shown in Table 4.

Table 4. Experimental conditions and results of Plackett–Burman designed experiment.

Run	X_1/%	X_2/min	X_3/%	X_4/min	X_5/°C	X_6/(mL/min)	Y_1	Y_2/%	Y_3/min
1	78.0	9.0	74.0	30.0	31.0	0.59	14	90.05	26.60
2	78.0	8.0	73.0	31.0	31.0	0.61	13	89.78	25.36
3	79.0	8.0	74.0	31.0	29.0	0.61	9	93.69	26.09
4	78.0	9.0	74.0	31.0	29.0	0.61	10	93.16	26.59
5	78.0	9.0	73.0	30.0	29.0	0.61	12	91.10	25.66
6	79.0	9.0	73.0	31.0	31.0	0.59	12	91.34	26.39
7	78.5	8.5	73.5	30.5	30.0	0.60	14	89.26	26.00
8	78.0	8.0	73.0	30.0	29.0	0.59	13	90.37	25.46
9	78.5	8.5	73.5	30.5	30.0	0.60	14	89.85	25.99
10	79.0	8.0	74.0	30.0	29.0	0.59	11	91.85	26.08
11	79.0	8.0	73.0	30.0	31.0	0.61	12	91.86	25.03
12	79.0	9.0	73.0	31.0	29.0	0.59	12	92.46	26.40
13	78.5.0	8.5	73.5	30.5	30.0	0.60	13	90.67	25.94
14	79.0	9.0	74.0	30.0	31.0	0.61	13	90.37	26.14
15	78.0	8.0	74.0	31.0	31.0	0.59	13	91.51	26.21

3.4. LC-Q-TOF-Ms Analysis

A LC-Q-TOF-MS (AB Sciex Triple TOF 5600+, AB Sciex, Framingham, MA, USA) was used to analyze the sugar components in Xiaochaihu capsules. The optimal condition within MODR was selected as the analysis condition. The specific analysis condition is described in Section 3.2. The mass spectrometry conditions are as follows. The electron spray ionization was chosen as ion source. The acquisition mode was set at negative ion. The scan mode was MS. The scan range was set at m/z 100–1500. The drying gas temperature was set at 320 °C. The drying gas flow rate was set at 8 L/min. The nebulizer pressure was set at 35 psi. The sheath gas temperature was set at 350 °C. The sheath gas flow rate was set at 11 L/min. The capillary voltage was set at 3500 V. The nozzle voltage was set at 1000 V. The crushing voltage was set at 175 V. The cone hole voltage was set at 65 V. The octapole radio frequency voltage peak value was set at 750 V.

3.5. Method Validation

Validation of the fingerprinting method was carried out in terms of precision, repeatability, and stability. The validation of the content determination method was carried out in terms of linear examination, precision, repeatability, stability, and recovery. Detailed experimental methods are shown in the supplementary material.

4. Results

4.1. Identification of CMAs

The peak number was chosen as a CMA to fully reflect the chemical composition of samples (Y_1). The percentage of common peak (Y_2) was chosen to ensure the representativeness of the fingerprint. The retention time of the last peak (Y_3) was chosen to optimize the separation time.

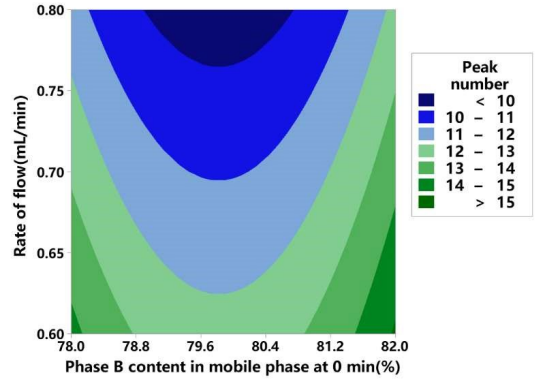
The experimental results of DSD are shown in Table 3. The peak number ranged from nine to 17. The percentage of common peak ranged from 85.26 to 93.88%. The retention time of the last peak ranged from 21.884 to 28.681 min. The value of CMAs varied considerably with different method parameters. Thus, method parameters need to be further optimized.

4.2. Influence of Method Parameters

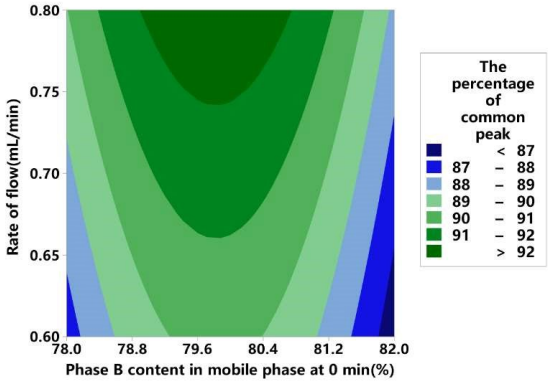
The quantitative mathematical models between each CMA and method parameters were established according to Equation (1). The regression coefficients and analyses of variance (ANOVA) of the models are shown in Table 5. The coefficients of determination (R^2) of the three models were 0.9322, 0.9925, and 0.9999, respectively. The adjusted coefficients

of determination (R^2adj) were 0.8712, 0.9795, and 0.9998, respectively. These indicated that the models were all well-fitted and could explain most of the variance.

Contour plots of each response variable can be obtained from the established mathematical models. Some of the contour plots are shown in Figure 2. The p value of each parameter was less than 0.1. X_1, X_2, X_5, and X_6 all showed significant effects on the peak number. X_1–X_6 all showed significant effects on the percentage of common peaks. X_1, X_2, X_3, X_4, and X_6 all showed significant effects on the retention time of the last peak.



(**a**)



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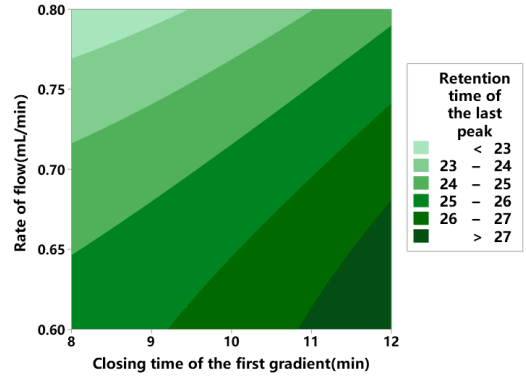

(**c**)

Figure 2. Contour plots of each response variable. In order to better show the relationship between the parameters and the response values, the other parameters are fixed (**a**) Peak number. Closing time of the first gradient was 10 min; closing time of the second gradient was 30 min; column temperature was 28 °C; (**b**) The percentage of common peak. Closing time of the first gradient was 10 min; column temperature was 28 °C; (**c**) Retention time of the last peak. Phase B content in mobile phase at 0 min was 80%; phase B content in mobile phase at the beginning of the second gradient was 73%; closing time of the second gradient was 30 min; column temperature was 28 °C.

Table 5. Regression coefficients and ANOVA for each model.

	Y_1		Y_2/%		Y_3/min	
Item	**Coefficient**	***p* Value**	**Coefficient**	***p* Value**	**Coefficient**	***p* Value**
Constants	10.942	0.000	91.463	0.000	25.254	0.000
X_1	0.429	0.036	−0.590	0.000	0.170	0.000
X_2	−0.571	0.009	0.443	0.001	1.223	0.000
X_3	-	-	−0.160	0.092	1.088	0.000
X_4	-	-	−0.170	0.077	0.682	0.000
X_5	0.357	0.073	−0.629	0.000	-	-
X_6	−1.429	0.000	1.227	0.000	−1.573	0.000
X_1^2	2.337	0.000	−3.323	0.000	−0.208	0.000
X_2^2	-	-	2.788	0.000	0.236	0.000
X_3^2	-	-	-	-	0.223	0.000
X_4^2	−1.288	0.020	0.543	0.044	−0.460	0.000
X_5^2	0.962	0.068	−1.690	0.000	0.554	0.000
X_6^2	-	-	-	-	−0.374	0.000
X_1 X_2	−1.125	0.001	1.106	0.000	−0.323	0.000
X_1 X_4	-	-	−0.401	0.008	−0.062	0.002

4.3. MODR and Validation

A larger peak number indicates more information in the fingerprint. Thus, the lower limit was set at 14. The percentage of the common peak should be larger. Therefore, the lower limit was set at 0.87. The upper limit of the retention time of the last peak was set at 27 min in order to shorten the analysis time.

To be able to better demonstrate the MODR, three of the parameters were fixed. The calculated MODR is shown in Figure 3.

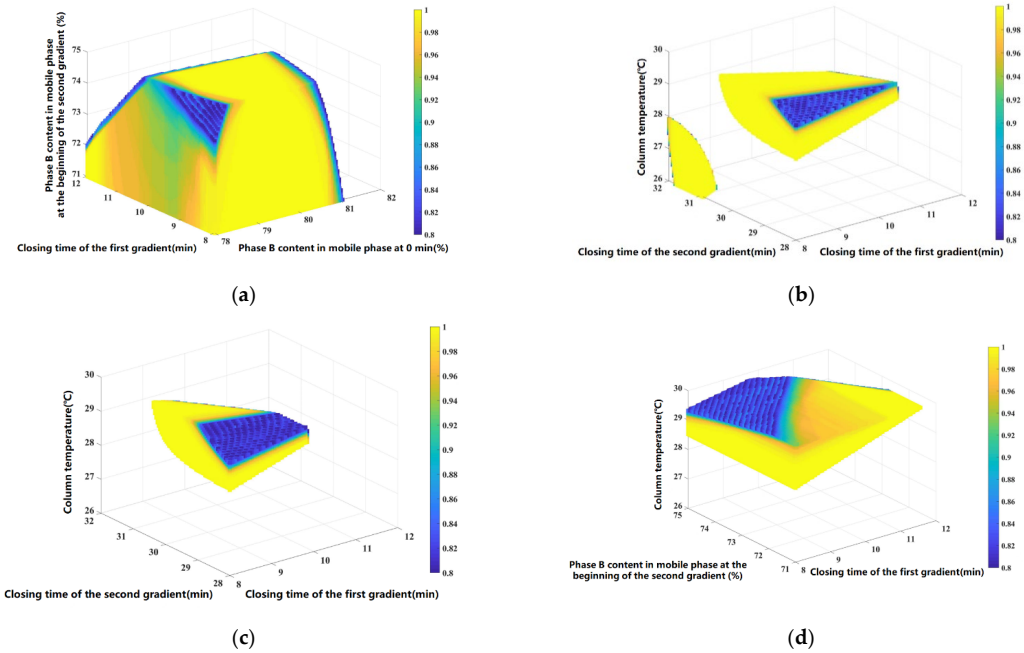

Figure 3. The MODR calculated using the experimental error simulation method. (**a**) Closing time of the second gradient was 28 min; column temperature was 30 °C; rate of flow was 0.6 mL/min; (**b**) Phase B content in mobile phase at 0 min was 81%; phase B content in mobile phase at the beginning of the second gradient was 74%; rate of flow was 0.6 mL/min; (**c**) Phase B content in mobile phase at 0 min was 81%; phase B content in mobile phase at the beginning of the second gradient was 75%; rate of flow was 0.6 mL/min; (**d**) Phase B content in mobile phase at 0 min was 81%; column temperature was 28 °C; rate of flow was 0.6 mL/min.

Within the MODR, three optimal combinations of method parameters were selected for validation experiments. The conditions and results of the validation method are shown in Table 6. Among them, the column temperature was set at 30 °C and the flow rate was set at 0.60 mL/min. The measured values were closer to the predicted values. Most of the indicators met the range requirements of the MODR, indicating that the established MODR is reliable.

Table 6. Validation of experimental conditions and results.

Methods	Gradient 1		Gradient 2		Peak Number		Percentage of Common Peak/%		Retention Time of the Last Peak/min	
	Phase B Content in Mobile Phase at the Beginning/%	Closing Time/min	Phase B Content in Mobile Phase at the Beginning/%	Closing Time/min	Predicted Value	Measured Value	Predicted Value	Measured Value	Predicted Value	Measured Value
A	78.0	10.0	74.0	28.0	14	14	88.26	91.72	26.025	26.683
B	78.5	8.5	73.5	30.5	14	14	90.19	91.50	26.236	25.504
C	78.0	9.0	74.0	30.0	15	14	89.43	88.65	26.515	26.445

4.4. Plackett–Burman Designed Experiment Result

The results of the Plackett–Burman designed experiment are shown in Table 4. Most groups of experiments showed that the peak number was greater than or equal to 12, the percentage of the common peak was greater than 89%, and the retention time of the last peak was less than 27 min. The CMAs obtained can still meet the analytical requirements when the analytical parameters are varied within MODR. In other words, the established analytical method has good robustness.

4.5. LC-Q-TOF-MS Analysis

The total ion chromatogram obtained using LC-Q-TOF-MS is shown in Figure S2. Based on the accurate relative molecular masses and chemical reference substances, seven compounds were inferred. Their numbers and inferred results are shown in Table S2. Peaks 2–9 were inferred to be ribitol, fructose, glucose, sucrose, maltose, raffinose, and stachyose, respectively.

4.6. Method Validation

4.6.1. Fingerprint Method Validation

Different batches of Xiaochaihu capsules numbered S1–S10 were studied. Seven common peaks were identified under the conditions. The peak of fructose (No. 3) was designated as the reference peak for its relatively large peak area and good separation from neighboring peaks. The results were expressed as the relative standard deviation (RSD) of the relative retention time and relative peak areas of each common peak with respect to the reference peaks. As shown in Tables S4 and S5, in the precision, repeatability, and stability tests of injection, the RSD values of relative retention time and relative retention peak area of each peak were less than 4%. The results met the requirements of chromatography fingerprinting, indicating that the test solution was stable within 24 h.

4.6.2. Application of Fingerprinting

Ten batches of Xiaochaihu capsules were prepared into sample solution according to Section 3.1. They were analyzed under the conditions of Section 3.2 to establish fingerprints. As shown in Figure 4, the original data of ten batches (S1–S10) of Xiaochaihu capsules were imported into the similarity evaluation system software (v2012.130723, Chinese Pharmacopoeia Commission, Beijing, China). The reference fingerprint was generated using the average method. The similarity results between the reference fingerprint and the sample fingerprints are shown in Table S3. The values of similarity were all above 0.90. They indicated that the sugar components of Xiaochaihu capsules from each batch had good-quality consistency.

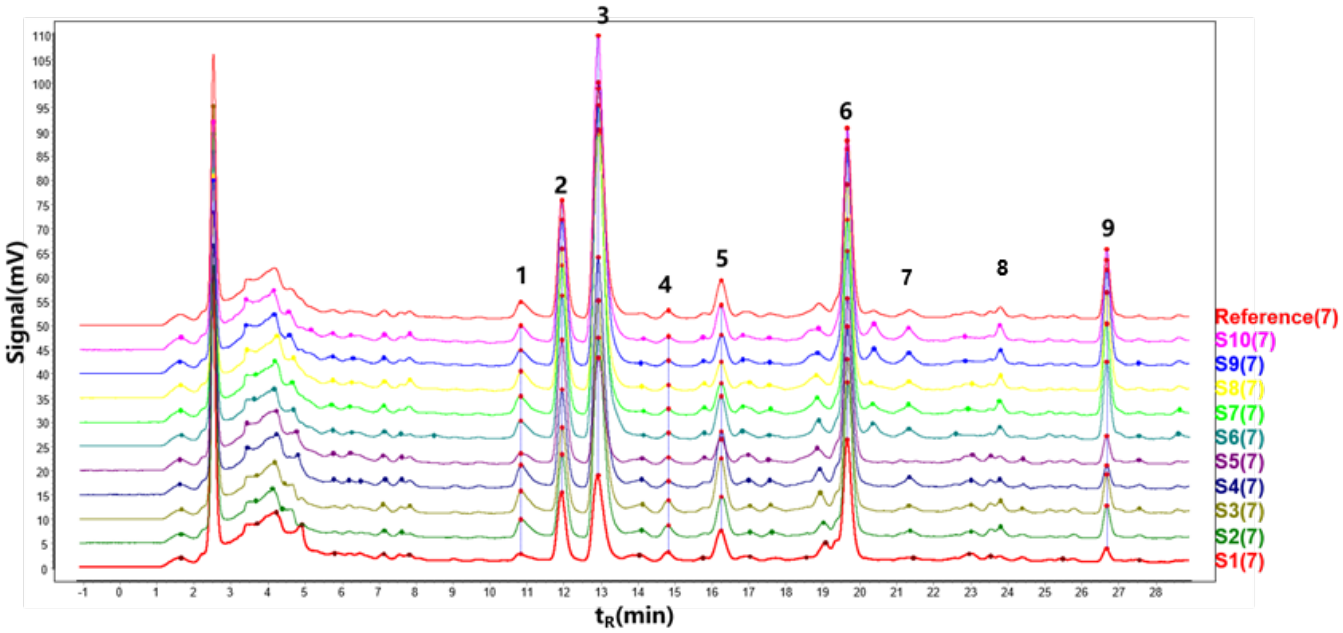

Figure 4. Reference fingerprint of Xiaochaihu capsule. Peaks 2, 3 and 5–9 were inferred to be ribitol, fructose, glucose, sucrose, maltose, raffinose, and stachyose, respectively.

4.6.3. Content Determination Method Validation

According to the retention times of the chemical reference substances and mass spectrometry, peaks 2, 3, 5, 6, 7, 8, and 9 were identified as ribitol, fructose, glucose, sucrose, maltose, raffinose, and stachyose, respectively. According to the principle of establishing fingerprint, fructose and sucrose are the main components of four components in Xiaochaihu capsules. Their detection can reflect the chemical composition of the Xiaochaihu capsule. This follows the principle of systematicity. Ribitol is a characteristic component of *Bupleurum* root. The detection of ribitol reflects the medicinal material of *Bupleurum* root. It is in line with the principle of characterization. Stachyose has the effect of regulating intestinal flora. This can be used to explain the effect of Xiaochaihu capsules in the treatment of loss of appetite, irritability, vomiting, etc. The detection of the effective ingredient is also in line with the principle of systematicity. In summary, ribitol, fructose, sucrose and stachyose were selected as the components for content determination.

The regression equations, linear range, limit of detection (LOD), and limit of quantitation (LOQ) of content determination components are shown in Table S6. The linear fit results were all greater than 0.999. The results of the injection precision experiments are shown in Tables S7 and S8. The results of the reproducibility experiments are shown in Table S9. The results of the solution stability experiments are shown in Table S10. The RSD values of precision, reproducibility, and stability were less than 3%. The results were in accordance with the requirements of the Chinese Pharmacopoeia. The results of the recovery experiments are shown in Table 7. The average recovery value of each component met the requirements, and the RSD values were less than 4%. These proved that the optimized method is accurate and reliable and can be used for the determination of sugar components in Xiaochaihu capsules.

The control strategy of the analysis method can be realized in the following two ways. First, the system suitability needs to be paid attention to before tests, including system precision, signal–noise ratio, tailing factor, and other parameters. Second, the parallel sample and reference substances can be used to observe whether the retention time is offset. When the chromatographic analyzer works abnormally, it is important to act in time.

Table 7. Results of recovery experiments.

Concentration Level	Ribitol	Fructose	Sucrose	Stachyose
Low level recovery rate (%)	102.4	105.4	106.6	103.5
	103.4	105.0	106.4	99.64
	105.3	103.8	105.4	99.71
Medium level recovery rate (%)	101.1	99.95	101.5	95.20
	102.9	103.3	103.9	102.1
	104.8	102.8	103.1	101.2
High level recovery rate (%)	97.39	97.59	100.6	98.58
	97.78	96.87	98.86	98.26
	97.14	94.50	99.28	98.82
Average recovery rate (%)	101.4	101.0	102.9	99.66
RSD (%)	3.142	3.896	2.880	2.414

4.6.4. Applications of Content Determination

Twelve batches of Xiaochaihu capsules were prepared into sample solution according to Section 3.1. They were analyzed under the conditions of Section 3.2. The content determination results of the quantitative component are shown in Table 8. In each batch of Xiaochaihu capsules, the content of ribitol ranged from 0.8985 to 2.281%, fructose ranged from 1.815 to 9.018%, and sucrose ranged from 2.054 to 5.320%. The content of stachyose was lower than 2.430%. Among them, the contents of fructose and sucrose were higher.

Table 8. Quantitative component content determination results for 12 batches of Xiaochaihu capsules.

Sample Number	Ribitol (%)	Fructose (%)	Sucrose (%)	Stachyose (%)
S1	2.004	2.012	4.666	0.5578
S2	1.205	4.819	3.504	0.6880
S3	1.206	5.021	3.413	0.8859
S4	1.411	5.737	3.750	0.4003
S5	1.854	2.263	3.877	0.5058
S6	2.200	9.018	4.549	1.815
S7	2.281	7.209	4.838	2.255
S8	2.209	6.478	5.109	2.411
S9	2.236	7.244	5.316	2.430
S10	2.185	7.935	5.320	2.310
S11	0.8985	1.815	2.735	0 *
S12	1.042	2.303	2.054	0.412

* 0 means the result is below LOQ.

5. Conclusions

In this study, a HPLC-CAD analytical method for quantitative fingerprinting of the sugar components of Xiaochaihu capsules was established based on AQbD. First, the peak number, the percentage of common peak, and the retention time of the last peak were chosen as CMAs. According to the results of definitive screening designed experiments, the critical parameters affecting the peak number were phase B content in mobile phase at 0 min, closing time of the first gradient, column temperature, and flow rate. The critical parameters affecting the percentage of common peak were phase B content in mobile phase at 0 min, closing time of the first gradient, phase B content in mobile phase at the beginning of the second gradient, closing time of the second gradient, column temperature, and flow rate. The critical parameters affecting the retention time of the last peak were phase B content in mobile phase at 0 min, closing time of the first gradient, phase B content in mobile phase at the beginning of the second gradient, closing time of the second gradient, and flow rate. Then, quantitative mathematical models between each CMA and each method parameter were established using multiple regression analysis. The R^2 of all three models exceeded 0.93, which could explain most of the variation. The MODR was calculated using the experimental error simulation method. Three experimental conditions within it were

selected and successfully validated, indicating that the established MODR was reliable. Considering various factors, ribitol, fructose, sucrose, and stachyose were identified as the content determination components. The HPLC conditions for quantitative fingerprint analysis were as follows: The mobile phase consisted of solvent A (water) and solvent B (acetonitrile). The gradient elution program was as follows: 0–10 min, 78–74% B; 10–28 min, 74–50% B; 28–33 min, 50% B. The flow rate was set at 0.6 mL/min. The injection volume was set at 10 µL. The column temperature was set at 30 °C. The evaporation temperature was set at 35 °C. The peak of fructose was chosen as a reference peak to establish the fingerprint with seven common peaks. The method validation results showed that the performance of the fingerprint and content determination methods were good. Twelve batches of Xiaochaihu capsule samples were determined using the developed analysis method. The results showed that the content of fructose and sucrose were higher. In the established analytical method, the influence of the analytical parameter variation on the method's performance has been investigated. Most groups of experiments showed that the CMAs obtained can still meet the analytical requirements when the analytical parameters are varied within MODR. The proposed method is expected to be robust in the quality control of Xiaochaihu capsules.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10010013/s1, Table S1: Source merchant and lot number information of Xiaochaihu capsules; Table S2: LC-Q-TOF-MS analysis of some sugar components of Xiaochaihu capsules; Table S3: Fingerprint similarity evaluation results of 10 batches of Xiaochaihu capsule sample solution; Table S4: The relative retention time of injection precision, method repeatability, and sample stability; Table S5: The relative peak areas of each common peak of injection precision, method repeatability, and sample stability; Table S6: The linear equation, coefficient of determination, and analytical range of each component; Table S7: Injection precision of the peak area; Table S8: Injection precision of retention time; Table S9: Method repeatability of content determination; Table S10: Sample stability of content determination; Figure S1: Fishbone diagram of potential critical method parameters; Figure S2 The total ion chromatogram of LC-Q-TOF-MS.

Author Contributions: Conceptualization, X.G. and G.W.; methodology, J.L.; software, J.L.; validation, J.L. and L.W.; formal analysis, J.L.; investigation, P.G., P.Z. and Y.X.; resources, Y.X., P.G. and P.Z.; data curation, J.L.; writing—original draft preparation, J.L.; writing—review and editing, J.L., L.W., X.G. and H.Q.; visualization, J.L. and P.Z.; supervision, X.G. and H.Q.; project administration, G.W. and X.G.; funding acquisition, H.Q. and X.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Innovation Team and Talent Cultivation Program of the National Administration of Traditional Chinese Medicine (ZYYCXTD-D-202002) and the Fundamental Research Funds for the Central Universities (226-2022-00226). The APC was funded by Xingchu, Gong.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are grateful for the support of Yanni Tai and Feng Ding.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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