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Multiresponse Optimization of Ultrasonic-Assisted Extraction for Aurantii Fructus to Obtain High Yield of Antioxidant Flavonoids Using a Response Surface Methodology

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Abstract: Aurantii fructus (zhiqiao, ZQ) is a traditional Chinese medicine (TCM) and raw material of TCM healthcare food (TCM-HF), mainly focused on the regulation of gastrointestinal disorders and the abundant application of antioxidants. Pharmacological investigations of ZQ flavonoids have identified them as the main bioactive components in recent years, but little has been reported on the extraction processes of antioxidant flavonoids (AFs). The aim of this study was to establish an efficient ultrasonic-assisted extraction (UAE) method for the extraction of AFs from ZQ using a response surface methodology (RSM), analyze the composition of AFs, and develop a qualitative evaluation method for ZQ. Flavonoid yield and antioxidant ability were selected as the responses to optimize the extraction of AFs, and the multiple effects of independent variables were investigated. The optimized conditions for the extraction of AFs based on the Box-Behnken design (BBD) were as follows: ethanol concentration, 58%; extraction temperature, 70 °C; and extraction time, 17 min. The flavonoid yield and antioxidant activity reached 241.70 mg/g and 59.42%, respectively, which matched the predicted values. Furthermore, optimized UAE processes were first established for the efficient and fast extraction of AFs. Flavanones and polymethoxyflavonoids (PMFs) were identified as potential AFs using time-of-flight mass spectrometry. Meanwhile, the quality of ZQ was evaluated using the criteria importance through intercriteria correlation (CRITIC) method for the first time, and *Yuanjiang* ZQ was considered as an excellent raw material of TCM-HF.

Keywords: Aurantii fructus; antioxidant flavonoids; ultrasonic-assisted extraction; response surface methodology; criteria importance through intercriteria correlation method

1. Introduction

The importance of traditional Chinese medicine (TCM) has been accepted as a sustainable health treatment resource around the world. In recent years, the conception of TCM healthcare food (TCM-HF) has fast developed from a traditional treatment to dietotherapy [1]. Therefore, it has become increasingly important to explore active compounds from natural sources using essential extraction and isolation procedures in the application of pharmaceutic preparations, functional food components, dietary supplements, nutraceuticals, and food additives [2]. Research and development regarding

TCM-HF mainly include formulae, quality standards and process procedures, and optimization of process procedures is essential. Moreover, antioxidant activity is one of the key indexes of TCM-HF and has been shown to eliminate or reduce the amount of free radicals and to decrease the incidence of diseases [3].

Aurantii fructus (zhiqiao, ZQ), a TCM and a raw material of TCM-HF, is harvested from the immature, green fruit of *Citrus aurantium* L., mainly focused on the treatment of gastrointestinal dysfunction, the improvement of qi stagnation, and the remission of chest pain in traditional therapies [4–6]. The secondary metabolites of ZQ include flavonoids, alkaloids, triterpenes, volatile oils, and coumarins [7–10]. Based on pharmacologic studies and clinical practice, flavonoids are considered as the main medicinal components with an enriched content and play an important role in pharmacological effects, such as anti-oxidation, anti-inflammation, the treatment of cardiovascular disease, and the promotion of gastrointestinal motility [11–14]. Although many studies have been carried out on the pharmacology and analytical chemistry of ZQ, there are few studies on the extraction technology of ZQ extract [15]. As a potential raw material of TCM-HF, it is important to develop an efficient and concise extraction procedure for the extraction of health-promoting compounds from ZQ. In pre-experiments for this study, we found that the flavonoid yield was positively correlated with antioxidant activity. According to the basic requirement of TCM-HF, extraction procedures for antioxidant flavonoids (AFs) from ZQ should be further studied [16].

In previous research, common extraction methods of ZQ flavonoids mainly included ultrasonic extraction [5,8], hot-water extraction [17,18], and reflux extraction [19]. Concerning the current study, there is no report on the ultrasonic-assisted hot-water extraction (UAE) method for the extraction of AFs from ZQ. In this study, UAE variables such as raw material concentration, sample size, extraction solvent, solvent concentration, extraction time, and extraction temperature were optimized using single-factor tests; among these, ethanol concentration, extraction temperature, and time were selected as the individual variables for the response surface methodology (RSM) by performing a three-level, three-variable Box-Behnken design (BBD) [20,21] to study the appropriate extraction conditions for AFs from ZQ. Then, we analyzed the main components of AFs from ZQ using liquid chromatography combined with quadrupole time-of-flight mass spectrometry (LC–Q–TOF–MS) [7] and evaluated the quality of ZQ from different habitats using the criteria importance through intercriteria correlation (CRITIC) method [22].

2. Materials and Methods

2.1. Materials, Chemicals, and Reagents

ZQ samples were collected from a series of raw materials of Hunan Province in China and identified by Prof. Qi Tang (Hunan Agricultural University). The fresh ZQs were continuously dried in 60 °C oven until a constant weight. The dried ZQ samples were milled with a grinder, sieved through a series of sieves, and stored in a desiccator at ordinary temperature (25 °C) until the tests. Standard substances (narirutin, naringin, eriocitrin, neoeriocitrin, poncirin, hesperidin, neohesperidin, nobiletin, and tangeretin) with high purities of over 98% were purchased from Yuan-ye Bio-Technology Co., Ltd. (Shanghai, China). Methanol, ethanol, acetone, ethyl acetate, ether, and petroleum ether were the analytical reagents (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Acetonitrile and formic acid were of the chromatographic grade for the mass analysis (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

2.2. Optimal Extraction of AFs from ZQ

An extractive procedure was optimized for the extraction of AFs; in short, 50 mg of each dried ZQ sample was added to 20 mL of 58% ethanol, and extracted for 17 min in a 70 °C water bath by use of a KM5200DV ultrasonic instrument with a constant power (200 W, 40 Hz; Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China).

2.3. Determination of Flavonoid Yield

The flavonoid yield of each extract was determined by Lay et al. [23], with few modifications. Each standard solution of naringin (200 μL) within a set concentration range (10, 40, 80, 120, 160, 200, 400 μg/mL) was added to 5.0 mL of 0.01 mol/L $AlCl_3$ solution (dissolved in methanol), respectively, incubated for 10 min in the dark at room temperature, and then measured at 310 nm on an 1800 UV spectrophotometer (Shimadzu Corp., Kyoto, Japan). A calibration curve was established: $Y = 0.0009X - 0.0039$, where Y was the absorbance (Abs), and X was the naringin concentration (μg/mL), $R^2 = 0.9969$.

An extract solution of 100 μL and 100 μL methanol solvent were mixed, and the same procedure was then repeated, as described above. The concentration of flavonoids was determined based on the calibration curve measured, and the flavonoid yield in the extract was calculated according to the naringin equivalent (mg of flavonoids/g of extract).

2.4. Analysis of Antioxidant Activity

The antioxidant activity of the ZQ extract was analyzed according to the free-radical scavenging activity and measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. This method was performed as proposed by Lay et al. [23], with a minor modification. An extract solution of 3.0 mL was added to a tube and then mixed with 3.0 mL of 80 μg/mL DPPH/methanol solution. The mixture was incubated for 30 min in dark conditions at room temperature. After the reaction, the absorbance was recorded at a wavelength of 517 nm on an 1800 UV spectrophotometer. Methanol was used as a blank control. The antioxidant activity of the tested sample was expressed as the DPPH radical scavenging rate (SR).

The calculated equation was:

$$\% \text{ SR} = [(A_0 - A_1)/A_0] \times 100\%, \quad (1)$$

where A_0 is the blank control and A_1 is the absorbance sample.

2.5. Experimental RSM Design

Based on the single-factor tests, several independent variables which had a significant influence on flavonoid yield and antioxidant activity were selected as the factor variables and studied using the BBD of RSM.

2.6. Identification of AFs

The identification of AFs was conducted on an Agilent 1290 HPLC system (Agilent Technologies, Palo Alto, CA, USA), combined with an accurate-mass mass spectrometer of Agilent 6530 Q-TOF-MS (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was carried out on an Agilent-ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, CA, USA), with a gradient elution (0–45 min, 10–90% acetonitrile). The other experimental conditions were consistent with our previous study [7].

2.7. Quality Evaluation of ZQ

Based on the optimal extraction conditions, the flavonoid yield and antioxidant activity of ZQ samples from 16 different habitats of Hunan Province were determined, and the criteria importance through intercriteria correlation (CRITIC) method was employed for the ZQ quality evaluation.

3. Results and Discussion

3.1. Selection of Optimization Factors

The solubility of AFs is affected by various variables. In this study, the ratio of solid/solvent was set to 50 mg/20 mL in the pre-test, which guaranteed an adequate dissolution of AFs, and a UAE with a consistent ultrasonic power of 200 W, 40 Hz was employed, which was mild for the molecular structure of the AFs. Furthermore, other independent variables were carefully screened using single-factor tests.

3.1.1. Selection of Organic Solvent for Extraction

Extraction solvents have a significant influence on extraction yield. The solubility of flavonoids is controlled by the polarity of the solvents used. Therefore, it is important to employ suitable solvents to ensure the optimal extraction of ZQ flavonoids. The ZQ flavonoids were extracted using different solvents from low polarity to high polarity, and their antioxidant activities were analyzed. The results showed that, compared to other solvents, the solubility of ZQ flavonoids in methanol and ethanol solution increased significantly, but there was no significant difference between them, and the antioxidant activity of ZQ extract in ethanol solution was better than in methanol (Figure 1A). In consideration of green environmental chemistry, which encourages low toxicity, environmental friendliness, and relative safety, ethanol solution was chosen as the extraction solution. Then, its concentration was tentatively set to 60% for further optimization (Figure 1E).

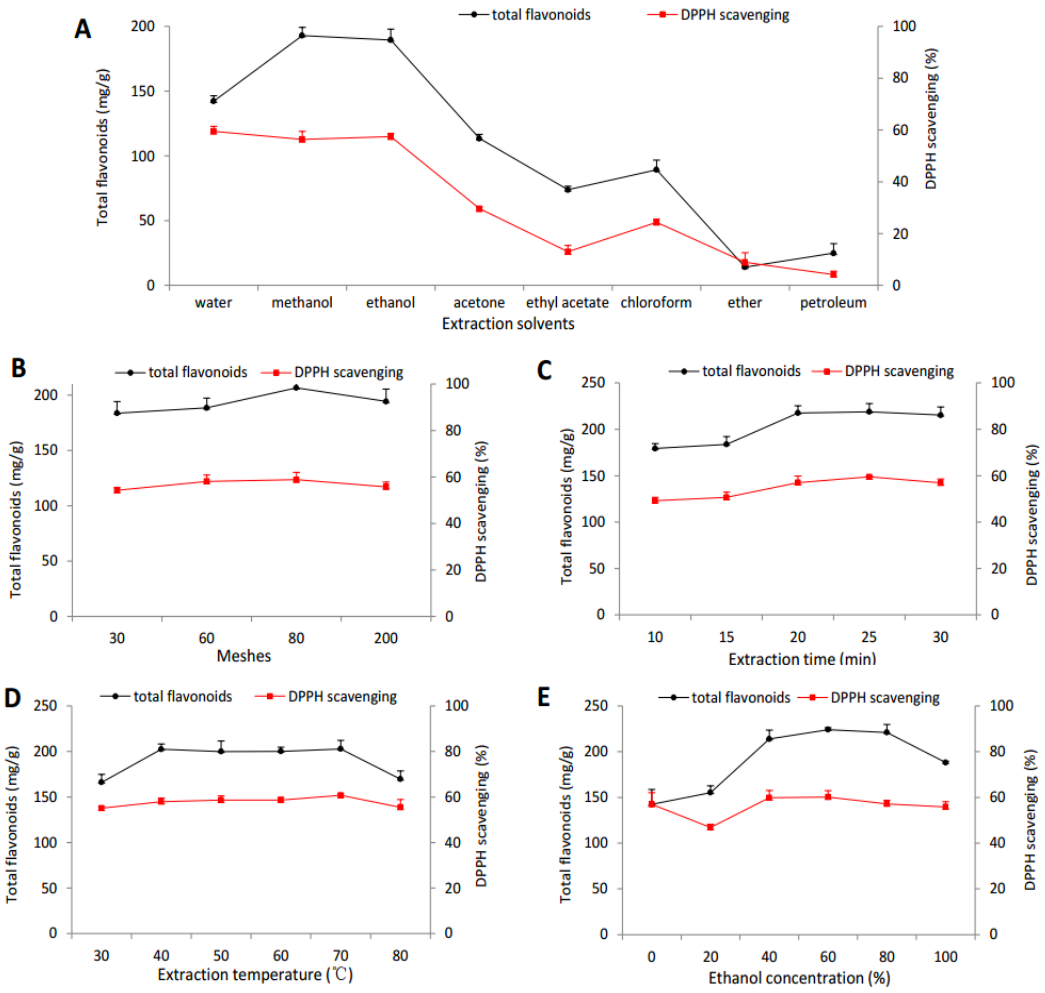

Figure 1. Influence of the main factors ((**A**) extraction solvents, (**B**) meshes, (**C**) extraction time, (**D**) extraction temperature, (**E**) ethanol concentration) on flavonoids and DPPH scavenging of Aurantii fructus.

3.1.2. Selection of Sieves

It was found that the sieve sizes were not very significant, being in the range of 30–200 meshes, but it was still clear that the flavonoid yield and antioxidant activity obtained from 80 meshes were suitable for ZQ extraction (Figure 1B).

3.1.3. Selection of Extraction Time

The effect of different extraction times on the flavonoid yield and antioxidant activity was studied. The results showed that both indexes first increased, and then decreased with longer extraction times (Figure 1C). An extraction time at 20 min was selected for further extraction optimization.

3.1.4. Selection of Extraction Temperature

Temperature had a great influence on flavonoid yield and antioxidant activity, but there was not a very significant difference in the range of 40–70 °C. As shown in Figure 1D, the composition of ZQ was relatively stable in this range, and there was a downward trend when the temperature rose. Therefore, the extraction temperature was tentatively selected at 50 °C for further optimization.

3.2. Optimization of Extraction Conditions Using RSM

Based on the single-factor tests mentioned above, three variables—ethanol concentration, extraction time, and extraction temperature—were selected as the guiding factors for further RSM optimization in the experiments, which affected the flavonoid yield and antioxidant activity in the extraction procedures.

A Box-Behnken design (BBD) of RSM was employed to investigate the effects of three variables—ethanol concentration (X_1), extraction temperature (X_2), and extraction time (X_3)—on the flavonoid yield (Y_1) and antioxidant activity (Y_2). The independent variables were coded at three levels (−1, 0 and 1), in detail, ethanol concentration (40%, 60%, and 80%), temperature (30, 50, and 70 °C) and extraction time (10, 20, and 30 min) were investigated (Table 1). This design was composed of 17 tested points, including five replications of the zero points (all variables were coded as zero), and the response results were obtained as shown in Table 2.

Table 1. Independent factors and their levels used in the response surface design.

Factors	Factor Level		
Coded levels	−1	0	1
A: Percentage of ethanol (%)	40	60	80
B: Extraction temperature (°C)	30	50	70
C: Extraction time (min)	10	20	30

Table 2. The experimental values for the responses of total flavonoids and 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging at different levels.

Std.	Run	A (%)	B (°C)	C (min)	Total Flavonoids (mg/g)	DPPH Scavenging (%)
7	1	−1	0	1	208.95	55.45
9	2	0	−1	−1	217.19	58.07
1	3	−1	−1	0	229.66	53.85
10	4	0	1	−1	232.18	59.78
4	5	1	1	0	215.29	58.45
17	6	0	0	0	235.11	58.92
3	7	−1	1	0	232.24	58.09
6	8	1	0	−1	211.85	59.28
2	9	1	−1	0	218.87	58.17

Table 2. *Cont.*

Std.	Run	A (%)	B (°C)	C (min)	Total Flavonoids (mg/g)	DPPH Scavenging (%)
8	10	1	0	1	213.07	59.34
16	11	0	0	0	235.94	59.56
14	12	0	0	0	235.70	58.36
11	13	0	−1	1	234.40	57.31
15	14	0	0	0	229.99	58.50
12	15	0	1	1	228.62	58.74
13	16	0	0	0	240.65	59.89
5	17	−1	0	−1	221.54	55.02

3.3. Effect of Extraction Conditions on Flavonoid Yield and Antioxidant Activity

The average flavonoid yield and antioxidant activity of each of the 17 tests under the various experimental UAE conditions are shown in Table 2. The highest flavonoid yield of 240.65 mg/g and antioxidant activity of 59.89% were obtained in experimental run number 16, with 60% ethanol, a temperature of 50 °C, and a time of 20 min. The yield from this test was excellent compared to previous data. Two second-order regression equations were established to fit with this experiment as follow:

$$\text{Flavonoids yield} = 235.5 - 4.2A + 1.0B + 0.3C - 1.5AB + 3.5AC - 5.2BC - 12.9A^2 + 1.4B^2 - 8.8C^2, \quad (2)$$

$$\text{Antioxidant activity} = 59.0 + 1.6A + 0.96B - 0.2C - 1.0AB - 0.1AC - 0.1BC - 1.6A^2 - 0.4B^2 - 0.2C^2. \quad (3)$$

The expected regression coefficients and analysis of variance (ANOVA) of the flavonoid yield and antioxidant activity were presented using the BBD. A quadratic regression model of flavonoid yield was significant ($p < 0.05$), while the lack of fit was not significant ($p > 0.05$), suggesting that this model was highly consistent with the experimental results of the flavonoid yield. Similarly, the quadratic regression model of antioxidant activity was also feasible. The regression coefficients of the two indexes were $R^2 = 0.87$ and $R^2 = 0.91$, respectively, which indicated a good degree of consistency between the experimental data and the predicted yield. The calibration coefficients of the index model were $R^2_{Adj} = 0.70$ and $R^2_{Adj} = 0.80$, respectively, which indicated that the results were reliable.

3.4. Response Surface Analysis

Based on the equations mentioned above, three-dimensional (3D) surface values were depicted to show the influences of the UAE variables (ethanol concentration, extraction temperature, and extraction time) on the flavonoid yield and antioxidant activity (Figure 2).

The results for the combined effect of ethanol concentration and extraction temperature suggested that the effects of low and high levels of ethanol concentration and extraction temperature on the extraction were significant. When the extraction temperature was at a constant value, the flavonoid yield initially increased and then decreased with the increase in ethanol concentration. However, at a constant ethanol concentration, it was not significant that the increase in extraction temperature impacted the flavonoid yield (Figure 2A). From Figure 2B, the results indicated that the interactional effects between ethanol concentration and extraction time were remarkable when the other variables were set at a fixed value. From Figure 2C, it can be seen that when the extraction time was set at a constant value, the increase in extraction temperature had little influence on the flavonoid yield. However, when the temperature was constant, the flavonoid yield underwent a significant change, from a low level to a high level, with the increase in extraction time. The response surface suggested that the flavonoid yield showed a significant correlation with the ethanol concentration and extraction time, but little influence was obtained in relation to the extraction temperature. This was highly consistent with previous data from single-factor tests.

Similarly, for the antioxidant activity analysis, the results suggested that ethanol concentration and extraction temperature had a significant influence on the antioxidant activity of ZQ extract, with a low influence for extraction time (Figure 2D–F).

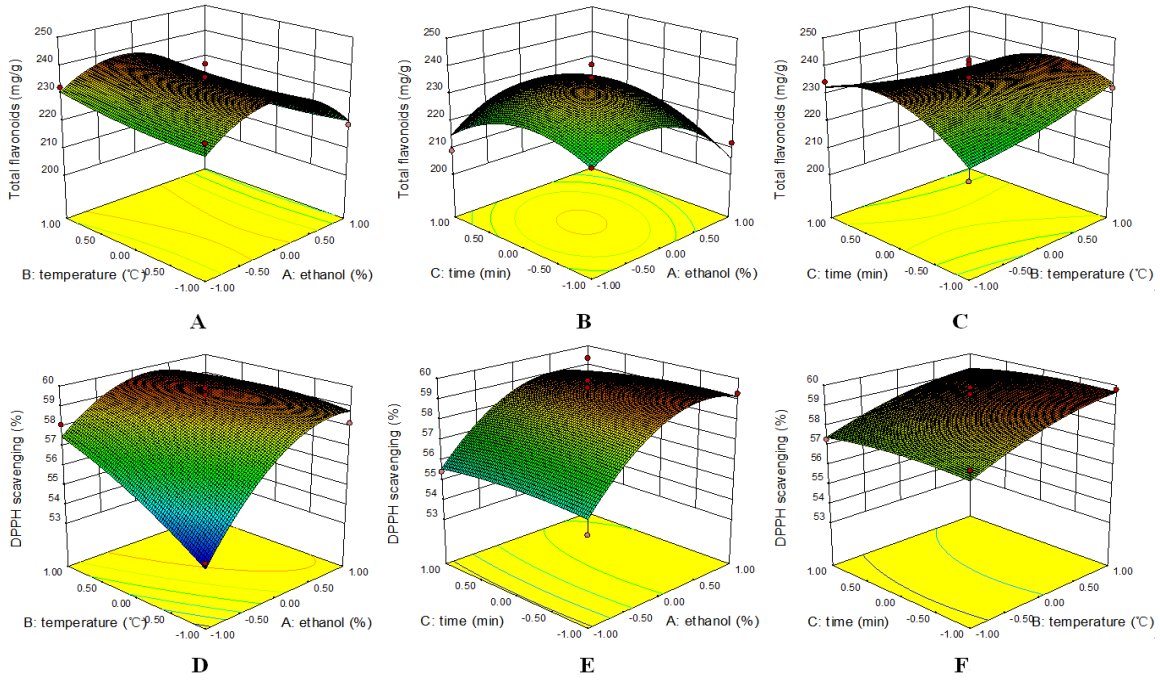

Figure 2. Response surface plots of ethanol concentration, extraction time, and temperature on the total flavonoids (**A–C**) and DPPH scavenging (**D–F**).

3.5. Theoretical Extraction Conditions and Verification

Based on the Design Expert software (Version10.0, Stat-Ease Inc., Minneapolis, MN, US), the desirability function of RSM was employed to obtain the optimal conditions for the flavonoid yield and antioxidant activity, and the optimum yield was achieved and set up with the following applied parameters: ethanol concentration, 58.4%; extraction temperature, 70 °C; and extraction time, 16.8 min. The estimated values were obtained (flavonoids, 239.04 mg/g; antioxidant activity, 59.59%).

The verification of the estimated results was validated using practical experiments under optimal conditions. The results indicated that the practical values (flavonoids, 241.70 mg/g; antioxidant activity, 59.42%) were consistent with the predicted values, the flavonoids yield was significant higher than previous reported data [15], and possessed high antioxidant activity at the same time. Therefore, the extraction conditions obtained using RSM were reliable and practical. The adjusted extraction conditions were: ethanol concentration, 58%; extraction temperature, 70 °C; and extraction time, 17 min. In contrast to traditional techniques, this model takes into account the interactions among several independent variables.

3.6. Identification of AFs from ZQ

The AFs from ZQ were identified using the HPLC–Q–TOF–MS method by comparing standards, fragmentation patterns and previously reported data [6] (Figure 3); flavanones and polymethoxyflavonoids (PMFs) were identified as the main AFs from ZQ (Table 3). In detail, flavanones including eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, and poncirin, and PMFs including isosinensetin, sinensetin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin were identified as the flavonoids with antioxidant properties. It was suggested that those antioxidant flavonoids might be effective ZQ ingredients for healthcare.

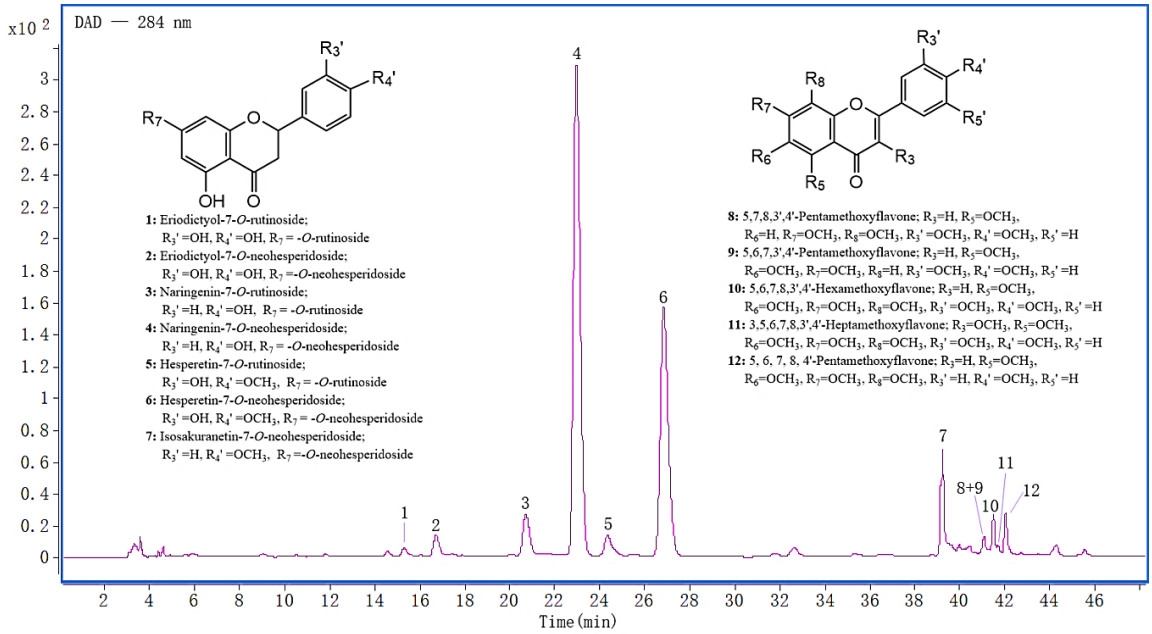

Figure 3. Representative chromatogram of flavonoids with antioxidant properties from ethanol extract of Aurantii fructus obtained using HPLC–DAD (λ = 284 nm) coupled with Q–TOF–MS.

Table 3. Mass spectrometry data of the main flavonoids with antioxidant properties from Aurantii fructus.

Number	Compound	$[M+H]^+/[M-H]^-$	Frag. (ESI+)	MW	Formula
Flavanones					
1	Eriodictyol-7-*O*-rutinoside (eriocitrin) [a]	597/595	435, 289	596	$C_{27}H_{32}O_{15}$
2	Eriodictyol-7-*O*-neohesperidoside (neoeriocitrin) [a]	597/595	435, 289	596	$C_{27}H_{32}O_{15}$
3	Naringenin-7-*O*-rutinoside (narirutin) [a]	581/579	419, 273	580	$C_{27}H_{32}O_{14}$
4	Naringenin-7-*O*-neohesperidoside (naringin) [a]	581/579	419, 273	580	$C_{27}H_{32}O_{14}$
5	Hesperetin-7-*O*-rutinoside (hesperidin) [a]	611/609	449, 303	610	$C_{28}H_{34}O_{15}$
6	Hesperetin-7-*O*-neohesperidoside (neohesperidin) [a]	611/609	449, 303	610	$C_{28}H_{34}O_{15}$
7	Isosakuranetin-7-*O*-neohesperidoside (poncirin) [a]	595/593	433, 287	594	$C_{28}H_{34}O_{14}$
Polymethoxyflavonoids (PMFs)					
8	5,7,8,3′,4′-Pentamethoxyflavone (isosinensetin)	373/—	358, 343, 315	372	$C_{20}H_{20}O_7$
9	5,6,7,3′,4′-Pentamethoxyflavone (sinensetin)	373/—	358, 343, 312	372	$C_{20}H_{20}O_7$
10	5,6,7,8,3′,4′-Hexamethoxyflavone (nobiletin) [a]	403/—	373, 355, 327	402	$C_{21}H_{22}O_8$
11	3,5,6,7,8,3′,4′-Heptamethoxyflavone	433/—	403, 388, 385	432	$C_{22}H_{24}O_9$
12	5, 6, 7, 8, 4′-Pentamethoxyflavone (tangeretin) [a]	373/—	358, 325, 297	372	$C_{20}H_{20}O_7$

[a] These compounds were accurately identified with reference standards.

3.7. Quality Evaluation of ZQ

The objective weight (W_j) according to the CRITIC method was expressed based on the characteristic conflict (R_j), the correlation of indicators (r_{ij}), the amount of information (C_j), and the standard deviation (σ_j). The calculated formulae were as follows:

$$R_j = \sum_{i=1}^{n}(1 - r_{ij}) \quad (4)$$

$$C_j = \sigma_j R_j \quad (5)$$

$$W_j = \frac{C_j}{\sum_{j=1}^{n} C_j} \quad (6)$$

The flavonoid yield and antioxidant activity of ZQs were determined, the data matrix was established according to the standardized data and formulae (experimental values − experimental minimum)/(experimental maximum − experimental minimum), and their objective weights were calculated according to the formulae mentioned above (Table 4).

Table 4. Comparison of intensity, conflict, information, and objective weight of evaluation indexes.

Evaluation Indexes	Intensity (σ_j)	Conflict (R_j)	Information (C_j)	Objective Weight (W_j)
Total flavonoids	0.231	0.358	0.083	0.440
DPPH scavenging	0.294	0.358	0.105	0.560

Then, according to the objective weight of the flavonoid yield and antioxidant activity, a qualitative evaluation method for ZQ was efficiently established, and the comprehensive scores of ZQ from different habitats were analyzed. As shown in Table 5, ZQ from *Sanyantang*, *Fuqiushan*, and *Chishanzhen* of Hunan Province showed excellent comprehensive scores and good quality levels.

Table 5. Comprehensive evaluation of Aurantii fructus from different areas in Hunan Province based on the intercriteria correlation (CRITIC) method (n = 3).

Samples	Region	Total Flavonoids (mg/g)	DPPH Scavenging (%)	Comprehensive Score	Ranking
S4	*Sanyantang, Yuangjiang*	356.53	75.34	97.33	1
S8	*Fuqiushan, Taojiang*	284.47	76.32	89.13	2
S6	*Chishanzhen, Yuangjiang*	264.36	79.11	88.63	3
S2	*Shijihu, Yuangjiang*	252.50	78.66	86.84	4
S9	*Heshanqu, Yiyang*	283.82	72.21	86.14	5
S11	*Nongda, Changsha*	277.24	71.82	85.05	6
S10	*Yangjixiang, Anren*	273.96	72.14	84.88	7
S7	*Longhushan, Yuangjiang*	249.72	74.54	83.58	8
S14	*Yanwanghuzhen, Hanshou*	242.64	75.77	83.58	9
S13	*Ningyuan, Yongzhou*	303.76	62.47	81.71	10
S15	*Bailuqiaozhen, Hanshou*	253.54	70.55	81.23	11
S5	*Tuanshanzhen, Yuangjiang*	231.88	74.06	81.04	12
S12	*Fenglinzhen, Lilin*	252.39	68.62	79.74	13
S16	*Xinning, Shaoyang*	212.36	61.70	69.88	14
S1	*Xinwanzhen, Yuangjiang*	182.99	55.25	61.69	15
S3	*Nanjuzhen, Yuangjiang*	151.23	51.24	54.94	16

4. Conclusions

This study clearly identified that the extraction processes of antioxidant flavonoids from ZQ could be improved by optimizing several key factors using RSM. Furthermore, the basic structures of potential antioxidant flavonoids were preliminarily illustrated using LC–Q–TOF–MS, and the comprehensive scores of AF quality from different habitats were then comparatively analyzed. As a raw material of TCM-HF, AFs of ZQ are an extract source with great potential for application in pharmaceutic preparations, functional food ingredients, dietary supplements, nutraceuticals, food additives, and so on.

Author Contributions: Y.H., H.X. and Q.T. conceived and designed the experiments; Y.H., Y.C., Y.S., H.T. performed the experiments and designed the figures; Y.H., Y.C., and Y.S. analyzed and helped in data interpretation; Q.T. collected the materials; Y.H. wrote the manuscript and K.Z. assisted language modification; J.Z. provided funding support; H.X. and Q.T. edited and supported suggestions for the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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