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Flavonoids from Fig (*Ficus carica* Linn.) Leaves: The Development of a New Extraction Method and Identification by UPLC-QTOF-MS/MS

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Abstract: Flavonoid-rich leaves of the *Ficus carica* L. plant are usually discarded as waste. In this work, ultrasonic enzyme-assisted aqueous two-phase extraction (UEAATPE) was proposed as an innovative method to estimate the total flavonoids present in *F. carica* L. leaves. Total flavonoids were analyzed qualitatively and quantitatively by UPLC-QTOF-MS. At 38% (*w*/*w*) ethanol/18% (*w*/*w*) ammonium sulfate, we achieved the optimum conditions in which to establish an easy-to-form aqueous two-phase extraction (ATPE) as the final system. The optimal UEAATPE conditions were set at an enzymatic concentration of 0.4 U/g, 150 min enzymolysis time, an enzymolysis temperature of 50 °C, a liquid–solid ratio of 20:1 (mL/g), and 30 min ultrasonic time. The yields of the total flavonoids, i.e., 60.22 mg/g, obtained by UEAATPE were found to be 1.13-fold, 1.21-fold, 1.27-fold, and 2.43-fold higher than those obtained by enzyme-assisted ATPE (EAATPE), ultrasonic-assisted ATPE (UAATPE), ATPE, and soxhlet extraction (SE) methods, respectively. Furthermore, eleven flavonoids from the leaves of the *F. carica* L. plant were completely identified and fully characterized. Among them, ten flavonoids have been identified for the first time from the leaves of the *F. carica* L. plant. These flavonoids are quercetin 3-*O*-hexobioside-7-*O*-hexoside, 2-carboxyl-1,4-naphthohydroquinone-4-*O*-hexoside, luteolin 6-C-hexoside, 8-C-pentoside, kaempferol 6-C-hexoside-8-C-hexoside, quercetin 6-C-hexobioside, kaempferol 6-C-hexoside-8-C-hexoside, apigenin 2″-*O*-pentoside, apigenin 6-C-hexoside, quercetin 3-*O*-hexoside, and kaempferol 3-*O*-hexobioside. Therefore, *F. carica* L. leaves contain new kinds of unidentified natural flavonoids and are a rich source of biological activity. Therefore, this research has potential applications and great value in waste handling and utilization.

Keywords: *Ficus carica* L.; flavonoids; ultrasonic enzyme co-assisted; aqueous two-phase extraction; UPLC-QTOF-MS/MS; identification

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1. Introduction

Plant-derived organic waste mainly includes crop stalks, leftover branches and wood strips, fallen leaves, dry vines, weeds, and nut shells from the production process. Among the large traditional agricultural countries, agricultural organic waste has the following four characteristics: large quantity, poor quality, low price, and harmful properties [1]. In most agricultural organic waste treatment processes, the treatment efficiency is not high, and the environmental damage caused by improper treatment methods is relatively serious [2]. Most agricultural organic waste, due to its relatively abundance, can help protect the environment and save energy while improving comprehensive utilization of agricultural organic wastes.

Ficus carica L., a fig plant, has a long history as a Moraceae [3]. Figs are native to the Mediterranean coast, from Turkey to Afghanistan, having been established in the region since ancient times [4]. In China, figs were introduced from Persia during the Tang Dynasty and have been cultivated in both the south and the north, especially in Xinjiang and Shandong provinces [5]. As a crop with a long history and due to its cultivation and high nutritional value, figs have always been a source of food for human survival. Regarding their nutritional value, figs have recently been used in food processing [6,7]. Figs also have extensive medicinal value, as well as having functions in the nourishment of the stomach, the clearance of the intestines, the reduction of swelling, and detoxification [8–10].

Currently, fig leaves with a high biomass and many bioactive compounds are usually discarded, resulting in a waste of resources [11]. Fig leaves also contain flavonoids, sugars, pectin, tannins, vitamin C, trace elements, and other bioactive components [12,13]. They have many pharmacological effects due to the large amounts of flavonoids contained in fig leaves. They can prevent cardiovascular diseases and are anti-osteoporotic and are used in the treatment of diarrhea, for scavenging oxidative free radicals and for blood lipid reduction, sore throats, and immune regulation [14–17]. However, most of the flavonoids have not been identified or characterized. Therefore, in order to develop and utilize fig resources reasonably, extraction methods of flavonoids should necessarily be developed.

At present, traditional extraction methods of flavonoids are commonly used. However, the traditional methods are inefficient and environmentally unfriendly [18]. In order to overcome these shortcomings, the aqueous two-phase extraction (ATPE) method could be used to replace conventional extraction methods. As an economic, mild, and simple separation method, it has been widely used in the field of natural product separation [19]. Additionally, enzyme-assisted extraction (EAE) is a better pretreatment method for separation. Cellulase can remove the pectin in the cell wall so that the material in the cell can be dissolved quickly and fully. This can improve the extraction yield of effective ingredients while reducing the consumption volume of solvents without destroying the structure of compounds. An enzymatic reaction is widely used in extracting various compounds from natural products due to its mildness, economical nature, and environmental protection [20]. Recently, UAE has been widely used in natural product extraction because of its time efficiency and reduced solvent usage [21–23]. For purposes of increasing the extraction efficiency of ATPE, a combination of the UAE and ATPE methods was developed [24,25].

In this study, on the basis of the advantages of UAE, EAE and ATPE [26,27], an ultrasonic enzyme-assisted ATPE (UEAATPE) method was developed for its environmental protection characteristics. Furthermore, the identification and characterization of eleven flavonoids was achieved using UPLC-QTOF-MS/MS from fig leaves. This research is of great significance for improving the development of fig leaves and promoting the rational utilization of this resource. This can be used not only to develop new resources, but also to effectively use green waste and further achieve recycling to promote agricultural development. In the current situation of environmental protection and sustainable development, it is also an effective waste management method.

2. Materials and Methods

2.1. Materials and Chemicals

We collected fig leaves in Chengshan Town, Rongcheng City. The fig leaves were thoroughly dried in a cool and dark place. For further study, we used a disintegrator to crush the dried leaves into powder (60 mesh). All chemicals used, unless stated otherwise, were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard solution was stored at −20 °C and used for the subsequent experiment.

2.2. Apparatus

Acquity^{TM} ultra-high-performance liquid chromatography (Waters, Milford, MA, USA); Triple TOF 5600+ time-of-flight mass spectrometer with electrospray ion source (AB SCIEX, Framingham, MA, USA); ASE350 Rapid Solvent Extraction Apparatus (Dionex,

Sunnyvale, CA, USA); AG135 Precision electronic balance (Mettler Toledo, Greifensee, Switzerland); KQ-100e Ultrasonic cleaning instrument (Kunshan Ultrasonic Instrument Co., LTD., Kunshan, China).

2.3. Ultrasonic Enzyme-Assisted Aqueous Two-Phase Extraction (UEAATPE)

ATPSs were screened on the basis of the formation described in reference [28]. Each of the salts tested (ammonium sulfate, dipotassium hydrogen phosphate, sodium carbonate, sodium sulfate, calcium chloride, potassium dihydrogen phosphate, and sodium chloride) was dissolved in deionized water. The salt solution was mixed with ethanol by a vortex stirrer. ATPS was formed when the mixture showed two-phase separation at the cloud point. Due to rapid phase formation and stratification, an ethanol/$(NH_4)_2SO_4$ system was chosen [29]. A diagram of UEAATPE is shown in Figure 1.

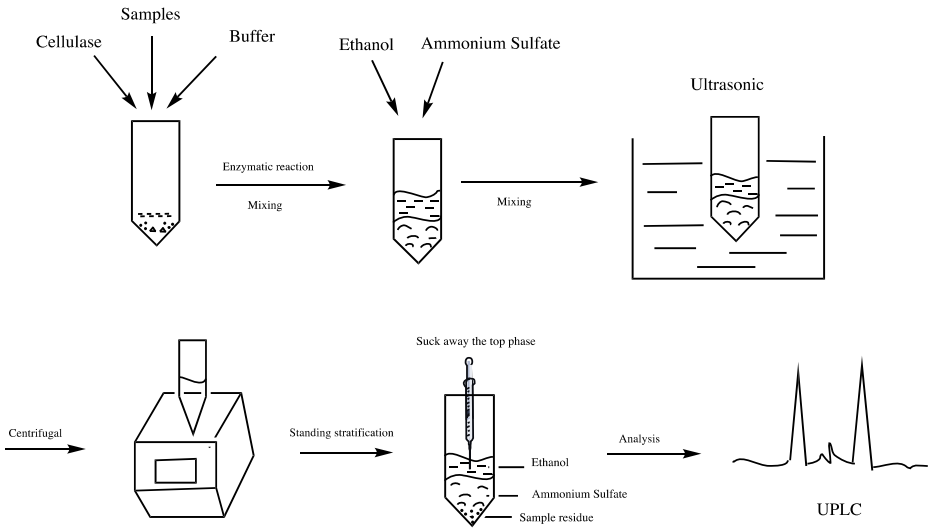

Figure 1. Diagram of UEAATPE.

To a total of 0.2 g fig leaf powder, 4.0 g disodium hydrogen phosphate–citric acid buffer solution and 0.3 U/g cellulase were first added in a 10 mL graduated test tube and then mixed evenly by a vortex mixer and placed in a water bath at a certain constant temperature. Then, ammonium sulfate and ethanol were added into the enzymatic slurry and vibrated for 10 min by a vortex mixer to completely dissolve the salt. The suspension was given ultrasonic (100 w) treatment for 30 min. After ultrasonic treatment, the mixture was mixed well and then set at room temperature for 30 min to form an aqueous two-phase system.

2.4. Determination of Total Flavonoids

The total flavonoids were determined by the method described in [28]. The extracted solutions (0.3 mL) were transferred to a 10 mL test tube, to which sodium nitrite solution (5%; 0.3 mL) was added. The mixture was allowed to stand for 6 min, and then 0.3 mL of 10% aluminum nitrate solution was added. After another 6 min, this was followed by the addition of 4 mL of 4% sodium hydroxide solution. The absorbance of the mixture was measured at 510 nm using a UV-Vis spectrophotometer (Perkin–Elmer Lambda 25, Waltham, MA, USA). After 15 min, the flavonoid contents in the extracts were determined in comparison to a standard curve that was plotted using rutin. The results were the averages of triplicate analyses. The calibration curve was obtained using rutin as the standard as shown in Table 1. Then, the extraction yield of total flavonoids was calculated according to Equation (1):

$$Y = C_t V_t / M_t \quad (1)$$

where Y (mg/g) represents the yield of total flavonoids; C_t (mg/mL) represents total flavonoid concentrations in the top phases; and V_t (mL) is the volume of the top phases. M_t (g) is the total mass of the fig leaf powder.

Table 1. Calibration curve, correlation coefficient, LOD, and LOQ of the total flavonoids.

Analytes	Calibration Curve	R^2	Linear Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
rutin	y = 5.5778 x + 0.0635	0.9929	3.901–250	2.196	7.032

2.5. Experiment Design of UEAATPE

After testing in a single-factor experiment, four factors were selected and combined in the proposed methods to assess the main role of BBD and its interactions. These were X_1: 36–40% ethanol concentration; X_2: 16–20% ammonium sulfate concentration; X_3: 10:1–30:1 (mL/g) liquid–solid ratio; X_4: 20–40 min ultrasonic time. Y represents the yield of total flavonoids in different ranges.

2.6. Comparison of Different Extraction Methods

The extraction yield of total flavonoids was compared with UEAATPE, EAATPE, UAATPE, ATPE, and SE. The mass fractions of ethanol and ammonium sulfate were 38% (w/w) and 18% (w/w), respectively. The other fixed extraction conditions under optimization were enzymatic hydrolysis for 180 min at a 0.4 U/g cellulase concentration, a fixed temperature of 50 °C, an extraction time of 30 min, and a liquid–solid ratio of 20:1 mL/g.

2.7. The Analysis of UPLC-QTOF-MS/MS

The supernatant was evaporated until dry with a rotary evaporator (RE-52AA, Shanghai Huxi Instrument, Shanghai, China) under reduced pressure in a 60 °C water bath. The suspended sample was re-dissolved in a methanol solution. Then, before the UPLC-QTOF-MS analysis, the solution was filtered through a 0.22 μm microporous membrane.

The samples were separated on an ACQUITY UPLC HSS T3 (150 mm × 2.1 mm i.d., 1.8 μm, Waters). The column temperature was maintained at 50 °C, and the injection volume was set as 2 μL with 0.3 mL/min as a fixed flow rate. The mobile phase was composed of acetonitrile (A) and 0.1% (v/v) formic acid in aqueous solution (B). The gradient elution conditions were as follows: 0–2 min, 5% A; 2–25 min, 5–40% A; 25–32 min, 40–95% A. The chromatogram was obtained at 254 nm, and semi-quantitative calculations were performed for each compound based on the relative peak area and rutin standard.

In the negative ion mode, m/z 100–1500 was used as the mass spectrum data acquisition condition. Atomizing air (GS1): 55 psi; atomizing air (GS2): 55 psi; source temperature (TEM): 550 °C; source voltage (IS): −4500 V. Level 1 scan: de-cluster voltage (DP) and focusing voltage (CE): 100 V and 10 V. Secondary scan: TOF MS~Product Ion~IDA mode was used to collect mass spectrum data. The CID energy was −20, −40, and −60 V. Before sample injection, a CDS pump was used for mass axis correction to make the mass axis error less than 2 ppm.

2.8. Statistical Analysis

The data are presented as mean ± standard deviation (SD). All data for this study were adopted for analysis of variance (ANOVA) to determine significant differences. The significance of such differences between mean values was determined using Duncan's test ($p < 0.05$). ANOVA and Duncan's multiple range tests were performed with SPSS19 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Screening of Phase Ratio in ATPS System

3.1.1. Selection of Ethanol Mass Fraction

Single-factor conditions were fixed to allow analysis of other factors. As shown in Table 2, the effect of the ethanol mass fraction on the yield of total flavonoids was studied. Before the ethanol mass fraction reached 38% (*w*/*w*), the yield of total flavonoids was positively correlated with it; then, the yield of flavonoids showed a downward trend with increasing ethanol concentration. The reason for this is that as the mass fraction of the ethanol in the system increased, the concentration of the ethanol in the top phase increased, and the polarity decreased. Under these conditions, it was more conducive to perform the extraction of flavonoids. As the mass fraction of ethanol exceeded 38% (*w*/*w*), the polarity of the upper phase decreased. This resulted in the immiscibility of the flavonoids and the salt of the precipitation [30]. At this time, the amount of some fat-soluble organic compounds also increased, which inhibited the leaching of total flavonoids. Therefore, the optimal parameter for the ethanol concentration was 38% (*w*/*w*).

Table 2. Effects of different influencing factors on the yield of total flavonoids.

No.	Mass Fraction of Ethanol (%)	Mass Fraction of Ammonium Sulfate (%)	Concentration of Enzymes (U/g)	Enzymolysis Time (min)	Enzymolysis Temperature (°C)	Ultrasonic Time (min)	Liquid–Solid Ratio (mL/g)	Yield of Total Flavonoids (mg/g)
1	32	18	0.3	180	50	30	20	56.83
2	34	18	0.3	180	50	30	20	58.92
3	36	18	0.3	180	50	30	20	59.46
4	38	18	0.3	180	50	30	20	60.02
5	40	18	0.3	180	50	30	20	58.18
6	38	14	0.3	180	50	30	20	47.79
7	38	16	0.3	180	50	30	20	57.03
8	38	18	0.3	180	50	30	20	60.19
9	38	20	0.3	180	50	30	20	58.41
10	38	22	0.3	180	50	30	20	55.40
11	38	18	0.3	180	50	30	20	47.13
12	38	18	0.4	180	50	30	20	60.00
13	38	18	0.5	180	50	30	20	59.03
14	38	18	0.6	180	50	30	20	57.25
15	38	18	0.7	180	50	30	20	57.17
16	38	18	0.3	90	50	30	20	46.13
17	38	18	0.3	120	50	30	20	50.24
18	38	18	0.3	150	50	30	20	53.20
19	38	18	0.3	180	50	30	20	59.89
20	38	18	0.3	210	50	30	20	50.00
21	38	18	0.3	180	35	30	20	41.55
22	38	18	0.3	180	40	30	20	44.99
23	38	18	0.3	180	45	30	20	58.09
24	38	18	0.3	180	50	30	20	60.12
25	38	18	0.3	180	55	30	20	52.53
26	38	18	0.3	180	50	10	20	56.11
27	38	18	0.3	180	50	20	20	57.99
28	38	18	0.3	180	50	30	20	60.91
29	38	18	0.3	180	50	40	20	59.23
30	38	18	0.3	180	50	50	20	57.10
31	38	18	0.3	180	50	30	10	55.01
32	38	18	0.3	180	50	30	20	60.01
33	38	18	0.3	180	50	30	30	58.32
34	38	18	0.3	180	50	30	40	58.29
35	38	18	0.3	180	50	30	50	58.10

3.1.2. Selection of Ammonium Sulfate Mass Fraction

It can be seen in Table 2 that as the ammonium sulfate mass fraction increased, the yield of flavonoids showed a trend of rising first and then falling. However, the yield of flavonoids changed little in the range 17~22% of ammonium sulfate. When the ammonium sulfate mass fraction reached 18% (*w*/*w*), the highest yield of total flavonoids was obtained.

This occurred in view of the mass fraction of ammonium sulfate directly affecting the phase ratio in the ATPS system. The volume ratio of the upper and lower phases and the total extraction capacity of the solvent were the factors that affected the extraction rate of total flavonoids [31]. Therefore, we selected 18% (*w*/*w*) of ammonium sulfate concentration as the optimal parameter.

3.2. Univariate Analysis of UEAATPE

3.2.1. Effects of Enzyme Concentration on Flavonoid Yield

When determining the enzyme concentration, economic effects should also be considered. The idea was to attain complete extraction and avoid excessive use of enzymes. It can be seen from Table 2 that the total flavonoid content changed under different cellulase concentrations of 0.3, 0.4, 0.5, 0.6, and 0.7 U/g. In the range of cellulase concentration from 0.30 to 0.4 U/g, the total flavonoid yield showed a positive correlation. However, the same change trend between 0.4 and 0.7 U/g was not clear. The reason for this is that cellulase destroyed the cell wall and released bioactive ingredients [32]. However, a superfluous enzyme concentration can saturate the substrate. Excess enzymes should not be combined with substances that cause waste [33]. In brief, a 0.4 U/g concentration of cellulase was chosen for further experimental optimization.

3.2.2. Effects of Enzymolysis Time on Flavonoid Yield

It can be seen from Table 2 that an obvious trend of flavonoid yield was observed between 90 and 180 min, and then the yield of flavonoids began to decrease after 210 min. Enzymatic digestion of the cell walls appeared to have occurred in the sample and the maximum amount of flavonoids was released in 180 min. Enzymatic hydrolysis time affected the yield of target components, and the enzymatic hydrolysis time was short, which did not allow the target components to fully dissolve. The long enzymatic hydrolysis time not only increased the extraction cost, but also led to an increase in impurities. It may be that as the time increases, some flavonoids are oxidized to form quinone compounds and reduce the yield of the target compounds of the top phase. This indicates that the enzymatic hydrolysis of 180 min is the optimal time to catalyze cell wall hydrolysis.

3.2.3. Effects of Enzymolysis Temperature on Flavonoid Yield

Table 2 shows the changing trend of total flavonoids. As the temperature reached between 35 °C and 50 °C, the extraction yield of flavonoids showed a positive correlation. Generally speaking, enzyme activity is closely related to temperature. Enzyme activity and reaction rate can be increased by raising the temperature of enzyme hydrolysis. However, at temperatures above 50 °C, the extraction yield of flavonoids decreased significantly. It may be that excessive temperatures denature the enzyme. It can be seen that the optimal enzymolysis temperature parameter was set to 50 °C.

3.2.4. Effects of Ultrasonic Time on Flavonoid Yield

Ultrasound is a crucial parameter influencing total flavonoid yield. As shown in Table 2, the parameter range of the extraction time was set from 10 to 50 min. Under the condition of a fixed power of 200 w, the yield of total flavonoids was studied. Before the ultrasound time reached 30 min, the extraction yield of total flavonoids was positively correlated with the ultrasound time. At 30 min, it reached the highest yield of 60.07 mg/g, then the yields of flavonoids decreased with the further increase in ultrasonic time [34]. This was mainly due to the large amount of time taken by the ultrasonic reaction, leading to some flavonoid oxidation or degradation, so the yield of flavonoids decreased. Thus, 30 min of extraction time was selected for the subsequent experiments.

3.2.5. Effects of the Liquid–Solid Ratio on Flavonoid Yield

The effect of different liquid–solid ratios on the yield of flavonoids is shown in Table 2. Economically speaking, an appropriate liquid–solid ratio is very important for flavonoid

extraction. The extraction yield of total flavonoids showed an upward trend as the liquid–solid ratio increased from 10:1 to 20:1 mL/g, and then began to decline after it exceeded 20:1 mL/g. This is because the larger the liquid–solid ratio, the more adverse the penetration of the solvent and solute diffusion, resulting in less sufficient flavone dissolution, and the extraction yield of total flavonoids will thus become lower. However, the addition of dried fig leaf powder can absorb water from the aqueous phase and increase the ethanol concentration. The total amount of leaching decreased with the increase in the amount of powder added due to the decrease in permeability and diffusion capacity. Considering this factor economically, in order to avoid significant wastage of the solvent, the liquid–solid ratio in the optimal test design was 20:1 mL/g.

3.3. Optimization of UEAATPE

As shown in Table 3, the test resulted in 29 uncontrolled runs. The correlation between the response and the independent variable can be visualized with a 3D surface plot. Figure 2 indicates the influence of the ethanol concentration %, (X_1), ammonium sulfate concentration %, (X_2), liquid–solid ratio mL/g, (X_3), and ultrasound time min, (X_4), on the yield of total flavonoids and their interaction. Furthermore, it is possible to predict the optimal value of the response and the corresponding experimental conditions through the F value (>7.84) and p value (<0.01), as shown in Table 4. This result shows that the model can describe the total flavonoid yield of UEAATPE well. The equation of the response variables and independent variables is as follows:

$$Y = 59.60 - 0.03X_1 + 0.48X_2 - 4.66X_3 - 1.63X_4 + 1.18\,X_1X_2 + 2.95\,X_1X_3 + 3.24\,X_1X_4 + 2.00\,X_2X_3 - 0.59\,X_2X_4 - 4.48X_3\,X_4 - 5.19\,{X_1}^2 - 3.37\,{X_2}^2 - 9.60\,{X_3}^2 - 8.41\,{X_4}^2 \quad (2)$$

where Y represents yields of total flavonoids (mg/g); X_1, X_2, X_3, and X_4, respectively, represent the ethanol concentration (%), ammonium sulfate concentration (%), liquid–solid ratio (mL/g), and ultrasonic time (min).

Table 3. Experimental data and total flavonoid extraction analyzed by the Box–Behnken approach.

Runs	Independent Variables				Yield of Total Flavonoids (mg/g)
	Ethanol Concentration (X_1, %)	Ammonium Sulfate Concentration (X_2, %)	Liquid—Solid Ratio (X_3, mL/g)	Ultrasonic Time (X_4, min)	
1	38	18	30:1	20	43.71
2	38	18	10:1	20	44.62
3	36	18	10:1	30	52.80
4	38	16	20:1	40	45.44
5	36	18	30:1	30	35.25
6	38	16	10:1	30	49.99
7	40	18	30:1	30	42.99
8	40	18	10:1	30	48.75
9	38	20	10:1	30	48.13
10	36	18	20:1	20	48.74
11	38	18	20:1	30	60.39
12	40	18	20:1	20	42.87
13	40	18	20:1	40	48.16
14	38	18	10:1	40	49.70
15	38	20	20:1	40	45.84
16	38	16	30:1	30	39.54
17	38	20	30:1	30	45.68
18	38	18	30:1	40	30.87
19	36	18	20:1	40	41.04
20	40	16	20:1	30	49.59

Table 3. *Cont.*

Runs	Independent Variables				Yield of Total Flavonoids (mg/g)
	Ethanol Concentration (X_1, %)	Ammonium Sulfate Concentration (X_2, %)	Liquid—Solid Ratio (X_3, mL/g)	Ultrasonic Time (X_4, min)	
21	38	18	20:1	30	58.94
22	38	18	20:1	30	59.11
23	36	20	20:1	30	51.40
24	40	20	20:1	30	51.11
25	38	16	20:1	20	48.90
26	38	18	20:1	30	61.08
27	36	16	20:1	30	54.60
28	38	18	20:1	30	58.50
29	38	20	20:1	20	51.70

Table 4. Analysis of variance for the extraction yield of total flavonoids by quadratic model.

Source	Sum of Squares	Mean Square	F-Value	*p*-Value	Significance
Model	1.40×10^3	100.32	28.36	<0.0001	significant
X_1	1.05×10^{-2}	1.05×10^{-2}	2.97×10^{-3}	0.9573	not significant
X_2	2.81	2.81	0.79	0.3882	not significant
X_3	260.89	260.89	73.76	<0.0001	significant
X_4	31.69	31.69	8.96	0.0097	not significant
X_1 X_2	5.57	5.57	1.58	0.2301	not significant
X_1 X_3	34.76	34.76	9.83	0.0073	not significant
X_1 X_4	42.11	42.11	11.91	0.0039	not significant
X_2 X_3	16.00	16.00	4.52	0.0517	not significant
X_2 X_4	1.44	1.44	0.41	0.5342	not significant
X_3 X_4	80.28	80.28	22.70	0.0003	not significant
X_1^2	175.31	1.75×10^2	49.57	<0.0001	significant
X_2^2	73.68	73.68	20.83	0.0004	not significant
X_3^2	598.54	5.99×10^2	1.70×10^2	<0.0001	significant
X_4^2	459.01	4.60×10^2	1.30×10^2	<0.0001	significant
Lack of Fit	44.82	4.48	3.82	0.1042	not significant
R^2			0.9659		

According to appropriate extraction conditions (independent variables) and actual operation analysis by Design Expert software, all these conditions were modified as follows: 38% (*w*/*w*) ethanol/18% (*w*/*w*) ammonium sulfate; liquid–solid ratio of 20:1 mL/g, and ultrasonic time of 30 min. Under these conditions, it was possible to obtain 60.22 mg/g flavonoids by UEAATPE. According to the RSM prediction model, the above experimental value matches the fitted value (RSD < 1.72%).

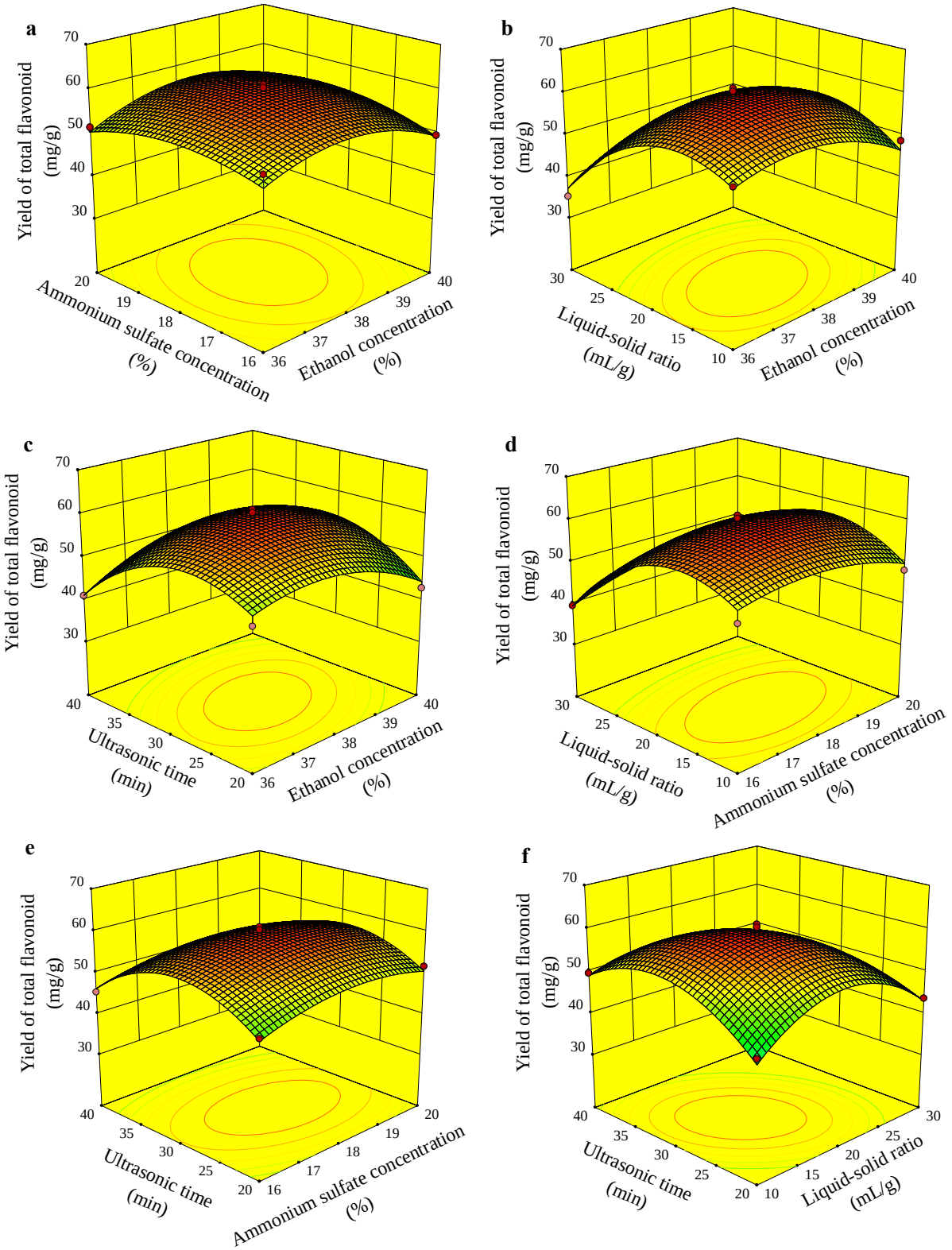

Figure 2. Response surface representations for total flavonoids in fig leaves (the variables are as follows: (**a**) interaction of ethanol concentration, % and ammonium sulfate concentration, %; (**b**) interaction of liquid–solid ratio, mL/g and ammonium sulfate concentration, %; (**c**) interaction of ultrasonic time, min and ethanol concentration, %; (**d**) interaction of liquid–solid ratio, mL/g and ammonium sulfate concentration, %; (**e**) interaction of ultrasonic time, min and ammonium sulfate concentration, %; (**f**) interaction of ultrasonic time, min and liquid–solid ratio, mL/g.

3.4. Comparison of Different Methods

The results shown in Figure 3 indicate the yields of flavonoids that were obtained by UEAATPE, EAATPE, UAATPE, ATPE, and SE. Among the above five methods, the highest yield of total flavonoids was extracted by UEAATPE. At the same time, the remaining four methods in order of extraction yield were UAE > EAE > ATPE > SE (53.48, 49.59, 47.46, and 24.79 mg/g, respectively). The extraction yield of total flavonoids with SE was lower than that with the other four methods. Therefore, UEAATPE is a prospective method for the extraction of flavonoids.

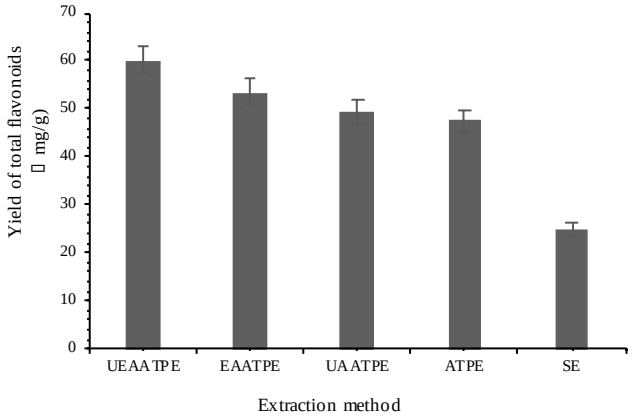

Figure 3. Effects of different methods on the yield of total flavonoids.

3.5. Identification of Flavonoids in Fig Leaves

The UPLC chromatography of flavonoid extraction by UEAATPE was detected at 254 nm, as shown in Figure 4. The identification and structure elucidation of the compounds from the leaves was completed by UPLC-QTOF-MS/MS in negative ion mode. Approximately 11 peaks were separated in the extract. Subsequently, the ESI-MS^1 and MS^2 were used to identify and characterize the flavonoids from the fig leaves. The mass spectra and fragmentation pathways can be seen in Figures S1–S11 (see the Supplementary Material). The identification of peaks was performed using reference data such as retention time and mass spectrum, as shown in Table 5. According to the structural characteristics, these compounds are all flavonoids. The chemical structure compounds identified are shown in Figure 5.

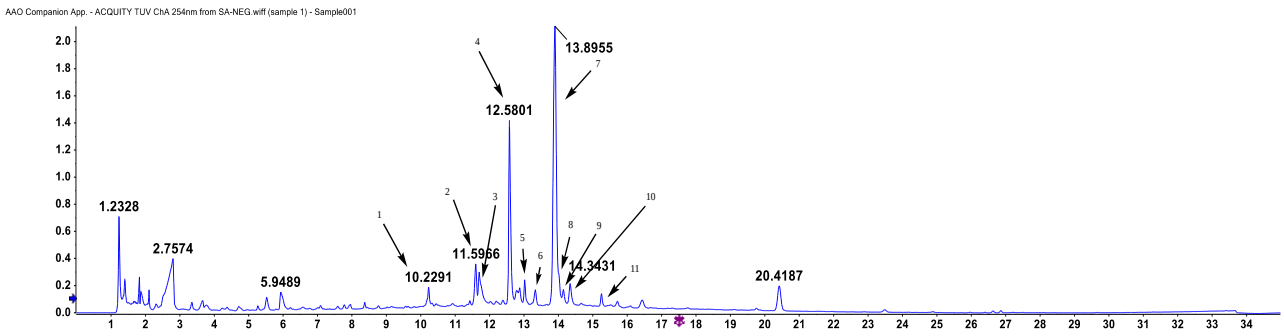

Figure 4. The UPLC chromatogram of flavonoid extraction from fig leaves detected at 254 nm.

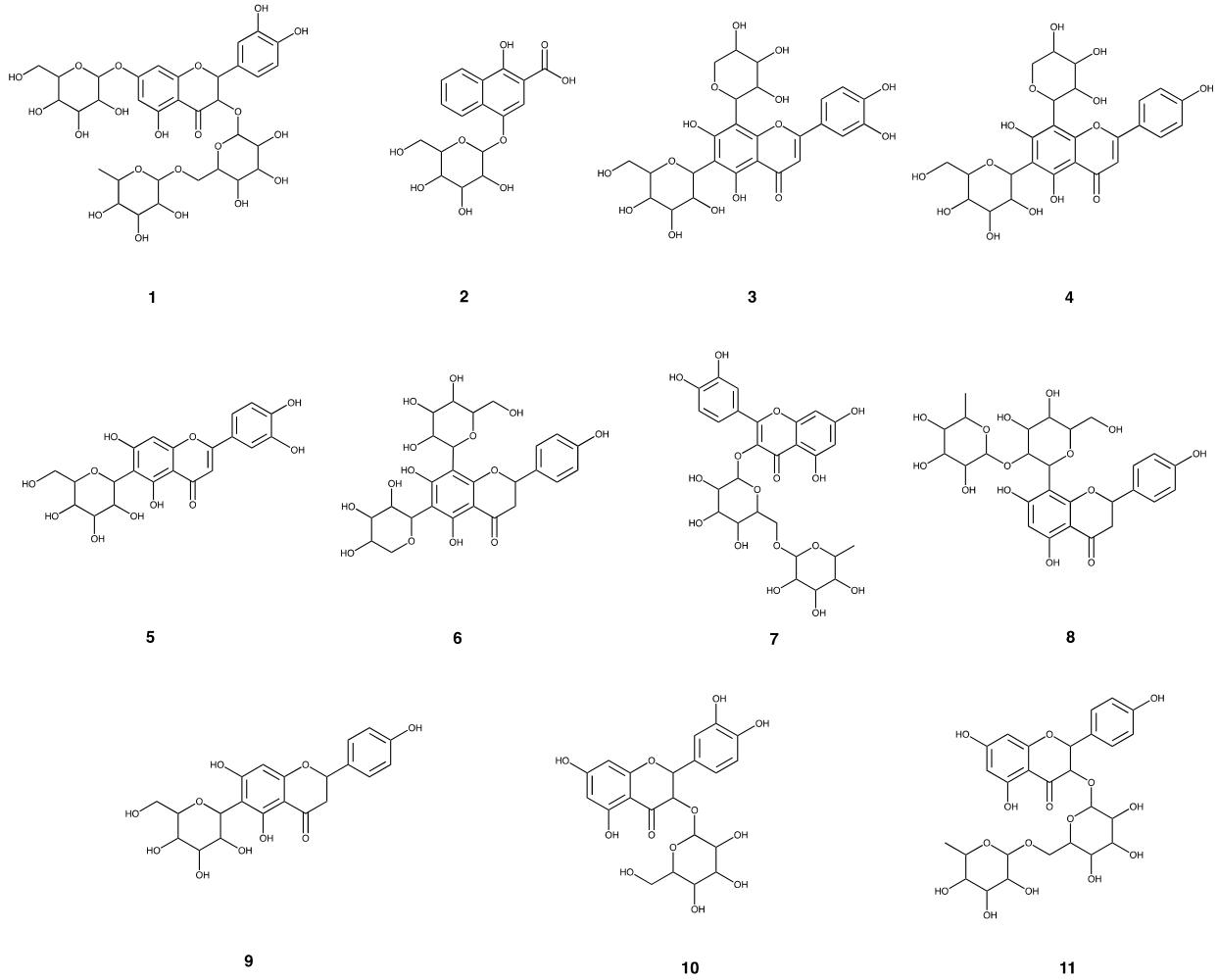

Figure 5. Chemical structures of the compounds identified from fig leaves (refer to Table 5): Compounds 1 through 11 are 3-*O*-(rhamnopyranosyl-glucopyranosyl)-7-*O*-(glucopyrnosyl)-quercetin; 2-carboxyl-1,4-naphthohydroquinone-4-*O*-hexoside; luteolin 6-C-hexoside, 8-C-pentoside; kaempferol 6-C-hexoside-8-C-hexoside; quercetin 6-C-hexobioside; kaempferol 6-C-hexoside-8-C-hexoside; quercetin 3-*O*-hexobioside; apigenin 2″-*O*-pentoside; apigenin 6-C-hexoside; quercetin 3-*O*-hexoside; kaempferol 3-*O*-hexobioside.

Table 5. Flavonoids identified in the fig leaf extracts using UPLC-QTOF-MS/MS.

Peak No.	t_R	MS	MS/MS	Molecular Weight	Molecular Formula	Identification
1	10.22	771.2027	609.1530, 462.0838, 301.0357	772.20621	$C_{33}H_{40}O_{21}$	3-*O*-(rhamnopyranosyl-glucopyranosyl)-7-*O*-(glucopyrnosyl)-quercetin
2	11.59	365.0881	203.0352, 159.0454, 130.0422	366.09508	$C_{17}H_{18}O_9$	2-carboxyl-1,4-naphthohydroquinone-4-*O*-hexoside
3	11.7	579.1366	519.1194, 489.1083, 429.0856, 369.0635	580.14282	$C_{26}H_{28}O_{15}$	luteolin 6-C-hexoside, 8-C-pentoside
4	12.58	563.1414	473.1115,443.1001, 353.0670	564.14791	$C_{26}H_{28}O_{14}$	kaempferol 6-C-hexoside-8-C-hexoside
5	12.87	447.0934	369.0615, 357.0622, 327.0511, 297.0397, 285.0396, 133.0280	448.10050	$C_{21}H_{20}O_{11}$	quercetin 6-C-hexobioside
6	13.02	563.1412	443.1001, 353.0670	564.14791	$C_{26}H_{28}O_{14}$	kaempferol 6-C-hexoside-8-C-hexoside
7	13.89	609.1470	301.0362, 151.0031, 257.0450, 273.0477	610.15338	$C_{27}H_{30}O_{16}$	quercetin 3-*O*-hexobioside
8	14.02	577.1577	457.1164, 293.0454	578.16356	$C_{27}H_{30}O_{14}$	apigenin 2″-*O*-pentoside
9	14.14	432.1056	341.0673, 311.0564, 283.0612	432.10565	$C_{21}H_{20}O_{10}$	apigenin 6-C-hexoside
10	14.34	463.0890	301.0357	464.09548	$C_{21}H_{20}O_{12}$	quercetin 3-*O*-hexoside
11	15.25	593.1524	285.0403	594.15847	$C_{27}H_{30}O_{15}$	kaempferol 3-*O*-hexobioside

The 11 compounds were divided into flavonoid oxygen glycoside compounds and flavonoid carbon glycoside compounds [35]. As shown in Figure 6, flavonoid glycosides mainly underwent Y^- type cleavage; that is, the glycosyl is removed, and the hydroxy is retained, which is represented by Y^-. The right subscript of the ion indicates the type of glycosyl. For example, Y_{H^-} means the cleavage of the hexose to remove the glycosyl. Flavonoid glycosides mainly underwent the cleavage of the sugar ring (X cleavage). When cleavage occurs, the position of the broken bond on the sugar ring is indicated by the left superscript, and the type of the cleavage glycosyl is indicated by the right subscript. This included hexose (H), pentose (*p*), and deoxyhexacarbonose (D). For example, ${}^{0,2}X_H$ represents a ring-opening cleavage caused by the breakage of the 0, 2 bond on the hexose [36,37].

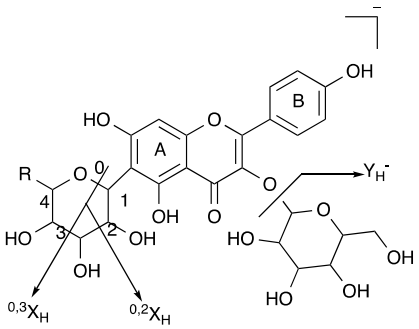

Figure 6. Mass spectrometric analysis of flavonoid glycosides in negative ion mode.

Compounds **1**, **2**, **7**, **10**, and **11** were all flavonoid oxygen glycoside compounds [38]. Quasi-molecular ion peaks can appear in negative ion modes of flavonoid oxygen glycoside compounds to cause glycosidic bond cleavage. This is characterized by a neutral loss of the glucosyl $C_6H_{10}O_5$ (162), rhamnosyl $C_6H_{10}O_4$ (146), or xylose $C_5H_8O_4$ (132). The aglycon structure can continue to lose -CH_3 and -CO_2 or RDA cleavage can occur in the C ring of the flavonoid aglycon.

The MS spectrum of Compound **1** (t_R = 10.22 min) showed an $[M - H]^-$ ion at m/z 771.2027, and the fragment ions of oxygen glycosides: m/z 609.1530, 462.0838, and 301.0357 were generated. Compound **1** was identified as quercetin 3-*O*-hexobioside-7-*O*-hexoside [39].

Compound **10** (t_R = 14.34 min) showed an $[M - H]^-$ ion at m/z 463.0890. According to m/z 301 $[M\text{-}162\text{-}H]^-$ secondary mass spectrometry, we speculated that there was glucose in the structure of the compound, and the molecular weight of the parent nucleus was 301, which was quercetin. Based on Scifinder and Reaxy database retrieval, Compound **10** was presumed to be quercetin 3-*O*-hexoside [40]. Compound **1** and Compound **10** had the same mass spectrum fragmentation pathway. The relative molecular mass of Compound **10** was 464. The fragment ion peak (m/z 301) was caused by the loss of a quasi-molecular ion peak and a neutral fragment of m/z 162. According to the relative molecular mass, the neutral fragment of m/z 162 was preliminarily judged to be a glucose group. Since $[M\text{-}H\text{-}162]^-$ (m/z 301) is a fragment ion of quercetin aglycone, this indicates that there was a glucosyl group in the compound. The fragmentation of m/z 301 ion generated two main characteristic ions: m/z 179 and m/z 151. The m/z 179 may be obtained by the fragmentation of m/z 301 and the transfer of 2 H ions. The m/z 151 fragment ion was generated by the RDA reaction of m/z 301. In addition, the loss of one molecule of CO at m/z 179 also generated m/z 151 ions. This is consistent with the cleavage pathway for quercetin 3-*O*-hexobioside quercetin aglycon fragment ions, and the cleavage law conformed to the structural characteristics of quercetin 3-*O*-hexoside.

Compound **2** (t_R = 11.59 min) showed an $[M - H]^-$ at m/z 365.0881 and product ions at m/z 203.0352, 159.0454, and 130.0422. Among them, m/z 203.0352 represented $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ obtained after the precursor ion lost glucose, which is the aglycon of the compound. The m/z 159.0454 represents the fragment ion obtained after the precursor ion

had lost 206 Da [M-$C_{11}H_{10}O_4$-H]$^-$. The m/z 130.0422 represents the fragment ion obtained after the precursor ion had lost 235 Da [M-$C_{12}H_{11}O_5$-H]$^-$. According to the fragment ions in the secondary mass spectrum and the related literature, we speculated that the compound was 2-carboxyl-1,4-naphthohydroquinone-4-*O*-hexoside [38].

Compound **7** (t_R = 13.89 min) was the main flavonoid in fig leaves. This showed the MS spectrum [M − H]$^-$ ion at m/z 609.1407, and the product ions at m/z 301.0362, 151.0031, 257.0450, and 273.0477 were generated. Under the bombardment of 25% energy, the quercetin 3-*O*-hexobioside aglycone was lost to form fragments of m/z 301.0362. In the tandem mass spectrometry of the m/z 301.0362 ion, there were three main ways of fragmentation. The first was fragmentation of m/z 301.0362 through RDA to form fragments of m/z 151. The second type was fragmentation of m/z 301. The loss of the carbonyl group from the C ring formed fragments of m/z 273.0477. The compound was identified as quercetin 3-*O*-hexobioside [41].

Compound **11** was eluted at 15.25 min. It showed the [M − H]$^-$ ion at m/z 593.1524 and the product ions at m/z 285 [M-162-146-H]$^-$ were generated. The quasi-molecular ion peak lost a neutral fragment of m/z 309, resulting in fragment ions [M-H-308]$^-$ (m/z 285). According to the relative molecular mass, the neutral fragment of m/z 308 was initially judged to be rutin. According to the literature, the substance was kaempferol 3-*O*-hexobioside [42].

Compounds **3**, **4**, **5**, **6**, **8**, and **9** are all flavonoid glycosides [38]. Quasi-molecular ion peaks appeared in negative ion modes for the flavonoid carboglycosides. The negative ion mode showed higher abundance for fragment ions. We observed the ring-opening and cleavage of the sugar ring and the subsequent neutral loss of sugar residues, CO, aglycon loss, CH_3, and other fragment ion peaks. The ring-opening cleavage of the sugar ring is the characteristic cleavage form of carbon glycosides. This mainly occurs in the sugar ring 0, 2 bond and 0, 3 bond, hexose neutral loss of $C_4H_8O_4$ or $C_3H_6O_3$, and the neutral loss of pentose $C_3H_6O_3$ or $C_2H_4O_2$.

Compound **3** (t_R = 11.7 min) had the [M − H]$^-$ ion at m/z 579.1366, and the product ions at m/z 519.1194, 489.1083, 429.0856, 399.0748, 369.0635, and 339.0521 were generated. At m/z 519.1194, a fragment ion was obtained after the precursor ion lost a $C_2H_4O_2$ fragment [M-H-$C_2H_4O_2$]$^-$. The m/z 489.1083 represents the fragment ion obtained after the precursor ion had lost 90 Da [M-$C_3H_6O_3$-H]$^-$. The m/z 429.0856 represents the fragment ion obtained after the precursor ion had lost 150 Da [M-$C_3H_6O_3$-$C_2H_4O_2$-H]$^-$. The m/z 399.0748 represents the fragment ion obtained by discarding 180 Da of the precursor ion [M-$C_2H_4O_2$-$C_4H_8O_4$-H]$^-$. The m/z 369.0635 represents the fragment ion obtained by losing 120 Da of the precursor ion [M-$C_7H_{14}O_7$-H]$^-$. The m/z 339.0521 represents the fragment ion obtained by discarding 240 Da of the precursor ion [M-$C_7H_{14}O_7$-CH_2O-H]$^-$. According to the fragment ions in the secondary mass spectrum and related literature, we speculated that the compound was luteolin 6-C-hexoside, 8-C-pentoside [43].

The retention time of Compound **4** was 12.58 min. Compound **4** had the [M − H]$^-$ ion at m/z 563.1414, and the product ions at m/z 473.1115,443.1001, and 353.0670 were generated. It was identified as kaempferol 6-C-hexoside-8-C-hexoside [44]. The m/z 563 [M − H]$^-$ is its quasi-molecular ion peak, and m/z 443.1001, 473.1115, and 353.0670 are the main fragments produced by its cracking. The most abundant fragment ion in MS_2 was m/z 443. There was a difference of 120 mass units between it and m/z 563. We speculated that it was produced by 0-2 cracking of the hexosyl part of [M − H]$^-$. Due to the difference of 90 mass units between m/z 563 → 473, we speculated that m/z 473 was caused by 0-3 cracking of the hexosyl part of [M − H]$^-$. The difference between m/z 563 → 353 was 120 + 90 mass units. We speculated that [M − H]$^-$ 0-2 cracking of hexose and pentose occurred simultaneously in the ESI source. The difference between m/z 563 → 383 was 120 + 60 mass units. We speculated that [M − H]$^-$ 0-2 cracking of pentose and 0-3 cracking of hexose may have occurred simultaneously in the ESI source. The difference between m/z 563 → 503 was 60 mass units. Presumably, [M − H]$^-$ 0-3 cracking of the pentose occurred in the ESI source. The main ion fragments appearing in the secondary

mass spectrum were similar to those in the description of the cleavage behavior of the sugar moiety of flavonoid glycosides in the literature. Compound **6** (t_R = 13.02 min) showed the $[M - H]^-$ ion at m/z 473.1115, and the MS/MS mainly produced characteristic fragment ions of carbon glycosides: m/z 443.1001, 353.0670. It was identified as kaempferol 6-C-hexoside-8-C-hexoside [44]. The cleavage rules for isochafotaside and kaempferol 6-C-hexoside-8-C-hexoside were the same, but the peak order was different. Kaempferol 6-C-hexoside-8-C-hexoside was in the front; isochafotaside was in the back.

Compound **5** (t_R = 12.87 min) showed an MS spectrum for the $[M - H]^-$ ion at m/z 447.0934 and the product ions at m/z 369.0615, 357.0622, 327.0511, 297.0397, 285.0396, and 133.0280. It was identified as quercetin 6-C-hexobioside [45]. The available molecular formula is $C_{21}H_{20}O_{11}$. Among these, m/z 369.0615 represents the fragment ion $[M\text{-}H\text{-}C_2H_6O_3]^-$ after the precursor ion had lost 78 Da. The m/z 357.0622 represents the fragment ion $[M\text{-}H\text{-}C_3H_6O_3]^-$ after the precursor ion had lost 90 Da. The m/z 327.0511 represents the fragment ion after the precursor ion had lost 120 Da, $[M\text{-}H\text{-}C_4H_8O_4]^-$. The m/z 297.0397 represents the fragment ion after the precursor ion had lost 180 Da, $[M\text{-}H\text{-}C_5H_{10}O_5]^-$. The m/z 285.0396 represents the fragment ion $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ after the precursor ion had lost 162 Da. It is the aglycon of the compound after removing glucose. The m/z 133.0280 represents the fragment ion of this compound after RDA fragmentation, $[M\text{-}H\text{-}C_7H_4O_4]^-$.

Compound **8** (t_R = 14.02 min) showed the $[M - H]^-$ ion at m/z 577.1577, and the product ions at m/z 457.1164 and 293.0454 were detected. We speculated that there was glucose in the structure of the compound and that the link between sugar and the parent nucleus was carbon glycoside. The molecular ion peak was m/z 577 $[M - H]^-$. Then, a fragment ion peak of $[M\text{-}H\text{-}120]^-$ appeared. We observed that it cleaved off the ion $C_4H_8O_4$, and the original structure may have been replaced by hexose. In addition, the position of the pentose phosphate pathway easily merged with the parent ring, and the hydroxyl group was dehydrated and broken and lost 164, forming m/z 413 $[M\text{-}H\text{-}164]^-$. When position 120 of hexose was closed again, m/z 293 $[M\text{-}H\text{-}120]^-$ was formed. Thus, Compound **8** was identified as apigenin 2″-*O*-pentoside [46].

Compound **9** (t_R = 14.14 min) showed the $[M - H]^-$ ion at m/z 432.1029, and the product ions at m/z 341.0673, 311.0564, and 283.0612 were generated. In the process of sugar chain cleavage, one molecule of $C_3H_6O_3$ was lost to generate fragment ions $[M\text{-}H\text{-}90]^-$. The m/z 311.0564 represents the fragment ion generated by losing one molecule of $C_4H_8O_4$ during the rupture of the glucosyl ring with the parent ion of m/z 431.0981 $[M\text{-}H\text{-}120]^-$. It continued to lose one molecule of CO to generate fragments of m/z 283.0612 $[M\text{-}H\text{-}C_4H_8O_4\text{-}CO]^-$. In addition, m/z 311.0564 lost one molecule of CH_2O to produce fragment ions m/z 281.0450 $[M\text{-}H\text{-}C_4H_8O_4\text{-}CH_2O]^-$. It was identified as apigenin 6-C-hexoside [47].

4. Conclusions

This study indicated that the proposed method could be successfully used for the pretreatment and identification of flavonoids in discarded fig leaves. Overall, an innovative extraction method was developed to extract flavonoids from discarded fig leaves by UEAATPE. The ATPE system of 38% (*w*/*w*) ethanol/18% (*w*/*w*) ammonium sulfate was established as the final system. Simultaneously, an enzymatic concentration of 0.4 U/g, 150 min enzymolysis, an enzymolysis temperature of 50 °C, a liquid–solid ratio of 20:1 (mL/g), and an extraction time of 30 min were obtained as the optimal UEAATPE conditions. Under these conditions, we obtained 60.22 mg/g flavonoids by UEAATPE. The yield of total flavonoids obtained by UEAATPE was 1.13-fold, 1.21-fold, 1.27-fold, and 2.43-fold higher than the yields obtained by the other four methods (EAATPE, UAATPE, ATPE, and SE), respectively. UEAATPE has been shown to be a promising method in the field of bioactive ingredient extraction. Among the eleven compounds characterized, ten flavonoids were first reported in fig leaves. The major flavonoid with the highest content was quercetin 3-*O*-hexobioside, at about 26.93%. The reuse of fig leaf waste and the development of biologically active ingredients can help to minimize the impact of agricultural waste on the environment. All of this is attributed to the potential medicinal

properties of fig leaf flavonoids. In the future, this method may be increasingly applied in the development and utilization of agricultural waste, acting as a bond between promoting economic balance and environmental protection.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11167718/s1. Figure S1: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 1; Figure S2: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 2; Figure S3: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 3; Figure S4: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 4; Figure S5: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 5; Figure S6: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 6; Figure S7: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 7; Figure S8: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 8; Figure S9: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 9; Figure S10: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 10; Figure S11: The first order mass spectrometry (a), the secondary mass spectrometry (b) and the cleavage pathway (c) of compound 11.

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

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