separations

MDPI

Article

An Ultra-Fast and Green LC-MS Method for Quantitative Analysis of Aesculin and Aesculetin in Cortex Fraxini

Xiaodong Wang [1,2,†], Wenhao Wang [2,†], Qinggui Lei [2], Zhengming Qian [2,*] and Wenbin Deng [1,*]

[1] School of Pharmaceutical Sciences, Sun Yat-sen University, No.66, Gongchang Road, Guangming District, Shenzhen 518107, China; xiaodongw420@163.com
[2] Key Laboratory of State Administration of Traditional Chinese Medicine, Dongguan HEC Cordyceps R&D Co., Ltd., No.368, Zhen'an Middle Road, Chang'an Town, Dongguan 523850, China
* Correspondence: qianzhengming1982@126.com (Z.Q.); dengwb5@mail.sysu.edu.cn (W.D.); Tel.: +86-0769-8531-5888 (Z.Q.); +86-0755-2326-0201 (W.D.)
† These authors contributed equally to this work.

Abstract: This study aims to develop a fast and eco-friendly liquid chromatography–mass spectrometry (LC-MS) method for the determination of aesculin and aesculetin in Cortex Fraxini. Ultrapure water was used as the solvent during the microwave-assisted extraction process to prepare the Cortex Fraxini sample. This extraction method reduces the cost of the harmful solvent (only ultrapure water was used) and microwave extraction time (1 min). The LC separation was conducted using an Agilent InfinityLab Poroshell 120 EC-C_{18} column (2.1 mm × 30 mm, 2.7 µm) with a mobile phase consisting of 0.1% formic acid and acetonitrile (90:10, *v*/*v*) at a flow rate of 0.6 mL/min. Isocratic elution was employed, and the analytes were detected by MS. Through careful optimization and selection of LC-MS conditions, the analysis time was reduced to 1 min, demonstrating the method's efficiency. The developed method was validated and exhibited excellent specificity, linearity, limit, precision, accuracy, and stability in quantifying aesculin and aesculetin in the Cortex Fraxini samples. The analysis result revealed the presence of aesculin (ranging from 3.55 to 18.8 mg/g) and aesculetin (ranging from 1.01 to 16.2 mg/g) in all ten batches of Cortex Fraxini samples. Compared to the reported LC methods, this approach substantially reduces the total analysis time and requires a minuscule volume of organic solvents. An "Analytical Eco-Scale" assessment was used to evaluate the different assay methods of Cortex Fraxini. The current LC-MS method scored an impressive 90; it was better than the other four reports' LC methods. Thus, the developed LC-MS method is rapid and green, which is helpful for the quality evaluation of Cortex Fraxini.

Keywords: LC-MS; Cortex Fraxini; aesculin; aesculetin; green; fast

Citation: Wang, X.; Wang, W.; Lei, Q.; Qian, Z.; Deng, W. An Ultra-Fast and Green LC-MS Method for Quantitative Analysis of Aesculin and Aesculetin in Cortex Fraxini. *Separations* **2023**, *10*, 515. https://doi.org/10.3390/separations10090515

Academic Editor: Paraskevas D. Tzanavaras

Received: 2 August 2023
Revised: 5 September 2023
Accepted: 13 September 2023
Published: 19 September 2023

1. Introduction

Cortex Fraxini has been utilized in traditional Chinese medicine to treat a variety of ailments for centuries. It is employed as an analgesic, antibacterial, and anti-inflammatory agent for conditions such as arthritis, bacillary dysentery, diarrhea, and hyperuricemia [1–4]. Cortex Fraxini is derived from the bark or branch bark of *Fraxinus chinensis* Roxb., *Fraxinus rhynchophylla* Hance, *Fraxinus chinensis* Roxb. var. *acuminate* Lingelsh., and *Fraxinus stylosa* Lingelsh., which are widely distributed in China. The therapeutic properties of Cortex Fraxini are attributed to its bioactive compounds, particularly aesculin and aesculetin (Figure 1), which have been extensively studied in recent years [5–8]. Aesculin possesses anticoagulant [9], anti-inflammatory [10], and antioxidant activities [11], while aesculetin exhibits anticancer [12], anti-inflammatory [13], and antibacterial effects [14]. Hence, it is critical to quantitatively analyze aesculin and aesculetin in Cortex Fraxini to evaluate its quality and therapeutic potential. With the increasing utilization of Cortex Fraxini as a raw material in pharmaceutical development, the significance of green chemistry is also growing due to global environmental concerns. However, current quantitative

methods predominantly employ substantial quantities of hazardous organic solvents, such as methanol [5–7]. Moreover, although a relatively environmentally friendly quantitative method has been developed, it still necessitates 75 min for the analysis process, which fails to fulfill the existing demand [8]. Consequently, there exists an imperative requirement to devise a more efficient and environmentally friendly quantitative method to bridge this gap.

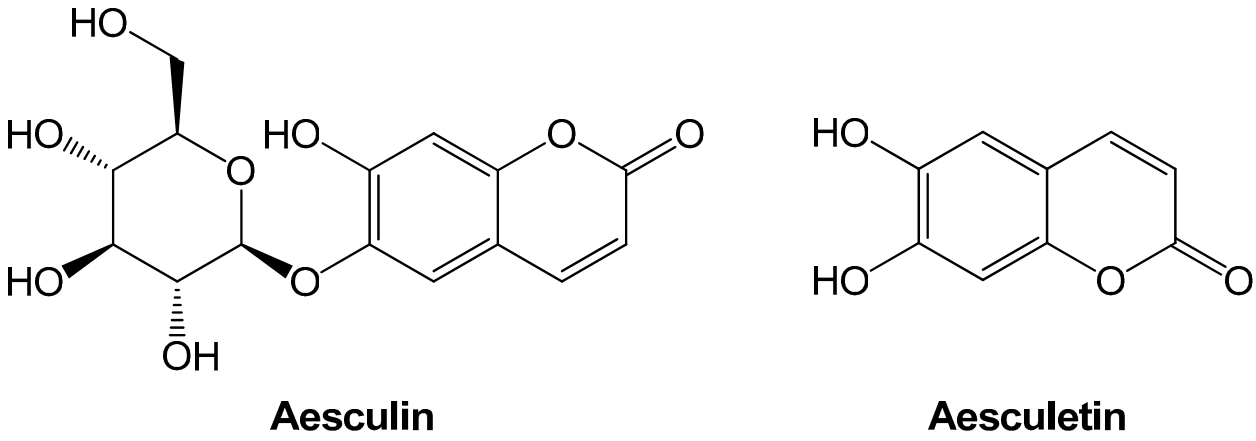

Figure 1. Chemical structures of aesculin and aesculetin.

Liquid chromatography (LC) gained popularity due to its ability to provide precise and rapid separations [15–17]. Mass spectrometry (MS) is a powerful analytical tool increasingly utilized in numerous scientific disciplines because of its high sensitivity, selectivity, accuracy, and high throughput capability. MS provides structural and content information about the analytes by measuring the mass-to-charge ratio (*m/z*) values of charged molecules [18]. As a result, MS enables the rapid detection of specific compounds, which is more efficient compared to conventional ultraviolet detection methods. Furthermore, MS is widely used for the rapid analysis of medicinal herbs due to its fast and efficient analytical capabilities [19–21]. Therefore, utilizing environmentally friendly extraction solutions and integrating the features and benefits of LC and MS assays present a good plan for rapid analytical methods for the determination of bioactive components in medicinal herbs.

In this study, a green and ultra-rapid LC-MS-based assay using ultrapure water as the sample extraction solvent was developed for the determination of aesculin and aesculetin in Cortex Fraxini. The new method was also applied to quantify ten batches of Cortex Fraxini samples to assess the effectiveness of the method in practical applications.

2. Materials and Methods

2.1. Chemicals and Reagents

Ultrapure water used in the study was purified by a Milli-Q Advantage A10 water purification system (Merck KGaA, Darmstadt, Germany). Formic acid [high-performance LC (HPLC) grade] was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Acetonitrile (HPLC grade) was procured from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China). Aesculin (99.77%) and aesculetin (99.94%) were sourced from Chengdu Push Bio-technology Co., Ltd. (Chengdu, Sichuan, China).

2.2. Sample of Cortex Fraxini

Ten batches of Cortex Fraxini samples were collected and labeled S1 to S10, all of which were identified as the dried bark or branch bark of *Fraxinus chinensis* Roxb., from the Oleaceae family, by Dr. Qian (Figure 2). Samples S1 to S3 were obtained from Kangmei Pharmaceutical Co., Ltd. (Shenzhen, Guangdong, China), samples S4 to S9 were commercially available from Dongguan, Guangdong, China, and sample S10 was procured from Lingnan Traditional Chinese Medicine Tablets Co., Ltd. (Foshan, Guangdong, China).

Figure 2. Cortex Fraxini.

2.3. Chromatographic Conditions

The parameters were optimized to achieve ultra-fast LC-MS conditions. Various conditions such as the mobile phase composition and ratio, monitoring mode, and quantitative ions were studied to obtain clear chromatograms without interfering peaks. The LC conditions used for optimization were as follows: an Agilent InfinityLab Poroshell 120 EC-C_{18} column (4.6 mm × 50 mm, 2.7 μm; Agilent Technologies, Inc., Santa Clara, CA, USA); the mobile phase comprised 0.1% formic acid (A) and acetonitrile (B) (94:6, *v*/*v*); the column temperature was set to 40 °C; and the injection volume was 5 μL. In addition, mobile phase ratios were optimized using an Agilent InfinityLab Poroshell 120 EC-C_{18} column (2.1 mm × 30 mm, 2.7 μm; Agilent Technologies, Inc., Santa Clara, CA, USA).

The optimized LC-MS conditions were as follows.

LC conditions: The LC separation was conducted using an Agilent 1260 Infinity LC System (Agilent Technologies, Inc., Santa Clara, CA, USA), consisting of a binary pump, a thermostatic column compartment, and an autosampler. The chromatographic column employed was an Agilent InfinityLab Poroshell 120 EC-C_{18} column (2.1 mm × 30 mm, 2.7 μm). The mobile phase was 0.1% formic acid (A) and acetonitrile (B) (90:10, *v*/*v*). The flow rate was set at 0.6 mL/min, the column temperature was maintained at 40 °C, and the injection volume was 1 μL.

MS conditions: The analytes were detected using an Agilent 6130 Single Quadrupole LC/MS System (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an electrospray ionization source operating in negative ionization mode. The selected ion monitor mode was employed to record the monitored ions, namely *m/z* 339 for aesculin and *m/z* 355 for aesculetin. The additional operating conditions were as follows: a drying gas (N_2) flow rate of 10.0 L/min, a gas temperature of 300 °C, a nebulizer gas pressure of 30 psig, a fragmentor setting of 70 V, and a capillary voltage of 4000 V.

2.4. Standard Solution Preparation

The aesculin (20.18 mg) and aesculetin (20.18 mg) were precisely weighed and dissolved in 100% ethanol, respectively. Subsequently, the solutions were adjusted to a final volume of 10 mL each, resulting in standard solutions with concentrations of 2.02 mg/mL and 2.02 mg/mL, respectively. The aesculin and aesculetin standard solutions were transferred to 100 mL volumetric flasks as necessary and then diluted with ultrapure water to obtain mixed standard solutions.

2.5. Sample Solution Preparation

The Cortex Fraxini samples were pulverized and passed through a 50-mesh screen with an inner sieve hole diameter of 355 μm ± 13 μm. Approximately 20 mg of Cortex Fraxini powder was precisely weighed and transferred to a 25 mL colorimetric tube. Then, 2 mL of ultrapure water was added to the tube. Following that, the tube was sealed with plastic wrap and subjected to microwave extraction at 700 W for 1 min using a Midea M1-L213B microwave oven (Midea Group Co., Ltd., Foshan, Guangdong, China). After the extraction, the tube was allowed to cool, and the volume was adjusted to 10 mL with ultrapure water. The suspension was thoroughly mixed, and then the resulting supernatant

was collected and filtered through a 0.22 μm pore-size membrane filter. The collected filtrate was then utilized as the sample solution.

2.6. Method Validation

To evaluate the effectiveness of the developed method, the method validation was operated according to the guidelines of the Chinese Pharmacopoeia, which included the specificity, linearity, limit, precision, accuracy, and stability [22].

2.6.1. Specificity, Linearity and Limit

To evaluate the specificity of the analytical method, the blank solutions of ultrapure water, mixed standard solutions containing aesculin (16.1 μg/mL) and aesculetin (30.3 μg/mL), and sample solutions were tested. By utilizing established extraction procedures and analytical conditions, we investigated the peak regions of aesculin and aesculetin to identify any potential interference. For the purpose of assessing linearity, standard solutions were accurately diluted with ultrapure water to obtain aesculin and aesculetin concentrations of 1.01, 2.02, 5.05, 10.1, 20.2, and 50.5 μg/mL, and then analyzed. A standard curve was constructed by plotting the peak area (*Y*-axis) against the concentration of the standard solutions (*X*-axis), in order to obtain a linear regression equation and the *R*-value for evaluating linearity. The limit of quantitation was calculated based on a signal-to-noise ratio of 10.

2.6.2. Precision

The precision of the analytical method was assessed by conducting intra-day and inter-day precision tests as well as repeatability tests. The intra-day precision test involved performing six consecutive injections of aesculin and aesculetin mixed standard solution at concentrations of 16.1 μg/mL and 30.3 μg/mL, respectively, within one day. The peak area's relative standard deviation (RSD%) was then calculated to assess the intra-day precision of the method. For the inter-day precision test, the same mixed standard solution was injected twice a day for three consecutive days, and the RSD% of the peak area was calculated to evaluate the method's inter-day precision. To confirm repeatability, six aliquots of S4 powder weighing 20 mg each were quantitatively analyzed using established extraction procedures and analytical conditions. The RSD% of the aesculin and aesculetin content in the six sample solutions was then calculated.

2.6.3. Accuracy

Six aliquots, each weighing 10 mg, of S4 powder were precisely weighed and transferred into 25 mL colorimetric tubes. Afterwards, 2 mL of a mixed standard solution, consisting of 80.7 μg of aesculin and 151 μg of aesculetin, was added to each tube. The quantification of the compounds was carried out using the established extraction procedure and analytical conditions. The accuracy of the analytical method was verified by calculating the amount, recovery rate, average recovery rate, and RSD% for both aesculin and aesculetin. The recovery rate was determined by using the formula: 100% × (found amount − original amount)/spiked amount.

2.6.4. Stability

S4 powder (20 mg) was weighed and prepared into samples for testing using the established extraction procedure. The samples were quantified at room temperature (25 ± 5 °C) at different time intervals: 0, 4, 6, 8, 16, and 24 h. The stability of the analytical method was ensured by calculating the RSD%.

3. Results and Discussion

3.1. Optimization of Extraction Conditions

Previous methods for measuring aesculin and aesculetin in Cortex Fraxini have frequently utilized organic solvents as extraction solvents [5–7]. However, these solvents are inconsistent with the principles of green chemistry. In this study, ultrapure water, a

green solvent, was utilized for the extraction of the target compounds from the sample. In order to obtain optimal extraction conditions, the extraction time and solvent volume were individually evaluated. Initially, different extraction times of 0.5, 1, 1.5, and 2 min were assessed. The results indicated no significant variation in the extraction rate at each time period. However, it must be noted that a time of 0.5 min was deemed too short, potentially leading to inconsistent heating of the sample. On the other hand, a time of above 1.5 min increased the risk of the solvent drying out. Thus, for practical purposes, the extraction time of 1 min was ultimately selected for this investigation. Subsequently, we examined solvent volumes of 1 mL, 2 mL, and 3 mL. The results indicated comparable extraction rates among the three volumes. Nevertheless, at a solvent volume of 1 mL, the higher temperature caused the solvent to evaporate rapidly, increasing the likelihood of it drying out. Hence, a 2 mL volume of solvent was used in current study.

In summary, the final extraction method for this study involved the following steps: Cortex Fraxini was crushed and sieved, accurately weighed (~20 mg), placed in a colorimetric tube, mixed with 2 mL of ultrapure water, sealed with cling film, microwaved for 1 min, cooled, diluted to 10 mL, and shaken thoroughly. The supernatant was then filtered through a 0.22 μm membrane to collect the filtrate as the sample solution. This developed extraction method offers a simplified operation and fast extraction and utilizes the eco-friendly solvent of ultrapure water. Hence, this extraction method may serve as an efficient and green technique for the quantification of aesculin and aesculetin in Cortex Fraxini, further promoting the concept of green analytical chemistry.

3.2. Optimization of Chromatographic Conditions

The LC and MS conditions were optimized to achieve ultra-fast LC-MS conditions. By integrating a fast separation column (superficially porous column) and an efficient MS detector for analysis, the analytical time was reduced to only 1 min. The superficially porous column offers comparable efficiency and speed to sub-2 μm particle columns while maintaining the low back pressure characteristic of conventional LC systems [23,24]. As a result, this method can be implemented cost-effectively on conventional LC platforms in various laboratory settings, facilitating its adoption and widespread use.

To investigate the analysis of Cortex Fraxini, an Agilent InfinityLab Poroshell 120 EC-C_{18} column was employed to optimize the LC conditions. During the optimization of LC conditions, different conditions were tested, which were commonly used in previous studies [5–8]. Three mobile phase combinations were compared: water–acetonitrile, 0.1% formic acid aqueous solution–acetonitrile, and 1 mmol/L ammonium acetate–acetonitrile. The results demonstrated that the utilization of a 0.1% formic acid aqueous solution–acetonitrile as the mobile phase yielded the greatest specificity and sensitivity for both target components (Figure S1). Consequently, 0.1% formic acid aqueous solution and acetonitrile was chosen as the optimal mobile phase. The choice of mobile phase ratio is essential. Three different ratios of 0.1% formic acid aqueous solution and acetonitrile (88:12, 89:11, and 90:10) were evaluated, and it was determined that the 90:10 ratio provided a fast elution time while maintaining a good peak shape and meeting the resolution criteria. In contrast, the other ratios resulted in lower resolution of the target compounds' peaks due to their higher organic solution content (Figure S2). Consequently, a mobile phase composed of a 0.1% formic acid aqueous solution and acetonitrile in a 90:10 (v/v) was utilized.

In the present study, the detection was operated on a single quadrupole MS. To achieve high specificity and sensitivity, the MS conditions were optimized, including the selection of the monitoring mode and quantitative ions. A comparison of the MS responses in the positive and negative ion modes revealed that both analytes exhibited better sensitivity in the negative ion mode (Figure S3). For quantitative analysis, $[M\text{-}H]^-$ (m/z 339) was chosen as the detection ion for aesculin, and $[2M\text{-}H]^-$ (m/z 355) was chosen for aesculetin, owing to their highest response (Figure S4). Additionally, we evaluated the impact of the interference peaks on the target compounds. The investigation employed analytical conditions with a long LC column and took 14 min separation time. The results displayed in Figure S5

demonstrate the chromatograms obtained through LC-MS and LC–ultraviolet detection. It was shown that there were no interference peaks with the target compounds in the LC-MS chromatogram. Therefore, the LC-MS can be further used for the ultra-fast analysis of two analytes by Agilent InfinityLab Poroshell 120 EC-C_{18} column (2.1 mm × 30 mm, 2.7 μm).

In conclusion, the final LC-MS condition was as follows: Agilent InfinityLab Poroshell 120 EC-C_{18} column (2.1 mm × 30 mm, 2.7 μm) was used for separation of the sample solution. The mobile phase consisted of 0.1% formic acid and acetonitrile (90:10). Additionally, the MS detector was set in negative ion mode. Selected ion monitor mode was used to record the monitored ions, *m/z* 339 for aesculin and *m/z* 355 for aesculetin.

3.3. Method Validation

As depicted in Figure 3, the specificity study revealed no interference at the elution positions of aesculin and aesculetin in the blank chromatogram. Additionally, the resolution of neighboring peaks was greater than 1.5, indicating excellent specificity of the method. The results for the linearity and the limit are presented in Table 1. The linearity study demonstrated that all compounds showed *R*-values above 0.9928. In the precision study, the intra-day precision test showed RSD% values of 1.46% for aesculin and 0.817% for aesculetin. Similarly, the inter-day precision test showed RSD% values of 1.97% for aesculin and 4.06% for aesculetin. These results indicate excellent intra-day and inter-day precision. In the repeatability study, the RSD% values of aesculin and aesculetin content in six sample solutions were found to be 2.01% and 1.45%, respectively. The accuracy study results revealed that the recovery rates of aesculin in six spiked sample solutions ranged from 99.9% to 104%, while those of aesculetin ranged from 96.0% to 103%. The average recovery rates were 102% and 101% for aesculin and aesculetin, respectively, with RSD% values of 1.34% and 2.47% (Table S1). Additionally, the investigation into the stability of the experiment revealed that the values of RSD% for the peak areas of aesculin and aesculetin were 2.40% and 2.06%, respectively. This suggests that the sample solutions were stable for 24 h.

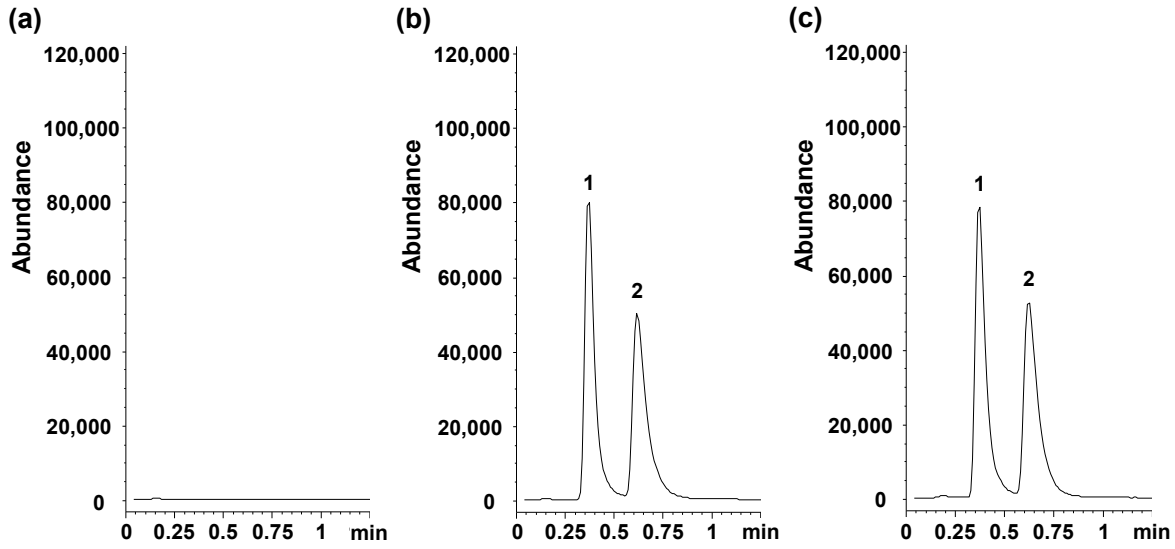

Figure 3. LC-MS chromatograms of the blank solution (**a**), the standard solution (**b**), and the sample solution (**c**), depicting: aesculin (1) and aesculetin (2).

Table 1. Method validation for linearity and limits.

Analytes	Linearity			Limit
	Calibration Curves	*R*	Range (μg/mL)	LOQ (μg/mL)
Aesculin	$Y = 11{,}928.8\ X + 30{,}325.3$	0.9929	1.01~50.5	0.101
Aesculetin	$Y = 7290.2\ X - 3581.4$	0.9997	1.01~50.5	1.01

LOQ: limit of quantification.

3.4. Sample Analysis

Ten batches of Cortex Fraxini samples were analyzed using the developed LC-MS method, and the corresponding results are presented in Table 2. The aesculin content ranged from 3.55 to 18.8 mg/g, while the aesculetin content ranged from 1.01 to 16.2 mg/g. The combined content of these two compounds ranged from 4.56 to 24.8 mg/g. Among all the samples, S6 exhibited the highest aesculin content, whereas S9 had the lowest. Similarly, S4 showed the highest aesculetin content, while S9 had the lowest. In terms of the total content, S4 had the highest, whereas S9 had the lowest. According to the Chinese Pharmacopoeia (2020 edition), the total content of aesculin and aesculetin in dried Cortex Fraxini should be higher than 10 mg/g [25]. Hence, eight batches of samples met the requirements, with the total content of the compliant samples ranging from 11.7 to 24.8 mg/g. However, two batches (S8 and S9) did not meet the criteria, as their total content was determined to be 9.95 mg/g and 4.56 mg/g, respectively. Further investigation is needed to determine the reasons for the non-compliance in different samples. In addition, a previous report revealed that the average content of aesculin and aesculetin in Cortex Fraxini was measured to be 16.5 mg/g and 7.81 mg/g, respectively [6]. This is similar to our quantitative results, which showed the average contents of 11.5 mg/g for aesculin and 4.03 mg/g for aesculetin. This confirms the precision and reliability of the newly established analytical method.

Table 2. Aesculin and aesculetin contents in Cortex Fraxini (mg/g; means ± standard deviation; $n = 2$).

Batch	Aesculin (mg/g)		Aesculetin (mg/g)		Total (mg/g)
S1	11.6	±0.239	1.12	±0.0325	12.7
S2	16.8	±0.202	2.39	±0.0427	19.2
S3	16.0	±0.407	2.67	±0.0275	18.6
S4	8.59	±0.223	16.2	±0.0467	24.8
S5	9.14	±0.208	2.53	±0.0680	11.7
S6	18.8	±0.0171	1.24	±0.0233	20.0
S7	8.00	±0.0901	3.97	±0.0446	12.0
S8	5.09	±0.0273	4.85	±0.0253	9.95
S9	3.55	±0.0374	1.01	±0.0119	4.56
S10	17.4	±0.501	4.33	±0.103	21.8
Average	11.5	±5.45	4.03	±4.49	15.5

In conclusion, the method established in this study is simple, rapid, and repeatable. It is suitable for the determination of aesculin and aesculetin and helps in the quality evaluation of Cortex Fraxini.

3.5. Comparison with the Reported LC Methods

The comparison between the present method and previously reported methods is summarized in Table 3. The total analysis time for the previous methods, including sample extraction and LC separation, all exceeded 74 min. Method 3, which had a complex extraction process, required more than 240 min, making it the slowest method. Although Method 4 was the fastest one, it still took 75 min to complete the sample analysis. In contrast, the current method requires approximately 4 min, consisting of the sample extraction time (1 min for microwaving and approximately 1.5 min for sample solution cooling and filtering) and the LC-MS separation time (0.5 min for sample injection and 1 min for LC separation). This is a significant improvement, as it is more than 10 times faster than Method 4. Therefore, this method is fit for the rapid determination of aesculin and aesculetin in Cortex Fraxini.

Table 3. LC methods for determination of aesculin and aesculetin in Cortex Fraxini.

No	Analysts	Sample Preparation				Sample Detection			Total Time	Average Content (mg/g)	References
		Methods	Solvents	Time	Other Steps	Methods	Mobile Phase	Time			
1	Aesculin Aesculetin	Ultrasonic ice water bath extraction	73% methanol; over 10 mL	75 min	Centrifugation	HPLC-MS/MS	A: methanol; 10.7 mL B: 0.05% acetic acid aqueous solution; 13.3 mL	24 min	99 min	24.6 3.79	[5]
2	Aesculin Aesculetin	Sonication extraction	80% methanol; 15 mL	45 min	Filtration	HPLC-DAD-ESI-MS	A: acetonitrile; 9.28 mL B: 0.3% acetic acid aqueous solution; 30.2 mL	40 min	85 min	16.5 7.81	[6]
3	Aesculin Aesculetin	Immersion extraction Ultrasonic extraction	70% ethanol; Approximately 2340 mL. Methanol; Approximately 10 mL. 0.1% phosphoric acid containing 0.2% triethylamine; Approximately 5 mL.	Over 190 min	Centrifugation. Evaporation. Filtration.	HPLC-UV	A: acetonitrile; 9 mL B: 0.1% phosphoric acid contained 0.2% triethylamine; 31 mL	50 min	Over 240 min	NC	[7]
4	Aesculin Aesculetin	Ultrasonic extraction	20% betaine/glycerol (1:3); 10 mL	50 min	Centrifugation Filtration	HPLC-UV	A: 0.1% formic acid aqueous solution; 8.75 mL B: methanol; 3.75 mL	25 min	75 min	36.3 1.87	[8]
5	Aesculin Aesculetin	Microwave extraction	Ultrapure water; 10 mL	2.5 min	Filtration	LC-MS	A: 0.1% formic acid aqueous solution; 0.54 mL B: acetonitrile; 0.06 mL	1.5 min	4 min	11.5 4.03	This work

HPLC-MS/MS: high-performance liquid chromatography–tandem mass spectrometry; HPLC-DAD-ESI-MS: high-performance liquid chromatography–diode array detector–electrospray ionization mass spectrometry; HPLC-UV: high-performance liquid chromatography–ultraviolet detection; LC-MS: liquid chromatography–mass spectrometry; NC: non-calculable (the quantitative samples used in this method consist of compounded herbs, making it difficult to accurately determine the specific content of Cortex Fraxini).

In addition, hazardous organic solvents, such as methanol and acetonitrile, were commonly employed for sample extraction and LC separation. The largest quantity of hazardous organic solvents used among the previously reported methods was Method 2, which consumed 10 mL of methanol and 9 mL of acetonitrile. Conversely, Method 4 had the lowest solvent consumption, using only 3.75 mL of methanol. In contrast, the developed LC-MS method only costs 0.06 mL acetonitrile. To further evaluate the environmental impact of the developed method and reported ones, an Analytical Eco-Scale assessment was carried out [26]. This assessment is a widely recognized metric used to gauge the environmental sustainability of analytical procedures. As listed in Table 4, the metric evaluated whether various parameters of the analytical procedure (such as reagent amount, hazards, energy, and waste) deviated from the criteria for an ideal environmentally sustainable analysis, which is assigned a maximum score of 100. A score greater than 75 indicates excellent green analysis, a score between 50 and 75 indicates acceptable green analysis, and a score below 50 indicates inadequate green analysis [26]. Among the five methods examined, only Method 4 and the current LC-MS method met the criteria for excellent green analysis. Notably, the method developed in this study exhibited the highest level of environmental friendliness, scoring an impressive 90 points. In comparison, Method 4 scores 7 points lower than the developed method, despite its already high level of environmental sustainability. This is attributed to the fact that the developed method solely employs ultrapure water for the extraction process, in addition to utilizing a minuscule volume of organic solution during the LC-MS analysis. Consequently, this method presents the greatest potential for environmental protection.

Table 4. Environmental impact assessment of developed and reported analytical methods.

Scoring Items	Penalty Points				
	Method 1	**Method 2**	**Method 3**	**Method 4**	**Proposed Method**
Reagents					
Acetic acid	4	4	/	/	/
Phosphoric acid	/	/	2	/	/
Triethylamine	/	/	6	/	/
Betaine	/	/	/	1	/
Glycerol	/	/	/	0	/
Formic acid	/	/	/	2	2
Acetonitrile	/	4	4	/	4
Methanol	12	12	12	6	/
Ethanol	/	/	6	/	/
Ultrapure water	0	0	0	0	0
Instrument energy					
Ultrasonic generator	2	1	1	1	/
Centrifuge	0	/	0	1	/
Heater	/	/	2	/	/
Microwave oven	/	/	/	/	0
HPLC-MS/MS	1	/	/	/	/
HPLC-DAD-ESI-MS	/	1	/	/	/
HPLC-UV	/	/	1	1	/
LC-MS	/	/	/	/	0
Occupational hazard	3	3	3	0	3
Waste	5	5	5	5	1
Total penalty points	27	30	42	17	10
Analytical Eco-Scale total score	73	70	58	83	90

HPLC-MS/MS: high-performance liquid chromatography–tandem mass spectrometry; HPLC-DAD-ESI-MS: high-performance liquid chromatography–diode array detector–electrospray ionization mass spectrometry; HPLC-UV: high-performance liquid chromatography–ultraviolet detection; LC-MS: liquid chromatography–mass spectrometry.

4. Conclusions

In this study, simultaneous determination of aesculin and aesculetin in Cortex Fraxini samples was performed by LC-MS. Ten batches of Cortex Fraxini samples were examined, and the findings demonstrated that all samples of Cortex Fraxini contained aesculin and

aesculetin. Moreover, among the samples, 80% met the criteria of Chinese Pharmacopoeia. Compared with previously reported methods, the established LC-MS method is simple, fast, and eco-friendly. The total time for sample extraction and chromatographic analysis was only 4 min, and no organic solvents were used during the sample extraction process. The established LC-MS method is an improved analysis method that can be used to determine the content of aesculin and aesculetin in Cortex Fraxini samples.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10090515/s1, Table S1: Recovery results of the developed LC-MS method; Figure S1: MS chromatograms of the mobile phase in water–acetonitrile (94:6, *v*/*v*) (a), 0.1% formic acid aqueous solution–acetonitrile (94:6, *v*/*v*) (b), and 1 mmol/L ammonium acetate–acetonitrile (94:6, *v*/*v*) (c); Figure S2: MS chromatograms of the mobile phase in 0.1% formic acid aqueous solution–acetonitrile (88:12, *v*/*v*) (a); 0.1% formic acid aqueous solution–acetonitrile (89:11, *v*/*v*) (b); 0.1% formic acid aqueous solution–acetonitrile (90:10, *v*/*v*) (c); Figure S3: MS chromatograms of the sample extraction solution detected in the positive ion mode (a) and negative ion mode (b); Figure S4: MS spectra of aesculin (a) and aesculetin (b) with selected *m*/*z* for the target component (★); Figure S5: LC-MS (a) and LC–ultraviolet detection (b) chromatograms of sample extraction solution aesculin (1), aesculetin (2), and interference peak (x).

Author Contributions: Conceptualization, Z.Q. and W.D.; Funding acquisition, Z.Q.; Investigation, W.W., X.W. and Q.L.; Methodology, Q.L.; Project administration, W.D.; Supervision, Z.Q. and W.D.; Writing—original draft, W.W. and X.W.; Writing—review and editing, W.W., X.W., Z.Q. and W.D.; Resources, Z.Q. In particular, the quantitative method development, method validation, and sample quantification were done by Q.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Science and Technology Innovation Project of Guangdong Drug Administration (Project Number: 2021ZDB05).

Data Availability Statement: All data, models, and code generated or used during the study are presented in the submitted article.

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

References

1. Liang, C.; Ju, W.; Pei, S.; Tang, Y.; Xiao, Y. Pharmacological activities and synthesis of esculetin and its derivatives: A mini-review. *Molecules* **2017**, *22*, 387. [CrossRef] [PubMed]
2. Fang, L.H.; Lv, Y.; Du, G.H. Progress in study of pharmacological effect of Cortex Fraxini. *China J. Chin. Mater. Medica* **2008**, *33*, 2732–2736.
3. Tsai, J.C.; Tsai, S.; Chang, W.C. Effect of ethanol extracts of three Chinese medicinal plants with anti-diarrheal properties on ion transport of the rat intestinal epithelia. *J. Pharmacol. Sci.* **2004**, *94*, 60–66. [CrossRef] [PubMed]
4. Li, J.M.; Zhang, X.; Wang, X.; Xie, Y.C.; Kong, L.D. Protective effects of cortex fraxini coumarines against oxonate-induced hyperuricemia and renal dysfunction in mice. *Eur. J. Pharmacol.* **2011**, *666*, 196–204. [CrossRef] [PubMed]
5. Fu, S.; Zhang, J.; Li, T.; Wang, S.; Ding, W.; Zhao, M.; Du, Y.; Wang, Q.; Jia, J. Multi-responses extraction optimization based on response surface methodology combined with polarity switching HPLC–MS/MS for the simultaneous quantitation of 11 compounds in Cortex Fraxini: Application to four species of Cortex Fraxini and its 3 confusable species. *J. Pharm. Biomed. Anal.* **2014**, *91*, 210–221.
6. Zhou, L.; Kang, J.; Fan, L.; Ma, X.C.; Zhao, H.Y.; Han, J.; Wang, B.R.; Guo, D.A. Simultaneous analysis of coumarins and secoiridoids in Cortex Fraxini by high-performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2008**, *47*, 39–46. [CrossRef]
7. Wang, Y.; Xu, Q.; Shen, Z.; Wang, Y.; Dong, L.; Fu, S.; Yang, L.; Qin, M.; Zhang, Y.; Guo, S. Simultaneous determination of eight active compounds in Baitouweng Decotion and its single herbs. *J. Chromatogr. Sci.* **2019**, *57*, 502–510. [CrossRef]
8. Wang, Y.; Hu, Y.; Wang, H.; Tong, M.; Gong, Y. Green and enhanced extraction of coumarins from Cortex Fraxini by ultrasound-assisted deep eutectic solvent extraction. *J. Sep. Sci.* **2020**, *43*, 3441–3448. [CrossRef]
9. Ahmad, I.; Sharma, S.; Gupta, N.; Rashid, Q.; Abid, M.; Ashraf, M.Z.; Jairajpuri, M.A. Antithrombotic potential of esculin 7, 3′, 4′, 5′, 6′-O-pentasulfate (EPS) for its role in thrombus reduction using rat thrombosis model. *Int. J. Biol. Macromol.* **2018**, *119*, 360–368. [CrossRef]
10. Yu, Y.; Chen, J.; Zhang, X.; Wang, Y.; Wang, S.; Zhao, L.; Wang, Y. Identification of anti-inflammatory compounds from Zhongjing formulae by knowledge mining and high-content screening in a zebrafish model of inflammatory bowel diseases. *Chin. Med.* **2021**, *16*, 42. [CrossRef]

11. Song, Y.; Wang, X.; Qin, S.; Zhou, S.; Li, J.; Gao, Y. Esculin ameliorates cognitive impairment in experimental diabetic nephropathy and induces anti-oxidative stress and anti-inflammatory effects via the MAPK pathway. *Mol. Med. Rep.* **2018**, *17*, 7395–7402. [CrossRef] [PubMed]
12. Zhang, J.; Feng, M.; Guan, W. Naturally occurring aesculetin coumarin exerts antiproliferative effects in gastric cancer cells mediated via apoptotic cell death, cell cycle arrest and targeting PI3K/AKT/M-TOR signalling pathway. *Acta Biochim. Pol.* **2021**, *68*, 109–113. [CrossRef] [PubMed]
13. Wang, S.K.; Chen, T.X.; Wang, W.; Xu, L.L.; Zhang, Y.Q.; Jin, Z.; Liu, Y.B.; Tang, Y.Z. Aesculetin exhibited anti-inflammatory activities through inhibiting NF-κB and MAPKs pathway in vitro and in vivo. *J. Ethnopharmacol.* **2022**, *296*, 115489. [CrossRef]
14. Liang, J.; Huang, X.; Ma, G. Antimicrobial activities and mechanisms of extract and components of herbs in East Asia. *RSC Adv.* **2022**, *12*, 29197–29213. [CrossRef]
15. Clifford, M.N.; Wu, W.; Kirkpatrick, J.; Kuhnert, N. Profiling the chlorogenic acids and other caffeic acid derivatives of herbal Chrysanthemum by LC–MS^n. *J. Agric. Food. Chem.* **2007**, *55*, 929–936. [CrossRef] [PubMed]
16. Choi, S.; Shim, W.S.; Yoon, J.; Choi, D.; Song, E.; Choi, Y.J.; Paik, S.H.; Lee, K.T. Method Development and Validation for the Simultaneous Quantitation of Pentoxifylline, Its Pharmacologically Active Metabolites, and Donepezil Using LC–MS/MS in Rat Plasma: Its Application to a Pharmacokinetic Study. *Separations* **2023**, *10*, 276. [CrossRef]
17. Haque, A.; Iqbal, M.; Alamoudi, M.K.; Alam, P. A Selective and Accurate LC–MS/MS Method for Simultaneous Quantification of Valsartan and Hydrochlorothiazide in Human Plasma. *Separations* **2023**, *10*, 119. [CrossRef]
18. Glish, G.L.; Vachet, R.W. The basics of mass spectrometry in the twenty-first century. *Nat. Rev. Drug Discov.* **2003**, *2*, 140–150. [CrossRef]
19. Li, W.; Qian, Z.; Lei, Q.; Lian, Y.; Zou, Y.; Wang, Y.; Lan, D. An ultra-rapid and eco-friendly method for determination of loganic acid and gentiopicroside from Gentianae Macrophyllae Radix by vortex-assisted matrix solid-phase dispersion extraction and LC–MS. *J. Pharm. Biomed. Anal.* **2023**, *222*, 115085. [CrossRef]
20. Li, L.; Huang, M.; Shao, J.; Lin, B.; Shen, Q. Rapid determination of alkaloids in Macleaya cordata using ionic liquid extraction followed by multiple reaction monitoring UPLC–MS/MS analysis. *J. Pharm. Biomed. Anal.* **2017**, *135*, 61–66. [CrossRef]
21. Lin, H.Y.; Lin, T.S.; Wang, C.S.; Chien, H.J.; Juang, Y.M.; Chen, C.J.; Lai, C.C. Rapid determination of bioactive compounds in the different organs of Salvia Miltiorrhiza by UPLC–MS/MS. *J. Chromatogr. B* **2019**, *1104*, 81–88. [CrossRef]
22. Committee of the Chinese Pharmacopoeia. *Pharmacopoeia of the People's Republic of China*, 2020th ed.; China Medical Science Press: Beijing, China, 2020; Volume 4, pp. 466–468.
23. Tanaka, N.; McCalley, D.V. Core–shell, ultrasmall particles, monoliths, and other support materials in high-performance liquid chromatography. *Anal. Chem.* **2016**, *88*, 279–298. [CrossRef] [PubMed]
24. Waterlot, C.; Ghinet, A.; Lipka, E. Core-shell particles: A way to greening liquid chromatography in environmental applications. *Curr. Chromatogr.* **2018**, *5*, 78–90. [CrossRef]
25. Committee of the Chinese Pharmacopoeia. *Pharmacopoeia of the People's Republic of China*, 2020th ed.; China Medical Science Press: Beijing, China, 2020; Volume 1, pp. 282–283.
26. Gałuszka, A.; Migaszewski, Z.M.; Konieczka, P.; Namieśnik, J. Analytical Eco-Scale for assessing the greenness of analytical procedures. *Trac-Trends Anal. Chem.* **2012**, *37*, 61–72. [CrossRef]