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The Simultaneous Determination of Nine Furocoumarins in *Angelica dahurica* Using UPLC Combined with the QAMS Approach and Novel Health Risk Assessment Based on the Toxic Equivalency Factor

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Abstract: Objective: This study aimed to provide data for the type and content of linear furocoumarins (FCs) in *Angelica dahurica* (AD) in order to assess their cumulative risks and provide a scientific basis for the rational use and quality evaluation of the medicinal AD to improve public health. Methods: A UPLC method was developed for the simultaneous determination of nine FCs initially by using imperatorin (Im) as the internal standard substance, including Im, phellopterin (Ph), isoimperatorin (Is), oxypeucedanin hydrate (Oh), byakangelicin (Bn), xanthotoxin (8-MOP), bergapten (5-MOP), byakangelicol (Bl), and oxypeucedanin (Op) in two species of *Angelica dahurica* (AD). And, the risk assessment for the total FCs in AD was explored using the hazard index combined with the toxic equivalency factor (TEF-HI) strategy for the first time. Results: The established method revealed acceptable applicability, and there were no significant differences compared with the external standard method (ESM). The quantitative results demonstrated that the total content of FCs in *Angelica dahurica* (BZ) were higher than that in *Angelica dahurica* var. *formosana* (HBZ), and there was a great difference between the Bl and Op. Moreover, the risk assessment data revealed that the risk of total FCs in AD to human health was low. Conclusions: The established UPLC method that determined nine FCs in AD using a single marker could solve the problem of difficulty in obtaining a chemical reference substance with high purity and requiring a long determination time. And, the TEF-HI risk assessment approach associated with FCs in ADs could guide the rational utilization of toxic FCs in ADs in the progress of improving public health safety. In short, the whole systematic strategy provides a scientific basis for rational quality evaluation and the healthy use of related herbal medicines.

Keywords: *Angelica dahurica*; furocoumarin; quantitative analysis of multicomponents by single marker (QAMS); risk assessment (RA); toxic equivalency factors (TEFs); hazard index (HI)

Citation: Wang, Z.; Zan, K.; Hu, X.-W.; Kang, S.; Li, H.-L.; Zuo, T.-T.; Jin, H.-Y.; Ma, S.-C. The Simultaneous Determination of Nine Furocoumarins in *Angelica dahurica* Using UPLC Combined with the QAMS Approach and Novel Health Risk Assessment Based on the Toxic Equivalency Factor. *Separations* **2023**, *10*, 508. https://doi.org/10.3390/separations10090508

Academic Editor: Petr Bednar

Received: 15 August 2023
Revised: 4 September 2023
Accepted: 5 September 2023
Published: 15 September 2023

1. Introduction

Furocoumarins (FCs) are a class of natural chemical compounds that widely exists in many herbs, including some commonly used as food, cosmetics, flavorings, or herbal medicines by humans [1]. Just as its name implies, these compounds have a skeleton formed via a coumarin unit fused to a furan ring and can be divided into linear or angular isomers depending on the position of the furan ring [2]. Although FCs are widely used for medicine and food purposes, every coin has two sides, and it is reported that linear furanocoumarins, which are predominant in natural sources such as 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP), are phototoxic and genotoxic [3–6]. And, furanocoumarin-induced hepatotoxicity has been reported to occasionally occur in humans [7]. It was once believed that FCs could interact with proteins, lipids in membranes,

ribosomes, mitochondria, and, in particular, mono- and diadducts with DNA or RNA, which are the main contributors to toxicity in humans and animals [8–10]. In addition, certain FCs have been publicized to act as greatly potent inhibitors of a quantity of cytochromes P450 mono oxygenases [11], which can be interpreted as hepatotoxicity. These phenomena are defined as co-carcinogenic conditions [12]. Therefore, the U.S. Food and Drug Administration (FDA) had proscribed the use of FCs as food additives. As some research has reported, the family of FCs comprises more than 90 individual compounds, and the individual FCs shown differ in photomutagenic potency [13]. FCs are abundantly found in herbal products, especially in herbal medicine belonging to *Angelica* L., and these various FCs occur in one-plant ingredients, inevitably raising the problem of combination effects or interactions. Therefore, it is urgently required to establish feasible and reliable methods for the combined risk assessment of FCs' components in herbal medicine.

Traditional Chinese medicine (TCM) *Angelica dahurica* (AD, called BaiZhi in Chinese) is the dried root of *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et. Hook. f. or *Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook. f. var. *formosana* (Boiss.) Shan et Yuan (Fam. Umbelliferae) [14]. It is a tall perennial herb mainly distributed in the provinces of Sichuan, Hebei, Henan, Zhejiang, Anhui, Shandong, Neimenggu, Fujian, and Taiwan of China [15]. As a typical medicinal and food plant, it has the effects of expelling wind, dehumidifying, relieving pain, and relieving itching [16]. The major chemical constituents of it comprise polysaccharide, coumarins, alkaloids, benzofurans, volatile oil, fatty acids, and amino acids, and the total content of coumarins in the active ingredient of AD is more than 60% [17–19]. Modern pharmacology has shown that these chemical constituents, especially FCs, have efficacy in treating tumors, antioxidants, etc. [20,21]. But, because of the photogenotoxicity and photocarcinogenicity of FCs, the application of AD for external use is limited in China [22]. And, in October 2007, the European Medicines Agency (EMA) suggested a risk-management strategy for herbal preparations, which is rich in FCs in the paper titled "Reflection Paper on the Risk Associated with Furocoumarins Contained in Preparations of *Angelica archangelica* L." [12]. However, there are limited reports about the relevant toxicity risk assessment of AD for oral usage, and no official proposal on the potential consumption of FCs is available in China.

Nowadays, a lot of research on the identification and isolation of FCs from AD has been reported. Components in AD have been authenticated or quantified via HPLC, GC, LC-MS, GC-MS, NMR, and high-resolution mass spectrometry, such as ion trap MS and TOF-MS [23–26]. However, there has been a deficiency report about the simultaneous determination of nine FCs in AD via a single marker combined with UPLC. And, information regarding the detailed quantitative analysis of FCs in the two pieces of AD is limited. As for the risk assessments about FCs contained in plants, although EMEA has proposed some strategies, currently, the available assessment is primarily based on the data of 5-MOP and 8-MOP, owing to the fact that toxicological figures on other individual FCs in these plants are almost lacking. But, using 5-MOP and 8-MOP as a model for the risk assessment of plants which FCs are rich in might make the assessment inaccurate. Fortunately, recent researchers have proposed the concept of the toxic equivalency factor (TEF) based on photomutagenic, photocytotoxic, and photoclastogenic properties in a V79 cells experiment, which suggested that the photogenotoxic and phototoxic properties of individual furocoumarins may diverge widely but showed relatively strict additivity once occurring as multifarious mixtures [27,28]. Moreover, in 2008, the European Food Safety Authority (EFSA) conducted a series of research in experimental animals, declared that the carcinogenic influence was not triggered via a genotoxic mechanism, and then proposed the tolerable daily intake (TDI) values (0.1 mg coumarin/kg bw/day) based on the non-observed adverse effect level (NOAEL) for liver toxicity in a 2-year dog study [29]. Thus, it is significant to determine the content of various FCs in AD and to evaluate the related health risks when using medicine in a more scientific approach.

In this study, a practical QAMS method was adopted, wherein nine FCs could be determined simultaneously by using a single internal standard reference substance. And, a

new risk assessment strategy for assessing the human health risk related to the consumption of AD via TEF-HI was provided to suggest the safety usage of FCs in TCM.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile (MS-grade) was obtained from Merck (Overijse, Belgium). Ultrapure water was prepared by a Milli-Q system (Millipore, Billerica, MA, USA). The rest of the chemicals (analytical grade) were obtained from Sinopharm Chemical Reagent Beijing (Beijing, China). The reference standard of imperatorin (Im) and isoimperatorin (Is) were provided by NIFDC. Standard phellopterin (Ph), oxypeucedanin hydrate (Oh), byakangelicin (Bn), bergapten (5-MOP), xanthotoxin (8-MOP), byakangelicol (Bl), and oxypeucedanin (Op) were obtained from Chengdu DeSiTe Biological Technology Co., Ltd. (Chengdu, China), all with the purity above 98%.

In total, 20 batches of AD plants including 10 batches of *Angelica dahurica* (Hoffm.) *Benth. & Hook. f. ex Franch. & Sav.* (BZ, called BaiZhi in Chinese, Species No. 1) and 10 batches of *Angelica dahurica* var. formosana (Boissieu) Yen (HBZ, called HangBaiZhi in Chinese, Species No. 2) were collected from Bozhou Anhui, Anguo Hebei, Shehong Sichuan, Suining Sichuan of China, and Hong Kong, respectively, in August 2021. They were originally authenticated by Dr. Kang Shuai of the National Institutes for Food and Drug Control (NIFDC, Beijing, China). All specimens had been stored in the Chinese Herb medicine museum of NIFDC.

2.2. Instrumentation and Chromatographic Condition

An ultra-high-performance liquid chromatographic (UHPLC) system (Shimadzu LC_30AD, Shimadzu, Tokyo, Japan) equipped with a DAD detector was used. An ultrasonic bath (300 W) was also used in the process.

Chromatography was performed with a Waters ACQUITY UPLC® CSH™ C_{18} Column (2.1 mm × 100 mm, 1.7 μm) at 25 °C with a flow rate of 0.3 mL·min^{-1}, and 254 nm was chosen as the detection wavelength. Mobile phases A and B consisted of water and acetonitrile. A gradient elution (0–10 min, 30–40% B; 10–17 min, 40–80% B; 17–20 min, 80% B) was adopted, and the injection volume was 2 μL.

2.3. Preparation of Standard Solutions

Nine standard FC substances were dissolved in methanol, respectively, and the stock solutions were made to be about 1 mg/mL. Then, the mixed reference stock solution was prepared comprising all standards including Oh, Bn, 8-MOP, 5-MOP, Bl, Op, Im, Ph, and Is at the concentration of 158.7, 49.6, 57.3, 53.6, 90.15, 258.75, 102.0, 60.1, and 102.5 mg/L, respectively. The working solutions were kept at −20 °C and were newly prepared each day via the appropriate dilution of the stock with methanol.

2.4. Preparation of BZ Samples

Before preparation, cut the 20 batches of BZ samples into small pieces and powder them through a No. 2 sieve, respectively. Then, accurately weigh 0.5 g of powdered sample into a 50 mL conical flask, add 20 mL of 70% methanol, and sonicate (300 W) the mixture for 20 min. Filter and transfer the filtrate to a 50 mL volumetric flask. Repeat the steps once. Then, wash the residue, filter paper, and the funnel with the appropriate volume of 70% methanol and combine the solutions into the same volumetric flask. Increase the volume of the volumetric flask up to the mark with 70% methanol. Filter the solution through a 0.22 μm nylon filter before analysis.

2.5. Method Validation

The established UPLC method was validated through complete methodology, including specificity, accuracy rate (using recovery rate), precision, reproducibility, linearity

(or calibration curves), covers, limits of detection (LOD), limits of quantification (LOQ), stability, and durability.

Among these, Radix Ophiopogonis (one commonly used TCM) was chosen as a blank matrix to perform the specificity, LOD, and LOQ tests by adding an appropriate amount of the relevant reference. The LOD and LOQ values for the nine FCs were defined as signal-to-noise (S/N) ratios of 3 and 10, respectively. The mixed reference stock solution was diluted to six concentration levels, and calibration curves (Y = aX + b) were established where the X- and Y-axes represented the peak area and the concentration of every furocoumarin, respectively. The correlation coefficient (r) was calculated to inspect the linearity of every calibration curve. The precision was evaluated using six consecutive injections of the same mixed standard solutions. The repeatability was measured by analyzing six independently prepared sample solutions of the same batch. The stability was assessed by injecting the same prepared sample solution at different times (0, 2, 4, 8, 12, 24 h). The relative standard deviation (RSD %) was analyzed at the same time to estimate the results of precision, repeatability, and stability. Finally, the recovery test was performed to verify the accuracy of the method. The mixed reference stock solution of nine FCs at certain levels were precisely added to 0.25 g of the above sample power. Then, six parallel solutions were created according to the above sample preparation requirements, calculating the average recovery rate (n = 6) and analyzing the RSD of every component.

2.6. Theory of QAMS

The principle of the QAMS method is that the content of the component is proportional to the detector response under particular conditions, and different effective components of TCM exist on intrinsic functions and proportional relations, which make the calculation of relative correction factors (RCFs) feasible [14]. Based on the RCFs, the content of each analyst can be determined independently according to an internal standard substance. In this case, the calculation of RCFs is crucial in this study. First, Im was chosen as the internal standard substance, and then the RCFs for the rest of the FCs were calculated using the following multipoint method (Equation (1)). Thereafter, the content of the other FCs was determined according to Equation (2) [30].

The equation for RCFs was calculated as follows,

$$f_{s/i} = f_i/f_s = A_i \times C_s/(A_s \times C_i) \quad (1)$$

The quantification of the other investigated FC components is calculated as follows,

$$C_i = (A_i \times C_s)/(f_{s/i} \times A_s) \quad (2)$$

where A_s and A_i represent the peak areas of the internal standard (Im) and other FCs in standard solutions or in the AD samples, respectively. $f_{s/i}$ represents the average of the RCFs between Im and each of the other FC components. C_s and C_i represent the concentrations of Im and the other investigated FC components in standard solutions or in the AD samples.

Additionally, for the location of target chromatographic peaks, the relative retention time (RRT) was introduced to position the chromatographic peaks of the other investigated FC components according to Equation (3) [31],

$$t_{i/s} = t_i/t_s \quad (3)$$

where $t_{i/s}$ is the RRT of the analysts and t_i and t_s are the retention times of the other investigated FC components and Im, respectively.

2.7. Quantification of AD Samples

Both the newly established QAMS and ESM methods were used to determine and analyze the samples. Each sample was determined 3 times, and then the mean was

calculated. The accuracy of the two methods was assessed using standardized mean difference (SMD), which was calculated according to Equation (4) [32],

$$SMD = [(C_{ESM} - C_{QAMS})/C_{ESM}] \times 100\% \quad (4)$$

where C_{ESM} and C_{QAMS} represent the mean content of the FCs determined by QAMS and ESM, respectively.

2.8. Healthy Risk Assessment

2.8.1. Assessment of Daily Intake of AD Based on the EQF

To examine the health risk of FCs in AD, a real-life exposure situation was considered by estimating the EDI (μg/kg bw/day) based on the EQF as in Equation (5) [33],

$$EDI = EF \times Ed \times IR \times C \times EQF/W \times AT \quad (5)$$

where EDI is the estimated daily intake of FCs in AD; EF is the abbreviation of exposure frequency, which is 90 days/year [33]; and Ed represents exposure time, which according to our previous study is 20 years [33]; and IR is short for the daily intake rate of AD (g/day). Based on the guidelines of ChP [14], the mean IR is 6.5 g/day. C represents the content of the FCs in AD (mg/g) and EQF is the equivalency factor. It was suggested that the value for 5-MOP should be set at 1.00, then the value for Oh, Bn, 8-MOP, Bl, Op, Im, Ph, and Is would be 0.16, 0.01, 0.20, 0.01, 0.076, 0.15, 0.01, and 0.15, respectively. Then, W symbolizes the average of body weight, which is 70 kg based on the date given in adults by the EFSA and AT represents the mean duration of exposure to AD, which is usually 365 days/year × 70 years.

2.8.2. Risk Characterization Based on Health-Based Guidance Values

Risk characterization is commonly expressed by calculating risk metrics including hazard index (HI), Margins of Exposure (MoE), or Reference Point Index (RPI). For hazardous substances with health-based guideline values, an HI strategy is recommended. As the EFSA had allocated a TDI for coumarins via diet, the HI value can be calculated according to Equation (6) [33].

$$HI = \left(\sum_{i=1}^{n} EDI_i \times 10\right)/TDI \quad (6)$$

In Equation (6), HI represents the hazard index. $\sum_{i=1}^{n} EDI_i$ is the calculated daily intake of the nine FCs in AD and 10 is the safety factor, which indicates that the daily intake of FCs from TCM and its products is not greater than 10% of the daily exposure (including diet). TDI is the tolerable daily intake value (mg/kg bw/day). The total TDI of nine FCs in each sample was calculated in this study. According to the research of EFSA, the TDI of coumarins is 0.1 mg/kg bw/day in this study. Once the HI value is >1, the exposure risks of the analyst should not be ignored.

3. Results and Discussion

3.1. Extraction Procedures and UPLC Condition Optimization

To extract coumarins from AD more completely, the extraction solvents (100%, 70%, 50%, and 30% ethanol), extraction methods (ultrasound and reflux), extraction times (20, 30, and 40 min), and number of extractions were investigated. The extraction efficiency showed that adding 20 mL of 70% methanol, sonication (300 W) for 20 min, and repeating the extraction once could extract the coumarins exhaustively.

First, for chromatographic conditions, the chromatographic columns of ACQUITY UPLC® BEH Amide (2.1 mm × 100 mm, 1.7 μm), ACQUITY UPLC® CSH™ C_{18} (2.1 mm × 100 mm, 1.7 μm), CORTECS® UPLC® C_{18} (2.1 mm × 100 mm, 1.6 μm), ACQUITY UPLC® BEH SHIELD (2.1 × 50 mm, 1.7 μm), Thermo Syncronis aQ (2.1 mm × 100 mm, 1.7 μm), and Phenomenex OOD-4475-AN Kinetex C_{18} 100A (2.1 mm × 100 mm, 1.7 μm) were investigated for studying the separation of complex components in AD. ACQUITY

UPLC® CSH™ C_{18}, CORTECS® UPLC® C_{18}, and Thermo Syncronis aQ were selected for the establishment of the QAMS method. A CSH column had the best separation effect and was selected for its stability and excellent reproducibility for both high- and low-pH compounds. The methodology and durability of this experiment were studied with a Shimadzu UPLC instrument (LC-30AD), an ultrahigh performance liquid chromatograph.

Second, the DAD was used to scan the combined working solution at full wavelength to obtain better sensitivity, and the maximum absorption wavelength for each of the nine FCs was obtained. It is shown that, at 254 nm, the nine FC components exhibited relatively good absorbance.

Third, different mobile phases, for example, 0.1% formic acidacetonitrile, wateracetonitrile, and watermethanol with different proportional gradient elutions, were compared. The baseline condition showed that the mobile phase system of acetonitrile was better than that of methanol. The adoption of a 0.1% formic acid solution had no obvious effect on the CSH column; therefore, wateracetonitrile was used as the mobile phase. At the same time, the design of several chromatographic gradient methods, flow rates (0.2, 0.3, and 0.5 mL/min), and column temperatures (25 °C, 30 °C, and 35 °C) were optimized. Finally, we confirmed an acetonitrile water system for the mobile phase at 25 °C with a flow rate of 0.3 mL/min for analysis. Under the optimized conditions, the nine FCs were all separated from the baseline within 20 min. The UPLC chromatograms of the nine mixed standard solutions and the representative AD samples are shown in Figure 1.

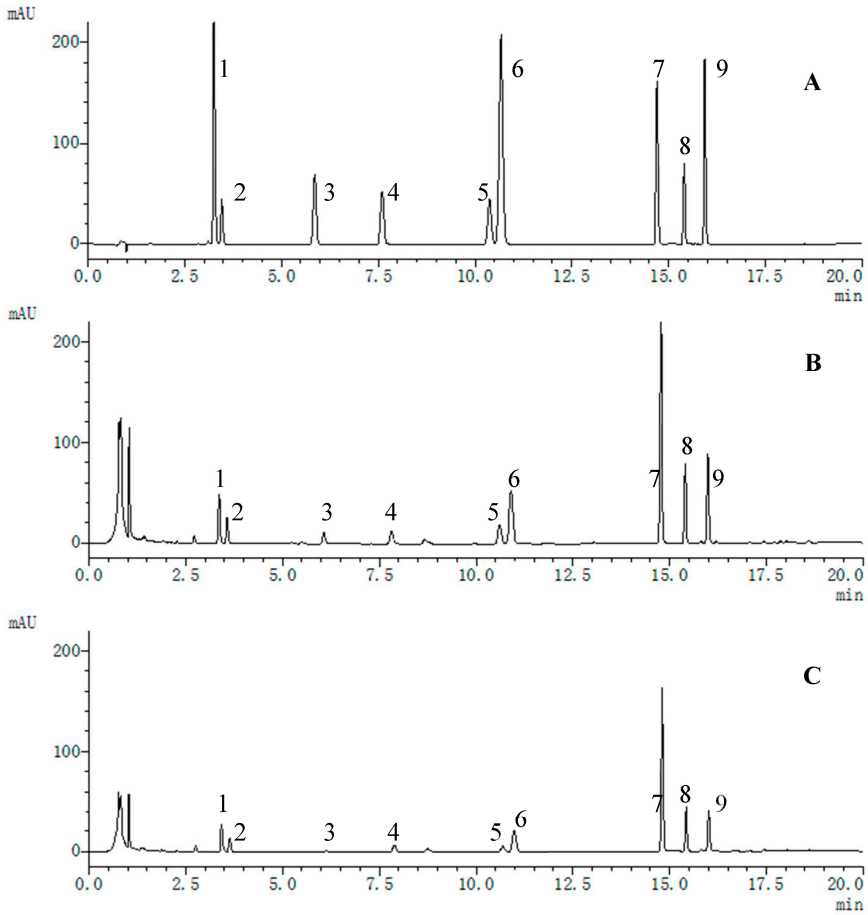

Figure 1. Representative UPLC chromatograms of the standards (**A**), the AD sample (BZ, Species No. 1) (**B**), and the AD sample (HBZ, Species No. 2) (**C**). Peak identification: oxypeucedanin hydrate (Oh, 1), byakangelicin (Bn, 2), xanthotoxin (8-methoxypsoralen, 8-MOP, 3), bergapten (5-methoxypsoralen, 5-MOP, 4), byakangelicol (Bl, 5), oxypeucedanin (Op, 6), imperatorin (Im, 7), phellopterin (Ph, 8), and isoimperatorin (Is, 9).

3.2. Method Validation

The established UPLC protocol was evaluated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, stability, and accuracy. The correlation coefficients of the nine FCs were >0.9999 within the respective detection test ranges. The LOD and LOQ values of each of the FCs varied from 0.01–0.02 mg/mL to 0.04–0.07 mg/mL (Table 1). The RSD values of precision, repeatability, and stability for all nine FCs were <2.75% (Table 2). The overall recovery rate (n = 6) ranged from 90.07% to 107.94%, with RSD values of <3.00% (Table 2). These results indicated that the proposed method was precise, accurate, and efficient for simultaneously qualifying the nine FCs.

Table 1. Linearity (regression equation, linear range, and correlation coefficient) and sensitivity (LOD and LOQ) of the method determination of the eight FCs.

Analytes	Calibration Curves	r	/μg·mL^{-1}	/μg·mL^{-1}	/μg·mL^{-1}
1	Y = 24489800X + 4561.67	0.9999	1.270–158.7	0.01	0.05
2	Y = 16185800X + 850.89	0.9999	0.3970–49.60	0.01	0.04
3	Y = 31411100X + 1986.14	0.9999	0.4580–57.28	0.01	0.04
4	Y = 29319800X + 1010.05	0.9999	0.4290–53.65	0.01	0.04
5	Y = 16335500X + 1443.08	0.9999	0.7210–90.15	0.02	0.07
6	Y = 26801600X + 5930.63	0.9999	2.070–258.8	0.02	0.07
7	Y = 27494800X + 3435.56	0.9999	0.8160–102.0	0.01	0.04
8	Y = 28897035X + 1351.04	0.9999	0.8000–100.50	0.02	0.07
9	Y = 28331800X + 597.95	0.9999	0.8200–102.50	0.01	0.04

'1–9' represents the analytes of oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), imperatorin (7), phellopterin (8), and isoimperatorin (9).

Table 2. Precision, stability, repeatability, and recovery results of nine FCs.

Analytes	Precision (RSD/%, n = 6)	Stability (RSD/%, n = 6)	Repeatability (RSD/%, n = 6)	Recoveries (n = 6)	
				Mean/%	RSD/%
1	0.02	0.82	2.01	97.57	2.98
2	0.09	0.76	1.89	107.9	2.59
3	0.52	1.10	2.75	107.9	2.98
4	0.19	1.14	2.07	106.9	2.91
5	0.23	1.07	1.93	90.07	2.98
6	0.02	0.93	1.91	92.09	2.66
7	0.01	1.04	1.97	93.76	3.00
8	0.21	0.89	2.10	101.5	2.62
9	0.01	0.63	1.98	91.97	2.16

'1–9' represents the analytes of oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), imperatorin (7), phellopterin (8), and isoimperatorin (9).

3.3. Quantitative Analyses of Multiple Components via Single Marker

The UPLC chromatograph detected nine FCs in the two species of AD. The chemical structures of the nine FCs are shown in Figure 2. For the QAMS method, selecting a proper internal standard substance for the calculation of RCFs and RRTs of the rest of the FCs in AD is vital. In this study, Im was selected as an internal standard substance owing to its high content, good stability, relatively moderate retention value, and easy availability in AD. Therefore, it met the requirements of QAMS. The calculated RCFs of eight FCs compared with Im are shown in Table 3. The results indicated that the RSDs of these FCs were <3%, calculated from the slope at various concentrations. Moreover, the mean RCFs were 0.891, 0.585, 1.136, 1.046, 0.587, 0.968, 1.051, and 1.015.

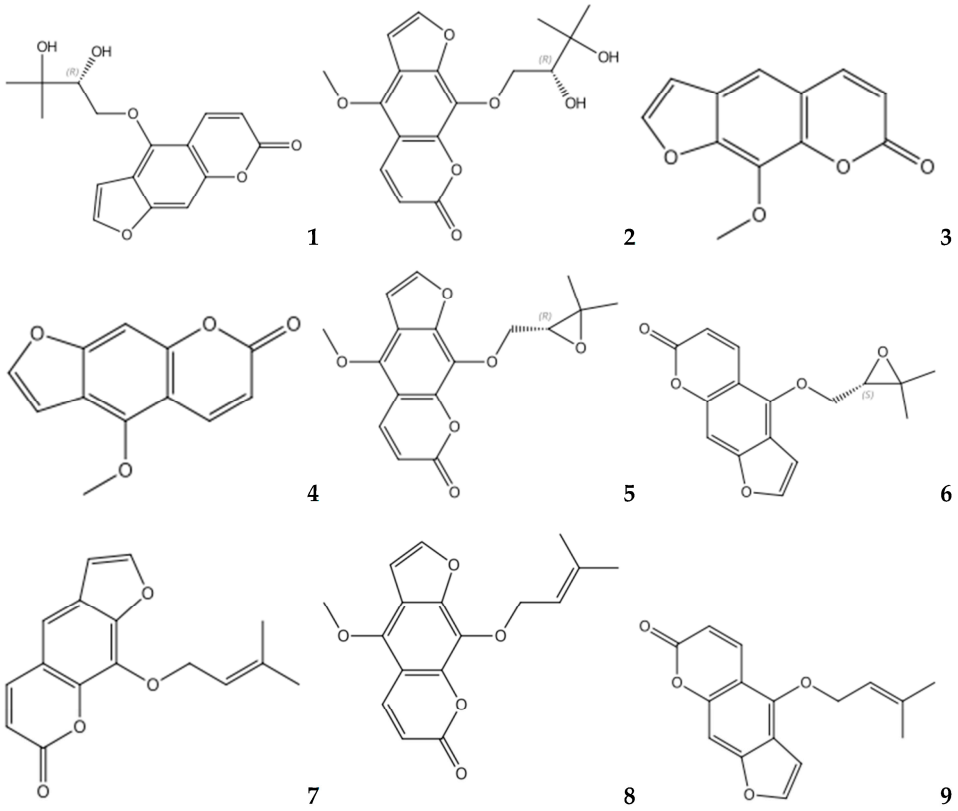

Figure 2. Chemical structures of FCs analyzed in AD: oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), imperatorin (7), phellopterin (8), and isoimperatorin (9).

Table 3. Relative correction factor (RCF) values of the eight FCs of the AD calculated via slope and concentration method (n = 3).

RCF	Calculated by Slope	a	b	c	d	e	f	Mean	RSD (%)
$f_{7/1}$	0.891	0.890	0.892	0.893	0.892	0.891	0.889	0.891	0.17
$f_{7/2}$	0.589	0.574	0.583	0.585	0.587	0.588	0.589	0.585	0.91
$f_{7/3}$	1.142	1.124	1.131	1.136	1.137	1.140	1.142	1.136	0.58
$f_{7/4}$	1.066	1.016	1.025	1.041	1.054	1.060	1.061	1.046	1.85
$f_{7/5}$	0.594	0.568	0.584	0.589	0.590	0.593	0.593	0.587	1.57
$f_{7/6}$	0.975	0.961	0.965	0.967	0.968	0.970	0.971	0.968	0.46
$f_{7/8}$	1. 051	1.055	1.053	1.052	1.049	1.048	1.051	1. 051	0.25
$f_{7/9}$	1.030	1.008	1.009	1.011	1.012	1.014	1.018	1.015	0.76

'f_i' represents the RCFs of oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), phellopterin (8), and isoimperatorin (9) compared with imperatorin (7). 'a–f' indicates six different concentration levels. Taking imperatorin (7) as an example, its concentration levels are 0.816 μg/mL, 2.04 μg/mL, 4.08 μg/mL, 10.2 μg/mL, 20.4 μg/mL, and 102 μg/mL, respectively.

Further, the system suitability and durability of QAMS were evaluated. First, different instruments and diverse columns were investigated by calculating the RSD values of each RCF. Subsequently, the Shimadzu UPLC instrument with a CSH column was selected to assess the effects of changed flow rates (0.28, 0.30, and 0.32 mL/min) and column temperatures (23 °C, 25 °C, and 27 °C) on RCFs. The results show that these potential factors, including instruments, columns, flow rates, and column temperatures, displayed no substantial influence on RCFs based on the RSDs of < 3% (Table 4), which demonstrated that Im and the other eight FCs were robust under these conditions. Finally, three chromatographic columns with two different UPLC instruments assessed the RRTs. The results

showed that all RSDs were <3%, implying that the RRT method could be used to locate the chromatographic peak component of nine FCs in AD (Table 5). The RRTs were 0.224, 0.237, 0.401, 0.518, 0.703, 0.726, 1.055, and 1.085.

Table 4. Effects of different instruments, columns, column temperatures, and flow rates on RCFs of nine FCs (n = 3).

Conditions	Items	$f_{7/1}$	$f_{7/2}$	$f_{7/3}$	$f_{7/4}$	$f_{7/5}$	$f_{7/6}$	$f_{7/8}$	$f_{7/9}$
Waters ACQUITY UPLC H-Class	Column 1	0.898	0.582	1.110	1.051	0.599	0.973	1.055	1.018
	Column 2	0.890	0.541	1.125	1.082	0.547	0.967	1.049	1.042
	Column 3	0.881	0.591	1.041	1.045	0.597	0.966	1.062	1.021
Shimadzu UPLC Instrument (LC-30AD)	Column 1	0.891	0.585	1.136	1.046	0.587	0.968	1.051	1.015
	Column 2	0.878	0.523	1.121	1.103	0.571	0.978	1.048	1.038
	Column 3	0.902	0.576	1.064	1.045	0.590	0.994	1.049	1.016
	Mean	0.890	0.566	1.100	1.062	0.582	0.974	1.052	1.025
	RSD (%)	1.05	4.88	3.47	2.32	3.39	1.10	0.51	1.16
Column temperature (°C)	23	0.890	0.603	1.141	1.024	0.577	0.971	1.0518	1.018
	25	0.891	0.585	1.136	1.046	0.587	0.968	1.050	1.015
	27	0.891	0.582	1.122	1.053	0.594	0.962	1.0475	1.014
	Mean	0.891	0.590	1.133	1.041	0.586	0.967	1.050	1.016
	RSD (%)	0.07	1.92	0.87	1.46	1.46	0.47	0.21	0.21
Flow rates (mL/min)	0.28	0.872	0.569	1.114	1.041	0.579	0.966	1.049	1.006
	0.30	0.891	0.585	1.136	1.046	0.587	0.968	1.050	1.015
	0.32	0.925	0.601	1.151	1.052	0.595	0.971	1.0524	1.034
	Mean	0.896	0.585	1.134	1.046	0.587	0.968	1.051	1.018
	RSD (%)	3.00	2.74	1.64	0.53	1.36	0.26	0.17	1.41

Column 1—ACQUITY UPLC® CSHTM C_{18}(2.1 mm × 100 mm, 1.7 μm). Column 2—CORTECS® UPLC® C_{18} Column (2.1 mm × 100 mm, 1.6 μm). Column 3—Thermo Syncronis aQ (2.1 mm × 100 mm, 1.7 μm).

Table 5. Relative retention values measured in different instruments and different columns (n = 3).

Instrument	Column	$t_{7/1}$	$t_{7/2}$	$t_{7/3}$	$t_{7/4}$	$t_{7/5}$	$t_{7/6}$	$t_{7/8}$	$t_{7/9}$
Waters ACQUITY UPLC H-Class	ZORBAX Eclipse XDB-C_{18}	0.225	0.239	0.401	0.509	0.701	0.730	1.049	1.087
	ZORBAX Eclipse plus C_{18}	0.221	0.235	0.397	0.517	0.706	0.725	1.051	1.084
	ACQUITY UPLC HSS, T3	0.223	0.235	0.399	0.529	0.703	0.719	1.064	1.081
Shimadzu UPLC Instrument (LC-30AD)	ZORBAX Eclipse XDB-C_{18}	0.229	0.241	0.414	0.511	0.705	0.726	1.052	1.089
	ZORBAX Eclipse plus C_{18}	0.222	0.236	0.398	0.518	0.706	0.726	1.052	1.084
	ACQUITY UPLC HSS, T3	0.223	0.234	0.398	0.522	0.698	0.728	1.063	1.083
mean		0.224	0.237	0.401	0.518	0.703	0.726	1.055	1.085
RSD/%		1.29	1.14	1.61	1.41	0.46	0.51	0.62	0.27

't_i' represents the relative retention time (RRT) of oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), phellopterin (8), and isoimperatorin (9) compared with imperatorin (7).

3.4. Quantification and Method Assessment

BZ and HBZ are the two original plant sources of AD in China. Both the ESM and QAMS methods were performed simultaneously to determine the nine FCs in 20 AD samples. Subsequently, the SMD was calculated to assess the differences between the two methods. The results are shown in Supplementary Table S1. The absolute SMD values ranged from 0.02 % to 1.32 %, demonstrating that the accuracy of the content results obtained using QAMS could be accepted. Hence, it is feasible to determine the nine FCs in AD using QAMS simultaneously.

The results show that all samples of AD contain nine FCs, and the mean content of Oh, Bn, 8-MOP, 5-MOP, Bl, Op, Im, Ph, and Is are 0.47, 0.37, 0.08, 0.19, 0.37, 1.11, 1.95, 0.57, and 0.66 mg/g, respectively. As shown in Figure 3, the total content of the nine FCs varied

from 4.38 to 9.37 mg/g, with those in BZ being relatively higher than in HBZ. Moreover, the most abundant ingredient of the nine quantified FCs in the two types of samples is Im. The content of Bl and Op were found to be substantially different in both samples.

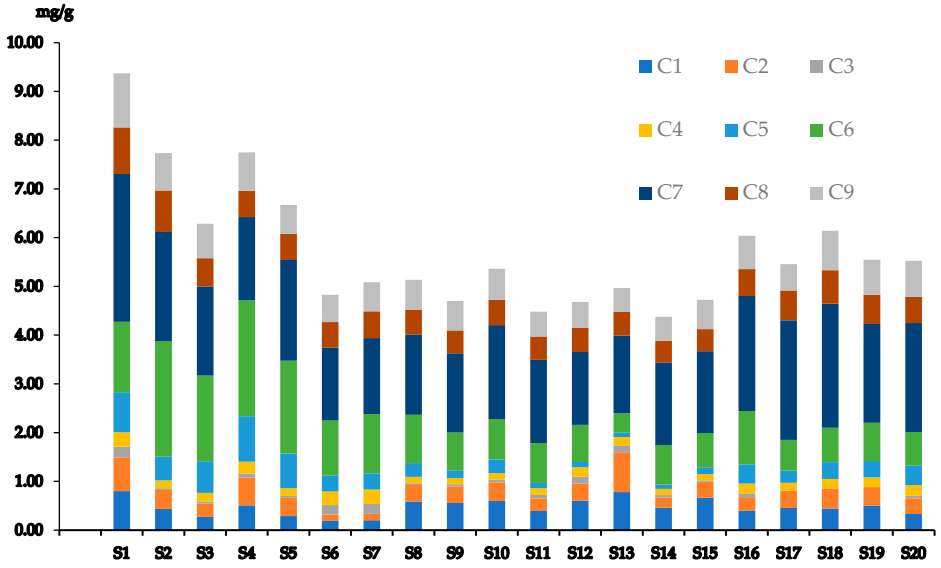

Figure 3. Comparison of FCs in 20 batches of samples. C_i represents the average content of oxypeucedanin hydrate (1), byakangelicin (2), xanthotoxin (3), bergapten (4), byakangelicol (5), oxypeucedanin (6), imperatorin (7), phellopterin (8), and isoimperatorin (9).

3.5. Risk Assessment

In 1986, the IARC3 working group concluded that 8-MOP combined with ultraviolet radiation was carcinogenic to humans, based on sufficient evidence from both animal and human data [34]. The safety evaluation of furocoumarin via diet has been a concern for a long time. However, because of the toxicological data on all individual FCs being almost absent, this assessment mainly used the data on 5-MOP and 8-MOP, which are both used as the basis for risk assessment. To the best of our knowledge, furocoumarin is never found in plants as a single compound but always as complex mixtures of many individual furocoumarins. An overvalued scenario may emerge if a risk assessment is performed using only one type of furocoumarin, such as 8-MOP, as a basis for calculating the total toxic equivalent. However, diverse results are obtained when genuine mixtures of FCs in given herbal preparations are studied. Recently, the Raquet and Schrenk Group proposed an equivalency factor for phototoxicity and genotoxicity of furocoumarin mixtures. This provides the possibility for a toxicity estimation of different FCs and can be considered as the TEF. Herein, we calculated the EDI values based on the TEF. 5-MOP was selected as a basis or called a reference compound for the total risk assessment of FCs in AD owing to its strong photomutagenicity, as testified in previous studies and through its abundance in medicinal plants and food [12]. The TEF values of Oh, Bn, 8-MOP, Bl, Op, Im, Ph, and Is calculated based on research in the literature were 0.16, 0.01, 0.20, 0.01, 0.076, 0.15, 0.01, and 0.15, respectively [28]. The results showed that the EDI values of the total FCs in AD calculated without the TEF (0.029–0.061 mg/kg bw/day) were very close to the TDI values (0.1 mg/kg bw/day). However, after correction with the TEF, the EDI ranged from 0.004 to 0.008 mg/kg bw/day, revealing a reduced order of magnitude. According to the established HI-TEF strategy, the HI values of all the samples were <1, indicating that AD posed a low risk, which is consistent with the clinical and animal toxicity research [35,36]; the details are presented in Table 6.

Table 6. Estimated daily intake (EDI) and hazard index (HI) in 20 batches of AD samples.

Batch No.	Source	Species	EDI_1	EDI_2	EDI_3	EDI_4	EDI_5	EDI_6	EDI_7	EDI_8	EDI_9	ΣEDI Based on TEF	ΣEDI without TEF	HI Values Based on TEF	HI Values without TEF
B1	Bozhou, Anhui	1	0.00084	0.00005	0.00029	0.00194	0.00005	0.00072	0.00298	0.00006	0.00109	0.0080	0.0613	0.80	6.13
B2	Anguo, Hebei	1	0.00045	0.00003	0.00005	0.00105	0.00003	0.00118	0.00220	0.00006	0.00075	0.0058	0.0506	0.58	5.06
B3	Anguo, Hebei	1	0.00028	0.00002	0.00006	0.00116	0.00004	0.00087	0.00179	0.00004	0.00069	0.0050	0.0411	0.50	4.11
B4	Anguo, Hebei	1	0.00053	0.00004	0.00011	0.00157	0.00006	0.00118	0.00168	0.00004	0.00077	0.0060	0.0507	0.60	5.07
B5	Anguo, Hebei	1	0.00030	0.00002	0.00005	0.00103	0.00005	0.00095	0.00203	0.00003	0.00059	0.0050	0.0436	0.50	4.36
B6	Jiaozuo, Hebei	1	0.00020	0.00001	0.00025	0.00185	0.00002	0.00056	0.00147	0.00003	0.00055	0.0049	0.0316	0.49	3.16
B7	Hong Kong	1	0.00021	0.00001	0.00027	0.00194	0.00002	0.00060	0.00153	0.00004	0.00059	0.0052	0.0333	0.52	3.33
B8	Hong Kong	1	0.00062	0.00002	0.00005	0.00085	0.00002	0.00049	0.00161	0.00003	0.00060	0.0043	0.0336	0.43	3.36
B9	Hong Kong	1	0.00058	0.00002	0.00006	0.00083	0.00001	0.00039	0.00160	0.00003	0.00059	0.0041	0.0308	0.41	3.08
B10	Hong Kong	1	0.00063	0.00002	0.00007	0.00086	0.00002	0.00041	0.00189	0.00003	0.00062	0.0046	0.0351	0.46	3.51
B11	Shehong, Sichuan	2	0.00041	0.00002	0.00010	0.00092	0.00001	0.00041	0.00167	0.00003	0.00050	0.0041	0.0293	0.41	2.93
B12	Suining, Sichuan	2	0.00063	0.00002	0.00018	0.00125	0.00001	0.00038	0.00147	0.00003	0.00053	0.0045	0.0306	0.45	3.06
B13	Suining, Sichuan	2	0.00082	0.00005	0.00019	0.00112	0.00001	0.00019	0.00157	0.00003	0.00048	0.0045	0.0325	0.45	3.25
B14	Suining, Sichuan	2	0.00048	0.00001	0.00007	0.00081	0.00001	0.00040	0.00166	0.00003	0.00048	0.0040	0.0286	0.40	2.86
B15	Suining, Sichuan	2	0.00070	0.00002	0.00004	0.00083	0.00001	0.00035	0.00164	0.00003	0.00059	0.0042	0.0309	0.42	3.09
B16	Suining, Sichuan	2	0.00041	0.00002	0.00010	0.00130	0.00003	0.00054	0.00232	0.00004	0.00067	0.0054	0.0395	0.54	3.95
B17	Hong Kong	2	0.00048	0.00002	0.00000	0.00108	0.00002	0.00031	0.00241	0.00004	0.00053	0.0049	0.0357	0.49	3.57
B18	Hong Kong	2	0.00046	0.00003	0.00000	0.00129	0.00002	0.00035	0.00250	0.00004	0.00079	0.0055	0.0402	0.55	4.02
B19	Hong Kong	2	0.00052	0.00003	0.00000	0.00130	0.00002	0.00039	0.00199	0.00004	0.00070	0.0050	0.0363	0.50	3.63
B20	Hong Kong	2	0.00034	0.00002	0.00007	0.00140	0.00003	0.00034	0.00219	0.00004	0.00073	0.0052	0.0361	0.52	3.61

'B1–B20' represents the batch numbers of AD samples. '1,2' represents the species numbers of AD samples. 1—*Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. 2—Angelica dahurica var. formosana (Boissieu) Yen.

The assessment in this study is quite conservative when compared with real clinical applications. AD as a part of TCM may rarely be used for a whole lifetime. In most cases, the duration of TCM usage will not exceed 2 years. In this exposure scenario, the EDI values of the total FCs in the 20 batches of AD samples are not more than 0.00023 mg/kg bw/day, which indicates that the risk of taking AD is extremely low. In addition, for the FCs with a TEF value of <0.01, we calculated under an assumption of a TEF equal to 0.01. Conversely, some uncertainties may cause underestimation in the risk calculation. For example, owing to the limited detection technology, some FCs of a low dose cannot yet be evaluated. In this study, we assumed that the toxicity of FCs in AD was the dose addition (DA) type. DA indicates that every single toxicant in the mixture contributes to the whole toxicity in proportion to its toxic unit, for example, its concentration or its potency. This implies that mixture toxicity will rise with the addition of individual agents. However, this is not the case. The joint toxicity of different FCs may be considerably larger or smaller than the toxicity (if any) associated with the NOAEL of each FC on its own [37,38]. Although there are numerous gaps and uncertainties in knowledge about the FCs from TCM, the established HI-TEF approach offers one possible strategy for the risk assessment of FCs originating from TCM.

4. Conclusions

In the present study, a fast, simple, credible, and sensitive evaluation method was developed for the accurate determination of nine FCs using single marks in 20 min based on UPLC. This method was successfully used for the content determination of AD samples in 20 batches. Thus, the problem of preparing a bulk of expense reference standards was effectively solved. Moreover, risk assessment methods for FCs from TCM in terms of toxicity were established for the first time, using AD as an example, based on the HI-TEF strategy. The cumulative risk assessment results revealed that the potential health risks of AD medicines were acceptable. Overall, the comprehensive application of the QAMS method alongside the HI-TEF risk assessment approach was meaningful, which could provide a significant reference for the rational utilization and quality control of FCs in TCM.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10090508/s1, Table S1: Contents of the 9 FCs in AD determined by ESM and QAMS methods (mg/g, n = 3).

Author Contributions: Z.W.: conceptualization, formal analysis, data acquisition, examination, writing—original draft. K.Z.: methodology, validation. X.-W.H.: investigation. S.K.: collection and identification of samples. H.-L.L.: investigation. H.-Y.J.: investigation, data curation. T.-T.Z.: writing—review and editing. S.-C.M.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Traditional Chinese Medicine Modernization Project, under grant numbers 2022YFC3501505.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Others come from previously reported studies and datasets, which have been cited.

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

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