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Determination of Biflavones from Trogopterus Feces by HPLC

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ABSTRACT

A reversed-phase high performance liquid chromatographic method was developed to simultaneously determine two major biflavones, amentoflavone and hinokiflavone, in Trogopterus Feces. The chromatographic separation was achieved with a linear gradient of acetonitrile in 1% acetic acid at a flow rate of 1.0 mL/min using an Alltima C-18 column (250 × 4.6 mm, 5.0 μm). Detection was carried out using a photodiode array detector at 330 nm. The calibration curves of all analytes showed good linearity over the investigated ranges ($R^2 > 0.999$). Repeatability was evaluated by intra-day and inter-day assays and RSD values were less than 2.40%. The recoveries were 103.5% and 97.3%. This developed method was simple and accurate. The results suggested that the HPLC method could be used to evaluate the quality of Trogopterus Feces from different producing areas.

Key words: HPLC, Quality assessment, Biflavone, Trogopterus Feces

INTRDUCTION

Trogopterus Feces, also known as Wulingzhi, are the dry seeds of *Trogopterus xanthipes* Milne-Edwars. Trogopterus Feces activate blood analgesia and are often used in the treatment of dysmenorrhea and angina pectoris caused by congestion clinically. Modern studies have indicated that Trogopterus Feces mainly consist of the chemical constituents of terpenoids, organic acids and flavonoids, and possess the pharmacological action of inhibiting platelet aggregation, enhancing immunity and anti-inflammation[1]. In our present study, different solvent extracts of Trogopterus Feces were actively screened. Note that the ethyl acetate extract could notably inhibit the frequency of mice uterine contraction induced by oxytocine *in vitro*, which demonstrated that the ethyl acetate extract was the bioactive part. The ethyl acetate extract was chemically investigated and two main biflavones, amentoflavone and hinokiflavone, were isolated (Figure1). The literatures reported that the two biflavones have various pharmacological functions such as anti-inflammation and inhibiting human platelet cyclic AMP phosphodiesterase[2-4], indicating amentoflavone and hinokiflavone are the potential active constituents of Trogopterus Feces. Trogopterus Feces are mainly distributed in Hebei and Shanxi provinces of China, and no quality assessment has been reported to date. Therefore, the analysis of major constituents is necessary and helpful to control their qualities. This study developed a simple and reliable solvent extraction and HPLC method to analyze amentoflavone (**1**) and hinokiflavone (**2**) in Trogopterus Feces from Hebei and Shanxi provinces.

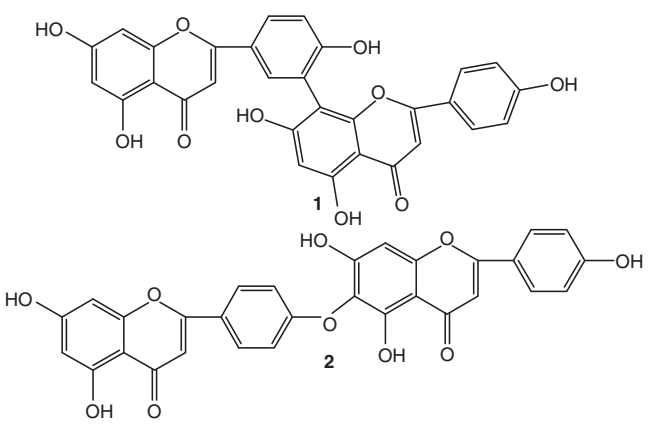

Figure 1. Structural formulas of biflavones based on the present study.

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MATERIALS AND METHODS

I. *Materials and Chemicals*

Trogopterus Feces samples were collected from Hebei and Shanxi provinces in June 2008 and identified as the dry seeds of *Trogopterus xanthipes* by Associate Professor Nianyun Yang of Nanjing University of Traditional Chinese Medicine.

Standard substances, amentoflavone (**1**) and hinokiflavone (**2**), were separated from the ethyl acetate extract of Trogopterus Feces from Hebei province by silica gel column chromatography eluted with $CHCl_3$-CH_3OH (20: 1, v/v) and purified by preparative HPLC in our lab. All purities were more than 98% as determined by HPLC. The structures were confirmed by their UV, MS, ^1H NMR and ^{13}C NMR data compared with the data from literatures[5,6] (Figure 1).

Acetonitrile for HPLC was purchased from Merck (Darmstadt, Germany). All solvents used for separation and extraction were of analytic grade and purchased from Shanghai Chemical Reagent Factory (Shanghai, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

II. *Standard Solutions Preparation*

A mixed standard stock solution was dissolved in ethanol, and prepared by accurately weighing the standard substances, amentoflavone (**1**) and hinokiflavone (**2**), at 23.5 μg/mL and 36.2 μg/mL, respectively. Working standard solutions were then prepared for the calibration curves by appropriate dilution of the mixed standard solution with ethanol. These prepared standard stock and working solutions were stored at 4°C in dark brown calibrated flasks.

III. *Sample Solutions Preparation*

The Trogopterus Feces samples were pulverized and dried to constant weight and then comminuted with a mill to pass through a 40-mesh sieve before use. The powders (40 mesh, 2 g, accurately weighed) were extracted with 30 mL of ethanol by Soxhlet method, concentrated under reduced pressure, transferred into a 25 mL volumetric flask which was made up to its volume with extraction solvent, and filtered through a 0.45 μm micropore. Ten microliters of sample were injected for HPLC analysis.

IV. *HPLC Apparatus and Conditions*

The Waters Alliance 2690 HPLC chromatographic system with a Waters 996 photodiode array detector was used for the analysis. Detection wavelength was set at 330 nm and the flow rate was 1.0 mL/min. The column temperature was maintained at 30°C. Different ratios of water-acetonitrile and water-methanol mobile phase compositions were investigated and the optimum separation was achieved using a gradient system with 1% acetic acid solution-acetonitrile (A-B). The gradient program was as follows: initial 0-10 min using isocratic elution A-B (65: 35, v/v), followed by 10-40 min of (65: 35, v/v) linear change to A-B (55: 45, v/v). Re-equilibration duration was 10 min between individual runs. Representative sample chromatograms are shown in Figure 2 and identification of the marker compounds in the sample was carried out by characterizing the sample peaks in terms of retention time and UV spectra.

The chromatographic column, Hypersil ODS-2 (250 × 4.6 mm, 5.0 μm, Dalian, China), Sunfire C-18 (250 × 4.6 mm, 5.0 μm, Ireland), and Alltima C-18 (250 × 4.6 mm, 5.0 μm, USA), were investigated for separation. The Alltima C-18 column was found to be the most efficient.

V. *Optimization of the Extraction Method*

To determine the optimized extraction conditions, extraction methods (ultrasonic, heating at reflux or Soxhlet extraction) and extraction solvents (ethanol or methanol) were investigated with Trogopterus Feces. Dried powder of the same batch of Trogopterus Feces from Hebei province was accurately weighed (2.0 g) into three portions, extracted with 30 mL of ethanol by ultrasonic. Afterwards, the extract was concentrated, transferred, filtered and analyzed as described above. The same experiments were done for ethanol extraction by heating reflux and Soxhlet method and methanol extraction by Soxhlet method so as to compare the analyte contents from different extraction method.

VI. *Linearity*

Ethanol stock solutions containing amentoflavone

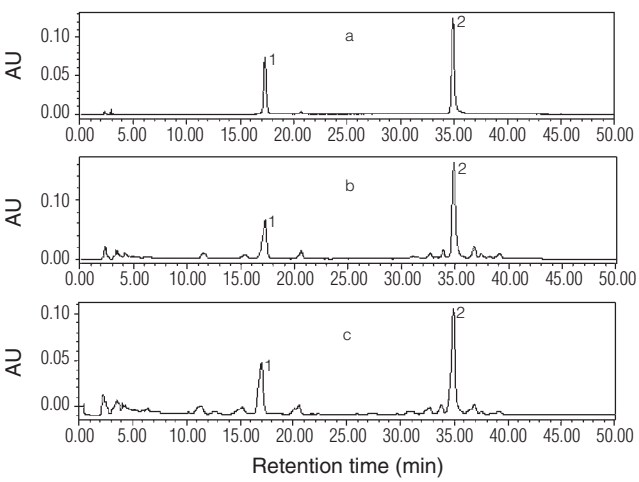

Figure 2. HPLC chromatograms: (a) standard substance mixture, (b) ethanol extract of Trogopterus Feces from Hebei province, (c) ethanol extract of Trogopterus Feces from Shanxi province **1:** amentoflavone; **2:**hinokiflavone.

(**1**) and hinokiflavone (**2**) were prepared and diluted to appropriate concentrations for the construction of calibration curves. Six concentrations of the solution were analyzed in duplicates under the above HPLC condition. The calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. Linear regressions and correlative coefficients were determined based on the calibration curves.

VII. *Limits of Detection and Quantification*

The stock solutions mentioned above were diluted to a series of appropriate concentrations with ethanol, and an aliquot of the diluted solutions was injected into HPLC-PDA for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

VIII. *Validation*

(I) *Precision and Repeatability*

Intra-day and inter-day variations were chosen to determine the precision of the developed assay. For intra-day variability test, the mixed standard stock solution [amentoflavone (**1**) (23.5 μg/mL) and hinokiflavone (**2**) (36.2 μg/mL)] were analyzed for six replicates within 1 day. For inter-day variability test, the solutions were examined in duplicates for three consecutive days. Variations were expressed by the relative standard deviations (RSD).

The reproducibility of the developed method was evaluated from six portions of the same batch of Trogopterus Feces of Hebei province. Each portion was accurately weighed (2.0 g), extracted and analyzed as mentioned above. Variations were also expressed by RSD.

(II) *Recovery*

Dried powder of the same batch of Trogopterus Feces of Hebei province was accurately weighed (1.0 g) into five portions. A ration of amentoflavone (**1**) and hinokiflavone (**2**) was added into each portion which was extracted and analyzed as mentioned above. The recovery rate was determined by the percentage of calculated amount versus theoretical amount.

IX. *Application*

The developed method was applied to evaluate the quality of Trogopterus Feces from Hebei and Shanxi provinces. Dried powder of Hebei Trogopterus Feces was accurately weighed (2.0 g) in triplicate which was extracted and analyzed as mentioned above, and the same experiments were done for Shanxi Trogopterus Feces.

RESULTS AND DISCUSSION

HPLC chromatograms of the standard compounds are shown in Figure 2. Optimization of the extraction method suggested that ethanol extraction by Soxhlet is the best method as shown from the determined contents of amentoflavone (**1**) and hinokiflavone (**2**) in Table 1. Linear regression and concentration ranges of the calibration curves for amentoflavone (**1**) and hinokiflavone (**2**) are shown in Table 2. Linearities of the calibration curves of these compounds were excellent. The LOD and LOQ data for each investigated compounds are shown in

Table 1. Determination of the two main biflavones (μg/g) in Hebei Trogopterus Feces by different extraction method

Analyte	Ethanol extraction by ultrasonic method	RSD (%)	Ethanol extraction by heating at reflux	RSD (%)	Ethanol extraction by Soxhlet method	RSD (%)	Methanol extraction by Soxhlet method	RSD (%)
Amentoflavone (**1**)	155.50	1.95	205.31	1.80	226.05	1.63	213.30	1.91
Hinokiflavone (**2**)	253.05	2.11	330.61	2.41	366.21	2.60	344.76	2.90

All experiments run in triplicate.

Table 2. Linear regression data(*) , LOD and LOQ of the investigated compounds

Analytes	Linear regression equation	Test range (μg/mL)	R^2	LOD (ng)	LOQ (ng)
Amentoflavone (**1**)	y = 5547287.8500x - 40076.7418	2.35-47.0	0.9999	0.46	1.70
Hinokiflavone (**2**)	y = 6760100.1249x - 45699.6697	3.62-72.4	0.9999	0.59	2.06

*All 6 concentrations were run in duplicates.

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Table 3. Determination of the two main biflavones (μg/g) in Trogopterus Feces samples

Sample	Amentoflavone (**1**)	RSD (%)	Hinokiflavone (**2**)	RSD (%)
Trogopterus Feces from Hebei	224.65	1.76	367.76	2.01
Trogopterus Feces from Shanxi	184.18	1.28	304.54	1.85

Each three samples from Hebei and Shanxi Provinces were analyzed.

Table 2. The intra-day and inter-day analytical precision of these compounds was also excellent, with intra-day RSD of 1.41% and 0.53% and inter-day RSD of 2.39% and 1.21%, respectively. The relative standard deviations of the repeatability of amentoflavone (**1**) and hinokiflavone (**2**) in Hebei Trogopterus Feces sample was less than 2.1% and 1.0% respectively. The average recovery rates of amentoflavone (**1**) and hinokiflavone (**2**) were 103.5% and 97.3%, and RSDs were 1.87% and 1.60%, respectively. So this study developed a simple and reliable solvent extraction and HPLC method to analyze the two main biflavones in Trogopterus Feces.

The average contents of amentoflavone (**1**) and hinokiflavone (**2**) in Trogopterus Feces from Hebei and Shanxi provinces are listed in Table 3. Trogopterus Feces are the dry seeds of *T. xanthipes*, which feeds on the leaves of cypress. Therefore, it could be concluded that the variations of the marker compounds amentoflavone (**1**) and hinokiflavone (**2**) were based on the cypress quality difference due to genetic variation, plant origin and environmental factors[7]. The two analyzed biflavones could be used as index components to evaluate the quality of Trogopterus Feces. A simple and accurate HPLC method with UV detection has been established for simultaneous analysis of two major biflavones in Trogopterus Feces within 40 min. The method was validated for linear range, LOD, LOQ, precision, repeatability, and separation efficiency. Thus, this HPLC method can be used to assess quality of the analysis for Trogopterus Feces and guide disease treatments with TCM products.

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