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Effects of Different Extraction Methods on the Extraction Rates of Five Chemical Ingredients of *Swertia mussotii* Franch by UPLC-ESI-MS/MS

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Abstract. Objective: To compare the effects of three extraction methods (ultrasound, reflux and percolation) on the contents of gentiopicroside, mangiferin, swertiamain, sweroside and oleanolic acid in *Swertia mussotii* Franch. Method: The contents of five components were determined by UPLC-ESI/MS. In the solvent system, eluent A was 0.05% (v: v) formic acid with 1mM/L ammonium acetate aqueous solution and eluent B was acetonitrile. Chromatographic separations were achieved using an Agilent EC-C18 column (4.6x100 mm, 2.7 um) at 30°C. The flow rate was set at 0.8mL/min. The compound ionization was adopted at negative ionization mode by electro spray ionization (ESI). The quantification was performed in multiple reaction monitoring (MRM). Results: The linear ranges of swertiamarin, gentiopicroside, mangiferin, sweroside and oleanolic acid are 80-7450 ng/mL, 103-6600 ng/mL, 130-8450 ng/mL, 100-7000 ng/mL, respectively. As a result, the content of oleanolic acid was the highest extracted by ultrasonic extraction and the content of mangiferin was the highest extracted by reflux extraction. For percolation extraction, the contents of five components were between ultrasound and reflux extraction. Conclusion: For five components, there are significant differences between the three different extraction methods. The results could provide a reference for the quality control of *Swertia mussotii* Franch and the research and development of new drugs.

1. Introduction

*Tibetan capillaris* (Songdi) is one of the 8 valuable Tibetan medicines, a kind of whole herb belonging to *Swertia chirayita* (Roxb ex Flemi) Karsten under Gentianaceae. The main chemical ingredients are xanthones, flavonoids, iridoid, triterpenes, etc.

Such medical literatures as Jingzhubencao record that *Swertia mussotii* Franch has the functions of clearing liver, promoting diuresis, joining sinew and bones and stanching bleeding [1-4]. Modern pharmacological studies show that *Swertia mussotii* Franch has many pharmacological activities, such as protecting liver, normalizing gallbladder function, antioxidation, reducing blood sugar, anti-inflammation and protecting blood vessel of heart and brain [5, 6].

Currently, the extraction methods for *Swertia mussotii* Franch are mainly ultrasonic method, reflux extraction method and percolation method. The contents of active ingredients vary in different...
extraction methods, especially for some heat sensitive active ingredients. The quality of the extraction will directly affect the efficacy and safety of drugs.

Based on literature investigation [7-9], five chemical ingredients (i.e. gentiopicroside, mangiferin, swertiamain, sweroside and oleanolic acid) in the extract of *Swertia mussotii* Franch through ultrasound, reflux and infiltration extraction were simultaneously determined by ultra-high performance liquid chromatography mass spectrometry (UPLC-ESI/MS). Meanwhile, the differences of five chemical ingredients in different extraction methods were studied, which could provide scientific basis for the quality control of *Swertia* mussotii Franch and development of new drugs.

2. Instruments and Results

2.1. Instruments
Chromatographic analysis was performed on an UPLC system (Shimadzu, Japan) with an on-line degasser, LC-30AD binary pump, an autosampler (STL-20A XR), and a temperature controller compartment for column (CTO-20AC). MS detections were performed on a triple quadruple mass spectrometer (QQQ 5500, SCIEX, USA) equipped with an ESI source.

2.2. Reagents
Standards of gentiopicroside, mangiferin, swertiamain, sweroside and oleanolic acid were purchased from Nanjing Spring & Autumn Biological Engineering Corporation (Nanjing, China) with purity of more than 98%; Acetonitrile (HPLC grade) was purchased from Merck company (Merck, Germany). Methanol (HPLC grade) and formic acid (LC-MS grade) were purchased from Thermo Fisher Scientific (Thermo, USA). Purified water was acquired from a Milli-Q system (Millipore, USA). All other reagents were of analytical grade.

*Swertia mussotii* Franch was collected in Dangxiong County, Lhasa city, Tibet, China and was authenticated by Lecturer Dawa from the Experimental Specimen Center under Tibet Traditional Medical College as *swertia chirayita* of genus Swertia under Gentianaceae genus.

3. Methods and Results

3.1. Analytical Condition: Chromatographic Condition
In the solvent system, eluent A was 0.05% (v: v) formic acid with 1 mM/L ammonium acetate aqueous solution and eluent B was acetonitrile. Chromatographic separations were achieved using an Agilent EC-C18 column (4.6x100 mm, 2.7 μm) at 30 °C. The flow rate was set at 0.8 mL/min. The optimized HPLC gradient elution conditions were as follows: 0.1~5 min, 10%~15% B; 5~7 min, 15%~95% B, 7~10 min, 95% B, 10.1~13 min, 10% B; The injection volume was 5 μL.

3.2. Analytical condition: mass spectrometry condition
The compound ionization was adopted at negative ionization mode by electro spray ionization (ESI). The quantification was performed in multiple reactions monitoring (MRM). For ESI-MS (+), the operating parameters were as follows: ion spray voltage floating (ISVF) ~4500 V; ion source GS1 55 psi; ion source GS2.55 psi; curtain gas (CUR). 35 psi; temperature (TEM) 600 °C. The declustering potential (DP) and collision energy (CE) conditions of five compounds are shown in Table 1. The MS/MS spectrometries and structures of five compounds are shown in Fig. 1.

| Compounds      | Adduct     | Precursor Ion (m/z) | Product Ion (m/z) | DP(V) | CE(eV) |
|----------------|------------|---------------------|-------------------|-------|--------|
| gentiopicrosin | [M+HCOO]^- | 401                 | 179.1             | -50   | -15    |
| mangiferin     | [M-H]^-    | 421                 | 331.1             | -100  | -15    |
| swertiamain    | [M+HCOO]^+ | 419.2               | 179.0             | -50   | -16    |
| sweroside      | [M+HCOO]^+ | 403.1               | 195.0             | -60   | -17    |
| oleanolic acid | [M-H]^+    | 455.2               | 407.3             | -50   | -53    |

Table 1. The declustering potential (DP) and collision energy (CE) conditions of five compounds.
3.3. Sample Solution Preparation

3.3.1 Preparation for control sample. As control sample, the mixed standard solution was prepared by precisely weighing appropriate amount of gentiopicrin, mangiferin, swertiamain, sweroside and oleanolic acid into acetonitrile solution and the final concentrations of gentiopicrin, mangiferin, swertiamain, sweroside and oleanolic acid were 132 μg/mL, 480 μg/mL, 149 μg/mL, 169 μg/mL and 140 μg/mL, respectively.

3.3.2 Preparation for sample solution for ultrasound extraction. The solution was made by weighing 0.50 g Swertia mussotii Franch powder into conical flask with 20mL methanol. Then it was carried out for 40mins by ultrasonic extraction (100 w, 40 kHz). The ultrasonic extraction solution was filtered by a 0.2 μm microporous membrane. It was diluted with acetonitrile and the final concentration of Swertia mussotii Franch was 0.005 g/mL.

3.3.3 Preparation for sample solution for reflux extraction. The reflux extraction was prepared by using 4 times, 6 times and 8 times amount of 160 g of Swertia mussotii Franch powder to extract with 85% ethanol for 3 times, respectively. Then it was made by merging supernatant to get 2500 mL extract [10]. The reflux extract was filtered by a 0.2 μm microporous membrane. It was diluted with acetonitrile and the final concentration of Swertia mussotii Franch was 0.005 g/mL.

3.3.4 Preparation for sample solution for percolation extraction. The percolation extraction was prepared by mixing 160 g Swertia mussotii Franch powder with the same amount of 85% ethanol into percolator. Then add solvent to exhaust bubbles and collect 400 mL percolation exhaust gas liquid.
Thirdly it was soaked for 36 h. The percolation liquid was made by being percolated at a speed of 2 mL/min/kg and it got 1400 mL. The total percolation liquid was the mixture of 400 mL and 1400 mL [11]. The percolation extract was filtered by a 0.2 μm microporous membrane. It was diluted with acetonitrile and the final concentration of *Swertia mussotii* Franch was 0.005 g/mL.

3.4. Methodological Evaluation

3.4.1. Linear relationship investigation. The precise control solution was prepared by the method of preparing control sample. A series of mixed standard solutions were prepared by multiple dilution method. Precisely absorb 5 μL injection liquid chromatography and record chromatogram. The standard curve was plotted with concentration as abscissa (X) and peak area as ordinate (Y). The detection limit was determined by 3 times of signal-to-noise ratio, and the quantitative limit was determined by 10 times of signal-to-noise ratio. The results are shown in Table 2.

### Table 2. Regression equation, correlation coefficient, linear range, detection limit and quantitative limit of the five components

| compounds     | Regression equation       | linear range (ng•mL⁻¹) | r      | LOD (ng•mL⁻¹) | LOQ (ng•mL⁻¹) |
|---------------|---------------------------|------------------------|--------|---------------|---------------|
| gentiopicrin  | y=10000•1.46×10⁷          | 103~6600               | 0.9999 | 19.10         | 47.51         |
| mangiferin    | y=3.34×10³x+1.16×10⁵      | 100~8000               | 0.9986 | 3.26          | 16.31         |
| swertiamain   | y=3.34×10³x+1.16×10⁵      | 80~7450                | 0.9977 | 0.72          | 5.76          |
| sweroside     | y=282x•2.6×10⁵            | 130~8450               | 1.0000 | 21.10         | 52.52         |
| oleanolic acid| y=2.27×10³x+2.75×10⁵      | 100~7000               | 0.9935 | 0.056         | 0.115         |

3.4.2. Precision test. Precisely absorb 5 μL of medium concentration under "C", 6 continuous insertions with stitches, calculate the intraday precision, and the RSD were 0.62% gentiopicroside, 1.06% mangiferin, 1.23% swertiamain, 1.14% sweroside, 2.00% oleanolic acid respectively. Samples were injected for 3 successive days, and then calculate the daytime precision, and the RSD were 2.26% gentiopicroside, 3.85% mangiferin, 3.59%, swertiamain, 1.53% sweroside and 2.75% oleanolic acid.

3.4.3. Stability test. Precisely absorb the medium concentration under "C", and samples were injected at 0, 2, 6, 8, 10 and 12 h respectively, and the RSD were 1.58% gentiopicroside, 2.40% mangiferin, 2.41% swertiamain, 2.11% sweroside and 2.92% oleanolic acid.

3.4.4. Sample recovery test. Take the samples of *Swertia mussotii* Franch prepared by different methods, and precisely add 50 μL of control sample solution, and test it. The recovery rates of gentiopicroside, mangiferin, swertiamarin, sweroside and oleanolic acid were 90.3%, 105.6%, 98.9%, 91.5% and 108%, respectively.
Figure 2. Chromatograms of mixed control sample (A), percolation sample (B), reflux sample (C), ultrasonic sample (D). The chromatographic peaks are swertiamain, mangiferin, gentiopicrin, sweroside and oleanolic acid in turn.

3.5 Determination of Sample Content
The test sample solutions were prepared according to different extraction methods under "C". The chromatographic and mass spectrometric conditions were determined under "C", and record the area of the chromatographic peak. Put the 5 peak areas of the to-be-measured ingredients into the corresponding standard curve equation, and calculate its mass fraction. The results are shown in Table 3.

Table 3. Content of five components got by different extraction methods in Swertia mussotii Franch

| components    | content by ultrasound extraction (mg/g) | content by reflux extraction (mg/g) | content by percolation extraction (mg/g) |
|---------------|-----------------------------------------|------------------------------------|----------------------------------------|
| gentiopicroside | 0.014                                   | 0.089                              | 0.047                                   |
| mangiferin    | 0.173                                   | 0.838                              | 0.668                                   |
| swertiamain   | 0.086                                   | 0.346                              | 0.304                                   |
| sweroside     | 0.016                                   | 0.085                              | 0.063                                   |
| oleanolic acid| 0.667                                   | 0.525                              | 0.390                                   |
4. Summary
A rapid and accurate method for UPLC-ESI/MS analysis was established in the experiment, then compared the differences of 5 kinds of chemical ingredients in extracts of different extraction technology in Tibetan medicine *Swertia mussotii* Franch and analyzed the influence of heating extraction (extraction) and normal temperature extraction (percolation extraction) on the chemical ingredients of *Swertia mussotii* Franch.

The ESI source was used in the mass spectrometry, and the dependence on mobile phase was relatively great. Therefore, the mobile phase of different buffer salt methanol system, different buffer salt acetonitrile system and different proportion formic acid water system were investigated. The results showed that acetonitrile has a good separation effect.

The peak shape of mangiferin would be better under the condition of acid water. The response of oleanolic acid would be better under the condition of ammonium acetate water. So formic acid-ammonium acetate water was preferred as aqueous phase. Then compare the peak shape and response after mixing different proportions of formic acid and ammonium acetate, it was discovered that excessive formic acid resulted in a decrease in the response of oleanolic acid. When ammonium acetate was at 1 mM/L, the oleanolic acid could achieve the best condition in the mixed mobile phase system. So, it was determined to adopt -0.05% formic acid -1 mM/L ammonium acetate water as the mobile phase. The contents of 5 chemical ingredients in the extracts were compared by 3 kinds of extraction techniques. The results showed that the content of oleanolic acid was higher than that of the other two methods. The content of gentiopicroside and mangiferin, swertiamarin and sweroside was significantly lower than that of percolation and reflux extraction process. The current 2015 edition of the Chinese Pharmacopoeia only limits the content of oleanolic acid in the quality standards of Tibetan herbal medicine, while the content of other chemical ingredients is not limited. However, the current actual production of Zangyinchen capsule, Zangyinchen tablet and related Chinese patent drugs, heat reflux extraction of 80% ethanol was used more often.

Therefore, it is suggested that a variety of chemical ingredients should be used as control indicators so as to improve the quality standard of *Swertia mussotii* Franch. In addition, by comparing the content of chemical ingredients extracted by heat extraction reflux extraction and cold extraction-infiltration, the results showed that the contents of 5 chemical ingredients constituent in reflux extracts were higher than those of percolation extraction, but there were no significant differences in the chemical composition, which shows that the extraction temperature contributes to the leaching of the chemical ingredients of *Swertia mussotii* Franch, and the 5 chemical ingredients did not lose in the reflux extraction process.

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