Development of assay method by HPLC-DAD for the quantitative determination of chromones in *Saposhnikovia divaricata* radices and its validation

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**Abstract.** Assay method using high performance liquid chromatography with diode array detection was developed and validated for the determination of chromones: prim-O-glucosylcimifugin, cimifugin, 4’-O-β-D-glucosyl-5-O-methylisaminol. Chromatographic method for the determination of chromones was validated for specificity, linearity, accuracy, repeatability and intermediate precision. The optimal conditions for the extraction of chromones from the *Saposhnikoviae divaricatae* radices were selected. It was found that these substances are better extracted with 50% ethanol using ultrasonic extraction at room temperature. The quantity of chromones in *Saposhnikoviae Radix* of the flora of Buryatia was also determined.

**1. Introduction**

*Saposhnikovia divaricata* (Turcz.) Schischkin. is a plant that belongs to *Umbelliferae*, or *Apiaceae* family of Russian flora. This species is distributed in the southern regions of Eastern Siberia and the southwestern part of the Far East on the territory of the Russian Federation, and is also found on the territory of China, the Korean Peninsula, Mongolia, and Japan. *Saposhnikovia divaricata* is a perennial and monocarpic plant with taproot system [1]. For several thousand years *Saposhnikoviae divaricatae radices* were used in the medicine of Asian countries as an effective anti-inflammatory agent. It is known as Fang Feng in Traditional Chinese Medicine, and it is used in decoction form that has antipyretic action and also in ethanol extract, which has anti-inflammatory and cough-depressant effects [2]. Also, herbal juice has antimicrobial activity against strains such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [3]. In Mongolian traditional medicine fruits of *Saposhnikovia divaricata* are used as a fortifying agent. The roots are included in the list of official medicinal products of the Republic of Korea called “Bangpung” and “Bofu” in Japan [4]. Proceeding from the above mentioned, *Saposhnikovia divaricata* is popular in Asia, but this plant is not included in the State Pharmacopoeia of the Russian Federation. It is necessary to analyze the quantity of biologically active substances for the development of regulatory documentation and standardization of medicinal plant raw material. According to the requirements of the Pharmacopoeia of China, the main active substances in the roots are chromones (prim-O-glucosylcimifugin and 4’-O-β-D-glucosyl-5-O-methylisaminol). Thus, the aim of this study was to develop a new method for the quantitative content of chromones in the roots of *Saposhnikovia divaricata* and to validate this method.
2. Models and Methods
The objects of the study were the samples of *Saposhnikoviae radix*, that were collected in the fruiting stage of development in 2016-2018. HPLC grade methanol (Ultra Gradient HPLC Grade, J.T. Baker) was used for chromatography. Prim-O-glucosylcimifugin (98.94%) (PGC) and 4'-O-β-D-glucosyl-5-O-methylvisammioside (99.19%) (MV) were purchased from ANPEL laboratory Technologies (Shanghai, China). The standard of cimifugin (95.00%) (C) was obtained in the laboratory of medical chemistry at the N N Vorozhtsov Novosibirsk Institute of Organic Chemistry SB RAS. For the extraction we used ethyl alcohol of "Constanta-Farm M" production. Deionized water for chromatographic analysis was obtained on a Millipore instrument.

3. Results and Discussion
Medicinal plant raw materials were crushed in particles passing through a sieve with not more than 2 mm holes in diameter and were extracted in ratios of 1:100 → 1:5 with various solvents (methanol with a concentration from 50% to 100%, ethanol from 30% to 90%). We made ultrasound assisted extraction (frequency - 22 kHz) at room temperature. Also, we studied the optimal terms of extraction, such as duration (from 20 to 50 minutes) and the multiplicity of extraction (single, double and triple extraction). The extract was filtered through a “blue ribbon” filter into a 50 mL volumetric flask and adjusted to the mark.

The obtained extracts were analyzed using Agilent 1200 (Agilent Technologies, USA) high performance liquid chromatograph with a diode array detector. Separation was achieved using Zorbax Eclipse XDB-C18 column (4.6*150mm, 5 μm). The mobile phase consisted of aqueous solvent A: water and organic phase solvent B: methanol, and a flow rate was 1.0 mL/min. The gradient program was applied as follows: 0-5 minutes 60-45% A; 5-10 minutes 45-40% A; 10-15 minutes 40-0% A; 15-20 minutes 0% A. Sample volume was 5 μl of solution and the temperature of column was maintained at 30°C. The wavelength of analysis was 300 nm. We conducted the analysis of the quantitative content of three chromones (prim-O-glucosylcimifugin, cimifugin and 4'-O-β-D-glucosyl-5-O-methylvisamminol). Identification was performed by retention time and absorption spectrum in comparison with standard samples.

About 2.5 g of crushed medicinal plant raw materials, passing through a sieve with not more than 2 mm holes in diameter, were placed in a flat-bottom flask (V=100 ml), 25 ml of 50% ethanol was added and extracted by ultrasonic extraction at a frequency of 22 kHz at room temperature for 40 minutes. Then extract was filtered through a “blue ribbon” filter into volumetric flask (50 ml). The filtration residue was transferred back to the same flask, 25 ml of 50% ethanol was added and the extraction was repeated under the same conditions. The obtained extract was again filtered through the same filter in the same volumetric flask and adjusted to the mark with 50% ethanol. This solution was chromatographed under the above conditions, and the content was calculated from the peak area.

The evaluation of the validity of the analytical methodology was carried out according to the following validation parameters: specificity, linearity, accuracy, repeatability and intermediate precision.

**Specificity.** The resolution coefficient between the peak of prim-O-glucosylcimifugin and cimifugin was 9.79, between cimifugin and 4'-O-β-D-glucosyl-5-O-methylisamminol - 8.26, while the resolution of the peak of the component being determined with other peaks was more than 1.5 (Figure 1).

**Linearity.** To determine this parameter, 6 solutions were prepared (1.50 mg/ml; 0.75 mg/ml; 0.50 mg/ml; 0.25 mg/ml; 0.15 mg/ml; 0.15 mg/ml; 0.05 mg/ml), the calibration graphs were constructed and the regression coefficient was calculated. The regression coefficient of a line graph of O-glucosylcymifugin standard sample was 0.9937, of cimifugin - 0.9910 and the regression coefficient of 4'-O-β-D-glucosyl-5-O-methylisamminol was 0.9935. This technique can be considered linear, because the correlation coefficient was not lower than 0.99.

**Accuracy.** The analytical accuracy indicates the proximity of the test results to the true value. 9 solutions were prepared by standard addition method to determine the accuracy of the method, and
they were made in three different concentrations and conducted in three replications for each standard solution and the recovery percentage was calculated. For the solution of prim-O-glucosylcimifugin with a concentration of 0.4 mg/ml the recovery percentage was from 98.45% to 100.35%, for the concentration of 0.5 mg/ml - from 99.82% to 100.34%, and for prim-O-glucosylcimifugin with concentration of 0.6 mg/ml the recovery percentage was from 99.90% to 100.05%; for cimifugin with 0.12 mg/ml concentration - from 99.67% to 100.33%, with 0.15 mg/ml - from 99.47% to 100.90% and 0.18 mg/ml - from 99.50% to 100.41%; for 4’-O-β-D-glucosyl-5-O-methylvisamminol solution with the concentration of 0.4 mg/ml the recovery was from 99.88% to 100.13%, 0.5 mg/ml -from 98.90% to 100.80% and for solution with 0.6 mg/ml concentration - from 99.87% to 100.18%. The results meet the requirements, since the recovery percentage should be between 98.0% and 102.0%.

Repeatability. To conduct this test, 6 samples with the same concentration were chromatographed and the coefficient of variation was calculated. It is known that the coefficient of variation is the ratio of the standard deviation to the arithmetic mean of the results of several measurements. The variation coefficients of prim-O-glucosylcimifugin and cimifugin standard samples were the same and amounted to 0.00001%, and the standard sample of 4’-O-β-D-glucosyl-5-O-methylvisamminol was 0.005%. The coefficient of variation of parallel samples for six measurements should be no more than 2%.

Intermediate precision. The analyses were conducted on a high-performance liquid chromatograph Milichrom A-02 (Econova, Russia). To determine this indicator, the coefficient of variation was also calculated: prim-O-glucosylcimifugin solution - 0.002%, cimifugin - 0.0006% and 4’-O-β-D-glucosyl-5-O-methylisumminol - 0.014%.

Figure 1. Chromatograms (A) of standard solution and (B) 50% ethanol extract of *Saposhnikoviae Radix*: a – prim-O-glucosylcimifugin; b – cimifugin; c - 4’-O-β-D-glucosyl-5-O-methylvisamminol.
Furthermore, samples of *Saposhnikovia divaricata radix* that were collected on the territory of Tarbagatai district in the Republic of Buryatia of the Russian Federation in 2016-2018 in the fruiting stage were analyzed. The content of prim-O-glucosylcimifugin ranged from 2.96 mg/g to 4.08 mg/g; cimifugin - from 0.10 mg/g to 1.08 mg/g; 4’-O-β-D-glucosyl-5-O-methylvisamminol from 1.42 mg/g to 1.91 mg/g. The content of chromones in samples collected in 2018 are characterized by the highest content of chromones which is more noticeable in the example of cimifugin (Table 1). A comparative analysis with the literature data revealed a slight difference in the content of prim-O-glucosylcimifugin and 4’-O-β-D-glucosyl-5-O-methylvisamminol depending on the place of collection.

| Place of collection / year of collection / phase of development | PGC   | C      | MV     |
|---------------------------------------------------------------|-------|--------|--------|
| Tarbagatai, Tarbagatai district, Buryatia, Russia / 2016 / fruiting phase | 2.96±0.23 | 0.14±0.02 | 1.42±0.10 |
| Tarbagatai, Tarbagatai district, Buryatia, Russia / 2017 / fruiting phase | 3.98±0.06 | 0.10±0.00 | 1.73±0.04 |
| Tarbagatai, Tarbagatai district, Buryatia, Russia / 2018 / fruiting phase | 4.08±0.31 | 1.08±0.01 | 1.91±0.16 |
| Chinese origin\(^{a}\) | 3.30±0.01 | ND     | 2.30±0.01 |
| German origin\(^{a}\) | 2.70±0.01 | ND     | 1.70±0.01 |

\(^{a}\) Scherubl R, Manns D, Heilmann J, Franz G 2013 Comparison of HPLC Versus HPTLC-Densitometry for the Quantification of Chromone Glucosides in Saposhnikoviae divaricatae Radix *Chromatographia* 76 1537–43.

4. Conclusion

Thus, we have developed assay method for determining the quantitative content of chromones (prim-O-glucosylcimifugin, cimifugin and 4’-O-β-D-glucosyl-5-O-methylvisamminol) in *Saposhnikoviae divaricatae radices* and validated it. We determined of chromones in medicinal plant raw materials of the flora of Buryatia.

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