Influence of extraction methods on the recovery of astragaloside IV from the roots of Astragalus mongholicus in Soxhlet- and Twisselmann-type apparatus

Abstract: Astragalus mongholicus, commonly used in Traditional Chinese Medicine (TCM), contains significant amounts of triterpene saponins – astragalosides, which possess a wide variety of pharmacological actions, including the treatment of cardiovascular and respiratory diseases. The present study was performed to develop a fast and effective extraction method of saponins from crude plant material using Twisselmann-type apparatus. Moreover, a detailed comparison between Soxhlet and Twisselmann apparatus was performed to explain the advantages and disadvantages of their applications. The content of astragaloside IV – the major saponin of A. mongholicus extracts and chosen as an extraction efficiency marker – was determined by means of RP-HPLC-ELSD chromatography in an herein optimized method. Twisselmann extraction shortened its duration from 4 to 2 hours in comparison with the Soxhlet method – a generally accepted method preparation for this extract. The effectiveness of both apparatus was alike (3.24 ± 0.44 mg and 3.48 ± 0.26 mg of astragaloside IV/4g of powdered root for 2 hours in Twisselmann and 4 hours in Soxhlet apparatus). Compared with a pharmacopoeial method, the application of Twisselmann apparatus enabled a decrease in analysis time of 50%, which is of high significance, especially for large, scaled-up industrial scale.

Keywords: Astragalus mongholicus, Twisselmann extraction, HPLC-ELSD detection, saponins, astragaloside IV

1 Introduction

Mongolian astragalus (Astragalus mongholicus) is a perennial herbaceous plant from the Fabaceae family which originates from north-eastern China, central Mongolia and Siberia [1]. The roots of Astragalus mongholicus are used in Traditional Chinese Medicine (TCM) as stimulating and anti-inflammatory remedies. They are valued adaptogens often comparable to ginseng [2-3]. The main secondary metabolites present in A. mongholicus extracts include: polysaccharides, triterpene saponins – e.g. astragalosides (I–VIII), flavonoids and isoflavones [4].

The authors, following the European Pharmacopoeia monograph, decided to choose astragaloside IV (A IV) – a steroidal saponin characterized by vast pharmacological activities [5-12] – as a marker to elaborate an efficient recovery protocol of active components from crude plant material. In the scheduled survey Soxhlet extraction, recommended in the pharmacopoeial procedure, was compared with Twisselmann extraction to elucidate the influence of the recovery protocol on the composition of extracts.

Both extraction techniques are available for pilot and batch production. In this case, the presented work may be perceived as a model for future industrial large scale development. To the authors’ knowledge, this is the first trial of saponins’ recovery in Twisselmann apparatus.
Moreover, HPLC chromatography with ELSD detection was applied and validated to elaborate an efficient fingerprinting method suitable for qualitative determination of astragaloside IV in the astragalus extracts.

## 2 Materials and Methods

### 2.1 Plant material

The root of *Astragalus mongholicus* was obtained from the Institute for Crop Sciences and Plant Breeding of Bavarian State Research Center for Agriculture, Freising-Weihenstephan, Germany in March 2014. The plant was cultivated in 2012. Its roots were collected in autumn 2012, washed, sliced and dried at 40°C for a week. The dried plant was stored in a cool, dry place until 2014. A voucher specimen (WKK002014) was deposited in the Chair and Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, Poland.

### 2.2 Chemicals and Reagents

Chemical standard of astragaloside IV (98% purity) was purchased from Sigma Aldrich. Ammonia, 1-butanol, methanol (gradient grade), were produced by Avantor Performance Materials. HPLC grade solvents: methanol, acetonitrile and acetic acid were obtained from J.T. Baker.

### 2.3 Extraction

#### 2.3.1 Soxhlet apparatus

Based on the suggestions presented in the *A. mongholici* monograph of European Pharmacopoeia [13], 8 g of root powder was packed into a paper thimble and macerated overnight in methanol. Then, it was moved to Soxhlet apparatus and extracted continuously with 160 mL of methanol (solute: liquid ratio of 1:20) for 4 hours. A 2- and 3-hour long extraction was also performed on freshly powdered plant material, in triplicate.

#### 2.3.2 Twisselmann apparatus

According to the previously described extraction pattern, 8 g of powdered root in a paper thimble was macerated overnight in methanol. Then, it was moved to Twisselmann apparatus and extracted by hot vapors of methanol (160 mL, solute: liquid ratio of 1:20) for 4 hours. A 2- and 3-hour long extraction was also performed on freshly powdered plant material, in triplicate.

### 2.4 Separation of saponins

The separation of saponins was performed after a liquid-liquid butanol-water extraction. According to the pharmacopoeial procedure [13], the obtained extract was diluted in 10 mL of water and subjected to further analysis. Firstly it was shaken with four portions of butanol, 40 mL each. Butanol fractions were joined and washed twice with 40 mL of 15% ammonia. This step enabled the creation of A IV in the solution [14]. Butanol layers were joined, evaporated to dryness, redissolved in 5 mL of methanol, filtered through a nylon syringe filter (nominal pore size 0.45 µm) and subjected to HPLC-ELSD analysis.

### 2.5 HPLC-ELSD analysis of extracts

HPLC-ELSD quantitative and qualitative analysis of extracts was performed using a Varian liquid chromatograph (920-LC) equipped in an electric light scattering detector (385-LC), DAD detector, a pump and an autosampler. The following gradient of acetonitrile (B) in water (A) was introduced for the separation of the extract constituents: 0–5 min 10% of B in A, 5–10 min 10–20% B in A, 10–20 min 20–25% B in A, 20–30 min 25–30% B in A, 30–40 min 30–50% B in A, 40–45 min 50% B in A, 45–66 min 50–10% B in A, and finally at 50 min 10% of B in A. Postrun was set to 15 minutes and the flow rate to 0.5 mL min⁻¹. For the DAD-based chemical profiling the UV chromatograms were recorded in 290 nm by monitoring spectra within a wavelength range of 200–400 nm, in room temperature. The separation was performed on a C18 column Discovery-Sigma Aldrich (250 mm × 4 mm, 5 µm). The described HPLC gradient composition was a modification of an HPLC method from the monograph of *Astragalus mongholicus* described in the European Pharmacopoeia [13]. The herein performed optimization was based on the desire to introduce a commonly applied chromatographic column with a diameter of 4.6 mm instead of a thinner and less often used – 3.2 mm one, as suggested by the pharmacopoeia. An ELSD detection was performed in the following settings: nebulizer temperature: 30°C, evaporator temperature: 50°C, gas flow (SLM): 1.5, LED%:
The Galaxie operational system was used for the data acquisition and control.

A calibration curve of a standard solution of A IV was prepared for different concentrations of a standard compound: 0.3, 0.6, 1.2, 1.75, 2.4, 3.0, 3.6, 4.2 mg mL\(^{-1}\). The injection volume was set at 10 µL for each solution. The qualitative analysis of astragaloside IV in the samples was conducted taking into account their weights, injection volumes and the peak areas of A IV. The limits of detection (LOD) and quantification (LOQ) under the set chromatographic conditions were calculated at a signal-to-noise ratio (S/N) of 3 and 10, respectively. Furthermore, the linear range value, intra-day and inter-day values (measured after a triple injection) were determined for the applied method.

2.6 Statistical analysis

The obtained data were analyzed in the Statistica (10.0) program. All analyses were done in six replicates, and were expressed as average values ± standard deviation. The significance of the results was determined at \( p < 0.05 \) using \( t \)-test for the applied methods.

3 Results and discussion

3.1 Extraction protocol

The description of the extraction protocol of *A. mongholicus* roots in a pharmacopoeial monograph [13] involves time-consuming procedures namely overnight maceration of the powdered root followed by its exhaustive extraction in the Soxhlet apparatus. The presented manuscript tends to increase the effectiveness of the recovery in order to decrease the production costs of astragalus extracts rich in saponins, which are commonly introduced to herbal drugs and dietary supplements around the world [15].

Based on the initial observations of a Twisselmann extraction procedure in the recovery of fatty acids, the authors herein present a new approach and target the saponins’ extraction from the roots of *Astragalus mongholicus* and namely the astragaloside IV enriched extracts.

In the performed study, a detailed comparison between Soxhlet and Twisselmann apparatus was shown to explain the advantages and disadvantages of their applications. Soxhlet 2-, 3-, and 4-hour long extraction (S2, S3, S4, respectively) has been compared to a Twisselmann 2-, 3- and 4-hour long extraction (T2, T3, T4) to check whether the procedure’s duration might be shortened, leaving a similar recovery rate of an investigated saponin. Moreover, the authors’ intention is to predict the influence of extraction temperature on the recovery of A IV and its quantity in the extracts to confirm the thermal stability of the compound and A IV – structure related saponins.

The performed studies showed marked differences in the content of A IV depending on the extractor type and extraction duration. These variations might have been evoked by the actual extraction temperature, which differs between these two apparatus. Cooled down, liquified solvent vapours wash out the constituents of plant material in Soxhlet extractor. On the other hand, the extraction conducted in Twisselmann apparatus enables the solvent vapours to pass directly through the plant material in a higher efficiency due to an increase in the solvent’s temperature (ca. 10°C higher than in Soxhlet apparatus) [16].

As shown in Table 1 and Fig. 1, comparing the average results of A IV extraction at different times and in different apparatus, the A IV content tended to increase within the prolongation of extraction time. The average concentration of the compound varied from 1.90 to 3.48 mg in 4 g of freshly dried and powdered root after Soxhlet extraction, while 3.24 to 3.79 mg – after Twisselmann extraction in the same batch. It seemed that the particular portion of solvent used in the process was capable to extract this amount of astragaloside IV and that longer

![Figure 1: The box and whisker plot obtained for all extracts obtained by Soxhlet and Twisselmann apparatus in different extraction conditions.](image)
Influence of extraction methods on the recovery of astragaloside IV

Extraction would not bring any increase of its content in the extracts. Apparently, the Soxhlet extractor achieved the state of saturation after 4 hours, and Twisselmann extractor after ca. 2 hours (Fig. 1).

A 4-hour long recovery yielded the highest quantity of the saponin: 3.48 mg (for Soxhlet) and 3.79 mg (for Twisselmann). However, the application of Twisselmann apparatus resulted in the ability to decrease the extraction process twice, and keep the same effectiveness as relevant to a pharmacopoeial procedure. The T2 extraction was characterized by higher SD values for A IV content and indicated some differences between the samples. However, according to Table S3 and Table S1 (‘Supplementary material’), assuming the value of \( p < 0.05 \), 2- and 3- hour-long Twisselmann extractions (T2 and T3) yield similar quantities of astragaloside IV than a 4-hour-long Soxhlet extraction (S4). Twisselmann extraction conducted at 4 hours (T4) was not correlated with pharmacopoeial conditions, which confirms a statistically significant higher content of A IV in these conditions.

Summing up, the \( t \)-test testing differences between the means revealed, that there was no statistically significant difference (\( p < 0.05 \)) between the methods: S4 and T2, but also between S4 and T3 (Table S3 and Table S1 from ‘Supplementary material’). The unity of the obtained results within a group were tested by a \( t \)-test and revealed no significant differences between the results in the same group (Table S2).

As compared to the pharmacopoeial method, the application of Twisselmann apparatus enabled a decrease in the analysis time of 1 or even 2 hours, which is of high significance especially on a large, scaled-up industrial scale. Except from a higher efficiency, Twisselmann apparatus offers the same loading capacity as Soxhlet, and additionally gives the ability to evaporate the extract on-line, during the extraction process. These help to save time and energy and may induce noticeable savings.

### 3.2 HPLC-ELSD analysis

In the conducted study, a modified HPLC-based method has been applied to well suit a commonly available chromatographic column (C18, 250 mm × 4.6 mm, 5 μm) with no loss in resolution, system selectivity and run time. Firstly, the gradient profile was adjusted together with the flow rate of a solvent system, and with the gas flow in the ELSD detector. The peak of A IV was eluted after 23.5 min (Fig. 2) in the obtained chromatographic conditions and was well separated from other accompanying compounds in the extract. The method enabled an effective separation of ca. 10 compounds in the methanolic extract of astragali root.

The quantitative analysis was performed based on a determined calibration curve equation for A IV:

\[
y = 2000000 x + 480542 \quad (R^2 \text{ value of } 0.9982)\]

The herein proposed method was sensitive for astragaloside IV determinations, with LOD values of 0.2 mg mL\(^{-1}\) and LOQ

---

**Table 1**: An average astragaloside IV content in the extracts obtained in different apparatus and extraction duration.

| Extraction time (h) | Soxhlet apparatus [mg of astragaloside IV/ 4 g of powdered root] | Twisselmann apparatus [mg of astragaloside IV/ 4 g of powdered root] |
|---------------------|------------------------------------------------------------------|------------------------------------------------------------------|
|                     | Mean | SD  | Mean | SD  |
| 2                   | 1.90 | 0.12 | 3.24 | 0.44 |
| 3                   | 2.55 | 0.21 | 3.69 | 0.20 |
| 4                   | 3.48 | 0.26 | 3.79 | 0.13 |

---

**Figure 2**: ELSD chromatogram of Astragalus mongholicus root extracts with astragaloside IV at 23.5 min
of 0.6 mg mL⁻¹. The linearity range calculated for a 10 µl injection, was 0.5 mg mL⁻¹ to 4.0 mg mL⁻¹ of A IV, and the intraday and interday precision of the runs was measured at 3.14% and 4.97%, respectively.

The identification of this saponin in the extracts was performed based on its retention time recorded at ELSD detector compared to an authentic standard. Due to a chemical character of A IV (lack of a chromophore), no UV spectrum of this compound was recorded.

4 Conclusions:

On the basis of the performed astragaloside IV content measurements in crude root extracts of Astragalus mongholicus obtained by Soxhlet and Twisselmann apparatus, it was concluded that the use of the latter enabled the extraction time to be shortened by 50% – from 4 hours to 2 hours, giving the possibility to evaporate the extractent during the extraction run. The applied in the study HPLC-ELSD analysis of astragaloside IV content, dependant on the extraction conditions, was selective and resulted in a satisfactory separation of mixture’s components.

Abbreviations

TCM – Traditional Chinese Medicine,
SD – standard deviation,
A IV – astragaloside IV,
ELSD – Evaporative Light Scattering Detector

Acknowledgements: The authors thank: Dr. Heidi Heuberger from the Institute for Crop Science and Plant Breeding in the Bavarian State Research Center for Agriculture for the identification of plant material and root samples used in this study, Ms. Monika Kolbuk and Ms. Anna Koprowska for their support.

References

[1] Anon. Astragalus membranaceus. Altern. Med. Rev., 2007, 8, 72-77.
[2] Pharmacopoeia of the People’s Republic of China, Vol. 1, People’s Medical Publishing House, Shenzen, 2001.
[3] Yesilada E., Bedir E., Calis I., Takahashi Y., Ohmoto Y., Effects of triterpene saponins from Astragalus species on in vitro cytokine release, J. Ethnopharmacol., 2005, 96, 71-77.
[4] Lin L.Z., He X.G., Lindenmaier M., Nolan G., Yang J., Cleary M., et al., Liquid Chromatography-Electrospray Ionization Mass Spectrometry Study of the Flavanoids of the Roots of Astragalus mongholicus and A. membranaceus, J. Chrom. A, 2000, 876, 1-2, 87-95.
[5] Ren S., Zhang H., Mu., Sun M., Liu P., Pharmacological effects of Astragaloside IV: a literature review, J. Tradit. Chin. Med., 2013, 15, 33, 3, 413-416.
[6] Hu J.Y., Han J., Chu Z.G., Song H.P., Zhang D.X., Zhang Q., et al., Astragaloside IV attenuates hypoxia-induced cardiomyocyte damage in rats by upregulating superoxide dismutase-1 levels, Clin. Exp. Pharmacol. Physiol., 2009, 36, 4, 351-357.
[7] Ji K.T., Tang J.F., Chai J.D. Effect of Astragaloside against the oxidative damage on endothelial cells. Zhong Guo Zhong Xi Yi Jie He Za Zhi 2011, 31, 6, 807-810.
[8] Li H.B., Gei Y.K., Zhang L., Astragaloside IV improved barrier dysfunction induced by acute high glucose in human umbilical vein endothelial cells. Life Sci. 2006; 79, 12, 1186-1193.
[9] Liu X., Min W., Protective effects of Astragaloside against ultraviolet a-induced photoaging in human fibroblasts, Zhong Xi Yi Jie He Xue Bao, 2011, 9, 3, 328-332.
[10] Meng L.Q., Tang J.W., Wang Y., Zhao J.R., Shang M.Y., Zhang M., et al., Astragaloside IV synergizes with ferulic acid to inhibit renal tubulointerstitial fibrosis in rats with obstructive nephropathy, Br. J. Pharmacol., 2011, 162, 8, 1805-1818.
[11] Liu H., Wei W., Sun W.Y., Li X., Protective effects of Astragaloside IV on porcine-serum-induced hepatic fibrosis in rats and in vitro effects on hepatic stellate cells, J. Ethnopharmacol., 2009, 122, 3, 502-508.
[12] Wang S., Li J., Huang H., Gao W., Zhuang C., Li B., et al., Anti-hepatitis B virus activities of Astragaloside IV isolated from radix Astragali, Biol. Pharm. Bull., 2009, 32, 1, 132-135.
[13] European Pharmacopoeia 8.0 edition, 2013.
[14] Monschein M., Ardjomand-woelkart K., Rieder J., Wolf I., Heydel B., Kunert O., et al., Accelerated sample preparation and formation of astragaloside IV in Astragali radix, Pharm Biol. (In press), DOI: 10.3109/13880209.2013.839712.
[15] Jalsrai A., Grecksch G., Becker A., Evaluation of the effects of Astragalus mongholicus Bunge saponin extract on central nervous system functions, J. Ethnopharmacol., 2010, 131, 3, 544-549.
[16] Kloeck G., Noke A., Christiansen T., Soxhlet or Twisselmann, Filtr. Separat., 2013, 33, 32-33.