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Hydrophilic interaction chromatography–tandem mass spectrometry for the determination of swainsonine in plants

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Hydrophilic interaction chromatography (HILIC) coupled with tandem mass spectrometry was developed for the quantification of swainsonine in plants. Optimized separation was performed on an Acquity UPLC BEH HILIC column using acetonitrile/water (90:10, v/v) with 10 mmol/L formic acid as the mobile phase. In comparison to the conventional reversed-phase separation mode, the HILIC technique provided a significantly better retentivity for swainsonine. The calibration curve showed a good linearity over the concentration range 5–750 μg/L corresponding to 0.75–112.5 μg/g in the dry matter (DM) of plant material. The limit of quantification for the method was 5.0 μg/L (0.75 μg/g DM). The mean recoveries of swainsonine in the two milkwetch matrices ranged from 85.5 to 104.4%. The method was applied to determine swainsonine in two milkvetch species (*Astragalus cicer* and *Astragalus glycyphyllos*) growing in Lithuania. None of the samples contained detectable amounts of swainsonine.

Keywords: swainsonine, milkvetch, HILIC, MS/MS

INTRODUCTION

Swainsonine (Fig. 1), an indolizidine alkaloid, is found in some *Astragalus*, *Oxytropis*, and *Swainsona* species of the Leguminosae family growing throughout the world [1]. Consumption of swainsonine containing plants by grazing animals leads to a chronic neurologic disease characterized by weight loss, depression, altered behaviour, decreased libido, infertility, abortion, birth defects, and death [2, 3]. Poisoning of animals by swainsonine containing plants is the most widespread poisonous plant problem in the North America and causes great economic losses to the livestock industry [4]. Rapid, sensitive and accurate analytical methods are therefore needed to detect and to determine swainsonine in plants.

The most commonly used technique is gas chromatography–mass spectrometry (GC–MS) due to its excellent selectivity and sensitivity [5, 6]. However, swainsonine is too polar for GC separation in its native form and derivatization is required prior to analysis. The requirement for the derivatization procedure makes this assay time consuming and may cause an unexpected loss of the analyte due to an incomplete derivatization.

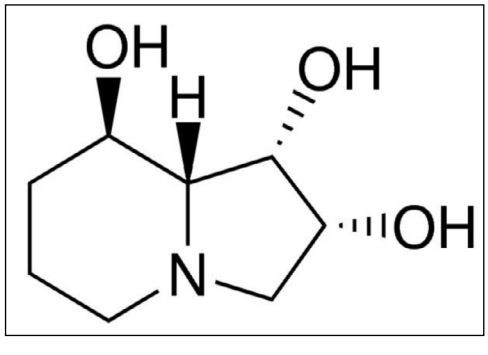

Fig. 1. Structure of swainsonine

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High-performance liquid chromatography (HPLC) techniques have also been reported [6], but the determination of swainsonine by HPLC is associated with two problems. The first one is related to detection: swainsonine cannot be detected using a conventional UV detector due to the lack of chromophore. The problem of detection has been overcome by use of an evaporative light scattering detector [7], but the detection limit of swainsonine was 4.0 μg/mL. Thus, for the determination of swainsonine in plant samples using this technique, the time-consuming preconcentration procedure is required. Another and perhaps the most useful approach to improve the detectability of swainsonine is the combination of HPLC with a MS detector [5, 8, 9]. The detection limit of swainsonine was 0.019 μg/mL, corresponding to 0.001% swainsonine by weight in dry plant material.

A more serious problem is that conventional reversed phase (RP) stationary phases do not provide sufficient retention of this very hydrophilic analyte. Highly aqueous mobile phases are therefore required for adequate retention. However, such approaches are associated with stationary phase de-wetting under highly aqueous conditions and poorer ionization when coupled to MS. In addition, the elution of co-extracted less polar compounds prolongs the analysis or gradient elution must be employed to accelerate the analysis.

The above-mentioned problems can usually be avoided by using hydrophilic interaction chromatography (HILIC) coupled to MS. The HILIC technique uses a polar stationary phase in conjunction with a mobile phase consisting of a polar organic solvent (typically acetonitrile) containing an appreciable amount of water and retains analytes with an increasing order of hydrophilicity [10, 11]. The large percentage of acetonitrile (≥60%) in the HILIC mobile phase facilitated solvent evaporation in the MS source and thus often an increase in the analyte response when compared to more aqueous based systems. Another significant advantage is that less polar interfering species requiring gradient elution by reversed phase HPLC are eluted early in an isocratic HILIC system, thereby simplifying and accelerating the overall analysis. Taking all these factors into account, the HILIC technique seems to be very promising for the determination of swainsonine. However, despite of the gained popularity of HILIC over the past decade, only one report on the present topic has been published so far [7]. The authors found that swaisonine was not retained on the bare silica stationary phase (Waters XBridge HILIC column) using the conventional HILIC mobile phase. Furthermore, they found that under HILIC conditions swainsonine exhibited the typical RP behaviour of increasing retention with increasing water content in the mobile phase.

In the present study, the HILIC technique combined with tandem mass spectrometry (MS/MS) was developed and validated for the rapid and sensitive quantification of swainsonine. The developed technique was employed for the determination of swainsonine in milkvetch (*Astragalus* sp.) growing in Lithuania.

EXPERIMENTAL

Water was obtained from a Mili-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN), formic acid, acetic acid, ammonium formate and ammonium acetate were of LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Swainsonine (≥99%) was also from Sigma-Aldrich.

The primary stock solution of swainsonine standard was prepared at a concentration of 250 mg/L in water. The stock solution was stored at 4 °C before use. Working solutions were prepared by diluting the standard stock solution in the mobile phase. Calibration standards of swainsonine were prepared by spiking the appropriate amount of the working solution into the blank plant extract.

Milkvetch species (*Astragalus cicer* and *Astragalus glycyphyllos*) were collected from the Central Lowland of Lithuania (55°23′49′N; 23°51′40′E) during the flowering stage of growth in 2014. The aerial part of each milkvetch species was divided into two subsamples. One of them was investigated as a sample of the whole aerial plant part and the other subsample was fractionated into morphological plant parts: stems, leaves and flowers. The samples were washed thoroughly with tap water, rinsed with distilled water and blotted on filter paper. Then they were chopped, oven-dried at (65 ± 5)°C and ground to pass a 1-mm screen using a cyclone mill.

The extraction was performed according to a previously published procedure [8]. The dried plant material (100 mg) was placed in a 10 mL screw-cap glass container and extracted with 5 mL of 2% acetic acid for 16 h with agitation. After extraction the samples were centrifuged for 5 min. An aliquot (0.50 mL) of the extract was added to 1.00 mL of ACN, thoroughly mixed, then filtered with a 0.2 μm nylon syringe filter into a glass sample vial and analyzed.

All separations were carried out on a 1290 Infinity UHPLC system connected to a 6410 triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source (Agilent Technologies, USA). The Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) column (Waters, Milford, USA) was employed for the separations in the reversed phase HPLC mode. The aqueous 10 mmol/L formic acid mobile phase was used at a flow rate of 0.25 mL/min.

The HILIC separation was performed on the Acquity UPLC BEH HILIC (2.1 × 100 mm, 1.7 μm) column (Waters). The mobile phase was a mixture of ACN and water (90:10, v/v) containing 10 mmol/L formic acid and set at a flow rate of 0.5 mL/min. For both separation modes the column temperature was 25 °C and the injection volume was 5 μL.

The ESI source operated in the positive ion mode and MS data acquisition was performed in the selected reaction monitoring (SRM) mode. Nitrogen was used as the nebulizer and collision gas. The parameters of the source were used with the following settings: capillary voltage 4000 V, nebulizer pressure 60 psi, drying gas flow 10 L/min, drying gas temperature 320 °C. The first and third quadrupoles

were operated at unit resolution. Data were acquired and processed using the MassHunter software (Agilent).

RESULTS AND DISCUSSION

Series of experiments were performed to optimize the separation conditions for swainsonine in both, RP and HILIC separation, modes using isocratic elution. In both techniques the column packed with sub-2 μm particles (ultra-high-pressure liquid chromatography) was employed, since this modern technology provides faster separations, better resolution, and lower solvent consumption than conventional HPLC. For RP separations the Acquity UPLC HSS T3 column was selected. The T3 bonding utilizes a trifunctional C18 phase bonded at a ligand density that promotes retention of very polar compounds and aqueous mobile phase compatibility. However, even with the 100% aqueous mobile phase containing 10 mmol/L HCOOH swainsonine was weakly retained and eluted shortly after the column void volume. Although by increasing pH of the mobile phase from 3 to 7 the retention time of swainsonine increased from 1.10 to 1.42 min, undesired peak broadening occurred at higher pHs precludes accurate quantification.

For HILIC separations the ethylene bridged hybrid silica column (Acquity UPLC BEH HILIC) was employed. The retention behaviour of swainsonine was studied with acetonitrile/water mobile phases containing appropriate (formic acid, ammonium formate, acetic acid, ammonium acetate) additives. As expected, swainsonine exhibited the typical HILIC behaviour of decreasing retention with increasing water content in the mobile phase. The retention decreased initially as the water content increased from 5 to 40%, but levelled off when the water content further increased to 50%. Additionally, the pH of the mobile phase was investigated in a range of pH 3.0–7.0 using appropriate buffer solutions. In contrast to RP-HPLC, in the HILIC mode the retention time of swainsonine increased as the pH of the mobile phase decreased.

As already mentioned in the introduction, quite contradictory results have been reported with respect to the retention behaviour of swainsonine under HILIC conditions [7]. The authors found that swaisonine was not retained on the bare silica stationary phase (Waters XBridge HILIC column) using the ammonium acetate buffer containing the acetonitrile/water (95:5, v/v) mobile phase. Furthermore, they found that under conventional HILIC conditions swainsonine exhibited the typical RP behaviour of increasing retention with increasing water content in the mobile phase and reaching the maximum retention at 50% H_2O. However, the explanation of such anomalous behaviour is difficult.

The final chromatographic conditions of the methods compared here are detailed in the Experimental section and the resulting chromatograms are shown in Fig. 2. Considering a twice higher mobile phase flow rate employed in HILIC, this technique provides a significantly better retentivity for swainsonine.

The major protonated molecular ion $[M+H]^+$ (m/z = 174) was observed in the full scan positive ion ESI-MS spectra of swainsonine. Negative ionization was also applied, but compared to the positive mode, the sensitivity was much lower. The product-ion mass spectrum of swainsonine contained a most abundant ion at m/z 156 and a less abundant ion at m/z 138, corresponding to the loss of one and two

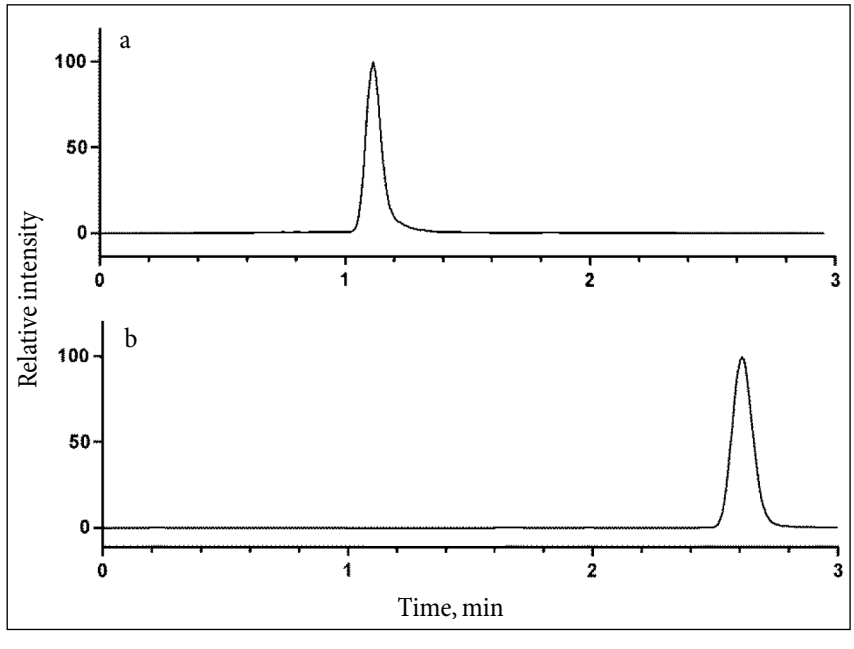

Fig. 2. SRM chromatograms illustrating the separation of swainsonine (0.25 mg/L, transition m/z 174 → 156) by (a) RPLC and (b) HILIC. For chromatographic conditions see the Experimental section

water molecules, respectively. The most intense product ion was used for quantification, whereas the second one was used to complete the identification. Fragmentor voltage and collision energies were optimized by direct infusion of the swainsonine standard. Fragmentor voltage was 80 V and collision energies were 6 and 8 eV for the transitions *m/z* 174 → 156 and *m/z* 174 → 138, respectively.

The method was evaluated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision (intraday and interday) and accuracy in the swainsonine-free milkvetch extract matrix. Linearity was measured with the seven-point calibration curve (three replicates) by linear regression of the peak area of swainsonine versus the concentration in mg/L. The LOD and LOQ of the proposed method were determined at signal-to-noise ratios of 3 and 10, respectively (Table 1).

Table 1. **Calibration data, LODs and LOQs for the HILIC–MS/MS method (n = 3)**

Parameter	Value
Linear range, µg/L	5.0–750.0
R^2	0.9985
LOD, µg/L	1.5
LOQ, µg/L	5.0

The calibration curve of the spiked swainsonine showed a good linearity (R^2 = 0.9985) over the concentration range 5.0–750.0 µg/L corresponding to 0.75–112.5 µg/g in the dry matter (DM) of the original plant material. The estimated LOD and LOQ for the method were 1.5 µg/L (0.23 µg/g DM) and 5.0 µg/L (0.75 µg/g DM), respectively. To the best of our knowledge, the proposed method has the lowest LOD among the reported methods.

The intraday and interday precision and accuracy of the method were estimated by analyzing spiked milkvetch samples (whole aerial plant part) at three different concentrations (1.5, 7.5 and 75.0 µg/g) in a single day and for three days, respectively. The precision was described as the relative standard deviation (RSD) of each assay. For evaluating the accuracy recoveries were determined. The data are summarized in Table 2. The intraday RSD values for both matrix types ranged from 5.1 to 11.3% and the interday RSDs were between 7.3 and 15.2%. The mean recoveries of swainsonine in the two matrices ranged from 85.5 to 93.1% at the low concentration and from 92.1 to 97.5% at the high concentration. The obtained results indicate that the proposed method has an acceptable precision and accuracy for the determination of swainsonine in milkvetch species.

The optimized HILIC–MS/MS method was applied to determine swainsonine in two milkvetch species (*Astragalus cicer* and *Astragalus glycyphyllos*) growing in Lithuania. The aerial part of each plant sampled in 2014 at the flowering stage was divided into two subsamples. One of them was investigated as a sample of the whole aerial plant part and the other subsample was fractionated into morphological parts: stems, leaves and flowers. None of the samples contained detectable amounts of swainsonine. The representative HILIC-MS/MS SRM chromatograms of the *Astragalus glycyphyllos* (whole aerial plant part) extract and the extract spiked with swainsonine at 10 µg/L are shown in Fig. 3.

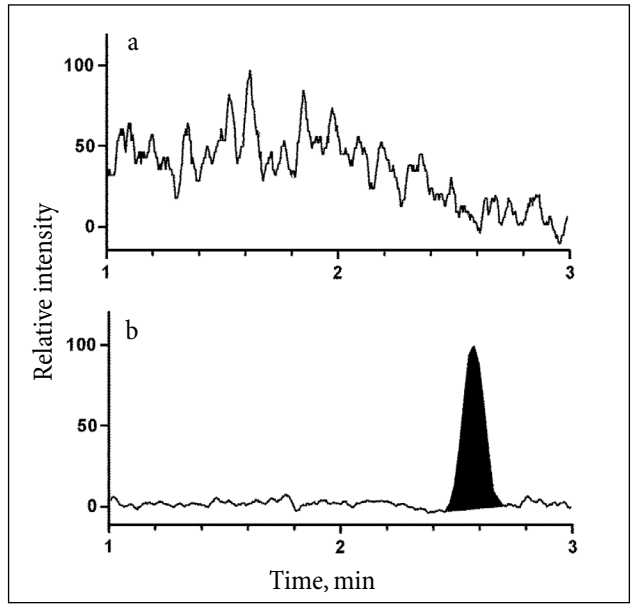

Fig. 3. HILIC-MS/MS SRM chromatograms of (a) the *Astragalus glycyphyllos* sample extract and (b) the *Astragalus glycyphyllos* sample extract spiked with 10 µg/L of swainsonine. Only the chromatograms for the quantifier transition *m/z* 174 → 156 are shown

Table 2. **The intraday and interday precision and accuracy for swainsonine in milkvetch (n = 5)**

Milkvetch species	Nominal concentration, µg/g	Intraday precision (RSD), %	Accuracy, %	Interday precision (RSD), %	Accuracy, %
Astragalus cicer	1.5	11.3	88.7	15.2	90.2
	7.5	5.5	96.0	7.4	95.5
	75.0	5.8	92.1	8.7	97.3
Astragalus glycyphyllos	1.5	9.4	93.1	13.5	85.5
	7.5	6.9	92.7	8.0	104.4
	75.0	5.1	96.3	7.3	95.8

In conclusion, the HILIC–MS/MS method developed in this study is rapid, sensitive and accurate, and may be easily extended for the determination of swainsonine in other types of plant materials.

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HIDROFILINĖS SĄVEIKOS CHROMATOGRAFIJA-TANDEMINĖ MASIŲ SPEKTROMETRIJA SVAINSONINUI AUGALUOSE NUSTATYTI

S a n t r a u k a

Hidrofilinės sąveikos chromatografijos-tandeminės masių spektrometrijos metodas pritaikytas svainsoninui augaluose nustatyti. Chromatografinis atskyrimas buvo atliekamas Acquity UPLC BEH HILIC kolonėlėje naudojant acetonitrilo / vandens (90:10, v/v) judrią fazę su 10 mmol/L metano rūgšties priedu. Palyginti su tradiciniu atvirkščių fazių chromatografijos metodu, hidrofilinės sąveikos chromatografijos sąlygomis svainsoninas sulaikomas žymiai stipriau. Išmatuota kalibracinė kreivė tiesinė 5–750 µg/L svainsonino koncentracijų intervale arba, perskaičiavus į sauso augalo masę, 0,75–112,5 µg/g koncentracijų intervale. Svainsonino nustatymo riba siekia 5,0 µg/L (0,75 µg/g sausos augalo masės). Įvertintos svainsonino standartinių priedų išgavos iš kulkšnės mėginių buvo nuo 85,5 iki 104,4 %. Metodas panaudotas svainsoninui augančioje kulkšnėje Lietuvoje nustatyti. Ištirtuose dviejų rūšių (*Astragalus cicer* ir *Astragalus glycyphyllos*) kulkšnės mėginiuose svainsoninas nebuvo aptiktas.