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Rapid sample preparation technique for the determination of pyrrolizidine alkaloids in plant extracts

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Abstract

To determine toxic pyrrolizidine alkaloids from various plant sources, a sample preparation technique is described that affords alkaloid fractions suitable for capillary GC or TLC determination. The procedure includes the reduction of the alkaloid N-oxides with the oxygen-absorbing resin Serdoxit and a clean-up with strong cation-exchange solid-phase columns. If desired an enrichment of the alkaloids can be obtained. The method was tested with methanolic extracts from *Senecio vulgaris*, *Petasites hybridus* and *Symphytum officinale*.

1. Introduction

Pyrrolizidine alkaloids (PAs) are ester alkaloids that occur in a wide range of plants, but especially in Boraginaceae, Asteraceae and Fabaceae. Those PAs with a 1,2-unsaturated pyrrolizidine moiety (see Fig. 4) are hepatotoxic and have shown carcinogenic and mutagenic potency in some animal feeding experiments [1]. PAs from toxic pasture plants have led to loss of cattle and transfer of these alkaloids into milk or honey has been reported [2]. Further, this kind of alkaloid occurs as minor constituents in some medicinal plants. Because of their high toxicity, the use of medicinal plants containing them has been severely restricted in Germany. It must be ensured that the daily contact with toxic PAs from phytopharmaceuticals does not exceed 1 or 100 μg by internal or external application, respectively [3]. Therefore, it is of interest to determine these alkaloids even at low concentrations. The usual techniques, such as capillary GC, HPLC or TLC, require a lengthy sample clean-up and concentration step. The sample preparation method proposed here using solid-phase columns avoids the use of separating funnels and allows an enrichment of the alkaloids.

2. Experimental

2.1. Reagents

Solutions used included the following: ammonia, 12.5% in distilled water; HCl–methanol (1), concentrated HCl–distilled water–methanol (25:135:40); HCl–methanol (2), concentrated HCl–distilled water–methanol (25:75:100); and sodium dithionite, 5% in distilled water. Serdoxit oxygen-absorbing resin was obtained from Serva (Heidelberg, Germany). Varian Bond Elut LRC SCX 1211-3039, 0.5- and 2-g strong cation-exchange solid-phase extraction columns were obtained from ICT (Vienna, Austria). HPTLC plates were silica gel 60 F-254 (10 × 20 cm) from Merck. Dipping solutions (1) was 0.1% *o*-chloranil in toluene and dipping solution (2) was

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2 g of 4-dimethylaminobenzaldehyde + 85 ml of acetic acid + 15 ml of 32% HCl. The mobile phase was CH_2Cl_2–MeOH–25% ammonia (85:14:1.5). The GC internal standard was 18 mg of caffeine in 100 ml of methanol. PAs used as reference substances were senecionine, seneciphylline, retrorsine and retrorsine-N-oxide, obtained from Roth (Karlsruhe, Germany).

2.2. Extraction of plant material

A 0.2–1-g amount of dry, finely powdered plant material was extracted in a 25-ml flask for 1 h with boiling methanol (65°C). After cooling to room temperature the extracts were diluted to 25 ml and filtered.

2.3. Preparation of extraction columns

The Serdoxit column was prepared by filling 1 g of the resin into an empty solid-phase extraction column fitted with a valve for flow regulation and rinsing intensively with MeOH and distilled water. As Serdoxit also reacts with oxygen from air, it has to be regenerated immediately before use with 4 ml of a solution of the reducing agent sodium dithionite. Serdoxit is provided with an indicator which turns from black to yellow when fully regenerated. Excess of sodium dithionite was washed out with 8 ml of distilled water and 4 ml of MeOH were added to displace the water. The SCX columns were first treated with 8 ml of HCl–MeOH (2) solution and then washed neutral with MeOH.

2.4. Clean-up procedure

The clean-up procedure is summarized in Fig. 1. It consists of three steps: the reduction of the N-oxides in the Serdoxit column, a first clean-up by retention and subsequent elution of the PAs from the SCX column, and extraction of the PAs in the alkaline phase with dichloromethane.

A 3–6-ml volume of the methanolic plant extract was applied to the freshly regenerated Serdoxit column (Fig. 1: 1). The dropping velocity was adjusted to 1 drop in 3 s to ensure a sufficient contact time for complete reduction of the N-oxides. MeOH (5 ml) was added to wash the extract completely through the Serdoxit column (Fig. 1: 2). In this step the Serdoxit column must not run dry. Extract coming from the outlet of the Serdoxit column dropped directly on to the top of the SCX column (with 0.5 g of packing material). The PAs were retained in the SCX column. The extract passed through the SCX column by action of gravity; there was no need for flow control. After all extract had passed through the SCX column, addition of 8 ml of MeOH was necessary to wash out other compounds (Fig. 1: 3).

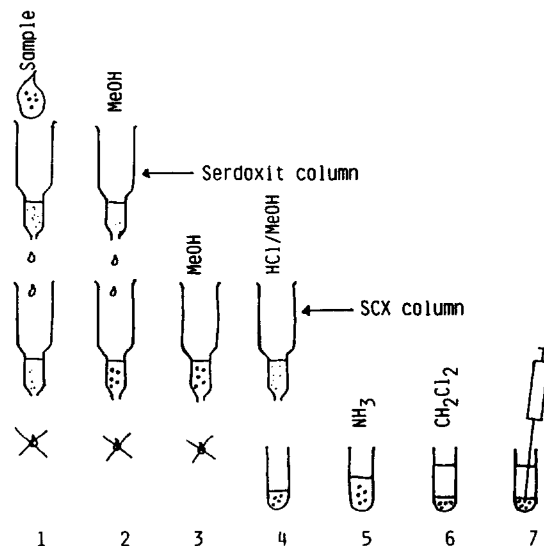


Fig. 1. Scheme for the clean-up of pyrrolizidine alkaloids in plant extracts. 1 = Applying 3–6 ml extract onto the Serdoxit column, reduction of the N-oxides to the free alkaloids; 2 = washing the alkaloids through the Serdoxit column; 3 = retention of the alkaloids, washing out other compounds from the SCX columns; 4 = elution of the alkaloids with HCl–MeOH; 5 = making the eluate alkaline; 6 = extraction of the alkaloids with CH_2Cl_2; 7 = taking the CH_2Cl_2 layer for analysis.

The PAs were eluted from the SCX resin with 8 ml of HCl–MeOH (1) solution (Fig. 1: 4). This eluate was made alkaline with 3 ml of ammonia solution (Fig. 1: 5) and then mixed with 1.5 ml of dichloromethane (Fig. 1: 6). After vigorous shaking, an aliquot of the CH_2Cl_2 layer was

taken (Fig. 1: 7) and evaporated to dryness. If larger sample volumes had to be analysed and greater enrichment was desired, larger SCX columns containing 2 g of packing material were used. In this event 12 ml of HCl–MeOH (1) solution were necessary to elute the PAs.

2.5. *Capillary gas chromatography*

The GC apparatus used was a Carlo Erba Vega Series 6000 equipped with a Carlo Erba DP 700 integrator and a Carlo Erba A200S autosampler and flame ionisation detector (Fisons Instruments, Milan, Italy). Various apolar capillary GC columns are suitable. For this work two different columns were employed. One was an SE-54 (Machery–Nagel, Düren, Germany) capillary column (50 m × 0.32 mm I.D.; 0.25-μm film thickness) operated with the following temperature programme: 1 min at 150°C, increased at 6.5°C/min to 260°C, 10 min at 260°C, increased at 10°C/min to 280°C. Most of the PAs eluted in this system at 260°C. Alternatively, an Rtx-5 column (30 m × 0.32 mm I.D.; 0.1-μm film thickness) was used (Restek; obtained from ICT). The temperature programme was 3 min at 120°C, increased at 6°C/min to 230°C, 8 min at 230°C, increased at 10°C/min to 270°C. The PAs eluted at 230°C. Hydrogen was used as the carrier gas for the first column and helium for the second column at the optimal velocity. The detector temperature was set at 280°C and the injector temperature at 260°C with a bottom split of *ca.* 12 ml/s.

The dried sample from the clean-up procedure was taken up to 100 μl of MeOH containing the internal standard caffeine and 1 μl of this solution was injected into the GC system. To establish the calibration graph, 5–150 μl of senecionine solution (7.3 mg in 50 ml) were evaporated to dryness and the residue was dissolved in 100 μl of internal standard solution 0.36 mg/ml. Each of the concentrations was injected at least three times. The ratios of the senecionine peak area to the caffeine peak area were calculated and plotted against the amount of senecionine in micrograms.

2.6. *High-performance thin-layer chromatography*

The dried PA fraction from the clean-up step was dissolved in 100 μl of MeOH and 2–10 μl of this solution were applied to the TLC plates in 2-mm bands using a Camag (Berlin, Germany) Linomat IV automatic application device. On each plate three spots of 0.15–0.85 μg of a reference alkaloid (senecionine, seneciphylline or retrorsine) were placed for calibration.

The developing chamber was fitted with a filter-paper to ensure better saturation. The plates were removed after 20 min when the front has reached 7 cm. For detection the plates were dipped for 3 s into dipping solution (1) using a DC-Tauchfix dipping device from Baron (Insel Reichenau, Germany). After heating at 100°C for 1 min and cooling to room temperature, the plates were dipped into dipping solution (2) for 3 s and dried in an air stream.

Quantitative evaluation was carried out by scanning the plates with a CS 9000 dual-wavelength flying spot scanner from Shimadzu (Kyoto, Japan). It was operated in the reflection mode at 580 nm with a zig-zag scan amplitude of 5 mm.

2.7. *Calculation of pyrrolizidine alkaloid content in the plant material*

The PA content in the plant material was calculated according to the following equations which result from sample preparation.

In GC analysis the amount A of PA in the dried plant is given by

$$A = (x g_c v_e)/(g_a v_u p)$$

where A = amount of PA in dried plant (μg/g), x = amount of the individual alkaloid in the cleaned fraction, obtained from the calibration graph, g_c = amount of CH_2Cl_2 used in the clean-up procedure (g) (1.98 g per 1.5 ml), g_a = mass (g) of the aliquot of the CH_2Cl_2 layer which is brought to dryness, v_u = volume (ml) of the plant extract cleaned up, v_e = total volume (ml)

of the plant extract and p = amount (g) of dried plant extracted.

In TLC analysis the equation is

$$A = (xg_c v_e v_a)/(v_t g_a v_u p)$$

where v_a = volume (μl) in which the PA fraction has been taken up after clean-up (100 μl) and v_t = volume (μl) of alkaloid fraction applied to the TLC plate.

3. Results and discussion

The usual clean-up of alkaloid extracts carried out in separating funnels is lengthy and requires large amounts of solvents. This procedure has been replaced by the use of strong cation-exchange columns which at neutral pH bind PAs [4,5]. The addition of methanol to HCl for elution of PAs from the SCX column was necessary to obtain complete recovery. Moreover, the samples usually contain other compounds which also may be retained by ionic or non-specific interactions in the column and can be eluted with HCl. Therefore, an additional cleaning of the HCl eluate is required by making it alkaline and extracting it with dichloromethane.

3.1. Reduction of N-oxides

To determine PAs by GC or TLC they must be present as free alkaloids. As PAs extracted from plants occur at least partly as N-oxides, a reduction step is needed. The prevalent reduction of N-oxides with zinc dust in acidic medium, which requires several hours, has been replaced by the use of the oxygen-absorbing resin Serdoxit, allowing the reduction to be performed in a few minutes. Serdoxit, an adsorbate of indigosulphonate on a highly porous anion exchanger, enables mild reduction conditions to be used [6].

To test the reduction step with Serdoxit, a 2.1-ml portion of methanol containing 22.8 μg (0.0565 μmol) of retrorsine-N-oxide was passed through a Serdoxit column. The eluate containing retrorsine from retrorsine-N-oxide reduction was evaporated to dryness and the residue was dissolved in 200 μl of methanol. Its retrorsine content was determined by HPTLC with authentic retrorsine as a standard. For four replicates a mean of 0.060 ± 0.004 μmol of retrorsine was recorded. This indicates that complete reduction of the N-oxides was achieved.

3.2. Capillary gas chromatography

The determination of PAs by capillary GC is possible without derivatization steps [7] but they elute at fairly high temperatures. To decrease the elution temperature thin-film columns are preferable. A column length of 30–50 m is necessary to separate closely related PAs [7].

The calibration graph was recorded with senecionine and was used to calculate the amounts of all PAs. The typical shape of this graph can be seen in Fig. 2. It shows good linearity between 3 and 25 μg of senecionine in 100 μl of internal standard. At lower concentrations, however, the graph became flatter, indicating decomposition of PAs to a small extent in the GC system, an effect which could be observed on both columns used. The injection of small amounts of senecionine showed that 0.5 μg in 100 μl of internal standard (i.e., 5 ng injected) was still were detected by flame ionization detection. Because of the poor linearity of the calibration graph in this low concentration range, exact quantitative results were difficult to obtain. Nevertheless, working with 6 ml of plant

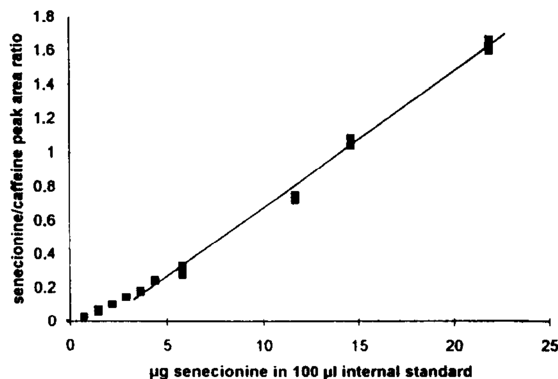


Fig. 2. Calibration graph for GC determination of pyrrolizidine alkaloids.

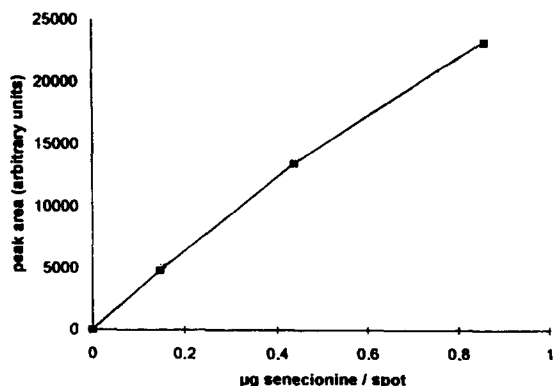
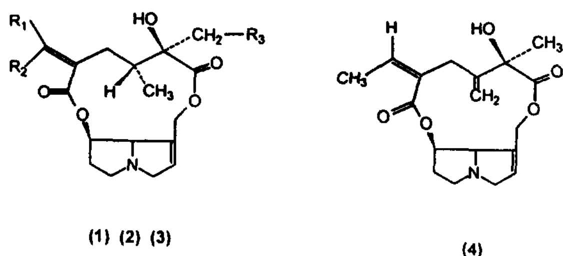


Fig. 3. Calibration graph for TLC determination of pyrrolizidine alkaloids.

extract, corresponding to 0.25 g of dried plant in the clean-up, a detection limit of 1.5–2 μg/g dried plant could be achieved. The use of larger SCX columns (with 2 g of packing material) allowed the preparation of larger sample volumes and greater enrichment. In this instance the detection limit could be decreased to 0.4–0.5



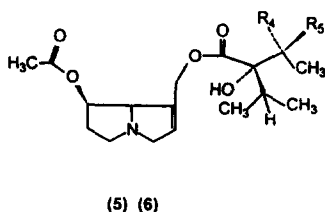


Fig. 4. Structures of some toxic pyrrolizidine alkaloids. 1 = Senecionine ($R_1 = R_3 = H$, $R_2 = CH_3$); 2 = integerrimine ($R_1 = CH_3$, $R_2 = R_3 = H$); 3 = retrorsine ($R_1 = H$, $R_2 = CH_3$, $R_3 = OH$); 4 = seneciphylline; 5 = acetyllycopsamine ($R_4 = H$, $R_5 = OH$); 6 = acetylintermedine ($R_4 = OH$, $R_5 = H$).

μg/g dried plant. Further enrichment was not appropriate as enriched impurities impaired the clarity of the chromatogram.

3.3. *High-performance thin-layer chromatography*

PAs were detected on the TLC plates through a reaction giving purple spots on a light yellow background. *o*-Chloranil corrects the PAs into pyrrole derivatives, which react with 4-dimethylaminobenzaldehyde to give purple complexes. N-Oxides do not react with *o*-chloranil [8,9]. This detection procedure was easier to carry out than those using H_2O_2 and acetic anhydride to generate the pyrrole derivatives [7].

Fig. 3 presents the calibration graph obtained by TLC scanning. It was necessary to record an individual calibration graph on each plate because the multitude of steps in the detection procedure did not allow exact, reproducible colour formation from plate to plate. The smallest spot that can still be seen or detected by the TLC scanner contained 20 ng of senecionine. With regard to sample clean-up with 0.5-g SCX columns, the detection limit of 2 μg/g dried plant was of the same order of magnitude as for GC analysis. There were, however, some differences between the individual PAs in colour formation on the TLC plate. Senecionine gave a 2.4-fold more intense colour than retrorsine.

3.4. *Recovery of the whole pyrrolizidine alkaloid analysis*

The equations for calculating the PA content of a plant sample were established on the assumption that quantitative extraction and enrichment of the alkaloids could be achieved. To test if this is so, 17.5 μg of senecionine were added to 5 ml of an extract from *Petasites hybridus* rhizomes. In the original extract 20.8 ± 1.00 μg of senecionine were recorded. After addition of senecionine, its content increased to 37.4 ± 4.6 μg (three replicates). Therefore, a recovery of more than 90% could be achieved.

3.5. Application to pyrrolizidine alkaloid-containing plants

The described sample preparation procedure was used to determine the PA content in *Senecio vulgaris*, a poisonous weed, *Symphytum officinale* and *Petasites hybridus*. The last two are medicinal plants, their use being impaired by the presence of toxic PAs as minor compounds. The structures of the PAs from the plants studied are

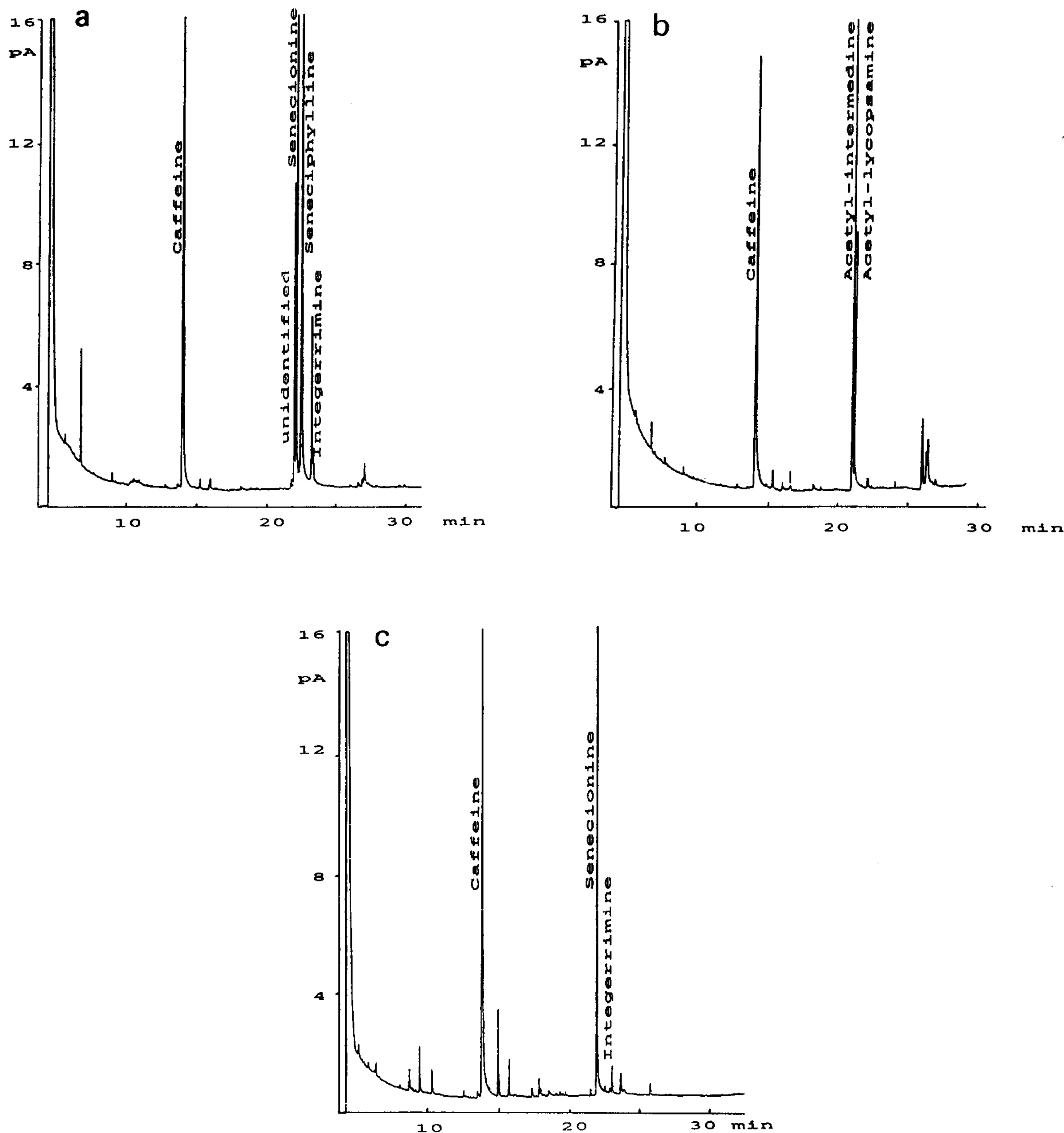


Fig. 5. Gas chromatograms from selected plant extracts after clean-up. (a) *Senecio vulgaris*; (b) *Symphytum officinale*; (c) *Petasites hybridus*.

Table 1
Pyrrolizidine alkaloid content of *Senecio vulgaris*, *Petasites hybridus* and *Symphytum officinale*.

Plant	Method	Compound	Concentration (μg/g)[a]
Senecio vulgaris			
(above-ground parts)	GC	Senecionine	354 (9.1%)
		Seneciphylline	632 (13.5%)
		Integerrimine	135 (16.7%)
	TLC		Not determined
Petasites hybridus			
(rhizomes)	GC	Senecionine	104 (4.8%)
		Integerrimine	12.4 (10.8%)
	TLC		161 (5.8%)
Symphytum officinale			
(roots)	GC	Acetylintermedine	463 (3.7%)
		Acetyllycopsamine	237 (6.8%)
	TLC		590 (6.9%)

[a] Means of 3–5 replicates in μg/g dried plant, with R.S.D. in parentheses.

shown in Fig. 4. The chromatograms shown in Fig. 5 were recorded on the Rtx-5 column, on which the internal standard caffeine eluted at a retention time of 14.00–14.05 min. *Senecio vulgaris* displayed an unidentified PA, senecionine, seneciphylline and integerrimine at retention times of 22.01, 22.16, 22.53 and 23.25 min, respectively (Fig. 5a). These alkaloids could not be separated by TLC. In *Symphytum officinale* the main PAs detected were the stereoisomers acetylintermedine and acetyllycopsamine, with retention times of 21.0 and 21.3 min, respectively (Fig. 5b). *Petasites hybridus* contained mainly senecionine and little integerrimine (Fig. 5c). Table 1 summarizes the amounts of individual PAs in the different plants. The highest PA content was in *Senecio vulgaris* and the lowest in *Petasites hybridus*. The analyses were carried out with 3–5 replicates for each plant. The standard deviation of the calculated PA content ranged from 3.7 to 16.7%. The discrepancies between GC and TLC analysis may arise from the different responses of the alkaloids in the colour formation on the TLC plate.

The proposed method is suitable for determining pyrrolizidine alkaloids from various plants over a wide concentration range.

4. Acknowledgement

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