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Quantification of Ptaquiloside and Pterosin B in Soil and Groundwater Using Liquid Chromatography−Tandem Mass Spectrometry (LC−MS/MS)

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The carcinogenic compound ptaquiloside is produced by bracken fern (*Pteridium aquilinum* L.). Ptaquiloside can enter the soil matrix and potentially leach to the aquatic environment, and methods for characterizing ptaquiloside content and fate in soil and groundwater are needed. A sensitive detection method has been developed using liquid chromatography−tandem mass spectrometry (LC−MS/MS) for analyzing ptaquiloside and its transformation product pterosin B. Detection limits are 0.19 μg/L (ptaquiloside) and 0.15 μg/L (pterosin B), which are 300−650 times better than previously published LC−UV methods. Sequential soil extractions are made using 5 mM ammonium acetate for extraction of ptaquiloside, followed by 80% methanol extraction for pterosin B. Groundwater samples are cleaned-up and preconcentrated by a factor of 20 using solid-phase extraction. The LC−MS/MS method enables quantification of ptaquiloside and pterosin B in soil and groundwater samples at environmentally relevant concentrations and delivers a reliable identification because of the structure-specific detection method.

KEYWORDS: Bracken fern (*Pteridium aquilinum* L. Kuhn); natural toxins; soil extraction; carcinogen; solid-phase extraction

INTRODUCTION

Bracken fern (*Pteridium aquilinum* L. Kuhn) is a cosmopolitan plant species and among one of the most abundant plants in the world (*1*). In temperate areas, the species is commonly found in forests and forest margins, on recently deforested areas, and on regressing farmland (*2*). Bracken has caused concern for many years, because a number of severe animal diseases have been observed in animals browsing on it. Such diseases in cattle and sheep may be caused by the presence of carcinogenic compounds, among which ptaquiloside, a norsesquiterpene glucoside, is by far the most important (*3, 4*) (**Figure 1**). Thiamine deficiency, acute hemorrhagic syndrome in sheep and cattle (acute bracken poisoning), retinal degeneration of sheep (bright blindness), tumors in the urinary bladder in sheep and cattle (bovine enzootic haematuria), and tumors in the upper gastrointestinal tracts of ruminants are among the diseases most often encountered (*5, 6*). There are indications that ptaquiloside may also cause human cancer (*3, 7*), e.g., through direct ingestion (*4*) or by consumption of milk from cows that have browsed on bracken (*8*). Another proposed route is ptaquiloside contamination of local water supplies, although no proof has been provided thus far (*9*).

The concentration of ptaquiloside in bracken stands is very variable; concentrations up to 37 mg/g dry weight in mature fronds have been reported (*10*). Ptaquiloside is very water-soluble and after transfer to soil, the sorption to humic matter or other soil sorbents is limited (*11*). The first degradation reaction, a hydrolysis causing the formation of pterosin B (**Figure 1**), is pH-dependent and may occur quickly (*12*). In soil and soil solutions, the pterosin B formation rate varies, and in sterile soil solutions, no ptaquiloside transformation was observed within 28 days (*11, 13*). The properties of ptaquiloside

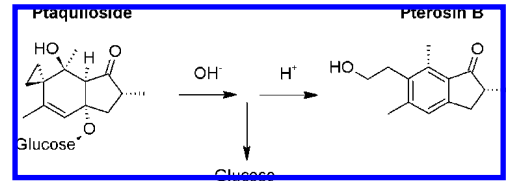

Figure 1. Structure of ptaquiloside and the transformation product pterosin B.

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indicate a high potential of the compound to leach to surface and groundwater, and preliminary laboratory experiments and measurements in upper groundwater from bracken-infested areas in Denmark confirm that this is the case (*11, 14*).

In existing methods, ptaquiloside in plant material and soil extracts is commonly quantified using LC−UV, with extracted ptaquiloside converted to pterosin B prior to determination and detected as such at 214, 220, or 260 nm (*13, 15, 16*). Extracts are usually cleaned-up by passage through a polyamide column to remove pterosin B before conversion of ptaquiloside to pterosin B (*11, 16*). Dependent upon the concentration in the soil or water samples, a preconcentration step may be needed (*17*). Ptaquiloside may also be determined directly at 220 or 214 nm. However, the indirect detection of ptaquiloside is preferred for two reasons: The conversion to pterosin B results in an approximate doubling of the UV response and will hence lower the limit of detection, and the difficult handling of ptaquiloside as a result of its high instability. LC−UV determination is not a structure-specific method, because substances with a similar retention and UV−vis spectrum may be misidentified as the compound in question. As a result, there is a risk for false positive as well as negative analytical results using unspecific detection, such as UV−vis. Hence, a more sensitive and specific method for quantification of ptaquiloside and pterosin B in soil and groundwater is needed. Mass spectrometric detection is preferable because it provides the required specificity and a lower limit of detection. Also, in combination with an optimized cleanup and chromatographic separation of the compounds from matrix components, a liquid chromatography−tandem mass spectrometry (LC−MS/MS) method may limit the need for a preconcentration step. To the best of our knowledge, only one method using mass spectrometry for quantification of ptaquiloside has been published (*18*). This method comprises transformation of ptaquiloside to bromopterosine or methoxypterosine and quantification using gas chromatography−mass spectrometry (GC−MS). The conversion step is undesirable because of the possibility of losing ptaquiloside during the laborious laboratory process.

The objective of the present study was to develop and validate a sensitive and specific method for quantification of ptaquiloside and pterosin B in soil and groundwater. The method is used with a new sequential extraction procedure for ptaquiloside and pterosin B in soil or with a new preconcentration and cleanup step by solid-phase extraction for low-concentration (<1 μg/L) groundwater samples. The performance of the new method is compared to the similar LC−UV method.

MATERIALS AND METHODS

Chemicals and Reagents. Pure ptaquiloside was isolated and purified from bracken material using the procedure described in Rasmussen et al. (*11*), with a few minor modifications. Pterosin B was prepared from ptaquiloside by the method of Agnew and Laureen (*16*). LC-grade acetonitrile, methanol, isopropanol, and ammonium acetate of analytical grade were purchased from Sigma Aldrich (St. Louis, MO). All solutions and eluents for LC−UV were prepared using triple-deionized water, whereas for LC−MS, MilliQ water was used.

Stock Solutions and Calibration Standards. Ptaquiloside stock solution (1000−2000 mg/L) was prepared using triple-deionized water and kept at −18 °C in the dark. The stock solution was only thawed briefly and shaken when calibration standards were to be made. Pterosin B stock solution (100−200 mg/L) was prepared as described above and kept at −18 °C in the dark. Standards for LC−UV experiments were diluted with triple-deionized water (250−100 000 μg/L ptaquiloside and 100−50 000 μg/L pterosin B), and standards for LC−MS/MS experiments were prepared in 50% (v/v) methanol (0.5−100 μg/L ptaquiloside and 0.3−60 μg/L pterosin B). Standards and samples for LC were kept in amber vials to avoid photo-degradation of ptaquiloside.

Soils and Groundwater. Three forest soils of varying types covered with bracken were used for the method development (pH 3−5). From a sandy soil (soil I), the litter layer and the A-horizon (5−20 cm) were used with organic carbon contents of 23 and 4%, respectively. The gravimetric water content of the two soils was 95−130 and 35%, respectively. From an organic soil (soil II), the A-horizon (5−20 cm) with an organic carbon content of 41−49% and gravimetric water content of 350−400% was sampled. From a loamy sand (soil III), the litter layer, A-horizon (5−20 cm), and C-horizon (30−50 cm) were all used. Organic carbon content of these soils was 31, 6, and 2%, respectively, and gravimetric water contents were 115, 37, and 23%, respectively. From areas without bracken cover but from the same locations as soils I and II, A-horizons were collected as well. These soils did not have a natural content of ptaquiloside and pterosin B and are labeled "blank soils". All soils were mixed thoroughly before use and kept frozen whenever not in use. Roots, leaves, and pebbles were removed before mixing. In addition, upper groundwater from 1.0 m (soil I), 0.3 m (soil II), and 2.2 m (soil III) below ground were sampled (pH 5.2−6.2). At all three locations, a groundwater well was augered to the uppermost groundwater level cased with a 40 mm PEDH casing and with a 1 m × 0.5 mm screen, enabling sampling of the most recent formed groundwater (age: days−month). The groundwater was sampled from all three locations using a peristaltic pump.

Soil Extraction. The A-horizons of soils I and II as well as the litter layer of soil I were used for development of the extraction method. The method was developed on the basis of the determination of the extent of extraction of the natural contents of ptaquiloside and pterosin B in the soil material. The general setup comprised extraction of 5 g of fresh weight (FW) soil with 20 mL of extraction solution in a 50 mL polypropylene centrifuge tube, which was shaken in the dark (horizontal, 100/min). The tubes were centrifuged at 1500*g* for 10 min, after which the supernatant was filtered by use of a plastic syringe through 0.20 μm RC-filter (La-Pha-Pack, Langenwehe, Germany) and analyzed by LC−MS/MS. Possible sorption of the compounds to the plastic syringe and the RC-filter was tested; no sorption was observed.

Extraction periods of variable length (5−30 min) were evaluated for ptaquiloside extraction. The completeness of the extraction was tested by carrying out two consecutive extractions of the same duration. Two different extraction solutions (5 mM aqueous ammonium acetate and pure MilliQ water) were tested for the extraction of ptaquiloside. The extraction of pterosin B was performed on previously aqueous ammonium acetate extracted soil by testing three extraction solutions: 20% (v/v), 40% (v/v), and 80% (v/v) aqueous methanol. The extraction time for pterosin B was set to 60 min. Two consecutive extractions of the same duration and the same soil material using the best extractant [80% (v/v) aqueous methanol] were tested to verify the extent of the first extraction of pterosin B.

The recovery was evaluated by spiking the two blank soils to obtain ptaquiloside concentrations of 30 or 300 μg/kg FW soil and pterosin B concentrations of 25 or 250 μg/kg FW soil. To allow for a homogeneous spiking, the blank soils were air-dried overnight prior to spiking. The soils were rewetted to previous water content during spiking. Spiked soils were thoroughly mixed to obtain a homogeneous spiking and stored at 5 °C until extraction in triplicate 1 h later.

Final Soil Extraction Method. A total of 5 g of FW soil and 20 mL of 5 mM ammonium acetate were added to a 50 mL polypropylene tube and shaken for 10 min in the dark (horizontal, 100/min). The tube was centrifuged at 1500*g* for 10 min, after which the supernatant was removed for later determination of ptaquiloside. Then, 20 mL of 80% (v/v) methanol was added, and the tube was shaken for 60 min in the dark (horizontal, 100/min). Again, the tube was centrifuged at 1500*g* for 10 min. Aliquots of the two supernatants were filtered through 0.20 μm RC-filters into amber LC vials by use of a plastic syringe. The filtered supernatants were kept at 5 °C until analysis by LC−MS/MS later the same day or kept at −18 °C if the analyses were performed later.

Preconcentration of Groundwater. For the groundwater solid-phase extraction (SPE) development, an Oasis Max SPE column from Waters

(Milford, MA) was used. A total of 10 SPE columns were spiked with 1 mL aqueous ptaquiloside and pterosin B solutions with concentrations of 1.5−1.9 mg/L. The effect of increasing modifier content on the elution of the analytes was evaluated using a 10 level parallel setup using 0, 10, 20, 30, 40, 50, 60, 70, 80, or 90% (v/v) aqueous methanol, respectively, to evaluate the retention and elution of the compounds.

In the final method, an Oasis Max SPE column (60 mg) was conditioned with 2 mL of methanol followed by 2 mL of MilliQ water. A total of 20 mL of groundwater sample was transferred to the column. The column was rinsed with 2 mL of MilliQ water followed by 2 mL of 15% (v/v) methanol. Elution was performed using 2× 0.25 mL of 80% (v/v) methanol. Before analysis, the eluate was diluted 1:1 by MilliQ water to make the injection solvent similar to the composition of the eluent of the LC method. Using this method, the samples were preconcentrated by a factor of 20. To evaluate the recovery of the final method, groundwater samples from all three locations were spiked to obtain concentrations of 0.4 μg/L ptaquiloside and 0.5 μg/L pterosin B; for the groundwater sample containing the highest concentration of dissolved organic matter (sampled from location II), nine replicates were performed to evaluate the reproducibility of the method.

Instrumentation. *LC−MS.* A model 2690 LC system (Waters, Milford, MA) was used for the chromatographic separation of ptaquiloside and pterosin B. The column temperature was set to 30 °C. The samples were kept at 5 °C in the dark in the autosampler. Detection was made using a Quattro Ultima triple quadrupole mass spectrometer from Micromass (Manchester, U.K.), equipped with an electrospray ionization probe.

LC−UV. A series 1100/1200 LC system (Agilent, Santa Clara, CA) with a diode array detector (DAD) was used. UV detection was performed at 214 nm for ptaquiloside and at 220 nm for pterosin B. The column temperature was kept at 35 °C.

LC Separation. Isocratic separation was performed using a 50 × 2.0 mm inner diameter, 3 μm, Gemini C6-hexyl column (Phenomenex, Torrance, CA) and an eluent of 40% (v/v) methanol and 3 mM ammonium acetate. The flow rate was set to 0.2 mL/min. The retention times were 3.0 min for ptaquiloside and 10.0 min for pterosin B.

MS Conditions. Electrospray ionization was used in positive mode (ESI^+) for both compounds. The capillary voltage was 3.0 kV; the desolvation gas flow (N_2) was 800 L/h; and the cone gas flow (N_2) was 80 L/h. The desolvation temperature was 250 °C, and the source temperature was 100 °C. Argon was used as collision gas for MS/MS; collision energies and cone voltages were optimized for each compound. The compounds were quantified by selective reaction monitoring (SRM), measuring a single characteristic fragment ion of each compound in the MS/MS mode. In the final method, ion traces of m/z 421.1 → 241.1 (ptaquiloside) and m/z 219.1 → 201.0 (pterosin B) were recorded. Cone voltages were set to 50 V (ptaquiloside) and 30 V (pterosin B), whereas collision energies of 17 eV (ptaquiloside) and 15 eV (pterosin B) were used.

Matrix Effect. To estimate the effect of the soil extracts on ionization in the MS/MS method, equal concentrations of the analytes were added to soil extracts and to 50% (v/v) methanol. Soil extracts were prepared by shaking soil and MilliQ water (1:8) for 5 min, after which the solution was filtered through a 0.20 μm RC-filter before spiking with the analyte. All six soil horizons from the bracken-covered soils were tested. For each soil extract, spiking was performed in triplicate at two concentration levels of the analytes (25 and 60 μg/L), after which the samples were analyzed by LC−MS/MS. Because the soils were all bracken-covered, all horizons had a natural content of ptaquiloside and pterosin B. The natural content of ptaquiloside and pterosin B in the soil extracts was analyzed and subtracted before the matrix effect was calculated.

Validation. Validation was performed for the LC−MS/MS method as well as for the LC−UV method for comparison of performance. The linear range of the LC−MS/MS method was determined by triplicate injections of nine standards in the range of 0.5−100 μg/L ptaquiloside and 0.3−60 μg/L pterosin B, whereas for the LC−UV method, triplicate injections were performed for nine standards covering the range of 250−100 000 μg/L ptaquiloside and 100−50 000 μg/L pterosin B. For determination of detection limits (LOD), the standard deviations (SD) were estimated at 0.22 μg/L ptaquiloside and 0.19 μg/L pterosin B in the LC−MS/MS method, and for the LC−UV method, the estimations were performed at 106 μg/L ptaquiloside and 50 μg/L pterosin B, respectively. Detection limits were calculated as $LOD = t_{(0.995,\ n=10)} \times SD = 3.25 \times SD$. Within- and between-day precision of the instruments were determined by injections of standard samples at two concentration levels: 50 versus 5 μg/L ptaquiloside and 27 versus 3 μg/L pterosin B (LC−MS/MS method) and 40 000 versus 4000 μg/L ptaquiloside and 20 000 versus 2000 μg/L pterosin B (LC−UV method). Within-day instrument variation was determined by nine (LC−MS/MS) or three (LC−UV) injections of the samples within the same day and based on the obtained concentrations. An analysis of variation (ANOVA) was used to evaluate the within- and between-day variation of the analytical method. Results were analyzed using a one-way ANOVA, between-groups design, and a Tukey's HSD test (SAS, proc glm, version 9.1 Win, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Analytical Method Development. *Soil Extraction: Ptaquiloside.* Volatile buffers are preferable for detection by MS/MS ESI^+, which is why previous used extraction solutions (0.01 M $CaCl_2$ or 0.1 M phosphate buffer (pH 6.4) (*11, 13*)) could not be used. A comparison of a buffered aqueous solution (5 mM ammonium acetate) with a nonbuffered solution (MilliQ water) revealed no significant difference in extraction efficiencies. To avoid possible transformation of ptaquiloside, during the extraction period or in the period from extraction to the LC−MS/MS analysis, 5 mM ammonium acetate was chosen as the extractant. No difference in extraction efficiency was observed between 5 and 30 min of extraction, and an extraction time of 10 min was used in the final method.

The efficiency of the ammonium acetate extractant was tested on three of the natural ptaquiloside containing soil and litter types, each subjected to two consecutive extractions. It was observed that almost all ptaquiloside was extracted with the first treatment; the highest efficiency was found using the A-horizon from soil I (92 ± 1%), followed by the A-horizon from soil II (83 ± 5%), and last the litter layer from soil I (78 ± 4%). Possibly the extraction efficiency may be reversely related to the content of soil organic matter; i.e., the higher the content of soil organic matter, the lower the extraction efficiency. In all cases, at least 78% ptaquiloside was extracted during the first extraction and only one extraction with the ammonium acetate solution was performed in the further work. In previous methods, up to four consecutive extractions were performed for extraction of ptaquiloside from soil (*11, 13*); this would result in an increase in the extraction efficiency but also an prolonged extraction time, causing higher detection limits because of dilution and increased risk of ptaquiloside degradation.

Soil Extraction: Pterosin B. To the best of our knowledge, no previous methods for extraction of pterosin B in soil have been published. Because of the much higher hydrophobicity of pterosin B compared to ptaquiloside, low extraction efficiencies were seen when using aqueous solutions for pterosin B extraction. In the present study, extraction by methanol/water mixtures was tested subsequent to the ammonium acetate extraction of ptaquiloside for the A-horizons of soils I and II. In **Table 1**, the pterosin B extraction efficiency obtained using different proportions of methanol is shown. It was observed that the amount of pterosin B extracted was proportional to the methanol content and because of that 80% (v/v) aqueous methanol was chosen for the final method.

To test the efficiency of the pterosin B extraction, two consecutive extractions were made using the 80% (v/v) methanol solution. Most of the pterosin B was extracted during the first

Table 1. Extracted Pterosin B Using Three Tested Extractants

	soil I, A-horizon (μg/kg DW)[a]	soil II, A-horizon (μg/kg DW)[a]
20% aqueous methanol	17.8 ± 1.1	797 ± 69
40% aqueous methanol	48.2 ± 2.6	1560 ± 190
80% aqueous methanol	66.8 ± 4.9	3830 ± 880

[a] Mean and standard deviation; $n = 3$; DW = dry weight.

Table 2. Recoveries for the Soil Extraction Procedure Using Spiked Soil Samples

	ptaquiloside		pterosin B	
	high 300 μg/kg FW (%)[a]	low 30 μg/kg FW (%)[a]	high 500 μg/kg FW (%)[a]	low 50 μg/kg FW (%)[a]
blank soil I	71 ± 1	69 ± 8	90 ± 1	84 ± 1
blank soil II	63 ± 2	48 ± 2	91 ± 2	85 ± 3

[a] Mean and standard deviation; $n = 3$; FW = fresh weight.

extraction: soil I, A-horizon (98 ± 1%); soil II, A-horizon (83 ± 1%); and soil I, litter (87 ± 1%). Hence, a single extraction using 80% methanol was chosen for the final method.

Recoveries from Spiked Soil. For evaluation of recoveries, spiked soil samples were extracted using the final method. Recoveries for spiked samples at levels of 30 or 300 μg/kg FW were found to be 48−71% for ptaquiloside (**Table 2**). The lowest recovery was found for soil II spiked with the low ptaquiloside concentration. No further ptaquiloside was found when analyzing the following 80% methanol extract. On the other hand, pterosin B was detected in the high level spiked soils, despite the soils having been spiked with ptaquiloside only. This indicated that some of the added ptaquiloside had been transformed to pterosin B between the time of spiking and extraction, e.g., because of acid hydrolysis. For the low-level ptaquiloside spiked soils, no quantification of pterosin B could be performed, because the concentrations were below the quantification limit for pterosin B. In addition to pterosin B, other transformation products of ptaquiloside may be formed in acidic solutions (*12*). Further explanation can relate to the very high organic carbon content of soil II, which may cause irreversible bonding of ptaquiloside to the soil (*10, 11, 13*). The loss of ptaquiloside seen in the spiking process is not relevant in soils naturally contaminated by ptaquiloside because it is the ptaquiloside concentrations at the time of sampling that are of importance and not the amount that may have been transformed or irreversibly sorbed. Thus, dependent upon the matrix, the real recovery may be higher than the values given here, and the observations emphasize the importance of using identical matrix samples for recovery estimation and quantification. The recoveries from pterosin B spiked samples were 84−91% (**Table 2**).

Groundwater SPE Development. Evaluating the elution using organic modifier contents ranging from 0 to 90% (v/v) methanol showed that ptaquiloside was not eluted from the SPE column using 20% methanol or less, while full recovery was obtained at methanol contents above 50−60% (data not shown). The elution of pterosin B required a relatively higher content of methanol in the solvent because the pterosin B recovery was negligible at solvent compositions below 50% methanol. Full recovery in the eluate was observed using eluent compositions with methanol content above 70−80%. Consequently, it was chosen to wash the SPE column using 15% (v/v) methanol, where neither ptaquiloside nor pterosin B was eluted, while 80% (v/v) methanol was chosen to elute the compounds to maximize recovery for both compounds.

The volume of 80% methanol required for elution was tested by sequential elution with 4 × 0.50 mL aliquots. Each 0.50 mL was quantified separately. Results showed that 96% of the eluted ptaquiloside and 84% of the eluted pterosin B was eluted within the first 0.50 mL. Hence, it was chosen to elute by 0.50 mL only to be able to quantify samples containing a lower initial concentration. Full recovery of pterosin B (>98%) would be possible if eluting in total 1.00 mL.

Recoveries for the SPE process were tested for groundwater from the three locations used in the method development. The relative standard deviations (RSDs) obtained by the nine replicates of groundwater (soil II) were 6−7%. Recovery for pterosin B was 84−89%, while the recovery for ptaquiloside was somewhat smaller (52−66%). The recoveries for ptaquiloside or pterosin B were within the same range using the three different groundwater samples. Other experiments demonstrated that the recovery related to the volume of groundwater used; the higher the volume, the lower recovery obtained (data not shown). Hence, a higher recovery would probably be feasible if the volume of sample loaded to the column was lowered or if the SPE volume was increased. The drawback would be a higher overall method detection limit because of the smaller preconcentration factor. Because the recovery for ptaquiloside was found to be at a quite constant level for all samples, the recovery for the method is considered satisfactory for use.

MS Optimization. The efficiency of the ionization and the detection of the precursor ions and the fragments were optimized by adjusting capillary and cone voltages for both compounds. Precursor ions were only detected using positive ionization. No signal of the protonated molecular ion of ptaquiloside was detectable at the expected *m/z* 399 under various MS conditions. For ptaquiloside, a precursor ion was detected at *m/z* 421.1, corresponding to the sodium adduct of the molecular ion $[M + Na]^+$. For pterosin B, the protonated molecular ion $[M + H]^+$ was detected at *m/z* 219.1. Fragmentation patterns of the precursor ions were examined at different collision energies. For *m/z* 421.1, dominant fragment ions were observed at 241.1 and to less extent 223.1 and 203.0 (**Figure 2**). The loss of 180 mass units (421.1 → 241.1) is proposed to correspond to the loss of the glucose moiety of ptaquiloside $[M-Glu + Na]^+$. The highest response for the fragment ion 241.1 was found using collision energies of 15−20 eV. For pterosin B *m/z* 219.1, the dominant fragment ion was observed at 201.0 (**Figure 2**), and the fragment was most abundant using a collision energy of 15 eV. For quantification, it was decided to use one daughter ion, because only one fragment ion was present for pterosin B, although if possible, the use of two daughter ions may be a way to further improve the detection quality aspects.

To stabilize and intensify the signal of $[M + Na]^+$ for ptaquiloside, substituting sodium acetate for 3 mM ammonium acetate in the eluent was investigated. A concentration of 0.5 mM was used, because sodium in the eluent may enhance the precipitation on the MS cone, which then may require more frequent cleaning. A signal intensification of ca. 50% was obtained for $[M + Na]^+$ for ptaquiloside. However, the addition of sodium ions did also change the signal from pterosin B to a sodium adduct (*m/z* 241.1), but no fragment ions could be obtained from that precursor ion. Hence, sodium acetate was omitted from the eluent again, and in the final method, $[M + Na]^+$ was chosen as the precursor ion for ptaquiloside quantification. If only ptaquiloside determination is of interest, a lower detection limit may be reached by adding sodium acetate to the eluent. It is stressed that the use of sodium adducts should

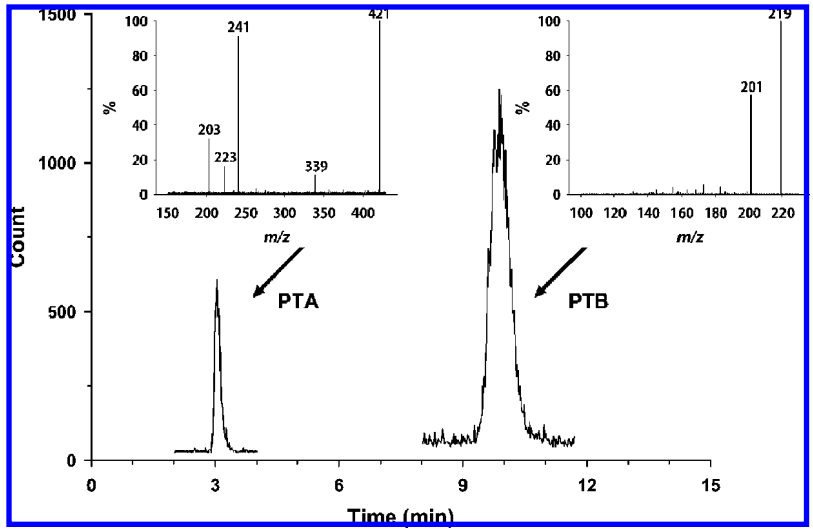

Figure 2. Chromatogram of an aqueous ammonium acetate (ptaquiloside) and 80% aqueous methanol (pterosin B) soil extract from a natural soil sample is shown. Concentrations in the extracts are 16 μg/L (rt = 3.1 min) and 19 μg/L (rt = 9.9 min) for ptaquiloside and pterosin B, respectively. The *m*/*z* traces shown in the chromatogram are 241.1 (ptaquiloside, 2−4 min) and 201.0 (pterosin B, 8−12 min), and the actual ion count values are shown. Fragmentation of ptaquiloside and pterosin B using a collision energy of 15 eV is shown in the two insets. The ions fragmented were 421.1 (ptaquiloside) and 219.1 (pterosin B).

Table 3. Recoveries in Spiked Soil Extracts Analyzed by LC−MS/MS

	ptaquiloside		pterosin B	
	high 60 μg/L (%)[a]	low 25 μg/L (%)[a]	high 60 μg/L (%)[a]	low 25 μg/L (%)[a]
soil I, litter layer	83 ± 5	87 ± 4	102 ± 3	97 ± 3
soil I, A-horizon	90 ± 4	87 ± 9	102 ± 2	98 ± 5
soil II, A-horizon	86 ± 4	86 ± 4	100 ± 3	95 ± 5
soil III, litter layer	76 ± 4	77 ± 7	100 ± 5	100 ± 3
soil III, A-horizon	81 ± 4	87 ± 5	104 ± 8	101 ± 3
soil III, C-horizon	88 ± 4	95 ± 6	99 ± 3	97 ± 4

[a] Mean and standard deviation; $n = 3$.

always be tested on the specific instrument used, because ionization and adduct formation may differ between instruments.

Because a sodium adduct was used for quantification of ptaquiloside and sodium adducts were observed for pterosin B when Na^+ was added to the eluent, the possible influence of the concentration of sodium ions in the samples was evaluated. Sodium acetate was added to ptaquiloside and pterosin B standards in MilliQ water at five concentration levels from 0 to 100 mg/L sodium ions. The range covered the concentrations of sodium found in surface and groundwater from the three locations. The MS/MS detection of $[M + Na]^+$ and $[M + H]^+$ for ptaquiloside and pterosin B, respectively, was evaluated. The detector response was constant, and no quantitative effect of the sodium ion addition was observed (data not shown). It was concluded that sodium concentrations at levels seen in soil solutions and groundwater do not influence the quantification of ptaquiloside and pterosin B.

It is well-known that the sample matrix may affect ionization (*19*−*21*). In the present method, matrix effects were evaluated by comparing the measured ptaquiloside and pterosin B concentrations in 50% (v/v) methanol to the concentrations measured in spiked extracts of soils (subtracting the natural content). The responses were 76−95% for ptaquiloside and 95−104% for pterosin B (**Table 3**). No matrix effect was seen to influence pterosin B determination, and because the negative matrix effect observed for ptaquiloside determination was relatively small and varied between the different soil extracts, it was chosen to prepare standards in 50% (v/v) methanol for the further experiments.

Validation. *Linear Range.* The linear range of the LC−MS/MS was found to be 0.5−100 μg/L for ptaquiloside and 0.3−60 μg/L for pterosin B, typically with correlation coefficients (r^2) better than 0.994. It might be possible to extend the linear range to higher concentrations, but this was not tested, because measurements within that concentration range were less relevant to natural soil and groundwater studies. Using LC−UV, the linear range was found to be 250−100 000 μg/L for ptaquiloside and 100−50 000 μg/L for pterosin B, with correlation coefficients better than 0.997. As before, the linearity may include higher concentrations. Linear ranges from 100 to 200 000 μg/L (ptaquiloside and pterosin B) have been reported for a similar LC−UV method (*16*).

Within- and Between-Day Variation. **Table 4** provides an overview of the quantitative performance of the LC−MS/MS method, and for comparison, a limited data set is included for the method using UV for detection. On the basis of the statistics for the MS/MS method, acceptable overall RSDs for both ptaquiloside and pterosin B were observed; i.e., 7% at the high concentration, and at the low concentration level, the RSDs were in the range of 11−16%. In comparison, the overall RSDs for the UV method were 0.4−2.1%. The within-day variation for the MS/MS and UV method is also shown in **Table 4**. Whereas the UV within-day RSDs of 0.1−2% were much smaller than for the LC−MS/MS method (3−20%), all within-day variations were in an acceptable range. Using LC−MS/MS, the day-to-day variation proved to be nonsignificant ($p < 0.05$), whereas for the UV detection method, a tendency to the effects of day-to-day variation was observed.

Limit of Detection. The LOD for the LC−MS/MS method was determined to be 0.19 μg/L for ptaquiloside and 0.15 μg/L for pterosin B. The detection limits for the LC−UV detection were about a factor 50−100 higher: for ptaquiloside, 19 μg/L and for pterosin B, 8.4 μg/L. In the literature, reports of LODs between 60 and 5000 μg/L for ptaquiloside determined by LC−UV have been reported (*12, 13, 16*). For pterosin B, LODs in the range of 100−130 μg/L for LC−UV have been

Table 4. Reproducibility of Ptaquiloside and Pterosin B Analysis Using UV or MS for Detection

		MS				
		n	mean (μg/L)	STD	*F*	*F* < *p*
			Ptaquiloside			
	day 1	9	52.2	2.3		
50 μg/L	day 2	9	51.7	4.0	1.92	0.17
	day 3	9	54.7	3.8		
	day 1	9	4.91	0.57		
5 μg/L	day 2	9	5.62	0.64	2.18	0.13
	day 3	9	5.62	1.14		
			Pterosin B			
	day 1	9	27.0	0.9		
27 μg/L	day 2	9	27.2	1.9	0.38	0.68
	day 3	9	27.8	2.5		
	day 1	9	2.76	0.07		
3 μg/L	day 2	9	3.08	0.19	2.68	0.0887
	day 3	9	2.95	0.47		

		UV				
		n	mean (mg/L)	STD	*F*	*F* < *p*
			Ptaquiloside			
	day 1	3	42.6	0.12		
40 mg/L	day 2	3	42.9	0.09	12.45	0.0073[a]
	day 3	3	42.5	0.03		
	day 1	3	4.05	0.01		
4 mg/L	day 2	3	4.14	0.01	112.72	<0.0001[a]
	day 3	3	3.96	0.02		
			Pterosin B			
	day 1	3	19.2	0.1		
20 mg/L	day 2	3	19.3	0.2	0.86	0.47
	day 3	3	19.2	0.1		
	day 1	3	1.76	0.02		
2 mg/L	day 2	3	1.69	0.04	7.08	0.026[a]
	day 3	3	1.75	0.01		

[a] Possible grouping is marked in cases where the Tukey's HSD test indicated that content measured at the 3 days was significantly different.

reported (*12, 16*). In comparison to the previously published methods, the LC−MS/MS method developed improves the LOD of ptaquiloside by about a factor of 300 and that of pterosin B by about a factor of 650.

For soil samples, when the soil extraction procedure is included, this will result in detection limits of 0.60 μg/kg FW soil (pterosin B) and 0.76 μg/kg FW soil (ptaquiloside) using the LC−MS/MS method. Groundwater samples, where the preconcentration step by SPE is performed, will have detection limits of 14−18 ng/L for ptaquiloside and 8.4−8.9 ng/L for pterosin B using the LC−MS/MS method. This includes a correction for the recovery.

A quantitative LC−MS/MS method has been developed for determination of ptaquiloside and the metabolite pterosin B in soil and groundwater. Detection limits are found to be in the range of 0.15−0.19 μg/L. The method is an improvement over existing LC−UV methods, where detection limits are 300−650 times higher. In combination with the developed method for soil extraction, the detection limits correspond to 0.6−0.8 μg/kg FW soil, which is suitable for the determination of the content of ptaquiloside and pterosin B in bracken-covered soils. For groundwater samples, the addition of the preconcentration SPE step results in detection limits of 8−18 ng/L. In addition, the use of MS/MS for detection improves the specificity of the method, resulting in a more reliable determination of ptaquiloside and pterosin B at trace levels. The method will be useful for studies of ptaquiloside and pterosin B levels in bracken-covered soil, and water wells and surface waters can be screened for contamination by ptaquiloside. Also, fate studies can be based on the present method because ptaquiloside as well as the transformation product pterosin B can be identified and quantified in soil and groundwater. Inclusion of pterosin B provides further options for interpretation of ptaquiloside loads and stability in soils and waters.

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