reduced by slight modifications in these protective garments, such as elastic in sleeves and exclusive use of butyl rubber gloves and boots.

The Midwestern applicators who were sampled indicated that they would never handle this product more than 10 days/season. Assuming this handling frequency, and use of the pumped-EC formulation in our best protection scenario, average exposure for these applicators should not exceed 2500 μg/season.

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A Simple Method for Purification and Determination of Miserotoxin

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A column chromatography method was developed to simplify miserotoxin (3-nitro-1-propyl β-D-glucoside) purification from timber milkvetch (*Astragalus miser* var. *serotinus*). The column eluate was then directly treated with diazotized *p*-nitroaniline to determine the concentration of the toxic glycoside in the forage. The column method agreed favorably ($r = 0.97$) with the GC procedure for miserotoxin determination, but erratic values were obtained when the glycoside was estimated with the Griess-Ilosvay reagent after KOH displacement of the nitro group. Aqueous extracts of fresh plant material contained factors that enhanced the yield of nitrite during KOH treatment. The interference was reduced in acid extracts of oven-dried plant samples, but the latter treatment also reduced the yield of miserotoxin.

Aliphatic nitro compounds occur in over 500 *Astragalus* species of the family Leguminosae (Williams, 1981a). The compounds also occur in other legume genera (*Coronilla*, *Indigofera*, and *Lotus*) and, less abundantly, in other families (Williams, 1981b). Derivatives of 3-nitropropanol (NPOH) or 3-nitropropionic acid (NPA) are usually detected. Glucose esters of NPA and the β-D-glucoside of NPOH (miserotoxin) have been isolated and identified, but NPA and NPOH do not occur together (Stermitz and Yost, 1978). Numerous studies have demonstrated the toxicity of NPA or NPOH to ruminant and nonruminant animals (James et al., 1980; Majak et al., 1981; Shenk et al., 1976). In British Columbia, timber milkvetch (*Astragalus miser* var. *serotinus*) is widely distributed on Interior grassland and forest range and miserotoxin can accumulate to levels exceeding 8% of the dry herbage weight (Majak et al., 1977).

Derivatives of NPA and NPOH can be determined spectrophotometrically by direct coupling of diazonium salts to the aci tautomers (Majak and Bose, 1974) or indirectly by measuring the nitrite ion after alkaline displacement of the nitro group. The Griess-Ilosvay reagent determines nitrite by coupling 1-naphthylamine to the diazonium salt formed with sulfanilic acid (Bose, 1931). When this reagent is used, the sensitivity of the procedure depends essentially on the yield of nitrite ion. An improved procedure for liberating nitrite at pH 9.5 (Matsumoto et al., 1961) is widely used for NPA because the yield of nitrite exceeds 90%. The yield is much lower if NPA is treated with 20% KOH (Cooke, 1955). In spite of this, a slightly modified version of Cooke's method (Williams and Norris, 1969) appears to be effective for screening purposes (Williams and Barneby, 1977), and it was also used for estimating miserotoxin levels (Parker and Williams, 1974).

As reported earlier (Majak and Bose, 1974), the Griess-Ilosvay reagent was not suitable for miserotoxin analysis due to interfering substances and low yields of nitrite in crude extracts of timber milkvetch. This led to the development of the direct coupling system for miserotoxin determination, but a partial TLC purification was required for plant extracts prior to spectrophotometric

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analysis (Majak and Bose, 1974). We now describe a simpler method for miserotoxin purification by column chromatography which eliminates the need for preparative TLC. The new method is then compared to the GC procedure for NPOH (Majak et al., 1977) and to the method of Williams and Norris (1969) utilizing the Griess-Ilosvay reagent in crude extracts of timber milkvetch.

EXPERIMENTAL SECTION

To provide standards, miserotoxin was isolated from timber milkvetch by a new method (Majak, 1982) and NPOH was synthesized by Dr. Gary Yost at the Chemistry Department Colorado State University, Fort Collins. The latter was shown to be pure by NMR spectroscopy and TLC. To determine the purity of the miserotoxin isolate, the glycoside was hydrolyzed enzymatically and the theoretical yield of NPOH was obtained by GC analysis (see below). An aliquot of the isolate was also derivatized with diazotized *p*-nitroaniline (Majak and Clark, 1980), and the absorbance at 405 nm agreed with the value for an authentic standard. The R_f values of the miserotoxin isolate and standard were identical after TLC.

Timber milkvetch samples were collected near Kamloops, British Columbia, during June and July (1981–1982). Plant samples were stored at −20 °C and subsamples were used for dry matter determinations. Each sample (10 g fresh weight) was extracted with 80% ethanol (500 mL) as described previously (Majak et al., 1974). Aliquots (2 × 100 mL) were concentrated to dryness, and each was resuspended in 50 mL of hot H_2O for duplicate column chromatography on charcoal.

Activated coconut charcoal (50–200 mesh, Fisher No. 5-690-B) was deactivated by treatment with 3 volumes of boiling 3 N HCl for 30 min (Lindberg and Wickberg, 1954). The acid was decanted and the charcoal was resuspended in water 5 times to remove fines. The charcoal was then poured into a large column and washed with water until the pH of the eluate was 4. The charcoal was stored in H_2O, and a slurry was used to prepare a small column (1.5 × 10 cm) supported on a glass wool plug. The hot H_2O extract was acidified with HCl to pH 4 and this was applied to the column. The column was washed with 200 mL of 10^{-4} N HCl and the wash was discarded. Methyl ethyl ketone (MEK) in 10^{-4} N HCl (20% v/v MEK) was then applied, a 10-mL void volume was discarded, and miserotoxin was eluted with 125 mL of 20% MEK. An aliquot (1 or 2 mL) of the MEK fraction was derivatized with the diazotized *p*-nitroaniline reagent (Majak and Clark, 1980). Miserotoxin concentration, determined from the relationship to absorbance at 405 nm, was expressed as a percentage of herbage dry matter. The recovery of miserotoxin was significantly reduced if columns were developed at pH 5–6. Problems could also develop with the use of pH indicator sticks which were not sufficiently accurate for estimating acidity of HCl solutions.

For comparison, miserotoxin was also determined in hot H_2O extracts by releasing the aglycon with β-glucosidase (almond emulsin) and by analyzing NPOH by GC as described previously (Majak et al., 1977). The operating temperatures were slightly modified for a Perkin-Elmer σ 2B GC (oven, 180 °C; injector, 230 °C; FID detector, 250 °C), the column length was increased to 6 ft (2-mm i.d.), and 2-nitroethanol was used as an internal standard to facilitate quantitative analyses. The retention time was 2.92 min for NPOH as compared to 2.04 min for nitroethanol. Each GC determination was based on the average of three sample injections from an incubated subsample of the H_2O extract. Determinations were repeated with replicate incubations. Extracts were also analyzed with the Griess-Ilosvay reagent as described by Williams and Norris (1969).

Table I. Comparison of the Charcoal Column and GC Methods

sample and date of collection (1981)	% miserotoxin[a]	
	charcoal column method[b]	GC method[c]
A, June 9	3.85	4.11
B, June 9	4.09	4.03
C, June 22	5.47	5.55
D, June 22	5.81	5.76
E, June 29	3.44	4.28
F, June 29	3.21	2.88
G, July 3	1.55	1.34
H, July 3	1.83	1.62

[a] Percent dry matter. [b] Values are averages of duplicate determinations. [c] Except for single determinations on E and G, values are averages of triplicate determinations.

RESULTS AND DISCUSSION

The charcoal column method for determining miserotoxin in timber milkvetch enables an experienced technician to analyze 30 prepared extracts per day. Results shown in Table I indicate that miserotoxin values determined by the column method agree favorably with those obtained by the GC procedure ($r = 0.97$). The average difference between the methods and its 95% confidence interval was −0.04 ± 0.32% miserotoxin. In agreement with previous studies (Majak et al., 1974), higher miserotoxin levels were associated with earlier sampling dates and prebloom stages of growth (samples A–D). Samples analyzed by both methods in 1982 were also in good agreement ($r = 0.97$, $n = 11$), and the average difference and its 95% confidence interval were 0.05 ± 0.09% miserotoxin.

Various concentrations of MEK and ethanol were tried, and 20% MEK proved to be the most efficient solvent for eluting miserotoxin from charcoal. When miserotoxin (10 mg) was applied to the column, the average recovery (± standard error) was 94.1 ± 1.5% ($n = 9$). Most of the glucoside was contained in the first 100 mL of 20% MEK with 2–3% occurring in the final 25 mL. When miserotoxin (5 mg) was added to plant extracts, the recovery was greater than 94% ($n = 6$). A single spot cochromatographed with authentic miserotoxin when each MEK fraction was concentrated, chromatographed (TLC), and visualized by using the diazotized *p*-nitroaniline spray system (Majak and Bose, 1974). In addition to miserotoxin (which can also be detected on cellulose TLC by quenching under short-wave ultraviolet light), the 20% MEK fraction contained other quenching compounds but these did not react with the diazonium spray. If the MEK fraction is concentrated to gain sensitivity, the fraction should be initially treated batchwise with DEAE-cellulose (Majak and Towers, 1973) to remove the HCl. Otherwise, the acid is concentrated during evaporation under reduced pressure, partial hydrolysis of the glucoside can occur, and the aglycon can be volatilized. The MEK fraction may also contain free NPOH which has been reported in *Astragalus* spp. (Stermitz and Yost, 1978), but in timber milkvetch the aglycon is a minor component usually not detectable on chromatograms of crude extracts. Nevertheless, to ensure that total NPOH (free and bound) was determined, NPOH (25 mg) was applied to the column and the recovery in 20% MEK exceeded 92%. Consistent recoveries were not obtained with NPA, however, since the compound showed extensive tailing on charcoal. Absorbance values shown in Table II illustrate the excellent reproducibility

Table II. Absorbance Values (at 405 nm) for Duplicate Determinations by Charcoal Column Method

sample	I	II
A	0.305	0.304
B	0.334	0.330
C	0.467	0.461
D	0.493	0.500
E	0.255	0.262
F	0.287	0.277
G	0.110	0.113
H	0.178	0.176

of the charcoal column method for miserotoxin determination. The standard deviation of duplicate determinations is 5.74×10^{-3}. The success of the analysis also depends on sample preparation: uniformly chopped, fresh-frozen samples are suitable for extraction, but oven-dried samples should be avoided due to losses incurred by drying. When the latter (dried at 60 °C for 48 h) were compared to the former, the average yield of miserotoxin (per 100 g dry matter) decreased by 0.73 g (range 0.29–1.12 g, $n = 18$).

An attempt was made to analyze the H_2O extracts by Cooke's method using KOH for displacing the nitro group. The same problems were encountered as described earlier (Majak and Bose, 1974): the yield of nitrite was low (<1%) for both NPOH and miserotoxin, in comparison to the reaction of the Griess-Ilosvay reagent with pure standards, red azo formation was altered in crude extracts (salmon pink color instead of cherry red), and exaggerated miserotoxin levels were obtained (for example, >30% miserotoxin for sample F). Interference by endogenous nitrite was not a contributing factor since nitrite was eliminated by treating samples with HCl before KOH addition (Cooke, 1955). When sample F was purified on charcoal and the MEK fraction was analyzed with the Griess-Ilosvay reagent, a 6% miserotoxin level was obtained, but this was almost twice the value obtained by the GC or direct coupling methods (Table I). Similar results were also obtained when samples were purified on polyamide.

Numerous trials suggested that the release of nitrite was enhanced in plant extracts and that the enhancement could be partially reduced by column chromatography. To test the enhancement theory, miserotoxin standards (1 mg) were determined in the presence and absence of hot H_2O extract (0.05 mL) by using Cooke's procedure and KOH treatment for 4–28 h. Table III clearly shows that absorbance values are enhanced 6–16-fold in the presence of extract. Enhancement (1300%) was also observed when nitrite was determined by the method of Schneider and Yeary (1973). The effect was also observed for smaller volumes of plant extract: 2- and 10-μL aliquots enhanced absorbances by 200 and 700%, respectively. Attempts to isolate the factor(s) that intensified the displacement of the nitro group were unsuccessful, but the enhancement effect appeared to be associated with a fraction sedimenting at 27000g. The enhancement effect was partly reduced when samples were oven-dried and extracted with HCl: samples A, C, E, and G (Table I) yielded values of 5.44, 5.48, 4.32, and 3.76% miserotoxin, respectively, when analyzed by the method of Williams and Norris (1969). With the exception of sample C, these values are still higher than the ones shown in Table I. The comparison is further complicated by the concomitant loss of miserotoxin when samples are dried at 60 °C.

Table III. Effect of Timber Milkvetch Extract on Azo Dye Formation[a] Using the Griess-Ilosvay Reagent

KOH treatment, h	miserotoxin (x)	extract (y)	miserotoxin[b] plus extract (z)	enhancement,[c] %
28	0.070	0.986	>2.0	1300
28	0.078	1.30	>2.0	800
28	0.069	0.719	1.94	1700
17	0.043	0.289	0.801	1100
15	0.053	0.234	0.638	660
15	0.041	0.226	0.562	700
4	0.026	0.255	0.556	1100

[a] Shown as absorbance values (x, y, z) recorded at 530 nm. [b] Miserotoxin (1 mg) was combined with 0.05 mL of timber milkvetch extract (equivalent to 20 mg of fresh herbage). [c] Enhancement (%) = $[z - (x + y)]/x \times 100$.

In short, miserotoxin determination by nitro group displacement was not an acceptable procedure for timber milkvetch analysis owing to endogenous components that affected the yield of nitrite. The enhancement effect may cause discrepancies when other miserotoxin-containing species are analyzed. Alternatively, the problem can be avoided by using the charcoal column (direct coupling) or GC procedures that compare favorably.

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