Analysis of Loline Alkaloids in Endophyte-Infected Tall Fescue by Capillary Gas Chromatography

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A capillary gas chromatographic method for routine analysis of loline-type alkaloids in tall fescue seed and forage has been developed. Filtered solvent extracts of seed, in CH_2Cl_2/MeOH/NH_4OH (75:25:0.5), with phenylmorpholine as an internal standard were normally suitable for direct GC analysis; however, forage extracts required additional cleanup by ion exchange to remove interfering substances. Peak identities were confirmed by mass spectrometry and comparison to known standards. The method should be useful in studies concerning the relationships between loline alkaloid concentration in grasses, insect resistance, and performance problems in cattle.

Tall fescue, *Festuca arundinacea* Schreb, is a cool-season pasture grass that is used extensively in the southeastern United States. Most existing pastures are infected with an endophytic fungus, *Acremonium coenophialum* Morgan Jones and Gams, and *A. coenophialum* infected tall fescue is known to contain a variety of alkaloids (Figure 1). The ergot-type alkaloids, such as ergovaline and ergonovine, and the loline-type alkaloids, mainly *N*-formyl- and *N*-acetylloline, have not been found in tall fescue when the endophyte is absent. These two classes of alkaloids have been associated with production losses in cattle (Sanchez, 1987), and economic losses to cattle producers have been estimated at $50 to $200 million annually (Siegel et al., 1984).

The quantity and identity of each individual toxic compound, including the alkaloids present in endophyte-infected tall fescue (EITF), must be known in order to conduct meaningful toxicity studies. Relative amounts of some representative alkaloids reported in the literature are given in Table I. Satisfactory analytical procedures for the detection and quantitation of all known alkaloids, except saturated pyrrolizidine alkaloids of the loline type, are published (see Table I). GC analysis of *N*-formylloline and *N*-acetylloline taken together has been described (Belesky et al., 1987), but the minor loline-type alkaloids present in EITF samples were not identified. The individual contribution of each loline-type alkaloid, to the total EITF toxicity, is unknown.

Saturated pyrrolizidine alkaloids of the loline type that are known to occur in tall fescue are shown in Figure 2. Only these alkaloids, from or produced during the workup of EITF, are sufficiently volatile for facile GC analysis. Names of substances will be abbreviated throughout the manuscript as follows: norloline, NL; loline, L; *N*-methylloline, NML; *N*-formylnorloline, NFNL; *N*-acetylnorloline, NANL; *N*-formylloline, NFL; *N*-acetylloline, NAL; phenylmorpholine, PM.

Recent studies have demonstrated positive associations between endophyte infection, the presence of volatile loline derivatives, and resistance to insect pests such as sod webworm, *Crambus spp.* (Funk et al., 1983); fall armyworm, *Spodoptera frugiperda* (Hardy et al., 1986); and aphids, *Rhopalosipum pali* and *Schizaphis graminum* (Johnson et al., 1985). Yates et al. (1989) reported that NFL is toxic to the large milkweed bug *Oncopeltus fasciatus*. The bird-cherry oat aphid *R. pali* avoided tall fescue plants infected with the endophyte *A. coenophialum* but settled on tall fescue infected with a *Phialophora*-like endophyte and on endophyte-free plants (Latch et al., 1985). The role of individual loline alkaloid derivatives in EITF insect pest resistance is a topic deserving further study.

We describe a convenient capillary GC method to tentatively identify each of the loline alkaloids present in EITF seed or forage samples and relate their concentrations to an internal standard. Peak identification may be confirmed by mass spectrometry or by cochromatography with known standards. The method should assist toxicologists engaged in animal feeding experiments and those interested in the study of EITF insect pest resistance.

EXPERIMENTAL SECTION

Instruments. Gas chromatography was performed with a Hewlett-Packard 5980A instrument equipped with flame ionization detectors. The oven temperature was held at 80 °C for 2 min and then programmed to 220 °C at 6 °C/min; the injector temperature was 220 °C, and the detector temperature was 250 °C. The helium flow rate for columns was 20 mL/min, and the auxiliary gas flow rate was 10 mL/min. The hydrogen flow rate was 30 mL/min, and the air flow rate was 400 mL/min. Wide-bore (0.53-mm) HP-1 columns (5 or 10 m), with a film thickness of 2.65 μm, were used. Split-vent flow rates were 1 mL/18 s for the 5-m column and 10 mL/19 s for the 10-m column.

For confirmation of peak identities, mass spectra were recorded in the electron impact mode at 70 eV in a Finnigan Model 4600 TSQ mass spectrometer with sample introduction through a gas chromatograph.

Plant Materials. Certified 100% EITF seed (*F. arundinacea*) was purchased from Lambert Seed Co., Camden, AL. Seed of endophyte-infested *Festuca versuta* was obtained from Dr. Daryl D. Rowan, DSIR, Palmerston North, New Zealand. Infection of *F. versuta* has been reported (White and Cole, 1986), but the endophyte has not been identified. Endophyte-free tall fescue and EITF forage were grown at this location. All seed and forage samples were ground to pass a No. 20 screen (0.9 mm) prior to extraction.

Standard Compounds. Saturated pyrrolizidine alkaloids native to EITF were prepared from loline (Petroski et al., 1989). Phenylmorpholine was purchased from Aldrich Chemical Co., Inc. Milwaukee, WI).

Sample Extraction. Routine extractions were conducted with solvent having the following composition: CH_2Cl_2:MeOH:NH_4OH = 75:25:0.5. In a typical analysis, extraction solvent (50 mL) was added to ground sample (5 g) contained in a 250-mL round-bottomed flask. Internal standard, PM (10 mg), was added; the flask was then stoppered and placed on an orbital shaker at 130 rpm for 22–24 h at 25 °C. The mixture was then filtered through Whatman No. 54 paper, and

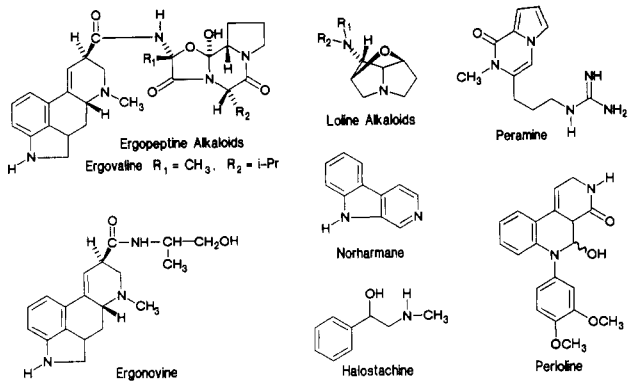


Figure 1. Typical alkaloids reported to occur in endophyte-infected tall fescue.

Table I. Reported Concentrations of Some Representative Alkaloids Present in Endophyte-Infected Tall Fescue

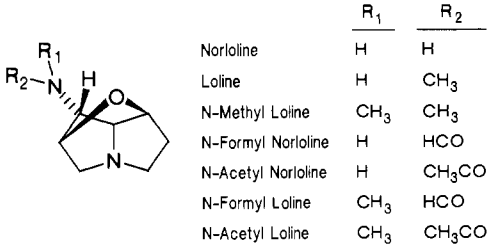
		concn, μg/g dry wt	
alkaloid	reference	seed	forage
ergovaline	Yates and Powell, 1988	3	
	Yates et al., 1985		0.3
ergonovine	Yates and Powell, 1988	detected	
loline alkaloids	Robbins et al., 1972	2000	1800–5000
	Petroski et al., 1989		
halostachine	Davis et al., 1983		1
perloline	Hovin and Buckner, 1983		2000–3000
	Yates et al., 1975		
peramine	Fannin, personal communication	1	10
	Tapper et al., 1989		

the filtrate, from seed samples, was used directly for GC analysis (5-μL injections). Forage sample extracts contained material that interfered with their GC analyses and required additional purification for satisfactory GC analysis.

Forage Sample Extracts. Extracts of forage samples were treated as follows to remove interfering materials: an aromatic sulfonic acid solid-phase extraction tube (7090-3, 3 mL; J. T. Baker Chemical Co., Phillipsburg, NJ) was preconditioned by passing 3–4 mL of CH_2Cl_2/MeOH (75:25) through the column. Forage sample extract (1.0 mL, representing 0.1 g of ground sample) was applied to the column. Interfering materials were removed by washing the column with CH_2Cl_2/MeOH (3 mL of 75:25) followed by 1 mL of CH_2Cl_2/MeOH (75:25) to which 50 μL of NH_4OH was added. The loline (saturated pyrrolizidine) alkaloids were then eluted from the tube with 2 mL of MeOH/NH_4OH (100:5). For GC analysis, 5-μL injections of the eluate were used.

Calculation of Response Factors and Alkaloid Concentrations. Response factors (RF) are defined as micrograms of alkaloid/micrograms of standard × area of standard/area of alkaloid. RF were determined by chromatographing mixtures of the pure individual alkaloids with PM in ratios of 1:3, 1:1, and 3:1; 1:1 ratios, which contained approximately 1 μg/μL, were injected in 0.5-, 2.0-, and 4.0-μL volumes. Response factors (standard deviation) were 1.51 (0.10) for NL, 1.45 (0.04) for L, 1.29 (0.04) for NML, 1.32 (0.13) for NFNL, 1.8 (0.17) for NANL, 1.90 (0.18) for NFL, and 2.25 (0.24) for NAL. Means and standard deviations (SD) were calculated from at least eight replicate analyses. The lower limit of detection was 10 ng of alkaloid. Quantities of individual alkaloids in samples were calculated by the formula: micrograms of alkaloid/gram of sample = RF × area alkaloid/area standard × micrograms of standard/gram of sample.

Recovery Experiments. Analysis of unspiked seed or forage samples revealed the amounts of endogenous NFL and NAL present. Identical seed or forage samples were prepared and spiked with approximately 20% of the total endogenous NFL and NAL. Standard alkaloids, spiked into samples for recovery studies, were added along with the internal standard. The spiked samples were then analyzed, and the percentage of each added alkaloid recovered was calculated. Recoveries (%) were calculated as the difference between the amounts of NFL and NAL found in the spiked and in the nonspiked samples, expressed as a percentage of the amount of NFL or NAL added.



	R_1	R_2
Norloline	H	H
Loline	H	CH_3
N-Methyl Loline	CH_3	CH_3
N-Formyl Norloline	H	HCO
N-Acetyl Norloline	H	CH_3CO
N-Formyl Loline	CH_3	HCO
N-Acetyl Loline	CH_3	CH_3CO

Figure 2. Loline-type saturated pyrrolizidine alkaloids.

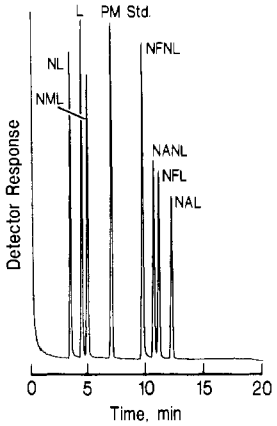


Figure 3. Capillary gas chromatographic analysis of standard loline alkaloids.

RESULTS AND DISCUSSION

Chromatography of a mixture of reference standards revealed the order of elution shown in Figure 3. Retention time increased with molecular weight for NL (140), L (154), and NML (168), respectively. Retention time also increased with molecular weight for the *N*-acylnorlolines, NFNL (168) and then NANL (182), as well as for the *N*-acyllolines, NFL (182) and then NAL (196). Phenylmorpholine was chosen as the internal standard because it was relatively inert and eluted in the wide gap between NML and NFNL. NFNL has not been reported to occur in EITF, but this synthetically prepared material (Petroski et al., 1989) was added to the standard alkaloid mixture for reference.

As with all types of GC analysis, some of the materials being analyzed are either trapped or destroyed on the column to a limited extent. Saturated pyrrolizidine alkaloids of the loline type and relatively crude extracts containing them are no exception. Columns should be conditioned with standard alkaloids, if possible, or with a pure alkaloid fraction until consistent results are obtained. Columns so conditioned should then be dedicated solely to the analysis of loline alkaloids. The condition of the column should be checked periodically (perhaps 1 in every 100 analyses, or sooner if a problem is detected) with a mixture of standard alkaloids or with a pure alkaloid fraction. Although both 5- and 10-m HP-1 columns gave satisfactory analyses, slightly better separations were obtained in the longer column.

During the determination of response factors, it became evident that chlorinated solvents reduce detector response to loline-type alkaloids. Mixtures of internal standard and single alkaloids gave higher response factors when $CHCl_3$ was used as the solvent for injection than when

Table II. Capillary GC Analysis of EITF Seed, Forage, and Endophyte-Infected *F. versuta* Seed Samples

source	alkaloid	mean,[a] μg/g sample	std dev	% total
EITF seed	NML	98	5	3
	NANL			
	NFL	2617	104	80
	NAL	547	38	17
total		3262	133	100
EITF forage	NML	50	4	3
	NANL	230	20	13
	NFL	1158	68	67
	NAL	285	32	17
total		1723	114	100
F. versuta seed	NML	160	8	3
	NANL	1186	65	22
	NFL	3576	89	67
	NAL	416	46	8
total		5338	193	100

[a] Injections of 5 μL representing 500 μg of EITF seed, 500 μg of endophyte-infected *F. versuta* seed, or 250 μg of EITF forage. Mean of eleven, eight, and six replicate analyses for EITF seed, EITF forage, and *F. versuta* seed samples, respectively.

Table III. Recovery of Standard Alkaloids

		μg/g sample[a]				
source	alkaloid	endogenous	added	found	% rec[b]	std dev for % rec
FITE seed	NFL	2617	420	3042	101	17
	NAL	547	100	629	82	9
EITF forage	NFL	1158	269	1456	111	4
	NAL	285	70	346	89	11

[a] Mean of eleven replicate analyses for EITF seed samples and eight replicate analyses for EITF forage samples. [b] Differences between the amounts found in spiked and control samples, expressed as a percentage of the amount of NFL or NAL added.

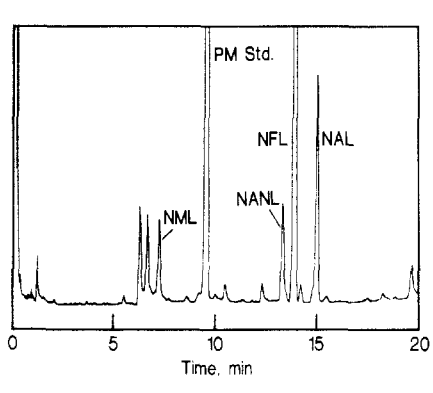


Figure 4. Typical capillary gas chromatographic analysis of an extract of endophyte-infected tall fescue seed.

MeOH was used. Fortunately, CH_2Cl_2 containing 25% methanol was found to be an acceptable solvent.

After experiments with many pure solvents and solvent combinations, loline-type alkaloids in EITF seed samples were found to be extracted efficiently, with a minimum of interfering materials, by using a mixture of CH_2Cl_2/MeOH/NH_4OH (75:25:0.5). Forage samples required an additional ion-exchange separation step to remove interfering materials. Direct injection of extracts (seed samples) or final column eluates (forage samples), without evaporation of solvent, will prevent loss of volatile sample alkaloids or internal standard.

A typical capillary GC record of EITF seed extract, with PM added as an internal standard, is shown in Figure 4. Although small peaks corresponding closely in retention time to NL, L, and NFNL are observed, mass spectral analysis revealed that the fragmentation patterns of these substances did not contain the m/z 82 ion characteristic of NL, L, or their N-acyl derivatives. It is probable that only NML, NANL, NFL, and NAL are native to EITF. The amount of NANL in EITF seed appears to be variable, ranging from undetected to as much as 3% of the total saturated pyrrolizidine alkaloid. The reason for such variation in different batches of EITF seed is unknown.

Results of analyses of EITF seed, forage, and endophyte-infected *F. versuta* seed samples are given in Table II. NANL was not detected in EITF seed but was 13% of the total saturated pyrrolizidine alkaloid in EITF forage and 22% in endophyte-infected *F. versuta* seed. NFL was the most abundant alkaloid in all cases. The highest concentration of total saturated pyrrolizidine alkaloids was found in endophyte-infected *F. versuta* seed (5338 μg/g of sample vs 3262 μg/g of sample for EITF seed and 1723 μg/g of sample for EITF forage). No detectable amounts of loline alkaloids were found in endophyte-free tall fescue seed or forage.

Recovery experiments, to test the analytical method, were conducted on EITF seed and forage (Table III). Good recoveries of added NFL and NAL for both seed and forage samples were obtained.

ACKNOWLEDGMENT

We thank Ronald Plattner (NRRC, USDA—ARS) for GC–MS analyses.

Registry No. Norloline, 4839-19-4; laline, 25161-91-5; *N*-methyllaline, 22143-50-6; *N*-formylnorlaline, 61391-10-4; *N*-acetylnorloline, 38964-35-1; *N*-formylloline, 38964-33-9; *N*-acetylloline, 4914-36-7.

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Received for review May 12, 1989. Accepted August 29, 1989. Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

A Simplified HPLC Method for the Determination of Phytoestrogens in Soybean and Its Processed Products

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By using the proposed procedure, phytoestrogens (daidzein, genistein, coumestrol) can be isolated from soybean and its processed products and subsequently quantitated by HPLC without defatting and cleanup of the samples prior to assay. The samples are extracted with acetonitrile–water, and the extract is filtered through a glass fiber filter. The analytes in the filtrate are in turn separated by HPLC on a C_{18} column and quantified by spectrometry. The method is sensitive to 2 ppm of isoflavones with UV detection and 0.5 ppm of coumestrol with fluorescent detection. The recoveries of phytoestrogens in spiked samples ranged between 75 and 110%. The rapidity, simplicity, and low cost of the method make feasible the assay of large numbers of samples in a regulatory laboratory.

Soybean and its processed products have been used as food in the Orient for centuries. They are known to contain high amounts of protein composed of all the essential amino acids. Soybean oil is also known to be rich in ω-3 fatty acids and fat-soluble vitamins. The carbohydrates of soybean are largely polysaccharides and indigestible fiber, which reduces the diseases of the lower gastrointestinal tract. The soybean has been, therefore, acclaimed as a health food. In the United States, people are now eating more health cautiously. Processed soy products such as tofu, soy milk, soy sauce, etc., are commonly sold in the oriental food store, as well as the local supermarket, and the consumption of these products continues to increase. However, soybeans contain estrogenic compounds, isoflavones, and coumestanes, and there is a significant carry-over of the soy phytoestrogens into its processed products (Murphy, 1982). This means more exposure to phytoestrogens by the consumer. These compounds after ingestion can induce estrus in immature animals or interfere with the normal reproductive processes (Thomson, 1975; Morley et al., 1966). Besides, coumestrol has been suspected to be a tumor-promotor (Verdeal et al., 1980). In order to assure that food containing appreciable quantities of phytoestrogens are not used for human consumption, the occurrence of phytoestrogens in soybeans and its products thus merits careful scrutiny. As a result, the search for more effective and simple monitoring methodologies are needed.

Naim et al. (1974) described a gas–liquid chromatographic (GLC) procedure for the quantitation of soybean genistein and daidzein after the preparation of the trimethylsilyl derivatives. West et al. (1978) developed a high-performance liquid chromatographic (HPLC) method for the analysis of soybean genistein and 4′,6,7-trihydroxyisoflavone. Since HPLC methodology can directly analyze these compounds in free and conjugated forms in samples without the need for derivatization, a number of HPLC methods have been developed during the past decade for the determination of soybean isoflavones (West et al., 1978; Murphy, 1981, 1982; Eldridge, 1982b) and coumestrol (Lookhart et al., 1978, 1979). Murphy (1982) investigated various extraction solvents for isoflavones and coumestrol with and without H_2O or hydrochloric acid and found that acetonitrile with H_2O or hydrochloric acid was superior to all other solvent systems. But, the recoveries for daidzein and coumestrol were lower than 63% for the best extraction systems. Recently, Pettersson and Kiessling (1984) described

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