such as the fiberoptics luminoscope skin monitor.

Registry No. 5-MOP, 484-20-8; 8-MOP, 298-81-7; psoralen, 66-97-7.

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Analysis of Ergopeptine Alkaloids in Endophyte-Infected Tall Fescue

Shelly G. Yates* and Richard G. Powell

A method has been developed for analysis of ergopeptine alkaloids in tall fescue by means of high-performance liquid chromatography (HPLC) with fluorescence detection. Ergopeptine alkaloids in methanol extracts of seed were routinely analyzed in an acetonitrile–pH 7.6 buffer gradient with dihydroergocristine as an internal standard. Measured recovery of ergovaline added at the 1.5 μg/g level was 98%, and the lower limit of detection was 150 pg. Ergopeptine alkaloids, primarily ergovaline, are presumed contributors to animal toxicity of tall fescue (*Festuca arundinacea*) infected with *Acremonium coenophialum*. The method complements tandem mass spectrometry (MS–MS) in that HPLC readily distinguishes physiologically active "ine" and inactive "inine" epimers.

Tall fescue (*Festuca arundinacea* Schreb.) was brought to the new world by early settlers and existed in relative obscurity for many years. A pure stand of a naturalized variety was observed in Kentucky in 1931, and this variety, KY-31, was released commercially in 1943 (Ball, 1984) and recommended for use by the Department of Agriculture. Tall fescue gained rapidly in popularity (Cowan, 1956), and recently forage scientists estimated that 35 million acres are grown in the southeastern United States (Ball, 1984). Much of the time, tall fescue compares favorably with other pasture grasses with respect to daily weight gain of cattle on pasture. Under heat stress during summer months, however, clinical signs of fescue toxicity, including elevated temperature, poor weight gain, problems with reproduction, and reduced lactation (Bush et al., 1979), are often observed. This phenomenon, referred to as summer slump or summer syndrome, has become accepted as normal by livestock producers who rely primarily on fescue forage. During periods of stress due to cold in winter, another syndrome known as fescue foot can become important (Bush et al., 1979). Characteristic signs of fescue foot are loss of weight, rough hair coat, arched back, trembling, and gangrene of the extremities in severe cases. Despite these reports and estimated economic losses of $50 to $200 million annually (Siegel et al., 1984), tall fescue is favored as a cool season pasture for year-around grazing systems. The problems with fescue can be largely avoided by proper management practices. Full understanding of the causes of these syndromes remains elusive due to lack of understanding of all contributing factors and the need for rapid and sensitive analytical procedures.

Symptoms of fescue toxicity often mimic those of ergotism; however, ergot (*Claviceps purpurea*) does not appear to be the major problem in fescue pastures. In recent years it has been recognized that 95% or more of the tall fescue grown in the United States is infected, to varying degrees, with *Acremonium coenophialum* and that the degree of toxicity of fescue forage is related to the degree of infection by this endophytic fungus. *A. coenophialum* has been demonstrated to produce ergopeptine alkaloids, notably ergovaline (Porter et al., 1981; Yates et al., 1985), and, though not present in endophyte-free fescue, ergovaline appears to be a normal constituent of endophyte-infected fescue leaf blades and sheaths at levels up to 2.8 ppm (Lyons et al., 1986).

The presence of ergovaline and other ergot alkaloids in fescue at reported levels is of concern due to their wide range of potent physiological activities (Berde and Schild, 1978). In addition, concentrations of these alkaloids in forage may fluctuate widely due to seasonal or environmental changes (Lyons et al., 1986), unrecognized analogues may be present, and additive or synergistic effects involving other types of compounds may be complicating factors. This report describes a rapid, sensitive, and reasonably quantitative method for analysis of ergot alkaloids in tall fescue. The procedure is a modification of an HPLC method for analysis of ergot alkaloids in flour (Scott and

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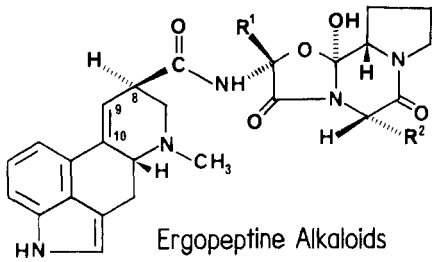
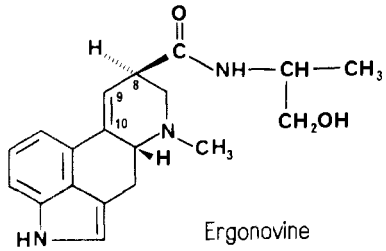
Lawrence, 1980) programmed for maximum separation of those compounds most important in tall fescue studies (ergonovine, ergovaline, ergosine, ergotamine). The use of dihydroergocristine as an internal standard allows for increased sensitivity and confidence levels.



Ergopeptine Alkaloids

	R^1	R^2
Ergotamine	CH_3	$PhCH_2$
Ergosine	CH_3	i-Bu
β-Ergosine	CH_3	sec-Bu
Ergovaline	CH_3	i-Pr
Ergostine	C_2H_5	$PhCH_2$
Ergoptine	C_2H_5	i-Bu
β-Ergoptine	C_2H_5	sec-Bu
Ergonine	C_2H_5	i-Pr
Ergocrystine	i-Pr	$PhCH_2$
α-Ergocryptine	i-Pr	i-Bu
β-Ergocryptine	i-Pr	sec-Bu
Ergocornine	i-Pr	i-Pr



Ergonovine

EXPERIMENTAL SECTION

Instruments. Programmed-gradient HPLC was performed with a Beckman 421 controller and 110A pumps, and detection was accomplished by a Kratos Spectroflow 980 fluorescence detector (excitation at 310 nm, 360-nm-long pass filter, 1-s rise time, range 0.025 μA full scale). Detector response was integrated by a computer. HPLC was conducted on DuPont Zorbax columns (4.6 × 250 mm, 5 μm ODS, or 4.6 × 250 mm, 5 μm C-8) fitted with a 20-μm Zorbax ODS guard column and a 4.6 × 250 mm precolumn filled with 200/425-mesh Adsorbosil (Alltech).

Standard Alkaloids. Ergotamine, α-ergocryptine, and ergonovine were purchased from Sigma Chemical Co., dihydroergocristine methanesulfonate was purchased from Atomergic Chemetals Corp., and ergovaline was a gift from Dr. George Rottinghaus, University of Missouri. Other standard alkaloids were gifts of Sandoz Ltd., Basel, Switzerland. TLC analyses, molecular extinction coefficients, and MS and NMR spectra of all alkaloids indicated that they were correctly identified and were of sufficient purity to be used as quantitative standards (Pollock and Stevens, 1965; Yates et al., unpublished Data). Dihydroergocristine, added at 100-fold the expected alkaloid concentration, or 0.4 mg/g of sample (plant material or feed ration), was used as an internal standard. In earlier experiments quantitation was achieved by measuring peak height, and nanograms of alkaloid per millimeter of peak height were established for pure alkaloids by repetitive analyses. Response factors = nanograms of alkaloid/nanogram of dihydroergocristine × area dihydroergocristine/area alkaloid, determined by repetitive analysis, were 22.7 × 10^{-3} for ergovaline, 23.9 × 10^{-3} for ergosine, 25.0 × 10^{-3} for ergotamine, and 8.2 × 10^{-3} for ergonovine. For routine analyses, an average response factor (R_f) of 23.9 × 10^{-3} was used for all ergopeptide alkaloids.

Quantities of individual alkaloids were calculated by the formula

$$\mu g \text{ alkaloid/g sample} = R_f \times \text{area alkaloid/area dihydroergocristine} \times \mu g \text{ dihydroergocristine/g sample}$$

Solvents and Gradient Program. Solvents consisted of (a) HPLC-grade acetonitrile and (b) 0.1 N ammonium acetate buffer (pH 7.6), refrigerated when not in use. Particulate matter and dissolved gases were removed by vacuum filtration using a 0.45-μm nylon 66 membrane. The gradient program, used for quantitation by the peak height method with a flow rate of 0.8 mL/min, was as follows:

time, min	event
0.0	35% CH_3CN
0.1	50% CH_3CN in 30 min
40.0	100% CH_3CN in 15 min
75.0	35% CH_3CN in 5 min
100.0	end

The gradient program used for quantitation by the internal standard method, with a flow rate of 0.8 mL/min, was as follows:

time, min	event
0.0	35% CH_3CN
0.1	50% CH_3CN in 30 min
35.0	100% CH_3CN in 15 min
70.0	35% CH_3CN in 5 min
85.0	end

During periods when the column was not in use, it was cleaned with a flow rate of 0.8 mL/min with the following buffer flushing program:

time, min	event
0.0	35% CH_3CN, 65% HPLC water
15.0	100% HPLC water in 10 min
65.0	100% CH_3CN in 15 min
120.0	85% CH_3CN, 15% HPLC water in 10 min
150.0	hold and stop flow

Sample Preparation and Recovery Experiments. Various recovery procedures were compared including exhaustive Soxhlet extraction with hexane followed by methanol (agitation), sequential extraction at room temperature with a solvent series (3× each; hexane, ethanol, and 50% aqueous ethanol), and simple extraction (3×) with either $CHCl_3$ or MeOH alone. The preferred method for HPLC analysis of ergot alkaloids was to extract samples of endophyte-free or infected seed either alone or mixed with Purina rat chow 1:1, 3× at room temperature with MeOH (10 mL of solvent/g of sample). Preparation of combined extracts for analysis involved filtering through Whatman No. 54 paper, reduction to an appropriate volume at 25 °C, and final filtration through a 0.45-μm membrane. Internal standard, when used, was added to the initial sample (0.4 mg/g) prior to extraction. All operations were conducted in subdued light. Endophyte-free and endophyte-infected seed were obtained through the courtesy of the University of Kentucky or purchased from Lambert Seed Co., Camden, AL. The latter seed was certified as 100% endophyte-infected.

RESULTS AND DISCUSSION

The relatively simple lysergic acid amide, ergonovine, and the 12 common ergot peptide alkaloids, along with their corresponding C-8 epimers, were all examined in this study. Chromatography of reference standards revealed

Table I. Order of Elution of Ergonovine, Ergopeptine Alkaloids, and Their Epimers on Reversed-Phase Columns[a]

alkaloid	MW
ergonovine	441
ergonovinine	441
ergovaline	533
ergosine & β-ergosine	547, 547[b]
ergonine	547
ergotamine	581
ergoptine & β-ergoptine	561, 561[b]
ergocornine & ergostine	561, 595[b]
ergovalinine	533
α-ergocryptine	575
β-ergocryptine & ergocristine	575, 609[b]
ergosinine & β-ergosinine	547, 547[b]
ergoninine	547
ergotaminine	581
ergoptinine, β-ergoptinine, ergocorninine, and dihydroergocristine[c]	561, 561, 561,[b] 611
ergostinine	595
α-ergocryptinine & β-ergocryptinine	575, 575[b]
ergocristinine	609

[a] In order of increasing retention time. [b] Compounds are not separated when gradient program described in the Experimental Section is used. [c] Semisynthetic internal standard.

Table II. Change in Ergovaline Response Factor with Time

day	response factor	% change
0	22.7×10^{-3}	0
1	23.6×10^{-3}	4
2	24.5×10^{-3}	8
7	25.7×10^{-3}	13
18	27.0×10^{-3}	19

the order of elution as shown in Table I. Elution time increased with molecular weight with two notable exceptions; those alkaloids having benzyl substituents (ergotamine, ergostine, ergocristine) eluted sooner than predicted based on their aliphatic counterparts having similar molecular weights. Those compounds considered to be naturally occurring all eluted earlier than the corresponding C-8 epimers. These C-8 epimeric compounds, assumed to be artifacts of isolation, exhibit markedly reduced physiological responses and are generally less soluble than their naturally occurring counterparts (Berde and Schild, 1978).

Dihydroergocristine proved to be an acceptable internal standard as it eluted in a region where interference from other fluorescent peaks was tolerable; it does not occur naturally, and it does not epimerize at C-8. Dihydroergocristine eluted later than the alkaloids of interest, and its fluorescence response proved to be much less than the response of the normal unsaturated alkaloids. Fluorescence response, expressed as millimeters of peak height/nanogram of alkaloid, of all the common ergopeptide alkaloids was similar on RP-8 and RP-18 columns (6.4 mm/ng, SD = 1.0 and 12.2 mm/ng, SD = 1.1, respectively); the response of ergonovine (30 mm/ng) was more intense. Fluorescence response factors for pure compounds were determined as listed in the Experimental Section. Response factors were determined immediately upon dissolving the samples in methanol as the compounds are somewhat unstable in solution. Ergovaline, kept in methanol at room temperature for 6 h during analysis and then stored at −18 °C, showed a continuous drop in response over an 18-day period (Table II). Isomerization of ergovaline, to ergovalinine, occurred readily and was observed to progress in methanol solution until an equilibrium mixture (near 1:1) was obtained. Loss of fluorescence response was assumed to be due to reaction of the alkaloids with solvent and exposure to light (Berde and Schild, 1978).

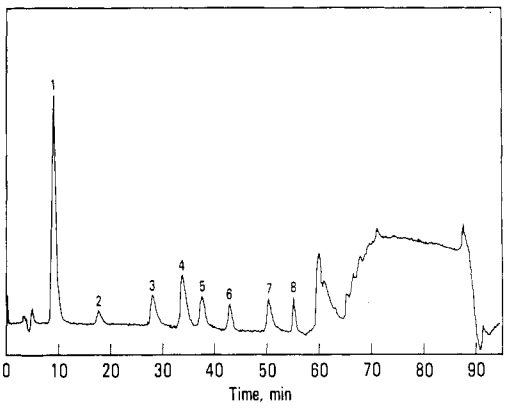


Figure 1. HPLC separation of standard alkaloids important to tall fescue research. Peaks: 1, ergonovine; 2, ergonovinine; 3, ergovaline; 4, ergosine; 5, ergotamine; 6, ergovalinine; 7, ergosinine; 8, ergotaminine.

Table III. Precision of HPLC Analysis of Ergot-like Alkaloids in Endophyte-Infected Tall Fescue Seed

	μg of alkaloid/g of seed[a]	
alkaloid	mean	SD
unknown[b]	1.23	0.10
unknown	0.95	0.07
ergovaline	2.89	0.22
unknown	0.15	0.02
ergosine	0.80	0.31
ergotamine	0.21	0.12
ergovalinine	2.51	0.28
ergosinine	0.47	0.12
ergotaminine	0.32	0.08

[a] Injections of 4 or 5 μL representing 1600 or 2000 μg of seed. Mean of seven analyses. [b] Peak contains both unknown and ergonovine. This pair is partially resolved if each is present in approximately equal amounts. SD = standard deviation.

Table IV. Recovery of Alkaloid Standards

	μg/g of seed		
	endogenous	added	found
ergovaline, ergovalinine	5.40	1.43	6.67
ergosine, ergosinine	1.27	0.23	2.20
ergotamine, ergotaminine	0.53	0.18	1.42

A study of the extraction process revealed that when rat rations spiked with ergonovine, ergocryptine, or ergotamine were extracted, 80–87% of the total extractable alkaloid was found in the first extract and that 0–5% was found in the third extract. Removal of ergot alkaloids from ground seed was more difficult; 61–73% of the total extractable alkaloid was found in the first extract, and 7–9% of the total alkaloid was in the third extract.

A typical HPLC run for standards is shown in Figure 1. This may be compared to HPLC runs of extracts equivalent to 1400 μg of endophyte-infected seed (Figure 2A) and to 1400 μg of endophyte-free seed (Figure 2B). A typical HPLC record of endophyte-infected seed, with dihydroergocristine added as an internal standard and a slightly different program gradient, is given in Figure 3. Peaks eluting in the first 5 min and peaks eluting after 53 min occur in both endophyte-free and endophyte-infected samples. The former peaks were unidentified compounds extracted from the seed, and the latter peaks were due to unidentified impurities in the solvent.

The precision obtained with seven representative samples from the same lot of fescue seed is given in Table III (samples are representative of 1600 or 2000 μg of seed). Recoveries of ergovaline, ergosine, and ergotamine added

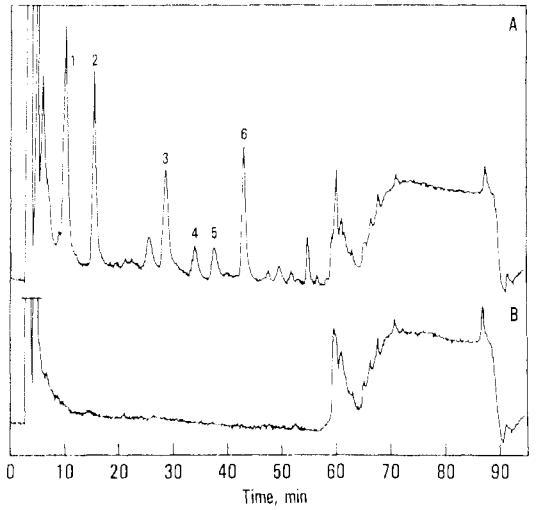


Figure 2. HPLC separation of an ethanol extract representative of 1400 μg of endophyte-infected (A) and endophyte-free (B) tall fescue seed. Peaks: 1, unknown plus ergonovine; 2, unknown; 3, ergovaline; 4, ergosine; 5, ergotamine; 6, ergovalinine.

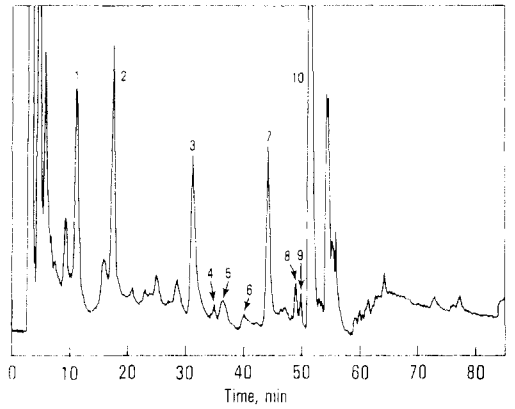


Figure 3. HPLC separation of ergopeptine alkaloids of endophyte-infected Ky-31 tall fescue seed with added internal standard, dihydroergocristine. Separation accomplished on a RP-18 analytical column with a gradient program with pH 7.6 ammonium acetate buffer and acetonitrile. Four microliters of methanol extract representing 1600 μg of seed was injected. Peaks: 1, unknown plus ergonovine (1.09 μg/g); 2, unknown (1.09 μg/g); 3, ergovaline (2.77 μg/g); 4, unknown (0.13 μg/g); 5, ergosine (0.43 μg/g); 6, ergotamine (0.10 μg/g); 7, ergovalinine (2.19 μg/g); 8, ergosinine (0.39 μg/g); 9, ergotaminine (0.24 μg/g); 10, dihydroergocristine.

to endophyte-infected seed, extracted into methanol, and analyzed with no further purification are indicated in Table IV. Recoveries of the latter two alkaloids were somewhat variable and appeared to be related to contamination of the seed samples with small amounts of ergot sclerotia. Careful examination of the seed lot revealed the presence of trace amounts of ergot sclerotia. Ergosine and ergotamine are characteristic alkaloids of ergot sclerotia (Porter et al., 1987), and their concentrations varied markedly from sample to sample. Recovery of ergovaline was more consistent as it is the predominant ergopeptide of the fescue endophyte and is only a minor constituent of ergot. Results of HPLC analysis for ergovaline were in reasonable agreement with those obtained by tandem mass spectrometry (Plattner et al., 1983); detailed comparisons however, were not made.

Progress in understanding the problem of fescue toxicity has been hampered by lack of knowledge concerning what chemical factors are responsible for the observed problems, general unavailability of rapid and accurate analytical methods for known contributing factors that are present only at the ppm level, and unavailability of an appropriate small animal model or other bioassay. The method described herein was developed for rapid and accurate analysis of ergopeptide alkaloids and may be used to analyze fescue samples or to monitor feeding trails. The necessary equipment for HPLC analysis is available in most chemical laboratories and is relatively inexpensive compared to mass spectrometric techniques.

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Registry No. Ergovaline, 2873-38-3; ergosine, 561-94-4; ergotamine, 113-15-5; ergovalinine, 3263-56-7; ergosinine, 596-88-3; ergotaminine, 639-81-6.

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