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Electrospray[+] tandem quadrupole mass spectrometry in the elucidation of ergot alkaloids chromatographed by HPLC: screening of grass or forage samples for novel toxic compounds†‡

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Ergot alkaloids are mycotoxins generated by grass and grain pathogens such as *Claviceps*, for example. Ergot alkaloid–poisoning syndromes, such as tall fescue toxicosis from endophyte-infected tall fescue grass, are important veterinary problems for cattle, horses, sheep, pigs and chickens, with consequent impact on food, meat and dairy industries. Damage to livestock is of the order of a billion dollars a year in the United States alone. HPLC with UV and fluorescence detection are the predominant means of ergot alkaloid determination, with focus on quantitation of the marker compound ergovaline, although ELISA methods are undergoing investigation. These techniques are excellent for rapid detection, but of poor specificity in defining new or poorly characterized ergot alkaloids and related compounds. This paper demonstrates the facility of using electrospray(+) mass spectrometry with multiple reaction monitoring (MRM) detection during chromatographic examination of ergot alkaloid standards of lysergic acid, lysergol, ergonovine, ergovaline, ergotamine, ergocornine, ergocryptine and ergocrystine by HPLC. Ergoline-8 position epimers could be separated on the gradient HPLC system for ergocornine, ergocrystine and ergonovine and appeared as shoulders for ergotamine and ergovaline; epimers generally showed different patterns of relative intensity for specific MRM transitions. There was reasonable correspondence between retention of standards on the 2-mm ESI(+)MS phenyl-hexyl-based reverse phase column and those on the 4-mm C18-based column. Since up to 10% of clinical cases involving toxin exposure display unidentified chromatographic peaks, 11 samples of feed components associated with such cases were studied with developed MRM methods to attempt elucidation of crucial components if possible. Ergotamine appeared in all, ergovaline appeared in five and ergocornine appeared in six; ergonovine, ergocryptine, ergocrystine and lysergol also appeared in several. In addition, molecular weights of compounds newly revealed by mass spectrometry suggested ergosine, ergostine and ergoptine in four samples, for which standards were not available. Dehydrated products of ergotamine, ergocrystine and ergocornine were discovered, along with dihydrogenated ergocrystine and ergocryptine in seven of the samples, and the issue was raised as to whether dehydration was strictly an instrument-derived artifact. Finally, five of the samples, along with fescue seed standard, evidenced one or more of 14 new ergot alkaloids ranging in size from 381 to 611 molecular weight and with key mass spectral characteristics of ergot alkaloids, specifically the pair of peaks *m/z* 223 and 208, corresponding to the ergoline ring system and its demethylated variant, respectively. It is anticipated that findings such as these will provide impetus to future development of analytical methodology for these heretofore relatively rare ergot alkaloid species. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: ergot alkaloids; ergotoxins; ergovaline; LC/MS/MS; electrospray(+) mass spectrometry

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INTRODUCTION

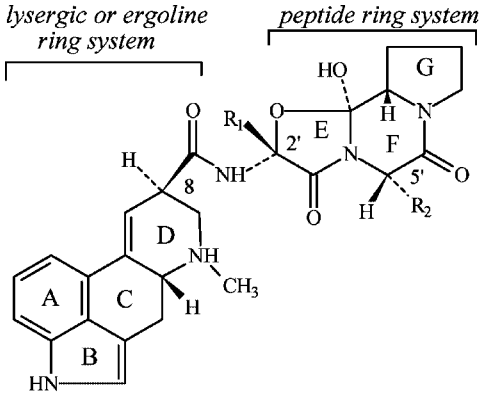
Ergot alkaloids or ergotoxins are mycotoxins that have contributed to human diseases since antiquity (for review see Ref. 1). Common grass pathogens known as *Claviceps* that contaminate grains are responsible for causing gangrenous or convulsive forms of ergotism, which affect the blood supply to extremities or to the central nervous system, respectively. Although modern methods of grain cleaning have largely eliminated ergotism as a human disease, it is still an important veterinary problem, with principal victims including cattle, horses, sheep, pigs and chickens. Clinical signs include gangrene, abortion, convulsions, suppression of lactation, hypersensitivity and ataxia.

In general, plant material including grasses contain a number of alkaloids of agricultural significance, some of which have been isolated and characterized and many others of which remain undefined. Of particular interest are perennial ryegrass (*Lolium perenne*) and tall fescue grass (*Festuca arundinacea* Schreb). For example, it has been found that perennial ryegrass contains not only the lolitrem alkaloids but also ergovaline, the common ergotoxin in tall fescue. Concentrations of the lolitrem alkaloids and ergovaline have shown positive correlation with one another ($r^2 = 0.7335$), i.e. if one is high, the other is also likely to be.[2] However, clinical cases of fescue toxicosis have also been associated with tall fescue grasses containing substances that produce undefined and unidentified HPLC chromatographic peaks, possibly ergotoxins with structures related to ergovaline.

The general structures of ergot alkaloids under consideration are presented in Fig. 1, with more complex analogs incorporating both lysergic, i.e. ergoline, and peptide ring systems as shown to give ergopeptides, and simpler analogs such as lysergic acid including only the lysergic ring system with far simpler C-8 substituents. Solvent-, pH- or temperature-induced epimerization between carbon-8 β and α versions occurs at the lysergic ring C-8 position, most likely arising as the result of keto-enol tautomerism at the central amide oxygen; see the section 'Discussion' for more information on this topic.

Barber *et al*.[3] first reported application of mass spectrometry to both ergopeptide (C9–C10 double bond) and non-peptide-containing clavine (saturated at C10, generally with C8–C9 double bond) alkaloids in which they observed relatively strong molecular ions and M-1 base peaks with the clavine-type compounds, and more extensive and sequential series of fragmentations with the ergopeptides, as visualized on a double-focusing high-resolution MS with a direct insertion probe and with a relatively mild 16-eV electron energy. Voigt *et al*.[4] applied electron impact mass spectrometry (EI-MS) to similar clavines and ergopeptides via direct insertion probe to compare positive and negative ion modes, and dependably found ergopeptide M and M-1 ions more easily by negative mode than by positive mode. Vokoun *et al*.[5] carried out similar direct insertion EI-MS of Δ^{8-9}, Δ^{9-10} and C8–9–10-saturated clavines, which displayed intense M^+ ions, and ergopeptides, which did not, confirming the work of Barber *et al*.;[3] spectra of C-8 epimers were considered indistinguishable from one another. Porter and Betowski[6] further expanded the repertoire of applied mass spectrometric techniques in reporting deconvolution of chemical ionization (CI) mass spectra of ergot alkaloids, claiming it a successful supplementary technique to EI-MS which typically eliminates the molecular ion.

Eckers *et al*.[7,8] applied HPLC coupled via moving belt interface to EI- and CI-MS to identify 11 known alkaloids in chloroform extracts of *Claviceps purpurea*; this represented a step forward since HPLC is more amenable to clavine and ergopeptide compounds than GC. In a similar chromatographic advance, Porter's group[9] combined thin-layer chromatography with both EI- and CI-MS by introduction of extracts from *C. purpurea* sclerotia by direct insertion to identify nine alkaloids in fescue, barley and wheat, with estimation of relative peptide alkaloid percentages from MS/MS data using the relative ratios of respective signals for the major daughter ion fragments. Porter[10] subsequently summarized isobutane CI-MS parameters for 15 ergot alkaloids



R_1	R_2	compound
CH_3	$CH(CH_3)_2$	ergovaline
CH_3	$CH_2C_6H_5$	ergotamine
$CH(CH_3)_2$	$CH(CH_3)_2$	ergocornine
$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	ergocryptine
$CH(CH_3)_2$	$CH_2C_6H_5$	ergocrystine

Other C-8 substituents (i.e. in place of peptide-NHC=O):

$(C=O)NHC(CH_3)CH_2OH$	ergonovine
COOH	lysergic acid
CH_2OH	lysergol
$(C=O)NH_2$	ergine

Figure 1. Ergot alkaloid general structure, including stereochemistry and crucial position numbering and ring labeling (rings A–D constitute lysergic or ergoline; E–G, peptide ring systems). R1 and R2 alkyl and aralkyl substituents are listed in tabular form with corresponding compound names; epimers with C-8 α substituents (rather than β as shown) are ergovalinine, ergotaminine, ergocorninine, ergocryptinine and ergocrystinine, respectively. Removal of the peptide ring and central amide and replacement with other substituents as shown provides the additional compounds listed. Of these four (ergonovine, lysergic acid, lysergol, ergine) only ergonovine is afforded a C-8 α diastereomer known as ergometrinine or ergonovinine.

and each of their three principal fragment ions. Klug *et al*.[11] took a different tack in developing HPLC methods with fluorescence detection for quantitation of 13 ergot alkaloids in cereal grains, followed by negative-mode GC-CI-MS confirmation of injector-temperature (250 °C) pyrolysis-derived peptide fragments released quantitatively; this group found selected ion-monitoring methods necessary for sufficient sensitivity in this confirmatory technique.

Czech groups represented by Shelby *et al*.,[12] Halada *et al*.[13] and Cvak *et al*.[14] have actively pursued a variety of mass spectrometric approaches to chemical analyses of ergot alkaloids. Shelby *et al*.[15] reported the LC/ESI(+)-MS analysis of semipreparative HPLC extracts of *Neotyphodium coenohialum* – infected tall fescue to identify six ergot alkaloids; their methodology involved electrospray interfaced to a double-sector (magnetic/electrostatic) instrument. Relevant techniques, spectra and fragmentation mechanisms of this group were explained in greater detail by Halada *et al*.[13] Cvak *et al*.[14] carried out low-temperature selective hydrogenation of ergopeptides with $LiAlH_4$ and reported characterization of derivatives with both EI-MS for fragment identification and FAB-MS for $[M + H]^+$ determination.

More recently, Stahl and Naegele[16] have reported nano-LC/MS/MS analysis of fungal extracts, with ion trap detection enabling MS^n experiments. The known alkaloids ergotamine and ergometrine were detected, and deconvolution of additional spectra enabled identification of three unknowns, dehydroergotamine, hydroxyergotamine and ergoval, the latter being a lysergic acid-valine esterification product. Durix *et al*.[17] have also demonstrated the value of ESI(+)MS/MS as an unequivocal detector for ergovaline during elaboration of an HPLC-fluorimetric detection method for analysis of mycotoxins in goat's milk.

The previous paper in this series[18] elucidated the ESI(+)-mass spectrometric fragmentation patterns of ergot alkaloids, including clavine-type (lysergic acid, lysergol), water-soluble lysergic acid derivatives (ergonovine), and water-insoluble lysergic acid peptide, i.e. ergopeptide, derivatives (ergotamine, ergocornine, ergocryptine, ergovaline, ergocrystine). Similar to Stahl and Naegele,[16] Durix *et al*.[17] and Shelby *et al*.,[15] the resultant understanding of fragmentation events facilitated HPLC-ESI(+)MS/MS examination and characterization of methanolic extracts of grasses for ergot alkaloids, the results of which are reported here. Development and initial testing of such methodology is of value in that they enable highly sensitive and specific detection of known ergot alkaloids as well as simple screening techniques for new or poorly characterized ergot alkaloids, which may be of particular importance in characterizing typical clinical events such as fescue toxicosis, as well as unusual syndromes such as Mare Reproductive Loss Syndrome (MRLS)[19] or related abortion syndromes.

EXPERIMENTAL

Compounds

Stock preparations of lysergic acid, lysergol, ergonovine, ergotamine tartrate, ergocryptine and ergocrystine (Sigma, St Louis, MO) were prepared at 1.0 mg/ml in methanol; ergocornine (Sigma) was prepared in ethyl acetate. Ergovaline was isolated in semipure form from tall fescue by established methods in the laboratory of Dr Morrie Craig.[20] 1.0 μg/ml solutions were prepared in methanol from these stock solutions. Methanol and acetonitrile were HPLC grade from Fisher Scientific (Fairlawn, NJ). Formic acid was from EM Science (Gibbstown, NJ).

Preparation of plant extracts

Grasses clipped just above the soil were air dried, ground with a Cyclotec Sample Mill (Foss Tecator, Hoeganaes, Sweden) and 1.0 g were combined with 10 ml HPLC-grade chloroform (Fisher, Fairlawn, NJ) and 1 ml 1.0 mM NaOH. Following 18 h of extraction on a Fisher rotorack, the extracts were centrifuged in a Beckman TJ-6 centrifuge (Beckman-Coulter, Pasadena, CA) 2000 rpm for 5 min and 5 ml of supernatants filtered through desiccated solvent-conditioned syringe filters containing 1.0 g silica and 0.5 g sodium sulfate (Fisher). After two 1-ml washes with chloroform : acetone (Fisher HPLC grade), 3 : 1 and 1.5-ml methanol (Fisher HPLC grade), extracts were eluted with 2.5-ml methanol, dried under a flow of nitrogen at 50 °C and reconstituted with 0.5-ml methanol.

High-pressure liquid chromatography

HPLC analysis of ergot alkaloid standards was carried out with gradient analyses as follows: HPLC-fluorescence chromatography was carried out on a PE Series 200 HPLC (Perkin-Elmer, Boston, MA) equipped with a PE LS40 fluorometer and a Phenomenex (Torrance, CA) Luna C18 column, 4.6 mm × 250 mm × 5 μm particle size, including an identical matrix guard column 4.0 mm × 3.0 mm × 5 μm. Elution proceeded by means of a gradient with a 1.0 ml/min flow rate utilizing solvent A = 200 mg/l ammonium carbonate in water, B = acetonitrile as follows: 0–5 min, 80% A, 20% B; 5–25 min, varying linearly to 30% A, 70% B; 25–30 min, held at 30% A, 70% B; 30–35 min, returned to 80% A, 20% B. The column was maintained at ambient temperatures. Detection was by means of excitation at 250 nm and emission at 420 nm.

The following considerations were applied in modifying the fluorescence-HPLC method for HPLC-ESI(+)-MS/MS: a smaller column and lower flow rate enable enhancement of sensitivity (J. Henion, Cornell University, personal communication) and proceeding to a 1 mm or smaller column would be recommended for further sensitivity. The near neutrality of the mobile phase helps prevent proton-catalyzed epimerization at the lysergic ring 8-position during analysis. Finally, ammonium carbonate volatilizes at 60 °C[21] and would be suitable for electrospray examination during HPLC. Rapid examination of standards in 0.05% formic acid : acetonitrile, 1 : 1 is a suitable alternative that can be used for direct infusion studies of protonated ergot alkaloids, since prolonged storage time in autosampler trays with acid is avoided. HPLC-ESI(+)-MS/MS was carried out on an Agilent (Palo Alto, CA)1050 HPLC equipped with a Micromass (Beverly, MA) Quattro II tandem quadrupole mass spectrometer and a Phenomenex Luna phenyl-hexyl column, 2 mm × 250 mm × 5 μm particle size, including a

4 mm × 2 mm phenylpropyl guard column. Elution proceeded by means of a gradient with a 0.5 ml/min flow rate utilizing solvent C = 2.5 mM ammonium carbonate, pH ~ 7, D = acetonitrile as follows: 0–1 min, 90% C, 10% D; 1–5 min, varying linearly to 30% C, 70% D; 5–8 min, held at 30% C, 70% D; 8–9 min, returned to 90% C, 10% D; 9–15 min, held at 90% C, 10% D. The column was maintained at 30 °C by means of a column heater.

Electrospray ionization (ESI) mass spectrometry

Ergot alkaloid standards or extracts were prepared for direct infusion ESI (+) MS analysis by dilution 1:5 or 1:10 with 0.05% formic acid (aq):acetonitrile, 1:1, and infused with a Harvard (Holliston, MA) syringe pump as described previously.[18] The mass spectrometer was a Micromass (Beverly, MA) Quattro II ESI-MS/MS tandem quadrupole instrument, and typical ESI-MS voltage settings for detection and analysis of various ergot alkaloids were as follows: capillary, 3.15 kV; HV lens, 0.5 kV; cone, 30 V; skimmer lens, 1.0 V; RF lens, 0.1 V; source temperature, 120 °C; argon pressure for collisionally induced dissociation (CID) experiments, 3–4 × 10^{-3} mbar; ionization energies: MS1, 0.5 V; MS2, 13.0 V. Collision energy was set between 24 and 32 eV. ESI-MS and MS/MS spectra were acquired as continuum data for a minimum of 1–2 min over the m/z 10–800 mass range, applying 1.8 s per scan duration. Resultant data were background subtracted and smoothed with the Micromass MassLynx version 3.4 software. Spectra were deconvoluted with the assistance of Mass Spec Calculator Pro software, version 4.03 (Quadtech Associates, Inc., 1998; published by ChemSW, Fairfield, CA).

HPLC detection parameters for specific compounds by multiple reaction monitoring (MRM) are listed in Table 1, including specific dwell times for each fragmentation and corresponding collision energies. The ergocrystine dehydrate was studied with settings similar to those of parental ergocrystine.

In order to screen grass extracts for ergot alkaloids with maximum sensitivity, MRM detection was carried out in a two-stage HPLC method, involving 0.01 s dwell on the following, with 23.5-eV collision energy over retention times 0–6 min: m/z 255.20 → 44.00; 255.20 → 196.90; 269.20 → 44.00; 269.20 → 192.00; 326.30 → 223.20 and 326.30 → 208.20; followed immediately by 0.01-s dwell on the following, with 23.5-eV collision energy over retention times 6–15 mins: m/z 534.40 → 223.20; 534.40 → 268.20; 562.40 → 223.20; 562.40 → 268.20; 576.40 → 268.20; 576.40 → 223.20; 582.20 → 223.20; 582.20 → 268.30; 592.40 → 223.20 and 610.40 → 223.20; 610.40 → 268.20. This arrangement afforded two diagnostic ions per compound.

Unusual ergot alkaloids and related compounds were screened by a single-stage MRM acquisition involving 0.01-s dwell on the following with 23.5-eV collision energy: m/z 452.40 → 251.00; 452.40 → 210.00; 484.40 → 269.00; 484.40 → 214.00; 548.50 → 223.20; 562.50 → 239.20; 562.50 → 237.20; 562.50 → 223.20; 564.50 → 225.00; 564.50 → 223.20; 578.50 → 225.00; 596.50 → 237.20; 596.50 → 223.20 and 612.60 → 225.20. This list refers to compounds cabergoline (m/z 452.40), nicergoline (m/z 484.40), ergosine/ergonine (m/z 548.50), ergoptine (m/z 562.50), ergotamine dehydrate/dihydroergocornine (m/z 564.50), dihydroergocryptine (m/z 578.50), ergostine (m/z 596.50), and dihydroergocristine (m/z 612.60), with predicted m/z values in parentheses.[18] Additional simpler HPLC screens for unanticipated ergot alkaloids were carried out by parent ion scans for m/z 223.20, 225.20 and 268.20 in the following respective m/z ranges: 220–620, 220–620 and 260–620. The cone voltage was 30 and the collision energy was 23.5 eV in these experiments. The respective ions represent recurring structural fragmentation motifs in the ergot alkaloids as described previously,[18] specifically m/z 223 = $[M + H]^+$ minus [peptide side chain-NH_2-HC=O]; m/z 225 = peptide ring system released by cleavage of M + H central amide minus alkyl and/or aralkyl side groups R_1 and R_2 (*cf* Fig. 1) and m/z 268 = [lysergic ring system-C=O-NH_2] + H. Nonchromatographic screening of unusual ergot alkaloids was accomplished by full scans followed by daughter ion scans on direct infused mixtures as described above.

Table 1. Multiple reaction monitoring (MRM) detection parameters for specific ergotoxin compounds; dwell time was 0.010 s for each transition

Compound	Parent	Daughter ions, m/z	Collision ion, m/z energy (eV)
Ergocrystine	610.40	592.30, 348.20, 325.20, 305.20, 268.20, 223.20	23.5
Ergocrystine dehydrate	592.40	348.20, 325.20, 305.20, 268.20, 223.20	23.5
Ergotamine	582.20	564.20, 320.20, 297.20, 292.40, 277.20, 268.20, 225.20, 223.20, 208.20	23.5
Ergocryptine	576.40	558.10, 348.20, 268.10, 223.00, 208.00	19.5
Ergocornine	562.40	544.30, 348.40, 305.4, 277.10, 268.20, 223.20	23.5
Ergovaline	534.40	516.50, 268.20, 223.00, 208.00	19.5
Ergonovine	326.30	223.00, 208.00, 180.00	23.5
Ergotoxin Contaminant[a]	270.00	240.10, 102.00, 86.00, 57.00	19.5
Lysergic acid	269.20	182.00, 192.00, 44.00	30.5
Lysergol	255.20	240.20, 196.90, 223.00, 44.00	19.5

[a] Ergotoxin contaminant 269 mw proposed as dihydrolysergamide.

RESULTS

Figure 2(A) depicts chromatography of ergocornine with MRM detection, including nested ion chromatograms each of which represent fragmentations of the M + H pseudomolecular ion. Solutions in methanol of ergot alkaloid standards refrigerated for 1 week or less often displayed additional peaks, most likely due to proton-catalyzed epimerization at the lysergic ring 8-position arising from keto-enol tautomerism. An example is shown in Fig. 2(B), wherein the 8α variant of ergocornine, or ergocorninine according to the Merck Index,[21] accrued at 6.79 min.

Ergocryptine chromatography is shown in Fig. 2(C), again with chromatographically nested sets of MRM transitions specific to ergocryptine. Full scan analysis of this methanolic standard (data not shown) revealed a fairly significant contaminant of ergocryptine, as revealed by MRM chromatography seen in Fig. 2(D). The pseudomolecular M + H ion of m/z 270 indicated a mw of 269, previously tentatively identified as dihydrolysergamide.[18]

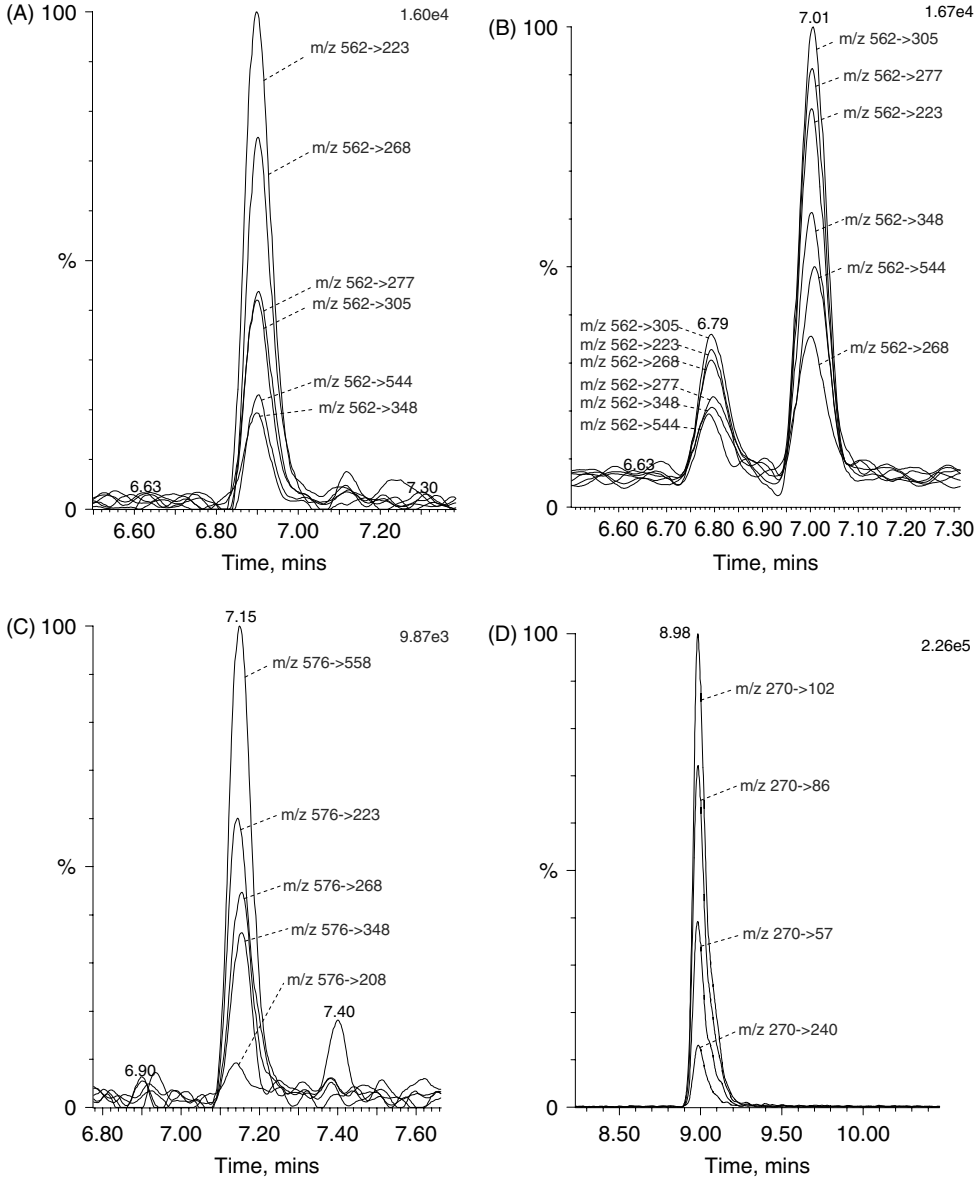


Figure 2. Ion chromatography of ergot alkaloids run by HPLC-ESI(+)-MS/MS. Fifteen nanograms of each compound was run, and the respective MRM transitions were labeled. (A), ergocornine standard; (B), an older preparation of ergocornine, run under significantly lower MS2 ionization energy than in (A); (C), ergocryptine standard; (D), contaminating component seen in many ergot alkaloid standards, possibly as a breakdown product, shown here in an ergocryptine preparation; (E), ergocrystine standard – note that a fresher preparation of this compound contained only the 7.1-min peak; (F), dehydrate of ergocrystine seen during study of the ergocrystine standard; (G), ergonovine standard; (H), older preparation of ergonovine, run under moderately different conditions than in (G); (I), ergotamine standard; (J), ergovaline standard; (K), lysergic acid standard; (L), lysergol standard. Instrument settings: cone volts (CV) = 30.0 V, collision energy (CE) = 23.5 V in (A), (B), (E), (F), (G), (I) and (L); CV = 30, CE = 19.5 in (C), (D) and (J); CV = 30, CE = 30.5 in (K); CV = 28, CE = 30 in (H). MS2 ionization energy settings: 0.4–0.5 in (B), (E), (F), (G) and (I); 0.9 in (K); 8.9 in (H); 13 in (A), (C), (D), (J) and (L).

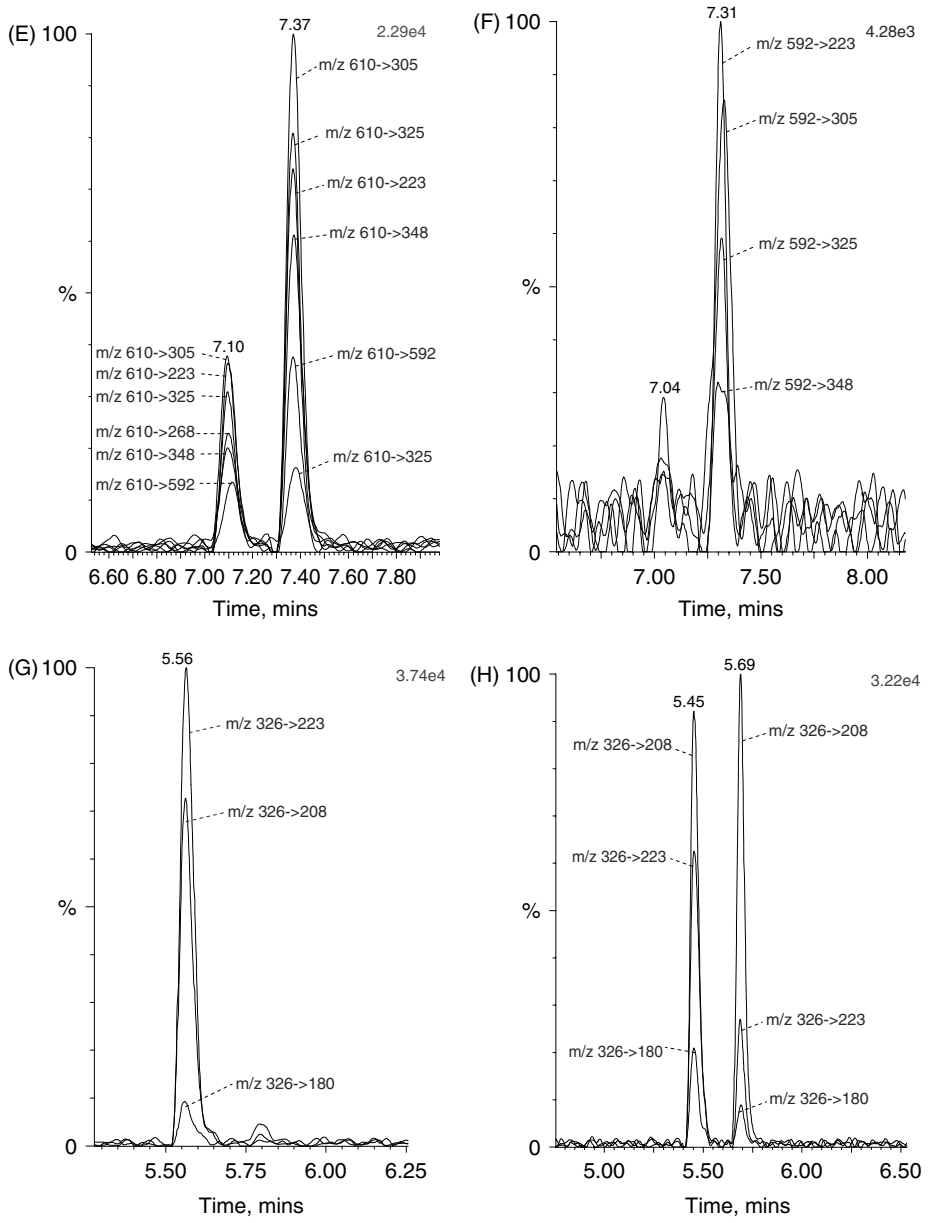
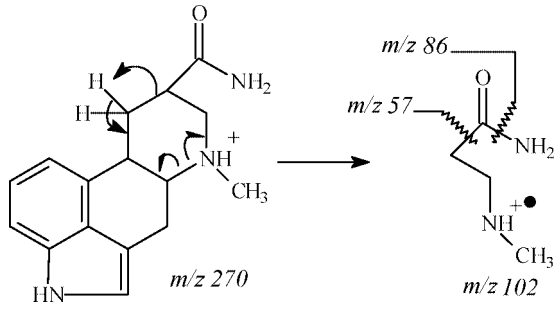


Figure 2. (*Continued*).

Examination of ergocornine, ergocrystine, ergonovine, ergotamine, ergovaline, lysergic acid and lysergol revealed the commonality of this contaminant and the overall uniformity of its chromatographic and mass spectrometric properties, as occurring at retention time $8.80 + 0.09$ min, with the predominance of m/z 270 daughter ions occurring in the order 102 > 86 > 57 > 240. Scheme 1 shows the proposed structure and a fragmentation scheme to account for the origin of these principal fragments.

Ergocrystine chromatography, shown in Fig. 2(E), displayed two peaks after storage of the standard in the refrigerator at 4 °C for 1 week. The 7.10-min peak was apparently the starting material, with the 8α-isomer being ergocristinine.[21]

Ergocrystine, in addition to certain other ergot alkaloids, had a property of forming a dehydrated product at the 12′-hydroxy group of the peptide ring system, with the reaction shown in Scheme 2. Figure 2(F) displays the product



Scheme 1. Structure of a 269-mw ergot alkaloid contaminant proposed as dihydrolysergamide and the suggested fragmentation scheme to account for its daughter ions. Cleavage of the saturated D-ring with shift of a proton are necessary to account for the *m/z* 102 fragment, after which alpha cleavages at the ketone function release the *m/z* 86 and 57 fragments, the latter requiring shift of a proton to release neutral formamide.

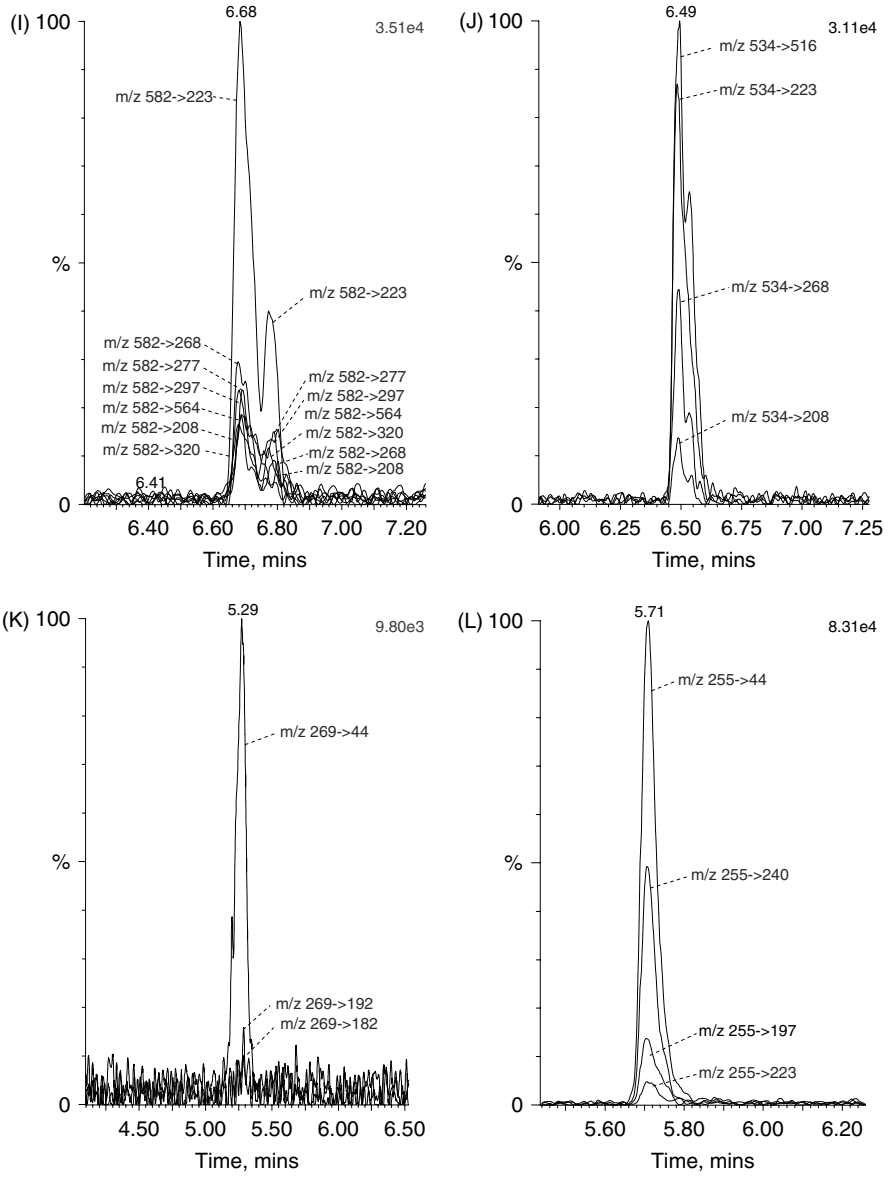
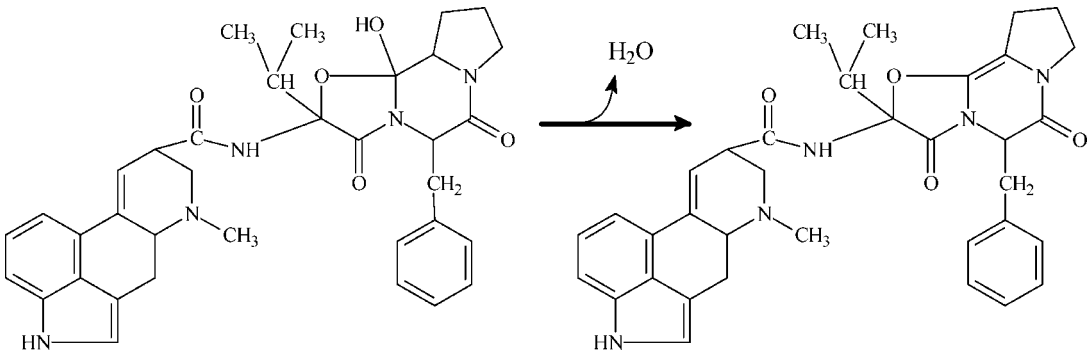


Figure 2. (*Continued*).



Scheme 2. Dehydration of the ergocrystine 12′-hydroxyl group in the peptide ring system.

of such conversion chromatographically as an $[M + H]^+ m/z$ 592 species, including overlap of MRM transitions.

Ergonovine standard is shown in Fig. 2(G), including its overlap of MRM transitions. Similarly, to ergocornine in Fig. 2(B), ergonovine formed a mixture of diastereomers, as shown in Fig. 2(H). The additional peak at 5.69-min retention time was the 8α-isomer ergometrinine also known as ergonovinine.[21]

Ergotamine chromatography is shown in Fig. 2(I). This compound showed a poorly resolved presumed pair of diastereomers which displayed differences in relative intensity of MRM transitions. The additional peak occurring

in ergotamine standard by stereoisomerization would be ergotaminine.[21]

Ergovaline chromatography is shown in Fig. 2(J), and these results also suggest a pair of highly overlapped unresolved peaks of quite similar relative responses of MRM transitions.

Lysergic acid chromatography is shown in Fig. 2(K). Daughter ion mass spectrometry had previously shown the m/z 192 and 182 fragments as being relatively significant components of lysergic acid in comparison with the m/z 269 → 44 transition; on chromatography, however, these transitions were only on the order of 5% or less by area, with the m/z 192 product perhaps twice as large as the m/z 182 product. In contrast, lysergol, shown in Fig. 2(L), had more significant daughter ion peaks which were readily visible on examining overlapping MRM transitions. Neither of these compounds evidenced additional peaks from stereoisomerization, as would be expected since isomerization at the 8-position would only form enantiomers unresolvable under the conditions of the chromatography, in contrast to diastereomers with the other compounds.

Table 2 compares HPLC-ESI(+)MS/MS with fluorescence HPLC under conditions not optimized for ESI(+)MS. HPLC fluorescence occasionally gave additional minor peaks which, if related structurally, could be explained to some extent as one of three things: the 269-mw contaminant or breakdown product described in Scheme 1; other similar contaminants or as diastereomer formation by isomerization at the 8-position. The general order of reverse-phase elution of the principal components correlated well with that seen with LC/MS/MS detection, with retention times also listed in Table 2 along with the primary MRM transitions utilized in each compound's identification. Figure 3 graphically illustrates a typical HPLC-fluorescence chromatogram performed under a more extended version of the program described in the 'Experimental' section as well as the good general correspondence of retention times using the two HPLC methods, which in turn verified the peak identifications made.

ESI(+)LC/MS/MS study of various biological extracts is shown in Fig. 4 as HPLC TICs (total ion chromatograms). One can glean from this assortment of MRM-acquired data that some of the samples, e.g. #4687, had relatively simple components, whereas others were quite complex, e.g. #154. Depending upon the availability of standards, assignments were made to the peaks in these arrays of LC chromatograms with varying degrees of assuredness. Table 3 lists the presence of the characterized components in each of the samples based on similar transitions and retention times. Table 4, on the other hand, lists additional ergot alkaloids for which standards were not available, but which are nevertheless described in the literature. Of those listed, ergostine had a unique molecular weight, ergosine could also have been assigned as its structural isomer ergonine and ergoptine was raised as a possibility on the basis of a unique retention time (7.26 min) that differentiated it from its structural isomer ergocornine seen in Fig. 2(A) (*cf* Lehner *et al*.[18] for discussion of screening new compounds). Table 5 lists simple derivatives of the compounds that have already been examined, all of which are also described in the literature.

With regard to ergot alkaloid identifications, Table 6 lists a number of compounds identifiable only by molecular weight which share common mass spectral fragmentations with the standards under investigation.[18] These were identified primarily by simple direct infusion-MS scans of methanolic sample extracts (data not shown), followed by daughter ion analysis of new or unusual peaks. Many of

Table 2. Comparison of chromatography by HPLC-fluorescence detection with LC/MS/MS (minor peaks in parentheses)

Standards	mw	Major peak by fluorescence-HPLC done in Oregon; RT (min)	Minor peak(s) by fluorescence-HPLC done in Oregon; RT (min)	Major peak by LC/MS/MS (isomer in parenthesis when present); RT (min)	Primary LC/MS/MS identification by MRM transition (m/z):
Ergocrystine	609	23.6	–	7.37 (7.10)	610 → 223
Ergotamine	581	17.8	(10.8, 26.4)	6.69 (6.78)	582 → 223
Ergocryptine	575	23.0	–	7.15 (7.40?)	576 → 223
Ergocornine	561	21.0	–	6.90 (6.80)	562 → 223
Ergovaline	533	15.1	(15.6, 23.0)	6.49 (6.53)	534 → 223
Ergonovine	325	9.0	(4.0)	5.56 (5.79)	326 → 223
Lysergic acid	268	2.6	(4.0)	5.28	269 → 44
Proposed dihydrolysergamide	269	–	–	9.0	270 → 102
Lysergol	254	11.0	(4.0, 23.6)	5.71	255 → 44
Other standards:					
Fescue seed standard: ergovaline;	533	15.1	(18.1, 21.4, 24.2, 8.2, 4.0, etc.)	6.58 (6.60) ergovaline; 6.88 (6.98) ergotamine	534 → 223; 582 → 223
NLC fescue hay control: ergovaline	533	14.8	(17.0, 22.4, 26.0, 4.0, 7.8, etc.)	6.55 ergovaline; 6.85 (6.96) ergotamine	534 → 223; 582 → 223
E^--fescue seed std: ergotamine	581	19.1	(24.8, 4.0, 9.3)	6.85 (6.96) ergotamine	582 → 223

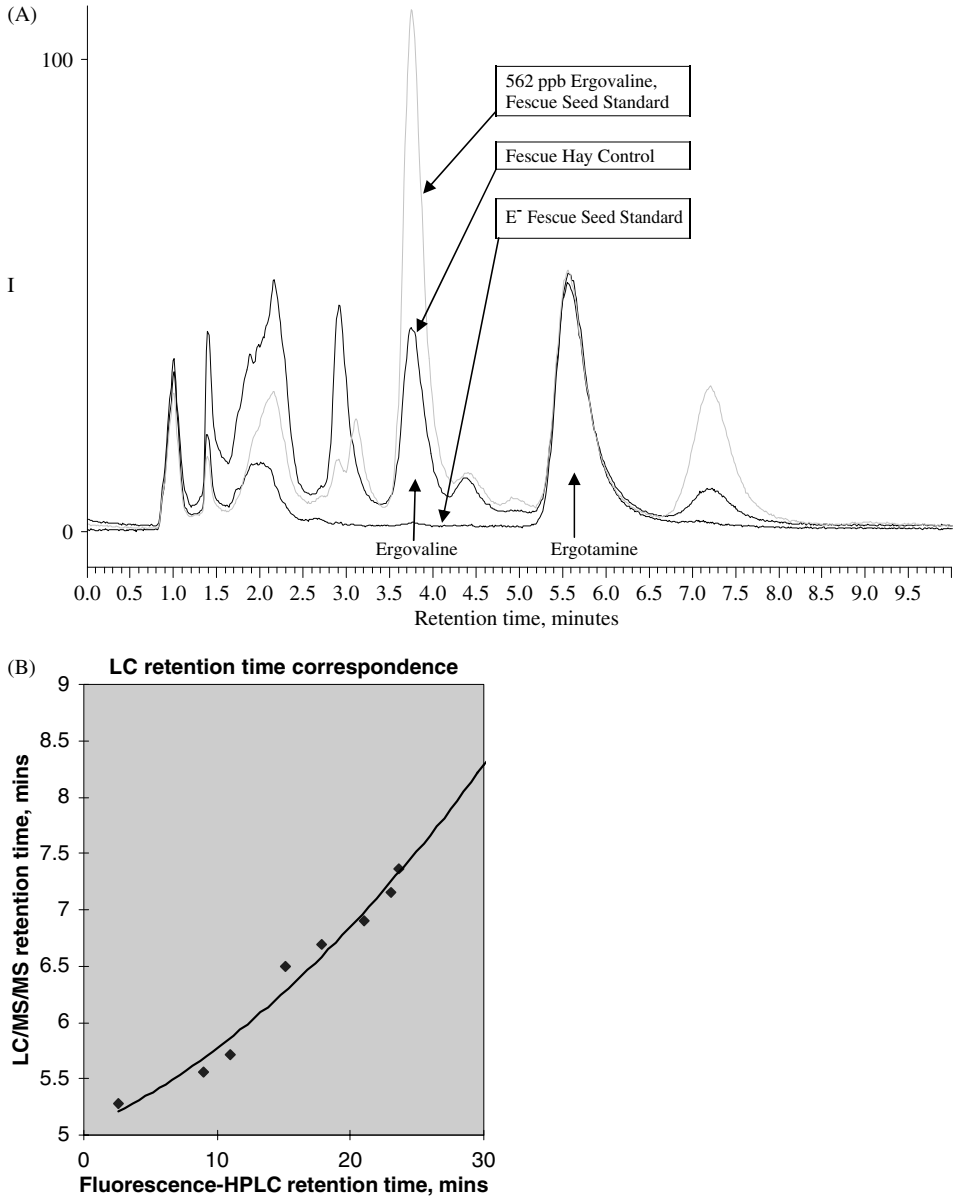


Figure 3. (A), Typical HPLC-fluorescence chromatograms of ergot alkaloids, overlaying results for E^+-fescue seed standard, E^+-fescue hay and E^--fescue seed standard; *y*-axis indicates fluorescence intensity in mv, with the 562 ppb ergovaline peak representing 110-mv intensity. (B) General correspondence between retention times of principal ergot alkaloid standards on two reverse-phase HPLC methods: LC/MS/MS on a 2 mm × 250 mm Luna phenyl-hexyl column *versus* fluorescence-HPLC on a 4.6 mm ×250 mm Luna C18 column, with otherwise similar conditions. The coefficient of determination (r^2) on the second-order trendline was calculated by Microsoft Excel software as 0.97 for these data.

the molecular weight species listed may be simple chemical derivatives of known ergot alkaloids, such as those seen with the dehydrated products and dihydrogenated species of Table 5. Others may be known in naturally occurring ergot alkaloids, not yet gleaned from the literature. Mass spectra of all unusual ergot alkaloids are tabulated graphically in Fig. 5 and labeled simply by molecular weight as in Table 6. All these spectra demonstrate significant m/z 223 and 208 as distinguishing features (compare predictions of Lehner *et al*.[18]). A few, especially 513 mw, were not present in sufficient quantity to provide detailed spectra, yet still showed the expected property of having m/z 223 and 208. HPLC parent ion scans for m/z 223 confirmed some of these findings, as shown in the footnotes in Table 6, and as shown graphically in Fig. 6.

DISCUSSION

Identification of ergot alkaloids and their specific subtype(s) is of tremendous value in achieving definitive diagnoses in certain clinical diseases having agricultural impact. For example, the presence of ergovaline is a crucial indicator upon which many cases are evaluated. Ergovaline is present in the majority of samples associated with cases of fescue foot or summer slump. Ergocornine is the second most common ergopeptide associated in this manner.[22]

Unidentified substances from grass or forage that are revealed by HPLC with UV or fluorescence detection are often suspected to have clinical veterinary significance. The present study has evaluated the HPLC chromatography of

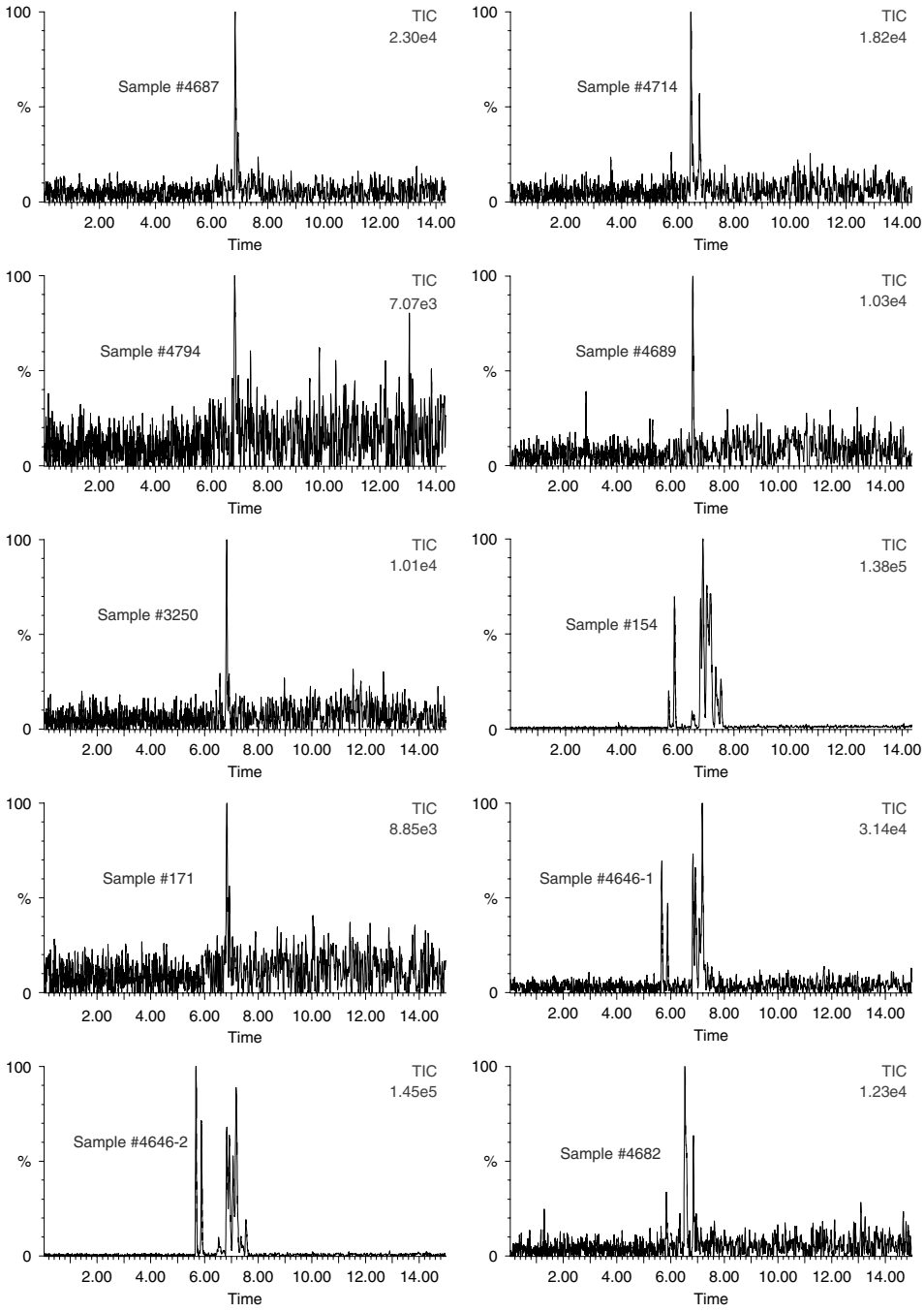


Figure 4. Collection of 14 ESI(+)LC/MS/MS TICs of grass sample extracts and standards, screened by MRM in two phases: 0–6 min: *m/z* 255.20 → 44.00, 255.20 → 196.90, 269.20 → 44.00, 269.20 → 192.00, 326.30 → 223.20, 326.30 → 208.20; and 6–15 mins: *m/z* 534.40 → 223.20, 534.40 → 268.20, 562.40 → 223.20, 562.40 → 268.20, 576.40 → 268.20, 576.40 → 223.20, 582.20 → 223.20, 582.20 → 268.30, 592.40 → 223.20, 610.40 → 223.20, 610.40 → 268.20.

ergot alkaloid standards as it relates to such clinical cases with the primary goal of determining how unidentified substances related to the ergots could be further explored and distinguished with ESI(+)-tandem mass spectrometry. It has exploited and further developed concepts of ergot alkaloid fragmentation revealed in the earlier paper in this series.[18]

About 5–10% of samples associated with clinical disease display chromatograms containing unidentified peaks suspected to be ergot alkaloids. Ergovaline may be present in these along with additional unidentified peaks; it may be present in low amounts or it may even be absent, with varying amounts of other alkaloids present. The present study sought to identify unknown peaks in chromatograms of 11 such clinical cases. Table 1 lists MRM parameters developed for the HPLC-MS/MS detection of ten ergot alkaloids, whereas Table 2 summarizes HPLC-MS/MS detection of ergots and their isomers and comparability with HPLC-fluorescence detection. Tables 3, 4 and 5 list known ergot alkaloids or their derivatives tentatively identified in case samples, while Table 6 lists unidentified compounds having ergot-like mass spectrometric properties which were also identified in six of these clinical cases.

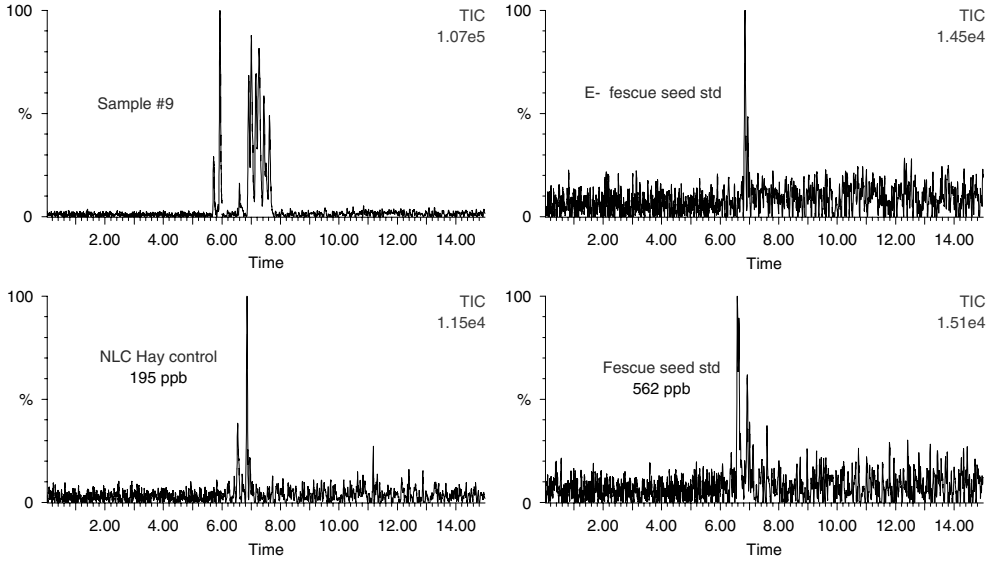


Figure 4. (*Continued*).

Table 3. Ergot alkaloids found in various samples by HPLC-two-phase MRM method: those corresponding to available standards (x = compound found)

Sample	Ergotamine	Ergovaline	Ergocornine	Ergonovine	Ergocryptine	Ergocrystine	Lysergol
#154	x	x	x	x	x	x	–
#171	x	–	x	–	–	–	–
#3250	x	–	–	–	–	–	–
#4646-1	x	–	x	x	x	x	–
#4646-2	x	x	x	x	x	x	–
#4682	x	x	–	–	–	–	x
#4687	x	–	–	–	–	–	–
#4689	x	–	–	–	–	–	–
#4714	x	x	x	–	–	–	–
#4794	x	–	–	–	–	–	–
#9 (10/23/01)	x	x	x	x	x	x	–
E^{-}-fescue seed std	x	–	–	–	–	–	–
NLC hay control	x	x	–	–	–	–	–
Fescue seed std 562 ppb	x	x	–	–	–	–	

In case #154, 160 horses died and both ergotamine and ergovaline were found, indicating ingestion of both *C. purpurea* and Neotyphodium grass. This was supported by botanical staining and identification of fungi. It was found (Tables 3 and 4) that a significant number of ergopeptides were present in the feed, including ergotamine, ergovaline, ergocornine, ergonovine, ergocryptine, ergocrystine, possibly ergosine and ergostine, and possibly dehydrated products of both ergocrystine and ergocornine and a dihydrogenated ergocryptine (Table 5). In addition, ergot alkaloids of mw 529, 547 and 563 were also present (Table 6), and their sizes and spectra (Fig. 5) suggest possible interrelatedness. For example, 529 could be related to the others by addition of a molecule of water to give 547 and an additional oxygen atom to give 563. Given the propensity for ergot alkaloids to differ by substituents in the peptide ring system 2′ and 5′ positions, this suggests that 529 is a dehydrated version (at the 12′ position) of 547, whereas 547 would include five alkyl carbons (such as 2′ -methyl, 5′ -butyl, or the equivalent). If related, 563 could then include an additional alcohol or, less likely, ether functionality. More rigorous analysis of new ergot molecular weight values is provided below. The roles of any of these alkaloids in producing disease have not previously figured in the pathology of vasoconstriction, and yet are likely, in our opinion, to have contributed measurably to the clinical disease.

In case #171, ergocornine appears to be the major ergot alkaloid present and in even greater amounts than ergovaline (Table 3).

Sample #3250 had little, if any, ergovaline and little in the way of ergot alkaloids in general with the exception of an unidentified 7.0-min 563-mw ergot alkaloid. HPLC-UV disclosed only an unusual peak at 3.4 min. Therefore, it appears that clinical diseases in the cattle affected in this case are not likely to have been the result of ergot alkaloids.

Case #4646 (two samples) involved eastern Oregon cattle with clinical signs of fescue foot and related syndromes. The associated samples contained a large number of ergot

Table 4. Ergot alkaloids found in various samples by HPLC-two-phase MRM method: those corresponding to known compounds from literature without available standards (x = compound possible based on mw)

Sample	Ergosine	Ergostine	Ergoptine
#154	x	x	–
#171	–	–	–
#3250	–	–	–
#4646-1	x	–	–
#4646-2	x	–	–
#4682	–	–	–
#4687	–	–	–
#4689	–	–	–
#4714	–	–	–
#4794	–	–	–
#9 (10/23/01)	–	–	x
E^--fescue seed std	–	–	–
Standard NLC hay control	–	–	–
Fescue seed standard 562 ppb	x	–	–

alkaloids (Tables 3 and 4), including ergotamine, ergovaline, ergocornine, ergonovine, ergocryptine, and ergocrystine, possibly ergosine, and possibly dehydrated products of both ergocrystine and ergotamine and a dihydrogenated ergocrystine (Table 5). In addition, ergot alkaloids of mw 381, 543, 545, 557, 559, 591, 593, 595 and 611 were also present in these samples (Table 6). This number pattern suggests simple differences involving dehydrogenation to double bonds, specifically when comparing the pairs 545/543, 559/557, 593/591, 595/593 and 611/609 (ergocryptine). The 381 species differs from ergonovine by only the addition of four alkyl substituents, 545 and 559 differ from one another by the addition of a methylene (CH_2 group) and 545 differs only by a site of unsaturation in the potential five alkyl carbon 547-mw compounds described in the discussion for case #154; the higher molecular weight species in this case must introduce some novel motif such as the benzyl group as they do not differ by simple addition of methylenes. As mentioned previously, more rigorous analysis of new ergot molecular weight values is provided below.

These types of findings are reminiscent of the HPLC and LC-ESI(+)MS studies of Shelby and Flieger,[12] who studied fescue plants and seeds. They found lysergamide, ergovaline, ergosine, ergonine and didehydroergovaline in endophyte-infected tall fescue seeds as well as possible ergot alkaloid glycosides. Major ergot alkaloid species were confirmed by epimerization of isolated peaks in their study, without apparent access to daughter ion spectra for mw determination and generation of epimer mass spectra.

The rest of this discussion considers fine details of ergot alkaloid HPLC-ESI(+)MS as it relates to identification of unique compounds. The ergot alkaloids with ergoline ring system 8-position substituents are capable of stereochemical isomerization (epimerization) at this position from β- to α-configuration, even on storage at refrigerator temperatures on the order of 1–2 weeks. This process converts ergovaline to ergovalinine, and ergotamine to ergotaminine, for example. Despite this property, ergotamine is still generally valuable as an internal standard, since it is found in the fungus *C. purpurea* but not in the Neotyphodium grasses.[10,23,24] Table 2 lists isomeric peaks visualized by LC/MS/MS for ergocrystine, ergotamine, ergocornine, ergovaline, ergonovine and probably ergocryptine.

Scheme 3 indicates a likely route to epimerization of the ergot alkaloid D-ring 8-position via keto-enol tautomerism of the amide carbon oxygen bond. The isomerism as shown [W → X → Y] would probably compete with double bond formation to the amide nitrogen [W → V], a rearrangement known to occur to some extent in peptide bonds depending upon backbone constraints.[25,26] Studies on such amide-iminol tautomerism represent a substantial literature that has repercussions on understanding biochemical events such as

Table 5. Ergot alkaloids found in various samples by the HPLC-two-phase MRM method: those corresponding to simple derivatives for which standards were unavailable (x = compound possible based on mw)

Sample	Ergotamine-dehydrated product	Ergocrystine-dehydrated product	Ergocornine-dehydrated product	Dihydrogenated ergocrystine	Dihydrogenated ergocryptine
#154	–	x	x	–	x
#171	–	–	–	–	–
#3250	–	–	–	–	–
#4646-1	x	x	–	–	–
#4646-2	x	x	–	x	–
#4682	x	x	–	–	–
#4687	x	–	–	–	–
#4689	–	–	–	–	–
#4714	x	–	–	–	–
#4794	x	–	–	–	–
#9 (10/23/01)	x	x	–	–	x
E^--fescue seed standard	–	–	–	–	–
NLC hay control	x	x	–	–	–
Fescue seed standard 562 ppb	x	–	–	–	–

Table 6. Ergot alkaloids screened from various samples primarily by the daughter ion–scanning method, with some supplementation by the LC-MRM method indicated in those cases below where LC retention times are appended: these ergot alkaloids correspond to unique unknown structures not predicted by reference to literature or existing structures (x = unusual ergot alkaloid found)

Sample	381 mw	513 mw	529 mw	531 mw	543 mw	545 mw	547 mw	557 mw	559 mw	563 mw	591 mw	593 mw	595 mw	611 mw
#154	–	–	x[b]	–	–	–	x	–	–	x	–	–	–	–
#171	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#3250	–	–	–	–	–	–	–	–	–	x	–	–	–	–
#4646-1	–	–	–	–	–	–	x	–	–	–	x	–	–	–
#4646-2	x	–	–	–	x	x	–	x	x	–	x	x	x	x
#4682	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#4687	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#4689	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#4714	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#4794	–	–	–	–	–	–	–	–	–	–	–	–	–	–
#9 (10/23/01)	x[a]	–	x[b]	–	–	–	–	–	–	x[d]	–	x[e]	–	–
E^--fescue seed standard	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NLC hay control	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fescue seed standard 562 ppb	–	x	–	x[c]	–	–	x	–	–	–	–	–	–	–

[a] RT = 6.76 min.
[b] RT = 6.68, 6.77 min.
[c] RT = 6.56, 6.65 min.
[d] RT = 7.03, 7.24 min.
[e] RT = 7.28, 7.64 min.

protein folding and DNA internal hydrogen bonding, among others. Theoretical studies generally favor the amidic form over iminolic tautomers,[27,28] yet spectroscopic studies and related calculations reveal differing ratios of amidic-iminolic tautomers, depending upon the system. For example, certain heterocyclic amides tautomerize with a preference for the iminolic form[29] and in certain cases with a zwitterionic structure, with charge separation dependent on solvent proton-donating power and dielectric constant as well as the nature of ring substituents adjacent to the nitrogen.[30] Noncyclic amides can also function as proton-donating acids that favor the iminolic form, depending upon factors such as solvent and neighboring substituents such as fluorines.[31,32] Peptide bond carbonyl oxygens have recently been studied as recipients for H atoms in rearrangements of peptide radicals and radical cations in which electron transfer through a pi-orbital system occurs in either the same or the opposite direction to proton migration, depending on the electronic properties of the protein chain, and leading to peptide N–C bond dissociation or side-chain loss and generation of iminolic products.[33,34] Rearrangement of the amide to an enol rather than iminol has precedents in the synthetic lab, as revealed in certain lithium complexes and, as with amide-iminol tautomerism, affected by the nature of adjacent functionalities.[35–38] It appears likely that amide conversion to iminol is energetically favored over amide conversion to enol owing to anticipated superior acidity of the amide *versus* C–H and stability of the transient polar iminol;[39] this may explain the relatively gradual appearance of diastereomeric peaks in Figs 2(B), (E), (F), (H), (I) and (J) by acid- or alkali-induced epimerization involving an intermediate potentially stabilized by conjugation of bonds 1 and 2 in intermediate X (Scheme 3).

Neither the Scheme 3 epimerization nor potential side reactions to iminol V or double bond migration product Z offer direct opportunities for interpreting the appearance of an ergot alkaloid side-product illustrated in Scheme 1. The proposed dihydrolysergamide requires retention of three nitrogen atoms and hydrogenation of a double bond to account for its odd 269 mw, but no direct route to its accumulation can be gleaned from Scheme 3 even upon introduction of various hydrolytic events.

A related question is whether double bond migration products of the Z type accumulate naturally. If W, Y and Z are possible isomeric products of X, then Z types may be difficult to distinguish from the others chromatographically. However, it is more likely that double bond configurations of the W type beginning with lysergic acid provided an evolutionary advantage that enabled ergopeptide synthesis through nonribosomal peptide synthetic pathways.[24,40–42] Thus, the simpler clavine-type ergot alkaloids generally have a Δ^{8-9} configuration in the pathway to the exclusively Δ^{9-10} ergopeptides,[24] although some side-product clavines have Δ^{9-10} configurations. Conversion of elymoclavine (Δ^{8-9}) to D-lysergic acid (Δ^{9-10}) occurs via the intermediate paspalic acid (Δ^{8-9}), a process believed to depend on cytochrome *cpP*450-1 monooxygenases.[41,43] One laboratory synthetic conversion occurs through treatment of paspalic acid with tetrabutylammonium hydroxide (TBAH) under patented processes.[44] Narasaka[45] considers similar TBAH-induced double bond movement in light of simultaneous transposition of hydroxyl groups, and a hydroxylated

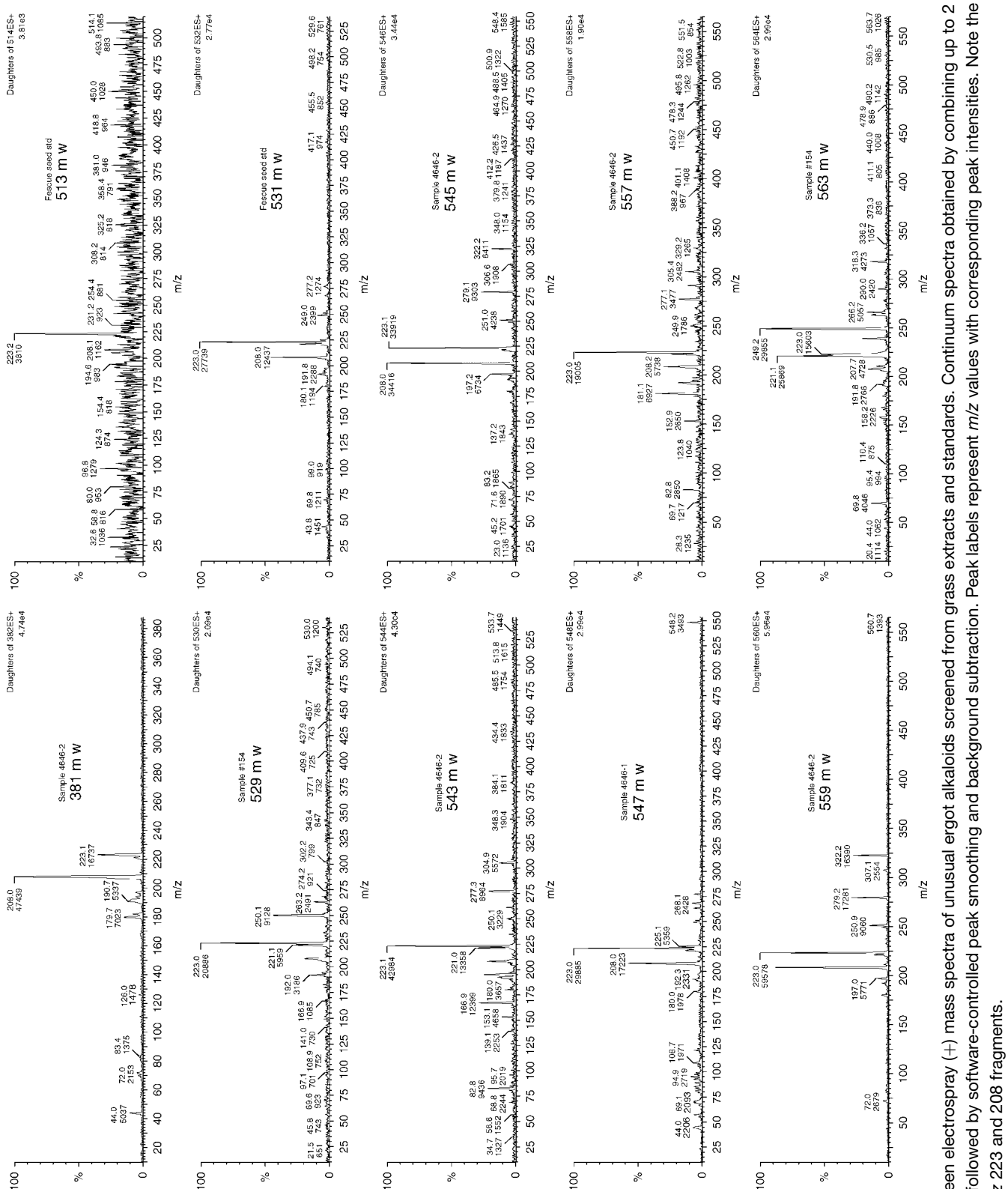


Figure 5. Fourteen electrospray (+) mass spectra of unusual ergot alkaloids screened from grass extracts and standards. Continuum spectra obtained by combining up to 2 min of acquired data, followed by software-controlled peak smoothing and background subtraction. Peak labels represent *m/z* values with corresponding peak intensities. Note the consistent presence of *m/z* 223 and 208 fragments.

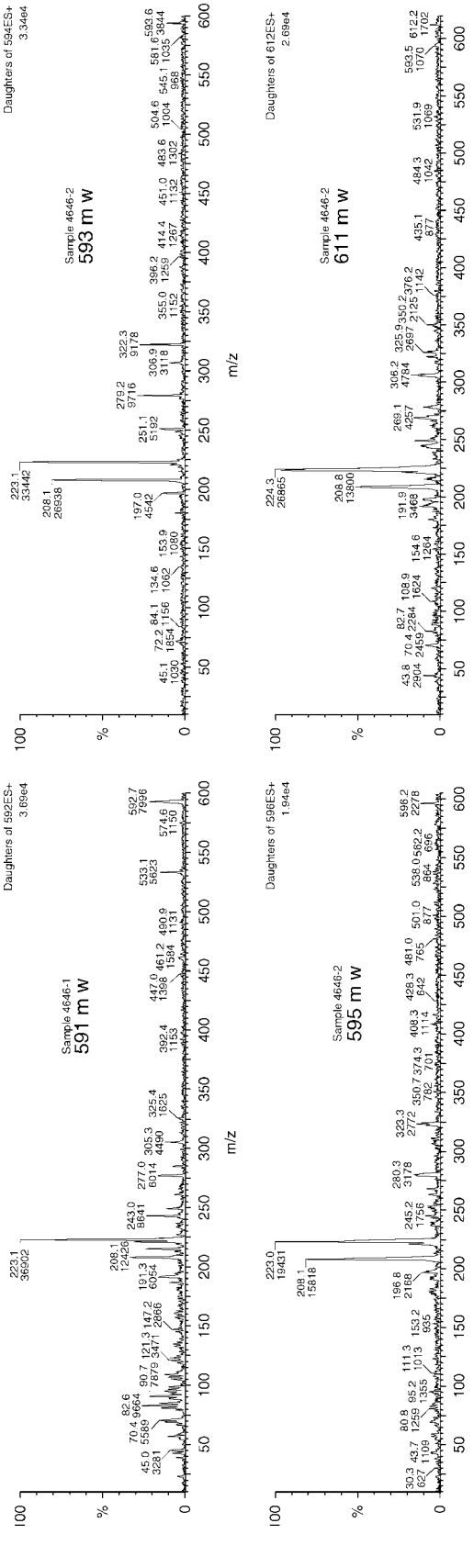


Figure 5. (*Continued*).

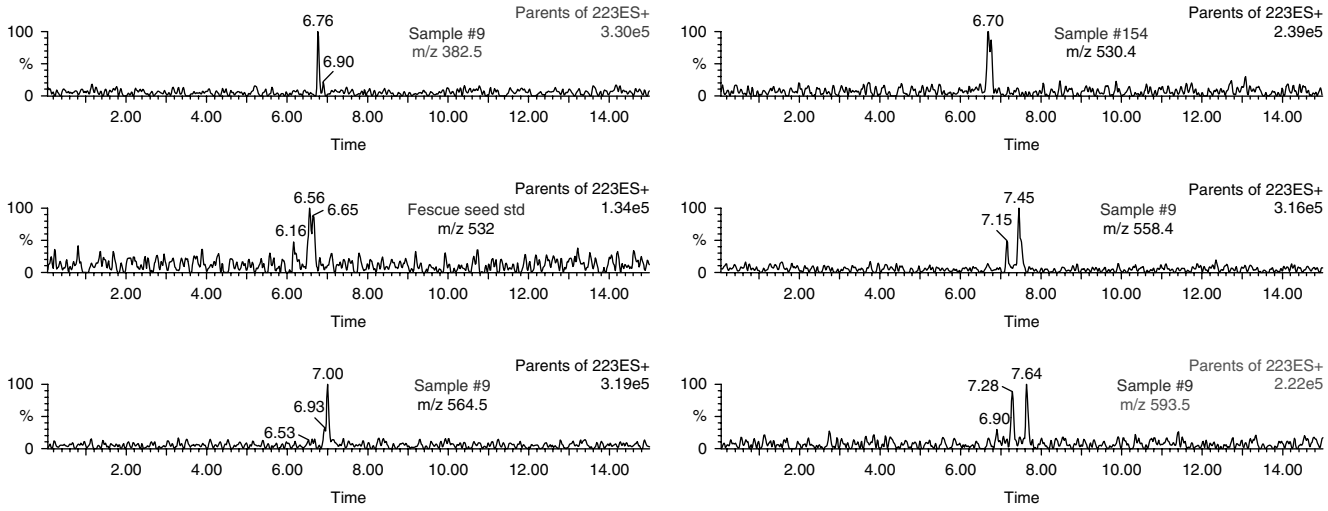
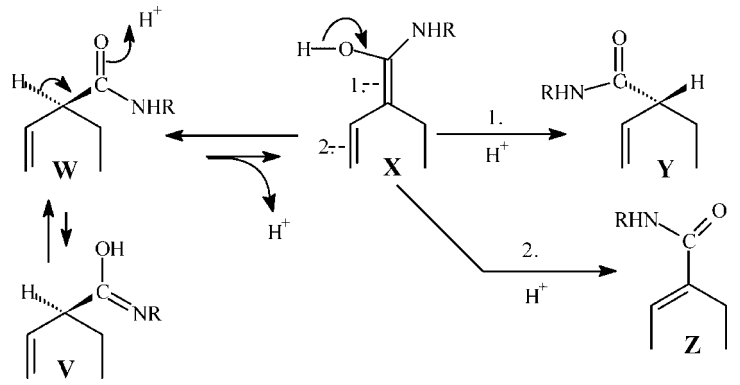


Figure 6. LC/MS/MS selected ion scans corresponding to unique ergot alkaloids. Scans for *m/z* 382.5, 530.4, 532.0, 558.4, 564.5 and 593.5 correspond to nominal molecular weights 381, 529, 531, 557, 563 and 593, respectively. Data collected as parent ion scans for a common mass spectral fragment, *m/z* 223.



Scheme 3. Proposed acid-catalyzed rearrangements of ergot alkaloid D-ring substituents. Acid attack at the amide oxygen in **W** enables generation of the keto-enol tautomer **X** stabilized by conjugation of double bonds 1 and 2. This process competes at some rate with amide tautomerization **W** → **V** (amide-iminol rearrangement). Rearrangement of **X** with acid attack at double bond 1 can return the original ergot alkaloid epimer **W** or lead to epimerization at C-8 with generation of **Y**. The question arises as to whether acid attack can occur at double bond 2 for generation of the conjugation-stabilized product **Z**. Acid-induced rearrangements and conceivable double bond migration appear to preclude generation of the 269-mw contaminant (structure in Scheme 1) in the absence of an additional hydrogenation step.

transition state may well be posited for the paspalic-lysergic acid conversion as well. In any case, current speculation is that the indole portion of the lysergic acid (rings A and B; Fig. 1) may not play a role in its recognition by the lysergylpeptidyl synthetase 2,[40] leaving the specific configuration of rings C and D crucial for the biosynthetic elaboration of the ergopeptides. In such a scenario, absence of the double bond (dihydrolysergamide precursors; Scheme 1) or its rearrangement (theoretical type Z compounds; Scheme 3) may be biosynthetic dead ends.

However, hydrogenation of the D-ring double bond may not necessarily be a dead end as revealed below. If one examines the molecular weights of unique ergot alkaloids identified in this paper (Tables 4–6), one derives the mw list 381, 513, 529, 531, 543, 545, 547, 557, 559, 563, 577, 591, 593, 595 and 611. If one, next, makes the assumption that only Fig. 1 substituents (methyl, isopropyl, isobutyl and benzyl corresponding to amino acid biosynthetic precursors alanine, valine, leucine and phenylalanine, respectively) contribute to ergot alkaloid structure, and then one calculates all possible combinations, one develops the mw list 505, 533, 547, 561, 575, 581, 589, 609, 623 and 657 with underlined values corresponding to structures tabulated in Fig. 1. However, mw 547 overlaps our previous list, encouraging us to examine further. If one allows the fact that dehydrated products arise by mechanisms similar to that in Scheme 2, then the above list becomes 487, 515, 529, 543, 557, 563, 571, 591, 605 and 639. Our original list of unknowns now overlaps with mw 529, 543, 557, 563 and 591. Of the remainder in our initial list of unknowns, mw 531, 545, 559, 593 and 611 differ from those deduced previously by addition of two hydrogen atoms, and the discovery of dihydrolysergamide (Scheme 1) as an ergot alkaloid by-product or contaminant therefore suggests ergot ring D as a likely site of such hydrogenation. Since the above

Table 7. Hypothetical structures proposed to accommodate new ergot alkaloid molecular weights seen principally in Table 6. Refer to Fig. 1 or Scheme 2 for location of substituents: 2′ or 5′ substituents based on those found in literature; C12′-C 11′ disposition refers to either normal hydroxylated [C(OH)-CH] structure or dehydrated double bond [C═C]; C9–C10 disposition refers to normal D-ring double bond [C═C] or its hydrogenated alternative [CH–CH_2]

mw	2′ [or 5′] substituent	5′ [or 2′] substituent	C12′–C11′ disposition	C9–C10 disposition	Supporting precedent(s) in scientific literature
513	Methyl	Isopropyl minus H_2	C═C	C═CH	Putative C ring dehydrogenation[16]
529	Methyl	Butyl[a]	C═C	C═CH	
531	Methyl	Butyl[a]	C═C	CH-CH_2	Alternative is didehydroergovaline[12]
543	Isopropyl	Isopropyl	C═C	C═CH	
545	Isopropyl	Isopropyl	C═C	CH-CH_2	
547	Methyl	Butyl[a]	C(OH)-CH	C═CH	Isomeric with ergonine and ergosine[46]
557	Isopropyl	Butyl[a]	C═C	C═CH	
559	Isopropyl	Butyl[a]	C═C	CH-CH_2	
563	Methyl	Benzyl	C═C	C═CH	Semisynthetic dihydroergocornine[21]
577	Isopropyl	Butyl[a]	C(OH)-CH	CH-CH_2	Semisynthetic dihydroergocryptine[21]
591	Isopropyl	Benzyl	C═C	C═CH	
593	Isopropyl	Benzyl	C═C	CH-CH_2	
595	Ethyl	Benzyl	C(OH)-CH	C═CH	Ethyl substituents in ergoxin group;[46] isomeric with ergostine
611	Isopropyl	Benzyl	C(OH)-CH	CH-CH_2	Semisynthetic dihydroergocristine[21]

[a] Butyl substituent can be either *sec*-butyl or isobutyl.

mw patterns thus offer internally consistent guidelines for their interpretation and require only the straightforward assumptions of similarity of amino acid precursors and possibilities of dehydration and/or hydrogenation, we felt encouraged to offer potential assignments of all the unknowns as listed in Table 7.

Please note that butyl groups in Table 7 can occur as either isobutyl (derived from leucine) or *sec*-butyl (derived from isoleucine) by comparison with known ergot structure.[46] To accommodate the three remaining unexplained mw's (381, 513 and 595) one might introduce the ethyl group as a possible substituent as found in the ergoxin group of ergopeptides, which includes ergonine, ergoptine and ergostine.[46] The ethyl group introduces mw's 519, 533, 547, 561 and 595 by our previous reasoning, thus accommodating 595 while also offering an alternative possibility for 547. Molecular weight 513, on the other hand, might require the possibility of a methyl/isopropyl substituent pair with an additional double bond and corresponding loss of H_2. Finally, since, by the reasoning introduced here, the smallest ergopeptide would be a methyl/methyl-dehydrated product at 487 mw, 381 would necessarily be a nonpeptide ergot alkaloid. One possibility that arises is substitution of ergonovine's side chain methyl group with isobutyl to give mw 367, followed by addition of an extra methyl group possibly by hydroxyl group methylation, for example.

CONCLUSIONS

Ergot alkaloid diastereomers, where they were found, displayed differences in the relative abundances of transitions between variants, e.g. the intensities of m/z 562 → 277 and m/z 562 → 223 for the ergocornine variants seen in Fig. 2(B), relative to the major m/z 562 → 305 fragmentation in each case. Another example of diastereomeric distinctions is seen with ergocrystine, Fig. 2(E), where, relative to the major m/z 610 → 305 fragmentation, m/z 610 → 325 and 610 → 223 basically switch relative positions under tuning conditions basically identical to those of ergocornine in Fig. 2(B). Differences of an unrelated type can be seen on comparing the 7.01-min ergocornine peak in Fig. 2(A) with that in Fig. 2(B), but in this case, they arise solely from differences in MS-tuning parameters between these experiments, particularly MS2 ionization energy. In general, one may attribute diastereomerization to effects of solution pH,[47] although it may not be possible to rule out heat or absorption of UV light as contributing factors.

Discernment of the mass spectrometric properties of a common 269-mw contaminant enabled its HPLC detection and demonstration in ergot alkaloid standards. Structural features in common between the parental compounds listed (ergocryptine, ergocornine, ergocrystine, ergonovine, ergotamine, ergovaline, lysergic acid and lysergol) led us to suspect a hydrogenated lysergamide as a likely explanation for this contaminant (Scheme 1), although this remains to be

proven by comparison with synthetic standards. The structure could then be a dihydrogenated variant of the breakdown product ergine, i.e. lysergic acid amide or lysergamide, the discovery of which as a methanolic-alkali-induced component of ergot alkaloid mixtures goes back to the 1930s (see Ref. 48 and references therein). This compound, dihydrolysergamide, does not appear to be a chromatography-induced artifact since (1) it can be distinguished chromatographically from the principal components at a consistent retention time under nonacidic autosampler storage and mobile-phase conditions (visualization under direct infusion ESI(+) with mild acid alone might have suggested otherwise); and (2) acid or alkali treatment alone could account for double bond migrations (Scheme 3) and specific cleavages, but an additional act of hydrogenation is likely required to achieve the compound's molecular weight.

Simple chemical variants of the ergot alkaloids are listed as possibilities in Table 5. The dihydrogenated variants of ergocrystine and ergocryptine are likely to be actually present as such, although the exact site(s) of hydrogenation is difficult to discern without further study. On the other hand, the dehydrated products, such as that described for ergocrystine in Fig. 2(F), carry with them the conundrum that they may have been formed accidentally during handling; a related possibility is that they were inadvertently formed in the instrument during analysis. Additional, almost anecdotal, information can be brought to bear on this latter possibility. Specifically, E^--fescue seed lacks ergotamine dehydrate, whereas Table 3 indicates that this sample does contain ergotamine, suggesting that ergotamine does not always spontaneously dehydrate under the same set of conditions. A similar point can be made with regard to ergocornine present in Samples #171, 4646-1 and 4646-2, and the absence of the dehydrated product in these samples. Also, Samples #4682 and NLC hay control contain ergocrystine dehydrate, whereas ergocrystine itself was not found, suggesting in this case that the presence of the dehydrated product was not ergocrystine dependent.

Molecular weight analysis for new ergot alkaloid species identified in the course of this research indicates that they are highly likely to follow precise rules including hydrogenation and dehydration and that they most likely reflect biosynthesis requiring combinations of amino acids alanine, valine, leucine, isoleucine or phenylalanine to give methyl, isopropyl, isobutyl, *sec*-butyl or benzyl 2′ or 5′ substituents, respectively.

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