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Development and Validation of an LC-MS Method for Quantitation of Ergot Alkaloids in Lateral Saphenous Vein Tissue

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A liquid chromatography−mass spectrometry (LC-MS) method for simultaneous quantitation of seven ergot alkaloids (lysergic acid, ergonovine, ergovaline, ergocornine, ergotamine, ergocryptine, and ergocristine) in vascular tissue was developed and validated. Reverse-phase chromatography, coupled to an electrospray ionization source, was used to separate and ionize alkaloids. Singly protonated molecular ions for each alkaloid and methysergide (internal standard), were detected by single-ion monitoring (SIM). Calibration curves were obtained over a linear range of 0.1 to 40 pmol on column with correlation coefficients better than 0.994. Method recoveries were 68.4% to 111.0%. Intra-assay precision was 3.4% to 16.1%. Matrix effects were observed and overcome by introducing matrix components into calibrant solutions to create matrix-diluted standards. Limits of detection and quantitation were 0.05 pmol and 0.1 pmol, respectively. Method ruggedness tests resulted in recoveries of 86.1 to 122% with an interassay precision of 7.9% to 22.8%. These results indicate that this method is suitable for quantitation of alkaloids extracted from in vitro-exposed vascular tissue.

KEYWORDS: Ergot alkaloids; LC-MS; quantitation; vein tissue

INTRODUCTION

Fescue toxicosis is a costly animal syndrome resulting in nearly one billion dollars in annual production losses for forage-animal (i.e., cattle, sheep, horses, and goats) enterprises within the United States of America (*1*). The syndrome has been linked to the consumption of tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh] containing ergot alkaloids produced by a symbiotic endophytic fungus (*Neotyphodium coenophialum*) located within the plant (*2*). The ergot alkaloids investigated share an ergoline ring in their basic chemical structure (**Figure 1**). Many ergot alkaloids have a tripeptide structure bonded to the ergoline ring at the C8 position by an amide bond between the two lactam rings and are known as ergopeptines (exceptions include lysergic acid and ergonovine). Various chemical moieties at two substituent sites, denoted as R_1 and R_2 in **Figure 1**, distinguish one ergopeptide from another. The ergoline ring structure, found in all ergot alkaloids, is similar to that of neurotransmitters including dopamine, norepinephrine, epinephrine, and serotonin (*3*). The structural similarities to neurotransmitters may account for the physiological responses (e.g., vasoconstriction and decreased prolactin secretion) noted in animals suffering from fescue toxicosis (*2*). To fully understand and mitigate the impact of these alkaloids on the physiology of the animal, identification and quantitation of these alkaloids within biological tissues and fluids are required.

Currently, the primary methods for quantitation of ergot alkaloids associated with fescue toxicosis include quantitating individual alkaloids using high performance liquid chromatography (HPLC) with fluorometric detection (*4*, *5*) and a competitive enzyme-linked immunosorbent assay (*6*) for total alkaloid level determination within a given matrix. In addition, HPLC-mass spectrometry (LC-MS) has been used to identify and quantitate individual alkaloids (*7*−*9*) within plant material. Recent research has focused on the study of these alkaloids and their effects on vascular activity in vitro using an isolated blood vessel assay (*10*, *11*). The more complex ergopeptines, specifically ergovaline (**Figure 1**), appear to bioaccumulate in vascular tissue exposed in vitro, as evidenced by sustained vascular contraction following the removal of alkaloids from the tissue media (*11*); no such response is apparent for the simpler ergot alkaloids, specifically lysergic acid, to support bioaccumulation (*12*). In order to determine if bioaccumulation is the cause of the sustained vascular contraction, a quantitative LC-MS method for the identification and quantitation of ergot alkaloids in vascular tissue was developed and validated.

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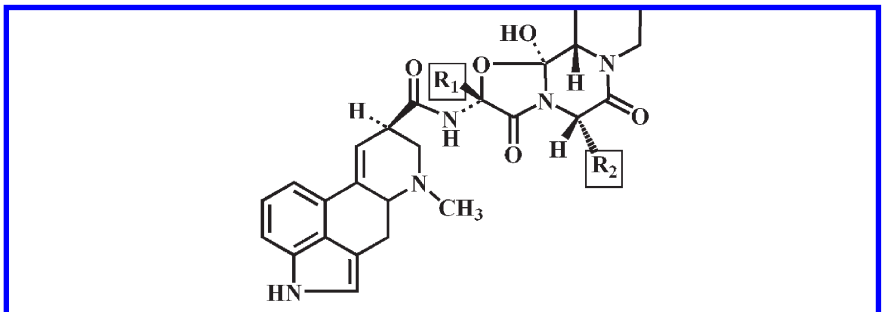
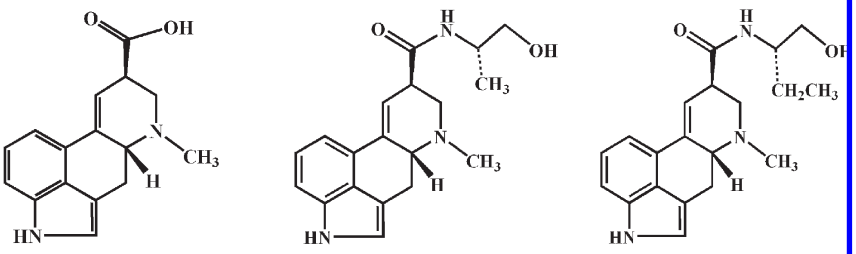


General Ergopeptine Structure



Lysergic Acid Ergonovine Methysergide

Ergot Alkaloid	$[M+H^+]^+$ (m/z)	R_1	R_2
Lysergic Acid / Lysergic Acid Epimer	269	NA	NA
Ergonovine	326	NA	NA
Methysergide	354	NA	NA
Ergovaline / Ergovalinine	534	$-CH_3$	$-CH-(CH_3)_2$
Ergocornine / Ergocorninine	562	$-CH-(CH_3)_2$	$-CH-(CH_3)_2$
Ergocryptine / Ergocryptinine	576	$-CH_2-CH-(CH_3)_2$	$-CH-(CH_3)_2$
Ergotamine / Ergotaminine	582	$-CH_3$	$-CH_2$-Phenyl
Ergocristine / Ergocristinine	610	$-CH-(CH_3)_2$	$-CH_2$-Phenyl

Figure 1. Chemical structures for ergot alkaloids and related compounds.

MATERIALS AND METHODS

Chemicals and Reagents. Ergonovine maleate, methysergide maleate, ergocornine, α-ergocryptine, ergotamine D-tartrate, and α-ergocristine were purchased in solid, purified forms (≥97%) from Sigma-Aldrich (St. Louis, MO, USA). D-Lysergic acid dihydrate was purchased from Acros Organics (95% purity; Geel, Belgium). Ergovaline tartrate was obtained as a custom synthetic product (≥93% purity with ~1% (w/w) ergotamine contamination) from F.T. Smith (Pharmaceutical Sciences, Auburn University, Auburn, AL, USA). Methanol (HPLC-grade), acetonitrile (Fisher Optima grade) and formic acid (reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Purified water was generated by a Milli-Q reagent water system (Millipore Corporation, Bedford, MA, USA).

Vein Collection, Processing, and Extraction. Segments (2 to 3 cm in length) of the cranial branch of the lateral saphenous vein were collected at local abattoirs from healthy cattle of mixed breed and gender immediately after slaughter as previously described in ref *10*. Briefly, venous tissue was placed in a modified-Krebs−Henseleit oxygenated buffer solution (95% O_2/5% CO_2; pH 7.4; mM composition = D-glucose, 11.1; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118.1; $CaCl_2$, 3.4; and $NaHCO_3$, 24.9; Sigma Chemical Co., St. Louis, MO, USA) and kept on ice for transport back to the laboratory where it could be processed. Excess fat and connective tissue were carefully removed from the exterior of the segments, which were subsequently sliced into 2- to 3-mm cross-sections. The cross-sections were placed in individual 1.5 mL microcentrifuge tubes and stored at −20 °C. Once frozen, samples were freeze-dried (Botanique Preservation Equipment Inc., Peoria, AZ, USA) and returned to storage at −20 °C until extraction for analysis.

For extraction, freeze-dried vein tissue segments (4 to 5 mg dry weight) were ground in liquid nitrogen with a mortar and pestle and transferred to a capped, glass 15 mL centrifuge tube. Methysergide (**Figure 1**) was chosen as the internal standard since it is a chemical analogue of the naturally occurring ergot alkaloids. The methysergide was added to the tissue (40 pmol), taking care to spike the solution directly on the tissue and not on the inner walls of the tube. One milliliter of methanol was added to each tube. The extraction was allowed to proceed for 2 h at 30 °C in an Innova 4300 incubator shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA) set at 350 rpm. The tubes were then centrifuged at 3500 rpm (2524*g*), 30 °C with a CR422 swinging bucket rotor (Jouan, Inc., Winchester, VA, USA) for 10 min. The supernatant was transferred to a 2-mL amber autosampler vial and dried under ultrahigh purity nitrogen in a heat block set to 60 °C. The residue was resuspended in 200 μL of initial mobile phase (5% (v/v) aqueous acetonitrile with 0.1% (v/v) formic acid) and transferred to a limited volume vial insert for analysis.

Chromatography and MS Analysis. Reverse-phase chromatographic separation of seven ergot alkaloids (lysergic acid, ergonovine, ergovaline, ergocornine, ergotamine, ergocryptine, and ergocristine) and the internal standard (methysergide) was achieved. The separation was performed on a liquid chromatograph, consisting of a binary pump and an autosampler, directly coupled to a Varian 1200 L triple quadrupole mass spectrometer with an electrospray ionization (ESI) source (Varian, Inc., Walnut Creek, CA, USA). The mobile phase was delivered at a constant flow rate of 0.2 mL/min. The column used was a 150 mm × 2.0 mm i.d., 5 μm, Gemini C18, preceded by a 4.0 mm × 2.0 mm i.d. guard column of the same material (Phenomenex, Torrance, CA, USA). The binary mobile phase consisted of 0.1% (v/v) aqueous formic acid (mobile phase A) and

0.1% (v/v) formic acid in acetonitrile (mobile phase B), each of which was degassed by helium sparging for 10 min prior to use. Sample extracts and standard solutions (50 μL) were injected into the initial gradient conditions of 95% mobile phase A/5% mobile phase B, which were maintained for 1 min following injection. Mobile phase B was increased at a linear rate to 20% over the next 9 min and then increased further to 30% over the subsequent 18 min to chromatographically resolve all of the ergot alkaloids. The gradient profile was then stepped to 95% B over 0.01 min and held for 5 min to wash the LC column of any residual nonpolar matrix components. At 34 min after injection, the initial mobile phase conditions were set to allow re-equilibration of the LC column in preparation for the next analysis. The total run time from one injection to the subsequent injection was 43 min, while data acquisition occurred from 5 to 35 min postinjection.

Chromatograms from single-ion monitoring (SIM) of singly protonated molecular ions ($[M + H^+]^+$) were used for quantitation of the ergot alkaloids. The mass spectrometer was operated in positive ion mode and unit mass resolution with the source housing held at 50 °C. The ESI needle voltage and shield and capillary potentials were set at 5000, 600, and 100 V, respectively. The drying and nebulizing gases were supplied at 18 and 44 psi, respectively, from the boil-off of a liquid nitrogen Dewar, with the drying gas temperature set at 200 °C. The collision-induced dissociation gas (argon) was not needed for these SIM experiments and thus was not turned on. Eight different scan channels were acquired simultaneously, corresponding to each of the singly protonated molecular ions for the analytes (**Figure 1**), with a dwell time of 1 scan/s and a multiplier setting of −1800 V.

Linearity and Matrix Effects. Two series of standard solutions containing known quantities of the ergot alkaloids, one set with and one set without matrix components, were analyzed to determine the degree of matrix effects on the overall signal. Individual stock solutions of ergonovine, ergovaline, ergocornine, ergocryptine, and ergocristine were prepared at 8 mM concentrations in methanol. Since lysergic acid and ergotamine were slightly insoluble in methanol at this concentration, lysergic acid was suspended in 80% (v/v) aqueous methanol to which 0.2 M acetic acid was added dropwise until lysergic acid was completely dissolved, while the ergotamine stock solution was prepared at 4 mM in methanol. Stock solutions were protected from light in amber vials and stored at −20 °C. From the concentrated stock solutions, a secondary stock mixture solution (known as a working stock solution) was prepared with a final concentration of 0.1 mM for each alkaloid by mixing 12.5 μL of each individual stock solution and 25 μL of 4 mM ergotamine stock solution with methanol for a final volume of 1000 μL. For the matrix-free calibration curve, the 0.1 mM working stock solution was serially diluted in the initial mobile phase solution, 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. Similarly, calibration curves containing tissue matrix components were prepared by serial dilution of the 0.1 mM working stock solution with reconstituted extract from vein tissue void of alkaloids (~5 mg freeze-dried tissue per calibrant solution). Methysergide was added as an internal standard to each calibrant solution at a final concentration of 0.2 μM, equivalent to 10 pmol injected on column with a 50 μL injection volume. Both sets of calibrant solutions were analyzed by LC-MS, and the resultant chromatographic peak areas were used to construct either matrix-free or matrix-diluted calibration curves for comparison.

Matrix effects were determined by statistically comparing the slopes of the calibration curves for matrix-free and matrix-diluted standards using a Student's t test (*13*). The pooled error of the slopes was calculated using eq .

$$s_{b(p)} = s_{y \cdot x(p)} \left(\left(1/\sum x^2 \right)_1 + \left(1/\sum x^2 \right)_2 \right)^{1/2} \quad (1)$$

where $s_{y \cdot x(p)}$ is the pooled standard error of the estimates, a measure of the uncertainty in the instrument response (y values on the calibration curves), and was calculated using eq .

$$s_{y \cdot x(p)} = [((n_1 - 2)s^2_{y \cdot x(1)}) + ((n_2 - 2)s^2_{y \cdot x(2)}))/(n_1 + n_2 - 4)]^{1/2} \quad (2)$$

where $s^2_{y \cdot x(1)}$ and $s^2_{y \cdot x(2)}$ are the variances in the matrix-free and matrix-diluted data sets, respectively, and the factor $(n_1 + n_2 - 4)$ represents the pooled number of degrees of freedom.

A t value was calculated by dividing the absolute value of the difference between the slopes by the pooled number of degrees of freedom. The calculated t value was then compared to the Student's t value at the 95% confidence level (*14*) for the corresponding number of degrees of freedom.

Method Recovery. Method recoveries at two different ergot alkaloid concentration levels, 4 and 40 pmol (equivalent to 1 and 10 pmol injected on column), were investigated. Briefly, segments of freeze-dried vein that were void of ergot alkaloids were spiked with methysergide (40 pmol) and all ergot alkaloids (4 or 40 pmol), taking care to spike the solutions directly on the tissue and not on the inner walls of the tube. The veins were then extracted using methanol and preconcentrated for LC-MS analysis. As a measure of method ruggedness, the developed method recovery steps were repeated in triplicate for 3 days (resulting in 9 separate vein extracts) and analyzed using the LC-MS analytical parameters in an independent laboratory on a Varian 1200 L triple quadrupole mass spectrometer. Finally, to ensure that the conditions of the extraction procedure and the sample preconcentration steps did not chemically degrade the ergopeptines to lysergic acid or ergonovine, a separate method recovery experiment was performed in which all ergot alkaloids except lysergic acid and ergonovine were added to the tissues.

RESULTS AND DISCUSSION

Chromatography and MS Analysis. Reconstructed ion chromatograms are reported in **Figure 2**, in which the detector current for a specific scan channel that corresponds to the singly protonated molecular ion of each ergot alkaloid is plotted. Singly protonated molecular ions were detected by setting the instrument to the particular m/z of the ergot alkaloid of interest, corresponding to $[M + H^+]^+$ (**Figure 1**), for a scan channel (i.e., SIM). Eight different scan channels, monitoring the seven individual ergot alkaloids and the internal standard, are recorded simultaneously over the course of one analytical run at a rate of 1 scan/s. Although a single m/z value was monitored per scan channel, some of the ion chromatograms produced two chromatographic peaks. It has been reported that ergot alkaloids can undergo epimerization around the peptide bond connected to the ergoline portion of the molecule (*15*). The epimer would have the same molecular weight and subsequent mass spectrometric information as a particular ergot alkaloid and was routinely observed for ergovaline, ergocryptine, ergotamine, and ergocristine. This isomerization reaction can occur rapidly, depending on the acidity or alkalinity of the extraction solvent and the storage conditions of the solutions (*15*, *16*). The -inine epimers are not thought to be physiologically active in the animal (*17*). However, given that the epimerization process occurs through a reversible reaction, the epimers could contribute to toxicity to the forage animal by converting back to the active form (*18*). Developed methods for quantitation on the accumulation of an ergot alkaloid in a given animal tissue should include both forms. Since the change in molecular conformation of the epimer resulted in a change in retention times when compared to the corresponding native alkaloid, chromatographic conditions were established to optimize separation and peak resolution of all of the ergot alkaloids and any corresponding epimers analyzed at a constant flow rate of 0.2 mL/min. The final gradient chromatographic conditions were insufficient to prevent coelution of ergocornine and ergotamine/ergotaminine. However, peak areas for these compounds can be isolated from each other using reconstructed ion chromatograms in which only the m/z of the singly protonated molecular ion of interest is plotted. The quantitation method reported uses the combined peak areas of the ergot alkaloids and corresponding epimers to determine a total alkaloid concentration. Reference standard material was not available for the epimeric forms of the alkaloids; thus, a separate and accurate quantitation of the individual stereoisomers was not possible.

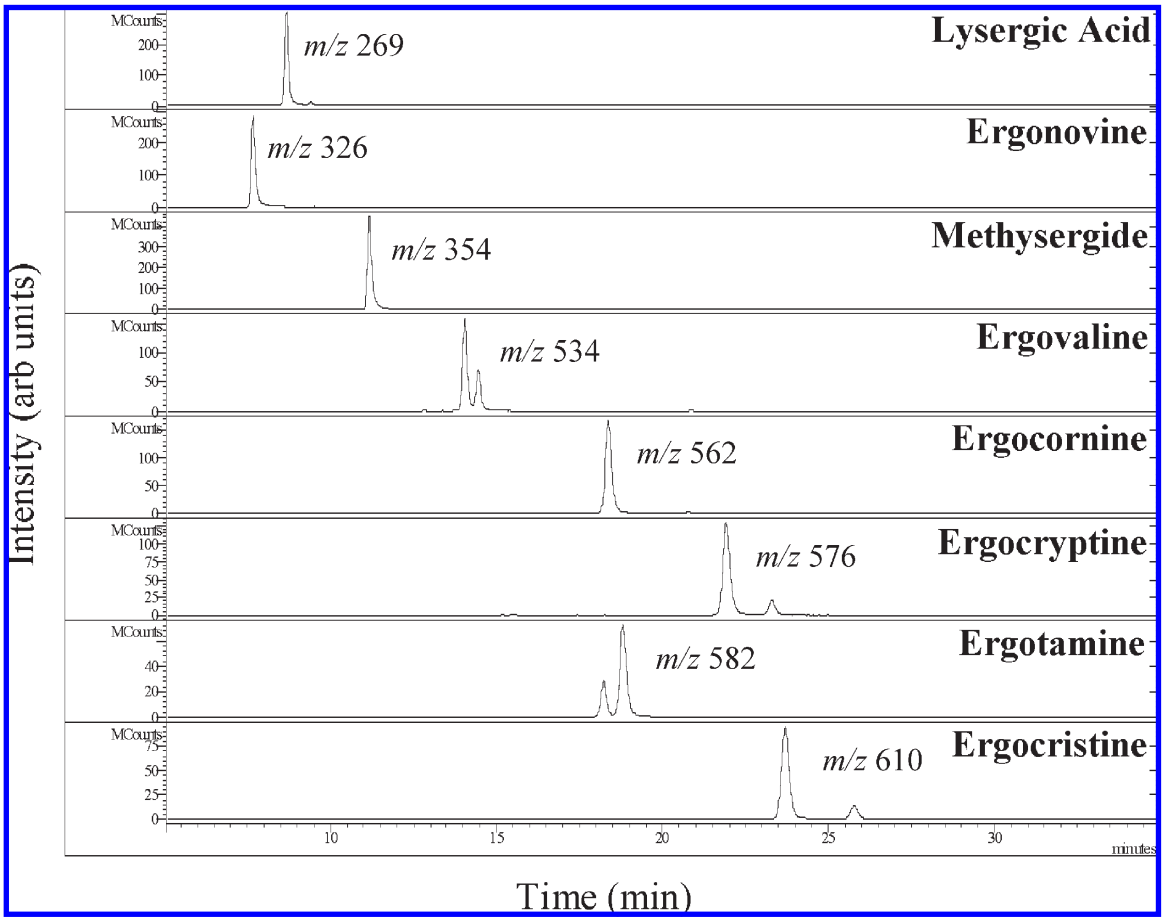


Figure 2. Reconstructed ion current at the detector for each singly protonated ($[M + H^+]^+$) form of the ergot alkaloids present in a standard solution mixture (10 pmol of each alkaloid injected on column).

Although several researchers have elected to use selected reaction monitoring (SRM) (*19*, *20*) or multiple reaction monitoring (MRM) (*9*, *21*) for ergot alkaloid detection, in which fragments of a given precursor ion are monitored, SIM of the precursor ions was utilized in the present study. While the SIM detection scheme is not as specific to each alkaloid as SRM or MRM, it is adequate for the goals of the developed in vitro method. The samples for which this method was developed are physically cleaned of most extraneous connective tissue and fat deposits initially and are then exposed to known concentrations of selected ergot alkaloids using an in vitro vascular bioassay (*10*). Vein tissue harvested from an animal that was not consuming alkaloid-containing forages immediately prior to the time of tissue collection was extracted and used as blank vein extract. It was confirmed that any prior consumption of endophyte-infected tall fescue and concomitant exposure to ergot alkaloids did not result in detectable background levels of alkaloids that would interfere with the ergot alkaloid analysis (data not shown). The very clean physical nature of these samples, as well as the absence of peak interferences in the blank vein extract, suggests SIM would be adequate. The combination of correlating chromatographic retention times and molecular ion *m*/*z* values between standard ergot alkaloid solutions and unknown extracts provides a 2-fold degree of peak identification. However, if this method is applied to biological samples that have been exposed to unknown quantities and types of alkaloids through in vivo experiments (i.e., alkaloid exposure through consumption of alkaloid-containing forages and/or seed in grazing and feedlot studies), then a greater degree of specificity may be required for accurate compound identification.

Assay Performance. *Linearity, Range, and Matrix Effects.* Using the developed SIM method, the linear dynamic range for each ergot alkaloid was determined by creating individual calibration curves over a range of ergot alkaloid concentrations. The working standards were prepared by serial dilution of a stock mixture solution comprising equimolar quantities of the seven individual ergot alkaloids, as previously described. Methysergide, the internal standard, was added to each working standard solution at a concentration equivalent to 10 pmol injected on column. The lower limit of the linear dynamic range in the presence of matrix components, also known as the limit of detection (LOD), was defined as the concentration at which a signal-to-noise ratio of 3 is routinely obtained. For this method, an alkaloid concentration of 1 nM (equivalent to 0.05 pmol injected on the column) is the LOD for all ergot alkaloids. The limit of quantitation (LOQ) was defined as the concentration at which a signal-to-noise ratio of 10 is routinely obtained. In the presence of matrix components, an alkaloid concentration of 2 nM (equivalent to 0.1 pmol injected on the column) is the LOQ for all ergot alkaloids. The LOQ for this method compares favorably to reported data (*19*) of 38 pg on column for ergonovine, ergotamine, ergocornine, ergocryptine (equivalent to 0.07 pmol to 0.12 pmol, depending on molecular mass), and 62 pg for ergocristine (equivalent to 0.10 pmol).

The typical dynamic linear range, for the method reported herein, was determined to be 0.1 to 40 pmol injected on column for lysergic acid and ergonovine, respectively. The ergopeptines showed a slightly wider range of linearity, 0.1 to 50 pmol injected on column. The upper limit of the linear dynamic range was determined on the basis of MS detector response. At concentrations

higher than the most concentrated standard, the detector signal was maximized, and the autointegration of peak areas by the instrument control software was routinely inaccurate by an order of magnitude or more. These ranges of linearity are narrower than those reported using SRM (*20*) and MRM (*9*) detection schemes, respectively. However, it is not expected that vein tissue samples will routinely have ergot alkaloid levels that exceed the upper concentration limits. Should this occur, the extraction sample could be diluted to achieve instrument responses within the linear dynamic range for quantitative analysis.

To determine if matrix components contribute to either suppression or enhancement of ion signal, a range of ergot alkaloid standards was prepared using blank vein extraction aliquots as the diluent and analyzed using the developed SIM method. The instrument response was plotted as the ratio of the chromatographic peak areas for a given alkaloid (and its epimer, if applicable) to the internal standard, methysergide, versus the ratio of the quantities of alkaloid to internal standard injected. An average instrument response of triplicate injections was used to create the calibration curve and determine a best-fit linear trendline. The slope of the trendline for matrix-diluted calibrants was then compared to the trendline slope for matrix-free calibrants that had been diluted with initial mobile phase solvent (5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid overall). While the addition of matrix components in calibration standard solutions did not cause deviation from linearity in instrument response, the response factor did decrease, resulting in a lower slope in the presence of matrix components. Correlation coefficients (r^2) for both sets of standards ranged from 0.9943 to 0.9998 and did not appear to be affected by the presence of matrix components. The slopes, intercepts and correlation coefficients for these linear trendlines for all of the ergot alkaloids are reported in **Table 1**.

To measure the presence of matrix effects, a signal suppression/enhancement percent (SSE (%)) (*9*) was calculated and also reported in **Table 1**.

$$\mathrm{SSE}(\%) = 100 \times (\mathrm{slope}_{\text{matrix-diluted}}/\mathrm{slope}_{\text{matrix-free}}) \qquad (3)$$

Table 1. Slopes, Intercepts, and Correlation Coefficients (r^2) from Linear Trendline Fits to Calibration Curves Produced from Either a Series of Matrix-Free Calibrant Standards or Standards Diluted with Blank Extracts Containing Vein Tissue Matrix Components

	matrix-free standards			matrix-diluted standards			
alkaloids	slope	intercept	r^2	slope	intercept	r^2	SSE (%)[a]
lysergic acid	0.5367	0.0735	0.9981	0.6091	0.0624	0.9972	113.5
ergonovine	0.4862	0.1005	0.9945	0.4852	0.0841	0.9943	99.8
ergovaline	0.4638	0.0505	0.9990	0.3971	0.0254	0.9995	85.6
ergocornine	0.4645	0.0407	0.9993	0.4295	0.0297	0.9994	92.5
ergotamine	0.1506	0.0139	0.9995	0.1407	0.0108	0.9998	93.0
ergocryptine	0.5081	0.0237	0.9996	0.4967	0.0171	0.9997	97.8
ergocristine	0.4127	0.0083	0.9991	0.3726	0.0036	0.9998	90.3

[a] SSE (%): signal suppression/enhancement percent (*9*).

While the SSE (%) factor was a tool by which the calibration curve slopes could be compared between the matrix-free and matrix-diluted data sets easily, the indications regarding degree of matrix effects from assessing the numerical value of the SSE (%) factors were ambiguous. There was no valid means by which a SSE (%) cutoff range could be defined beyond which it could be considered that a definite matrix effect was observed. The Student's *t* test was used to compare the trendline slopes of the calibration curves and determine in what numerical value range for SSE (%) factors a difference in instrument response, as a result of the presence of matrix components, was significant.

The *t* values, calculated from the pooled standard errors of the slopes and the pooled standard errors of estimates in the matrix-free and matrix-diluted data sets (using eqs and), are reported in **Table 2**. Comparison of the calculated *t* values to the Student's *t* values at the 95% confidence level (*14*) showed that ergonovine was the only ergot alkaloid in this method for which there was no statistically significant difference between the slopes for the matrix-diluted and the matrix-free calibration curves. The SSE (%) in **Table 1** for ergonovine was 99.8%, only 0.2% from being parallel to the matrix-free calibration curve slope. From the SSE (%) factor alone, ergocryptine with a value of 97.8% (**Table 1**), might be considered to have no matrix effect, yet the Student's *t* test has shown there is a statistically significant difference between the calibration curve slopes at the 95% confidence level with 8 degrees of freedom.

Method Recovery. Method recoveries at two different concentration levels, 4 and 40 pmol (equivalent to 1 and 10 pmol, respectively, injected on column), were determined. A calibration curve prepared from working standards that were diluted with vein extract matrix components was used to interpolate the known concentrations of ergot alkaloids spiked into the sample vials prior to extraction and recovered throughout the extraction and sample processing steps. Method recovery results are reported in **Table 3**. Complete method recovery for ergot alkaloids using the developed LC-MS method ranged from 68.4% to 111.0%, independent of spike concentration levels. Intra-assay coefficients of variation ranged from 3.4% to 16.1%, although there was no clear correlation between alkaloid structure and

Table 3. Method Recoveries and Intra-Assay (Analyses Performed within One Day) Precision for Two Concentrations of Ergot Alkaloids Spiked into Saphenous Veins for Extraction

	4 pmol spike			40 pmol spike		
alkaloid	*n*	recoveries (%) ± SD	CV (%)	*n*	recoveries (%) ± SD	CV (%)
lysergic acid	3	110.0 ± 15.0	13.6	3	109.2 ± 7.7	7.0
ergonovine	3	111.0 ± 11.7	10.6	3	106.9 ± 3.6	3.4
ergovaline	3	85.7 ± 13.8	16.1	6	90.3 ± 6.8	7.6
ergocornine	3	74.2 ± 5.7	7.7	6	83.1 ± 9.3	11.2
ergotamine	3	74.1 ± 8.5	9.9	6	109.1 ± 5.3	4.9
ergocryptine	3	75.0 ± 5.1	6.8	6	80.5 ± 7.1	8.8
ergocristine	3	68.4 ± 5.0	7.2	6	69.4 ± 3.5	5.0

Table 2. Pooled Standard Errors and *t*-Test Results for the Statistical Comparison of Linear Trendline Fits to Calibration Curves Produced from Either a Series of Matrix-Free Calibrant Standards or Standards Diluted with Blank Extracts Containing Vein Tissue Matrix Components

alkaloids	pooled standard error ($s_{y\cdot x\,(p)}$)	pooled error of slopes ($s_{b(p)}$)	calculated-*t*	table-*t* (95% confidence)[c]	matrix-diluted = matrix-free slope?
lysergic acid	0.051	1.56e-2	4.652	2.447[a]	No
ergonovine	0.066	2.02e-2	0.049	2.447[a]	Yes
ergovaline	0.027	5.62e-3	11.870	2.306[b]	No
ergocornine	0.025	5.27e-3	6.642	2.306[b]	No
ergotamine	0.012	1.28e-3	7.751	2.306[b]	No
ergocryptine	0.020	4.24e-3	2.686	2.306[b]	No
ergocristine	0.021	4.35e-3	9.216	2.306[b]	No

[a] Number of degrees of freedom (df) = $n_1 + n_2$-4; df = 6 for Lysergic acid and Ergonovine. [b] df = 8 for all ergopeptines. [c] Ref *14*.

degree of precision. These findings are within ranges reported by other researchers (*12*, *19*, *20*, *22*) and are acceptable for the application intended.

For complete method validation, it was necessary to determine the degree of precision for method recovery experiments performed on different days (i.e., interassay precision). These experiments were combined with a test of the method ruggedness by performing the interassay recoveries in an independent laboratory. The method recovery/ruggedness experiments were performed in triplicate over 3 days. The LC-MS analyses were performed using the same Varian 1200 L instrument (Varian, Inc., Walnut Creek, CA, USA) with determined instrument parameters. Method recovery results are reported in **Table 4**. The developed LC-MS method used in the independent laboratory generated ergot alkaloid method recoveries ranging from 77.9% to 108.7% for the lower concentration spike (4 pmol) and fell within the recovery range reported for a single day's analyses in **Table 3**. The upper concentration spike (40 pmol) produced recoveries in the range of 91.9% to 122%, values that are much higher than those for a single day's analyses (69.4% to 109.2%; **Table 3**). While these recovery values are much higher for the independent laboratory analyses, the true discrepancy between data sets may lie in the comparison of intra-assay to interassay precisions. For both concentration spike levels, intra-assay coefficients of variation (CV%) ranged from 3.4% to 16.1%, with the higher CV% values usually corresponding to recovery of the lower concentration levels. The interassay precision for the 4 pmol spike level falls within the range of the intra-assay CV% values (7.9% to 17.5%, **Table 3**) and was considered acceptable. However, the variability of between day analyses showed a much larger deviation for the 40 pmol spike from the intra-assay CV% values (3.4% to 11.2%, **Table 2**, vs 13.5% to 22.8%, **Table 4**). Thus, the recovery values are not truly higher for the independent laboratory because there was a higher degree of variation within the sample set. Though it is difficult to differentiate the contributions of transferring the method to a secondary laboratory and general interassay imprecision to the overall variation in the recovery results reported in **Table 4**, one factor was thought to greatly affect the reliability of the interassay results. While all instrument parameters and settings were transferred to an identical instrument in the independent laboratory, care could not be taken to optimize the chromatographic resolution parameters specific to that instrument's hardware (e.g., hesitation of independent laboratory to minimize transfer tubing lengths to reduce reported discrepancies). At lower flow rates, band diffusion within transfer tubing can degrade the overall quality of the separation. As a consequence, peak resolution was compromised for all ergot alkaloids and is most apparent as represented in **Figure 3** for lysergic acid and its epimer. Not only was the baseline resolution of the two peaks lost upon method transfer but also peak tailing becomes problematic for peak area integration. The asymmetry factor for the lysergic acid peak in **Figure 3** was calculated to be 2, which is higher than the typical asymmetry factor upper limit of 1.5 (*23*). The autointegration of these peak areas by the instrument control software was inconsistent, and as a result, manual integration of peak areas was used.

Table 4. Method Recoveries and Inter-Assay (between Day) Precision for Two Concentrations of Ergot Alkaloids Spiked into Saphenous Veins for Extraction and Analyzed by an Independent Laboratory

	4 pmol spike			40 pmol spike		
alkaloid	*n*	recoveries (%) ± SD	CV (%)	*n*	recoveries (%) ± SD	CV (%)
lysergic acid	8	108.7 ± 10.1	9.3	6	91.9 ± 13.2	14.3
ergonovine	9	86.5 ± 15.1	17.5	9	100.7 ± 13.6	13.5
ergovaline	9	105.8 ± 8.3	7.9	9	122.0 ± 20.4	16.7
ergocornine	9	97.2 ± 9.0	9.2	9	113.9 ± 20.0	17.6
ergotamine	9	77.9 ± 7.6	9.7	9	103.1 ± 19.2	18.6
ergocryptine	9	89.8 ± 10.0	11.1	9	110.3 ± 22.6	20.5
ergocristine	9	86.1 ± 12.9	15.0	9	97.5 ± 22.2	22.8

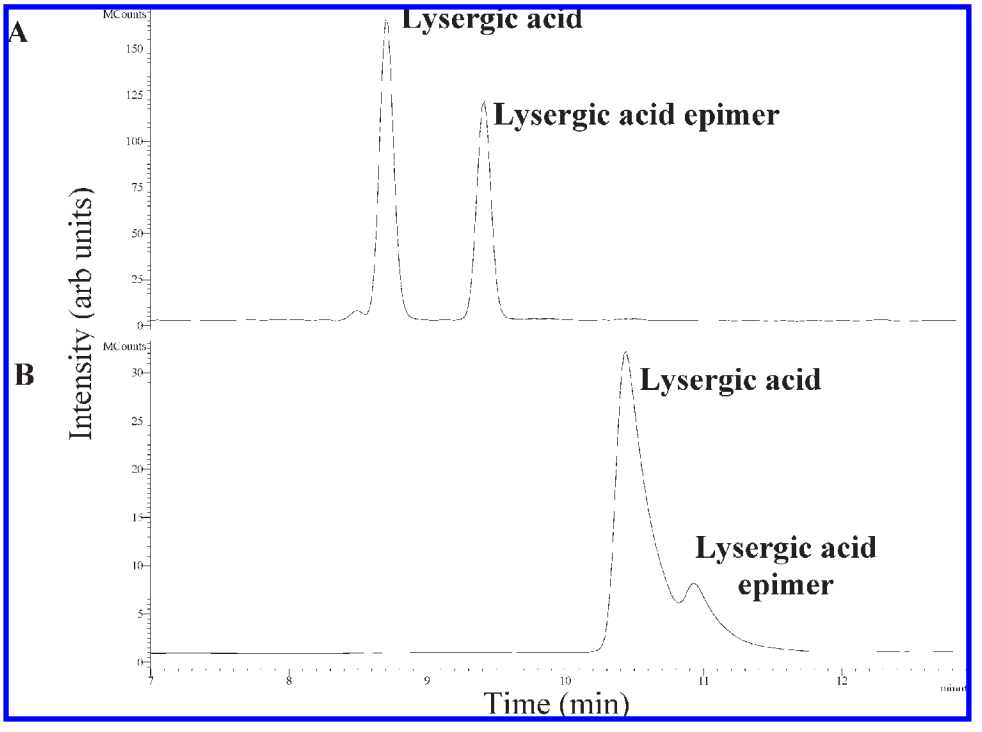


Figure 3. Reconstructed ion chromatograms of the total detector current for singly protonated ($[M + H^+]^+$) lysergic acid and lysergic acid epimer (*m*/*z* 269) generated by data recorded on (**A**) a chromatographically optimized system for maximal peak resolution and (**B**) an independent system to which the reported LC-MS parameters were transferred.

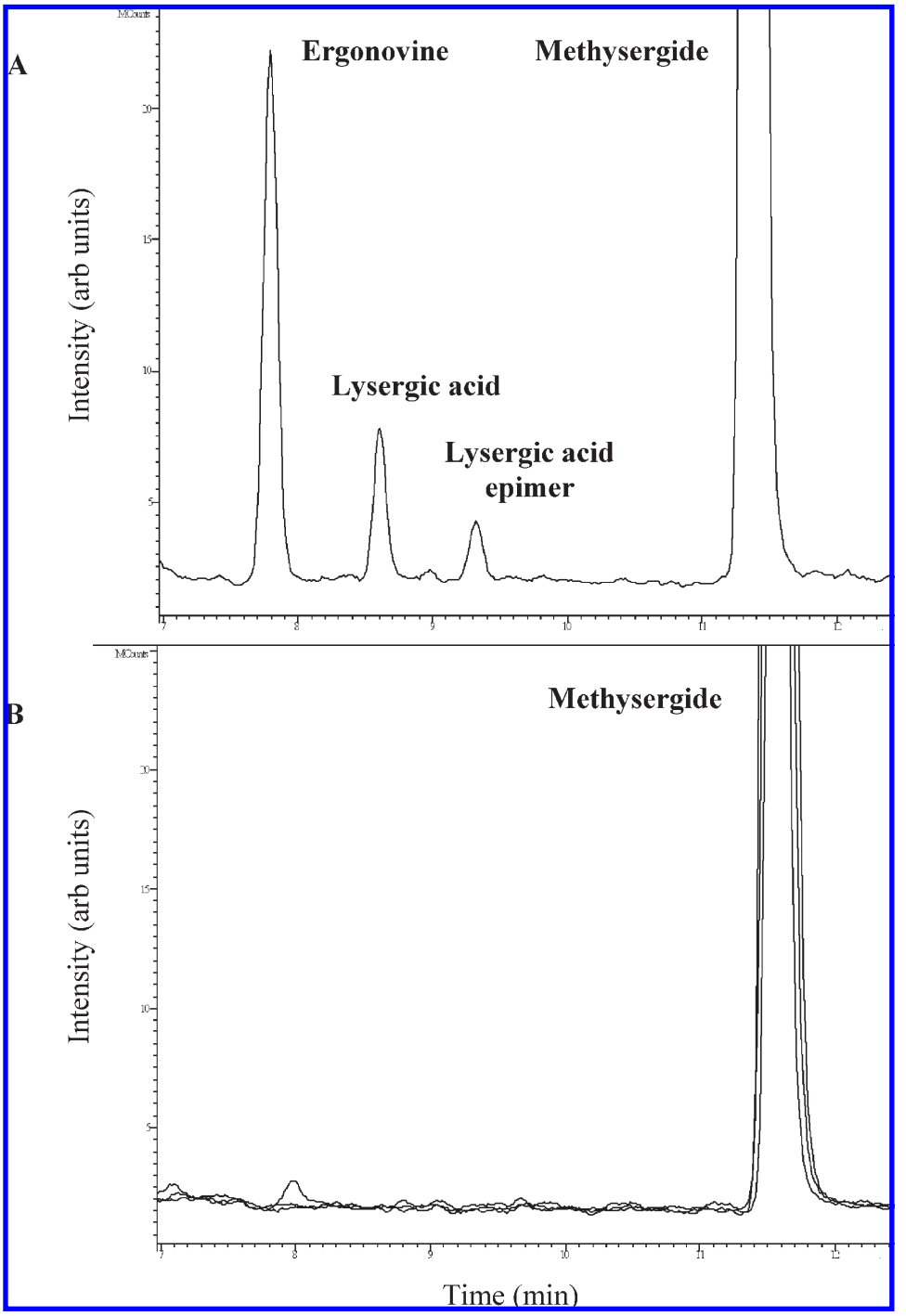


Figure 4. Reconstructed ion chromatograms of the total detector current for ions of *m*/*z* 269, 326, and 354 (equivalent to singly protonated lysergic acid, ergonovine, and methysergide, respectively) for (**A**) 1 pmol of standard solution injected on column and (**B**) triplicate analyses of the method recovery equivalent to 10 pmol of each ergot alkaloid in the absence of lysergic acid and ergonovine.

With such a degree of peak tailing, it was difficult to define the beginning and ending points of a peak consistently for replicate samples. Because the combined method recovery/ruggedness experimental results were consistent for the lower spike concentration (4 pmol), this method is suitable for the analyses of vascular tissues that are expected to contain trace levels of ergot alkaloids. In the event that higher concentrations of alkaloids will be measured, optimization of chromatographic resolution is critical to the accuracy and precision of the results using this method.

There was some concern that the relatively high method recoveries for lysergic acid and ergonovine (ranging from 106.9%−111.0%) may have been the result of other ergot alkaloids in the sample decomposing into either lysergic acid or ergonovine during the sample extraction and processing steps (**Table 3**). To determine if alkaloid decomposition was possible, a set of blank vein samples was spiked with all of the ergot alkaloids (including the internal standard) except lysergic acid and ergonovine. The samples were then extracted and analyzed as previously described. It was observed that the chromatographic peaks corresponding to lysergic acid and ergonovine at retention times of 7.8, 8.6, and 9.3 min are not detected at quantities greater than the developed method's limit of detection (**Figure 4**). Thus, the extraction and sample handling conditions of the developed

SIM method were not inducing decomposition of ergot alkaloids into lysergic acid or ergonovine.

In conclusion, a method for the effective separation and simultaneous quantitation of seven ergot alkaloids using LC-MS in bovine vascular tissue was developed and validated. The extraction process of the ergot alkaloids from lateral saphenous vein tissue required no solution preparation and was relatively simplistic. The resulting method recoveries were within extraction efficiencies typically reported in the literature for this class of compounds (*9*, *19*, *20*, *22*). Single ion monitoring of the singly protonated molecular ions produced by electrospray ionization for the target alkaloids allowed a 2-fold degree of peak identification in the form of chromatographic peak retention time and molecular mass. In the event that this method should be extended to vascular tissue exposed to unknown quantities and combinations of ergot alkaloids in vivo (e.g., tissues collected from animals exposed to alkaloids through consumption of toxic endophyte-infected tall fescue), a greater degree of specificity may be required for accurate alkaloid identification, at which time selected reaction monitoring may be a better choice for detection. Nevertheless, using this method, it is now possible to conduct routine monitoring for these alkaloids in vein tissue exposed to ergot alkaloids in vitro for correlation to vascular function measurements. Furthermore, the developed method will serve as a platform from which extraction and sample cleanup protocols for other types of biological tissues and fluids may be developed.

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