Comparison of electrospray ionization and atmospheric pressure photoionization liquid chromatography mass spectrometry methods for analysis of ergot alkaloids from endophyte-infected sleepygrass (*Achnatherum robustum*)

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Abstract:

Ergot alkaloids are mycotoxins with an array of biological effects. With this study, we investigated for the first time the application of atmospheric pressure photoionization (APPI) as an ionization method for LC–MS analysis of ergot alkaloids, and compared its performance to that of the more established technique of electrospray ionization (ESI). Samples of the grass *Achnatherum robustum* infected with the ergot producing *Epichloë* fungus were extracted using cold methanol and subjected to reserved-phase HPLC–ESI–MS and HPLC–APPI–MS analysis. The ergot alkaloids ergonovine and lysergic acid amide were detected in these samples, and quantified via external calibration. Validation parameters were recorded in accordance with ICH guidelines. A triple quadrupole MS operated in multiple reaction monitoring yielded the lowest detection limits. The performance of APPI and ESI methods was comparable. Both methods were subject to very little matrix interference, with percent recoveries ranging from 82% to 100%. As determined with HPLC–APPI–MS quantification, lysergic acid amide and ergonovine were extracted from an *A. robustum* sample infected with the *Epichloë* fungus at concentrations of 1.143 ± 0.051 ppm and 0.2822 ± 0.0071 ppm, respectively. There was no statistically significant difference between these concentrations and those determined using ESI for the same samples.

Keywords: Electrospray ionization | Atmospheric pressure photoionization | Ergot | *Achnatherum robustum* | Endophyte | Lysergic acid amide | Ergonovine

Article:

1. Introduction

Ergot alkaloids, secondary metabolites produced by several *Ascomycetes* fungal species, are of considerable biological and medical importance. The medicinal and toxic effects of these

mycotoxins have been known for thousands of years [1]. Ergot alkaloids have been employed as pharmaceuticals. For example, Cafergot is used to treat migraine headaches [2] and Methergine is used for its oxytocic effects [3]. Cafergot is a mixture of caffeine and the ergot alkaloid ergotamine, and Methergine is a semi-synthetic derivative of ergonovine [4]. Ergot alkaloids also have significant effects on circulation and neurotransmission, due in large part to their structural similarities to serotonin, dopamine, norepinephrine, and epinephrine [5], [6]. Ergot alkaloids and their derivatives can also lead to poisoning. For example, ergotism, which is caused by consumption of grain infected by fungi such as *Claviceps purpurea*, was responsible for killing tens of thousands of Europeans during the Middle Ages [1]. Ergotism is an uncommon malady today, due in large part to food supply screening that prevents consumption of infected grain. However, ergot alkaloids produced by fungal endophytes that inhabit pasture and turf grasses have strong toxic and deterrent effects on vertebrate grazers, including livestock (cattle, sheep and horses) [7], and insect herbivores [8]. Livestock production losses due to endophyte-produced ergot alkaloids in turf and pasture grasses in North America were estimated at $600 million in 1993 [9]. These fungal endophytes have been found to infect the cool season grass *Achnatherum robustum* and produce ergot alkaloids, often at very high levels [10], [11]. Previous studies have indicated that ergonovine and lysergic acid amide are among the most abundant ergot alkaloids produced by endophytic fungi infecting this grass [8], [10], and these alkaloids were the focus of the studies presented herein.

A number of analytical methods have been developed for analysis of ergot alkaloids [12], including enzyme linked immunosorbent assays (ELISA) [13], gas chromatography–mass spectrometry (GC–MS) with chemical ionization (CI) [14]and direct exposure probe–electron ionization (DEP–EI) [15], high performance liquid chromatography–fluorimetric detection (HPLC–FLD) [16], and HPLC–electrospray ionization mass spectrometry (HPLC–ESI–MS) [17], [18], [19], [20]techniques. Our goal with these studies was to evaluate the effectiveness of two different ionization methods for analysis of ergot alkaloids, electrospray ionization (ESI) and atmospheric pressure photoionization (APPI). These ionization techniques are similar. For both techniques, the sample is ionized at atmospheric pressure before entering the mass spectrometer. The techniques differ in that ESI is achieved by application of a voltage to the spray tip, while APPI involves the reaction of aerosol droplets with photons produced by a UV lamp. APPI is generally considered most applicable to the analysis of non-polar species, and has been shown to demonstrate better sensitivity than ESI in some such applications [21]. However, APPI has also been applied to the analysis of polar, chargeable species, such as various pharmaceuticals [22]. Thus, we predicted that APPI would be applicable to the analysis of ergot alkaloids, and sought to compare its effectiveness to ESI for this purpose.

2. Experimental

2.1. Synthesis of lysergic acid amide (1)

Lysergic acid (Farmitalia, Milan, Italy) (100 mg, 0.372 mmol), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; Aldrich) (213 mg, 0.410 mmol) and triethylamine (94 mg, 0.93 mmol) were suspended and stirred in CH_2Cl_2 (15 mL). Solid, powdered NH_4Cl (30 mg, 0.56 mmol) was then added to the mixture, which was stirred at 0 °C in an ice bath for 10 min. Diisopropylethylamine (96 mg, 0.075 mmol) was then added and

stirring was continued at 0 °C for 30 min and then at room temperature for 3.5 h. The mixture was quenched by addition of H_2O (5 mL) followed by 10% aqueous K_2CO_3 soln. (10 mL) and stirred for 15 additional min. CH_2Cl_2 (25 mL) was added, and the layers were separated. The aqueous layer was washed with CH_2Cl_2 (2 × 15 mL) and the combined organic extracts were washed with H_2O and brine. A small amount of activated charcoal was added to the CH_2Cl_2 solution, with swirling to adsorb colored contaminants, and the solution was dried over $MgSO_4$. After filtration the solvent was removed by rotary evaporation. Any remaining diisopropylethylamine was removed by azeotropic distillation following addition of H_2O (2 mL) and reduction to dryness again by rotary evaporation. The residue was pumped under high vacuum to remove any remaining volatiles.

The crude product was purified by centrifugal thin layer preparative chromatography (Chromatotron, Harrison Research) over a 2 mm silica rotor and elution with 92.5:7.5CH_2Cl_2/MeOH under an atmosphere of N_2 that had been bubbled through conc. NH_4OH. The purpose of the NH_4OH is to decrease the pH of the separation, thereby increasing the mobility of the alkaloid on the TLC plate. The faster moving blue fluorescent band (seen under long wave UV) was collected and the solvent was removed by rotary evaporation. Traces of tripyrrolidinophosphine oxide (a PyBOP by-product) that coeluted with the lysergamide proved extremely difficult to remove completely by chromatography. Therefore, the crude lysergamide base, contaminated with a small amount of tripyrrolidinophosphine oxide, was dissolved in *tert*-BuOH (3.0 mL), which was added to a vial containing (+)-tartaric acid (28 mg, 0.186 mmol) dissolved in a minimum volume of MeOH. A white precipitate immediately formed, which was dissolved by gentle heating on a steam bath. The solution was then stored overnight at 0 °C, causing it to solidify completely. Ethyl acetate was layered onto the frozen solvent, which was broken up with a spatula, dissolving in the EtOAc in the process and leaving a suspension of the crystalline product. The material was collected by filtration and washed on the filter with Et_2O. The white product was dried overnight under high vacuum to afford lysergic acid amide hemitartrate (64 mg, 50.1%) mp 138 °C. ^{1}H NMR (300 MHz; free base) ($CDCl_3$): δ 2.59 (s, 3H), 2.68 (t, J = 12.8 Hz, 1H), 2.74 (dd, J = 10.8, 3.40 Hz, 1H), 3.09–3.24 (m, 3H), 3.59 (dd, J = 15.0, 5.47 Hz, 1H), 5.25 (bs, 1H), 6.62 (d, J = 6.0, 1H), 6.94 (t, J = 1.8 Hz, 1H), 7.15–7.26 (m, 3H), 7.96 (bs, 1H), 8.50 (bs, 1H). MS (+ESI) 268.1 ($[M + H]^+$).

2.2. Analytical standards

Solutions of the tartrate salts of the lysergic acid amide epimers (**1**) (Fig. 1) (98% pure by HPLC–MS) and the maleate of ergonovine (**2**) (Fig. 1) (Sigma–Aldrich, 100% pure by TLC) were prepared in water at a stock concentration of 0.01 mg/mL. The standard stock and serial dilutions thereof were stored at −20 °C. Identities of the standards were supported by observation of protonated molecules ($[M + H]^+$) at *m*/*z*268.1 and 326.1, respectively, for lysergic acid amide and ergonovine and by comparison of collision induced dissociation (CID) fragmentation patterns for both alkaloids with literature [23].

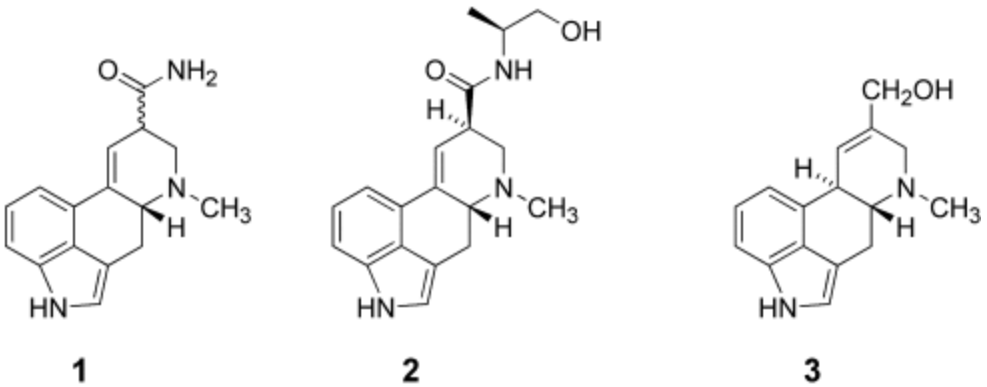

Figure 1. Structures of ergot alkaloids detectable in *Epichloë*—infected sleepygrass [15], lysergic acid amide (**1**), ergonovine (**2**), and chanoclavine (**3**). The compounds referred to as "lysergic acid amide" throughout the text are epimers of compound **1** (lysergic acid amide and isolysergic acid amide).

2.3. Optimization of method parameters

Dilute analytical standards (10 µM of the free base) were used to optimize chromatographic conditions. Solvent composition and gradient were adjusted to optimize resolution of the alkaloids and minimize peak broadening. MS ion optics and transmission were tuned using ESI and the autotune features included with the Xcalibur software for the triple quadrupole instruments used. The MRM mode was tuned by adjusting the collision energy and tube lens values to maximize the selected fragment ions for each alkaloid.

2.4. HPLC–ESI–MS

A triple quadrupole mass spectrometer (TSQ Quantum, Thermo, San Jose, CA, USA) fitted with an ESI source was used for this study. Analyses were conducted in the positive ionization mode using a dependent scan with a range of 150–1500 Da. Collision induced dissociation MS–MS spectra were acquired using 20% collision energy from the protonated molecules ($[M + H]^+$) of lysergic acid amide (268.1) and ergonovine (326.1). Additional components of the extracts also were selected for fragmentation by data dependent scanning and fragmentation of the most abundant peak. Source and ion optics parameters were 4000 V spray voltage, 300 °C capillary temperature, 87 V capillary offset, and sheath gas, ion sweep gas, and auxiliary gas pressures of 30, 0, and 20 (arbitrary units), respectively.

The mass spectrometer was interfaced to an HPLC system with photodiode array (PDA) detector and quaternary pump (Agilent HP1100 series). A binary solvent composition of aqueous 0.1% formic acid (solvent A) and 0.1% formic acid in methanol (solvent B) was employed with a flow rate of 0.20 mL/min, with a 3 µL injection volume on a C18 column (50 × 2.1 mm, 3 µm particle size, 110 Å pore size, Prevail packing, Grace, Deerfield, IL, USA). The gradient initiated at 95% A:5% B (v/v) and remained isocratic from 0 to 4 min; decreased linearly to 90% A:10% B from 4 to 5 min; to 70% A:30% B from 5 to 11.5 min; to 10% A:90% B from 11.5 to 11.6 min and held isocratic to 16 min; increasing to 95% A:5% B from 16 to 16.1 min and remained isocratic to 24 min. Absorbance was measured at 300, 307, 310, and 312 nm using the online PDA detector.

2.5. HPLC–APPI–MS

An APPI source (Thermo, San Jose, CA) was used in place of the ESI source on the triple quadrupole mass spectrometer. A vaporizer temperature of 350 °C was employed, and other source parameters and HPLC conditions were consistent with those described for the HPLC–ESI–MS mode.

2.6. Multiple reaction monitoring (MRM)

For multiple reaction monitoring (MRM) experiments, the mass spectrometer was operated in the positive ion mode with a 0.1 s dwell time and a scan window of *m/z*0.5 for the first quadrupole. The following transitions were monitored: *m*/*z* 268.13 ($[M + H]^+$, lysergic acid amide) to *m/z* 180.2, 207.2, 208.2, 223.3 and 225.1; *m*/*z* 326.2 ($[M + H]^+$, ergonovine) to *m*/*z* 180.2, 181.9, 207.1, 208.1, and 223.2. Quantification for the lysergic acid amide epimers was performed using the *m*/*z* 268 to 208 transition, and *m*/*z* 326 to 223 transition for ergonovine.

2.7. Plant growth and endophyte detection

A freshly cut *Epichloë* spp.-infected sample (E+) of *A*. *robustum* was obtained from an experimental field plot at the Arboretum of Flagstaff, Flagstaff, AZ, USA. This *A*. *robustum* plant originated from seed collected at a field site in the Lincoln National Forest near Cloudcroft, NM (N: 32° 57.5′ W: 105° 43.1′), as described previously [8]. A voucher specimen for this population is preserved at the University of North Carolina Herbarium (NCU 616703), and was authenticated by Dr. Stanley Faeth. The sample was shipped to the laboratory overnight on ice and freeze-dried in preparation for extraction. An *Epichloë*-free sample (E^-) was obtained by soaking seeds for 4 h in a microcentrifuge tube at 25 °C, then transferring the tubes to a water bath at 55 °C, an approach modified from [24]. These seeds were germinated and the plants were grown in a greenhouse for more than one year. To test for endophyte infection, samples were initially analyzed with the Phytoscreen© Immunoblot Kit (Agronostics, Ltd., Watkinsville, GA, USA) and later by *Epichloë* spp. Genetic analysis using DNA primer sets for *tefA* and several alkaloid genes [8], [25], [26]. DNA was extracted from the samples as described elsewhere [26].

2.8. Extraction and alkaloid analysis

Freeze-dried plant material from the E+ and E− *A*. *robustum* plants was weighed to the nearest 0.1 mg and extracted for 48 h at 4 °C at a ratio of 40 mg dry plant material: 1 mL HPLC grade methanol. Extracts were centrifuged and an aliquot (100 μL) of the supernatant was evaporated to dryness under vacuum and resuspended in 95% water:5% methanol (1 mL). These solutions were diluted 1:1000 in 95% water:5% methanol prior to analysis. The remaining supernatant was decanted and stored at −20 °C for future analysis. Alkaloid concentration was determined by linear regression of the relevant calibration curves. The calibration curves were plotted as log (area) for the relevant ion or transition versus log (concentration). These log–log plots enhanced the linearity and extended the linear dynamic range as compared to that achieved when plotting untransformed area versus concentration. The peak area for diluted samples fell within the linear range of the calibration curve. The lysergic acid amide epimers were quantified as a mixture.

2.9. Matrix effects

An extract from an endophyte-negative (E$^-$) *A. robustum* plant (prepared as described in Section 2.8) was used as the matrix. Samples were prepared at two concentrations (10 ppb and 50 ppb) in neat extraction solvent and the supernatant from the E− *A. robustum* extract. The alkaloid standards were added to dried matrix after extraction and evaporation, so comparisons reflect matrix interference, not differences in extraction efficiency. Percent matrix effect (%ME) was calculated for the spiked samples according to Eq. (1), where A_{solvent} and A_{matrix} are defined as the chromatographic peak area for the relevant standard in neat solvent and matrix, respectively. Positive values for %ME indicate that the signal was higher in the matrix than in neat solvent, while negative values indicate that signal was lower in the matrix.

$$\%\text{ME} = \left(1 - \frac{A_{\text{matrix}}}{A_{\text{solvent}}}\right) \times 100 \quad (1)$$

Table 1. Regression parameters, limits of detection, and quantification of lysergic acid amide and ergonovine standards using a triple quadrupole mass spectrometer. Data were collected using different scan modes (full scan and MRM) and different ionization modes (ESI and APPI). All calibration curves were plotted as log (area) for the relevant ion or transition versus log (concentration).

	Lysergic acid amide (1)			**Ergonovine (2)**		
	ESI[a]	**ESI–MRM[b]**	**APPI–MRM[b]**	**ESI[a]**	**ESI–MRM[c]**	**APPI–MRM[c]**
LOD (ppb)[d]	4.6	1.5	0.7	6.0	1.4	1.3
LLOQ (ppb)[e]	15	5.0	2.5	20	4.7	4.4
ULOQ (ppb)[f]	1000	250	250	1000	250	250
Correlation coefficient	0.998	0.998	0.989	0.984	0.998	0.994
Standard error	0.058	0.12	0.071	0.160	0.025	0.047
y-intercept	4.49 ± 0.08	3.5 ± 0.2	3.9 ± 0.1	4.63 ± 0.22	4.43 ± 0.04	4.06 ± 0.08
Slope of regression line	0.99 ± 0.04	1.3 ± 0.1	1.1 ± 0.7	0.96 ± 0.10	1.01 ± 0.02	1.01 ± 0.04

[a] ESI represents the full scan mode for electrospray ionization, where quantification is accomplished on the $[M + H]^+$ ion.
[b] ESI–MRM represents the multiple reaction monitoring mode for electrospray ionization. Quantification was accomplished for lysergic acid amide using the *m*/*z* 268–208 transition and *m*/*z*326–223 for ergonovine.
[c] APPI–MRM represents the multiple reaction monitoring mode for atmospheric pressure photoionization. Quantification was accomplished for lysergic acid amide using the *m*/*z* 268–208 transition and *m*/*z* 326–223 for ergonovine.
[d] Limit of detection (LOD) was calculated as the analyte concentration necessary to achieve a signal-to-noise ratio of 3:1, with noise determined by injection of a solvent blank.
[e] Lower limit of quantitation (LLOQ) was calculated similarly to LOD, except that the signal-to-noise ratio chosen was 10:1.
[f] Upper limit of quantitation (ULOQ) was selected as the maximum analyte concentration for which linearity was maintained so that the residual (calculated as described for Table 2) was <±15%.

3. Results and discussion

3.1. Synthesis of lysergic acid amide

The synthesis of lysergic acid amide has previously been reported using a solid phase synthesis on a 3 mg scale, but the product was characterized only by HPLC and mass spectrometry [27]. A

compound thought to be lysergic acid amide was also isolated by HPLC from a hydrolysis mixture of lysergic acid α-hydroxyethylamide, but was not chemically characterized [28]. Thus, we report here the first preparative synthesis with characterization of this compound. The product of the lysergic acid amide synthesis was identified based on NMR and LC–MS data as a mixture of at least two diastereoisomers of (**1**; normal + isolysergic acid amide), which could be partially resolved with the chromatographic method described here. This mixture is referred to throughout this text as lysergic acid amide.

3.2. Comparison of scan mode and ionization techniques for ergot alkaloid analysis

Comparisons were made between scan mode and multiple reaction monitoring (MRM), and two ionization modes, APPI and ESI, for analysis of ergot alkaloids (Table 1). All methods yielded linear calibration curves; however, the upper limit of quantitation for ESI–MRM and APPI–MRM modes was lower than that observed in the full scan mode, with a maximum concentration of 250 ppb.

As expected, a significant improvement in detection limit (~5 times lower) was observed when switching from full scan to MRM mode. However, both ESI and APPI ionization modes demonstrated similar performance in terms of limit of detection (LOD) and upper and lower limits of quantitation.

Table 2. Precision and accuracy data for lysergic acid amide and ergonovine using LC–APPI–MS with MRM. The transitions monitored were *m*/*z* 268–208 (lysergic acid amide) and *m*/*z* 326–223 (ergonovine).

Lysergic acid amide (1)				
Theoretical concentration (ppb)	**Measured concentration (ppb)**	**Residuals (%)[a]**	**Repeatability (%)[b]**	**Intermediate Precision (%)[c]**
10	8.7	−13	2.4	12
25	27	9.2	6.2	14
50	56	13	4.2	6.8
100	107	7.3	2.1	3.7
250	218	−13	3.0	6.8
Ergonovine (2)				
Theoretical concentration (ppb)	**Measured concentration (ppb)**	**Residuals (%)**	**Repeatability (%)**	**Intermediate precision (%)**
10	9.0	−10	5.6	6.7
25	27	8.8	1.2	7.7
50	54	8.0	2.0	2.0
100	104	4.1	3.0	3.4
250	228	−8.6	1.8	2.0

[a] Residuals were calculated by the following equation: (measured concentration − theoretical concentration)/theoretical concentration × 100.
[b] Repeatability was assessed by calculating the percent relative standard deviation of the average ($N = 3$) back-calculated concentration of the standard for three analyses performed within a single run (on one day).
[c] Intermediate precision was determined by calculating the relative standard deviation of the average ($N = 9$) back-calculated concentration obtained for three separate analyses performed on three different days.

To facilitate comparison between APPI–MRM and ESI–MRM methods, validation parameters were measured according International Committee of Harmonisation Guidelines [29], as described in more detail elsewhere [30] (Table 2, Table 3, respectively). As a measure of the

accuracy of the methods, the theoretical concentrations agreed with measured concentrations, with residuals of ≤18% for both APPI–MRM and ESI–MRM. Comparison of the data in Table 2, Table 3demonstrates that APPI–MRM yielded slightly better intermediate precision and better accuracy (lower residuals) than was achieved with ESI–MRM. However, the data were generally comparable, showing that either APPI or ESI are effective ionization methods for ergot alkaloid analysis by LC–MS. Importantly, both methods also demonstrated very little matrix interference (Table 4), with matrix effects (defined according to Eq. (1)) ranging from −0.2% to 17.4%.

Table 3. Precision and accuracy data for lysergic acid amide and ergonovine using LC–ESI–MS with MRM. The transitions monitored were *m*/*z* 268–208 (lysergic acid amide) and *m*/*z* 326–223 (ergonovine).

Lysergic acid amide (1)				
Theoretical concentration (ppb)	**Measured concentration (ppb)**	**Residuals (%)**	**Repeatability (%)**	**Intermediate precision (%)**
10	8.2	−18	22	19
25	29	17	7.7	18
50	59	18	1.0	9.9
100	106	6	3.8	3.6
250	211	−16	0.26	1.6
Ergonovine (2)				
Theoretical concentration (ppb)	**Measured concentration (ppb)**	**Residuals (%)**	**Repeatability (%)**	**Intermediate precision (%)**
10	9.4	−5.5	9	6.7
25	26	4.5	2	5.0
50	52	3.7	5	2.3
100	103	2.8	3	2.5
250	237	−5.0	0.9	3.6

Table 4. Evaluation of matrix interference in *A*. *robustum* extracts with ESI and APPI. Ergot alkaloid standards were spiked into an endophyte-negative (E^-) extract of *A*. *robustum* (post extraction) and peak areas were compared for the standard in extraction solvent versus the standard in the extract.

Standard	**Concentration (ppb)**	**Matrix effect (ESI)[a] ± SD[b] (%)**	**Matrix effect (APPI)[a] ± SD[b] (%)**
Lysergic acid amide	10	17.4 ± 1.5	14.4 ± 0.6
Lysergic acid amide	50	2.1 ± 0.1	6.7 ± 0.3
Ergonovine	10	17.1 ± 0.7	10.4 ± 0.3
Ergonovine	50	−0.20 ± 0.01	3.6 ± 0.1

[a] % matrix effect was calculated by the following equation: [1-(peak area in matrix/peak area in neat solvent)] × 100.
[b] Standard deviation (SD) values are calculated for three separate samples prepared by individually spiking separate aliquots of the matrix with the standards.

3.3. Identification and quantification of alkaloids in grass samples

Several ergot alkaloids (Fig. 1) were detected in extracts from *Epichloë* spp.-infected sleepygrass (*A*. *robustum*) using both HPLC–APPI–MS and HPLC–ESI–MS methods (Fig. 2). Lysergic acid amide (**1**) and ergonovine (**2**) were identified by comparison of fragmentation patterns and retention times with those of standards, and were subsequently quantitated. Additionally, chanoclavine (**3**) was tentatively identified based on the presence of the putative $[M + H]^+$ ion at *m*/*z* 257.1. Several compounds with the same nominal mass of lysergic acid amide (*m*/*z* 268) and ergonovine (*m*/*z*326) also were detected. These compounds produce similar

fragmentation patterns as ergonovine and lysergic acid amide, and are likely isomers of these compounds. Availability of an ergonovine standard enabled it to be distinguished from its putative isomer on the basis of retention time. Chemical interference complicated the chromatograms for *A. robustum* extracts (Fig. 2). To minimize interference, the MRM mode was employed for quantitative analysis, yielding chromatograms consisting only of peaks corresponding to lysergic acid amide (**1**), ergonovine (**2**), and one putative isomer (Fig. 3). Note that the peaks corresponding to lysergic acid amide (Fig. 2, Fig. 3) include two partially resolved diastereoisomers of **1** (lysergic acid amide and C-8 epimer isolysergic acid amide). The presence of lysergic acid amide diastereoisomers in endophyte-infected grass samples has previously been documented [31].

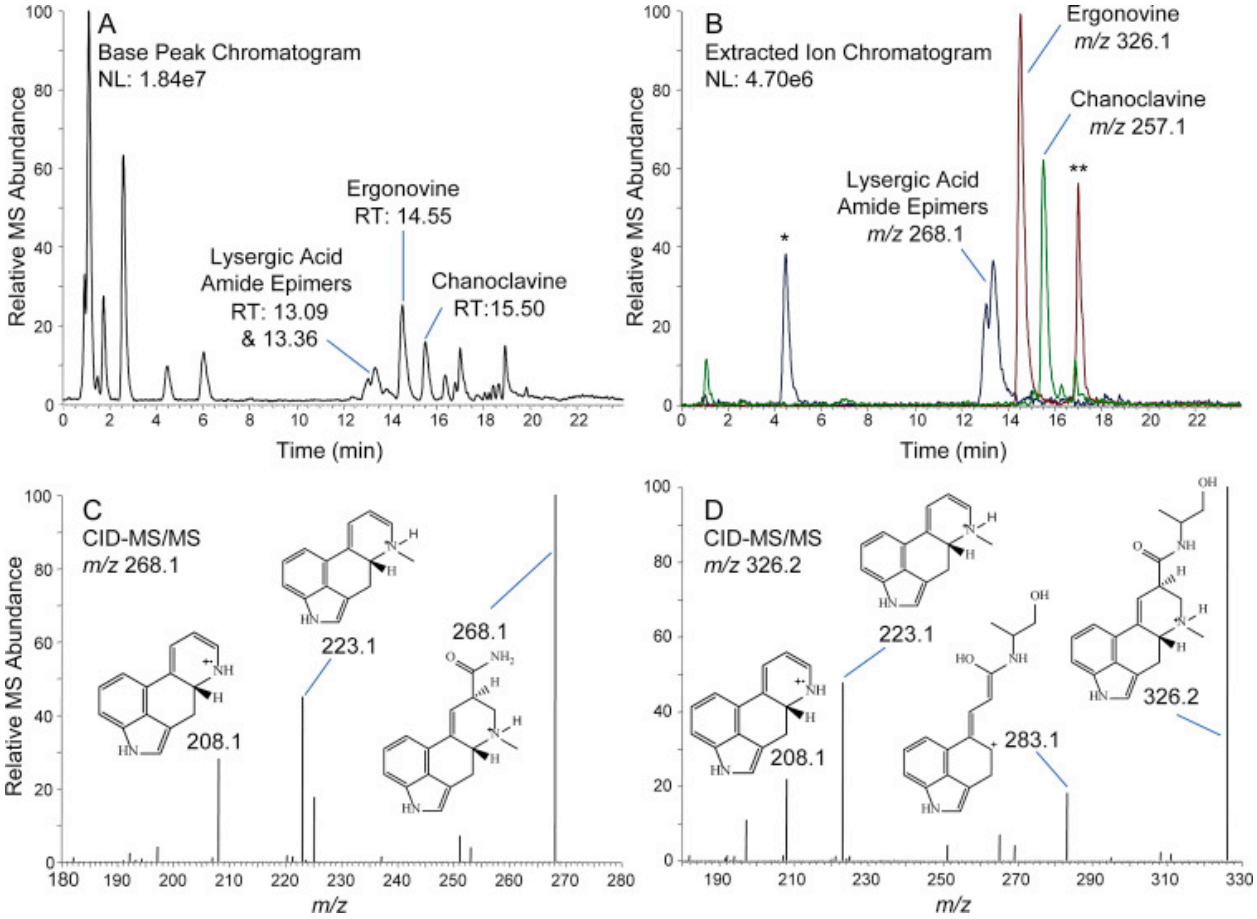

Figure 2. (A) Base peak chromatograms of an endophyte-infected *Achnatherum robustum* obtained using a triple-quadrupole mass spectrometer with positive mode electrospray ionization. (B) Extracted ion chromatogram displaying only compounds **1**–**3**. (C and D) The MS/MS spectra corresponding to the latter eluting lysergic acid amide stereoisomer and ergonovine are displayed with proposed fragment ion structures, both spectra were collected using 20% relative collision energy on a triple quadrupole mass spectrometer. These alkaloids were not detected in an endophyte-negative (E^-) samples of *A. robustum* (Fig. 3) *Indicates putative lysergic acid amide isomer, **indicates putative ergonovine isomer. Note that conclusive identification of isomers is not possible without confirmation with standards and/or NMR analyses, which were not conducted as part of these studies.

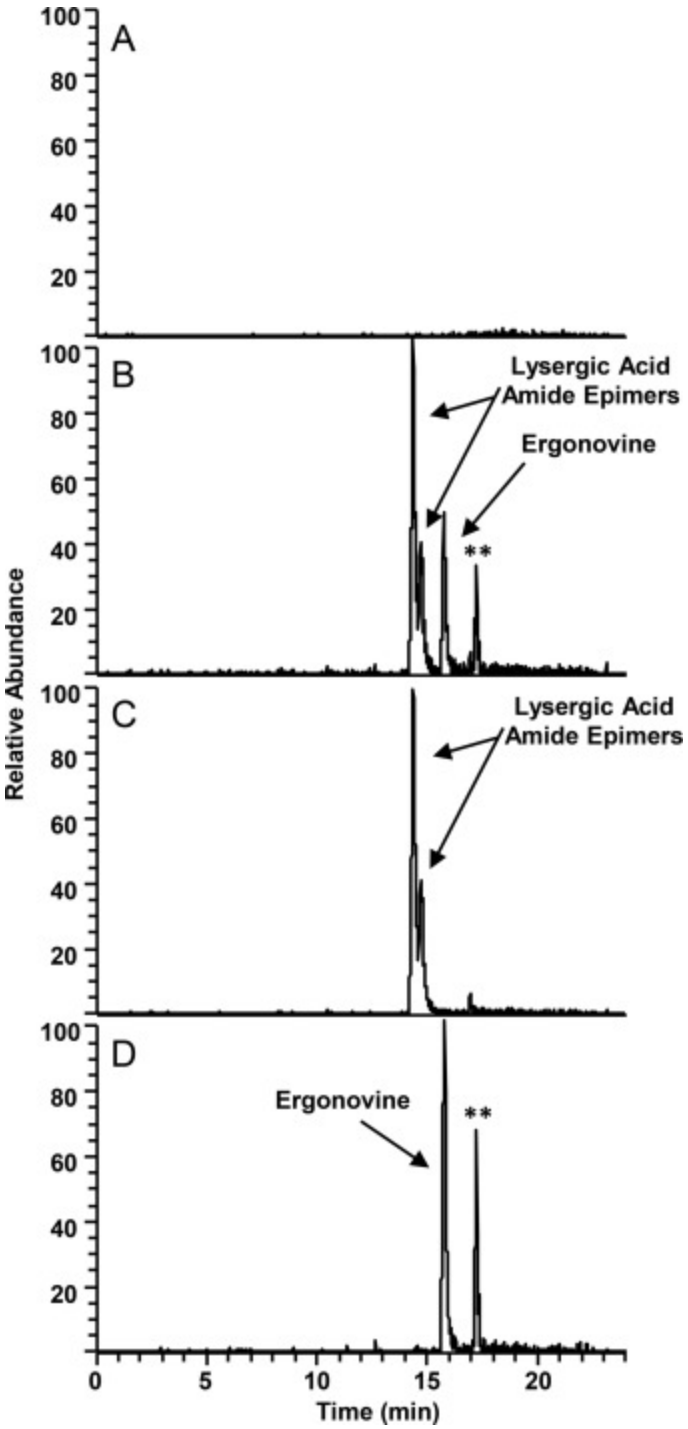

Figure 3. (A) ESI–MRM chromatogram of an endophyte negative (E^-) sample of sleepygrass (*Achnatherum robustum*) sample, signal summation of all monitored MRM transitions. (B) ESI–MRM chromatogram collected under the same conditions as (A) for an extract of an endophyte-infected sample of sleepygrass (*A*. *robustum*). (C) ESI–MRM chromatogram with lysergic acid amide (**1**) transition filter applied (*m*/*z* 268 to 180, 207, 208, 223, and 225). (D) ESI–MRM base peak chromatogram with ergonovine (**2**) transition filter applied (*m*/*z* 326 to 180, 182, 207, 208, and 223). **indicates putative ergonovine isomer.

To compare the effectiveness of APPI and ESI for quantifying alkaloids in a mixture, the HPLC–ESI–MS and HPLC–APPI–MS methods were applied for quantitative analysis of alkaloids in an endophyte-infected sample of *A. robustum* (Table 5). Very similar results were obtained for ESI–MS and APPI–MS (no statistically significant difference in concentration of either lysergic acid amide or ergnovine by *t*-test at the 95% confidence interval). An endophyte-free sample did not contain ergot alkaloids above the limit of detection for the method (Fig. 3A).

Table 5. Quantitative analysis of alkaloid levels extracted from an endophyte-infected sample of *Achnatherum robustum*. A negative control sample (without endophyte infection) was analyzed in the same analysis, and lysergic acid amide and ergonovine were not present above the limit of detection.

Ionization mode	Lysergic acid amide (ppm)[a, c]	Ergonovine (ppm)[a, c]
APPI[b]	1.143 ± 0.051	0.2822 ± 0.0071
ESI[b]	1.053 ± 0.085	0.2207 ± 0.0083

[a] Concentrations are reported as ppm or mg of alkaloid extracted per kg of plant material (mean ± SD).
[b] No statistically significant difference (*t*-test) in concentration was observed between the concentrations of lysergic acid amide or ergonovine as determined with APPI and ESI at the 95% confidence level.
[c] Note that extraction efficiency was not evaluated in this study; thus, the concentrations in this table represent extracted alkaloid levels but may not reflect absolute concentrations present in the original sample. They are, nonetheless, useful for comparative purposes.

4. Conclusion

The results of this study indicate that APPI, an ionization method typically considered best for non-polar species, can be employed for the analysis of polar alkaloids in complex botanical extracts. ESI and APPI demonstrated similar performance in this study, and matrix interference did not constitute a problem in either the ESI or APPI ionization modes. However, a number of literature reports suggest that for some samples, matrix interference is a more significant problem with ESI than with APPI [21], [22]; thus, it is possible that APPI would be better than ESI for analysis of ergot alkaloids in more complex matrices, such as biological fluids. Furthermore, it has been shown in a number of literature reports that the use of a dopant can significantly improve signal with APPI, typically as much as 10-fold [32]. The applicability of a dopant to improve sensitivity of ergot alkaloid analysis with APPI would be an interesting topic for future investigation.

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