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Determination of the Ergot Alkaloid Ergovaline in Tall Fescue Seed and Straw Using a QuEChERS Extraction Method with High Performance Liquid Chromatography-Fluorescence Detection

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Abstract

Ergovaline is an ergot alkaloid produced by the symbiotic endophyte *Epichloë coenophiala* which can colonize varieties of the cool-season grass tall fescue (*Festuca arundinacea*). It is the principle toxicant responsible for the vasoconstrictive and reproductive sequelae seen in "fescue toxicosis" in livestock which consume forage exceeding the threshold of toxicity established for this compound. A new method for extraction of ergovaline from tall fescue seed and straw was optimized and validated, based upon the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, with high performance liquid chromatography-fluorescence detection. Fourteen extraction solvents were tested; 2.1 mM ammonium carbonate: acetonitrile (50:50, v/v) had the highest and most consistent recovery (91-101%). Linearity, limit of detection, limit of quantitation, accuracy, intra- and inter-day precisions for tall fescue seed and straw were 100-3500 µg/kg; 37 and 30 µg/kg; 100 µg/kg; 98%; 3.0 and 1.6%; and 3.8 and 1.0%, respectively. When applied to 17 tall fescue straw samples, there was good agreement between the currently used solid phase extraction (SPE) and QuEChERS methods (correlation coefficient = 0.9978). The QuEChERS method achieved the goals of eliminating chlorinated solvents, and developing a fast, efficient, reliable method for quantitating ergovaline in tall fescue forage that can be applied in a high-throughput food safety laboratory.

Keywords: tall fescue, ergovaline, ergot alkaloids, HPLC-fluorescence

Introduction

The Pacific Northwest, USA is the world's center for grass seed production, with most of the acreage devoted to this crop concentrated in the Willamette Valley, Oregon. Tall fescue (*Festuca arundinacea* (Schreb.) seed was harvested off of 134,680 acres of Oregon farmland in 2013, making it the most productive cool-season grass species, followed closely by annual (*Lolium multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.) (126,040 and 104,790 harvested acres, respectively).[1] Tall fescue is also grown extensively for pasture, silage, and straw, and is used for reducing soil erosion, recycling nutrients from manure and biosolids, and turf. The symbiotic fungal endophyte *Epichloë coenophiala* has been intentionally propagated in tall fescue as it improves seed survival and growth, and enhances stress tolerance and disease resistance in the plant. Some endophyte strains exert these benefits through the production of ergot alkaloids, which, unfortunately, also cause deleterious effects in cattle and other herbivore species when endophyte-infected grasses are grazed or fed as hay/straw; this is manifested in the diseases "fescue foot" or "summer syndrome."[2,3] However, it is possible to utilize tall fescue straw safely in forage/livestock systems if the concentration of the principle ergot alkaloid causing disease, ergovaline, **1** (Figure 1), is known, and tall fescue is fed below established thresholds of toxicity for the livestock species in question.[4–6]

Quantitation of **1** can be assessed through qualified service laboratories and typically involves the use of solid phase extraction (SPE)[7,8] for sample clean-up, which can be both costly and time-consuming. The search for a simpler, more efficient, and more environmentally friendly extraction procedure lead us to evaluate a QuEChERS (Quick Easy Cheap Effective Rugged Safe)-based method. QuEChERS is an analytical approach that has vastly simplified the analysis of multiple pesticide residues in fruit, vegetables, cereals and processed products.[9,10] To date, it

has also been successfully employed in the analysis of mycotoxins in a variety of food matrices, including aflatoxins in noodles [11] trichothecenes in wheat flour[12] and cereals,[13] and for multiple mycotoxin detection (coupled with LC-MS/MS) in silage,[14] cereals,[15] and other food matrices.[16–19] The purpose of this study was to simplify the extraction of **1** from tall fescue seed and straw, and eliminate the use of chlorinated solvents through application of the QuEChERS method, coupled with HPLC-fluorescence for detection.

Materials and methods

Chemicals and reagents

Acetic acid, acetonitrile (MeCN), isopropyl alcohol, and methanol (MeOH) were HPLC grade and produced by EMD Chemicals (Gibbstown, NJ). Ethyl acetate was HPLC grade and obtained from Mallinckrodt (Phillipsburg, NJ). Sodium chloride (NaCl) crystalline powder, 99+%, was supplied by Alfa Aesar (Ward Hill, MA). Ammonium carbonate, HPLC grade and formic acid, A.C.S. reagent, were both supplied by J.T. Baker (Phillipsburg, NJ). The DL-lactic acid sodium salt was purchased from Sigma Aldrich (St. Louis, MO). Anhydrous magnesium sulfate ($MgSO_4$), 99.5%, was purchased from UCT (Bristol, PA). Ammonium hydroxide, A.C.S. grade, was purchased from VWR BDH Prolabo (West Chester, PA). Ultrapure water (18.2 MΩ cm^{-1}) was purified with a PURELAB Ultra Genetic system (Elga, Marlow, Buckinghamshire, UK).

Ergovaline tartrate was prepared by Dr. Forrest Smith, Department of Pharmacal Sciences, Auburn University, Auburn, AL. Chemical characterization and assessment of purity of the crystalline standard was established by nuclear magnetic resonance (NMR) and HPLC/mass spectrometry (HPLC/MS). It was established that the ergovaline tartrate was comprised of ergovaline:tartrate:water in the ratio 2:1:1. The **1** standard was weighed on an analytical balance,

diluted in MeOH, dispensed in 10 µg aliquots, evaporated under nitrogen, and stored frozen at -20 °C. The working standard was prepared by dilution of a 10 µg aliquot in MeOH.

Sample preparation

Tall fescue seed and straw samples were ground in a Cyclotec 1093 sample mill (Foss, Hilleroed, Denmark)) to pass through a 0.5 mm screen and stored at -20 °C prior to analysis. Details on collection of a representative straw or seed sample for forage testing can be found on-line.[20]

Optimization of extraction solvent

The first step of the extraction/partitioning process of the QuEChERS method was evaluated in a tall fescue seed sample with a known high **1** level (1998 µg/kg, *n* = 3/solvent). The extraction solutions were selected from published procedures that were used to extract mycotoxins via the QuEChERS method, or used in the extraction of ergot alkaloids via other techniques and were as follows: Extraction solvent 1: 1% acetic acid in MeCN (v/v);[11] extraction solvent 2: MeOH:MeCN (15:85, v/v);[11] extraction solvent 3: MeOH:MeCN (85:15, v/v);[11] extraction solvent 4: MeOH:water (60:40, v/v);[11] extraction solvent 5: MeOH:water (80:20, v/v);[11] extraction solvent 6: 2.1 mM ammonium carbonate:MeCN (16:84, v/v)-modification from a method for the quantitation of ergot alkaloids in cereals and foodstuffs; [20] extraction solvent 7: isopropyl alcohol: 1% lactic acid (50:50, v/v)-method developed to quantitate **1** and peramine in grass tillers;[21] extraction solvent 8: MeCN: 0.1% formic acid (57:43, v/v)-modification of a method developed for detection of *Fusarium* mycotoxins in cereals;[13] extraction solvent 9: ethyl acetate:MeOH:4.1 mM ammonium carbonate (62.5:25:12.5, v/v)-modification of a method for

ergot alkaloid extraction from food and feed sources;[22] extraction solvent 10: water:0.5% acetic acid in MeCN (50:50, v/v- modification of a QuEChERS method developed for the analysis of 17 mycotoxins in cereals;[15] extraction solvent 11: ethyl acetate:MeOH:25% ammonium hydroxide solution (75:5:7, v/v)-modification of a method developed for analysis of ergot alkaloids in rye and rye products;[23] extraction solvent 12: MeCN:1.0% acetic acid (80:20, v/v); extraction solvent 13: MeCN: 1mM ammonium carbonate (80:20, v/v); extraction solvent 14: MeOH: 1% acetic acid (80:20, v/v).

For the initial survey of solvent systems, 1.0 g samples of ground tall fescue seed were placed into 16 x 125 mm glass screw top tubes in triplicate. Five mL of the solvent mixture was added; the tubes were capped and vortexed for 10 s. The tubes were placed on an end-over-end rotary mixer for 30 min after which 0.5 g of $MgSO_4$ and NaCl (4:1, w/w) [9] was added. The tubes were capped and vortexed immediately to prevent agglomeration of the salts for 10 s, four times during a 10 min period. The samples were then centrifuged at 913 x *g* for 10 min. One mL of the supernatant was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The samples were reconstituted in 0.5 mL of MeOH, and after vortexing and sonication for 20 s each, transferred to a centrifugal filter tube with a 0.45µm modified nylon membrane (VWR, Radnor, PA)). The tubes were centrifuged at 8161 x *g* (10,000 rpm) for 5 min; the filtrate was then transferred to an amber HPLC vial for analysis.

Final extraction procedure

Five ml of 2.1 mM ammonium carbonate:MeCN (50:50, v/v) extraction solvent was added to 0.5 g of ground plant material and mixed by vortexing for 30 s, then placed on the rotary mixer for 30 min. Subsequently, 0.4 g of pre-weighed $MgSO_4$ and 0.1 g NaCl was added and the tubes

were capped and vortexed immediately for 10 s, four times during a 10 min period. Samples were centrifuged at 913 x *g* for 10 min. A 2.0 mL aliquot of the separated MeCN phase was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The samples were reconstituted in 0.5 mL of MeOH, and after vortexing and sonication for 20 s each, were transferred to a centrifugal filter tube. The tubes were centrifuged at 8161 x *g* for 5 min and the filtrate was transferred to an amber HPLC vial for analysis.

HPLC analysis

A Series 200 HPLC system (Perkin Elmer, Waltham, MA) was used throughout the experiment. This system consisted of an autosampler, binary pump, column oven, and a fluorescence detector (λ_{ex} = 250 nm, λ_{em} = 420 nm). Chromatographic data were managed using TotalChrom™ Workstation Software version 6.3.2 (Perkin Elmer). HPLC separations were performed with a 100 mm x 4.6 mm i.d., 2.7 µm Brownlee SPP C18 HPLC column (Perkin Elmer), with a 4.6 mm i.d. SecurityGuard ULTRA C18 guard column (Phenomenex (Torrance, CA)) maintained at 35 °C. The mobile phase consisted of 30% MeCN in 200 mg/L ammonium carbonate in purified water (solvent A) and 100% MeCN (solvent B), run at a flow rate of 1.8 mL/min. Initial gradient conditions were 5% B held for 1.0 min, increasing linearly to 65% B over 0.8 min and held for 0.8 min. Finally, there was a linear return to initial conditions over 0.3 min, which was then held for 1.3 min for a total run time of 4.2 min. Injection volume was 10 µL.

Method validation

Performance characteristics were evaluated using guidelines developed by the Food and Drug Administration for bioanalytical analysis.[24] Validation of the optimized extraction method included linearity, selectivity, recovery, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and stability. Linearity was assessed using matrix-matched calibration curves established using extracts of endophyte-free (E-) tall fescue seed and straw fortified with the working **1** standard at 50, 100, 500, 1000, 2500 and 3500 µg/kg. Selectivity was established using comparison of non-infected seed and straw sample chromatograms with **1**-fortified extracts. Interference (visual examination of the chromatogram for co-elution of other peaks) at the retention time of **1** (1.75 min) and ergovalinine (**2**) (3.60 min) was checked.

For recovery investigations, the **1** standard was added to E- tall fescue seed and straw at fortification levels of 100, 1000 and 2500 µg/kg (n = 10/level). To avoid epimerization of **1**, the MeOH in the spiked sample was evaporated under a gentle steam of nitrogen, then the tube was capped and frozen at -20 °C until extraction. The LOD and LOQ were estimated by measurement of the peak-to-peak signal-to-noise ratio (S/N) and calculated at S/N > 3 and S/N > 10, respectively. LOQ values must meet an accuracy of ± 20% and a precision of < 20% relative standard deviation (RSD).

The accuracy and precision of the method was determined using in-house tall fescue seed and straw reference material. The **1** content of the reference material was established using the Ergosil silica gel solid phase extraction (SPE) method.[5,8] The intra-day precision was established using five replicates at four levels of **1**. Inter-day precision and accuracy were evaluated over three separate days of analysis of five replicates at the four concentration levels. The stability of the sample extracts was investigated by repeat analysis of the samples (n = 48) after 24 h at room temperature.

Statistical analysis

Statistical analyses (averages, standard deviations (SD), relative standard deviations (RSD), *t*-tests (two-tailed, two-sample equal variance) and ANOVA) were performed in Excel (Microsoft Office 2010, Redmond WA) or GraphPad Prism (Version 5.01, La Jolla, CA). Results were considered significant when the *p*-value was less than 0.05.

Results

Optimization of extraction procedure

The 14 solvent mixtures were significantly different from each other in their ability to extract **1** (Kruskall-Wallis test, P = 0.001) (Figure 2). The maximum amount of extracted **1** was obtained with water: 0.5% acetic acid in MeCN (50:50), which extracted 98% of the 1998 µg/kg **1** in the seed that was being used, with a low standard deviation of 12 µg/kg (RSD 0.6%). The MeCN:0.1% formic acid (57:43) mixture showed a similar ability to extract **1**, extracting 95% of the 1998 µg/kg **1** in seed, but had a higher standard deviation of 182 µg/kg (RSD 9.6%). While the two solvent combinations were not statistically different from each other ($p = 0.611$), the water: 0.5% acetic acid in MeCN (50:50) mixture was chosen for its lower standard deviation which would ensure greater throughput under the rigorous quality control standards in a diagnostic laboratory setting. Once this was established, different types of aqueous solution, namely, 0.5% and 1% acetic acid, 1% formic acid, 2.1 mM ammonium carbonate, 1 mM ammonium carbonate or water, were studied in both seed (1998 µg/kg **1**) and high **1** content straw (850 µg/kg). It was found that the highest and most consistent recoveries were achieved with 2.1 mM ammonium carbonate (Figure 3). This solvent mixture extracted 90.5±1.0% of the

194 **1** in the tall fescue seed (1998 µg/kg) and 100.5±5.3% in the tall fescue straw (850 µg/kg) that
195 were used. The addition of the 2.1 mM ammonium carbonate/MeCN extraction solution added as
196 a mixture or separately as two components to tall fescue seed containing 1998 µg/kg **1** was
197 investigated; no statistical difference was found between either method (n = 3 replicates/method,
198 t-test p = 0.656). Thus, combining the 2.1 mM ammonium carbonate:MeCN (50:50, v/v) as one
199 addition to the sample was chosen.
200 The optimum mixing time for the sample and extraction solvent was evaluated at 30, 60, 90,
201 and 120 min in both seed and straw samples (Table 1). Thirty min was found to be a sufficient
202 amount of time for the sample and extraction solvent to mix to maximize the amount of **1**
203 extracted, with low RSD values. Following addition of the 0.4 g of anhydrous $MgSO_4$ and 0.1 g
204 NaCl, immediate vortexing and mixing four times during a 10 min period was required to give
205 the most consistent mixing and to promote homogeneity.
206 Figure 4 shows representative HPLC-fluorescence chromatograms of the final, optimized
207 QuEChERS procedure for tall fescue seed and straw, as compared to the Ergosil SPE method
208 currently used in our laboratory. [5]
209
210 *Method Validation*
211 *Linearity*
212 A calibration curve was constructed by plotting the peak area of **1** against the nominal
213 concentrations of **1** at 50, 100, 500, 1000, 2500 and 3500 µg/kg. The correlation coefficient (R^2)
214 generated from the calibration curve was >0.999 in both seed and straw from six replicate
215 standard sets. The linear dynamic range was found to be 100-3500 µg/kg.
216

Selectivity

Non-infected seed and straw samples were extracted to verify that there were no peaks interfering with the fluorescent detection of **1** or its epimer **2** at their expected retention times (1.75 and 3.60 min, respectively).

Recovery

1 was spiked into E- seed and straw samples at three levels, 100, 1000 and 2500 µg/kg (*n* = 10/level). The mean recoveries varied from 91.3-97.2% in tall fescue seed and 89.6-98.1% in tall fescue straw.

LOD and LOQ

The LOD was determined to be 37 µg/kg and 30 µg/kg in tall fescue seed and straw, respectively. A LOQ level of 100 µg/kg was established in both seed and straw, with a demonstrated accuracy of 98% and 91%, and a precision of 9% and 6%, respectively.

Precision and Accuracy

Precision and accuracy were determined by the extraction of **1** from tall fescue seed (143-1998 µg/kg **1**) and straw (181-1061 µg/kg) reference material. The intra-day precision, expressed in RSD, ranged from 1.8-5.8% in tall fescue seed and 0.5-3.4% in tall fescue straw. The inter-day precision ranged from 1.9-6.4% in seed and 0.7-4.8% in straw. The results from the experiments to establish accuracy ranged from 97.9-98.6% in tall fescue seed and 97.3-99.2% in tall fescue straw.

Stability

The extracted samples demonstrated comparable stability with the Ergosil SPE method upon repeat analysis after storage at room temperature for 24 h.

Application of the method to real samples

The validated method was applied to 17 tall fescue straw samples which were previously analyzed using the Ergosil SPE method.[5,8] Including the standard reference material, controls, and samples run in duplicate, the run size was 48 individual extractions, which is a typical sample run in our laboratory. There was good agreement between the two different methods. Both methods found that seven of the samples had less than a quantitative amount of **1** (<100 µg/kg). The results from the remaining ten samples had a RSD of 10% or better agreement. **1** values for the QuEChERS/Ergosil SPE methods were: 119/119, 156/173, 203/224, 237/244, 238/240, 339/318, 499/469, 523/530, 526/518, 535/482 and 632/603 µg/kg, respectively. Comparative statistics showed that application of the two extraction methods to the 17 samples were not different from each other (t-test, $p = 0.258$), and that the methods were very well correlated (correlation coefficient = 0.9978).

Discussion

Endophyte (*Epichloë coenophiala)*-infected tall fescue (*Festuca arundinacea*) is used as forage for livestock in the form of pasture, straw, silage and pellets. Some endophyte strains produce ergot alkaloids as a protective mechanism against herbivory which cause vasoconstrictive and reproductive abnormalities that present as the diseases known as “summer syndrome” and “fescue foot” in livestock. [2,3] **1** is the ergopeptide alkaloid produced in highest

concentration in tall fescue[25] and is a potent vasoconstrictor with the potential to bioaccumulate in vascular tissue,[26,27] and, as such, has been used to establish thresholds of toxicity for these diseases in relevant livestock species.[5] When used safely with these thresholds in mind, tall fescue can be fed as an important nutritional component in livestock feeding programs. To achieve this, accurate testing of **1** concentration in tall fescue material is vital. While we currently employ a robust SPE method for **1** quantitation,[5,8] we were interested in application of a QuEChERS method to make the assay more efficient, and eliminate the use of chlorinated solvents.

Fourteen extraction solvents were examined and 2.1 mM ammonium carbonate:MeCN (50:50, v/v) was found to have the highest and most consistent recovery (90.5±1.0% and 100.5±5.3% for tall fescue seed and straw, respectively) (Figures 2 and 3). Thirty min was found to be an adequate amount of time for mixing, and produced the best recovery of **1** from tall fescue seed and straw (Table 1). Total extraction time was improved to 4.4 h, as compared to 5.9 h using traditional SPE, giving a time savings of 1.5 h.

When validating the method, performance characteristics were similar to existing extraction procedures using HPLC-fluorescence for detection. The QuEChERS procedure had an LOD of 37/30 µg/kg in tall fescue seed/straw, respectively; a LOQ level of 100 µg/kg in both seed and straw; a linear dynamic range of 100-3500 µg/kg; and recoveries of spiked **1** from 91.3-97.2% for seed and 89.6-98.1% for straw. The Ergosil SPE method[5,7] has an LOD of 31 µg/kg, LOQ of 100 µg/kg, linear dynamic range of 100-2000 µg/kg and recovery of 91% and is the method currently in use by the Endophyte Service Laboratory at Oregon State University. Previous extraction methods upon which the Ergosil SPE method was based also showed similar values with an LOD of 50 µg/kg and recovery of 85%[28] or 93%.[8] These parameters are in line with the

ability to determine clinically relevant concentrations of **1** in feed, based upon the established thresholds of toxicity (300-800 µg/kg **1**, depending on species and climatic conditions).[5] Another method using 2-propanol-lactic acid as an extraction solvent to examine **1** distribution throughout the plant established an LOQ of 100 µg/kg; a linear dynamic range of 400-10,000 µg/kg; and recoveries of 93-105%, and had the advantage of being able to use small (2-5 mg) sample amounts for quantitation.[21] More recently, a SPE method using sodium-neutralized strong cation exchange columns was developed which accounted for both the native toxicologically active (*R*)-epimer of ergot alkaloids as well as their biologically inactive –inine (*S*)-epimer forms in rye flour and wheat germ oil samples, while minimizing epimer formation during the extraction itself.[29] They achieved LODs of 0.3-0.8 µg/kg; LOQs of 0.7-2.0 µg/kg; linear dynamic ranges of 8-200 µg/kg; and recoveries of 71-120%.

Advancements in LC-MS have made it increasingly common in diagnostic laboratories. Application of the QuEChERS extraction method to clean-up samples, particularly for those destined for screening of multiple ergot alkaloids via LC-MS/MS, is a logical next step, as this mode of detection will likely allow the limits of detection and quantitation to be lowered. Two methods using electrospray ionization (ESI) (+) for detection of **1** and other ergot alkaloids analyzed by selected reaction monitoring (SRM) laid out a robust framework which also allowed for elucidation of "unusual" ergot alkaloid compounds and metabolites.[30,31] Another LC-MS method, using extraction procedures developed in Spiering et al.,[21] employed SRM for the transition *m*/z 534-268 and found concentrations of 240-3480 µg/kg **1** in five populations of tall fescue from Iran.[32] While **1** was not specifically included, LC-MS/MS determination of multiple ergot alkaloids in the following methods had performance characteristics of: LOQs of 0.17-2.78 µg/kg (with LODs similar to LOQs), recoveries of 69-105% and concentration ranges of 0.5-200

309 µg/kg;[33] and LODs of 0.04-0.29 µg/kg, LOQs of 0.1-1 µg/kg and recoveries of 88-110%.[34]
310 Lastly, a method using saphenous vein tissue as a matrix obtained an LOD of 1 nM; LOQ of 2
311 nM; linear dynamic range of 0.1-50 pmol; and recovery of 86-90% for spiked **1**.[35]
312 We have observed epimerization of **1** to **2** in SPE extracted samples at room temperature after
313 approximately 12 h. Based on this, a complete batch of samples, validated reference materials
314 and controls are either always run immediately after extraction via HPLC-fluorescence, or stored
315 in a freezer which halts epimerization until samples can be analyzed.[36] For quantitation, only **1**
316 and not **2** are analyzed for total concentration in this method, as the Ergosil silica material in the
317 SPE appears limited in its ability to bind **2**. Further, the thresholds of toxicity for fescue toxicosis
318 in livestock[5] were established using analysis of feed samples for **1** (and not its epimer) via the
319 SPE method; thus, measurement of **1** in feed samples which will be used in livestock feeding
320 programs to avert fescue toxicosis remains relevant. On the other hand, the QuEChERS method
321 had a significant peak for **2** in both seed and straw samples (Figures 4A and C); this technique
322 appears to be able to extract more of this stereoisomer than the SPE method, and/or
323 epimerization from the (*R*) to the (*S*) isomer configuration is occurring at a greater rate over the
324 course of performing this method. However, quantitation was again performed using only **1**, to
325 compare extractability of **1** alone between the two methods. When the QuEChERS extraction
326 procedure was applied to 17 tall fescue straw samples that had also been quantitated for **1** using
327 the Ergosil SPE method, a correlation coefficient of 0.9978 was obtained when the two methods
328 were compared. The concentrations tested ranged from seven that were below the LOQ (100
329 µg/kg) to an additional ten from 119-632 µg/kg, giving a good representation of the **1**
330 concentrations that are typically seen in samples submitted to the Endophyte Service Laboratory.

In summary, a method following the QuEChERS extraction procedure was developed for determination of **1** in tall fescue seed and straw material using HPLC-fluorescence for detection. The method has eliminated the need for halogenated/chlorinated solvents; is faster and as equally efficient as compared to the Ergosil SPE method currently in use in our laboratory; and was validated to perform across a linear range of 100-3500 µg/kg **1** in tall fescue seed and straw material. These features make it ideal for application in a high-throughput food safety laboratory.

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FIGURE CAPTIONS

Figure 1. Chemical structures of ergovaline (**1**) and ergovalinine (**2**).

Figure 2. Evaluation of different solvent mixtures for extraction of **1** from endophyte-infected tall fescue seed containing 1998 µg/kg **1** using the QuEChERS method (*n* = 3/solvent). For solvents tested refer to Materials and Methods.

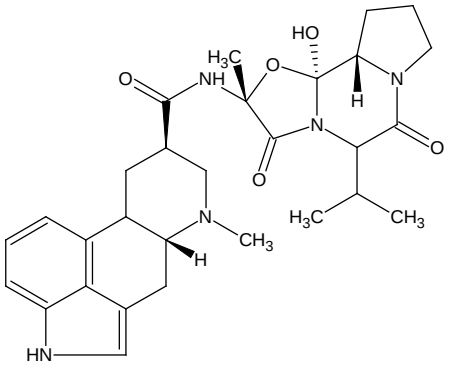
Figure 3. Additions to the aqueous component of the extraction solution evaluated for recovery of **1** from tall fescue seed (1998 µg/kg **1**) and tall fescue straw (850 µg/kg **1**) (*n* = 3/sample type/solvent mixture). 1) 1% acetic acid:MeCN (50:50), 2) 2.1 mM ammonium carbonate:MeCN (50:50), 3) 1 mM ammonium carbonate:MeCN (50:50), 4) 1% formic acid:MeCN (50:50), 5) 0.5% acetic acid:MeCN (50:50), 6) water:1% acetic acid in MeCN (50:50), 7) water:MeCN (50:50).

Figure 4. Representative HPLC-fluorescence chromatograms comparing extraction of tall fescue seed and straw between the optimized QuEChERS method and an Ergosil SPE method for **1** and its epimer **2**. A) QuEChERS extract of tall fescue seed, B) Ergosil SPE extract of tall fescue seed, C) QuEChERS extract of tall fescue straw, and D) Ergosil SPE extract of tall fescue straw.

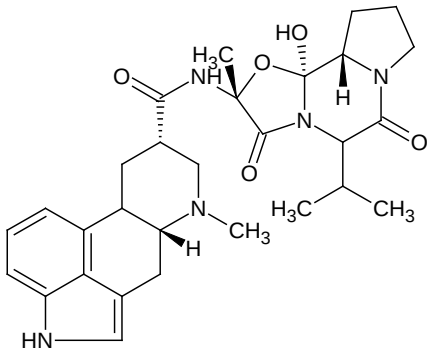
Table 1. Comparison of Mixing Times for Solution of 2.1 mM Ammonium Carbonate:MeCN (50:50, v/v) from Tall Fescue Seed and Straw for Extraction of Ergovaline, **1**, Using the QuEChERS Method.

	30 min	60 min	90 min	120 min
	(µg/kg Ergovaline, **1**)[a]			
Tall fescue seed (1998 µg/kg)	1901 (3)	1771 (4)	1637 (4)	1644 (3)
Tall fescue straw (776 µg/kg)	712 (8)	675 (8)	568 (11)	620 (9)

[a]RSD values are given in brackets (n = 5)



1: Ergovaline



2: Ergovalinine

Figure 1.

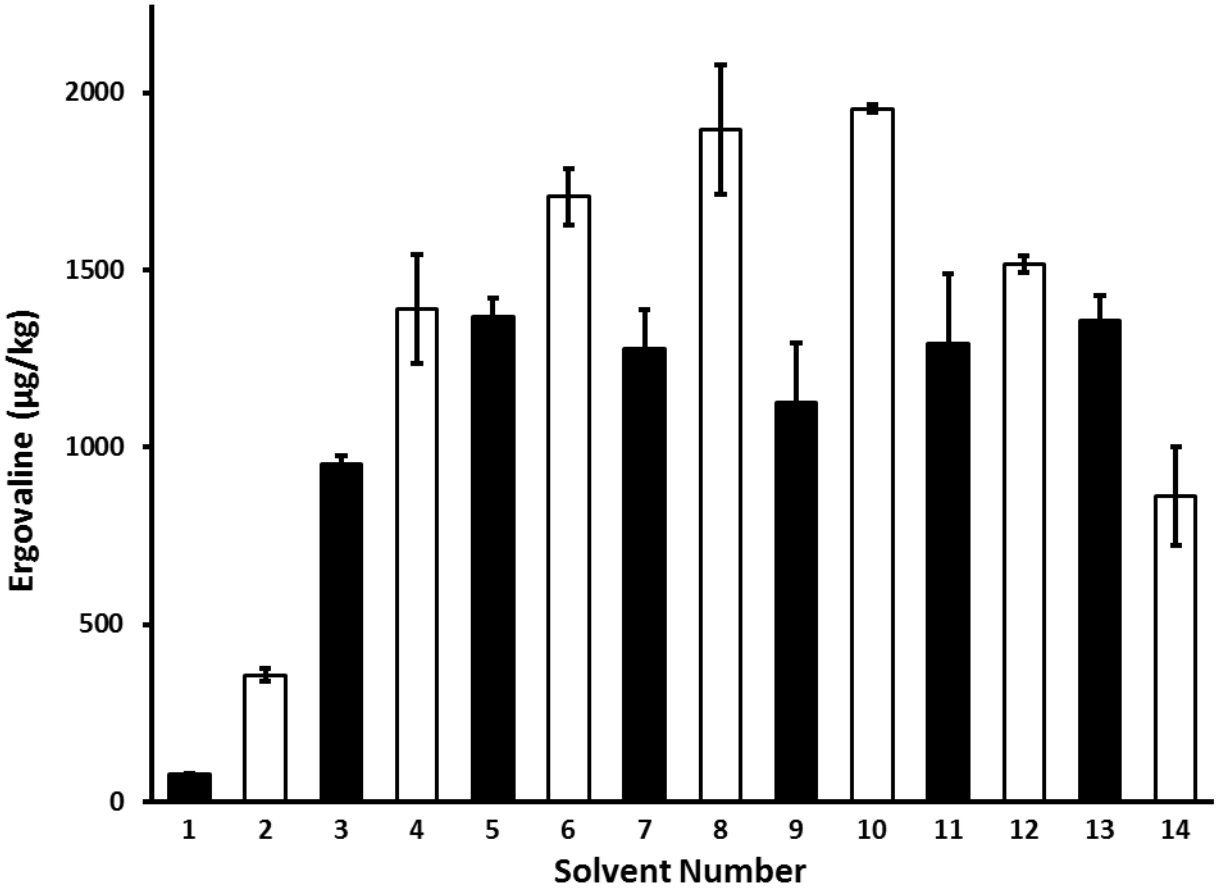

Ergovaline (µg/kg)
2000
1500
1000
500
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Solvent Number

Figure 2

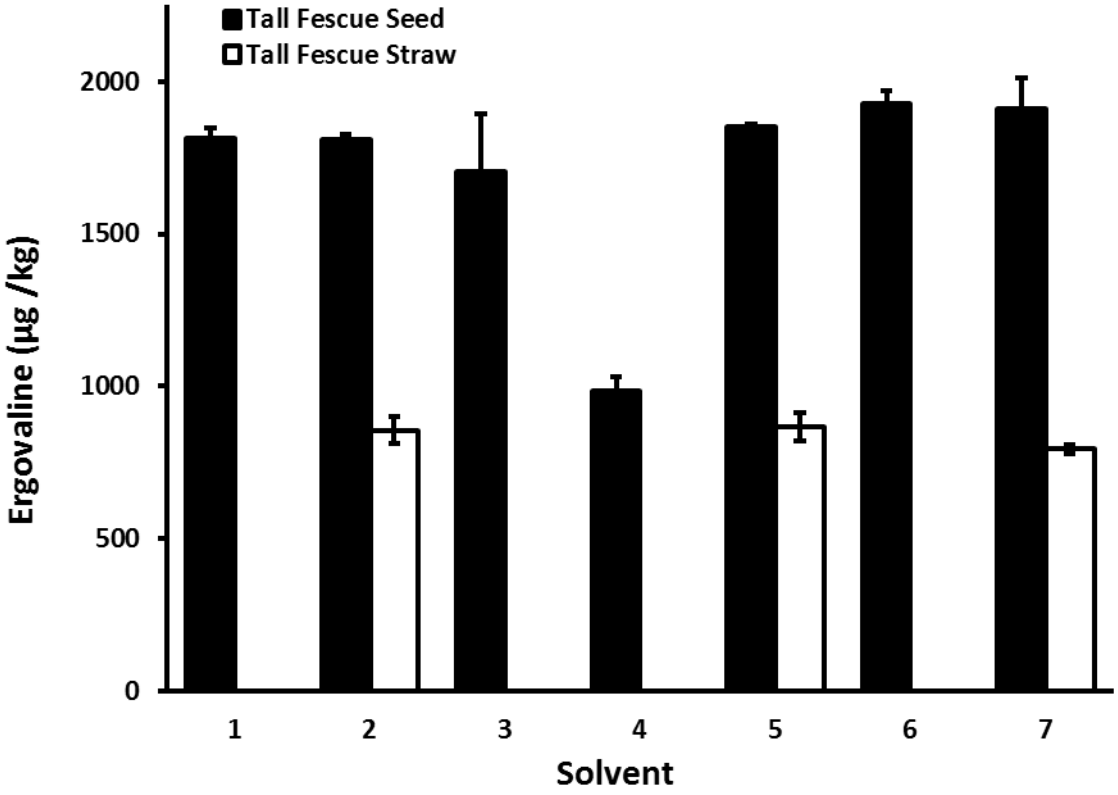

Tall Fescue Seed
Tall Fescue Straw
Ergovaline (µg /kg)
2000
1500
1000
500
0
1
2
3
4
5
6
7
Solvent

Figure 3

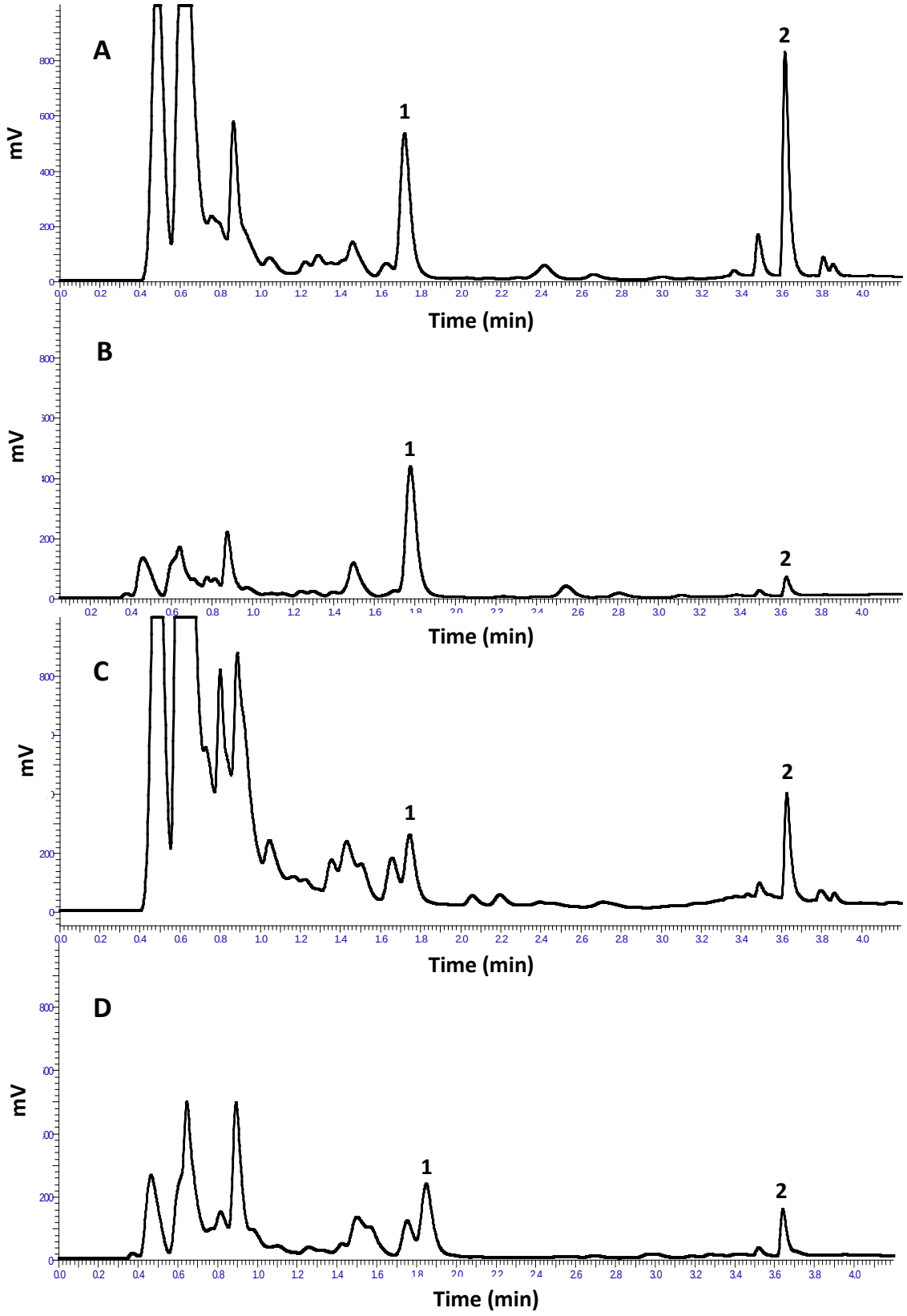


Figure 4.

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