Alkaloids of the sorghum ergot pathogen (*Claviceps africana*): assay methods for grain and feed and variation between sclerotia/sphacelia

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Abstract. Assay methods for the alkaloids of sorghum ergot (*Claviceps africana*) are described and compared. Sorghum ergot bodies (sclerotia/sphacelia) from various regions of Queensland and New South Wales were collected in 1997 and 2001 and assayed by spectrophotometry, thin layer chromatography, or high performance liquid chromatography (HPLC). All contained dihydroergosine (DHES) as the main alkaloid component (about 80%), with smaller amounts of dihydroelymoclavine and festuclavine. The preferred method of assay for infected sorghum and mixed feeds involved extraction into dichloromethane:methanol:ethyl acetate:ammonium hydroxide (50:5:25:1) using an ultrasonic bath. After solvent removal, the extract was dissolved in diethyl ether and partitioned into 0.5 M hydrochloric acid. After adjusting the pH to 8–10 with ammonium hydroxide, the alkaloids were extracted into dichloromethane, the solvent evaporated, and the residue dissolved in methanol. HPLC separation was on a C18 column, 150 by 3.9 mm, run isocratically at 40°C, with acetonitrile:0.1% ammonium acetate:methanol (31:50:20) as the mobile phase. Detection was either by UV at 280 nm or by fluorescence with excitation at 235 nm and absorbance at 340 nm. Levels of quantitation for DHES in sorghum approached 0.1 mg/kg (UV) and 0.01 mg/kg (fluorescence). Method recoveries for DHES in the range of 0.025–7 mg/kg averaged 75%. The total alkaloid content of ergot bodies (sclerotia/sphacelia) from different batches of grain varied from 100 to 7900 mg/kg (0.79%). Within batches, there was much less variation in the alkaloid content of ergot bodies, but larger ergots tended to contain more alkaloid than smaller ergots, and those infected with *Cerebella* species contained even less; this probably related to the ratio of sclerotial/sphacelial tissue present. Honeydew also contained DHES (1–10 mg/kg) and might contaminate clean grain at significant levels. Tests on 4 farms showed that substantial amounts of ergot bodies and alkaloids were removed during grain harvesting.

Additional keywords: mycotoxin, fungus, sclerotium, sphacelium.

Introduction

Ergot disease of sorghum (*Sorghum bicolor* (L.) Moench) was first identified in Australia in April 1996, and is now endemic in all sorghum-producing regions in Queensland and New South Wales, as well as seed production areas in the Ord River irrigation area (Western Australia) and southern New South Wales (Ryley *et al.*2001). The disease affects unfertilised ovaries. Ovarian tissue is replaced by a white sporulating fungal mass, the sphacelium, from which sticky, conidia-containing honeydew oozes and facilitates rapid spread of infection. Subsequently, further tissue differentiation can occur to eventually produce a hard sclerotium, a 'resting' or dormancy structure able to withstand degradation in the environment. Sphacelial and sclerotial tissues exist side by side in infected ovaries, but sphacelial tissues become inactive as the sclerotium develops and finally separates to form a clear boundary from the sphacelial tissues, which become dry and shrunken (Frederickson *et al.* 1999). Fully formed sclerotia are usually <6 mm long, cylindrical with rounded ends, comprising tightly compacted hyphal cells, with a thin orange-brown rind and a white interior. By contrast, sphacelia (which can contain some sclerotial tissue) are often pointed, comprising loosely woven hyphae, white-cream, do not possess a hard rind, and often have floral elements (paleas, lemmas, and glumes) attached after harvest. The honeydew-oozing sphacelia are often overgrown by the saprophytes *Cerebella* spp., which develop powdery, black, convoluted fruiting bodies (sporodochia) on the surface of the sphacelia.

Three species of fungi are currently recognised as causing sorghum ergot disease: *Claviceps africana* Frederickson, Mantle and DeMilliano; *Claviceps sorghi* Kiulkarni, Seshadri and Hegde; and *Claviceps sorghicola* Tsukiboshi, Shimanuki and Uematsu. *C. africana* can be distinguished by the production of the ergot alkaloid dihydroergosine (DHES), whereas the others produce little or none of this alkaloid (Frederickson *et al.* 1991; Bandyopadhyay *et al.* 1998). Actually, it has recently been shown that the alkaloid produced by *C. sorghicola* is caffeine and that *C. sorghi* also produces some caffeine (Bogo and Mantle 2000). Isolates from Australia appeared to be *C. africana*, based on morphology (Ryley and Henzell 1997), production of DHES (Maryam *et al.* 1997), and molecular analysis (Pazoutova *et al.* 2000), and this was recently confirmed by Komolong *et al.* (2002).

There were no reports of disease in livestock caused by sorghum ergot anywhere in the world (Mantle 1977) until cases of impaired milk production and feed refusal in sows and cows were identified in Queensland (Blaney *et al.* 2000). Feeding trials have since demonstrated that sorghum ergot alkaloids have similar toxic effects to rye ergot alkaloids (Blaney *et al.* 2001). In order to establish the degree of toxicity of sorghum ergot to livestock, reliable assay methods were required for the alkaloids present. A method was described for the assay of sorghum ergot alkaloids in sclerotia (Frederickson 1990), and methods have been published for the assay of rye ergot alkaloids at trace concentrations in wheat, barley, and flour (Scott and Lawrence 1980), but none has been assessed for detection of trace concentrations in sorghum and mixed feeds.

It was important to determine the extent to which ergot bodies (sclerotia/sphacelia) varied in alkaloid content, whether alkaloid content could be predicted from their size and shape, and whether harvesting reduced the ergot and alkaloid content of grain.

Consequently, the objectives of this study were: to assess methods for alkaloid detection in grain and feed, including sampling; to identify the alkaloids and amounts present in different sizes of sclerotia/sphacelia from different regions, and in honeydew; and to determine if alkaloid concentration was reduced during grain harvest.

Materials and methods

Reagents

All reagents and the solvents diethyl ether, ethyl acetate, and dichloromethane were analytical reagent grade (Ajax Chemicals, Australia). Other solvents (chloroform, methanol, acetonitrile) were HPLC grade (Malinckrodt Baker, Inc., Paris, Kentucky).

Van Urk's reagent (0.125 g *p*-dimethlyaminobenzaldehyde) was dissolved in 100 mL of 65% sulfuric acid and 0.1 mL of 5% ferric chloride was added.

Ehrlich's reagent (1 g *p*-dimethlyaminobenzaldehyde) was dissolved in 25 mL concentrated hydrochloric acid and 75 mL methanol.

Alkaloid reference standards

Dihydroergosine (methane sulfonate salt) was supplied by Dr Danka Pericic of the Ruder Boscovik Institute in Croatia; this appeared chromatographically pure on TLC and HPLC and was assumed to be 100% DHES. Samples of festuclavine and dihydroelymoclavine were supplied by Dr Miroslav Flieger (Institute of Microbiology, Academy of Sciences of the Czech Republic), and were about 95% pure with each containing about 5% of the other alkaloid. The standards were dried over silica gel and stored at –20°C. Quantitative results were corrected for purity of standards and for the mass of the methane sulfonate group, and expressed in terms of the base alkaloids. The stability of these compounds either in methanol solution or in methanolic sample extracts containing these compounds was tested under the following regimens of light and temperature exposure: 24 h exposure to indirect sunlight (in glass containers on the laboratory bench near a window; 30 min heating at 100°C; 14 days at 10°C (refrigerator); and alkaline conditions of pH 10–11 for 1–2 h.

Alkaloid detection by spectrophotometry

Extracts of ergot sclerotia/sphacelia (5–20) in 2% tartaric acid were assayed for total alkaloid by adding 2 mL extract to 4 mL of van Urk's reagent (see extraction Method A below). After 15 min reaction in a waterbath at 30°C, optical density was read at 580 nm and compared with a standard curve prepared with 2-mL aliquots of solutions containing 0, 10, 20, 40, and 60 mg alkaloid/L.

Alkaloid detection by thin layer chromatography

Aliquots (1–10 µL) of sample extracts, each equivalent to 1 g of sample dissolved in 100 µL dichloromethane, were spotted onto silica gel 60 F_{254} plates (0.2-mm layer thickness on aluminium sheets, Merck). The plates were developed in dichloromethane:methanol (9:1), or chloroform:methanol (9:1). After air-drying, the plates were either examined under a UV lamp at 254 nm, or sprayed with Ehrlich's reagent and heated at 80°C until alkaloids appeared as blue spots. Quantitation of alkaloids was by visual comparison of spot intensity with that of standards.

Alkaloid detection by high-performance liquid chromatography

The system used was a Novapac (Waters) C18 column 150 by 3.9 mm, used isocratically at 40°C. This was evaluated with various solvent mixtures, including methanol:8 mM ammonium hydroxide (7:3); methanol:water (1:1); acetonitrile:water (1:1 and 7:3); and acetonitrile:0.1% ammonium acetate (38:62). The mixture ultimately chosen was acetonitrile:methanol:0.1% ammonium acetate (31:20:50). Both UV and fluorescence detection systems were compared and wavelengths optimised using standard techniques.

Sampling and milling of grain

Sampling can be a major source of error when dealing with extraneous contaminants in grain. Ergot bodies were observed to flow differently from grain, producing an uneven distribution in bulk grain. Techniques used to sample bulk grain for weed seeds were adopted when sampling bulk grain for ergot. Observations were made about the distribution of ergot in bulk grain samples by collecting 7 sequential samples (each 1 kg) as 2 t of grain were unloaded from a 5-t silo, and assaying these samples in duplicate or triplicate. Observations were also made of the precision of alkaloid estimation by replicate assay when the grain was ground to 2 different degrees of fineness (firstly, hammer-milled to pass a 1-mm screen and, secondly, shatter-box milled to pass a 0.1-mm screen).

Alkaloid extraction and clean up of sorghum and mixed feeds

Several extracting solvents were evaluated for extraction efficiency, cleanliness of extract, compatibility with subsequent clean-up steps, and detection methods and safety. These included: diethyl ether; methanol; methanol:water (70:30); dichloromethane; chloroform; and dichloromethane:methanol:ethyl acetate:ammonium hydroxide (50:5:25:1). The following 2 procedures were evaluated.

Method A (adapted from Frederickson 1990): 10 g of sorghum or feed sample was mixed with 2 g sodium bicarbonate and twice extracted with 50 mL diethyl ether for 3 min in an ultrasonic bath. After filtration, alkaloids were twice partitioned into 50 mL 2% tartaric acid. The pH was adjusted to 8–10 with ammonium hydroxide, and alkaloids twice partitioned into 50 mL dichloromethane. After evaporation to dryness, the extract was dissolved in 1 mL of methanol, transferred into a sealed vial, and stored in the dark at –10°C.

Method B (adapted from Scott and Lawrence 1980): 4–10 g of sample was extracted 3 times with 30 mL dichloromethane:methanol:ethyl acetate:ammonium hydroxide (50:5:25:1) in an ultrasonic bath for 3 min for each extraction. Extracts were filtered and the residue twice washed with 50 mL dichloromethane. Extracts were combined and evaporated to dryness, and re-dissolved in 40 mL diethyl ether:methanol (35:5). Alkaloids were partitioned twice into 60-mL aliquots of 0.5 M HCl. The pH was adjusted to 8–10 with ammonium hydroxide, and alkaloids partitioned 3 times into 25 mL of dichloromethane. The extract was evaporated to dryness, transferred into a sealed vial with 1 mL of methanol, and stored in the dark at –10°C.

Alkaloid recoveries and extraction efficiency

Definitive quantities of alkaloids, dissolved in methanol, were added to milled sorghum grain and to mixed feeds, both based on ergot-free sorghum. The samples were well mixed, excess methanol was allowed to evaporate, and samples held for at least 12 h before assay. Samples were extracted using Methods A and B and alkaloids quantified by HPLC by direct comparison with the concentrations of standard alkaloids in the spiking solutions.

Completeness of extraction (as opposed to recovery efficiency of added standard) was assessed by exhaustive extraction of naturally contaminated samples using favoured solvents combined with Soxhlet extraction, shaking, high-speed blending, and an ultrasonic bath. Some solvents were compared using a single extraction with a 10:1 ratio of solvent to sample and 3 min in an ultrasonic bath.

Alkaloid concentrations in sclerotia/sphacelia, infected grain, and honeydew

Ergot bodies were collected from commercial sorghum crops at various locations in central and southern Queensland in 1997. All fitted published descriptions of *C. africana* (Frederickson *et al.* 1991). Samples selected for assay were mostly composed of fully formed sclerotia in that they were separated from the glumes and enclosed with a hard rind. From other sorghum samples collected in 1997, a few 'typical' ergot bodies, larger ergot bodies, and ergot bodies infected with *Cerebella* spp. (probably sphacelia) were selected for separate assay.

At one site on the Darling Downs (Norwin) in 1997, 2 types of sclerotia were identified, one of which was 'atypical', being larger and more cylindrical (5–8 mm long and 1.8–2.5 mm wide) than the typical shape (4–5.5 mm long and 2–4 mm wide) (Ryley and Henzell 1997).

Alkaloids were extracted from the sclerotia/sphacelia collected in 1997, using a slight variation of Method A. Sclerotia/sphacelia (1 g) were ground together with 0.2 g sodium bicarbonate using a mortar and pestle, and then extracted twice with 15 mL of diethyl ether, in an ultrasonic bath for 3 min. Combined extracts were filtered and alkaloids were partitioned twice into 15-mL aliquots of 2% tartaric acid. An

aliquot (2 mL) of this extract was taken for spectrophotometric determination of total alkaloids. The remaining 28 mL was made alkaline (pH 8–10) with ammonium hydroxide, and twice partitioned into 15-mL aliquots of methylene chloride. This extract was evaporated to dryness, dissolved in 1 mL methanol, and examined by TLC and/or HPLC. The relative precision of these 3 detection systems was compared by duplicate estimations of the same ergot extracts, originating from the ergot samples collected in 1997.

In 2001, sorghum crops infected with ergot were identified on farms in 3 locations in Queensland and one in New South Wales. Before harvest, 50 panicles were collected from each of 6 sites (replicates) within each crop and threshed using a small stationary thresher. Subsequently, during harvest, approximately 2-kg samples of grain were collected from the harvesters as they passed over the same sites from which the panicles had been previously collected. On 2 sites, clumps of grain stuck together with honeydew were also collected. DHES was assayed in subsamples (500 g) of the pre- and post-harvest grain, and in the grain clumps. In separate subsamples (100 g) of the pre- and post-harvest grain, ergots (whether sclerotia, sphacelia, or covered with *Cerebella* spp.) were separated by hand and weighed to determine percentage total ergot in the samples. In a second investigation, subsamples from each of 3 replicates of the pre-harvest grain were individually separated into ergot separated from glumes (mostly sclerotia), ergot still within the glumes (mostly sphacelia), ergot with *Cerebella* infection (mostly sphacelia), remnants of the panicle (trash), and apparently clean grain. The DHES concentration of each fraction was determined using Method B.

Pure honeydew was collected on 2 occasions in 2001 from ergot-infected panicles growing in a glasshouse at Toowoomba, Qld (honeydew approx. 1 and 3 weeks old), and in March and April 2001 from infected panicles in a field trial at Hermitage Research Station, Warwick, Qld.

Several other sources of naturally contaminated sorghum were used to develop the assay methods; some of these had been associated with toxicity to livestock (Blaney *et al.* 2000), and others had been submitted from farmers for testing before feeding to livestock.

Results and discussion

Stability of alkaloids

DHES in methanol solutions proved stable during several months of storage at –10°C. Extracts containing DHES did not appear to be affected by any of the temperature, light exposure, and pH regimen tested. It was also noted that contaminated sorghum samples had similar alkaloid concentration before and after storage for 3 years in silos, as did milled samples held at 4°C for long periods. This is in accord with Mantle's observation (1977) that the 9,10 dihydro-ergot alkaloids are much more stable than their unsaturated counterparts.

Alkaloid detection by spectrophotometry

The method was satisfactory for rapid screening of sclerotia/sphacelia, with a detection limit of about 5 mg alkaloid/kg. However, colour development tended to be variable and unreliable in sorghum and mixed feed. Close control of temperature and time of colour development improved the precision of assay, but the method could only be described as semi-quantitative for sorghum and mixed feed.
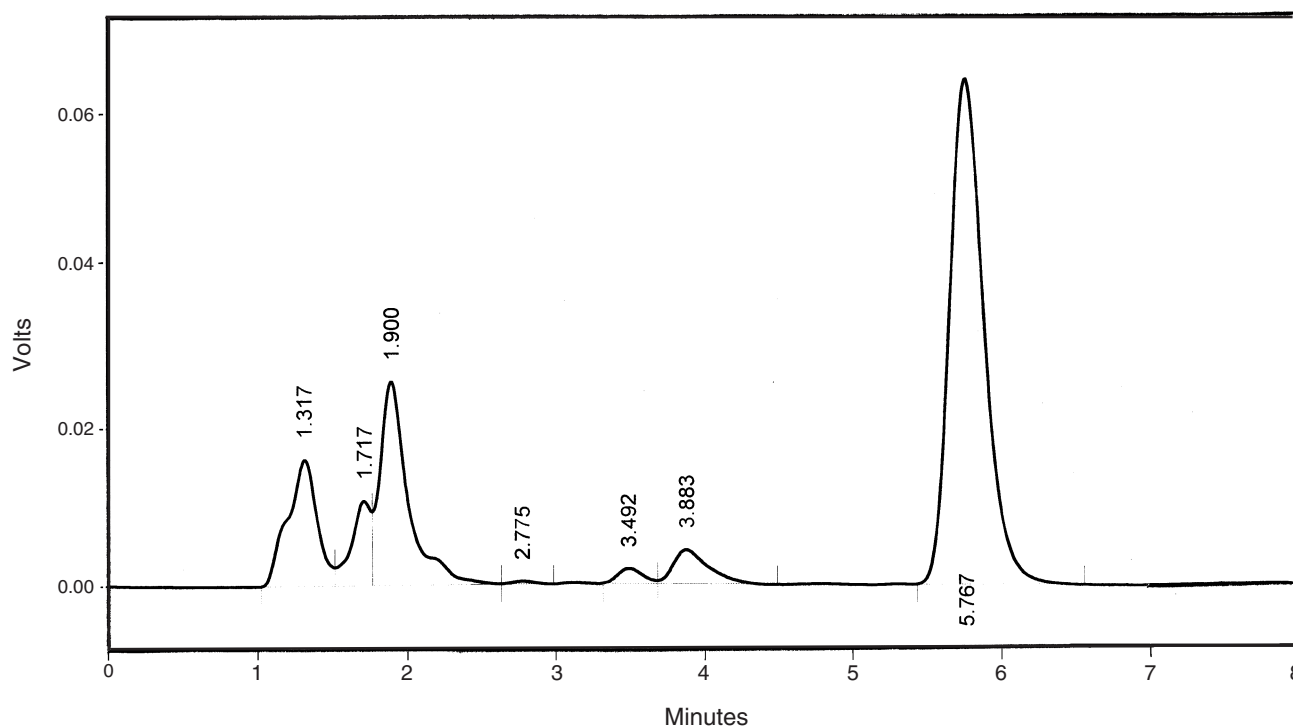
Fig. 1. High performance liquid chromatogram of an extract of sorghum ergot sclerotia, showing dihydroergosine (5.8 min), festuclavine (3.9 min), and dihydroelymoclavine (1.9 min). Column C18, 150 by 3.9 mm. Mobile phase acetonitrile:methanol:0.1% ammonium acetate (31:20:50) at 1 mL/min. Detection by fluorescence with excitation at 235 nm and absorption at 340 nm.

Alkaloid detection by thin layer chromatography

All samples of ergot showed a similar pattern of spots, with the dominant spot being coincident with standard DHES (Rf = 0.31, methylene chloride:methanol, 9:1). The other 2 prominent spots were coincident with festuclavine (Rf = 0.23) and dihydroelymoclavine (Rf = 0.15). In some samples, there were minor spots at Rf = 0.20 and Rf = 0.16 (see next section). Detection limits for DHES were 25 ng per spot, and ranged from approximately 0.2 mg/kg for sclerotia to 2 mg/kg for samples with a high degree of interference such as sorghum and mixed feed. The precision of TLC estimation is heavily dependent on the skill of the analyst, and it is difficult for the eye to discriminate between spots varying in intensity by less than 20%, at best. The need for an even application of spray reagent introduces another variable. TLC estimation is therefore satisfactory for screening and general diagnostic purposes, and also as a confirmatory test to support the HPLC procedure.

Alkaloid detection by high performance liquid chromatography

A typical chromatogram of an ergot extract is shown in Fig. 1. Under the operating conditions described and the solvent mix of acetonitrile:methanol:0.1% ammonium acetate (31:20:50), DHES, festuclavine, and dihydroelymoclavine appeared at retention times of 5.8, 3.9,

and 1.9 min, respectively (these retention times do vary from day to day with column, pre-column, and temperature). This is consistent with the work of Barrow *et al.* (1974), who showed that dihydroelymoclavine and festuclavine are the major precursors of DHES in sorghum ergot, and that alkaloids with the 9,10 position unsaturated are not involved in biosynthesis of DHES. There were minor peaks at 3.5 and 2.1 min that when collected from the HPLC eluent and tested by TLC corresponded to the minor spots first detected by TLC. Based on reaction with Erhlich's reagent on TLC, and UV and fluorescent characteristics on HPLC, these are related alkaloids. The peak at 3.5 min is likely to represent pyroclavine and one of the peaks shown at 1.7 and 1.3 might represent chanoclavine, based on comparisons with the chromatograms of Frederickson (1990). Agroclavine was not detected in any sclerotia/sphacelia; standard agroclavine appeared in the HPLC trace at 4.2 min.

Detection was achieved either by UV or fluorescence spectrophotometry. We found the following conditions to be optimal for detection of DHES in reference solutions: using a UV detector at 280 nm, injection volume of 10 µL, the limit of detection (LOD) was 2 ng (Signal/Noise Ratio = 3) and the limit of quantitation (LOQ) was 7 ng. Using a fluorescence detector with excitation at 235 nm and detection at 340 nm, the LOD was 0.02 ng and LOQ was 0.1 ng in respect to standards.
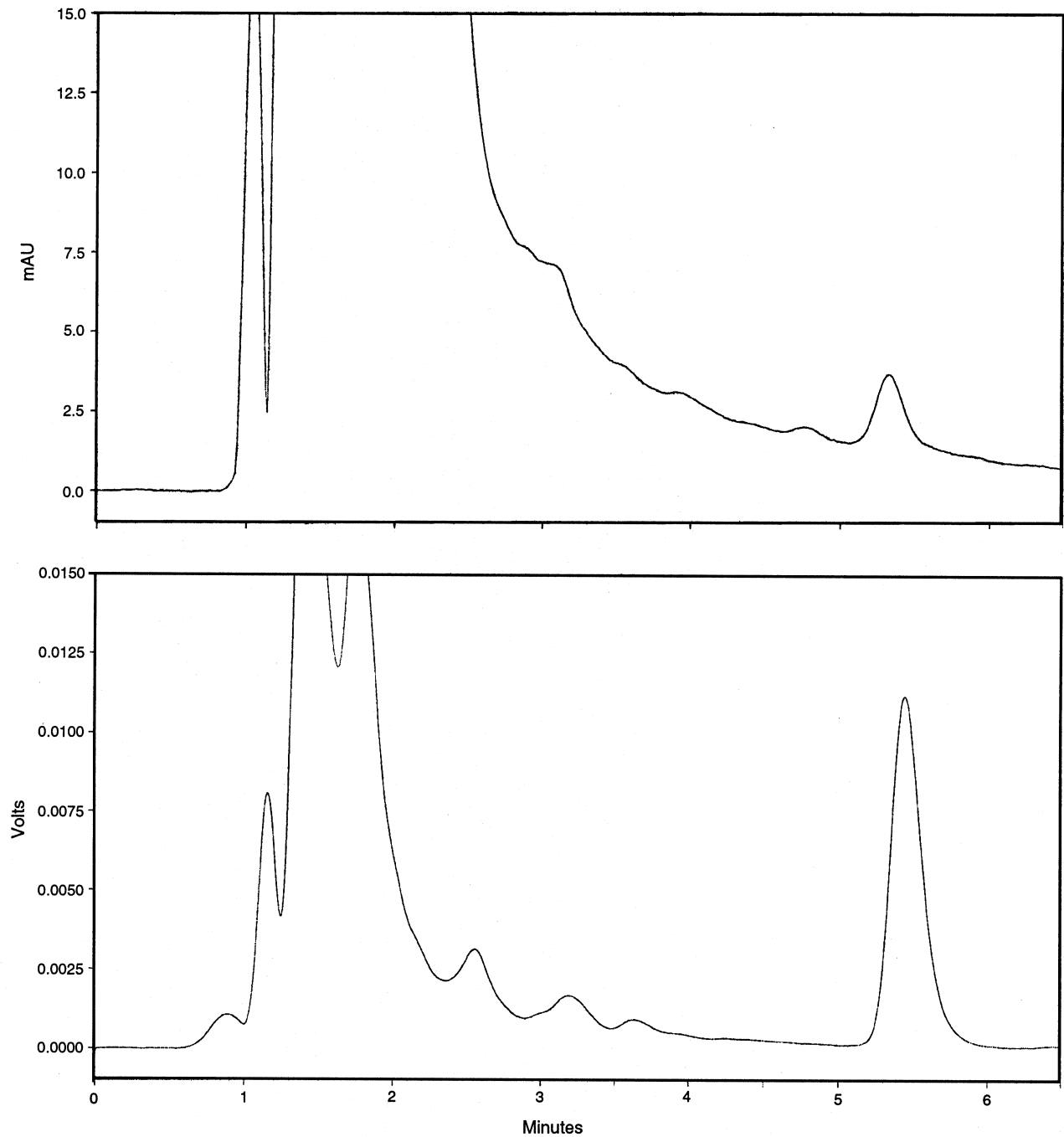
Fig. 2.   High performance liquid chromatograms of extracts of a sorghum-based pig feed containing 1.4 mg DHES/kg, as detected by ultra-violet absorbance (top, 5.4 min) and fluorescence (bottom, 5.5 min). Column C18, 150 by 3.9 mm. Mobile phase acetonitrile:methanol:0.1% ammonium acetate (31:20:50) at 1 mL/min.

The extraction and purification methods described above for sorghum samples allow for a 10-fold concentration from initial sample to final extract (10 g–1 mL), which implies a LOD of about 0.001 mg/kg. In practice, these detection limits are restricted by impurities in sorghum and mixed feeds. Recovery data given below support a LOQ of about 0.01 mg/kg in sorghum grain. Fig. 2 shows chromatograms of an extract of mixed pig feed containing 1.4 mg DHES/kg. It can be inferred from the signal to noise ratios in these chromatograms that a LOD of 0.2 mg DHES/kg using the UV detector, and 0.02 mg DHES/kg using the fluorescence detector, are more realistic for complex samples such as

Table 1. Dihydroergosine (DHES, mg/kg) and alkaloid (mg/kg) concentrations in ergot sclerotia/sphacelia collected in 1997 from various regions of Queensland, assayed by different methods

Sampling site	DHES		Total alkaloid
	TLC	HPLC	spectrophotometry
Moreton (south-east Queensland)			
Mutdapilly sclerotia[A]	1100	1200	1400
Grantham sclerotia	6000	5700	6200
Darling Downs			
Toowoomba sclerotia	1300	1600	1900
Wyreema sclerotia	1300	1100	1400
Norwin typical sclerotia	2400	2400	2000
Norwin, atypical (larger) sclerotia	6000	7000	7200
Central Queensland coast			
Monto A sclerotia	6200	5200	7900
Monto B sclerotia	1100	2400	3100
Monto C sclerotia	1000	1800	2700
Monto D sclerotia	700	1500	1900
Wowan A sclerotia	900	1200	1000
Monto E (typical sclerotia)			340
Monto E (larger sclerotia)			780
Monto E (ergot body with *Cerebella*)[B]			115
Biloela (typical sclerotia)			1150
Biloela (ergot body with *Cerebella*)			66
Wowan B (typical sclerotia)			194
Wowan B (ergot body with *Cerebella*)			128
Central highlands			
Emerald sclerotia	7000	6400	7200
Capella sclerotia	200	100	200

[A]Samples described as sclerotia were predominantly composed of ergots fully separated from the glumes and with a hard rind. Some retained glumes and might have residual sphacelial tissue.

[B]Samples infected with *Cerebella* still had adhering glumes, and were probably mostly sphacelial tissue.

mixed feed, with the LOQ rising in proportion. It can also be seen that it is only possible to detect festuclavine and dihydroelymoclavine in sclerotia/sphacelia samples (Fig. 1), or in infected sorghum at high concentrations using fluorescence detection; otherwise there is too much interference.

The dissolution of sample extracts and treatment prior to HPLC was identified as a critical step in achieving reproducible results. We found that sample extracts were difficult to dissolve in the original mobile phase used (acetonitrile : 0.1% ammonium acetate) without formation of fine precipitates that adsorbed alkaloids, which were then removed during final filtration prior to injection (alkaloids could be reclaimed from the filters with methylene chloride). Dissolving extracts in methanol largely overcame this problem, but then precipitates could be formed after injection onto the column, eventually blocking the pre-column. The addition of methanol to the mobile phase appeared to overcome the latter problem. However, we also found that some extracts needed to be dissolved in methanol : methylene chloride (4 : 1) before injection, rather than in methanol alone. These steps overcame a major problem of poor reproducibility.

Sampling and milling of grain and feeds

On the basis of our data (presented below in Table 1) that some sclerotia can contain up to 7900 mg/kg total alkaloid, as few as 12 such sclerotia might be sufficient to contaminate a 1-kg sample at a concentration of 1 mg/kg. In order to obtain a representative final analytical sample of 10 g, those 12 sclerotia would have to be broken into 100 equal pieces and distributed very evenly through the grain. Moreover, sclerotia/sphacelia are not as brittle as sorghum grain, tend to squash, and resist some milling operations, which is likely to be another source of imprecision in the assay method.

For screening assays where some accuracy could be sacrificed for speed, we found it satisfactory to mill about 500 g through a 1-mm screen before taking a 10-g final

sample. To improve accuracy and precision, a double milling process was adopted. Firstly, the entire 1-kg sample was hammer-milled through a 1-mm screen. After thorough mixing, a riffle divider was used to withdraw a subsample of at least 100 g, which was then ground to pass a 0.1-mm screen using a shatter-box mill.

The order of sampling versus method variation was shown by replicate assay of samples taken sequentially from a silo as it was unloaded. The assay results ranged from 12 to 29 mg DHES/kg, and the mean (± s.d.) was 23 (± 4.3) mg/kg. The variation expressed as the s.d. was thus 18.7% of the mean value. The variation between replicate assays was then determined after normalisation of results for the 7 samples to the overall mean. This showed that the variation (s.d.) due to the assay was 11.5% of the mean, leaving the remaining variation of 7.2% as due to the sampling variation.

Alkaloid extraction and clean up of sorghum and mixed feeds

Method A

Diethyl ether has been a preferred solvent for alkaloid extraction for many years. It combines good extraction efficiency for alkaloids with less co-extracting interferences than other solvents produce, and we used this during our earlier studies. It produced the cleanest extract of those solvents tested for sclerotia/sphacelia. However, it is extremely flammable and has been phased out of use in many laboratories for this reason. It did not perform as well with mixed feeds as with ergot, due to serious emulsion problems. High evaporation rates also made it difficult to use in blenders and shakers.

Method B

This used a dense solvent extracting mixture, which appeared to penetrate the sample better than ether and, probably for this reason, gave slightly better recoveries. The method still uses diethyl ether in a partitioning step, which is a disadvantage. Emulsions remain a problem with this method during the partition steps, and we have observed some association between their occurrence and poor recoveries, but they can sometimes be overcome via centrifugation and/or freezing.

Alkaloid recoveries and extraction efficiency

We found that diethyl ether, methylene chloride, and methanol all extracted 80–90% of the alkaloid from sclerotia and grain in a single extraction with 10:1 solvent:sample ratio, compared with estimates obtained by exhaustive extraction with the same solvents. With sorghum and mixed feeds, the mixture recommended in Method B, and methanol, recovered about 5% more than methylene chloride and diethyl ether. Barring the possibility of some intractably bound alkaloid fraction, extraction of alkaloids from naturally contaminated sorghum appeared to be quite

efficient. The double extraction using a 3:1 solvent:sample ratio recommended by Scott and Lawrence (1980) also appeared to recover over 80%. However, we found that a third extraction improved recoveries by about 5%, so we recommend this modification to the method.

Using Method B, recoveries of DHES added to sorghum grain ('spiking') at concentrations ranging from 0.9 to 7 mg/kg were 74 ± 9.4% (mean ± s.d.) ($n = 10$). For spikes ranging from 0.12 to 0.25, recoveries were 69 ± 11.1% ($n = 7$). For spikes of 0.025 mg/kg, recoveries were 97 ± 13.3% ($n = 7$). A second analyst obtained recoveries of 66 ± 4.5% ($n = 10$) from sorghum spiked at 0.75–1.5 mg/kg. Recoveries from a formulated sow feed spiked at 5 mg/kg were 83 ± 17.0% ($n = 5$). Recoveries of dihydroelymoclavine and festuclavine were 98 ± 7.4% and 54 ± 6.2% ($n = 4$), respectively. Overall, these recoveries appear somewhat inferior to those reported by Scott and Lawrence (1980) from spiked wheat flour; those averaged 70–88% for a range of different rye ergot alkaloids at concentrations around 0.01 mg/kg, despite the greater instability of rye ergot alkaloids. We have checked all of the transfer and partitioning steps in the methods, and all were highly efficient for DHES. When poorer recoveries occurred, they seemed to be associated with occurrence of persistent emulsions during partitioning steps, and with precipitates in the final extracts (see comments above on HPLC). Sorghum grain contains very large amounts of pigments and other interferences compared with wheat, which increase the clean-up problems and may reduce recoveries through adsorption onto surfaces.

We did not perform exhaustive recovery experiments with Method A, but on several occasions when they were compared under conditions of replicate analysis, they achieved similar mean results (within 5%) for the assay of naturally contaminated samples.

Alkaloid concentrations in sclerotia/sphacelia, infected grain, and honeydew

The assay results using different assay methods for sclerotia/sphacelia collected in 1997 are shown in Table 1. The DHES concentration of sclerotia/sphacelia varied from 100 to 7000 mg/kg (0.01–0.70%) and total alkaloids from 100 to 7900 mg/kg. DHES was the dominant alkaloid seen during TLC for all samples. The 3 methods were fairly consistent in the estimates made, but only HPLC was sufficiently precise and reproducible to be recommended for routine assay. Subsequent examination of the HPLC profile of a large number of samples indicated that DHES usually comprised about 80% of the total alkaloid content of sclerotia/sphacelia and infected sorghum samples, with about 14% dihydroelymoclavine and 4% festuclavine. If the unknown peaks represent related alkaloids such as pyroclavine and chanoclavine (and have a similar order of molar absorptivity), then their concentrations would be <2% of the total alkaloid content of sclerotia/sphacelia.

Table 2.   Reductions in ergot and dihydroergosine contents of infected sorghum during the harvesting process in samples from farms in three regions of Queensland and one region in New South Wales during 2001
Values presented are range and mean

	No.	Kingaroy (South Burnett, Qld)	Dalby (Darling Downs, Qld)	Warwick (southern Downs, Qld)	Willowtree (Liverpool Plains, NSW)
Grain before harvest					
Ergot[A] (%)	6	0.24–1.41; 1.02	0.1–0.32; 0.22	0.28–0.64; 0.43	0.29–1.01; 0.60
DHES (mg/kg)	6	2.9–11; 6.0	0.1–0.8; 0.4	0.5–4.5; 1.8	0.9–4.3; 2.3
Grain after harvest					
Ergot (%)	6	0.08–0.48; 0.29	0.06–0.14; 0.09	0.02–0.54; 0.22	0.08–0.76; 0.26
DHES (mg/kg)	6	0.1–3.4; 1.5	<0.1–0.2; 0.13	<0.1–1.7; 1.1	<0.1–0.7; 0.3
Grain clumps with honeydew[B]					
DHES (mg/kg)	4	1.7–65; 23	<0.1–0.1; <0.1		

[A]Including ergot either fully separated or still attached to glumes.
[B]Clumps of grain adhering with honeydew, taken from harvested grain.

A few samples were separated into larger and smaller sclerotia/sphacelia and those with *Cerebella* infection (Table 1). There was a tendency for larger sclerotia/sphacelia to have more alkaloid than small sclerotia, and for *Cerebella*-infected sclerotia/sphacelia to have less, but this variation was far less than that between samples, so size of ergot would not be a dependable means of assessing relative toxicity.

Results obtained on infected grain before and after harvest on 4 farms in 2001 are shown in Table 2. There was a substantial reduction in ergot content and alkaloid concentration during the harvesting process, presumably as light-weight material was blown out of the header. Although 3 out of 4 samples exceeded the regulated limit of 0.3% sclerotia/sphacelia by weight in Queensland stockfood (Anon. 1997) before harvest, all were below that limit after harvest. It is noted, however, that cattle in feedlots have been adversely affected by only 0.8 mg DHES/kg in grain (Blaney *et al.* 2001), and this level was exceeded by 2 of the 4 crops (at Kingaroy and Warwick) even after harvest.

The presence of alkaloids in the grain clumped together with honeydew was inconsistent with our prior assumption that alkaloids would be present mainly in sclerotia and that consumption of grain at the early infection or 'honeydew' stage (when only sphacelia were present) would be safe for livestock. However, we had previously assayed a panicle covered with honeydew and detected 70 mg DHES/kg (B. J. Blaney, unpubl. data). This caused us to investigate whether this was due to developing sclerotia in the mass or to the honeydew itself. Consequently, 2 samples of 'pure' honeydew collected from inoculated panicles of *S. bicolor* in the Toowoomba glasshouse were assayed and found to contain 5.6 mg DHES/kg, and 10 mg/kg 2 weeks later. A third sample that was collected from a field trial at Warwick contained 1 mg DHES/kg. The alkaloid could be from the spores contained in the honeydew, or from alkaloids diffused

from the developing sclerotium into the honeydew. The risks associated with consumption of honeydew need to be re-assessed.

The results obtained on different fractions of the pre-harvest samples are given in Table 3. Alkaloid content of sclerotia/sphacelia varied greatly within samples, and varied even more from farm to farm. Fully formed sclerotia from only one farm (Kingaroy) in 2001 contained concentrations >1000 mg DHES/kg (0.1%), whereas a few sclerotia collected in 1997 (Table 1) contained up to 7000 mg DHES/kg. One of the reasons for this difference was that fully formed sclerotia were selected from standing crops in 1997, and possibly were larger than the average sclerotia/sphacelia in those crops, whereas in 2001 the sclerotia/sphacelia were taken from representative

Table 3.   Dihydroergosine (mg/kg; range and mean) in ergot bodies and different fractions of infected sorghum in samples from farms in three regions of Queensland and one region in New South Wales, collected in 2001

No.	Kingaroy (South Burnett, Qld)	Dalby (Darling Downs, Qld)	Warwick (southern Downs, Qld)	Willowtree (Liverpool Plains, NSW)
Clean grain				
3	0.1–0.4; 0.3	<0.1	0.1–1.6; 0.7	<0.1
Trash				
3	<0.1–14; 5	<0.5–6; 3	<0.1–0.7; 0.4	<0.1–0.1; <0.1
Ergot in glumes				
3	40–1050; 410	2.5–15; 9	8.3–49; 24	16–113; 49
Ergot separated from glumes				
3	900–1760; 1340	12–14; 13	19–74; 47	60–600; 240
Ergot with Cerebella				
2	12–57; 35		<0.3–27; 14	

subsamples of infected crops. The lack of control measures in 1997 and weather conditions also allowed more time for sclerotia to develop before harvest.

As was shown in some samples from 1997 (Table 1), structurally mature sclerotia contained far more alkaloid than developing sclerotia/sphacelia remaining in the glumes, but the content of the latter was still substantial and cannot be ignored. Fungi tend to invest more chemical defences in dormancy structures, and it has been expected that most of the alkaloid would be present in sclerotia. Sclerotia/sphacelia infected with *Cerebella* species had even lower alkaloid concentrations, and it seems very likely that infection with *Cerebella* is limiting alkaloid accumulation, either by competition for plant-obtained nutrients or by direct parasitism on the sclerotia/sphacelia.

Some samples of apparently clean grain from crops originally with higher alkaloid contents still had sufficient contamination to affect lot-fed cattle. Given the alkaloids detected in honeydew, it seems possible that honeydew dried on the surface might be responsible for this contamination of clean grain.

Conclusions

We have validated assay methods, and used these to identify the primary alkaloids contained in sclerotia/sphacelia from several sorghum-growing regions of Queensland and New South Wales as dihydroergosine, dihydroelymoclavine, and festuclavine. This is consistent with the known pattern of production of *C. africana* as described in other countries. All sclerotia/sphacelia examined showed a very similar spectrum of alkaloids, but the concentration present varied greatly between sclerotia and could not be reliably predicted from size and shape, although there were tendencies for larger sclerotia to contain more alkaloid and *Cerebella*-infected sclerotia/sphacelia to contain less. More investigations are required in order to assess factors influencing production of alkaloids, and whether the large differences in alkaloid accumulation in sclerotia can be related to climatic factors or to different genetic lines of either *C. africana* or host.

Acknowledgments

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