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Reducing the risk of ergot poisoning of flot cattle

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Abstract

Both rye grass ergot and sorghum ergot have been shown to produce heat stress, reduced feed intake and poor growth of cattle in feedlots, even at very low concentrations in the feed. Serious losses have occurred in feedlots in the past, but diagnosis and prevention of the problem has been seriously hampered by the lack of analytical methods to detect the poisonous alkaloids contained in the ergot. This project demonstrated that the toxicity of ergot varied greatly between batches, and that physical methods were inadequate for predicting toxicity. Subsequently, a new ELISA method was developed which is able to detect the main alkaloids of both rye grass and sorghum ergot. The method is very sensitive, potentially quite cheap, of high throughput, and moderately rapid (between 30 minutes and 2 hours, depending on the sensitivity and accuracy required). It currently requires modest laboratory facilities, but there is potential to adapt it for use outside the laboratory. Producers can now quickly determine whether the cause of reduced production in a feedlot is due to ergot. In seasons when ergot is widespread in grain, it would be possible to test all grain for alkaloids prior to purchase. The risk of ergot poisoning in feedlots has been greatly reduced.

Executive summary

Sorghum ergot is a fungal disease with the potential to affect a large proportion of Australia's sorghum crop. Studies showed that feedlot cattle were very sensitive to sorghum ergot alkaloids, mainly represented by dihydroergosine (DHES). Depressed feed intakes and growth were measured in Hereford steers given feed containing ergot and these effects were most pronounced during hot, humid weather, when affected animals were apparently unable to dissipate heat and showed signs of severe heat stress. The tolerance was < 1 mg/kg (1ppm) of alkaloid in the feed. Rye grass ergot (main alkaloid ergotamine) is also of concern to lot-feeders, as it regularly contaminates wheat and barley crops in southern states and has been associated with mortalities in cattle, eg in WA, at Forbes in late 1999, and in SA in 2001. As with sorghum ergot, the effects of rye ergot greatly increase susceptibility to heat stress. Both sorghum and rye ergot sclerotia vary greatly in alkaloid content, and good analytical methods are essential if tolerances are to be firmed up, and feed-monitoring regimens implemented. To reduce the risk of ergot poisoning in feedlots, this project investigated means of physically identifying those ergot sclerotia that might contain high concentrations of alkaloid, and whether physical segregation by grading could remove all of the alkaloid from contaminated loads. The project also aimed to develop rapid tests for ergot alkaloids.

Effectiveness of Near-Infra-Red Spectroscopy for detecting ergot alkaloids. A previous study supported by MLA (FLOT.115) evaluated Near Infra-red spectroscopy (NIR) for detecting ergot alkaloids, as this could potentially allow very rapid screening of grain samples. The results indicated that NIR can predict high alkaloid concentrations, but the detection limit of about 20 mg/kg was nowhere near the required detection limit of <1mg/kg alkaloids. The major differences in the spectra of samples were in the 1700 and 2100 nm regions of the NIR spectrum, which is where fat absorbs. We assayed a selection of the samples for fat content, and found that there was a high correlation between fat content predicted by NIR and alkaloid content. Consequently, we assayed

sclerotia of rye and sorghum ergot and compared this to sorghum grain. These results showed that ergot sclerotes have a much higher total fat and fibre content than sorghum grain. This clarified the reasons for apparent discrepancies between our findings and those reported for predicting related alkaloids in tall fescue – fat and fibre in tall fescue probably increase with plant maturity, and alkaloid content does likewise – the NIR correlation was not detecting alkaloid itself. The problem is different for ergot in sorghum grain, since grain is fairly uniform in composition and does not change in composition as alkaloid content of sclerotia increases. This explains the observed correlation between NIR and fat content of ergot-infected grains, and why the correlation is poor as it approaches the normal fat level of grain.

Effectiveness of predicting alkaloid content from size and shape of ergot. There had been no previous studies of the time course of alkaloid production by ergot fungi in plants, although it has been assumed that alkaloid is usually confined to the hard sclerotium ('ergot') that forms towards the end of the infection cycle. Also, there is no information available on whether alkaloid increases with size or age of sclerotium. Recent studies have shown that during infection of the sorghum flower by ergot, ovarian tissue is replaced by a white sporulating fungal mass, the sphacelium, from which sticky, conidia-containing honeydew oozes. Subsequently, further tissue differentiation can occur to eventually produce a hard sclerotium. Fully-formed sclerotia are usually <6 mm long, cylindrical with rounded ends, comprised of 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, comprised of 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. It has been assumed that sphacelial tissue does not contain much alkaloid.

Ergot bodies were collected from commercial sorghum crops at various locations in central and southern Queensland and NSW in 1997 and 2001. These were separated into 'typical' ergot bodies, larger ergot bodies, and ergot bodies infected with *Cerebella* spp. (probably sphacelia) before assay. All sclerotia/sphacelia examined showed a similar spectrum of alkaloids, but the concentration varied greatly between sclerotia and could not be reliably predicted from size and shape. Fully formed sclerotia, separated from the glumes, contained between 10 to 10,000 mg/kg, although 1,000 mg/kg was typical. 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 with these samples. In a given crop, the majority of ergot bodies are likely to be immature sphacelia and these will typically contain 10 to 400 mg alkaloids/kg. *Cerebella* infection appears to hinder development of the sclerote and minimise alkaloid accumulation. Pure honeydew was also collected for assay and was shown to contain 1-10 mg/kg of alkaloid. Because crops apparently free of ergot might still contain 1 mg alkaloid/kg if they had become contaminated with honeydew, it is highly advisable to assay grain originating from any crops infected with ergot, before purchase for use in feedlots.

Effectiveness of physical separation methods for removing ergot from grain. In 2001, sorghum crops infected with ergot were identified on farms in three locations in Queensland and one in New South Wales. To determine if much ergot was removed from grain during the harvesting process, samples of infected grain were taken before and after harvest from farms at four locations. There was a substantial reduction in ergot content and alkaloid concentration during the harvesting process, presumably as lightweight material was blown out of the header. While 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, and this level was exceeded by 2 of the 4 crops (at Kingaroy and Warwick) even after harvest. Once harvested, studies showed that a shaking gravity table and screening was quite effective in removing rye grass ergot from barley, because of the disparity in sizes of the grain and ergot from infected rye grass seed. Grading was much less effective with sorghum ergot, since the ergots are not greatly different in size from grain. This indicates that grading will not be completely effective with crops that are heavily contaminated.

Development of a rapid test for ergot alkaloids in grain. The project successfully developed competitive enzyme-linked immunosorbent assays (ELISA) for measuring the DHES concentration in grains and mixed animal feed. The assays were developed using a DHES-specific mouse monoclonal antibody and rabbit polyclonal antibodies raised against DHES conjugated to bovine serum albumin. Good recoveries were obtained from spiked grain using a simple, one step extraction with 70% methanol. Extraction of naturally-contaminated samples was complete after 30 to 60 min but at least 75% of the alkaloid was extracted within the first 5 min. An extraction time of 30 min was selected for routine use. Forty-four samples of sorghum grain previously assayed for DHES content by the HPLC method and ranging between 0 and 246 mg/kg were analysed by the monoclonal antibody ELISA and the results compared. Overall ELISA and HPLC results were in excellent agreement. Both the monoclonal and polyclonal assays are capable of detecting DHES concentrations above 0.01 mg/kg but quantification is most reliable at concentrations of 0.1 mg/kg or higher. The polyclonal test was also effective for detecting ergotamine, the main rye ergot alkaloid at similar concentrations.

Conclusions and Recommendations. To reduce the risk of poisoning of cattle in feedlots, it is essential to monitor feed for ergot and its alkaloids. Ergot infection is quite evident in sorghum crops, and suppliers of grain to feedlots should be required to state if there was any such evidence. The Department of Primary Industries has produced brochures and extension notes that describe rapid means for detecting ergot in grain, and lot-feeders should take note of such material. In seasons when ergot is common, it would be highly advisable for samples of grain to be tested for ergot alkaloids before purchase. Using the ELISA method developed here, very large numbers of samples could be tested within 24 hours, and at quite acceptable cost. Where a lot-feeder elects not to do this, samples of all grain deliveries should be retained for later testing, should any batch be associated with hyperthermia and poor production.

Main research report

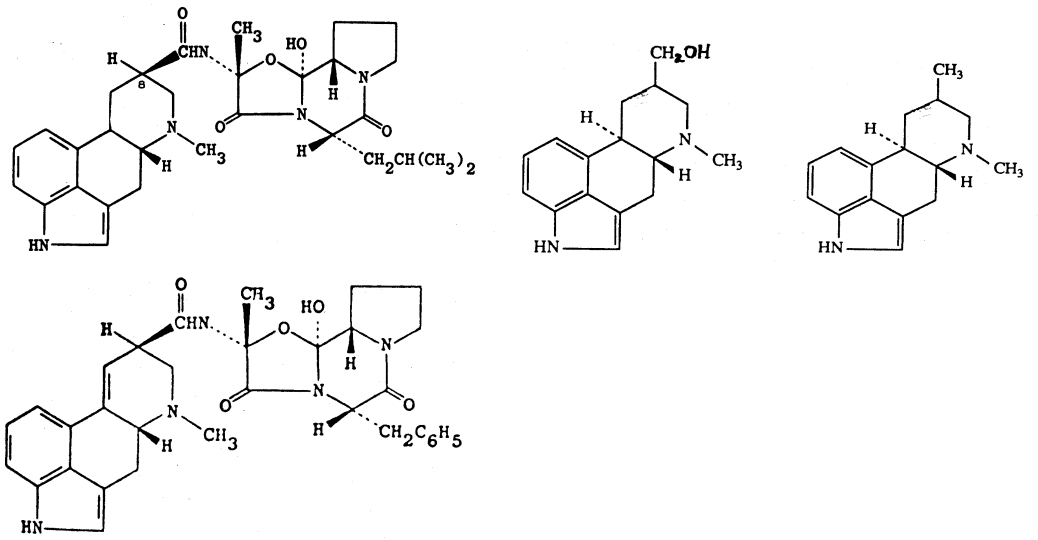
Background to project and industry context

Sorghum ergot is a fungal disease first identified in Australia in 1996, with the potential to affect a large proportion of Australia's sorghum crop. Three studies conducted by DPI (Blaney, McLennan, et al) with support from GRDC (DAQ 412) and MLA (FLOT.112; FLOT.114) showed that feedlot cattle were very sensitive to sorghum ergot alkaloids, mainly dihydroergosine (DHES) with lesser amounts of dihydroelymoclavine and festuclavine. The structures of these alkaloids are shown in Figure 1. Depressed feed intakes and growth were measured in Hereford steers given feed containing ergot and these effects were most pronounced during hot, humid weather, when affected animals were apparently unable to dissipate heat and showed signs of severe heat stress. These studies have shown that the tolerance was < 1 mg/kg (1ppm) of alkaloid in the feed under these conditions. This is of great concern, as high temperature and humidity are already an important limitation to intensive cattle production in Australia, and even low ergot concentrations may exacerbate this.

Rye grass ergot (main alkaloid ergotamine, with lesser amounts of ergocristine, ergocryptine and several others) is also of concern to lot-feeders in Australia, as it regularly contaminates wheat and barley crops in southern states and has been associated with mortalities in cattle, eg in WA, at Forbes in late 1999, and in SA in 2001. As with sorghum ergot, the effects of rye ergot greatly increase susceptibility to heat stress. Both sorghum and rye ergot sclerotia vary greatly in alkaloid content, and good analytical methods are essential if tolerances are to be firmed up, and feed-monitoring regimens implemented.

To reduce the risk of ergot poisoning in feedlots, two main issues needed to be addressed. Firstly, if there are any means of physically identifying those ergot sclerotia that might contain high concentrations of alkaloid, and whether physical segregation by grading could remove all of the alkaloid from contaminated loads. Secondly, to develop rapid tests for detecting and measuring alkaloid concentrations in sclerotes and in grain. A previous study supported by MLA (FLOT.115) evaluated Near Infra-red spectroscopy (NIR) for detecting ergot alkaloids. The results indicated that NIR can predict high alkaloid concentrations, but the detection limit of about 20 mg/kg was nowhere near the required detection limit of <1mg/kg alkaloids. However, some further investigation seemed justified as detailed below. The most promising approach towards a rapid assay was a newly developed immunoassay for DHES, the main alkaloid of sorghum ergot, which also appeared able to detect ergotamine from rye ergot. All of the issues were addressed in this project.

Figure 1. Structures of the sorghum ergot alkaloids (clockwise from top left): dihydroergosine DHES; festuclavine; dihydroelymoclavine; and the main rye ergot alkaloid, ergotamine.



Project objectives

1) To investigate reasons for lack of sensitivity of NIR for detecting ergot alkaloids and whether NIR can detect if sorghum ergot sclerotia contain high concentrations of alkaloids.
2) To determine the extent of variation in alkaloid content of sclerotia in particular batches of infected grain, and whether alkaloid content correlates with shape/size of sclerotia.
3) To determine how effectively physical separation procedures such as gravity separation and grading can remove ergot alkaloids from sorghum grain.
4) To establish immunoassays for sorghum and rye ergot alkaloids in grain.
5) To validate the accuracy and precision of an immunoassay for the alkaloids of sorghum and rye grass ergot sclerotia in grain samples.
6) To adapt the immunoassay to make it as fast and robust as possible.
7) If needed, to develop immunoassays based on monoclonal antibodies that are more specific and ensure an ongoing supply of antibodies.

Investigations approaching these objectives are reported in sequence below.

Investigation 1. To Investigate reasons for lack of sensitivity of NIR for detecting ergot alkaloids and whether NIR can detect if sorghum ergot sclerotia contain high concentrations of alkaloids.

Methodology

Detection of pure alkaloids: Two series of samples spiked with known concentrations of pure alkaloid (either dihydroergosine or dihydroergotamine) were prepared (0.1 to 40 mg/kg) and examined by NIR. Dihydroergotamine was used instead of ergotamine as it is very closely related to ergotamine, but is far more stable and readily available.

Testing of sclerotes: A few samples of sclerotes were scanned by NIR.

Sample preparation: Samples containing a range of alkaloid contents were finely milled to pass a 0.1 mm screen using a shatter box, and scanned by NIR.

Reasons for the discrepancy between our results and those of other workers: Initial attempts to contact Craig Roberts (USA researcher who published a paper on detection of ergovaline by NIR) were unsuccessful. Don Law met him at the International NIR Conference held in Korea in June. Roberts promised to send data from his research via e-mail. Despite several e-mail messages these data were not provided. It appears very doubtful that Roberts was actually detecting alkaloid, based on the results obtained below. His published scans are in the region of fat absorption, but at many orders of magnitude greater than we found in the same region with sorghum grain, which is highly unusual in itself. We investigated this by testing the fat composition of sorghum ergot sclerotia.

Results & Discussion

Detection of pure alkaloids: Both series showed very low sensitivities to the alkaloids themselves, but high correlations were found between NIR absorption and alkaloid concentration for many wavelengths. However, when the two series were combined, the high correlations were markedly reduced, suggesting that the relationship may have resulted from impurities in the alkaloids.

Testing of sclerotes: No promising absorption bands were detected. This approach really requires about 40 – 100 samples of ground sclerotes of known alkaloid content, which we were unable to acquire.

Sample preparation: Fine grinding of samples did not improve the prediction. In view of this and the results above, other methods of sample preparation were not pursued.

Reasons for the discrepancy between our results and those of other workers: The final report on FLOT 115 showed that the major differences in spectra of samples was in the 1700 and 2100 nm regions of the NIR spectrum, which is the region where fat absorbs. We have since assayed a selection of the samples for fat content, and found that there

was a high correlation between fat content predicted by NIR and alkaloid content. Consequently, we assayed sclerotia of rye and sorghum ergot and compared this to sorghum grain. These results are in Table 1.1. It can be seen that ergot sclerotes have a much higher total fat content than sorghum grain, and also that most of this difference is due to the C16 and C18 acids.

Table 1.1 Fatty Acid Composition (g/kg) of ergot sclerotia versus sorghum grain

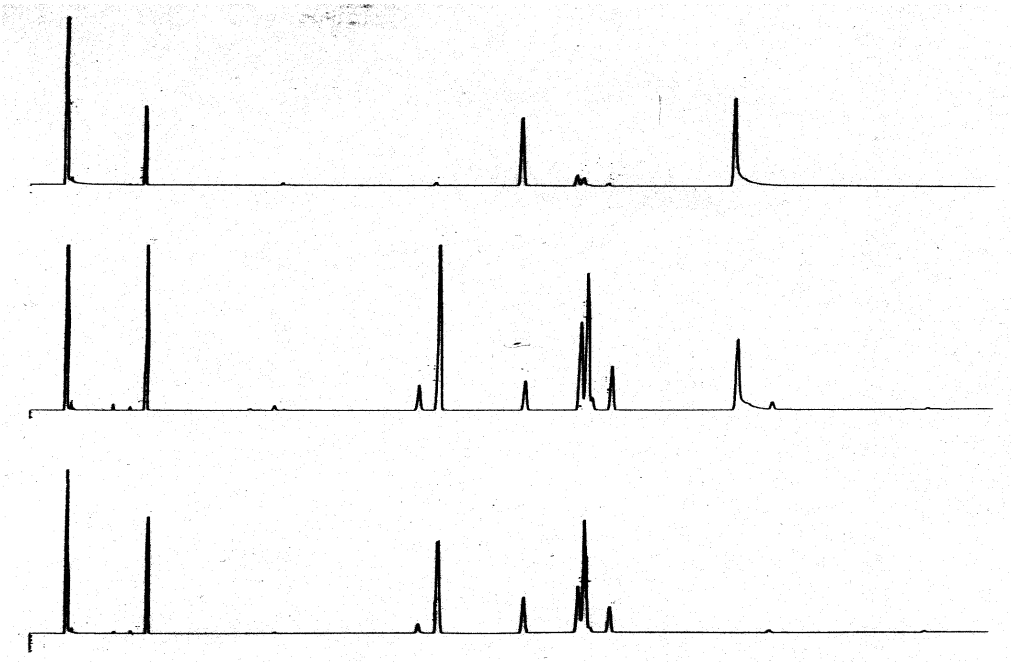
Fatty Acid	Sorghum Ergot	Rye Ergot	Sorghum Grain
C14	0.5	0.8	0
C15	0	0.1	0
C16	29	36.5	4.6
C16:1n-7	2.7	4.6	0.2
C18	8.9	8.8	0.5
C18:1n-9	33.8	26.6	11.6
C18:1n-7	1.6	2.6	0.4
C18:2n-6	16.2	18.1	15.2
C18:3n-3	7.5	0.6	0.5
C20	0.9	1.6	0.1
C20:1n-9	0	0.5	0.1
C22	0.5	0.4	0
C24	0.4	0.2	0.1
Total	**102**	**101**	**33**

This confirmed that the major differences in the spectra were due to the increasing wax (fat) contents with increasing sclerotia content. However, the correlation between the NIR absorptions and alkaloid concentration was appreciably higher than that between NIR fat content and alkaloid concentrations suggesting that the NIR was detecting something other than fat concentration. This conclusion is supported by the fact that the significant wavelengths in the NIR prediction tend to be outside the 'fat' area.

We speculated that a major component of castor oil, the hydroxy C18 fatty acid, ricinoleic acid (a purgative) might be present in sorghum ergot, as has been reported overseas for rye ergot. This is of interest as other scientists (Chris Bourke, Aust Vet J) have speculated that all of the toxicity of rye ergot to cattle might not be caused by alkaloids. We have since confirmed (by capillary gas-liquid chromatography, Figure 1.1) the presence of ricinoleic acid (0.34, 0.4 and 1.6%) in 3 samples of Australian rye ergot sclerotes, and its absence from sorghum ergot sclerotes (<0.1%). (Castor oil contained 62%). This could have some bearing on the comparative toxicity of these two ergots.

However, this indicated that ricinoleic acid was not augmenting the NIR correlation with fat. Subsequently, we also conducted some fibre analyses, and found the fibre content of sclerotia also to be higher than grain. We now conclude that the original correlation was due to a combination of higher fat and fibre in sclerotia.

Figure 1.1. Capillary gas-liquid chromatogram of the triglycerides of castor oil (top), rye ergot sclerotes (middle) and sorghum ergot sclerotes (bottom). Ricinoleic acid is the tailing peak on right of the top two chromatograms.



This clarified the reasons for apparent discrepancies between our findings and those reported for predicting related alkaloids in tall fescue – fat and fibre in tall fescue probably increase with plant maturity, and alkaloid content does likewise – the NIR correlation was not detecting alkaloid itself. The problem is different for ergot in sorghum grain, since both grain is fairly uniform in composition and does not change in composition as alkaloid content of sclerotia increases. This explains the observed correlation between NIR and fat content of ergot-infected grains, and why the correlation is poor as it approaches the normal fat level of uninfected grain.

Conclusions

None of the additional studies improved the predictability of NIR for sorghum ergot alkaloids. Work with spiked samples suggests that the sensitivity of NIR to the alkaloids is quite inadequate to achieve the target detection level, and there appear to be no other components in grain or sclerotia that correlate with alkaloid at the target detection levels.

Investigation 2: To determine the extent of variation in alkaloid content of sclerotia in particular batches of infected grain, and whether alkaloid content correlates with shape/size of sclerotia.

Methodology

There have been no previous studies of the time course of alkaloid production by ergot fungi in plants, although it has been assumed that alkaloid is usually confined to the hard sclerotium that forms towards the end of the infection cycle. Also, there is no information available on whether alkaloid increases with size or age of sclerotium.

Recent studies by Frederickson have shown that during infection of the sorghum flower by ergot, 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, comprised of 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, comprised of 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. It has been assumed that sphacelial tissue does not contain much alkaloid.

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, two 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).

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 three 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 three locations in Queensland and one in New South Wales. To determine if much ergot was removed from grain during the harvesting process, samples of infected grain were taken before and after harvest from farms at four locations. 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. DHES was assayed in subsamples (500 g) of the pre-and post-harvest grain.

The samples were next separated into fractions and then assayed for alkaloids. On two sites, clumps of grain stuck together with honeydew were also collected. 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 three 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 also determined.

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

Results & Discussion

The assay results using different assay methods for sclerotia/sphacelia collected in 1997 are shown in Table 2.1. The DHES concentration of sclerotia/sphacelia varied from 100 to 7000 mg/kg (0.01 to 0.70%) and total alkaloids from 100 to 7900 mg/kg. DHES was the dominant alkaloid seen and usually comprised about 80% of the total alkaloid content of sclerotia/sphacelia and infected sorghum samples, with about 14% dihydroelymoclavine and 4% festuclavine.

A few samples were separated into larger and smaller sclerotia/sphacelia and those with *Cerebella* infection (Table 2.1). 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 with these samples.

Table 2.1 Dihydroergosine (DHES) and alkaloid concentrations in ergot sclerotia/sphacelia collected in 1997 from various regions of Queensland, assayed by different methods

Sampling Site	DHES mg/kg		Total alkaloid mg/kg
	TLC	HPLC	Spectro-photometry
Moreton (south-east Queensland)			
Mutdapilly sclerotiaA	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.

Results obtained on infected grain before and after harvest on four farms in 2001 are shown in Table 2.2. There was a substantial reduction in ergot content and alkaloid concentration during the harvesting process, presumably as lightweight material was blown out of the header. While 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 results obtained on different fractions of the pre-harvest samples are in Table 2.3.

Table 2.2 Reductions in ergot and dihydroergosine contents of infected sorghum during the harvesting process in samples from farms in three regions of Queensland and one in New South Wales during 2001.

	No	Kingaroy (south Burnett, Qld)	Dalby (Darling Downs, Qld)	Warwick (southern Downs, Qld)	Willowtree (Liverpool Plains, NSW)
Grain before harvest					
Ergot[A] (%):range; mean	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):range; mean	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 (%):range; mean	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):range; mean	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):range: mean	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

Table 2.3 Dihydroergosine (mg/kg) in ergot bodies and different fractions of infected sorghum in samples from farms in three regions of Queensland and one 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 DHES (mg/kg):range; mean	3	0.1 - 0.4; 0.3	<0.1	0.1 - 1.6; 0.7	<0.1
Trash DHES (mg/kg):range; mean	3	<0.1 – 14; 5	<0.5 – 6; 3	<0.1 - 0.7; 0.4	<0.1–0.1; <0.1
Ergot in glumes DHES (mg/kg):range; mean	3	40 – 1050; 410	2.5 – 15; 9	8.3 – 49; 24	16 – 113; 49
Ergot separated from glumes (sclerotia) DHES (mg/kg):range; mean	3	900 – 1760; 1340	12 – 14; 13	19 – 74; 47	60 – 600; 240
Ergot with Cerebella DHES (mg/kg):range; mean	2	12 – 57; 35		<0.3 – 27; 14	

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 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 2.1), 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-sourced 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.

Alkaloids in honeydew: The presence of alkaloids in the grain clumped together with honeydew (Table 2.2) 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 (Blaney, unpublished data). This caused us to investigate whether this was due to developing sclerotia in the mass or to the honeydew itself. Consequently, two samples of 'pure' honeydew collected from inoculated panicles of sorghum in the Toowoomba glasshouse were assayed and found to contain 5.6 mg DHES/kg, and 10 mg/kg two weeks later. A third sample that was collected from a field trial at Warwick contained 1 mg DHES/kg. . It seems likely that the alkaloid is associated with spores contained in the honeydew, but this is an academic point. Given the detection of alkaloid in apparently clean grain in the sample from Kingaroy (Table 2.3), it seems that honeydew presents some risk of poor production to grazing livestock, and also might cause low contamination levels in apparently clean grain from the same crop.

Conclusions

All sclerotia/sphacelia examined showed a similar spectrum of alkaloids, but the concentration present varied greatly between sclerotia and could not be reliably predicted from size and shape. Fully formed sclerotia, separated from the glumes, contained between 10 to 10,000 mg/kg, although 1,000 mg/kg was typical. In a given crop, the majority of ergot bodies are likely to be immature sphacelia and these will typically contain 10 to 400 mg alkaloids/kg. *Cerebella* infection appears to hinder development of the sclerote and minimise alkaloid accumulation. More investigations are required to assess factors influencing production of alkaloids, such as climatic factors or different genetic lines of either C. africana or host. Because crops apparently free of ergot might still contain 1 mg alkaloid/kg if they were originally infected, it is

recommended that grain originating from any crop infected with ergot be assayed before purchase to minimise risk of ergot poisoning in feedlots.

Investigation 3. To determine how effectively physical separation procedures such as gravity separation and grading can remove ergot alkaloids from grain.

Rye ergot: In November 2001, Grainco Ltd procured 4000 tonne of barley from a bunker store in NSW. This contained low concentrations of rye ergot sclerotes (about 0.002%), but a zero tolerance was in place for malting barley. Consequently, this material was cleaned at the Grainco (Globex) facility at Fisherman Island. The project leader inspected the operation, and acquired samples taken before and after screening. It appeared that the grading process was highly effective in concentrating all ergot into 1% of lightweight screenings, which then contained about 0.3% rye ergot. The equipment used was a large, multiple storey, vibrating screen assembly. In comparison, the adjacent Container Link grading facility was much slower and graded out 2-3% offal containing 0.11% ergot on average. However, it must be noted that rye ergot sclerotia resulting from infected rye grass are much smaller than barley seeds, which facilitates the cleaning. If the rye ergot fungus were to directly infect the barley (or wheat), then the effectiveness of cleaning could be greatly reduced. Alkaloids were not detected in the cleaned grain.

Rye ergot: On a smaller scale, a farmer located at Turill (between Newcastle and Dubbo) graded 35 tonne of wheat and oats containing infected rye grass seed. He used an aspirator first (removed the curved ergots) but then screens to remove the rest. The latter was much easier with wheat than oats. We assessed the original contamination to be 0.03% ergot, and the screenings at 2.8%. The screenings were assayed to contain about 20 mg/kg alkaloids. The background to this was that the farmer had lost 12 out of 112 heifers fed the grain as a supplement in hot weather, which was diagnosed as ergotism. He then graded the grain, but accidentally fed two bags of gradings to a batch of ewes as a supplement – these displayed panting and lying in shade for 3 to 4 days, although the weather was mild (27-28^{0}C), but eventually recovered. Upon request from a neighbouring piggery interested in the grain, we advised that the ungraded material could probably be fed to grower pigs, since our assays indicated only about 0.2 mg alkaloids/kg, even if rye ergot alkaloids were 10 times more toxic than sorghum ergot alkaloids. The piggery accepted our advice, and the ergotised grain was fed after dilution with clean grain.

Sorghum ergot: One batch of heavily-infected sorghum was graded by Grainco at Biloela in 1997, and samples assayed at ARI. The results were:

Ungraded sorghum:	9.2% ergot	21 mg alkaloids/kg
Graded sorghum:	1.3% ergot	3 mg alkaloids/kg
Gradings	98% ergot	221 mg alkaloids/kg

Conclusion: Grading of rye ergot from grain is quite effective, because of the disparity in sizes of the grain and ergot from infected rye grass seed. Grading is much less

effective with sorghum ergot, since the ergots are not greatly different in size from grain. Grading will not eliminate the risk of sorghum ergot poisoning in feedlots.

Investigation 4. To establish immunoassays for sorghum and rye ergot alkaloids in grain.

This investigation was highly successful. Full details of the methodology used to develop an immunoassay able to detect the main alkaloids of sorghum ergot (dihydroergosine) and rye ergot (ergotamine) are given in the draft of a scientific paper intended for publication, inserted below.

An Immunoassay for the Detection of Dihydroergosine,

the Principal Alkaloid of Sorghum Ergot (*Claviceps africana*).

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ABSTRACT

Dihydroergosine (DHES) is the principal toxic alkaloid produced by sorghum ergot (*claviceps africana*). It has recently been shown that DHES levels as low as 1 mg/kg in animal feed can cause significant production losses in cattle. Quantitative immunoassays for detecting the related rye ergot alkaloid, ergotamine, are described in the literature but those assays are relatively insensitive for DHES. This paper describes competitive enzyme-linked immunosorbent assays (ELISA) for measuring the DHES concentration in grains and mixed animal feed. The assays were developed using a DHES-specific mouse monoclonal antibody and rabbit polyclonal antibodies raised against DHES conjugated to bovine serum albumin. Recoveries of between 77 and 103% were obtained from spiked grain using a simple, one step extraction with 70% methanol. Both the monoclonal and polyclonal assays are capable of detecting DHES concentrations above 0.01 mg/kg but quantification is most reliable at concentrations of 0.1 mg/kg or higher.

INTRODUCTION

Sorghum ergot (*Claviceps africana)* is widespread in Africa and Asia and has recently been introduced into Australia (*1*). The fungus infects the ovary of flowering plants, and the fungus eventually produces hard sclerotia (ergots), which contain toxic alkaloids. The major alkaloid produced by sorghum ergot is the ergo-peptide, dihydroergosine (DHES), which usually represents at least 80% of the total alkaloids present (*2*). Minor alkaloids include festuclavine and dihydroelymoclavine, which lack a peptide moiety. These three alkaloids are saturated at the 9,10 position, in which respect they differ from ergotamine and the various other ergo-peptides produced by rye ergot (*C. purpurea*) (*3*).

Until recently sorghum ergot had not been associated with disease in livestock, but cases of poisoning in pigs and dairy cattle occurred in 1997 in Queensland (*4*). Subsequent research has shown marked depression of milk production in sows fed diets containing 3-6 mg DHES/kg for only a few days prior to farrowing, and reduced growth rates in beef cattle fed diets containing as little as 1mg DHES/kg (*5*). These effects were associated with a profound depression in plasma prolactin.

Quantitative high performance liquid chromatography (HPLC) and semi-quantitative thin layer chromatography (TLC) methods have been developed for detecting DHES in extracts from affected grain (*6*). TLC is simple and has a high throughput but is relatively insensitive. HPLC is sensitive and specific but requires expensive equipment and trained staff and is therefore most suited to reference laboratories. HPLC methods also typically involve lengthy extraction and clean-up procedures, thus limiting throughput. Compared to HPLC, immunoassays usually have lower precision but greater sensitivity and much higher throughput, and are consequently cheaper and more practical for screening large numbers of samples. Immunoassays have been developed for rye ergot alkaloids and shown to be very sensitive (*7*, *8*), but the assays are relatively insensitive to DHES (R. A. Shelby, personal communication).

This paper describes the development and validation of immunoassays for DHES using polyclonal antibodies raised in rabbits and a mouse monoclonal antibody. The assays employ a simple one-step extraction in 70% methanol and are reliable for quantifying DHES concentrations of 0.1 mg/kg or higher in grain or mixed feed.

MATERIALS AND METHODS

Alkaloid standards. Alkaloids were purchased or acquired from other laboratories, generally as methanesulfonate derivatives. They produced single peaks or spots in HPLC and TLC respectively, and were assumed to be pure. In quantitative calculations, allowance was made for the mass of the methanesulfonate group, so that results are quoted in terms of the concentration of the base alkaloids.

Alkaloid standards ranging from 0.1 to 10,000 ng/ml were prepared in 70% methanol. DHES standards were also prepared by spiking the 70% methanol extracts of ergot-free sorghum grain or a typical mixed feed formulated with ergot-free sorghum.

Conjugation of hapten. DHES was conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) using a Mannich reaction as described previously (9).

Animal inoculation and antibody production. Three rabbits each received three subcutaneous injections containing approximately 500 μg of BSA-DHES conjugate dialysed into sterile phosphate buffered saline (PBS), at approximately two week intervals. The first injection only, included 100 μg of saponin as adjuvant. After the third injection, blood was collected from the ear vein and tested for antibodies to DHES in an ELISA using the OVA-DHES conjugate as antigen. All rabbits were then exsanguinated by heart puncture under general anaesthetic and euthanased by lethal injection before recovery.

Mice received three intraperitoneal injections each containing approximately 125 μg of the BSA-DHES conjugate, at approximately two weekly intervals. The first injection only, included 10 μg saponin as adjuvant. After the third injection blood was collected from the tail and tested for antibodies to DHES as described above. The mice

were left for at least one month and then given a final intraperitoneal booster injection with OVA-DHES (125 μg) three days before harvesting the spleen for fusion. Monoclonal antibodies were produced by conventional hybridoma technology (10). Hybridomas were screened for DHES-specific antibody production in an ELISA using OVA-DHES as test antigen and OVA as control antigen. For use in ELISAs, monoclonal antibodies were affinity purified from cell culture supernatant using prepacked Protein G Sepharose columns (Pharmacia Biotech, Upsala, Sweden) according to the manufacturer's instructions.

ELISA. Grain and feed samples were hammer-milled to pass a 1mm screen. A 4 g sample was extracted in 40 ml of 70% methanol for 30 min in a sealed plastic tube with constant agitation and then allowed to settle briefly before sampling the supernatant for ELISA.

Competitive ELISAs were developed using both the rabbit polyclonal antibodies and the mouse monoclonal antibody. For ELISAs using the monoclonal antibody, 96-well microtitre plates (MaxiSorp, Nunc, Naperville, IL.) were coated with 100 μl/well of OVA-DHES diluted to a concentration of 40μg/ml in 0.1 M sodium carbonate buffer (pH 9.6). Blank wells were included on each plate and were coated with a similar amount of OVA in 0.1 M sodium carbonate buffer (pH 9.6). After an overnight incubation at 4oC, excess antigen was removed and 200 μl of a 2% solution of low fat skim milk powder (SMP) in PBS containing 0.1% Tween 20 (PBST) was added to each well. The plate was then incubated for 1 h at room temperature after which the wells were washed with PBST (5 x 200 μl rinses followed by 1 x 200 μl soak for 5 min with agitation). Next, 25 μl of 2% SMP in PBST was added to each well, followed by 25 μl of either undiluted sample extract, DHES standard (0, 0.1, 1, 10, 100, 1000 or 10000 ng/ml in 70% methanol) or 70% methanol (blank wells), and 50 μl of monoclonal antibody diluted 1/4000 in 2% SMP in PBST. The plate was then incubated for 30 min at room temperature with gentle agitation. Wells were then washed as described previously, 100 μl of peroxidase-labelled goat anti-mouse IgG conjugate (KPL, Gaithersburg, MD) diluted 1/10000 in 2% SMP in PBST added to each well and the plate again incubated for 30 min at room temperature with gentle agitation. Wells were again washed as described previously and 100 μl of peroxidase substrate 3',3',5',5'-tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD) added to each well. Colour was allowed to develop until the absorbance of the wells containing the 0 ng/ml DHES standard was between one and two absorbance units, at which time the reaction was stopped by addition of 50 μl of 2M phosphoric acid. Absorbance was read at 450 nm. Sample extracts were tested in duplicate. On each plate, the 10, 100 and 1000 ng/ml DHES standards were included in quadruplicate and the remaining standards were included in duplicate. DHES concentrations were quantified using SOFTmax Pro software (Molecular Devices Corporation, Menlo Park, CA) with standards fitted to a four-parameter curve and expressed as mg/kg. Sample extracts with concentrations of DHES in excess of 200 ng/ml were diluted with 70% methanol so that they fell within the optimum range for quantification in the ELISA (10-100 ng/ml).

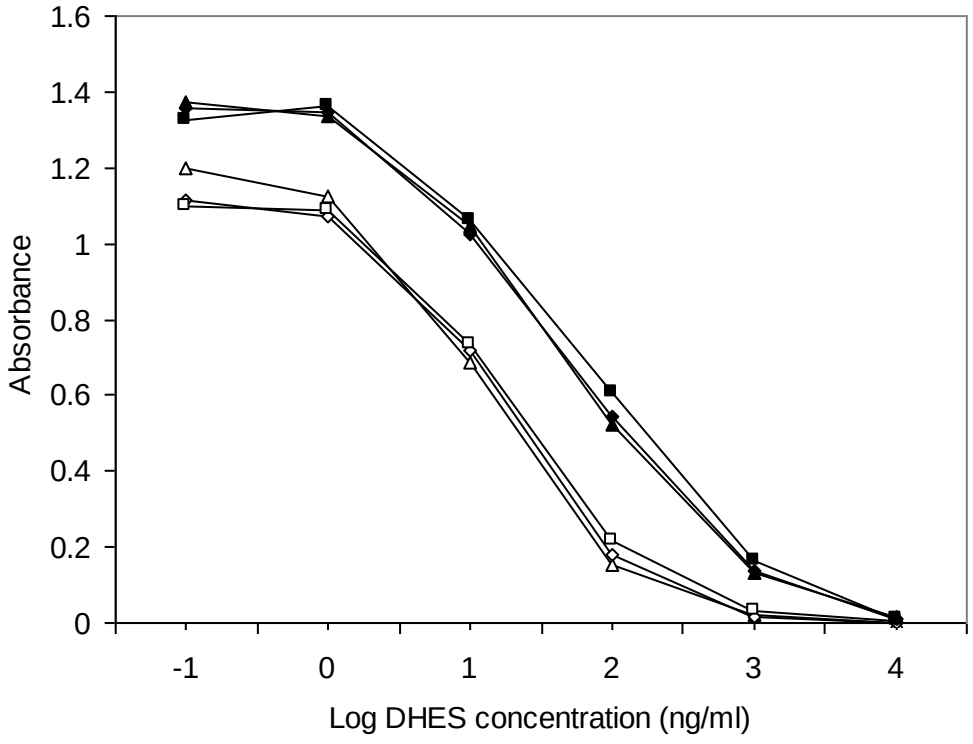
ELISAs using polyclonal antibodies were conducted in the same manner except that the rabbit anti-serum was used at a dilution of 1/3000 and peroxidase labelled goat anti-rabbit IgG conjugate (KPL, Gaithersburg, MD) was diluted 1/5000.

HPLC. Grain samples were analysed for ergot alkaloids in duplicate or triplicate using HPLC (6). In brief, the method involves: a triple extraction with a mixture of methylene chloride, ethyl acetate, methanol and 25% aqueous ammonia; dissolution in diethyl ether and partition into 0.5M hydrochloric acid; and partition back into methylene chloride after alkalisation with ammonia. Separation is by reverse phase HPLC using an acetonitrile, ammonium acetate, methanol mobile phase, with fluorescence and/or UV detection. Recoveries for the method are quoted at about 75% (6), but the comparison between HPLC and ELISA shown below was made using uncorrected assay figures.

RESULTS

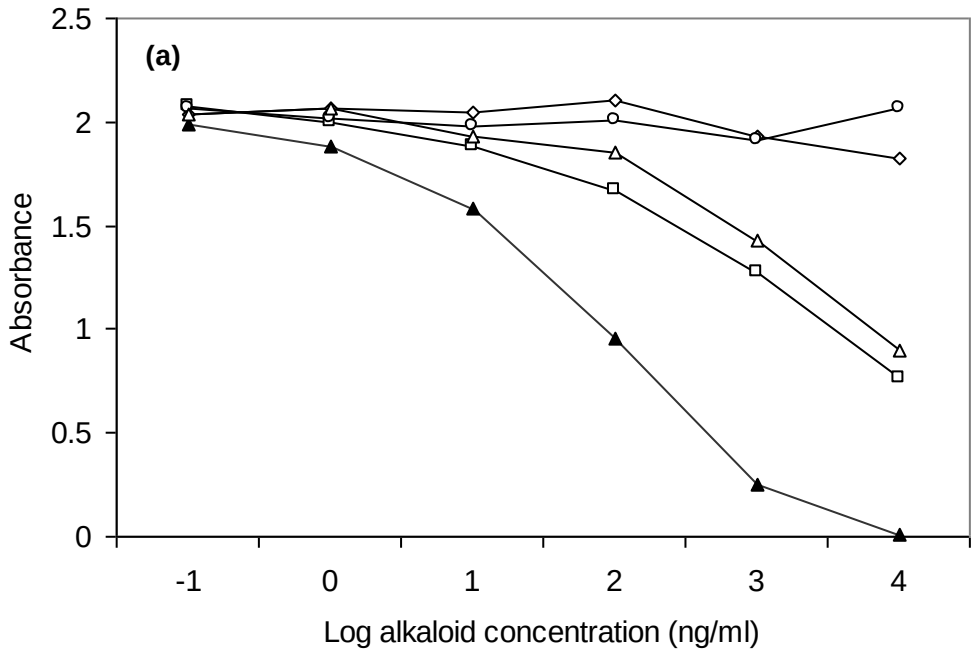
Sensitivity. Sensitivity was estimated by assaying DHES standards prepared in 70% methanol and in the 70% methanol extracts of ergot-free sorghum and mixed feed (Figure 4.1). The assays using the monoclonal and polyclonal antibodies were both capable of detecting DHES concentrations above 1 ng/ml and could reliably be used to quantify concentrations above 10 ng/ml. Accounting for a 10-fold dilution during the extraction process, these figures equate to 0.01 and 0.1 mg/kg in a grain or mixed feed sample. The assays were unaffected by pigments and other compounds present in 70% methanol extracts from ergot-free grain or mixed feed.

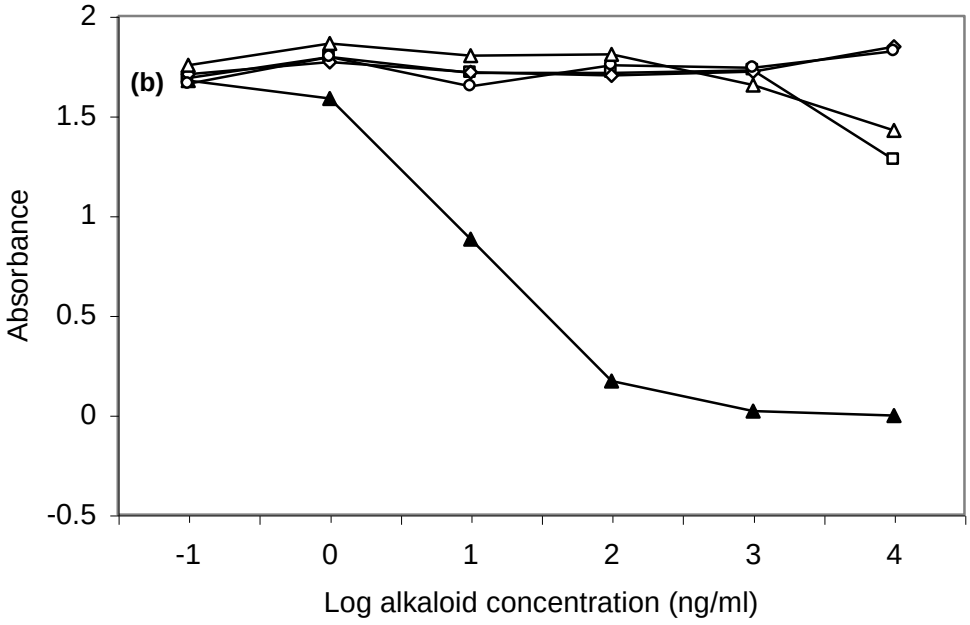
Figure 4.1 Sensitivity of ELISAs using polyclonal rabbit antibodies (filled symbols) and mouse monoclonal antibody (open symbols) for detecting varying concentrations of dihydroergosine standard diluted in 70% methanol (Δ), 70% methanol extract of ergot-free sorghum (◊) and 70% methanol extract of an ergot-free sorghum-based mixed feed (□).



Specificity. The monoclonal and polyclonal antibodies were tested for cross-reactivity with the minor sorghum ergot alkaloids, festuclavine and dihyroelymoclavine, the rye ergot alkaloid ergotamine, and synthetic dihydroergotamine (Figure 4.2). Neither showed any evidence of cross-reaction with festuclavine or dihyroelymoclavine. The polyclonal antibodies cross-reacted with both ergotamine and dihydroergotamine but the sensitivity of the assay for these alkaloids was at least 10-fold lower than for DHES. The monoclonal antibody showed some evidence of cross-reaction with ergotamine and dihydroergotamine but only at concentrations of 10,000 ng/ml or higher.

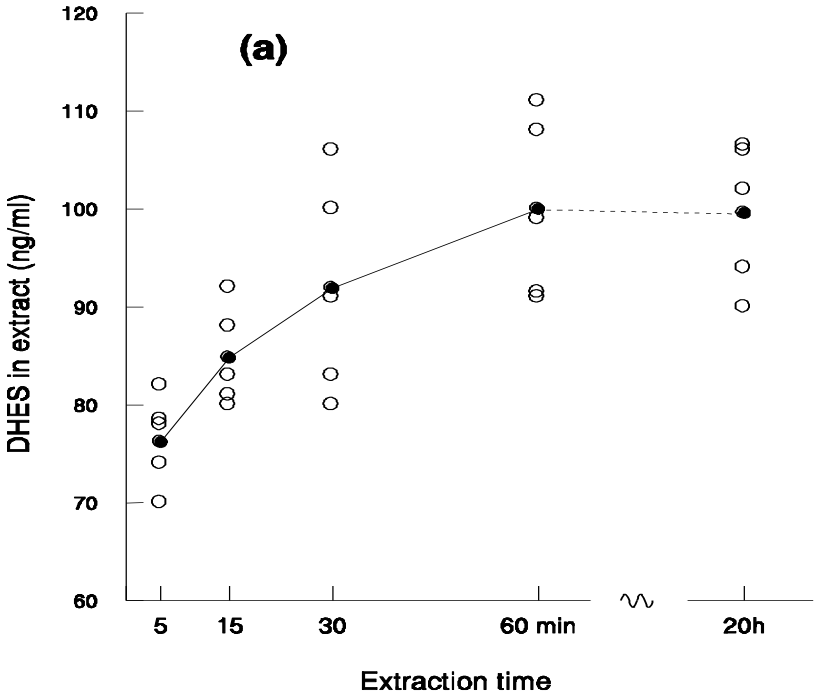
Figure 4.2 (a) Specificity of ELISAs using (a) polyclonal rabbit antibodies and (b) mouse monoclonal antibody. Graphs show inhibition of antibodies by varying concentrations of dihydroergosine (▲), dihydroelymoclavine (◊), festuclavine (○), dihydroergotamine (□) and ergotamine (Δ).





Recovery of DHES from spiked grain. DHES in 70% methanol was added to milled ergot-free sorghum grain at levels of 0.1, 1 and 10 mg/kg and allowed to absorb into the milled grain for at least 2 h. The spiked grain was then extracted and assayed in quadruplicate using the monoclonal antibody ELISA. Percentage recoveries were 77 (+/-3.5), 103 (+/- 1.3) and 88 (+/- 3.3) %, respectively.

Figure 4.3 Efficiency of extraction of dihydroergosine (DHES) with 70% methanol from sorghum grains with moderate levels of ergot infestation. Grains were extracted in five replicate tubes and the extraction supernatant sampled after 5, 15, 30 and 60 min and after approximately 20h. Data points for individual tubes are shown as open circles (o) and the averages for the five tubes as filled circles (●).

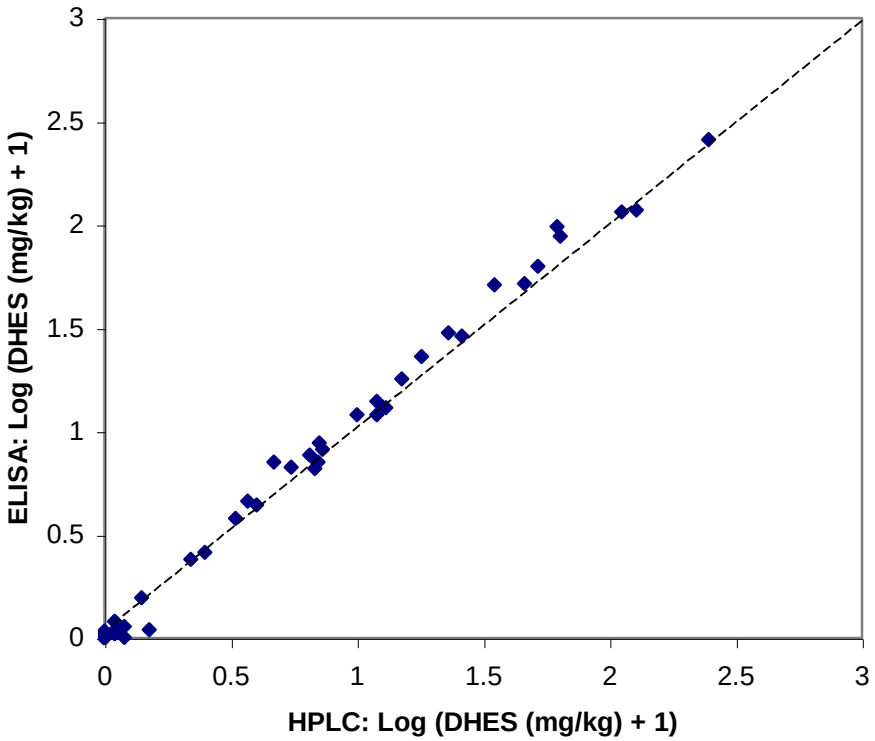


Extraction efficiency. A sample of grain with moderate levels of ergot infestation was extracted with 70% methanol in five replicate tubes and a 200 µl aliquots of the 70% methanol extract removed from each tube for later testing in the monoclonal antibody ELISA after 5 min, 15 min, 30 min, 1h and overnight for approximately 20 h (Figure 4.3). Extraction was complete after 30 to 60 min but at least 75% of the alkaloid was extracted within the first 5 min. An extraction time of 30 min was selected for routine use.

Comparison with HPLC. Forty-four samples of sorghum grain previously assayed for DHES content by the HPLC method, were analysed by the monoclonal antibody ELISA and the results compared (Figure 4.4). DHES concentrations determined by HPLC ranged between 0 and 246 mg/kg. Overall ELISA and HPLC results were comparable although the ELISA did tend to give slightly higher results for most samples. However that difference would be entirely accounted for once the ELISA results were corrected for a recovery of 90%, and the HPLC results for the established recovery of 75% (6).

Repeatability. Estimates of ELISA repeatability were based on results obtained for a grain sample containing approximately 1 mg/kg DHES that was included on each plate. The coefficient of variation over eight plates run over five days was 15.5% (mean = 1.27, SD = 0.20).

Figure 4.4 Comparison of dihydroergosine concentrations in 44 samples of sorghum grain with varying levels of ergot infestation as measured by HPLC and ELISA. Log_{10} (DHES concentration + 1) was plotted to better represent values over the full range assayed (1 was added to each value to avoid complications arising from 0 values). The diagonal line represents perfect agreement between the two assay methods.



DISCUSSION

Detrimental effects of sorghum ergot on livestock have been reported at DHES concentrations as low as 1 mg/kg in feed (*4, 5*). A sensitive test is therefore required for screening grain and mixed feed. The ELISAs we have developed are capable of detecting DHES at concentrations above 0.01 mg/kg and are reliable for quantifying concentration of 0.1 ng/ml or higher. The monoclonal antibody assay is specific for DHES with measurable cross-reactivity with the most closely related naturally occurring alkaloid (ergotamine) and synthetic dihydroergotamine occurring only at concentrations above 1000 mg/kg. In contrast, when rabbit polyclonal antibodies were used the assay detected the rye ergot alkaloid, ergotamine, at concentrations of 1 mg/kg or higher and could therefore be useful for screening for ergot infestation in both sorghum and rye. The specificity of the assays suggests that the monoclonal antibody and the majority of the polyclonal antibodies recognise an epitope on the unique peptide domain of the molecule.

Extraction of DHES with 70% methanol appears to be remarkably efficient with the majority of alkaloid being extracted within 5 min. The repeatability of the ELISA between plates, measured as the coefficient of variation, was 15.5%. Variability of that order is almost inevitable when using a log scale for quantification and is similar to that recently reported for a competitive ELISA for ergot alkaloids in tall fescue (*11*). Recovery of DHES from spiked grain was about 90%, as far as can be estimated given the precision of the assay. We recognize however that this may not reflect the true recovery from naturally contaminated grain and we have therefore relied mainly on comparison with the established HPLC method to validate our results. Once allowance was made for the different recoveries of the two methods (75% for HPLC and 90% for

ELISA), there was excellent agreement between the two methods over a wide range of DHES concentrations (0.01 – 250 mg/kg).

The ELISAs reported here are suitable for screening sorghum grain and mixed feeds for the presence of DHES. The specificity of the monoclonal antibody should make it the reagent of choice when testing for DHES alone but substitution with the polyclonal antibodies would be advantageous when screening for a range of ergot alkaloids, including ergotamine. The assays are more robust and more sensitive than HPLC and require only a rapid one-step extraction. The precision of ELISA is inferior to HPLC, but recoveries are apparently higher. For regulatory work, ELISA and HPLC methods should complement one another.

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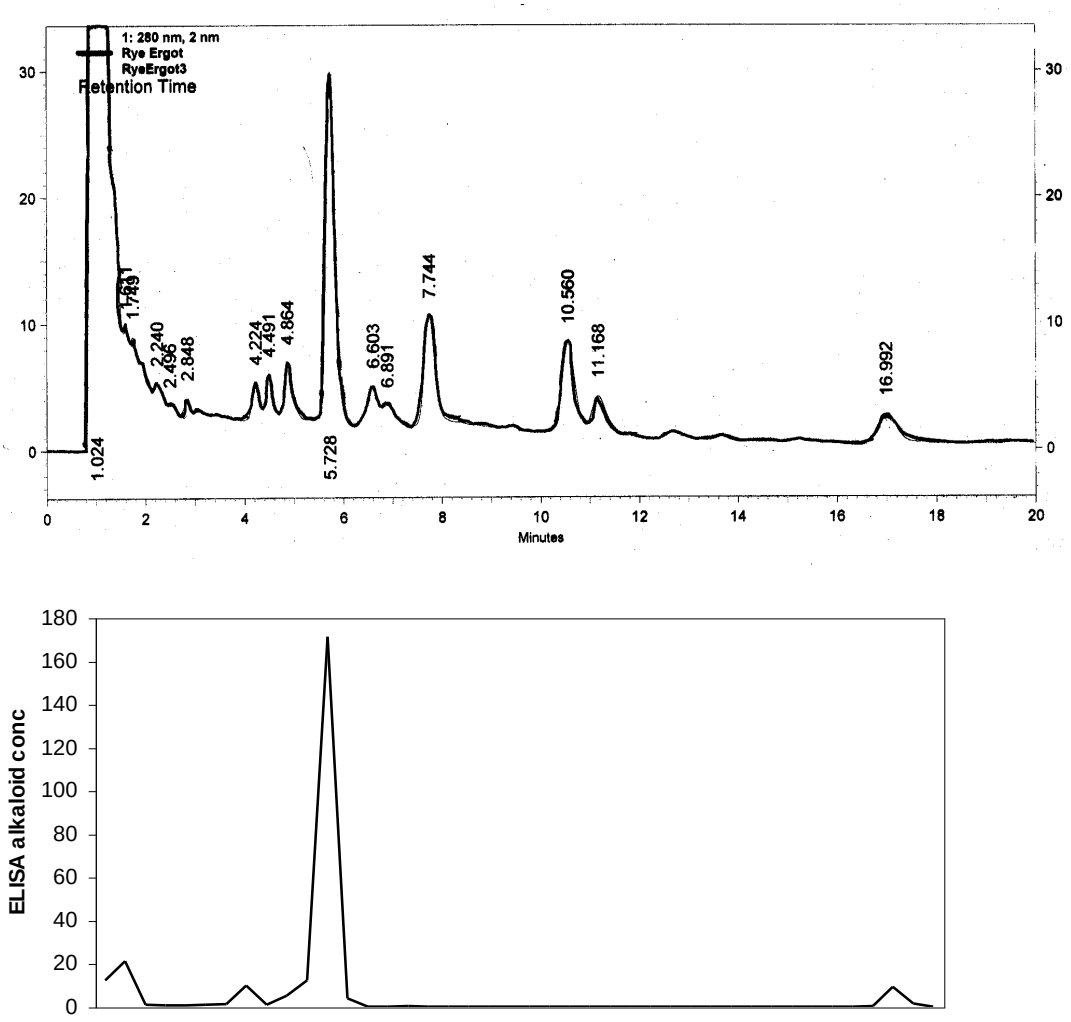
Investigation 5. To validate the accuracy and precision of an immunoassay for the alkaloids of sorghum and rye grass ergot sclerotia in grain samples.

The validation of the ELISA is also detailed in the draft paper above. For samples containing sorghum ergot, there was an excellent correlation between the rapid and specific ELISA method and the slower, but more precise HPLC method for both grain and mixed feed samples over a wide range of DHES concentrations.

We were unable to obtain sufficient samples naturally contaminated with rye ergot for a detailed comparison of ELISA detection of ergotamine with the HPLC method. However, the ELISA was in agreement with HPLC at least in a qualitative sense, in that it correctly differentiated those samples containing >0.2 mg ergotamine/kg from those containing <0.2 mg/kg. Its specificity was shown in the draft paper above, but was also confirmed as follows: An extract of rye ergot sclerotia was injected into the HPLC and fractions of 0.5 ml were collected from the effluent after the separation of sample components. These aliquots were all assayed by ELISA. The results are shown in Figure 5.1.

Figure 5.1 HPLC separation of alkaloids in a rye ergot sample, with corresponding activity in ELISA screening of the eluate.



It can be seen that over 75% of the activity was associated with the ergotamine peak which emerged at about 5.7 minutes. The other fractions with some activity in the ELISA were eluted under the initial solvent front (about 4 minutes) and after 17 minutes.

It was significant that the peak at 17 minutes was also present in our ergotamine standard. There were several other peaks in the chromatogram, eg at 7 and 10 minutes, which had a similar UV spectrum to ergotamine and are likely to be ergocristine, ergocryptine, etc, which are very similar structurally to ergotamine, but these did not react strongly in the ELISA. Given this high level of specificity, the most likely identity of the peak at about 17 minutes was ergotaminine, the D-isomer of ergotamine. We cannot suggest an identity for the other peaks showing minor activity.

To fully complete the validation for rye ergot as a quantitative assay, we still need to acquire more naturally contaminated samples. However, we have demonstrated that the ELISA as such is, at very least, a very effective screening method for rye ergot alkaloids.

Investigation 6. To adapt the immunoassay to make it as fast and robust as possible.

This was approached by directly labelling the monoclonal antibody with the enzyme horseradish peroxidase thus eliminating the conjugate incubation step and one washing step from the ELISA. We also tested the extraction process and determined that about 70% of alkaloid was extracted within 5 minutes. We next tested the monoclonal antibody incubation step to see if it could be shortened. We found that reducing the incubation time below 30 min compromised the sensitivity of the assay. Incubation times of 5 min or less were definitely not feasible. Given that it was clearly not going to be possible to reduce the total assay time to the 5 or 10 min required for monitoring bulk grain during delivery, we decided to retain the 30 min incubation step. The general procedure is as follows:

1. Mill sample (2-5 minutes, depending on sample size – large samples will be more accurate but small samples could be satisfactory for screening).
2. Weigh 4 g into plastic tube, add 40 ml 70% methanol and shake for a period of 5 – 30 minutes, depending on extraction efficiency required.
3. Pippette extract into pre-coated ELISA plate wells (1min), add labelled antibody, incubate 30 min. An incubation of 5 min greatly reduced sensitivity of the assay, but less than 30 min might be OK in some circumstances. Wash plate (5 min). We think the washing step might possibly be shortened but this was not evaluated.
4. Add colour development reagent and incubate for 5 min. Stop reaction with phosphoric acid and read optical density. It is possible that the result could be read by eye if rapid screening was the intent.

In summary, it may be possible to test 1-5 samples within about 30 min, provided that the samples were already milled but it must be realised that this would involve sacrifice of sensitivity and accuracy. The real strength of the ELISA is that it is possible to at least 500 samples in a single day.

Investigation 7. To develop immunoassays based on monoclonal antibodies that are more specific and ensure an ongoing supply of antibodies.

We successfully developed ELISAs using rabbit polyclonal antibodies that could detect both sorghum and rye ergot alkaloids with great sensitivity. However, the amount of rabbit antibody we have is finite and it would eventually be necessary to inoculate more rabbits to produce a new batch of antibody that may or may not perform as well as the original batch. To guarantee an ongoing supply of reliably high quality antibody and to make the assays even more specific, we embarked on a program to produce monoclonal antibodies against DHES and ergotamine.

A monoclonal antibody for dihydroergosine was successfully developed, and incorporated into the ELISA as is described in the draft paper above. The monoclonal antibody assay was both more sensitive and more specific than the rabbit antibody assay.

We then turned our attention to producing a monoclonal antibody to ergotamine but this has proved somewhat more difficult. To date six mice have been inoculated with a dihydroergotamine-BSA conjugate and all have developed high titre antibodies to dihydroergotamine but despite repeated attempts we have not been able to isolate a monoclonal antibody that detects ergotamine. A further three mice have now been inoculated and will be processed early in 2003. It is difficult to understand why we have not been successful in producing a monoclonal antibody that recognizes ergotamine given that its chemical structure is so similar to that of DHES. In the end it may come down to the fact that monoclonal antibody production is very much a hit and miss process and we may simply have been unlucky.

Success in achieving objectives

It is considered that all of the objectives were effectively addressed during this project.

a) Investigations showed that NIR was not suitable for rapid screening of grain for ergot alkaloids, and clarified the reasons for this.
b) Sorghum ergot bodies of similar sizes showed a wide variation in alkaloid content, but larger ergot bodies that were fully separated from glumes contained more alkaloid that those still encased in the glumes and/or infected with *Cerebella* fungus. Size would not be a reliable indicator of alkaloid content. Different batches of rye ergot of identical appearance also varied greatly in alkaloid content.
c) The sorghum ergot content of grain can be greatly reduced during harvesting provided that the air blower is set to remove lighter material. Shaking gravity grading can effectively remove rye ergot from wheat and barley, but this is much less effective with sorghum ergot.

d) An immunoassay was successfully developed for detecting the main alkaloids of sorghum and rye ergots.
e) A monoclonal antibody was successfully prepared for sorghum ergot alkaloid, but not as yet for rye ergot alkaloid.
f) The test was made as rapid as possible by adaptation to allow an ELISA to be performed within about 30 minutes, provided the sample is already milled.

In total, it is considered that the work has achieved the project goal of reducing the risk of ergot poisoning in feedlots. While the project was unable to identify a rapid physical means of detecting samples containing higher concentrations of ergot alkaloids at grain intake (5-10 minutes test), alternative processes such as testing all suspect grain before purchase would be quite feasible using the ELISA test.

Impact on Meat and Livestock Industry

A highly specific, sensitive and rapid test is now available for detecting the main alkaloids of sorghum ergot (dihydroergosine) and rye ergot (ergotamine) in grain and feed mixes. This will provide unambiguous proof of involvement of ergot or otherwise in future cases of hyperthermia in feedlots. Although the test is probably not fast enough to accept/reject batches on delivery, it can easily be applied to grain under consideration for purchase. As contamination can be seasonal, the test will also make it possible to screen grain from certain areas of high risk of contamination as an early alert system. This will greatly reduce the risk of ergot poisoning of cattle in feedlots, and the serious economic consequences of such events.

Conclusions and Recommendations

To reduce the risk of ergot poisoning of cattle in feedlots, it is essential to monitor feed for its presence. Ergot infection is quite evident in sorghum crops, and suppliers of grain to feedlots should be required to state if there was any such evidence. The Department of Primary Industries has produced brochures and extension Notes that describe rapid means for detecting ergot in grain, and lot-feeders should take note of such material. In seasons when ergot is common, it would be highly advisable for samples of grain to be tested for ergot alkaloids before purchase. Using the ELISA method developed here, very large numbers of samples could be tested within 24 hours, and at quite acceptable cost. Where a lot-feeder elects not to do this, samples of all grain deliveries should be retained for later testing, should any batch be associated with hyperthermia and poor production.

Administrative details report

Budget

MLA contribution

Item	Cost ($)
Investigations:	
1. Further NIR Investigations	4,000
2. Variation in alkaloid content of sclerotia	15,000
3. Effectiveness of ergot separation in cleaning grain	11,000
4. Validation of Immunoassay	18,500
5. Increasing speed of immunoassay	22,500
6. Developing assays based on monoclonal antibodies	27,000
Total	**$98,000**

DPI contribution

Year	Salaries	Name	Operating	Use of facilities
2001	20,000	B Blaney		15,000
	5,000	M Ryley		
	15,000	J Molloy		
	5,000	C Moore		
2002	10,000	B Blaney		15,000
	10,000	J Molloy		
	3,000	others		
Total	**68,000**			**30,000**

Intellectual property arising

The antibodies, particularly the monoclonal antibody for DHES, have a moderate value.

Commercial Exploitation

The possibility of incorporating the ELISA test into a commercial package is being investigated. Mr Des Rinehart is acting for MLA in the preliminary negotiations.