final report

Project code: **FLOT.115**

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Date published: **April 2001**

ISBN: **9781741917697**

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Near Infra Red (NIR) detection of sorghum ergot alkaloids in grain

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

NEAR INFRA RED (NIR) DETECTION OF SORGHUM ERGOT ALKALOIDS IN GRAIN

Abstract

This project investigated the feasibility of using the instrumental technique known as Near Infra Red Spectroscopy (NIR) for rapidly scanning sorghum samples to detect sorghum ergot alkaloids and measure the concentration present. Ergot alkaloids at concentrations over 1 mg/kg in feed have been shown to reduce feed intake and growth of cattle in feedlots. Over 40 samples with alkaloid contents ranging from zero up to 250 mg/kg were assayed by accurate laboratory methods (high performance liquid chromatography) and then tested by NIR. A reasonable correlation was obtained over the whole range, but it was too insensitive below 25 mg/kg to show promise of being a practical method for use in detecting alkaloids around the concentration of 1 mg/kg.

Executive summary

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. At first sorghum ergot was thought to be relatively harmless to livestock, but this situation changed when cases of reduced milk production in sows and dairy cows were caused by sorghum containing up to 30% ergot and 50 mg/kg ergot alkaloids (Blaney *et al.* 2000). Experiments conducted by DPI with funding support from PRDC, GRDC and MLA (Blaney 1999, McLennan 2000a,b) have shown that growth of Hereford steers in feedlots is inhibited with alkaloid concentrations of 1.5 mg/kg. This is roughly equivalent to the current limit for sorghum ergot in stockfood regulations of 0.3%.

As investigations proceeded, it became clear that ergot and alkaloid contents are not well correlated. This means that a sample containing the regulated limit of 0.3% ergot most commonly would contain about 1 mg/kg, but in principal might contain anything from 0.3 to 24 mg alkaloids/ kg. Looked at in reverse, the tolerated limit of 1 mg alkaloid/kg for feedlot cattle might conceivably be present in samples containing as little as 0.01% ergot! If climatic conditions increase ergot contamination in future seasons, bulk grain marketers and end-users such as feedlotters will need to detect alkaloids rather than ergot, to detect loads with higher toxicity (>1 mg/kg).

One technique that might be fast enough is a physical test like Near Infra-red Spectroscopy (NIR). The quantification of ergovaline (an ergot alkaloid very closely related to dihydroergosine) in tall fescue pasture by NIR was reported by Roberts et al (1997). They reported several wavelengths from 2300 to 2400nm where absorbance was correlated with ergovaline concentrations ranging from 0.07 – 0.94 mg/kg. The project reported investigated the possibility of measuring dihydroerosine in sorghum by similar means. The specific objective was to evaluate the possibility of using NIR to detect sorghum ergot alkaloid in sorghum grain at levels of 0.1 mg/kg and greater. If this was successful, there were plans to move into a second stage of assessing the test for use with whole grain and then to see how effective grading processes were for removing ergot from sorghum.

a preliminary evaluation of the use of NIR to measure ergot alkaloid was made. Using a NIR Systems 5500 Spectrophotometer with a spinning sample cup, the maximum absorption wavelength of the pure sorghum ergot alkaloid (dihydroergosine) was determined to be 2262 nm (D. Law and B. Blaney, 2000, unpublished data). Three sorghum samples containing about 0, 10 and 40 mg/kg alkaloid were also assayed and showed differences at 2262 nm as well as other wavelengths, which was quite promising.

About 34 samples of sorghum containing a range of ergot alkaloid contents were acquired from various parts of southern and central Queensland. Some had been associated with problems in piggeries and dairies, while others were from ergot-infection sorghum experimental plots.

The resultant samples were analysed for ergot alkaloids in duplicate or triplicate. The method involved a triple extraction into ethyl acetate: methylene chloride: ammonia: methanol (25:50:1:3) facilitated by an ultrasonic bath. Solvent was removed and the alkaloids were semi-purified by partition between diethyl ether and 0.5N HCl, and back-extraction into methylene chloride after rendering basic again with ammonia. After dissolution in methanol and filtration, samples were injected into a high performance liquid chromatograph using an RP-18 column and a mobile phase of 0.1% aqueous ammonium acetate: methanol: acetonitrile (50:20:30). Detection was by both ultraviolet and fluorescence.

Ergot alkaloid results are based on the main alkaloid present, dihydroergosine, which accounts for about 80% of the total alkaloid content. The minor alkaloids are festuclavine and dihydroelymoclavine (subject to mass-spectral confirmation), and appear in constant proportions relative to dihydroerosine in all samples. Results are quoted in terms of the dihydroergosine content alone, which ranged from <0.01mg/kg to 246 mg/kg.

The duplicate samples were examined in the NIR Systems 5500 Spectrophotometer with a spinning sample cup over a wavelength range of 1180-2460nm. Multiple regression analyses provided in WINISI software was used to develop the calibration.

The relationship between the actual and predicted values appeared visually accurate over the wide range of concentrations, but there appeared little correlation when samples contained less than 45 mg/kg. The predicted standard error of validation (SECV) was 9.5 mg/kg. The calibration equation selected contained 5 wavelengths after converting to first derivative and using a scatter correction. Equations using different transformations and fewer wavelengths gave slightly higher standard errors of calibration though probably not significantly different from the one selected. The plot of the second derivative spectra (peaks reversed) in the 1700 -1740nm region demonstrated that samples with high values were clearly distinguishable, but those with lower concentrations were not.

These results suggest a detection limit for the NIR method of about 25 mg/kg, which falls far short of the objective of detecting 0.1 (or even 1) mg/kg. Additionally, it cannot even be concluded that the technique is actually measuring the concentration of the alkaloid. NIR is a statistical technique and in this case it could be measuring a substance that was correlated with the toxin. It is noteworthy that the wavelength (2260nm) identified as a main peak for the dihydroergosine standard did not appear in any of the equations developed.

This result is in stark contrast with that reported by Roberts et al (1997) for ergovaline in tall fescue. We re-examined our data in the range reported by those authors. The range for our scan of sorghum samples in this range is from –0.02 to +0.015 while Robert's (reproduced from their publication) is from –0.8 to +0.4. This indicates a response factor 30 times greater than ours, while their alkaloid concentrations are lower by a factor of 1000! We cannot conceive of any logical reason for this difference, and have been trying to contact the authors for access to their raw data, so far without success. Unless they can provide substantiation of these results, we must discount the report in entirety.

In the case of sorghum ergot, it would seem that the only other possibility for use of the NIR technique might be to assess whether small amounts of ergot physically separated from grain were 'hot' or not. In such a case, up to five gram of lightweight material taken from 100 g of grain might be assayed by NIR. If the result was <20 mg/kg, then the parent grain would contain <1 mg/kg. This presumes that all of the alkaloid is in the ergot sclerotes and lightweight material, which seems reasonable but requires more verification. If that approach is pursued, the next step would be to obtain and analyze about 150 samples of ergot with various concentrations of the alkaloid to enable a rugged calibration to be developed and validated. It should be noted that further work is unlikely to significantly lower the detection limit or improve the precision of the estimate.

A small amount of further work is justified in determining whether the NIR procedure can be applied to ergots in lightweight material, but otherwise the technique appears to hold little promise. The objectives of this project were clearly achieved in that the possibility of using NIR was carefully and rigorously assessed. Unfortunately the answer appears to be negative.

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. At first sorghum ergot was thought to be relatively harmless to livestock, but this situation changed when cases of reduced milk production in sows and dairy cows were shown to be caused by sorghum ergot (Blaney *et al.* 2000).

Experiments conducted by DPI with funding support from PRDC, GRDC and MLA MLA (Blaney 1999, McLennan 2000a,b) have shown that the alkaloid toxins in sorghum ergot inhibit lactation of sows at alkaloid concentrations of 3 mg/kg. Growth of Hereford cattle in feedlots was inhibited with alkaloid concentrations of 1.5 mg/kg. Lactation of dairy cows is also affected by prolonged feeding with concentrations exceeding 1 mg/kg. The current limit for sorghum ergot in stockfood regulations is 0.3%.

As investigations proceeded, it became clear that ergot and alkaloid contents are not well correlated. This means that a sample containing the regulated limit of 0.3% ergot most commonly would contain about 1 mg/kg, but in principal might contain anything from 0.3 to 24 mg alkaloids/ kg. In fact, DPI has already detected 6 mg/kg alkaloids in one sample of sorghum with only 0.3% ergot that was suspected of affecting pigs in NSW. Looked at in reverse, the tolerated limit of 1 mg alkaloid/kg for feedlot cattle might conceivably be present in samples containing as little as 0.01% ergot! To further complicate the issue, estimates of sorghum ergot are very imprecise and subjective when applied to bulk grain.

While the bulk of grain sorghum crops have escaped heavy contamination over the past two seasons, many late-planted crops in central Queensland in 1997 contained up to 30% ergot and >40 mg/kg alkaloids. If climatic conditions increase ergot contamination in future seasons, bulk grain marketers and end-users such as feedlotters will need to detect alkaloids rather than ergot, to distinguish loads with higher toxicity. The need is for a rapid test that can distinguish samples containing >1 mg/kg of alkaloids. Feedlot operations need to make a decision about accepting grain within about 10 – 20 minutes. Bulk grain marketers like Grainco need to test within 5-10 minutes. One technique that might be fast enough is a physical test like Near Infra-red Spectroscopy (NIR).

The quantification of ergovaline (an ergot alkaloid very closely related to dihydroergosine) in tall fescue pasture by NIR was reported by Roberts et al (1997). Using freeze-dried and milled samples, they reported several wavelengths from 2300 to 2400nm where absorbance was correlated with ergovaline concentrations ranging from 0.07 – 0.94 mg/kg. An investigation into the possibility of measuring dihydroerosine in sorghum by similar means is reported here.

Project objectives

Stage 1 objective. To evaluate the possibility of using Near Infra-red Spectroscopy (NIR) to detect sorghum ergot alkaloid in sorghum grain at levels of 0.1 mg/kg and greater.

This report deals only with Stage 1, but if this had been successful, then there were plans to pursue the Stage 2 objectives:

> to determine whether it might be possible to use NIR for rapid screening of whole grain for ergot alkaloids, and;
>
> to determine whether physical separation procedures such as gravity separation and grading can effectively remove ergot alkaloids from sorghum grain.

Preliminary Investigation

Prior to commencement of this project, a preliminary evaluation of the use of NIR to measure ergot alkaloid was made. Using a NIR Systems 5500 Spectrophotometer with a spinning sample cup, the maximum absorption wavelength of the pure sorghum ergot alkaloid (dihydroergosine) was determined to be 2262 nm (Figures 1 and 2) (D. Law and B. Blaney, 2000, unpublished data).

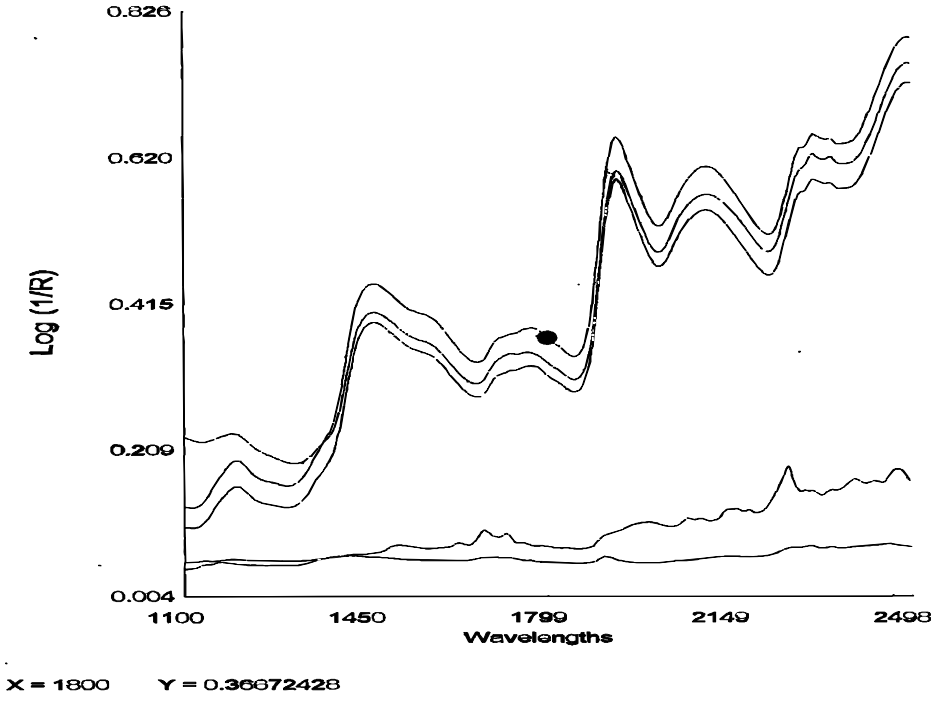

Figure 1. Spectra of 3 sorghum samples and dihydroergosine standard

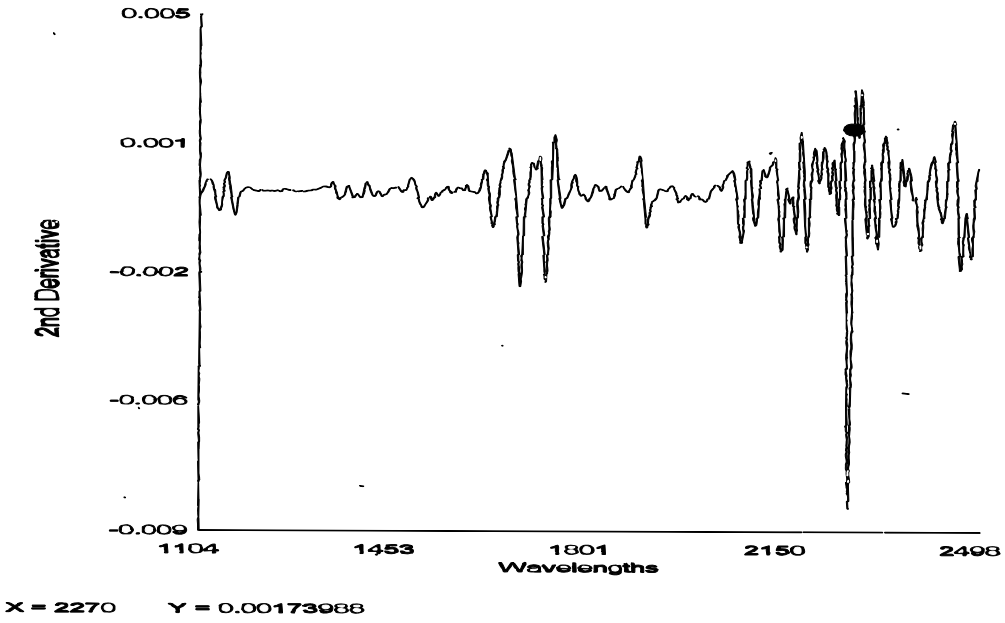

Figure 2. Second derivative spectra of dihydroergosine standard showing major peak at 2262 nm.

Three sorghum samples containing about 0, 10 and 40 mg/kg alkaloid were also assayed and showed differences at 2262 nm as well as other wavelengths (Figure 3).

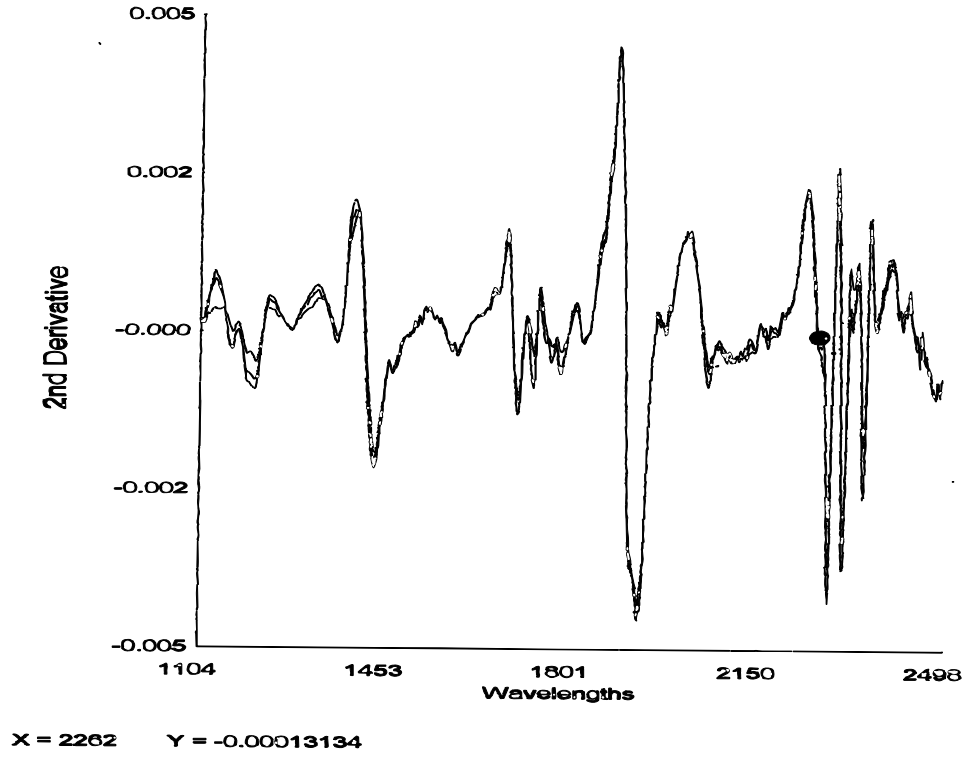

Figure 3. Spectra of 3 sorghum samples - second derivative, dot indicates 2262 nm.

This result was considered promising, and the next step was to evaluate the sensitivity and precision of the procedure using ground sorghum samples with a range of alkaloid contents.

Methodology

About 34 samples of sorghum containing a range of ergot alkaloid contents were acquired from various parts of southern and central Queensland. Some had been associated with problems in piggeries and dairies, while others were from ergot-infection sorghum experimental plots.

Samples of around 1 kg were milled through a Cristie Norris hammer mill with a 1mm screen. After thorough mixing, two sub-samples were taken, one for alkaloid assay and the other for NIR assessment. In addition, two samples containing higher concentrations of alkaloid were diluted with two different samples of sorghum containing little or none alkaloid, in concentrations of 50, 25, 12.5, 6.25 and 3.1% in order to test potential sensitivity without variation in matrix effects.

The resultant samples were analysed for ergot alkaloids in duplicate or triplicate. The method involved a triple extraction into ethyl acetate: methylene chloride: ammonia: methanol (25:50:1:3) facilitated by an ultrasonic bath. Solvent was removed and the alkaloids were semi-purified by partition between diethyl ether and 0.5N HCl, and back-extraction into methylene chloride after rendering basic again with ammonia. After dissolution in methanol and filtration, samples were injected into a high performance liquid chromatograph using an RP-18 column and a mobile phase of 0.1% aqueous ammonium acetate: methanol: acetonitrile (50:20:30). Detection was by both ultraviolet and fluorescence.

The duplicate samples were examined in a NIR Systems 5500 Spectrophotometer with a spinning sample cup over a wavelength range of 1180-2460nm. Multiple regression analyses provided in WINISI software was used to develop the calibration.

Results and discussion

Ergot alkaloid results are based on the main alkaloid present, dihydroergosine, which accounts for about 80% of the total alkaloid content. The minor alkaloids are festuclavine and dihydroelymoclavine (subject to mass-spectral confirmation), and appear in constant proportions relative to dihydroerosine in all samples. Results are quoted in terms of the dihydroergosine content alone, which ranged from <0.01mg/kg to 246 mg/kg. The list of samples tested and HPLC results are in Table 1.

Table 1. Sorghum Samples for NIR Assessment with Dihydroergosine contents

	Code	DHES (mg/kg)	Sorted by alkaloid contents	
Mutdapilly Clean grain	A1	0	A1	0
Clifton Clean grain	A2	0.2	A3	0
Jimboure Clean grain	A3	0	I1	0
Rocklea outside silo 2	B1	5.5	I2	0
Rocklea outside silo 3	B2	3.7	J3	0
Rocklea outside silo 6 (conc.ergot)	B3	111	K1	0
Rocklea (L) inside bin 3	C1	14	E	0.1
Rocklea (L) inside bin 11	C2	45	F1	0.1
Rocklea (V) inside bin 14	C3	45	F4	0.1
Rocklea (P) inside bin 8	C4	11	H1	0.1
Rocklea (McL) inside	C5	63	H2	0.1
Pac seeds gravity sep ergot	D	6.1	J2	0.1
Ingham piggery	E	0.1	A2	0.2
Hermitage BH Buster	F1	0.1	F2	0.2
Hermitage BH Hybrid	F2	0.2	K4	0.4
Hermitage BH A-line	F3	6.3	K2	0.5
Hermitage BH DK4817	F4	0.1	N5	1.2
Emerald (McC)	G	12	N10	1.5
Downs trial plot PL site 3	H1	0.1	N4	2.3
Downs trial plot PL site 4	H2	0.1	K3	2.7
Downs trial plot C site 1	I1	0	N9	3
Downs trial plot C site 2	I2	0	B2	3.7
Casino piggery Bin E (lightweight)	J1	5.8	N3	4.5
Casino piggery Bin D	J2	0.1	B1	5.5
Casino piggery Bin G	J3	0	J1	5.8
Casino piggery ex S Downs(G)	K1	0	N8	6
Casino piggery ex S Downs(W)	K2	0.5	D	6.1
Casino piggery ex S Downs(H)	K3	2.7	F3	6.3
Casino piggery ex S Downs(M)	K4	0.4	N2	9
Monto (J) (conc ergot)	L1	246	C4	11
Wowan (L) (conc ergot)	L2	61	N7	11
Mutdapilly dairy trial	M	51	G	12
Dilution of O with A2 50%	N1	17	C1	14
25%	N2	9	N1	17
12.50%	N3	4.5	N6	22
6.25	N4	2.3	O	34
3.10%	N5	1.2	C2	45
Dilution of C3 with A3 50%	N6	22	C3	45
25%	N7	11	M	51
12.50%	N8	6	L2	61
6.25%	N9	3	C5	63
3.10%	N10	1.5	B3	111
McL (used 2000/01 pig trial)	O	34	P	127
Mutdapilly lightweight (conc ergot)	P	127	L1	246

0 = <0.1mg/kg

Figure 4 shows the relation for the calibration between the actual and predicted values. The predicted standard error of validation (SECV) was 9.5 mg/kg. In Figure 4, Sample C2 was an outlier and K2 was on the limit. The reason for this is not known.

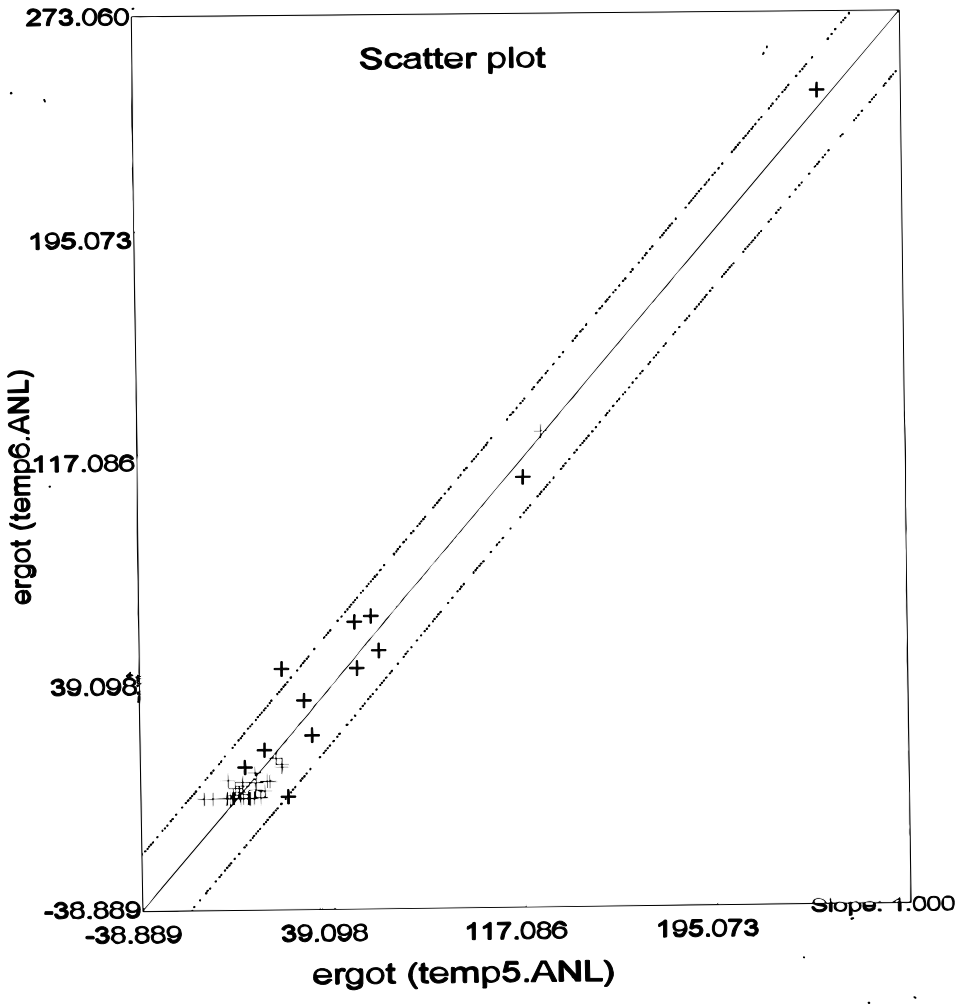

Figure 4. Relation between actual (Y-axis) dihydroergosine content (mg/kg) and predicted values (X-axis) for all samples used in the calibration (n=44).

While the relation in Figure 4 appears visually accurate, this is a result of the wide range of concentrations. Figure 5 illustrates the relation for results less than 45 mg/kg.

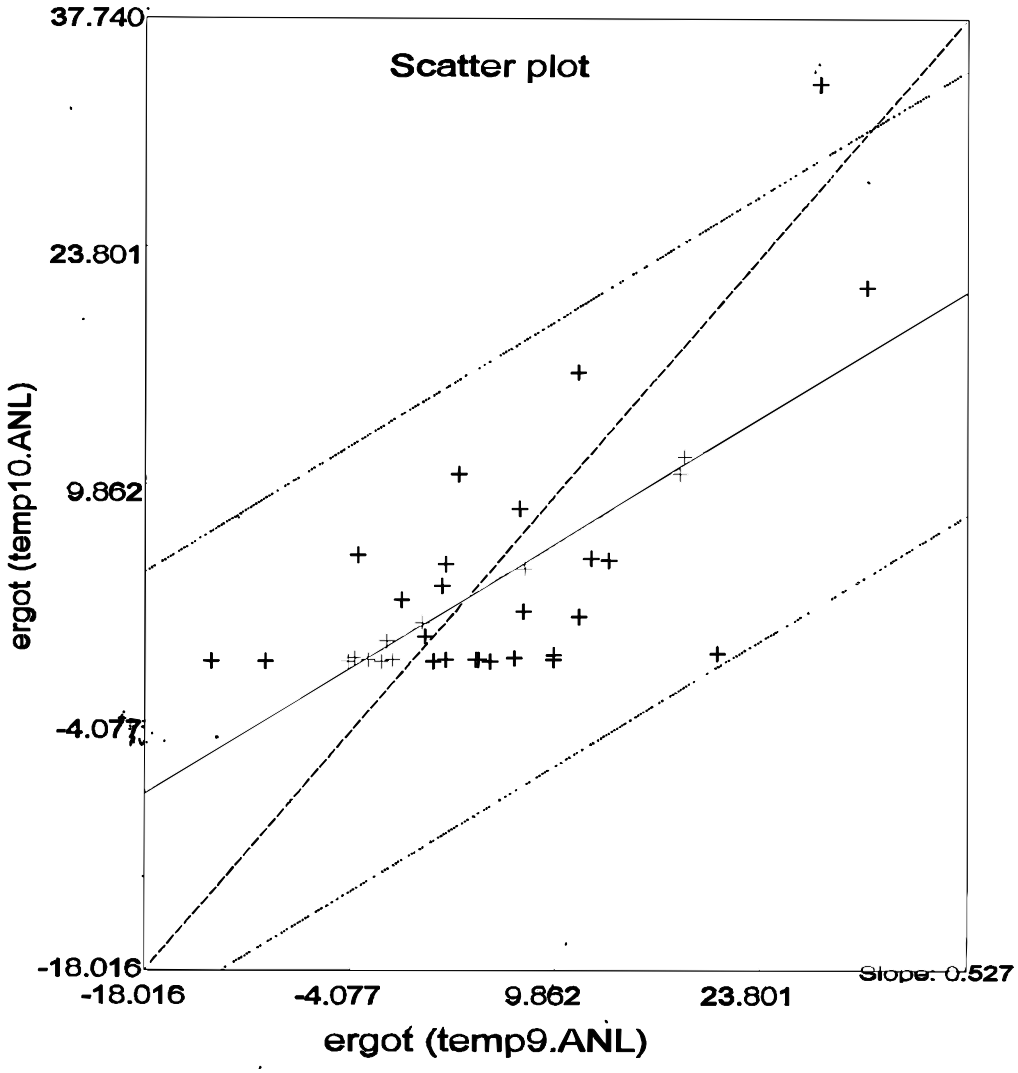

Figure 5. Relation between actual (Y-axis) dihydroergosine content (mg/kg) and prediced values (X-axis) for samples with less than 45 mg/kg (n=36)

The calibration equation used in Figure 4 contained 5 wavelengths after converting to first derivative and using a scatter correction. Equations using different transformations and fewer wavelengths gave slightly higher standard errors of calibration though probably not significantly different from the one selected.

Figure 6 shows the plot of the second derivative spectra (peaks reversed) in the 1700 -1740nm region, peak at 1720nm and demonstrates that while samples with high values are clearly distinguishable, those with lower concentrations are not.

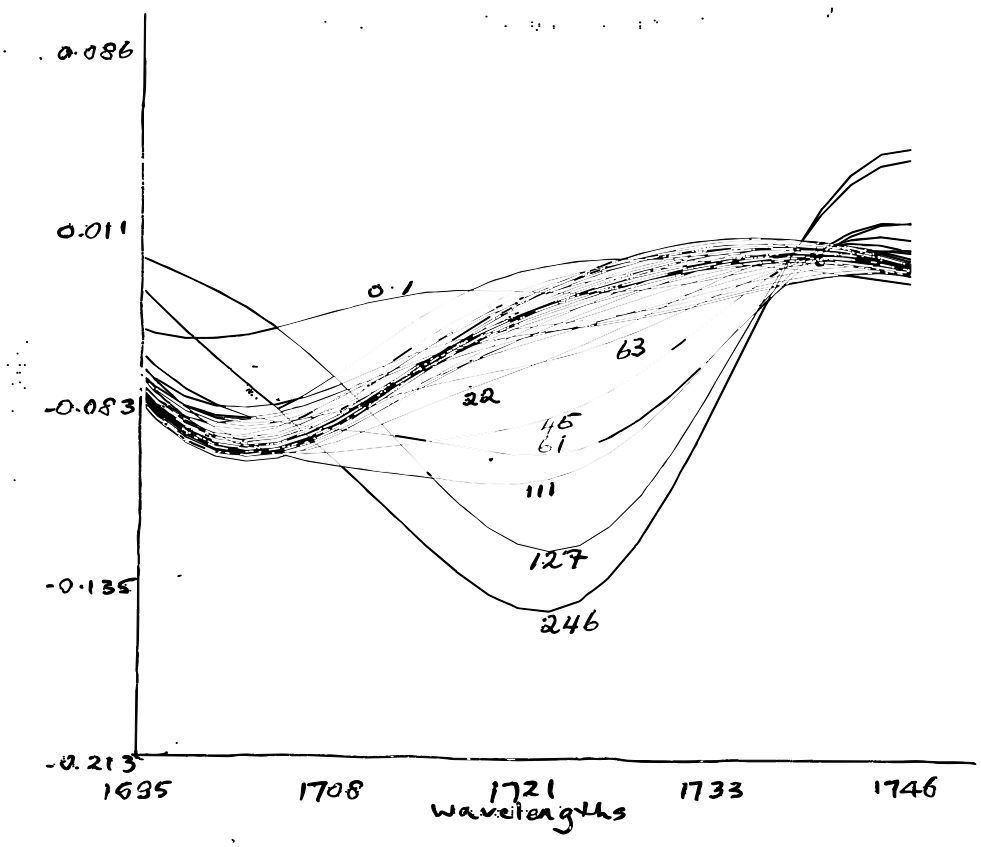

Figure 6. Second derivative spectra over the range 1700 - 1740 nm with dihydroergosine contents marked for the highly contaminated samples.

These results suggest a detection limit for the NIR method of about 25 mg/kg, which falls far short of the objective of detecting 0.1 (or even 1) mg/kg. Additionally, it cannot even be concluded that the technique is actually measuring the concentration of the alkaloid. NIR is a statistical technique and in this case it could be measuring a substance that was correlated with the toxin. It is noteworthy that the wavelength (2260nm) identified in the preliminary study did not appear in any of the equations developed.

This result is in stark contrast with that reported by Roberts et al (1997) for ergovaline in tall fescue. We re-examined our data in the range reported by those authors and compare them in Figure 7.

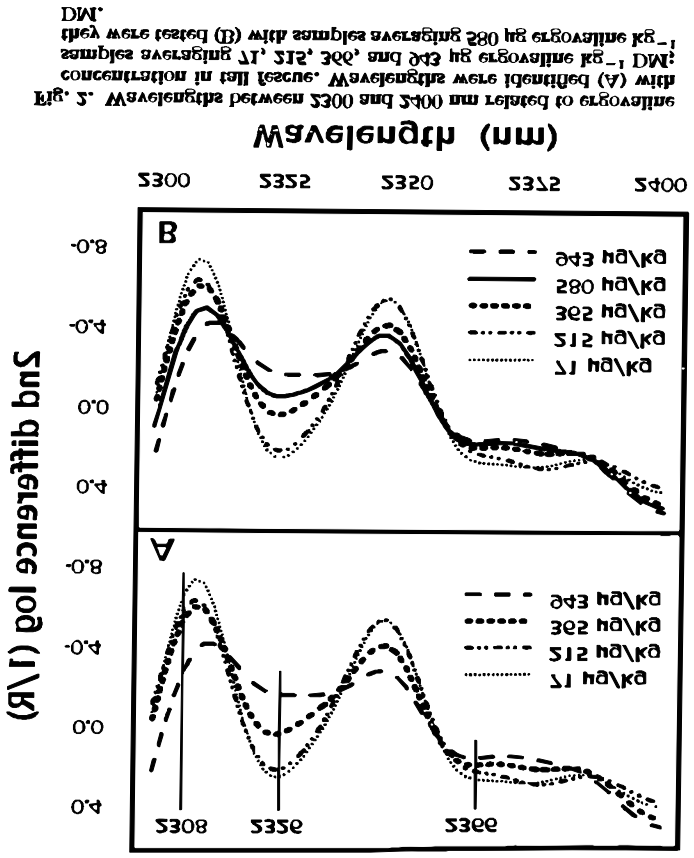

Fig. 5. Wavelengths between 2500 and 2400 nm related to ergovaline concentration in leaf fescue. Wavelengths were identified (A) with samples averaging 17, 212, 308, and 482 μg ergovaline kg^{-1} DM; they were tested (B) with samples averaging 082 μg ergovaline kg^{-1} DM.

Figure 7a. Scan of fescue from Roberts et al 1997

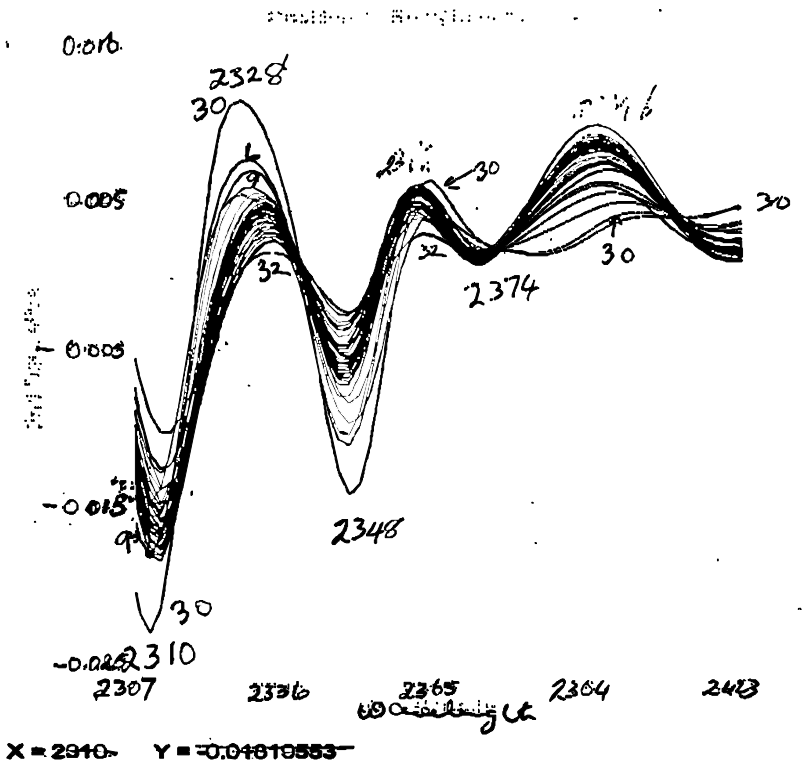

Figure 7b. Scan of sorghum samples

The range for our scan of sorghum samples in this range is from –0.02 to +0.015 while Robert's (reproduced from their publication) is from –0.8 to +0.4. This indicates a response factor 30 times greater than ours, while their alkaloid concentrations are lower by a factor of 1000! We cannot conceive of any logical reason for this difference, and will try to contact the authors for access to their raw data. Unless they can provide substantiation of these results, we must discount the report in entirety.

In the case of sorghum ergot, it would seem that the only other possibility for use of the NIR technique might be to assess whether small amounts of ergot physically separated from grain were 'hot' or not. In such a case, up to five gram of lightweight material taken from 100 g of grain might be assayed by NIR. If the result was <20 mg/kg, then the parent grain would contain <1 mg/kg. This presumes that all of the alkaloid is in the ergot sclerotes and lightweight material, which seems reasonable but requires more verification. If that approach is pursued, the next step would be to obtain and analyze about 150 samples of ergot with various concentrations of the alkaloid to enable a rugged calibration to be developed and validated. It should be noted that further work is unlikely to significantly lower the detection limit or improve the precision of the estimate.

Success in achieving objectives

The objectives were clearly achieved in that the possibility of using NIR was carefully and rigorously assessed. Unfortunately the answer was negative.

Impact on Meat and Livestock Industry

The results of this project will have no impact on livestock industry. However, they have clearly shown that NIR is not likely to be a useful test for rapid assay of ergot alkaloids by feedlotters. In the broader sense, ergot alkaloids are likely to have a major impact on feedlots in future, and projects such as this are part of a systematic enquiry into means of reducing that impact.

Conclusions and Recommendations

It is concluded that NIR cannot be used for rapid scanning of sorghum samples to detect samples containing 1 mg/kg or less of ergot alkaloids. The detection limit for the NIR method was about 25 mg/kg, which falls far short of the objective of detecting 0.1 (or even 1) mg/kg. It is recommended that some further research be conducted into determining whether the NIR procedure can be applied directly to ergots, in the case that these can be rapidly separated from grain. It is also recommended that research be conducted into alternative rapid assay methods for ergot alkaloids, such as immunoassay.

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Appendices

None

Administrative details report

Budget

MLA contribution

Item	Cost ($)
Labour for sample collection and preparation	2,300
HPLC assay of 50 – 60 samples in duplicate	6,000
NIR assessment by Mr Don Law	1,200
Travel / car lease	300
Total	**$9,800**

DPI contribution

Year	Salaries	Name	Operating	Use of facilities
2000	6,000	B Blaney		500
(Stage 1)	2,000	M Ryley		
	1,300	Several others*		
Total	**9,300**			**500**

*Mainly involved in collection and processing of samples

Intellectual property arising

None apparent

Commercial Exploitation

Not applicable