Development of an enzyme-linked immunosorbent assay for the detection of lolines in pastures

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**ABSTRACT**

Polyclonal antibodies were produced in sheep for the development of a competitive enzyme-linked immunoassay for use in quantifying loline alkaloids in pasture samples. Lolines are aminopyrrolizidine secondary metabolites produced by fungal endophytes present in tall fescue and meadow fescue grasses, and confer increased resistance to a range of grass pests. Immunizing and plate coating antigens were prepared with derivatized loline dihydrochloride. Cross-reactivity studies indicated that the assay developed has broad specificity and antibody binding to loline analogues is affected by structural changes in the side chain of the loline molecule. Results obtained using the optimized immunoassay were compared with those determined by gas chromatography. The assay provides a sensitive and rapid analytical method that detects the loline analogues of interest and has been applied in pasture breeding programmes. The assay limit of quantitation for lolines in pasture is 3 µg/g in dried herbage.

**1. Introduction**

The loline group of alkaloids are saturated aminopyrrolizidine derivatives that are fungal secondary metabolites (Powell & Petroski, 1992). A number of these compounds are produced by the endophytes *Epichloë coenophiala* and *E. uncinata* when infecting tall fescue (*Lolium arundinaceum*) and meadow fescue (*L. pratense*), respectively. In such associations, the most abundant loline analogues found in herbage are *N*-formyl loline (NFL), *N*-acetyl loline (NAL), and *N*-acetyl norloline (NANL) (Adhikari, Boelt, & Fomsgaard, 2016; Bush, Fannin, Siegel, Dahlman, & Burton, 1993; Justus, Witte, & Hartmann, 1997). Lolines possess bioactivity against a broad range of herbivorous insects, and increased resistance of loline-containing pastures to grass pests has been widely reported with effects on livestock and mammalian wildlife, however, considered to be negligible (Finch, Munday, Munday, & Kerby, 2016; Gooneratne, Patchett, Wellby, & Fletcher, 2012; Scharl, Grossman, Nagabhryu, Faulkner, & Mallik, 2007). It is possible to artificially infect ryegrass (*L. perenne*) with *E. coenophiala* taken from tall fescue although such associations show comparatively less production of loline than that found in tall fescue (Easton, 2007). High-producing,
robust and persistent pastures that are non-toxic to livestock and have broad-spectrum resistance to grass insects are of major importance to agriculture. Plant breeders are able to provide protection to pasture against insects by increasing mean loline concentrations in grasses by selection for high loline production within ryegrass families (Easton, Lyons, Cooper, & Mace, 2009). Such breeding programmes require that large numbers of progeny infected with *E. coenophiala* are screened for loline content.

A number of suitable methods have been reported for the detection and quantification of lolines (Adhikari et al., 2016; Powell & Petroski, 1992; Yates, Petroski, & Powell, 1990). Enzyme-linked immunosorbent assays (ELISA), however, offer an alternative methodology ideally suited for rapid screening. Preparation of samples usually only involves dilution of the extracts and the immunoassays can be sensitive, provide high sample throughput and require relatively low-cost equipment. ELISAs developed for the quantification of many endophyte secondary metabolites have had application in a wide range of research and pasture breeding programmes (Garthwaite, Sprosen, Briggs, Collin, & Towers, 1994; Hill & Agee, 1994). Here we describe the extraction and derivatization of loline compounds of interest, production of polyclonal antibodies to loline, and their use in an indirect competitive ELISA for detection of lolines in herbage. Methods for sample extraction and assay conditions were developed to overcome assay matrix effects and to allow analysis after minimal preparation of samples. The antibodies have good recognition for the loline analogues found in herbage samples and provide a sensitive and robust immunoassay.

2. Materials and methods

2.1. Materials and reagents

Bovine serum albumin (BSA) Fraction V, (fatty acid poor), manufactured by Gibco, New Zealand, was supplied by Invitrogen Corp. Freund’s complete (containing heat-killed *Mycobacterium tuberculosis*) and incomplete adjuvants (without *M. tuberculosis*) were from Sigma Chemical Co. (St. Louis, MO). Cationized BSA (cBSA) was prepared by the method of Hermanson (1996). Ovalbumin (OVA) was supplied by Inovatech Inc. (BC, Canada). Donkey anti-sheep immunoglobulin–horseradish peroxidase conjugate (anti-sheep–HRP) was from Dako Ltd. (Victoria, Australia). Horseradish peroxidase substrate solution was BioFX from Surmodics, MN, USA. Maxisorp 96-well immunoplates were from Nunc (Roskilde, Denmark).

N,N-dimethylformamide (DMF) and 1,1’-carbonyldiimidazole (CDI) were supplied by Sigma-Aldrich, MO, USA. All inorganic chemicals and organic solvents were of reagent grade or better. Coating buffer was carbonate buffer (50 mM, pH 9.6). Phosphate-buffered saline (PBS) contained NaCl (150 mM), Na₂HPO₄ (8.4 mM), and NaH₂PO₄ (17 mM, pH 7.4). ELISA washing buffer (PBST) consisted of 0.05% Tween 20 in PBS. Blocking/antibody buffer consisted of 1% BSA (w/v) in PBST and standard/sample buffer was PBS.

2.2. Detection, characterization and estimation of lolines

Initial detection of lolines was achieved by thin layer chromatography on silica gel (Merck SIL G-25) with chloroform/methanol (3:1 v/v) as eluent. Compounds were visualized by exposure to iodine vapour.
The presence of lolines in extracts and fractions was monitored by liquid chromatography mass spectrometry (LC-MS/MS, LTQ Linear Ion Trap Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) (Harrison, Fraser, Lane, Villas-Boas, & Rasmussen, 2012). Ions selected included m/z+ 141 (norloline), 155 (loline), 169 (N-methyl loline), 183 (NANL and NFL), and 197 (NAL). NANL and NFL could be distinguished by MS2 fragments of m/z+ 155 and 165, respectively.

The purity of the lolines was estimated by quantitative proton NMR (500 MHZ Bruker Advance, Bruker BioSpin GMBH, Billerica, MA, USA). 10–15 mg of each loline was accurately weighed into vials and dissolved in 0.5 mL of deuterium oxide containing 0.5 μL of 1,4-dioxane. Proton spectra were recorded, and the loline signals were integrated and compared to the integral of the 1,4-dioxane signal for calculation of purity. This showed purities of 96 ± 2% for NAL, 101 ± 2% for NANL and 82 ± 2% for NFL. The NMR of NFL showed signals for two conformers and the spectrum indicated that the major impurity was residual water.

2.3. Isolation of lolines

Meadow fescue seed (endophyte-infected Northland ecotype, Grasslanz Technology Ltd, Lincoln, New Zealand) was ground to a fine powder, and batches of 400 g extracted with 3 × 3 L of water/ethanol (2:1 v/v) at ambient temperature for 24 h and with occasional manual mixing. Anionic materials were removed from the extract by stirring with Amberlite IR45 resin (OH form, Rohm and Hass, Pennsylvania, USA). After decanting from the resin, the solution was passed through a column of 1.5 L of pre-swollen FPC3600 cation exchange resin (Rohm and Hass). The column was washed with 2 L of water and the lolines and other cationic materials were eluted with 2 × 1 L of water/formic acid (19:1 v/v) followed by 3 × 1 L of water/formic acid (49:1 v/v). Fractions were analysed as described above, and those containing lolines were freeze dried, yielding a gum-like resin.

The gum was dissolved in 60 mL of water and adjusted with aqueous sodium hydroxide (10 M). Precipitated material was removed by centrifugation and subsequently extracted with 70 mL of chloroform. The supernatant was also extracted with 3 × 70 mL of chloroform, and the extracts were combined and dried by rotary evaporation.

2.4. Preparation of loline dihydrochloride

The pale syrup recovered from dried extracts was characterised as described above and shown to contain a mixture of NFL, NANL, and NAL. Loline dihydrochloride was prepared from this mixture by treatment with an excess of 4 M hydrochloric acid (25 mL) (Figure 1). The solution was warmed with mixing and left to stand for 2 h before drying by rotary evaporation. The residue was rinsed with ethanol and loline hydrochloride obtained by crystallization from aqueous ethanol.

2.5. Isolation of NANL

Ground tall fescue seed (Flecha MaxP*, PGG Wrightsons Seeds, Christchurch, New Zealand), which contains predominantly NANL, was extracted with aqueous ethanol
and the extract purified by ion exchange as described above. The syrup obtained containing NAL was distilled under vacuum in a sublimation apparatus (100°C at 1.2 torr to 150°C at 0.65 torr) on to a cold finger cooled with solid carbon dioxide. The core of the viscous distillate partially crystallized after storage at 4°C for 2 weeks, and the harvested crystals were used to seed further crystallizations of NAL from an ethyl acetate/toluene/hexane mixture.

2.6. Isolation of NFL and NAL

Aqueous ethanolic extracts of meadow fescue seed, prepared as described above, were dried under vacuum. The residue was subjected to liquid–liquid partitioning (in a manual counter-current scheme) using chloroform/water (1:1 v/v). The system consisted of a series of 12 capped tubes each containing 6 mL of chloroform with the mixed lolines sample added to the first three tubes. Water saturated with chloroform (6 mL) was then added to the first tube, which was mixed by mechanical rotation for 5 min, followed by standing for 5 min to permit phase separation. The aqueous phase was transferred to the next tube in the sequence until it had passed across all 12 tubes. Water (6 mL) added in this way 24 times to yield 24 aqueous solutions, with 12 extracted chloroform solutions remaining. All samples were analysed by mass spectrometry or thin layer chromatography as described above. Fractions found to be pure in one single loline species were evaporated to dryness. These were then dissolved in a small volume of chloroform, dried

Figure 1. Synthesis of immunogen (cBSA-N-hemisuccinyl loline, (1) and coating conjugate (OVA-N-hemisuccinyl loline, (2) by preparation of derivatized loline and conjugation to carrier proteins.
with sodium sulphate, and concentrated by evaporation. The fraction containing NAL crystallized on standing at 4°C and these crystals were used to seed samples for subsequent recrystallization. The NANL fraction also crystallized when seeded with the NANL crystals previously obtained, although crystallization of NFL was not possible following this procedure.

2.7. Synthesis of N-hemisuccinyl loline (hapten)

Hapten was prepared as illustrated in Figure 1. Loline dihydrochloride (230 mg) was dissolved in 2 mL of water. Sodium hydroxide (10 M, 220 µL) was added and loline extracted with 4 × 5 mL of chloroform. After drying the pooled extracts with anhydrous sodium sulphate, 140 µL of triethylamine and 150 mg of succinic anhydride were added and dissolved by shaking. The volume was then reduced to 5 mL by boiling. The solution was left to stand at ambient temperature for 2 d, then 1.5 mL of hexane was added and the mixture was cooled at 4°C. Crystals of 91 mg of hapten were produced, which were purified by recrystallization from methanol/propan-2-ol.

This material was characterized using a range of 1- and 2-dimensional NMR experiments. Approximately 10 mg of hapten was dissolved in deuterated methanol. The proton spectrum showed no significant peaks, other than those attributable to the loline derivative, indicating high purity.

2.8. Synthesis of protein–hapten conjugates

The hapten was conjugated to cBSA or OVA using the carbodiimide method (Bauminger & Wilchek, 1980). N-hemisuccinyl loline (3.97 mg) and 15.3 mg of CDI were dissolved in 0.5 mL of anhydrous DMF. After 30 min, 150 µL of water was added, and half the solution was added to 20 mg of OVA and half to 20 mg of cBSA. Both proteins were previously dissolved in 3 mL of phosphate buffer (250 mM, pH 7.4). The resulting solutions were stirred for 6 h at ambient temperature and then stored at 4°C for 15 h. After equilibrating to ambient temperature, the conjugates were purified by desalting (Bio-Rad Econo-Pac 10DG column) followed by dialysis (Pierce SnakeSkin, 10 kDa cutoff) against PBS. Aliquots (1 and 5 mg) were stored at −20°C.

2.9. Immunization

Five sheep were each immunized with 300 µg of cBSA-hapten. For primary immunizations, immunogen was prepared as a water-in-oil emulsion by injecting conjugate dissolved in filter-sterilized PBS into Freund’s complete adjuvant, adding one part to two parts of complete adjuvant followed by vortex mixing. Immunogens for secondary and subsequent immunizations were prepared as above but as emulsions in Freund’s incomplete adjuvant (adding one part to three parts of incomplete adjuvant). Primary immunization was administered to two sites (500 µL each) on the muscle on one side of the neck while subsequent immunizations were administered intramuscularly to two sites on the upper hind legs (500 µL each). Test-bleeds (10 mL) were taken from the jugular vein of each sheep 1 week after immunization and the sera screened by ELISA for antibodies suitable for use in competitive ELISAs for lolines. Within 2 weeks after immunization, blood
was collected from selected sheep into blood bags (Pharmaco) under negative pressure and the antisera were stored at −20°C. All animal experiments were approved by the AgResearch Ruakura Animal Ethics Committee (Application 11832).

2.10. ELISA

2.10.1. Non-competitive ELISA
All assay procedures were carried out at 21°C. Microtiter plates were coated 100 µL/well with OVA-hapten (3 µg/mL) in coating buffer. After incubation for 16 h, plates were washed four times with PBST and blocked for 1 h with 1% BSA in PBST (200 µL/well). This was followed by four washes with PBST. To each well was added 50 µL of sample/standard buffer (PBS) followed by 50 µL of various dilutions of antiserum in blocking/antibody buffer and the plate was incubated for 1 h. After four washes (PBST), 100 µL of anti-sheep–HRP (diluted 1:4500 in antibody buffer) was added; plates were incubated for 2 h and washed four times with PBST. Substrate solution (100 µL/well) was added and after incubation with shaking for 30 min the enzyme reaction was stopped by addition of sulphuric acid (0.3 M, 100 µL/well). The absorbance of wells was determined at 450 nm using a Versa_max microplate reader (Molecular Devices Corporation, California, USA).

2.10.2. Competitive ELISA
Plates were coated and then blocked with 1% BSA as above. A standard of NFL (2.2 mg/mL) in 80% methanol in water (v/v) was diluted in sample buffer to 1 µg/mL, and further 2.5-fold dilutions were made in sample buffer to give 10 standards (0.1–400 ng/mL). To each well was added 50 µL of standard or diluted sample, followed by 50 µL of antibody at a dilution such that the maximum absorbance (in the absence of analyte) in the assay was approximately 1.0 absorbance. All samples were analysed in duplicate wells. ELISA standard curves were prepared for each assay using NFL as the reference standard and results were reported in NFL immunoreactive equivalents. The assay was completed as described for the non-competitive ELISA. Data analysis was performed using SOFTmax PRO data analysis software (Molecular Devices Corporation). Curve fits of mean absorbance versus the logarithm of the analyte concentration were performed by four-parameter curve fit.

2.11. Herbage extraction
Herbage was freeze dried and milled using an UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, USA) and approximately 30 mg of the ground material was weighed accurately into an Eppendorf tube and extracted with 1 mL of PBS for 1 h by end-over-end rotation. The extracts were centrifuged (Eppendorf Microfuge 5415C) at 8600 g for 5 min and an aliquot (10 µL) of the supernatant diluted to a minimum dilution of 1 in 300 with PBS, with a second dilution of 1 in 3000 also being made.

2.12. Assay validation
Milled samples of endophyte-free tall fescue, meadow fescue, and perennial ryegrass were obtained, which did not contain loline analogues. Several extracting solvents were
investigated and the effects of dilutions of loline-free grass extract on ELISA maximum absorbance ($A_{\text{max}}$) were studied for each solvent system. The extracting solvent selected was one requiring the least dilution of these grass extracts to remove inhibition of $A_{\text{max}}$ while giving the best assay signal (inhibition of colour development) for the loline-containing samples extracted in the same system and at the same dilution.

Absence of ELISA matrix effects was confirmed by comparing the response of the assay to NFL standards in buffer and NFL standards in extracts of loline-free herbage diluted as previously determined to remove matrix interference. When curves were coincidental it was concluded that no further sample treatment or assay modification was required. The optimized method was validated by comparing results obtained using the standard GC-FID method (Baldauf, Mace, & Richmond, 2011) with those obtained by cELISA. Herbage samples with different profiles of loline analogues were included in the study.

Intra-assay variation was determined by duplicate extraction and analysis of 39 samples with each replicate analysed on the same plate. Inter-assay variation was determined by extraction and analysis of a single herbage sample on 10 different occasions.

3. Results

3.1. Characterization of NFL-specific antisera

After six immunizations with cBSA–hemisuccinyl loline, antisera from sheep 6139 gave the highest titre in ELISA (Table 1). Sheep 6140 antiserum gave the most sensitive ELISA with NFL although the steepness (accuracy) of the dose–response curve for NFL was less than that of antiserum 6139 (data not shown) and recognition of NANL was approximately 5-fold less than with sheep 6139 antiserum (Table 1). ELISA cross-reactivity studies using antibodies collected from sheep 6139 and loline analogues that were available showed that cross-reactivity with NFL and NAL in the assay was approximately three times greater than that with NANL (Table 2).

3.2. Assay optimization and matrix studies

Assay optimization was carried out using bulk collection of antiserum taken from sheep 6139 after four immunizations. Optimal concentrations of plate coating antigen, antiserum, and anti-sheep–HRP were determined by checkerboard assays. Solvents investigated for extraction of ryegrass, meadow fescue, and tall fescue were: 10%, 50%, 70%, or 90% methanol in water; PBS; or PBST. PBS and PBST were also investigated as suitable extractants. PBS extracts were found to require the lowest dilution of loline-free pasture

| Animal | Titer$^a$ ($\times 10^3$) | IC$_{50}$ (ng/mL) | IC$_{50}$ (ng/mL) |
|--------|--------------------------|------------------|------------------|
| 6032   | 30                       | 90               | 1406             |
| 6116   | 20                       | 6                | 58               |
| 6139   | 70                       | 6                | 12               |
| 6140   | 40                       | 2                | 97               |
| 6209   | 20                       | 68               | 4939             |

$^a$Antiserum dilution required to give 1.0 absorbance in the ELISA (no analyte present).
$^b$Concentration of NFL (ng/mL).
$^c$Concentration of NANL (ng/mL) required to inhibit ELISA absorbance to 50% of the maximum absorbance.
Table 2. Cross-reactivities of antisera\textsuperscript{a} with loline analogues.

| Compound              | Structure | IC\textsubscript{50} (ng/mL) | Cross-reactivity\textsuperscript{b} (%) |
|-----------------------|-----------|-----------------------------|----------------------------------------|
| N-formyl loline       |           | 7.2                         | 100                                    |
| N-acetyl loline       |           | 7.5                         | 104                                    |
| N-acetyl norloline    |           | 24                          | 30                                     |
| loline dihydrochloride|           | 214                         | 3                                      |

\textsuperscript{a}Bulk bleed collected from sheep 6139.

\textsuperscript{b}Cross-reactivity = (IC\textsubscript{50} reference compound/IC\textsubscript{50} test analogue) × 100, where IC\textsubscript{50} is the molar concentration of compound giving 50% inhibition in maximum ELISA absorbance obtained in the absence of reference compound or analogue. Cross-reactivities of loline analogues were determined in ELISAs performed on three separate occasions. Mean inter-assay variation (CV) ranged from 3.1% to 7.6%.

Figure 2. Effect of pasture extracts (PBS) on ELISA maximum absorbance (A\textsubscript{max}). Extracts diluted in PBS, tall fescue without endophyte and free of lolines (□), tall fescue with endophyte and containing lolines (●).
extract to remove inhibition of $A_{\text{max}}$ while at the same time giving the highest assay signal with loline-containing samples extracted in PBS and at the same dilution. As shown in Figure 2, dilution of extracts 300-fold in PBS was sufficient to remove inhibition of $A_{\text{max}}$. ELISA response curves for NFL standards prepared in PBS and curves for standards in loline-free perennial ryegrass, tall fescue, and meadow fescue extracts diluted 1 in 300 in PBS were coincidental (Figure 3).

Based on the means from 10 assays, the optimized assay had a working range of 1.2–32 ng/mL, the concentrations giving 20–80% inhibition of $A_{\text{max}}$ (IC$_{20}$–IC$_{80}$) and the mean IC$_{50}$ was 6.4 ng/mL. After 300- and 600-fold dilution of extracts, the limit of quantitation

Figure 3. Loline ELISA standard curves in the absence (□) and presence of extracts (PBS) of loline-free perennial ryegrass (○), tall fescue (Δ), and meadow fescue (∇). All samples were diluted 300-fold in PBS. ELISA absorbance values shown are the mean of duplicate determinations with error bars representing standard deviation.

Figure 4. Correlation between ELISA and GC-FID determination of total lolines in perennial ryegrass with AR525 endophyte, samples containing NFL only. Regression data: $n = 17$; $R^2 = 0.8$; slope = 0.8.
(IC\textsubscript{20}) for loline immunoreactive equivalents in pasture samples determined by ELISA (mean of 10 assays) was 3 µg/g in dried herbage.

Mean intra-assay coefficient of variation was 1.3% (range: 0.2–6.2%), while mean coefficient of variation for inter-assay variation was 2.6% (range: 0.1–4.8%).

**Assay validation**

Two groups of herbage samples were analysed for total loline immunoreactive equivalents by ELISA and compared with results determined for total lolines (sum of NFL, NANL, and NAL) by GC-FID. Correlation between ELISA and GC-FID results for individual plant samples of perennial ryegrass infected with AR525 endophyte was high ($R^2 = 0.8$). Values determined by ELISA were 79% of those determined by GC-FID (Figure 4). The GC-FID analysis also indicated that NFL was the only loline analogue present in the plant samples.

Correlation between the two methods for the tall fescue samples, however, was less ($R^2 = 0.7$). In this case, bulk pasture samples of Flecha cultivar infected with AR542 and common-toxic (wild-type) tall fescue endophytes were collected from the field and samples were shown by GC-FID to contain a variable profile of loline analogues. They predominantly contained NANL and in some samples there was a small amount of NFL and/or a trace of NAL. Values determined by ELISA were 40% of those determined by GC-FID (Figure 5).

**4. Discussion**

Polyclonal antibodies against loline were produced and used in the development of an immunoassay for the quantification of loline analogues in pasture. These compounds are found in grasses containing loline-producing endophytes and have similar structures except for differences on the side chain grouping at position C-1 (Table 2) (Bush et al.,...
The aim of the project was to produce antibodies suitable for use in an ELISA detecting all loline structures of interest. The side chain on the generic loline structure was derivatized so that immunization with the protein–hapten conjugate would expose the cyclic regions of the molecule, common to all analogues, to the immune system. As anticipated, antibodies could be selected with cross-reactivity to all of the loline analogues available for testing (Table 2). Cross-reactivity studies demonstrated that the presence and nature of the amide linkage on the side chain of each analogue influenced the degree of cross-reactivity in the assay. As is the case with the immunogen, NFL and NAL contain a secondary amide on the side chain and these analogues demonstrated the highest cross-reactivity in the assay (100% and 104%, respectively). NANL, which has a primary amide group at the same position, has lower cross-reactivity (30%) while loline dihydrochloride, with a secondary amine group and least like the immunogen, has the lowest cross-reactivity (3%). Unlike NFL, NAL, and NANL, the synthetic analogue loline dihydrochloride does not occur in herbage.

The sensitivity of the ELISA compares well with that for the standard GC-FID method (limit of quantitation 12 and 25 µg/g, respectively), making the immunoassay developed a suitable method for the analysis of plant material. Frequently, herbage samples require dilutions much greater than 1 in 600 to bring the loline concentration into the working range of the assay, for example, 1 in 3000.

There was good correlation ($R^2 = 0.8$) between ELISA and the GC-FID method when samples contained only NFL, as in the case of individual perennial ryegrass plants infected with AR525 endophyte (Figure 4). When NFL is used as the reference standard, the ELISA gives a quantitative measure for total lolines when NFL and/or NAL is present, as the cross-reactivities for both analogues are close to 100% in the ELISA. A mixed and variable profile of loline analogues was found in the tall fescue samples containing AR542 endophyte that were collected from the field. This was because there was contamination with plants containing other loline-producing endophytes. In this situation, the ELISA and GC-FID method did not correlate as well ($R^2 = 0.7$) and the ELISA signal did not give a quantitative measure of total lolines because of varying cross-reactivities of the analogues (Figure 5). The broad specificity of the antibodies and the sensitivity of the ELISA, however, enable the method to provide a sensitive and useful signal for the presence or absence of lolines in mixed pasture samples.

The development of an ELISA for lolines provides a rapid and high throughput analytical method that uses relatively inexpensive instrumentation. The methodology has been applied in plant breeding programmes and used as a tool in research particularly where analysis of a large numbers of samples was required or when sample size was limited. With further validation, the assay could also be extended to other matrices of interest, for example, seeds, culture media. The assay could also be used on animal body fluids to determine uptake and elimination of loline analogues and metabolites in animal studies. This would depend on antibody recognition of metabolized lolines. Application of the antibodies is also possible in the preparation of immunoaffinity columns to provide sample cleanup as required for other analytical methods such as LC–MS. The antibodies may be used for immunolocalization studies of loline analogues and loline metabolites in a wide range of tissue types.
5. Conclusion

A sensitive cELISA was developed for quantifying loline alkaloids in pasture samples. The assay detects the loline analogues of interest in dried herbage and has a limit of quantitation for lolines of 3 µg/g. Antibodies produced for the assay can now also be used for the development of other immunotechnologies such as immunoaffinity chromatography and immunolocalization studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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