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Development and Validation of a Potentiometric Biosensor Assay for Tylosin with Demonstrated Applicability for Detection of Two Other Antimicrobial Growth Promoter Compounds in Feedstuffs

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Abstract

A potentiometric biosensor assay based on a commercially available polyclonal antibody has been developed to detect tylosin residues in animal feed. The method can be used as a rapid (less than 45 minutes) laboratory-based procedure or, as a portable field-test for the simultaneous measurement of up to 12 different samples. For both procedures the qualitative detection capability (CCβ) for tylosin was determined as 0.2 mg kg^{-1} in a range of animal feeds with a measurement repeatability at concentrations between 0.2 and 4 mg kg^{-1} of ≤13% %Coefficient of Variation (%CV). The field-test format was capable of detecting tylosin residues at operating (external air) temperatures ranging between +4 to 37°C although some reduction in signal was observed at the lower temperatures. The laboratory-based tylosin assay was evaluated using 16 medicated and 22 non-medicated feeds and found to give comparable data to a confirmatory method based upon Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS). The potential to develop a multi-probe format assay for the simultaneous detection of tylosin, spiramycin and virginiamycin was also demonstrated. Cross-validation in a second laboratory showed the assay to be transferable, reliable and robust.

Keywords

Potentiometric biosensor; antimicrobial growth promoters; tylosin; feed; veterinary drug residues; screening assay.

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1. Introduction

Antibiotics have been routinely used worldwide in animal production for decades. Various antimicrobial compounds have been found to be effective growth promoting agents when added in sub-therapeutic doses to the feed of farm animals [Wegener 2003; Schwarz *et al*., 2001]. Previously, the EC banned certain antibiotics used in human medicine from being added to animal feeds as Antimicrobial Growth Promoters (AGPs) for safeguarding measures. As from 1st January 2006 this was extended and an EU-wide ban on the use of antibiotics as growth promoters in animal feed became effective [EC 2003]. This ban is the final step in the phasing out of antibiotics used for prophylactic purposes and is part of the overall European strategy to address the increasing emergence of bacteria and other microbes resistant to antibiotics, due to their over-exploitation or misuse.

In order for this ban to be applied effectively, analytical systems are required to detect potential abuse. Traditionally, residue analysis has been a laboratory based activity however, the possibility of undertaking analysis *in situ, e.g.* on farm is highly attractive and an increased trend in the development of rapid testing without the need for laboratory-based equipment has resulted [Alfredsson *et al*, 2005]. For example, multiple feed types may be present and the collection, labelling and transportation of these samples all have cost implications and it is likely that only a limited number would be sampled by inspection officials. The ability to carry out a rapid test *in situ* would allow a more rigorous sampling regime to be used. A positive (non-compliant) screening result on farm could also trigger a more reactive response than laboratory testing alone allows for example, additional sampling of feedingstuffs and livestock to prove the banned substance was indeed being abused.

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Rapid bioassay based approaches, including surface plasmon resonance (SPR) biosensors, lateral flow devices (LFDs) and enzyme-linked immunosorbent assays (ELISAs) have been reported in the scientific literature [Caldow *et al* (2005); Campbell *et al.*, 2007; Situ *et al*., 2005] for use as biosensors for the detection of tylosin. Whilst these methods have been studied for a number of years, the validation has been targeted towards animal tissues, milk and honey rather than animal feeds. An alternative platform is the commercially available potentiometric biosensor (Vantix™ system) which claims to provide a reproducible platform on which sensitive and robust assays can be developed [Purvis *et al*., 2003]. In such devices, enzyme labelled antibody/ antigen complexes are formed at the surface of a polypyrrole-coated electrode. The analyte detection is mediated by a secondary, enzyme-linked reaction resulting in the formation of electrically charged products. The charged products of the enzymic reaction can be rapidly and accurately measured as a change in potential at the electrode surface [Tudorache *et al*., 2007].

The aim of the research reported here was to carry out a feasibility study to assess the applicability of a commercially available potentiometric biosensor (using the Vantix™ Research system) for the qualitative analysis of AGP compounds in animal feedingstuffs applicable for operation as either a rapid laboratory, or field-based assay. Tylosin, (Figure 1a) a member of the macrolide class of antimicrobial compounds was selected due to its availability as a medicated feed approved for veterinary usage and its previous widespread usage as an AGP in pig and poultry production. A single analyte assay for tylosin was developed and validated in accordance with Commission Decision 2002/657 guidelines for qualitative screening assays [EC, 2002] in feedingstuffs.

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A prototype multi-probe sensor for the simultaneous detection of tylosin and two other macrolide compounds banned for use as feed additives, virginiamycin (Figure 1b) and spiramycin (Figure 1c) was also developed and evaluated. A target detection limit of 1 mg kg^{-1} [VMD 2007] was identified as an appropriate concentration for AGP residues in feeds, thus a Screening Target Concentration (STC) [CRL, 2010] of 0.5 mg kg^{-1} was selected to provide an inherent 50% safety factor.

[Insert Figures 1a,b,c about here]

2. Materials and Methods

2.1. Chemical and Reagents

All the chemicals and reagents used were of analytical grade and obtained from Sigma Aldrich, (Dorset, UK) unless otherwise stated.

2.1.1. Phosphate buffered saline containing 1% bovine serum albumin

Sodium chloride (9.0 g), disodium hydrogen phosphate anhydrous (7.57 g), potassium dihydrogen phosphate (1.82 g), thimerosal (0.1 g) and bovine serum albumin (BSA) fraction V (0.5 g) was dissolved in HPLC grade water (750 mL) and adjusted to a pH between 7.2 and 7.4, the final volume was adjusted to 1000 mL using HPLC grade water.

2.1.2 HRP-labelled antigen conjugates

The stock horseradish peroxidase (HRP) labelled analyte (tylosin, spiramycin or virginiamycin) conjugates (Randox, Northern Ireland, UK) were diluted using an enzyme stabilisation reagent,

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Stabilzyme® (Diarect AG, Freiburg, Germany) to give working dilutions of 1:75, 1:30 and 1:150, respectively. The working solutions of the HRP reagents were prepared on a weekly basis and stored excluded from light at +4-8°C.

2.1.3. HRP substrate solution (basic substrate)

One tablet of the o-phenylenediamine (OPD) substrate was added to citrate buffer (0.05 M, 200 mL). The substrate solution was prepared on a weekly basis and stored excluded from light at +4-8°C.

2.1.4. Enhancer solution

Sodium perborate monohydrate powdered material (15 mg, ±3 mg) was added to an aliquot (50 mL) of the basic substrate solution. The solution was mixed by manual shaking for *ca*. 30 s. The solution was prepared immediately prior to use and protected from ambient light. This volume was sufficient for the measurement of up to 96 sensors.

2.1.5. Binding reagents

The stock polyclonal antibody solution (anti-tylosin, anti-spiramycin and anti-virginiamycin) was obtained from Randox Laboratories, (Crumlin, Northern Ireland, UK). The protein concentrations for the anti-tylosin, anti-spiramycin and anti-virginiamycin antibody solutions was determined as 7.6, 13.0, 9.2, respectively. The stock antibody solutions were diluted in potassium phosphate buffer (0.05 M, pH 8.0) to give working dilutions of 1:200.

2.1.6. Pre-coated screen printed electrodes (Vantix™ biosensors)

The potentiometric biosensor employs proprietary single use disposable screen-printed electrodes (PPY1) incorporating a working carbon electrode and an integral reference silver/silver

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chloride electrode. The sensor elements are printed layer by layer starting with conducting tracks followed by reference and working electrodes. Finally, a dielectric is printed on top of the other layers leaving defined openings on the reference and working electrodes and insulating the conducting tracks. A conducting polymer is deposited electrochemically onto the working electrode forming the sensing element and immobilisation matrix of the working electrode.

During the assay optimisation phase the diluted antibody solution (from 2.1.3) (*ca*. 3 µL) was manually dispensed directly onto the carbon working electrode surface whereas an automated liquid dispensing system was used to prepare the electrodes for the validation experiments. In both cases the working electrode was then coated using the biomolecule stabilisation solution, sealed in an airtight container and stored in the refridgerator at +4-8°C until use (up to 3 months).

2.2. Instrumentation

2.2.1. Bench-top ball mill

A bench-top ball mill instrument (MM400) and stainless steel screw top grinding jars (50 mL capacity) and beads were purchased from Retsch GmbH (Haan, Germany) and used to dry mill samples of pelleted and compound feed prior to the laboratory based solvent extraction process.

2.2.2. Orbital shaker

The orbital shaker instrument (ELMI Shaker DOS-10L) was purchased from Progen Scientific (London, UK).

2.2.3. Potentiometric Vantix^{TM} biosensor (Vantix™ Research system)

The Vantix™ Research system (VAN RES 1) consisting of a potentiometric reader head unit containing 12 sensor docking positions, a multi-plate holder base unit, Vantix^{TM} Research application software and USB cable was obtained from Universal Sensors Limited (Ickleton, Cambridgeshire, UK).

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The Vantix™ Research system was powered by a portable PC *via* a USB connection and the electrical potential was measured and recorded *via* the Vantix™ Research software.

2.2.3.1 Potentiometric biosensor – instrument settings for the single and multiplex format assays

The instrument settings used throughout the assay development phase and validation study (unless otherwise stated) are detailed in Table 1 for both the single and multiplex format assays.

[Insert Table 1 about here]

3. Experimental

3.1. Feed extraction protocols

3.1.1. Laboratory-test procedure

The extraction procedure was developed as a generic method applicable for the recovery of water soluble AGP compounds from feedingstuffs, requiring only basic equipment and reagents and minimising the use of organic solvents. In the case of the pelleted and compound feed samples, a representative sample (*ca*. 100 to 150 g) of the bulk material was taken and milled (2 min at a vibrational frequency of 1500 min^{-1}) using the bench-top ball mill to produce a fine and homogeneous powder.

The laboratory based extraction protocol developed for feed is detailed below;

Extraction buffer, PBS containing 1% Bovine Serum Albumin (BSA) and thimerosal at neutral pH (2.1.1), (20 mL) was added to pre-milled feed samples (1 g, ±0.05 g) and the sample was shaken using an orbital shaking device (500 rpm, for 10 min). The sample was ultra-sonicated (5 min) and

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centrifuged (4200 g, for 10 min at 10°C). An aliquot of the supernatant was removed for subsequent biosensor analysis.

3.1.2. Field-test procedure

To minimise the requirement for laboratory equipment in the field, a simplified procedure was also developed and the performance compared to the laboratory based method.

The field test procedure is detailed below;

Pre-ground (manually ground using a mortar and pestel) feed samples (1 g ±0.05 g) were extracted using the phosphate buffer (2.1.1), (20 mL). The samples were shaken vigorously by hand for 60-70 s then allowed to sediment on standing for *ca*. 5-10 min. Any remaining particulate matter was removed from the sample by passing approximately 5 mL through a 70 micron 'cell strainer' (BD Biosciences, Bedford, MA, USA.).

3.2. Potentiometric biosensor - measurement

All measurements were performed in a 96 well multi-plate format. Feed extract (100 µL) and an equal volume of the diluted HRP conjugate (2.1.2) was added to each well using a pipette. Pre-coated Vantix™ biosensors were placed in a holder device and soaked in water (1 min), and washed twice in water prior to use (to remove the bio-stabilising surface coating). The Vantix™ biosensors (12 in parallel) were then inserted into the appropriate sample wells and incubated at room temperature for 20 min (±5 min). The Vantix™ biosensors were removed from the reaction mixture washed twice using water and wiped dry. Finally, the Vantix™ biosensors were inserted into the Vantix™ Research system reader head, the carbon working electrode and silver reference electrodes dipped into the enhancer solution (2.1.4) and the potentiometric measurement taken.

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Throughout the validation experiments the biosensor response was expressed as a relative response in millivolts (mV). The instrument relative response setting was used whereby the absolute responses recorded were automatically normalised against the negative control (blank buffer) pre-positioned in the first channel of the reader head unit.

4. Results and Discussion

The biosensor assays for feed were developed using a commercially available Vantix™ Research system. The assay principle is comparable to a conventional inhibition format ELISA. The antibody recognition element is physically adsorbed *via* the carboxyl terminal to the polypyrrole surface (forming a self assembled monolayer) at the carbon-working electrode. Sample extract is pre-mixed with an aliquot of HRP enzyme-linked analyte conjugate. A competition for antibody binding occurs in a single step between the target analyte present in the extract and the HRP conjugate. The HRP conjugate catalyses an electrochemical reaction resulting in the transfer of electrons between the electrochemically reactive electrode and the substrate molecule mediated *via* the enzyme active site. The potentiometric reader device measures the potential difference between the working and reference electrodes. The difference in potential is created by the reaction which takes the place on, or near the surface of the working electrode. The measured response (mV) is inversely proportional to analyte concentration.

The performance of the laboratory format, single analyte procedure for tylosin was characterised in a range of animal feedingstuffs. The following parameters were determined as part of the optimisation and validation study;

- Intra- and inter-laboratory measurement repeatability
- Determination of detection capability (CCβ)

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- Comparison with LC-MS/MS using a range of different medicated and non-medicated feeds
- Assay selectivity (cross-reactivity) to selected other antibiotics
- Assay ruggedness and inter-batch variation
- Extract stability

The applicability of the assay for operation as a field-test format was also evaluated and the performance compared to that of the laboratory based procedure.

The potential to develop a multi-probe assay for the simultaneous detection of three AGP compounds, tylosin and two other compounds (spiramycin and virginiamycin) was also demonstrated. The performance of the prototype multi-probe format biosensor was evaluated in feeds.

4.1. Tylosin potentiometric biosensor measurement intra-laboratory repeatability

To determine the intra-laboratory repeatability (r) of the biosensor measurement an experiment was performed whereby known blank porcine feed extract was spiked post-extraction (termed "feed standards") at four concentrations bracketing the target detection limit of 1 mg kg^{-1}. At each concentration, seven replicates were analysed on each of three days giving a total of 21 replicates. These results (Table 2) show that the inter-day repeatability of the Vantix™ biosensor measurement, expressed as % CV was less than 13% for all the concentrations tested.

[Insert Table 2 about here]

In a second experiment, 22 different types of known blank samples of porcine feed (listed in Table 3 non-medicated samples) were spiked pre-extraction with increasing amounts of tylosin to give concentrations equivalent to 0.2, 0.5 and 1 mg kg^{-1} in feed. The individual relative response

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values for the 22 samples (both the blank and spiked sets) are shown graphically (Figure 2). The mean relative response for the 22 different blank feed samples included in this experiment were combined with the results obtained for the negative control samples included within every experiment conducted during the validation study (in total n=212 measurements). These combined results obtained were used to calculate the overall mean response value and associated standard deviation (σ) for non-medicated feed. In Figure 2, data from the 22 individual blank feed samples are plotted alongside the mean blank response (n=212). The 95% (2 x σ) and 99% (3 x σ) confidence limits are also shown. These results reveal that;

(i) none of the 22 different blank feed samples tested would be assessed as false positive (non-compliant)

(ii) even at the lowest concentration tested (0.2 mg kg^{-1}) all results would be recorded as 'screening positive' although, at the 99% confidence limit, two samples were borderline case.

[Insert Figure 2]

Based on these data the STC for feed was established at 0.5 mg kg^{-1} to reduce the risk of recording false compliant results at the lowest concentration level.

An inter-laboratory repeatability experiment was performed at a second laboratory whereby replicates (n=7) at the STC (0.5 mg kg^{-1}) were analysed on each of three days according to the same protocol used for the intra-laboratory validation (4.1). These results (Table 2) show the Vantix™ biosensor precision and relative response at 0.5 mg kg^{-1} is consistent between both test laboratories.

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4.2. Determination of detection capability (CCβ)

To estimate the qualitative detection capability of the assay (with a β error of 5%) representative portions of each of the 22 different blank feeds (listed in Table 3 non-medicated feeds) were individually spiked with tylosin pre-extraction. Based on the findings of the measurement repeatability experiment multiple blank feed samples (n=22) were spiked pre-extraction at the lowest concentration (to give a feed concentration of 0.2 mg kg^{-1}), which was found to give a strong positive test response. The spiked feed samples were then extracted and analysed and the results were plotted graphically along with the negative control responses obtained from the analysis of the corresponding unspiked feed samples (Figure 3). The negative control and spiked sample responses are clearly resolved on the y-axis even when the mean response plus two times the standard deviation is plotted. This finding indicates that all the spiked samples were correctly indentified as non-compliant therefore the CCβ is equal to 0.2 mg kg^{-1} with a false non-compliant rate of less than 5%.

[Insert Figure 3 about here]

The typical composition of the feed samples evaluated in this study was as follows; wheat, barley, rapemeal, soya oil, limestone. In addition to these components certain feeds, pre-mixes and supplements containing essential amino acids, minerals and vitamins were present at varying concentrations. The biosensor was found to be applicable to this wide range of different feed types and compositions.

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4.3. Comparison of the tylosin potentiometric biosensor with LC-MS/MS for the analysis of a range of blank and mediated feed samples

A wide range of medicated and non-medicated porcine and poultry feed samples were analysed using the Vantix™ biosensor assay and a chemical confirmatory method (LC-MS/MS) based on the method previously reported by Wang [Wang 2004] to assess the performance of the biosensor assay using a wide cross-section of feed types. These results (Table 3) reveal that a good agreement between the LC-MS/MS measurement and qualitative potentiometric biosensor response was achieved. All of the 16 medicated samples containing tylosin at a concentration greater than 1 mg kg^{-1} were recorded as positive (non-compliant) by both techniques. All of the 22 non-medicated samples were found to be negative (compliant) by both techniques. From these findings, a false non-compliant and compliant rate of 0% was observed at n=16 and 22, respectively.

[Insert Table 3 about here]

4.4. Tylosin assay selectivity (cross-reactivity)

The tylosin assay response to a selected range of structurally related (tilmicosin and spiramycin) and structurally diverse (virginiamycin M_1 and sulfathiazole) antibiotic compounds was investigated. Known blank feed was spiked individually with the compounds under investigation to give the following concentrations 0.5, 1.0 and 20 mg kg^{-1}. The feed was extracted and analysed according to the laboratory based protocol (3.1.1). The relative response was plotted against the analyte concentration for each compound. These results (Figure 4) reveal that for tylosin, a concentration dependent increase in response was observed. A similar but weaker trend was observed in the presence of tilmicosin. At the target detection concentration (1 mg kg^{-1}) the estimated assay cross-reactivity to tilmicosin was determined as 75%. Tilmicosin shares a degree of structural homology with tylosin and may possess part of the same epitope region for antibody recognition. Based on

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these findings, the Vantix™ biosensor assay is applicable for the simultaneous screening of tylosin and tilmicosin residues in feed.

No cross-reactivity was observed in the presence of the other three compounds tested.

[Insert Figure 4 about here]

4.5. Assay ruggedness and inter-batch variation

The optimisation of the sensor response to the analyte (tylosin) was performed by varying both the instrument settings and assay parameters in order identify the critical factors and set the appropriate tolerances.

4.5.1. Instrument parameters

A series of small procedural changes were introduced to the optimised protocol (2.2.3.1) and the effect on the assay performance was observed. The ruggedness of the following instrument parameters was investigated including, the potential (mV) applied during the potential step, the duration of the applied potential (s) and the current limit setting (nA).

These results (not shown) reveal the Vantix™ biosensor assay performance is generally tolerant of small variations in the three instrument settings investigated. In all three cases, no significant change in response was recorded for the 0 and 0.5 mg kg^{-1} standards; however, some minor variation in the highest (20 mg kg^{-1}) standard response was observed either side of the optimised settings. Based on these findings the tolerance limits for the instrument parameters was fixed at ± 10% of the recommended setting.

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4.5.2. Assay parameters

The antibody dilution factor and concentration of the HRP conjugate had been previously characterised for the Randox immunoreagents during the initial assay optimisation phase. Both the antibody and HRP dilution factors (1:200 and 1:75, respectively) were found to be critical thus, were not further investigated here.

The concentration of the BSA blocking reagent in the extraction buffer was varied. For each parameter a range of settings both above and below the recommended concentration were investigated using a set of matrix matched standards, equivalent to 0, 0.5 and 20 mg kg^{-1}. These results (data not shown) show that the presence of BSA in the extraction buffer serves to stabilise the response by reducing signal interference caused by non-specific binding effects from the matrix. The optimum percentage of BSA was found to be 1% (w/v).

The assay incubation time was varied to determine how critical this step is and the appropriate tolerance factor. The incubation step was performed for periods of 5, 10, 15, 20, 25 or 30 min at room temperature (+18-22°C) under normal lighting conditions using a single antibody dilution factor (2.1.5) and the results compared. A range of feed standards at 0, 1, 2, 20 and 100 mg kg^{-1} were included. These results (not shown) indicate that even after a 5 min incubation period the response signals obtained for the range of feed standards are clearly resolved. However, some variability in response was observed between the 5 and 10 min incubation periods. The measured responses were found to be more consistent between 15 and 25 min. Based on these findings the recommended incubation time is 20 (± 5) min. The assay incubation time is related to the antibody coating concentration on the sensor surface and the compromise between incubation time and

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antibody cost is primary consideration. The incubation time could be further reduced (if required) by increasing the antibody coating concentration.

4.5.3. Inter-batch variation

To assess the variation between different production batches of sensor surfaces pre-coated with antibody, the performance of two batches (produced *ca*. 3 months apart) was compared using identical assay and instrument protocols. Feed standards at 0, 0.2, 0.5 and 20 mg kg^{-1} were prepared and replicates (n=6) were analysed using both batches and the mean responses compared. These results (data not shown) reveal that no significant difference in the performance of the two batches was observed at 0, 0.2 and 0.5 mg kg^{-1}. However, some variation was observed at the highest concentration standard with the second production batch giving a *ca*. 20% higher response signal than the first production batch.

4.6. Method performance and false compliant and non-compliant rates

During the course of the validation study known blank, medicated and spiked feed check samples (n≥1) were included in every experiment and the relative responses were plotted using a Stewhart style control chart. An average of the total historical blank sample responses were used as the baseline for the control chart. These results (Figure 5) show that all the 191 blank measurements were found to give a response below both the screening target level and the CCβ. The responses for the medicated samples and spiked samples were all above the STC. The mean responses for the CCβ at 0.2 mg kg^{-1} and the STC of 0.5 mg $kg^{-1}$are also displayed. No false compliant or non-compliant response was recorded during the course of the study.

[Insert Figure 5 about here]

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4.7. Extract stability

The stability of tylosin in feed extract was monitored over a 70 day period. Known blank feed was extracted in bulk and spiked post-extraction to give a solution concentration of 1 µg mL^{-1} (equivalent to 20 mg kg^{-1} in feed). The spiked extract was stored under four different conditions (room temperature in the light, room temperature in the dark, fridge +4 to 8°C and freezer –26 to 20°C). Aliquots of the extracts were removed from their storage locations and analysed according to the optimised protocol (n=6) at the following time points, day 0, 1, 2, 7, 14, 21, 28 and 70. Samples of the blank feed and a medicated feed were freshly prepared on the day of analysis and analysed (n=1) to monitor the Vantix™ biosensor performance over the duration of the stability experiment.

These results (data not shown) reveal that the spiked extract was stable over the full duration of the stability experiment. Some analytical variability in response was observed within the four storage location conditions over the 10-week period, which was more pronounced in the extracts stored at room temperature. As a general observation, the mean relative response (n=6) remained consistent between the four storage locations at each time point, the precision as expressed by %CV ranged from 0.9 to 16.3. Based on these findings it is recommended that the feed extract be retained in the fridge or freezer if longer-term storage (up to 70 days) is required.

4.8. Applicability to field-test conditions

To determine whether the Vantix™ biosensor assay for tylosin had the potential for future development into a 'field' test format, preliminary investigations focused on two key parameters; the extraction protocol and the effect of external temperature on the overall assay performance.

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Tylosin positive samples were prepared by spiking bulk non-medicated feed samples with tylosin to give 0.5 mg kg^{-1}.

4.8.1. Simplified extraction procedure (field-test format)

To minimise the requirement for laboratory equipment in the field, it was important to determine whether suitable extraction was possible without the use of laboratory equipment including the orbital shaker, ultra-sonicator and centrifuge equipment. This required comparison of the laboratory extraction protocol to a manual extraction process. Feed samples (n=7) were extracted at room temperature (RT) (+18 to 22°C) using both the laboratory and the manual extraction protocols. Subsequent Vantix™ biosensor reading was also performed at RT. These results (Table 4) show that at RT manual extraction performed consistently with a CV of 12.3%. Furthermore, the recovery efficiency of tylosin by manual extraction (%RE) as indicated by relative response (mV), was 91% of that seen following the laboratory extraction procedure.

4.8.2. Effect of ambient temperature

To determine whether the ambient temperature was influential on the extraction and subsequent biosensor measurement, samples, reagents, sensors, and Vantix™ Research system reader head were allowed to equilibrate for two hours prior to the extraction and analysis at a range of temperatures (+4 to 37°C) likely to be encountered during *in-situ* operation.

Seven extractions and analyses were performed at each temperature for the manual extraction protocol, with subsequent Vantix™ biosensor reading also performed at the given temperature. These data presented in Table 4 reveals that for a given temperature, manual extraction and subsequent analysis is consistent with %CVs of 7, 12, 12 and 6 for 4°C, 13°C, RT, and 37°C,

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respectively. However, relative to the laboratory extraction procedure at RT, apparent recovery of tylosin was suppressed at reduced temperatures (60% and 67% at 4°C and 13°C, respectively) but increased at elevated temperatures (110% at 37°C).

These data are not unexpected as both the kinetics of antibody-antigen binding and enzymatic conversion of substrate will be influenced by temperature. Increased incubation times at reduced temperatures would likely overcome this finding. However, with suppressed apparent recovery the spiked samples could still clearly be separated from blank samples, with relative responses in excess of 20 mV. The reproducibility of the response also suggests that a correction factor for assay temperature could be used, (if required). These data indicate that, for qualitative analysis of feedstuffs the biosensor system and manual extraction methodology is applicable at a range of temperatures likely to be encountered during operation under field test conditions.

[Insert Table 4 about here]

4.9. Multi-probe format assay for tylosin, spiramycin and virginiamycin

A prototype multi-probe format for the simultaneous in parallel detection of tylosin, spiramycin and virginiamycin was developed and evaluated. The evaluation was performed using aliquots of known blank feed spiked with the individual analytes at three concentrations, 1, 4 and 10 mg kg^{-1} (n=3) prior to extraction. The dose response curves (data not shown) reveal that all three analytes are detected at 1 mg kg^{-1} following fortification into feed. Tylosin and spiramycin generated similar response profiles. The magnitude of response for virginiamycin was found to be less than that of tylosin and spiramycin. Based on the outcome of these findings a further experiment was performed

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at a lower concentration range 0.2, 0.5 and 1 mg kg^{-1} to characterise the assay sensitivity. A known blank feed sample was spiked using a combined mixture of the three analytes to determine the effect of analyte "cross-talk". These results (Figure 6) show that the all three analytes were clearly detected at the lowest concentration tested of 0.2 mg kg^{-1} (equivalent to the CCβ concentration previously determined for tylosin). A dose dependent increase in response was obtained for all three analytes. In the presence of the multi-analyte spike the response for virginiamycin was enhanced, indicative of cross-reactivity between spiramycin and virginiamycin for the virginiamycin antibody.

[Insert Figure 6 about here]

5. Conclusions

A potentiometric biosensor assay suitable for the screening of single and multiple AGPs in animal feedingstuffs has been developed and validated using a commercially available instrument (Vantix™ Research system) and immunoreagents. The Vantix™ biosensor assay has been found to be a sensitive and robust method for the qualitative screening of feed samples and capable of the simultaneous screening of up to 12 samples (including positive and negative controls) within 45 min. The qualitative CCβ (β error =5%) for tylosin in feed was found to be 0.2 mg kg^{-1}. A wide range of non-medicated and medicated feed samples including pellets, pre-mixes, supplements and compound formulations were screened using the biosensor assay and the performance compared to LC-MS/MS. Results from both techniques were found to be in close agreement. A multiplex format assay for the simultaneous detection of tylosin, spiramycin and virginiamycin was developed and it's applicability demonstrated in feed. These findings indicate that this technique has the potential to detect a wide range of antimicrobial growth promoters in feeds.

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The Vantix™ biosensor is a low-cost, high throughput and versatile format. If good quality, well characterised immunoreagents are available the time required to develop and validate new applications is relatively short (a few weeks). Furthermore the Vantix™ biosensor was shown to be appropriate for future development into a multiplex field-based assay, employing a more portable format requiring minimal sample preparation.

6. Acknowledgements

The financial support of the Veterinary Medicines Directorate, an executive agency of the UK Department for Environment, Food and Rural Affairs (project VM02154) is gratefully acknowledged.

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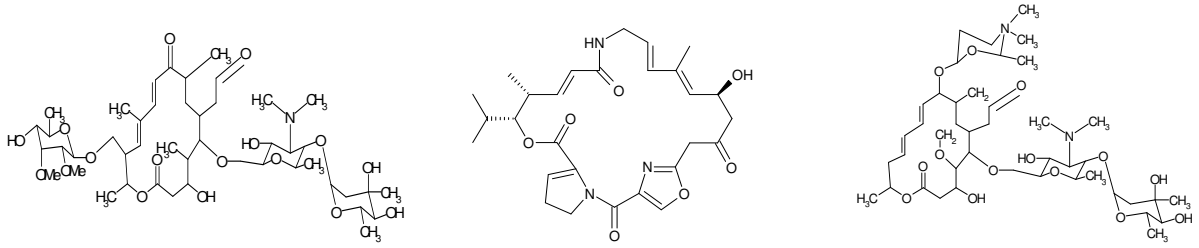

(a) Tylosin A (b) Virginiamycin M_1 (c) Spiramycin 1

Figure 1. The chemical structures of (a) tylosin A, (b) virginiamycin M_1 and (c) spiramycin 1

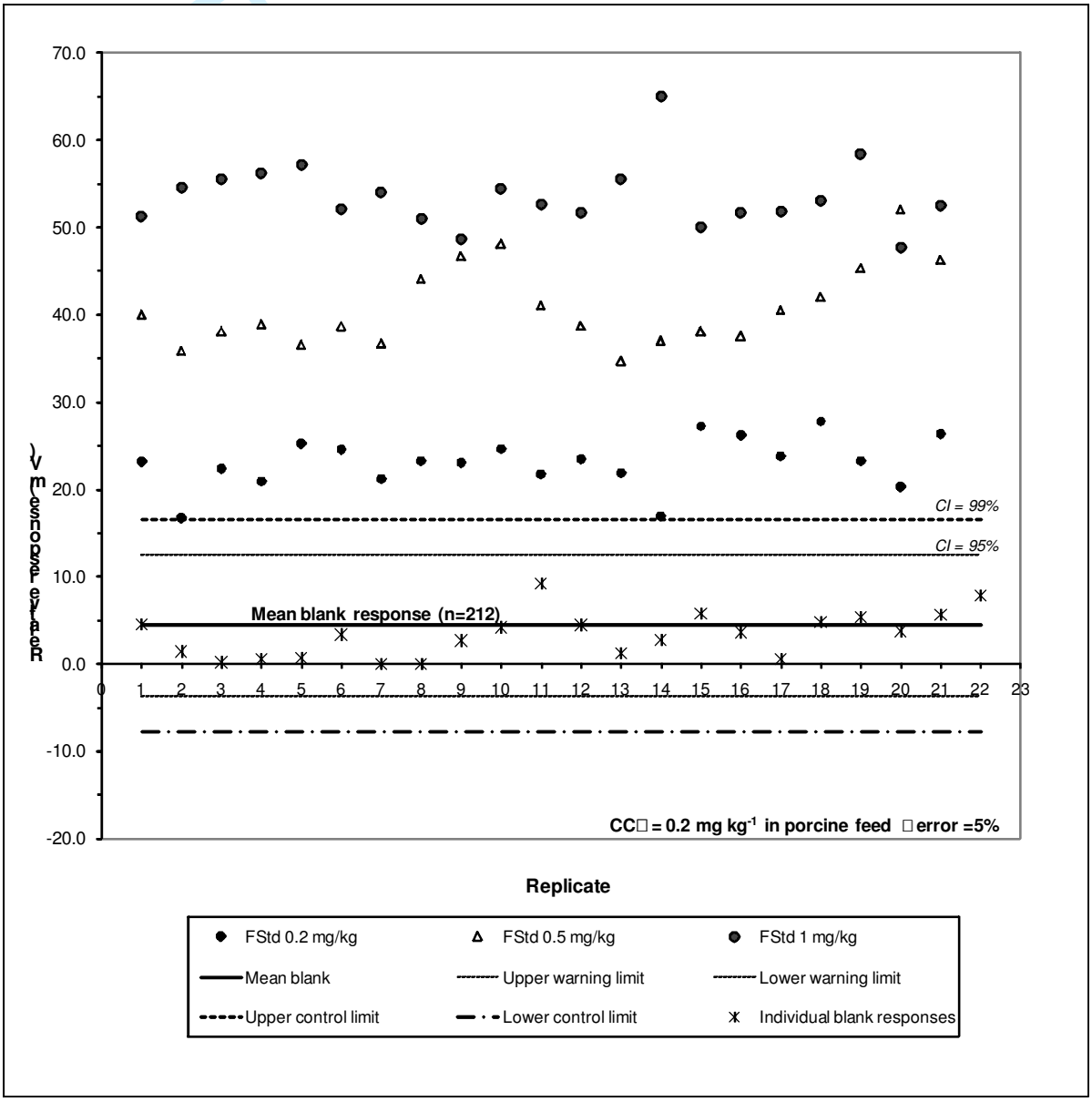

Figure 2. Intra-laboratory measurement repeatability for the Vantix™ biosensor assay for tylosin in feed and determination of the screening threshold concentration.

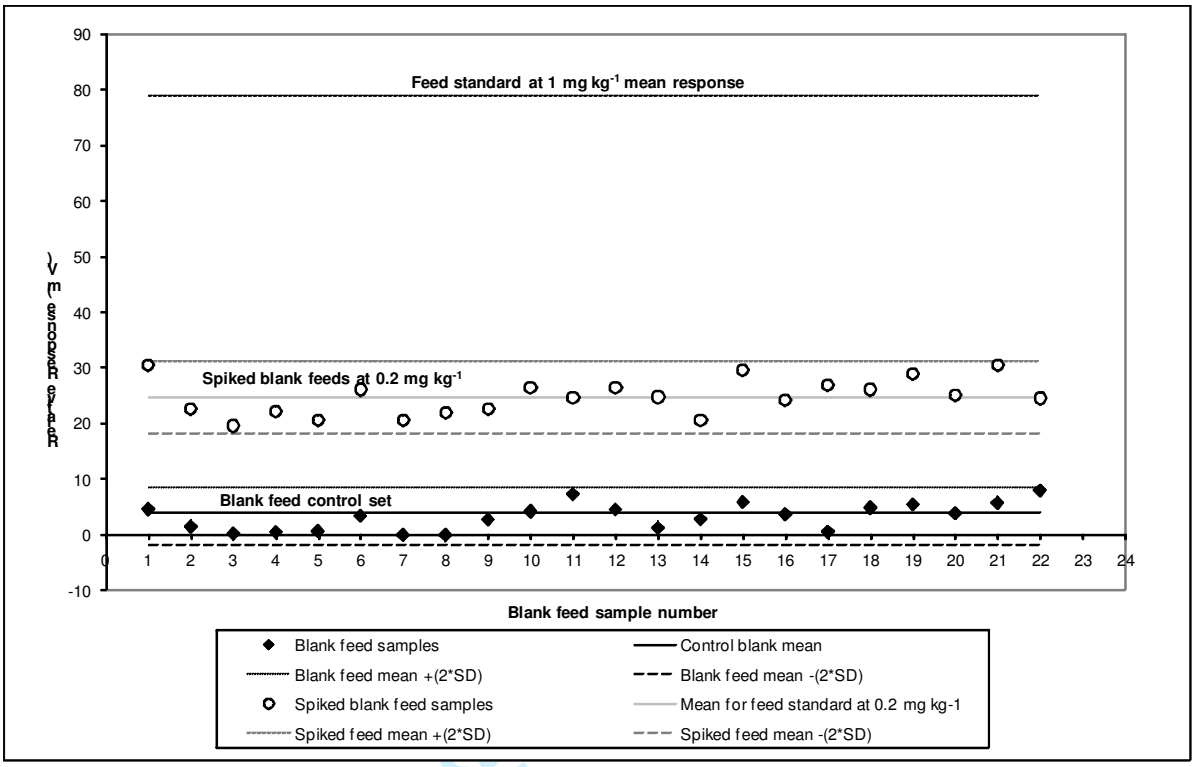

Figure 3. Tylosin Vantix™ biosensor assay response to a range of 22 different known negative feed samples (diamonds) and spiked with tylosin at the CCβ concentration 0.2 mg kg^{-1} (circles).

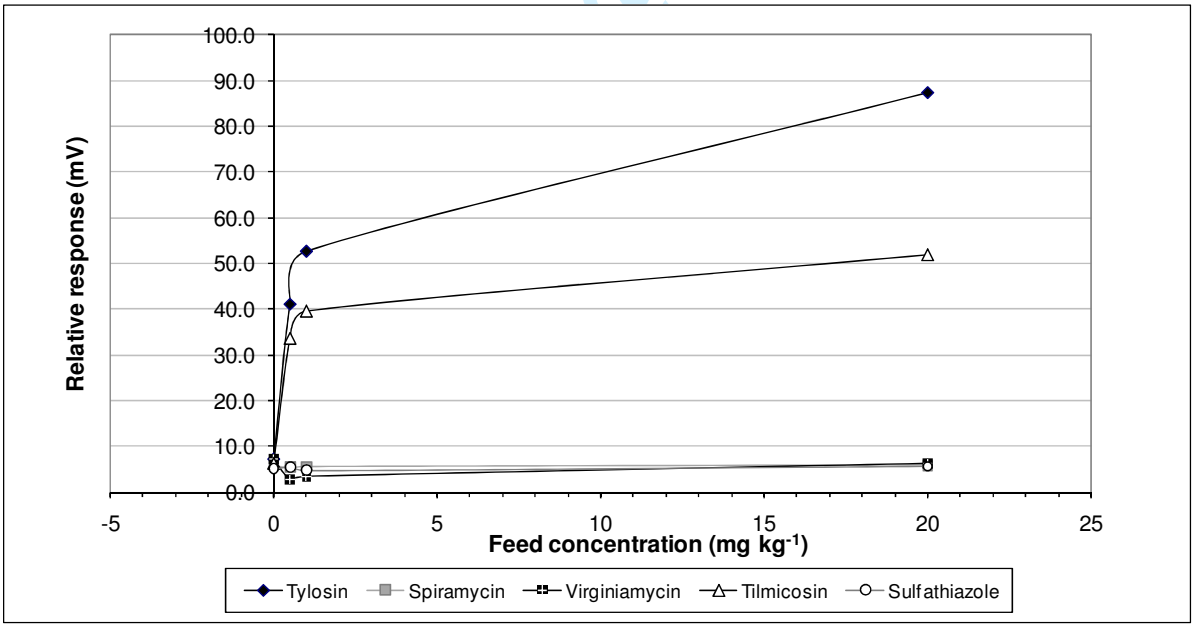

Figure 4. Tylosin Vantix™ biosensor assay cross-reactivity towards a selected range of structurally related (tilmicosin and spiramycin) and diverse (virginiamycin M_1 and sulfathiazole) antibiotics spiked into feed.

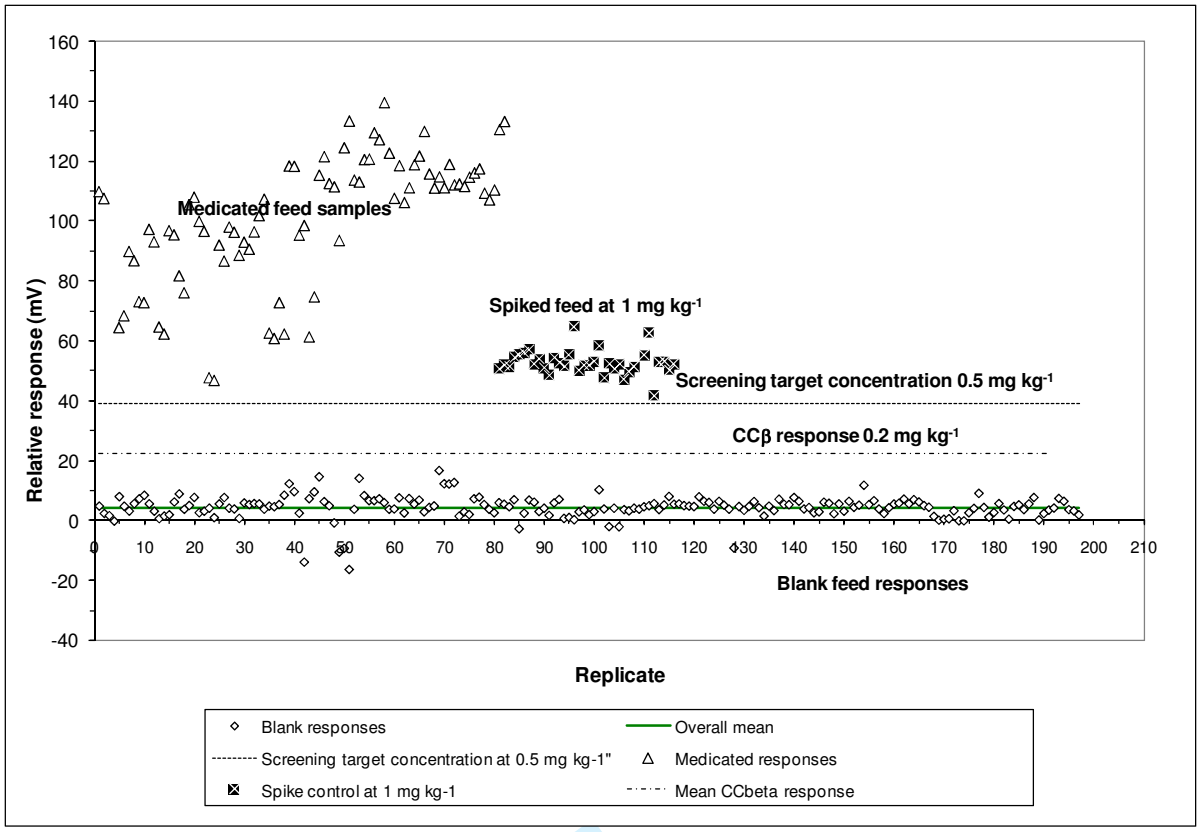

Figure 5. Shewhart style control chart showing longer-term Vantix™ biosensor response to a range of blank, tylosin medicated / spiked feed control samples.

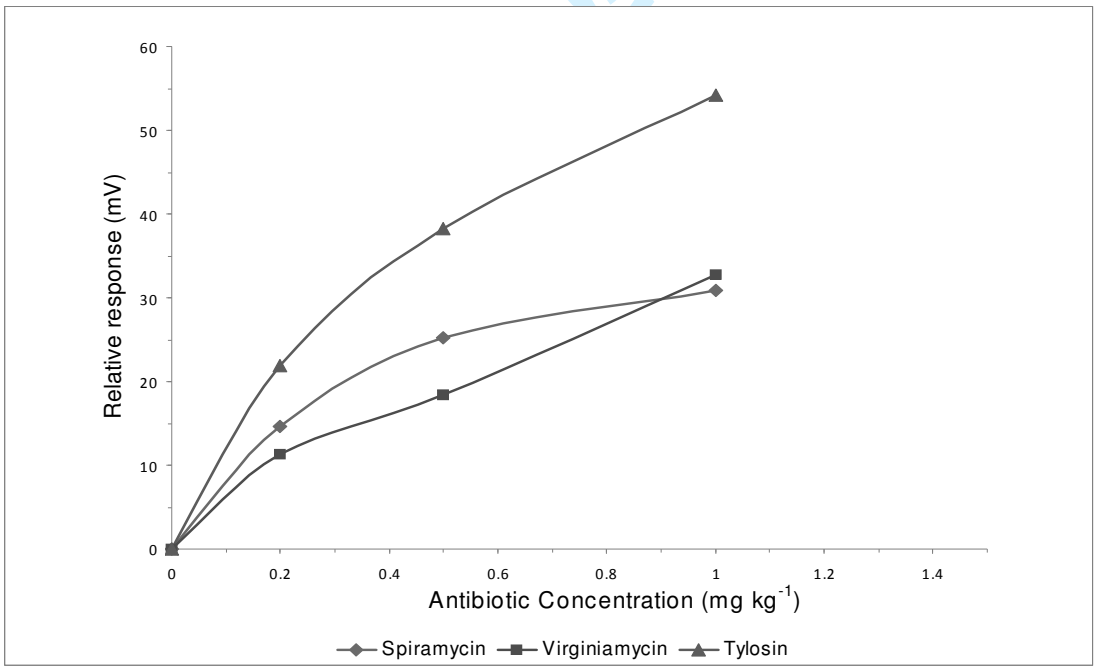

Figure 6. Multi-probe format assay dose response curves generated using feed samples spiked using a mixed solution of tylosin, spiramycin and virginiamycin at 0,0.2, 0.5 and 1 mg kg^{-1} prior to extraction (n=3).

Table 1. The optimised Vantix™ biosensor parameters for the tylosin single and the multiplex format assays

Parameter	Value	
	Tylosin single assay	**Tylosin, spiramycin and virginiamycin multiplex assay**
Measurement length (s)	**90** Comprising control point 1 at 15 s and control point 2 at 88 s	**60**
Number of potential steps	**1**	**1**
Applied potential voltage (mV)*	**-115** (±15)	**-100** (±10)
Duration of the potential voltage (s)*	**10** (±1)	**10** (±1)
Current limit (nA)*	**20** (±2)	**20** (±2)

*Indicates the instrument parameters subject to ruggedness testing

Table 2. Mean intra-laboratory measurement repeatability for the tylosin Vantix™ biosensor assay performed at five different concentrations and the mean inter-laboratory measurement repeatability performed at a single concentration in feed (0.5 mg kg^{-1}) on three different days.

Summary	**Concentration of tylosin in spiked feed extract expressed as units equivalent to mg kg^{-1} in feed**					
	0.2	**0.5**	**0.5**[1]	**1**[2]	**2**	**4**
	Vantix™ biosensor **relative response (mV)**					
Replication	21 (7 *per* day)					
Inter-day mean relative response (mV)	23.0	40.8	37.3[1]	53.6	66.0	76.5
SD	2.9	4.6	3.7[1]	3.7	5.1	9.7
%CV	12.6	11.3	10.0[1]	7.0	7.8	12.7

[1] Inter-laboratory measurement repeatability data
[2] Target detection concentration

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Table 3. The comparative results of the analysis of 16 different medicated feed samples and 22 non-medicated feeds by both LC-MS/MS and Vantix™ biosensor, (n=2)

Feed type	Feed Description	Mean LC-MS/MS concentration (mg kg^{-1}) n=2	Vantix™ biosensor result n=2
Tylosin medicated feeds	1. Tylosin medicated pig finisher premix	63	Positive
	2. Antibiotic medicated pig pellets 1*	<1 *(0.66)*	Negative
	3. Antibiotic medicated pig pellets 2	2	Positive
	4. Tylosin medicated pig pellets	270	Positive
	5. Tylosin medicated pig weaner	20	Positive
	6. Tylosin medicated pig grower	113	Positive
	7. Tylosin medicated pig weaner	49	Positive
	8. Tylan medicated premix pig grower	76	Positive
	9. Tylosin mediated premix	40	Positive
	10. Mineral supplement plus tylosin	860	Positive
	11. Tylosin medicated pig weaner and grower	171	Positive
	12. Tylosin medicated pig finisher meal	62	Positive
	13. Tylosin medicated balancer meal	506	Positive
	14. GCFG mineral plus tylosin	1782	Positive
	15. Tylosin medicated pig fattener	90	Positive
	16. Tylosin medicated pig grower	143	Positive
Non-medicated feeds	1. Pig pellets 1	<RL	Negative
	2. Pig pellets 2	<RL	Negative
	3. Poultry layer premix	<RL	Negative
	4. Sow breeder premix	<RL	Negative
	5. Pig finisher premix	<RL	Negative
	6. Broiler withdrawal premix	<RL	Negative
	7. Pig finisher premix	<RL	Negative
	8. Dry sow Pellets	<RL	Negative
	9. Pig finisher	<RL	Negative
	10. Dry sow	<RL	Negative
	11.Lactating sow	<RL	Negative
	12.Lactating sow	<RL	Negative
	13. Pig pellets 3	<RL	Negative
	14. Pig pellets 4	<RL	Negative
	15. In house compound pig feed mix 1	<RL	Negative
	16. Probiotic pre-mix	<RL	Negative
	17. In house compound pig feed mix 2	<RL	Negative
	18. Dry sow Pellets	<RL	Negative
	19. Grower pig pellets	<RL	Negative
	20. Weaner Pellets	<RL	Negative
	21. Pig pellets 5	<RL	Negative
	22. Gilt Pellets	<RL	Negative

*Feed medicated with oxytetracycline, low concentration cross-contamination with tylosin was detected.
Italic text indicates the value quoted is indicative only.
<RL means tylosin was not detected at the lowest calibration concentration of 0.1 mg kg^{-1}
Positive biosensor result means a response greater than the cut-off level at 0.5 mg kg^{-1} was recorded for both replicates.
Negative Vantix™ biosensor result means a response below cut-off level at 0.5 mg kg^{-1} was recorded for both replicates.

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Table 4. Comparison of the laboratory and field-test extraction protocols and the effect of ambient temperature on the Vantix™ biosensor for tylosin relative response to feed fortified at 0.5 mg kg^{-1}

Replicate	Concentration of tylosin in spiked feed extract expressed in units equivalent to (mg kg^{-1}) in feed at 0.5 mg kg^{-1}				
	Vantix™ biosensor relative response (mV)				
	Laboratory extraction	Field test extraction			
	RT (+18-22°C)	RT (+18-22°C)	+4°C	+13°C	+37°C
1	39.5	41.7	21.6	27.7	42.7
2	34.3	32.5	23.1	23.0	41.2
3	40.7	32.8	24.7	27.4	45.1
4	40.7	33.4	26.5	24.6	47.9
5	40.1	36.2	23.7	23.3	42.8
6	38.0	32.7	24.1	25.7	42.0
7	43.1	42.7	22.5	32.3	40.9
Mean	39.5	36.0	23.7	26.3	43.2
SD	2.7	4.4	1.6	3.2	2.5
%CV	6.9	12.3	6.7	12.2	5.7
%Recovery (*Vs* laboratory based procedure at normal room temperature)	*N/a*	91	60	67	110

N/a = not applicable

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