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# Development and Validation of an Optical SPR Biosensor Assay for Tylosin Residues in Honey

MARIANNE CALDOW,*,† SARA L. STEAD,† JOANNA DAY,† MATTHEW SHARMAN,† CHEN SITU,‡ AND CHRIS ELLIOTT‡,§

Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom, Department of Veterinary Science, Queen's University Belfast, Stormont, Belfast BT4 3SD, United Kingdom, and Veterinary Sciences Division, Department of Agriculture and Rural Development, Stormont, Belfast BT4 3SD, United Kingdom

In recent years there has been an increase in the use of tylosin in apiculture as bacterial brood diseases become resistant to oxytetracycline. Confirmatory mass spectrometry based methods have been developed but up until now there has been no complementary screening method available capable of sub 10 $\mu$g kg$^{-1}$ detection limits. In this paper the development and validation of a screening method using optical biosensor technology is presented. The honey was first dissolved in a phosphate buffer and following solid-phase extraction (SPE) cleanup was analyzed using a Biacore Q instrument. Using the criteria specified in European Commission Decision 2002/657/EC for qualitative screening methods, the detection capability (CC$\beta$) of the method was determined to be 2.5 $\mu$g kg$^{-1}$. Honey samples containing trace residue levels of tylosin were analyzed by both the biosensor screening method and a LC-MS/MS confirmatory procedure; the results were in good agreement.

**KEYWORDS: Tylosin; biosensor; honey; veterinary drug residues; food safety**

## INTRODUCTION

Tylosin is a macrolide bacteriostatic antibiotic (made naturally by the bacterium *Streptomyces fradiae*), active against most Gram-positive bacteria, mycoplasma, and certain Gram-negative bacteria.

The mode of action of tylosin is via inhibition of bacterial protein synthesis by selective binding to the 50S ribosome, a cellular structure only present in some bacteria (*1*). The macrolide class of antibiotics, including tylosin, have been widely used as therapeutic agents and growth-promoting antibiotics in veterinary medicine. However, the use of tylosin as a feed additive was banned by the European Union in 1999 (*2*).

The chemical structure of tylosin A (the major bioactive component) is shown in Figure 1.

Oxytetracycline (OTC) is commonly used worldwide and is the only legally approved antibiotic registered in the USA for the control of the bacterial pathogens, American Foulbrood (AFB) (*Paenibacillus larvae White*) and European Foulbrood (EFB) (*Melissococcus plutonius*) within bee colonies (*3*). The emergence of bacterial resistance to oxytetracycline is now widespread within the United States and oxytetracycline is no longer a fully effective treatment for the control of AFB. Therefore, new antibiotics have been investigated for the control of AFB (*3*). The most active antibiotics which are used in the control of OTC-resistant AFB are erythromycin, lincomycin, monensin, and tylosin (*4*).

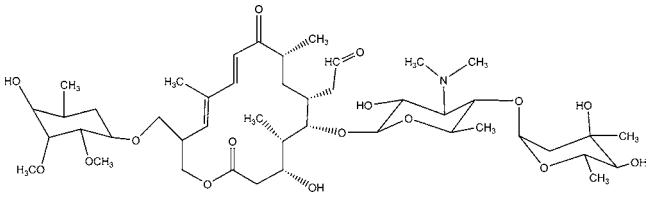

**Figure 1.** Structure of tylosin A.

The usage trends of these other antibiotics in apiculture appear to be linked to geographical origin, which can be correlated to the spread of bacterial resistance to OTC. It has been reported that tylosin can be administered by veterinarians as an "Extra-Label Use Privilege" (when suffering or death of the animal may result from failure to treat) (*5*). Tylosin residues in honey in the range 0.0012−0.1156 mg kg$^{-1}$ have recently been reported by the Canadian Food Inspection Agency (CFIA) (*6*).

EU Maximum Residue Limits (MRLs) exist for tylosin in tissues of all food-producing species; however, there is currently no MRL set for tylosin in honey (*7*). Therefore, there must be no detectable residues of tylosin in honey resulting from its "Extra-Label" or unauthorized use (*5*).

Within this laboratory sub 10 $\mu$g kg$^{-1}$ tylosin residues have been detected in retail honey samples as part of the Department for Environment Food and Rural Affairs (Defra) funded UK Non-Statutory Surveillance scheme. With the possible increase

* To whom correspondence should be addressed. Fax: +44 1904 462311. Tel.: +44 1904 462000. E-mail: m.caldow@csl.gov.uk.
† Central Science Laboratory.
‡ Department of Veterinary Science, Queen's University Belfast.
§ Department of Agriculture and Rural Development.

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in unauthorized usage of antibiotics in apiculture, it has therefore become essential to develop rapid and sensitive analytical methodology to monitor for potential residues of tylosin in honey samples. Work has been conducted and previously reported by both Wang (*8*) and Thompson et al. (*9*, *10*) concerning the development of LC-MS/MS methodology to quantify and confirm tylosin residues in honey with associated low-level detection limits. At present, there are no reports in scientific literature of a screening method capable of detecting tylosin residues in honey at sub 10 $\mu$g kg$^{-1}$ to complement the detection limits achievable by LC-MS/MS. Charm Sciences Inc. markets a semiquantitative test for tylosin in honey using the Charm II 1600 scintillation analyzer; however, the detection limit reported is 15−20 $\mu$g kg$^{-1}$ (*11*).

The aim of this investigation was to develop and validate a sensitive complementary screening method for the analysis of tylosin using SPR biosensor technology.

## MATERIALS AND METHODS

**Apparatus and Reagents.** The SPR-based biosensor system Biacore Q was obtained from Biacore AB (Uppsala, Sweden). Antibody and immobilized sensor chips were provided by Queen's University Belfast. HBS-EP buffer (HBS) was purchased from Biacore AB. Biacore control software, version 3.0.1, was used for instrument operation. Standards were purchased from Sigma (Poole, UK). Strata-X 33 $\mu$m Polymeric sorbent SPE cartridges were purchased from Phenomenex (Macclesfield, UK). Potassium dihydrogen orthophosphate, sodium hydroxide, HPLC-grade water, methanol, and acetonitrile were purchased from Fisher Scientific (Loughborough, UK).

**Honey.** Yorkshire Blossom Honey (both runny and set), known to be free from tylosin, was obtained from the UK National Bee Unit and was used as blank controls throughout the method development and validation experiments. Retail honey from a variety of geographical locations and floral origins were purchased for use within the matrix specificity experiment. Honey samples from retail surveillance programs known to contain residues of tylosin were also included in the experiments.

**Antibody Production.** Protein conjugates of tylosin-HSA were prepared and mixed with Freund's adjuvant and used as immunogens for raising polyclonal antibodies in New Zealand White rabbits by the method of McCaughey et al. (*12*). The first injections of animals were performed using Freund's complete adjuvant which contained heat-killed *Mycobacterium tuberculosis*. Subsequent injections were performed using Freund's incomplete adjuvant. The emulsions containing 1 mg of immunogen were injected subcutaneously into four sites of the animal (left and right front quarters and left and right hind quarters). The animals received immunization every 2 weeks and a blood sample was collected at the same time as the injection assessment of antibody activity with a competitive ELISA using drug-HRP as label. When a suitable titer was achieved, antiserum was harvested from the rabbit and stored at −20 °C. The specificity of the polyclonal antibody was assessed by cross-reactivity studies with a comprehensive range of antimicrobial agents (*13*).

**Immobilization of the Sensor Chip.** Tylosin was immobilized onto the surface of a CM5 sensor chip. The chip surface was activated with 40 $\mu$L of a 1:1 mixture of 0.4 mol dm$^{-3}$ 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC):0.1 M *N*-hydroxysuccinimide (NHS) for 20 min followed by 50 $\mu$L of 10 mmol dm$^{-3}$ hydrazine hydrate for 1.5 h at room temperature. The unreacted sites were blocked by the addition of 50 $\mu$L of 1 mol dm$^{-3}$ ethanolamine for 20 min. The reactants were removed and 50 $\mu$L of tylosin (2 mg mL$^{-1}$ in 10 mmol dm$^{-3}$ sodium carbonate/bicarbonate buffer, pH 9.6) was added and allowed to remain in contact with the sensor chip surface overnight at room temperature. The reactant was removed and the chip surface was washed with deionized water, then dried using a stream of nitrogen gas, and stored desiccated at +4 °C when not in use.

**Extraction Procedure.** (Based on the method of extraction previously reported by Wang (*8*)). Aliquots of honey (5 g) were dissolved in 20 mL of 0.1 mol dm$^{-3}$ potassium buffer (pH adjusted to 8). Nonpolar interferences (such as waxes) were partitioned into hexane (10 mL). An aliquot (15 mL) of the aqueous fraction was collected and further cleaned up using a Strata-X solid-phase extraction (SPE) cartridge; the cartridge was preconditioned with methanol (10 mL), water (10 mL), and extraction buffer (5 mL). The extract was loaded at a flow rate of 1 mL min$^{-1}$; the loaded cartridge was first washed with 40% methanol in water (5 mL) and eluted with methanol (5 mL). Following evaporation (at 40−50 °C) the residue was reconstituted in HBS-EP buffer (500 $\mu$L), prior to analysis using the Biacore Q. For comparative quantitation by LC-MS the residue was reconstituted in methanol (500 $\mu$L).

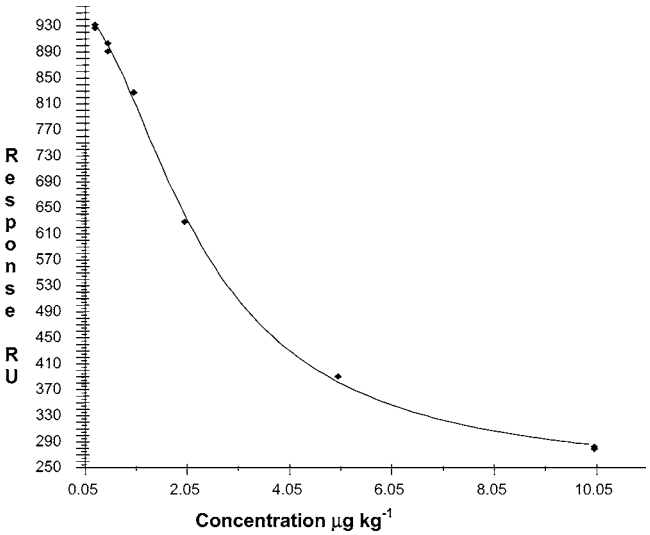


**Figure 2.** SPR biosensor calibration curve using matrix-matched standards in the range 0.05–10 $\mu$g kg$^{-1}$.

**Biosensor Assay.** The extracts were mixed within the autosampler with an equal volume of stock antibody diluted in running buffer (1 in 300) and injected for 180 s over the sensor chip surface at a flow rate of 40 $\mu$L min$^{-1}$. The sensor surface was regenerated between cycles with a 90 s pulse of 20% acetonitrile in 0.25 mol dm$^{-3}$ sodium hydroxide at a flow rate of 40 $\mu$L min$^{-1}$.

**Calibration.** For each batch of samples a set of five matrix-matched standards at 0.5, 1.0, 2.0, 5.0, and 10 $\mu$g kg$^{-1}$ were prepared by fortifying blank extract. The use of matrix-matched standards compensated for any nonspecific binding effects. Duplicate injections at each concentration were performed and the mean response was used to construct a calibration curve. A typical calibration curve is given in Figure 2. The working range (80 to 20% inhibition) of the sigmoidal curve is between 0.5 and 4.0 $\mu$g kg$^{-1}$.

Batch recovery was determined by the fortification of honey samples prior to extraction. The concentration was determined against the matrix-matched calibration curve. Percentage recovery was calculated accordingly (observed concentration/added concentration) × 100.

**LC-MS/MS Determination.** (Based on the LC-MS/MS conditions previously reported Wang (*8*)). The LC-MS/MS system used was a Quattro Ultima Platinum Triple Quadrupole coupled to an Alliance 2695 Separations Module (Waters). The software used was Mass Lynx version 4.0. A gradient separation was performed on a Thermo Electron Corporation HyPurity $C_{18}$ 5 $\mu$m particle size, 150 × 2.1 mm with a $C_{18}$ guard column installed. The transitions monitored were 917>174, 917>773, and 917>156.

**Method Validation.** The validation of the SPR biosensor method and determination of CC$\beta$ were conducted in accordance with the European Commission Decision, 2002/657/EC (*14*); details of those performance criteria required for screening analysis can be found in Table 9 of the document. Both analyte and matrix specificity experiments were conducted to determine the method cross-reactivity and its applicability to a wider variety of honey types from different geographical locations. Honey, known to contain residues of tylosin, has been analyzed using both biosensor and LC-MS/MS quantification and the results compared.

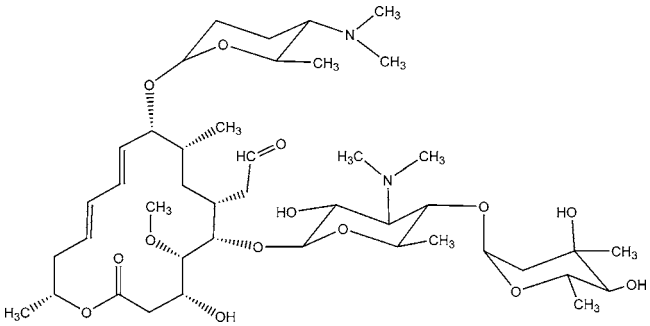

**Figure 3.** Structure of spiramycin.

## RESULTS AND DISCUSSION

**Method Validation. Determination of CC$\beta$.** Since no MRL currently exists for tylosin in honey, an initial assessment of method performance was made via the calibration graph (Figure 2). Whilst this data indicated that the developed method had a "limit of detection" of approximately 0.5 $\mu$g kg$^{-1}$, a formal experiment was required to calculate the "detection capability" (CC$\beta$) of the biosensor screening method (*14*). To calculate the CC$\beta$, 20 samples of known honey blank were spiked with tylosin at 2.5 $\mu$g kg$^{-1}$. This concentration was chosen to minimize the occurrence of false positives. During initial method validation some honey varieties, when tested, elicited a low-level biosensor response which would be equivalent to tylosin in the range 0.3−1.98 $\mu$g kg$^{-1}$. However, these potential residues did not confirm by LC-MS/MS. All recoveries were in the range 47.6−70.4% with an average of 60.3% and a % CV of 9.1. From these results it can be concluded that the CC$\beta$ for this screening procedure is less than or equal to 2.5 $\mu$g kg$^{-1}$.

**Intra-laboratory Repeatability.** An intra-laboratory repeatability experiment was conducted by a second analyst. Replicate samples ($n$ = 7) were fortified with tylosin at 2.5 $\mu$g kg$^{-1}$ (equivalent to the CC$\beta$). The calculated recoveries were in the range 54.8−70.0% with an average of 61.5% and a % CV of 7.5, which compared well to the primary validation results.

**Ruggedness.** A series of key parameters such as the capacity of the SPE cartridges and the pH and molarity of the extraction/loading buffer were investigated. SPE cartridges with sorbent bed capacity of both 200 and 60 mg were tested with no significant difference noted in the results obtained. Variations in the extraction/loading buffer specifications were investigated with each of the following tried: water (pH unadjusted), water at pH 8, 0.1 mol dm$^{-3}$ buffer (native pH approximately 4.4) unadjusted in pH, buffer adjusted to pH 8 with ±10% variation in molarity (0.08, 0.09, 0.10, 0.11, and 0.12 mol dm$^{-3}$). These experiments were not exhaustive but indicated that variations in those conditions tested did not significantly affect the performance of the method.

**Analyte Specificity.** The specificity of the entire procedure was investigated against some structurally related compounds and compounds which may be administered concurrently: tilmicosin, spiramycin, erythromycin, bacitracin, and lincomycin. To determine the cross-reactivity of the five compounds, blank honey samples were fortified with each of the different compounds in the range 0.5−10 $\mu$g kg$^{-1}$. The $IC_{50}$ values were calculated and expressed as a percentage relative to the $IC_{50}$ value for tylosin. As expected, tilmicosin, erthromycin, bacitracin, and lincomycin did not show any measurable cross-reactivity. Spiramycin (Figure 3) produced a measurable response on the biosensor and its cross-reactivity was found to be approximately 60%.

**Table 1.** Comparative Analysis of Different Honey Samples by SPR Biosensor and LC-MS/MS

| | concentration ($\mu$g kg$^{-1}$) | |
|---|---|---|
| country of origin/honey variety | LC-MS/MS | SPR biosensor |
| Scottish Blossom | <0.5 | <2.5 |
| Yorkshire Spring Blossom | <0.5 | <2.5 |
| Hungarian Acacia | <0.5 | <2.5 |
| New Zealand Clover | <0.5 | <2.5 |
| French/Spanish Lavender | <0.5 | <2.5 |
| European Sunflower | <0.5 | <2.5 |
| Scottish Lowlands | <0.5 | <2.5 |
| Spanish Rosemary | <0.5 | <2.5 |
| Spanish Eucalyptus | <0.5 | <2.5 |
| Spanish honeycomb (Balearic) - 1 | <0.5 | <2.5 |
| Blend[a] Orange Blossom | <0.5 | <2.5 |
| Mexican Wildflower | <0.5 | <2.5 |
| Turkish Pine Forest | <0.5 | <2.5 |
| Tasmanian Leatherwood | <0.5 | <2.5 |
| English Dorset Heathland | <0.5 | <2.5 |
| Australian Eucalyptus | <0.5 | <2.5 |
| Scottish Heather | <0.5 | <2.5 |
| Australia/New Zealand Manuka Clear | <0.5 | <2.5 |
| Greek | <0.5 | <2.5 |
| Spanish honeycomb (Balearic) - 2 | <0.5 | <2.5 |
| USA Clear | 2.3, 3.8 ($n$ = 2) | 4.1 ($n$ = 20) |
| Pure Natural[b] | 3.5, 2.5 ($n$ = 2) | 2.7 |
| Hungarian Acacia | 6.1, 7.7 ($n$ = 2) | 9.1, 8.5 ($n$ = 2) |
| Italian Chestnut | 2.3, 1.3 ($n$ = 2) | 5.6, 5.7 ($n$ = 2) |

[a] Blend of EC and non-EC honey. [b] Country of origin unknown (imported into Ireland).

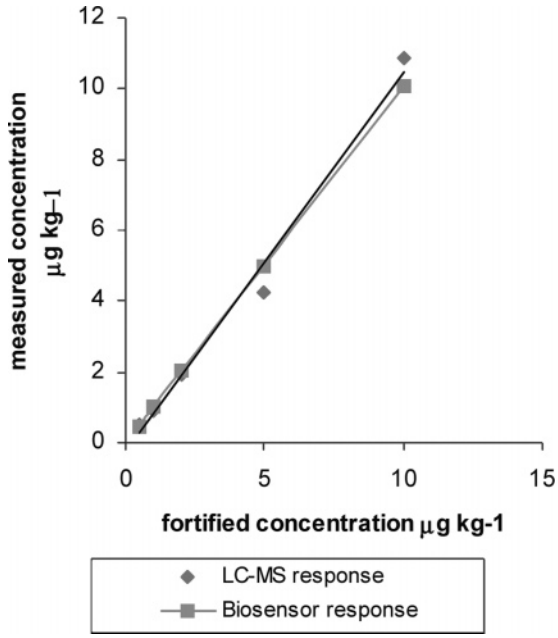


**Figure 4.** Comparison of SPR biosensor and LC-MS/MS calibration curves using matrix-matched standards in the range 0.5–10 $\mu$g kg$^{-1}$.

**Matrix Specificity/False Positive and Negative Rate Determination.** Twenty confirmed blank honey samples, including honey originating from different floral types and different geographical locations, were analyzed by both the biosensor and LC-MS/MS. Honey samples, which had previously been identified as containing sub 10 $\mu$g kg$^{-1}$ residue levels of tylosin (as part of the Defra funded UK Non-Statutory Monitoring Programme) were also quantified by both techniques. The types of honey tested with associated residue concentrations are shown in Table 1.

All of the 20 different "blank" samples analyzed by the biosensor produced a response less than the CC$\beta$. When the

same samples were analyzed by LC-MS/MS, no residues (>0.5 $\mu$g kg$^{-1}$) were found. No false positive results were recorded following biosensor analysis at or greater than the method CC$\beta$ of 2.5 $\mu$g kg$^{-1}$. For those samples known to contain tylosin residues the biosensor results generated comparable data to that obtained by LC-MS/MS (Table 1). Replicate biosensor analyses ($n = 20$) were performed on a single noncompliant (positive) honey sample obtained from the United States. The average residue concentration found was 4.1 $\mu$g kg$^{-1}$ with a % CV of 4.1 with no false negative results recorded. The duplicate LC-MS/MS data for this sample were comparable at 2.3 and 3.8 $\mu$g kg$^{-1}$.

**Comparison of Instrument Performance.** Figure 4 shows a comparison of the calibration curves achieved from both methods: the biosensor screening assay and the confirmatory LC-MS/MS method. The correlation coefficients for the calibration graphs are 1.00 and 0.99, respectively.

**Concluding Remarks.** The SPR biosensor method is an assay suitable for use as a rapid screening method for the detection of low-level tylosin residues in honey. The assay shows 60% cross-reactivity toward spiramycin; however, no significant cross-reactivity to the other analytes tested was observed. As with all screening methodologies, suspect positive samples would require mass spectrometric confirmatory analysis. The CC$\beta$ for tylosin was determined to be 2.5 $\mu$g kg$^{-1}$, which is complementary to existing LC-MS/MS confirmatory methods. The false positive and negative rates for the assay are <5% and the results obtained compare well to those produced using a confirmatory mass spectrometric technique, indicating the reliability and robustness of the biosensor method.

Using the methodology presented in this article, it is possible to extract and analyze up to 25 samples within a working day. The extraction method employs a simple SPE format, which lends itself well to automation; the biosensor instrument is a fully automated system allowing "out of hours" operation. The reprocessing is fully automated and does not require specialist data handlers.

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