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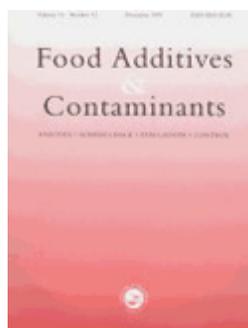
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A new method for the rapid identification of tetracycline residues in foods of animal origin - using the Premi®Test in combination with a metal ion chelation assay

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Methods/Techniques:	antimicrobial screening, Premi®Test , metal ion chelation
Additives/Contaminants:	Veterinary drug residues - tetracycline, Antimicrobial compounds
Food Types:	Honey, Meat

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Table 1. Results obtained using the extended integrated antimicrobial assay during a blind trial with chicken muscle fortified with various antimicrobials ($n=2$).

Sample ID	Premi® Test scanner Z-score				Analyst interpretation	Identification assessment
	Control Response	Plus TCN	Plus β -lactamase	Plus p-ABA		
Unknown 1 (Doxycycline 1000 $\mu\text{g kg}^{-1}$)	4.66	-0.38	2.13	3.34	Tetracycline	Deleted: ¶
Unknown 2 (Amoxicillin 10 $\mu\text{g kg}^{-1}$)	2.56	0.44	-5.74	12.64	β -lactam	Correct
Unknown 3 (Chlortetracycline 100 $\mu\text{g kg}^{-1}$)	4.29	-4.86	4.44	4.35	Tetracycline	Correct
Unknown 4 (Blank)	-7.67	-13.97	-7.24	-3.17	Blank	Correct
Unknown 5 (Oxytetracycline 200 $\mu\text{g kg}^{-1}$)	4.46	-2.13	3.08	3.64	Tetracycline	Correct
Unknown 6 (Mixed spike - sulfadiazine 200 $\mu\text{g kg}^{-1}$ & chlortetracycline 100 $\mu\text{g kg}^{-1}$)	7.49	5.46	5.00	4.92	<i>Multiple residue sulphonamide, β-lactam or tetracycline class OR an alternative Gram positive inhibitor.</i>	Correct
QC Sample 1 Blank	-8.03	-15.20	-7.75	-3.49	Blank	Correct
QC sample 2 Penicillin G 10 $\mu\text{g kg}^{-1}$	6.60	7.29	-7.15	5.95	β -lactam	Correct
QC sample 3 Chlortetracycline 100 $\mu\text{g kg}^{-1}$	4.91	-1.75	3.27	4.32	Tetracycline	Correct
QC sample 4 Sulfadiazine 200 $\mu\text{g kg}^{-1}$	5.39	2.89	2.11	-4.10	Sulphonamide	Correct

Figures in bold text indicates a negative test response.

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A new method for the rapid identification of tetracycline residues in foods of animal origin - using the Premi[®]Test in combination with a metal ion chelation assay

Abstract

22 A post-screening classification assay for tetracycline compounds has been developed
23 and integrated into the previously reported optimised Premi[®]Test methodology (Stead
24 *et al.* 2004 and Stead *et al.* 2005) The new post-antimicrobial screening assay is based
25 on a metal ion chelation using calcium and sodium ions and has been shown to be
26 specific towards the tetracycline class. The assay is both cost-effective and
27 complementary to the post-screening procedures that have previously been developed
28 for the β -lactam and sulfonamide compounds. A validation study was conducted in
29 accordance with 2002/657/EC (Commission Decision). The method was demonstrated
30 to be rugged and applicable to a range of tetracyclines of differing antimicrobial
31 potencies over a wide concentration range. A blind trial was undertaken in which all
32 antimicrobial residues in the unknown samples were successfully identified by the
33 analyst following the integrated Premi[®]Test procedure for the classification of
34 antimicrobial compounds.
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55 **Keywords:** Tetracyclines, Premi[®]Test, metal ion chelation, antimicrobial screening
56 assay, food safety.
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Introduction

Tetracyclines are antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A). Tetracycline molecules comprise a tetracyclic nucleus to which various groups are attached, the general structure is shown in Figure 1.

[Insert Figure 1 about here]

Tetracyclines are broad-spectrum agents that exhibit activity against a wide range of Gram-positive and Gram-negative bacteria; they have been widely used in both human and veterinary medicine. In some countries the use of antibiotics has been expanded to include addition, at sub-therapeutic levels, to animal feeds for the purpose of growth promotion. The prevalence of bacterial resistance to the tetracycline class of antibiotics has increased following the widespread usage of these compounds within clinical, veterinary and agricultural practice. The spread of bacterial resistance has major implications to human health (World Health Organisation, 2006).

Under EU Legislation, members of the tetracycline class of antimicrobial compounds are permitted for use in the treatment of bacterial diseases in food-producing animal species. Maximum Residue limits (MRLs) have been set under Council Regulation (EEC) No. 2377/90 (as amended) ranging from 100 – 600 $\mu\text{g kg}^{-1}$ dependent on the species and tissue type. The current MRL for tetracycline residues in muscle tissue of

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4 food producing animals is $100 \mu\text{g kg}^{-1}$ and no MRLs currently exist for honey. During
5
6 Defra funded surveillance exercises conducted in the UK between 2001 and 2004
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8 residues of tetracyclines have been found in pig, poultry and calf kidney in the range
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10 of $780 - 4020 \mu\text{g kg}^{-1}$ (Veterinary Medicines Directorate, 2006). It is therefore,
11
12 important that effective screening and confirmatory procedures are available for
13
14 detecting tetracyclines in foods of animal origin in order to provide legislators and
15
16 consumers with confidence that the food products entering the food chain are
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18 compliant with the current legislation regarding permissible MRLs.
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26 Previous studies (Stead *et al.* 2004 and Stead *et al.* 2005) have shown that the
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28 Premi[®]Test is a cost-effective screening tool capable of detecting penicillins,
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30 cephalosporins, sulfonamides, tetracyclines and some macrolides in a wide range of
31
32 tissues from a variety of species; including muscle, liver and kidney along with eggs
33
34 and fish. The Premi[®]Test is a commercially available, agar diffusion test based on the
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36 inhibition of growth of *Bacillus stearothermophilus*. The agar contains a standardized
37
38 number of bacterial spores, selected nutrients and the pH indicator, bromocresol
39
40 purple. After adding the sample extract directly onto the agar, the test is incubated for
41
42 approximately 3 hours at 64°C . During incubation, microbial metabolism will result
43
44 in a change in pH and hence in a change of colour from purple to yellow. By contrast,
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46 if the sample contains sufficiently high concentrations of inhibitory substances, the
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48 colour remains purple. The tests can either be read visually or by using the recently
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50 developed scanner technology, Premi[®]Scan.
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4 A limitation of this type of broad-range antimicrobial test is the generic nature of a
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6 positive result. Without the use of a secondary diagnostic assay it is not possible to
7
8 attribute the positive response as being elicited by a particular class of compound. For
9
10 two other classes of antimicrobial compounds we have previously demonstrated that
11
12 class specific tests can be employed. The enzyme, β -lactamase can selectively identify
13
14 the β -lactam class. A competitive binding reaction, using the natural agonist of the
15
16 prokaryotic enzyme, (dihydropteroate synthetase), para-aminobenzoic acid (p-ABA),
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18 can selectively identify the sulfonamide class.
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26 The ability of the tetracycline compounds to chelate polyvalent metal cations is a
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28 well-established property of this class. There are many extraction, clean-up and
29
30 analysis methods for tetracyclines reported in the scientific literature based on
31
32 transition metal ion chelation columns, for example, Chelating Sepharose Fast Flow
33
34 columns (Carson 1993). Calcium forms a 2:1 metal-ion to ligand complex.
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36 Formation of the calcium complex involves the addition of one metal ion to the C-10,
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38 C-11 site with subsequent addition of a second metal ion at the C-12 and C-1 site
39
40 (Newman 1976). Using a calcium and sodium containing buffer, metal ion chelation
41
42 was investigated as a mechanism for disrupting the antimicrobial activity of
43
44 tetracyclines and thus selectively reversing the primary Premi[®]Test positive response
45
46 into a secondary negative response. It is postulated that, on formation of the metal-
47
48 ion chelation complex tetracyclines can no longer permeate the bacterial cell
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50 membrane via binding to the active transport mechanism, the Tet(R) efflux pump
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52 (Chopra and Roberts, 2001).
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4 The Premi[®]Test methodology incorporating a solvent extraction and post-screening
5 assays for β -lactam and sulphonamide classes of antimicrobials has been previously
6 validated (Stead *et al.* 2004 and Stead *et al.* 2005). Within this study additional
7 experiments based on the 2002/657/EC guidelines for the validation of qualitative
8 methods were conducted in order to prove the novel post-screening assay for
9 tetracyclines was effective. The individual experiments conducted include;
10 repeatability, determination of the Premi[®]Test assay CC β for
11 chlortetracycline, determination of false positive and false negative rates, analyte
12 specificity and ruggedness. A single blind trial was also conducted whereby six
13 samples (a mixture of spikes and blanks) were prepared and blind coded by one
14 analyst and analysed by a second analyst in accordance with the integrated
15 antimicrobial screening procedure (Stead *et al.* 2004 and Stead *et al.* 2005), with the
16 addition of the newly developed tetracycline assay. The tetracycline selective assay
17 was transferred to a second laboratory, DSM (Delft, The Netherlands) for an inter-
18 laboratory assessment of the method in combination with the previously reported fluid
19 expression extraction procedure (EuroResidue IV, Offereins *et al.* 2000).
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45 This paper reports the development and validation of a third post-screening assay,
46 selective for tetracyclines. The use of these simple secondary-screening assays allows
47 a suspect positive sample to be reliably directed to the appropriate chemical method
48 for confirmatory analysis.
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54 **Materials and methods**

55 *Chemicals and reagents*

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4 All solvents and reagents were of analytical grade and purchased from Sigma-Aldrich
5 (Poole, Dorset, UK) with the exceptions of; Lab Lemco broth and β -lactamase, which
6
7 were purchased from Oxoid (Basingstoke, Hampshire, UK), acetonitrile, acetone and
8
9 HPLC grade water were purchased from Fisher Scientific (Loughborough,
10
11 Leicestershire, UK). The Premi[®]Test kits were provided by DSM (Delft, The
12
13 Netherlands).
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21 *Instrumentation - The scanner system*

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23 The Premi[®]Test end-point was measured instrumentally using a flatbed scanner in
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25 combination with the Premi[®]Scan (custom version) software, provided by DSM
26
27 (Delft, The Netherlands). A scanner value (Z score) of greater than zero was classified
28
29 as a positive result and a scanner value of zero or lower was classified as a negative
30
31 result.
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37 *Samples*

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39 The validation experiments were undertaken using known blank chicken muscle. The
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41 UK National Bee Unit, based at CSL, York, supplied honey (wild-flower blossom set
42
43 honey), for the matrix specificity experiments.
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49 *TCN buffer (calcium containing buffer) preparation*

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51 TRIS HCL (7.88 g) 50 mM, calcium chloride (2.19 g) 10 mM, sodium chloride (8.76
52
53 g) 150 mM dissolved in 1 litre of water and adjusted to pH 7.5 with 1 M sodium
54
55 hydroxide. (Stored in the dark between 4 - 8 °C and used within 1 month.)
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Lab Lemco Broth

Powdered Lab Lemco media (8 g) dissolved in 1 litre of HPLC grade water and shaken by hand for approximately 5 min. The broth solution was stored in the dark between 4 – 8°C and used within 1 week.

Sample extraction procedure

To tissue (4 g), acetonitrile: acetone 70:30% (10 mL) and anhydrous sodium sulphate (6 g) were added prior to homogenisation for 45 seconds. The extract was centrifuged (4500 rpm, 4 °C, 10 minutes), the supernatant (8 mL) was removed and evaporated to near dryness under a stream of nitrogen at 35-40 °C and then re-suspended in Lab Lemco Broth (600 µL). The extract (100 µL) was then applied directly to the Premi[®]Test, the ampoules were sealed using plastic film and the incubation was carried out according to the test kit instructions (DSM webpage, Premi[®]Test demo).

Post screening method for tetracyclines (TCN assay)

To a portion of extract (100 µL) TCN buffer (50 µL) [or water (50 µL) in the case of blank and spiked controls] was added, vortex mixed for approximately 30 seconds and allowed to stand at room temperature for 10 min. Treated extract (100 µL) was applied to the Premi[®]Test and incubated as described above.

Figures 2a and 2b outline the existing antimicrobial screening procedure – including the post-screening class specific assays for β -lactam and sulfonamide antimicrobial compounds and the new tetracycline post-screening assay respectively.

[Insert Figures 2a and 2b about here]

Results and discussion

Initial experiments showed that the incubation of the sample extract with calcium and sodium ions in TCN buffer selectively reversed the primary screen positive response for tetracyclines observed on the Premi[®]Test. In these initial experiments the quantity of TCN buffer required was also optimised (data not shown). On the basis of this mechanism the validation study was conducted.

The result of the specificity experiment is summarised in graphical format in Figure 3. These data show the Z score values recorded using the Premi[®]Scan scanner system following incubation of extracts on the Premi[®]Test with and without the addition of TCN. This experiment was conducted for a variety of primarily Gram-positive inhibitory antimicrobial compounds including; penicillin G, cloxacillin, sulfadiazine, tylosin and a range of tetracycline compounds. The primary matrix was chicken muscle, although a set of honey samples was also included to assess matrix-to-matrix variations.

These data indicate that, in the presence of the TCN buffer, the control (positive) response recorded for the tetracyclines; oxytetracycline, doxycycline, tetracycline and chlortetracycline spiked into chicken muscle at the MRL concentration of 100 µg kg⁻¹ is reversed to a negative response. In the case of the non- tetracycline class compounds spiked into muscle (at or above their respective MRL concentrations) the control positive response is not significantly affected by the addition of the TCN buffer. This finding demonstrates that the addition of TCN buffer to sample extract

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4 selectively reverses the positive control response of the tetracycline class and does not
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6 significantly affect the positive response caused by a range of other antimicrobial
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8 compounds.
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14 Currently there are no approved antibiotics for the treatment of European Foul Brood
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16 within the EU. Nevertheless oxytetracycline has been detected in retail honey
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18 samples. Therefore, in order to investigate the wider applicability of this assay honey
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20 was spiked with oxytetracycline at $100 \mu\text{g kg}^{-1}$, following the extraction procedure the
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22 extracts were treated with TCN buffer and applied to the Premi[®]Test. These results,
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24 shown in Figure 3, indicate that TCN assay performs comparably in both muscle
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26 tissue and the second matrix type, honey.
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33 [Insert Figure 3 about here]
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38 Figure 4 presents the results of the investigation conducted to determine the efficiency
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40 of TCN at reversing the tetracycline control response across a wider analyte
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42 concentration range. The Premi[®]Test response to chicken muscle spiked with either
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44 chlortetracycline (a weaker potency tetracycline) or doxycycline (a stronger potency
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46 tetracycline) at concentrations representing one half, one, two and twelve times the
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48 MRL was found to be effectively reversed by the addition of 50 μL of TCN buffer to
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50 100 μL of the sample extract.
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57 To determine the Premi[®]Test primary screening procedure (Figure 2a) $\text{CC}\beta$ for the
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59 tetracyclines, 21 different samples of known muscle blank fortified with
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4 chlortetracycline (the weakest potency tetracycline) at the MRL concentration of 100
5 $\mu\text{g kg}^{-1}$ were analysed. Twenty samples out of the twenty-one were found to give a
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7 positive response, therefore the qualitative $\text{CC}\beta$ (β error of 5 %) was determined to
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9 be less than or equal to 100 $\mu\text{g kg}^{-1}$, equivalent to the MRL for tetracyclines in muscle
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11 tissue. This finding also indicates the false negative rate of the Premi[®]Test primary
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13 screen for tetracyclines is equivalent to 5%.
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21 The 21 sample extracts were also treated according to the TCN assay procedure
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23 (Figure 2b) and applied to the Premi[®]Test. In the presence of TCN, all 21 samples
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25 gave a negative test response identifying the presence of a tetracycline. This result
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27 suggests that the probability of falsely recording a tetracycline positive using the TCN
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29 assay is zero at the 95% confidence interval.
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35 An inter-laboratory assessment of the TCN post-screening assay was conducted at
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37 DSM (Delft, The Netherlands) using the alternative fluid expression procedure (*data*
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39 *not shown*). This simple extraction procedure, which is based on the use of a garlic
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41 press to release meat juices, can be performed without the use of any laboratory
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43 reagents or equipment and is applicable for use as a field test. A total of 10 known
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45 blank chicken muscle samples were fortified with oxytetracycline at 200 $\mu\text{g kg}^{-1}$
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47 (twice the MRL in muscle tissue) extracted following the fluid expression procedure
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49 and 100 μL of the resulting fluid treated with 50 μL of TCN as detailed in Figure 2b.
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52 All 10 spiked replicates showed a strong positive control response on the Premi[®]Test.
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55 However, following the addition of TCN only 8 out of the 10 extracts produced a
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57 negative response. This suggests that the TCN post-screening assay was only 80%
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4 efficient at reversing the response of a tetracycline following the crude fluid
5 expression procedure. It may be that tissue derived substances in the raw fluid
6
7 expressed are competing for the divalent calcium ions. Further method optimisation is
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9 required in order to improve the efficiency of the TCN assay in combination with the
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11 fluid expression extraction.
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18 Results of the blind trial are shown in Table 1. Using the fully integrated antimicrobial
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20 procedure for β -lactams, sulfonamides and tetracyclines all 6 blind coded samples
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22 were correctly characterised by the analyst, showing the procedure to be applicable to
23
24 routine screening. Following treatment with TCN, the strong positive control
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26 responses seen for samples 1, 3 and 5 were selectively reversed, the responses
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28 remained unaffected by the other post-screening procedures. From these observations
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30 the analyst correctly identified the class responsible for eliciting the positive control
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32 response as tetracycline. In presence of β -lactamase, the strong positive control
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34 response seen for sample 2 was selectively reversed, indicating the presence of a β -
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36 lactam compound. The test response for sample 4 was found to be negative and not
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38 significantly affected by any of the post-screening assays, therefore this sample was
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40 recorded as a blank. In the case of sample 6, the strong positive control was not
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42 significantly affected by any of the post-screening assays. Following the three post-
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44 screening treatments, the strong positive response observed in the control is reduced
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46 from approximately 7.5 units to between approximately 5 and 5.5 units. The analyst
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48 concluded that this was either the result of multiple classes of compound being
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50 present in the extract and the combined antimicrobial potency reduced in the presence
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4 of a post-screening treatment or another Gram-positive inhibitory antimicrobial class,
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6 for example a macrolide compound.
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11 [Insert Table 1 about here]
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14 15 16 **Conclusions**

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18 The recently developed and validated TCN assay complements the existing integrated
19 antimicrobial screening strategy in combination with the Premi[®]Test and scanner
20 system. The fully integrated strategy offers a rapid, broad-spectrum primary screen for
21 Gram-positive inhibitors with a secondary identification of penicillin, sulphonamide
22 and now tetracycline residues in foods of animal origin.
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32 The metal ion chelation (TCN buffer) approach is cost-effective, requiring the
33 preparation of only basic laboratory reagents and is simple to use. The concentration
34 of calcium and sodium ions and the ratio of TCN buffer to extract were optimised in
35 order to compensate for the differences in the antimicrobial potency within the
36 tetracycline class and the potential for very high residue levels. Thus, a fixed volume
37 of TCN buffer is combined with a fixed volume of extract following the solvent
38 extraction procedure.
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52 The TCN assay has been successfully validated in accordance with the 2002/657/EC
53 guidelines for qualitative screening methods. The method was shown to be
54 applicable to two different matrix types; class specific and applicable to the post-
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4 screening detection of a range of tetracyclines when present below, at and above the
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6 MRL concentration in both chicken muscle tissue and a secondary matrix, honey.
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9 10 **Acknowledgments**

11
12 We acknowledge the funding of this work by the Defra Veterinary Medicine
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14 Directorate.
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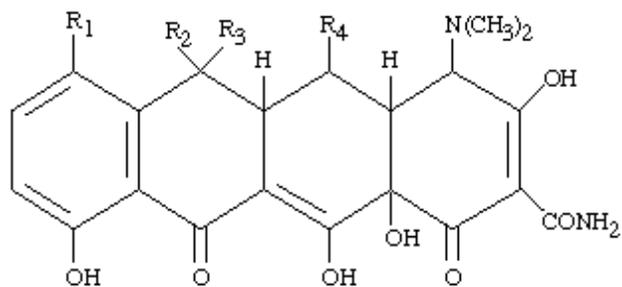
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Figure 1. The chemical structure of some of the tetracycline compounds



Conserved structure

Compound	R ₁	R ₂	R ₃	R ₄
Tetracycline	H	OH	CH ₃	H
Chlortetracycline	Cl	OH	CH ₃	H
Oxytetracycline	H	OH	CH ₃	OH
Doxycycline	H	CH ₃	H	OH

Figure 2a. The existing integrated antimicrobial screening procedure –including the post-screening class specific assays for β -lactam and sulfonamide antimicrobial compounds.

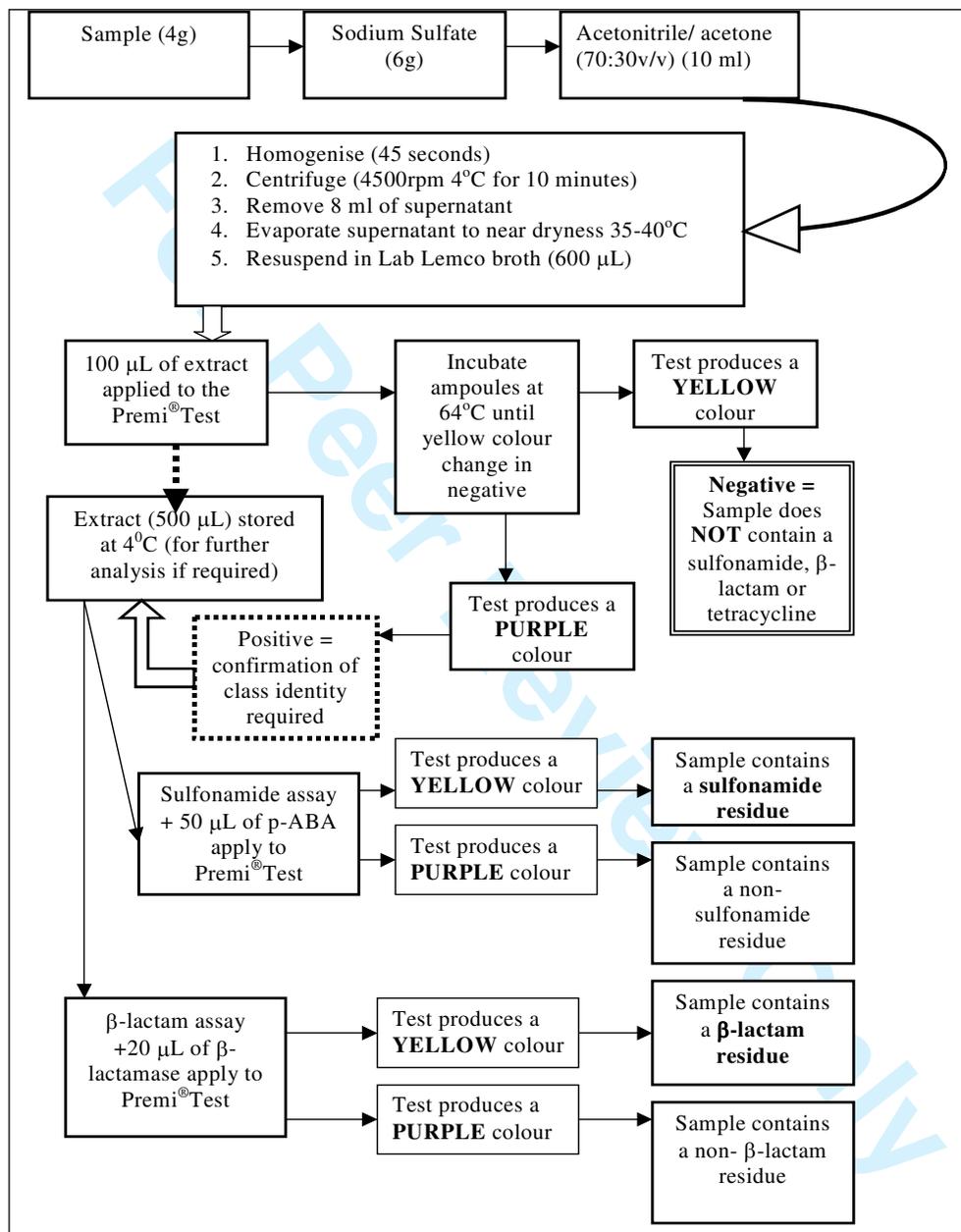


Figure 2b. The integrated antimicrobial screening procedure –including the new post-screening class specific assay for tetracyclines.

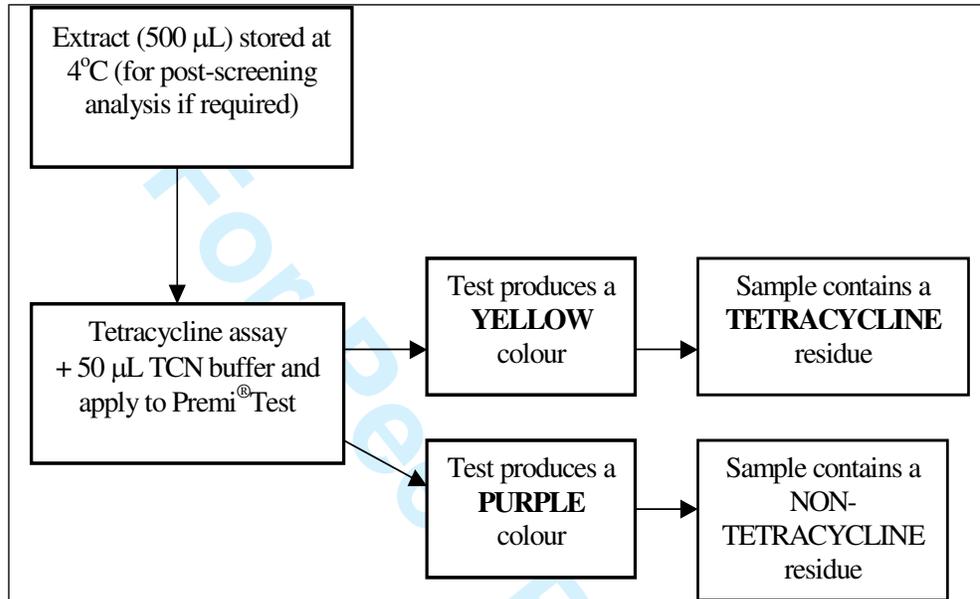


Figure 3. Effect of TCN on the Premi[®]Test response to a range of antimicrobial compounds; 200 µg kg⁻¹ sulfadiazine, 600 µg kg⁻¹ cloxacillin 100 µg kg⁻¹ tylosin 10 µg kg⁻¹ penicillin-G and a range of tetracycline compounds; doxycycline, tetracycline, oxytetracycline and chlortetracycline at 100 µg kg⁻¹ - fortified chicken muscle and honey (n =6).

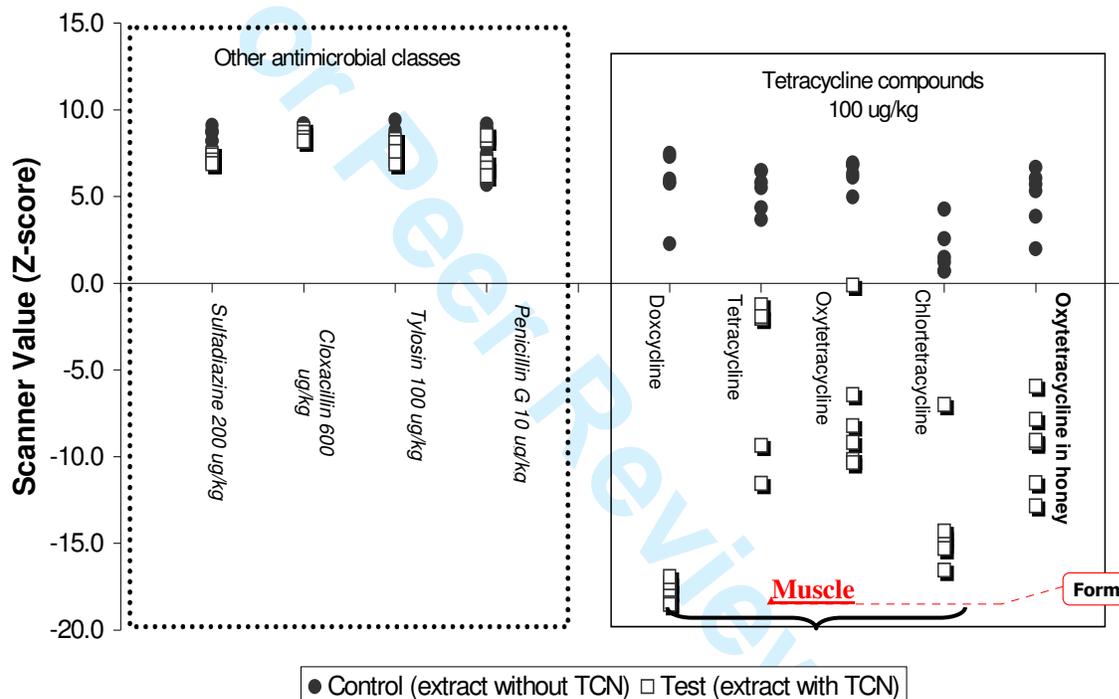
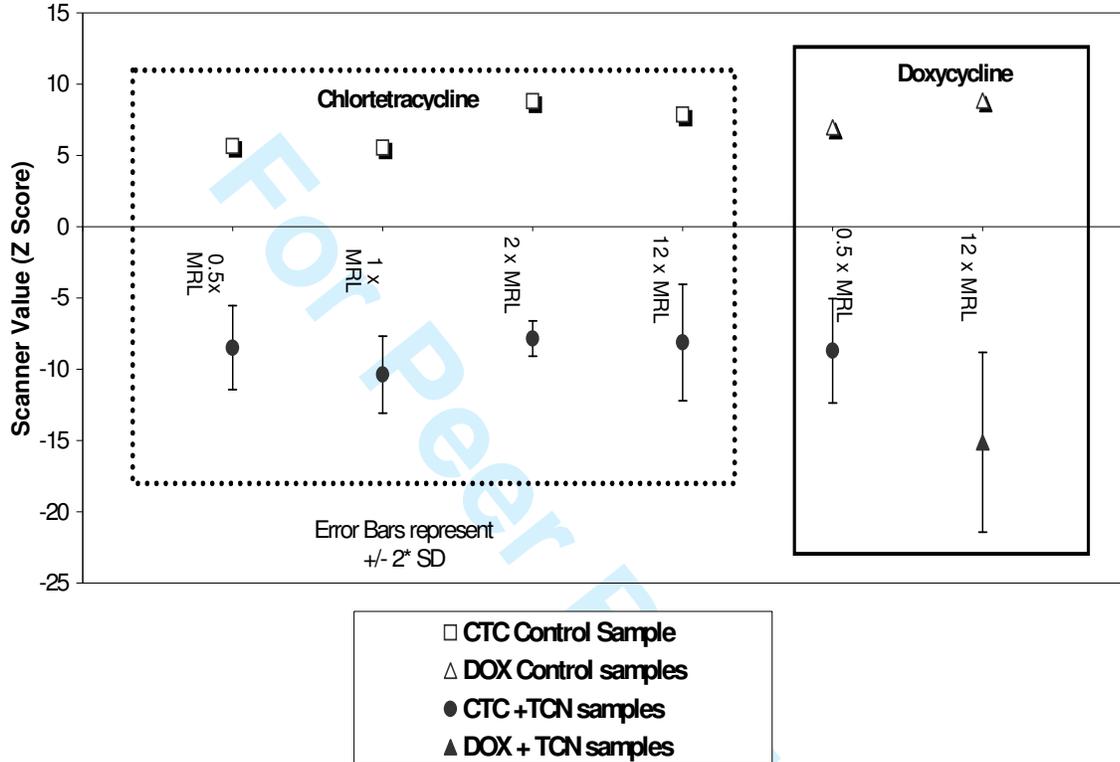


Figure 4. Effect of varying the concentration of two tetracycline compounds on the Premi[®]Test response to control and TCN treated chicken muscle extracts ($n=4$)



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