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Manuscripts

**Validation of two enzyme immunoassays for aminoglycoside**

**residues according to European Decision 657/2002**

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For Peer Review Only

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56 **Abstract**  
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8 Aminoglycoside antibiotics are commonly used in the treatment of bacterial infections  
9  
10 in human and veterinary practice. Because of their toxicity, the European Community  
11  
12 has established Maximum Residue Limits (MRL) in foodstuffs of animal origin (EEC  
13  
14 No 2377/90). In the present work the performance of two new enzyme immunoassays  
15  
16 (EIA), *I'screen Gentamicin* and *I'screen Neomycin*, for the quantitative detection of the  
17  
18 aminoglycosides gentamicin and neomycin in milk and tissue are described. The  
19  
20 validation of these EIAs has been performed in accordance to criteria of the European  
21  
22 Decision 657/2002. Assays sensitivity at the MRLs was 95% for milk samples and  
23  
24 100% for tissue samples, while specificity was 100% at 33% and 25% of the MRLs for  
25  
26 milk and tissues, respectively. The performance of these EIAs indicates that they can be  
27  
28 used as easy screening methods in the analysis of aminoglycosides in milk and tissue  
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30 samples.  
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4041 **Keywords:** gentamicin, neomycin, EIA, drug residues  
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#### 44 Introduction

46 The aminoglycosides are closely-related antibiotics produced by *Streptomyces spp.* and  
47 *Micromonospora spp.* (Salisbury 1995). They have similar antibacterial properties, i.e.  
48 inhibition of protein synthesis at the 30S ribosomal subunit, and because of their  
49 toxicity towards both gram positive and gram negative bacteria (Schenck 1998), are  
50 broad spectrum bactericidal antibiotics, widely used in human and veterinary practice.  
51 In food-animal production, the most commonly used aminoglycosides are gentamicin,  
52 neomycin, streptomycin and dihydrostreptomycin. Since 1970 EU Directives regulated  
53 the use of antibiotics as additives in feed (Council Directive 70/524 EEC and following  
54 amendments), progressively banning the use of these molecules for auxinic purposes.  
55 Aminoglycosides, intended as therapeutics, are preferably administered by injection. In  
56 this case, they accumulate in tissues in high and persistent residues. In fact, since they  
57 are eliminated by renal filtration, they tend to accumulate in the kidney where they bind  
58 to tissue proteins and macromolecules via ionic bonds (Isoherranen and Soback 1999),  
59 causing nephrotoxicity (Salisbury 1995). Besides urines and kidney, aminoglycosides  
60 can be found in cochlea, in serum, in milk and in other tissues, depending on plasma  
61 levels. Antibiotic levels in milk are dependent on a number of physico-chemical  
62 parameters, and their concentration is higher in cases of mastitis (Debackere 1995;  
63 Saran 1995), following intramammary treatment.

64

65 For humans, aminoglycosides in food hold the risk of undesirable health effects, as  
66 nephrotoxicity and ototoxicity (Saran 1995). Council Regulation (EEC) No 2377/90 of  
26 June 1990 (Woodward 1995) lays down a procedure for the establishment of

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2  
3 68 Maximum Residue Limits (MRLs) of veterinary drugs in foodstuffs of animal origin.  
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5

6 The wide use of aminoglycosides in veterinary medicine requires therefore suitable  
7  
8 70 screening and confirmatory methods for their detection in edible tissues. For the  
9  
10 detection of aminoglycosides in food, microbial inhibition assays are widely used as  
11  
12 72 screening methods (Schenck 1998), but even if they are simple and relatively cheap,  
13  
14 they are time consuming, lack sensitivity (Isoherranen and Soback 1999) and do not  
15  
16 74 allow substance identification. On the other hand, chromatographic analysis (LC/MS),  
17  
18 is used as confirmatory method (Salisbury 1995), because it provides unequivocal  
19  
20 76 identification of the analyte and is aimed at preventing false positive results (Woodward  
21  
22 1995), but it is rather expensive.  
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28  
29 In recent years, many enzyme immunoassays (EIA) for the detection of aminoglycoside  
30  
31 80 residues in animal tissues have been developed (Haasnoot et al. 1999; Loomans et al.  
32  
33 2003; Jin et al. 2005; Jin et al. 2006). In order to identify the contaminant following the  
34  
35 82 positive findings from microbiological screening methods, EIAs are very suitable,  
36  
37 thanks to their ease of use, sensitivity, rapidity and specificity. Nevertheless, the  
38  
39 84 sensitivity and specificity of quantitative EIA kits for gentamicin and neomycin  
40  
41 detection do not always fit with the EU MRLs for these two antibiotics.  
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48 The present work describes the performance evaluation of two new quantitative enzyme  
49  
50 88 immunoassays for the detection of gentamicin and neomycin in milk and tissues,  
51  
52 meeting the need for rapid sample preparation and test implementation, and having the  
53  
54 90 dosing range around the EU MRLs. The validation of the two EIAs was carried out  
55  
56 according to European Decision 657/2002 for quantitative screening methods.  
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58 92 Therefore, performance characteristics such as detection capability (CC $\beta$ ), precision,  
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3 specificity and ruggedness were determined. The decision limit ( $CC\alpha$ ) and recovery for  
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6 94 milk and tissue samples in both assays were also investigated.  
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## 10 96 **Materials and methods**

### 11 98 *Materials*

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17 Gentamicin sulfate salt, neomycin trisulfate salt hydrate, gentamicin solution, neomycin  
18  
19  
20 100 solution, streptomycin sesquisulfate, kanamycin, bovine serum albumin (BSA),  
21  
22 horseradish peroxidase (HRP), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide  
23  
24  
25 102 hydrochloride (EDC) were purchased from Sigma.  
26  
27  
28

29  
30 104 *N*-hydroxysulfosuccinimide (sulfo-NHS) was obtained from Pierce. Protein A  
31  
32 Sepharose CL-4B was obtained from GE Healthcare Europe GmbH. The EIA kits  
33  
34 106 *I'screen Gentamicin* and *I'screen Neomycin* (Tecna Srl, Trieste, Italy) contain all the  
35  
36 necessary materials and methods for the assay.  
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39 108

### 40 41 *Test samples*

42  
43  
44 110 Twenty raw milk samples, derived from untreated cows, were kind gifts from AAFVG  
45  
46 (Associazione Allevatori del Friuli Venezia Giulia). Twenty tissue samples (ten bovine  
47  
48 112 and ten swine muscles) were purchased in a supermarket, guaranteed as residue free  
49  
50 ("Prodotti con Amore", COOP Italia).  
51  
52

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### 54 55 *Gentamicin EIA*

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58 116 The anti-gentamicin antibody was raised in rabbit by three cycles of immunization with  
59  
60 gentamicin-BSA conjugates, obtained using EDC and Sulpho-NHS (Hermanson 1996).

1  
2  
3 118 Microtiter plates were coated with anti-gentamicin antibodies purified on a Protein A  
4  
5 Sepharose CL-4B column. The assay design is a direct competitive enzyme  
6  
7  
8 120 immunoassay, because during the first incubation, standards/samples and gentamicin-  
9  
10 HRP-conjugate (obtained by EDC and Sulpho-NHS mediated conjugation procedures,  
11  
12  
13 122 Hermanson 1996) compete for the antibodies binding sites on the microtiter plate (30  
14  
15 minutes, room temperature). The amount of enzyme conjugate which remains bound  
16  
17  
18 124 after washings is inversely proportional to the amount of the analyte in  
19  
20 standards/samples and is determined by measuring the absorbance after a developing  
21  
22  
23 126 reaction step of 30 minutes using tetramethyl benzydine (TMB) as chromogenic HRP-  
24  
25 substrate. To stop the reaction, a sulphuric acid solution is then added, and the  
26  
27  
28 128 absorbance is measured at 450 nm by a microplate reader (Sunrise, Tecan).

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31  
32 130 All the absorbance values were transformed into relative signal (OD/OD of zero  
33  
34 standard =  $B/B_0$ ). Results were elaborated through the software “Magellan” (Tecan).

35  
36  
37 132 The four parameter logistic was chosen as algorithm to fit the calibration curve  
38  
39 according to the following formula:  $y = (A - D) / [1 + (x/C)^B] + D$ , where A is the  
40  
41  
42 134 maximal absorbance, D is the minimum absorbance, C is the concentration that  
43  
44 produces a response halfway between A and D, while B is the slope at the inflection  
45  
46  
47 136 point of the sigmoidal curve.

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50  
51 138 Gentamicin in tissue extracts was determined through ready-to-use buffer standard  
52  
53 solutions, while for milk samples a calibration curve in matrix was used. Milk standard  
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56 140 solutions were freshly prepared at each analysis.

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59  
60 142 *Neomycin EIA*



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3 The anti-neomycin antibody was raised in rabbit by three cycles of immunization with  
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5  
6 144 neomycin-BSA conjugates, obtained using EDC and Sulpho-NHS (Hermanson 1996).

7  
8 Microtiter plates were coated with anti-neomycin antibodies purified on a Protein A  
9  
10  
11 146 Sepharose CL-4B column. As the gentamicin assay, the *I'screen Neomycin* test kit is a  
12  
13 competitive enzyme immunoassay. The assay is carried out in the same way, except that  
14  
15  
16 148 the first incubation is for 20 min and the second for 10 min. Data handling was also  
17  
18 performed with the same instrument, the same software, the same curve fitting. Also in  
19  
20 150 this case two different calibration curves for the analysis of milk or meat samples were  
21  
22 used, but both curves were freshly prepared at each analysis.  
23  
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152

#### 27 *Cross-reactivities determination*

28  
29 154 Calibration curves of different aminoglycosides antibiotics (gentamicin, neomycin,  
30  
31 streptomycin, kanamicin) were prepared using the test kit dilution buffers. The cross-  
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33  
34 156 reactivities values were calculated from the calibration curves obtained, according to the  
35  
36 following equation:  $(IC_{50} \text{ of gentamicin or neomycin} / IC_{50} \text{ of the tested compound}) \times$   
37  
38 158  $100$ ;  $IC_{50}$  is the concentration producing the 50% of the maximal absorbance ( $B/B_0 =$   
39  
40  $50\%$ ).  
41  
42

160

#### 46 *Sample preparation*

47  
48 162 Sample preparation was the same for both EIAs. The milk samples were refrigerated  
49  
50 and centrifuged at 4°C for 10 minutes at 3000g. The fat was discarded and the skimmed  
51  
52  
53 164 milk was diluted 10 times with the dilution buffer provided by the kit. The tissues  
54  
55 samples were extracted according to protocols previously reported (Haasnoot et al.  
56  
57 166 1999; Brown et al. 1988; Fox 1989), with some modifications. Briefly, to 1 g of tissue 4  
58  
59 ml of a 3% trichloroacetic acid solution (TCA) were added. After homogenization for 1  
60

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3 168 minute, the sample was mixed head over head for 30 minutes, then centrifuged 10  
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5 minutes at 2000g at 4°C. The extracts were diluted 2 times with dilution buffer and pH  
6  
7  
8 170 was adjusted to 7.4 with a 0.1M NaOH solution.  
9

## 172 **Results and discussion**

### 173 *Immunoassays performance*

174 *I'screen Gentamicin*. Figure 1 shows calibration curves obtained (10 runs) for the  
175 gentamicin enzyme immunoassay, i.e. the buffer and the milk calibration curves. In the  
176 preliminary work carried out during the development of the assay, no matrix effect was  
177 observed for tissue samples when analysed in respect to a calibration curve made from  
178 gentamicin in buffer solutions (data not shown), obtaining a specificity of 100% for  
179 negative tissue samples. On the contrary, the spiking of milk samples using a buffer  
180 calibration curve determined an over-estimation of the analyte content because of  
181 matrix interference. Therefore, in order to compensate for matrix effects and to achieve  
182 higher specificity, it was necessary to provide two different calibration curves. Table I  
183 summarizes the parameters of these calibration curves; limits of detection (LOD),  
184 calculated as the zero standard OD minus 3 standard deviations (SD), were so low that  
185 spiking ranges could start at a few ng/ml. Slopes and R<sup>2</sup> values show a high quality of  
186 calibration. The low values for standard deviations of LOD, IC<sub>50</sub> and R<sup>2</sup> show the high  
187 repeatability of assay results. [Insert Figure 1 and Table I about here]

188

189 According to European Commission Regulation No 2377/90 and followings (EC No  
190 2377/90; EC No 1960/2000; EC No 868/2002), maximum residue limits of gentamicin  
191 (MRL) are 50 µg kg<sup>-1</sup> for muscle, 750 µg kg<sup>-1</sup> for kidney and 100 µg L<sup>-1</sup> for milk. On  
192 the basis of LODs reported in Table I, the calibration ranges chosen were 2.5-250 µg L<sup>-1</sup>

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3 for the buffer calibration curve and 5-250  $\mu\text{g L}^{-1}$  for the milk calibration curve. Since  
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6 194 sample preparation procedures determine a ten folds dilution factor, these calibration  
7  
8 curves allow the analysis of samples contaminated with amounts of gentamicin in the  
9  
10  
11 196 range of 25-2500  $\mu\text{g kg}^{-1}$  for tissue and 50-2500  $\mu\text{g L}^{-1}$  for milk. Therefore  
12  
13 contamination levels around MRLs can be easily detected without the need of excessive  
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15  
16 198 sample dilution, providing an accurate measurement.

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19  
20 200 The intra-assay precision of *I'screen Gentamicin* (calculated as Coefficient of Variation  
21  
22 %, CV) was obtained testing three duplicates of buffer and milk standard solutions. CVs  
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24  
25 202 of the mean absorbances were always less than 5%. The intra-assay and inter-assay dose  
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27 CVs, evaluated in three runs, were below 10% in between 5 and 100  $\mu\text{g L}^{-1}$ , both for  
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29  
30 204 milk and buffer standard solutions.

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34 206 The gentamicin EIA is highly specific, since cross reactivities against other  
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36 aminoglycosides (neomycin, streptomycin, kanamycin) are below 0.1%. It is stable after  
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39 208 storage at +4°C for 1 year, since after incubation of the kit at 37°C for 1 week, assay  
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41 performances (OD values and calibration curve  $\text{IC}_{50}$ ) are not significantly changed (data  
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43  
44 210 not shown) (Deshpande 1996).

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49 212 *I'screen Neomycin*. Figure 2 shows buffer and milk calibration curves obtained for  
50  
51 neomycin assay (mean of 10 runs) and Table II summarizes their parameters. As for the  
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53  
54 214 gentamicin assay, the milk calibration curve is necessary to compensate matrix effect of  
55  
56 milk samples, while tissue samples can be analysed using a buffer calibration curve.  
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58  
59 216 The values obtained for LODs,  $\text{IC}_{50}$ , slope and  $R^2$  show also for this assay a high quality  
60  
of calibration, as well as a high repeatability of results in case of buffer calibration,

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4 218 while milk calibration shows similar sensitivity but lower repeatability. [Insert Figure 2  
5  
6 and Table II about here]  
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9 220

10 According to EU Regulations (EC No 2377/90; EC No 1960/2000; EC No 1181/2002)  
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12  
13 222 MRL of neomycin are  $500 \mu\text{g kg}^{-1}$  for muscle,  $5000 \mu\text{g kg}^{-1}$  for kidney and  $1500 \mu\text{g L}^{-1}$   
14  
15 for milk. On the basis of LODs reported in Table II, the calibration ranges chosen were  
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17  
18 224 10-1000 ng/ml for the buffer calibration curve and  $50-1000 \mu\text{g L}^{-1}$  for the milk  
19  
20 calibration curve. Considering the ten folds dilution factor of samples (see  
21  
22  
23 226 experimental), these calibration curves allow the detection of amounts of neomycin in  
24  
25 the range of  $100-10\ 000 \mu\text{g kg}^{-1}$  in tissue and  $500-10\ 000 \mu\text{g L}^{-1}$  in milk. The use of two  
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28 228 different calibration curves allows therefore the identification of samples contaminated  
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30 with levels of antibiotic around MRLs; moreover, MRLs were in both cases in the linear  
31  
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33 230 part of the calibration curves.  
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36

37 232 The intra-assay precision of *I'screen Neomycin* was determined testing the standard  
38  
39 solutions with three duplicates. CVs of the mean absorbance were always  $< 10\%$ . The  
40  
41  
42 234 intra-assay dose CV testing buffer standard solutions was below  $10\%$  in between 10 and  
43  
44 100 ng/ml. The intra-assay dose CV testing milk standard solutions was below  $5\%$  in  
45  
46  
47 236 between 100 and  $500 \mu\text{g L}^{-1}$ . Dose inter-assay CVs, evaluated in three different  
48  
49 experiments, were less than  $15\%$  for buffer standard solutions and less than  $20\%$  for  
50  
51  
52 238 milk standard solutions.  
53  
54  
55

56 240 The specificity of the assay was studied by testing other aminoglycoside antibiotics  
57  
58 (gentamicin, streptomycin, kanamycin): cross-reactivities were in all cases below  $0.1\%$ .  
59  
60

242 The stability of the reagents was evaluated incubating the test kit at  $37^\circ\text{C}$  for one week:

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2  
3 neither the OD values, nor the calibration curve  $IC_{50}$  were significantly different from  $t_0$ ,  
4  
5  
6 244 meaning that the kit is stable at  $+4^{\circ}C$  after 1 year storage (data not shown) (Deshpande  
7  
8 1996).

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### 13 *Assays Validation*

15 248 According to European Decision 657/2002, for the validation of *I'screen Gentamicin*  
16  
17 and *I'screen Neomycin* the following performance characteristics were determined:  
18  
19  
20 250 Decision Limit ( $CC\alpha$ ), specificity, Detection Capability ( $CC\beta$ ), recovery, precision, and  
21  
22 ruggedness.  
23

252

27 *Milk samples.* According to EU regulations, a screening method for the detection of  
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29  
30 254 veterinary drug residues must guarantee 5% or less false negative results  
31  
32 (2002/657/EC). To be cost effective, this method must guarantee that a food sample  
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34 256 containing residues at a concentration lower than the MRL should be classified as  
35  
36 “negative” (compliant). The cut-off values, i.e. the Limit of Decision ( $CC\alpha$ ), should be  
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38  
39 258 established taking into account both requirements. In order to keep the false non-  
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41 compliant occurrence at a low rate,  $CC\alpha$  values were determined as the  $B/B_0$  value of  
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43  
44 260 MRL spiked samples + 1.64 SD.

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48  
49 262 To calculate the probability of false positive and false negative results, 20 blank  
50  
51 samples were employed; these samples were fortified at MRL concentrations ( $100 \mu g$   
52  
53  
54 264  $kg^{-1}$  of gentamicin and  $1500 \mu g kg^{-1}$  of neomycin), as well as at lower concentrations.

55  
56 Table III shows  $B/B_0\%$  values for blank and fortified samples, false compliant rates ( $\beta$   
57  
58  
59 266 errors) and false non-compliant rates ( $\alpha$  errors). [Insert Table III about here]

60

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4 268 At the established Limit of Decision, in *I'screen Gentamicin* no false non-compliant  
5  
6 sample was obtained (0%  $\alpha$  error) evaluating the blank milk samples, as well as spiked  
7  
8  
9 270 at 33  $\mu\text{g L}^{-1}$  (0.33 MRL) and at 50  $\mu\text{g L}^{-1}$  (0.5 MRL), indicating an assay specificity of  
10  
11 100%.

12  
13 272

14  
15 Regarding *I'screen Neomycin*, no false non-compliant were obtained testing blanks or  
16  
17  
18 274 0.33 MRL spiked samples, but the specificity decreased with samples fortified at 0.66  
19  
20 MRL, generating 50% of false non-compliant results.  
21

22  
23 276

24  
25 Sensitivity evaluated at MRL contamination values for both kits was 95% (5%  $\beta$  error).  
26  
27  
28 278 The detection capabilities (CC $\beta$ ) were therefore 100  $\mu\text{g kg}^{-1}$  for *I'screen Gentamicin*  
29  
30 and 1500  $\mu\text{g kg}^{-1}$  for *I'screen Neomycin*. It can be concluded that both assays had a  
31  
32  
33 280 good diagnostic specificity and a high sensitivity at the MRL.

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37 282 Accuracy was studied by fortification of 20 blank milk samples with four  
38  
39 concentrations of both analytes above and below respective MRLs (50, 100, 250 and  
40  
41  
42 284 500  $\mu\text{g L}^{-1}$  of gentamicin and 500, 100, 1500 and 2000  $\mu\text{g L}^{-1}$  of neomycin). Results are  
43  
44 shown in Figure 3. The correlation obtained between spiked and measured  
45  
46  
47 286 concentrations was quite good for gentamicin ( $r^2 = 0.979$ ,  $S_{y.x} = 32.49 \mu\text{g L}^{-1}$ ), while it  
48  
49 was weaker for neomycin ( $r^2 = 0.710$ ,  $S_{y.x} = 398.9 \mu\text{g L}^{-1}$ ). The slope of the linear  
50  
51  
52 288 regression was, on the opposite, close to 1 in case of neomycin (1.10), higher in case of  
53  
54 gentamicin (1.27). [Insert Figure 3 about here]  
55

56  
57 290

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59 Mean recovery at the MRL was 128 $\pm$ 14% for gentamicin and 132 $\pm$ 22% for neomycin.

60  
292 Both assays overestimated the true content of analyte, particularly in the lower part of

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2  
3 dosing ranges, but the bias was higher in case of neomycin: the mean recoveries at each  
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6 294 spiking ranged in fact from 128% to 152% for gentamicin, and from 132% to 201% for  
7  
8 neomycin.

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11  
12 *I'screen Gentamicin* precision was calculated as Coefficient of Variation % (CV) of the  
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14  
15 298 intra- (n=3 duplicates) and inter-assay measurements (n=3 days) of a blank sample  
16  
17 fortified with amounts of analyte corresponding to 0.5 MRL, MRL, and 5 MRL (50,  
18  
19 300 100 and 500  $\mu\text{g kg}^{-1}$ ). The intra-assay CVs were all below 7% (4.90, 6.84 and 0.25%,  
20  
21 respectively). The inter-assay CVs were all below 8% (7.22, 0.45 and 3.57%,  
22  
23  
24 302 respectively).

25  
26  
27  
28  
29 304 In the same way was calculated *I'screen Neomycin* precision. A blank sample fortified  
30  
31 with amounts of analyte at 0.66 MRL and MRL levels (1000 and 1500  $\mu\text{g kg}^{-1}$ ) was  
32  
33  
34 306 tested. The intra-assay CVs were always below 5% (3.50 and 1.93, respectively); inter-  
35  
36 assay CVs were 22.99 for and 18.74 % respectively.

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41 *Tissue samples.* A successful tissue extraction method should make the tissue-bound  
42  
43 310 aminoglycosides soluble, remove most of the proteins, eliminate other matrix  
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45 interferences and provide satisfactory and reproducible recoveries. Homogenization of  
46  
47 312 samples with TCA solutions is commonly used to precipitate tissue proteins and to  
48  
49 obtain high recoveries of analytes. This method was applied in the analysis of twenty  
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52 314 blank muscle samples (ten bovine and ten swine).

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57 316 The limits of decision and the detection capabilities were established as for milk testing.

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59  
60 Table IV shows B/B<sub>0</sub> %, and  $\alpha$  and  $\beta$  errors for both kits. Evaluating the blank muscle

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3 318 samples by the established limits of decision, no false non-compliant occurred by both  
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5 immunoassays (0%  $\alpha$  error). No false non-compliant incurred even testing blanks  
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9 320 spiked at 0.25 MRL (12.5  $\mu\text{g kg}^{-1}$  of gentamicin and 125  $\mu\text{g kg}^{-1}$  of neomycin) by both  
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11 kits. The false compliant rate ( $\beta$  error) was 0% testing the samples spiked at the MRLs  
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14 322 with both test kits, indicating a 100% sensitivity at the MRLs. Considering the results  
15  
16 shown, detection capabilities ( $\text{CC}\beta$ ) were 50  $\mu\text{g kg}^{-1}$  for *I'screen Gentamicin*, and 500  
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18 324  $\mu\text{g kg}^{-1}$  for *I'screen Neomycin*. [Insert Table IV about here]

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23 326 Accuracy in tissue testing was evaluated in the range 100-1000  $\mu\text{g kg}^{-1}$  for neomycin  
24  
25 and in the range 50-500  $\mu\text{g kg}^{-1}$  for gentamicin. The correlations between spiked and  
26  
27 measured concentration were quite good for both analytes ( $r^2 > 0.95$ ). The slope was  
28  
29 close to 1 in case of gentamicin, lower in case of neomycin (see Figure 4).  $S_{y,x}$  values  
30  
31 were also quite low: 27.78 and 46.66  $\mu\text{g kg}^{-1}$  for gentamicin and neomycin,  
32  
33 330 respectively. In case of gentamicin the recovery at the MRL was 160 $\pm$ 40%, in case of  
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37 332 neomycin it was 94  $\pm$  9%. [Insert Figure 4 about here]

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42 334 The precision of *I'screen Gentamicin* and *I'screen Neomycin* assays in tissue testing  
43  
44 was studied analysing three times the same fortified muscle sample. Gentamicin was  
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46 spiked at MRL (50  $\mu\text{g kg}^{-1}$ ) and 2 MRL (100  $\mu\text{g kg}^{-1}$ ), obtaining intra assay CVs (n=3  
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48 336 duplicates) of 5.35 and 6.84%, respectively and inter-assay CVs (n=3 days) of 12.33  
49  
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52 338 and 14.56%, respectively. In the same way neomycin was spiked at MRL (500  $\mu\text{g kg}^{-1}$ )  
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54 and 2 MRL (1000  $\mu\text{g kg}^{-1}$ ): intra-assay CVs were 10.44 and 5.51%, respectively and  
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57 340 inter-assay CVs were 16.39 and 15.58%, respectively.



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3 342 *Ruggedness. I'screen Gentamicin* and *I'screen Neomycin* ruggedness was determined  
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5 using the Youden's approach (Youden and Steiner 1975; 2002/657/EC). The method  
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8 344 implies the deliberate introduction of minor reasonable simultaneous variations of  
9  
10 parameters in the test and the observation of their consequences. In both tests, variations  
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13 346 in seven parameters in respect to established procedure were introduced: the enzyme  
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15 conjugate dilution factor, the enzyme conjugate batch, the assay temperature, the assay  
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18 348 incubation time, the development incubation time, the number of washings and the kit  
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20 storage temperature. According to Youden's approach, the effect of each variation was  
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23 350 determined by a set of eight combinations, where the parameters were alternated in their  
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25 "control mode" (according to the established procedure) and "changed mode". For each  
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27 352 parameter, the difference between the mean result obtained in the "control mode" and  
28  
29 the mean result obtained in the "changed mode" was determined ( $D_i$ ). The analyses  
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32 354 were carried out with a muscle sample, spiked with  $100 \mu\text{g kg}^{-1}$  of gentamicin and  $1000$   
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34  $\mu\text{g kg}^{-1}$  of neomycin. As shown in Table V, the effect of each parameter is given by the  
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36  
37 356 calculated difference  $D_i$ . The standard deviation of the differences ( $S_{D_i}$ ) for each set of  
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39 experiments was calculated by the formula:  
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INSERT  $S_{D_i}$  FORMULA HERE

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49 When the calculated value for the standard deviation of the differences is significantly  
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52 362 larger than the inter-assay precision, the test is considered not to be robust. Furthermore,  
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54 the application of a  $t$ -test for each variable gives the opportunity to identify the most  
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57 364 disturbing variation factors (Scortichini et al. 2005); the experimental  $t$  was calculated  
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59 by the formula:  
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INSERT  $t$  FORMULA HERE

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where  $n$  ( $n=4$ ) is the number of experiments carried out for each parameter in the  
“control mode” or in the “changed mode”, and  $CV$  is the inter-assay precision ( $n=9$ )  
(Forti et al. 2005).

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As shown in Table V, the standard deviations of the differences (10.79% for *I'screen*  
*Gentamicin* and 14.70% for *I'screen Neomycin*) were lower than the inter-assay  
precisions obtained for the spiked level considered (14.56% and 15.58% respectively),  
indicating that both EIAs are robust. Moreover, experimental  $t$  values were always  
lower than the two-tailed  $t$  critical value ( $t_{crit}=2.3$ ,  $v=9-1$ , 95% confidence level).

378 [Insert Table V about here]

From the results obtained it can be concluded that none of the changes introduced in the  
considered parameters negatively influenced the results of the tests; however, results  
shown in Table V indicate that kits storage temperature is the most critical factor for the  
performance of *I'screen Gentamicin* and *I'screen Neomycin* and should be strictly  
controlled.

## 386 **Conclusions**

Immunoassays previously reported in the literature for neomycin and gentamicin  
detection in milk and meat did not provide data to estimate the percentage of false  
positive and false negative results at or close to the MRL (Haasnoot et al. 1999;  
Loomans et al. 2003). It has been shown that commercially available kits are easy to use  
and very sensitive, but quite inaccurate (Jànosì et al. 2004). Moreover, the approach

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3 392 employed by test kit manufacturers was not adequate for regulatory controls of  
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5 registered antimicrobials. In order to determine the appropriate decision limits to be  
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8 394 applied, as far as we are aware no validation study was performed. In accordance with  
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10 the European Decision 657/2002, screening methods have to be sensitive enough to  
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13 396 detect 95% of non-compliant samples, but, on the other hand, it is also necessary that  
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15 false positive rates are kept low. Without an appropriate limit of decision, the rate of  
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18 398 false non-compliant samples could be excessive and therefore a high number of  
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20 confirmatory analyses would be required. Confirmatory analyses for aminoglycosides  
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22 400 are quite expensive and a very small number of laboratories are accredited to perform it.  
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27 402 From the results presented, we can claim that the two *I'screen* test kits can satisfy both  
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29 the specificity and the sensitivity requirements. For milk, as well as for meat testing,  
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32 404  $CC\alpha$  have been established close enough to the EU MRLs to minimize false non-  
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34 compliant results. The  $\alpha$  error was actually very low even with food samples  
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36 406 contaminated at concentrations lower than violating levels. At the same time, violating  
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38 samples, contaminated at the MRL, have been correctly classified as non-compliant in  
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41 408 95% of milk samples, and in 100% of meat samples.  
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46 410 The use of two different calibration curves in the analysis of milk or tissue samples and  
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48 a moderate dilution factor contribute to the accuracy of the assays, which, at the MRL  
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51 412 level, was in the range between 94% and 160%.  
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56 414 Taking into account the matrices tested and the procedures of their preparation,  
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58 screening by the test kits here presented meets the quality criteria of EU Decision  
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60 416 657/2002, guaranteeing, at the same time, a low rate of false compliant results. The

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3 performance of these EIA kits indicates that they can be therefore used as easy and cost-  
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6 418 effective screening methods in the analysis of aminoglycosides in milk and tissue  
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8 samples.  
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### 12 **Acknowledgements**

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14  
15 422 AAFVG (Associazione Allevatori del Friuli Venezia Giulia, Codroipo, Italy), for  
16  
17 providing milk samples.  
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$$S_{D_i} = \sqrt{2 \times \sum \left( \frac{D_i^2}{7} \right)}$$

SDi formula to be inserted in the Manuscript at line n. 343



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$$t = \frac{\sqrt{n} \times |D_i|}{\sqrt{2} \times CV}$$

t formula to be inserted in the Manuscript at line 351

Table I  
Parameters of gentamicin EIA. Reported values are the mean  $\pm$  standard deviation relative to 10 calibration curves run on different days

Parameter	Buffer calibration curve	Milk calibration curve
OD max (zero st.)	1.32 $\pm$ 0.23	1.19 $\pm$ 0.21
LOD <sup>a</sup> , ng ml <sup>-1</sup>	3.02 $\pm$ 0.08	6.53 $\pm$ 0.11
IC <sub>80</sub> , ng ml <sup>-1</sup>	8.17 $\pm$ 1.18	12.32 $\pm$ 2.17
IC <sub>50</sub> , ng ml <sup>-1</sup>	36.60 $\pm$ 8.75	64.16 $\pm$ 19.26
slope	- 0.953 $\pm$ 0.117	- 0.774 $\pm$ 0.143
R <sup>2</sup>	0.9995 $\pm$ 0.0005	0.9990 $\pm$ 0.0006

<sup>a</sup> LOD: zero standard OD – 3 standard deviations

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Table II

Parameters of neomycin EIA. Reported values are the mean  $\pm$  standard deviation relative to 10 calibration curves run on different days

Parameter	Buffer calibration curve	Milk calibration curve
OD max (zero st.)	1.13 $\pm$ 0.19	1.54 $\pm$ 0.14
LOD, ng ml <sup>-1</sup>	10.20 $\pm$ 0.40	48.47 $\pm$ 3.32
IC <sub>80</sub> , ng ml <sup>-1</sup>	22.54 $\pm$ 1.67	91.35 $\pm$ 19.35
IC <sub>50</sub> , ng ml <sup>-1</sup>	96.53 $\pm$ 10.80	334.07 $\pm$ 80.67
slope	- 0.925 $\pm$ 0.088	- 0.971 $\pm$ 0.242
R <sup>2</sup>	0.9998 $\pm$ 0.0003	0.9988 $\pm$ 0.0014

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Table III

B/B<sub>0</sub> values of blank and spiked milk samples (n = 20)

parameter	sample	<i>I'</i> screen Gentamicin	<i>I'</i> screen Neomycin
B/B <sub>0</sub> (%)	blanks	106.95 ± 4.11	96.16 ± 2.82
	0.33 MRL	93.81 ± 3,92	81.60 ± 3.58
	0.5 MRL	85.77 ± 3.70	ND <sup>a</sup>
	0.66 MRL	ND <sup>a</sup>	69.86 ± 3.25
	MRL	76.63 ± 2.53	63.17 ± 4.54
	CC $\alpha$	80.78	70.61
$\beta$ error (%)	MRL	5	5
	blanks	0	0
$\alpha$ error (%)	0.33 MRL	0	0
	0.5 MRL	0	ND <sup>a</sup>
	0.66 MRL	ND <sup>a</sup>	50

<sup>a</sup> ND: not determined

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Table IV

B/B<sub>0</sub> values of blank and spiked muscle samples (n = 20)

Parameter	Sample	<i>I</i> 'screen Gentamicin	<i>I</i> 'screen Neomycin
	blanks	98.53 ± 2.61	93.39 ± 2.96
B/B <sub>0</sub> (%)	0.25 MRL	95.03 ± 2.40	82.64 ± 1.96
	MRL	84.04 ± 3.07	67.87 ± 2.07
	CC $\alpha$	89.07	71.26
$\beta$ error (%)	MRL	0	0
$\alpha$ error (%)	blanks	0	0
	0.25 MRL	0	0

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Table V  
Ruggedness test results

Parameter	<i>I'screen Gentamicin</i>		<i>I'screen Neomycin</i>	
	Difference (D <sub>i</sub> ) in % recovery (absolute value)	<i>t</i> - value	Difference (D <sub>i</sub> ) in % recovery (absolute value)	<i>t</i> - value
Enzyme conjugate dilution factor	8.30	1.05	9.93	0.90
Enzyme conjugate batch	4.44	0.56	12.45	1.13
Assay temperature	0.01	0.00	2.30	0.21
Assay incubation time	8.89	1.13	4.92	0.45
Development incubation time	2.61	0.33	8.55	0.78
Number of washings	3.01	0.38	10.73	0.98
Kit storage temperature	14.97	1.90	16.92	1.54
<i>S</i> <sub>D<sub>i</sub></sub>	10.79%		14.70%	

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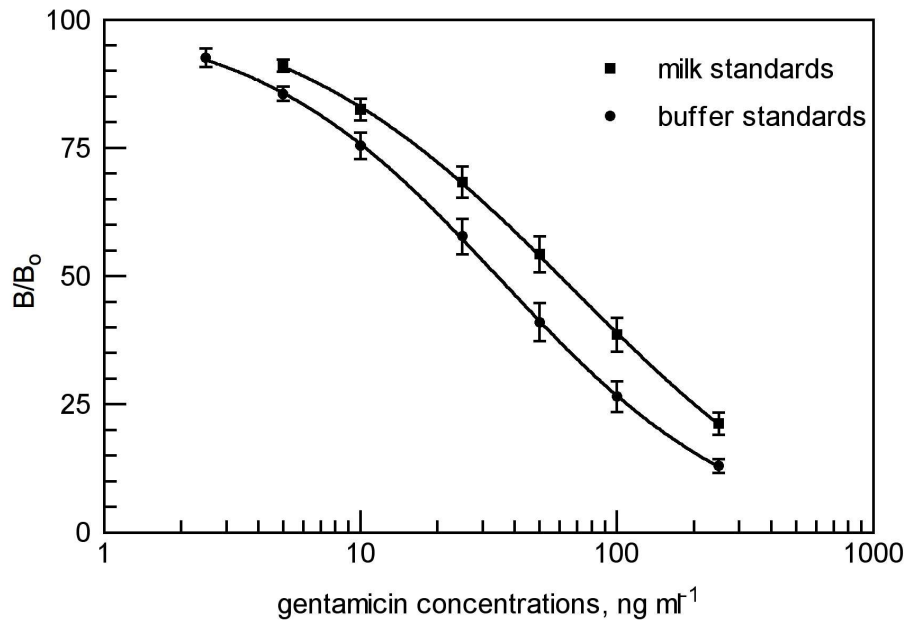


Figure 1. I'screen Gentamicin EIA calibration curves (mean  $\pm$  SD,  $n=10$ ). Two different calibration curves were applied depending on the samples: the milk standard curve in case of milk and the buffer standard curve in case of tissue.

79x55mm (600 x 600 DPI)

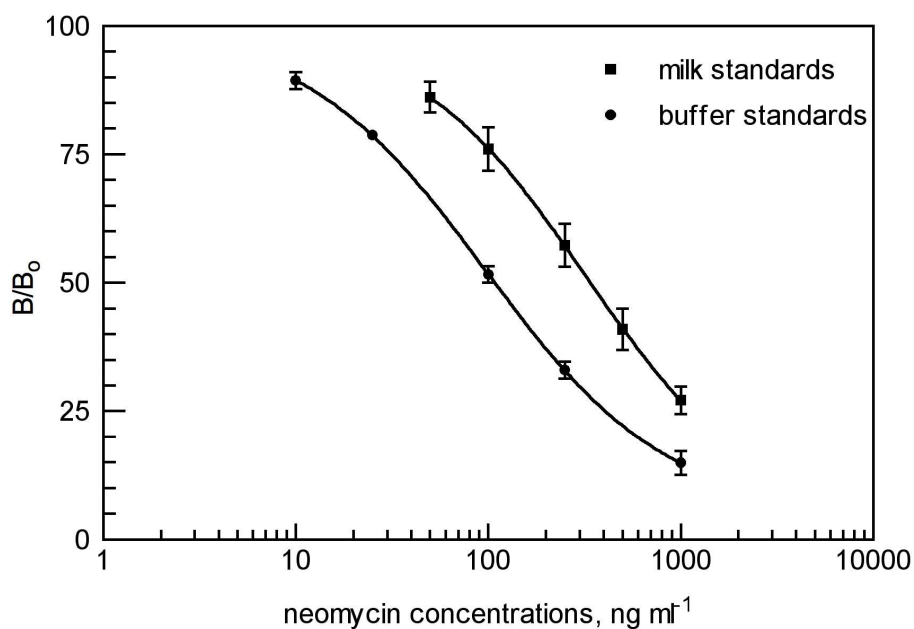


Figure 2. I'screen Neomycin EIA calibration curves (mean  $\pm$  SD,  $n=10$ ). Two different calibration curves were applied depending on the samples: the milk standard curve in case of milk and the buffer standard curve in case of tissue.

79x55mm (600 x 600 DPI)



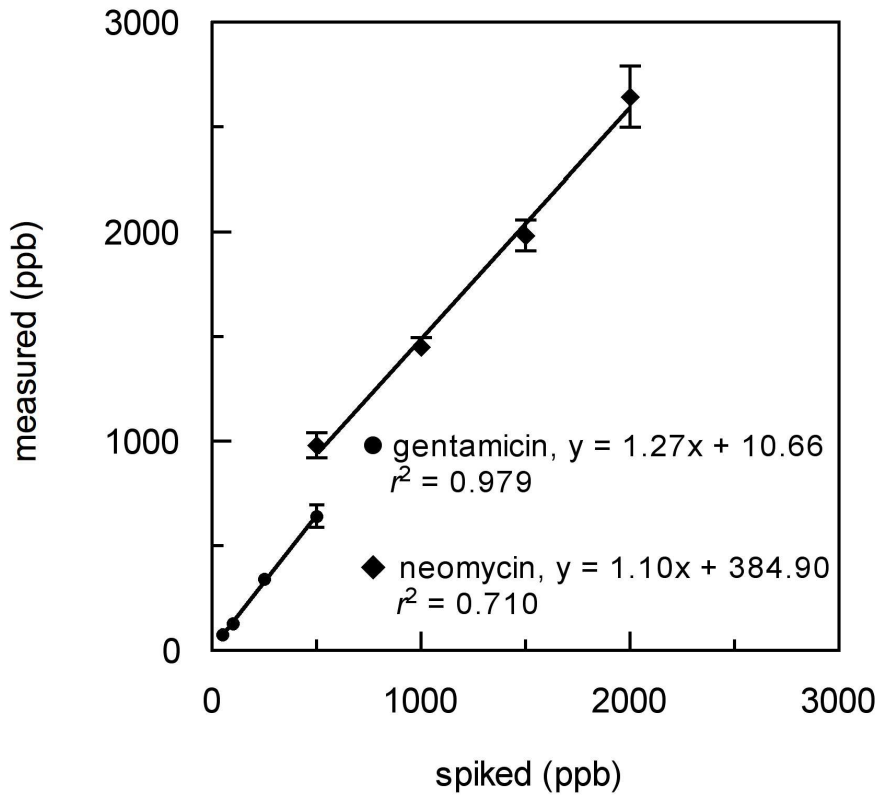


Figure 3. Recoveries studies to assess the accuracy of milk samples testing (mean  $\pm$  SD, n=20).  
81x72mm (600 x 600 DPI)

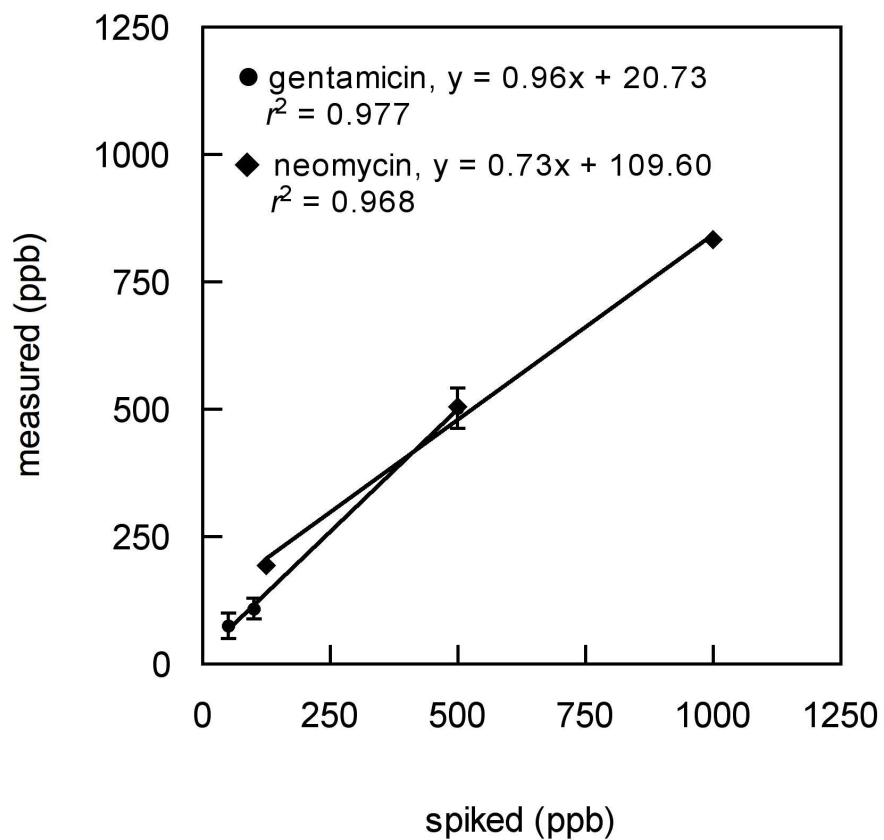


Figure 4. Recoveries studies to assess the accuracy of muscle samples testing (mean  $\pm$  SD, n=20).  
79x74mm (600 x 600 DPI)

**Figure Captions**

Figure 1. *I'screen Gentamicin* EIA calibration curves (mean  $\pm$  SD, n=10).

Two different calibration curves were applied depending on the samples: the milk standard curve in case of milk and the buffer standard curve in case of tissue.

Figure 2. *I'screen Neomycin* EIA calibration curves (mean  $\pm$  SD, n=10).

Two different calibration curves were applied depending on the samples: the milk standard curve in case of milk and the buffer standard curve in case of tissue.

Figure 3. Recoveries studies to assess the accuracy of milk samples testing (mean  $\pm$  SD, n=20).

Figure 4. Recoveries studies to assess the accuracy of muscle samples testing (mean  $\pm$  SD, n=20).