



**HAL**  
open science

# Comparative study of 3 screening tests, ExplorerÒ test, PremiÒTest2 microbiological tube tests and a multi-sulphonamides ELISA kit, for the detection of antimicrobial and sulphonamide residues in eggs

Valerie Gaudin, Celine Hedou, Annie Rault, Pascal Sanders, Eric Verdon

## ► To cite this version:

Valerie Gaudin, Celine Hedou, Annie Rault, Pascal Sanders, Eric Verdon. Comparative study of 3 screening tests, ExplorerÒ test, PremiÒTest2 microbiological tube tests and a multi-sulphonamides ELISA kit, for the detection of antimicrobial and sulphonamide residues in eggs. *Food Additives and Contaminants*, 2009, 26 (04), pp.427-440. 10.1080/02652030802527626 . hal-00577344

**HAL Id: hal-00577344**

**<https://hal.science/hal-00577344>**

Submitted on 17 Mar 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**Comparative study of 3 screening tests, ExplorerÒ test, PremiÒTest2 microbiological tube tests and a multi-sulphonamides ELISA kit, for the detection of antimicrobial and sulphonamide residues in eggs**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-065.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	22-Sep-2008
Complete List of Authors:	Gaudin, Valerie; AFSSA, LERMVD Hedou, Celine; AFSSA, LERMVD Rault, Annie; AFSSA, LERMVD Sanders, Pascal; AFSSA, LERMVD Verdon, Eric; afssa, lermvd
Methods/Techniques:	In-house validation, Method validation, Screening - immunoassays, Screening - microbial screening
Additives/Contaminants:	Veterinary drug residues - antibiotics, Veterinary drug residues - antimicrobials, Veterinary drug residues - sulphonamides, Veterinary drugs
Food Types:	Eggs

SCHOLARONE™  
Manuscripts

1  
2 1 **Comparative study of three screening tests, two microbiological tube**  
3  
4  
5 2 **tests and a multi-sulphonamide ELISA kit, for the detection of**  
6  
7  
8 3 **antimicrobial and sulphonamide residues in eggs**  
9

10 4  
11  
12 5  
13  
14 6 V. GAUDIN\*, C. HEDOU, A. RAULT, P. SANDERS & E. VERDON  
15

16 7 *Community Reference Laboratory for Antimicrobial Residues, AFSSA Fougères – LERMVD, La*  
17

18 8 *Haute Marche – BP 90203, 35302 FOUGERES Cedex, France*  
19  
20  
21 9

22  
23 10  
24  
25 11  
26  
27 12 \*To whom correspondence should be addressed.  
28

29 13 e-mail: v.gaudin@fougeres.afssa.fr  
30  
31 14  
32  
33 15  
34  
35 16  
36  
37  
38 17  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 18 **Abstract**

3  
4 19 The screening of antimicrobial residues in eggs is especially an important subject. Three different  
5  
6 20 commercial kits for the screening of sulphonamides and other antimicrobials in eggs have been  
7  
8 21 validated in accordance with the decision 2002/657/EC (EC 2002): one ELISA kit multi-  
9  
10 22 sulphonamides (from RAISIO Diagnostics) and 2 microbiological tests (Premi®Test from DSM and  
11  
12 23 Explorer® kit from Zeu-Inmunotec). The false positive rates were lower than 2 % for all kits. The  
13  
14 24 detection capabilities ( $CC\beta$ ) have to be as low as possible for banned substances and lower than  
15  
16 25 the MRL when MRLs have been set. The sensitivity of the Premi®Test was better than that of  
17  
18 26 Explorer® test, probably because of the dilution of the eggs before Explorer® test. The  $CC\beta$  values  
19  
20 27 towards most of the tested sulphonamides were satisfactory with Premi®Test ( $\leq 100 \mu\text{g kg}^{-1}$ ). The  
21  
22 28 performance in a proficiency test for the detection of sulphonamides in eggs with Premi®Test  
23  
24 29 confirmed these results. The detection capability of tetracycline and doxycycline were at the level  
25  
26 30 of the MRL or twice the MRL maximum. The detection capabilities for chlortetracycline and  
27  
28 31 oxytetracycline were higher (4 to 6 times the MRL). The detection capabilities for amoxicillin,  
29  
30 32 neomycin, tylosin and erythromycin were lower than their respective MRLs. Detection capabilities  
31  
32 33 for sulphonamides were much lower for ELISA kit than for microbiological tests. The ELISA kit  
33  
34 34 (from RAISIO Diagnostics) could be recommended for the targeted screening of sulphonamides in  
35  
36 35 eggs. On the other hand, Explorer® test and Premi®Test could be used as wide screening tests  
37  
38 36 allowing to detection of most of the antimicrobial families.

39  
40  
41  
42  
43  
44 37  
45  
46 38 **Keywords:** *Validation, inhibition test, ELISA test, screening assay, egg samples.*

## 39 Introduction

40 Eggs are a staple food in the world for many populations providing a vital sources of animal  
41 protein. Antimicrobials are used as feed additives or in drinking water in poultry breeding to prevent  
42 animal diseases (enteric diseases) (Dahiya et al. 2006) or as growth promoting factors (Donoghue  
43 2003; Stolker et al. 2007). The use of antimicrobials in laying hens could result in the persistence  
44 of residues in eggs. Different studies of the excretion of tetracyclines and their epimers have been  
45 performed after oral medication of laying hens (Roudaut et al. 1989; Zurhelle et al. 2000; Donkova  
46 et al. 2005), macrolides (Roudaut et al. 1990); sulphonamides (Shaikh et al. 2000; Roudaut 2002;  
47 Shaikh et al. 2004), ampicillin (Donoghue et al. 1997). The conclusion was that residues of  
48 antimicrobials could persist in eggs several days after medication in drinking water. Furthermore,  
49 Kan (2000) reviewed several studies on drug residues in eggs and their distribution between yolk  
50 and white, after administration to laying hens. Some models were established to evaluate the  
51 persistence of antimicrobial residues in eggs. A sulfamethazine model was presented by Tansakul  
52 (2007) which demonstrated that even low doses of sulfamethazine could result in the presence of  
53 residues in eggs and therefore could present a risk for consumer safety. As a conclusion, nearly all  
54 veterinary drugs and feed additives available on the market may result in residues in eggs (Kan et  
55 al. 2007).

56  
57 Therefore, the detection of antimicrobial residues in eggs is especially of great interest both for its  
58 impact on public health. Moreover, the presence of residues could lead to bacterial resistance to  
59 different antimicrobials (i.e. streptomycin and tetracycline) (Adesiyun et al. 2007). A study  
60 conducted by the Canadian Food Inspection Agency (CFIA) reported the level of contamination of  
61 eggs with antimicrobials (Quon 2000). This study was conducted over a period of 2 years on both  
62 domestic and imported eggs. More than 99% of the samples screened were found to be free of any  
63 veterinary drug residue. The other samples contained tetracyclines, sulphonamides, ciprofloxacin,  
64 macrolides, and streptomycin. Another study conducted in Trinidad concluded that the  
65 antimicrobial residues detected in table eggs were particularly sulphonamides and then  
66 macrolides, tetracyclines, and beta-lactam (Adesiyun et al. 2005). Sulphonamides are still effective  
67 tools in the treatment of coccidiosis in broiler chicken and are widely used in intensive poultry

1  
2 68 farms. The Committee for Veterinary Medicinal Products recommended that the sulphonamides  
3  
4 69 are entered into Annex I of Council Regulation (EEC) 2377/90 (EC 1990). The MRL of  
5  
6 70 sulphonamides for meat and milk is 100 µg kg<sup>-1</sup>. However, there is no MRL set for sulphonamide in  
7  
8  
9 71 egg products. In fact, there are no sulphonamides and no beta-lactams approved for use in laying  
10  
11 72 hens. Among macrolides, tylosin is intended to be administered to laying hens, in the drinking  
12  
13 73 water or in premix formulations, for the treatment of respiratory diseases and necrotic enteritis.  
14  
15 74 Erythromycin is used for the treatment of chronic respiratory diseases due to mycoplasma in  
16  
17 75 poultry. In veterinary medicine, tetracyclines are allowed for medication of laying hens and used as  
18  
19 76 a broad spectrum antibiotic. Neomycin is used to treat bacterial gastrointestinal infections of poultry  
20  
21 77 by the oral route. The previously cited antimicrobials are authorised to be administered to laying  
22  
23 78 hens. The European Union has identified Maximum Residue Limits (MRL) for some molecules but  
24  
25  
26 79 very few antimicrobials have MRL in eggs (Table I).

27  
28 80 Insert Table I here.

29  
30 81 When the use of some antimicrobials is forbidden for all animal species (i.e. chloramphenicol), they  
31  
32 82 are entered in Annex IV of the Regulation EC 2377/90 (EC 1990). Furthermore, some  
33  
34 83 antimicrobials are forbidden to be administered to laying hens produced for human consumption  
35  
36 84 only (i.e. doxycycline, spectinomycin, enrofloxacin). However, other antimicrobials (i.e. penicillins,  
37  
38 85 sulphonamides) have no MRL set for eggs (thus a “zero” tolerance). Therefore, in both cases, the  
39  
40 86 detection capabilities of the methods have to be as low as possible as a consequence of no  
41  
42 87 authorisation of these antimicrobials in eggs and no MRL set for this product.  
43  
44  
45 88

46  
47 89 Due to the possible persistence of antimicrobial residues in eggs, the presence of antibiotics must  
48  
49 90 be controlled with relevant analytical methods to meet the EU regulations. There are very few  
50  
51 91 publications at this time describing screening methods for the detection of antimicrobial in eggs.  
52  
53 92 However, the screening step is the first step of the control and thus is an essential step. There is a  
54  
55 93 need of wide spectrum screening tests, quick, easy to use and cheap. Different screening tests  
56  
57 94 were developed for the screening of antimicrobials in eggs: microbiological plate tests (Kabir et al.  
58  
59 95 2004; Bendix et al. 2005; Pikkemaat et al. 2007), tube tests like Premi®Test (Hussein et al. 2005)  
60  
96 and Explorer® test, enzyme linked immunosorbent assay (ELISA) (Huet et al. 2006), receptor test

1  
2 97 (Lamar et al. 2007), dipstick-test for tetracyclines (Alfredsson et al. 2005), Charm II test ((Adesiyun  
3  
4 98 et al. 2005)) or physicochemical methods like HPLC (Horii et al. 1990; Herranz et al. 2007) and  
5  
6 99 LC/MS-MS methods (Wang et al. 2005; Heller et al. 2006; Stolker et al. 2007). To our knowledge,  
7  
8  
9 100 there is no scientific publication at this time describing the use of Explorer® test for the screening  
10  
11 101 of antimicrobials in eggs.

12  
13 102  
14  
15 103 An interlaboratory study was organised for the screening and the confirmation of different  
16  
17 104 sulphonamides in eggs by our laboratory as Community Reference Laboratory. On this occasion, a  
18  
19 105 study was conducted to evaluate the performances of 3 different commercial kits for their ability to  
20  
21 106 detect sulphonamides and other antimicrobials in eggs: one ELISA kit multi-sulphonamides (from  
22  
23 107 RAISIO Diagnostics, Diffchamb, Lyon, France) and 2 microbiological tests (Premi®Test from DSM,  
24  
25 108 Delft, The Netherlands and Explorer® kit from Zeu-Immunotec, Saragossa, Spain). Premi®Test  
26  
27 109 and Explorer® test are 2 commercial microbiological based tests. These kits, based on the  
28  
29 110 inhibition of bacterial growth (*Bacillus stearothermophilus* strain), contain a redox indicator that  
30  
31 111 changes colour in the absence of antibiotics in the sample. They are simple and fast methods for  
32  
33 112 the detection of antibiotics in fresh meat, eggs and feed samples. Technical guidelines and  
34  
35 113 performance criteria (e.g., detection capability  $CC\beta$ , selectivity and specificity) for residue control in  
36  
37 114 the framework of Directive 96/23/EC (EC 1996) are described in Commission Decision  
38  
39 115 2002/657/EC (EC 2002). This paper reports the validation of 3 kits for the detection of  
40  
41 116 sulphonamides and other antimicrobials in egg samples according to the Commission Decision  
42  
43 117 2002/657/EC (EC 2002).  
44  
45  
46  
47  
48  
49

## 50 119 **Materials and methods**

### 51 52 53 120 *Explorer® test*

54  
55  
56 121 Explorer® test is a commercial kit for detection of inhibitory substances in raw meat and eggs. The  
57  
58 122 test, a 96-well microtiter plate, is based on the inhibition of microbial growth. Each well contains  
59  
60 123 specific agar medium containing a redox indicator and spread with *Geobacillus stearothermophilus*  
124 spores.

1  
2 125  
3  
4 126 *Preparation of egg samples.* Whole eggs (white and egg yolk) were homogenised and diluted (v: v  
5  
6 127 1:3) with distilled water (2 g egg + 6 ml distilled water). The diluted sample was heated for 12 min  
7  
8  
9 128 at 100 ° C in a water bath and stirred vigorously 20-30 seconds to prevent clotting. The sample  
10  
11 129 was allowed to cool at room temperature.

12  
13 130  
14  
15 131 *Test procedure.* 100 µl of diluted egg was added to each well. The wells were sealed with a plastic  
16  
17 132 film and the microplate was pre-incubated at 65°C for 30 min in a block heater (Zeu-Inmunotec,  
18  
19 133 Spain) and the sample allowed to diffuse through the well (pre-diffusion). Afterwards the wells were  
20  
21  
22 134 washed by filling the wells up with distilled water, using a squeeze bottle. The washing step was  
23  
24 135 repeated 3 times to 4 times. The plate was emptied by turning the plate upside down on top of an  
25  
26 136 absorbent paper to remove the water in excess. NB: It should not remain traces of coagulated  
27  
28 137 egg!! The plate was sealed again with plastic wrap and incubated at 65°C until the negative control  
29  
30 138 sample has turned orange (approximately 3h-3h30min).

31  
32 139  
33  
34 140 *Readings and interpretation of Explorer® results.*

35  
36  
37 141 The photometric reading of microplates is based on the reading at 2 wavelengths (590 and 650  
38  
39 142 nm) on a microplate reader. The results were interpreted as the difference between the values of  
40  
41 143 the two readings. On each day of validation, one negative control was analysed and also one  
42  
43 144 positive control (penicillin G at 25 µg kg<sup>-1</sup>) (Quality Control QC). The plate is ready to be read when  
44  
45 145 the result for the negative control sample (difference of absorbance NA590nm - NA650 nm) is  
46  
47  
48 146 between 0.15 and 0.25 OD (optical density units), where NA: Negative control absorbance. A  
49  
50 147 sample is declared positive when: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15,  
51  
52 148 where : SA: Sample absorbance.

53  
54 149  
55  
56 150 One single day of validation is presented as an example of interpretation. The result of each  
57  
58 151 unknown sample (SA = Sample Absorbance) is compared to the line of negative control sample  
59  
60 152 absorbance (NA) + 0.15. When: SA 590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15, the



1  
2 153 unknown sample was declared positive. An example of interpretation of quantitative results  
3  
4 154 (Optical Density) is presented on Figure 1.

5  
6 155 Insert figure 1

7  
8 156

9  
10  
11 157 *Premi® test*

12  
13 158 Premi®Test is a commercial test for the screening of antimicrobial residues in fresh meat as well  
14  
15  
16 159 as in fish and eggs. Premi®Test gives a result in less than four hours.

17  
18 160 *Preparation of egg samples.* Egg sample was homogenised (white and yolk).

19  
20 161 *Test procedure.* A syringe with a set volume of 100 µl is supplied with the kit. 100 µl of  
21  
22 162 homogenized egg fluid was transferred onto the agar in the ampoule, by pressing the syringe and  
23  
24 163 releasing it. The ampoule is closed with the plastic foil supplied with the kit. The ampoule(s) was  
25  
26 164 placed in a water bath at 80°C for 10 minutes. After this heat pre-treatment, the ampoules were  
27  
28  
29 165 incubated at the required temperature of 64°C (± 0,5 °C). The ampoules were incubated until the  
30  
31 166 negative control (egg fluid what has been tested before negative) changed colour (turned yellow).

32  
33 167 *Readings and interpretation of Premi®Test results.* When the negative control changes colour from  
34  
35 168 purple to yellow (approx. 3 hours), the results could be read from the bottom 2/3 part of the  
36  
37 169 ampoule. A clear colour change purple to yellow indicates that the antimicrobial compounds were  
38  
39  
40 170 below the Premi®Test detection limits. A purple colour indicates the presence of antibiotics at or  
41  
42 171 above the detection limits of the Premi®Test.

43  
44 172  
45  
46 173 *Multi-sulphonamides ELISA kit (RAISIO Diagnostics)*

47  
48  
49 174 This commercial multi-sulphonamide test is a competitive enzyme immunoassay (EIA) for  
50  
51 175 quantitative screening of tissue, milk, honey, egg and urine samples. The method should detect a  
52  
53 176 wide range of sulphonamides. It is based on monoclonal antibodies (21C7) raised against protein  
54  
55 177 bound sulfamethazine. The manufacturer claimed that it offers high sensitivity for sulfamethazine,  
56  
57 178 sulfadiazine, sulfamerazine, sulfisoxazole, sulfachloropyridazine and sulfachloropyrazine. This kit  
58  
59 179 is suitable for screening tissue, egg, milk, honey and urine for sulphonamides.

1  
2 180 *Preparation of egg samples.* Egg sample was homogenised (white and yolk). 1 g was weighted  
3  
4 181 into a clean tube. 5 ml of ethyl acetate were added and thoroughly mixed with a vortex, followed by  
5  
6 182 mixing head over head for at least half an hour. After centrifugation (5 min., 2000xg), 1 ml of  
7  
8 183 supernatant (ethyl acetate) was pipetted into a clean tube and evaporated to dryness under a mild  
9  
10 184 stream of nitrogen at 50°C. The residue was dissolved in 1 ml of phosphate buffered saline (PBS\*).  
11  
12 185 Then 1 ml of iso-octane/trichloromethane (2:3; v/v) was added and mixed using a vortex. After  
13  
14 186 centrifugation (5 min., 2000xg), 100 µl of supernatant was pipetted into a clean tube and 300 µl of  
15  
16 187 PBS\* was added. Finally 50 µl portions were used for ELISA.

17  
18  
19 188 \*PBS is not provided in the kit. The buffer is prepared as follows: Na<sub>2</sub>HPO<sub>4</sub> 0.77 g, KH<sub>2</sub>PO<sub>4</sub> 0.18  
20  
21 189 g, NaCl 8.94 g, distilled water qsp. 1000 ml. pH 7.4 ± 0.2

22  
23  
24 190 *Test procedure.* 100 µl of the zero standard in duplicate (wells A1, A2, blank), 50 µl of the zero  
25  
26 191 standard in duplicate (wells B1, B2, maximal signal), 50 µl of each of the sulfamethazine standard  
27  
28 192 solutions in duplicate (wells C1,2 to H1,2 i.e. 0.125, 0.25, 0.5, 0.8, 2.5 and 5 ng ml<sup>-1</sup>) and 50 µl of  
29  
30 193 each extracted sample in duplicate were pipetted. Then 25 µl conjugate (sulphonamide-HRPO)  
31  
32 194 and 25 µl antibody solution were added to all wells, except wells A1 and A2. The microtiter plate  
33  
34 195 was sealed and shaken for a few seconds. The plate was incubated for 1 hour in the dark between  
35  
36 196 +2°C and +8°C). The microtiter plate was washed 3 times with rinsing buffer. Then 100 µl  
37  
38 197 substrate/chromogen solution was added to all wells. The plate was again incubated, 30 min at  
39  
40 198 room temperature in the dark. Finally the reaction was sopped by adding 100 µl of stop solution to  
41  
42 199 each well.  
43  
44

45 200 *Readings and interpretation of ELISA Test results.* Immediately after adding the stop solution, the  
46  
47 201 absorbance values at 450 nm was read. The values (% maximal absorbance) calculated for the  
48  
49 202 standards are plotted (on the Y-axis) versus the sulphonamide equivalent concentration (ng ml<sup>-1</sup>)  
50  
51 203 on a logarithmic x-axis. The amount of sulphonamide in the samples is expressed as  
52  
53 204 sulphonamide equivalents. The sulphonamide equivalents in the extracts (ng ml<sup>-1</sup>) corresponding  
54  
55 205 to the % maximal absorbance of each extract can be read from the calibration curve.  
56  
57

58 206  
59  
60 207 *Quality controls (QC)*

1  
2 208 As it was strongly recommended by the manufacturer ((Premi®Test, DSM and Explorer®, Zeu-  
3  
4 209 Immunotec), a negative control sample (antibiotic-free egg) was used to determine the optimal  
5  
6 210 incubation time of each assay. Moreover, a positive sample (egg sample spiked with high  
7  
8 211 concentration of antibiotic, ie. Penicillin G 25 µg kg<sup>-1</sup>) was tested at each day of validation to check  
9  
10  
11 212 the performance of the kit.

### 13 14 213 *Validation*

15  
16 214 *Specificity and false positive rate.* To determine these parameters, at least 20 egg samples from  
17  
18 215 different origins were analysed in blind duplicate for each of the 3 kits. Finally, even more than 20  
19  
20 216 blank egg samples were analysed with Premi®Test (33 blank egg samples) and with Explorer®  
21  
22 217 test (55 blank egg samples) (1 to 3 were analysed at each day of validation). However, only 12  
23  
24 218 blank samples have been analysed at the end with the ELISA kit. In practice, some experiments  
25  
26 219 failed because of stability issues of the conjugate. The instability of the conjugate was observed  
27  
28 220 when some days the optical density of the blank standard was lower than 0.6 OD, which was the  
29  
30 221 validity limit for the kit. For this reason, the number of negative samples that could be analysed  
31  
32 222 should be reduced to 12 samples only.

33  
34 223  
35  
36 224 *Detection capabilities.* The concentration level, where only ≤ 5 % false compliant results remain,  
37  
38 225 equals the detection capability of the method (EC 2002).

39  
40 226 A preliminary study allowed us to choose for all the 3 kits, the target concentrations for each  
41  
42 227 sulphonamide and each antimicrobial. It is the least detectable concentration in eggs. Then, at  
43  
44 228 least 20 egg samples were spiked for each sulphonamide and each antimicrobial, for at least one  
45  
46 229 concentration level (MRL or claimed detection capability). 20 analyses have to be carried out in  
47  
48 230 order to ensure a reliable basis for this determination. These samples were analysed in blind  
49  
50 231 duplicate with each test.

51 232  
52  
53 233 The RAISIO Diagnostics ELISA kit was a multi-sulphonamides ELISA kit which was not able to  
54  
55 234 detect other antimicrobial residues. Therefore only sulphonamides have been tested for their  
56  
57 235 detection by the ELISA kit.

1  
2 236  
3  
4 237 *Selection of the antimicrobial compounds and concentrations.* The sulphonamides (sulfamethazine  
5  
6 238 (SMZ), sulfadiazine (SDZ), sulfamethoxazole, sulfadoxine, sulfamerazine, sulfadimethoxine,  
7  
8 239 sulfisoxazole, sulfachloropyridazine, sulfamethizole, sulfathiazol) and the tested concentrations  
9  
10 240 were selected by the CRL according to the data given by the 3 manufacturers on detection limits.  
11  
12 241 The other antimicrobials were chosen because some of them have MRL in eggs (chlortetracycline  
13  
14 242 (CTC), oxytetracycline (OTC), tetracycline (TTC), erythromycin, tylosin and neomycin). The 2  
15  
16 243 others (doxycycline, amoxicillin) were selected because they are from the main families of  
17  
18 244 antimicrobials (Table II).  
19  
20

21  
22 245 Insert Table II here  
23

24 246 For preparation of antibiotic stock solutions, antibiotic standards with known purity were used (from  
25  
26 247 Sigma Aldrich, France; Cluzeau InfoLabo, France). The stock solutions were diluted with distilled  
27  
28 248 water to prepare spiked samples.  
29

30 249  
31  
32 250 *Cross-reactivities.* The percentages of cross-reactivities between SMZ and other sulphonamides  
33  
34 251 were only calculated for the multi-sulphonamides ELISA kit when determining the detection  
35  
36 252 capability  $CC_{\beta}$ . The cross-reactivity percentages (%) will be compared with those claimed by the  
37  
38 253 manufacturer.  
39  
40

41 254  
42  
43 255 *Precision.* The precision was determined as it is written in the European Decision EC/2002/657  
44  
45 256 (EC 2002) for the RAISIO Diagnostics ELISA kit which was the only quantitative test. In this  
46  
47 257 decision, the precision has to be determined for quantitative screening tests. For authorised  
48  
49 258 substances (MRL), the precision has to be determined at 0.5, 1 and 1.5 times the MRL. For  
50  
51 259 banned substances, the precision has to be determined at 1, 1.5 and 2 times the MRPL, where  
52  
53 260 MRPL is the Minimum Required Performance Limit. Concerning the detection of sulphonamides in  
54  
55 261 eggs, we have no MRL and no MRPL. In the literature, validation studies for sulphonamides in  
56  
57 262 eggs are commonly based on a target concentration of  $100 \mu\text{g kg}^{-1}$  (Tarbin et al. 1999; Heller et al.  
58  
59 263 2006; Wang et al. 2007). Therefore, the evaluation of precision was performed with a target  
60  
264 concentration of  $100 \mu\text{g kg}^{-1}$ , as it was the MRL value at. Two samples of blank egg samples were

1  
2 265 spiked with sulfamethazine solutions to yield concentrations equivalent to 0.5, 1 and 1.5 times the  
3  
4 266 MRL. At each level, the analysis at least six replicate analyses were performed each day of  
5  
6 267 analysis. These steps were repeated on 4 days. The SMZ concentration detected in each sample  
7  
8  
9 268 was calculated. Finally, the mean concentration, standard deviation (SD) and the coefficient of  
10  
11 269 variation (CV %) of the fortified samples were calculated. Different experiment were performed:  
12  
13 270 Same batch, same technician (repeatability conditions) (2 days) and other batch, same technician  
14  
15 271 (reproducibility conditions) (2 days).

16  
17 272  
18  
19 273 *Preparation of spiked egg samples.* Egg samples were spiked on each day of validation. 20 µl of  
20  
21 274 each antimicrobial solution was added to 2 g of egg sample. All the samples were codified to  
22  
23  
24 275 perform blind analyses.

25  
26 276  
27  
28  
29 277 *Stability.* No experiments have been carried out to determine the stability of sulphonamides or  
30  
31 278 other antimicrobials in eggs. A stability study of different antibiotics in solution and in kidney juices  
32  
33 279 was already published (Cantwell et al. 2006). However, because the 3 tests could not distinguish  
34  
35 280 between all sulphonamides, the stability study should have been performed for all detected  
36  
37  
38 281 sulphonamides in separate experiments. Furthermore, Premi® Test and Explorer® test could not  
39  
40 282 distinguish between all detected antibiotics. The number of experiments would have been high.  
41  
42 283 This kind of study would have become expensive and the workload would have been very high.  
43  
44 284 Finally, the ELISA kit (from RAISIO Diagnostics) allowed to report quantitative results. However,  
45  
46 285 our experience showed that ELISA kits and other immunological methods like BIACORE methods  
47  
48  
49 286 (Gaudin et al. 2007) are not fitted to test stability of drugs because of their high variability.  
50  
51 287 Therefore, the stability of the analytes in the egg matrix was studied by a bibliographical study.

52  
53 288  
54  
55 289 *Participation to a proficiency test*  
56  
57  
58 290 A proficiency test (PT) was organised by the Community Reference Laboratory (CRL) in Fougères  
59  
60 291 to evaluate the participants' methods to detect, confirm and quantify residues of sulphonamides in  
292 whole eggs. The participants were free to use the method of their choice. Therefore several

1  
2 293 different methods have been implemented (in house methods or commercial kits). The participants  
3  
4 294 were National Reference Laboratories (NRLs) from Member States and Third Countries. As the  
5  
6 295 French NRL, our laboratory has participated to the PT with the Premi@Test. However, the  
7  
8 296 technicians who implemented the Premi@Test were not involved in the organisation of the PT and  
9  
10  
11 297 did not know the content of samples. Six samples were sent to the participants in December 2006,  
12  
13 298 2 blank samples and 3 spiked samples (materials 3, 4, 5). 2 blank samples were sent (1 laying  
14  
15 299 hens (material 1) and 1 quail egg (material 2)). Material 3 contained sulfadiazine (SDZ) at 50  $\mu\text{g}$   
16  
17 300  $\text{kg}^{-1}$  and sulfadimethoxine (SDMX) at 60  $\mu\text{g}$   $\text{kg}^{-1}$ , material 4 contained sulfamethazine (SMZ) at 50  
18  
19 301  $\mu\text{g}$   $\text{kg}^{-1}$  and material 5 contained sulfadiazine at 30  $\mu\text{g}$   $\text{kg}^{-1}$ . Material 4 (a/b) was sent in blind  
20  
21 302 duplicate.  
22  
23  
24 303  
25  
26 304  
27  
28 305  
29

## 30 306 **Results and discussion**

### 31 32 33 307 *Specificity and false positive rate*

34  
35 308 *Explorer® test.* Only one false positive result was obtained out of 55 blank egg samples.  
36  
37  
38 309 Therefore, the false positive rate was satisfactory (1.82%). 2 different batches of Explorer® were  
39  
40 310 used during this study. Moreover, 2 different batches of eggs were also analysed.  
41  
42 311  
43

44 312 *Premi@Test.* 33 blank egg samples have been tested finally. The false positive rate was 0%.  
45  
46 313  
47

48  
49 314 *ELISA kit (RAISIO Diagnostics).* For the 12 blank egg samples, calculated SMZ concentrations  
50  
51 315 were very low (between 0.0 +/- 0.0 and 4.5 +/- 1.9  $\mu\text{g}$   $\text{kg}^{-1}$  over the 6 days). The overall mean was  
52  
53 316 2.2 +/- 2.0  $\mu\text{g}$   $\text{kg}^{-1}$ . The distribution of the SMZ concentrations of the 12 blank samples was  
54  
55 317 represented on figure 1.  
56

57 318   
58

59 319 The difference of calculated SMZ concentrations between blank samples and spiked SMZ samples  
60  
320 at 25  $\mu\text{g}$   $\text{kg}^{-1}$  is clearly observable. No false positive results were observed.

1  
2 321 The pre-incubation at 80°C during 10 minutes with Premi®Test and during 12 minutes from  
3  
4 322 Explorer® test allowed to reduce the false positive rate due to the possible presence of naturally  
5  
6 323 inhibitors in eggs. The extraction prior the ELISA test also contributed to reduce the false positive  
7  
8 324 rate.

9  
10  
11 325  
12  
13 326 *Detection capabilities.*

14  
15 327 The detection capabilities were calculated with one tested concentration for each test. Therefore, if  
16  
17  
18 328 at least 95% of the spiked samples were found positive with the test, the detection capability was  
19  
20 329 lower or equal to the tested concentration. If less than 95 % of the spiked samples were found  
21  
22 330 positive with the test, the detection capability was higher to the tested concentration.

23  
24 331  
25  
26 332 *Sulphonamides.* The CC<sub>β</sub> values obtained for the 3 kits for sulphonamides are compared in Table

27  
28  
29 333 III.

30  
31 334 Insert Table III here

32  
33 335 *Explorer® Test.*

34  
35  
36 336 Sensitivity of Explorer® test was calculated in eggs by the manufacturer according to the EN ISO  
37  
38 337 13969:2003 regulation. Samples without any antibiotics or sulphonamides were spiked with  
39  
40 338 different concentrations of antimicrobial compounds, and at least eight replicates of every  
41  
42 339 concentration were analysed on different days. The concentration of antibiotic producing 95%  
43  
44 340 positive samples was considered as the limit of detection for that antibiotic. Therefore, this  
45  
46 341 parameter is comparable to the detection capabilities calculated in this study.

47  
48  
49 342 Only one detection limit was claimed by the manufacturer for sulfadiazine (400 µg kg<sup>-1</sup>). The  
50  
51 343 detection capability determined here was even lower (300 µg kg<sup>-1</sup>).

52  
53 344 The detection capabilities of Explorer® test for sulphonamides were highThe target concentration  
54  
55 345 in our study was 100 µg kg<sup>-1</sup> for sulphonamides in eggs, like for other edible tissues. Therefore, the  
56  
57 346 detection capabilities of the 6 tested sulphonamides were between 3 (sulfadiazine,  
58  
59 347 sulfadimethoxine) and 8 times (sulfamethazine) this target concentration.

1  
2 348 As a conclusion, the detection capabilities for sulphonamides of the Explorer® test are  
3  
4 349 unsatisfactory regarding the target concentration of 100 µg kg<sup>-1</sup>.

5  
6 350  
7  
8  
9 351 3 different batches of Explorer® test as well as different batches of egg negative samples were  
10  
11 352 used for the determination of detection capabilities and specificity. There was no observed  
12  
13 353 influence of the different batches on the results.

14  
15 354  
16  
17 355  
18  
19  
20 356 *Premi®Test.*

21  
22  
23 357 The detection capabilities calculated for the Premi®Test were lower than those found for the  
24  
25 358 Explorer® test for sulfadoxine, sulfadimethoxine, sulfamethoxazole, and probably for  
26  
27 359 sulfamerazine. The detection capabilities of sulfamethazine and sulfadiazine were determined at  
28  
29 360 200 µg kg<sup>-1</sup>. Therefore, the detection capabilities of Premi®Test for sulfamethazine and  
30  
31 361 sulfadiazine seemed to be lower than those of Explorer® test. Finally, Premi®Test was generally  
32  
33 362 more sensitive for sulphonamides than Explorer® test. One reason could be that egg samples  
34  
35 363 were diluted before analyses with the Explorer® test as it was recommended by the manufacturer.

36  
37  
38 364 The detection limits claimed by the manufacturer were 25 µg kg<sup>-1</sup> for sulfamethazine, sulfadiazine  
39  
40 365 and sulfamethoxazole and 100 µg kg<sup>-1</sup> for sulfathiazol. The calculated detection capabilities were  
41  
42 366 higher than those claimed by the manufacturer for SMZ and SDZ. The detection capability for  
43  
44 367 sulfamethoxazole and sulfathiazole (< 100 and <400 µg kg<sup>-1</sup>, respectively) could be in accordance  
45  
46 368 with manufacturer data (25 and 100 µg kg<sup>-1</sup>, respectively). Like for Explorer® test, the definition of  
47  
48 369 detection limits was not given by the manufacturer. The calculation of detection limits could be  
49  
50  
51 370 different from the calculation of detection capabilities.

52  
53 371 3 different batches of Premi®Test as well as different batches of egg negative samples were used  
54  
55 372 for the validation. There was no influence of the different batches on the results.

56  
57 373  
58  
59 374 *ELISA kit (from RAISIO Diagnostics)*  
60



1  
2 375 The calculated concentrations for blank samples and all the spiked samples tested with the ELISA  
3  
4 376 kit were presented in figure 2. The optical densities (OD) (y-axis) are represented in relation to the  
5  
6 377 number of analyses (x-axis). The 40 analyses per sulphonamide' concentration are duplicate  
7  
8  
9 378 analyses of 20 samples over 4 days. A quick visual reading of the figure showed that the detection  
10  
11 379 of S6 (sulfamethizole 200  $\mu\text{g kg}^{-1}$ .) was unsatisfactory because Optical Denisites were similar to  
12  
13 380 those of the blank samples.

14  
15 381 Insert figure 2

16  
17 382 In the ELISA kit leaflet, detection limits (LOD) in eggs were presented. The way to determine the  
18  
19 383 LOD was to carry out sample blank determinations on at least 20 representative blank samples.  
20  
21 384 The limit of detection was calculated as:  $X_n + 3SD$  ( $n \geq 20$ ) where  $X_n$  is the mean response for the  
22  
23  
24 385 20 blank samples. Limits of detection (EC 1993) and detection capabilities (EC 2002) are different  
25  
26 386 performance characteristics. The decision 2002/657/EC repealed the decision 93/256/EC. These  
27  
28 387 kits were validated before 2002 and therefore detection limits have been calculated. On the  
29  
30 388 contrary, detection capabilities are determined by the analysis of at least 20 samples spiked at the  
31  
32  
33 389 lowest detectable concentration, which would give at least 95 % of positive results. Therefore,  
34  
35 390 limits of detection and detection capabilities could not be compared directly. The parallel that could  
36  
37 391 be done between LOD and  $CC\beta$  is that both of them should be lower than the MRL when MRL  
38  
39 392 exists and as low as possible when the substance is banned. The  $CC\beta$  calculated for the multi-  
40  
41 393 sulphonamides ELISA test (RAISIO Diagnostics) were equal to or lower than  $25 \mu\text{g kg}^{-1}$  for 3 of the  
42  
43  
44 394 6 tested sulphonamides (sulfamethazine, sulfamerazine and sulfisoxazole) and lower than  $50 \mu\text{g}$   
45  
46 395  $\text{kg}^{-1}$  for sulfadiazine and sulfachloropyridazine. As a conclusion, the lowest LOD ( $3 \mu\text{g kg}^{-1}$ )  
47  
48 396 (sulfamethazine, sulfamerazine and sulfisoxazole) corresponded to the lowest detection  
49  
50 397 capabilities ( $\leq 25 \mu\text{g kg}^{-1}$ ) and the LOD of  $5 \mu\text{g kg}^{-1}$  (sulfadiazine and sulfachloropyridazine)  
51  
52  
53 398 corresponded to the  $CC\beta$  of  $\leq 50 \mu\text{g kg}^{-1}$ . The detection capability of sulfamethizole was higher  
54  
55 399 than  $200 \mu\text{g kg}^{-1}$ . The detection capabilities for sulfamethazine and sulfadiazine were lower than  
56  
57 400  $CC\beta$  of Premi@Test and especially  $CC\beta$  of Explorer@ test.

1  
2 401 For each of the sulphonamide determination of detection capability, 2 different batches were used  
3  
4 402 during the analyses of the 20 egg samples. There was no observed influence of the batch on the  
5  
6 403 calculated concentrations (see figure 2). Moreover different boxes of the same batch kit were used.  
7

8 404  
9  
10  
11 405 *Other antimicrobials.* The detection capabilities obtained for Premi®Test and Explorer® test for  
12  
13 406 other antimicrobials are compared in Table IV.

14  
15 407 Insert Table IV here  
16

17 408  
18  
19 409 *Explorer® Test*  
20

21  
22 410 The calculated  $CC\beta$  ( $> 4000\mu\text{g kg}^{-1}$ ) was higher than claimed by the manufacturer ( $2800\mu\text{g kg}^{-1}$ ) for  
23  
24 411 oxytetracycline. However, the calculated detection capabilities were lower than those claimed for  
25  
26 412 tylosin and tetracycline. The detection capabilities were near the MRL (2 to 2.5 times the MRL) for  
27  
28 413 macrolides (erythromycin and tylosin). On the contrary, the detection capabilities for tetracyclines  
29  
30 414 were unsatisfactory regarding the MRL values (7.5 to 20 times the MRL). The detection capability  
31  
32 415 of doxycycline was very high ( $800\mu\text{g kg}^{-1}$ ) while its use is forbidden for laying hens.

33  
34 416 Prior to validation, some analyses were performed at 700 and 1000  $\mu\text{g kg}^{-1}$  with TTC. The  
35  
36 417 conclusion was that the  $CC\beta$  was higher than 1000  $\mu\text{g kg}^{-1}$  (data not shown). Then, 20 repeated  
37  
38 418 analyses at 1000  $\mu\text{g kg}^{-1}$  and at 1250  $\mu\text{g kg}^{-1}$  with TTC confirmed that  $CC\beta$  was higher than 1250  
39  
40 419  $\mu\text{g kg}^{-1}$  (Table IV). Therefore,  $CC\beta$  for tetracycline should be equal to 1500  $\mu\text{g kg}^{-1}$ . Oxytetracycline  
41  
42 420 at 700, 1000 and 2000  $\mu\text{g kg}^{-1}$  were also tested and negative results were obtained at these  
43  
44 421 concentrations. The detection capability of oxytetracycline was higher than 4000  $\mu\text{g kg}^{-1}$ .  
45  
46 422

47  
48 423 There was no MRL for amoxicillin but the detection capability was low ( $40\mu\text{g kg}^{-1}$ ).  
49

50  
51 424 To our knowledge, no scientific publications exist at this time concerning performance studies of  
52  
53 425 Explorer® test, neither in eggs nor in other edible tissues. Therefore it was impossible to compare  
54  
55 426 our results to other scientific data.  
56

57 427  
58  
59  
60 428 *Premi®Test*

1  
2 428 The calculated detection capabilities were very similar to those claimed by the manufacturer. The  
3  
4 429 detection capabilities for tetracycline, oxytetracycline, amoxicillin, erythromycin, tylosin and  
5  
6 430 neomycin were even lower than those claimed. Regarding the regulation, the detection capabilities  
7  
8  
9 431 of Premi®Test were satisfactory for 3 antimicrobial residues (tetracycline, erythromycin, tylosin and  
10  
11 432 neomycin) because  $CC\beta$  were lower than existing MRLs. For oxytetracycline, the  $CC\beta$  was equal  
12  
13 433 to  $400 \mu\text{g kg}^{-1}$  and therefore lower than 2 times the MRL. At the MRL, no positive results (out of 20  
14  
15 434 analyses) were obtained at  $200 \mu\text{g kg}^{-1}$ . The detection capability of chlortetracycline was high ( $\leq$   
16  
17 435  $2000 \mu\text{g kg}^{-1}$ ), when the manufacturer claimed  $600 \mu\text{g kg}^{-1}$ . Some preliminary assays were  
18  
19 436 performed to select the antibiotic concentrations to be validated. Chlortetracycline was tested at  
20  
21  
22 437  $600$  and  $800 \mu\text{g kg}^{-1}$ . The results were negative. This is the reason why we have decided to  
23  
24 438 validate at  $2000 \mu\text{g kg}^{-1}$ . Therefore, the detection capability of chlortetracycline should be included  
25  
26 439 between  $1000$  and  $2000 \mu\text{g kg}^{-1}$  which was too high regarding the MRL. Microbiological activity  
27  
28 440 profile was quite different for chlortetracycline from oxytetracycline and tetracycline. The detection  
29  
30  
31 441 capability of amoxicillin was very low ( $5 \mu\text{g kg}^{-1}$ ) and lower than the  $CC\beta$  of Explorer® test. This  
32  
33 442 result was satisfactory because no MRL was set for amoxicillin in eggs. Finally, the detection  
34  
35 443 capability for doxycycline was 4 times lower with Premi®Test ( $200 \mu\text{g kg}^{-1}$ ) than with Explorer® test  
36  
37 444 ( $800 \mu\text{g kg}^{-1}$ ). However, considering that this antimicrobial is banned for laying hens, a lower  
38  
39 445 detection capability would have been preferable.  
40  
41 446 Like for sulphonamides, the detection capabilities of Premi®Test were much lower than those of  
42  
43  
44 447 Explorer® test. One reason could be that egg samples were diluted before analyses with the  
45  
46 448 Explorer® test as it was recommended by the manufacturer.  
47  
48 449 To our knowledge, only 2 studies have been published on the detection of antimicrobial residues in  
49  
50 450 eggs with Premi®Test (Hussein et al. 2005; Lohajova et al. 2006). Therefore, it was difficult to  
51  
52 451 compare our results with previous studies. Hussein (2005) reported a comparative study between  
53  
54 452 a Four Plate Test (FPT) and Premi®Test for the detection of sulfamethazine (SMZ) in laying eggs  
55  
56  
57 453 after treatment of animals. No false positive results were obtained with both tests. Premi®Test was  
58  
59 454 more sensitive than FPT. The FPT gave positive results when the SMZ concentration was at least  
60  
455 equal to  $31860 \mu\text{g kg}^{-1}$ . Negative results were observed at  $1720 \mu\text{g kg}^{-1}$ . However Premi®Test  
456 gave positive results, even at  $96 \mu\text{g kg}^{-1}$  of SMZ (8 days after administration). Finally, high

1  
2 457 correlation was observed between results of the Premi®Test and HPLC results. In our study, the  
3  
4 458 detection capability of SMZ in eggs with Premi®Test was higher than  $100 \mu\text{g kg}^{-1}$ .

5  
6 459 A comparison of STAR microbiological method and Premi®Test for the detection of amoxicillin  
7  
8 460 residues in laying eggs after treatment of animals was also published (Lohajova et al. 2006). Egg  
9  
10 461 samples were extracted before analyses with the STAR protocol, while no extraction was  
11  
12 462 performed before Premi®Test. The conclusion was that Premi®Test was a little more sensitive for  
13  
14 463 amoxicillin than the STAR protocol. In fact, Premi®Test allowed to detect amoxicillin 96h after the  
15  
16 464 end of the animal treatment in egg yolk and 72h in egg white, while the STAR protocol allowed to  
17  
18 465 detect amoxicillin after 72h in egg yolk and 48h in egg white after the end of the treatment. Our  
19  
20 466 validation study also proved that the detection capability of amoxicillin in eggs with Premi®Test  
21  
22 467 was lower than  $5 \mu\text{g kg}^{-1}$  which was also the detection limit claimed by the manufacturer.  
23  
24  
25  
26 468

#### 27 28 469 *Cross-reactions.*

29  
30  
31 470 The percentages (%) of cross-reactivities (CR) were calculated in eggs and were compared with  
32  
33 471 those claimed by the manufacturer for the ELISA kit (RAISIO Diagnostics) (see Table V). The  
34  
35 472 calculated % of cross-reactivities were lower than those given by the manufacturer for sulfadiazine,  
36  
37 473 sulfamerazine and sulfisoxazole. The % CR for sulfachloropyridazine and sulfamethizole are  
38  
39 474 similar. The % CR of cross-reactivities given by the manufacturer were probably calculated in  
40  
41 475 buffer, which could explain the differences.  
42  
43

44 476 Insert Table V here  
45  
46 477

#### 47 48 478 *Precision.*

49  
50  
51 479 The evaluation of precision was performed with the hypothesis that the MRL for sulphonamides in  
52  
53 480 eggs would be set at  $100 \mu\text{g kg}^{-1}$ . The concentrations were lower than those expected because no  
54  
55 481 recovery was applied to the calculated concentrations given here. The 4 days of analyses were  
56  
57 482 spread out over 6 months. Moreover, 2 different batches (1 and 2) of RAISIO Diagnostics ELISA  
58  
59 483 kits were tested (determination of detection capabilities) and different boxes of the same batch kit  
60  
484 were used. There was no observed influence of the batch on the calculated concentrations. The

1  
2 485 overall coefficients of variation were included between 30 and 40 % (Table VI). It is quite high  
3  
4 486 regarding the criteria of the decision EC/2002/657. However it is acceptable for an ELISA test  
5  
6 487 which is considered as only a semi-quantitative test.  
7

8  
9 488 

Insert Table VI here

10  
11 489 These results confirmed that the ELISA kit should have been used to study the stability of  
12  
13 490 sulphonamides in eggs because of the high variability of quantitative results.  
14

15 491  
16

17 492 *Stability.*  
18

19  
20 493 As it is required in the decision EC/2002/657 (EC 2002), stability studies should be performed  
21  
22 494 during validation studies. However, the tests implemented in this study were qualitative tests  
23  
24 495 (Premi®Test, Explorer® test) or only semi-quantitative tests (RAISIO Diagnostics ELISA kits). Our  
25  
26 496 experience proved that this kind of tests are not suited to perform stability studies. Therefore,  
27  
28 497 stability data if possible have to be collected in the literature. However, few publications about  
29  
30  
31 498 validation of quantitative methods reported stability data of antimicrobials in food of animal origin,  
32  
33 499 for example after storage at  $-20^{\circ}\text{C}$ . Data on the stability of antimicrobials in eggs were much more  
34  
35 500 difficult to find than in more common matrices like milk or muscle. None of the validation studies  
36  
37 501 found in the literature, even for confirmatory methods (physico-chemical), included evaluation of  
38  
39 502 analyte stability for sulphonamides in eggs. Therefore, stability data of sulphonamides and other  
40  
41 503 antimicrobials in milk and tissue samples were presented hereafter when no data was found in  
42  
43  
44 504 eggs.

45  
46 505 In all studies, the stability of sulphonamide residues in milk at  $-20^{\circ}\text{C}$  was proved over 95 days.  
47  
48 506 Juhel-Gaugain (2005) also demonstrated that there was a problem of stability of sulphonamides in  
49  
50 507 tissues during storage at  $-20^{\circ}\text{C}$ , particularly in liver. This result confirmed the observations from  
51  
52 508 other studies (Parks et al. 1994). A depletion of the residues at the beginning of the storage at  $-20$   
53  
54 509  $^{\circ}\text{C}$  (during the first month) was observed. Then, sulfa drugs were stable for a long time (Juhel-  
55  
56  
57 510 Gaugain et al. 2005). Sulfamethazine (SMZ) was found stable in bovine milk and piglet muscle  
58  
59 511 samples at  $-20$  and  $-75^{\circ}\text{C}$  during at least 3 and 5 months, respectively (Papapanagiotou et al.  
60  
512 2005). However, a trend of decreasing SMZ levels during prolonged storage at  $-20^{\circ}\text{C}$  was clear in  
513 most studies.

1  
2 514 The effects of cold storage on the residues of 5 antimicrobials (ampicillin, chloramphenicol,  
3  
4 515 oxytetracycline, streptomycin and sulfamethazine) in meat at +4°C or -20°C were very variable  
5  
6 516 (O'Brien et al. 1981). However, the ampicillin concentration in kidney and meat tissues stored at +4  
7  
8 517 +8C or at -20°C decreased. Stability studies showed that penicillin G and oxytetracycline were  
9  
10 518 quite stable in incurred tissue samples.

11  
12  
13 519 At -20°C, oxytetracycline, chloramphenicol and sulfamethazine seemed to be stable. Moreover, in  
14  
15 520 another study, after 8 months of storage at -20°C, a significant decrease for ampicillin in muscle  
16  
17 521 arose and was never observed at -75°C (Verdon et al. 2000).

18  
19 522 Some articles have been published about the stability of antimicrobials to heat process (cooking).  
20  
21 523 For example, it was observed that neomycin and streptomycin residues in eggs were quite stable  
22  
23 524 to normal egg cooking procedures (Katz et al. 1978; Inglis et al. 1978). However, there was no  
24  
25 525 information about the stability when eggs were stored at +4°C or -20°C for example.  
26  
27

28 526 The conclusions on this bibliographical study was that there is a huge lack of data on the stability  
29  
30 527 of antibiotics in eggs. Moreover, when looking at stability studies in other matrices, we could  
31  
32 528 observe a great variability in the results.  
33

#### 34 529 35 36 530 *Participation to a proficiency test*

37  
38  
39 531 The results of our lab were satisfactory (Table VII). Firstly because the 2 blank samples were found  
40  
41 532 negative: hen and quail eggs. Secondly, the samples containing sulphonamide residues have been  
42  
43 533 found doubtful (material 4a and 4b (SMZ 50 µg kg<sup>-1</sup>) and material 5 (SDZ 30 µg kg<sup>-1</sup>)) or even  
44  
45 534 highly positive (material 3). is the great positivity was a logical result because material 3 contained  
46  
47 535 a mix of 2 sulphonamides (SDZ 50 µg kg<sup>-1</sup> / SDMX 60 µg kg<sup>-1</sup>). Therefore, the cumulative  
48  
49 536 concentration was the highest concentration. As a conclusion, the Premi®Test was able to  
50  
51 537 discriminate between negative blank egg samples and egg samples containing sulphonamide  
52  
53 538 residues at levels equal or below 50 µg kg<sup>-1</sup>.  
54  
55  
56  
57 539  
58  
59  
60

1  
2 540 **Conclusion**

3  
4  
5 541 The majority of approved antibiotics are screened by microbiological methods. Screening tests  
6  
7 542 have to be very fast, easy to perform, with high throughput, enough sensitivity to meet  
8  
9 543 requirements of regulations (EC 1990) and low-cost. Moreover, wide screening tests have to be  
10  
11 544 implemented for routine control.

12  
13 545  
14  
15 546 The detection capability is the most important characteristic to be determined for a screening test.

16  
17 547 The detection capabilities have to be as low as possible for banned substances and lower than

18  
19 548 MRL when MRLs have been set. The sensitivity of Premi®Test was better than sensitivity of

20  
21 549 Explorer® test for all the tested sulphonamides and the other tested antimicrobials, probably

22  
23 550 because of the eggs dilution before Explorer® test. A dilution of egg samples (1/4) before

24  
25 551 Explorer® analysis could explain that the detection capabilities are too high for most of the

26  
27 552 antimicrobials. The results of our participation to the proficiency test confirmed the performance of

28  
29 553 Premi®Test for the detection of sulphonamides in eggs. The detection capabilities for the following

30  
31 554 antimicrobials were lower than their respective MRLs: amoxicillin, neomycin, tylosin and

32  
33 555 erythromycin. Therefore, Premi®Test seemed to be more suitable to the wide screening of

34  
35 556 antimicrobials in eggs as a routine method. Complementary experiments should be implemented to

36  
37 557 validate Explorer® test and Premi®Test for more antimicrobial residues.

38  
39 558  
40  
41  
42 559 The second characteristic of a screening test should be to minimise the % of false positive results

43  
44 560 to reduce the number of confirmatory analyses. In this case, the false positive rate was very low for

45  
46 561 all kits (lower than 2 %) and thus the specificity was satisfactory.

47  
48 562  
49  
50  
51 563 The multi-sulphonamide ELISA kit is of great interest because generally ELISA tests could detect

52  
53 564 only one antibiotic (Spinks et al. 2001). The CC<sub>β</sub> values for sulphonamides (25 to 50 µg kg<sup>-1</sup>) were

54  
55 565 much lower than for microbiological tests. This ELISA test should be considered only as a semi-

56  
57 566 quantitative test because of high coefficient of variation (precision) (between 30 and 40 %). The

58  
59 567 extraction of egg samples was one disadvantage of ELISA kit. It was more time and money

60

1  
2 568 consuming than microbiological plate tests and also needed a more skilled technician. So this kind  
3  
4 569 of ELISA kit could be recommended for the targeted screening of eggs, for sulphonamides  
5  
6 570 exclusively.  
7

8 571  
9  
10  
11 572 Finally, optical reading and microplate format of Explorer® test were advantages versus visual  
12  
13 573 reading and ampoule format of Premi®Test. High throughput screening could be reach easily.  
14  
15 574 However, optical reading is also possible for Premi®Test with scanner technology (Stead et al.  
16  
17 575 2005).  
18

19 576  
20  
21 577 As a conclusion, the control of eggs for the presence of antimicrobials could be reinforced with  
22  
23 578 these kind of tests because they are simple, rapid and sensitive enough (Premi®Test and ELISA  
24  
25 579 kit from RAISIO Diagnostics).  
26  
27

## 28 580 29 30 581 **Acknowledgements**

31  
32  
33 582 Thanks to Zeu-Inmunotec (Saragossa, Spain) for providing us with different batches of Explorer®  
34  
35 583 test, to DSM (Delft, The Netherlands) for providing us with different batches of Premi® test.  
36

## 37 584 38 39 585 **References**

- 40  
41  
42 586 Adesiyun AA, OYah N, Lashley V, Seepersadsingh N, Rodrigo S, Georges K. 2005. Prevalence of  
43  
44 587 antimicrobial residues in table eggs in Trinidad. *Journal of Food Production* 68:1501–1505.  
45  
46 588 Adesiyun AA, OYah N, Seepersadsingh N, Rodrigo S, Lashley V, Musai L. 2007. Antimicrobial  
47  
48 589 resistance of *Salmonella* spp. and *Escherichia coli* isolated from table eggs. *Food Control*  
49  
50 590 18:306–311.  
51  
52  
53 591 Alfredsson G, Branzell C, Granelli C, Lundstrom A. 2005. Simple and rapid screening and  
54  
55 592 confirmation of tetracyclines in honey and egg by a dipstick test and LC-MS/MS. *Analytica*  
56  
57 593 *Chimica Acta* 529:47–51.  
58  
59  
60



- 1  
2 594 Bendix A, Ja"hne J, Ku"hne M. 2005. Solid-phase extraction coupled with microbial inhibition tests  
3  
4 595 for the sensitive detection of veterinary drugs in full-egg. *Archiv fur Lebensmittelhygiene* 56  
5  
6 596 (6):132-135.  
7
- 8  
9 597 Cantwell H & O'Keeffe M. 2006. Evaluation of the Premi®Test and comparison with the One-Plate  
10  
11 598 Test for the detection of antimicrobials in kidney. *Food Additives and Contaminants*  
12  
13 599 23(2):120-125.  
14
- 15 600 Dahiya JP, Wilkie DC, Van Kessel AG, Drew MD. 2006. Potential strategies for controlling necrotic  
16  
17 601 enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology* 129  
18  
19 602 (1-2):60-88  
20
- 21  
22 603 Donkova NV. 2005. Residues of tetracycline in poultry meat and eggs due to the use of antibiotics.  
23  
24 604 *Gigiena i sanitariia* 2:41-43.  
25
- 26 605 Donoghue DJ, Hairston H, Henderson M, McDonald M, Gaines S. 1997. Modeling drug residue  
27  
28 606 uptake by eggs: yolks contain ampicillin residues even after drug withdrawal and non  
29  
30 607 detectability in the plasma. *Poultry Science* 76 (3):458-462.  
31
- 32  
33 608 Donoghue DJ. 2003. Antibiotic residues in poultry tissues and eggs: Human health concerns?.  
34  
35 609 *Poultry Science* 82 (4):618-621.  
36
- 37 610 EC 1990. Council Regulation (EEC) N° 2377/90 of 26 June 1990: laying down a Community  
38  
39 611 procedure for the establishment of maximum residue limits of veterinary medicinal products  
40  
41 612 in foodstuffs of animal origin. *Official Journal of European Communities* L224:1-8.  
42
- 43 613 EC 1993. Commission Decision 93/256/EC of 14 April 1993: laying down the methods to be used  
44  
45 614 for detecting residues of substances having a hormonal or a thyrostatic action. *Official*  
46  
47 615 *Journal of European Communities* L 118:64-74.  
48  
49
- 50 616 EC 2002. Commission Decision 2002/657/EC of 12 August 2002: implementing Council Directive  
51  
52 617 96/23/EC concerning the performance of analytical methods and the interpretation of results.  
53  
54 618 *Official Journal of European Communities* L221:8-36.  
55
- 56 619 Gaudin V, Hedou C, Sanders P. 2007. Validation of a Biacore method for screening eight  
57  
58 620 sulfonamides in milk and porcine muscle tissues according to European Decision  
59  
60 621 2002/657/EC. *Journal of AOAC International* 90(6):1706-1715.

- 1  
2 622 Heller DN, Nochetto CB, Rummel NG, Thomas MH. 2006. Development of multiclass methods for  
3  
4 623 drug residues in eggs: hydrophilic solid-phase extraction cleanup and liquid  
5  
6 624 chromatography/tandem mass spectrometry analysis of tetracycline, fluoroquinolone,  
7  
8 625 sulfonamide, and beta-lactam residues. *Journal of Agriculture and Food Chemistry* 54 (15):  
9  
10 626 5267-78.  
11  
12  
13 627 Herranz S, Moreno-Bondi MC, Marazuela MD. 2007. Development of a new sample pre-treatment  
14  
15 628 procedure based on pressurized liquid extraction for the determination of fluoroquinolone  
16  
17 629 residues in table eggs. *Journal of Chromatography A* 1140 (1-2):63-70.  
18  
19 630 Horii S, Momma C, Miyahara K, Maruyama T, Matsumoto M. 1990. Liquid chromatographic  
20  
21 631 determination of three sulfonamides in animal tissue and egg. *Journal of AOAC International*  
22  
23 632 73 (6):990-992.  
24  
25  
26 633 Huet AC, Charlier C, Tittlemier SA, Singh G, Benrejeb S, Delahaut P. 2006. Simultaneous  
27  
28 634 determination of (fluoro)quinolone antibiotics in kidney, marine products, eggs, and muscle  
29  
30 635 by enzyme-linked immunosorbent assay (ELISA). *Journal of Agriculture and Food Chemistry*  
31  
32 636 54 (8):2822-2827.  
33  
34  
35 637 Hussein, K.; Marcincak, S.; Mate, D.; Kozarova Ivona; Sokol, J.; Zdolec, N. 2005 *Acta Veterinaria*  
36  
37 638 (Beograd) 55 (5-6) 493-500 Use of premi Test for the detection of sulphonamide residues in  
38  
39 639 chicken eggs  
40  
41 640 Inglis JM, Katz SE. 1978. Determination of streptomycin residues in eggs and stability of residues  
42  
43 641 after cooking. *Journal of AOAC International* 61(5):1098-1102.  
44  
45 642 ISO 1369 Regulation. 2003. Milk and milk products. Guidelines for a standardized description of  
46  
47 643 microbial inhibitor tests In: ISO/IDF International Standard (ISO/IDF), no. 13969, 183 /  
48  
49  
50 644 International Organization for Standardization, Geneva (Switzerland); International Dairy  
51  
52 645 Federation, Brussels (Belgium).  
53  
54 646 Kabir JU, Umoh VJ, Auduokoh E, Umoh JU, Kwaga JKP. 2004. Veterinary drug use in poultry  
55  
56 647 farms and determination of antimicrobial drug residues in commercial eggs and slaughtered  
57  
58 648 chicken in kaduna state, nigeria. *Food control* 15 (2):99-105.  
59  
60 649 Kan CA, Petz M. 2000. Residues of veterinary drugs in eggs and their distribution between yolk  
650  
650 and white. *Journal of Agriculture and Food Chemistry* 48 (12):6397-6403.

- 1  
2 651 Kan CA, Meijer GAL. 2007. The risk of contamination of food with toxic substances present in  
3  
4 652 animal feed. *Animal Feed Science and Technology* 133 (1-2):84-108.  
5  
6 653 Katz SE, Levine PR. 1978. Determination of neomycin residues in eggs and stability of residues  
7  
8 654 after cooking. *Journal of AOAC International* 61(5):1103-6.  
9  
10  
11 655 Juhel-Gaugain M, Fourmond MP, Delepine B, Laurentie M, Brigitte R, Sanders P. 2005. European  
12  
13 656 Proficiency testing of national reference laboratories for the confirmation of sulfonamide  
14  
15 657 residues in muscle and milk. *Food Additives and Contaminants* 22 (3): 221-3.  
16  
17 658 Lamar J, Petz M. 2007 Development of a receptor-based microplate assay for the detection of  
18  
19 659 beta-lactam antibiotics in different food matrices. *Analytica Chimica Acta* 586 (1-2):296-303.  
20  
21  
22 660 Lohajova L, Nagy J, Rozanska H, Popelka P, Jevinova P. 2006. Suitability of star and Premi@Test  
23  
24 661 for the detection of amoxicillin residues in laying hens. *Bulletin of the Veterinary Institute in*  
25  
26 662 *Pulawy* 50 (3):367-371.  
27  
28 663 O'Brien JJ, Campbell N, Conaghan T. 1981. Effect of cooking and cold storage on biologically  
29  
30 664 active antibiotic residues in meat. *Journal of Hygiene (Cambridge)* 87:511-523.  
31  
32 665 Papapanagiotou EP, Fletouris DJ, Psomas EI. 2005. Effect of various heat treatments and cold  
33  
34 666 storage on sulphamethazine residues stability in incurred piglet muscle and cow milk  
35  
36 667 samples. *Analytica Chimica Acta* 529:305-309.  
37  
38  
39 668 Parks OW. 1994. Stability of sulfaquinoxaline, sulfadimethoxine, and their N4-acetyl derivatives in  
40  
41 669 chicken tissues during frozen storage. *Journal of AOAC International* 77(2):486-488.  
42  
43 670 Pikkemaat MG, Mulder PPJ, Elferink JWA, De Cocq A, Nielen MWF, Van Egmond HJ. 2007.  
44  
45 671 Improved microbial screening assay for the detection of quinolone residues in poultry and  
46  
47 672 eggs. *Food Additives and Contaminants* 24(8):842 – 850.  
48  
49  
50 673 Quon DJ. 2000. Monitoring of domestic and imported eggs for veterinary drug residues by the  
51  
52 674 Canadian Food Inspection Agency. *Journal of Agricultural and Food Chemistry* 48 (12):6421-  
53  
54 675 6427  
55  
56 676 Roudaut B, Moretain JP, Boisseau J. 1989. Excretion of tetracycline and chlortetracycline in eggs  
57  
58 677 after oral medication of laying hens. *Food Addit Contam* 6(1):71-8.  
59  
60  
678 Roudaut B, Moretain JP. 1990. Residues of macrolide antibiotics in eggs following medication of  
679  
laying hens. *British Poultry Science* 31(3):661-75.

- 1  
2 680 Roudaut B. 2002. Sulphonamide residues in eggs following drug administration via the drinking  
3  
4 681 water. Food Additives and Contaminants 19 (4):373-378.  
5
- 6 682 Shaikh B, Chu PS. 2000. Distribution of total <sup>14</sup>C residue in egg yolk, albumen, and tissues  
7  
8 683 following oral [<sup>14</sup>C]sulfamethazine administration to hens. Journal of Agriculture and Food  
9  
10 684 Chemistry 48(12):6404-8.  
11
- 12  
13 685 Spinks CA, Schut CG, Wyatt GM, Morgan M. R. 2001. Development of an ELISA for  
14  
15 686 sulfachlorpyridazine and investigation of matrix effects from different sample extraction  
16  
17 687 procedures. Food Additives and Contaminants 18(1):11-8.  
18
- 19 688 Stead S, Richmond S, Sharman M, Stark J, Geijp E. 2005. A new approach for detection of  
20  
21 689 antimicrobial drugs in food PremiTest coupled to scanner technology. Analytica Chimica Acta  
22  
23 690 529:83-88.  
24
- 25  
26 691 Stolker AAM, Zuidema T, Nielen MWF. 2007. Residue analysis of veterinary drugs and growth-  
27  
28 692 promoting agents. Trends in Analytical Chemistry 26 (10):967-979.  
29
- 30 693 Tansakul N, Niedorf F, Kietzmann M. 2007. A sulfamethazine model to evaluate pharmacokinetics  
31  
32 694 and residues at various concentrations in laying hen. Food Additives and Contaminants  
33  
34 695 24(6):1-7.  
35
- 36  
37 696 Tarbin JA, Clarke P, Shearer G. 1999. Screening of sulphonamides in egg using gas  
38  
39 697 chromatography-mass-selective detection and liquid chromatography-mass spectrometry. J  
40  
41 698 Chromatogr B Biomed Sci Appl. 729 (1-2):127-38.  
42
- 43 699 Verdon E, Fuselier R, Hurtaud-Pessel D, Couedor P, Cadieu N, Laurentie M. 2000. Stability of  
44  
45 700 penicillin antibiotic residues in meat during storage: Ampicillin. Journal of Chromatography A 882  
46  
47 701 (1-2):135-143.  
48
- 49  
50 702 Wang J, Leung D, Butterworth F. 2005. Determination of five macrolide antibiotic residues in eggs  
51  
52 703 using liquid chromatography/electrospray ionization tandem mass spectrometry. Journal of  
53  
54 704 Agricultural and Food Chemistry 53 (6):1857-1865.  
55
- 56 705 Zhang H, Wang L, Zhang Y, Fang G, Zheng W, Wang S. 2007. Development of an Enzyme-Linked  
57  
58 706 Immunosorbent Assay for Seven Sulfonamide Residues and Investigation of Matrix Effects from  
59  
60 707 Different Food Samples. Journal of Agricultural and Food Chemistry 55 (6):2079 –2084.

1  
2 708 Zurhelle G, Petz M, Mueller Seitz E, Siewert E. 2000. Metabolites of oxytetracycline, tetracycline,  
3  
4 709 and chlortetracycline and their distribution in egg white, egg yolk, and hen plasma. Journal of  
5  
6 710 Agricultural and Food Chemistry 48 (12):6392-6396.  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

For Peer Review Only

1  
2 711 **Figure 1. Example of interpretation of quantitative results (Optical Density) with the**  
3  
4 712 **Explorer® test.**

5  
6 713 *This figure represents one day of validation. The optical densities (OD) (difference between the*  
7  
8  
9 714 *values of the 2 readings at 2 wavelengths (590 and 650 nm)) are represented on y-axis in relation*  
10  
11 715 *to the number of analyses on x-axis. The difference of OD of blank samples (negative control NA)*  
12  
13 716 *are represented with a square shape ■. The difference of OD of positive control (penicillin G at 25*  
14  
15 717 *µg kg<sup>-1</sup>) are represented with a triangle shape ▲. The negative control sample absorbance (NA) +*  
16  
17 718 *0.15 is represented with a cross shape ×. The result (SA = Sample Absorbance) of each unknown*  
18  
19  
20 719 *sample (diamond shape ◆) was compared to this line. A sample was declared positive when: SA*  
21  
22 720 *590nm - SA 650 nm ≥ NA590nm - NA 650nm + 0.15.*  
23

24 721  
25  
26 722 **Figure 2. Determination of detection capabilities for the multi-sulphonamides ELISA kit**  
27  
28 723 **(RAISIO Diagnostics).**

29  
30  
31 724 *This figure represents the optical densities (OD) (y-axis) in relation to the number of analyses (x-*  
32  
33 725 *axis). The OD of blank samples are represented with a diamond shape ◆ and the points are linked*  
34  
35 726 *by a line.*

36  
37 727 *The 40 analyses per sulphonamide' concentration are duplicate analyses of 20 samples over 4*  
38  
39 728 *days. The 6 sulphonamides are as follows: S1 = sulfamethazine 25 µg kg<sup>-1</sup>, S2 = sulfadiazine 50*  
40  
41 729 *µg kg<sup>-1</sup>, S3 = sulfamerazine 25 µg kg<sup>-1</sup>, S4 = sulfisoxazole 25 µg kg<sup>-1</sup>, S5 = sulfachloropyridazine*  
42  
43  
44 730 *50 µg kg<sup>-1</sup> and S6 = sulfamethizole 200 µg kg<sup>-1</sup>.*  
45

Figure 1.

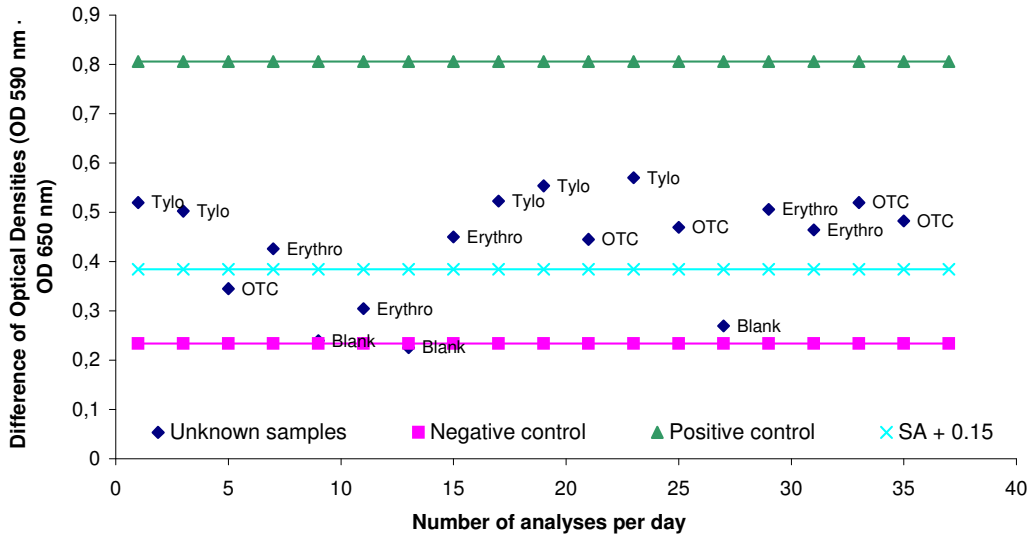
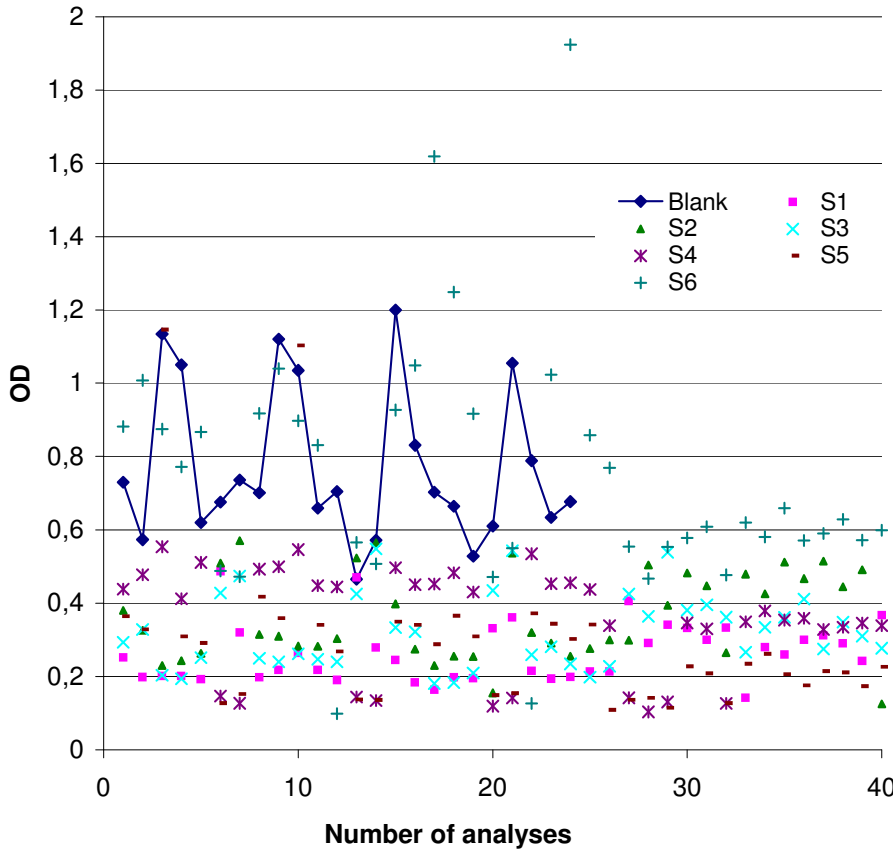


Figure 2.



Deleted: ¶





4 Table II. Antimicrobial substances tested with the 3 kits.

		Tested concentrations (ng g <sup>-1</sup> )		
SULPHONAMIDES	MRL	Premi®Test	Explorer®	ELISA (RAISIO Diagnostics)
Sulfamethazine	/*	100	800	25
Sulfadiazine	/	100	200	50
Sulfamerazine	/	100	500	25
Sulfisoxazole	/	/	/	25
Sulfachloropyridazine	/	100	/	50
Sulfamethizole	/	100	/	200
Sulfathiazol	/	400	/	/
Sulfadoxine	/	100	500	/
Sulfadimethoxine	/	100	500	/
Sulfamethoxazole	/	100	500	/

		Tested concentrations (ng g <sup>-1</sup> )		
OTHER ANTIMICROBIALS	MRL	Premi®Test	Explorer®	ELISA (RAISIO Diagnostics)
Chlortetracycline (CTC)	<b>200</b>	2000	/	/
Tetracycline (TTC)	<b>200</b>	200	800	/
Oxytetracycline (OTC)	<b>200</b>	400	3000- 4000	/
Doxycycline	/	200	1500	/
Amoxicillin	/	5	40	/
Erythromycin	<b>200</b>	50	300-400	/
Tylosin	<b>100</b>	50	200-250	/
Neomycin	<b>500</b>	300	/	/

5 *No MRL has been set for sulphonamides in eggs at this time.*

7 **Table III. Comparison of the detection capabilities calculated for the 3 kits for sulphonamides and**  
 8 **comparison with the announced detection limits.**

	Premi®Test			Explorer®			RAISIO Diagnostics®	
	Levels of fortification ( $\mu\text{g kg}^{-1}$ )	nb + results /spiked samples	CC $\beta$	Levels of fortification ( $\mu\text{g kg}^{-1}$ )	nb + results /spiked samples	CC $\beta$	Levels of fortification ( $\mu\text{g kg}^{-1}$ )	CC $\beta$
<b>Sulfamethazine</b>	100 200	25/40 20/20	>100 <b>200</b>	<b>800</b>	19/20	<b>800</b>	<b>25</b>	$\leq 25$
<b>Sulfadiazine</b>	100 200	35/40 20/20	>100 <b>200</b>	200 <b>300</b>	16/20 <b>19/20</b>	>200 <b>300</b>	<b>50</b>	$\leq 50$
<b>Sulfamerazine</b>	/	NT	NT	250 <b>350</b> 500	15/20 20/20 20/20	>250 <b>350</b> $\leq 500$	<b>25</b>	$\leq 25$
<b>Sulfadoxine</b>	100	20/20	$\leq 100$	<b>500</b>	16/20	> <b>500</b>	/	NT
<b>Sulfadimethoxine</b>	100	20/20	$\leq 100$	250 <b>300</b> 500	18/20 <b>20/20</b> 20/20	>250 <b>300</b> $\leq 500$	/	NT
<b>Sulfamethoxazole</b>	100	25/25	$\leq 100$	<b>500</b>	19/20	<b>500</b>	/	NT
<b>Sulfachloropyridazine</b>	/	NT	NT	/	NT	NT	<b>50</b>	$\leq 50$
<b>Sulfamethizole</b>	/	NT	NT	/	NT	NT	<b>200</b>	> <b>200</b>
<b>Sulfathiazol</b>	400	20/20	$\leq 400$	/	NT	NT	/	NT
<b>Sulfisoxazole</b>	/	NT	NT	/	NT	NT	25	$\leq 25$

\* 20/20 (number of positive results / number of spiked samples analysed) for all tested sulphonamides.

1  
2 13 **Table IV. Comparison of the detection capabilities calculated for Premi®Test and Explorer® test for**  
3  
4 14 **the other antimicrobial residues and comparison with the announced detection limits.**  
5  
6 15

	Premi®Test					Explorer®			
	MRL	Announced LOD (DSM) ( $\mu\text{g kg}^{-1}$ )	Levels of fortification ( $\mu\text{g kg}^{-1}$ )	nb + results /spiked samples	CC $\beta$ ( $\mu\text{g kg}^{-1}$ )	Announced LOD (Zeu) ( $\mu\text{g kg}^{-1}$ )	Levels of fortification ( $\mu\text{g kg}^{-1}$ )	nb + results /spiked samples	CC $\beta$ ( $\mu\text{g kg}^{-1}$ )
<b>CTC</b>	<b>200</b>	600	2000	20/20	$\leq 2000$	/	/	/	/
<b>TTC</b>	<b>200</b>	200	200	20/20	$\leq 200$	<b>2800</b>	1000 1250 <b>1500</b>	8/20 16/20 <b>20/20</b>	>1000 >1250 <b>1500</b>
<b>OTC</b>	<b>200</b>	400	200 <b>400</b>	0/20 <b>20/20</b>	<b>400</b>	<b>2800</b>	3000 4000	14/20 15/20	>4000
<b>Doxycycline</b>	/	200	100 <b>200</b>	0/20 <b>20/20</b>	100 <b>200</b>	/	800	19/20	<b>800</b>
<b>Amoxicillin</b>	/	5	5	19/20	<b>5</b>	/	40	20/20	$\leq 40$
<b>Erythromycin</b>	<b>200</b>	50	50	20/20	$\leq 50$	<b>400</b>	200 300 <b>400</b>	7/20 14/20 <b>20/20</b>	>200 >300 <b>400</b>
<b>Tylosin</b>	<b>100</b>	50	50	20/20	$\leq 50$	<b>400</b>	200 <b>250</b>	15/20 <b>20/20</b>	>200 <b>250</b>
<b>Neomycin</b>	<b>500</b>	300	300	19/20	<b>300</b>	<b>1600</b>	/	/	/

16  
17 *LOD: detection limit*  
18

1  
2 19 **Table V. Cross-reactivity percentages.**

	Announced % CR	Calculated % CR
<b>S1 : Sulfamethazine (SMZ)</b>	100*	100*
<b>S2 : Sulfadiazine</b>	68	36.6
<b>S3 : Sulfamerazine</b>	108	77.9
<b>S4 : Sulfisoxazole</b>	99	68.6
<b>S5 : Sulfachloropyridazine</b>	64	52.3
<b>S6 : Sulfamethizole</b>	5.3	3.6
<b>Sulfathiazol</b>	7	NT
<b>Sulfadoxine</b>	<1	NT
<b>Sulfadimethoxine</b>	<1	NT
<b>Sulfachloropyrazine</b>	97	NT

18 20 *S1 to S6 were tested with the multi-sulphonamides ELISA kit.*

20 21 \* SMZ is the reference sulphonamide to calculate cross-reactivity percentages.

22 Table VI. Precision determined for the multi-sulphonamides ELISA kit (RAISIO Diagnostics).

		D3+D5	D2	D4+D6	D7	
Batch		1	2	1	2	
Box		4	9	5	9-10	
						TOTAL
0.5 * target concentratio n	Mean	20	27	43	22	28
	SD	8	1	5	1	10
	CV	40	4	12	5	37
target concentratio n	Mean	41	51	69	32	50
	SD	9	10	6	3	16
	CV	23	20	8	8	31
1.5 target concentratio n	Mean	51	51	80	NC*	61
	SD	19	11	8	NC	19
	CV	38	21	10	NC	31

24 Target concentration was  $100 \mu\text{g kg}^{-1}$ .

26 D2: Day 2 of validation

28 Mean: mean concentration (intra-day  $n=6$  ; inter-days  $n=36$ )

30 SD: standard deviation (intra-day  $n=6$  ; inter-days  $n=36$ )

32 CV: Coefficient of variation (%)

34 \* NC: not calculated because no quantitative results (results were out of the standard curve)

36 Batch: 2 different batches supplied by the manufacturer have been tested (different batch number, different validity date)

38 Box: From each batch, different boxes (each containing a microplate 96 wells and reagents) were also tested (different boxes with the same batch number and the same validity date).

33 **Table VII. Results of our participation to the proficiency test for the detection of sulphonamides in**  
 34 **eggs with Premi®Test.**

	Material 1	Material 2	Material3	Material 4a	Material 4b	Material 5
<b>AB</b>	Blank laying hen	Blank quail egg	SDZ / SDMX	SMZ	SMZ	SDZ
<b>Concentration</b> ( $\mu\text{g kg}^{-1}$ )	/	/	50 / 60	50	50	30
<b>PT results</b>	-	-	++	D	D	D

36 *sulfadiazine (SDZ), sulfadimethoxine (SDMX), sulfamethazine (SMZ)*

37 *D: Doubtful*