



WETENSCHAPPELIJK INSTITUUT
VOLKSGEZONDHEID
INSTITUT SCIENTIFIQUE
DE SANTÉ PUBLIQUE



**FOOD, MEDICINES AND CONSUMER SAFETY
FOOD SECTION**

Tim Reyns, Stéphanie Fraselle, Désiré Laza, Joris Van Loco

(Tim.Reyns@wiv-isp.be)

June 2010

NRL Proficiency Study Report

Analysis of macrolide and sulfonamide
antibiotics in porcine muscle tissue

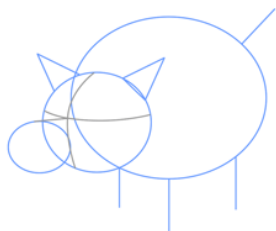




Table of contents

1.	Introduction	4
2.	Planning of the study	5
3.	Test material	6
3.1.	<i>Preparation of the incurred samples</i>	6
3.2.	<i>Stability studies</i>	6
3.3.	<i>Homogeneity studies</i>	6
4.	Sample overview	7
5.	Analytical methodologies	9
5.1.	<i>Macrolides</i>	9
5.2.	<i>Sulfonamides</i>	10
6.	Results	11
6.1.	<i>Macrolides</i>	11
6.2.	<i>Sulfonamides</i>	12
7.	Conclusion	16
8.	Acknowledgements	16



1. Introduction

Sulfonamides are widely used as antibacterials in veterinary medicine for the treatment and prevention of respiratory and gastro-intestinal tract infections. However, large applications of these drugs raise the risk of the persistence of residues in food products, mostly the result of improper observance of withdrawal times. Therefore, the European Union (EU) has defined maximum residue limits (MRLs) of $100 \mu\text{g kg}^{-1}$ for all sulfonamides in edible tissues of all food-producing animals.

Macrolide antibiotics are extensively used in veterinary medicine against Gram-positive bacteria and mycoplasma. Macrolides are used therapeutically for the treatment of e.g. dysentery and pneumonia in pigs. To protect the consumer's health, the EU has fixed MRLs for different tissues and animal species (see Table 1).

Table 1. Overview of the different MRLs for macrolide antibiotics

Active substance	Animal species	MRL ($\mu\text{g kg}^{-1}$)	Target Tissue
Erythromycin	All food producing animals except:	200	Muscle, Fat, Liver, Kidney
	Chicken	300	Skin and Fat
		400	Liver
	Porcine (as spiramycin 1)	250	Muscle
		2000	Liver
		1000	Kidney
Tilmicosin	All food producing animals	50	Muscle, Fat
		1000	Liver, Kidney
	Poultry	75	Muscle, Skin and Fat
		1000	Liver
		250	Kidney
Tulathromycin	Bovine	100	Fat
		3000	Liver and Kidney
	Porcine	100	Skin and Fat
		3000	Liver and Kidney
Tylosin	All food producing animals	100	Muscle, Skin and Fat, Liver, Kidney



As National Reference Laboratory, the Scientific Institute of Public Health (IPH) organized, in collaboration with the Federal Agency for the Safety of the Food Chain, in April 2010 a proficiency test for the analysis (screening and/or confirmation) of sulfonamide and macrolide antibiotics in porcine muscle tissue. The participants were free to use any reliable method of their choice. Each participating laboratory received two batches of five porcine muscle tissue samples. One batch should be analysed for macrolide antibiotics, the other for sulfonamides.

Samples contain one of the sulfonamides or macrolides listed in Table 2.

Table 2. Overview of the sulfonamide and macrolide antibiotics possibly present in the samples

Sulfonamides	Macrolides
Sulfadiazine	Spiramycin
Sulfathiazole	Erythromycin
Sulfamerazine	Josamycin
Sulfapyridine	Tilmicosin
Sulfamethizole	Tylosin
Sulfadimidine	
Sulfamethoxypyridazine	
Sulfamonomethoxine	
Sulfachlorpyridazine	
Sulfadoxine	
Sulfadimethoxine	
Sulafquinoxaline	
Sulfamoxole	
Sulfanilamide	
Sulfaguanidine	

2. Planning of the study

- January 2010: Animal experiment
- January – April: Stability and homogeneity study by IPH
- 8 March 2010: Sending Invitation to laboratories
- 29-31 April 2010: Dispatching of the frozen samples to the participants by IPH courier
- 22 April 2010: Deadline for the reporting of the results to IPH
- May-June 2010: Sending of the final report to the participating laboratories and copy to the Federal Agency for Food Safety together with identification of the participants



3. Test material

3.1. Preparation of the incurred samples

The choice of the two groups of antibiotics was based on the high presence of macrolides and sulfonamides in suspected injection sites taken by the Federal Agency for Food Safety. Moreover, tylosin and sulfadiazine were mostly found after confirmation of this samples.

In January 2010, incurred samples were prepared. Two pigs were treated separately with an intramuscular injection with tylosin at a dosage of 10 mg kg⁻¹ BW (Tylan 200[®], Eli Lilly) and sulfadiazine/trimethoprim (Duphatroxim[®], Fort Dodge A.H.) at a dosage of 20/4 mg kg⁻¹ BW, respectively. Twenty four hours after dosage, pigs were euthanized and injection sites were collected (\pm 500g). After homogenization, samples were stored at \leq -20°C.

3.2. Stability studies

To ensure stability of tylosin and sulfadiazine in the incurred samples, long-term stability studies were performed over the period January - April 2010. Blank porcine tissue samples were spiked at the MRL level of tylosin (100 μ g kg⁻¹) and other blanks were spiked at the MRL of sulfadiazine (100 μ g kg⁻¹). Samples were stored at \leq -20 °C. Each week, 2 samples were analyzed to investigate possible degradation of the compounds. The recovery of tylosin and sulfadiazine in porcine muscle after 3 months at \leq -20 °C was 89.0 % (\pm 4.0%) and 88.4 % (\pm 0.1%), respectively. These results showed acceptable stability of the compounds in spiked tissue samples during the whole proficiency test. Moreover, no significant loss after several freeze/thaw cycles was noticed.

3.3. Homogeneity studies

Since the incurred samples were diluted with blank matrix to obtain the final concentration, a homogeneity study was also performed. The initial concentration of tylosin and sulfadiazine in the injection sites were determined using our validated method. Several dilutions were made with blank muscle tissue (from Coprosain, Ath, Belgium) to obtain the final concentration. The dilutions were performed in different steps using a Robot-Coupe[®] mixer (Robot-coupe, Mont-Ste-Geneviève, Belgium) to obtain good homogeneity. After each dilution, at least four aliquots of the total volume of tissue were taken and analysed for its homogeneity. For the final dilution (= samples for the proficiency test), six aliquots were taken. Table 3 gives an overview of the several dilutions and corresponding concentrations of tylosin and sulfadiazine in porcine muscle tissue.



Table 3. Different dilutions of the incurred injection site for tylosin and sulfadiazine

Dilution of injection site	Mean concentration in $\mu\text{g kg}^{-1}$ (RSD in %)
<i>Tylosin</i>	
No dilution	25041.4
1/10 (n=4)	1568.8 (3.3)
1/100 (n=6)	387.4 (5.1)
1/250 (n=6)	119.6 (8.8)

Dilution of injection site	Mean concentration in $\mu\text{g kg}^{-1}$ (RSD in %)
<i>Sulfadiazine</i>	
No dilution	2877.1
1/10 (n=4)	4087.9 (7.7)
1/100 (n=6)	350.7 (2.7)

As can be seen from the table, all dilutions were homogeneous for tylosin and sulfadiazine. The RSD (%) values of all dilutions were < 10%.

The final dilutions of 1/250 and 1/100 for tylosin and sulfadiazine, respectively, were sent (in duplicate) to the participants. Together with the four incurred samples, one blank sample was also included.

4. Sample overview

During the last year, sulfonamide and macrolide antibiotics were frequently found in suspected injection sites. After further confirmatory purposes of the samples at the IPH, sulfadiazine and tylosin were mostly confirmed. Therefore, these antibiotics were used in the proficiency test.

All participants received five unknown samples. Two of them were non-compliant for tylosin, two for sulfadiazine and one blank. Laboratories were free to subscribe for sulfonamide and/or macrolide screening and/or confirmation. An overview of the samples with corresponding laboratory and their performed analysis is given in Table 4. The last column gives the randomisation of the different samples, together with their expected result of the participants. Laboratories had no access to this information during the whole duration of the test.



Table 4. Overview of the samples dispatched to the different participants

Lab code of participant	Performed analysis	Sample Code	Expected result
A	Screening sulfonamides and macrolides	SM.2010-A-1	Containing sulfadiazine
		SM.2010-A-2	Containing tylosin
		SM.2010-A-3	Containing sulfadiazine
		SM.2010-A-4	Containing tylosin
		SM.2010-A-5	Blank
B	Screening macolides and screening + confirmation sulfonamides	SM.2010-B-1	Containing tylosin
		SM.2010-B-2	Blank
		SM.2010-B-3	Containing tylosin
		SM.2010-B-4	Containing sulfadiazine
		SM.2010-B-5	Containing sulfadiazine
C	Screening and confirmation sulfonamides	SM.2010-C-1	Containing sulfadiazine
		SM.2010-C-2	Containing sulfadiazine
		SM.2010-C-3	Containing for tylosin
		SM.2010-C-4	Blank
		SM.2010-C-5	Containing tylosin
D	Screening sulfonamides and macrolides	SM.2010-D-1	Blank
		SM.2010-D-2	Containing sulfadiazine
		SM.2010-D-3	Containing tylosin
		SM.2010-D-4	Containing sulfadiazine
		SM.2010-D-5	Containing tylosin
E	Screening	SM.2010-E-1	Containing tylosin
		SM.2010-E-2	Containing tylosin
		SM.2010-E-3	Blank
		SM.2010-E-4	Containing sulfadiazine
		SM.2010-E-5	Containing sulfadiazine
F	Confirmation	SM.2010-F-1	Blank
		SM.2010-F-2	Containing tylosin
		SM.2010-F-3	Containing tylosin
		SM.2010-F-4	Containing sulfadiazine
		SM.2010-F-5	Containing sulfadiazine



5. Analytical methodologies

One participant did not submit their results of the proficiency test (Lab D). Nevertheless the inscription was sent to the IPH.

Six laboratories participated in this proficiency study. All participants were free to use their proper analytical method to perform the proficiency test. The different methods for screening and confirmatory analysis of macrolides and sulfonamides are summarized in Table 5 and 6, respectively.

5.1. Macrolides

For the macrolide analysis, four laboratories participated (Table 5). Three of the laboratories participated for a screening (Lab A, B and E) (Table 4). Laboratory F confirmed the macrolide antibiotics with LC-MS/MS after an extraction with a TRIS buffer and further SPE extraction. Two participants used a microbiological and/or receptor assay to perform a screening of the samples (participants B and E). While laboratory A used rapid acetonitrile extraction followed by evaporation and subsequent UPLC-MS/MS analysis (Lab A).

Table 5. Summary of the used methods for macrolide analysis

Lab	Analysis	IS	Technique	Extraction/purification	CC _α μg kg ⁻¹	CC _β μg kg ⁻¹
A	Screening	---	UPLC-MS/MS	ACN extraction, Centrifugation and Concentration	---	--- / 50
B	Screening	---	Premi-test [®]	→ Solvent extraction	---	≤ 12.5
			Charm II [®] Test	→ Protocol Charm Sciences	---	400
			ELISA kit tylosin	→ Protocol Tecna S.r.l.	---	10
C	Did not participate in the macrolide analysis					
D	Did not submit their results					
E	Screening	---	Premi-test [®]	---	---	---
F	Confirmation	Roxithromycin	LC-MS/MS	Extraction TRIS buffer Acidic deproteinisation SPE (OASIS HLB [®])	123	147



5.2. Sulfonamides

Five laboratories participated in the proficiency test for sulfonamide analysis (Table 6). Two laboratories (Lab A and E) performed a screening, while three confirmed the samples (Lab B, C and F) (Table 4). Laboratories B and C performed both a screening and a confirmation. A fast and simple extraction method with acetonitrile was performed by laboratory A and C to screen the samples. Afterwards, a more extensively clean-up by a liquid-liquid extraction with water and methyl tertiary butyl ether was performed to confirm the positively screened samples by participant C. Participant F used a combination with dichloromethane and acetone, followed by an SPE extraction for confirmation. Laboratory B used a combination of microbiological and receptor assay for screening. For their confirmatory purposes, an acetonitrile extraction was performed. The Premi-test[®] was used by laboratory E.

Table 6. Summary of the used methods for sulfonamide analysis

Lab	Analysis	IS	Technique	Extraction/purification	CC _α μg kg ⁻¹	CC _β μg kg ⁻¹
A	Screening	---	UPLC-MS/MS	ACN extraction, Centrifugation and Concentration	---	25*
B	Screening	---	Premi-test [®] Charm II [®] Test	→ Solvent extraction → Protocol Charm – Sciences	---	40
	Confirmation	Sulfachloropyridazine	LC-MS/MS	Sodium sulphate drying ACN extraction Centrifugation and Concentration	---	LOD 5
C	Screening	---	LC-MS/MS	ACN extraction and direct injection	< 50	< 50
	Confirmation	Sulfadimidine-d4	LC-MS/MS	LLE with water and methyl tertiary butyl ether	122	133
D	Did not submit their results					
E	Screening	---	Premi-test [®]	---	---	---
F	Confirmation	Sulfadiazine-C ₁₃ Sulfadimidine-C ₁₃ Sulfanilamide-C ₁₃ Sulfadimethoxine-D ₆	LC-MS/MS	Extraction dichloromethane/acetone SPE (SCX, Varian [®])	113	126

* CC_β of trimethoprim



6. Results

6.1. Macrolides

For macrolide analysis, three laboratories performed a screening, i.e. A, B and E. While laboratory F confirmed the macrolides. Results are summarized in Table 7.

Table 7. Results for the macrolide analysis reported by the participants

Lab code	Sample Code	Reported result	Expected results
A	SM.2010-A-1	Compliant	No macrolides
	SM.2010-A-2	Non-compliant for tylosin	Containing macrolides (tylosin)
	SM.2010-A-3	Compliant	No macrolides
	SM.2010-A-4	Non-compliant for tylosin	Containing macrolides (tylosin)
	SM.2010-A-5	Compliant	No macrolides
B	SM.2010-B-1	Suspect for Tylosin (Spiramycine)	Containing macrolides (tylosin)
	SM.2010-B-2	Nihil	No macrolides
	SM.2010-B-3	Suspect for Tylosin (Spiramycine)	Containing macrolides (tylosin)
	SM.2010-B-4	Nihil	No macrolides
	SM.2010-B-5	Nihil	No macrolides
C	Did not participate in the macrolide analysis		
D	Did not submit their results		
E	SM.2010-E-1	Suspect	Containing macrolides (tylosin)
	SM.2010-E-2	Suspect	Containing macrolides (tylosin)
	SM.2010-E-3	Compliant	No macrolides
	SM.2010-E-4	Suspect	No macrolides
	SM.2010-E-5	Suspect	No macrolides
F	SM.2010-F-1	Compliant for macrolides	No macrolides
	SM.2010-F-2	113.3 $\mu\text{g kg}^{-1}$ tylosine	Containing macrolides (tylosin)
	SM.2010-F-3	114.1 $\mu\text{g kg}^{-1}$ tylosine	Containing macrolides (tylosin)
	SM.2010-F-4	Compliant for macrolides	No macrolides
	SM.2010-F-5	Compliant for macrolides	No macrolides

All laboratories have found the suspected samples for macrolides. No other compound(s) has (have) been reported (false positive). Moreover, all laboratories have identified the blank sample as compliant.

Only one laboratory performed a screening by UPLC-MS/MS after a rapid extraction with acetonitrile (labo A). This participant identified tylosin as unknown macrolide.



Participant B performed a cascade of screening techniques i.e.: Premi-test[®], Charm II[®] and ELISA to investigate the presence of macrolide antibiotics in the samples. These tests were antimicrobial and receptor assay test and are able to detect the non-compliant samples. Moreover, this participant was able to identify the macrolide as tylosine. It can be concluded that laboratory B performed a very specific screening.

Laboratory E performed a screening test with the Premi-test[®] and could distinguish the suspected samples from the blank one. This test was not able to distinguish between sulfonamides and macrolides. Therefore, the non-compliant samples for sulfadiazine were also reported as non-compliant for macrolides. It is already known that the Premi-test[®] is less specific in comparison with more specific receptor assays. Laboratory F confirmed a mean concentration of 113.7 $\mu\text{g kg}^{-1}$ tylosine with LC-MS/MS.

6.2. Sulfonamides

Five laboratories analyzed the samples for sulfonamides, i.e. A, B, C, E and F. Results are summarized in Table 8.

As for the macrolide analysis, all laboratories were able to detect the non-compliant sulfonamide samples. No false positives results were reported. The blank samples were also reported as negative.

One participants detected trimethoprim instead of a sulfonamide. Indeed, the samples were also non-compliant for trimethoprim, since it is always given i.m. together with sulfadiazine. It could be mentioned that laboratory A submitted a false negative result for sulfadiazine, but due to the presence of trimethoprim, the sample was also reported as non-compliant.

Screening methods were performed by (UP)LC-MS/MS after simple extraction with acetonitrile by participants A and C. Laboratories B and E used microbiological and receptor assay tests. Participant E used the Premi-test[®] and this test is not able to differentiate between macrolides and sulfonamides. Therefore, as for the macrolide samples, the non-compliant samples for tylosine were also reported as non-compliant for sulfadiazine.

Three labs performed a confirmation (Laboratory B, C and F) and found good comparable (mean) concentrations, i.e. 353.5, 330.0 and 356.2 $\mu\text{g kg}^{-1}$ of sulfadiazine, respectively. Due to a limited number of participants, it's obvious that a full statistical evaluation of the data could not be performed.

The Z-scores for sulfadiazine have been calculated for information purposes only.



Table 8. Results for the sulfonamide analysis reported by the participants

Lab code	Sample Code	Reported results		Expected result s
		Screening	Confirmation ($\mu\text{g kg}^{-1}$)	
A	SM.2010-A-1	Non-compliant for trimethoprim		Containing sulfonamides (sulfadiazine)
	SM.2010-A-2	Compliant		No sulfonamides
	SM.2010-A-3	Non-compliant for trimethoprim	---	Containing (sulfadiazine)
	SM.2010-A-4	Compliant		No sulfonamides
	SM.2010-A-5	Compliant		No sulfonamides
B	SM.2010-B-1	Compliant for sulfonamides	---	No sulfonamides
	SM.2010-B-2	Compliant for sulfonamides	---	No sulfonamides
	SM.2010-B-3	Compliant for sulfonamides	---	No sulfonamides
	SM.2010-B-4	Sulfonamides found	390.0	Containing sulfonamides (sulfadiazine)
	SM.2010-B-5	Sulfonamides found	317.0	Containing sulfonamides (sulfadiazine)
C	SM.2010-C-1	Sulfonamides found	330.0	Containing sulfonamides (sulfadiazine)
	SM.2010-C-2	Sulfonamides found	330.0	Containing sulfonamides (sulfadiazine)
	SM.2010-C-3	Compliant	---	No sulfonamides
	SM.2010-C-4	Compliant	---	No sulfonamides
	SM.2010-C-5	Compliant	---	No sulfonamides
D	Did not submit their results			



E	SM.2010-E-1	Suspect		No sulfonamides
	SM.2010-E-2	Suspect		No sulfonamides
	SM.2010-E-3	Compliant	---	No sulfonamides
	SM.2010-E-4	Suspect		Containing sulfonamides (sulfadiazine)
	SM.2010-E-5	Suspect		Containing sulfonamides (sulfadiazine)
F	SM.2010-F-1	---	No sulfonamides found	No sulfonamides
	SM.2010-F-2	---	No sulfonamides found	No sulfonamides
	SM.2010-F-3	---	No sulfonamides found	No sulfonamides
	SM.2010-F-4	---	356.5	Containing sulfonamides (sulfadiazine)
	SM.2010-F-5	---	355.9	Containing sulfonamides (sulfadiazine)



The calculation of the Z-scores for the different laboratories was used to assess the accuracy of the results.

$$Z - \text{score} = \frac{x - X}{\sigma}$$

x = Result of each participant

X = Assigned value (median value or spiked value)

$$\sigma = \text{Target standard deviation} = \frac{0.02 \times \text{assigned value}^{0.8495}}{10^{-9}}$$

Evaluation of the Z-score can be summarized as followed

- |Z| ≤ 2 → satisfactory
- 2 < |Z| < 3 → questionable
- |Z| ≥ 3 = → unsatisfactory

Table 9. Results and Z-scores for the sulfadiazine analysis

Assigned Value (Median of the results) = 353.5 µg kg⁻¹
σ = 66.13

Labo	Result (µg kg ⁻¹)	Z-score
B	353.5	0
C	330.0	0.36
IPH	356.2	0.04



7. Conclusion

Incurred samples were used in this proficiency study of macrolide and sulfonamides in porcine tissues. The samples were non-compliant for sulfadiazine or tylosin. Each participant was free to inscribe for one or both analysis. One laboratory did not report their results, nevertheless an inscription was send to the IPH.

Three laboratories performed a screening for macrolides. Two of them found tylosin as 'unknown' macrolide (Lab A, B and F). Participant E performed a Premi-test[®] and was not able to identify distinguish between sulfonamides and macrolides. Nevertheless, their method was able to detect the non-compliant samples from the blank ones. Laboratory B performed subsequently a Premi-test[®], Charm II[®] test and an ELISA test to identify tylosin in the incurred samples. It seems to be a time consuming screening method, but a very good specificity was obtained by the participant. Laboratory F confirmed the samples and found a mean concentration of 113.7 $\mu\text{g kg}^{-1}$ tylosin.

Five laboratories participated in the sulfonamide analysis. No false positive results were reported. Two of them performed only a screening, the others also confirmed the samples. One screening laboratory (Lab A) reported trimethoprim in the samples without any sulfonamide. Indeed, samples contain also trimethoprim since it is given i.m. in combination with sulfadiazine, but unfortunately a false negative result for sulfonamides was reported. As for the macrolide analysis, laboratory E used a Premi-test[®] and was not able to identify distinguish between sulfonamides and macrolides. The Premi-test[®] is a good technique to screen samples, but lacks some specificity to differentiate between groups of antibiotics. On the other hands, immunological and receptor assays are more specific and are able to distinguish between several groups of antibiotics.

On a quantitative point of view, laboratories who confirmed the sulfonamides (Lab B, C and F) found all sulfadiazine and obtained good results regarding their Z-scores. Nevertheless due to very few results, Z-scores were given for information purposes.

Most screening methods are based on microbiological, immunological and receptor assays. A microbiological assay (Premi-test[®]) is not able to distinguish between several antibacterial groups. Nevertheless it is a very good technique for a fast and inexpensive screening to detect non-compliant samples. When the latter technique is followed by subsequent receptor and/or immunological methods, more specificity can be obtained, which we recommend as procedure for screening of antimicrobials. Nevertheless, (UP)LC-MS/MS can also be used for a first screening. Liquid chromatography combined with electrospray ionization - tandem mass spectrometry is the method of choice to confirm antibiotics in animal tissues.

8. Acknowledgements

Special thanks to Samira, Khari and Karine for the excellent technical assistance during stability and homogeneity studies. The IPH also thank Pieter-Jan and Els from the CODA-CERVA for their help with the animal experiments.