Proficiency study for macrolides in porcine tissue

B.J.A. Berendsen, A.A.M. Stolker

Business Unit: Analysis & Development
Group: Veterinary Drugs

Mailing list:
- 13 participating labs, among them 2 from The Netherlands.
- Food and Consumer Product Safety Authority (VWA); J.A. van Rhijn.

Summary

The proficiency study for macrolides in porcine tissue was organized in accordance with ISO/IEC Guide 43-1 and 43-2 and ILAC-G13, and under accreditation (Dutch Accreditation Board, ILAC-G13).

For this proficiency study, four test materials were prepared:
- A blank porcine muscle material;
- A porcine muscle material containing about 80 µg/kg tylosin, 300 µg/kg josamycin, 100 µg/kg lincomycin and 150 µg/kg tulathromycin (spiked);
- A blank porcine kidney material;
- A porcine kidney material containing about 80 µg/kg tylosin, 300 µg/kg josamycin and 50 µg/kg tilmicosin (spiked).

During homogeneity testing, all materials proved to be sufficient homogenous for proficiency testing. The stability test demonstrated that no significant loss of any of the compounds occurred during the timescale of the proficiency test.

Thirteen laboratories subscribed for participation in the proficiency study. Eleven laboratories managed to submit valid results within the timeframe of the stability study. For muscle, seven and for kidney three of the participating laboratories applied a validated method.

Some false negatives and false positives occurred in this proficiency study. Although spiramycin was not present in the samples, one laboratory found spiramycin in both of the kidney materials. The same laboratory missed tylosin in the muscle samples. Two laboratories did not detect tilmicosin in the sample, although this compound was included in their method.

The laboratory's performance for the materials containing macrolides are summarized in Table 1.

Table 1. Summary of the laboratory's performance of the materials containing macrolides

Matrix	Compound	Assigned value (X) (µg/kg)	Uncertainty of X (µg/kg)	No. of labs that reported results	No. of satisfactory results
Muscle	Tylosin	38.3	4.3	10	7
	Josamycin	197	41	8	6
	Lincomycin	120	11	8	8
	Tulathromycin	217	42	5	5
Kidney	Tylosin	66.7	11.5	10	8
	Josamycin	177	24	7	5
	Tilmicosin	36.5	4.6	6	6

For lincomycin, tulathromycin and tilmicosin all reported results were satisfactory. For tylosin and josamycin some questionable and unsatisfactory results are observed. The occurrence of questionable or unsatisfactory results could not be explained by the applied detection or sample preparation technique

nor by the use of different reference standards or the fact that some laboratories reported tylosin A and some reported the total amount of tylosin.

In this proficiency study 45% of the laboratories showed acceptable performance in terms of accuracy and the absence of false positive and false negative findings.

Based on the results of this proficiency study it is concluded that:
- Although regulations for most macrolides are established before 2005, many laboratories do not have a validated and accreditated method for the analysis of all relevant macrolides.
- For tylosin and josamycin more effort is needed for an accurate and more precise quantification of macrolides in porcine muscle. The elimination of ion suppression and the use of a well characterized tylosin reference standard could be important issues.
- In general, more effort is needed to control food safety with respect to the occurrence of macrolide residues.

Contents

1   Introduction

1.1   Proficiency testing

Proficiency testing is conducted to provide laboratories with a powerful tool to evaluate and demonstrate the reliability of the data that is produced. Next to validation and accreditation, proficiency testing is an important requirement of the EU Additional Measures Directive 93/99/EEC [1] and is increasingly important in the new ISO 17025:2005 [2].
No internationally focused broad range proficiency studies regarding the analysis of macrolides in porcine muscle or kidney that focused on the quantitative aspect were organized during the last years: an inter-laboratory quality control for this analyte-matix combination was lacking. Therefore, RIKILT decided to organize a proficiency study regarding this subject.

The aim of this proficiency study was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of macrolides in porcine tissues. Two different tissues were included in the proficiency study giving the opportunity to compare method performances for both matrices. This study also provided an evaluation of the methods applied for quantitative and confirmatory analysis of macrolides in porcine tissue.

This proficiency study was conducted in accordance with guidelines ISO/IEC 43-1 [3], ISO/IEC 43-2 [4] and ILAC-G13 [5] and was organized under accreditation by RIKILT - Institute of Food Safety.

1.2   Macrolides

The macrolides are antimicrobial agents consisting of one or more deoxy sugars bound to a 14, 15 or 16-membered macrocyclic ring. The first macrolide, erythromycin, was isolated in 1952 from *Streptomyces erythreus* [6].

Macrolides have a very broad clinical application in livestock, poultry and domestic animals in the treatment of infections such as respiratory tract and soft tissue infections, being more effective towards Gram-positive than Gram-negative bacteria. The mechanism of action of the macrolides is inhibition of bacterial protein biosynthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby blocking translocation of peptidyl tRNA [6] or causing dissociation of the peptidyl-tRNA [7]. Tylosin and lincomycin are the most commonly used macrolides for controlling dysentery and *Mycoplasma* infections in swine [7].

Macrolide resistance is an emerging problem [8]. Especially because macrolides have been used in the treatment of food producing animals for decades [7], control of food products for the presence of macrolide residues is of importance.

The use of macrolides as veterinary drug is regulated within the European Union. Macrolides are included in Annex I: pharmacologically active veterinary products for which a Maximum Residue Limit (MRL) is established [10]. Regarding macrolides MRLs for several species and tissues are established.

This proficiency study focused on tylosin, lincomycin (a lincosamide, closely related to macrolides), josamycin, tilmicosin and tulathromycin in porcine muscle and kidney. The MRLs for these compounds in porcine muscle and kidney are presented in Table 1.

Table 2. MRL in porcone muscle and kidney of macrolides included in the inter-laboratory study

Compound	MRL in porcine muscle (µg/kg)	MRL in porcine kidney (µg/kg)	Reference
Tylosin	100	100	[10]
Lincomycin	100	1500	[10]
josamycin	200	400	[11]
Tilmicosin	50	100	[10]
tulathromycin	-	3000	[12]

2   Test materials

2.1   Sample preparation

For muscle one blank material and one material containing tylosin (TYL), josamycin (JMC), lincomycin (LMC) and tulathromycin (TMC) were prepared. For kidney one blank material and one material containing tylosin, josamycin and tilmicosin (TMS) were prepared. The macrolide containing materials were prepared by adding methanolic solutions of these compounds to blank materials. The materials presented in Table 2 were obtained. Each of the materials was homogenised under cryogenic conditions according to in-house standard operating procedures.

Table 3.Target amount of macrolides in the inter-laboratory study test materials

Material code	Target amount (µg/kg)				
	TYL	JMC	LMC	TMC	TMS
M-A	-	-	-	-	-
M-B	80	300	100	130	
K-A	-	-	-	-	-
K-B	90	300	-	-	45

2.2   Sample identification

The materials were stored in polypropylene containers containing at least 25 gram of sample, yielding a total of 38 containers of material M-A and K-A, 80 containers of material M-B and 60 containers of material K-B. The muscle samples were randomly coded with a code from MACRO/2008/MUSCLE/001 through 118. The kidney samples were randomly coded with a code from MACRO/2008/KIDNEY/001 through 098.

For homogeneity and stability testing, 20 randomly selected containers of material M-B and K-B were assigned. For each laboratory a sample set was prepared consisting of one randomly selected sample of material M-A, K-A and K-B and two randomly selected samples of material K-B. The codes of the samples belonging to each sample set are presented in Annex 1.

2.3   Homogeneity study

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [13] and ISO/DIS 13528 [14], taking into account the insights discussed by Thompson [15] regarding the Horwitz equation.

With this procedure the between-sample standard deviation (s_s) is compared with the target standard deviation derived from the Horwitz equation (σ_H, §4.3). A material is considered adequately homogeneous if $s_s \leq 0.3\sigma_H$.

Ten containers of materials M-B and K-B were each analyzed in duplicate for TYL, JMC, LMC, TMC, TMS, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin to determine the homogeneity of the materials. The results of the homogeneity study and their statistical evaluation are presented in Annex 2a through f. For TMC no data were obtained during the homogeneity study. Because all materials demonstrated to be sufficiently homogeneous for use in the proficiency study for TYL, JMC, LMC and TMS it was concluded that also TMC was sufficiently homogeneous. The amounts determined during the homogeneity study are presented in table 3.

No extensive homogeneity study was carried out for materials M-A and K-A. The homogeneity of these materials is not relevant because the results of these materials will not be evaluated in a quantitative way. Furthermore, it is assumed that the homogeneity of material M-A and K-A are comparable with the homogeneity of the other materials because all materials are homogenized in the same way. Nevertheless, three randomly selected samples of material M-A and K-A were analyzed for 12 macrolides. No aivlosin, erythromycin, gamithromycin, JMC, LMC, pirlimycin, tiamulin, TMS, TMC, TYL, spiramycin or valnemulin was detected. It was concluded that materials M-A and K-A are suited to use as blank materials in the proficiency study.

Table 4. Determined amount of macrolides in the proficiency study test materials

Material code	Amount of TYL (µg/kg)	Amount of JMC (µg/kg)	Amount of LMC (µg/kg)	Amount of TMS (µg/kg)
M-A	-	-	-	-
M-B	52.6	245	145	-
K-A	-	-	-	-
K-B	68.4	242	-	30.9

2.4 Participants

Thirteen laboratories subscribed for participation in the proficiency study macrolides in porcine tissue. Most participating laboratories are situated in Europe.

2.5 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code (1 through 13). The sample sets with the corresponding number, consisting of five coded samples (Annex 1) were sent to the participating laboratories during the first half of August 2008. The sample sets were packed in an insulating box containing dry ice or cool packs and were dispatched to the participants immediately by courier. Three laboratories reported that the samples were not sufficiently frozen at arrival. A new

sample set was sent to each of these laboratories. For one sample set a severe delay at customs occurred. Therefore the corresponding laboratory was not able to analyze the samples within the time frame of the study. All other laboratories confirmed the receipt of the samples in good condition (frozen). The samples were accompanied by a letter (Annex 3) describing the requested analyses, an acknowledgement of receipt form and a results form.

The laboratories were asked to store the samples until analysis according to their own laboratory's procedure. A duplicate analysis of each sample was requested, resulting in two results for materials M-A, K-A and K-B, and four results for material M-B. The deadline for sending in results was October 17th 2007, allowing the participants at least six weeks for analysis.

2.6   Stability

Just after preparation of the materials three randomly selected samples of each material were stored at <-70 °C. It is assumed that the macrolides in the samples are stable at these storage conditions. The remaining samples were stored at -20 °C. On November 10th three randomly selected samples of each material were moved from -20 °C to room temperature to thaw. On November 20th, after the deadline of the inter-laboratory study, the samples stored at <-70 °C, three randomly selected samples of each of the materials stored at -20°C and the thawed samples were analyzed. For each set of samples, the average of the results and the standard deviation was calculated.

First it was determined if a consequential instability occurred [13, 14]. A consequential instability occurs when the average value of the samples stored at -20°C is more than $0.3\sigma_H$ below the average value of the samples stored at <-70 °C. If so, the instability has a significant influence on the calculated z-scores. Second, it was determined if a statistically significant instability occurred using a Students t-test [14]. The hypothesis for this test is:

$$E(x_0) = E(x_d)$$

where:
$E(x_0)$ = the expected amount of macrolides for the samples stored at <-70 °C;
$E(x_d)$ = the expected amount of macrolides for the samples stored at -20 °C.

The value t is calculated by:

$$t = \frac{\bar{x}_0 - \bar{x}_d}{s\sqrt{\frac{1}{n_0} + \frac{1}{n_d}}}$$

where:
$\bar{x}_0$ = the average amount calculated for the samples stored at <-70 °C;
$\bar{x}_d$ = the average amount calculated for the samples stored at 20 °C;
s = pooled standard deviation;

n_0 = number of results of the samples stored at <-70 °C;
n_d = number of results of the samples stored at 20 °C;

The calculated value t is compared to a critical value (t_{crit}) derived from a Students-t table with t having $n_0 + n_d$ - 2 degrees of freedom [14]. If t < t_{crit} it is demonstrated that no statistically significant difference between the average amount of the samples at both storage conditions is found.

The results and statistical evaluation of the stability test are presented in Annex 4. For tulathromycin a severe variation in the replicate results was obtained and therefore evaluation of the stability is not possible for this compound. For the other compounds in all materials no statistically significant instability was observed when the samples are stored at -20 °C. For josamycin in muscle a severe increase in the standard deviation of the results is observed after 92 days of storage at -20°C. Using an F test it was shown that the difference between the standard deviation of the results of storage at <-70°C and -20°C is significant. Therefore the t test is not a valid test for comparing both of the averages for josamycin in muscle.

When looking at the absolute difference between the averages at both storage conditions a consequential instability was only observed for josamycin in muscle. Therefore, the evaluation of laboratories that obtained a z-score just outside the 2s or 3s limits for josamycin in muscle should not be used for evaluation purposes but for information only. For tulathromycin in muscle no conclusions regarding the quantitative aspect can be drawn from the results.

For all analytes in the thawed samples a severe decrease of the level was observed ranging from a loss of 32% for josamycin in muscle to 74% for tylosin in muscle. This indicates that if the samples arrived thawed, no conclusions can be drawn from this proficiency study.

3   Applied methods of chemical analysis

The participating laboratories applied different sample preparation procedures for the analysis of macrolides in porcine tissues. All laboratories apply the same method for muscle as for kidney, with the exception of lab 7. Lab 7 added an additional extraction using hexane to remove fat. A schematic overview of the methods applied is presented in Annex 5.

For the analysis of macrolides in porcine tissue many different extraction solvents or mixtures of solvents were used at various pH's. Four laboratories apply an aqueous buffer for extraction. Three of them apply a pH of 4.0, the other a pH of 10.5. Three laboratories use only acetonirile as the extraction solvent. A phase exchange or dilution with water is needed to make the extract suitable for analysis.

For the sample clean up also several different techniques were applied. Five laboratories applied solid phase extraction using either the reversed phase or ion exchange principle. Three laboratories only diluted and/or filtrated the raw extract before analysis.

Two detection techniques were applied for the quantitative analysis of macrolides in porcine tissues. One laboratory applied LC combined with photo diode array detection (PDA). The other eight labs used MS/MS as the detection techniques. This detection technique is suited for confirmation of the identity of group B substances according to 2002/657/EC [17].

Of the participants that used LC-MS/MS as a detection technique, six used one or more internal standards for the quantification of the macrolides. The internal standards used are:
- roxithromycin
- clindamycin
- oleandomycin
- erythromycin-^{13}C-d$_3$

The laboratories that did not analyze for one or more of the macrolides mentioned in the invitation letter are presented in Table 4. It is noted that especially aivlosin, gamithromycin, tiamulin and tulathromycin are not included by most laboratories. This is not very surprising, because no regulations are set for gamithromycin and for aivlosin and tulathromcin regulations are only established in 2007 [16] and 2004 [12] respectively. For tiamulin regulations were established in 1999 [17] but tiamulin is considered a pleuromutilin instead of a macrolide. Nevertheless it has a structural relation with macrolides.

Table 5. Overview of laboratories that did not include all macrolides in the analysis.

Compound	Not included by lab
Aivlosin	1, 2, 3, 5, 7, 8, 12
Erythromycin	3, 9
Gamythromycin	1, 2, 3, 5, 7, 8, 9, 12, 13
Josamycin	3, 9
Lincomycin	3, 8, 9
Pirlimycin	2,3,7
Tiamulin	1, 2, 3, 8, 9, 12
Tilmicosin	3, 9
Tulathromycin	2, 3, 7, 8, 9, 12
Tylosin	
Spiramycin	3, 8
Valnemulin	2,3,7,8,9,12

An overview of the method performance characteristics of the participating laboratories is presented in Annex 6. All values are presented as reported by the laboratories without any adjustments. Seven of the eleven laboratories that submitted results reported to have applied a validated method for the analysis of muscle. Of these laboratories five have an accreditation for this method. For the analysis of kidney only three laboratories reported to have applied a validated method.

4   Statistical evaluation

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [13], elaborated by ISO, IUPAC and AOAC and ISO/DIS 13528 [14] in combination with the insights published by the Analytical Methods Committee [21, 22] regarding robust statistics.

4.1   Calculation of the assigned value

The assigned value (*X*) was determined using robust statistics [14,20,21]. The advantage of robust statistics is that all values are taken into account: outlying observations are retained, but given less weight. Furthermore, it is not expected to receive normally distributed data in a proficiency test. When using robust statistics, the data does not have to be normally distributed in contrast to conventional outlier elimination methods.

The robust mean of the reported results of all participants, calculated from an iterative process that starts at the median of the reported results using a cut-off value depending on the number of results, was used as the assigned value [14,20]. The assigned value is therefore a consensus value.

4.2   Calculation of the uncertainty of the assigned value

The uncertainty of the assigned value is calculated to determine the influence of this uncertainty on the evaluation of the laboratories. A high uncertainty of the assigned value will lead to a high uncertainty of the calculated participants z_a-scores. If the uncertainty of the assigned value and thus the uncertainty of the z_a-score is high, the evaluation could indicate unsatisfactory method performance without any cause within the laboratory. In other words, illegitimate conclusions could be drawn regarding the performance of the participating laboratories from the calculated z_a-scores if the uncertainty of the assigned value is not taken into account.

The uncertainty of the assigned value (the robust mean) is calculated from the estimate of the standard deviation of the assigned value and the number of values used for the calculation of the assigned value:

$$u = \frac{\hat{\sigma}}{\sqrt{n}}$$

where:
u = uncertainty of the assigned value;
n = number of values used to calculate the assigned value;
$\hat{\sigma}$ = The estimate of the standard deviation of the assigned value resulting from robust statistics.

According to ISO/DIS 13528 [14] the uncertainty of the assigned value (*u*) is negligible and therefore does not have to be included in the statistical evaluation if:

$$u \leq 0.3\sigma_p$$

where:
u = The uncertainty of the assigned value;
σ_p = target standard deviation (§ 4.3).

In case the uncertainty of the assigned value does not comply with this criterion, the uncertainty of the assigned value should be taken into account when evaluating the performance of the participants regarding the accuracy (§ 4.4).

4.3   Calculation of the target standard deviation

According to Commission Decision 2002/657/EC [19], the coefficient of variation for the repeated analysis of a reference or fortified material under reproducibility conditions, shall not exceed the level calculated by the Horwitz equation. The Horwitz equation, $\sigma_H = 0.02c^{0.8495}$, presents a useful and widespread applied relation between the expected standard deviation under reproducibility conditions, σ_H and the concentration, *c* (g/g). It expresses inter-laboratory precision expected in inter-laboratory trials. Therefore, this relation is suitable for calculating the target standard deviation, σ_p in proficiency studies.

Thompson [13] demonstrated that the Horwitz equation is not applicable to the lower concentration range (<120 µg/kg) as well as to the higher concentration range (>138 g/kg). Therefore a complementary model is suggested:

For analyte concentrations <120 µg/kg:
$$\sigma_H = 0.22c$$

For analyte concentrations >138 g/kg:
$$\sigma_H = 0.01c^{0.5}$$

where:
σ_H = expected standard deviation in inter-laboratory trials;
c = concentration of the analyte (g/g).

The target standard deviation (σ_p) of tylosin and tilmicosin were determined using the equation for analyte concentrations <120 µg/kg. The target standard deviation (σ_p) of josamycin, lincomycin and tulathromycin were determined using the Horwitz equation. In these calculations *c* = the assigned value (*X*) expressed in g/g and $\sigma_H = \sigma_p$.

4.4   Performance characteristics with regard to the accuracy

For illustrating the performance of the participating laboratories with regard to the accuracy a z_a-score is calculated. For the evaluation of the performance of the laboratories, the Guidelines of ISO/IEC Guide 43-1 [3] and ISO/DIS 13528 [14] are applied. According to these guidelines z_a-scores are classified as presented in Table 5.

Table 6: Classification of z_a-scores

$|z| \leq 2$	Satisfactory
$2 < |z| < 3$	Questionable
$|z| \geq 3$	Unsatisfactory

If the calculated uncertainty of the assigned value complies with the criterion mentioned in § 4.2, the uncertainty is negligible. In this case the accuracy z-score is calculated from:

$$z_a = \frac{\bar{x} - X}{\sigma_p}$$

where:
z_a = accuracy z-score;
$\bar{x}$ = the average result of the laboratory[*];
X = assigned value;
σ_p = target standard deviation.

However, if the uncertainty of the assigned value does not comply with the criterion mentioned in § 4.2, it could influence the evaluation of the laboratories. Therefore in this case, the uncertainty is taken into account by calculating the accuracy z-score [14]:

$$z'_a = \frac{\bar{x} - X}{\sqrt{\sigma_p^2 + u^2}}$$

where:
z'_a = accuracy z-score taking into account the uncertainty of the assigned value;
$\bar{x}$ = the average result of the laboratory[*];
X = assigned value;
σ_p = target standard deviation;
u = uncertainty of the assigned value.

---

[*] In the evaluation $\bar{x}$ is an average of two or four values whereas σ_p is defined for a single analysis. This results in slightly optimistic z-scores.

5 Results and discussion

Thirteen laboratories subscribed for the participation in the inter-laboratory study for macrolides in porcine tissue. Eleven laboratories managed to submit valid results for muscle and ten laboratories managed to submit valid results for kidney. Not all laboratories did include all macrolides present in the sample. The amount of laboratories submitting results for each macrolide present in the muscle and the kidney materials is presented in table 6.

Table 7: Amount of laboratories that reported results for each macrolide in both muscle an kidney.

Matrix	Compound	No. of labs that reported a result
Muscle	Tylosin	10
	Josamycin	8
	Lincomycin	8
	Tulathromycin	5
Kidney	Tylosin	10
	Josamycin	7
	Tilmicosin	6

For the materials for which less than seven laboratories reported quantitative results, the data is only evaluated for information. The assigned value and the z-scores are calculated, but no conclusions should be drawn from this regarding the performance of the laboratories.

Some false negatives and false positives occurred in this proficiency study. An overview is given in Annex 7. Laboratory 4 and 8 did not detect tilmicosin in the kidney material (K-B) although tilmicosin is included in their method. It is noted that both labs did not yet validate their method for tilmicosin in kidney. Nevertheless, both finding are considered as false negatives.
Laboratory 12 did not detect tylosin in the muscle samples (M-B) although tylosin was included in their validated method and their LoD was 3 µg/kg which is far below the level of tylosin present in the samples. This is considered to be a false negative result. Furthermore, this laboratory detected spiramycin in both kidney samples (K-A and K-B) at significant levels. These findings are considered to be false positive results.

5.1 Evaluation of the results of tylosin

Tylosin was present in both the muscle and the kidney material. All laboratories that reported results have tylosin included in their method. Laboratory 12 did not detect tylosin in the muscle samples. Therefore, the evaluation of tylosin in muscle and kidney are both based on ten results. The results for tylosin as well as the evaluation of it are presented in Annex 8.

For muscle the lowest value reported for tylosin is 24 µg/kg and the highest value is 76 µg/kg. The assigned value of tylosin in muscle is 38.3 µg/kg with an uncertainty of 4.3 µg/kg.

The uncertainty of the assigned value of tylosin exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for tylosin obtained by each laboratory were calculated. The results are presented in Appendix 8a. Graphical representations of the z'_a-scores are included.

With respect to the accuracy the results of three laboratories (lab 2, 8 and 13) are questionable. The difference in accuracy among laboratories could not be attributed to differences in the applied sample preparation or detection technique.

For kidney the lowest value reported for tylosin is 23 µg/kg and the highest value is 128 µg/kg. The assigned value of tylosin in muscle is 66.7 µg/kg with an uncertainty of 11.5 µg/kg. The uncertainty of the assigned value of tylosin exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for tylosin obtained by each laboratory were calculated. The results are presented in Appendix 8b. Graphical representations of the z'_a-scores are included. With respect to the accuracy the results of laboratory 9 and 12 are questionable. It is noted that both laboratories only filtered or diluted their raw kidney extract before analysis. The same accounts for laboratory 7, but they applied an additional hexane extraction. It might be possible that due to the limited sample clean up procedure, ion suppression results in an enhanced signal.

The spreading in the reported results could be caused by the use of different reference standards or a different way of reporting results. According to regulations [10] the marker for tylosin is tylosin A. Therefore, laboratories should determine and report the amount of tylosin A solely. In table 7 the reference standard used by each of the laboratories are reported including whether the laboratory reported tylosin A or the total amount of tylosin. Of all of the used reference standards the certificates only indicate the purity of the total amount of tylosin. No specific information is given on the purity of tylosin A. Therefore, when determining the amount of tylosin A in an unknown sample, an extra error is introduced due to the unknown amount of tylosin A in the reference standard. This is an important complicating factor in the control of tylosin.

Table 8: Overview of the used reference standards for tylosin and the way of reporting

Lab code	Manufacturer	Catalogue number	Lot	Tylosin A / total	z' -scores muscle
1	Sigma	T6134	108H1073	Total	-1,04
2	Sigma	T6134	086K2004	Total	**2,06**
3	Sigma			Total	-0,56
4	Sigma	T6134	047K1639	Tylosin A	-0,93
5					-1,34
6	Fluka	93806	1304363	Tylosin A	-0,06
7	Riedel de Haen	33864	7045X	Tylosin A	-1,36
8	Sigma	T6134	108H1073	Tylosin A	**2,16**
9	Fluka	93806	1304363	Tylosin A	0,47
12	Riedel de Haen	33847	7190X	Tylosin A	**2,55**
13					-1,04

In some cases laboratories used the same reference standard (even the same lot number), but no correlation was found between the z'_a-scores and the reference standard used. Surprisingly, there is also no corrolation between the z'_a-scores and whether laboratories reported the amount of tylosin A or the total amount of tylosin.

Comparing the reported results for tylosin of the muscle and the kidney samples it is clear that both matrices result in a severe spreading of the laboratory results. No significant difference in the uncertainty of the assigned values for both matrices was demonstrated using the F-test.

When comparing results for muscle and kidney within a laboratory, it is expected that if a laboratory reports a deviating value for muscle, the value for kidney will deviate as well. Surprisingly, no correlation was found between the reported results for muscle and kidney for most laboratories. Only laboratory 3, 7 and 8 obtained comparable z'_a-scores for muscle and kidney.

5.2 Evaluation of the results of josamycin

Josamycin was present in both the muscle and the kidney material. Josamycin was not included in the method by laboratories 3 and 9. No false negatives or false positives for josamycin occurred. Therefore, the evaluation of josamycin in muscle and kidney is based on eight and seven results respectively. The results for josamycin as well as the evaluation of it are presented in Annex 9.

For muscle the lowest value reported for josamycin is 74.6 µg/kg and the highest value is 524 µg/kg. The assigned value of josamycin in muscle is 196.6 µg/kg with an uncertainty of 40.8 µg/kg. The uncertainty of the assigned value of josamycin exceeds σ_p. Therefore, for this material, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for josamycin obtained by each laboratory were calculated. The results are presented in Appendix 9a. Graphical representations of the z'_a-scores are included. With respect to the accuracy the results of laboratories 8 and 13 are questionable. Laboratory 12 obtained a z'_a-score just below -2: an unsatisfactory result. However, due to the consequential instability observed for josamycin in muscle, this result is not suited for evaluation purposes. The difference in accuracy among laboratories could not be attributed to differences in the applied sample preparation or detection technique.

For kidney the lowest value reported for josamycin is 121 µg/kg and the highest value is 441 µg/kg. The assigned value of josamycin in muscle is 176.5 µg/kg with an uncertainty of 24.4 µg/kg. The uncertainty of the assigned value of josamycin exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for josamycin obtained by each laboratory were calculated. The results are presented in Appendix 9b. Graphical representations of the z'_a-scores are included. With respect to the accuracy the results of laboratories 8 and 13 are questionable. The difference in accuracy among laboratories could not be attributed to differences in the applied sample preparation or detection technique.

Comparing the results of the muscle and the kidney analysis for josamycin it is clear that both matrices result in a severe spreading of the results. No significant difference in the uncertainty of the assigned values for both matrices was demonstrated using the F-test. When comparing results for muscle and kidney within a laboratory, it is expected that if a laboratory reports a deviating value for muscle, the

value for kidney will deviate as well. Surprisingly, no correlation was found between the reported results for muscle and kidney for most laboratories. Only laboratory 7 and 8 obtained comparable z'_a-scores for muscle and kidney.

5.3 Evaluation of the results of lincomycin

Lincomycin was present only in the muscle material. Lincomycin was not included in the method by laboratory 3, 8 and 9. No false negatives or false positives for lincomycin occurred. Therefore, the evaluation of lincomycin in muscle is based on eight results. The results for lincomycin as well as the evaluation of it are presented in Annex 10.

For lincomycin the lowest value reported is 68 μg/kg and the highest value is 208 μg/kg. The assigned value of lincomycin is 120.2 μg/kg with an uncertainty of 11.3 μg/kg. The uncertainty of the assigned value of lincomycin exceeds $0.3\sigma_p$ (§4.2). Therefore, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for lincomycin obtained by each laboratory were calculated. The results are presented in Appendix 10. A graphical representation of the z'_a-scores is included. With respect to the accuracy all laboratories obtained satisfactory results.

5.4 Evaluation of the results of tulathromycin

Tulathromycin was present only in the muscle material. Tulathromycin was not included in the method by laboratory 2, 3, 7, 8, 9 and 12. No false negatives or false positives for tulathromycin occurred. Therefore, the evaluation of tulathromycin in muscle is based on five results. Because this number is below seven and because no stability information was obtained for tulathromycin, the evaluation of tulathromycin is for information only: no conclusions can be drawn regarding the performance of the laboratories. The results for tulathromycin as well as the evaluation of it are presented in Annex 11.

For tulathromycin the lowest value reported is 105 μg/kg and the highest value is 408 μg/kg. The assigned value of tulathromycin is 217.2 μg/kg with an uncertainty of 42.5 μg/kg. The uncertainty of the assigned value of tulathromycin is about σ_p. Therefore, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores for tulathromycin obtained by each laboratory were calculated. The results are presented in Appendix 11. A graphical representation of the z'_a-scores is included.

5.5 Evaluation of the results of tilmicosin

Tilmicosin was present only in the kidney material. Tilmicosin was not included in the method by laboratory 3 and 9. Laboratory 1 only reported one result for tilmicosin. Laboratory 4 and 8 did not detect tilmicoin in the kidney sample, although tilmicosin was included in their method. Therefore, the evaluation of tilmicosin in kidney is based on six results. Because this number is below seven, the

evaluation is for information only: no clonclusions can be drawn regarding the performance of the laboratories. The results for tilmicosin as well as the evaluation of it are presented in Annex 12.

For tilmicosin the lowest value reported is 22.8 µg/kg and the highest value is 83.7 µg/kg. The assigned value of tilmicosin is 36.5 µg/kg with an uncertainty of 4.6 µg/kg. The uncertainty of the assigned value of tilmicosin exceeds $0.3\sigma_p$ (§4.2). Therefore, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'$_a$-scores for tilmicosin obtained by each laboratory were calculated. The results are presented in Appendix 12. A graphical representation of the z'$_a$-scores is included.

5.6   Overall evaluation

If not taking the results of tulathromycin in muscle and tilmicosin in kidney into account due to the low number of laboratories that reported results, from the 11 laboratories that submitted results 5 (i.e. 45%) showed optimal performance for the analysis of macrolides in muscle and kidney with respect to the accuracy and the occurrence of false positive and false negative results. An overview of the amount of satisfactory results is presented in table 8. A complete overview of z'$_a$-scores is given in Annex 13.

Table 9: Overview of the amount of satisfactory results for accuracy

Matrix	Compound	No. laboratories that reported results	No. of satisfactory results for accuracy	No. of questionable results for accuracy	No. of unsatisfactory results for accuracy
Muscle	Tylosin	10	8	2	0
	Josamycin	8	5	1	2
	Lincomycin	8	8	0	0
	Tulathromycin	5	5	0	0
Kidney	Tylosin	10	7	3	0
	Josamycin	7	5	1	1
	Tilmicosin	6	6	0	0

The amount of participating laboratories in the proficiency test for macrolides in porcine tissues is low. Many invited laboratories reported not to have a (validated) method available. Of the laboratories that did participate, many did not have all macrolides for which regulations are established included in their method.
Furthermore a severe variation in the results is observed for all compounds.

6   Conclusions

Thirteen laboratories subscribed for participation in the proficiency study macrolides in porcine tissue. Eleven laboratories managed to submit results for muscle. Ten of them were also able to report results for the kidney samples. Seven of the laboratories that reported results applied a validated method. The majority of labs applied the same method for muscle and kidney. Only one lab carried out an additional extraction for the kidney analysis using hexane.

In this proficiency test three laboratories reported false negative results. These involved tylosin in muscle and tilmicosin in kidney. One of these labs also reported a false positive result: spiramycin in muscle.

Table 10: Overview of the amount of satisfactory results for accuracy

Matrix	Compound	No. laboratories that reported results	No. of satisfactory results for accuracy	No. of questionable results for accuracy	No. of unsatisfactory results for accuracy
Muscle	Tylosin	10	8	2	0
	Josamycin	8	5	1	2
	Lincomycin	8	8	0	0
	Tulathromycin	5	5	0	0
Kidney	Tylosin	10	7	3	0
	Josamycin	7	5	1	1
	Tilmicosin	6	6	0	0

In all cases $u > 0.3\sigma_p$. This indicates that there is a severe variation among the laboratories. For several compounds the difference between the lowest and the highest reported value is a factor 5. As a result of this variation 6 of the 11 laboratories obtained questionable or unsatisfactory results.

Based on the results of this proficiency study it is concluded that:
- Although regulations for most macrolides are established before 2005, many laboratories do not have a validated and accreditated method for the analysis of all relevant macrolides.
- For all compounds in both matrices the variation among the laboratories is severe.
- For tylosin and josamycin more effort is needed for an accurate and more precise quantification of macrolides in porcine muscle. The elimination of ion suppression and the use of a well characterized tylosin reference standard could be important issues.
- In general, more effort is needed to control food safety within Europe with respect to the occurrence of macrolide residues.

7   References

1 Council directive 93/99/EEC of 29 October 1993 on the subject of additional measures concerning the official control of foodstuffs. Official Journal L 290, 24/11/1993, 0014 - 0017.

2 ISO/IEC 17025:2005(E). 2005. General Requirements for the Competence of Calibration and Testing Laboratories

3 ISO/IEC Guide 43-1. 1997. Proficiency testing by inter-laboratory comparisons - Part 1: Development and operation of proficiency testing schemes, 2nd edition.

4 ISO/IEC Guide 43-2. 1997. Proficiency testing by inter-laboratory comparisons - Part 2: Selection and use of proficiency testing schemes by laboratory accreditation bodies, 1st edition.

5 ILAC-G13:2007. 2007. ILAC Guidelines for the Requirements for the Competence of Providers of Proficiency Testing Scemes.

6 Swords WE, Rubin BK. 2003. Macrolide antibiotics, bacterial populations and inflammatory airway disease. The journal of medicine 61 (7): 242-248.

7 Engberg J, Aarestrup FM, Taylor DE, Gerner-Smidt P, Nachamkin I. 2001. Quinolone and Macrolides resistance in Campylobacter jejuni and C. coli: Resistance Mechanisms and Trends in human Isolates. CDC Emerging Infectious Diseases 7(1):24-34.

8 Klugman KP, Lonks JR. 2005. Hidden Epidemic of Macrolide-resistant Pneumococci. CDC Emerging Infectious Diseases 11(6).

9 Council Regulation (ECC) No 2377/90. 26 June 1990. Laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Commun. L224: 1

10 Corrigendum to Commission Regulation (EC) No 1181/2002 of 1 July 2002 amending Annex I of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. 2002. Official Journal of the European Communities. L 172.

11 Commission Regulation (EC) No 953/1999. 5 May 1999. Amending Annexes II and III of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal. L 118: 0023 - 0027

12 Commission Regulation (EC) No 1101/2004. Amending Annexes I and II to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue

limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal. L 211: 0003 - 0005

13 Thompson M, Wood R. 1993. The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories. J. AOAC Int. 76(4):926-940.

14 ISO/DIS 13528:2005(E). 2005. Statistical methods for use in proficiency testing by inter-laboratory comparison, Reference number of working document ISO/TC 69/SC 6 N 459.

15 Thompson M. 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst. 125:385-386.

16 Commission Regulation (EC) No 1353/2007. 20 November 2007. Amending Annex I to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards Monensin, Lasalocid and Tylvalosin. Official Journal. L 303. 6 - 8.

17 Commission Regulation (EC) No 2728/1999. 20 December 1999. Amending Annexes I, II and III to Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal L 328. 23 - 34.

18 Miller JN, Miller JC. Statistics and Chemometrics for Analytical Chemistry. 4th edition.

19 Commission Decision 2002/657/EC. 12 August 2002. Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal. L 221:67A-76A.

20 Analytical Methods Committee. 1989. Robust statistics - How not to reject outliers Part 1. Basic concepts. Analyst 114:1693-1697.

21 Analytical Methods Committee. 1989. Robust statistics - How not to reject outliers Part 2. Inter-laboratory trials. Analyst. 114:1699-1702.

22 ISO 5725-2. 1994. Accuracy (trueness and precision) of measurement methods and results - part 2: Basic methods for the determination of repeatability and reproducibility of a standard measurement method. 1st edition.

23 Official Methods of Analysis Program Manual [Internet]. c 2002. Annex D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis. AOAC International; [cited 2007 Dec 1] Available form: http://www.aoac.org/vmeth/Manual_Part_6.pdf

Annex 1   Codification of the samples

Sample set	Material M-A*	Material M-B*	Material K-A*	Material K-B*
1	109	031	077	008
		069		
2	057	050	039	035
		084		
3	079	066	031	047
		104		
4	052	047	054	056
		091		
5	072	060	015	028
		099		
6	115	095	004	057
		103		
7	114	005	016	044
		094		
8	088	018	011	079
		026		
9	020	013	098	034
		107		
10	043	042	070	055
		076		
11	062	006	012	066
		038		
12	102	014	068	082
		063		
13	100	068	076	027
		101		
14	040	046	019	032
		097		
15	093	070	083	042
		092		
16	022	048	078	010
		105		
17	067	055	071	075
		081		
18	117	051	002	087
		087		
19	023	064	009	089
		078		
20	090	037	006	001
		098		

* all muscle sample codes start with MACRO/2008/MUSCLE/ and the kidney sample codes with
  MACRO/2008/KIDNEY/

Annex 2a   Statistical evaluation of homogeneity data of material M-B for tylosin

Sample No.	Tylosin (µg/kg) Replicate 1	Replicate 2
1	44.9	48.5
2	50.8	59.0
3	50.7	56.9
4	62.3	58.0
5	61.8	50.0
6	52.9	51.2
7	49.2	62.0
8	54.5	49.7
9	52.3	42.9
10	46.9	47.5
Grand mean	52.6	
Cochran's test		
C	0.297	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 11.57	
s_x	4.38	
s_w	5.25	
s_s	2.32	
Critical = 0.3 σ_H	3.47	
s_s < critical?	ACCEPTED	

No tilmicosin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2b Statistical evaluation of homogeneity data of material M-B for josamycin

Sample No.	Josamycin (µg/kg) Replicate 1	Replicate 2
1	223	225
2	224	269
3	261	256
4	281	268
5	274	213
6	242	229
7	251	263
8	253	261
9	237	231
10	227	220
Grand mean	245	
Cochran's test		
C	0.581	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 48.5	
s_x	16.7	
s_w	17.9	
s_s	10.8	
Critical = 0.3 σ_H	14.6	
s_s < critical?	ACCEPTED	

No tilmicosin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2c   Statistical evaluation of homogeneity data of material M-B for lincomycin

	Lincomycin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	144	101
2	136	137
3	141	138
4	151	138
5	145	137
6	162	156
7	162	157
8	162	153
9	161	156
10	151	110
Grand mean	145	
Cochran's test		
C	0.481	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 31.0	
s_x	13.2	
s_w	14.0	
s_s	8.78	
Critical = 0.3 σ_H	9.30	
s_s < critical?	ACCEPTED	

No tilmicosin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2d Statistical evaluation of homogeneity data of material K-B for tylosin

Sample No.	Tylosin (µg/kg) Replicate 1	Replicate 2
1	73.9	54.3
2	54.4	55.0
3	61.2	71.9
4	71.9	70.3
5*	74.5	65.5
6	80.2	74.7
7	77.4	59.3
8	69.9	60.1
9	59.6	87.0
10	73.6	73.8
Grand mean	68.4	
Cochran's test		
C	0.420	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 15.1	
s_x	6.38	
s_w	9.46	
s_s	0	
Critical = 0.3 σ_H	4.52	
s_s < critical?	ACCEPTED	

No lincomycin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2e   Statistical evaluation of homogeneity data of material K-B for josamycin

Sample No.	Josamycin (µg/kg)	
	Replicate 1	Replicate 2
1	284	180
2	192	192
3	191	254
4	264	255
5*	301	238
6	293	259
7	258	185
8	237	226
9	194	295
10	289	258
Grand mean	242	
Cochran's test		
C	0.299	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 48.0	
s_x	27.3	
s_w	42.9	
s_s	0	
Critical = 0.3 σ_H	14.4	
s_s < critical?	ACCEPTED	

No lincomycin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages

s_w = within-sample standard deviation

s_s = between-sample standard deviation

Annex 2f   Statistical evaluation of homogeneity data of material K-B for tilmicosin

Sample No.	Tilmicosin (µg/kg) Replicate 1	Replicate 2
1	38.4	23.9
2	23.9	23.6
3	25.3	28.7
4	29.6	28.4
5*	30.3	39.8
6	39.3	28.8
7	40.6	27.3
8	30.6	27.1
9	27.9	41.4
10	33.1	29.8
Grand mean	30.9	
Cochran's test		
C	0.259	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 6.8	
s_x	3.7	
s_w	6.4	
s_s	0	
Critical = 0.3 σ_H	2.0	
s_s < critical?	ACCEPTED	

No lincomycin, aivlosin, erythromycin, gamithromycin, pirlimycin, tiamulin, spiramycin and valnemulin were detected in the samples.

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 3   Instruction letter

RIKILT
INSTITUTE OF FOOD SAFETY
WAGENINGEN UR

Dear participant,

Thank you very much for your interest in the proficiency study for macrolides in porcine tissue.

Hereby I send you a parcel containing five randomly coded samples; three muscle and two kidney samples. The samples may contain one or more of the following macrolides (in alphabetical order):

Aivlosin (Acetylisovaleryltylosin)	Tiamulin
Erythromycin	Tilmicosin
Gamothromycin	Tulathromycin
Josamycin	Tylosin
Lincomycin	Spiramycin
Pirlimycin	Valnemulin

Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately, preferably by fax.

Your laboratory code is:

Instructions:
After arrival store the samples according to your laboratory's procedure.
Defrost the samples before analysis and homogenize them according to your laboratoy's procedure.
Please analyze the samples according to the standard protocol of your laboratory. The samples should be treated as if they are routine samples.
Please make use your own reference standards. Unfortunately RIKILT – Institute of Food Safety, can not supply any of these reference standards.
Carry out a duplicate quantitative analysis for each sample. Please confirm the identity of any detected residues of macrolides according to 2002/657/EC.
Each sample consists of at least 25 g tissue. Please contact me if this is not sufficient for a duplicate quantitative analysis.
The results should be reported before October 17th 2008.
Please use the results form for reporting the results.
The evaluation will primarily focus on the quantitative part of this study.

Please contact me if you have any questions or need any assistance.

Kind regards,

Bjorn Berendsen

Annex 4a   Statistical evaluation of stability data of material M-B

Statistical evaluation for tylosin in material M-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	35.90	31.16	12.18
	41.17	37.77	13.31
	39.44	32.52	16.07
	44.06	32.22	15.40
	32.69	33.11	0.64
	34.27	56.81	0.79
Average amount (µg/kg)	37.9	37.3	7.1
n	6	6	6
st. dev (µg/kg)	4.4	9.8	
Difference		0.65	28.2
$0.3\sigma_H$	2.5		
Consequential difference?		NO	YES
Diff $< 0.3\sigma_H$			
t		0.15	8.3
t crit		2.23	2.23
Statistical difference?		NO	YES
T $<$ t crit			

Statistical evaluation for josamycin in material M-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	183.54	143.29	151.09
	181.31	152.14	144.80
	175.70	162.73	145.79
	188.96	139.00	155.44
	177.25	152.18	64.00
	184.78	216.37	82.24
Average amount (µg/kg)	181.9	161.0	123.9
n	6	6	6
st. dev (µg/kg)	4.9	28.3	39.9
Difference		21.0	58.0
$0.3\sigma_H$	11.3		
Consequential difference?		YES	YES
Diff $< 0.3\sigma_H$			
t		1.78	3.53
t crit		2.23	2.23
Statistical difference?		NO	YES
T $<$ t crit			

Annex 4a continued   Statistical evaluation of stability data of material M-B

Statistical evaluation for lincomycin in material M-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	102.86	105.20	67.84
	104.02	122.77	76.93
	113.55	111.45	61.03
	117.88	100.08	58.61
	95.76	108.31	51.74
	100.08	113.58	46.76
Average amount (µg/kg)	105.7	110.2	60.5
n	6	6	6
st. dev (µg/kg)	8.4	7.8	10.9
Difference		-4.54	45.2
$0.3\sigma_H$	7.0		
Consequential difference?		NO	YES
Diff < $0.3\sigma_H$			
t		0.97	8.1
t crit		2.23	2.23
Statistical difference?		NO	YES
T < t crit			

Annex 4b Statistical evaluation of stability data of material K-B

Statistical evaluation for tylosin in material K-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	69.8	67.1	29.7
	62.7	69.2	25.7
	73.0		37.5
	86.8	93.2	21.7
	55.0	73.0	
	85.6	57.1	26.6
Average amount (µg/kg)	72.2	71.9	28.2
n	6	5	5
st. dev (µg/kg)	12.5	13.2	18.0
Difference		0.23	44.0
$0.3\sigma_H$	4.8		
Consequential difference?		NO	YES
Diff < $0.3\sigma_H$			
t		0.03	7.2
t crit		2.26	2.26
Statistical difference?		NO	YES
T < t crit			

Statistical evaluation for josamycin in material K-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	190.6	198.9	58.6
	144.4	176.7	42.0
	150.0	178.9	81.4
	197.1	209.4	38.7
	152.8	159.6	31.0
	204.8	168.5	51.0
Average amount (µg/kg)	173.3	182.0	50.5
n	6	6	6
st. dev (µg/kg)	27.1	18.7	18.0
Difference		-8.72	122.8
$0.3\sigma_H$	10.9		
Consequential difference?		NO	YES
Diff < $0.3\sigma_H$			
t		0.65	9.3
t crit		2.23	2.23
Statistical difference?		NO	YES
T < t crit			

Annex 4b continued   Statistical evaluation of stability data of material K-B

Statistical evaluation for tilmicosin in material K-B

Storage temp	-80 °C	-20°C	Thawed
Time at -20°C (days)	0	91	
Calculated amounts (µg/kg)	47.3	45.7	8.7
	40.4	45.3	9.1
	32.1	43.1	12.8
	38.0	41.6	11.2
	39.0	37.8	9.2
	35.7	43.3	11.9
Average amount (µg/kg)	38.8	42.8	10.5
n	6	6	6
st. dev (µg/kg)	5.1	2.9	1.7
Difference		-4.0	28.3
$0.3\sigma_H$	2.6		
Consequential difference?		NO	YES
Diff < $0.3\sigma_H$			
t		1.68	12.9
t crit		2.23	2.23
Statistical difference?		NO	YES
T < t crit			

Annex 5 Overview of the applied methods

Lab code	Extraction	Sample purification	Internal standard	Detection method	Macrolides not analysed for
1	ACN	Evaporation of solvent and reconstitution in TRIS buffer pH=7, extraction with hexane, on-line SPE (hydrophilic-lipophilic polymer)	Roxithromycin, clindamycin, oleandomycin	MS/MS	Aivlosin, gamithromycin, tiamulin
2	TRIS buffer pH=10.5	SPE (OASIS HLB)		MS/MS	Aivlosin, gamithromycin, tiamulin, tulathromycin, valnemulin, pirlimycin
3	NM	NM	NM	PDA	Aivlosin, erythromycin, gamithromycin, josamycin, lincomycin, tiamulin, tilmicosin, tulathromycin, spiramycin, valnemulin, pirlimycin
4	*Tylosin, tilmicosin: ACN *Lincomycin: 2,5% TCA *Tulathromycin: K2HPO4/CAN (75:25) + HCl	*Dilution, SPE (C$_{18}$) *Centrifugation *Hydrolisys at 60°C, SPE (OASIS MCX)		*PDA *LC-MS/MS *LC-MS/MS	Aivlosin, erythromycin, gamithromycin, josamycin, tulathromycin in muscle
5	Phosphate buffer pH=4	SPE (OASIS HLB), evaporation of solvent, reconstitution	Erythromycin-C13, roxithromycin	MS/MS	Aivlosin, gamithromycin
6	McIlvain/EDTA buffer, pH=4.0	Adjust pH to 2.0, SPE (OASIS MCX), evaporation of solvent, reconstitution		MS/MS	
7	ACN (+hexane for kidney)	Filtration	Roxithromycin	MS/MS	Aivlosin, gamithromycin, tulathromycin, pirlimycin
8			Roxithromycin	MS/MS	Aivlosin, gamithromycin, lincomycin, tiamulin, tulathromycin, spiramycin, valnemulin, pirlimycin
9	70% MeOH + EDTA	Dilution with water		MS/MS	Erythromycin, gamithromycin, josamycin, lincomycin, tiamulin, tilmicosin, pirlimycin
10					
11					
12	ACN	Filtration	Roxithromycin	MS/MS	Aivlosin, gamithromycin, tiamulin, tulathromycin, pirlimycin
13	McIlvain/EDTA buffer, pH=4.0	Adjust pH to 2.0, SPE (OASIS MCX), evaporation of solvent, reconstitution	Roxithromycin	MS/MS	

NM = not mentioned

RIKILT Report 2009.003

Annex 6a Overview of method characteristics for muscle as reported by the participants

Lab code	Validation / accreditation	Tylosin CCα (µg/kg)	Tylosin CCβ (µg/kg)	Josamycin CCα (µg/kg)	Josamycin CCβ (µg/kg)	Lincomycin CCα (µg/kg)	Lincomycin CCβ (µg/kg)	Tulathromycin CCα (µg/kg)	Tulathromycin CCβ (µg/kg)
1	NM	50*	7*	100*	25*	50*	18*	50*	50*
2	Yes / Yes	125	152	220	240	80	99		
3	Yes / Yes	25*	30*						
4	Yes / Yes	113	126			5*	10*		
5	Yes / NM	137.1		277.0		138.6		4695	
6	Yes / Yes	129	158	255	311	73	95	124	148
7	Yes / Yes	111	122	48	55	118	136		
8	No / No								
9	On going / No	118	135						
10									
11									
12	Yes / Yes	109	118	223	247	114	128		
13	No / No								

* Reported values are LOD and LOQ respectively instead of CCα and CCβ.

Annex 6b   Overview of method characteristics for kidney as reported by the participants

Lab code	Validation / accreditation	Tylosin CCα (µg/kg)	Tylosin CCß (µg/kg)	Josamycin CCα (µg/kg)	Josamycin CCß (µg/kg)	Tilmycosin CCα (µg/kg)	Tilmycosin CCß (µg/kg)
1*		50	7	200	25	500	
2							
3*	Yes / Yes	25	30				
4	Yes / Yes	6*	13*				
5	Yes / NM	137.1		277.0		138.6	
6	No / No						
7	No / No	109	117	60	70	1073	1146
8	No / No						
9	No / No						
10							
11							
12		115	130	444	487	1023	1046
13	No / No						

* Reported values are LOD and LOQ respectively instead of CCα and CCβ.

Annex 7   Overview of false positive and false negative results

False positive results

Lab code	Sample code	Material	Analyte found	Replicate 1 (μg/kg)	Replicate 2 (μg/kg)
Lab 12	MACRO/2008/KIDNEY/068	K-A	Spiramycin	192.5	192.7
Lab 12	MACRO/2008/KIDNEY/082	K-B	Spiramycin	86.7	90.7

False negative results

Lab code	Sample code	Material	Analyte	LoD (μg/kg)
Lab 4	MACRO/2008/KIDNEY/056	Tilmicosin	K-B	8
Lab 8	MACRO/2008/KIDNEY/079	Tilmicosin	K-B	NM
Lab 12	MACRO/2008/MUSCLE/014	Tylosin	M-B	3
Lab 12	MACRO/2008/MUSCLE/063	Tylosin	M-B	3

NM: Not mentioned

Annex 8a   Results for the analysis of tylosin in muscle

Tylosin								
Assigned value: 38.3 µg/kg								
Uncertainty of assigned value: 4.3 µg/kg								
Target standard deviation (Horwitz, Thompson): 8.4 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z'_a-score
1	27	28	28	31	28,5	1,29	1,68	-1,04
2	45	64	60	62	57,8	7,80	7,80	**2,06**
3	30	31	35	36	33,0	0,58	3,56	-0,56
4	33	28	25	32	29,5	3,51	3,51	-0,93
5	24,3	24,0	29,1	25,1	25,6	1,64	2,39	-1,34
6	41,2	40,2	32,3	37,5	37,8	2,16	4,38	-0,06
7	25	27	24	26	25,5	1,15	1,15	-1,36
8	76	62	41	56	58,8	8,38	15,66	**2,16**
9	44,5	41,1	43,9	41,7	42,8	1,65	1,65	0,47
13	59,1	55,4	59,7	75,4	62,4	6,59	8,64	**2,55**

Figure a: Graphical representation of the reported results

The chart plots Result (μg/kg) on the y-axis against Lab code on the x-axis, with reference lines labelled $X + 2\sqrt{\sigma_p^2 + u^2}$, X, and $X - 2\sqrt{\sigma_p^2 + u^2}$.

Figure b: Graphical representation of z'$_a$-sc

The bar chart plots z-score against Lab code (ordered 7, 5, 1, 4, 3, 6, 9, 2, 8, 13).
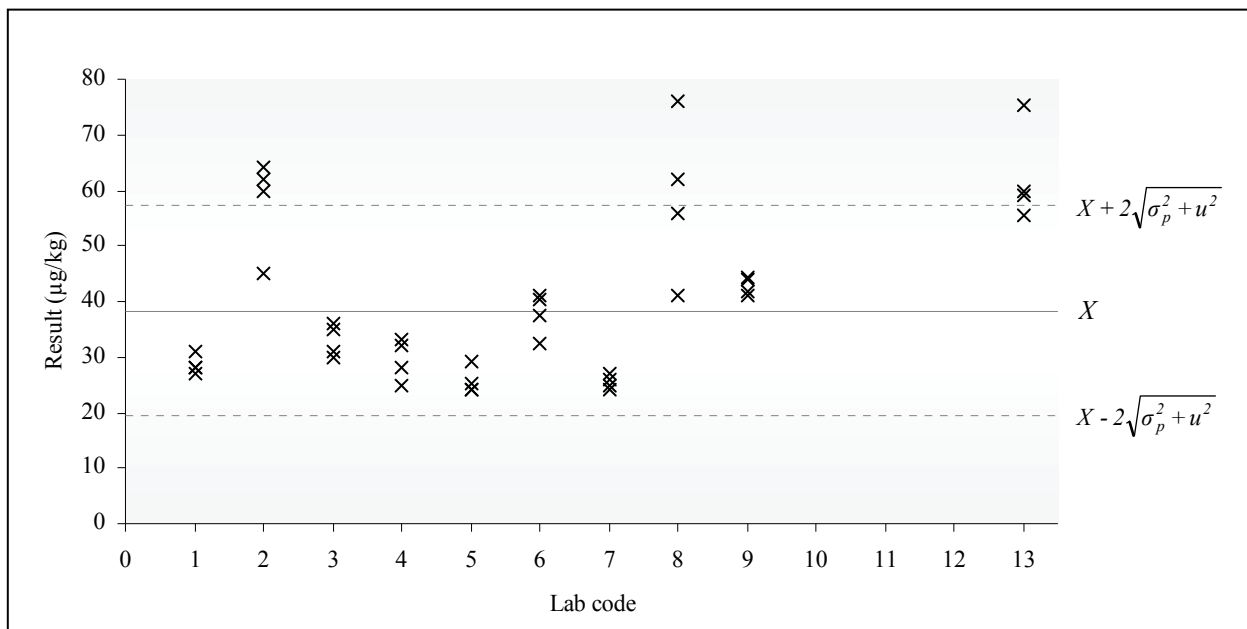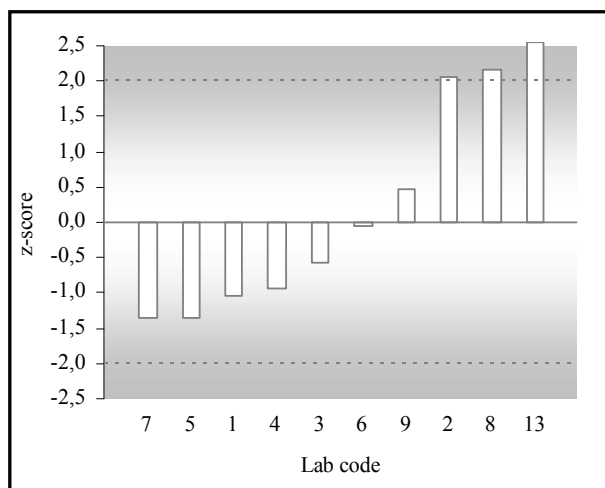
Annex 8b   Results for the analysis of tylosin in kidney

Tylosin

Assigned value: 66.7 µg/kg

Uncertainty of assigned value: 11.5 µg/kg

Target standard deviation (Horwitz, Thompson): 14.7 µg/kg

Code	Replicate 1	Replicate 2	Average	z_a-score
1	68	56	62,0	-0,25
3	57	61	59,0	-0,41
4	36	23	29,5	-1,99
5	83,7	74,6	79,2	0,67
6	30,1	38,2	34,2	-1,74
7	41	36	38,5	-1,51
8	75	121	98,0	1,68
9	128	112	120,0	**2,86**
12	109,4	113,5	111,5	**2,40**
13	42	44,5	43,3	-1,26

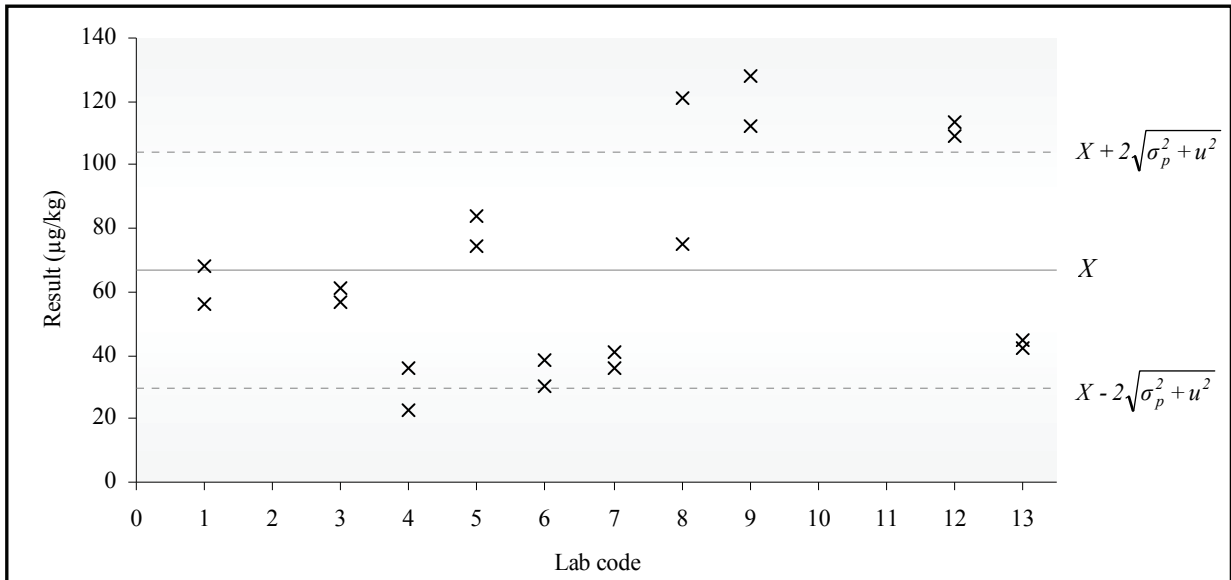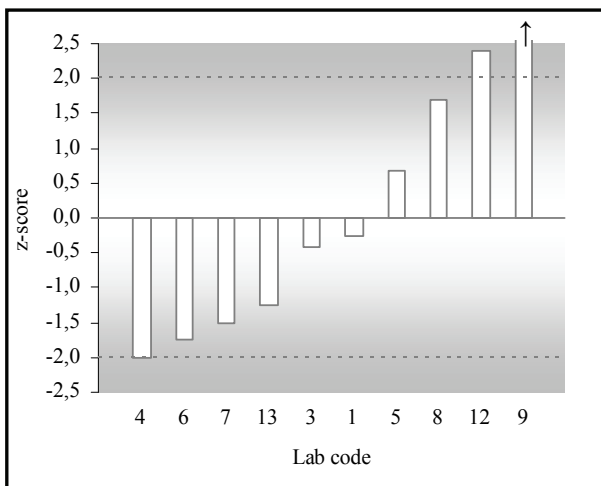*Figure a: Graphical representation of the reported results*

Figure b: Graphical representation of z′$_a$-score

Annex 9a   Results for the analysis of josamycin in muscle

Josamycin

Assigned value: 197 µg/kg

Uncertainty of assigned value: 41 µg/kg

Target standard deviation (Horwitz, Thompson): 40 µg/kg

Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z'_a-score
1	112	113	112	117	113,5	2,08	2,08	-1,45
2	238	281	274	307	275	22,13	26,93	1,37
5	123,5	117,8	121,3	120,6	120,8	2,34	2,34	-1,33
6	237	224	190	226	219,3	15,63	19,37	0,40
7	121	122	119	117	119,8	0,91	2,56	-1,34
8	434	380	492	437	435,8	31,47	46,35	**4,18**
12	76,1	81,5	74,6	88,3	80,1	6,01	6,01	**-2,04***
13	371	331	320	524	386,5	84,87	84,87	**3,32**

* Due to the consequential instability found this result is not suited for evaluation purposes.

Annex 9a continued   Results for the analysis of josamycin in muscle

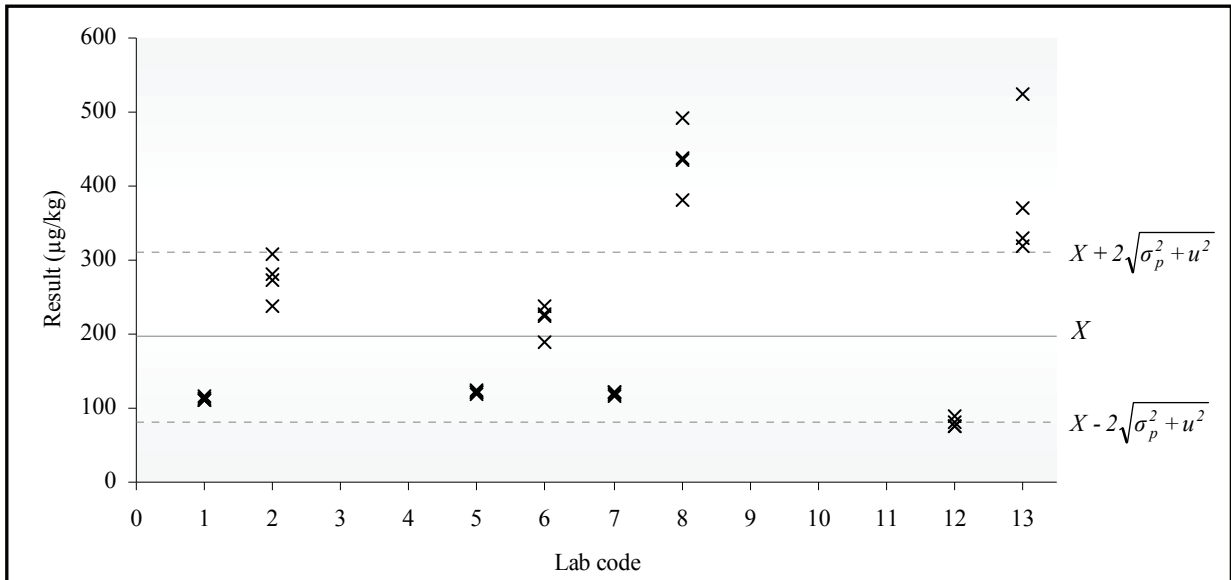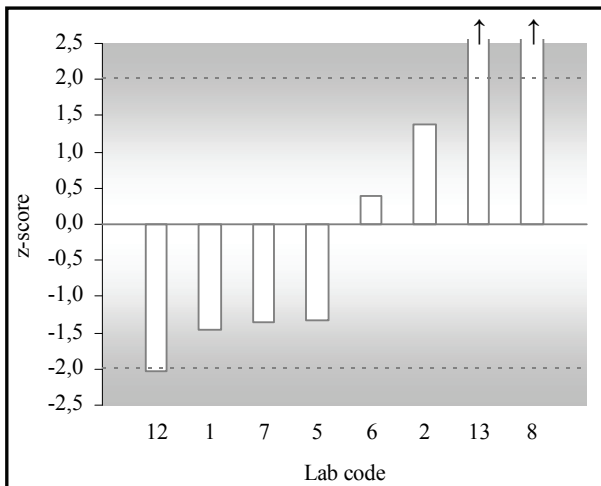*Figure a: Graphical representation of the reported results*

Figure b: Graphical representation of z'ₐ-score

Annex 9b Results for the analysis of josamycin in kidney

Josamycin				
Assigned value: 177 µg/kg				
Uncertainty of assigned value: 24 µg/kg				
Target standard deviation (Horwitz, Thompson): 37 µg/kg				
Code	Replicate 1	Replicate 2	Average	z'_a-score
1	181	161	171,0	-0,13
5	151,8	137,4	144,6	-0,72
6	159	174	166,5	-0,23
7	121	122	121,5	-1,25
8	372	441	406,5	**5,22**
12	124,3	123,6	124,0	-1,19
13	259	283	271,0	**2,14**

Figure a: Graphical representation of the reported results

The figure shows a scatter plot of Result (µg/kg) versus Lab code, with horizontal reference lines labelled $X + 2\sqrt{\sigma_p^2 + u^2}$, X, and $X - 2\sqrt{\sigma_p^2 + u^2}$.

Figure b: Graphical representation of z'_a-score

The figure shows a bar chart of z-score versus Lab code (7, 12, 5, 6, 1, 13, 8).

Annex 10 Results for the analysis of lincomycin in muscle

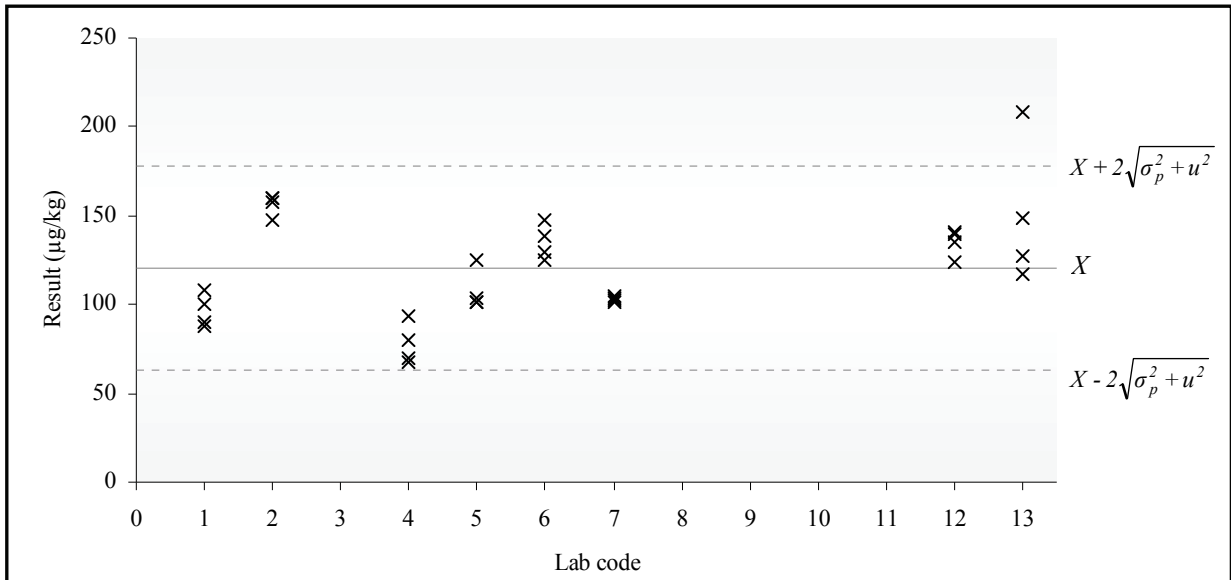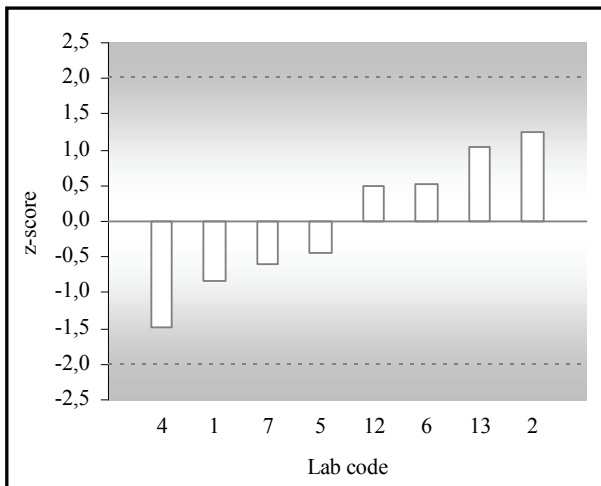|								
Lincomycin								
Assigned value: 120 µg/kg								
Uncertainty of assigned value: 11 µg/kg								
Target standard deviation (Horwitz, Thompson): 26 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z'_a-score
1	88	90	100	108	96,5	3,37	10,87	-0,82
2	158	160	147	160	156,3	5,37	5,44	1,25
4	93	80	70	68	77,8	5,37	12,94	-1,48
5	103,7	125,0	100,9	100,9	107,6	8,70	11,33	-0,44
6	130	147	125	139	135,3	8,99	8,99	0,52
7	104	105	101	103	103,3	0,91	1,88	-0,59
12	124,1	134,6	139,5	140,6	134,7	4,31	8,16	0,50
13	149,1	127	117	208	150,3	38,23	38,23	1,05

Figure a: Graphical representation of the reported results

Figure b: Graphical representation of z'$_a$-score

Annex 11   Results for the analysis of tulathromycin in muscle

Tulathromycin								
Assigned value: 217 µg/kg								
Uncertainty of assigned value: 42 µg/kg								
Target standard deviation (Horwitz, Thompson): 43 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z'_a-score
1	126	127	108	105	116,5	1,29	14,17	-1,65
5	303,1	311,4	304,4	319,7	309,7	7,11	7,11	1,52
6	159	224	188	243	203,5	34,76	34,76	-0,22
9	173	163	145	175	164,0	12,91	12,91	-0,87
13	276	250	235	408	292,3	71,42	71,42	1,23

These results are not suited for evaluation purposes, but for information only.

Annex 11 continued   Results for the analysis of tulathromycin in muscle
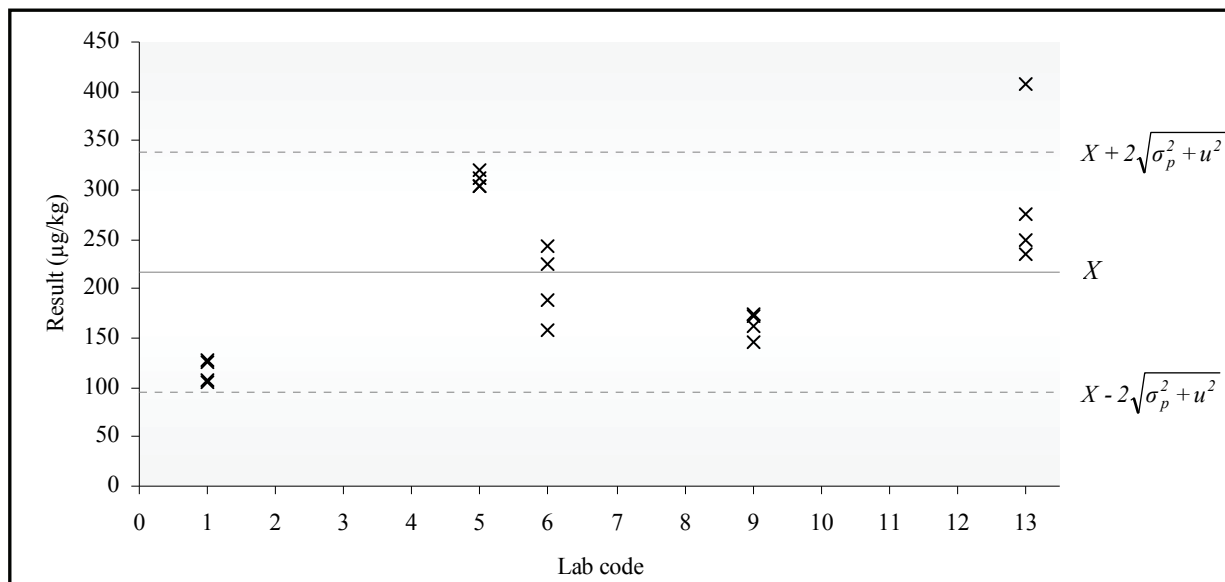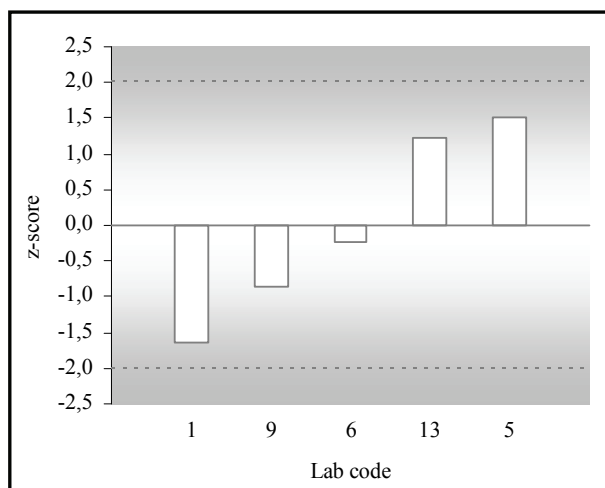
Figure a: Graphical representation of the reported results

Figure b: Graphical representation of z'_a-score

Annex 12   Results for the analysis of tilmicosin in kidney

Tilmicosin

Assigned value: 35.2 µg/kg

Uncertainty of assigned value: 3.5 µg/kg

Target standard deviation (Horwitz, Thompson): 7.7 µg/kg

Code	Replicate 1	Replicate 2	Average	z'_a-score
1	28		28,0	-0.85
5	42.3	43.6	43.0	0.91
6	42,6	42,0	42,3	0.84
7	38	36	37,0	0.21
12	25,0	22,8	23,9	-1.33
13	36,8	35,9	36,4	0.14

RIKILT Report 2009.003

Figure a: Graphical representation of the reported results
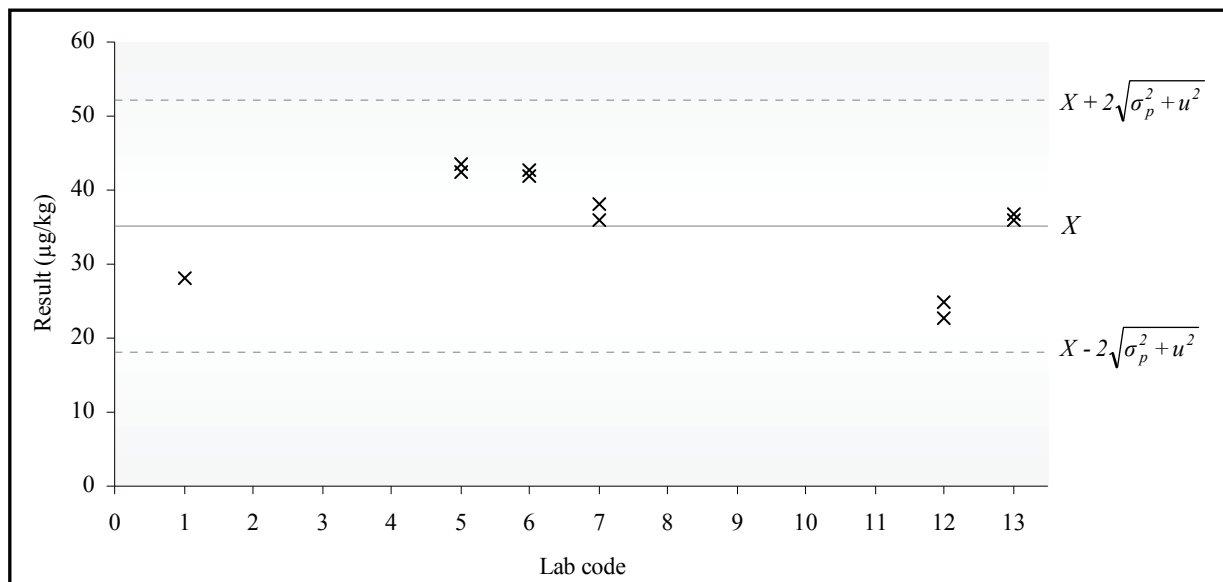
Figure b: Graphical representation of z'$_a$-score
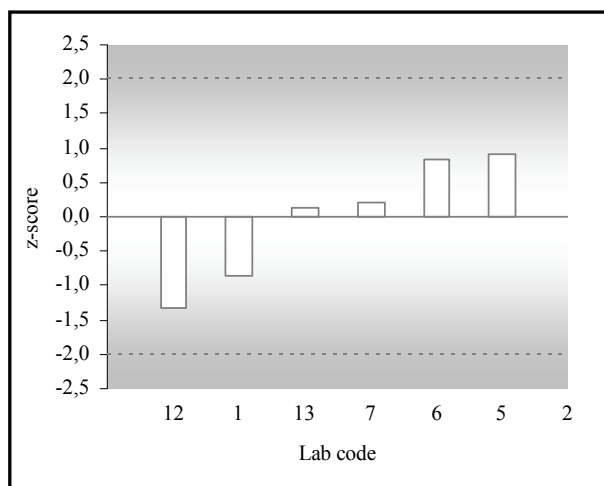
Annex 13   Overview of obtained z'$_a$ -scores

	1	2	3	4	5	6	7	8	9	12**	13
Muscle TYL	-1,04	2,06	-0,56	-0,93	-1,34	-0,06	-1,36	2,16	0,47	FN	2,55
Muscle JMC	-1,45	1,37	—	—	-1,33	0,40	-1,34	4,18	—	-2,04*	3,32
Muscle LMC	-0,82	1,25	—	-1,48	-0,44	0,52	-0,59	—	—	0,50	1,05
Muscle TMC	-1,65	—	—	—	1,52	-0,22	—	—	-0,87	—	1,23
Kidney TYL	-0,25	—	-0,41	-1,99	0,67	-1,74	-1,51	1,68	2,86	2,40	-1,26
Kidney JMC	-0,13	—	—	—	-0,72	-0,23	-1,25	5,22	—	-1,19	2,14
Kidney TMS	-0,85	—	—	FN	0,91	0,84	0,21	FN	—	-1,33	0,14
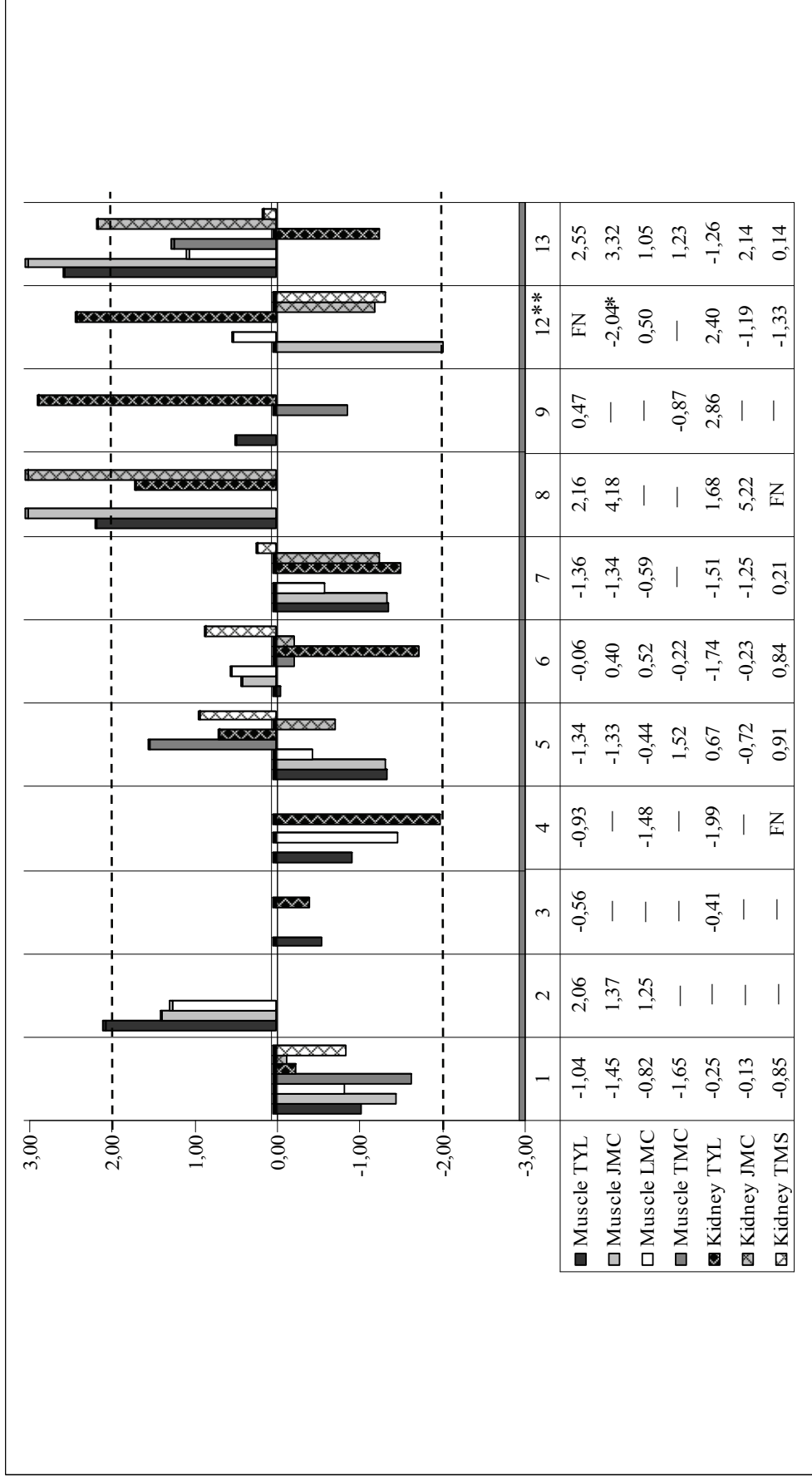
— = Not analyzed   FN = False negative

*   Due to a consequential instability observed, this result is not suited for evaluation purposes.

**   Two false positive results (spiramycin in K-A and K-B)