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Proficiency test for antibiotics in bovine muscle

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Mailing list:

- 36 participating laboratories, among them two from The Netherlands
- Food and Consumer Product Safety Authority (nVWA)

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Summary

The proficiency test for antibiotics in bovine muscle was organized by Rikilt - Institute of Food Safety and in accordance with ISO/IEC Guide 43-1 and 43-2 and ILAC-G13. The quantitative and confirmatory part was carried out under accreditation (Dutch Accreditation Board, ILAC-G13).

For this proficiency study, three test materials were prepared:

- a blank bovine muscle material;
- a bovine muscle material containing oxytetracycline aimed at 120 µg/kg;
- a bovine muscle material containing sulfachlorpyridazine aimed at 90 µg/kg, sulfadimidine aimed at 120 µg/kg and dapson aimed at 5 µg/kg.

The materials containing antibiotics were all prepared by spiking blank bovine muscle materials followed by cryogenic homogenization. During homogeneity testing, all materials proved to be sufficiently homogenous for proficiency testing. The stability test demonstrated that no statistically significant loss of oxytetracycline and sulfadimidine occurred during the timescale of the proficiency test. For sulfachloropyridazine and dapson a minor loss occurred during the thaw-freeze cycle that was included in the stability test.

The participating laboratories were first asked to carry out a screening analysis. After reporting the screening results they were asked to carry out a quantitative confirmatory analysis for the compounds found suspect and at least for tetracyclines and sulfonamides including dapsone. Thirthy-six laboratories subscribed for participation in the proficiency study but for one of them it was not possible to get the samples through customs. Within the timeframe of the study 35 laboratories submitted results: 34 laboratories submitted results for the screening analysis and 27 for the quantitative confirmatory part.

Three laboratories (labs 2, 19, 26) did not detect any antibiotics using their screening methodology. Seventeen laboratories (labs 3, 4, 5, 9, 11, 12, 15, 16, 18, 21, 23, 25, 28, 30, 34, 35 and 37) characterized all three samples correctly (compliant or suspect) based on the screening analysis and of these fourteen laboratories (3, 4, 5, 11, 12, 15, 16, 18, 21, 25, 28, 30, 35 and 37) indicated the correct compound groups for all samples.

The false positive and false negative rate were determined for all the individual laboratories and for all individual methods applied. A result is considered to be a false negative result if an antibiotic group/compound present in the sample is not detected. When evaluating the results for the individual labs (that in some cases carried out several different methods) fifteen false positive results out of 102 results occurred and twenty-one false negative results out of 64 results occurred.

After evaluating the results for all individual methods applied it became clear that the majority of false negative results was caused by using microbiological methods and the failure to detect sulfachloropyridazine in targeted instrumental screening methods. An overview of the screening analysis results evaluated on basis of all individual methods applied is presented in table 1. Dapson was left out of the calculations, because it was not found in any of the screening analyses.

If each method is considered separately, the false negative rate for the microbiological methods is 38%, for biochemical methods this is 25%, both caused by the Charm II test, and for instrumental analysis this is 23% all caused by missing sulfachloropyridazine. The proficiency test of 2009 organised by RIKILT included macrolides, quinolones and aminoglycosides in bovine muscle. The test of 2009 organised by RIKILT showed a false positive rate of 7%, in 2010 this is 15%.

Regarding the applied methods it is concluded that:

- many combinations of screening tests are used to cover the broad range of antibiotic groups;
- many false negative results are obtained, especially for microbiological screening methods.
- all false negative results obtained by instrumental methods can be explained by not including sulfachloropyridazine in the method.

Table 1: Overview of correct, false negative and false positive results for microbiological, biochemical and instrumental screening methods.

Material	A	B	C	
False positives	7	4	7	
Microbiology methods	7	3	4	
Biochemical methods	0	1	0	
Instrumental methods	0	0	3	
		Oxytetracycline	Sulfadimidine	Sulfachloropyridazine
No. of methods applied for the compound groups included*		38	37	37
Correct results	41	29	30	23
Microbiology methods		14	9	9
Biochemical methods		3	3	3
Instrumental methods		12	18	11
False negatives		9	7	14
Microbiology methods		8	6	6
Biochemical methods		1	1	1
Instrumental methods		0	0	7

* Because some laboratories applied several different methods and some laboratories do not have all compounds relevant for this proficiency test included in their method, this number is different from the number of laboratories.

Twenty-five laboratories carried out a quantitative and confirmatory analysis for tetracyclines and twenty-seven for sulfonamides including dapsone. Twenty-seven labs included sulfadimidine in their quantitative/confirmatory method, 19 labs included sulfachloropyridazine and 16 labs included dapson. False negatives occurred during the confirmatory analysis due to the absence of sulfachloropyridazine

and/or dapson in the method. One laboratory detected 63 µg/kg sulfaclozin which is considered as a false positive result.

For the quantitive analysis of oxytetracycline 20 out of 25 laboratories (80%) obtained satisfactory results. For sulfadimidine this was 26 out of 27 laboratories (96%), for sulfachloropyridazine 17 out of 18 (94%) and for dapsone 12 out of 13 (92%).

Based on the results of this proficiency test it is concluded that:

- considering the high percentage of false negative results, effort is needed to improve the effectiveness for the screening of veterinary drugs in muscle samples;
- microbiological screening methods relatively often cause false positive results
- for effectively applying targeted instrumental screening methods (LC-MS/MS or LC-UV) effort is needed to include a wider range of compounds;
- the quantification of especially oxytetracycline is not satisfactory for some laboratories.

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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 demanded by ISO 17025:2005 [2].

The aim of this proficiency study was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of antibiotics in bovine muscle, including the screening analysis. This study also provided an evaluation of the methods applied for screening and quantitative confirmatory analysis of antibiotics in bovine muscle.

This proficiency study was conducted in accordance with guidelines ISO/IEC 43-1 [3], ISO/IEC 43-2 [4] and ILAC-G13 [5]. The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative results were carried out under accreditation by Rikilt - Institute of Food Safety.

1.2 Previous results

In2009 Rikilt organized a proficiency test that focused on both the screening and confirmation part of antibiotic analysis in muscle focussing on flumequine, lincomysin and spectinomycin. Results showed that a huge effort was needed to improve the effectiveness of the screening of antibiotics in muscle samples. In the 2009 proficiency test, only fifteen out of twenty-six laboratories screened the samples correctly (compliant or suspect) and only three labs indicated the correct compound groups for all samples.

2 Test materials

This proficiency study focused on oxytetracycline (a tetracycline) and the combination of sulfadimidine (also called sulfamethazine or sulfadimerazine), sulfachloropyridazine (both sulfonamides) and dapson (a compound closely related to sulfonamides). The maximum residue limits (MRLs) for these compounds in bovine muscle are presented in table 2; dapson is a banned substance [14].

Table 2: MRL in bovine muscle of the compounds included in the proficiency test [6].

Compound	MRL in bovine musle (µg/kg)
Oxytetracycline	100
Sulfadimidine	100
Sulfachloropyridazine	100
Dapson	-

2.1 Sample preparation

One blank material (A), one material (B) containing oxytetracycline (OTC) and one material (C) containing a combination of sulfadimidine (SDD), sulfachloropyridazine (SCP) and dapson (DAP) were prepared. Material B and C were prepared by adding methanolic solutions of the selected compounds to blank bovine muscle aiming at the levels as presented in table 3. Each of the materials was homogenized under cryogenic conditions according to in-house standard operating procedures [15].

Table 3: Target amount of antibiotics in the proficiency test materials.

Material code	Target amount (µg/kg)			
	OTC	SDD	SCP	DAP
A	-	-		-
B	120	-		-
C	-	120	90	5

2.2 Sample identification

After homogenization, the sample materials were divided in subportions and stored in polypropylene containers. Each contained contained at least 50 gram of sample, yielding a total of 51 containers of material A and 113 containers of both material B and C. The samples for the participants were randomly selected and coded from AB1/2010/MUSCLE/001 through 135. For each laboratory a sample set was prepared consisting of one randomly selected sample of material A, B and C. The codes of the samples belonging to each sample set are presented in Annex 1.The remaining samples were used for

homogeneity and stability testing. For homogeneity and stability testing, 20 randomly selected containers of material B and C were assigned [15].

2.3 Participants

Thirty-six laboratories subscribed for participation in the proficiency study of which 30 are situated within Europe. One lab, situated outside Europe, did not get the samples through customs and was thus unable to participate.

2.4 Sample distribution and instructions

Each of the participating laboratories received a randomly assigned laboratory code (1 through 37). The sample sets with the corresponding number, consisting of three coded samples (Annex 1) were sent to the participating laboratories on May 5th, 2010. The sample sets were packed in an insulating box containing dry ice or cool packs and were dispatched to the participants immediately by courier. One laboratory reported that the samples were not sufficiently frozen at arrival. New samples were sent to this laboratory. The samples of two labs were returned to RIKILT after two days without a reason, so new sample sets were sent to these laboratories.
Finally all laboratories confirmed the receipt of the samples in good condition. The samples were accompanied by a letter (Annex 3) describing the requested screening analyses, an acknowledgement of receipt form and a screening results form. Three labs asked for additional sample material for the confirmatory analysis.

The laboratories were asked to store the samples until analysis according to their own laboratory's procedure. A single analysis of each sample was requested, resulting in one result for each material A, B and C. The deadline for sending in the screening results was May 29th 2010, allowing the participants 3 weeks for screening analysis. After the screening results were returned, the participants received new instructions for the quantitative confirmatory analysis (Annex 4). The deadline for the confirmatory analysis was August 1st 2010.

2.5 Homogeneity study

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [**7**] and ISO 13528 [**8**], taking into account the insights discussed by Thompson [**9**] regarding the Horwitz equation. With this procedure the between-sample standard deviation (s_s) and the within-sample standard deviation (s_w) are compared with the target standard deviation derived from the Horwitz equation (σ_H, §4.2.3). The method applied for homogeneity testing is considered suitable if $s_w \leqslant 0.5\sigma_H$ and a material is considered adequately homogeneous if $s_s \leqslant 0.3\sigma_H$.

Ten containers of materials B were analyzed in duplicate for oxytetracycline and ten containers of sample C were analyzed in duplicate for sulfadimidine, sulfachloropyridazine and dapson to determine the homogeneity of the materials. The results of the homogeneity study and their statistical evaluation are presented in Annex 2a through d. All materials demonstrated to be sufficiently homogeneous for use in the proficiency study.

No extensive homogeneity study was carried out for material A. The homogeneity of this material 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 A is comparable to the homogeneity of the other materials because all materials are homogenized in the same way. Nevertheless, three randomly selected samples of material A were analyzed for tetracyclines and sulfonamides. None of these antibiotics were detected. It was concluded that material A is suitable to use in the proficiency study.

2.6 Stability

Just after preparation of the materials six randomly selected samples of each material were stored at <-70 °C. It is assumed that the antibiotics included in this proficiency test are stable at these storage conditions. The remaining samples were stored at -20 °C. Of these, six at random selected samples were subjected to a thaw-freeze cycle to verify if thawing and freezing samples, as is likely to occur when a screening and confirmatory analysis is carried out, does not affect the stability.

On May 7th two sets of six samples were selected and stored at <-70°C. In the morning of September 8th two sets of six samples were selected from the samples stored at -20°C and thawed. After four hours at room temperature these samples were again stored at -20°C. On September 22nd, 138 days after preparation of the samples, six samples that had been stored at -20°C, six samples that were subjected to a thaw-freeze cycle and six samples that had been stored at <-70°C were analyzed for oxytetracycline. On September 24th, 140 days after preparation of the samples, a similar procedure was applied sulfadimidine, sulfachloropyridazine and dapsone. 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 [7, 8]. A consequential instability occurs when the average value of the samples stored at -20°C or the samples subjected to the thaw-freeze cycle 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 [8]. The results and statistical evaluation of the stability test are presented in Annex 5.

For oxytetracycline and sulfadimidine no consequential nor a statistical significant difference was observed between the samples stored at <-70°C, the samples stored at -20°C and the samples that were subjected to a thaw-freeze cycle. The samples are considered sufficiently stable.

For sulfachloropyridazine and dapson no consequential nor a statistical significant difference was observed between the samples stored at <-70°C and the samples stored at -20°C. However, a

consequential and a statistical difference were observed between the samples stored at <-70°C and the samples subjected to a thaw-freeze cycle. For both compounds the thaw-freeze cycle resulted in an average that is below the average of the samples stored at <-70°C. Therefore, for sulfachloropyridazine and dapson the observed instability is incorporated in the calculation of the z_a-scores (§4.2.4).

3 Applied methods of analysis

The participating laboratories applied biological, biochemical or instrumental methods or a combination of these methods for screening analysis. An overview of applied screening methods is presented in Annex 6. Seventeen laboratories applied a microbiological plate test ranging from four to twelve plates among which two laboratories applied the EU plate test, eight laboratories used the EU 4 plate test with an additional plate for quinolones and/or tetracyclines, two laboratories applied the Nouws Antibiotic Test (NAT) and two applied the STAR test. Five laboratories applied the Premi®test (three with a preceding solvent extraction) either or not in combination with other microbiological, biochemical or instrumental methods.
Seven laboratories applied biochemical methods (Charm II, Tetrasensor, SPR, RIA, ELISA, beta-STAR) and twenty laboratories applied an instrumental method (LC-MS/MS, LC-ToF/MS, LC-FLD, TLC, LC-UV, HPTLC or LC-DAD) for the screening analysis.

Twenty-seven laboratories carried out one or more confirmatory analyses. The substance groups for which a confirmatory analysis was carried out were selected based on the screening results and on additional information that was given to the participants (Annex 4) after the screening analyses. An overview of the quantitative confirmatory methods applied and the compounds included in the methods is presented in Annex 7.

For the quantitative and confirmatory analysis of tetracyclines in bovine muscle several different methods are applied. An overview of the applied confirmatory analyses for oxytetracycline is presented in Annex 7b. For the analysis of oxytetracycline in bovine muscle tissue many different extraction solvents or mixtures of solvents were used. For the sample clean up also several different techniques were applied: sixteen laboratories applied solid phase extraction using phases based on C_{18} , cyclohexyl or polymers. One laboratory used liquid-liquid extraction to clean up their raw extract. The other laboratories only filtered/diluted/evaporated their extract before injection. Several detection techniques were applied for the quantitative analysis of oxytetracycline in bovine muscle: four laboratories applied LC combined with diode array detection (DAD), seventeen laboratories used MS/MS as the detection technique and one laboratory combined LC-FLU and LC-MS/MS, one combined LC-Orbitrap and LC-MS/MS and one combined LC-UV and LC-DAD.

Of the participants that used mass spectrometric or DAD detection, eleven used an internal standard for the quantification of oxytetracycline. The internal standards used are:

- Demeclocycline (demethylchlortetraycline);
- Methacycline;
- 4-Epi-demethylchlortetracycline;
- Ciprofloxacin-d_8

For the quantitative and confirmatory analysis of sulfonamides including dapson in bovine muscle several different methods are applied. An overview of the applied quantitative confirmatory methods is presented in Annex 7c. One lab used a specific method for dapson.

For the analysis of sulfonamides including dapson in bovine muscle tissue many different extraction solvents or mixtures of solvents were used. For the sample clean up also several different techniques were applied: nine laboratories applied solid phase extraction using phases based on silica, cation exchange or polymers. Other laboratories used liquid-liquid extractions, filtration, dilution or evaporation of the extraction solvent to clean up their raw extract. Several detection techniques were applied for the quantitative analysis of sulfonamides including dapson in bovine muscle: two laboratories applied LC combined with UV detection, twenty laboratories used MS/MS as the detection technique and one laboratory applied LC-FLU. One lab combined LC-Orbitrap and LC-MS/MS and one lab combined LC-DAD and LC-MS/MS.

Of the participants that used mass spectrometric or FLU detection, eighteen used an internal standard for the quantification of sulfonamides and dapsone. The internal standards used are:

- Sulfadiazine-$^{13}C_6$;
- Sulfadimidine-$^{13}C_6$ or d_4 or d_7;
- Sulfanilamide-$^{13}C_6$
- Sulfadimethoxine-d_6
- Sulfachloropyridazine-$^{13}C_6$;
- Dapson-d_8;
- Sulfadiazine-d_4 or $^{13}C_6$;
- Sulfadoxine-d_3;
- Sulfapyridine;
- Sulfaphenazole;
- Sulfadimidine-^{13}C, 3-aminophenylsulfone;
- Sulfamethoxazole-$^{13}C_6$;
- Sulfameter;
- Sulfachloropyridazine;
- Ciprofloxacin-d_8;
- Sulfathiazole-$^{13}C_6$.

4 Statistical evaluation

The evaluation of the screening and quantitative analysis are carried out separately. The screening analysis is evaluated in a qualitative way resulting in a false negative and false positive rate [10]. The statistical evaluation of the quantitative part of the study was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [7], elaborated by ISO, IUPAC and AOAC and ISO 13528 [8] in combination with the insights published by the Analytical Methods Committee [11, 12] regarding robust statistics.

4.1 Screening analysis

First, all laboratories were evaluated separately regarding the screening results in which the number of false positives and false negatives is determined for each laboratory. The number of false positives is the number of samples in which growth inhibition or an antibiotic was detected although no antibiotic was present. A result is assigned as false negative if an antibiotic present is not detected although it is added to the bovine muscle.
After the individual evaluation of the laboratories an overall evaluation was carried out. In this the overall false positive and false negative rates were calculated for all laboratories that submitted results for the screening analysis [10]. Next it was studied if any relation exists between false negatives occurring and applied screening methods.

4.2 Quantitative analysis

For the evaluation of the quantitative results the assigend value, the uncertainty of the assigned value, a target standard deviation and z-scores were calculated.

4.2.1 Calculation of the assigned value

The assigned value (X) was determined using robust statistics [8,11,12]. 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 [8,11]. The assigned value is therefore a consensus value.

4.2.2 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 estimation 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 13528 [8] 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 (§ 4.2.3).

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 (§ 4.2.4).

4.2.3 Calculation of the target standard deviation

According to Commission Decision 2002/657/EC [13], 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 of a singular analysis result 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 tests.

Thompson [7] 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 oxytetracycline was determined using the regular Horwitz equation. In this calculation *c* = the assigned value (*X*) expressed in g/g and $\sigma_H = \sigma_p$.

4.2.4 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 13528 [8] are applied. According to these guidelines z_a-scores are classified as presented in table 4.

Table 4: 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 §4.2.2, the uncertainty is negligible. In this case the accuracy z-score is calculated from:

$$z_a = \frac{\bar{x} - X}{\sigma_p} \qquad \text{Equation I}$$

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 § 4.2.2, it could influence the evaluation of the laboratories. Therefore in that case, the uncertainty is taken into account by calculating the accuracy z-score [8]:

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

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.

If a consequential instability of the proficiency test materials is observed, this can influence the evaluation of the laboratory performance. Therefore, in that case the consequential instability should be taken into account when calculating z-scores. Because instability only regards one side of the confidence interval (in most cases a decrease of the amount is expected) this correction only applies to the lower 2s limit and results in an asymmetrical confidence interval.

In the case of a consequential instability the accuracy z-score for the laboratories that reported an amount below the assigned value is corrected for this instability by:

$$z_{ai} = \frac{\bar{x} - X}{\sqrt{\sigma_p^2 + \Delta^2}}$$ Equation III

where:

z_{ai} = accuracy z-score taking into account the instability of the assigned value;

$\bar{x}$ = the average result of the laboratory;

X = assigned value;

σ_p = target standard deviation;

Δ = difference between average concentration of compound stored at -70°C and average concentration after thaw-freeze cycle.

In some cases the uncertainty of the assigned value does not comply with the criterion in §4.2.4 ánd a consequential instability is observed. In this case the z'_a score for the laboratories that reported an amount below the assigned value is corrected for this instability by:

$$z'_{ai} = \frac{\bar{x} - X}{\sqrt{\sigma_p^2 + \Delta^2 + u^2}}$$ Equation IV

where:

z'_{ai} = accuracy z-score taking into account the uncertainty and instability of the assigned value;

$\bar{x}$ = the average result of the laboratory;

X = assigned value;

σ_p = target standard deviation;

Δ = difference between average concentration of compound stored at -70°C and average concentration after thaw-freeze cycle;
u = uncertainty of the assigned value.

5 Results and discussion

Thirty-six laboratories subscribed for the participation in the proficiency test for antibiotics in bovine muscle. Of these laboratories, 34 carried out a screening analysis and 27 carried out at least one confirmatory analysis (Table 5). For laboratories that carried out a screening and a confirmatory analysis the choice of the applied confirmatory analysis was based on the screening analysis results together with the additional information that was sent to the participants after reporting the screening analyses. The confirmation instructions (Annex 4) contained all compound groups found in the screening analyses plus tetracyclines and sulfonamides including dapson (if not reported in the screening results yet).

Table 5: Number of laboratories that reported results for each analysis.

Analysis	Compound	No. of labs that reported a result
Screening		34
Quantitative / confirmatory	Total	27
	Oxytetracycline	25
	Sulfadimidine	27
	Sulfachloropyridazine	18
	Dapson	13

5.1 Evaluation of the screening analysis

In the ideal case each laboratory that carried out a screening analysis would find the sample of material A compliant, the sample of material B and C suspect (for tetracyclines/OTC and sulfonamides/sulfadimidine, sulfachloropyridazine and dapson respectively). The actual screening results are presented in Annex 8a.

In this proficiency test for the screening analysis fifteen false positive results out of 102 results occurred, based on the overall results of materials A, B and C. Twenty-one false negative results out of 64 results occurred, based on the final results of materials B and C, which is caused by using microbiological methods and the failure to detect sulfachloropyridazine in targeted instrumental screening methods.

For material B, out of the 32 laboratories that screened for the presence for tetracyclines, 25 reported material B as a suspect sample for tetracyclines, oxytetracycline or a growth inhibitor (22% false negative). For material C, out of the 32 laboratories that screened for the presence of sulfonamides, 25 reported material C as a suspect sample for sulfonamides, sulfadimidine and/or sulfachloropyridazine or a growth inhibitor (22% false negative). However, when the failure to detect sulfachloropyridazine is taken into account, 18 out of 32 (44% false negative) laboratories correctly screened this material.

For the microbiological methods the false negative rate is highest with 38%. For biochemical methods the false negative rate is 25% and for instrumental screening methods 23%, the latter entirely caused by failure to detect sulfachloropyridazine.

For oxytetracycline, the false negative results were caused by using the EU 4 plate test (labs 2, 19, 20, 26 and 32), the STAR test (lab 7), the Charm II test (lab 12), a *B. cereus* pH=6 plate (lab 32) and the Premi®test without solvent extraction (lab 13). It stands out that the *B. subtilis* plate at pH=6 in this method is not suited for the screening of bovine muscle for the presence of tetracyclines at relevant levels. Including a *B. cereus* plate at pH=6-6.5 is an often applied strategy to fix this deficiency and appears to be effective; only 2 (labs 7 and 32) out of the 11 laboratories applying this bacterium, reported a false-negative result.

Five labs used the Premi®test as a screening method. The results of this method are somewhat ambiguous. The three labs that applied a solvent extraction prior to the Premi®test, to enhance the sensitivity of the test, all reported growth inhibition for material B, however, two of them also reported growth inhibition for the blank material A. Of the two labs that used Premi-test without solvent extraction, one reported a positive result and the other a negative result for material B, while both found material A negative.

It is concluded that instrumental methods (12 labs), Tetrasensor (2 labs) and the *B. cereus* pH=6-6.5 plate (11 labs, 9 correct results) are suited for screening of oxytetracycline in bovine muscle at relevant levels. Concerning the *B. cereus* based microbiological methods it may also be assumed that this result implies that the test will also be capable to detect the other veterinarily relevant tetracyclines, since oxytetracycline is considered to be the least detectable compound in this antibiotic group. Conclusions considering the suitability of the Charm II test for detection of tetracyclines remain uncertain, since only one of the two laboratories using the test reported a positive result.

For sulfonamides, it should be concluded that most of the applied microbiological methods are not capable of effectively detecting this antibiotic group at relevant levels. The only microbial plate test which appears sensitive enough for screening of sulfonamides is the *B. pumilis* at pH=7 +TMP (used by labs 23 and 36). Additionally, all five laboratories using Premi-test reported growth inhibition for material C, though as mentioned above, two of them also reported false positive results for material A. It stands out that many laboratories have already recognized the problematic microbial detection of sulfonamides, since 9 out of 22 laboratories that use microbial screening methods, have implemented alternative screening methods for sulfonamides, like TLC, LC-MS/MS, SPR, Charm II, HPLC.

In general it can be concluded that most of these methods appear suitable. Two laboratories that used LC-UV or HPTLC reported sulfaclozine and sulfaquinoxaline respectively, which are false positive results. Analogous to tetracyclines, suitability of the Charm II test for detection of sulfonamides also remains unclear, since also with material C only one of the two laboratories using this test reported a positive result. Seven laboratories that used an instrumental screening method missed the presence of sulfachloropyridazine, all because the compound is not included in the method. All laboratories reporting individual compounds, reported the presence of sulfadimidine. For screening analysis using targeted methods it is evidently of importance that all relevant compounds are included.

5.2 Evaluation of the quantitative analysis

Twenty-seven laboratories carried out one or more quantitative confirmatory analyses. An overview of the compounds found in the samples is presented in Annex 9a. Annex 9b gives an overview of false positive results that occurred during the quantitative analysis. One laboratory (lab 9) reported sulfaclozine with an amount of 63 µg/kg in the sample belonging to material C. Sulfaclozine only differs from sulfachloropyridazine in the position of the N-atom (ortho or para).
This is considered as a false positive result. False negatives only occurred in material C, caused by the exclusion of sulfachloropyridazine and/or dapson in the instrumental method. Nine laboratories missed the presence of sulfachloropyridazine and fourteen laboratories missed the presence of dapson.

Twenty-five laboratories carried out a quantitative confirmatory analysis for tetracyclines. All of these laboratories confirmed the presence of oxytetracycline and reported a quantitative result (Annex 10). The lowest value reported is 83.8 µg/kg and the highest value is 247.5 µg/kg. The assigned value of oxytetracycline is 122.0 µg/kg with a robust standard deviation of 30.1 µg/kg expressing the reproducibility within this proficiency test. This is very much comparable to the value suggested by Horwitz: 26.8 µg/kg. The uncertainty of the assigned value is 6.0 µg/kg which does not exceed $0.3\sigma_p$ (§4.2.2) and no consequential instability was observed (§4.2.4). Therefore z_a-scores were calculated (Annex 10, a graphical representation of the z_a-scores is included). With respect to the accuracy two results were questionable and three were unsatisfactory (Table 4).

Twenty-seven laboratories carried out a quantitative confirmatory analysis for sulfonamides. All of these laboratories confirmed the presence of sulfadimidine and reported a quantitative result (Annex 11). The lowest value reported is 33 µg/kg and the highest value is 125 µg/kg. The assigned value of sulfadimidine is 90.1 µg/kg with a robust standard deviation of 16.9 µg/kg expressing the reproducibility within this proficiency test. This is very much comparable to the value suggested by Horwitz: 19.8 µg/kg. The uncertainty of the assigned value is 3.3 µg/kg which does not exceed $0.3\sigma_p$ (§4.2.2) and no consequential instability was observed (§4.2.4). Therefore z_a-scores were calculated (Annex 11, a graphical representation of the z_a-scores is included). With respect to the accuracy all but one results are satisfactory. The deviating result is questionable (Table 4).

Of the twenty-seven laboratories that carried out a quantitative confirmatory analysis for sulfonamides, nineteen laboratories included sulfachloropyridazine in their confirmatory method (Annex 12). All but one laboratories confirmed the presence of sulfachloropyridazine. The lowest value reported is 18 µg/kg and the highest value is 89 µg/kg. The assigned value of sulfachloropyridazine is 64.3 µg/kg with a robust standard deviation of 14.8 µg/kg expressing the reproducibility within this proficiency test. This is very much comparable to the value suggested by Horwitz: 14.1 µg/kg. The uncertainty of the assigned value is 3.5 µg/kg which does not exceed $0.3\sigma_p$ (§4.2.2). Therefore the uncertainty of the assigned value is not taken into account in the evaluation of the laboratories. However, a consequential instability was observed caused by the thaw-freeze cycle and thus the instability observed was taken into account by calculating z_{ai}-scores (Annex 12, a graphical representation of the z_{ai}-scores is included). With respect to the accuracy all but one results are satisfactory. The deviating result is questionable (Table 4).

Sixteen labs included dapson in their confirmatory method and thirteen laboratories reported a quantitative confirmatory analysis for dapson (Annex 13). The lowest value reported is 1.42 µg/kg and

the highest value is 4.8 µg/kg. The assigned value of dapson is 3.35 µg/kg with a robust standard deviation of 1.0 µg/kg expressing the reproducibility within this proficiency test. This is very much comparable to the value suggested by Horwitz: 0.74 µg/kg. The uncertainty of the assigned value is 0.29 µg/kg which does exceed $0.3\sigma_p$ (§4.2.2). Therefore the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. Furthermore a consequential instability was observed caused by the thaw-freeze cycle and thus also the instability observed was taken into account calculating z'_{ai}-scores (Annex 13, a graphical representation of the z'_{ai}-scores is included). With respect to the accuracy all but one results are satisfactory. This result is questionable.

In general it can be concluded that most of the quantitative methods used are suitable for quantification of sulfadimine, sulfachloropyridazine and dapson. However, the quantification of oxytetracycline is more difficult, since 5 labs obtained z-scores > 2 . Furthermore, it is important to include a wider range of compounds in the instrumental methods to avoid false negative results.

6 Conclusions

Thirty-five laboratories reported results for the proficiency study of antibiotics in bovine muscle. Out of these three laboratories (labs 16, 21 and 35) showed optimal performance by screening/detecting all compounds, the absence of false positives and false negatives and a correct quantification of oxytetracycline, sulfadimidine, sulfachloropyridazine and dapson. Lab 16 used the Premi-test and LC-MS/MS for the screening part. Lab 21 used SPR and 6 microbiological plates (EU 4 pt + *E. coli* at pH=8 and *B. cereus* at pH=6) for the screening part. Lab 35 used LC-MS/MS for the screening of the samples. Three other laboratories (labs 10, 27 and 36) also quantified/confirmed all 4 compounds correctly, but reported false positive (10 and 36) or false negative (10 and 27) screening results.

The proficiency test of 2009 organised by RIKILT discussed macrolides, quinolones and aminoglycosides in bovine muscle. The test of 2009 showed a false negative rate of 53%, which is 33% in 2010. The false positive rate was 7% in 2009, which is 15% in 2010. For microbiological methods the overall false negative rate was 73% in 2009, which is 38% in 2010. For biochemical it was 50% and is 25% in 2010 and for instrumental methods it was 22% and is 23% in 2010.

For the microbiological methods the false negative rate is 38%, for biochemical tests this is 25% and for instrumental methods this is 23%. The false negative rate for microbiological methods is mainly caused by applying the EU four plate test, which relies on a *B. subtilis* plate at pH=6 for the screening of tetracyclines (assigned value of 122.0 µg/kg oxytetracycline) and a *B. subtilis* (+TMP) plate at pH=7.2 for sulfonamides (assigned values of 90.1 µg/kg sulfadimine and 64.3 µg/kg sulfachloropyridazine) . The false negative rate for biochemical methods is caused by using the Charm II test. The false negative rate for instrumental methods is caused by the use of methods in which sulfachloropyridazine is not included.

For the quantitative and confirmatory analysis 25 laboratories reported results for oxytetracycline, 27 for sulfadimidine, 18 for sulfachloropyridazine and 13 for dapson. For oxytetracycline 20 out of 25 laboratories obtained satisfactory results. For sulfadimidine 26 out of 27 laboratories obtained a satisfactory result, for sulfachloropyridazine this is 17 out of 18 and for dapson 12 out of 13. One false positive result and four false negatives were reported although the specific compounds (sulfachloropyridazine and/or dapson) were included in the method. Eighteen laboratories did not detect sulfachloropyridazine and/or dapsone because they were not included in the method.

Based on the results of this proficiency test it is concluded that:

- especially the screening part of the proficiency test demonstrates the drawbacks in the analytical approach for the analysis of antibiotics in muscle samples;
- considering the high percentage of false negative results, effort is needed to improve the effectiveness for the screening of veterinary drugs in muscle samples;
- microbiological screening methods relatively often cause false positive results
- the EU 4 plate test is not suited for the screening of oxytetracycline, sulfadimidine and sulfachloropyridazine in bovine muscle at relevant levels.

- for effectively applying targeted instrumental screening methods like LC-MS/MS, effort is needed to include a much wider range of compounds.

References

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15 RSVA0989 - De bereiding van referentiematerialen en referentiemonsters - RIKILT

Annex 1 Codification of the samples

Sample set no.	Material A*	Material B*	Material C*
1	044	032	014
2	021	031	074
3	005	036	035
4	071	017	134
5	088	030	013
6	053	041	065
7	048	020	004
8	010	058	067
9	007	016	129
10	026	123	046
11	028	055	057
12	018	118	019
13	037	131	094
14	085	110	011
15	003	083	068
16	053	041	065
17	006	120	087
18	023	096	101
19	029	090	112
20	073	086	082
21	126	095	075
22	102	051	022
23	039	038	125
24	063	099	133
25	121	072	081
26	008	066	098
27	108	024	117
28	042	009	033
29	104	061	111
30	113	047	100
31	106	127	130
32	052	080	119
33	093	077	103

Annex 1 continued Codification of the samples

Sample set no.	Material A*	Material B*	Material C*
34	045	128	056
35	084	135	002
36	043	122	027
37	062	060	089

** all sample codes start with AB1/2010/MUSCLE/*

Annex 2a Statistical evaluation of homogeneity data of material B for oxytetracycline

	Oxytetracycline (µg/kg)	
Sample No.	Replicate 1	Replicate 2
Hom/B001	124.3	108.5
Hom/B002	118.0	127.2
Hom/B003	111.7	122.1
Hom/B004	*	110.9
Hom/B005	83.2	112.6
Hom/B006	115.7	120.3
Hom/B007	116.1	113.2
Hom/B008	116.8	116.4
Hom/B009	123.5	112.7
Hom/B010	115.9	111.6
Grand mean	114.77	
Cochran's test		
C	0.585	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 25.25	
s_x	6.88	
s_w	9.04	
s_s	2.54	
Critical = $0.3\sigma_H$	0.32	
s_s < critical?	ACCEPTED	
$s_w < 0.5\sigma_H$?	ACCEPTED	

*value was 231.9 µg/kg → outlier

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 C for sulfadimidine

	Sulfadimidine (µg/kg)	
Sample No.	Replicate 1	Replicate 2
Hom/B001	116.5	109.5
Hom/B002	115.4	116.4
Hom/B003	109.0	107.4
Hom/B004	114.2	113.0
Hom/B005	113.0	114.7
Hom/B006	116.3	118.9
Hom/B007	112.0	111.4
Hom/B008	122.1	109.9
Hom/B009	100.4	107.5
Hom/B010	114.5	116.7
Grand mean	112.9	
Cochran’s test		
C	0.559	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 24.85	
s_x	4.13	
s_w	3.65	
s_s	3.22	
Critical = $0.3\sigma_H$	7.45	
s_s < critical?	ACCEPTED	
$s_w < 0.5\sigma_H$?	ACCEPTED	

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 C for sulfachloropyridazine

Sample No.	Sulfachloropyridazine (µg/kg) Replicate 1	Replicate 2
Hom/B001	86.7	80.0
Hom/B002	85.3	88.7
Hom/B003	90.5	86.2
Hom/B004	80.1	90.2
Hom/B005	88.1	83.3
Hom/B006	87.3	89.9
Hom/B007	78.0	79.0
Hom/B008	95.8	82.1
Hom/B009	85.0	81.8
Hom/B010	89.0	86.8
Grand mean	85.7	
Cochran’s test		
C	0.460	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 18.85	
s_x	3.27	
s_w	4.54	
s_s	0.62	
Critical = 0.3 σ_H	5.66	
s_s < critical?	ACCEPTED	
$s_w < 0.5 \sigma_H$?	ACCEPTED	

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 C for dapson

Sample No.	Dapson (µg/kg) Replicate 1	Replicate 2
Hom/B001	4.7	4.7
Hom/B002	4.9	5.0
Hom/B003	4.5	4.8
Hom/B004	4.7	4.7
Hom/B005	5.1	4.4
Hom/B006	4.8	4.3
Hom/B007	4.7	5.0
Hom/B008	4.9	5.3
Hom/B009	4.9	4.8
Hom/B010	4.5	5.0
Grand mean	4.8	
Cochran's test		
C	0.347	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 1.05	
s_x	0.16	
s_w	0.26	
s_s	0.00	
Critical = $0.3\sigma_H$	0.32	
s_s < critical?	ACCEPTED	
$s_w < 0.5\sigma_H$?	ACCEPTED	

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

Annex 3 Instruction letter

Dear participant,

Thank you very much for your interest in the proficiency study for the analysis of antibiotics in bovine muscle.

Hereby I send you a parcel containing three randomly coded samples. Each sample consists of at least 50 g bovine muscle. The samples may contain one or more analytes belonging to one ore more of the following groups (in alphabetical order):

Aminoglycosides	Quinolones
ß-lactams	Sulfonamides
Macrolides	Tetracyclins

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

Your laboratory code is: 37

Return the screening results before May 28th 2010

Instructions:

- After arrival store the samples according to your laboratory's procedures.
- Defrost the samples before analysis and homogenize them according to your laboratory's procedures.
- Please analyze the samples according to the **predefined screening methods** mentioned on the registration form. The samples should be treated as routine samples.
- In order to make the sample suitable for methods that use muscle disks, we propose the following:
  Take a few grams of the sample and let it thaw on a (clean) flat surface, press (e.g. with the back of a spoon) to a compact layer with a thickness approaching a regular muscle disk and take out a sample using your cork borer. To enhance diffusion, add 50 µl of water to the artificial disk, after you have placed it on the test plate.
- Please make use of your own reference standards. Unfortunately RIKILT – Institute of Food Safety, cannot supply antibiotic reference standards.
- Carry out a **single analysis** for each sample. Please report the screening results before **May 28th 2010**. After reporting the screening results instructions will be given on the quantitative and confirmatory analysis.
- Please use the results form for reporting the results.

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

Kind regards,
Ingrid Elbers

Annex 4 Confirmation instructions

Dear participant,

Thank you for reporting the screening results.
Hereby I send you the instructions for the confirmatory and quantitative part of the proficiency test.

- Please confirm and quantify all the compounds that are mentioned in the table below:

Sample 000	Antibiotic group(s)
Sample 000	Antibiotic group(s)
Sample 000	Antibiotic group(s)

Carry out a **single analysis** for each sample. Please confirm the identity of any detected residues according to 2002/657/EC.

- The results should be reported before the **1st of August 2010**.
- Please use the result form for reporting the results.

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

Kind regards,
Ingrid Elbers

Annex 5 Statistical evaluation of stability data of material B and C

Statistical evaluation for oxytetracycline in material B

Storage temp	-70 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	138	138
Calculated amounts (µg/kg)	125.25	108.93	116.57
	104.86	114.46	101.12
	110.77	100.61	107.32
	106.86	108.99	116.05
	109.60	107.83	110.54
	112.17	102.80	110.27
Average amount (µg/kg)	111.6	107.3	110.3
n	6	6	6
st. dev (µg/kg)	7.20	4.94	5.76
Difference		-4.31	-1.27
$0.3\sigma_H$	7.37		
Consequential difference? Diff < $0.3\sigma_H$		No	No
t		1.21	0.34
t crit		2.23	2.23
Statistical difference? T < t crit		No	No

Statistical evaluation for sulfadimidine in material C

Storage temp	-70 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	140	140
Calculated amounts (µg/kg)	100.13	99.73	91.43
	107.87	111.05	99.31
	95.28	109.05	103.72
	99.59	101.77	103.15
	110.07	96.76	99.51
	103.1	96.63	90.27
Average amount (µg/kg)	102.7	102.5	97.9
n	6	6	6
st. dev (µg/kg)	5.52	6.19	5.76
Difference		-0.18	-4.78
$0.3\sigma_H$	6.78		
Consequential difference? Diff < $0.3\sigma_H$		No	No
t		0.05	1.47
t crit		2.23	2.23
Statistical difference? T < t crit		No	No

Statistical evaluation for sulfachloropyridazine in material C

Storage temp	-80 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	140	140
Calculated amounts (µg/kg)	64.62	57.02	54.57
	63.39	*	59.84
	63.49	66.25	61.62
	62.54	62.8	61.9
	62.59	62.81	60.99
	67.93	56.72	55.26
Average amount (µg/kg)	64.1	61.1	59.0
n	6	5	6
st. dev (µg/kg)	2.03	4.13	3.27
Difference		-2.97	-5.06
$0.3\sigma_H$	4.23		
Consequential difference? Diff < $0.3\sigma_H$		No	Yes
t		1.56	1.47
t crit		2.26	2.23
Statistical difference? T < t crit		No	Yes

*value was 99.9 µg/kg → outlier

Statistical evaluation for dapson in material C

Storage temp	-80 °C	-20°C	Thaw - freeze
Time at -20°C (days)		140	140
Calculated amounts (µg/kg)	4.24	3.55	3.37
	4.23	4.35	3.61
	3.76	4.06	3.7
	3.84	3.82	3.7
	3.96	3.61	3.82
	4.25	3.64	3.39
Average amount (µg/kg)	4.0	3.8	3.6
n	6	6	6
st. dev (µg/kg)	0.221	0.312	0.182
Difference		-0.21	-0.45
$0.3\sigma_H$	0.267		
Consequential difference? Diff < $0.3\sigma_H$		No	Yes
t		1.33	3.83
t crit		2.23	2.23
Statistical difference? T < t crit		No	Yes

Annex 6 Overview of the applied screening methods

Lab	Aminoglycosides	ß-lactams	Macrolides	Quinolones	Sulfonamides	Tetracyclines
2	EU 4 plate test	EU 4 plate test	EU 4 plate test	EU 4 plate test	EU 4 plate test	EU 4 plate test
3	not tested	not tested	LC-MS	LC-FLD	LC-MS	Tetrasensor
4	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli*, pH=8	TLC	*B. cereus*, pH=6
5	LC-MS solvent extraction + Premi-test	LC-MS solvent extraction + Premi-test	LC-MS solvent extraction + Premi-test	LC-MS solvent extraction + Premi-test	LC-MS solvent extraction + Premi-test	LC-MS solvent extraction + Premi-test
6	not tested	not tested	not tested	not tested	not tested	*B. subtilis* ATCC 6633 pH=7.9 *B. staerothermophilus* ATCC 10149, pH=6.55 *K. rhizophila* ATCC 9341 *E. coli* ATCC 11303, pH=6 *B. megaterium* ATCC 9885, pH=7.3 + TMP *B. cereus* ATCC 11778, pH=5.85 LC-UV
7	STAR without *E.coli*	STAR exclusive *E.coli*	STAR without *E.coli*	LC-FLD	ELISA	STAR without *E.coli*
9	LC-MS/MS	LC-UV	LC-UV	LC-FLD	LC-UV	not tested

Annex 6 continued Overview of the applied screening methods

Lab	Aminoglycosides	ß-lactams	Macrolides	Quinolones	Sulfonamides	Tetracyclines
10	*B. subtilis* BGA, pH 6,0+ penicillinase *B. subtilis* BGA, pH 7,2+ TMP *B. subtilis* BGA, pH 7,2+TMP+PABA	*B. subtilis* BGA, pH=6.5 + penicillinase *B. subtilis* BGA, pH=6.0 + penicillinase *M. luteus* ATCC 15957, pH=6 *M. luteus* ATCC 15957, pH=8 *M. luteus* ATCC 15957, pH=8 + penicillinase	*B. subtilis* BGA, pH=6.5 + penicillinase *B. subtilis* BGA, pH=6.0 + penicillinase *M. luteus* ATCC 15957, pH=6 *M. luteus* ATCC 15957, pH=8 *M. luteus* ATCC 15957, pH=8 + penicillinase	*E. coli* ATCC 11303, pH=7.2	*B. subtilis* BGA, pH=7.2 + TMP *B. subtilis* BGA, pH=7.2 + TMP + PABA	*B. cereus* ATCC 11778, pH=6.5 *B. cereus*, tetracycline resistant, pH=6.5
11	not tested	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
12	STAR Charm II	STAR Charm II	STAR Charm II	STAR	STAR LC-DAD	STAR Charm II
13	Premi-test	Premi-test	Premi-test	Premi-test	Premi-test	Premi-test
14	*M. luteus*, pH=7 *B. subtilis*, pH=6 *B. subtilis*, pH=8	*M. luteus*, pH=7 *B. subtilis*, pH=6 *B. subtilis*, pH=8	*M. luteus*, pH=7	*M. luteus*, pH=7 *B. subtilis*, pH=6 *B. subtilis*, pH=8	*M. luteus*, pH=7 *B. subtilis*, pH=6 *B. subtilis*, pH=8	*M. luteus*, pH=7 *B. subtilis*, pH=6 *B. subtilis*, pH=8
15	LC-HRMS	LC-HRMS	LC-HRMS	LC-HRMS	LC-HRMS	LC-HRMS
16	LC-MS/MS Premi-test	EU 4 plate test Premi-test	LC-MS/MS Premi-test	*E. coli,* pH=6 Premi-test	LC-MS/MS Premi-test	LC-MS/MS Premi-test
17	*B.subtilis*, pH=8 *S. epidermidis*, pH=8	*B. subtilis*, pH=8 *K. rhizophila*, pH=8	*B. subtilis*, pH=8 *K. rhizophila*, pH=8	*E. coli*, pH=8 *Y. ruckeri*, pH=6	HPTLC	*B. cereus*, pH=6
18	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
19	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli*, pH=8	EU 4 plate test	EU 4 plate test
20	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli,* pH=8	EU 4 plate test	EU 4 plate test
21	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli*, pH=8	Surface plasma resonance	*B. cereus*, pH=6
22	not tested	Solvent extraction + Premi-test LC-TOF/MS	Solvent extraction + Premi-test LC-TOF/MS	Solvent extraction + Premi-test LC-TOF/MS	Solvent extraction + Premi-test LC-TOF/MS	Solvent extraction + Premi-test LC-TOF/MS

Annex 6 continued Overview of the applied screening methods

Lab	Aminoglycosides	ß-lactams	Macrolides	Quinolones	Sulfonamides	Tetracyclines
23	NAT: *B. subtilis* BGA, pH=8.5	NAT: *K. rhizophila*, pH=8	NAT: *K. rhizophila*, pH=8	NAT: *Y. ruckeri*, pH=6.5	NAT: *B. pumilus*, pH=7	NAT: *B. cereus*, pH=6.
25	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
26	EU 4 plate test	EU 4 plate test	EU 4 plate test	E. coli pH=8	EU 4 plate test	EU 4 plate test
27	not tested	not tested	Charm II	not tested	Charm II	Charm II
28	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
29	LC-MS/MS	RIA	LC-MS/MS	LC-MS/MS	not tested	not tested
30	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
31	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli,* pH=8	EU 4 plate test	*B. cereus*, pH=6
32	EU 4 plate test *B. subtilis*, pH=8 *S. epidermis*, pH=8	EU 4 plate test *K. rhizophila*, pH=6.5	EU 4 plate test *K. rhizophila*, pH=7.9	*E.coli*, pH=8 HPLC-FLD	LC-MS/MS	EU 4 plate test *B. cereus* , pH=6 LC-MS/MS
33	Solvent extraction + Premi-test Charm II streptomycins assay	Solvent extraction + Premi-test ß-s.t.a.r. tissue	Solvent extraction + Premi-test Charm II	Solvent extraction + *E. coli*	Solvent extraction + Premi-test Charm II	Solvent extraction + Premi-test Tetrasensor
34	EU 4 plate test	EU 4 plate test	EU 4 plate test	*E. coli,* pH=8	HPTLC	*B. cereus*, pH=6
35	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
36	NAT: *B. subtilis BGA*, pH=8.5	NAT: *K. rhizophila*, pH=8	NAT: *K. rhizophila*, pH=8	NAT: *Y. ruckeri*, pH=6.5	NAT: *B. pumilus*, pH=7	NAT: *B. cereus*, pH=6
37	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS

Kocuria rhizophila = *Micrococcus luteus* ATCC 9341

The EU 4 plate test comprises *B. subtilis* at pH 6, 7.2 or 7.4 (+ trimethoprim) and pH 8, and *K. rhizophila*

NAT = Nouws Antibiotic Test

Annex 7a Overview of compounds included in the quantitative methods applied

Lab	Sulfonamides	Tetracyclines
1	sulfadiazine, sulfapyridine, sulfanilamide, sulfamethoxypyridazine, sulfadoxine, sulfadimethoxine, **dapson**, **sulfadimidine**, sulfamonomethoxine, sulfamoxole, sulfaquinoxaline, **sulfachloropyridazine**, sulfathiazole, sulfaguanidine, sulfamethizole, sulfamerazine, sulfamethoxazole, sulfamerazine, sulfamethoxazole, sulfacetamide, sulfisoxazole	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites
4	sulfaguanidine, sulfaanilimide, sulfacetamide, sulfasomidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, **dapson**, sulfamoxole, sulfameter, sulfamethizole, **sulfametazine**, sulfamethoxypyridazine, **sulfachloropyridazine**, sulfamethoxazole, sulfatroxazole, sulfamonomethoxine, sulfadoxine, sulfaisoxazole, sulfabenzamide, sulfadimethoxine, sulfaquinoxaline	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
5	**sulfamethazine, sulfachloropyridzine**	-
6	**sulfamethazine**, **sulfachloropyridazine**, sulfamerazine, sulfadiazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfathiazole, sulfadoxine, sulfamethosazole, sulfisoxazole, sulfachlorpyrazine, sulfadimethoxine, sulfaquinoxaline, sulfapenazole	-
9	sulfadiazine, sulfathiazol, sulfamerazin, sulfamethizol, **sulfamethazin**, sulfaclozin, **dapson**, sulfamethoxazol, sulfadoxin	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites
10	sulfacetamide**, sulfachloropyridazine**, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfametoxypyridazine, sulfamonomethoxine, sulfamoxol, sulfanilamide, sulfapyridine, sulfaquinidine, sulfaquinoxaline, sulfathiazole, sulfisoxazole**,** **dapson**, **sulfamethazine**	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
11	sulfadoxin**, sulfametazin**, sulfatiazol, sulfadiazine, trimetoprim	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
12	**dapson**, sulfabenzamide, sulfacetamide, **sulfachloropyrazine**, sulfachloropyridazine, sulfadiazine, sulfadimethoxin, **sulfadimidin**, sulfadoxin, sulfamerazin, sulfameter, sulfamethizol. sulfamethoxazol, sulfamethoxypyridazin, sulfamonomethoxin, sulfamoxol, sulfaphenazol, sulfapyridin, sulfaquinoxalin, sulfathiazol, sulfatroxazol, sulfisomidin, sulfisoxazol, trimethorprim	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites, except for doxycycline

Annex 7a continued Overview of compounds included in the quantitative methods applied

Lab	Sulfonamides	Tetracyclines
14	sulfadiazine, **sulfamethazine**, sulfadoxin	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
15	**sulfachloropyridazin**, **sulfadimidin**, sulfabenzamide, sulfacetamid, sulfachlorpyrazin, sulfadiazin, sulfadimethoxin, sulfadoxin, sulfaguanidin, sulfamerazin, sulfameter, sulfamethizol, sulfamethoxazol, sulfamethoxipyridazin, sulfamonomethoxin, sulfamoxol, sulfanilamid, sulfanitran, sulfapyridin, sulfaquinoxalin, sulfasalazin, sulfathiazol, sulfisomidin, sulfisoxazol	**oxytetracycline**, chlortetracycline, demeclocycline, doxycycline, minocycline, tetracycline
16	**sulfamethazine**, **sulfachloropyridazine**, sulfathiazole, sulfadiazine, sulfadoxine, sulfamethoxazole, sulfisoxazole, sulfamerazine, sulfamethoxypyrodazine, sulfamonomethoxine, sulfaquinoxaline, sulfadimethoxine, sulfachlorpyrazine, sulfachlorpyrazine, sulfaphenazole, trimethoprim, **dapson**	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline, minocycline
17	**sulfamethazine, sulfachloropyridazine**, sulfaguanidin, sulfanilamid, sulfadiazine, sulfatiazol, sulfamerazine, sulfamoxole, sulfamonomethosine, sulfamethizole, sulfadoxine, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, sulfaquinoxaline, sulfaphenazole	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
18	sulfadimethoxin, **sulfadimidin**, **sulfachlorpyridazine**, sulfamethiazol, sulfathiazol	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
19	sulfadimethoxine, **sulfamethazine**, sulfamethoxypyridazine, sulfapyridine, sulfatiazol, sulfaquinoxaline, sulfadiazine, sulfamerazine, sulfacetamide	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
20	**sulfamethazine**, **sulfachloropyridazine**, sulfathiazole, sulfamethoxipiridazine, sulfaquinoxaline, sulfapyridine, sulfadiazine, sulfamethoxazole, sulfamethizole, sulfamerazine, sulfamoxole	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
21	sulfadiazine, sulfathiazole, sulfapyridine, trimethoprim, sulfamerazine, **sulfamethazine**, sulfamethizole, sulfamethoxypyridazine, sulfamonomethoxine, **dapson**, **sulfachloropyridazine**, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, sulfaquinoxaline	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
22	**sulfachloropyridazine**, sulfadiazine, sulfadimethoxine, sulfamerazine, **sulfamethazine**, sulfamethoxypyridazine, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, **dapson**	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites, except for doxycycline
23	**sulfadimidine**, **sulfachloropyridazine**	**oxytetracycline**

Annex 7a continued Overview of compounds included in the quantitative methods applied

Lab	Sulfonamides	Tetracyclines
25	sulfapyridine**, sulfamethazine**, **sulfachloropyridazine**, sulfadoxine, sulfaquinoxaline, sulfamethoxazole, sulfadiazine, sulfamerazine, sulfamethoxypyridazine, sulfaguanidine, sulfanilamide, sulfathiazole, sulfadimethoxine, sulfisoxazole, sulfamethizole, sulfamonomethoxine	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
27	**sulfadimidine, sulfachloropyridazine**, **dapson**	**oxytetracycline**
28	trimethroprime, **dapsone**, sulfadiazine, sulfathiazole, sulfamerazine, **sulfadimidine**, sulfadoxine, sulfamethoxazole, sulfadimethoxine, sulfamethizole, sulfameter, sulfamethoxypyridazine, sulfamonomethoxine, **sulfachloropyridazine,** sulfaquinoxaline, sulfisoxazole, sulfapyridine	demeclocycline, tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites
30	sulfacetamide, sulfadiazine, sulfapyridine, sulfathiazole, sulfamerazine, **sulfametazine**, sulfamethizole, sulfamethoxazole, sulfabenzamide, sulfadimethoxin**, dapson**	**oxytetracycline** and epi-metabolite, tetracycline and epi-metabolite, chlortetracycline, methacycline, doxycycline
32	sulfatiazol, sulfadiazine, **sulfametazine**, sulfamethoxipiridazine, sulfadimethoxine	**oxytetracycline**
33	sulfaquinoxaline, **sulfamethazine**, sulfamerazine, sulfathiazole, sulfamethoxazole, sulfadiazine, sulfapyridine, **dapson**	tetracycline, **oxytetracycline**, chlortetracycline and epi-metabolites
35	**sulfadimidine**, **sulfachloropyridazine**, **dapson,** sulfanilamide, sulfadimethoxine, sulfadiazine, sulfamerazine, sulfaquinoxaline, sulfaclozine, sulfadoxine, sulfamethoxypyridazine, sulfamethoxazole, sulfathiazole	**oxytetracycline,** tetracycline, chlorotetracycline, doxycycline
36	sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamoxole, **sulfadimidine**, sulfamethizole, sulfamethoxypyridazine, sulfamonomethoxine, **sulfachloropyridazine**, sulfadoxine, sulfamethoxazole, sulfisoxazole, sulfadimethoxine, sulfaquinoxaline, **dapson**	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline
37	Sulfaguanidine, sulfadiazine, sulfathiazole, **sulfadimerazine**, sulfamethoxypyridazine, sulfamonomethoxine, sulfadoxine, sulfaquinoxaline, sulfadimethoxine, **dapson**	tetracycline, **oxytetracycline**, chlortetracycline, doxycycline and epi-metabolites, except for doxycycline

Annex 7b Overview of the applied quantitative methods for tetracyclines

Lab code	Extraction	Sample purification	Internal standard	Detection method
1	McIlvain buffer + TCA	SPE (C_{18}), evaporation	Demethylchlortetracycline	LC-MS/MS
4	Glycine/HCl	Glass wool, SPE (CH), evaporation		LC-FLU and LC-MS/MS
5	-	-	-	-
6	-	-	-	-
9	McIlvain buffer	SPE (Oasis HLB), evaporation	-	LC-DAD
10	EDTA McIlvain buffer	Centrifugation, SPE (Oasis HLB)	-	LC-UV LC-DAD
11	EDTA buffer + 70% MeOH	Dilution with water	-	LC-MS/MS
12	Succinic buffer	Cu^{2+} Sepharose column, SPE (C_{18})		LC-DAD
14	Water	SPE (C_{18})	-	LC-MS/MS
15	Two solvents	Reversed phase SPE	-	UPLC-Orbitrap and LC-MS/MS
16	ACN + ethylacetate	Filtration	-	LC-MS/MS
17	McIlvain buffer	SPE (Strata-X, Polymer Reverse Phase), evaporation	Demeclocycline	LC-MS/MS
18	TCA/ACN	Evaporation	-	LC-MS/MS
19	McIlvain buffer	SPE (Oasis HLB)	-	LC-DAD
20	MeOH/water	Filtration, dilution	Demeclocycline	LC-MS/MS
21	EDTA buffer + ACN	Evaporation	Demeclocycline	UPLC-MS/MS
22	Acidic EDTA buffer	SPE	-	LC-MS/MS
23				
25	5% TCA	Hexane wash, SPE (C_{18}), evaporation	demeclocycline	LC-MS/MS

Annex 7b continued Overview of the applied quantitative methods for tetracyclines

Lab code	Extraction	Sample purification	Internal standard	Detection method
27				
28	Succinate buffer + EDTA	Hexane wash, SPE (Oasis)	methacycline	LC-MS/MS
30	TCA + McIlvain buffer	SPE (Oasis HLB), evaporation	demeclocycline	LC-DAD
32	Buffer pH 7.4	Filtration		LC-MS/MS
33	TCA	Filtration, SPE (Oasis HLB), evaporation	4-epi-demethylchlortetracycline	LC-MS/MS
35	ACN + McIlvain buffer	Liquid-liquid with ethylacetate, evaporation	ciprofloxacin-d_8	LC-MS/MS
36	EDTA+ McIlvain buffer pH 4	Filtration, SPE (Oasis HLB), evaporation	Demeclocylin	LC-MS/MS
37	McIlvain buffer + EDTA	Add TCA, freezer, SPE (C_{18} Bond Elut), filtration	Demeclocylin	LC-MS/MS

Annex 7c Overview of the applied quantitative methods for sulfonamides including dapson

Lab code	Extraction	Sample purification	Internal standard	Detection method
1	Sodium sulfate + dichloromethane/acetone	Acidified for SPE (SCX), evaporate	sulfadiazine-$^{13}C_6$ sulfadimidine-$^{13}C_6$ sulfanilamide-$^{13}C_6$ sulfadimethoxine-d_6	LC-MS/MS
4	Ethylacetate	Hexane wash	sulfadimethoxine-d_6 sulfamethazine-$^{13}C_6$ sulfachloropyridazine-$^{13}C_6$	UPLC-MS/MS
5	Water	Add tertiairy methyl butyl ether, ultrasonic bath, evaporate, dissolve and add iso-octane	sulfamethazine-d_4	LC-MS
6	ACN	Add C_{18}, evaporate, add ACN/water, evaporate, add acetic acid, filtration	-	LC-UV
9	ACN pH 6	Add heptane, SPE	-	LC-UV
10	EDTA + water + ACN	Evaporation	sulfamethazine-$^{13}C_6$ sulfachloropyridazine-$^{13}C_6$ dapson-d_8	UPLC-MS/MS
11	EDTA + 70% MeOH	Dilution with water	-	LC-MS/MS
12	ACN	Hexane wash, SPE (Oasis MCX)	-	LC-DAD LC-MS/MS
14	ACN/MeOH	Hexane wash	sulfadiazine-d_4 sulfamethazine-d_4 sulfadoxine-d_3	LC-MS/MS
15	Two solvents	Reversed phase SPE	-	UPLC-Orbitrap and LC-MS/MS
16	ACN	Filtration	-	LC-MS/MS
17	Ethylacetate	Ultrasonic bath, evaporate, SPE (Strata-X), evaporate, derivatisation with fluorescamine	sulfapyridine	LC-FLU

Annex 7c continued Overview of the applied quantitative methods for sulfonamides including dapson

Lab code	Extraction	Sample purification	Internal standard	Detection method
18	TCA/ACN	Evaporation	-	LC-MS/MS
19	ACN	SPE (Bond Elut SCX)	sulfadimethoxine-d_6	LC-MS/MS
20	ACN/water	Evaporation	sulfadiazine-d_4	LC-MS/MS
21	EDTA buffer + ACN	Evaporation	sulfaphenazole	UPLC-MS/MS
22	Sodium sulphate + ACN + ammonium acetate	SPE (silica)	sulfamethazine-d_7	LC-MS/MS
22*	Dilute TCA	SPE (cation exchange), evaporate	-	LC-MS/MS
23				
25	ACN/water	Hexane wash, evaporate	sulfaphenazole	LC-MS/MS
27				
28	Succinate buffer + EDTA	Hexane wash, SPE (Oasis)	sulfadimidine-$^{13}C_6$ sulfadiazine-$^{13}C_6$ trimetroprim-$_{d9}$ 3-aminophenyl sulfone	LC-MS/MS
30	ACN	Evaporation, filtration	sulfamethoxazole-$^{13}C_6$ sulfametazine-$^{13}C_6$	UPLC-MS/MS
32	Buffer pH 7.4	Filtration	sulfameter	LC-MS/MS
33	Na_2SO_4 + ACN	Evaporation	sulfachloropyridazine	LC-MS/MS
35	ACN + McIlvain buffer	Liquid-liquid with ethylacetate, evaporation	ciprofloxacin-d_8	LC-MS/MS
36	Water	Filtration, ultrafiltration	sulfadimidine-d4	UPLC-MS/MS
37	ACN	Evaporation, dilution in ammonium acetate, filtration	sulfaphenazole sulfadiazine-$^{13}C_6$ sulfathiazole-$^{13}C_6$ sulfadimerazine-$^{13}C_6$ sulfadoxine-d_3 sulfadimethoxine-d_6	LC-MS/MS

** for dapson only*

Annex 8a Overview of screening results

Lab	Material A	Material B	Material C
2	-	-	-
3	-	OTC	sulfachloropyridazine sulfadimidine
4	-	tetracyclines	sulfonamides
5	-	growth inhibition/ OTC	growth inhibition/ sulfachloropyridazine sulfadimidine
6	-	tetracyclines	-
7	ß-lactams macrolides	ß-lactams macrolides quinolones	ß-lactams macrolides tetracyclines quinolones sulfonamides
9	-	not tested	sulfaclozine sulfadimidine
10	ß-lactams	tetracyclines	ß-lactams
11	-	OTC	sulfadimidine
12	-	tetracyclines	sulfonamides/ sulfachloropyridazine sulfadimidine
13	-	-	growth inhibition
14	tetracyclines	tetracyclines	tetracyclines
15	-	OTC	sulfachloropyridazine sulfadimidine
16	-	growth inhibition/ tetracyclines	growth inhibition/ sulfonamides
17	aminoglycosides	tetracyclines	sulfonamides
18	-	tetracyclines	sulfonamides
19	-	-	-
20	-	-	sulfonamides
21	-	tetracyclines	sulfonamides
22	growth inhibition	growth inhibition/ tetracyclines	growth inhibition/ sulfonamides
23	-	tetracyclines sulfonamides	sulfonamides
25	-	OTC	sulfachloropyridazine sulfadimidine
26	-	-	-
27	-	tetracyclines	-

Lab	Material A	Material B	Material C
28	-	OTC	sulfadimidine
29	-	not tested	not tested
30	-	OTC	sulfadimidine
31	-	tetracyclines	-
32	-	-	sulfadimidine
33	growth inhibition	growth inhibition/ tetracyclines	growth inhibition/ sulfonamides
34	-	tetracyclines ß-lactams	sulfadimidne sulfaquinoxaline
35	-	OTC	sulfachloropyridazine sulfadimidine
36	ß-lactams	tetracyclines	sulfonamides
37	-	OTC	sulfadimidine

- = not detected

Annex 8b False positives and false negatives in screening analysis

False positive results

Lab code	Sample code	Material	Suspect for
7	048	A	ß-lactams macrolides
7	020	B	ß-lactams macrolides quinolones
7	004	C	ß-lactams macrolides quinolones tetracyclines
9	129	C	sulfaclozine
10	026	A	ß-lactams
10	046	C	ß-lactams
12	019	C	ß-lactams
14	085	A	tetracyclines
14	011	C	tetracyclines
17	006	A	aminoglycosides
22	102	A	growth inhibition
23	038	B	sulfonamides
33	040	A	growth inhibition
34	128	B	ß-lactams
34	056	C	sulfaquinoxaline
36	043	A	ß-lactams

False negative results

Lab code	Sample code	Tetracyclines/oxytetracycline	Sample code	Sulfonamides/sulfadimidine and sulfachloropyridazine
2	031	X	074	X
7	020	X		
9			129	X*
10			046	X
11			057	X*
12	118	X		
13	131	X		
14			011	X
19	090	X	112	X
20	086	X		
26	066	X	098	X
27			117	X
28			033	X*
30			100	X*
31			130	X
32	080	X	119	X*
37			089	X*

X =not detected
** missed sulfachloropyridazine with LC-MS or LC-UV*

Annex 9a Overview of quantitative/confirmatory results

Lab	Material A	Material B	Material C
1		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
4		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
5		no method	Sulfadimidine Sulfachloropyridazine
6		no method	Sulfadimidine Sulfachloropyridazine
9		Oxytetracycline	Sulfadimidine
10		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
11		Oxytetracycline	Sulfadimidine
12		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
14		Oxytetracycline	Sulfadimidine
15		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
16		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
17		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
18		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
19		Oxytetracycline	Sulfadimidine
20		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
21		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
22		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
23		Oxytetracycline	Sulfadimidine Sulfachloropyridazine
25		Oxytetracycline	Sulfadimidine Sulfachloropyridazine

Annex 9a continued Overview of quantitative/confirmatory results

Lab	Material A	Material B	Material C
27		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
28		Oxytetracycline	Sulfadimidine Dapson
30		Oxytetracycline	Sulfadimidine Dapson
32		Oxytetracycline	Sulfadimidine
33		Oxytetracycline	Sulfadimidine Dapson
35		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
36		Oxytetracycline	Sulfadimidine Sulfachloropyridazine Dapson
37		Oxytetracycline	Sulfadimidine Dapson

Annex 9b False positives and false negatives in quantitative/confirmatory analysis

False positive results

Lab code	Sample code	Material	Compound confirmed
09	129	C	Sulfaclozine

False negative results

Lab code	Sample code	Material	Compound confirmed
01	014	C	Dapson*
05	013	C	Dapson
06	065	C	Dapson
09	129	C	Dapson*
11	057	C	Sulfachloropyridazine Dapson
14	011	C	Sulfachloropyridazine Dapson
15	068	C	Dapson
17	087	C	Dapson*
18	101	C	Dapson
19	112	C	Sulfachloropyridazine Dapson
20	082	C	Dapson
23	125	C	Dapson
25	081	C	Dapson
28	033	C	Sulfachloropyridazine*
30	100	C	Sulfachloropyridazine
32	119	C	Sulfachloropyridazine Dapson
33	103	C	Sulfachloropyridazine
37	089	C	Sulfachloropyridazine

** included in method*

Annex 10 Results for the analysis of oxytetracycline

Oxytetracycline Assigned value: 122.0 µg/kg Uncertainty of assigned value: 6.0 µg/kg Target standard deviation (Horwitz, Thompson): 26.8 µg/kg		
Lab code	Result (µg/kg)	z_a-score
1	83.8	-1.43
4	91	-1.16
9	124	0.07
10	124.03	0.07
11	132.1	0.38
12	247.5	4.68
14	96	-0.97
15	94	-1.05
16	112	-0.37
17	112	-0.37
18	217.2	3.55
19	87	-1.31
20	93	-1.08
21	120.5	-0.06
22	205	3.10
23	99.9	-0.83
25	190	2.54
27	119.5	-0.09
28	159.4	1.40
30	121.9	0.00
32	200	2.91
33	115	-0.26
35	126	0.15
36	109	-0.49
37	119.5	-0.09

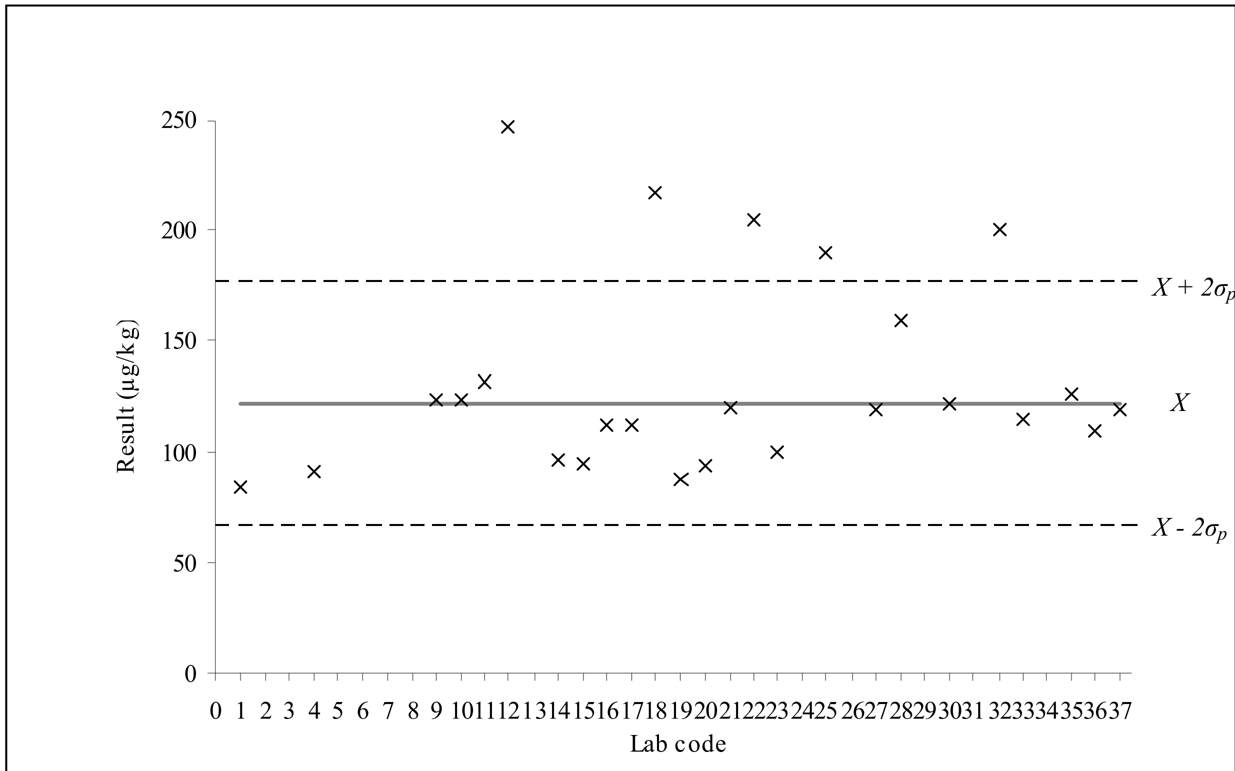

Figure a: Graphical representation of the reported results. The $X \pm 2\sigma_p$ lines are calculated according to equation I in §4.2.4.

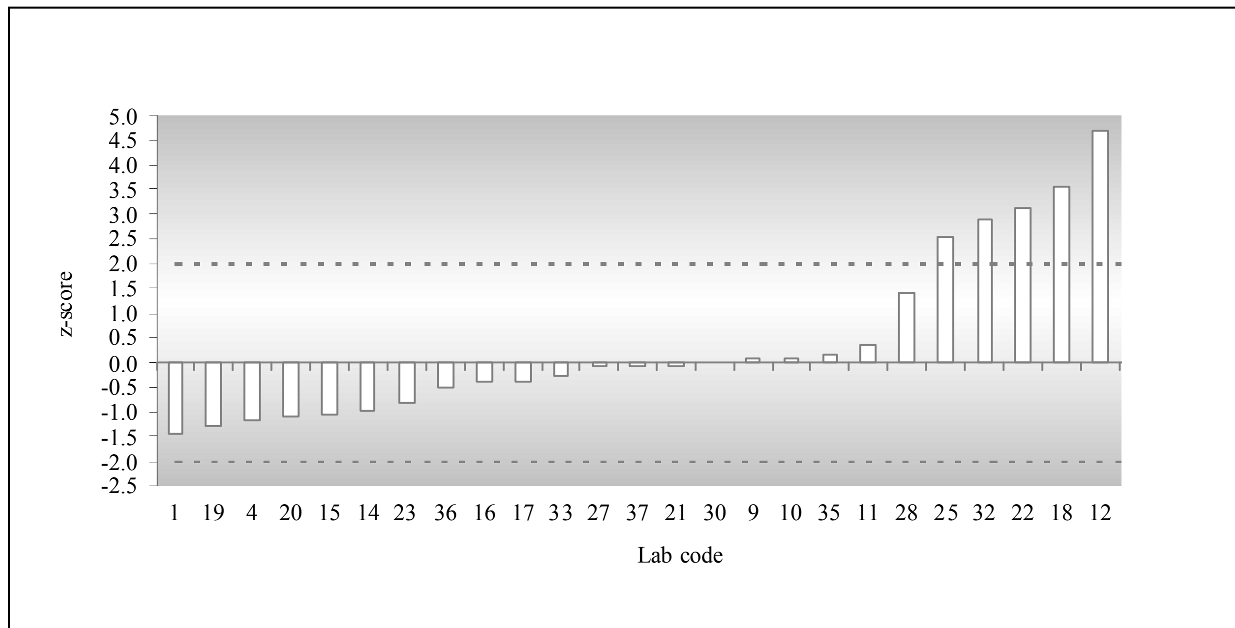

Figure b: Graphical representation of z_a-scores.

Annex 11 Results for the analysis of sulfadimidine

Sulfadimidine Assigned value: 90.1 µg/kg Uncertainty of assigned value: 3.25 µg/kg Target standard deviation (Horwitz, Thompson): 19.8 µg/kg		
Lab code	Result (µg/kg)	z_a-score
1	86	-0.21
4	85.8	-0.22
5	77	-0.66
6	33	-2.88
9	88	-0.10
10	93.5	0.17
11	74.2	-0.80
12	82.4	-0.39
14	99	0.45
15	94	0.20
16	81	-0.46
17	64.3	-1.30
18	67.6	-1.13
19	112	1.11
20	125	1.76
21	72.1	-0.91
22	124	1.71
23	125	1.76
25	91.5	0.07
27	83.3	-0.34
28	91.5	0.07
30	110.7	1.04
32	91	0.05
33	75	-0.76
35	91	0.05
36	94	0.20
37	124.5	1.74

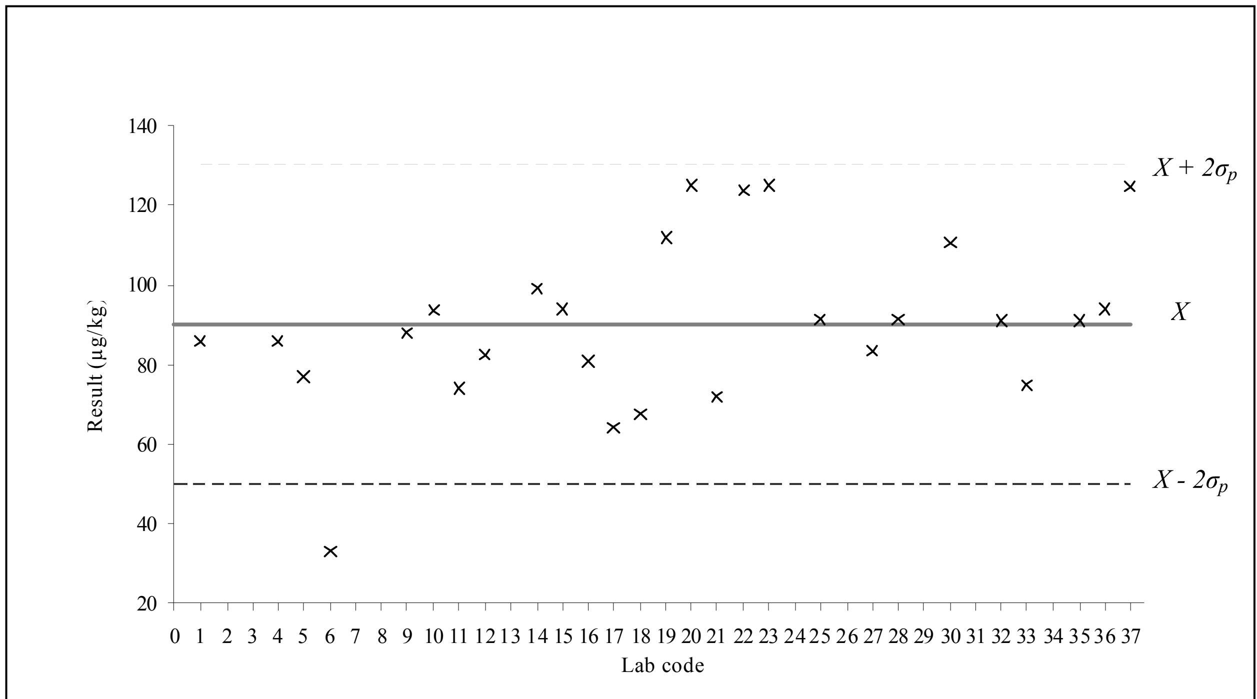

Figure a: Graphical representation of the reported results. The $X \pm 2\sigma p$ lines are calculated according to equation I in §4.2.4.

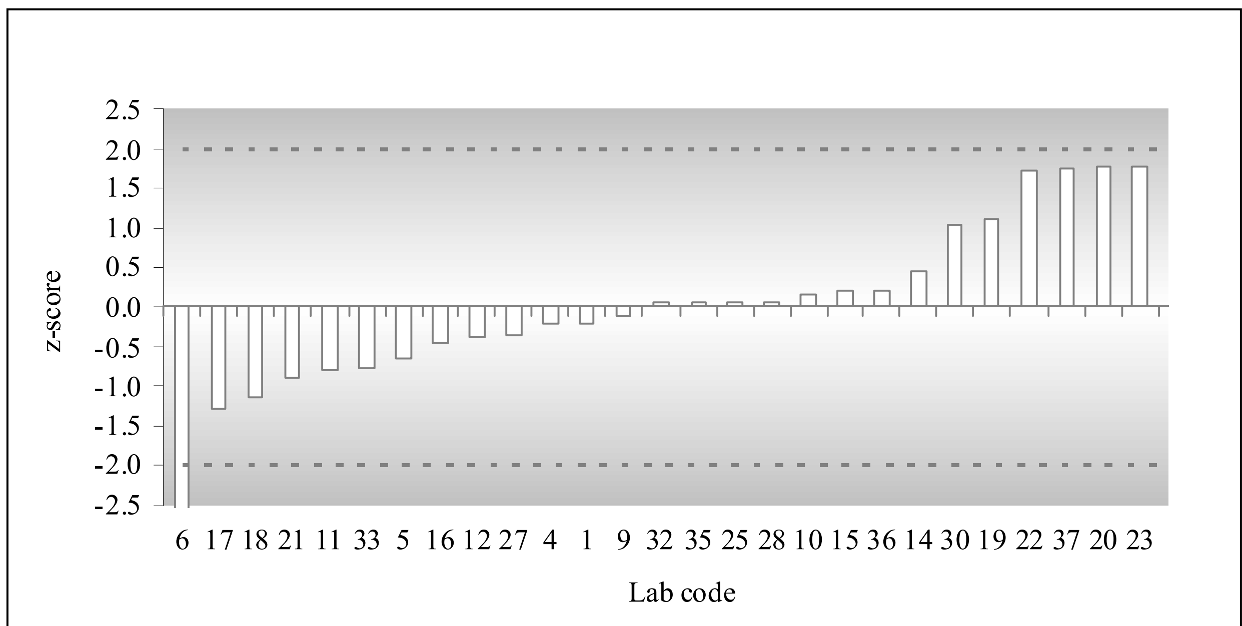

Figure b: Graphical representation of z_a-scores.

Annex 12 Results for the analysis of sulfachloropyridazine

Sulfachloropyridazine Assigned value: 64.3 µg/kg Uncertainty of assigned value: 3.49 µg/kg Target standard deviation (Horwitz, Thompson): 14.1 µg/kg		
Lab code	Result (µg/kg)	z_{ai}-score
1	73	0.62
4	66.7	0.17
5	63	-0.09
6	18	-3.08
10	73.1	0.62
12	63.3	-0.07
15	69	0.33
16	49	-1.02
17	36.5	-1.85
18	34.7	-1.97
20	83	1.32
21	51.7	-0.84
22	89	1.75
23	74.4	0.71
25	61.1	-0.21
27	82.8	1.31
35	57	-0.49
36	68	0.26

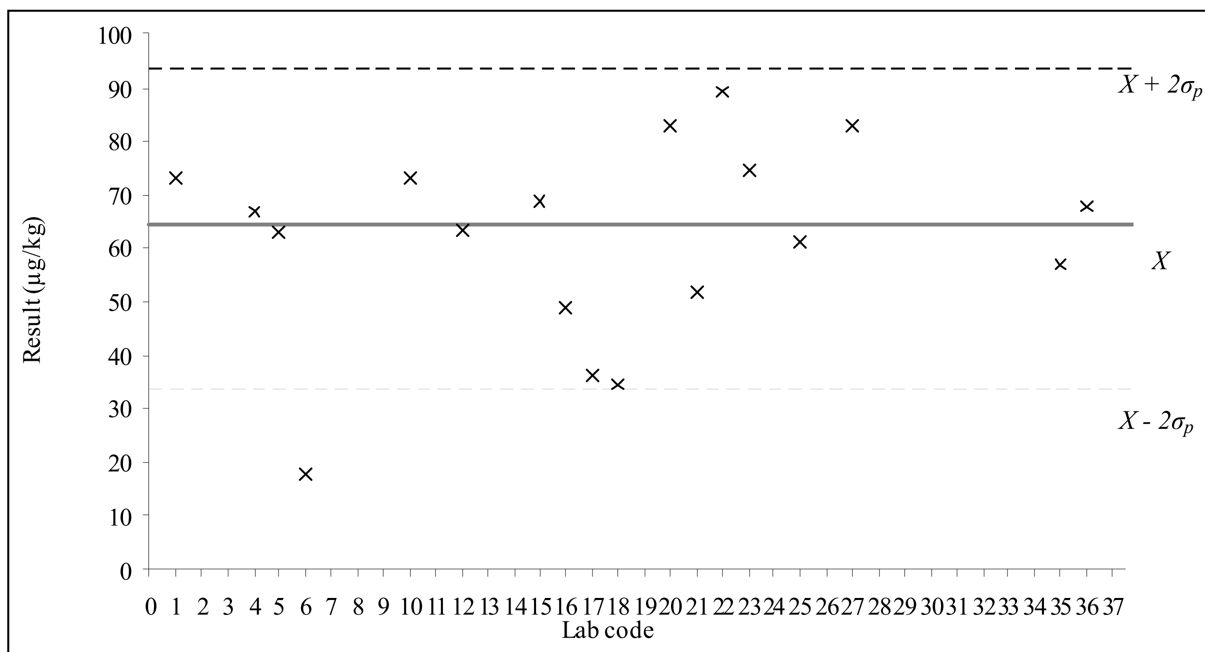

Figure a: Graphical representation of the reported result. The $X \pm 2\sigma_p$ lines are calculated according to equation III in §4.2.4.

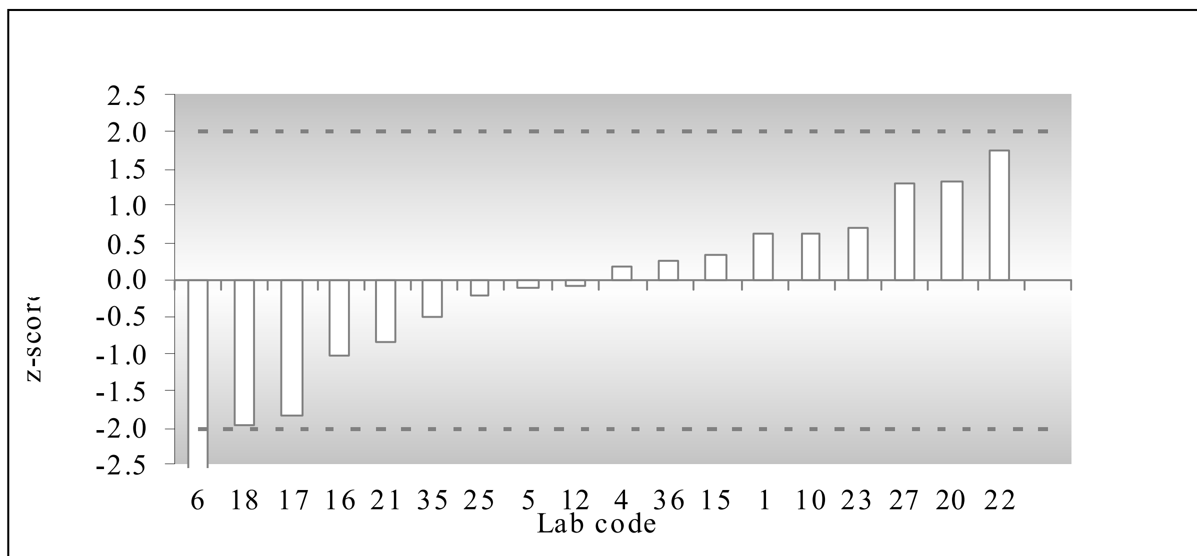

Figure b: Graphical representation of z_{ai}-scores.

Annex 13 Results for the analysis of dapson

Dapson Assigned value: 3.35 µg/kg Uncertainty of assigned value: 0.29 µg/kg Target standard deviation (Horwitz, Thompson): 0.74 µg/kg		
Lab code	Result (µg/kg)	z'_{ai}-score
4	1.42	-2.12
10	3.25	-0.11
12	4.1	0.95
16	1.62	-1.90
21	3.6	0.32
22	3.7	0.44
27	4.25	1.14
28	3.7	0.44
30	4.8	1.83
33	2	-1.48
35	3.0	-0.38
36	2	-1.48
37	4.3	1.20

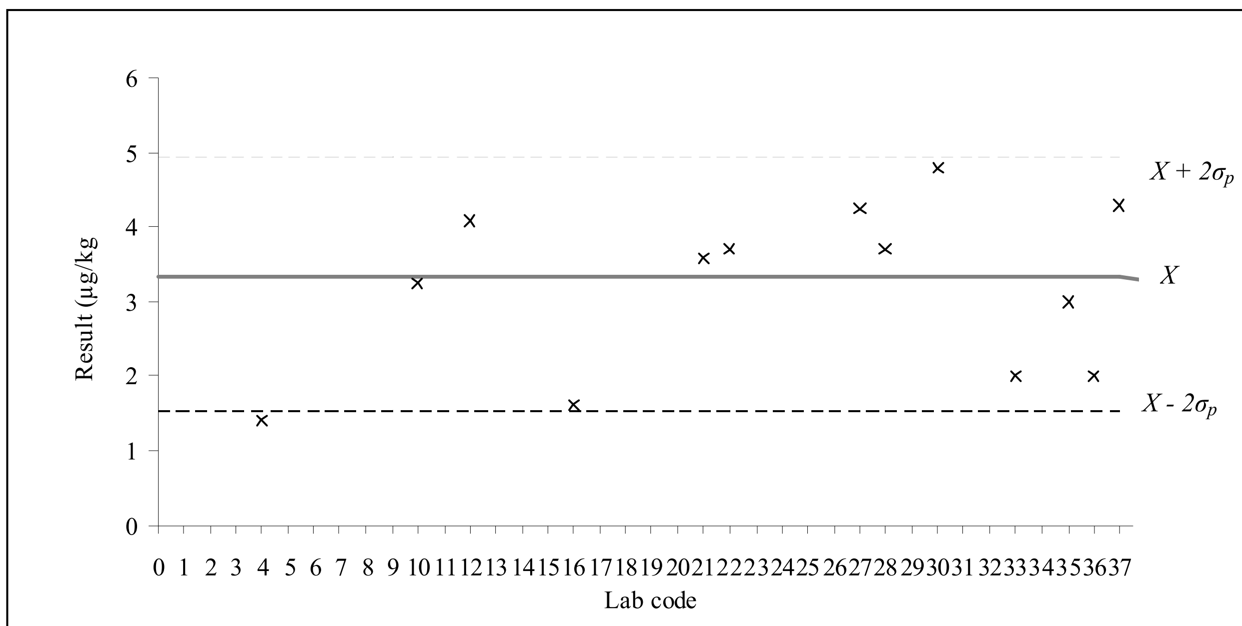

Figure a: Graphical representation of the reported results. The $X \pm 2\sigma_p$ lines are calculated according to equation IV in §4.2.4.

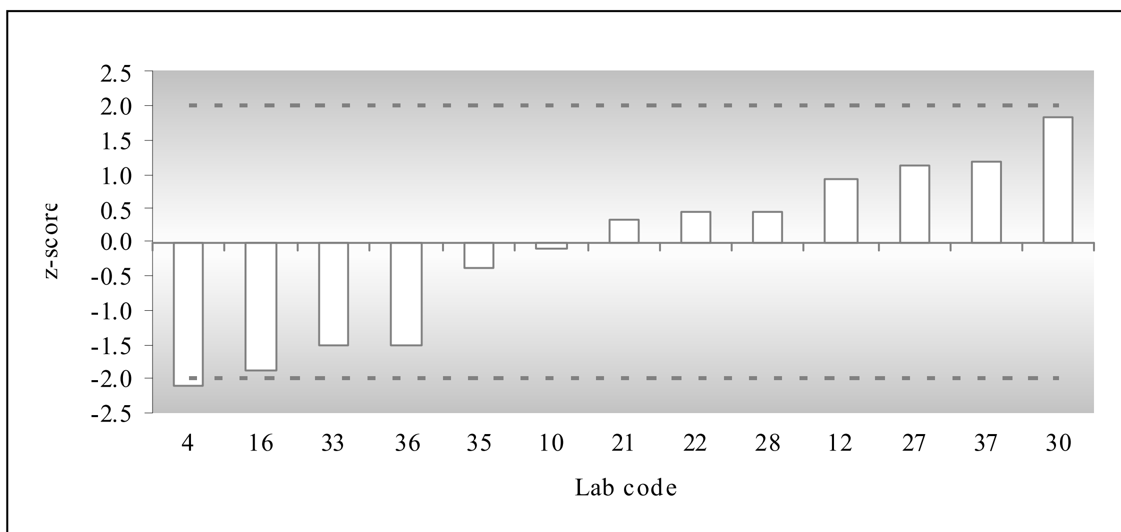

Figure b: Graphical representation of z'_{ai}-scores.