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# Inter-laboratory study for tetracyclines in poultry muscle

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# SUMMARY

The inter-laboratory study regarding tetracyclines in poultry muscle was performed following ISO/IEC Guide 43-1 and 43-2.

For the inter-laboratory study regarding tetracyclines in poultry muscle, three test materials were prepared:

- A blank material (A);
- A material containing oxytetracycline and 4-epioxytetracycline, the sum of both being just above the MRL and doxycycline at about 0.5*MRL (B);
- A material containing an amount of oxytetracycline and doxycycline just above the MRL (C).

Homogeneity and sufficient stability of the materials was demonstrated.

Seventeen laboratories were invited to participate in the inter-laboratory study for tetracyclines in poultry muscle of which sixteen laboratories, i.e. 94%, subscribed.
Each laboratory received six randomly coded samples including two duplicates of materials A, B and C. The laboratories were asked to analyze the samples in duplicate, resulting in four results for each material.

Eleven participating laboratories reported their results. The results of those laboratories were included in the report without any modifications. Two laboratories analysed the samples for oxytetracycline only. Five laboratories notified RIKILT not being able to participate due to different reasons.

The majority of the laboratories extracted the poultry muscle samples using an EDTA-McIlvain buffer. By far the most common sample clean-up procedure is Solid Phase Extraction (SPE) using a polymer based material (e.g. OASIS® HLB). Most of the participants used LC–MS/MS for the confirmatory analysis. Two laboratories applied LC–DAD as a confirmatory technique.
Four of the participating laboratories include 4-epiOTC, 4-epiTC and 4-epiCTC in their analysis. Those laboratories comply with the definition of the MRL as stated in the Commission Regulation No. 281/96 [11]. The laboratories that have those 4-epitetracyclines included in their method cope in different ways regarding the quantification of these analytes.

Most participating laboratories determined values for CCα and CCß and, hence, the majority already complies with Commission Decision 2002/657/EC regarding the way to report results for registered veterinary drugs as from the 1st of August 2007 [16].
For some laboratories the reported values for CCα and CCß are not in agreement with the reproducibility of the analysis calculated from the reported results in this inter-laboratory study.

In this inter-laboratory study, both false negative and false positive results occurred.

The calculated assigned value of material B is 104 µg/kg 4-epiOTC + OTC and 53 µg/kg DC, with an uncertainty of 12 µg/kg and 1.4 µg/kg respectively. The uncertainty of the assigned value of 4-epiOTC + OTC in this material is quit high (above $0.3\sigma_p$).
Although, all participants reported satisfactory results with regard to the accuracy for both 4-epiOTC + OTC and DC, it is stated that a considerable variation is observed for the reported amount of 4-epiOTC

+ OTC (average results ranging from 63.5 to 146.7 µg/kg). One laboratory reported results with a questionable reproducibility.

The calculated assigned value of material C is 141 µg/kg OTC and 181 µg/kg DC, with an uncertainty of 10 µg/kg for both analytes. The uncertainty of the assigned value of OTC in this material is quit high (above $0.3\sigma_p$).
82% of the participants reported satisfactory results with regard to the accuracy of OTC. A considerable variation is observed for the reported amount of OTC in this material (average results ranging from 85.5 to 231.5 µg/kg).
For DC, all laboratories reported satisfactory results.

The performance regarding accuracy, reproducibility, false positives and false negatives was expressed in a laboratory performance score for each laboratory. 72% of the laboratories obtained the maximum score.

Although most laboratories obtained satisfactory results regarding the accuracy and the reproducibility, it is concluded that extra effort is needed regarding the analysis of tetracyclines in poultry muscle:

- 4-epiOTC, 4-epiTC and 4-epiCTC should be included in the method for the analysis of tetracyclines by all laboratories;
- Reconsideration of values determined for CCα and CCß with respect to their accuracy may be necessary in some case;
- An effort should be made regarding the quantitative analysis of especially OTC in poultry muscle;
- Some laboratories should make an effort to prevent false positives and false negatives in the future.

# 1 INTRODUCTION

## 1.1 Inter-laboratory testing

Inter-laboratory 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, inter-laboratory testing is an important requirement of the EU Additional Measures Directive 93/99/EEC [1] and is increasingly important in the revised draft ISO 17025 [2].
The aim of this inter-laboratory study was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of tetracyclines in poultry muscle. This study also provided an evaluation of the methods applied for quantitative and confirmatory analysis of tetracyclines.
The inter-laboratory study was carried out according to guidelines ISO/IEC 43-1 [3] and ISO/IEC 43-2 [4].

## 1.2 Tetracyclines in poultry muscle

Tetracyclines are a very important group of antibiotic agents in human and veterinary medicine. Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) are the most well known tetracycline compounds.

CTC and OTC are produced by *Streptomyces aureofaciens* and *Streptomyces rimosus* respectively. TC is produced semisynthetically from CTC. DC is a semisynthetic derivate [5, 6].

Tetracyclines have a broad-spectrum activity which includes Gram-positive and Gram-negative bacteria. They interfere with the bacterial protein synthesis in rapidly growing and reproducing bacterial cells and inhibit the metabolism of the bacteria by interfering with the protein synthesis [5, 6].

Tetracyclines have a clinical application in the treatment of rickettsia and colibacillosis. At the beginning of administration success in combating infections is remarkable but in due course a resistance is built up and the effect may be reduced to zero [5-7].
The toxicity of tetracyclines is low, but after prolonged therapy or contact, infections with resistant organisms, allergic reactions and vitamin B deficiencies may occur. The use of tetracyclines during pregnancy and by young children and animals has adverse effects on skeleton formation [7].
In addition to the antibiotic use, tetracyclines are applied as growth-stimulants and as preservatives to lengthen the shelf-life of poultry and meats [7].

After oral administration adequate absorption of the tetracyclines takes place and they can persist at high concentrations [5]. Absorption of tetracycline is impaired by milk products, calcium and magnesium salts, and iron preparations. The mechanisms responsible for decreased absorption appear to be chelation and an increase in pH [6]. After absorption, tetracyclines are widely distributed in the body, with the highest concentrations in liver and kidney.
The metabolism of tetracyclines was extensively studied in several species including dogs and rats [6]. The results indicated that, with the exception of metal chelate formation, no chemical transformation occurred in the body. Small amounts of 4-epimers were detected. This was contributed to chemical instability at physiological conditions rather than to metabolic transformation [6].

According to EU regulations, all substances for veterinary use need to be included in Annexes I, II or III of Council Regulation (ECC) No 2377/90. Tetracyclines are included in Annex I: pharmacologically active veterinary products for which a Maximum Residue Limit (MRL) is established. The MRL for OTC, TC and CTC is established as the sum of the tetracycline and its 4-epimer and is 100 µg/kg in muscle for all food producing species. For DC, the MRL is established at 100 µg/kg in muscle tissue for DC only for all food producing species [8-10].

This inter-laboratory study focuses on OTC, 4-epiOTC and DC only. The structure of OTC, 4-epiOTC and DC are presented in figure 1.

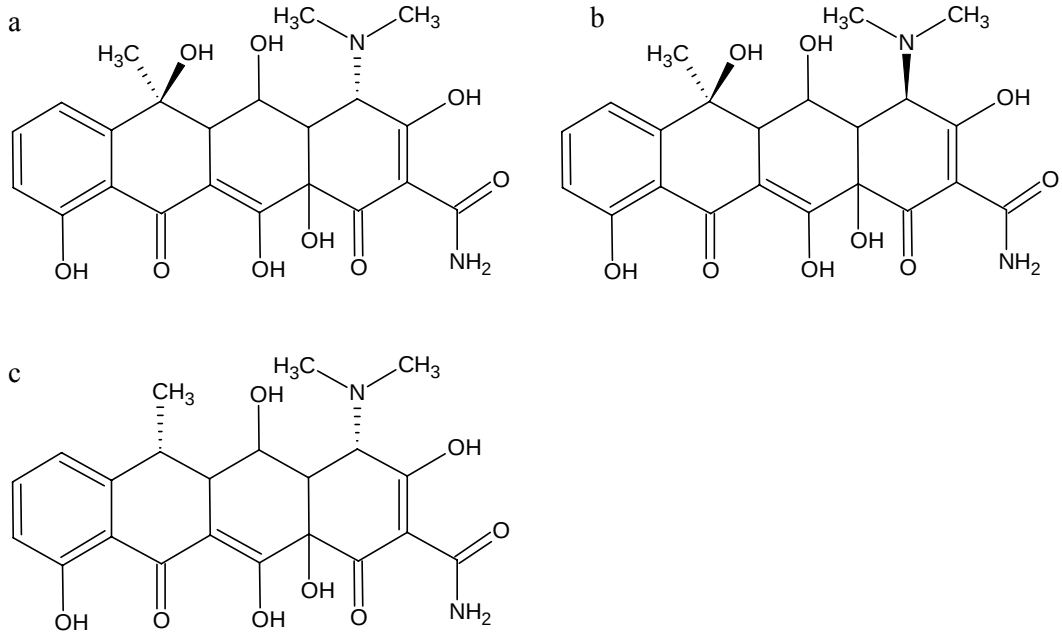


Figure 1. Molecular structure of (a) oxytetracycline, (b) 4-epioxytetracycline and (c) doxycycline.

# 2 TEST MATERIALS

## 2.1 Sample preparation

Three materials were prepared at different concentrations of OTC, 4-epiOTC and DC by adding solutions of OTC, 4-epiOTC and DC to blank poultry muscle. The materials were homogenised under cryogenic conditions. The materials presented in table 1 were obtained.

Table 1.Target amount of tetracyclines in the inter-laboratory test materials

| Material code | Amount of OTC (µg/kg) | Amount of DC (µg/kg) |
|---|---|---|
| A | Blank | Blank |
| B | Just above MRL* | Just below MRL |
| C | Just above MRL | About twice the MRL |

* Contains both OTC and 4-epiOTC.

## 2.2 Sample identification

Materials A, B and C were stored in containers containing at least 20 grams of muscle, yielding a total of 60 containers per batch. Per material, 20 randomly chosen containers were used for homogeneity and stability testing. The other samples were randomly coded with a code from TETRA/2005/001 through TETRA/2005/150.
Twenty sets consisting of two samples of each material were randomly prepared as presented in Appendix I.

## 2.3 Homogeneity study

Ten containers of materials B and C were each analyzed in duplicate for OTC, TC, CTC, their 4-epimers and DC for the determination of the homogeneity of the materials. The homogeneity study was carried out according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [11] and ISO/DIS 13528 [12], taking into account the insights discussed by Fearn *et al*. [13] and Thompson [14].
The results of the homogeneity study and their statistical evaluation is presented in Appendices II and III for materials B and C respectively.
All materials were demonstrated to be sufficiently homogeneous for use in inter-laboratory trials.
Simultaneous with the analysis of each of the materials, at least two samples of material A were analyzed. Those analyses demonstrated that the material was free of tetracycline residues (< 10 µg/kg) and is therefore suited to use as a blank material in the inter-laboratory study.

## 2.4 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code. The sample sets with the corresponding number, consisting of six coded samples, were sent to the participating laboratories in the beginning op Arpil. The sample sets were packed in an insulating box, containing dry ice and were dispatched to the participants immediately by courier.

The samples were accompanied by a letter describing the requested analyses, an acknowledgement of receipt form and a results form. The laboratories were asked to analyse each of the samples in duplicate, resulting in four results for each material. The deadline for sending in results was established at the 1st of June, allowing seven weeks for analysis. Receipt of the samples in good condition (frozen) was confirmed by all participants except for laboratory 10. Due to some shipment difficulties, the samples of laboratory 10 did only arrive after six days. The laboratory reported that the samples arrived thawed. A new set (set No. 17 in Appendix I) was sent to the laboratory on the 19th of April. This sample set did arrive in good condition.

## 2.5 Stability

From the homogeneity data, the amount of tetracycline residues in the materials, just after preparation, is calculated from the average of the 20 results.
The materials were stored at -80 °C until the 17th of May. On this day, 71 days after the initial analysis, three containers of material B and C were analysed. After this, the containers were stored at -20 degrees. On the 31st of May, 85 days after the initial analysis and at the end of the proficiency test, the containers were analysed again. For both points of time, the average of the results was calculated.
The results of the initial analysis were compared to the results of the 17th and 31st of May, using a Students t-test [15]. The hypothesis for this test is:

$$H_0 : x_0 = x_d$$

where: $x_0$ = the average of the initial analysis;
$x_d$ = the average of the analysis at time=d.

The standard deviation of both populations are considered the same, because the same analytical procedure is applied to obtain the results. Therefore the value *t* is calculated by:

$$t = \frac{x_0 - x_d}{s\sqrt{\frac{1}{n_0} + \frac{1}{n_d}}}$$

where: $x_0$ = the average amount calculated for the initial analysis;
$x_d$ = the average amount calculated for the analysis at time=d;
$n_0$ = number of results of the initial analysis;
$n_d$ = number of results of the analysis at time=d;

$$s = \sqrt{\frac{(n_0 - 1)s_0^2 + (n_d - 1)s_d^2}{(n_0 + n_d - 2)}}$$

where: s = pooled standard deviation;
$n_0$ = number of results of the initial analysis;
$n_d$ = number of results of the analysis at time=d;
$s_0$ = standard deviation of the initial analysis calculated from the CV% resulting from the validation procedure;
$s_d$ = standard deviation of the analysis at time=d calculated from the CV% resulting from the validation procedure.

The calculated value $t$ is compared to a critical value ($t_{crit}$) derived from a table with $t$ having $n_0$+$n_d$-2 degrees of freedom [15]. If $t < t_{crit}$ it is demonstrated that no significant difference between the average amount of the analysis at time=d and the initial analysis at time=0 is found. In this case the material is considered stable.

The results and statistical evaluation of the stability test are presented in appendix IV. It was demonstrated that no significant loss of 4-epiOTC, OTC or DC occurred during the timescale of the inter-laboratory study at the chosen storage conditions.

# 3 APPLIED METHODOLOGIES

The participating laboratories applied different sample preparation methods and detection techniques for the analysis of tetracyclines in poultry muscle. A schematic overview of the methods applied is presented in Appendix V.

The majority of the participants extracted the poultry muscle samples using an aqueous and slightly acidic extraction medium. An EDTA-McIlvain buffer is the most general applied medium. Others are a sodium succinate solution, acetonitril and heptane, or a trichloroacetic acid solution.
By far the most common sample clean-up procedure is Solid Phase Extraction (SPE) using a polymer based phase like OASIS® HLB. In one case this step is preceded by a clean-up using a chelated sepharose column loaded with copper ions. Two participants used a $C_{18}$ based material for the SPE procedure. Two participants only filtered their extract before analysis without further purification.

Most of the participants used LC–MS/MS for the confirmatory analysis. Two laboratories applied HPLC–DAD for their confirmatory method. This is in accordance with Commission Decision 2002/657/EC [16] regarding the identification of detected compounds. For one laboratory the confirmatory analysis (LC–MS/MS) was preceded by a quantitative analysis using HPLC–UV. One laboratory did not confirm the identity of the analytes but merely carried out a quantitative analysis.

Four laboratories used an internal standard for their quantitative analyses. In three cases demeclocycline (demethylchlortetracycline) was used.

A majority of the participating laboratories included OTC, TC, CTC and DC in their analysis. One laboratory tested for OTC only. One laboratory did not include DC in their method. The number of analytes included in the method is dependant on regional differences and could therefore be related to differences in registration of tetracyclines for veterinary use.
Only four of the participating laboratories did pay specific attention to the presence of 4-epiOTC, 4-epiTC and 4-epiCTC. Two other laboratories include just one of the 4-epimers in their method. These laboratories report several ways of coping with the quantitative analysis of 4-epiOTC:

- In most cases 4-epiOTC co-elutes with OTC. In this case the sum of 4-epiOTC and OTC is quantified using OTC calibrants.
- In some cases 4-epiOTC and OTC are chromatographically separated. Some laboratories quantify these compounds using separate calibrants for both 4-epiOTC and OTC. Others quantify the sum of both compounds using OTC calibrants.

For some laboratories 4-epiOTC and OTC coelute, especially at low retention times. It could well be possible that some laboratories that do not pay specific attention to 4-epiOTC, did include 4-epiOTC in the quantitative analysis because of this without knowing.
It is noted that the analysis of 4-epimers of tetracyclines is not yet generally applied. Seven of the eleven participating laboratories (i.e. 63%) do not fully comply with Commission Regulation No. 281/96 [10] regarding the definition of the MRL (inclusion of the 4-epimers).

An overview of the method performance of the participating laboratories is presented in Appendix VI. Amongst the participating laboratories, four did not report values for CCα and CCß. Hence, not all participating laboratories are yet ready to report their results as required by Commission Decision

2002/657/EC [16] regarding CCα and CCß that apply for registered veterinary drugs as from the 1st of August 2007.
It is noted that some laboratories (No. 1 and 12) report relatively low values for CCα. This suggests very good method reproducibility. Laboratory 10, on the other hand, reports relatively high values for CCα and CCß for DC, indicating variable method performance for this compound.

# 4 STATISTICAL EVALUATION

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [11], elaborated by ISO, IUPAC and AOAC and ISO/DIS 13528 [12] in combination with the insights published by the Analytical Methods Committee [17, 18] regarding robust statistics.

## 4.1 Calculation of the assigned value

The assigned value ($X$) was determined using robust statistics [14, 17, 18]. 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 an inter-laboratory proficiency test. When using robust statistics, the data does not have to be normally distributed, which is necessary for applying classical outlier elimination methods.
The robust mean of the reported results of all participants was calculated as the assigned 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. 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, is it legitimate to draw any conclusion regarding the performance of the participating laboratories from the calculated assigned value and $z_a$-scores?
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 [12] the uncertainty of the assigned value 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;
$\sigma_p$ = 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.

## 4.3 Calculation of the target standard deviation

According to Commission Decision 2002/657/EC [16], the inter-laboratory 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, $\sigma_H$ and the concentration, c. It expresses inter-laboratory precision expected in inter-laboratory trials. Therefore, this relation is suitable for calculating the target standard deviation, $\sigma_p$ in inter-laboratory trials.
Thompson [11] demonstrated that this Horwitz equation is not applicable to the lower concentration range (<120 µg/kg) as well as at high concentrations (>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 $\sigma_H$ = expected standard deviation in inter-laboratory trials;
$c$ = concentration of the analyte.

The target standard deviation, $\sigma_p$, was determined using the equation for analyte concentrations <120 µg/kg, with $c$ = the assigned value 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 43-2 [4] and ISO/DIS 13528 [12] are applied. According to these guidelines, $z_a$-scores are classified as presented in table 2.

Table 2: Classification of $z$-scores

| | |
|---|---|
| $\|z\| \leq 2$ | satisfactory |
| $2 < \|z\| < 3$ | questionable |
| $\|z\| \geq 3$ | unsatisfactory |

When 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:

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

where $z_a$ = accuracy *z*-score;
$x$ = mean result of the laboratory;
$X$ = assigned value;
$\sigma_p$ = target standard deviation.

However, if the uncertainty of the assigned value exceeds the value mentioned in § 4.2, it could influence the evaluation of the laboratories. Therefore this uncertainty is taken into account by calculating a $z_a$'-score [13]:

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

where $z_a'$ = accuracy *z*-score taking into account the uncertainty of the uncertainty;
$x$ = mean result of the laboratory;
$X$ = assigned value;
$\sigma_p$ = target standard deviation;
$u$ = uncertainty of the assigned value.

## 4.5 Performance characteristics with regard to the reproducibility

In addition to the evaluation of the accuracy, it is useful to inform the participants about the reproducibility of the results.
In the design of this inter-laboratory study, two unidentified duplicate samples each material were submitted to the participants. Therefore, every laboratory reported two pairs of results for each material. From the results of the blind pairs of material B and C the repeatability ($s_r$) and the within-lab-reproducibility ( $s_{R_L}$ ) were calculated [19].

The repeatability standard deviation is calculated from:

$$s_r = \sqrt{\frac{\sum d_i^{\,2}}{2p}}$$

where $s_r$ = repeatability standard deviation;
$d_i$ = difference between the individual values for a pair;
$p$ = number of pairs.

The within-lab-reproducibility standard deviation is calculated from:

$$s_{R_L} = \sqrt{1/2\,(s_p{}^2 + s_r{}^2)}$$

where $s_{R_L}$ = within-lab-reproducibility standard deviation;

$s_r$ = repeatability standard deviation;

$$s_p{}^2 = \frac{\sum (T_i - \bar{T})^2}{2(p-1)}$$

where $T_i$ = sum of the individual values for the pair;

$\bar{T}$ = mean of the $T_i$ across all pairs;

$p$ = number of pairs.

To inform a laboratory about the performance regarding the reproducibility, the Horwitz-ratio (*HORRAT*) is a suitable value [20]. In this report, the Horwitz ratio is calculated from the within-lab reproducibility, because it is not possible to calculate a reproducibility standard deviation from the laboratory data. The reproducibility ($s_R$ ) includes inter-laboratory variation and must therefore always be higher than the within-lab reproducibility ( $s_{R_L}$ ).
Because the *HORRAT* value is calculated from $s_{R_L}$ instead of $s_R$, this value is not for evaluation purposes but for information only.

The *HORRAT* is calculated from:

$$HORRAT = \frac{s_{R_L}}{\sigma_p}$$

where *HORRAT* = Horwitz ratio;

$s_{R_L}$ = within-lab reproducibility standard deviation;

$\sigma_p$ = target standard deviation (§ 4.3).

In this formula, a *HORRAT* value equal to 1.0 indicates that the within-lab reproducibility is equal to the predicted maximum reproducibility standard deviation resulting from the Horwitz equation. However, the latter refers to reproducibility between laboratories and, hence, would normally be higher than the within-lab reproducibility. Therefore it is within reason that the HORRAT value calculated from the within-lab reproducibility, as done in this report, should be substantially below 1.0.
Nonetheless in this report, a *HORRAT* value is not regarded as a questionable result unless it exceeds 1.0.

## 4.6 Calculation of laboratory performance scores

In the evaluation of this proficiency test a score is calculated to demonstrate the performance of the participating laboratories. This score accounts for the accuracy and reproducibility of the results, and the occurrence of false positive and false negative results.
For each satisfactory result regarding the accuracy ($|z_a$-score$|<2.0$) and for each satisfactory result regarding the reproducibility (*HORRAT*<1.0), 1 point is earned. For each false positive or false negative result per material, 1 point is subtracted from the score. The maximum attainable score is 8.

# 5 RESULTS AND DISCUSSION

Sixteen out of seventeen invited laboratories subscribed for the participation in the inter-laboratory study for tetracyclines in poultry muscle. Ten laboratories managed to submit valid results before the dead-line of the 1st of June. Laboratory 16 reported their results on the 27th of June. It is noted that no statement can be made regarding the stability of the materials over the period after the dead-line. Nevertheless, these results were included in the report.
Five laboratories were not able to report data due to different causes.
The majority of laboratories analysed the samples in duplicate. The laboratories 1 and 16 reported only one result per sample. The number of laboratories included in the statistical evaluation is 11 for 4-epiOTC and OTC and 9 for DC.
All results are presented as reported by the laboratories, without any correction.

## 5.1 Evaluation of the results of material A

Material A is a blank sample. Each laboratory received two randomly coded blank samples, which were not identified as such. Laboratory 5 detected OTC in both samples originating from material A. The identity of the analyte was confirmed by LC–MS/MS. This result is considered as a false positive result. None of the other participants detected any tetracycline residues in the samples originating from material A.
An overview of false negative and false positive results is presented in appendix VII.

## 5.2 Evaluation of the results of material B

Eleven participants reported results for the samples originating from material B. Laboratory 1 and 16 reported only a single value for both samples. Laboratory 3 and 7 did not include DC in their analyses and were therefore not able to report any values for this analyte.
No false negative or false positive results were reported.

The assigned value and the uncertainty of the assigned value were calculated according to § 4.1 and § 4.2.
The uncertainty of the assigned value of 4-epiOTC + OTC for material B exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account with regard to the evaluation of the laboratories. The $z_a$'-scores and *HORRAT* values for 4-epiOTC + OTC obtained by each laboratory were calculated. The results are presented in appendix VIII. Graphical representations of the $z_a$'-scores and *HORRAT* values are included.

The uncertainty of the assigned value of DC for material B is below $0.3\sigma_p$ (§4.2). Therefore, the uncertainty of the assigned value is considered to be negligible. The $z_a$-scores and *HORRAT* values for DC obtained by each laboratory were calculated. The results are presented in appendix IX. Graphical representations of the $z_a$-scores and *HORRAT* values are included.

The quantitative results of all of the laboratories were satisfactory regarding the accuracy. Surprisingly, no correlation was found between low results and laboratories that did not include 4epi-OTC in their analysis. The number of satisfactory $z_a$-scores for both analytes is presented in table 3.

The calculation of the *HORRAT* value results in a value above 1.0 for laboratory 5 for 4-epiOTC + OTC. This indicates questionable performance of the applied method with regard to repeatability. No *HORRAT* value could be calculated for laboratory 1 and 16, because only one result per sample was reported.

Table 3. Number and percentage of satisfactory $z_a$-scores for material B.

| Analyte | No. of satisfactory $z_a$-scores | % of satisfactory $z_a$-scores | No. of satisfactory *HORRAT* values | % of satisfactory *HORRAT* values |
|---|---|---|---|---|
| 4-epiOTC+OTC | 11 of 11 | 100% | 8 of 9 | 89% |
| DC | 9 of 9 | 100% | 7 of 7 | 100% |

Some laboratories report relatively small values for CCα. The CCα reported by laboratories are compared to the reproducibility calculated from the results in this inter-laboratory study. This comparison could not be made for laboratory 1 and 16 due to the lack of a duplicate analysis. Laboratory 1, however, reports surprisingly low CCα and CCß compared to the other participants. For laboratories 5 and 12, the reproducibility for 4epiOTC + OTC is higher than is suggested by the reported CCα for this analyte. For laboratory 9 the reproducibility for DC is higher than is suggested by the reported CCα for this analyte.

## 5.3 Evaluation of the results of material C

Eleven participants reported results for the samples originating from materials C. Laboratory 1 and 16 reported only a single value for each sample. Laboratory 6 did not detect any tetracycline residues in sample TETRA/2005/088 originating from material C. In both samples originating from material A, also no tetracycline residues were found. This excludes a switch of samples. This result is considered as a false negative result.
No other false negative or false positive results were reported.
An overview of false negative and false positive results is presented in appendix VII.

The uncertainty of the assigned value of 4-epiOTC + OTC for material C exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account with regard to the evaluation of the laboratories. The $z_a$'-scores and *HORRAT* values for OTC obtained by each laboratory were calculated. The results are presented in appendix X. Graphical representations of the $z_a$'-scores and *HORRAT* values are included.

The uncertainty of the assigned value of DC for material C is below $0.3\sigma_p$ (§4.2). Therefore, the uncertainty of the assigned value is considered to be negligible. The $z_a$-scores and *HORRAT* values for DC obtained by each laboratory were calculated. The results are presented in appendix XI. Graphical representations of the $z_a$-scores and *HORRAT* values are included.

The results of almost all of the laboratories were satisfactory regarding accuracy. The number of satisfactory $z_a$ -scores for both of the analytes is presented in table 4.

All calculated *HORRAT* values were considered satisfactory. No *HORRAT* value could be calculated for laboratory 1, 6 and 16, because only one result per sample was reported.
Table 4. Number and percentage of satisfactory *z*-scores for material C.

| Analyte | No. of satisfactory $z_a$-scores | % of satisfactory $z_a$-scores | No. of satisfactory *HORRAT* values | % of satisfactory *HORRAT* values |
|---|---|---|---|---|
| OTC | 9 of 11 | 82% | 8 of 8 | 100% |
| DC | 9 of 9 | 100% | 6 of 6 | 100% |

Some laboratories report relatively small values for CCα. The CCα reported by laboratories are compared to the reproducibility calculated from the results in this inter-laboratory study. This comparison could not be made for laboratory 1 and 16 due to the lack of a duplicate analysis.
For laboratory 5 the reproducibility for OTC is higher than is suggested by the reported CCα for this analyte.

## 5.4 Laboratory scores

The performance of each participating laboratory is expressed in a laboratory performance score (§4.6). The maximum attainable score is 8 points. In case DC is not included in the method or only a single value for each sample was reported, the maximum attainable score is lower. The laboratory performance score and the maximum attainable score per lab are presented in appendix XII.
From the 11 laboratories 8 (i.e. 72%) showed optimal performance for the analysis of tetracyclines in poultry muscle regarding the accuracy, the repeatability and the occurrence of false positive and false negative samples,.

# 6 CONCLUSION

Seventeen laboratories were invited to participate in the inter-laboratory study for tetracyclines in poultry muscle, of which sixteen laboratories subscribed.
Ten laboratories reported their results within the given timescale. One laboratory reported their results with a delay of 23 days. All reported results were included in the report.

Three laboratories did not report a value for CCα and CCß. Apparently some laboratories are not yet ready to comply with the requirements of Commission Decision 2002/657/EC [16] regarding CCα and CCß that apply for registered veterinary drugs as from the 1st of August 2007.
Some laboratories report relatively low values for CCα, suggesting good method reproducibility. One laboratory reports relatively high values for CCα for DC, indicating variable method performance for this compound.
The results regarding the reproducibility of some laboratories in this inter-laboratory study in some cases indicate higher reproducibility than is suggested by the reported CCα. In those cases, the accuracy of the reported CCα can be doubted.

Only four of the participating laboratories include 4-epiOTC, 4-epiTC and 4-epiCTC in their analysis, as stated in the Commission Regulation No. 281/96 [10]. The laboratories that have these 4-epitetracyclines included in their method cope in different ways regarding the quantification of these analytes.

One laboratory found OTC residues in a significant amount in the samples originating from the blank material. The identity of the analyte was confirmed by the laboratory. This is considered as a false positive result.

The calculated assigned value of material B is 104 µg/kg 4-epiOTC + OTC and 53 µg/kg DC, with an uncertainty of 12 µg/kg and 1.4 µg/kg respectively. The uncertainty of the assigned value of 4-epiOTC + OTC in this material is quit high (above $0.3\sigma_p$).
Although, all participants reported satisfactory results with regard to the accuracy, it must be noted that a considerable variation for the reported amount of 4-epiOTC + OTC is observed (average results ranging from 63.5 to 146.7 µg/kg).
One laboratory reported results with a questionable reproducibility.
No false positive or false negative results were repported for material B.

The calculated assigned value of material C is 141 µg/kg OTC and 181 µg/kg DC, with an uncertainty of 10 µg/kg for both analytes. The uncertainty of the assigned value of OTC in this material is quit high (above $0.3\sigma_p$).
82% of the participants reported satisfactory results with regard to the accuracy of OTC. A considerable variation is observed for the reported amount of OTC in this material (average results ranging from 85.5 to 231.5 µg/kg).
For DC, all laboratories reported satisfactory results.
One laboratory did not detect any tetracycline residues in material C. This was considered as a false negative result. No other false positive or false negative results were reported for material C.

The performance regarding accuracy, reproducibility, false positives and false negatives was expressed in a laboratory performance score for each laboratory. 72% of the laboratories obtained the maximum score.

Although most laboratories obtained satisfactory results regarding the accuracy and the reproducibility, it is concluded that extra effort is needed regarding the analysis of tetracyclines in poultry muscle:

- 4-epiOTC, 4-epiTC and 4-epiCTC should be included in the method for the analysis of tetracyclines by all laboratories;
- Reconsideration of values determined for CCα and CCß with respect to their accuracy may be necessary in some cases;
- An effort should be made regarding the quantitative analysis of especially OTC in poultry muscle;
- Some laboratories should make an effort to prevent false positives and false negatives in the future.

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## APPENDIX I: Codification of the samples

| Set No. | Material A | Material B | Material C |
|---|---|---|---|
| 1 | TETRA/2005/068 | TETRA/2005/095 | TETRA/2005/049 |
| | TETRA/2005/109 | TETRA/2005/030 | TETRA/2005/059 |
| 2 | TETRA/2005/112 | TETRA/2005/128 | TETRA/2005/144 |
| | TETRA/2005/004 | TETRA/2005/082 | TETRA/2005/132 |
| 3 | TETRA/2005/146 | TETRA/2005/036 | TETRA/2005/001 |
| | TETRA/2005/005 | TETRA/2005/023 | TETRA/2005/083 |
| 4 | TETRA/2005/002 | TETRA/2005/011 | TETRA/2005/057 |
| | TETRA/2005/107 | TETRA/2005/029 | TETRA/2005/008 |
| 5 | TETRA/2005/006 | TETRA/2005/007 | TETRA/2005/126 |
| | TETRA/2005/085 | TETRA/2005/025 | TETRA/2005/051 |
| 6 | TETRA/2005/120 | TETRA/2005/079 | TETRA/2005/092 |
| | TETRA/2005/110 | TETRA/2005/140 | TETRA/2005/088 |
| 7 | TETRA/2005/040 | TETRA/2005/087 | TETRA/2005/037 |
| | TETRA/2005/041 | TETRA/2005/043 | TETRA/2005/106 |
| 8 | TETRA/2005/034 | TETRA/2005/084 | TETRA/2005/104 |
| | TETRA/2005/105 | TETRA/2005/116 | TETRA/2005/108 |
| 9 | TETRA/2005/123 | TETRA/2005/035 | TETRA/2005/131 |
| | TETRA/2005/101 | TETRA/2005/046 | TETRA/2005/050 |
| 10 | TETRA/2005/100 | TETRA/2005/015 | TETRA/2005/145 |
| | TETRA/2005/130 | TETRA/2005/016 | TETRA/2005/133 |
| 11 | TETRA/2005/013 | TETRA/2005/058 | TETRA/2005/142 |
| | TETRA/2005/062 | TETRA/2005/135 | TETRA/2005/027 |
| 12 | TETRA/2005/134 | TETRA/2005/121 | TETRA/2005/143 |
| | TETRA/2005/042 | TETRA/2005/010 | TETRA/2005/096 |
| 13 | TETRA/2005/147 | TETRA/2005/076 | TETRA/2005/021 |
| | TETRA/2005/091 | TETRA/2005/115 | TETRA/2005/056 |
| 14 | TETRA/2005/127 | TETRA/2005/064 | TETRA/2005/071 |
| | TETRA/2005/136 | TETRA/2005/089 | TETRA/2005/054 |
| 15 | TETRA/2005/024 | TETRA/2005/111 | TETRA/2005/048 |
| | TETRA/2005/039 | TETRA/2005/077 | TETRA/2005/019 |
| 16 | TETRA/2005/033 | TETRA/2005/094 | TETRA/2005/137 |
| | TETRA/2005/044 | TETRA/2005/124 | TETRA/2005/148 |
| 17 | TETRA/2005/061 | TETRA/2005/117 | TETRA/2005/065 |
| | TETRA/2005/075 | TETRA/2005/067 | TETRA/2005/022 |
| 18 | TETRA/2005/099 | TETRA/2005/129 | TETRA/2005/081 |
| | TETRA/2005/113 | TETRA/2005/055 | TETRA/2005/028 |
| 19 | TETRA/2005/078 | TETRA/2005/032 | TETRA/2005/017 |
| | TETRA/2005/026 | TETRA/2005/119 | TETRA/2005/098 |
| 20 | TETRA/2005/014 | TETRA/2005/114 | TETRA/2005/072 |
| | TETRA/2005/060 | TETRA/2005/090 | TETRA/2005/102 |

**APPENDIX IIa: Statistical evaluation of homogeneity data of material B for OTC + 4-epiOTC**

| | OTC + 4-epiOTC (µg/kg) | |
|---|---|---|
| Sample No. | Replicate 1 | Replicate 2 |
| 1 | 131 | 133 |
| 2 | 130 | 140 |
| 3 | 127 | 156 |
| 4 | 128 | 128 |
| 5 | 129 | 123 |
| 6 | 115 | 130 |
| 7 | 127 | 138 |
| 8 | 126 | 123 |
| 9 | 124 | 130 |
| 10 | 127 | 115 |
| Grand mean | 129 | |
| Cochran's test | | |
| C | 0.527 | |
| $C_{crit}$ | 0.602 | |
| $C < C_{crit}$? | NO OUTLIERS | |
| Target sd ($\sigma_p$) | Horwitz: 28.1 | |
| $s_{an}^2$ | 78.1 | |
| $s_{sam}^2$ | 0.0 | |
| $\sigma_{all}^2$ | 71.0 | |
| critical | 212.4 | |
| $S_{sam}^2 <$ critical? | ACCEPT | |

No 4-epiTC, TC, 4epi-CTC or CTC was detected in the samples (LoD=10 µg/kg).

$s_{an}^2$ = estimate of analytical variance;
$s_{sam}^2$ = estimate of sampling variance;
$\sigma_{all}^2$ = allowable sampling variance.

## APPENDIX IIb: Statistical evaluation of homogeneity data of material B for DC

| | DC (µg/kg) | |
|---|---|---|
| Sample No. | Replicate 1 | Replicate 2 |
| 1 | 58 | 59 |
| 2 | 58 | 66 |
| 3 | 61 | 63 |
| 4 | 58 | 59 |
| 5 | 60 | 61 |
| 6 | 55 | 58 |
| 7 | 58 | 55 |
| 8 | 58 | 59 |
| 9 | 63 | 58 |
| 10 | 57 | 47 |
| Grand mean | 58 | |
| Cochran's test | | |
| C | 0.435 | |
| $C_{crit}$ | 0.602 | |
| $C < C_{crit}$? | NO OUTLIERS | |
| Target sd ($\sigma_p$) | Horwitz <120 ppb: 12.8 | |
| $s_{an}^2$ | 10.8 | |
| $s_{sam}^2$ | 3.6 | |
| $\sigma_{all}^2$ | 14.8 | |
| critical | 38.8 | |
| $S_{sam}^2 <$ critical? | ACCEPT | |

No 4-epiTC, TC, 4epi-CTC or CTC was detected in the samples (LoD=10 µg/kg).

$s_{an}^2$ = estimate of analytical variance;
$s_{sam}^2$ = estimate of sampling variance;
$\sigma_{all}^2$ = allowable sampling variance.

## APPENDIX IIIa: Statistical evaluation of homogeneity data of material C for OTC

| | OTC (µg/kg) | |
|---|---|---|
| Sample No. | Replicate 1 | Replicate 2 |
| 1 | 150 | 153 |
| 2 | 152 | 156 |
| 3 | 140 | 151 |
| 4 | 156 | 161 |
| 5 | 168 | 154 |
| 6 | 155 | 148 |
| 7 | 144 | 163 |
| 8 | 158 | 148 |
| 9 | 160 | 132 |
| 10 | 144 | 151 |
| Grand mean | 152 | |
| Cochran's test | | |
| C | 0.455 | |
| $C_{crit}$ | 0.602 | |
| $C < C_{crit}$? | NO OUTLIERS | |
| Target sd ($\sigma_p$) | Horwitz: 32.3 | |
| $s_{an}^2$ | 82.4 | |
| $s_{sam}^2$ | 0.0 | |
| $\sigma_{all}^2$ | 94.0 | |
| critical | 259.9 | |
| $S_{sam}^2 <$ critical? | ACCEPT | |

No 4epi-OTC, 4-epiTC, TC, 4epi-CTC or CTC was detected in the samples (LoD=10 µg/kg).

$s_{an}^2$ = estimate of analytical variance;
$s_{sam}^2$ = estimate of sampling variance;
$\sigma_{all}^2$ = allowable sampling variance.

## APPENDIX IIIb: Statistical evaluation of homogeneity data of material C for DC

| | DC (µg/kg) | |
|---|---|---|
| Sample No. | Replicate 1 | Replicate 2 |
| 1 | 205 | 210 |
| 2 | 215 | 225 |
| 3 | 192 | 184 |
| 4 | 207 | 189 |
| 5 | 206 | 201 |
| 6 | 190 | 188 |
| 7 | 181 | 190 |
| 8 | 178 | 186 |
| 9 | 199 | 171 |
| 10 | 190 | 195 |
| Grand mean | 195 | |
| Cochran's test | | |
| C | 0.499 | |
| $C_{crit}$ | 0.602 | |
| $C < C_{crit}$? | NO OUTLIERS | |
| Target sd ($\sigma_p$) | Horwitz: 42.9 | |
| $s_{an}^2$ | 73.6 | |
| $s_{sam}^2$ | 107.2 | |
| $\sigma_{all}^2$ | 143.4 | |
| critical | 343.9 | |
| $S_{sam}^2 <$ critical? | ACCEPT | |

No 4-epiOTC, 4-epiTC, TC, 4epi-CTC or CTC was detected in the samples (LoD=10 µg/kg).

$s_{an}^2$ = estimate of analytical variance;
$s_{sam}^2$ = estimate of sampling variance;
$\sigma_{all}^2$ = allowable sampling variance.

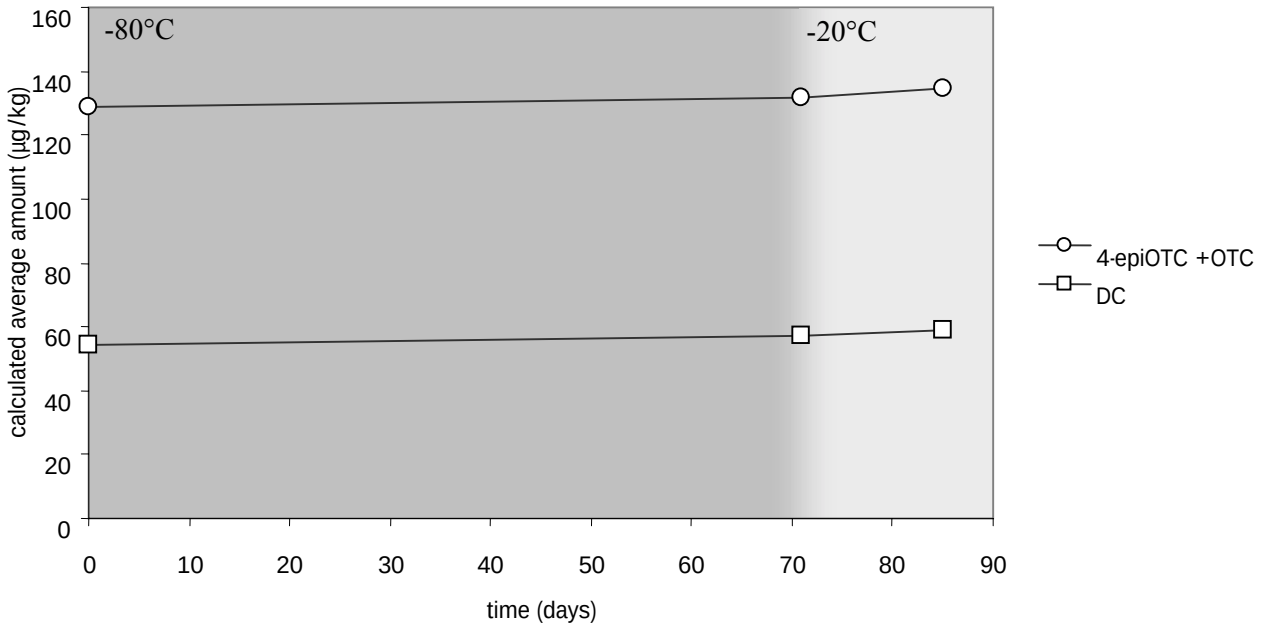
## APPENDIX IVa: Statistical evaluation of stability data of material B

Statistical evaluation for 4-epiOTC + OTC

| Date of analysis | Time (days) | 4-epiOTC + OTC (µg/kg) | Pooled st. dev (µg/kg) | $t$ | $t_{crit}$ | $t < t_{crit}$ |
|---|---|---|---|---|---|---|
| 03/07/2005 | 0 | 129 | | | | |
| 05/17/2005 | 71 | 132 | 10.9 | 0.43 | 2.08 | ACCEPT |
| 05/31/2005 | 85 | 135 | 10.9 | 0.83 | 2.08 | ACCEPT |

Statistical evaluation for DC

| Date of analysis | Time (days) | DC (µg/kg) | Pooled st. dev (µg/kg) | $t$ | $t_{crit}$ | $t < t_{crit}$ |
|---|---|---|---|---|---|---|
| 03/07/2005 | 0 | 55 | | | | |
| 05/17/2005 | 71 | 57 | 7.5 | 0.52 | 2.08 | ACCEPT |
| 05/31/2005 | 85 | 59 | 7.5 | 0.92 | 2.08 | ACCEPT |



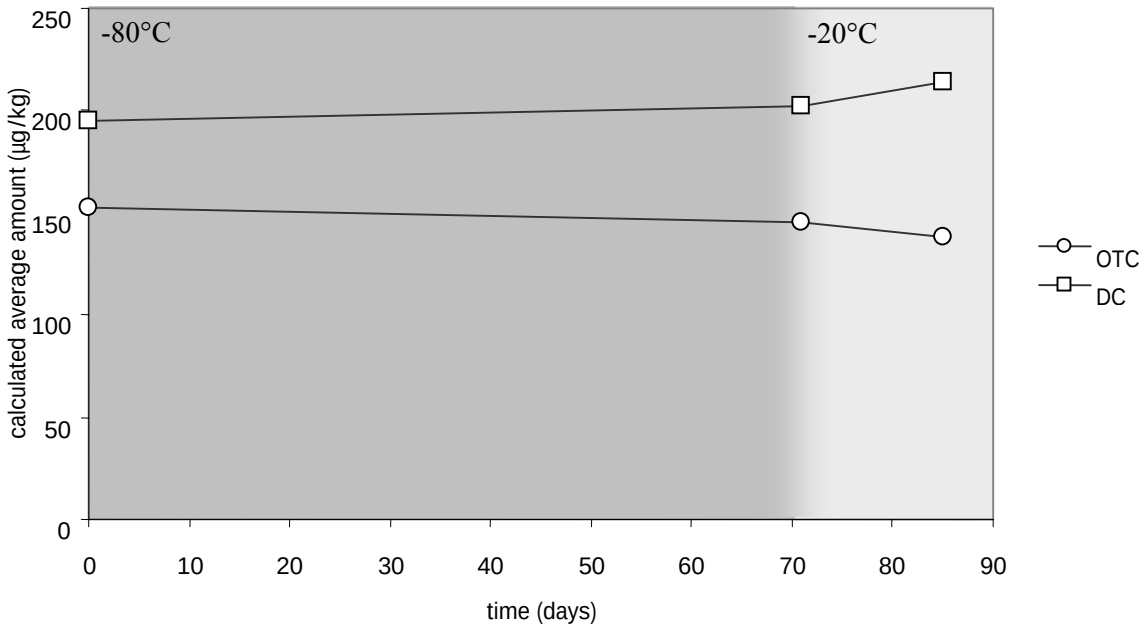
## APPENDIX IVb: Statistical evaluation of stability data of material C

Statistical evaluation for OTC

| Date of analysis | Time (days) | OTC (µg/kg) | St. dev (µg/kg) | $t$ | $t_{crit}$ | $t < t_{crit}$ |
|---|---|---|---|---|---|---|
| 03/07/2005 | 0 | 152 | | | | |
| 05/17/2005 | 71 | 146 | 12.7 | 0.84 | 2.08 | ACCEPT |
| 05/31/2005 | 85 | 138 | 12.7 | 1.77 | 2.08 | ACCEPT |

Statistical evaluation for DC

| Date of analysis | Time (days) | DC (µg/kg) | St. dev (µg/kg) | $t$ | $t_{crit}$ | $t < t_{crit}$ |
|---|---|---|---|---|---|---|
| 03/07/2005 | 0 | 195 | | | | |
| 05/17/2005 | 71 | 202 | 26.8 | 0.44 | 2.08 | ACCEPT |
| 05/31/2005 | 85 | 214 | 27.0 | 1.16 | 2.08 | ACCEPT |



## APPENDIX V: Overview of the applied methods

| Lab code | Extraction | Sample purification | Internal standard | Detection method | Compound analysed for | 4-epiOTC and OTC separated? | Quantification of 4-epiOTC | 2002/657/EC applied? |
|---|---|---|---|---|---|---|---|---|
| Lab1 | EDTA-McIlvain, pH=4.0 | Filter, SPE: OASIS® HLB | - | LC–UV LC–MS/MS | OTC, TC, CTC, DC | | | Yes |
| Lab2 | Sodium succinate (0.1 M), pH=4.0 | Filter, SPE: OASIS® HLB | demeclocycline | LC–MS/MS | OTC, TC, CTC, DC and 4-epimers | Yes | Separate quantification | Yes |
| Lab3 | EDTA-McIlvain | Filter, SPE: $C_{18}$ | - | LC–DAD | OTC, TC, CTC | | | No |
| Lab5 | PBS-EDTA buffer / acetonitrile | Filter | - | LC–MS/MS | OTC, TC, CTC, DC | | | Yes |
| Lab6 | McIlvain | Filter, SPE: OASIS® HLB | - | LC–MS/MS | OTC, TC, CTC, DC and 4-epiCTC | No | Sum quantified | Yes |
| Lab7 | EDTA-McIlvain, pH=4.0 | SPE: $C_{18}$ | - | LC–DAD | OTC | | | No |
| Lab8 | EDTA-McIlvain (0.1 M), pH=4.0 | Filter, SPE: OASIS® HLB | demeclocycline | LC–MS/MS | OTC, TC, CTC, DC and 4-epimers | Yes | Sum quantified | Yes |
| Lab9 | NM | Chelating sepharose column with copper ions, SPE: polymer based | - | LC–UV | OTC, TC, CTC, DC and 4-epimers | No | Sum quantified | Yes |
| Lab10 | TCA 5% | Filter | tetracycline | LC–MS/MS | OTC, TC, CTC, DC and 4-epiOTC | No | Sum quantified | Yes |
| Lab12 | EDTA-McIlvain (0.1 M) | SPE: OASIS® HLB | - | LC–MS/MS | OTC, TC, CTC, DC and 4-epimers | Yes | Separate quantification | Yes |
| Lab 16 | Acetonitrile / heptane with 0.15% formic acid | Evaporation of acetonitril, SPE: OASIS® HLB | demeclocycline | LC–MS/MS | OTC, TC, CTC, DC | | | Yes |

NM = not mentioned

## APPENDIX VI: Overview of method characteristics

| | 4-epiOTC | | OTC | | DC | |
|---|---|---|---|---|---|---|
| Lab code | CCα (µg/kg) | CCß (µg/kg) | CCα (µg/kg) | CCß (µg/kg) | CCα (µg/kg) | CCß (µg/kg) |
| Lab1 | | | 104.3 | 108.6 | 103.5 | 106.9 |
| Lab2 | 122.9 | 147.1 | 124.2 | 144.4 | 118.5 | 135.4 |
| Lab3 | | | | | | |
| Lab5 | | | 115 | 129 | 129 | 158 |
| Lab6 | | | | | | |
| Lab7 | | | | | | |
| Lab8 | | | 115 | 130 | 125 | 154 |
| Lab9 | | | 112 | 126 | 119 | 146 |
| Lab10 | | | 111 | 121 | 153 | 206 |
| Lab12 | 113 | 137 | 107 | 120 | 112 | 133 |
| Lab 16 | | | | | | |

| | 4-epiOTC | | OTC | | DC | |
|---|---|---|---|---|---|---|
| Lab code | LoD (µg/kg) | LoQ (µg/kg) | LoD (µg/kg) | LoQ (µg/kg) | LoD (µg/kg) | LoQ (µg/kg) |
| Lab1 | | | | | | |
| Lab2 | 0.21 | 10 | 0.15 | 10 | 2.5 | 10 |
| Lab3 | | | 50 | 50 | | |
| Lab5 | | | | | | |
| Lab6 | | | 8 | 25 | 2 | 10 |
| Lab7 | | | 25 | 50 | | |
| Lab8 | | | | | | |
| Lab9 | | | | | | |
| Lab10 | | | | | | |
| Lab12 | | | | | | |
| Lab 16 | | | | | | |

## APPENDIX VII: 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) |
|---|---|---|---|---|---|
| Lab5 | TETRA/2005/006 | A | OTC | 113 | 40 |
| | TETRA/2005/085 | A | OTC | 46 | 52 |

False negative results

| Lab code | Sample code | Material | Analyte |
|---|---|---|---|
| Lab6 | TETRA/2005/088 | C | OTC |
| | TETRA/2005/088 | C | DC |

## APPENDIX VIII: The result for the analysis of 4-epiOTC+OTC in poultry muscle (material B)

| 4-epiOTC + OTC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assigned value 104.0 µg/kg | | | | | | | | | |
| Uncertainty of assigned value 12.0 µg/kg | | | | | | | | | |
| Target standard deviation (Horwitz, Thompson) 22.9 µg/kg | | | | | | | | | |
| Code | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Average | $s_r$ | $s_{R_L}$ | $z_a$'-score | *HORRAT* |
| Lab1 | 65 | | 62 | | 63.5 | | | -1.6 | |
| Lab2 | 138.1 | 130.6 | 135.9 | 127.6 | 133.1 | 5.6 | 4.4 | 1.1 | 0.2 |
| Lab3 | 152.7 | 141.5 | 139.9 | 129.9 | 141 | 7.5 | 10.1 | 1.4 | 0.4 |
| Lab5 | 132 | 110 | 176 | 154 | 143 | 15.6 | 33.0 | 1.5 | **1.4** |
| Lab6 | 64 | 56 | 67 | 70 | 64.3 | 4.3 | 6.7 | -1.5 | 0.3 |
| Lab7 | 98.8 | 101.1 | 97 | 101.5 | 99.6 | 2.5 | 1.9 | -0.2 | 0.1 |
| Lab8 | 110 | 119 | 119 | 121 | 117.3 | 4.6 | 5.1 | 0.5 | 0.2 |
| Lab9 | 62 | 62 | 65 | 68 | 64.3 | 1.5 | 3.4 | -1.5 | 0.1 |
| Lab10 | 104.1 | 99 | 96.5 | 100.4 | 100 | 3.2 | 3.2 | -0.2 | 0.1 |
| Lab12 | 141.1 | 142.3 | 149 | 154.5 | 146.7 | 2.8 | 7.4 | 1.7 | 0.3 |
| Lab 16 | 70 | | 73 | | 71.5 | | | -1.3 | |

The bold values indicate a questionable performance (*HORRAT*>1)

Figure VIIIa: Graphical representation of the reported results

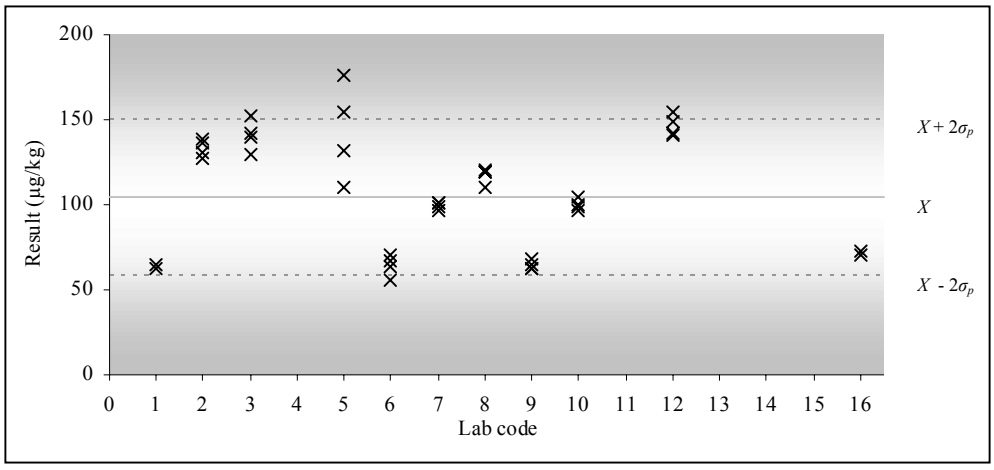



Figure VIIIb: Graphical representation of $z_a$'-score

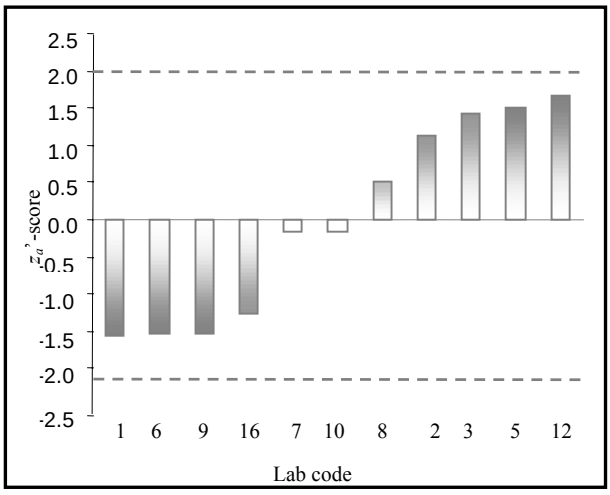
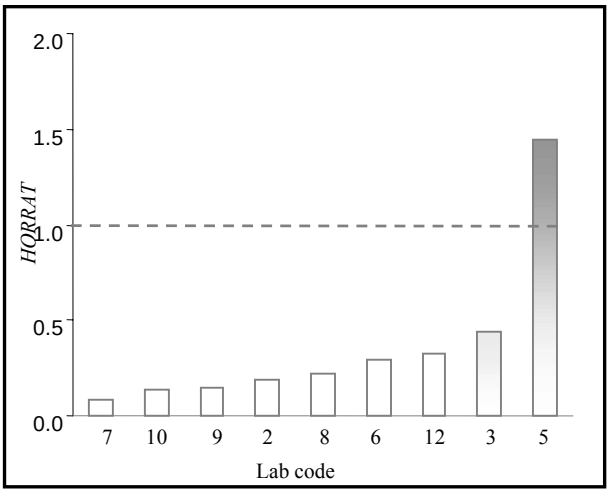



Figure VIIIc: Graphical representation of *HORRAT*



## APPENDIX IX: The result for the analysis of DC in poultry muscle (material B)

| DC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assigned value 53.3 µg/kg | | | | | | | | | |
| Uncertainty of assigned value 1.4 µg/kg | | | | | | | | | |
| Target standard deviation (Horwitz, Thompson) 11.8 µg/kg | | | | | | | | | |
| Code | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Average | $s_r$ | $s_{R_L}$ | $z_a$-score | *HORRAT* |
| Lab1 | 55.7 | | 52.9 | | 54.3 | | | 0.1 | |
| Lab2 | 55.6 | 57.6 | 54.4 | 58.3 | 56.5 | 2.2 | 1.6 | 0.3 | 0.1 |
| Lab5 | 46 | 54 | 53 | 61 | 53.5 | 5.7 | 6.4 | 0.0 | 0.5 |
| Lab6 | 41 | 36 | 44 | 52 | 43.3 | 4.7 | 7.5 | -0.9 | 0.6 |
| Lab8 | 59 | 58 | 58 | 60 | 58.8 | 1.1 | 0.9 | 0.5 | 0.1 |
| Lab9 | 44 | 50 | 65 | 57 | 54.0 | 5.0 | 10.5 | 0.1 | 0.9 |
| Lab10 | 44.6 | 42.7 | 38.2 | 37.4 | 40.7 | 1.0 | 4.2 | -1.1 | 0.4 |
| Lab12 | 52.5 | 57.9 | 57.9 | 57 | 56.3 | 2.7 | 2.5 | 0.3 | 0.2 |
| Lab16 | 56 | | 39 | | 47.5 | | | -0.5 | |

Figure IXa: Graphical representation of the reported results

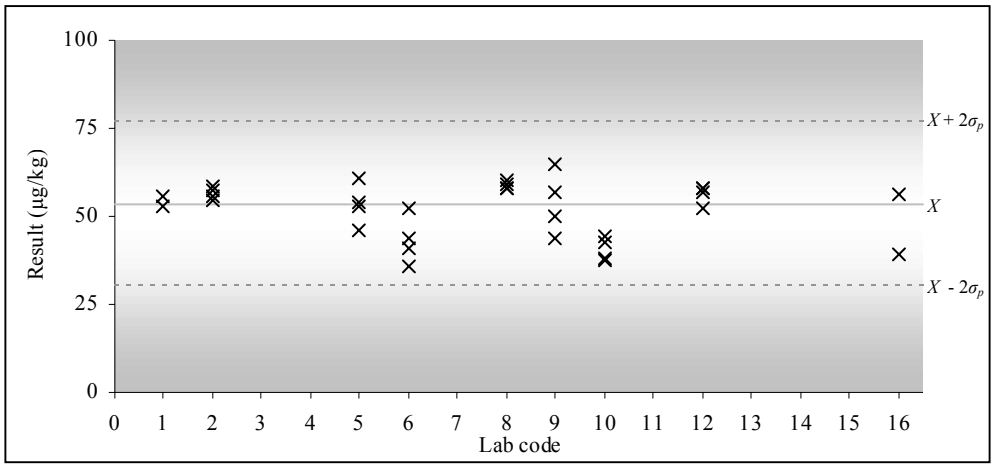



Figure IXb: Graphical representation of $z_a$-score

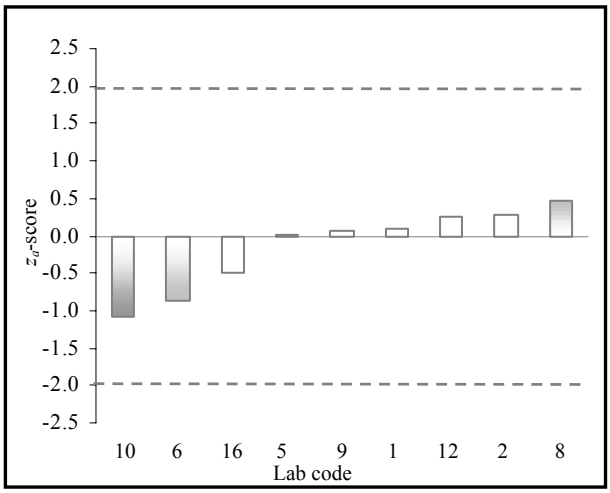
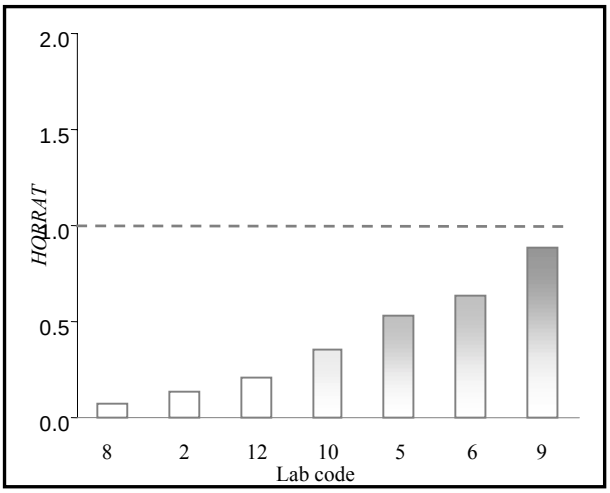



Figure IXc: Graphical representation of *HORRAT*



## APPENDIX X: The result for the analysis of OTC in poultry muscle (material C)

| OTC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assigned value 140.7 µg/kg | | | | | | | | | |
| Uncertainty of assigned value 9.6 µg/kg | | | | | | | | | |
| Target standard deviation (Horwitz) 30.2 µg/kg | | | | | | | | | |
| Code | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Average | $s_r$ | $s_{R_L}$ | $z_a$'-score | *HORRAT* |
| Lab1 | 144.4 | | 131.1 | | 137.8 | | | -0.1 | |
| Lab2 | 128.2 | 116.1 | 129.7 | 126.7 | 125.2 | 6.2 | 6.1 | -0.5 | 0.2 |
| Lab3 | 194.9 | 189.8 | 168.7 | 186.9 | 185.1 | 9.5 | 12.3 | 1.4 | 0.4 |
| Lab5 | 250 | 242 | 236 | 198 | 231.5 | 19.4 | 24.7 | **2.9** | 0.8 |
| Lab6 | | | 78 | 93 | 85.5 | | | -1.7 | |
| Lab7 | 235.2 | 242 | 228 | 235.3 | 235.1 | 5.0 | 6.0 | **3.0** | 0.2 |
| Lab8 | 146 | 161 | 165 | 148 | 155.0 | 11.3 | 8.3 | 0.5 | 0.3 |
| Lab9 | 138 | 151 | 158 | 156 | 150.8 | 6.6 | 10.0 | 0.3 | 0.3 |
| Lab10 | 124.4 | 126.4 | 120.6 | 117 | 122.1 | 2.1 | 4.9 | -0.6 | 0.2 |
| Lab12 | 107.5 | 99.3 | 101.1 | 97 | 101.2 | 4.6 | 4.5 | -1.2 | 0.2 |
| Lab 16 | 129 | | 134 | | 131.5 | | | -0.3 | |

The bold values indicate a questionable performance ($|z|>2$)

Figure Xa: Graphical representation of the reported results

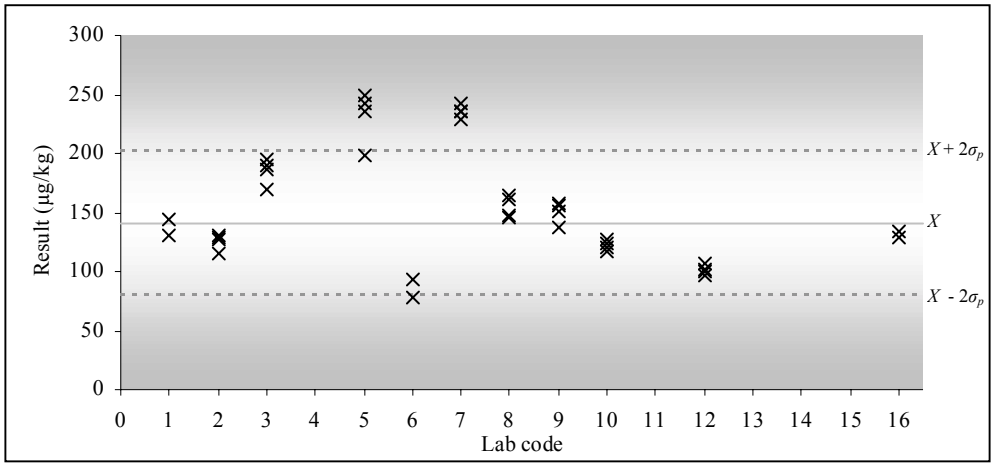



Figure Xb: Graphical representation of $z_a$-score

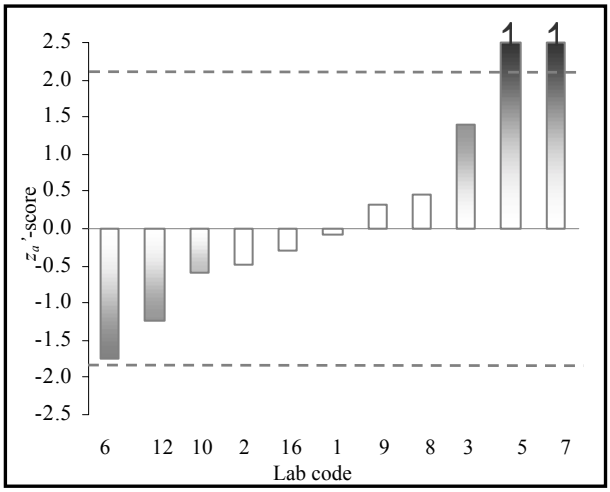
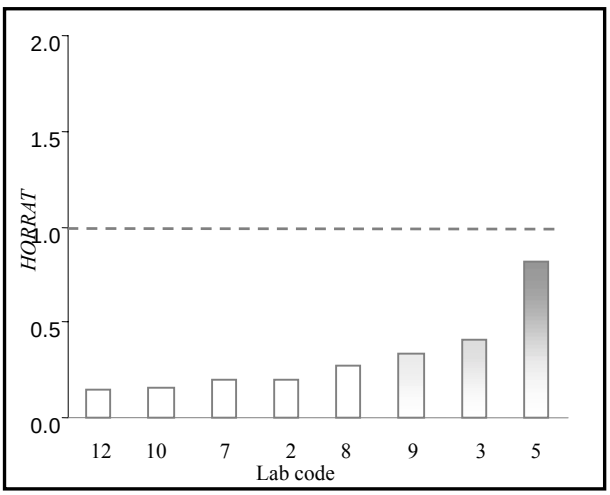



Figure Xc: Graphical representation of *HORRAT*



## APPENDIX XI: The result for the analysis of DC in poultry muscle (material C)

| DC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assigned value 180.5 µg/kg | | | | | | | | | |
| Uncertainty of assigned value 9.7 µg/kg | | | | | | | | | |
| Target standard deviation (Horwitz) 37.4 µg/kg | | | | | | | | | |
| Code | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Average | $s_r$ | $s_{R_L}$ | $z_a$-score | *HORRAT* |
| Lab1 | 205 | | 183.9 | | 194.5 | | | 0.4 | |
| Lab2 | 205.9 | 186.9 | 189.4 | 186.2 | 192.1 | 9.6 | 9.1 | 0.3 | 0.2 |
| Lab5 | 169 | 168 | 175 | 167 | 169.8 | 4.0 | 3.4 | -0.3 | 0.1 |
| Lab6 | | | 143 | 162 | 152.5 | | | -0.8 | |
| Lab8 | 218 | 240 | 218 | 220 | 224.0 | 11.0 | 10.5 | 1.2 | 0.3 |
| Lab9 | 186 | 178 | 197 | 206 | 191.8 | 6.0 | 14.4 | 0.3 | 0.4 |
| Lab10 | 128.7 | 142.9 | 157 | 154.8 | 145.9 | 7.2 | 15.1 | -0.9 | 0.4 |
| Lab12 | 195.8 | 200.3 | 197.3 | 195.8 | 197.3 | 2.4 | 2.0 | 0.5 | 0.1 |
| Lab16 | 134 | | 183 | | 158.5 | | | -0.6 | |

Figure XIa: Graphical representation of the reported results

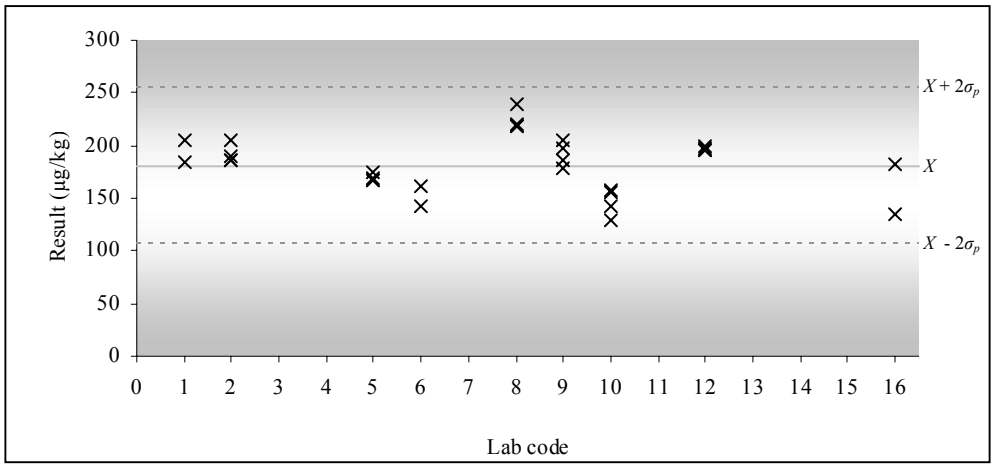



Figure XIb: Graphical representation of $z_a$-score

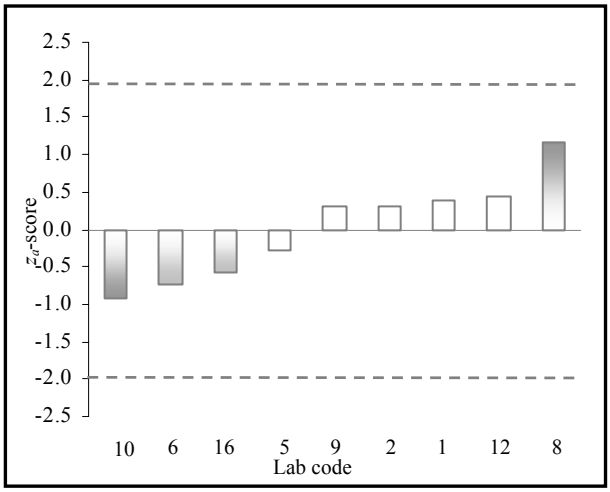
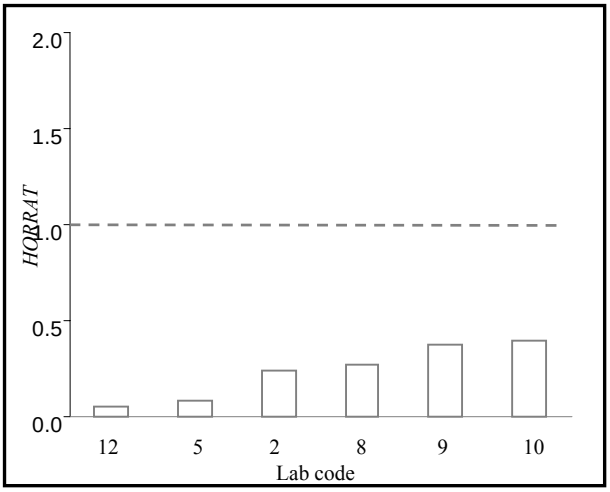



Figure XIc: Graphical representation of *HORRAT*



## APPENDIX XII: The calculation of the laboratory performance score

| Code | False positives | False negatives | Material B accuracy / reproducibility OTC | DC | Material C accuracy / reproducibility OTC | DC | Total | Maximum |
|---|---|---|---|---|---|---|---|---|
| Lab1 | | | 1 / - | 1 / - | 1 / - | 1 / - | 4 | **4*** |
| Lab2 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |
| Lab3 | | | 1 / 1 | - / - | 1 / 1 | - / - | 4 | **4*** |
| Lab5 | -1 | | 1 / 0 | 1 / 1 | 0 / 1 | 1 / 1 | 5 | 8 |
| Lab6 | | -2 | 1 / 1 | 1 / 1 | 1 / - | 1 / - | 4 | 6** |
| Lab7 | | | 1 / 1 | - / - | 0 / 1 | - / - | 3 | 4* |
| Lab8 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |
| Lab9 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |
| Lab10 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |
| Lab12 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |
| Lab 16 | | | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 | 8 | **8** |

The bold figures indicate laboratories that obtained the maximum attainable laboratory performance score.

* No HORRAT value could be calculated, because only one result per sample was reported.

** Because of the false negative result for material C, no HORRAT value could be calculated for this material.