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Proficiency study for quinolones in egg

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PROF. TESTING
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Distribution list:

- All participants of the proficiency study for quinolones in egg.

Summary

The proficiency study for quinolones in egg was organized in accordance with ISO/IEC Guide 43-1 and 43-2 and ILAC-G13, and under accreditation.

For this proficiency study, four test materials were prepared:
- A blank egg material;
- A blank egg material containing possibly interfering compounds to test the selectivity of the applied methods;
- An egg material containing about 70 µg/kg oxolinic acid (incurred) and about 50 µg/kg of both ciprofloxacin and enrofloxacin (spiked);
- An egg material containing about 125 µg/kg flumequine (incurred).

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

Eighteen laboratories subscribed for participation in the proficiency study quinolones in egg including three National Reference Laboratories. Fifteen laboratories managed to submit valid results within the timeframe of the stability study. Five of the participating laboratories applied a validated method which was accredited in all cases.

The minority of participants applied a validated method for the analysis of quinolones in egg. Only three laboratories reported values for CCα and CCß. It is noted that reported values for CCα and CCß severely differ among the laboratories. Most likely these differences are due to different ways of calculation. From the reported values of CCα it is concluded that some laboratories calculated CCα based on a self set MRL, others applied the zero tolerance approach. From this it is concluded that laboratories cope in very different ways with the non existence of MRL values for quinolones in egg. MRLs are set for other matrices. Discussion on this issue resulting in clear legislation is of main importance for obtaining a uniform approach within Europe.

None of the laboratories detected any quinolones in the blank material nor in the material containing possibly interfering compounds. It is concluded that the applied methods are all satisfactory specific for the quantitative and confirmative analysis of ciprofloxacin (CIF), enrofloxacin (ENF), oxolinic acid (OXA) and flumequine (FLU) in egg.
One laboratory detected norfloxacin instead of ciprofloxacin in the two samples that contain quinolones. This is considered as a false positive as well as a false negative result because CIF was included in their method.

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

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

Compound	Assigned value (X) (µg/kg)	Uncertainty of X (µg/kg)	No. of labs that reported results	No. of satisfactory results
				Accuracy
CIF	46.4	1.10	13	10
ENF	48.0	1.47	15	14
OXA	73.2	1.99	11	11
FLU	124.9	4.27	13	13

For OXA and FLU all reported results were satisfactory. For CIF and ENF some questionable and unsatisfactory results are observed. The occurrence of questionable or unsatisfactory results could not be explained by the applied detection or sample preparation technique.
However, 75% of the total of calculated z_a-scores is between -0.5 and 0.5 indicating excellent accuracy for most laboratories. Therefore it is concluded that the performance of most laboratories is excellent regarding the quantification of quinolones in egg.

One laboratory detected norfloxacin in one of the duplicate analysis of both samples of material Egg-03. This is considered as a false positive result. The same laboratory did not detect CIF in the samples of material Egg-03. This is considered as a false negative result.

In this proficiency study 73% of the laboratories showed optimal performance in terms of accuracy and the absence of false positive and false negative findings.

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

- regarding B group substances for which no MRL is set in a specific matrix, legislation should be clarified to obtain a uniform way for the determination of CCα and CCß within the EU and with this a uniform way of characterizing the samples in terms of compliant and non compliant..
- for most laboratories additional effort is needed to validate the analysis of quinolones in egg to be able to report results including a value for measurement uncertainty.
- Additional effort is needed by some laboratories to include oxolinic acid in the method of analysis for quinolones in egg because, officially, oxolinic acid is the only quinolone registered for medication of laying hens in the EU and for which an MRL is established.

Contents

1 Introduction

1.1 Proficiency testing

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

The aim of this proficiency study was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of quinolones in egg. Furthermore the specificity of the applied methods is evaluated by including possibly interfering compounds in the proficiency study.
This study also provided an evaluation of the methods applied for quantitative and confirmatory analysis of quinolones in egg. Additionally, this proficiency study was organized to get an overview of how laboratories are dealing with legislation of group B substances in matrices for which no maximum residue limit (MRL) is set.

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

1.2 Quinolones

The discovery of the synthetic antibacterial agent nalidixic acid in 1962 marks the beginning of decades of quinolone development for human and veterinary use [6, 7, 8]. Nalidixic acid was discovered as a by-product of the production of the anti-malaria drug chloroquine. Nalidixic acid was found to be a rapid bactericidal agent by inhibition of the bacterial DNA gyrase synthesis [9]. Nalidixic acid is active against the majority of Gram-negative bacteria. Unfortunately it is not active against Pseudomonas aeruginosa (responsible for causing numerous infections), Gram-positive organisms and anaerobes. In addition, the clinical use of nalidixic acid is limited, because administration results in low drug concentrations in serum and tissues. Furthermore, resistance to nalidixic acid developed rapidly in numerous organisms. Derivatisation products of nalidixic acid, like oxolinic acid represented only marginal improvements over nalidixic acid.

In 1976, the development of flumequine, the first fluoroquinolone, offered significant improvement. This monofluoroquinolone indicated that the addition of a fluor atom in the molecule improved Gram-positive activity. In 1978 norfloxacin, a monofluorinated quinolone with a piperazinyl side-chain was developed. This fluoroquinolone has a longer half-time, less protein binding and improved Gram-negative activity compared to the earlier developed compounds. Still the pharmacokinetic profile and activity were not adequate for systemic use [10].

Very successful and widely used compounds of the fluoroquinolone group are ciprofloxacin, developed in 1981 and its metabolite enrofloxacin that is mainly used as veterinary drug [11]. These compounds are active against a broad spectrum of Gram-positive as well as Gram-negative species, including Pseudomonas aeruginosa. Following oral administration, the drug is well distributed through the body with high concentrations in most tissues.

Gram-positive staphylococci became a major problem with increasing resistance to antibiotic compounds like ß-lactams and macrolides. Also for quinolones resistance in human pathogens was demonstrated [12]. Therefore, the search for new fluoroquinolones continued, aiming for improved activity for ß-lactam, macrolide and quinolone resistant strains, and activity against Gram-positive staphylococci and anaerobes. This resulted in the development of fourth-generation quinolones.

1.2.1 Quinolones in animal health

The most notable fluoroquinolones used in veterinary medicine worldwide include ciprofloxacin, danofloxacin, enrofloxacin, marbofloxacin, norfloxacin and sarafloxacin [9]. Data gathered by The World Health Organization indicate that the use of quinolones differs greatly as regards animal species and geographical spread [13, 14].
Quinolones have a very broad clinical application in livestock, poultry, fish and domestic animals in the treatment and prevention of respiratory, enteric and urinary tract infections [14].

Quinolone resistance has multiple mechanisms and significant clinical impact. Mutations may occur rapidly during fluoroquinolone therapy and may be the most significant factor limiting the use of these antimicrobials [15]. The toxicity of quinolones is mild at therapeutic doses and generally consists of gastrointestinal disturbances such as nausea and diarrhoea. At higher doses the central nervous system is affected resulting in dizziness, depression or insomnia [9].

The distribution and metabolism of enrofloxacin was studied in rats [15]. After oral administration enrofloxacin was well absorbed. The substance was widely distributed to all tissues with the highest concentration in liver and kidney. Elimination was rapid via both urine and faeces. Ciprofloxacin was indicated as the major metabolite of enrofloxacin [15]. The occurrence of metabolism of other quinolone compounds was not demonstrated.

1.2.2 Quinolones in egg

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 [13]. Quinolones are included in Annex I: pharmacologically active veterinary products for which a Maximum Residue Limit (MRL) is established. However, regarding quinolones in the matrix egg, only a MRL for oxolinic acid is established. Therefore, officially the use of other quinolones in laying hens is prohibited. A zero tolerance is applicable and the characterization of samples in terms of ‘compliant’ or ‘non compliant’ should be made based on this. However, the absence of MRLs for quinolones in egg is subject to discussion. The zero tolerance approach is in contrast with the establishment of MRLs for quinolones in poultry muscle and other matrices because toxiticity and occurrence of resistance for quinolones mainly depend on the intake of the quinolones, not on the matrix they are in. Because the intake of egg and poultry can be considered to be comparable, maybe equivalent MRLs should be set. Nevertheless, until MRLs are set for quinolone residues in egg specifically, a zero tolerance is officially applicable.

This inter-laboratory study focuses on oxolinic acid, enrofloxacin (and its metabolite ciprofloxacin) and flumequine in egg. The MRLs for these compounds in egg and poultry muscle are presented in Table 2. The structures of these fluoroquinolones are presented in Figure 1.

Table 2. MRL in poultry muscle of fluoroquinolones included in the inter-laboratory study [13]

Compound	MRL in egg (µg/kg)	MRL in poultry muscle (µg/kg)
Oxolinic acid	50	100
Ciprofloxacin	-	100*
Enrofloxacin	-	100*
Flumequine	-	400

* The sum of enrofloxacin and ciprofloxacin should be considered.

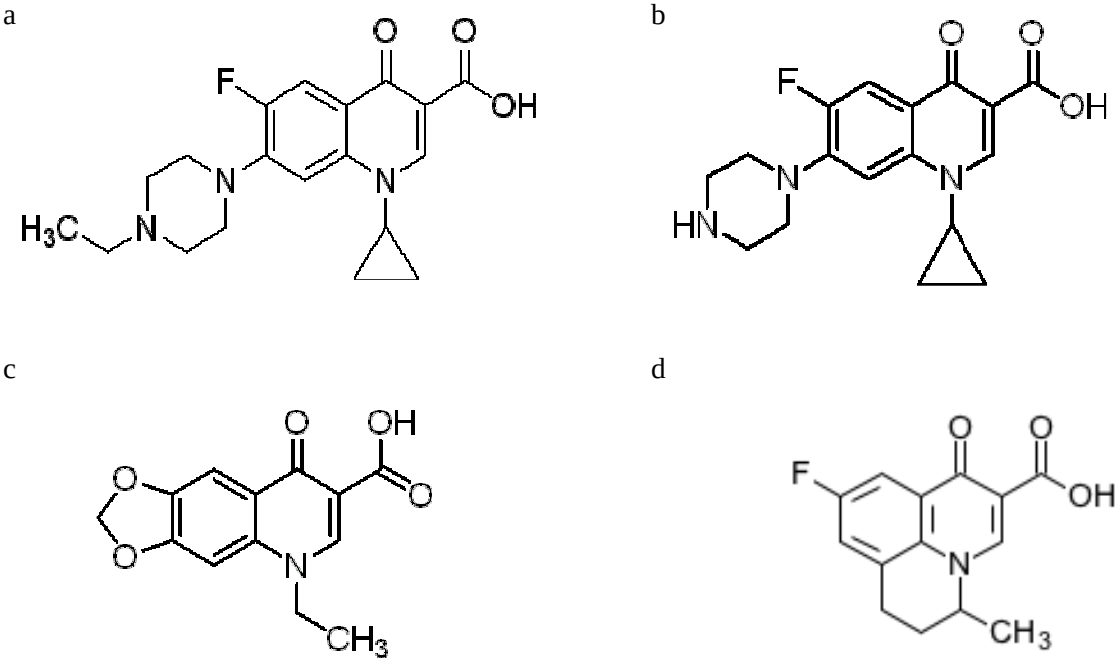

Figure 1. Molecular structure of (a) enrofloxacin, (b) ciprofloxacin, (c) oxolinic acid and (d) flumequine.

2 Test materials

2.1 Sample preparation

One blank material, one material containing oxolinic acid (OXA), enrofloxacin (ENF) and ciprofloxacin (CIF), one material containing flumequine (FLU) and one material containing possibly interfering substances were prepared.

The material used for testing the specificity was prepared by adding methanolic solutions of possibly interfering compounds to a blank material. Fenbendazole sulfon, piroxicam and triclabendazole were selected as possibly interfering substances based on their molecular mass. This material is referred to as Egg-02.

The material containing OXA, CIF and ENF was prepared from incurred samples containing high levels of oxolinic acid. These samples were mixed with blank samples to obtain a relevant level of OXA. CIF and ENF were added to this material by addition of methanolic solutions of these compounds. This material is referred to as Egg-03.
The material containing FLU was prepared from incurred samples containing high levels of FLU. These samples were mixed with blank samples to obtain a relevant level of FLU. This material is referred to as Egg-04.
Because the MRL for oxolinic acid in egg is half of the MRL of oxolinic acid in poultry muscle, the levels of the other compounds in the materials were aimed for about half of the established MRL in poultry muscle.

Each of the materials was homogenized by mixing according to in-house standard operating procedures. The target amounts of the quinolones in each material is presented in table 3.

Table 3.Target amount of quinolones in the proficiency study test materials

Material code	Amount of material (g)	Compound	Level (µg/kg)
Egg-01	1200	-	-
Egg-02	1200	Fenbandazole sulfon	50
		Piroxicam	30
		Triclabendazole	100
Egg-03	2400	Oxolinic acid	70
		Enrofloxacin	50
		Ciprofloxacin	50
Egg-04	1400	Flumequine	250

2.2 Sample identification

The materials were stored in polypropylene containers containing at least 25 gram of sample, yielding a total of 40 containers of material Egg-01 and Egg-02, 80 containers of material Egg-03 and 50

containers of material Egg-04. The egg samples were randomly coded with a code from QUIN/2007/EGG/001 through 210.

For homogeneity and stability testing, 20 randomly selected containers of material Egg-03 and Egg-04 were used.
For each laboratory a sample set was prepared consisting of one randomly selected sample of material Egg-01, Egg-02 and Egg-04 and two randomly selected samples of material Egg-03. The sample numbers belonging to each sample set are presented in Annex 1.

2.3 Homogeneity study

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [16] and ISO/DIS 13528 [17], taking into account the insights discussed by Thompson [18] regarding the Horwitz equation.
With this procedure the between-sample standard deviation (s_s) is compared with the target standard deviation derived from the Horwitz equation (σ_H , §4.3). The materials are considered adequately homogeneous if $s_s \leq 0.3\sigma_H$.

Ten containers of materials Egg-03 and Egg-04 were each analyzed in duplicate for OXA, ENF, CIF and FLU 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 were demonstrated to be sufficiently homogeneous for all quinolones for use in the proficiency study. The amounts determined during the homogeneity study are presented in table 4.
During the homogeneity study the amount of FLU in material Egg-04 proved to be lower than expected. This is probably due to a deviation in the estimated level of the incurred material used for preparation of this material. Nevertheless, the determined level of FLU in material Egg-04 is still relevant. Therefore, material Egg-03 and Egg-04 were found suitable for application in the proficiency study.
No extensive homogeneity study was carried out for materials Egg-01 and Egg-02. The homogeneity of these materials is not relevant because the results of these materials will not be evaluated in a quantitative way. Furthermore, it is assumed that the homogeneity of material Egg-01 and Egg-02 are comparable with the homogeneity of the other materials because all materials are homogenized in the same way. Nevertheless, three at random selected samples of material Egg-01 and Egg-02 were analyzed for ten quinolones. No CIF, ENF, danofloxacin, difloxacin, FLU, marbofloxacin, nalidixic acid, OXA or sarafloxacin was detected. It was concluded that materials Egg-01 and Egg-02 are suited to use as blank materials in the proficiency study.

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

Material code	Amount of OXA (µg/kg)	Amount of CIF (µg/kg)	Amount of ENF (µg/kg)	Amount of FLU (µg/kg)
Egg-01	-	-	-	-
Egg-02	-	-	-	-
Egg-03	72.2	50.9	50.4	-
Egg-04	-	-	-	114.4

2.4 Participants

Eighteen laboratories subscribed for participation in the proficiency study quinolones in egg. Most participating laboratories are situated in Europe. Of these laboratories three are National Reference Laboratories (NRL).

2.5 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code (1 through 18). The sample sets with the corresponding number, consisting of five coded samples (Annex 1), were sent to the participating laboratories at September 17th 2007. The sample sets were packed in an insulating box, containing dry ice or cool packs and were dispatched to the participants immediately by courier. All laboratories confirmed the receipt of the samples in good condition (frozen).

The samples were accompanied by a letter (Annex 3) describing the requested analyses, an acknowledgement of receipt form and a results form. Furthermore, a reference standard of CIF, including a certificate of analysis, was included in the package. The participants were asked to use this reference standard in their analysis. With this, the influence of the reference standard on the deviation of laboratory results can be determined in the evaluation process.

The laboratories were asked to store the samples until analysis according to their own laboratory's procedure. A duplicate analysis of each sample was requested, resulting in two results for materials Egg-01, Egg-02 and Egg-04, and four results for material Egg-03. The deadline for sending in results was November 2nd 2007, allowing the participants at least six weeks for analysis.

2.6 Stability

From the homogeneity data, the amount of quinolone residues in the materials, just after preparation, is calculated from the average of the 10 duplicate results.
The samples for the stability study were stored at -20 °C. On October 9th three containers of material Egg-03 and Egg-04 were analyzed in duplicate. On November 19th, after the deadline of the inter-laboratory study, again three containers of material Egg-03 and Egg-04 were analyzed. For the two points in time, the average of the results was calculated.
The results of the initial analysis were compared with the results of the analyses after the deadline of the study, using a Students t-test [20]. The hypothesis for this test is:

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

where:
$E(x_0)$ = the expected amount of quinolones at the time of the initial analyses;
$E(x_d)$ = the expected amount of quinolones at time=d.

The value t is calculated by:

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

where:
$\bar{x}_0$ = the average amount calculated for the initial analyses;
$\bar{x}_d$ = the average amount calculated for the analyses at time=d;
s = pooled standard deviation;
n_0 = number of results of the initial analyses;
n_d = number of results of the analyses at time=d;

The calculated value t is compared to a critical value (t_{crit}) derived from a Students-t table with t having $n_0 + n_d - 2$ degrees of freedom [20]. If $t < t_{crit}$ it is demonstrated that no significant difference between the average amount of the analyses at time=d and the initial analyses 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 CIF, ENF, OXA or FLU occurred at -20°C during the timescale of the inter-laboratory study. Therefore it is concluded that during the timescale of the proficiency study the samples were suitable for the purpose.

3 Applied methodologies

The participating laboratories applied different sample preparation procedures for the analysis of quinolones in egg. Fourteen laboratories applied a quantitative instrumental analysis. A schematic overview of the methods applied is presented in Annex 5.

For the instrumental quantitative analysis of quinolones in egg many different extraction solvents or mixtures of solvents were used. Six laboratories used a water based solvent (either without addition or set at a specific pH) for the extraction. Four laboratories used acetonitrile as the extraction solvent. The pH was set at low pH with formic acid or trifluoro acetic acid or at high pH using ammonia. Two laboratories used acified ethanol as the extraction solvent. The other laboratories used mixtures of several solvents among which acetonitrile, ethanol and methanol.

For the sample clean up also several different techniques were applied. Seven laboratories applied solid phase extraction of which five used C_{18} material, one a cation exchange material and one an amine material. Three laboratories applied a liquid-liquid extraction using ethyl acetate, hexane or dichloromethane.
Four labs only diluted, (ultra)filtered or concentrated (by evaporation) the extracts before analysis.

Two detection techniques were applied for the quantitative analysis of quinolones in egg. Eight laboratories applied LC-fluorescence (FLD), in some cases combined with photo diode array detection (PDA). Seven laboratories applied LC-MS/MS. Both detection techniques are suited for confirmation of the identity of group B substances according to 2002/657/EC [20].

Of the participants that used LC-MS/MS as a detection technique, four used one or more internal standards for the quantification of the quinolones. The internal standards used are:

- deuterated internal standards (d_5-norfloxacin, d_8-ciprofloxacin)
- lomefloxacin
- cinchophen

The laboratories that did not analyze for one or more of the quinolones mentioned in the invitation letter are presented in Table 5. It is noted that especially OXA is not included by all laboratories. This compound is the only quinolone for which a MRL is established. Therefore, OXA can be expected to be used in laying hens. Therefore, this compounds should be included in a method for analysis used in the framework of EU regulatory control of residues in egg.

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

Compound	Not included by lab
Danofloxacin	3, 5, 11
Difloxacin	3, 5, 11
Flumequine	3, 5
Marbofloxacin	3, 5, 6, 11, 12, 15
Nalidixic acid	3,5, 7, 11, 12
Norfloxacin	3, 6, 11, 12
Oxolinic acid	3, 5, 11, 12
sarafloxacin	3, 5, 11

An overview of the method performance characteristics of the participating laboratories is presented in Annex 6. All values are presented as reported by the laboratories without any adjustments. Five of the 15 participating laboratories (i.e. 36%) reported to apply a validated method. All of these laboratories have an accreditation for this method.

Amongst the participating laboratories, only three laboratories (2, 8, 12) did report values for CCα for quinolones in egg. Hence, only a minority of participating laboratories is able to report their results as required by Commission Decision 2002/657/EC [20].
It is noted that reported values for CCα and CCß severely differ among the laboratories. Most likely these differences exist due to different interpretation of the regulations. Because no MRLs are established for quinolones in egg (except for OXA) a zero tolerance applies. Therefore, the value of CCα for all quinolones except for OXA should be as low as reasonably possible (Limit of detection of the applied method). This complies with the values of CCα reported by lab 8. From the reported values of laboratory 2 it is derived that this laboratory calculated CCα based on a reporting limit of 10 µg/kg. From this it is concluded that laboratories cope in very different ways with the non existence of MRL values for quinolones in egg. MRLs are established for other matrices. Discussion on this issue resulting in clear legislation is of main importance for obtaining an uniform approach within Europe.

4 Statistical evaluation

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [16], elaborated by ISO, IUPAC and AOAC and ISO/DIS 13528 [17] in combination with the insights published by the Analytical Methods Committee [21, 22] regarding robust statistics.

4.1 Calculation of the assigned value

The assigned value (X) was determined using robust statistics [21-23]. 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 in contrast to conventional outlier elimination methods.
The robust mean of the reported results of all participants, calculated from an iterative process that is based on the median of the reported values, was used as the assigned value [21]. The assigned value is therefore a consensus value.

4.2 Calculation of the uncertainty of the assigned value

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

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

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

According to ISO/DIS 13528 [21] the uncertainty of the assigned value (u) is negligible and therefore does not have to be included in the statistical evaluation if:

$$u \leq 0{,}3\sigma_p$$

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

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

4.3 Calculation of the target standard deviation

According to Commission Decision 2002/657/EC [20], 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, σ_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, σ_p in inter-laboratory trials.

Thompson [18] 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.

The target standard deviation (σ_p) of CIF, ENF and OXA was determined using the equation for analyte concentrations <120 µg/kg. The target standard deviation (σ_p) of FLU was determined using the Horwitz equation. In these calculations c = the assigned value (X) and $\sigma_H = \sigma_p$.

4.4 Performance characteristics with regard to the accuracy

For illustrating the performance of the participating laboratories with regard to the accuracy a z_a-score is calculated. For the evaluation of the performance of the laboratories, the Guidelines of ISO/IEC Guide 43-1 [3] and ISO/DIS 13528 [17] are applied. According to these guidelines z_a-scores are classified as presented in Table 6.

Table 6: Classification of z_a-scores

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

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

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

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

However, if the uncertainty of the assigned value does not comply with the criterion mentioned in § 4.2, it could influence the evaluation of the laboratories. Therefore in this case, the uncertainty is taken into account by calculating the accuracy z-score [13]:

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

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

5 Results and discussion

Eighteen laboratories subscribed for the participation in the inter-laboratory study for quinolones in egg. Thirteen laboratories (i.e. 72 %) managed to submit valid results before the deadline of November 2nd. Laboratory 18 analyzed the samples on November 15th and laboratory 13 on November 16th. This was still within the time frame of the stability study and thus the results of these laboratories were included in the evaluation. Therefore, in total the results of 15 (83%) laboratories are included in the evaluation. Laboratory 17 carried out a screening analysis only. Therefore the results of this laboratory are not included in the calculation of the assigned value and the uncertainty of the assigned value. However, the results of this laboratory are evaluated in perspective of accuracy by calculating z-scores.

All laboratories analyzed the samples in duplicate. The number of laboratories included in the statistical evaluation is 12 for ciprofloxacin, 15 for enrofloxacin, 11 for oxolinic acid and 13 for flumequine. All results are used as reported by the laboratories, without any correction or adjustments. However, for each reported result only one decimal is presented. Furthermore, laboratory 2 and 18 detected small traces of oxolinic acid in the sample of material Egg-04. This is not included in the evaluation.

None of the laboratories detected any quinolones in the blank materials (Egg-01 and Egg-02). Because possibly interfering compounds were added to material Egg-02 it is concluded that the applied methods are all satisfactory specific for the quantitative and confirmative analysis of CIF, ENF, OXA and FLU in egg.
Laboratory 5 detected norfloxacin in one of the duplicate analysis of both samples of material Egg-03. This is considered as a false positive result.

5.1 Evaluation of the results of ciprofloxacin

All laboratories, except for laboratory 17 included CIF in their analysis. Laboratory 5 did not detect CIF in both samples of material Egg-03, however CIF was included in their method. This is considered as a false negative results. Therefore, the evaluation of CIF is based on the results of 13 laboratories. The results of CIF as well as the evaluation of it are presented in Annex 8.

The lowest value reported for CIF is 13.5 µg/kg and the highest value is 346.8 µg/kg. The assigned value of CIF is 46.4 µg/kg with an uncertainty of 1.10 µg/kg. The uncertainty of the assigned value of CIF does not exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the influence of the uncertainty of the assigned value on the calculated z-scores is negligible. The z_a-scores for CIF obtained by each laboratory were calculated. The results are presented in Annex 8a. Graphical presentations of the z_a-scores are included.

With respect to the accuracy the results of one laboratory (lab 18) is questionable and the result of two laboratories (lab 3 and 7) are unsatisfactory. Laboratory 7 reported results of CIF that exceed the assigned value by a factor 7. It is noted that this laboratory carried out the analysis of quinolones in egg under (a flexible scope) accreditation.
From the laboratories that reported results, eight used the supplied reference standard of CIF. Based on only the results of the laboratories that used the supplied reference standard, the assigned value would

be 46.9 µg/kg with an uncertainty of 1.22 µg/kg. Based on only the results of the laboratories that did not use the supplied reference standard, the assigned value would be 45.2 µg/kg with an uncertainty of 1.40 µg/kg. The assigned value as well as the uncertainty of the assigned value is not significantly different. Also no significant difference was observed between these values and the assigned value and uncertainty calculated from all laboratory results. From this it is concluded that, in this case, the applied reference standard is not a main source of uncertainty.

5.2 Evaluation of the results of enrofloxacin

All laboratories that sent in results included ENF in their analysis. Therefore the evaluation of ENF is based on the results of 15 laboratories. The results of ENF as well as the evaluation of it are presented in Annex 9.

For ENF no false negative results occurred.

The lowest value reported for ENF is 17 µg/kg and the highest value is 66.9 µg/kg. The assigned value of ENF is 48.0 µg/kg with an uncertainty of 1.47 µg/kg. The uncertainty of the assigned value of ENF does not exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the influence of the uncertainty of the assigned value on the calculated z-scores is negligible. The z_a-scores for ENF obtained by each laboratory were calculated. The results are presented in Annex 9. Graphical representations of the z_a-scores are included.

With respect to the accuracy the result of one laboratory (lab 3) is questionable. The difference in accuracy among laboratories could not be attributed to differences in the applied sample preparation or detection technique.

5.3 Evaluation of the results of oxolinic acid

Eleven laboratories that sent in results included OXA in their analysis. The results of OXA as well as the evaluation of it are presented in Annex 10.

For OXA no false negative results occurred.

The lowest value reported for OXA is 52 µg/kg and the highest value is 100 µg/kg. The assigned value of OXA is 73.2 µg/kg with an uncertainty of 1.99 µg/kg. The uncertainty of the assigned value of OXA does not exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the influence of the uncertainty of the assigned value on the calculated z-scores is negligible. The z_a-scores for OXA obtained by each laboratory were calculated. The results are presented in Annex 10. Graphical representations of the z_a-scores are included.

With respect to the accuracy the results of all laboratories are satisfactory; all z-scores (except for laboratory 17) are between -1.0 and 1.0 indicating excellent performance regarding the accuracy.

5.4 Evaluation of the results of flumequine

Thirteen laboratories that sent in results included FLU in their analysis. The results of FLU as well as the evaluation of it are presented in Annex 11.

For FLU no false negative results occurred.

The lowest value reported for FLU is 80.0 µg/kg and the highest value is 147.2 µg/kg. The assigned value of FLU is 124.9 µg/kg with an uncertainty of 4.27 µg/kg. The uncertainty of the assigned value of FLU does not exceeds $0.3\sigma_p$ (§4.2). Therefore, for this material, the influence of the uncertainty of the assigned value on the calculated z-scores is negligible. The z_a-scores for FLU obtained by each laboratory were calculated. The results are presented in Annex 11. Graphical representations of the z_a-scores are included.

With respect to the accuracy the results of all laboratories are satisfactory.

5.5 Overall evaluation

From the 15 laboratories 11 (i.e. 73%) showed optimal performance for the analysis of quinolones in egg with respect to the accuracy and the occurrence of false positive and false negative results. An overview of the amount of satisfactory results is presented in table 7. A complete overview of z-scores is given in Annex 12.

Table 7 Overview of the amount of satisfactory results for accuracy

Compound	No. laboratories that reported results	No. of satisfactory results for accuracy	No. of questionalble results for accuracy	No. of unsatisfactory results for accuracy
Ciprofloxacin	13	10	1	2
Enrofloxacin	15	14	0	1
Oxolinic acid	11	11	0	0
Flumequine	13	13	0	0

For CIF one questionable and two unsatisfactory results for accuracy are obtained. For ENF one laboratory obtained questionable results regarding reproducibility.
Laboratory 7 reported an unsatisfactory result for CIF that is a factor 7 above the assigned value, . The results of the other quinolones of this laboratory are satisfactory with z-scores between -0.41 and 0.29. Laboratory 18 reported questionable results for CIF. However, the results for the other quinolones are satisfactory with z-scores between -0.03 and 0.36. In this proficiency test laboratory 3 and 13 have a bias to lower results for all compounds included in their analysis.

In 2006 RIKILT organized a proficiency test regarding the analysis of quinolones in poultry muscle. In this study, fifteen laboratories reported results of which seven also participated in the proficiency test regarding the matrix egg presented in this report. It is noted that in 2006 the majority of laboratories reported to have a validated (87%) and accreditated (80%) method for the analysis of quinolones in poultry. In 2007 only 38% of the laboratories reported to have a validated method for quinolones in egg.

In the proficiency study of quinolones in poultry muscle of 2006 CIF and ENF were also included in the materials. In this former study in some materials the levels of CIF and ENF were comparable to the levels of CIF and ENF in the present study regarding the matrix egg.
The assigned values and relative standard deviation of the assigned values, as well as the amount of participants and satisfactory scores are presented in table 8.

Table 8 Overview of the results of the proficiency study of quinolones in poultry muscle (2006) and quinolones in egg (2007)

Description	PT quinolones in poultry muscle 2006		PT quinolones in egg 2007	
	Ciprofloxacin	Enrofloxacin	Ciprofloxacin	Enrofloxacin
Assigned value (X) (µg/kg)	38.1	68.1	46.4	48.0
Relative standard deviation of X (%)	33.0	28.9	8.6	11.5
No. of results	15	15	13	15
No. of satisfactory results (accuracy) (%)	73	80	77	92

It is clear that the relative standard deviation of the assigned value of CIF and ENF in the proficiency study of quinolones in poultry muscle is significantly higher compared to the proficiency study of quinolones in egg. It is concluded that in general the accuracy of the method of analysis of egg is better than for the analysis of poultry muscle or much development took place during the last year to increase the performance of the analysis of quinolones in general.

6 Conclusions

Eighteen laboratories subscribed for participation in the proficiency study quinolones in egg including three are National Reference Laboratories. Fifteen laboratories managed to submit valid results within the timeframe of the stability study. Five of the participating laboratories applied a validated method which was accredited in all cases.

The minority of participants applied a validated method for the analysis of quinolones in egg. Only three laboratories reported values for CCα and CCß. It is noted that reported values for CCα and CCß severely differ among the laboratories. Most likely these differences are due to different ways of calculations. From the reported values of CCα it is concluded that some laboratories calculated CCα based on a self set MRL, others applied the zero tolerance approach. From this it is concluded that laboratories cope in very different ways with the non existence of MRL values for quinolones in egg. MRLs are set for other matrices. Discussion on this issue resulting in clear legislation is of main importance for obtaining a uniform approach within Europe.

None of the laboratories detected any quinolones in the blank material nor in the material containing possibly interfering compounds. It is concluded that the applied methods are all satisfactory specific for the quantitative and confirmative analysis of CIF, ENF, OXA and FLU in egg.
One laboratory detected norfloxacin instead of ciprofloxacin in the two samples that contain quinolones. This is considered as a false positive result. The same laboratory did not detect CIF in the material that contains CIF. However, CIF was included in their method. This is considered as a false negative result.

Unless the fact that for CIF and ENF some non satisfactory results are observed, 75% of the total of calculated z_a-scores is between -0.5 and 0.5 indicating excellent accuracy. The occurrence of questionable or unsatisfactory results could not be explained by the applied detection or sample preparation technique. For oxolinic acid and flumequine all reported results were satisfactory.
In this proficiency study 73% of the laboratories showed optimal performance in terms of accuracy and the absence of false positive and false negative findings.

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

- Regarding B group substances for which no MRL is set in a specific matrix, legislation should be clarified to obtain a uniform way for the determination of CCα and CCß within the EU and with this a uniform way of characterizing the samples in terms of compliant and non compliant.
- For most laboratories additional effort is needed to validate the analysis of quinolones in egg to be able to report results including a value for measurement uncertainty.
- Additional effort is needed by some laboratories to include oxolinic acid in the method of analysis for quinolones in egg because, officially, oxolinic acid is the only quinolone registered for medication of laying hens in the EU and for which an MRL is established.

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Annex 1 Codification of the samples

Sample set	Material Egg-01	Material Egg-02	Material Egg-03	Material Egg-04
1	194	195	120	012
			167	
2	025	119	98	038
			165	
3	159	022	086	100
			202	
4	121	208	047	026
			085	
5	103	092	131	196
			163	
6	68	044	024	052
			035	
7	123	197	153	142
			156	
8	013	182	011	034
			058	
9	050	093	029	127
			160	
10	008	064	097	095
			112	
11	094	032	019	130
			199	
12	188	105	007	081
			189	
13	110	028	076	117
			178	
14	136	115	031	184
			140	
15	053	063	010	144
			161	
16	027	183	099	060
			125	
17	016	045	155	055
			200	
18	141	179	059	048
			192	
19	175	082	001	201
			170	
20	111	177	018	137
			069	

** all sample number start with QUIN/2007/EGG/*

Annex 2a Statistical evaluation of homogeneity data of material Egg-03 for oxolinic acid

	Oxolinic acid (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	63.8	66.6
2	68.4	69.2
3	72.1	72.6
4	71.6	74.7
5	76.7	70.6
6	73.6	70.8
7	70.3	73.0
8	73.9	72.0
9	74.4	77.6
10	77.9	74.0
Grand mean	72.2	
Cochran's test		
C	0.371	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 15.9	
s_x	3.21	
s_w	2.24	
s_s	2.79	
Critical = 0.3 σ_H	4.76	
s_s < critical?	ACCEPTED	

No danofloxacin, difloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin or sarafloxacin were detected in the samples.

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

Annex 2b Statistical evaluation of homogeneity data of material Egg-03 for ciprofloxacin

	ciprofloxacin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	48.9	43.6
2	54.3	57.1
3	64.1	50.2
4	50.8	46.5
5	52.3	48.4
6	56.3	54.1
7	59.1	46.7
8	45.2	52.3
9	39.3	52.2
10	43.7	53.8
Grand mean	50.9	
Cochran's test		
C	0.262	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 11.2	
s_x	4.04	
s_w	6.09	
s_s	0.00	
Critical = $0.3\,\sigma_H$	3.36	
s_s < critical?	ACCEPTED	

No danofloxacin, difloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin or sarafloxacin was detected in the samples.

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

Annex 2c Statistical evaluation of homogeneity data of material Egg-03 for enrofloxacin

	Enrofloxacin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	57.0	54.4
2	56.6	51.6
3	52.0	45.5
4	52.3	48.7
5	50.4	43.6
6	48.8	40.3
7	52.9	48.8
8	47.6	48.0
9	44.3	49.7
10	62.9	54
Grand mean	50.5	
Cochran's test		
C	0.240	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 11.1	
s_x	4.39	
s_w	4.08	
s_s	3.30	
Critical = $0.3\,\sigma_H$	3.33	
s_s < critical?	ACCEPTED	

No danofloxacin, difloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin or sarafloxacin was detected in the samples.

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

Annex 2d Statistical evaluation of homogeneity data of material Egg-04 for flumequine

	Flumequine (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	116.9	120.1
2	111.5	107.5
3	113.6	109.5
4	111.0	114.0
5*	130.1	110.2
6	112.1	110.4
7	113.4	109.1
8	120.8	116.1
9	115.3	117.5
10	122.6	118.1
Grand mean	115.0	
Cochran's test		
C	0.765	
Ccrit	0.602	
C < Ccrit?	OUTLIER sample no. 5	
After removal of Sample no. 5		
Grand mean	114.4	
Cochran's test		
C	0.187	
Ccrit	0.638	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 25.2	
s_x	4.01	
s_w	2.59	
s_s	3.56	
Critical = $0.3\,\sigma_H$	7.55	
s_s < critical?	ACCEPTED	

No ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid or sarafloxacin was detected in the samples.

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

Annex 3 Instruction letter

RIKILT
INSTITUTE OF FOOD SAFETY
WAGENINGEN UR

DATE
13 September 2007

OUR REFERENCE
07/RIK0866

HANDLED BY
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CHAMBER OF COMMERCE REGISTRATION NO.
09098104 te Arnhem

THE INTERNET
www.rikilt.wur.nl

Dear participant,

Thank you very much for your interest in the proficiency study for quinolones in egg.

Hereby I send you a parcel containing five randomly coded samples of egg. The samples may contain one or more of the following quinolones (in alphabetical order):

Ciprofloxacin*	Marbofloxacin
Enrofloxacin	Nalidixic acid
Danofloxacin	Norfloxacin
Difloxacin	Oxolinic acid
Flumequine	Sarafloxacin

A reference standard of ciprofloxacin (and certificate) is included in the parcel.
Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately, preferably by fax.

Your laboratory code is:
Instructions:

- After arrival store the samples according to your laboratory's procedure. This is part of the proficiency test.
- Defrost the samples before analysis and homogenize them according to your laboratory's procedure.
- Please analyze the samples according to the standard protocol of your laboratory. The samples should be treated as if they are routine samples.
- Please **make use of the supplied reference standard ciprofloxacin**, combined with your own reference standards for the other compounds. Only then, the influence of the reference standards on the quantitative results can be part of the evaluation.
- Carry out a **duplicate quantitative analysis** for each sample. Please confirm the identity of any detected residues of quinolones according to 2002/657/EC.
- Each sample consists of at least 25 g egg. Please contact me if this is not sufficient for a duplicate quantitative analysis.
- The results should be reported before **November 2nd 2007**.
- Please use the result forms we sent to you accompanying the samples.
- The evaluation will primarily focus on the quantitative part of this study.

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

Kind regards,

Bjorn Berendsen

RIKILT is accredited based on ISO 17025. These tests are described in detail on www.rva.nl (no. L014).

Wageningen University and DLO have combined forces in Wageningen UR (Wageningen University and Research Centre).

Annex 4a Statistical evaluation of stability data of material Egg-03

Statistical evaluation for ciprofloxacin in material Egg-03

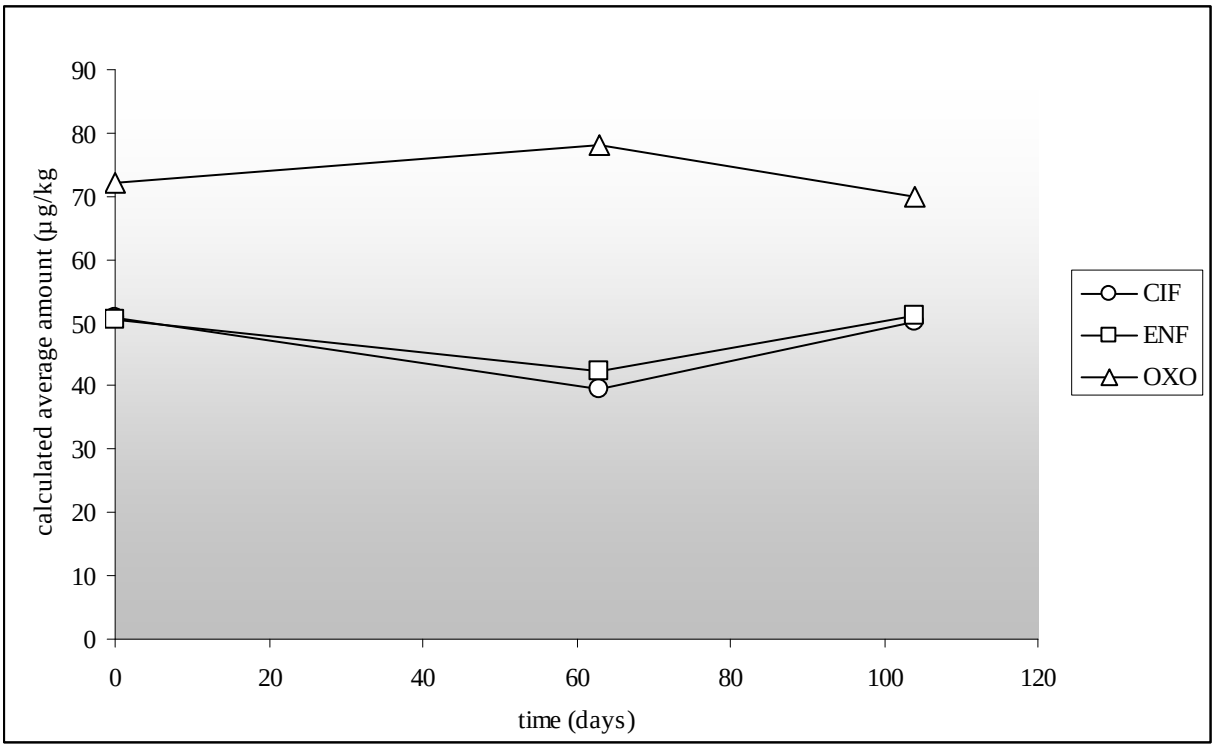
Date of storage at -20 °C	Time at -20°C (days)	Average amount (µg/kg)	n	st. dev (µg/kg)	t	tcrit	t < tcrit
Aug 7, 2007	0	50.9	20				
Nov 19, 2007	104	50.3	6	9.27	0.14	2.06	ACCEPTED

Statistical evaluation for enrofloxacin in material Egg-03

Date of storage at -20 °C	Time at -20°C (days)	Average amount (µg/kg)	n	st. dev (µg/kg)	t	tcrit	t < tcrit
Aug 7, 2007	0	50.5	20				
Nov 19, 2007	104	51.0	6	10.90	0.10	2.06	ACCEPTED

Statistical evaluation for oxolinic acid in material Egg-03

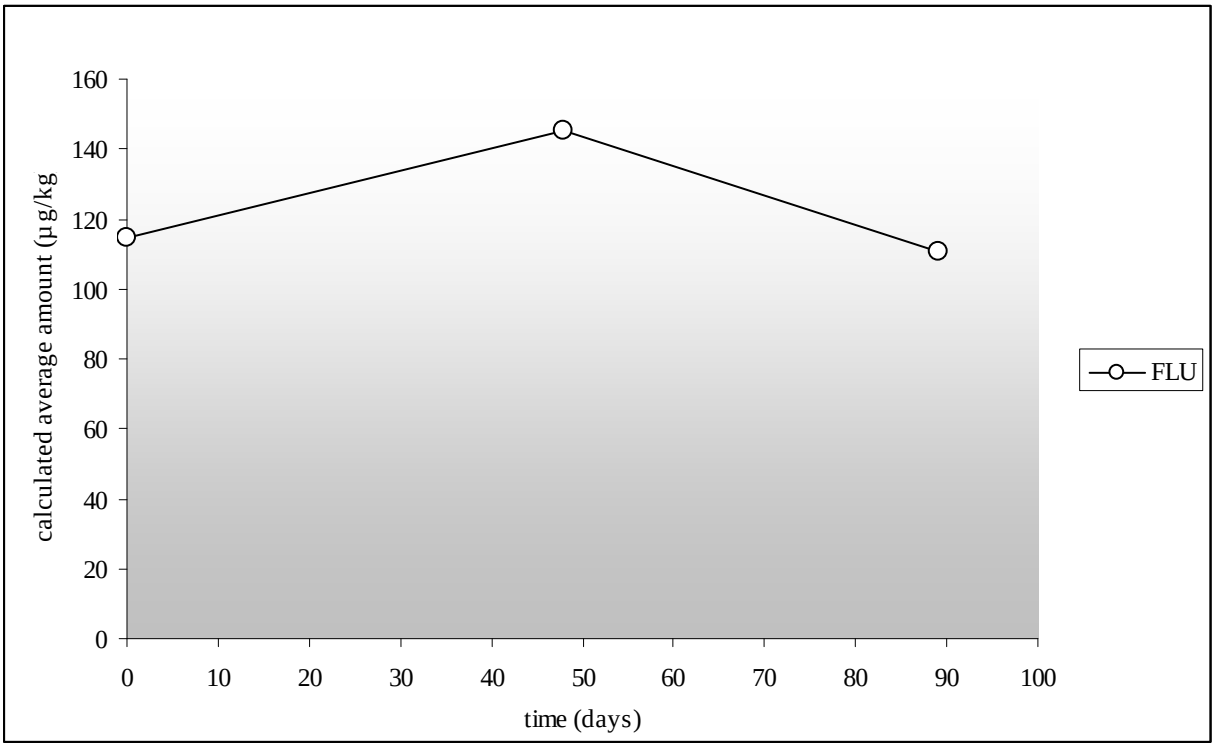
Date of storage at -20 °C	Time at -20°C (days)	Average amount (µg/kg)	n	st. dev (µg/kg)	t	tcrit	t < tcrit
Aug 7, 2007	0	72.2	20				
Nov 19, 2007	104	70.1	6	5.09	0.90	2.06	ACCEPTED



Annex 4b Statistical evaluation of stability data of material Egg-04

Statistical evaluation for flumequine in material Egg-04

Date of storage at -20 °C	Time at -20°C (days)	Average amount (µg/kg)	n	st. dev (µg/kg)	t	tcrit	t < tcrit
Aug 22, 2007	0	114.4	18				
Nov 19, 2007	89	110.6	6	6.24	1.31	2.07	ACCEPTED



Annex 5 Overview of the applied methods

Lab code	Extraction	Sample purification	Internal standard	Detection method	Quinolones not analysed for
2	McIlvain buffer	Add NaCl, LLE (ethyl acetate), evaporation of solvent, reconstitution, membrane filtration		LC-MS/MS	
3	acetonitrile, acetic acid, ethanol	Evaporation of solvent, reconstitution, LLE (hexane), evaporation of solvent, reconstitution, filter		LC-FLD	Danofloxacin, difloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin
4	HCl	SPE (C18), partial evaporation of solvent, adjustment of solvent volume		LC-MS/MS LC-PDA/FLD	
5	Ammonia (25%), Acetonitrile	LLE (dichloromethane)		LC-FLD	Danofloxacin, difloxacin, flumequine, marbofloxacin, nalidixic acid, oxolinic acid, sarafloxacin
6	Acetic acid, ethanol (2x)	Partial evaporation of solvent, adjustment of solvent volume, dilution		LC-FLD	Marbofloxacin, norfloxacin
7	Water	SPE (OASIS HLB), evaporation of solvent	d5-norfloxacin	LC-MS/MS	Nalidixic acid
8	Acetonitrile, trifluoroacetic acid	SPE (C18), evaporation of solvent, reconstitution		LC-FLD	
9	Acetonitrile, formic acid	Evaporation of solvent, reconstitution, SPE (Oasis MCX)	d8-ciprofloxacin d5-norfloxacin	LC-MS/MS	
11	Water	Filter		LC-FLD	Danofloxacin, difloxacin, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin
12	Phosphate buffer (pH=7.4)	Protein precipitation (phosphoric acid), filtration, SPE (DSC 18), evaporation of solvent, reconstitution		LC-FLD	Marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid
13	Ethanol, acetic acid	SPE (NH_2, PRS), evaporation of solvent, reconstitution		LC-MS/MS	
15	Water, methanol, acetonitrile, phosphoric acid, ultrasonic bath, 45 °C	Evaporation of organic solvent, SPE (OASIS HLB), evaporation of solvent, reconstitution		LC-FLU-PDA	Marbofloxacin
16	Water	Filtration, ultrafiltration	lomefloxacin, cinchophen	LC-MS/MS	
17				ELISA	
18	Acetonitrile	Evaporation of solvent, reconstitution	lomefloxacin, cinchophen	LC-MS/MS	

NM = not mentioned

Annex 6 Overview of method characteristics as reported by the participants

		Ciprofloxacin		Enrofloxacin		Oxolinic acid		Flumequine	
Lab code	Validation / accreditation	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)
2	Yes / Yes	12.23	13.77	12.19	13.70	10.77	11.31	11.15	11.95
3	No / No								
4	Yes / Yes								
5	No / No								
6	Yes / Yes								
7	Yes / Yes								
8	Yes / Yes	1.017	1.832	1.095	1.964	0.10	0.176	0.323	0.534
9	No / No								
11	No / No								
12	No / No	10	20	10	20			40	50
13	No / No		≤50		≤50		≤50		≤50
15	No / No								
16	No / No								
17	No / No								
18	No / No								

Annex 6 Overview of method characteristics as reported by the participants (continued)

		Ciprofloxacin		Enrofloxacin		Oxolinic acid		Flumequine	
Lab code	Validation / accreditation	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
2	Yes / Yes	0.23	0.47	0.50	1.00	0.21	0.42	0.07	0.14
3	No / No	1	2	1	2				
4	Yes / Yes	10	20	10	20	10	20	10	20
5	No / No		10		10				
6	Yes / Yes		6		6		10		10
7	Yes / Yes		5		5		5		5
8	Yes / Yes	0.06	0.15	0.03	0.06	12.53	22.31	4.06	8.58
9	No / No		20		20		20		20
11	No / No	3		6				8	
12	No / No								
13	No / No	<10		<10		<10		<10	
15	No / No								
16	No / No								
17	No / No			9	12	4	6	3.5	4
18	No / No	1		1		1		1	

Annex 7 Overview of false positive and false negative results

False positive results

Lab code	Sample code	Material	Analyte found	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)
Lab5	QUIN/2007/EGG/131	Egg-03	Norfloxacin	-	39
	QUIN/2007/EGG/163	Egg-03	Norfloxacin	-	43

False negative results

Lab code	Sample code	Material	Analyte
Lab5	QUIN/2007/EGG/131	Egg-03	Ciprofloxacin
	QUIN/2007/EGG/163	Egg-03	Ciprofloxacin

Annex 8 Results for the analysis of ciprofloxacin in egg (material Egg-03)

Ciprofloxacin								
Assigned value: 46.4 µg/kg								
Uncertainty of assigned value: 1.10 µg/kg								
Target standard deviation (Horwitz, Thompson): 10.2 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z_a-score
2	41.0	50.1	44.6	42.7	44.6	3.80	3.80	-0.18
3	16.7	14.6	13.5	13.8	14.7	0.87	1.54	**-3.11**
4	43.6	44.9	50.9	47.7	46.8	1.41	3.71	0.04
6	43.7	50.0	43.1	50.5	46.8	3.97	3.97	0.04
7	323.5	346.8	317.0	316.7	326.0	9.51	14.58	**27.38**
8	43.6	46.1	44.2	46.2	45.0	1.31	1.31	-0.14
9	43.0	45.0	46.0	42.0	44.0	1.83	1.83	-0.24
11	51.4	48.3	48.1	48.6	49.1	1.28	1.40	0.26
12	46.8	47.7	45.4	46.1	46.5	0.47	1.11	0.01
13	17.0	41.0	35.0	62.0	38.8	14.75	17.29	-0.75
15	44.2	46.5	45.4	44.5	45.2	1.01	1.01	-0.12
16	53.2	50.9	42.8	50.8	49.4	3.40	4.42	0.29
18	61.0	81.0	61.0	75.0	69.5	9.97	9.97	**2.26**

Annex 8 Results for the analysis of ciprofloxacin in egg (material Egg-03) (continued)

Figure a: Graphical representation of the reported results

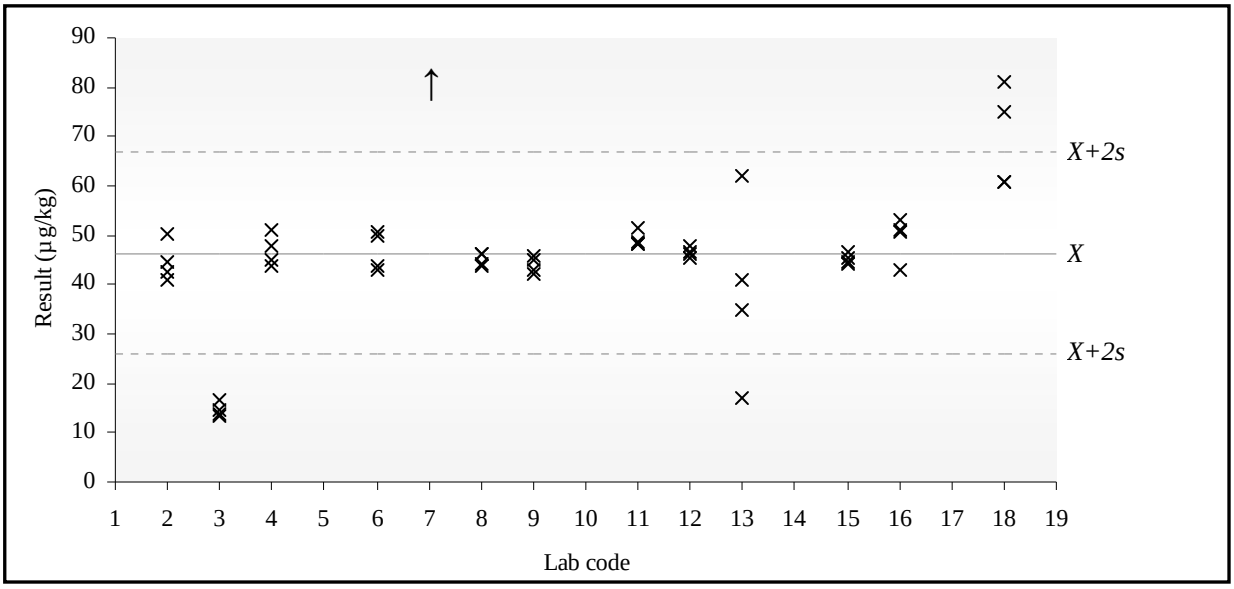
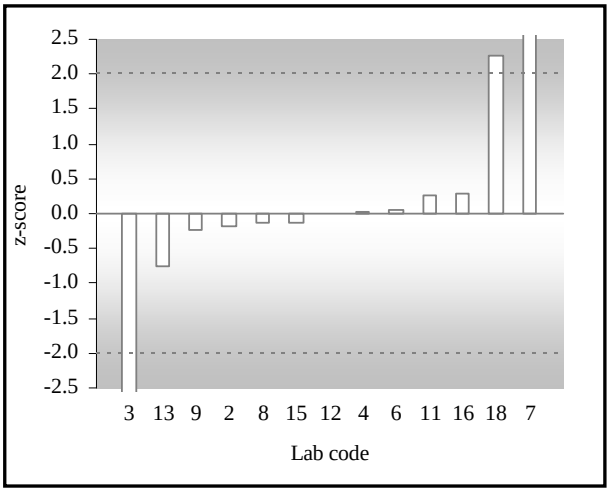


Figure b: Graphical representation of z_a-score



Annex 9 Results for the analysis of enrofloxacin in egg (material Egg-03)

Enrofloxacin								
Assigned value: 48.0 µg/kg								
Uncertainty of assigned value: 1.47 µg/kg								
Target standard deviation (Horwitz, Thompson): 10.6 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z_a-score
2	49.0	51.3	48.6	46.5	48.9	1.27	2.05	0.08
3	25.9	23.4	21.5	23.6	23.6	1.33	1.76	**-2.31**
4	47.4	47.5	59.7	56.2	52.7	1.43	7.49	0.44
5	53.0	51.0	52.0	54.0	52.5	1.15	1.15	0.42
6	59.0	66.9	57.0	66.1	62.3	4.92	4.92	1.35
7	54.8	45.9	51.6	50.8	50.8	3.65	3.65	0.26
8	40.3	43.0	41.1	43.0	41.9	1.35	1.35	-0.58
9	62.0	57.0	37.0	52.0	52.0	6.45	11.55	0.38
11	50.8	47.4	47.2	47.1	48.1	1.39	1.69	0.01
12	47.0	49.2	45.7	51.9	48.5	2.69	2.69	0.04
13	17.0	34.0	31.0	47.0	32.3	9.53	11.69	-1.49
15	40.3	45.1	43.9	41.9	42.8	2.12	2.12	-0.49
16	61.1	50.6	36.2	47.2	48.8	6.21	10.93	0.07
17	50.0	50.0	50.0	50.0	50.0	0.00	0.00	0.19
18	54.0	49.0	46.0	42.0	47.8	2.61	5.62	-0.03

Annex 9 Results for the analysis of enrofloxacin in egg (material Egg-03) (continued)

Figure a: Graphical representation of the reported results

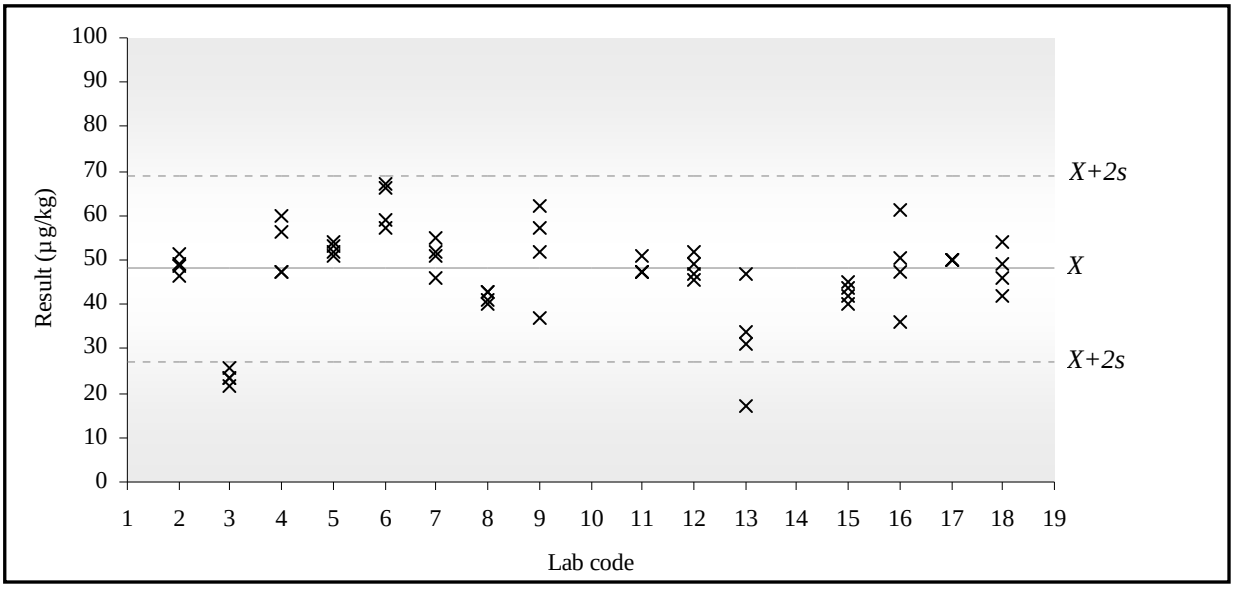
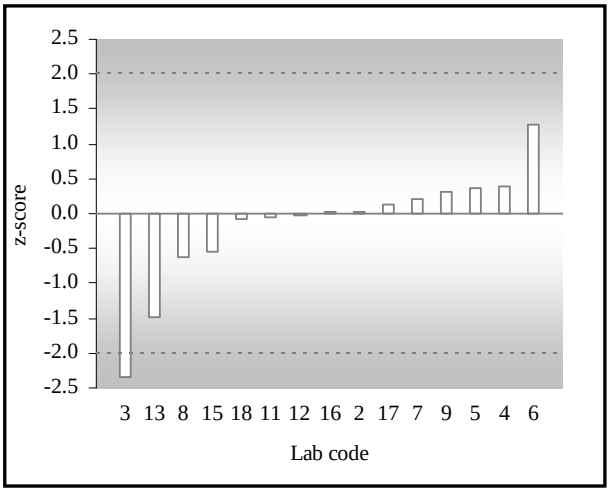


Figure b: Graphical representation of z_a-score



Annex 10 Results for the analysis of oxolinic acid in egg (material Egg-03)

Oxolinic acid								
Assigned value: 73.2 µg/kg								
Uncertainty of assigned value: 1.99 µg/kg								
Target standard deviation (Horwitz, Thompson): 16.1 µg/kg								
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_W	z_a-score
2	66.4	69.9	69.8	73.7	70.0	2.14	2.96	-0.20
4	82.8	78.9	91.7	85.4	84.7	3.02	5.85	0.72
6	69.0	87.6	67.1	88.9	78.2	11.70	11.70	0.31
7	61.7	71.0	69.5	63.9	66.5	4.43	4.43	-0.41
8	76.0	73.8	70.1	76.0	74.0	2.57	2.57	0.05
9	81.0	72.0	68.0	69.0	72.5	3.70	6.23	-0.04
13	52.0	62.0	65.0	64.0	60.8	4.10	6.04	-0.77
15	72.9	75.1	75.9	72.4	74.1	1.69	1.69	0.06
16	71.8	74.6	72.2	65.9	71.1	2.81	3.55	-0.13
17	100	100	80	80	90	0.00	14.14	1.04
18	86.0	75.0	81.0	74.0	79.0	5.32	5.32	0.36

Annex 10 Results for the analysis of oxolinic acid in egg (material Egg-03) (continued)

Figure a: Graphical representation of the reported results

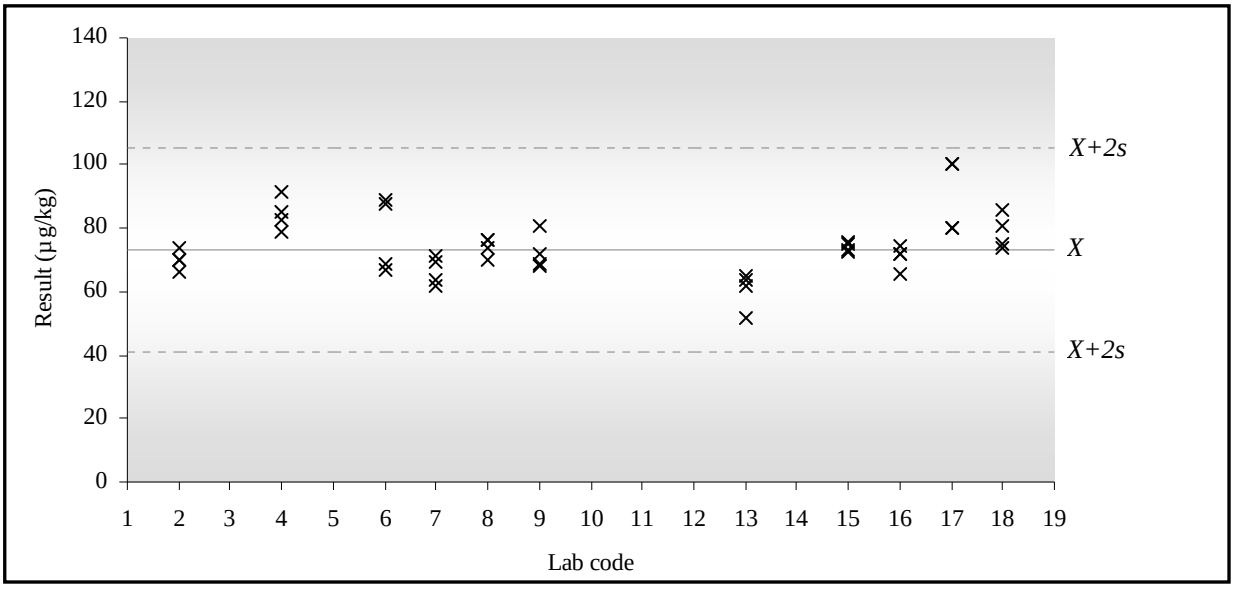
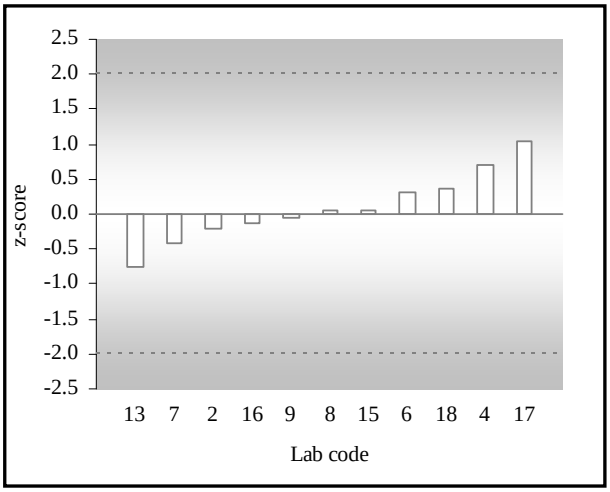


Figure b: Graphical representation of z_a-score



Annex 11 Results for the analysis of flumequine in egg (material Egg-03)

Flumequine				
Assigned value: 124.9 µg/kg				
Uncertainty of assigned value: 4.27 µg/kg				
Target standard deviation (Horwitz, Thompson): 27.3 µg/kg				
Code	Replicate 1	Replicate 2	Average	z_a-score
2	120.0	116.0	118.0	-0.25
4	137.0	128.0	132.5	0.28
6	129.6	139.9	134.8	0.36
7	130.4	135.2	132.8	0.29
8	103.1	125.9	114.5	-0.38
9	99.0	93.0	96.0	-1.06
11	143.2	133.8	138.5	0.50
12	111.0	113.0	112.0	-0.47
13	98.0	111.0	104.5	-0.75
15	122.3	128.2	125.3	0.01
16	142.0	147.2	144.6	0.72
17	80	80	80	-1.64
18	143.0	121.0	132.0	0.26

Annex 11 Results for the analysis of flumequine in egg (material Egg-03) (continued)

Figure a: Graphical representation of the reported results

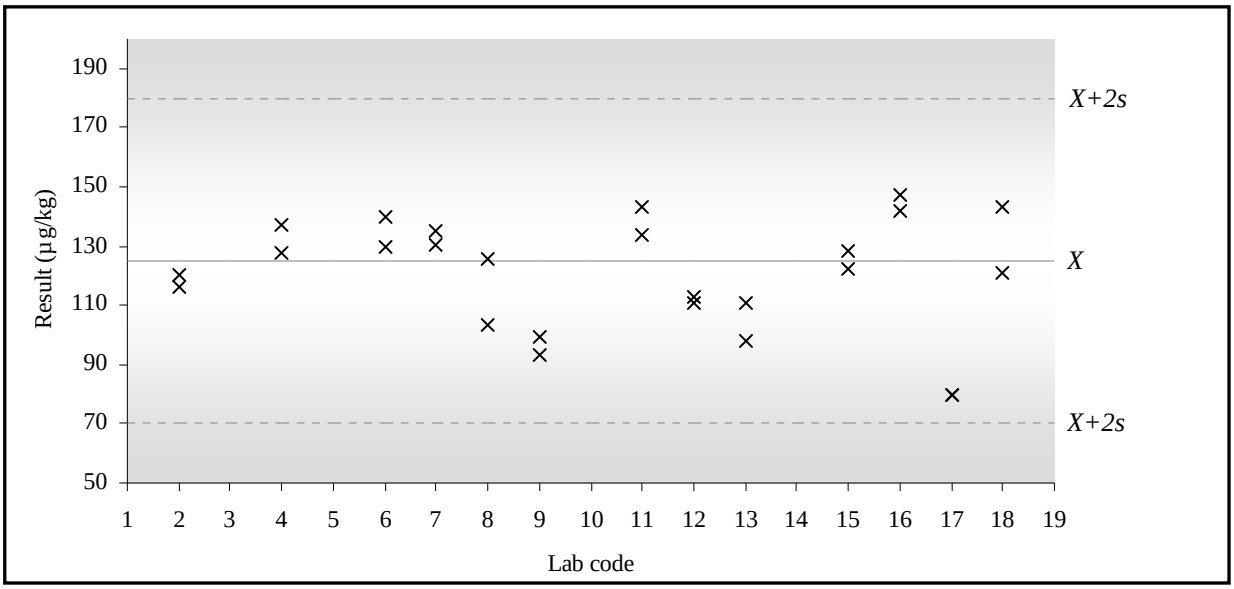
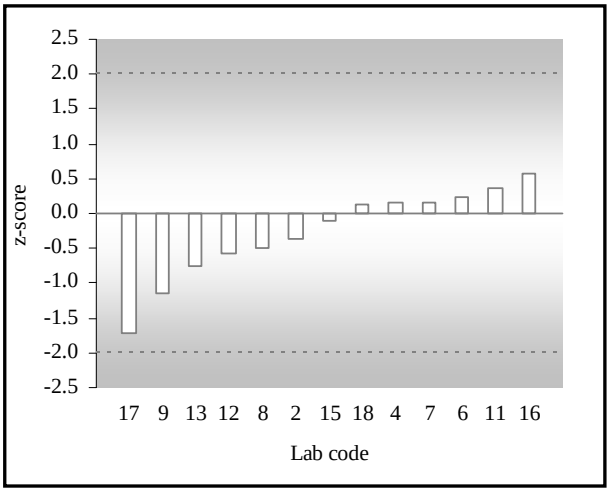
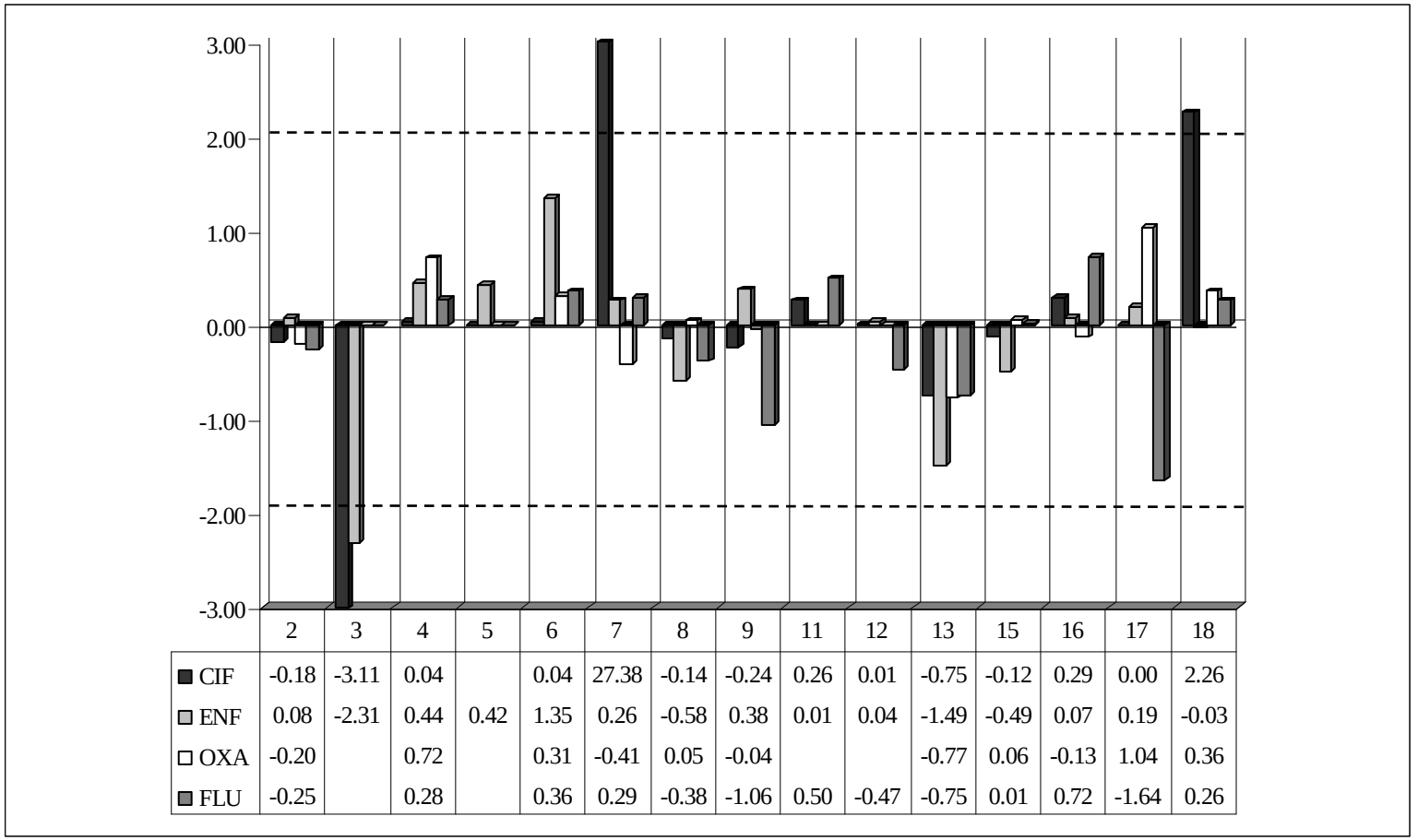


Figure b: Graphical representation of z_a-score



Annex 12 Overview of obtained z_a-scores



	2	3	4	5	6	7	8	9	11	12	13	15	16	17	18
CIF	-0.18	-3.11	0.04		0.04	27.38	-0.14	-0.24	0.26	0.01	-0.75	-0.12	0.29	0.00	2.26
ENF	0.08	-2.31	0.44	0.42	1.35	0.26	-0.58	0.38	0.01	0.04	-1.49	-0.49	0.07	0.19	-0.03
OXA	-0.20		0.72		0.31	-0.41	0.05	-0.04			-0.77	0.06	-0.13	1.04	0.36
FLU	-0.25		0.28		0.36	0.29	-0.38	-1.06	0.50	-0.47	-0.75	0.01	0.72	-1.64	0.26