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Proficiency study for penicillins in porcine tissues

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All participants of the proficiency study for penicillins in porcine tissues.

Summary

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

For this proficiency study, four test materials were prepared:

- A blank porcine muscle material (M-A);
- A porcine muscle material containing about 200 µg/kg cloxacilline and a trace of ampicillin (M-B);
- A blank porcine kidney material (K-A);
- A porcine kidney material containing about 100 µg/kg cloxacillin, 30 µg/kg ampicillin and about 20 µg/kg penicillin G (K-B).

During homogeneity testing, all materials proved to obtain sufficient homogeneity for proficiency testing.

Forty-four laboratories were invited to participate in the proficiency study for penicillins in porcine tissues of which 21 laboratories, i.e. 48%, subscribed. Each laboratory received six randomly coded samples. The laboratories were asked to analyze the samples in duplicate according to their own laboratory procedures. It was mentioned that maintaining the stability of the samples (storage and pre-treatment) was part of the proficiency test.

Eighteen laboratories managed to submit results within the timeframe of the study of which 15 reported quantitative results for both the muscle and the kidney samples. Two laboratories reported only results for the muscle samples and one laboratory only reported screening results. The majority of the laboratories applied a validated and accredited method for the analyses.

The stability of penicillins can, according to literature, be maintained by storing the samples below -70 °C. Within this proficiency study a stability experiment at -20 °C was carried out. This stability study showed a degradation of ampicillin, cloxacillin and penicillin G above 75 % in the kidney material during the timeframe of the proficiency study. The penicillins in kidney proved to be instable even after stabilization by buffering the material at pH=6. Therefore, the kidney samples are not suited for evaluation purposes.
During storage at -20 °C the muscle material showed a degradation of 31 % for ampicillin and 27% for cloxacillin was observed. However, the penicillins in the muscle material showed to be stable at -20 °C after buffering at pH=6. Therefore, the muscle materials are suitable for this proficiency study, because maintaining the stability was mentioned as a part of this proficiency study.

In the stability study a degradation of penicillins during storage at -20 °C was observed, which is in agreement with literature. According to the information supplied by the participants, four laboratories stored their samples at -20 °C. Therefore it can be assumed that the samples of these laboratories were instable during the timeframe of the proficiency study. However, no statistically significant difference was observed between the results of the laboratories that stored the samples at -20 °C and the results of the laboratories that stored the samples at a temperature below -70 °C. Therefore, also the stability of the samples, even if stored at -70°C can be questioned. Probably, other factors than storage temperature are of influence on the stability. Based on these observations, the correctness of the calculated assigned

values (consensus values) can be questioned. Therefore, this proficiency study is evaluated for information only.

The participants applied different methodologies for carrying out the analysis of penicillins in porcine tissues. Almost every laboratory applied identical procedures for muscle and kidney.
The mainly applied extraction solvent is a phosphate buffer at pH 8 to 9, sometimes combined with an organic solvent. The majority of laboratories applied Solid Phase Extraction as a sample clean up technique. In all cases a C_{18} material or OASIS^{TM} HLB was used as stationary phase.
Two laboratories applied a derivatization procedure. One laboratory applied a derivatization at the end of the sample clean up procedure using benzoic acid anhydride in combination with triazole and mercury chloride solution. The other laboratory applied a derivatization using piperidin during the extraction.

For all compounds and materials a considerable variation of the reported results is observed, possibly caused by the instability of the materials. No relations were observed between the laboratories results and the storage temperature, storage time (date of analysis) or a combination of these factors.

Based on the results, it is concluded that additional effort is needed to develop a robust method for the analysis of penicillins in porcine tissues. Stability of the compounds during storage seems to be an underestimated factor. Based on the results of the stability study, adequate storage and/or the use of a stabilization procedure at the time of arrival of the samples is required for obtaining reliable results.

Contents

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 new ISO 17025:2005 [2].
No internationally focused proficiency studies regarding the analysis of penicillins in porcine tissues were organized during the last years. Therefore an inter-laboratory quality control for this method 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 penicillins in porcine muscle and kidney. Maintaining the stability of the provided samples was underlined as one of the important factors of this study.
This study also provided an evaluation of the methods applied for quantitative and confirmatory analysis of penicillins.

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 Penicillins

The discovery of penicillins is attributed to Dr. Flemming in 1928 [6]. Penicillins are ß-lactam antibiotics used in the treatment of bacterial infections caused by Gram-positive organisms [7]. The narrow spectrum of activity of the penicillins, initiated the search for derivatives of penicillins which were able to treat a wider range of infections. Ampicillin, which offered a broader spectrum of activity than either of the original penicillins, was the first breakthrough. Later, ß-lactamase resistant (§1.3) penicillins like dicloxacillin were developed.

Penicillins are widely used in swine, cattle and poultry to treat infections. Furthermore they are used as a feed additive in the prevention of diseases. Penicillins are rapidly cleared from the blood via the kidney into the urine. Therefore, the amount of penicillin residues in kidney is found to be much higher compared to muscle [8].

According to EU regulations, all substances for veterinary use need to be included in Annexes I, II or III of Council Regulation (EEC) No 2377/1990 [13], (EC) No 508/1999 [14]. Penicillins are included in Annex I: pharmacologically active veterinary products for which a Maximum Residue Limit (MRL) is established.

This proficiency study focuses on ampicillin, cloxacillin and penicillin G (benzylpenicillin) in porcine muscle and kidney. For these penicillins the use and medication of swine is described [9-10]. The MRL for these compounds in porcine muscle and kidney is presented in Table 1.

Table 1. The MRL of penicillins in porcine tissues of penicillins included in the proficiency study [11-12]

Compound	MRL in muscle (µg/kg)	MRL in kidney (µg/kg)
Ampicilline	50	50
Cloxacillin	300	300
Penicillin G	50	50

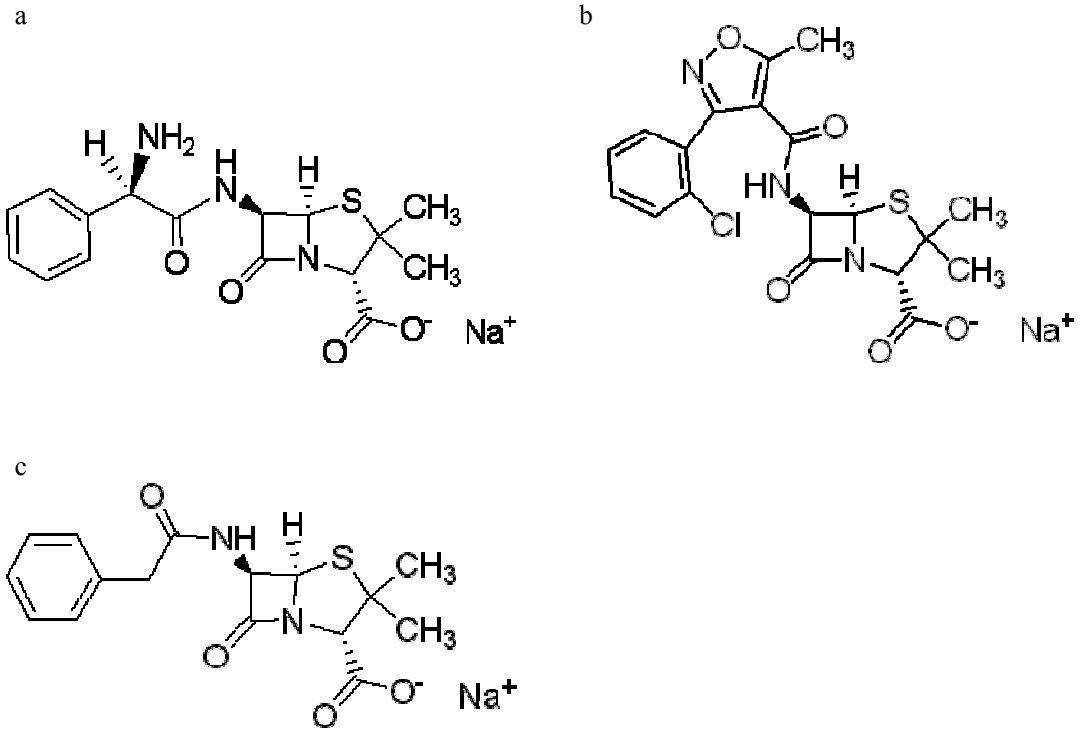

Figure 1. Molecular structure of (a) ampicillin, (b) cloxacillin and (c) penicillin G

1.3 Stability of penicillins

The stability of penicillins in especially tissues is a main issue in the analysis of this compound group. Several studies pointed out that penicillins have limited stability at low and high pH. Maximum stability is obtained in the range of pH 6 to 7 [13-15]. pH is therefore an important factor in maintaining the stability of penicillins.

Furthermore, the storage condition of tissue samples is an important factor in the stability. Several studies showed degradation of penicillins at a storage temperature of -20 °C. At approximately -75 °C the stability proved to be satisfactory. [16-18].

Already in 1940 an enzyme called penicillase, a ß-lactamase enzyme, was discovered that is able to break open the four-atom ß-lactam ring that is specific for penicillins [19]. Penicillase is formed by the bacteria *E. coli* and can be present in animal tissue. To prevent penicillins from degradation, most penicillins are administered in combination with a ß-lactamase inhibitor like clavulanic acid. Clavulanic acid contains a four-atom ß-lactam similar to penicillins. This results in a inhibition of the degradation of penicillins. In this way the penicillins can remain intact.

Next to the pH, storage temperature and the presence of penicillase, the occurrence of the tissue is relevant regarding penicillin stability [18]. The use of ground muscle showed to result in more stability for penicillins compared to bulk or diced material.

2 Test materials

2.1 Sample preparation

Four fresh test materials were prepared containing different amounts of ampicillin (AMP), cloxacillin (CLOX) and penicillin G (PENG) by adding methanolic solutions of these compounds to blank porcine muscle and kidney according to table 2. The materials were homogenized under cryogenic conditions according to in-house standard operating procedures.

Table 2.Target amount of penicillins in the proficiency study test materials

Material code	Matrix	Target amount of AMP	Target amount of CLOX	Target amount of PENG	Amount of material (g)
M-A	Porcine muscle	-	-	-	1500
M-B	Porcine muscle	ca. 0.1*MRL	ca. MRL	-	3000
K-A	Porcine kidney	-	-	-	1300
K-B	Porcine kidney	ca. MRL	ca. 0.5*MRL	ca. 0.5*MRL	2600

2.2 Sample identification

Materials M-A, M-B, K-A and K-B were stored in polypropylene containers containing at least 30 gram of matrix, yielding a total of 42 containers of material M-A and K-A, and 79 containers of material M-B and K-B. The muscle samples were randomly coded with a code from M/2006/001 through M/2006/121. The kidney samples were randomly coded with a code from K/2006/001 through K/2006/121.

For homogeneity and stability testing, 12 randomly chosen containers of material M-A and K-A, and 19 randomly chosen containers of material M-B and K-B were used.

For each laboratory a sample set was at randomly prepared consisting of one sample of material M-A and K-A, and two samples of material M-B and K-B. The sample numbers belonging to each sample set are presented in Annex 1.

2.3 Homogeneity study

Ten containers of materials M-B and K-B were each analyzed in duplicate for ampicillin, cloxacillin and penicillin to determine the homogeneity of the materials. The homogeneity study was carried out according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [20] and ISO/DIS 13528 [21], taking into account the insights discussed by Fearn et al. [22] and Thompson [23].
The results of the homogeneity study and their statistical evaluation is presented in Annex 2a through e. All materials were demonstrated to be sufficiently homogeneous for use in the proficiency study. During the homogeneity study the amount of all penicillins in both materials M-B and K-B proved to be lower than expected. Nevertheless, the determined levels were relevant and the samples were found suitable

for application in the proficiency study. The amounts determined during the homogeneity study are presented in table 3.
The at random chosen samples of material M-A and K-A were analyzed for seven penicillins. No amoxycillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G and phenoxymethylpenicillin (penicillin V) were detected in the materials M-A and K-A (< 5 µg/kg). It was concluded that materials M-A and K-A are suited for use as blank materials in the proficiency study.

Table 3. Determined amount of penicillins in the proficiency study test materials

Material code	Matrix	Target amount of AMP (µg/kg)	Target amount of CLOX (µg/kg)	Target amount of PENG (µg/kg)
M-A	Porcine muscle	-	-	-
M-B	Porcine muscle	Trace (< 10)	192	-
K-A	Porcine kidney	-	-	-
K-B	Porcine kidney	32	116	22

2.4 Participants

Forty-four laboratories were invited to participate in the proficiency study for penicillins in porcine tissues. These were mainly European laboratories, but also several inter-continental laboratories were invited. Twenty-one laboratories, i.e. 48%, subscribed for participation.

2.5 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code (001 through 021). The sample sets with the corresponding number, consisting of six coded samples, were sent to the participating laboratory at the 12th of December. The sample sets were packed in an insulating box, containing dry ice and were dispatched to the participants immediately by courier. Due to customs regulations the samples did not arrive at one of the laboratories (001). This laboratory was therefore not able to participate. One laboratory (017) reported that the samples were cold but partially thawed at arrival. In agreement with this laboratory, it was decided not to send a new sample set. All other laboratories confirmed the receipt of the samples in good condition (frozen).

The samples were accompanied by a letter describing the requested analyses, an acknowledgement of receipt form and a results form. Furthermore, a reference standard of ampicillin, including a certificate of analysis, was included in the packages. 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 during the evaluation process.

The laboratories were informed that maintaining the stability of the samples was part of the proficiency study. Furthermore, the laboratories were advised to store the samples at < -70 °C until analysis. A duplicate analysis of each sample was requested, resulting in two results for materials M-A and K-A, and four results for material M-B and K-B. The deadline for sending in results was the 31st of January, allowing the participants at least six weeks for analysis.

2.6 Stability

After preparation of the samples nine samples of each material were at randomly picked for use in the stability study. Each of these samples was split in two aliquots. The first aliquots were stabilized (addition of phosphate buffer pH=6) whilst the second aliquots did not undergo any further treatment. Two samples of each material (both with and without stabilization) were stored at -80°C directly after preparation (t=0). The other samples were stored at -20 °C. In this experiment the samples are assumed to be stable at a storage temperature of -80 °C.

On the 7th of February, just after the deadline of the proficiency study, two samples of each material stored at -20 °C (both with and without stabilization) were analyzed together with the samples stored at -80 °C. For each point in time the average of the results was calculated.
The results of both storage conditions were compared using a Student's t-test [24]. The hypothesis for this test is:

$$H_0 : E(\bar{x}_{-80}) = E(\bar{x}_{-20})$$

where:
$E(\bar{x}_{-80})$ = expected average of the samples stored at -80 °C;
$E(\bar{x}_{-20})$ = expected average of the samples stored at -20 °C.

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

$$t = \frac{\bar{x}_{-80} - \bar{x}_{-20}}{s\sqrt{\frac{1}{n_{-80}} + \frac{1}{n_{-20}}}}$$

where:
$\bar{x}_{-80}$ = the average amount calculated for the samples stored at -80 °;
$\bar{x}_{-20}$ = the average amount calculated for the samples stored at -20 °C;
n_{-80} = number of samples stored at -80 °C;
n_{-20} = number of samples stored at -20 °C;

$$s_p = \sqrt{\frac{(n_{-80} - 1)s^2 + (n_{-20} - 1)s^2}{(n_{-80} + n_{-20} - 2)}}$$

where:
s_p = pooled standard deviation;
n_{-80} = number of samples stored at -80 °C;
n_{-20} = number of samples stored at -20 °C;
s = standard deviation of the analyses calculated from the CV% resulting from the validation procedure.

The calculated value *t* is compared to a critical value (t_{crit}) derived from a Students-*t* table using a confidence level of 95% with *t* having $n_{-80} + n_{-20} - 2$ degrees of freedom [17]. If $t < t_{crit}$ it is demonstrated that no statistically significant degradation of the penicillins during storage at -20 °C occurs.

The results and evaluation of the stability test are presented in Appendix 3a through e.
It is demonstrated that a tremendous loss of ampicillin, cloxacillin and penicillin G in kidney occurs. The loss of these penicillins in the samples that did not undergo any treatment was at least 75%. The loss of these penicillins in the samples that were stabilized by addition of phosphate buffer pH=6 differed from 21% for cloxacillin to 47% for ampicillin. It is concluded that the penicillins in kidney degrade during storage at -20 °C. Adjustment of the pH to 6 can slow down the degradation, but will not stop it.
Also for the muscle samples that did not undergo any treatment a severe loss of both ampicillin and cloxacillin was observed. The degradation of ampicillin and cloxacillin in muscle was significantly lower compared to the kidney samples: ampicillin decreases with 31% and the loss of cloxacillin was 27%. For ampicillin the degradation is statistically significant according to the Students-*t* test. According to the calculation the degradation of cloxacillin is not statistically significant. This is caused by the low amount of degrees of freedom which results in a high critical value (t_{crit}). Nevertheless, a degradation of 27 % is considered as a significant difference. It is concluded that ampicillin as well as cloxacillin degraded in the porcine muscle material during the timeframe of the proficiency study if stored at -20 °C.
For the muscle samples that are stabilized by addition of phosphate buffer pH=6, no significant loss is observed during the period of storage.

This experiment demonstrates the instability of penicillins in mainly the porcine kidney material but also in the muscle material at a storage temperature of -20 °C. The necessity to store the compounds at a temperature below 70 °C and/or to apply a procedure to stabilize the compounds is demonstrated by the present study.

3 Applied methodologies

The participating laboratories applied different sample preparation procedures for the analysis of penicillins in porcine tissues. Almost every laboratory applied identical procedures for muscle and kidney. A schematic overview of the methods applied is presented in Annex 4.

Fourteen of the eighteen laboratories stored the samples at -90 to -70 °C before analysis. Four laboratories stored the samples at approximately -20 °C. Only one laboratory (015) applied a stabilization procedure by buffering the sample at pH=6 at arrival.

Eight laboratories used a phosphate buffer at pH 8-9 as the extraction solvent, sometimes combined with an organic solvent. The other laboratories applied water, methanol, acetonitrile, petroleum ether and mixtures of those solvents for the extraction. In all cases the pH of the solvent was neutral or slightly basic. This is in agreement with literature regarding stability of penicillins in solution (§1.3).

Thirteen laboratories applied solid phase extraction as a sample clean up technique. In all cases a C_{18} material or OASIS^{TM} HLB was used as stationary phase, either silica bound or on polymeric basis. Four laboratories (009, 010, 011 and 016) only evaporated the extraction solvent, in some cases followed by (ultra)filtration.

Two laboratories applied a derivatization procedure. One laboratory (005) applied a derivatization at the end of the sample clean up procedure using benzoic acid anhydride in combination with triazole and mercury chloride solution. The other laboratory (015) applied a derivatization using piperidine during the extraction.

Two detection techniques are applied for the quantitative analysis of penicillins in porcine tissues. Fifteen laboratories applied LC-MS/MS as a detection technique according to 2002/657/EC [25] regarding criteria for confirmation of the identity. One laboratory (005) applied LC-DAD as a detection technique.

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

- $^{13}C_2$-penicillin G and d_{10}-piperidinepenicillins, the only deuterated standard used
- Penicillin V, after a screening procedure or in case penicillin V is not included in the method
- Sulfamethazin
- Methicillin
- Nafcillin

The laboratories that did not analyze for one or more of the penicillins mentioned in the invitation letter are presented in Table 4. It is noted that amoxycillin, cloxacillin, dicloxacillin, oxacillin and especially phenoxymethylpenycillin are not included by all laboratories. These compounds however, are registered for medication in swine within the EU and a MRL for swine tissues is established. Therefore, these compounds should be included in a method of analysis used in the framework of EU regulatory control of residues in porcine tissues.

Table 4. Overview of laboratories that did not include all quinolones in the analysis.

Compound	Not included by lab
Amoxycillin	17
Ampicillin	
Cloxacillin	21
Dicloxacillin	21
Oxacillin	17, 21
Penicillin G	
Phenoxymethylpenicillin	10, 19, 21

An overview of the method performance characteristics of the participating laboratories is presented in Annex 5. All values are presented as reported by the laboratories without any adjustments. Eight of the 17 participating laboratories (i.e. 47%) applied a validated method whilst three laboratories reported that their validation was on going. Eleven of the participating laboratories (i.e. 65%) laboratories reported to have their method accredited. It was noticed that 65% of the participating laboratories reported that their method is accreditated whilst only 47% of these laboratories carried out a validation study. Probably some laboratories interpreted the question about validation and accreditation differently.

Amongst the participating laboratories, five laboratories (005, 008, 011, 015, 019) did report values for CCα. Hence, the minority of participating laboratories is already able to report their results as required by Commission Decision 2002/657/EC [25] coming in force from the 1st of August 2007.

4 Statistical evaluation

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [20], elaborated by ISO, IUPAC and AOAC and ISO/DIS 13528 [21] in combination with the insights published by the Analytical Methods Committee [26, 27] regarding robust statistics.

4.1 Calculation of the assigned value

The assigned value (X) was determined using robust statistics [26-28]. 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 was used as the assigned value. 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. 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 [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 [25], 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 [23] 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, was determined using the equation for analyte concentrations <120 µg/kg for ampicillin in muscle and for ampicillin, cloxacillin and penicillin G in kidney. The Horwitz equation was used for determining the target standard deviation, σ_p, for cloxacillin in muscle. 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 [21] are applied. According to these guidelines z_a-scores are classified as presented in Table 5.

Table 5: Classification of z-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 this uncertainty is taken into account by calculating the accuracy z-score [11]:

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

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

4.5 Performance characteristics with regard to 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 blind samples of material

M-B and K-B were submitted to the participants. Therefore, every laboratory reported multiple results for these materials. From the results of the blind samples of material M-B and K-B the repeatability (s_r) and an estimate of the within-lab-reproducibility (s_{R_L}) were calculated [28].

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.

An estimate of the within-lab-reproducibility standard deviation is calculated from:

$$s_{R_L} = \sqrt{s_L^2 + s_r^2}$$

where:
s_{R_L} = estimate of the within-lab-reproducibility standard deviation;
s_r = repeatability standard deviation;

$$s_L = \sqrt{\frac{p\sum(\bar{x}_p)^2 - (\sum\bar{x}_p)^2}{p(p-1)} - \frac{s_r^2}{2}}$$

where:
s_L = between sample variance (if s_L <0 this value is assumed to be zero)
p = number of pairs;
$\bar{x}_p$ = average result of the duplicates;
s_r = repeatability standard deviation.

Because the samples are not analyzed under true within-lab reproducibility conditions, the estimate of the within-lab reproducibility standard deviation (s_{R_L}) will always be lower than the true within-lab reproducibility standard deviation.
To inform a laboratory of its performance for reproducibility, the Horwitz-ratio (HORRAT) is a suitable value [29]. In this report, the HORRAT is calculated from the estimate of the within-lab reproducibility, because it is not possible to calculate a reproducibility standard deviation from the laboratory data. The reproducibility standard deviation (s_R) includes inter-laboratory variation and must therefore always be higher than the estimate of 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} = estimate of the within-lab reproducibility standard deviation;
σ_p = target standard deviation (§ 4.3).

In this formula, a HORRAT value equal to 1.0 indicates that the estimate of 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 estimate of 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.

5 Results and discussion

Twenty-one out of 44 (i.e. 47%) invited laboratories subscribed for the participation in the inter-laboratory study for penicillins in porcine tissues. Due to customs regulations, it was not possible to ship the samples to one of the laboratories (001). Therefore, this laboratory was not able to participate. Eighteen laboratories (i.e. 90 %) managed to submit valid results before the deadline of the 31st of January. Laboratory 14 carried out a screening analysis only. Therefore this laboratory is not included in the quantitative evaluation.

Because of the noticed instability at -20 °C of ampicillin, cloxacillin and penicillin G in the kidney material and ampicillin in the muscle material, for these compounds only the assigned value is calculated. The data of cloxacillin in the muscle material is more extensively evaluated (§5.1). The laboratories results and the calculated assigned value are presented in Annex 6a for ampicillin in muscle, 6b for cloxacillin in muscle, 6c for ampicillin in kidney, 6d for cloxacillin in kidney and 6e for penicillin G in kidney.

None of the laboratories detected any penicillins in the blank materials (material M-A and K-A). No false positive results occurred.

5.1 Evaluation of the results of cloxacillin in muscle

Three laboratories that sent in results did not report quantitative results for cloxacillin in material M-B. Therefore the evaluation of cloxacillin in muscle is based on the results of 15 laboratories. The laboratory results as well as the statistical evaluation of cloxacillin in the muscle material are presented in Annex 6b.

Of the laboratories that reported quantitative results for cloxacillin in the muscle material, four laboratories stored the samples at approximately -20 °C before analysis and eleven laboratories stored the samples at temperatures below -70 °C. In the stability study a small degradation (27%) of cloxacillin in the muscle material was observed at -20 °C. In literature [16-18] it is reported that penicillins are stable at storage temperatures below -70 °C. Therefore a difference in the laboratory results of laboratories that stored the samples at -20 °C versus the laboratory results of the laboratories that stored the samples below -70 °C is expected.
The effect of the storage temperature of the samples on the reported results was explored. An overview of the storage temperature versus the laboratories average result for cloxacillin in muscle is presented in annex 7. No statistically significant relation is found between these two parameters. This is in disagreement with the expectations, literature [16-18] and the stability study. Probably more factors have influence on the stability like storage time, presence of enzymes and the amount of defrosting cycles.

One laboratory (004) analyzed the samples within one week after shipment of the samples (19th of December). The other laboratories analyzed the samples between the 4th and the 31st of January (i.e. at least three weeks after shipment). Laboratory 004 reports the highest average result which may indicate a relation between the storage time and the laboratories results. Therefore, the effect of the storage time

on the laboratories results was explored. An overview of the date of analysis versus the reported results is presented in Annex 8. Unfortunately, only one laboratory analyzed the samples in December. This results in only one data point for the first three weeks after shipment of the samples. Therefore, no statistical conclusions can be drawn from the data regarding the effect of the storage time on the laboratories results.

Also the combination of the storage time and the storage temperature could be of influence on the results. Therefore, both factors were combined in several ways to find out if any relation between the combination of these factors and the reported results could be found. None of these combinations resulted in a clear relation with the reported results.

For material M-B the lowest value reported is 72.6 µg/kg and the highest value is 390 µg/kg. The assigned value of cloxacillin in material M-B, the consensus value resulting from the robust statistics, is 135 µg/kg with an uncertainty of 9.5 µg/kg. The target standard deviation, σ_p, is 29.2 µg/kg. The uncertainty of the assigned value of cloxacillin in material M-B exceeds $0.3\,\sigma_p$ (§4.2). Therefore, for this material, the uncertainty of the assigned value is taken into account in the evaluation of the laboratories; z_a'-scores are calculated. The z_a'-scores and HORRAT values for cloxacillin obtained by each laboratory were calculated. The results are presented in Annex 6b. A graphical representation of the z_a'-scores and HORRAT values is included.

The results of the participating laboratories are not in agreement with the expectations based on the storage conditions applied by the laboratories. It is observed that the two laboratories that obtained 'questionable' results (004, 013) both reported results above the assigned value. This could indicate better stability at these laboratories. Therefore, the correctness of the assigned value, which is a consensus value of all laboratory results, can be questioned.

Based on these observations, no conclusions regarding the performance of the laboratories can be drawn from this evaluation. Figures are given for information only, not for evaluation of the participating laboratories.

6 Conclusions

Forty-four laboratories were invited to participate in the inter-laboratory study for penicillins in porcine tissues, of which 21 laboratories subscribed. Eighteen laboratories reported their results within the given timeframe. Two laboratories reported results for the muscle samples only and one laboratory only reported screening results. The majority of the laboratories applied a validated and accredited method for the analysis of penicillins in porcine tissues.

For this proficiency test, four test materials were prepared: two porcine muscle samples containing ampicillin and cloxacillin and two porcine kidney samples containing ampicillin, cloxacillin and penicillin G. During homogeneity testing, all materials proved to obtain sufficient homogeneity for proficiency testing.

The stability of penicillins can, according to literature, be maintained by storing the samples below -70 °C. Within this proficiency study a stability study at -20 °C was carried out. This stability study showed a severe degradation of ampicillin, cloxacillin and penicillin G in the kidney material during the timeframe of the proficiency study. Even after stabilization by buffering the material at pH=6, the penicillins in kidney proved to be instable. Therefore, the kidney samples are not suited for evaluation purposes.
In the muscle material a degradation of 31 % for ampicillin and 27% for cloxacillin was observed. However, after buffering the muscle material at pH=6 the penicillins showed to be stable at -20 °C Because maintaining stability was mentioned as a part of this proficiency study, the muscle materials are suitable for this proficiency test.

According to the information supplied by the participants, four laboratories stored their samples at -20 °C. These laboratories did not apply a specific treatment for stabilization. Therefore it can be assumed that the samples of these laboratories were instable during the timeframe of the proficiency study. However, no statistically significant difference was observed between the results of the laboratories that stored the samples at -20 °C and the results of the laboratories that stored the samples at a temperature below -70 °C. Therefore, also the stability of the samples, even if stored at -70°C can be questioned. Probably, other factors than storage temperature are of influence on the stability. Based on these observations, the correctness of calculated assigned values, which are a consensus values, can be questioned. Therefore, this proficiency study is evaluated for information only.

For all compounds and materials a considerable variation of the reported results is observed, probably caused by the instability of the materials. No statistical relation was observed between the results and the storage temperature, storage time (date of analysis) or a combination of these factors.

Amongst the participating laboratories, five laboratories (005, 008, 011, 015, 019) reported values for CCα. Hence, the minority of participating laboratories is able to report their results as required by Commission Decision 2002/657/EC [25] from the 1st of August 2007. Therefore extra effort is needed regarding the validation of the applied methods for the analysis of penicillins in porcine tissues.

Based on the results, it is concluded that extra effort is needed regarding the analysis of penicillins in porcine tissues. Stability of the compounds seems to be an underestimated factor. It is concluded that extra effort is needed to obtain more information regarding the stability of penicillin in porcine tissues. Next to this, it is recommended to each laboratory to include stability testing of penicillins in porcine tissues in the validation procedure.

Furthermore, it is concluded that the organization of a proficiency study for penicillin analysis in tissues, working with fresh materials is complex. To be able to guarantee stability of the penicillin compounds during storage at each of the participating laboratories stringent guidelines for storage and pretreatment of the samples is necessary.

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Annex 1 Codification of the samples

Sample set	Material M-A	Material M-B	Material K-A	Material K-B
1	M/2006/014	M/2006/020	K/2006/007	K/2006/021
		M/2006/099		K/2006/055
2	M/2006/077	M/2006/107	K/2006/115	K/2006/064
		M/2006/061		K/2006/071
3	M/2006/060	M/2006/062	K/2006/111	K/2006/062
		M/2006/047		K/2006/088
4	M/2006/053	M/2006/003	K/2006/027	K/2006/041
		M/2006/104		K/2006/052
5	M/2006/042	M/2006/118	K/2006/024	K/2006/025
		M/2006/038		K/2006/001
6	M/2006/068	M/2006/058	K/2006/012	K/2006/018
		M/2006/064		K/2006/040
7	M/2006/051	M/2006/033	K/2006/099	K/2006/118
		M/2006/008		K/2006/120
8	M/2006/041	M/2006/109	K/2006/110	K/2006/106
		M/2006/100		K/2006/028
9	M/2006/106	M/2006/056	K/2006/054	K/2006/107
		M/2006/111		K/2006/068
10	M/2006/094	M/2006/036	K/2006/029	K/2006/013
		M/2006/039		K/2006/049
11	M/2006/089	M/2006/044	K/2006/117	K/2006/082
		M/2006/087		K/2006/009
12	M/2006/088	M/2006/019	K/2006/035	K/2006/087
		M/2006/091		K/2006/092
13	M/2006/016	M/2006/067	K/2006/095	K/2006/097
		M/2006/085		K/2006/048
14	M/2006/012	M/2006/079	K/2006/103	K/2006/079
		M/2006/066		K/2006/096
15	M/2006/032	M/2006/046	K/2006/014	K/2006/069
		M/2006/022		K/2006/080
16	M/2006/049	M/2006/070	K/2006/046	K/2006/008
		M/2006/120		K/2006/085
17	M/2006/002	M/2006/054	K/2006/036	K/2006/086
		M/2006/092		K/2006/047
18	M/2006/050	M/2006/017	K/2006/015	K/2006/077
		M/2006/084		K/2006/003
19	M/2006/098	M/2006/018	K/2006/075	K/2006/020
		M/2006/103		K/2006/017
20	M/2006/011	M/2006/021	K/2006/104	K/2006/005
		M/2006/083		K/2006/108
21	M/2006/013	M/2006/001	K/2006/066	K/2006/011
		M/2006/040		K/2006/109

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

	Ampicillin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	6.2	7.1
2	6.7	5.9
3	9.1	7.5
4	5.9	8.1
5	5.6	6.5
6	6.7	6.4
7	6.6	6.0
8	4.9	6.6
9	5.9	6.7
10	6.8	6.0
Grand mean	6.6	
Cochran's test		
C	0.339	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target sd (σp)	Horwitz: 1.44	
s_{an}	0.845	
s_{sam}	0.359	
σ_{all}	0.432	
critical	1.074	
s_{sam}^2 < critical?	ACCEPTED	

No amoxicillin, dicloxacillin, penicillin G, oxacillin and phenoxymethylpenicillin were detected in the samples (< 0.1 MRL µg/kg).

s_{an} = estimate of analytical variance
s_{sam} = estimate of sampling variance
σ_{all} = allowable sampling variance

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

	Cloxacillin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	182	188
2	181	180
3	198	232
4	180	177
5	192	200
6	216	199
7	218	186
8	200	177
9	192	190
10	184	171
Grand mean	192	
Cochran's test		
C	0.352	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target sd (σp)	Horwitz: 39.4	
s_{an}	12.8	
s_{sam}	9.02	
σ_{all}	11.8	
critical	428	
s_{sam}^2 < critical?	ACCEPTED	

No amoxicillin, dicloxacillin, penicillin G, oxacillin and phenoxymethylpenicillin were detected in the samples (< 0.1 MRL µg/kg).

s_{an} = estimate of analytical variance
s_{sam} = estimate of sampling variance
σ_{all} = allowable sampling variance

Annex 2c Statistical evaluation of homogeneity data of material K-B for ampicillin

	Ampicillin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	33.8	31.1
2	30.7	31.3
3	33.9	29.4
4	29.4	30.6
5	32.0	31.2
6	33.4	31.5
7	31.7	33.8
8	32.1	29.9
9	32.1	32.2
10	37.1	31.5
Grand mean	31.9	
Cochran's test		
C	0.423	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target sd (σp)	Horwitz: 7.03	
s_{an}	1.926	
s_{sam}	0.00	
σ_{all}	4.44	
critical	2.11	
s_{sam}^2 < critical?	ACCEPTED	

No amoxicillin, dicloxacillin, oxacillin and phenoxymethylpenicillin were detected in the samples (< 0.1 MRL µg/kg).

s_{an} = estimate of analytical variance
s_{sam} = estimate of sampling variance
σ_{all} = allowable sampling variance

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

	Cloxacillin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	109	112
2	117	115
3	120	114
4	108	108
5	118	117
6	121	117
7	119	116
8	118	115
9	122	117
10	116	111
Grand mean	116	
Cochran's test		
C	0.269	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target sd (σp)	Horwitz: 25.4	
s_{an}	2.59	
s_{sam}	3.25	
σ_{all}	7.62	
critical	116	
s_{sam}^2 < critical?	ACCEPTED	

No amoxicillin, dicloxacillin, oxacillin and phenoxymethylpenicillin were detected in the samples (< 0.1 MRL µg/kg).

s_{an} = estimate of analytical variance
s_{sam} = estimate of sampling variance
σ_{all} = allowable sampling variance

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

	Penicillin G (µg/kg)	
Sample No.	Replicate 1	Replicate 2
1	21.5	21.5
2	21.5	21.5
3	22.6	21.7
4	21.6	20.6
5	22.3	23.5
6	20.8	21.4
7	22.1	23.0
8	22.7	23.0
9	22.5	23.6
10	21.7	22.3
Grand mean	22.1	
Cochran's test		
C	0.237	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target sd (σp)	Horwitz: 4.86	
s_{an}	0.55	
s_{sam}	0.64	
σ_{all}	1.46	
critical	4.30	
s_{sam}^2 < critical?	ACCEPTED	

No amoxicillin, dicloxacillin, oxacillin and phenoxymethylpenicillin were detected in the samples (< 0.1 MRL µg/kg).

s_{an}^2 = estimate of analytical variance
s_{sam}^2 = estimate of sampling variance
σ_{all}^2 = allowable sampling variance

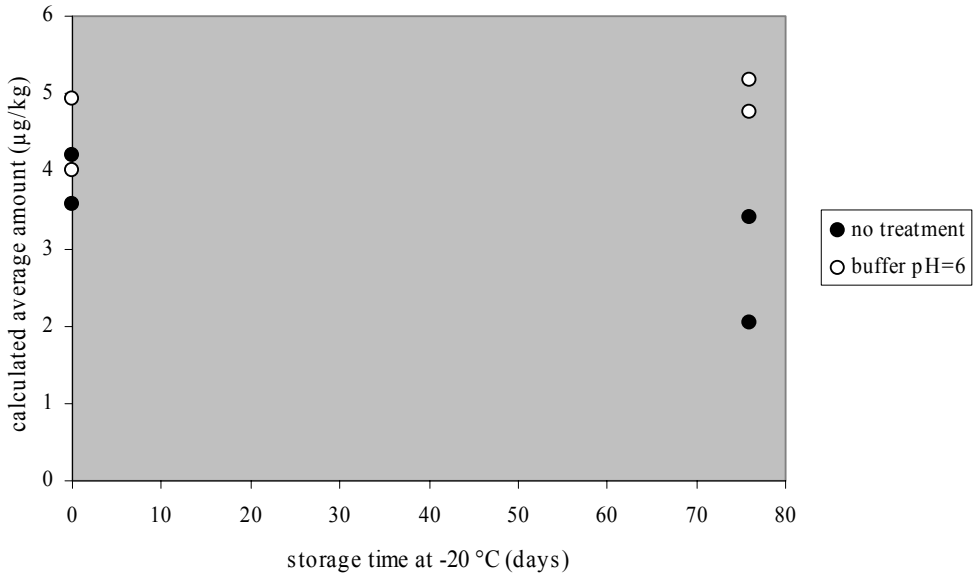
Annex 3a Statistical evaluation of stability data of material M-B for ampicillin

Statistical evaluation for ampicillin in material M-B without treatment

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 23, 2006	0	3.9	2				
Feb 07, 2007	76	2.7	2	0.22	5.4	4.3	NOT ACCEPTED

Statistical evaluation for ampicillin in material M-B after adjustment to pH=6

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 23, 2006	0	4.5	2				
Feb 07, 2007	76	5.0	2	0.31	1.6	4.3	ACCEPTED



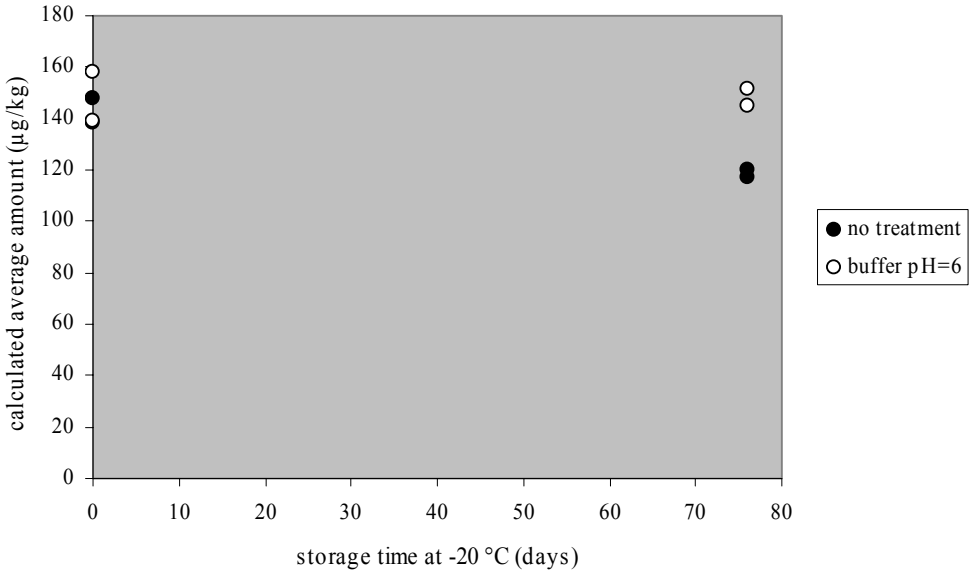
Annex 3b Statistical evaluation of stability data of material M-B for cloxacillin

Statistical evaluation for cloxacillin in material M-B without treatment

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 23, 2006	0	143	2				
Feb 07, 2007	76	105	2	8.9	4.2	4.3	ACCEPTED*

Statistical evaluation for cloxacillin in material M-B after adjustment to pH=6

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 23, 2006	0	149	2				
Feb 07, 2007	76	148	2	10.5	0.10	4.3	ACCEPTED



* Accepted due to the low amount of data. The difference between the amount on t=0 and t=76 is >25%. Therefore the material without treatment can not be characterized as stable.

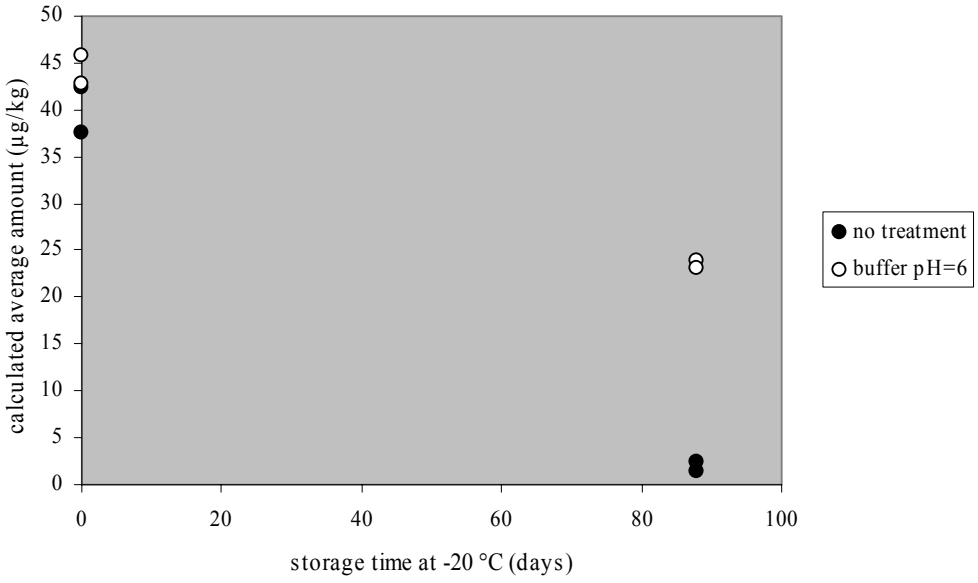
Annex 3c Statistical evaluation of stability data of material K-B for ampicillin

Statistical evaluation for ampicillin in material K-B without treatment

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	39.9	2				
Feb 07, 2007	88	1.9	2	3.78	10.0	4.3	NOT ACCEPTED

Statistical evaluation for ampicillin in material K-B after adjustment to pH=6

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	44.3	2				
Feb 07, 2007	88	23.4	2	2.34	8.9	4.3	NOT ACCEPTED



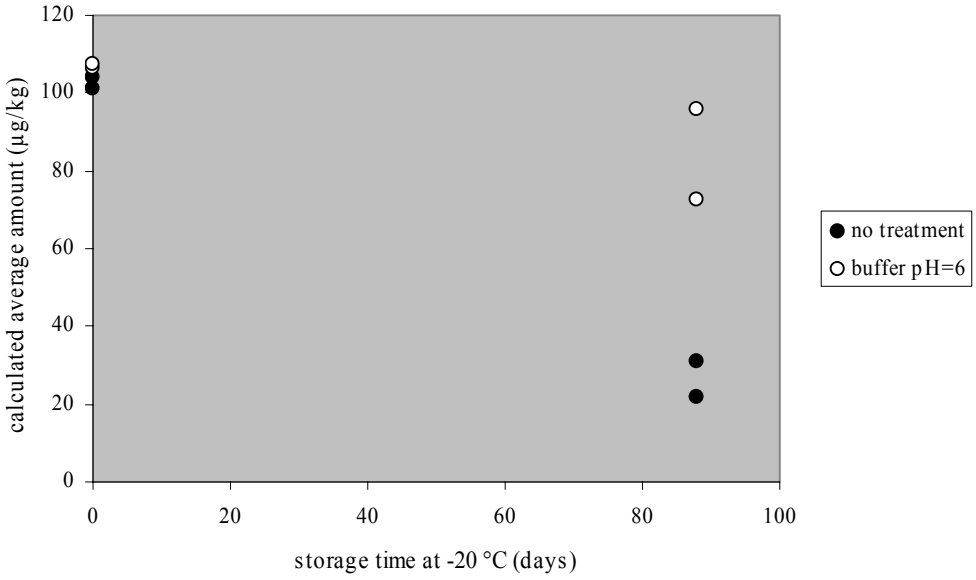
Annex 3d Statistical evaluation of stability data of material K-B for cloxacillin

Statistical evaluation for cloxacillin in material K-B without treatment

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	103	2				
Feb 07, 2007	88	26	2	3.97	19	4.3	NOT ACCEPTED

Statistical evaluation for cloxacillin in material K-B after adjustment to pH=6

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	107	2				
Feb 07, 2007	88	84	2	5.1	4.5	4.3	NOT ACCEPTED



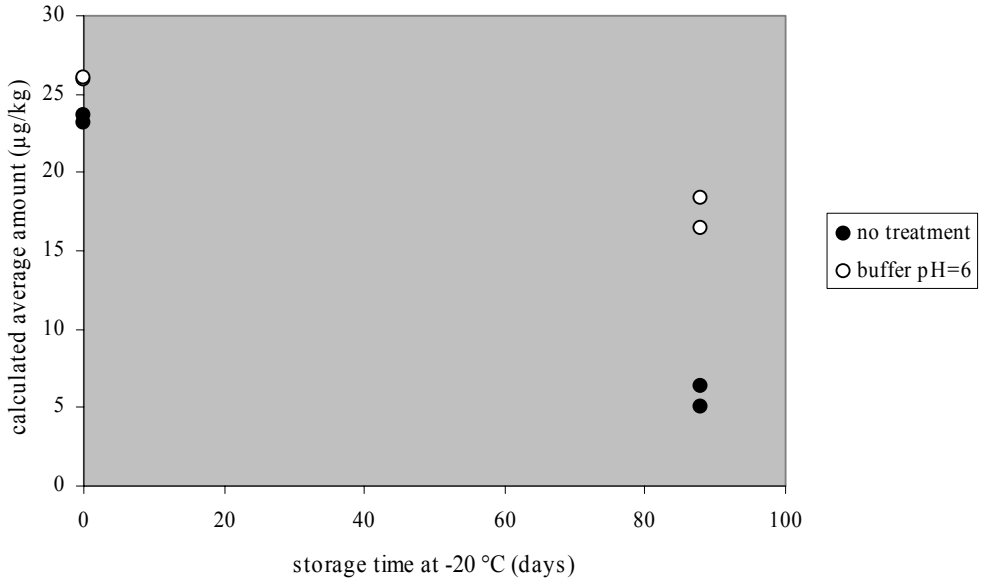
Annex 3e Statistical evaluation of stability data of material K-B for penicillin G

Statistical evaluation for penicillin G in material K-B without treatment

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	23.5	2				
Feb 07, 2007	88	5.8	2	0.87	20.3	4.3	NOT ACCEPTED

Statistical evaluation for penicillin G in material K-B after adjustment to pH=6

Date of storage at -80 °C	Time at -20°C (days)	Average amount (µg/kg)	n	Pooled st. dev (µg/kg)	t	tcrit	t < tcrit
Nov 11, 2007	0	26.0	2				
Feb 07, 2007	88	17.4	2	1.13	7.62	4.3	NOT ACCEPTED



Annex 4 Overview of the applied methods

Lab code	Stabilization / storage	Extraction	Sample purification	Derivatization	Internal standard	Detection method	Penicillins not analysed for
3	-70 °C	Phosphate buffer (pH=8), hexane	SPE (C_{18}), elution with H_2O/ACN (1:1)	-	-	LC-MS/MS	-
4	-18 °C	Phosphate buffer, hexane	SPE (C_{18}), elution with H_2O/ACN (1:1)	-	$^{13}C_2$-Pen G	LC-MS/MS	-
5	-70 °C	Phosphate buffer (pH=8), iso-octane	SPE (C_{18}), elution with Phosphate buffer/ACN (1:1), derivatization	Benzoic acid anhydride, triazole, $HgCl_2$, pH=9, 10 min, 65 °C	-	LC-DAD	-
6	-80°C	H_2O/ACN (1:9), evaporation of solvent, add phosphate buffer (pH=8.5)	SPE (OASIS HLB), elution with ACN/H_2O (7:3), partial evaporation of solvent	-	-	LC-MS/MS	-
7	-18°C	ACN/H_2O (4:1)	$RP_{18,}$ evaporation of solvent, filter 0.45 µm	-	-	LC-MS/MS	-
8	-70 °C	Phosphate buffer (pH=9), hexane	SPE (C_{18}), elution with MeOH, partial evaporation of solvent	-	-	LC-MS/MS	-
9	-20 °C	ACN/acetone (7:3)	Evaporation of solvent, reconstitution, ultrafiltration (30 kD)	-	sulfamethazin	LC-MS/MS	-
10	-80 °C	Screening: DSM Confirmatory: ACN	Evaporation of solvent, reconstitution	-	Pen V	LC-MS/MS	Pen V
11	-80 °C	ACN	Evaporation of solvent, reconstitution, filter 0.45 µm	-	Pen V	LC-MS/MS	
13	-89 °C	Phosphate buffer (pH=8.5), ios-octane	SPE (C_{18}), elution with ACN/H_2O (1:1)	-	-	LC-MS/MS	-
14	-80 °C	Screening: Ec 6-plate test	-	-	-	Plate test	-
15	Buffer pH=6, -80 °C	Phosphate buffer (pH=8), derivatization	SPE (OASIS HLB), elution with H_2O/methanol (1:4), evaporation of solvent, reconstitution	Piperidin, phosphoric acid, 5 min, 85 °C	Piperidinpenicillins-d_{10}	LC-MS/MS	-
16	-20 °C	ACN, petrolium ether	Partial evaporation of solvent, freezing, microfiltration	-	methicillin	LC-MS/MS	-
17	-80 °C	ACN	Kidney: none Muscle: SPE (OASIS HLB)	-	nafcillin	LC-MS/MS	Amoxycillin, Oxacillin

18	-85 °C	Phosphate buffer (pH=8.2)	SPE (OASIS HLB), elution with MeOH, evaporation of solvent, reconstitution	-	-	UPLC-MS/MS	-
19	-70 °C	Phosphate buffer (pH=8), hexane	SPE (C_{18}), elution ACN, evaporation of solvent, reconstitution	-	Pen V	LC-MS/MS	Pen V
20	-80 °C	Muscle: Phosphate buffer Kidney: sulphuric acid, sodium wolframate	SPE (C_{18})	-	Pen V	LC-MS/MS	-
21	-70 °C	Methanol/H_2O (85:15), dilution	SPE (C_{18})	-	-	LC-MS/MS	Cloxacillin, Dicloxacillin, Oxacillin, Pen V

NM = not mentioned

Annex 5 Overview of method characteristics as reported by the participants

	Muscle					Kidney						
		Ampicillin		Cloxacillin			Ampicillin		Cloxacillin		Penicillin G	
Lab code	Validation / accreditation	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)	Validation / accreditation	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)	CCα (µg/kg)	CCß (µg/kg)
3	No / Yes		50		300	No / Yes		50		300		50
4	Yes / Yes					Yes / Yes						
5	Yes / Yes	72	82	310	326	No / No						
6	No / No					No / No						
7	Yes / Yes					Yes / Yes						
8	Yes / Yes	62	74	357	416	Yes / Yes	62	74	386	473	60	70
9	No / Yes					No / Yes						
10	On going / No					On going / No						
11	On going / No	53	56.1	320.4	340.8	On going / No	57.8	65.6	365.0	429.9	65.0	80.0
13	Yes / Yes					Yes / Yes						
14												
15	Yes / Yes	56	62	324	353	Yes / Yes	55	59	349	379	58	63
16	On going / Yes					On going / Yes						
17	No / No					No / No						
18	No / No					No / No						
19	Yes / Yes	54.9	59.8	324.3	348.5	Yes / Yes	54.9	59.8	324.3	348.5	35.6	57.2
20	No / No					No / No						
21	Yes / Yes		< 50		< 300	Yes / Yes		< 50		< 300	58	67

	Muscle					Kidney						
		Ampicillin		Cloxacillin			Ampicillin		Cloxacillin		Penicillin G	
Lab code	Validation / accreditation	LoD (µg/kg)	LoQ (µg/kg)	LoD (µg/kg)	LoQ (µg/kg)	Validation / accreditation	LoD (µg/kg)	LoQ (µg/kg)	LoD (µg/kg)	LoQ (µg/kg)	LoD (µg/kg)	LoQ (µg/kg)
3	No / Yes	0.5	1.0	2.0	4.0	No / Yes	0.5	1.0	2.0	4.0	0.5	1.0
4	Yes / Yes	2	5	5	10	Yes / Yes	2	5	5	10	2	5
5	Yes / Yes	6	21	10	32	No / No						
6	No / No	2	10	5	10	No / No	2	10	5	10	5	15
7	Yes / Yes	1.3	7.5	1.3	7.5	Yes / Yes	5.0	19	3.0	11	2.0	8.0
8	Yes / Yes					Yes / Yes						
9	No / Yes	1	1.5	1	1.5	No / Yes	1	1.5	1	1.5	1	1.5
10	On going / No		25		150	On going / No		25		150		25
11	On going / No	<1	25	~2	150	On going / No	2	25	~2	150	~2	25
13	Yes / Yes	5	10	5	10	Yes / Yes	5	10	5	10	5	10
14												
15	Yes / Yes	5		15		Yes / Yes	5		15		5	
16	On going / Yes	0.5	0.5	0.5	0.5	On going / Yes	0.5	0.5	0.5	0.5	0.5	0.5
17	No / No		5		5	No / No		5		5		5
18	No / No					No / No						
19	Yes / Yes		25		150	Yes / Yes		25		150		25
20	No / No					No / No						
21	Yes / Yes					Yes / Yes					3.4	10

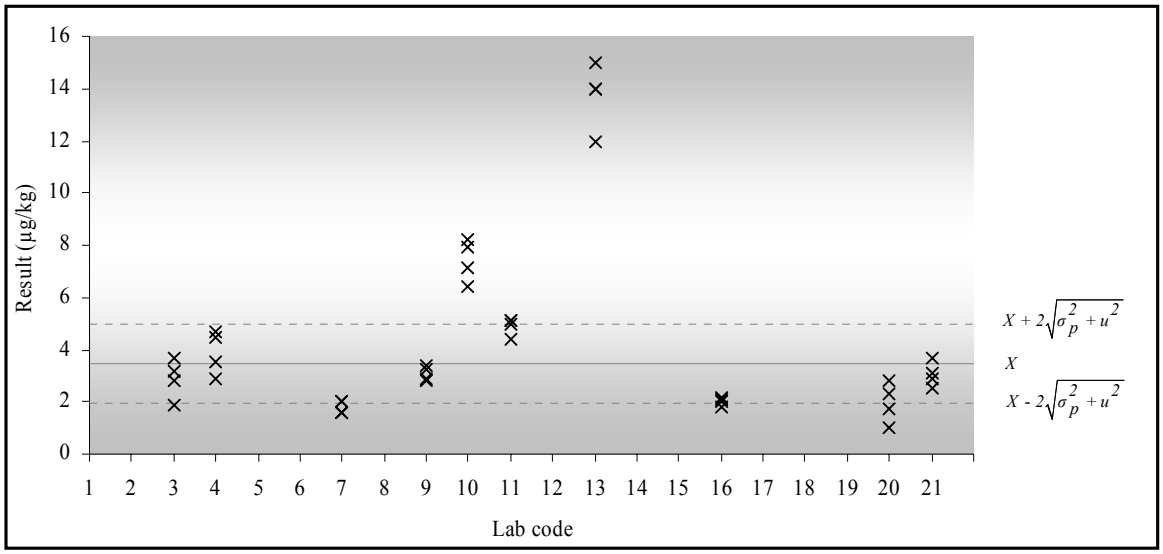
Annex 6a Results for the analysis of ampicillin in muscle (material M-B)

Ampicillin									
Assigned value: 3.4 µg/kg									
Uncertainty of assigned value: 0.5 µg/kg									
Target standard deviation (Horwitz, Thompson): 0.75 µg/kg									
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_{R_L}	z_a'-score	HORRAT
2									
3	2.8	3.2	3.7	1.9	2.9	0.75	0.75	-0.58	1.00
4	3.5	2.9	4.5	4.7	3.9	0.26	1.01	0.52	1.33
5									
6	<10	<10	<10	<10					
7	1.6	1.6	2.0	2.0	1.8	0.00	0.28	-1.80	0.37
8	<25	<25	<25	<25					
9	3.3	3.4	2.9	2.8	3.1	0.06	0.34	-0.39	0.45
10	7.1	7.9	6.4	8.2	7.4	0.80	0.80	4.38	1.07
11	5.1	4.4	5.0	5.1	4.9	0.29	0.29	1.62	0.39
12									
13	14.0	15.0	12.0	14.0	13.8	0.91	1.24	11.40	1.65
14									
15	<12.5	<12.5	<12.5	<12.5					
16	1.8	2.1	2.1	2.1	2.0	0.11	0.16	-1.56	0.21
17									
18									
19	<25	<25	<25	<25					
20	1.7	2.8	1.0	2.3	2.0	0.70	0.70	-1.63	0.92
21	2.5	2.9	3.7	3.1	3.1	0.29	0.54	-0.42	0.71

Results are for information only.

Annex 6a Results for the analysis of ampicillin in muscle (material M-B) (continued)

Figure a: Graphical representation of the reported results



Annex 6b Results for the analysis of cloxacillin in muscle (material M-B)

Cloxacillin									
Assigned value: 135 µg/kg									
Uncertainty of assigned value: 9.5 µg/kg									
Target standard deviation (Horwitz, Thompson): 29.2 µg/kg									
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_{R_L}	z_a'-score	HORRAT
2									
3	142.0	159.0	110.0	118.0	132.3	7.67	7.67	-0.10	0.90
4	351.0	341.0	390.0	338.0	355.0	21.62	21.62	7.15	0.74
5	136.1	141.7	120.3	123.2	130.3	2.57	2.57	-0.16	0.42
6	126.5	127.2	118.2	120.7	123.2	1.06	1.06	-0.39	0.18
7	102.0	105.0	119.0	112.0	109.5	3.11	3.11	-0.84	0.30
8	201.0	205.0	165.0	191.0	190.5	10.74	10.74	1.80	0.66
9	135.0	127.0	121.0	118.0	125.3	3.49	3.49	-0.33	0.29
10	148.9	166.3	111.5	121.2	137.0	8.13	8.13	0.06	1.02
11	176.0	158.0	188.0	167.0	172.3	11.29	11.29	1.20	0.39
12									
13	160.0	170.0	230.0	240.0	200.0	5.77	5.77	2.11	1.70
14									
15	126.0	138.0	136.0		133.3	55.74	55.74	-0.06	
16	84.3	79.2	92.1	80.5	84.0	5.17	5.17	-1.67	0.18
17	80.0	81.0	100.0	99.0	90.0	0.58	0.58	-1.47	0.46
18	72.6	80.2	129.9	141.0	105.9	5.49	5.49	-0.96	1.43
19	<150	<150	<150	<150	<150				
20	161.8	144.5	121.4	143.6	142.8	11.49	11.49	0.25	0.57
21									

Results are for information only

Annex 6b Results for the analysis of cloxacillin in muscle (material M-B) (continued)

Figure a: Graphical representation of the reported results

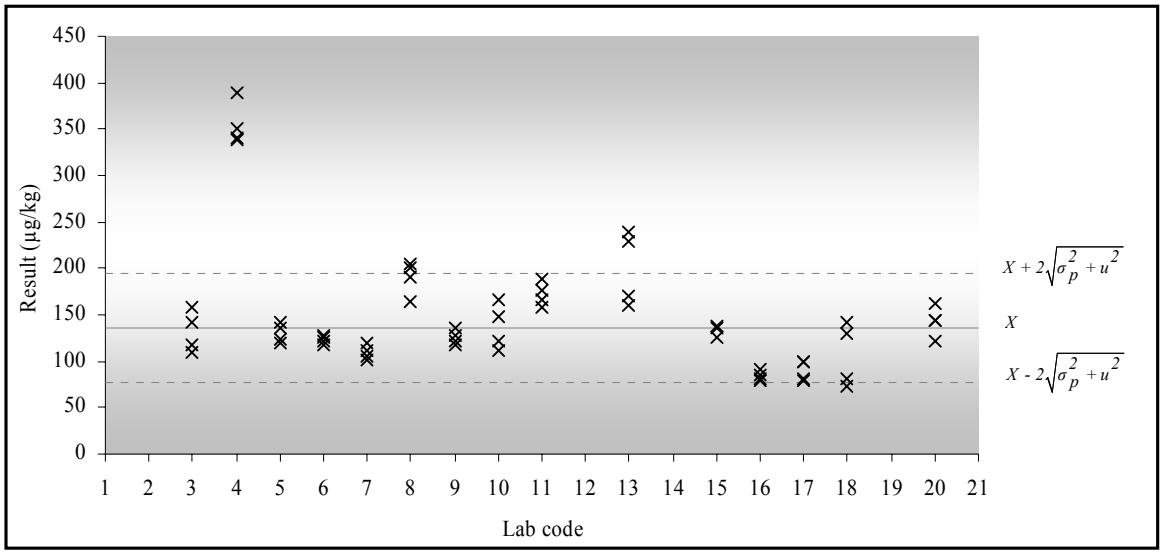


Figure b: Graphical representation of z_a'-score

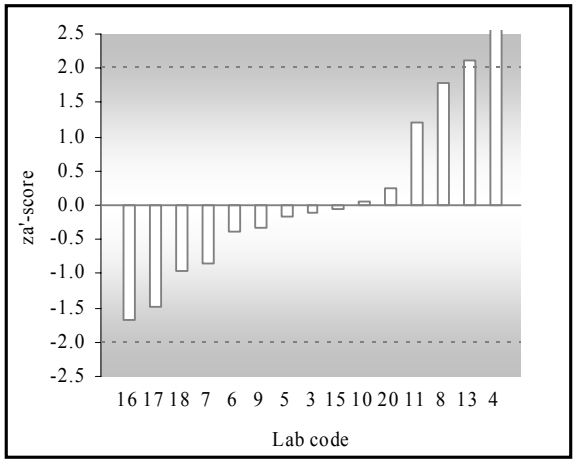
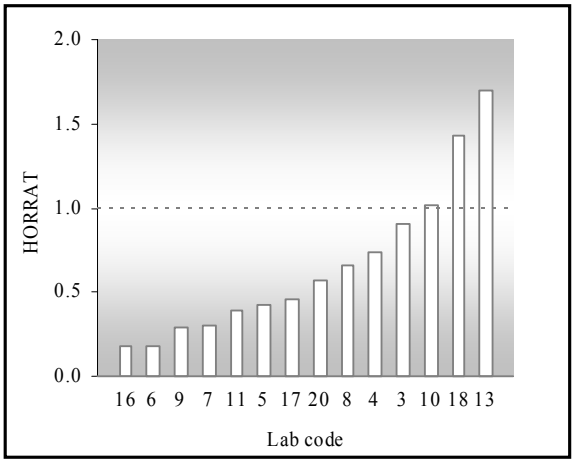


Figure c: Graphical representation of HORRAT



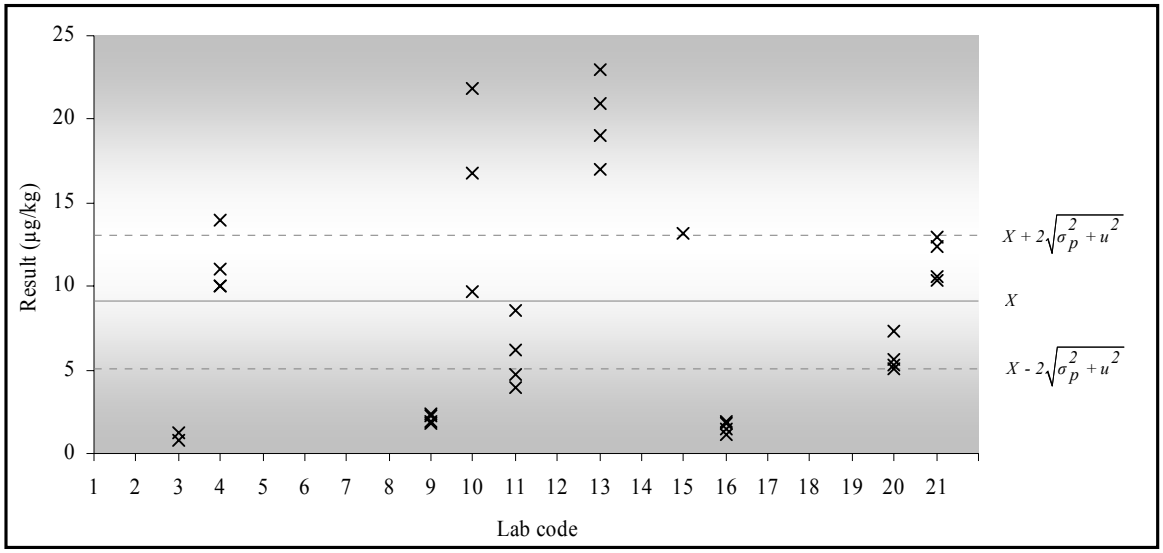
Annex 6c Results for the analysis of ampicillin in kidney (material K-B)

Ampicillin							
Assigned value: 9.1 µg/kg							
Uncertainty of assigned value: 2.5 µg/kg							
Target standard deviation (Horwitz, Thompson): 2.0 µg/kg							
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_{R_1}
2							
3			0.8	1.2			
4	11.0	10.0	10.0	14.0	11.3	1.68	1.68
5							
6	<10	<10	<10	<10			
7							
8	<25	<25	<25	<25			
9	2.3	2.2	1.8	2.0	2.1	0.08	0.29
10	16.8	9.7	21.9		16.1	9.40	9.40
11	3.9	4.7	8.6	6.2	5.9	1.03	2.31
12							
13	17.0	19.0	21.0	23.0	20.0	1.15	2.94
14							
15	<12.5	13.2	<12.5	<12.5	13.2		
16	1.9	1.8	1.1	1.5	1.6	0.13	0.41
17							
18							
19	<25	<25	<25	<25			
20	5.3	5.1	7.3	5.6	5.8	0.70	1.01
21	12.4	10.6	13.0	10.4	11.6	1.29	1.29

Results are for information only

Annex 6c Results for the analysis of ampicillin in kidney (material K-B) (continued)

Figure a: Graphical representation of the reported results



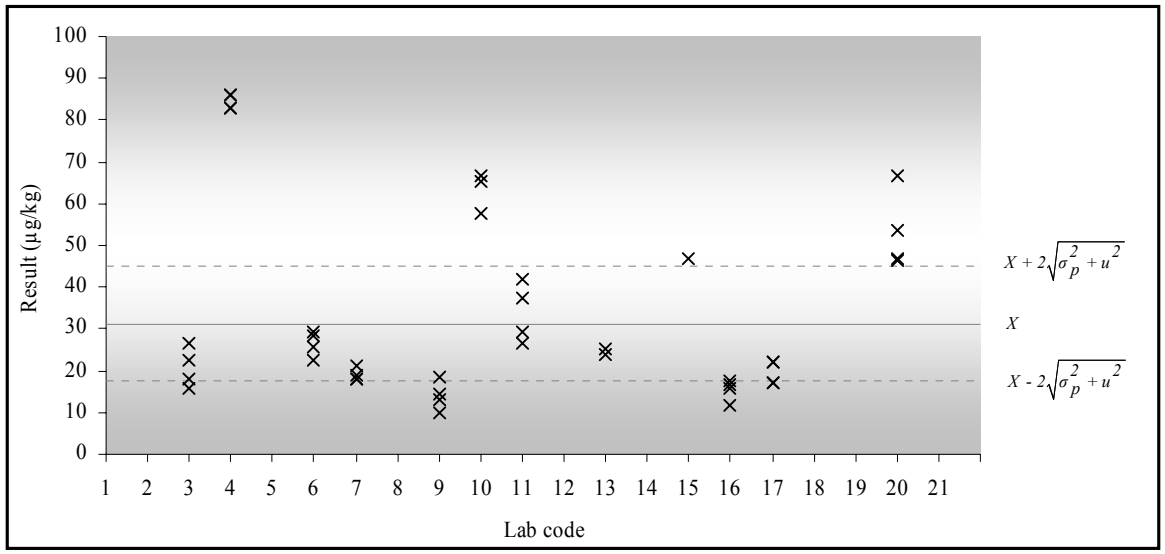
Annex 6d Results for the analysis of cloxacillin in kidney (material K-B)

Cloxacillin							
Assigned value: 31.3 µg/kg							
Uncertainty of assigned value: 5.2 µg/kg							
Target standard deviation (Horwitz, Thompson): 6.9 µg/kg							
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_{R_L}
2							
3	26.4	17.9	22.6	15.8	20.7	4.44	4.44
4	86.0	83.0	86.0	83.0	84.5	1.73	1.73
5							
6	29.2	28.2	25.5	22.3	26.3	1.37	3.53
7	18.0	19.0	18.0	21.0	19.0	1.29	1.29
8	<150	<150	<150	<150			
9	18.6	14.3	9.9	12.9	13.9	2.15	3.89
10	57.6	65.2	66.8		63.2	27.45	27.45
11	26.4	29.2	41.7	37.6	33.7	2.03	8.50
12							
13	<10	<10	24.0	25.0			
14							
15	47.0	<30	<30	<30	47.0		
16	17.5	16.8	11.6	15.6	15.4	1.66	2.77
17	22.0	22.0	17.0	17.0	19.5	0.00	3.54
18							
19	<150	<150	<150	<150			
20	46.9	46.4	66.6	53.8	53.4	5.23	10.27
21							

Results are for information only

Annex 6d Results for the analysis of cloxacillin in kidney (material K-B) (continued)

Figure a: Graphical representation of the reported results



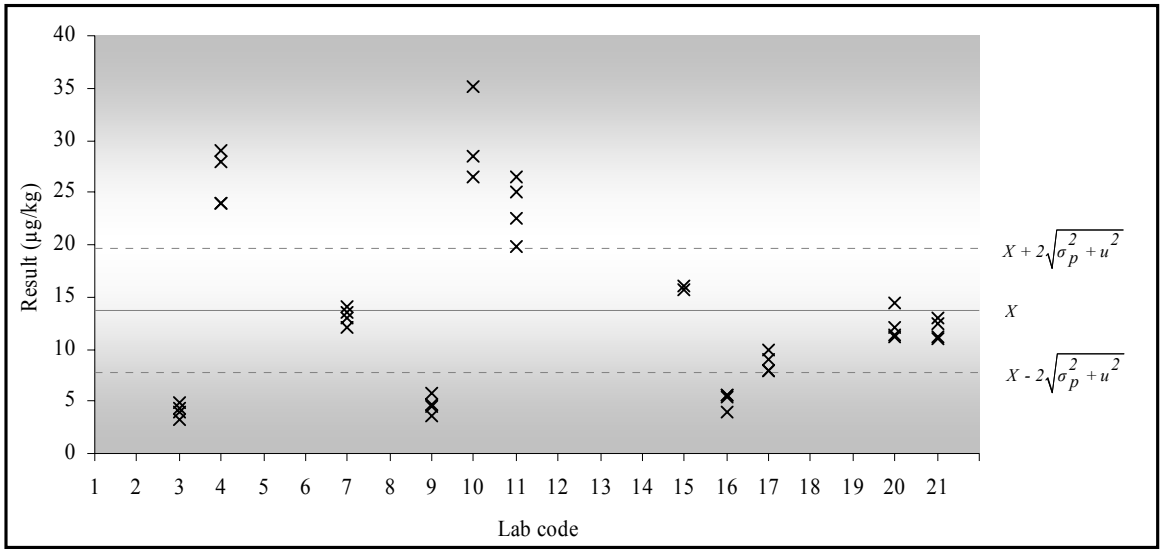
Annex 6e Results for the analysis of penicillin G in kidney (material K-B)

Penicillin G							
Assigned value: 13.7 µg/kg							
Uncertainty of assigned value: 3.2 µg/kg							
Target standard deviation (Horwitz, Thompson): 3.0 µg/kg							
Code	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average	s_r	s_{R_L}
2							
3	4.3	4.0	3.3	4.8	4.1	0.62	0.62
4	24.0	29.0	28.0	24.0	26.3	2.61	2.61
5							
6	<15	<15	<15	<15	<15		
7	12.0	13.0	13.5	14.0	13.1	0.46	0.94
8							
9	5.7	4.6	3.6	4.5	4.6	0.54	0.87
10	28.4	26.5	35.2		30.0	14.39	14.39
11	19.8	22.6	26.4	25.0	23.5	1.28	3.31
12							
13	<10	<10	<10	<10			
14							
15			15.7	16.0			
16	5.7	5.5	4.0	5.3	5.1	0.57	0.78
17	10.0	9.0	8.0	8.0	8.8	0.41	1.10
18							
19	<25	<25	<25	<25			
20	12.1	11.2	14.4	11.3	12.3	1.32	1.32
21	11.2	11.0	12.9	12.5	11.9	0.18	1.14

Results are for information only

Annex 6e
Results for the analysis of penicillin G in kidney (material K-B) (continued)

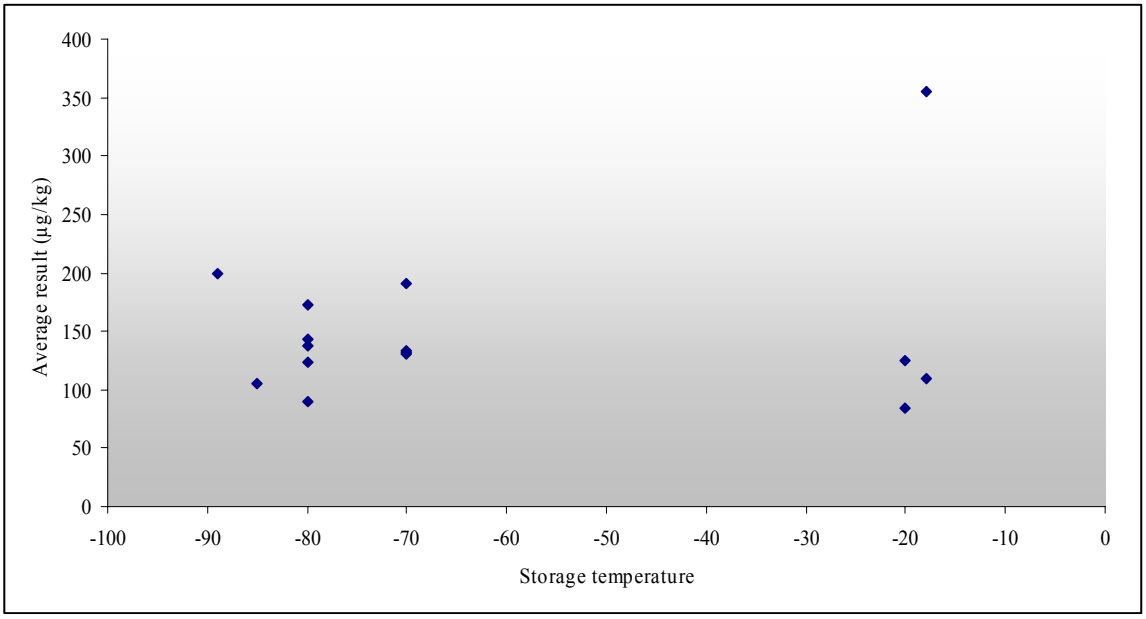
Figure a: Graphical representation of the reported results



Annex 7
Storage temperature versus laboratory results for cloxacillin in muscle (material M-B)

Cloxacillin		
Assigned value: 31.3 µg/kg		
Code	Storage temperature (°C)	Average result (µg/kg)
2		
3	-70	132.3
4	-18	355.0
5	-70	130.3
6	-80	123.2
7	-18	109.5
8	-70	190.5
9	-20	125.3
10	-80	137.0
11	-80	172.3
12		
13	-89	200.0
14		
15	-70	133.3
16	-20	84.0
17	-80	90.0
18	-85	105.9
19		
20	-80	142.8
21		

Figure a: Graphical representation of the average result versus the storage temperature



Annex 8
Date of analysis versus laboratory results for cloxacillin in muscle (material M-B)

Cloxacillin		
Assigned value: 31.3 µg/kg		
Code	Date of analysis	Average result (µg/kg)
2		
3	19 January 2007	132.3
4	19 December 2006	355.0
5	09 January 2007	130.3
6	22 January 2007	123.2
7	19 January 2007	109.5
8	31 January 2007	190.5
9	19 January 2007	125.3
10	10 January 2007	137.0
11	22 January 2007	172.3
12		
13	29 January 2007	200.0
14		
15	11 January 2007	133.3
16	11 January 2007	84.0
17	25 January 2007	90.0
18	09 January 2007	105.9
19		
20	04 January 2007	142.8
21		

Figure a: Graphical representation of the average result versus the date of analysis