Antibiotics

Keeping a close eye on validation!

- Are the tests you are using well validated?
- How do you recognize a reliable validation?
- How can you perform validation in accordance with the CRL Guideline?

More information:

https://r-b.io/antibio

R-Biopharm • An der neuen Bergstraße 17, 64297 Darmstadt, Germany • E-mail: sales@r-biopharm.de • food.r-biopharm.com

Antibiotics – keeping a close eye on validation!

Why test for antibiotics?

Dangers arise from residues like antibiotics, hormones and toxins for every consumer. These residues pose a risk to consumer health because of their **toxicity** and side effects. Furthermore, they can lead to allergic reactions and increase the promotion of bacterial resistance (e.g. multi-drug resistant bacteria).
On the other side, the presence of residues has **consequences** especially for the food producing and exporting industry.

In case residues are found in food, these products are destroyed and the Rapid Alert System RASFF is activated. This can lead to an exclusion of products from the import into e.g. the EU and will result in more screening of food.

Note

- Toxicity of residues
- Financial loss for food industry

How are antibiotics regulated?

In order to keep our food free from harmful substances, residues are regulated worldwide. Very sensitive tests have been developed in the recent years for this purpose. For many substances, a **Maximum Residue Level (MRL)** was set by authorities. This level was defined, based on the currently available data regarding toxicity. Some substances were banned, meaning their use is completely forbidden. Unfortunately, these substances are still applied in the food production. Therefore, **Minimum Required Performance Levels (MRPL)** of the test system were set by authorities. These define the minimum performance of the test systems.
If the MRPL is for example 500 ng/kg, it means that the test must be able to detect and confirm the residue at this level. In other words, the MRPL is a value that describes, how good a test has to be for the detection of that residue. For harmful residues, the requirements have become more strict. This is currently the case for the nitrofurans (in food) and chloramphenicol (in food and feed).

In the Commission Regulation (EU) 2019/1871 and the US Food and Drug Administration (Program 7304.018) the MRPLs were lowered to the values as described in table 1. Furthermore, the metabolite of nifursol (DNSH) was added to the list.

Table 1: MRPLs for nitrofuran metabolites and chloramphenicol

Substance	New MRPL
Chloramphenicol	150 ng/kg
AOZ = metabolite of furazolidone	500 ng/kg
AMOZ = metabolite of furaltadone	500 ng/kg
SC/SEM = metabolite of nitrofurazone	500 ng/kg
AHD = metabolite of nitrofurantoin	500 ng/kg
DNSH = metabolite of nifursol	500 ng/kg

Note

- Maximum Residue Level (MRL) in food
- Minimum Required Performance Levels (MRPL) for test systems
- New MRPLs for nitrofurans and chloramphenicol

Antibiotics – keeping a close eye on validation!

Validation

At R-Biopharm the validation of the tests is performed with three pilot batches. The summary of the most important results (limit of detection (LOD), the detection capability (CCß), the recovery and the cross-reactivity) can be found in the manual. Further information is given in the validation reports.

Limit of detection (LOD)

To illustrate the dimension of a concentration of 500 ng/kg, we can compare it to 500 sugar cubes, which are distributed in a lake of 2.7 billion liters.

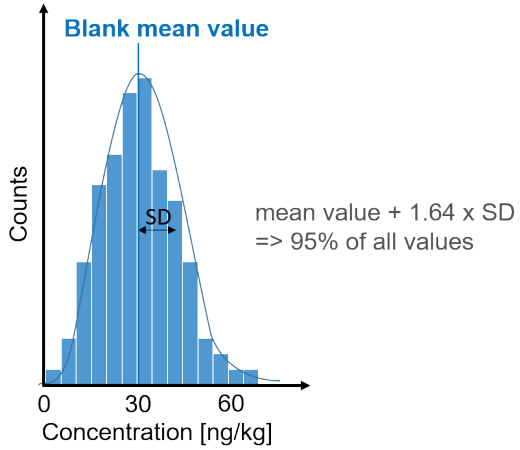
The test has to be very sensitive to detect this little amount of residues without being influenced by interfering organic and inorganic substances from the matrix and solvents.

In the first step, the background of the test needs to be measured in the desired matrix. It is very important to stress that this has to be performed in the matrix of interest, as inhibiting substance may vary from matrix to matrix. These inhibiting substances have a negative effect on the signal-to-noise ratio, resulting in a less sensitive test. The measured LOD values based on buffer samples is much lower, as these are “clean” and will not contain any substances that will interfere with the test!

As an example, 120 **shrimp blank samples** were analysed. The measured signals are used to calculate the concentrations.

For illustration, these concentrations are plotted in a histogram (see figure 1).
In this example the mean value is 30 and the measured concentrations scatter around this value. A probability function describes the frequency

Fig. 1: Histogram of determined concentration of blank shrimp samples. For illustration, a probability function is shown. The mean value provides the blank.



Antibiotics – keeping a close eye on validation!

(counts) of the measured concentration. The more the values scatter around the mean value, the bigger the width of the probability function is. Given by the fundamentals of statistics, the confidence interval of the mean value plus 1.64 times the standard deviation (SD) includes 95 % of all values.

Note

- LOD is determined by measuring blank samples
- Different samples will give (slightly) different results
- LOD depends on the matrix

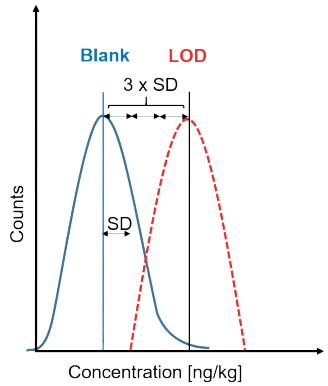
The limit of detection (LOD) is the lowest quantity of a residue that can be distinguished from the blank value with a confidence of approximately 95 %. As approximation, the LOD is calculated as mean value of 20 blank samples + 3 x SD (as described in figure 2).

The LOD is depending on the matrix and is only an estimation, because the measurement is performed at the lowest limit of the testsystem. No analyte is present!

Note

LOD = mean value + 3 x standard deviation (SD)

Fig. 2: Probability function of the blank and the calculated LOD according to the equation LOD = mean value + 3 x SD.



Detection capability (CCß)

The use of a screening method should assure a low rate of false-negative samples. A low rate of false-positive samples is acceptable, because these samples will be retested using a confirmatory method. But false-negative samples would slip through the net!

When an ELISA is used as a screening method, it is important to know at which concentration an unknown sample is positive with a certain probability.

This concentration is called detection capability or CCβ. All concentrations, which are equal or higher than this concentration indicate a positive sample. As shown in figure 3, the CCβ usually is below the official limits.

For example, the MRPL for a nitrofuran is 500 ng/kg. It means that the ELISA has to have a LOD of maximum 500 ng/kg. However, the nitrofuran is completely forbidden and should not be present in the sample at all.

Antibiotics – keeping a close eye on validation!

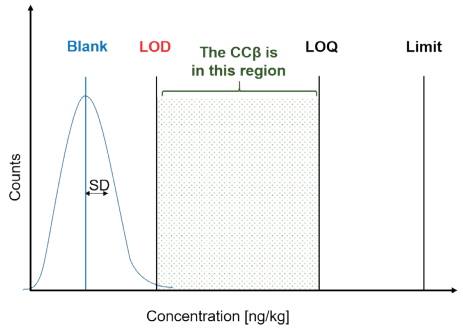
Therefore, it is important to know at which concentration it is unlikely (< 5 %) to have false-negative results. False-negative results are dangerous, because they are not observed by the tester and lead to the export of contaminated food. Authorities or incoming controls might check this food and find out that it is positive and activate the rapid alert system (RASFF). Therefore, the CCβ gives the tester the certainty that no positive samples are overlooked.

If the CCß was confirmed to be 350 ng/kg, then all shrimp samples which show a concentration larger than 350 ng/kg, have to be handled as positive sample.

Note

- Which concentration of antibiotics can be detected for sure? Test under real conditions!
- At and above the concentration of CCß, the probability to have false-negative result is < 5 %.
- Avoiding the rapid alert system (RASFF)

Fig. 3: Exemplary representation of the blank, the LOD and the LOQ in relation to the official limit. The range at which the CCßβis expected is marked (green dots).



In order to prove the ability of the test system, samples containing the analyte at a defined concentration have to be analyzed. The aim is to identify the lowest concentration, at which the smallest content of the residue may be detected with the error probability of 5 % (β-error or false-negative rate).

How to determine the CCß?

At R-Biopharm we choose a graphical approach to visualize the CCβ (see figure 4). At least 20 blank samples and 20 spiked samples are analyzed. The expected CCβ value is approximated after the finished LOD calculation (mean value + 6 x SD). A number of at least 20 samples is chosen, because a β error (false-negative rate) of 5 % would mean that for one out of 20 samples it is not possible to distinguish the blank from the spiked sample. It is important to mention that the blank sample and the corresponding spiked sample are a pair in the plot. Meaning that one sample is measured as blank and from the same sample, the spiked sample is prepared.

There are two different options to show the confirmation of the CCβ graphically:

1. Plotting the B/B_0 values of the blank and spiked samples.
2. Plotting the calculated concentrations of the blank and spiked samples.

Antibiotics – keeping a close eye on validation!

Option 1:

In case one (see figure 4), the B/B_0 values are directly plotted. The B/B_0 values, corresponding to the blank samples (see the blue dots in figure 4) scatter around 90 %. The B/B_0 values corresponding to the spiked values scatter around 60 %. The blank and the spiked samples can be distinguished easily. Thus, the CCβ value is confirmed and the minimal CCβ value would be even lower than 25 ng/kg. With further experiments the CCß value could be even decreased.

Option 2:

It is also possible to convert the B/B_0 values into a concentration first, by using a software like the RIDASOFT® Win.NET. The CCβ value is confirmed as well and the calculated concentrations are shown in the same graph. However, option 1 is a more general approach, which leaves out the calculation of the concentration.

Fig. 4: Comparison of the B/B_0 values of blank samples (blue) and spiked samples (red) at a spiking level of 25 ng/kg. There is no overlap of data points of blank and spiked samples. Thus, the CCβ is confirmed.

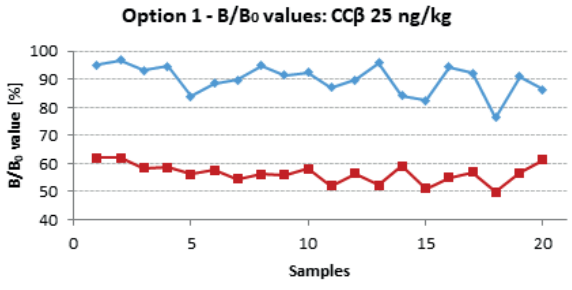
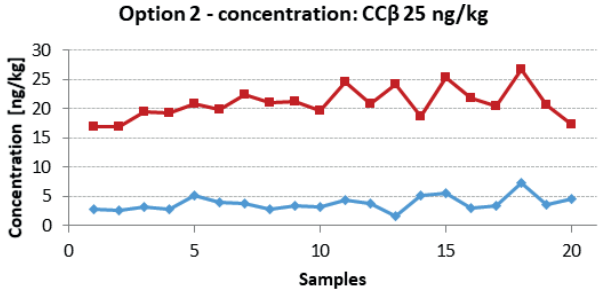


Fig. 5: Comparison of the calculated concentration of blank samples (blue) and spiked samples (red) at a spiking level of 25 ng/kg. There is no overlap of data points of blank and spiked samples. Thus, the CCβ is confirmed.



Antibiotics – keeping a close eye on validation!

Validation according to the Community Reference Laboratories Residues (CRL) Guidelines

In case of nitrofuran metabolites, for example, the new MRPL is 500 ng/kg. The validation according to the CRL Guidelines defines the number of samples required for the validation of the CCβ. In general, the lower the chosen CCβ in comparison with the MRPL, the fewer replicates are required. The aim is to correctly identify truly contaminated samples and to demonstrate that the CCβ is less than the MRPL. For example:

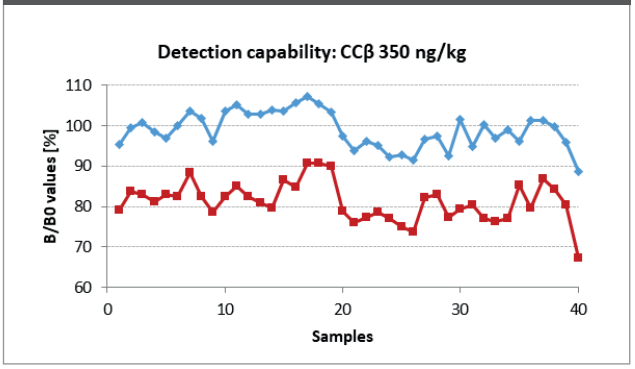
If the screening target concentration is up to 50 % of the MRPL = maximum 250 ng/kg, a number of at least 20 blank and spiked samples have to be analyzed. The occurrence of one or no false-compliant is enough to confirm this CCβ value.

If the screening target concentration is set between 50 % and 90 % of the MRPL = 250 - 450 ng/kg, a number of at least 40 blank and spiked samples have to be analyzed. The occurrence of no more than two false-compliant is enough to confirm this CCβ value. Please see figure 6 as example.

If the screening target concentration is set between 90 % and 100 % of the MRPL = 450 - 500 ng/kg, a number of at least 60 blank and spiked samples have to be analyzed. The occurrence of no more than three false-compliant is enough to confirm this CCβ value.

As these studies take a lot of time, they can be performed over several days. Furthermore, one matrix can be divided into different species (meat = pork, bovine, poultry).

Fig. 6: Comparison of the B/B_0 values of blank samples (blue) and spiked samples (red) at a spiking level of 350 ng/kg. There is no overlap of data points of blank and spiked samples. Thus, the CCß is confirmed.



Note

CRL Guidelines to confirm the CCβ:

- CCβ = up to 50 % MRPL -> 20 samples per matrix
- CCβ = 50 - 90 % MRPL -> 40 samples per matrix
- CCβ = 90 - 100 % MRPL -> 60 samples per matrix

Antibiotics – keeping a close eye on validation!

Recovery

The recovery is the percentage of the true concentration of a substance recovered during the analytical procedure. In other words, if a sample is spiked to a certain concentration, the recovery shows, how much of the residue can be found by the test. The recovery is depending on the matrix, sample preparation and the spike concentration. If possible, it is advisable to choose a concentration in the middle of the standard curve.
Furthermore, the recovery is depending on the cross-reactivity of the antibody, which was used in the test system. The antibodies have a different specificity for structural similar residues.

If the antibody binds to the target residue and equally to similar residues, then false-positive results can occur. These test systems are well suited as screening for a group of substances, but not for the quantification of one residue.

Note

- How much of spiked antibiotics can be found by the test!
- The recovery is the percentage of the true concentration of a substance recovered during the analytical procedure.

Conclusion

In order to keep our food free from harmful residues, sensitive test systems are necessary. During the development, three pilot batches are extensively validated. The summary of the most important results can be found in the manual, which is the limit of detection (LOD), the detection capability (CCß), the recovery and the cross-reactivity. Especially the detection capability (CCß) is an important parameter, because it gives a certainty at which concentration a sample is positive. That is the reason, why the CCß is mandatory according to the Community Reference Laboratories Residues (CRLs) 20/1/2010 and Commission Decision 2002/657/EC.

For e.g. exporting companies, the worst case are false-negative results. Because, if these are tested positive by authorities, the rapid alert system (RASFF) is activated and the exclusion of products from the import to the EU is following. Thus, the CCß is of big value to avoid false-negative results.

Note

- Look for the detection capability (CCß) in manuals and validation reports.
- CCß is mandatory according to regulation.
- CCß is important for exporting companies.