R-Biopharm AG

Good ELISA Practice

Manual

R-Biopharm – Innovative Soutions for the Food & Feed Analysis

Disclaimer

The Good ELISA Practice (GEP) manual represents a guideline for the establishment of reasonable framework conditions and conditions of use, which shall be complied with, when using test-kits of R-Biopharm AG and performing ELISA-analysis.

The GEP manual makes no claim to completeness, but describes only certain minimum standards. The compliance with such minimum standards does not guarantee the achievement of correct analytical results, but serves to increase the quality of assessment at ELISA-analysis. The manual applies in addition to the respective detailed instructions of the test-kits. For the proper use of test-kits, the respective instruction manual is decisive and shall take priority over this GEP manual.

The GEP manual is continuously up-dated. Please make sure that you have the newest version of the manual prior to performing the ELISA-analysis. The manual can be retrieved, printed and downloaded under the website *www.r-biopharm.com/products/food-feed-analysis*.

Introduction

The Good ELISA Practice (GEP) manual provides a comprehensive overview for both beginners and advanced analysts in order to improve the quality of performed ELISA analysis.

The manual is divided into three chapters, which follow the work flow of an ELISA based analysis:

In the **first** chapter, basic knowledge about the test principle, ELISA components and required laboratory equipment is given.

The **second** chapter describes the implementation of the analysis consisting of sample preparation and ELISA procedure.

The **third** chapter explains the process of data evaluation from measurement to interpretation.

Inhalt

1 ELISA Basics

1.1 Antibody – Antigen Detection

Antibodies are proteins produced in plasma cells of vertebrates as part of the adaptive immune system against structures (antigens) which are recognized as foreign to the body.

Antibodies bind to their antigens by a distinct pattern of ionic and hydrophobic interactions, hydrogen bridge linkages and Van-der Waals forces. The interaction between antibody and its antigen is selective and highly specific, similar to a lock and key. The Enzyme Linked Immunosorbent Assay (ELISA) is based on this selective and specific antibody-antigen recognition. Many formats of qualitative or quantitative ELISA tests have been established, a selection of them are illustrated in the following chapters.

Performing an ELISA involves at least one specific antibody for a particular antigen. A main principle is that one of these immunological components is immobilized to a solid phase, the cavities of a microtiter plate. The analyte from the sample interacts with the antibody-antigen system. This interaction can be visualized by enzymes, linked to secondary antibodies or antigens, and indicate if an antibody-antigen binding has occurred. An added substrate is converted by the coupled enzyme resulting in a change of colour, which can be measured with a spectrophotometer.

1.2 Analyte

The claimed analyte of an ELISA could be (1) a defined chemical substance e.g. aflatoxin B1, (2) a group of defined chemical substances e.g. aflatoxins B1, B2, G1 and G2, (3) a specific protein e.g. staphyloenterotoxin A, (4) a group of specific proteins e.g. staphyloenterotoxins A, B, C, D, and E, (5) a more or less defined group of proteins from a food commodity e.g. caseins (as a part of milk proteins), (6) a food commodity e.g. peanut protein.

1.3 ELISA Formats

Currently the following three different systems exist. In every system, the result is measured on the basis of the optical density.

1.3.1 Sandwich-ELISA

In this method a specific antigen (analyte) in the sample binds to antibodies attached to the solid phase of a microtiter plate. After washing, conjugated antibody is added which binds to a second epitope of the antigen (analyte). After washing, substrate is added and the results can be measured via the optical density (OD). The signal is proportional to the amount of antigen (analyte) (Figure 1).

1.3.2 Competitive ELISA (formats)

There are two possible formats for a competitive ELISA. The first system consists of a microtiterplate where the antibody is bound to the surface. The competition is between the analyte and an enzyme-analyte conjugate. The second system is based on a microtiter plate with a fixed amount of analyte which is bound to the surface. The analyte from the sample is added and the competition on antibody binding sites starts after adding the antibody. After washing, substrate is added and the measured OD value is inversely proportional to the amount of analyte in the sample. The more analyte that is present, the smaller the OD value. This method is suitable to measure samples with just one epitope as well as small analytes such as mycotoxins or antibiotics (Figure 2).

For the second system (with an antibody bound on the surface) a capture antibody is often used (Figure 3). The advantage of this system is that the reaction starts at the point of time the capture antibody is filled in, so there will be nearly the same incubation time over the whole plate.

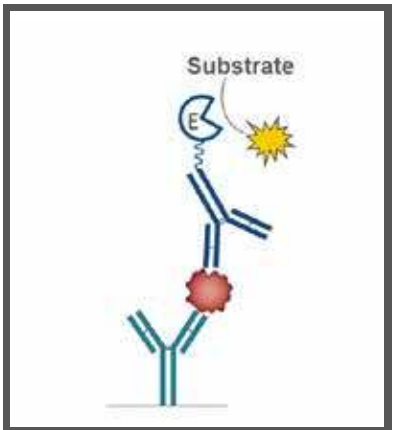


Fig. 1: Schematic structure of sandwich ELISA

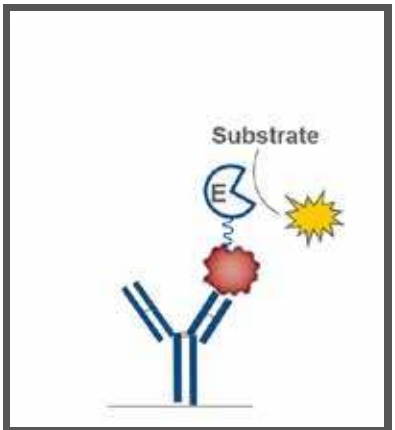


Fig. 2: Schematic structure of competitive ELISA

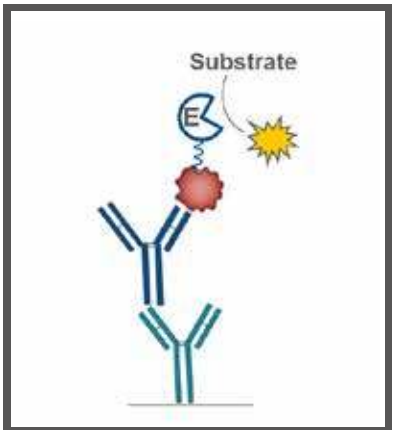


Fig. 3: Schematic structure of indirect competitive ELISA

1.4 ELISA components

As already mentioned above, several components are needed for an ELISA test system: Microtiter plates (MTP), enzyme-conjugates, standards (calibrators), substrate, stop-solution, and buffers. Furthermore, it is essential to use controls to be sure that the test procedure is working correctly.

1.4.1 Microtiter plate (MTP)

The MTP (96 or 48 wells), is the basis for the analysis. In every well the antibody or antigen (depends on the format) is bound to the surface. A common plate material is polystyrene though other materials can be used. The material is activated by β- or γ-radiation by the manufacturer of these plates. Without this activation no or only minor binding of antibodies or antigens occurs. In every well the antigen-antibody reaction and the conjugate-substrate reaction takes place. Lastly the reaction is stopped with a specific stop-solution and the optical density is measured.

1.4.2 Conjugate and Substrate

Antibodies or analytes linked to an enzyme are called conjugates. The linked enzyme converts its specific substrate into a colored bluish product. The substrate is usually a hydrogen peroxide/ chromogenic mix which reacts with the conjugate enzyme. The result is a coloured solution of which the optical density can be measured.

1.4.3 Standards (Calibrator)

All quantitative ELISA systems are calibrated by the use of standards. Therefore, the samples with unknown concentrations and a set of standards with known concentrations are analyzed in parallel on one plate. The result will be a calibration curve (with the associated mathematic formula) built out of the measured OD values and the concentrations of the standard (Figure 4). Based on this, the analyte concentration in the sample can be calculated.

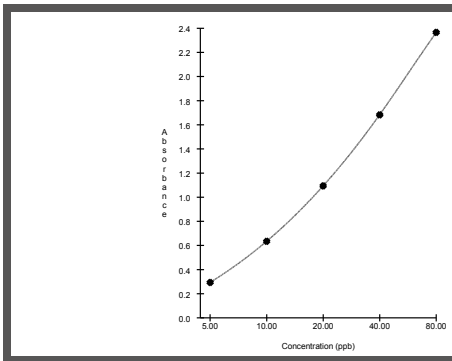


Fig. 4: Calibration curve

1.4.4 Buffers

All ELISA systems contain components of biological origin. For long term storage of these components and for proper function during the testing procedure, pH-value and ionic strength needs to be constant. Often the kind of buffer component also has an influence on test kit performance. For the convenience of test kit users, these buffer are included ready-to-use or as concentrates. They are also used for sample preparation and washing procedures of the microtiter plates.

1.4.5 Stop-solution

The stop-solution terminates the enzyme-conjugate-reaction. In most cases, sulphuric acid at low concentrations is used. By stopping the reaction the colour will change from blue to yellow and will remain stable until measurement within 10 min.

1.4.6 Additional components

For most systems a positive control is recommended, for example a spiking solution. This gives the opportunity to control your test system.

1.5 Lab equipment and its maintenance

Depending on the requirements of the test system, various specialized laboratory equipment is needed for the different steps e.g. pipettes, equipment for plate washing, incubator for constant temperature, ELISA reader and a computer to calculate concentrations. However, not all of the equipment mentioned here is needed for all test systems. In every case regular maintenance and calibration is required for machine and lab equipment which are used.

1.5.1 Pipettes

A pipette is used to transfer a precisely defined volume of a solution to e.g. the wells of a microtiter plate. Pipettes are crucial for results with a high precision. Therefore, special attention should be set on maintenance by experts and proper pipetting technique. Different kinds of pipettes exist:

- Single-channel pipettes with fixed volumes e.g. 50 µl
- Single-channel pipettes (e.g. with variable volumes between 10 and 100 µl); used normally for samples and standards
- Multistepper pipettes (with the possibility to pipette multiple times of a specific volume); used normally for addition of antibody or conjugate solutions
- Multi-channel pipettes (with the possibility to pipette the solution in 8 or 12 cavities at the same time); used normally for washing steps or addition of antibody or conjugate solutions
- Bottle top dispenser (used normally for washing steps)
- Fully automatic machines (all pipetting and incubation steps are done automatically)

1.5.2 Washer

After every incubation step (except the incubation with the substrate) the ELISA plate has to be washed with washing buffer. Washing is a crucial step when performing an ELISA to obtain results with high precision.

1.5.3 ELISA reader

The ELISA reader is a spectrophotometer which allows to measure the optical density (OD) and to calculate the concentration of your sample. Regular maintenance by experts is essential for exact results.

1.5.4 Automation

One possibility for working with an ELISA is the use of an automated system which allows you to test your samples without any manual steps. Therefore, the automated system has to be validated and calibrated for your test system. Possibilities for that kind of automation are the ChemWell® and the GEMINI. For further information please contact: *sales@r-biopharm.de.*

1.5.5 Additional equipment

In some tests an incubator is required to guarantee a stable temperature while the test is running. Sometimes, a seal or a protecting plate cover are necessary to prevent evaporation or contamination.

1.5.6 General words of advice

To ensure a high precision of the results, the equipment should be calibrated regularly. Please ask the manufacturer for the calibration interval and include this in quality control plans.

1.6 Good laboratory practice (GLP)

Dependent on the toxicities and contagiousness of the used materials, different levels of protective actions are necessary to guarantee health and safety of the user. However, basic protective clothing is already required to avoid contamination of the samples, which would lead to incorrect results. The following equipment is a minimum requirement for every lab:

- Lab coat
- Eye protection
- Gloves
- Fume hood (occasionally)

However, it is necessary to study the material safety data sheets (MSDSs) carefully for all chemicals substances used. The MSDS contains information about the dangers when working with a particular substance, required protective measures, as well as required actions in case of emergencies. The MSDS are available on request for each product. Please contact: *sales@r-biopharm.de*.

1.7 Test kit labeling

To ensure the correct handling and storage of your components please read the Instructions for Use (IFU) or the test kit insert. On all components the follow information is stated (if applicable):

- Product name
- Article number
- Name of the component
- Lot number
- Storage temperature
- Concentration

The expiry date is particularly important, as the specified behavior can only be guaranteed until then. After expiration the ingredients can degrade and the results can decrease in accuracy.

2 Sample preparation and test implementation

2.1 Pipetting techniques

Accurate and precise pipetting is crucial in ELISA analytics, particularly at high sensitivity levels where a small mistake in pipetting can induce large differences in the final test results. Be consistent during pipetting and do not change your technique while pipetting an assay. Be prepared before starting pipetting e.g. put all standards in a row and arrange all samples in a consistent way to allow uniform pipetting. Ensure that enough pipette tips are prepared and a waste container is in place.

The two pipetting techniques used for ELISA are forward pipetting (standard pipetting) and reverse pipetting. The forward pipetting technique is recommended for aqueous solutions. Using this technique, some liquids may induce bubbles or foam during pipetting. As an alternative, reverse pipetting lowers this risk and is recommended for liquids with higher viscosity. It requires more liquid volume (dead volume) and is more error-prone at high volume transfers. Familiarize yourself with pipetting techniques before you start the experiments.

2.1.1 General pipetting instructions

If you use an adjustable pipette please adjust it to the desired volume before you start pipetting.

Always check the adjusted volume directly before starting to pipette (Figure 5).

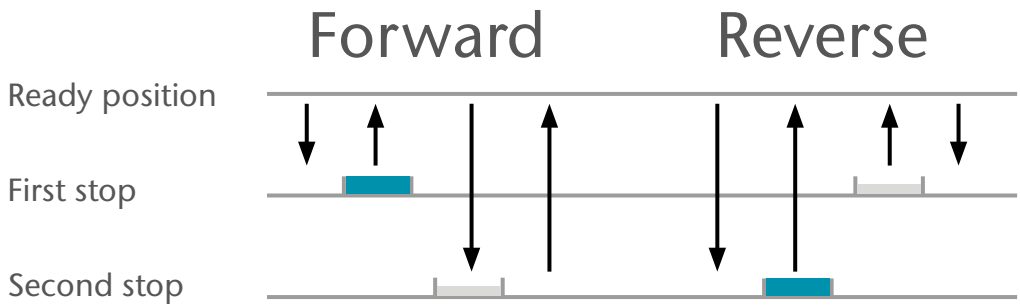


Fig. 5: Schematic illustration of forward and reverse pipetting techniques when using a single channel pipette (piston pipette). Blue blocks indicate the steps where the pipette needs to be placed in liquid to aspirate. Grey blocks indicate the target wells.

2.1.2 Forward pipetting

1. Put a new tip on your pipette and check for a firm fit.
2. Press the operation button to the first stop.
3. Pipette tips from some manufacturers need to be flushed before aspiration and dispensing of the appropriate liquid. Please check the according manual. In case of any doubt, flush the tip before pipetting.
4. Put the pipette tip approx. 1 cm deep into the liquid. Slowly release the operation button to the ready position and wait until the desired liquid volume has been aspirated. Ensure that no bubbles or foam occurs in the pipette.
5. Remove excessive liquid from the outside of the tip by touching the test tube with the tip.
6. Dispense the liquid into the desired well by pressing the operation button to the second stop.
7. Remove the tip to waste.

2.1.3 Reverse pipetting

1. Put a new tip on your pipette and check for a firm fit.
2. Press the operation button to the second stop.
3. Put the pipette tip approx. 1 cm deep into the liquid. Slowly release the operating button to ready position and wait until the desired liquid volume has been aspirated. Ensure that not bubbles or foam occurs in the pipette.
4. Remove excessive liquid from the outside of the tip.
5. Dispense the liquid into the desired well by pressing the operation button to the first stop. Ensure that no liquid remains on the outside of the tip.
6. For repetitive liquid pipetting, press the operation button to the first stop and repeat steps 3 - 5.
7. Remove the tip to waste.

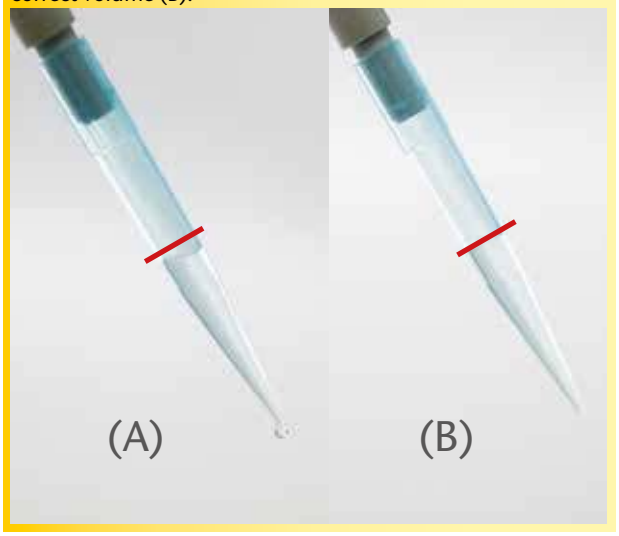
2.1.4 Pipetting of organic solvents

Organic solvents show high vapour pressures which can affect precise pipetting. The use of pipettes with air displacement technique to transfer organic solvents may lead to evaporation of the solvent or a leaking out of the tip (Figure 6). For the pipetting of organic solvents we recommend:

1. multistep pipettes which use the positive displacement technique
2. serological pipettes for larger sample volumes, since the graduation allows for the pipetting of exact volumes
3. bottle top dispensers
4. pipettes designed especially for the handling of organic solvents

If single channel pipettes are used to transfer organic solvents, the pipette tip and the air inside the pipette needs to be saturated with organic solvent vapour before pipetting the desired volume. For this aspirate and dispense the organic solvent at least 3 times before the desired volume is transferred. Use appropriate quality control procedures to monitor the correctness of these kinds of pipetting steps.

Fig. 6: Pipetting of organic solvents with pipettes using the air displacement technique. Particular care should be taken to prevent evaporation into and leaking out of the tip (A). Flushing the pipette before liquid transfer helps to transfer the correct volume (B).



2.2 Handling of samples

2.2.1 Storage of unprepared samples

Unwanted contamination of samples can influence test results significantly. If there are signs of unwanted contamination or spoilage do not use the sample and request a new one. Store the sample according to the test kit manufacturer or according to the best scientific knowledge.

Generally, samples should be prepared and tested immediately whenever possible. If storage can't be avoided check for optimal storage conditions and analyze them as soon as possible. All samples need to be correctly labelled and sealed to avoid evaporation or dry-out. Inappropriate storage conditions may influence later analysis and alter test results.

2.2.2 Sample drawing

The drawing of a representative sample is a crucial step. For some parameters, legislations apply on how to take a representative sample (e.g. mycotoxins). Please follow these rules precisely. If there are no rules, samples should be homogenized as much as possible. Inform a customer beforehand about a minimum sample amount. State in the test report that the result refers to the sample as it was sent.

2.2.3 Sample preparation

For sample preparation please follow the instructions for use provided with the test kit. Changes or variations may lead to incorrect test results. Make sure to use only suitable and maintained equipment for sample preparation. For any related questions please contact R-Biopharm AG.

Depending on the parameter to be tested, instructions for use may contain information on how prepared samples can be stored for later analysis. Please follow these instructions carefully or prepare samples directly before analysis.

2.2.4 Use of frozen samples

Before further use, samples must be completely thawed. Thawing of frozen samples should be performed at 4°C or at room temperature, dependent on analyte stability.

In case of unprepared, non-liquid samples check the instructions for use for further sample preparation and homogenization.

Liquid samples must be thoroughly mixed before they can be used for analysis. To achieve a homogeneous sample carefully vortex or invert the sample. Foam formation or intensive mixing should be avoided since it may denature proteins. A further sample preparation of liquid samples (e.g. in case of milk) may be necessary before analysis. Please check the instructions for use for further information.

Depending on the sample, freezing and thawing can lead to crystallization or coagulation. Avoid freeze-thaw cycles wherever possible, since it can change sample integrity and alter test results. If possible, aliquot liquid samples before storage at -20°C to avoid freeze-thaw cycles.

2.2.5 Certified reference material

Certified reference materials (CRM) are naturally contaminated, homogeneous matrices whose analyte content has been exactly and reliably determined (Figure 7). The regular use of CRMs is recommended to perform internal quality controls. This allows checking for the trueness and precision of experimental procedures and for the testing of handling skills. If no reference material is available, the use of matrices spiked with defined analyte concentrations is recommended. R-Biopharm can help you to find suitable certified reference materials and spiking solutions.

Fig. 7: Certified reference materials and standard solutions are essential quality control tools.

2.3 Preparation and handling of components

2.3.1 Storage of kits

The expiry date printed on the outer label applies to all reagents contained in the kit and is also valid after first use. To maintain the shelf life, store the kit at conditions noted on the outside label of the package (Figure 8).

Most kits have to be stored in a dry place and at 4°C. Freezing of components needs to be avoided. If you use a refrigerator for storage, make sure the kits are not stored close to the back wall to avoid freezing. Improper storage of kits or components may lead to damage. Freezing of test components may decrease test performance and lead to invalid test results or –as an extreme- non-functional test kits. Indicators for false storage are decreased or non-detectable optical density and the alteration of test results.

Fig. 8: Storage conditions and expiry date are printed on the outer label of each ELISA test kit.

2.3.2 First in – first out

The expiry date of the kit is labelled on the outer label of the kit package. At least until this date the kit will perform within specifications. Additionally every kit component has its own expiry date which is identical or even exceeds the expiry date of the test kit.

If you have more than one kit to hand it is recommended to use the first in – first out principle. This means the kit with the shortest expiry date on the outer label should be used first. We recommend indicating the date of the first use on the outer label to avoid a mix-up of this principle.

The exposure of the ELISA kit to cold/warm cycles should be kept as low as possible. We recommend that samples should be collected. Testing of higher sample numbers at once reduces the expenditure of time per sample. Rather test higher sample numbers at once than test only a few samples consecutively. Please check the instructions for use for relevant limitations.

2.3.3 Pre-Warming

All reagents need to be at room temperature before they can be used in the test. Take all components out of the kit package before use and allow them to reach room temperature (20 - 25 °C). Larger bottles and higher volumes may require more time to reach room temperature. Check the temperature of the components in any case of doubt (Figure 9).

After use it is recommended that all components are put back into the kit package to avoid the unintended mixture of components with components from other kits or lots. Return the kit back to the recommended storage conditions as soon as possible (see outer kit label for storage conditions).

Fig. 9: Bring all test kit components to room temperature before use and perform the test at 20 - 25 °C (68 - 77 °F).

2.3.4 Temperature control

ELISA tests are sensitive to temperature fluctuations. Therefore try to stabilize and control laboratory conditions. This includes the temperature during photometric analysis. Perform ELISA tests between 20 and 25 °C and avoid conditions that are able to drastically change the temperature or increase evaporation. ELISA tests should be prevented from direct exposure to sunlight and ventilations. Cold lab ware and cold benches may also influence the temperature. It is helpful to isolate the microtiter plate from the bench surface by performing the test on a suitable underlay. A cheap and easy solution is the use of paper towels.

2.3.5 Avoiding of contamination and sample mix-up

A clean and reproducible way of working is crucial for optimal results in food and feed analysis. A very common source of contamination is insufficiently cleaned re-usable lab ware. To avoid this, it is highly recommend to use solvent-resistant single-use lab ware. If this is not possible, re-usable lab ware should be laboratory sterile and free of contamination. We recommend using a laboratory dishwasher or equivalent. Use quality control blank samples to check for contamination.

Reagents should be handled with calibrated devices, clean pipettes and containers. Only take the amount of reagent needed and do not put reagents back into the container once it has been removed. Use separate containers and pipette tips for every reagent to prevent cross contamination. Make sure to label all containers correctly (Figure 10). Exchange your single use lab ware as often as needed.

Fig. 10: Carefully label all used containers and document your labelling.

2.3.6 General test handling

Before you start, read the instructions for use enclosed in the kit. Prepare all extraction solutions and buffers according to these instructions and follow the described procedures to obtain optimal results. To allow an unobstructed test procedure it is helpful to prepare a pipetting scheme before you start with your experiment.

Depending on the ELISA, antibody and conjugate solution may need to be diluted prior to use. These dilutions should be prepared directly before use and shall not be stored for further use. Contaminated or incorrectly stored conjugate solutions may have a reduced enzyme activity or may induce a background signal.

To obtain optimal results, test samples and control samples (standards, reference samples) have to be available in the same diluent. Strictly following the sample preparation protocol ensures this. The pipetting onto the plate needs to be performed quickly and without interruptions at every step of the test procedure. For the transfer of samples we recommend single channel pipettes, or, if a pre-dilution plate is used, a multi-channel pipette. For the pipetting of antibody and enzyme solutions, multistep pipettes are the best option.

Between handling steps a dry-out of the wells has to be prevented.

2.3.7 Time management for pipetting

Pipetting of standards and samples is general the most time consuming and laborious step when performing an ELISA. In general it is advised to keep the pipetting technique as constant as possible and the absolute pipetting time to a minimum. This is extremely important when the last component is added and the analytical reaction starts (e.g. addition of antibody in a competitive ELISA when sample and conjugate is already added). To avoid an 'optical density (OD) shift' from the first to the last well, it is recommended to use multichannel or stepper pipettes for components which are added to all wells such as antibody, conjugate, substrate/ chromogen or stop solution. However, it is not recommended to pipette in a rush as mistakes such as to pipetting into wrong wells, etc. may occur.

It is very important that all samples are handled in a comparable way. Strictly follow the pipetting order and the incubation times noted in the instructions for use. To obtain comparable results in all wells, the incubation time of each single well needs to be identical.

To achieve this, start the clock after you have pipetted a component starting a reaction (standards or samples, antibody solution, conjugate solution) into the last well. In case of substrate/chromogen solution, start the clock before you pipette the solution into the first well. Stop the substrate/chromogen reaction after the defined time by adding stop solution in the same order substrate/chromogen solution has been added. It is very important to meet the incubation times noted in the instructions for use.

The activity of the chromogen may be influenced by light. Protect the chromogen from light and store it in the brown flask in which it is delivered. To stop the reaction use the stop solution delivered with the kit.

2.3.8 Correct washing

Washing is a crucial step to remove all unbound components that might influence reactions or lead to false results. For washing only use washing buffers recommended in the instruction for use. Many kits contain washing buffer salts or solutions that can be used to prepare ready to use washing buffers. This removes the necessity to weigh reagents to prepare your own buffers. For stability and storage information on the individual washing buffers delivered with the kits, see the instructions for use. As all other components, washing buffers need to be at room temperature before use. Follow the specific recommendation of the instructions for use regarding the number of washing steps.

At the end of the incubation steps pour the liquid out of the wells and tap the microtiter plate holder vigorously upside down on absorbent paper to ensure the complete removal of the liquid from the well. All liquid has been successfully removed when no signs of liquid remains on the paper towel. Most ELISA tests require 250 - 300 µl of washing buffer per washing step and well. Add the washing buffer firmly and remove the liquid by pouring out and tapping. Repeat the washing step 3 - 5 times (see instructions for use). For washings steps we recommend to use a bottle-top dispenser with manifold.

If you use such an automatic washer, service, maintain and use the device according to the manufacturer's instructions. Ensure that the device does not show salt residues that may prevent liquid flow. Buffers, tubes, manifolds and washing needles need to be kept free from contamination of microorganisms. Keep your device clean and if necessary remove contaminated parts. In case of any doubt, use the manual washing procedure or contact the manufacturer of the washer.

Automated washing systems are not available at all testing sites. Due to this we highly recommend the use of the relatively cheap and easy to handle bottle top dispensers with an 8 - or 12 -fold manifold for your washing procedure. Other manual washing techniques like washing bottles may not allow to treat every well exactly the same and should not be applied.

Washing steps should be performed fast but efficiently and accurate. Make sure that the time between addition of washing buffer to the first and the last well is as short as possible. This ensures that wells do not dry-out and it minimizes differences in incubation times. Despite fast working speed pay attention to accuracy. Spillover of liquid from one well to another needs to be avoided. In case of any doubt add an additional washing step.

Fig. 11:

2.3.9 Storage of unused components for further experiments

Microtiter plates are delivered in a re-selable bag with a pouch containing a desiccant (Figure 12). In case not all wells of the plate are needed, store the rest of the wells in this bag. Put the wells and the microtiter plate together with the desiccant into the bag and close it. Close all flasks and make sure to screw the lids on firmly. This is especially important for components such as standard solutions that may contain organic solvents with a high vapour pressure. We recommend putting all components back into the kit package for storage. Store all components upright and under the indicated conditions (Figure 13).

Fig. 12: Unneeded plate strips should be stored together with the desiccant in the re-sealable pouch in which they are delivered.

Fig. 13: Until further use all components should be stored in the kit package in an upright position.

2.3.10 Interchange of reagents between tests and batches

The components of each lot are thoroughly adjusted to deliver ELISA kits that show the optimal performance. The exchange of one or more of these components between different lots will change the performance of the tests and is not allowed. An exchange of components of kits with the same product number is possible if the lot number of the kit is identical. However, we recommend using only components delivered with the particular kit.

2.3.11 Security references

ELISA test kits may contain hazardous substances. For information on hazards contained in the substances take note of the warnings on the labels of the components and refer to the appropriate material safety data sheets (MSDS). Generally handle all components with care and take all usual laboratory security precautions. While performing the test procedure use laboratory gloves, wear a lab coat, do not eat, drink, smoke and keep all components away from sources of ignition. Disposal of waste may differ from country to country. Please refer to local disposal rules.

2.4 Stopping and measuring of the ELISA

Most ELISAs are measured at a wavelenght of 450 nm. The correct wavelength for reading can be found in the test kit manual under point 3. test principle. At the end of the test implementation, stop solution which contains sulfuric acid is added to each microtiter well. The acid denatures all proteins including the antibodies and thus stops the reaction (Figure 14).

Nevertheless it is recommended to read the microtiter plate directly after addition of stop solution or at least within the time stated in the instructions for use. A large delay may still cause a shift of the absolute values measured.

Fig. 14: Addition of stop solution causes a color change from blue to yellow.

2.5 Parallel performance of tests

If several ELISA tests are performed in parallel, extraordinary care should be taken. Label microtiter plates and reagents properly to avoid mistakes and mixing of reagents between assays. Use a separate lab timer for each microtiter plate (Figure15). Take care, that handling steps of different assays do not overlap, e.g. washing of one microtiter plate when a second one has to be stopped. Schedule your pipetting, washing and OD reading activities before starting multiplex analysis.

Automation of parallel analysis is possible by using biochemistry analyzers such as ChemWell® (Awareness Technology Inc.) or Gemini analyzer (Stratec Biomedical AG). Please contact us for a list with tests, which are already validated on biochemistry analyzers.

Fig. 15: Parallel performance of several ELISA tests must be carefully scheduled and organized.

3 Data evaluation and interpretation of results

3.1 Determination of unknown samples by standard curve

The concentration of the analyte in an “unknown” sample can be determined by comparing the measured signal of the sample with the signal of standards containing known concentrations of the analyte. In ELISAs usually 5 - 7 standards are used to generate a standard curve covering the concentration range of interest. There are also ELISAs which use single calibration technology (SC), where a single standard is used to check compliance with a deposited standard curve.

3.2 Standard curve fittings

Depending on the assay, the standard curve is calculated by different curve fittings: linear regression, logit-log, cubic spline and 4 parameter 2nd order polynomial (Figure 16)

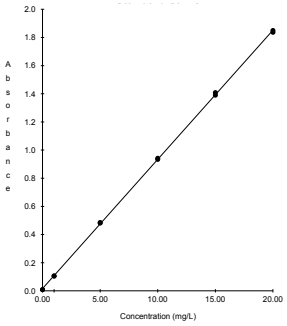
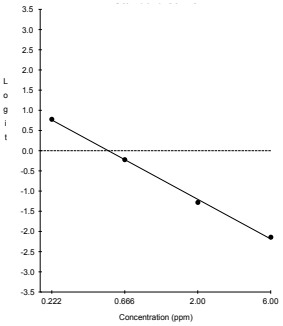
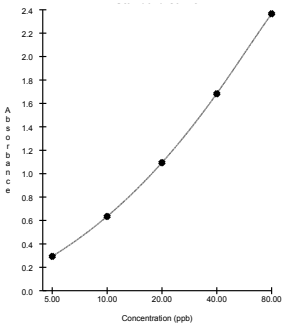
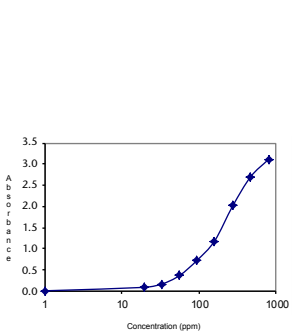
Linear regression	Logit-log	Cubic spline	4 parameter
			
X-axis			
Concentration	Concentration (logarithmic)	Concentration (logarithmic)	Concentration (logarithmic)
Y-axis			
OD	Logit of concentration	B/B0 in %	OD
The relationship between variable y and variable x is linear. Thus, simple linear regression is applied using the formula: y = mx + b	In Logit-log is used to linearize curved plots for further analysis by linear regression models.	Cubic spline is a numeric function that is piecewise-defined by 3 polynomial functions with high degree of smoothness at the connections of the polynomial pieces (knots).	4 parameter is a nonlinear regression model with 4 parameters: • bottom of the curve • top of the curve • EC50 • slope at the inflection point of the curve =EC50

Fig. 16: Overview about standard curve fittings

3.3 Standard curves of sandwich and competitive ELISAs

The correct algorithm for the respective ELISA is depicted on the certificate of analysis and is preset in the RIDA®SOFT Win.net-Software after choosing the method. Depending on assay format (sandwich or competitive) the standard curve is calculated differently.

In sandwich ELISAs the concentration of the standards is plotted on the horizontal x-axis, while OD is plotted on the vertical y-axis (figure 2). Sometimes B/Bmax, a percentage value is plotted on the vertical y-axis. For B/Bmax calculation, the standard with the highest analyte concentration is set as 100 % and B/Bmax of the further standards is calculated by dividing the absorbance of the respective standard by the absorbance of the highest standard. The result is multiplied by 100 to obtain percentage units (Figure 17).

For competitive ELISAs, the concentration of the standards is plotted on the horizontal x-axis, while B/B0, a percentage value is plotted on the vertical y-axis. For B/B0 calculation standard 1 which contains no analyte is set as 100 % and B/B0 of the further standards is calculated by dividing the absorbance of the respective standard by the absorbance of standard 1. The result is multiplied with 100 to obtain percentage units (Figure 18).

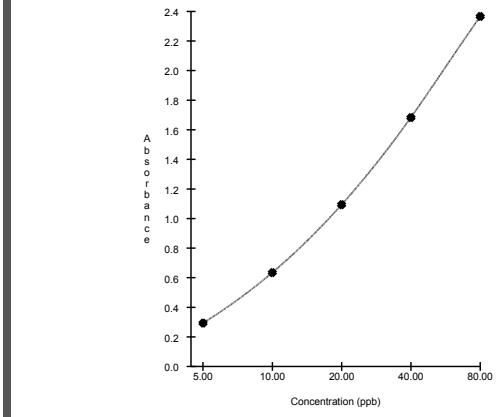


Fig. 17: Standard curve of an ELISA in sandwich format, where absorbance is plotted over concentration.

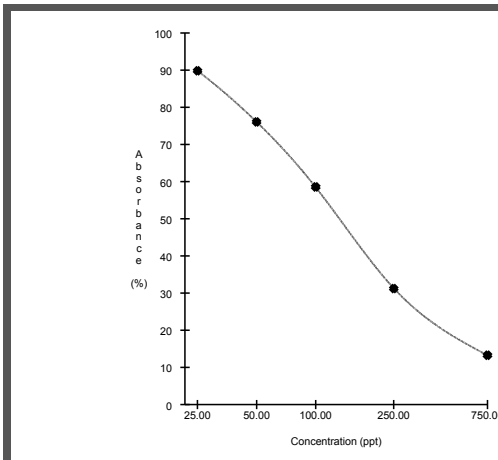
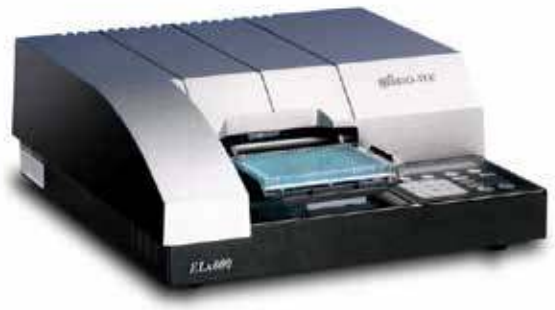


Fig. 18: Standard curve of an competitive ELISA, where B/B0 is plotted over concentration

3.4 Spectrophotometer and Software

The optical density is read by a microtiter plate spectrophotometer at a certain wavelength. There are many different spectrophotometer from different manufacturers available. R-Biopharm offers spectrophotometers and a software, called RIDA®SOFT Win.net, which is tailor-made for the analysis of ELISAs from R-Biopharm (Figure 19, 20). For the use of the software a manual (Art. No. R9996) is available on request. If a spectrophotometer is already available, please do not hesitate to contact us, to check if your spectrophotometer can be used in conjunction with the software and our assays.

Fig. 19: Biotek ELx800 microtiter plate spectrophotometer.



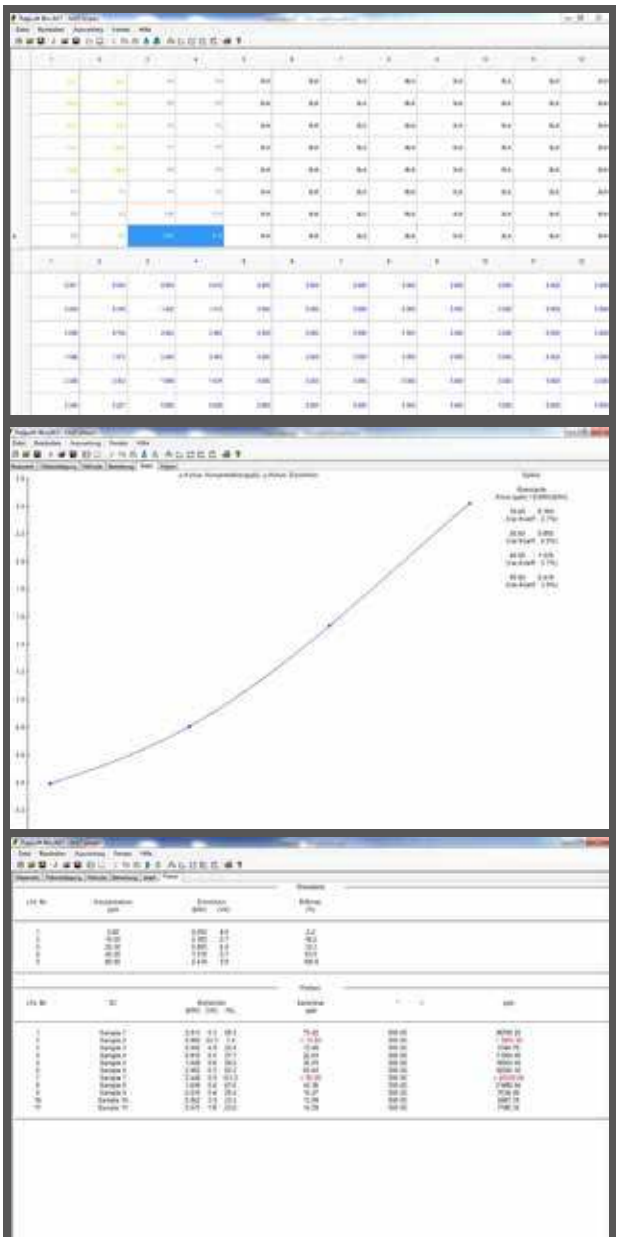


Fig. 20: Screenshots of RIDA®SOFT Win.net: plate layout, standard curve, results

3.5 Determination of analyte concentration

The software creates the standards curve and calculates the concentration of an analyte in an unknown sample as shown in figure 21. Outside the measurement range the software will calculate no values.

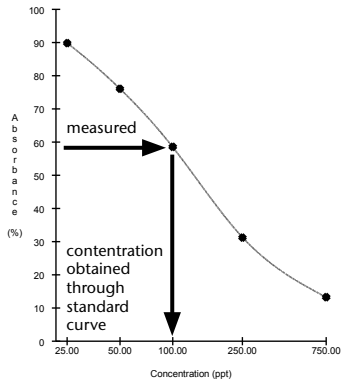


Fig. 21: Determination of analyte concentration from unknown sample through standard curve

3.6 Measuring range and dilution factor

If an absorbance or a B/B0 value is obtained which is below or above the standard curve, the RIDA®SOFT Win.net software gives a results '<' (below) or '>' (above) standard range. The software provides the option to extrapolate the concentration value. In general, these extrapolated values are only estimates and are not reliable. The further the sample is below the lowest or above the highest standard, the bigger the uncertainty of the calculated concentration is. It is advised, to dilute samples which are above the largest standard and to repeat the analysis until the result is within the concentration range of the standard curve.

The measuring range of the ELISA is determined by the concentration of the standards, and by the dilution factor of the sample preparation method. For example, if milk is diluted 1:4 (1+3) before applying to the microtiter well, the dilution factor is 4. This means, that the results read from the standard curve have to be multiplied by 4 to obtain the correct concentration of the analyte in the sample. If the analyte in a sample is concentrated by a factor of 2 during sample preparation, e.g. by evaporation or column clean-up, the dilution factor is 0.5. This means, that the results read from the standard curve have to be multiplied by 0.5 to obtain the correct concentration of the analyte in the sample (Figure 22, 23).

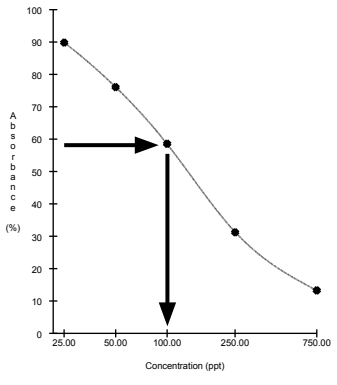


Fig. 22: A milk sample was diluted 1:4 before applying to the microtiter well. The results have to multiplied by 4 to obtain the correct concentration: The concentration of the analyte in the sample is 100 ng/kg x 4 = 400 ng/kg.

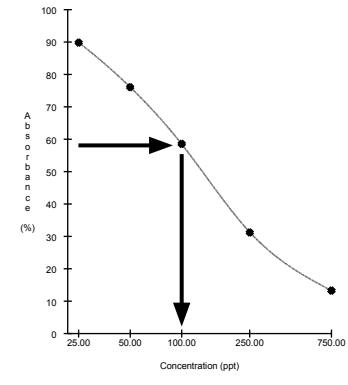


Fig. 23: A meat sample was concentrated by factor 2 during sample preparation 1. The result has to be multiplied with 0.5 to obtain the correct concentration: The concentration of the analyte in the sample is 100 ng/kg x 0.5 = 50 ng/kg.

3.7 Units and dimensions

Concentrations are sometimes not expressed in SI units, but in miscellaneous dimensionless quantity ‘part per’ annotation (Figure 24). We recommend using SI- units.

SI-units	decimal	parts per	symbol
g/kg	10^{-3}	parts per mille	‰
mg/kg	10^{-6}	parts per million	ppm
µg/kg	10^{-9}	parts per billion	ppb
ng/kg	10^{-12}	parts per trillion	ppt
pg/kg	10^{-15}	parts per quadrillion	ppq

Fig. 24: Overview about SI-unit and the miscellaneous dimensionless quantities ‘part per’ annotation

3.8 Limit of detection and quantification

The Limit of Detection (LOD) is the lowest concentration of an analyte which can be clearly distinguished from blank sample readings. The LOD is determined experimentally by measuring the concentration of at least 20 blank matrix samples and then calculated by the formula: Mean concentration of blank samples + 3-fold standard deviation of the concentrations of blank samples.

The Limit of Quantification (LOQ) is the lowest concentration of an analyte which can be detected quantitatively. The LOQ is determined experimentally by measuring the concentration of at least 20 blank matrix samples and then calculated by the formula: Mean concentration of blank samples + (most often but not necessarily 9-fold standard deviation of the concentrations of blank samples (Figure 25).

It is recommended to verify these values by spiking experiment with your sample matrix and laboratory equipment.

Results below the LOD indicate that a sample is negative or that the concentration of the analyte(s) is below the LOD.

Results, which are above the LOD and below the LOQ are qualitative (negative/positive) results only. This means, that the sample contains the analyte, but the exact amount can’t be quantified, as value is below the LOQ.

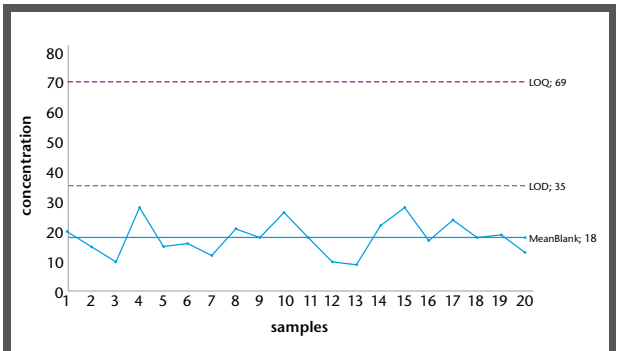


Fig. 25: 20 blank samples were analyzed. Mean of blank samples = 18 ng/kg with a standard deviation of 5,7 ng/kg
LOD = 18 ng/kg + 3 x 5,7 ng/kg = 35 ng/kg
LOQ = 18 ng/kg + 9 x 5,7 ng/kg = 69 ng/kg

3.9 Trueness and recovery

Trueness means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness can only be established by means of certified reference material (CRM). Trueness is calculated by dividing the measured concentration of the CRM by the assigned concentration of the CRM. The result is multiplied by 100 to obtain a percentage unit. The absolute difference between both values is called 'bias'.

If no CRM is available, e.g. for antibiotics in food of animal origin, instead of trueness, the recovery can be determined. Recovery is determined experimentally by measuring samples which are spiked prior to sample preparation. Recovery is calculated by dividing the measured concentration of the spiked sample by the spiking concentration. The result is multiplied by 100 to obtain a percentage unit. Please note, that spiking on the surface of a matrix may be not identical to naturally contaminated samples, which can lead to deviations in results between spiked and naturally incurred samples.

Recovery or trueness is given in the instructions for use and represents the mean of the measured samples The recovery of single samples can vary in accordance to the precision of the assay.

If samples were measured in areas where the standard curve is relatively flat, the accuracy of the results may be low especially at high concentrations, because small differences in OD or B/B0 may cause large differences in concentration (Figure 26).

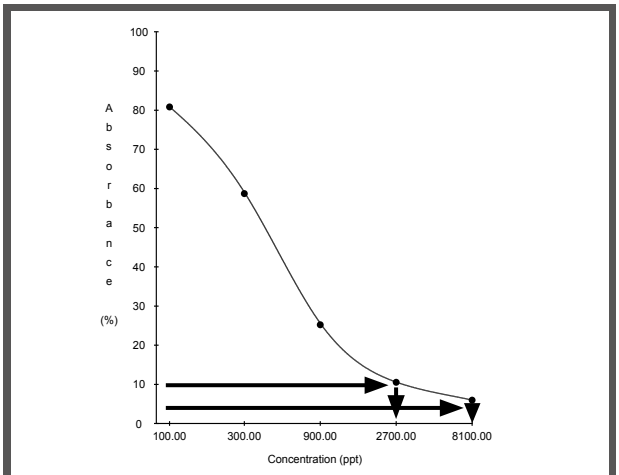


Fig. 26: In flat areas of the standard curve small differences in B/B0 produces large differences in concentration: 5 % B/B0 gives an 3 times higher results as 10 % B/B0.

3.10 Specificity and cross reactivity

Antibodies are highly specific to a single analyte, but sometimes they can also bind other molecules with different affinities.

The specificity or cross reactivity is determined by the measurement of a standard curve consisting of the analyte or cross reactive substance in a suitable concentration series (Figure 27). After calculation of the 50 %-dose of the analyte or cross reactive substance, the specificity or cross reactivity is

$$\text{Specificity or cross reactivity} = \left(\frac{\text{50 \%–dose of standard substance}}{\text{50 \%–dose of analyte or cross reactive substance}} \right) \text{x 100 \%}$$

calculated as follows:

If the binding of the antibody affects a substance that is included in the scope of the method, this is called specificity of the method. If this binding is related to unwanted substances that are not within the scope of the method it is called cross reactivity.

1st Example: The scope of an ELISA describes that the system was developed to quantify aflatoxin M1 in milk. Therefore, the specificity for aflatoxin M1 is 100 %. Furthermore it is stated that a cross-reactivity of 10 % to aflatoxin M2 exists.

Due to the principle of an ELISA system, the antibody is not able to discriminate between the specificity to aflatoxin M1 and the cross-reactivity related to aflatoxin M2. If both are present in an unknown sample, the result is the sum of both substances. But, due to the low cross-reactivity of 10 %, a 10-fold higher concentration of aflatoxin M2 (compared to M1) is necessary to resulting in a signal comparable to aflatoxin M1. An exact quantification is only possible if only one analyte or one cross-reacting substance is present in the sample.

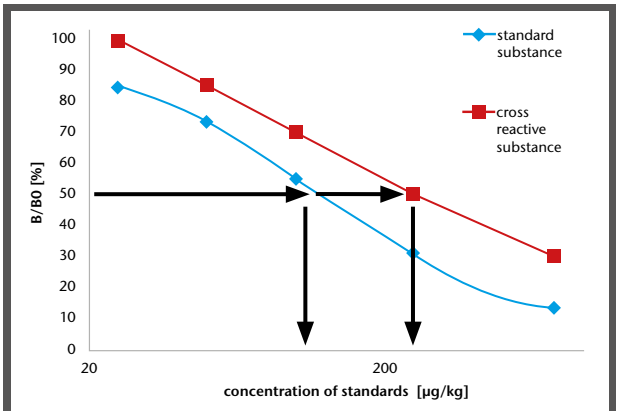


Fig. 27: The 50%-dose of the standard curve of the standard substance (blue) is more sensitive (170 µg/kg) as the 50%-dose of the standard curve of the cross reactive substance (210 µg/kg).

The cross reactivity of the cross reactive substance is therefore: (170 µg/kg / 210 µg/kg) x 100 % = 81 %

$$\text{Concentration of analyte or cross reactive substance} = \frac{\text{measured concentration}}{\frac{\text{specificity or cross reactivity of substance in \%}}{\text{100 \%}}}$$

In this case, the concentration can be calculated as follows:

2nd example: The scope of a Tetracyclin ELISA describes that the assay is specific for the determination of tetracycline, chlortetracycline, rolitetracycline and demeclocycline in different matrices. The calibrator material is tetracycline with a specificity of 100 %. The analysis of a chlortetracycline containing milk sample resulted in a concentration of 10 µg/L. Since the specificity of chlortetracycline is 70 % in this system, the concentration is calculated to 14 µg chlortetracycline per L of milk.

Nevertheless, ELISA test are used as screening methods. Any positive results or concentration higher than specified threshold should be verified by a confirmatory method e.g. LC-MS/MS.

In case of an unknown matrix and/or another specific analyte which are not included in the scope of the method, the user must determine the Limit of Detection and the Recovery of the specific analyte in the particular sample matrix. Please note that the specificities and cross reactivity's were experimentally determined in the buffer system only, as it is very time consuming and laborious to determine every specificity or cross reactivity of any analyte or cross reactive substance in every matrix.

3.11 Interferences and matrix effects

In general, food and feed are considered as 'highly difficult' sample matrices, due to their complex composition. For example, besides its natural ingredients, (carbohydrates, proteins and water) honey may contain lots of environmental contaminants such as heavy metals, radioactive isotopes, organic pollutants, pesticides, pathogenic bacteria and their toxins and genetically modified organisms. Consequently, it cannot be excluded that natural components or any contaminant of a sample interferes with the reagents used during sample preparation or in the ELISA test and cause so called 'matrix-effects'.

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