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GUIDELINES FOR THE VALIDATION OF ANALYTICAL METHODS USED IN RESIDUE STUDIES IN ANIMAL TISSUES

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1. Introduction

1.1. Objective of the guidance

This document is aimed at providing a general description of the criteria considered acceptable for the validation of analytical methods employed in residue depletion studies of veterinary drugs in animal tissues or other biological matrices.
During the veterinary drugs development or adaptation process, pharmacokinetic studies of tissues depletion or bioequivalence can be conducted in order to determine and analyze analyte concentrations in different biological matrices (tissue, plasma, milk, eggs or honey) in treated animals. This information is used in regulatory submissions around the world.

The validation of the methodology used during studies in biological matrices warranty the reliability of the experimental data obtained. The submission of validated methods and their requirements are well defined in various recognized international organisms and can even be defined by law.

This guideline is aimed at addressing the validation of analytical methods for the determination of the administered active principles and its metabolites in the different biological matrices, considering the recommendations of the associations of analytical chemistry and the health authorities.

1.2. Background

This document is based on VICH GL 49 "Guideline for the Validation of Analytical Methods used in Residue Depletion Studies – November 2009".

This work, based on the mentioned background, is intended to propose protocols adapted to Argentine requirements and needs, which can also be useful for other countries in the region.

2. Scope

Analytical procedures that have been developed to evaluate:

-Residue depletion studies aimed at determining withdrawal periods or 0-day withdrawal periods.

-Pharmacokinetic studies and tissue distribution studies.

-Bioequivalence studies *in vivo*.

This guidance is not aimed at defining the criteria for the validation of the procedures for residue monitoring by the official regulatory agencies.

The intent is that methods validated according to this guidance provide residue data that are acceptable to the regulatory agencies in determining appropriate withdrawal periods.

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3. Glossary

Analyte: Chemical entity involved.

Bioequivalence: Two medical products are bioequivalent if they are pharmaceutically equivalent or if they are pharmaceutical alternatives and their bioavailabilities after administration in the same molar dose are similar in such degree that their effects can be expected to be essentially the same (WHO 1996).

Accuracy Grade of agreement between the value measured and the real or expected value. (CAMEVET)

Pharmacokinetics: The branch in pharmacology that studies the pass of drugs through the organism, considering the time and dose. It includes the drugs absorption, distribution metabolism or biotransformation and excretion processes (CAMEVET).

Limit of Quantitation (LOQ): It is the smallest concentration of an analyte that can be quantified with a specific degree of accuracy and precision, within statistically defined limits.

Limit of Detection (LOD): It is the smallest concentration of an analyte from which it is possible to deduce the presence of the analyte in the test sample but it is not possible to quantify it, within statistically defined limits.

Linearity Ability of an analytical method to obtain results those are directly, or through a defined mathematical transformation, proportional to the concentration of the analyte in the sample.

Matrix: Predominant material, component or substrate that contains the analyte of interest. Used in residue depletion studies, it is the animal food product or byproduct (tissues, eggs, milk or honey) that includes or may include the residue under study.

Control sample: Tissue, plasma, milk, eggs, honey or any other biological material from an animal that has not been treated with the veterinary drug under investigation.

Processed sample: A sample that has been processed using a specific analytical procedure in order to extract the analyte of interest.

Real sample: Tissue, plasma, milk, eggs, honey or any other biological material from an animal that has not been treated with the veterinary drug under investigation.

Precision: It is the agreement degree between the results obtained during the repetitive use of an analytical procedure in a homogeneous sample, under the same conditions. It includes repeatability and reproducibility.

Repeatability (or intra test precision): It is the precision degree obtained using an analytical procedure in a defined laboratory, during a short time period, with the same equipment used by the same analyst. (USP 23) (CAMEVET).

Reproducibility: It is the agreement degree between the results obtained using the same analytical procedure and the same simple in different laboratories.

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Intermediate precision: Precision that reflects the variations inside a laboratory. It includes the same measurement procedure, the same location and repeated measurements of the same or similar objects during an extended period of time; it may include other changing conditions. Changes may include different days, new measuring calibrations, calibrators, operators and new systems of measurement.

Robustness It is a measure of the analytical method fiability, against little but intentional variations of the method parameters, giving a measure of its fiability during the habitual use. The EU defines it as the susceptibility of an analytical method to changes of experimental conditions (Dec. 657/2002)

Selectivity Ability of a method to distinguish between the analyte being measured and other substances that are expected to be present in the analyzed sample. It is also called "specificity".

4. Parameters to take into account for the validation of the analytical method

The validation of an assay method has specific parameters to bear in mind; it should be performed in the selected matrix/matrices and include, within the analytical range, the Maximum Residue Limit (MRL) for the studied substance. The parameters to be considered in a validation process are the following:

Linearity

Accuracy

Precision

Limit of detection

Limit of quantitation

Selectivity

Stability in matrix

Processed sample stability

Robustness

Each of the validation parameters will be described below.

4.1. Linearity

A calibration curve should be generated in which the linear relationship is demonstrated across the working range. The concentrations used have to be similar to the expected concentrations in the tissues in which the assay will be performed (e.g. plasma, tissue, milk, eggs, honey). In other words, the concentrations have to be around the MRL, being it in the middle point of the curve. The calibration curves can be generated in three formats depending upon methodology:

a) Standards in solutions (solvent/buffer),

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b) Matrix processed through extraction procedure and subsequently fortified into standard.

c) Matrix fortified into standard and subsequently processed through extraction procedure.

Linearity should be described through a linear regression plot of known concentration vs. response using a minimum of 5 different concentrations, triplicate. The next parameters are defined using statistical treatment: intercept, slope (Sensitivity), regression coefficient and repeatability for each concentration level. The linear relationship is generally best described by unweight linear regression, but it may be fit to a weighted linear regression with appropriate weighting factors in case of non-homogeneous variance of the experimental data (heteroscedasticity)

The recommended acceptance criterion for a standard curve depends on the format of the curve. Calibration curves generated in accordance to item c) are subjected to the same acceptance criteria as the fortified samples (see section 4.3. Precision). Calibration curves generated as described in items a) or b) require more stringent acceptance criteria (repeatability ≤15% in all concentrations and regression coefficient ≤0.98).

Some assays (e.g. microbiological assays) may require log transformations to achieve linearity, while other assays (e.g. ELISA, RIA) may require a more complex mathematical function to establish the relationship between concentration and response.

4.2. Accuracy

It is generally expressed in terms of percentage of recovery or percentage of error. Accuracy is closely related to systematic error (analytical method bias) and analyte recovery (measured as percent recovery). Recommended accuracy for residue methods will vary depending upon the concentration of the analyte. Recommended mean accuracies based on the concentration of the analyte provided by the CODEX[1] are listed above:

Analyte Concentration*	Acceptable Range
< 1 µg/kg	-50 % to +20 %
≥ 1 µg/kg < 10 µg/kg	-40 % to +20 %
≥ 1 µg/kg < 10 µg/kg	-30 % to +10 %
≥ 100 µg/kg	-20 % to +10 %

* µg/kg =ng/g = ppb

4.3. Precision

The precision in a laboratory validation should include an intra-test study (repeatability) and an inter test study (intermediate precision). In general, it is not necessary to determine inter-laboratory reproducibility in order to conduct a residue depletion study, since the laboratory that develops the method is usually the same laboratory that assays the samples of the residue study. Repeatability and intermediate precision should be determined through an evaluation of

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a minimum of three replicates at three different concentrations representative of the range of the intended validation range (which should include the LOQ) across three days of analysis.

For the purposes of the assay method validation, acceptable variability depends on the concentration of the analyte. Recommended acceptable precision as provided by the CODEX Guideline[2] are listed in the following table:

Analyte Concentration*	Intermediate Precision CV%
< 1 µg/kg	35%
≥ 1 µg/kg < 10 µg/kg	30%
≥ 1 µg/kg < 10 µg/kg	20%
≥ 100 µg/kg	15%

The Coefficient of Variation (CV) of the repeatability determined for each concentration point should not exceed 15%; except for the LOQ, that should not exceed 20%[3]. The CV is calculated by the following equation:

$$CV = \frac{DesvíoEstándar}{Media} * 100$$

4.4. Limit of detection

There are various scientifically valid ways to determine the LOD and any of these may be used as long as a scientific justification is provided for its use. See Annex I and Annex 2 for examples of acceptable methods to determine the LOD, and Annex 3 for a suggested protocol to determine accuracy, precision, LOD, LOQ and selectivity in a single study.

4.5. Limit of quantitation

As with the LOD, there are several scientifically valid ways to determine the LOQ and any of these may be used as long as a scientific justification is provided for its use. See Annex I and Annex 2 for examples of acceptable methods to determine the LOD, and Annex 3 for a suggested protocol to determine accuracy, precision, LOD, LOQ and selectivity in a single study.

4.6. Selectivity

In the case of the methods employed in residue studies, selectivity is primarily defined in relation to endogenous substances present in the matrix, and metabolites other than the marker residue. Since residue studies are well controlled, the exogenously administered components (i.e. other veterinary drugs or vaccines) are either known or not allowed during the study.

A good measure of selectivity of an assay is the determination of the response of control samples. That response should be no more than 20% of the response at the LOQ. See Annex 3

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for a suggested protocol to determine accuracy, precision, LOD, LOQ and selectivity in a single study.

4.7. Stability in matrix

Samples collected from residue studies are generally frozen and stored until assayed. It is necessary to determine how long these samples can be stored under the proposed storage conditions without undergoing excessive degradation prior to analysis. As part of the validation procedure or as a separate study, a stability study should be conducted to establish the appropriate storage conditions (e.g., 4º C, -20º C or -70º C) and the length of time the samples can be stored prior to analysis.

To conduct the assay, control samples (analyte-free) should be fortified with known quantities of the analyte and stored under the adequate conditions. Samples will be periodically assayed at specific intervals (i.e., initially, 1 week, 1 month and 3 months). If samples are frozen, freeze/thaw studies should be conducted (3 freeze/thaw cycles, one cycle per day at a minimum). Alternatively, real samples can be used with assays conducted to determine the starting concentrations. The protocol recommended for assaying stability in matrix is the analysis of two different concentrations in triplicate near the high and low end of the validation range. Stability in matrix is considered acceptable if the mean concentration obtained at the specified stability time point agrees with the acceptance criteria for accuracy, in agreement with the initial assay results or with freshly fortified control sample assay results.

4.8. Processed sample stability

Often, samples are processed one day and assayed on a second day or, due to an instrument failure, are stored additional days, e.g. weekend. The stability of the analyte in the processed sample extract can be examined when necessary to determine stability under processed sample storage conditions. Some examples of storage conditions would be 4 to 24 hours at room temperature and 48 hours at 4ºC. Other storage conditions can be investigated consistent with the method requirements.

The protocol recommended for assessing stability in matrix is the analysis of two different concentrations in triplicate near the high and low end of the validation range. Stability in processed samples is considered acceptable if the mean concentration obtained at the specified stability time point agrees with the acceptance criteria for accuracy, in agreement with the initial assay results or with freshly fortified control sample assay results.

4.9. Robustness

Evaluation of robustness of the analytical methods is of major importance. It should be evaluated particularly for areas of the method which could undergo changes or modifications over time. These areas may include variation of reagent lots or reagent lots of different ages, incubation temperatures, extraction solvent composition and volume, extraction time and number of extraction, solid phase extraction, cartridge brands and lots, analytical column brand and lots and HPLC elution solvent composition. During the development, validation or use of the assay, method sensibility to any or all of these conditions may become apparent and variations in the ones most likely to affect the method performance should be evaluated.

See Annex 4 for a suggested example to conduct this assay.

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Annex 1

Examples of methods to determine LOD and LOQ

One commonly used approach is referred to as the IUPAC definition[4]. In that procedure the LOD is estimated as mean of 20 control sample (from at least 6 separate sources) assay results plus 3 times the standard deviation of the mean. The LOQ then becomes the mean of the same results plus 6 or 10 times the standard deviation of the mean. Testing of the accuracy and precision at the estimated LOQ will provide the final evidence for determination of the LOQ. If the %CV for the repeatability measurement at that concentration is less than or equal to the accuracy and precision acceptance criteria (Section 2.2 and 2.3), then the estimated LOQ is acceptable.

In pharmacokinetic, bioequivalence or residue studies, values below the LOQ and above the LOD should not be taken into account for analysis, unless their use is properly justified.

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Annex 2

Codex alternative methods for determining LOD and LOQ

An alternative method for determining LOD and LOQ has been recommended by Codex Alimentarius[5] The method is said to overcome the problems associated with the IUPAC defined method (i.e. the high variability at the limit of measurement can never be overcome) in Annex 1. In this approach, the LOD is determined by a rounded value of the reproducibility relative standard deviation (RSD) when it goes out of control (i.e. where 3 X RSD = 100%; RSD = 33%, rounded to 50% because of the high variability). This method is then directly related to the analyte in matrix and not just the analyte.

The Limit of Quantitation (LOQ) then corresponds to the LOD and becomes defined as where the RSD = 25%. This is consistent with where the upper limit of detection merges with the lower limit of quantitation. As in the IUPAC method defined in Annex 1, testing of the accuracy and precision at the estimated LOQ will provide the final evidence for determination of the LOQ. If the %CV for the repeatability measurement at that concentration is less than or equal to the accuracy and precision acceptance criteria (Section 3.2 and 3.3), then the estimated LOQ is acceptable.

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Annex 3

Protocol for validation

Selectivity, LOD and LOQ are all interrelated and are affected by endogenous interferences that may be present in the matrix being assayed. LOD is often time difficult to determine particularly in LC/MS assays where control samples actually provide zero response at the retention time of the analyte. Without a response, it is impossible to calculate a standard deviation and therefore impossible to determine the LOD based on the mean plus 3 times the SD of the mean. Even if a mean plus 3 times the SD of the mean can be determined, it is often related to the instrument limit of detection rather than the method limit of detection. The following protocol is designed to determine specificity, LOD, LOQ, precision and accuracy in one study.

1. Collect 6 control samples from different animals and conduct a study of detection for any possible analyte contamination.

2. Fortify with the analyte each one of a minimum of 3 samples of the 6 control samples at 0. Each source should be randomly selected so that each source is represented at least once at each concentration.

   Concentrations to fortify the samples are the following:

   b1) the estimated LOD (determined during assay development)

   b2) 3 times the estimated LOD (equivalent to the estimated LOQ)

   b3) 3 other concentrations that will encompass the expected concentration range and should include the MRL, for example: 0.5 MRL; MRL and 2 MRL (Table 1).

   Repeat the fortification process for Day 2 and Day 3 using a second and third set of 3 samples each (randomly selected) so that each selected sample is represented at least once at each concentration of the 6 control samples.

Table 1. Example of Minimum Study Design to Allow Determination of LOD, LOQ, Accuracy and Precision (Six Sources/Animals: A, B, C, D, E, and F) Within One Study A, B, C, D, E y F) en un studio

Fortification Concentration	Animal/Source ID†		
	Day/Run 1	Day/Run 2	Day/Run 3
0 (Control)	B, F, D	A, C, C	B, E, F
eLOD*	B, C, E	D, F, F	A, B, E
eLOQ (3 X eLOD)*	C, C, E	A, B, E	D, F, D
Lower part of Validation Range	A, B, E	A, C, D	B, E, F

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Middle of Validation Range	B, C, E	C, E, F	A, D, F
Upper part of Validation Range	A, B, B	D, F, F	A, C, E

* eLOD = estimated LOD is generally determined from preliminary studies conducted during method development. eLOQ = estimated LOQ is determined as 3 times eLOD.

† each sample is randomly selected so that each source is represented at least once at each concentration across the 3 validation runs.

3. Assay the 18 samples each day and evaluate the results against a calibration standard curve.

4. Plot the results of concentration found against concentration added across all three days of assays. This will normalize the data results across days and allow all the data from the 3 runs to be used in the determination of the LOD and LOQ.

5. Establish a decision limit by calculating prediction intervals around both sides of the values of the estimated weighted regression line. (See following graphic)

   The upper prediction interval will be based upon the probability α (false positive) error.

   The lower prediction interval will be based upon the probability β (false negative) error[6].

   Normally, the prediction interval of the linear regression corresponds to a 90% confidence interval, that is to say an α error of 5% and a β error of 5%.

   The point in the Y-axis crossed by the upper limit of the confidence interval is called the decision limit (Y_C), and can be converted to concentration by extrapolating that value to the point corresponding to the regression line and from there to the X-axis (L_C). This is the critical point where 50% of the responses are real.

   The limit of detection (LOD) can be determined estimating the concentration derived from the extrapolation of the Y_C value to the lower confidence interval limit (β) and from there to the X-axis, point named L_D.

6. Establish a determination limit (Y_Q) by multiplying the limit of detection (Y_C) by 3 (commonly accepted ratio between the LOD and LOQ is 3). The LOQ (L_Q) can be determined calculating the point where the Y_Q line crosses the lower confidence limit β that reduces the false negative rate for the determination of LOQ to what level is assigned to β (typically 5%).

7. Internal reproducibility can be determined calculating the %CV at each concentration evaluated. Accuracy can be determined by comparison of the results obtained to the fortification levels. Acceptance criteria for accuracy and precision are provided in Sections 2.2. and 2.3 respectively.

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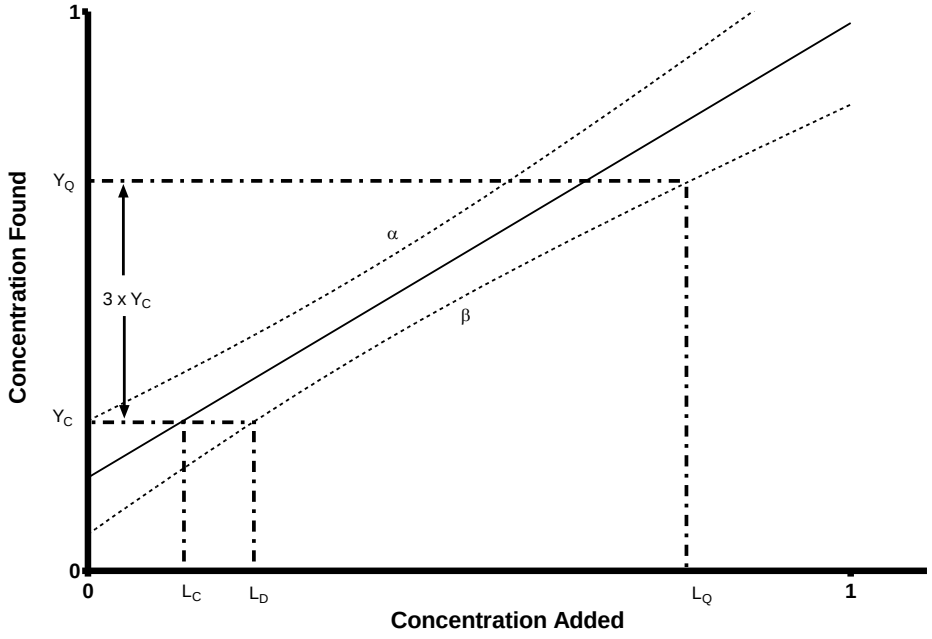

Concentration found	Concentration found
Concentration added	Fortification Concentration

This approach takes into consideration the interrelationship between specificity, LOD and LOQ By determining LOD and LOQ using 6 different sources of matrix, the variability due to the matrix as well as the variability of the assay is taken into account. Since specificity for residue methods is dependent upon the possible interference of matrix components this approach also addresses specificity and insures that specificity is acceptable at the LOD and LOQ determined. This approach is consistent with the determination of the LOD and the LOQ specified in VICH GL2 (Validation Methodology) Guideline.

Data Set Examples:

A validation procedure based on the above methodology was conducted on an ELISA assay

Control swine serum obtained from six different animals were each fortified with the analyte at 0, 50, 150, 300, 600 and 1200 ng/mL giving a total of 36 samples. Because this was a serum assay and it was relatively easy to run, all six fortification levels were run on each of three days. Had this been tissue samples, we would have randomly chosen 3 of the 6 animals (insuring that each of the 6 animals were run at least once) at each of the fortification levels to run on each of the 3 days of assay for a total of 18 samples per day

Based on these three days of analyses which consisted of 108 assays total (for tissue assays it would have been 54 assays total) the following determinations were done: repeatability (intra-day precision), inter-day precision, LOD and LOQ. The raw data and the results of the statistical analyses are listed below.

Run	Fortification Level, ng/mL	Results, ng/mL					
		Animal A	Animal B	Animal C	Animal D	Animal E	Animal F
1	0	nr	nr	nr	nr	nr	nr
	50	9	32	59	18	18	25
	150	162	160	148	145	133	128
	300	251	303	331	295	270	260
	600	508	514	592	513	568	609
	1200	907	1186	1162	1037	1050	1097
2	0	nr	nr	nr	nr	nr	nr
	50	26	41	40	36	37	27
	150	155	168	130	144	143	177
	300	234	251	335	307	251	247
	600	504	522	553	516	650	580
	1200	999	1030	1037	1020	985	996
3	0	1	nr	8	nr	nr	1
	50	39	60	71	50	68	48
	150	157	179	159	167	172	148
	300	290	277	336	319	299	278
	600	565	572	611	586	648	579
	1200	1071	1190	1218	1262	1246	1160

nr = no response

For tactical evaluation of the above data a simple model was used, which included the fixed effect of treatment, the random effects of run, sample preparation, etc. Such analysis was conducted as follows:

- In order to assess method accuracy, Percentage Recovery (%R) was calculated for each sample by dividing the found concentration by the fortification concentration prior to analysis (fortification level or nominal concentration) and multiplying then by 100.
- In order to assess within-day variability (Repeatability), the Percentage Coefficient of Variation (%CV) was calculated, dividing the standard deviation by the mean, taking into consideration data for all animals for each fortification level and multiplying by 100. Global repeatability was also calculated as the %CV taking into consideration values for all levels and all animals always for a same day (treatment).

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- In order to assess across-day variability (Intra-laboratory reproducibility) the %CV was calculated for a same fortification level but using %R values obtained for all animals and all days (using a total of 18). Also global internal repeatability was calculated in terms of the %CV, taking into account all levels from all days and all animals (using in this case a total of 72 samples). This last result (%CV for all levels and all days) is a good measurement of the variation the method will have in the run, independently from the fortification level, given that all factors that can affect the method accuracy are taken into consideration.

The Percentage Recovery values obtained are the following:

Run	Fortification Level, ng/mL	Results, % Recovery					
		Animal A	Animal B	Animal C	Animal D	Animal E	Animal F
1	0	nr	nr	nr	nr	nr	nr
	50	18,0	64,0	118,0	36,0	36,0	50,0
	150	108,0	106,7	98,7	96,7	88,7	85,3
	300	83,7	101,0	110,3	98,3	90,0	86,7
	600	84,7	85,7	98,7	85,5	94,7	101,5
	1200	75,6	98,8	96,8	86,4	87,5	91,4
2	0	nr	nr	nr	nr	nr	nr
	50	52,0	82,0	80,0	72,0	74,0	54,0
	150	103,3	112,0	86,7	96,0	95,3	118,0
	300	78,0	83,7	111,7	102,3	83,7	82,3
	600	84,0	87,0	92,2	86,0	108,3	96,7
	1200	83,3	85,8	86,4	85,0	82,1	83,0
3	0	1,0	Nr	8,0	Nr	Nr	1,0
	50	78,0	120,0	142,0	100,0	136,0	96,0
	150	104,7	119,3	106,0	111,3	114,7	98,7
	300	96,7	92,3	112,0	106,3	99,7	92,7
	600	94,2	95,3	101,8	97,7	108,0	96,5
	1200	89,3	99,2	101,5	105,2	103,8	96,7

Note: 50 ng/mL fortification level was below the LOD, and neither %R values nor %CV comply with the acceptance criteria and therefore were not used to determine precision.

Results of repeatability for the %R values are the following:

Run	Fortification Level, ng/mL	Repeatability per Level			Total Repeatability		
		SD	Mean	%CV	DS	Mean	%CV
1	0						

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	50						
	150	9,2	97,3	9,4	**8,8**	**93,4**	**9,4**
	300	10,1	95,0	10,6			
	600	7,5	91,8	8,1			
	1200	8,4	89,4	9,4			
2	0						
	50						
	150	11,6	101,9	11,4	**11,4**	**92,2**	**12,3**
	300	13,4	90,3	14,9			
	600	9,1	92,4	9,8			
	1200	1,7	84,3	2,1			
3	0						
	50						
	150	7,5	109,1	6,8	**7,6**	**101,8**	**7,4**
	300	7,8	99,9	7,9			
	600	5,2	98,9	5,2			
	1200	5,8	99,3	5,8			

Results of Intra-laboratory Repeatability for the %R values are the following:

Fortification Level, ng/mL	**Inter-laboratory Reproducibility per Level**			**Total Internal Repeatability**		
	SD	**Mean**	**%CV**	**SD**	**Mean**	**%CV**
0						
50						
150	10,3	102,8	10,0	**10,2**	**95,8**	**10,6**
300	10,8	95,1	11,4			
600	7,7	94,4	8,2			
1200	8,5	91,0	9,4			

Note: Since there is no guidance where a minimum percentage of recovery is determined, this could be low (e.g.: 40%) but if the %CV for all concentrations is below 20%, it is accepted. The opposite example would be: If we have a molecule with a 95% recovery but a %CV above 20%, it means there is a methodological problem.

The results obtained for the LOD and LOQ are the following:

LOD = 62 ng/ml

LOD = 112 ng/ml

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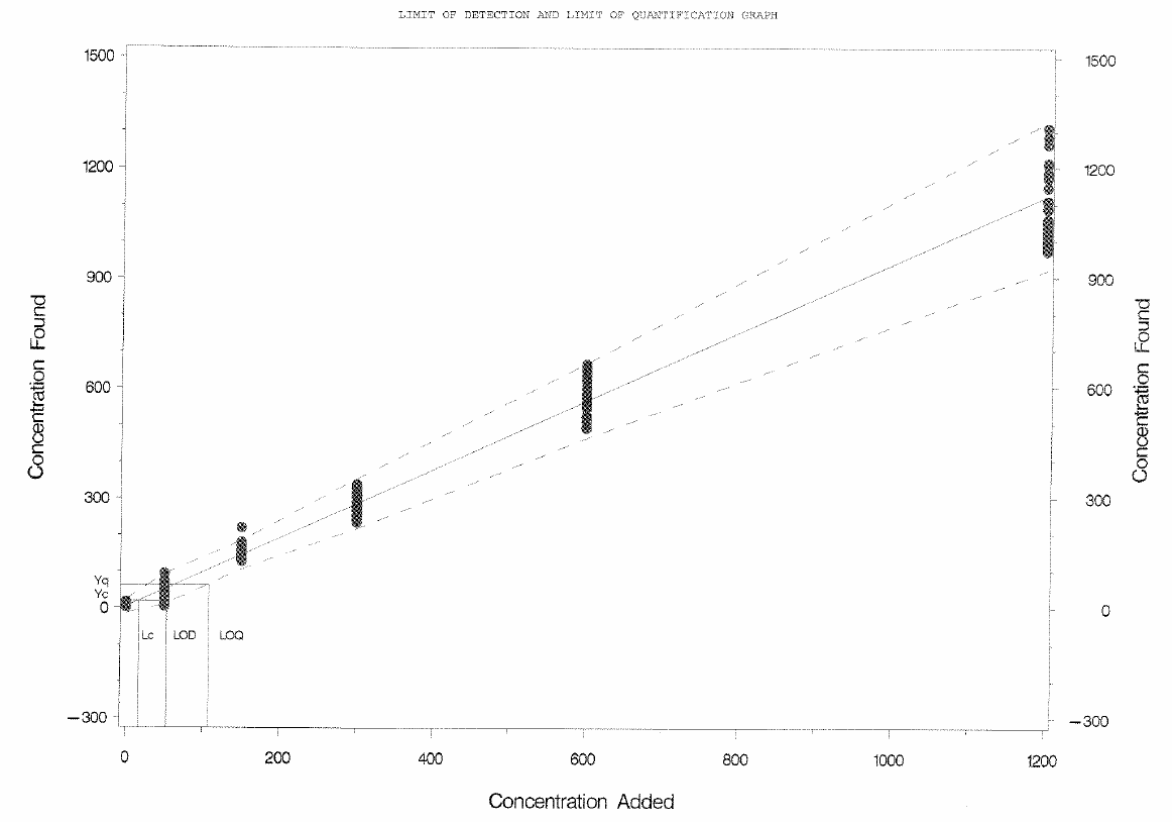
A graphical representation of the determination of the LOD and LOQ is provided below:



Limit of detection and limit of quantification graph	Limit of detection and limit of quantification graph
Concentration found	Concentration found
Concentration added	Concentration added

This is a straightforward way of accurately determining precision, accuracy, LOD and LOQ in a study during three days of validation.

Accuracy can also be determined as the gradient of the graphic of Found Concentration vs. Fortification Concentration.

The LOD and the LOQ agree with the estimate of a subjective evaluation of the data and based on these results it is logical not to have taken into consideration the fortification level of 50ng/mL in the calculation of method precision, given that below 112 ng/mL (LOQ) it is impossible to conduct a quantitation with a statistically acceptable level of confidence, that is experimentally verified when observing the values of found concentration (or %R) and the precision obtained for such level, which cannot comply with the established criteria of acceptance.

Precision obtained is considered more than acceptable if we take into account that it is an ELISA procedure that complies with the acceptance criteria described in this document. During the method run, after 50 to 100 new results of %R (for different fortification levels), have been obtained, precision, accuracy, LOD and LOQ values can be updated.

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Annex 4

Robustness

The Youden and Steiner procedure, which allows evaluation of up to seven variables with the analysis of only eight samples, can be used. The method is a fractional factorial design and does not allow the detection of interactions between the diverse factors.

Each variable is studied through a high (A, B,...G) and a low (a, b,...g) value (or quality when that is not possible) and eight samples are designed following the example shown in Table 1. Results are represented with letters from "s" to "z".

Table 1: Youden Robustness Test for analytical method

VALOR DE LAS VARIABLES	ANALISIS							
	1	2	3	4	5	6	7	8
A, a	A	A	A	A	a	a	a	a
B, b	B	B	b	b	B	B	b	b
C, c	C	c	C	c	C	c	C	c
D, d	D	D	d	d	d	d	D	D
E, e	E	e	E	e	e	E	e	E
F, f	F	f	f	F	F	f	f	F
G, g	G	g	g	G	g	G	G	g
Resultados	s	t	u	v	w	x	y	z

Based on the results from samples analysis, each variable effect can be determined by calculating the media of the four analysis containing the variable in its higher value (capital letter) and those presenting it in its the lower value (lower case letter). Thus, the effect of the change from Factor "A" to "a" is measured through the difference:

$$Dif = \frac{s+t+u+v}{4} - \frac{w+x+y+z}{4}$$

That is to say, the mean of the results (s+t+u+v) is equivalent to “A” because the remaining variables present in these four results neutralize each other because there are always two upper case and two lower case of each variable. In an analogue way, the mean of results (w+x+y+z) is equivalent to “a”.

The effect of each factor is calculated. Finally, the effect of change from “G” to “g” is measured by the difference (s+v+x+y)/4 - (t+u+w+z)/4.

When comparing both middle values, the influence of the variable in the study is known.

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For any other variable, the following similar procedure, as shown in Table 1, can be applied.

Establishing the seven possible comparisons (A-a,...G-g), the effect of each variable can be known; the bigger the difference, the greater the influence that such variable will have in the analytical method. If any of the differences between the mean of the subgroups of four is higher than $\sqrt{2} * DS$, these variables will receive special attention when drafting the method, highlighting the need of a strict control to obtain quality results, that is to say if:

$$Dif > \sqrt{2} * DS$$

Where SD= standard deviation between the replicates conducted under inter-laboratory reproducibility conditions (validation) at the same fortification level, then such variable will be considered critical.

Note 1: The factors being studied should not necessarily be seven; a lower number of variables can be considered. This will not affect the balance of the trial design as long as the eight indicated assays are conducted.

Note 2: An additional information of this Youden Test is that standard deviation of results "s" to "z" constitutes an excellent measurement of the estimated imprecision of the method when the routine analysis is used, since this procedure deliberately introduces the type of variation of the variables that can be expected to occur during the normal use of the method.

Frequency of revision
5 years

Codex Guidelines for the Establishment of a Regulatory Program for Control of Veterinary Drug Residues in Foods, Part III Attributes of analytical Methods for Residue of Veterinary Drugs in Foods, p. 41, CAC/GL 16-1993.

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[4] IUPAC: International Union of Pure and Applied Chemistry.

[5] Codex Alimentarius Procedural Manual, 15th Ed., Twenty-eight Session of the Codex Alimentarius Commission, Rome, 2005, p 81.

[6] Zorn ME, Gibbons RD, Sonzogni WC. Weighted Least-Squares Approach to Calculating Limits of Detection and Quantification by Modeling Variability as a Function of Concentration, *Anal Chem* 1997, 69, 3069-3075.

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