International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

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STUDIES TO EVALUATE THE METABOLISM AND RESIDUE KINETICS OF VETERINARY DRUGS IN FOOD PRODUCING ANIMALS: VALIDATION OF ANALYTICAL METHODS USED IN RESIDUE DEPLETION STUDIES

Revision at step 9

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This Guideline has been revised by the appropriate VICH Expert Working Group. At Step 7 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA.

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1. INTRODUCTION

1.1. Objective of the guidance

This guidance document is intended to provide a general description of the criteria that have been found by the European Union (EU), Japan, United States of America (USA), Australia, New Zealand and Canada to be suitable for the validation of analytical methods used in veterinary drug residue depletion studies.

1.2. Background

During the veterinary drug development process, residue depletion studies are conducted to determine the concentration of the residue or residues present in the edible products (tissues, milk, eggs or honey) of animals treated with veterinary drugs. This information is used in regulatory submissions around the world. Submission of regulatory methods (i.e., post approval control methods) and the validation requirements of the regulatory methods are usually well defined by various regulatory agencies worldwide and might even be defined by national or regional law. However, the residue depletion studies are generally conducted before the regulatory methods have been completed. Often times the in-house validated residue methods provide the framework for the methods submitted for regulatory monitoring. Harmonization of the validation requirements for methodology used during residue depletion studies and submitted to the regulatory agencies in support of the maximum residue limits (MRLs) and withdrawal periods should be achievable. It is the intent of this document to describe a validation procedure that is acceptable to the regulatory bodies of the VICH regions for use in the residue depletion studies. This validated method could continue on to become the "regulatory method" but that phase of the process will not be addressed in any detail in these guidelines.

A variety of validation guidelines exist for analytical methodology and many of the aspects of those validation procedures are incorporated in this document (VICH GL1 (Validation Definition), October 1998 and VICH GL2 (Validation Methodology), October 1998). However, there are aspects of residue validation procedures that are addressed in this guidance document that are not addressed in previous documents. The guidance provided here is intended to specifically address the validation of veterinary drug residue methods.

2. GUIDANCE

2.1 Purpose

The purpose of this document is to provide a general description of procedures that can be used for the validation of the analytical methods developed for the analysis of tissue samples obtained in residue depletion studies.

For purposes of this guidance, "acceptable" refers to the scientific evaluation of the analytical method in terms of the described validation criteria.

2.2. Scope

This guidance is only intended to apply to analytical procedures that have been developed for the evaluation of veterinary drug residue methods (assays developed to determine residues in marker residue depletion studies). It is not intended to define the criteria needed for validation of regulatory monitoring assay procedures.

This document provides performance characteristics of the residue assays that if followed would generally be considered acceptable by the regulatory agencies in the VICH regions. The intent is that methods validated according to this guidance will provide residue data that would generally be considered acceptable by the regulatory agencies in determining appropriate withdrawal periods.

While it is recognized that residue studies must be conducted under GLP using validated methodology, the actual method validation experiments do not fall within the scope of the GLP regulations. However, raw data generated as a result of a method validation should be archived as appropriate and be available for submission to regulatory authorities upon request.

3. PERFORMANCE CHARACTERISTICS

In general, there are specific performance characteristics of a method validation. Those performance characteristics are defined as follows:

Linearity

Accuracy

Precision

Limit of Detection

Limit of Quantitation

Selectivity

Stability in Matrix

Process Sample Stability

Robustness

Each of the characteristics will be described below as they apply to the validation of methods intended for use in veterinary drug residue depletion studies.

3.1. Linearity

A calibration curve should be generated in which the linear relationship is evaluated across the range of the expected matrix (tissue, milk, egg or honey) concentrations. Calibration standard curves can be generated in three formats depending upon the methodology: standards in solvent/buffer, standards fortified into control matrix extract and standards fortified into control matrix and processed through the extraction procedure. Linearity should be described by a linear, polynomial or other (as appropriate) regression plot of known concentration vs. response using a minimum of 5 different concentrations. Acceptability of weighting factors should be determined by evaluation of the residuals across three runs to determine if the residuals are randomly distributed. Evaluation of the residuals should be carried out across at least three separate runs.

The recommended acceptance criterion for a standard curve is dependent upon the format of the standard curve. Calibration standard curves generated by fortification of control matrix and processed through the procedure are subject to the same acceptance criteria as the samples (see

Section *3.3. Precision*). Calibration standard curves generated by standards in solvent/buffer or by fortification of control matrix extract would require more stringent acceptance criteria (Repeatability ≤ 15% at all concentrations except at or below LOQ where it can be ≤ 20%).

Some assays (e.g. microbiological assays) could require log transformations to achieve linearity where other assays (e.g., ELISA, RIA) could require a more complicated mathematical function to establish the relationship between concentration and response. Again, acceptability of the function selected should be verified by evaluation of the residuals generated when that function is used.

3.2. Accuracy

Accuracy refers to the closeness of agreement between the true value of the analyte concentration and the mean result that is obtained by applying the experimental procedure. 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. The accuracy should meet the range listed below:

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

* µg/kg =ng/g = ppb

3.3. Precision

Precision of a method is the closeness of agreement between independent test results obtained from homogenous test material under stipulated conditions of use. Analytical variability between different laboratories is defined as reproducibility, and variability from repeated analyses within a laboratory is repeatability. Single-laboratory validation precision should include a within-run (repeatability) and between-run component.

The within- and between-run precision of the analytical method can be determined as part of the validation procedure. There is generally not a need to determine reproducibility (between-laboratory precision) in order to conduct a residue depletion study, because the laboratory that is developing the method is often the same laboratory assaying the samples from the residue study. Instead of establishing reproducibility of the assay, a within-run precision, can be determined. Within- and between-run precision should be determined by the evaluation of a minimum of three replicates at three different concentrations representative of the intended validation range (which should include the LOQ) across three days of analysis.

For the purposes of the residue method validation, acceptable variability is dependent upon the concentration of the analyte. The precision should meet the range listed below.:

Analyte Concentration	Acceptable within-run precision (Repeatability), %CV	Acceptable between-run precision %CV*
< 1 µg/kg	30 %	45%
≥ 1 µg/kg < 10 µg/kg	25 %	32%
≥ 10 µg/kg < 100 µg/kg	15%	23%
≥ 100 µg/kg	10 %	16%

* as determined by the Horwitz equation $CV = 2^{(1-0.5 \log C)}$ where C = concentration expressed as a decimal fraction (e.g. 1 µg/kg is entered as 10^{-9}).

3.4. Limit of Detection

The limit of detection (LOD) is the smallest measured concentration of an analyte from which it is possible to deduce the presence of the analyte in the test sample with acceptable certainty. There are several scientifically valid ways to determine LOD and any of these could be used as long as a scientific justification is provided for their use. See Annex 1 and Annex 2 for examples of acceptable methods for determining LOD and Annex 3 for a suggested protocol for determining accuracy, precision, LOD, LOQ and selectivity in a single study.

3.5. Limit of Quantitation

The LOQ is the smallest measured content of an analyte above which the determination can be made with the specified degree of accuracy and precision. As with the LOD, there are several scientifically valid ways to determine LOQ and any of these could be used as long as scientific justification is provided. See Annex 1 and Annex 2 for examples of acceptable methods for determining LOQ and Annex 3 for a suggested protocol for determining accuracy, precision, LOD, LOQ and selectivity in a single study.

3.6. Selectivity

Selectivity is the ability of a method to distinguish between the analyte being measured and other substances which might be present in the sample being analyzed. For the methods used in residue depletion studies, selectivity is primarily defined relative to endogenous substances in the samples being measured. Because the residue depletion studies are well controlled, exogenously administered components (i.e., other veterinary drugs or vaccines) could either be known or not be allowed during the study. If it is the intent to submit the validated method as a regulatory method, it might be prudent for the investigator to test known products used in the animals being tested for possible interference.

A good measure of the selectivity of an assay is the determination of the response of control samples (see section 3.5 above). That response should be no more than 20% of the response at the LOQ. See Annex 3 for a suggested protocol for determining accuracy, precision, LOD, LOQ and selectivity in a single study.

3.7. Stability in Matrix

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

Samples should be fortified with known quantities of analyte and stored under the appropriate conditions. Samples should be periodically assayed at specified intervals (e.g. initially, 1 week, 1 month, 3 months). If the samples are frozen, freeze/thaw studies should be conducted (3 freeze/thaw cycles – one cycle per day at a minimum). Alternatively, incurred samples can be used with initial assays conducted to determine the starting concentrations. The recommended protocol 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 matrix is considered acceptable if the mean concentration obtained at the specified stability time point agrees with the initial assay results or freshly fortified control sample assay results within the accuracy acceptance criteria established in Section 3.2.

3.8. Processed Sample Stability

Often, the samples are processed one day and assayed on a second day or because of an instrument failure are stored additional days, e.g. over a weekend. The stability of the analyte in the process sample extract might be examined as necessary to determine stability under processed sample storage conditions. Examples of storage conditions would be 4 to 24 hours at room temperature and 48 hours at 4°C. Other storage conditions might be investigated consistent with the method requirements. The recommended protocol for assessing processed sample stability is the analysis of two different concentrations in triplicate near the high and low end of the validation range. Processed sample stability is considered adequate if the mean concentration obtained at the specified stability time point agrees with the initial assay results or with freshly fortified and processed control sample assay results within the accuracy acceptance criteria established in Section 3.2.

3.9. Robustness

Evaluation of the robustness of regulatory methods is of major importance. Evaluation of robustness for residue methodology is less of a concern for residue methods as these are usually conducted within a single laboratory using the same instrument. However, robustness should still be evaluated particularly for areas of the method that could undergo changes or modifications over time. These might include reagent lots, incubation temperatures, extraction solvent composition and volume, extraction time and number of extractions, solid phase extraction (SPE) cartridge brand and lots, analytical column brand and lots and HPLC elution solvent composition. During the development, validation or use of the assay, method sensitivity to any or all of these conditions can become apparent and variations in the ones most likely to affect the method performance should be evaluated.

4. GLOSSARY

Accuracy – The accuracy of an analytical procedure expresses the closeness of agreement between the true value of the analyte concentration and the mean result that is obtained by applying the analytical procedure. This is generally expressed as % recovery or % bias.

Control sample – Tissue, milk, egg or honey from an animal that has not been treated with the veterinary drug under investigation.

Between-run Precision – Between-run precision expresses within-laboratory between-run variations.

Incurred sample – Tissue, milk, egg or honey from an animal treated with the veterinary drug under investigation that has a residue concentration of the analyte of interest.

Limit of Detection – The limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected with acceptable certainty but not quantitated as an exact value.

Limit of Quantitation – The limit of quantitation of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy.

Linearity – The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Marker residue – The residue whose concentration is in a known relationship to the concentration of total residue in an edible tissue.

Matrix – The matrix is basic edible animal products (tissue, egg, milk or honey) that contains or could contain the residue of interest.

Precision – The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Processed Sample – A processed sample is a sample that has been extracted or otherwise processed to remove the analyte from much of the original sample matrix.

Repeatability – Repeatability expresses the precision under the same operating conditions over a short interval of time.

Reproducibility – Reproducibility expresses the precision between laboratories.

Residue – Veterinary drug (parent) and/or its metabolite.

Robustness – The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage**.**

Selectivity – Selectivity is the ability to assess the analyte in the presence of components (endogenous materials, degradation products, other veterinary drugs) that might be expected to be present.

Within-run Precision – Within-run precision expresses within-laboratory within-run variations.

Annex 1

Examples of Methods for Determining LOD and LOQ

One commonly used approach is referred to as the IUPAC definition.[1] 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 3.2 and 3.3), then the estimated LOQ is acceptable.

Annex 2

U.S. Environmental Protection Agency Method for Determining LOD and LOQ

The procedure described below is a slight modification of a procedure used by USDA's Interregional Project No. 4 program which is published, in 40 CFR Part 136, Appendix B).[2] This modified procedure can be found in Appendix 1 of the U.S. Environmental Protection Agencies document entitled "Assigning Values to Non-detected/Non-quantified Pesticide Residues in Human Health Food Exposure Assessments[3]. The procedure is provided below with minor modifications making it more representative of a tissue marker residue assay procedure example.

In this procedure, the estimation of the LOD and LOQ of a specific method for a specific analyte in a specific matrix can be done in the following two steps.

- The first step is to produce a preliminary estimate of the LOD and LOQ and to verify that a linear relationship between concentration and instrument response exists. These preliminary estimates correspond to what some term the IDL (Instrument Detection Limit) and IQL (Instrument Quantitation Limit), respectively. The matrix of interest will be fortified (spiked) at the estimate LOQ in the next step for the actual estimation of LOD and LOQ of the method.
- The second step is to use the initial estimate of the LOD and LOQ determined in Step 1 to estimate the method detection limit and the method quantitation limit in the matrix of interest.

An illustrative example follows:

Step 1. The analyst derives a standard curve for the method of interest. In this particular instance, the analyst prepares the standard solution in buffer or water with the following concentrations of the analyte of interest: 0.005, 0.010, 0.020, 0.050 and 0.100 µg/mL. For each concentration in the sample solution, the following instrument responses (measure peak height) are recorded:

Concentration (µg/mL)	Instrument Response (peak height)
0.100	206,493
0.050	125,162
0.020	58,748
0.010	32,668
0.005	17,552

In order to verify that a linear response is seen throughout the tested range, the instrument response is plotted as a function of injected concentration. The results (and associated statistics) are shown in Figure 1. Note from these results that the instrument response appears to be adequately linear throughout the range of tested concentrations (0.005 to 0.100 µg/mL), and that the R^2 value from the "Summary of Fit" box in Figure 1 as the Root Mean Square Error) is 8986.8. The equation which describes this relationship (provided in the "Parameter Estimates" box of Figure 1) is as follows:

$$Y = 15{,}120 + 1{,}973{,}098 * (\text{Concentration})$$

Where Y is the instrument response (peak height)

The estimated LOD and LOQ are calculated as follows (assuming these values are set to 3 and 10 standard deviations above the blank response, respectively):

1. The Peak Height at the LOD (Y_{LOD}) is calculated at 3 times the standard deviation while the Peak Height at the LOQ (Y_{LOQ}) is calculated at 10 times the standard deviation

   $$Y_{LOD} = 15120 + 3 * (8987) = 42{,}081$$

   $$Y_{LOQ} = 15120 + 10 * (8987) = 104{,}990$$

2. These values (peak height at LOD and peak height and LOQ) are then used to calculate the concentrations associated with these peak heights as follows:

   $$Y = 15{,}120 + 1{,}973{,}098 * (\text{Concentration})$$

   Rearranging,

   $$\text{Concentration} = (Y - 15{,}120) / 1{,}973{,}098$$

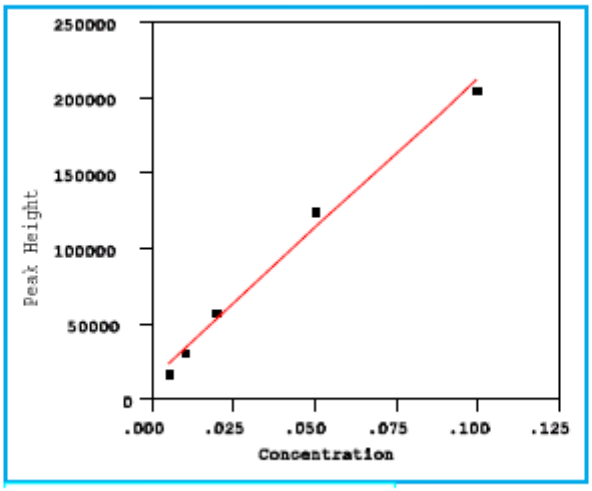
   Therefore,

   $$LOD = Y_{LOD} - 15{,}120 / 1{,}973{,}098 = (42{,}081 - 15{,}120) / 1{,}973{,}098 = 0.014 \text{ µg/mL}$$

   $$LOQ = Y_{LOQ} - 15{,}120 / 1{,}973{,}098 = (104{,}990 - 15{,}120) / 1{,}973{,}098 = 0.046 \text{ µg/mL}$$

Thus, the initial estimated LOD and LOQ are 0.014 and 0.046 µg/mL, respectively which correspond to the IDL and IQL.

These estimated LODs (or IDLs) and LOQs (or IQLs) are expressed in terms of the solution concentration and not in terms of the matrix concentration. At this stage, the solution concentration (µg/mL solution) should be converted to the effective concentration in the matrix (e.g., µg/g of matrix).



Summary of Fit

RSquare	0.99003
RSquare Adj	0.986707
Root Mean Square Error	8986.837
Mean of Response	88124.6
Observations (or Sum Wgts)	5

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Intercept	15119.954	5834.672	2.59	0.0810	-3448.891	33688.799
Concentration	1973098.5	114317.5	17.26	0.0004	1609283.2	2336913.9

Figure 1. Statistical Results

Step 2. With the initial estimate of LOD (or IDL) and LOQ (or IQL) obtained and linearity verified, Step 2 involves estimating the LOQ and LOD in spiked matrix samples. This procedure uses the estimated instrumental LOQ and the procedure detailed in 40 CFR Part 136, Appendix B[4] to provide a better estimate of LOQ and verifies that method recoveries are acceptable.

The method calls for the analysis of 7 or more untreated control samples spiked at the estimated LOQ. The standard deviation of these samples is measured and the LOD and LOQ are determined as follows:

$LOD = t_{0.99} * S$

$LOQ = 3 * LOD$

where t = one-tailed t-statistic at the 99% confidence level for n-1 replicates

S = Standard Deviation of n sample spikes at the estimated LOQ

The following is a set of t-values for use in the above equation:

# of Replicates (n)	Degrees of Freedom (n-1)	$t_{0.99}$	# of Replicates (n)	Degrees of Freedom (n-1)	$t_{0.99}$
3	2	6.965	13	12	2.681
4	3	4.541	14	13	2.650
5	4	3.747	15	14	2.624
6	5	3.365	16	15	2.602
7	6	3.143	17	16	2.583
8	7	2.998	18	17	2.567
9	8	2.896	19	18	2.552
10	9	2.821	20	19	2.539
11	10	2.764	21	20	2.528
12	11	2.718	22	21	2.518

In this example, the analyst prepated 7 untreated control samples spiked at the above estimated LOQ of 0.05 µg/g. The following results were obtained:

Concentration detected (µg/g)	% Recovery
0.0397	79.4
0.0403	80.6
0.0400	80.0
0.0360	72.0
0.0498	99.6
0.0379	75.8
0.0388	77.6

Average Concentration: 0.0404 µg/g

Standard Deviation: 0.0044 µg/g

Average Recovery: 80.7%

Given that recoveries are adequate at the LOQ (average = 80.7%, range = 72.0% to 99.6%), the LOD and LOQ for the method are estimated as follows:

LOD = $t_{0.99}$ * S (for 7-1 = 6 degrees of freedom)

= 3.143 * 0.0044 µg/g

= 0.0138 µg/g

LOQ = 3 * LOD

= 3 * 0.0138 µg/g

= 0.0414 µg/g

Annex 3

Protocol for Residue Method Validation

Selectivity, LOD and LOQ are all interrelated and are affected by endogenous interferences that might 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 drug free matrix from 6 separate sources (animals) and screen for any possible analyte contamination.
2. Fortify (spike) 1 each of a minimum of 3 samples (each source randomly selected such that each source is represented at least once at each concentration) of the 6 control samples at 0, at the estimated LOD (determined during assay development), at 3 times the estimated LOD (estimated LOQ), and 3 other concentrations that will encompass the expected concentration range (Table 1). Repeat the fortification process for Day 2 and Day 3 using a second and third set of 3 each (each source randomly selected such that each is represented at least once at each concentration) of the 6 control samples.

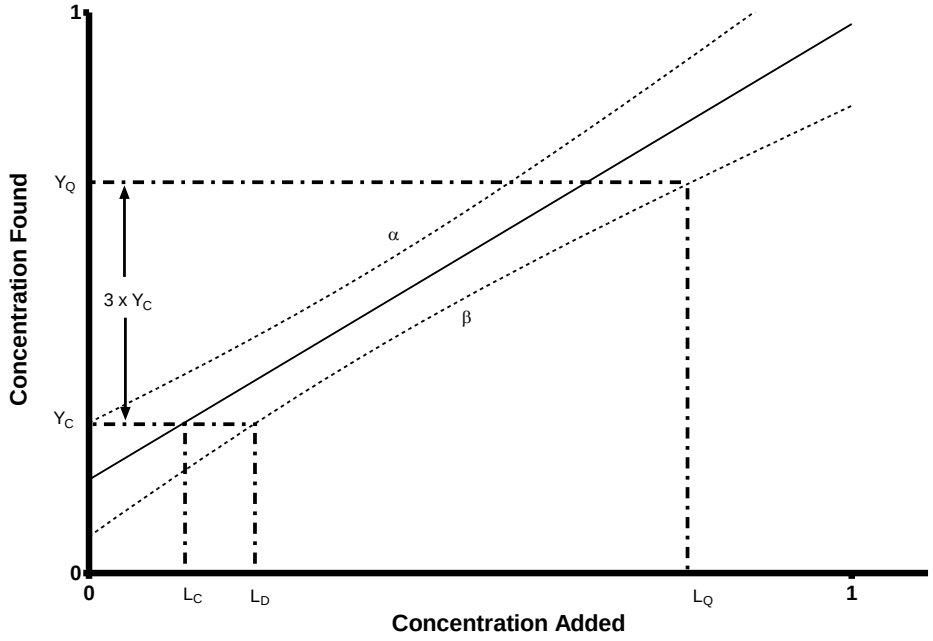
Table 1. Example of Minimum Study Design to Allow Determination of LOD, LOQ, Accuracy and Precision (Six Sources/Animals: A, B, C, D, E, and F) Within One Study

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
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 source randomly selected such 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 the weighted regression line with the upper confidence interval line based upon the probability α (false positive) and the lower confidence interval line based upon the probability β (false negative)[4]. The decision limit (Y_C) then becomes the point at which the upper confidence limit crosses the Y-axis and can be converted to concentration by estimating from the regression line to the x-axis (L_C). This is the critical point where 50% of the responses are real. The L_D or LOD can be determined by estimating concentration from the lower confidence limit β that reduces the false negative rate to what level is assigned to β. Typically, both α and β are set equal to 5%.
6. Establish a determination limit (Y_Q) by multiplying the detection limit (Y_C) by 3 (commonly accepted ratio between LOD and LOQ is 3). The LOQ (L_Q) can then be determined by estimating where the line Y_Q 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. Inter-day precision can be determined by 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 3.2 and 3.3, respectively.



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 detection limit and quantitation limit specified in VICH GL2 (Validation Methodology) Guideline.

Data Set Example:

A validation procedure based on the above methodology was conducted on an LC-MS/MS milk assay procedure.

Control bovine milk obtained from six different animals were each fortified with the analyte at 0, 4.2, 14.0, 35, 140 and 400 ng/mL giving a total of 36 samples. Milk samples from 3 of the 6 animals (insuring that each of the 6 animals were run at least once) were randomly chosen at each of the fortification levels to be 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 54 assays total the following determinations were done: repeatability (within-day precision), between-day precision, LOD and LOQ. The raw data and the results of the statistical analyses are listed below:

Concentration of Analyte in Control Milk Fortified at 0, 4.2, 14.0, 35.0, 140 and 400 ng/mL Across Three Days of Analysis						
Conc. Added, ng/mL	**Run 1**		**Run 2**		**Run 3**	
	Animal ID	**Conc. Found, ng/mL**	**Animal ID**	**Conc. Found, ng/mL**	**Animal ID**	**Conc. Found, ng/mL**
0	B	0.494	A	0.233	B	0.154
	F	0.654	C	0.012	E	0.120
	D	0.588	C	0.117	F	0.313
4.2	B	4.38	D	4.97	A	3.80
	C	4.13	F	3.85	B	4.12
	E	4.33	F	4.41	E	3.67
14.0	C	13.2	A	11.1	D	11.8
	C	13.5	B	12.0	F	10.5
	E	11.9	E	12.8	D	11.7
35.0	A	31.5	A	51.0	B	27.3
	B	32.7	C	33.2	E	29.4
	E	34.4	D	32.9	F	25.5
140	B	131	C	137	A	118
	C	147	E	124	D	106
	E	127	F	131	F	118
400	A	396	D	396	A	335
	B	394	F	390	C	316
	B	384	F	373	E	344

The statistical evaluation of the above data was conducted as follows: The percentage recovery was calculated for each sample using the concentration obtained and the fortification concentrations prior to analysis. A model which included the fixed effect of treatment (fortification level) and the random effects of run (day), run by treatment interaction and residual was used to obtain the least squares means and estimates of variation. To obtain the data in the table, the method calibration curve was calculated by weighted (1/variance) regression. Other weighting factors (*e.g.* $1/x$, $1/x^2$) may be used, as appropriate, in calculating the regression equation of the calibration curve (see Zorn reference).

In order to assess within-day variability, the residual variance was used in calculating the CV for each treatment and across treatments. The CVs were calculated by dividing the square root of the residual variance by the mean and multiplying by 100.

In order to assess across-day variability, the sum of the residual variance, the variance due to run, sample within run and run by treatment was used as the estimate of variance when calculating CVs for each treatment and overall treatments.

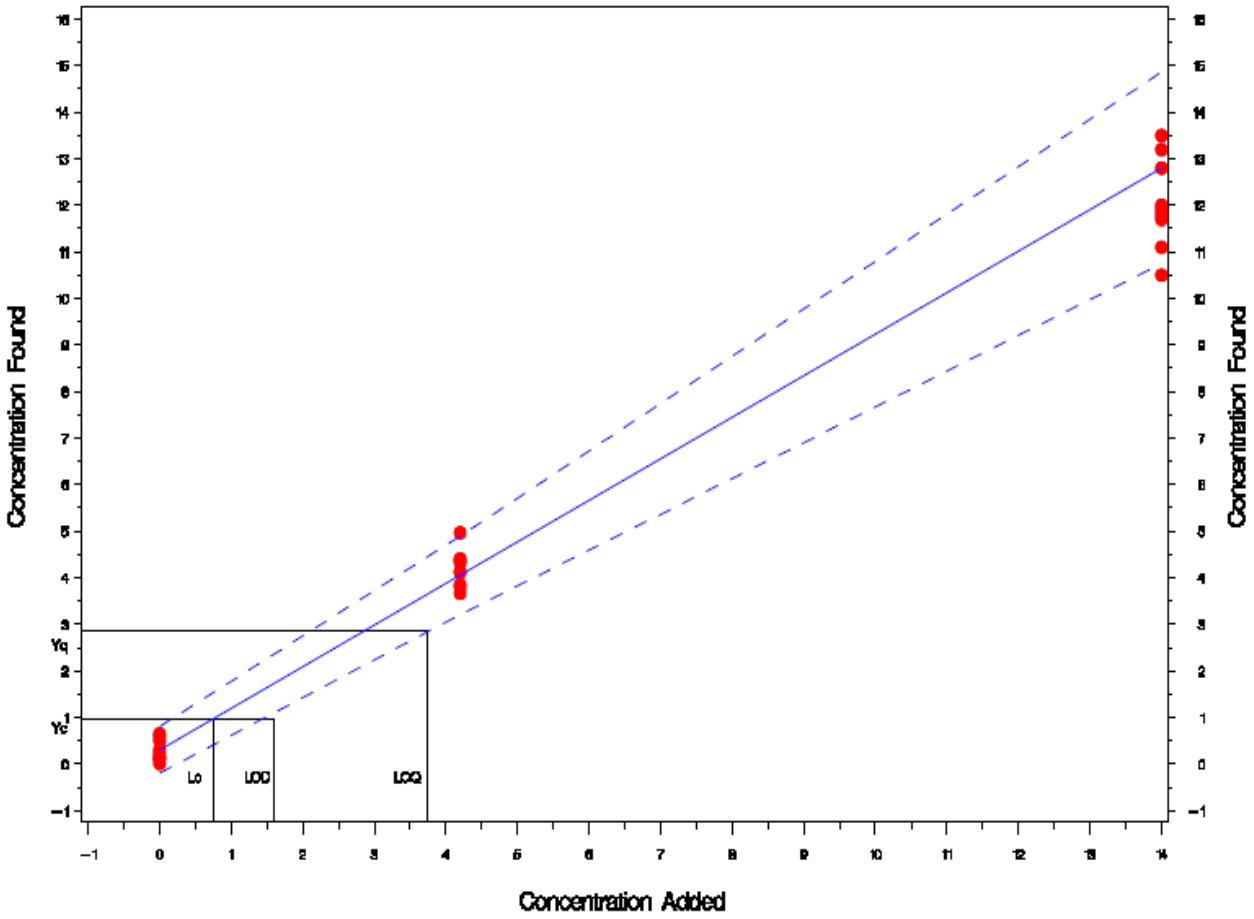
The results of the analysis were as follows:

Within- and Between-Run Assay Precision and Accuracy Determination*

Theoretical Concentration, ng/mL	n	Mean* Recovery, %	95% Confidence Interval	Precision, %CV	
				Within-Run	Between-Run
4.2	9	99.6	87.9 – 111.4	7.8	10.2
14.0	9	86.1	75.0 – 97.2	7.1	7.5
35.0	9	94.6	77.3 – 111.9	19.3	22.6
140	9	90.4	79.5 – 101.3	5.8	9.2
400	9	92.4	82.1 – 102.8	3.0	8.2

* The reported data in the above table were derived using statistical software capable of mixed-model analysis (*e.g.* SAS)

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



LOD = 1.6 ng/mL

LOQ = 3.7 ng/mL

This is a straightforward way to accurately determine precision, accuracy, LOD and LOQ within one study across three days of validation.

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

[2] U.S. Code of Federal Regulations, Title 40: Protection of Environment , Part 136 – Guidelines Establishing Test Procedures for the Analysis of Pollutants, Appendix B to Part 136 – Definition and Procedure for the Determination of the Method Detection Limit – Revision 1.11.

[3] U.S. Environmental Protection Agency, Office of Pesticide Programs, March 23, 2000, "Assigning Values to Non-detected/Non-quantified Pesticide Residue in Human Health Food Exposure Assessments" Appendix 1, A-1 through A-8.

[4] 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.