CONCLUSIONS AND RECOMMENDATIONS

XX Seminar on Harmonization of Registration and Control of Veterinary Medicines
Americas Committee on Veterinary Medicines (CAMEVET)
Ottawa, Ontario, Canada
August 26-29, 2014

Opening Speeches

Dr Luis Barcos, OIE Regional Representative for the Americas welcomed the participants, along with Dr. Montserrat Arroyo, Sub-Regional representative of the OIE for Central America, Dr. Enrique Argento, Secretary of CAMEVET, Dr. Glen Gifford, OIE Focal Point on Veterinary Medicines for Canada, Mrs. Jean Szkotnicki, Staff President of the Canadian Animal Health Institute (CAHI), Dr. Martine Dubuc, OIE Delegate and Vice-President of CFIA, and Dr. Harpreet Kochhar, Chief of the Canadian Veterinary Services.

Dr. Barcos highlighted the importance of the Veterinary Products and the priority that OIE gives to the antimicrobials and their resistance.

Mrs. Jean Szkotnicki, also emphasized the similarities between Latin America and Canada, in trade agreements

Dr. Martine Dubuc mentioned that there is a growing attention given to the use of Veterinary products, which applies both for the authorities and the general public.

President Assumption

Dr. Glen Gifford took the chair as President of the Seminar.

Session I – Relations of CAMEVET and implementation of harmonized documents

Report from the 82nd General Session of the OIE

Dr. Martín Minassian, Technical Assistant of the OIE Regional Representation for the Americas, presented the topics discussed during the 82nd OIE General Session which are relevant to CAMEVET.

Dr Minassian mentioned that World Trade Organization agreement on Sanitary and Phytosanitary recognizes the OIE as the reference authority in the solution of controversies on animal health and zoonoses, pointing that that the General Session defines the OIE policies and actions.

In reference to the antimicrobial resistance, Dr Minassian highlighted the advance in the creation of a global database on the use of antimicrobials in animals, as a follow up to the recommendations of the OIE World Conference on the responsible and prudent use of antimicrobials.

Regarding the possibility of having a more active participation of CAMEVET in the analysis of the OIE Standards under discussion, and due that the meetings of the Specialized Commissions are celebrated in August/September and February/March, Dr. Luis Barcos proposed the change in the date for the next CAMEVET to November. In that sense, this would allow to prepare recommendations for the Delegates from each country to be presented to the OIE.

The proposal was approved unanimously.

Status in the implementation of harmonized documents in the member countries

Dr. Enrique Argento presented the results of a survey that was sent to the countries from the Americas, in which the stage of implementation of harmonized documents was reflected.

He remained the participants on the need of having the answers from the countries, as this survey allows to have knowledge on the impact of the Committee´s work.
It was highlighted that, even when harmonized documents are not included in the national regulations, they are used as a technical reference or they are applied without being included in the regulations. Hence, it is very important to have this information.
For that, the criteria of the use of harmonized documents as reference documents, leaving the concept of implementation, was proposed.

Differences between the information provided about the use of some harmonized documents against the reality were observed by the industry sector. Labeling is an example, because it is mentioned as "applied" in almost all of the member countries which answered the survey, but keeps presenting a major problem.

Modification of the harmonized document concerning the steps in the harmonization of CAMEVET documents

The Secretariat informed that a project for the modification of the harmonized document named" Procedure for the elaboration, study, approval, adoption, identification and follow up of the Harmonized Documents" was elaborated in order to improve the interpretation of the status in the approval process. For that, the Executive Board will assume this work.

Communications and Web Site

After a question regarding the update in the CAMEVET website actualization, the OIE Regional Representative commented that the delay will be solved during next November.

Presentation of the results of the National Focal Points Workshop

Dr. Glenn Gifford presented the conclusions of the workshop for OIE National Focal Points for Veterinary Products that was held previously to the CAMEVET Seminar.
Dr. Gifford commented that different topics were presented, including antimicrobial resistance, the registration of diagnostic kits, and antiparasitic resistance. He highlighted that this last topic is very important, given the regional experience.

Also, the importance of measures to avoid products counterfeiting was highlighted, keeping in mind what happens in the human pharmaceutical industry.

Presentation of the results of the meeting of the industrial sector

Dr. Guillermo Leonel Rodas President of the Latin American Federation of Veterinary Products, FITVECA, presented the results of the meeting held by industry participants. They are included as **Annex**

Different issues concerning the need to avoid the duplication of tests were presented, especially referred to residues, efficacy, and safety tests, while adequate previous tests or from other countries were available, and also when sufficient bibliographical information on the topic was at hand.

Sesion II- Working Documents

Dr. Argento insisted to the members that it's very important to comply with the deadlines for the submission of comments, since this produces delays in the circulation and approval of the working documents.

Registration of homeopathic products for veterinary use

The SINDAN representation took on the coordination of the Working Group that will present the final document, given the impossibility of the Colombian official sector to continue with such task.

Dr. Milson da Silva Pereira explained registration of products is mandatory by law, and referred that there are eight veterinary laboratories producing that kind of products. He commented that not registering such products increased the risk of counterfeiting.

Due to the delays in the advancement in the draft, the circulation of the document containing the observations to the CAMEVET members, and a period of 90 days for new comments were proposed and accepted.

Also, it was also proposed the setting up of an electronic mechanism allowing the Focal Points to approve the drafts, in case of the absence of comments after the circulation.

The representative from the Official sector from Canada requested the formal communication whether there were no comments, in order to agree with the drafts.

Registration of Nutraceutics/Dietary Supplements (Guideline)

Dr. Carlos Rufrano, Coordinator of the Working Group, presented the final version of the document, in status of Step III, which was circulated for 60 days, according to the conclusions of the XIX Seminar in Panama.

Some countries highlighted that they could not approve nor implement this document, as it is out of their authority scope, for which it was agreed that each Focal Point shall

send the guideline to the corresponding areas in their countries for their review.

Guideline for the Potency test for bovine vaccines containing Bovine ParainIfuenza type 3 virus

Due to the absence of the nominated speaker, the discussion of this item was postponed. The document will remain in the Step IV status, for its presentation in the next Seminar.

Guide of good practices of warehousing, transport and distribution of products for veterinary use

Dr. Milson da Silva Pereira, Coordinator of the working Group presented the final document, in Step III status.

After the presentation, representatives from the official sectors from Mexico and Uruguay expressed that some of their comments were not included in the document.

Based on that, coordinators from Working Groups were reminded on their responsibility for the justification in the cases where the received comments were not included in the documents, and communicate on that to the senders.

After that, the document, including the comments provided by Mexico and Uruguay, will be circulated again, with a 90 days deadline for the reception of comments.

Guidelines related to withdrawal periods

1st. *Technical guideline to conduct metabolism and residue kinetic studies of veterinary pharmacological agents in food-producing animals - Marker residue depletion studies to establish veterinary drug withdrawal periods.*
2nd. *Guidelines for the validation of analytical methods used in residue studies in animal tissues.*
3rd. *Technical guideline to conduct metabolism and residue kinetic studies of veterinary pharmacological agents in food-producing animals - Marker residue depletion studies to establish veterinary drug withdrawal periods.*

Dr. Carlos Francia, Coordinator of the Working Group presented the final version of the documents, in Step III status. These drafts had a 90 days first circulation within the Working Group, and a second circulation for 60 days among all of the CAMEVET members.

As no comments were received and the established deadlines were fulfilled, the document was submitted for voting and approved by unanimity.

The Harmonized Guidelines are included as Annex

Bovine vaccines safety. Guideline for studies with inactivated vaccines

Due to the absence of the nominated speaker, the discussion of this item was postponed.

The document will remain in the Step IV status, for its presentation in the next Seminar.

Potency testing for bovine vaccines containing inactivated bovine rotavirus Group A-causal agent of calf neonatal diarrhea

Due to the absence of the nominated speaker, the discussion of this item was postponed. The document will remain in the Step IV status, for its presentation in the next Seminar.

Labeling

Dr. Niels Scherling made a presentation that highlighted the problems due to the lack of harmonization of the labeling requisites by the countries. This has not been solved, despite the availability of the labeling guideline, harmonized for more than 10 years ago.

He informed that a survey had been sent, which was answered only by eight countries. The need of providing such information by the countries was highlighted.

The Working Group which was already formed and coordinated by CAPROVE and had the participation of ALANAC, ANVET, AVISA, INFARVET/CANIFARMA, CLAMEVET, FENALCO and SINDAN will continue in the development of this project.
This project has the purpose of providing orientation on the applicability of the harmonized guideline on Veterinary Products Labelling by CAMEVET, and design a solution to a grievous problem affecting the industry from all of the member countries.

Stability

The delegation of SINDAN (Brazil) presented a proposal for the modification of the Stability Guideline recently approved in the XVIII Seminar celebrated in 2012.
The existence of mistakes in the text was highlighted, which were proposed for correction, and also some modifications to the document were proposed.

These proposals were sent to the Working Group (Official sector from Argentina, Uruguay and Colombia, and the private sector, CAPROVE, CLAMEVET, APROVET, CEV and ADIPRAVE) for their review and the circulation of a proposal.
After that, the document under revision shall be in Step VI status.

CAMEVET participation in the VICH Outreach Forum

Dr Enrique Argento made a presentation concerning his participation representing the Committee, reviewing the meetings of this Forum since 2011, and informed about the November 2013 meeting in Auckland, New Zealand, and June 2014 meeting in Brussels, Belgium.

He highlighted the importance of the participation of the Americas countries in the meetings, as well as the CAMEVET participation in the Working Group formed for the coordination of the VICH training activities, and the participation of Dr Laura Sbordi in the Expert Working Groups on residues in honey, and combined products.

Concerning the review of the VICH Guideline on stability testing of new veterinary drug substances, he commented that the CAMEVET harmonized Guideline, covering tropical climatic zones not included in the VICH document was sent to VICH, and that the concept paper made by the Task Force was circulated among the CAMEVET members with modifications, for comments.

He highlighted that the interest of VICH in the recognition of the guidelines was expressed in the Brussels Meeting, and that the objective is not the mandatory application in the non VICH countries.

It was raised that after the initiative made by the Argentina and CAMEVET delegations, the need of different channels of communication for both the technical and the political levels was detected. This is important, because the political level has the possibility of assigning resources for the tasks of registration and control of Veterinary Products.

The delegations from Mexico and Brazil which were invited to the Outreach Forum expressed their interest in the continuity in the participation.

Also, reaching a consensus previous to the participation in meetings was considered important in order to hold a regional position was considered as important. For that, the secretariat shall take the responsibility in coordinating such mechanism.

The official delegation from Argentina delegation expressed their satisfactory experience in the Outreach Forum, and manifested that they use the guidelines as a reference, keeping their sovereignty and their local regulations.

Dr. Argento informed about the invitation made by USA to host the next Steering Committee and Outreach Forum Meetings in 2015 February in Buenos Aires. The CAMEVET Executive Board approved this proposal as this invitation was considered very important. He also reported that in the Brussels meeting, the Steering Committee decided to postpone the invitation to February 2017

Round Table: VICH Guideline Implementation

USA and Canada Private delegations and Dr. Barbara Freischem, Chargée de mission at the OIE Scientific and Technical Department and OIE Representative at the VICH, exposed the criteria for the use of the VICH Guidelines and their utility.

It was highlighted that the use of the Guidelines are voluntary in the non VICH member countries, acting as technical documents which are not included into regulations in order to facilitate their update, based on the scientific advancement. The value of the Guidelines is recognized as a permanent reference material.

Policy on Generic Products

The Canadian policy on generic products was presented from both the official and industry point of view, and also from the perspectives from a global view, and a single company producing that category of products.

It was highlighted that the VICH adopted guideline for that kind of products facilitated and gave transparency to the process of registration, allowing the access to market for many products.

Brazilian industry delegation (SINDAN) expressed their country´s situation, where they a have law which has not been enacted yet which is not adequate for the Veterinary Industry, and highlighted the need of the support by the government for the development of such kind of industry, as happened in the human medicines industry.

Dr. Margarita Pinto, private sector member of the Executive Board, presented the process of development and registration of veterinary products classified as innovative.

After the presentations, it was proposed and accepted to create a Working Group that could review the existing legislation and develop a draft containing aspects related to that topic. Considering that bioequivalence is strongly related to generic products, it was suggested that both topics should be addressed in parallel by the same Working Group.

The working Group will be coordinated by the official representatives of Mexico, and shall have the participation of the official sectors of Guatemala, Chile, Uruguay, Costa Rica, and also INFARVET, CEV, ADIPRAVE, ALANAC, CLAMEVET, SINDA and FIVETCA.

Round Table and discussion: Present and future of CAMEVET

CAMEVET Strategic Plan

Dr. Federico Luna, Coordinator of the Working Group for the development of the CAMEVET 2015-2020 Strategic Plan, made a presentation showing the advances in this issue.

Dr Luna commented that a survey was distributed, and answers were received for only 18% of the members, which is insufficient for the development of such plan.
After that, a new circulation of the survey was proposed and accepted, asking the participation in its answering. Participants were also reminded that the elaboration of such project shall consider the future OIE Strategic plan, currently under development.

III Session - Working Documents

Use of Veterinary Products in Aquaculture

A presentation on that topic was considered in the Agenda, due to the growing importance of that category of products.

Topics included the programs for the development of products for the control of ectoparasites in salmonids, amongst others.

Given, the growth of fish farming in the countries of the region and the high level reached by Chile, the Working Group formed in 2013 Seminar in Panama was reactivated.

The group shall be coordinated by the official sector from Canada and formed by the official sectors from Chile, Costa Rica, México, Peru, the Cuban Center for Genetic Engineering and Biotechnology. Also, participants from ANVET, ALANAC, CAPROVE, FENALCO y SINDAN shall take part.

Proposal of new working topics

New working topics, as described below, were proposed and the respective Working Groups were formed. These Working Groups will develop the first documents, which shall be in Step II status.

Criteria to be applied in the registration of innovative, generic, similar and new products.

According to what was decided regarding the drafting of a working document on generic veterinary products, it was considered that the same working group shall have the responsibility of drafting a paragraph concerning the criteria to be applied in each category.

Minor Species and Minor Uses.

The Working Group will be coordinated by SINDAN and formed by representatives from the official sectors of Chile, Cuba, Ecuador, and Uruguay. Also ADIPRAVE, ALANAC, CEV, CLAMEVET, and FENALCO from the private sector

Growth Promoters

The Working Group will be coordinated by Brazil and formed by representatives from Chile, Ecuador, the Cuban Center for Genetic Engineering and Biotechnology, and also representatives from ADIPRAVE, ALANAC, ANVET, AVISA, CEV, CLAMEVET, and the Costa Rican Chamber of Agricultural Supplies.

Bioequivalence

The Working Group which is has been already formed and coordinated by CAPROVE, and formed by the official representatives from Chile, México, Peru and Uruguay, and representatives from ADIPRAVE, ALANAC, CEV, CLAMEVET, FENALCO, SINDAN and INFARVET, shall continue with the work in this topic.

Guide for filling the CAMEVET forms for the registration of biological and pharmacological products

Dr. Federico Luna, Representative from Argentina and coordinator of the Working Group, informed that a first draft is ready to be circulated within the Working Group, formed by SINDAN. CLAMEVET, Ecuador (Official) CAPROVE and Dr. Liliana Revolledo.

Dr. Benigno Alpizar added that a second draft concerning Pharmaceutical Products is ready to be delivered to the Working Group.

Diagnostic Kits

A new Working Group was created, coordinated by the official sector from USA, with the participation of the official representatives from Canada, Mexico, Guatemala, Cuba, and also INFARVET, SINDAN, and CLAMEVET.

Expenses report, financial state and 2013/2014 budget
Election of members of the Executive Board

Resources available to September 24, 2014		54.092,00 USD
Income		
XX Seminar registration (USD 300/UDS 400 for participant)	40.000,00 ARG	26.700,00 USD
Resources available at the end of the Seminar	8.171,92 ARG[1]	80.792,00 USD
Budget for the financial year 2014/ 2015		
Expenditures		
Fixed expenses		
⊸ Personal		8.400,00 USD
Variable expenses		
⊸ Support Fund for Focal Points		3.000,00 USD
⊸ Participation at meetings of the VICH Washington February, 2015		3.500,00 USD

According to the provisions established in the Statute, the new Executive Board members were elected, and shall hold office for two (2) years.

- **President:** Dr. María Eugenia Paz
- **Members from the Official Sector**:

  Honorary Member: Dr. Glenn Gifford

  1. **Argentina**: Dr. Federico Luna
  2. **Mexico**: Dr. Ofelia Flores
  3. **Paraguay**: Dr. Gloria Alarcon
  4. **Chile**: Dr. Fernando Zambrano
  5. **El Salvador**: Dr. Delfy Mariana Gochez Alvarenga - Substitute

- **Representatives from Supporting Members**:

  1. **CEV:** Dr. Mercedes Etcheverry
  2. **FIVETCA:** Mr. Guillermo Rodas Serrano

[1] 31828.08 ARG in concept of ticket Seminar XX for Assistant and Secretary CAMEVET

3. **CAPROVE:** Dr. Carlos Francia
4. **CLAMEVET:** Dr. Carlos Rufrano
5. **SINDAN:** Dr. Milson da Silva Pereira

The charges will be in effective until the end of the XXII Seminar to be held in 2016.

Venue for the next meeting

The proposal to hold the next seminar in Guatemala in the second week of November 2015 was accepted

In addition, it was accepted the Mexico proposal to organize 2016 Seminar.

List Annexes:

I. Minute of the official sector meeting
II. Harmonized Documents:
    i. Technical guideline to conduct metabolism and residue kinetic studies of veterinary pharmacological agents in food-producing animals - Marker residue depletion studies to establish veterinary drug withdrawal periods
    ii. Guidelines for the validation of analytical methods used in residue studies in animal tissues
    iii. Guideline for the estimation of the withdrawal period in edible tissues

List of acronyms used in the document

ADIPRAVE:	Asociación de las Industrias de Productos Agroquímicos y Veterinarios (Uruguay)
ALANAC:	Asociación de Laboratorios Farmacéuticos Nacionales (Brasil) (Associação dos Laboratórios Farmacêuticos Nacionais)
ANVET:	Asociación Nacional de Laboratorios Veterinarios (Chile)
APROVET:	Asociación Nacional de Laboratorios de Productos Veterinarios (Colombia)
AVISA:	Asociación Venezolana de la Industria de Salud Animal
	Cámara Nacional de la Industria Farmacéutica (México)
CAHI:	Canadian Animal Health Institute
CAMEVET:	Comité de las Américas de Medicamentos Veterinarios
CAPROVE:	Cámara Argentina de la Industria de Productos Veterinarios
CEV:	Cámara de Especialidades Veterinarias (Uruguay)
CLAMEVET:	Cámara de Laboratorios Argentinos Medicinales Veterinarios
FDA:	US Food and Drug Administration
FIVETCA:	Federación de Industria veterinaria Centroamericana
INFARVET:	Industria Farmacéutica Veterinaria – Canifarma (México)
OIE:	Organización Mundial de Sanidad Animal
OIRSA:	Organismo Internacional Regional de Sanidad Agropecuaria
SENASA:	Servicio Nacional de Sanidad y Calidad Agroalimentaria (Argentina)
SINDAN:	Sindicato Nacional da Industria de productos para Saúde Animal
VICH:	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

ANNEX I

REUNIÓN PLENARIA DE LA INDUSTRIA
Ottawa, Canadá agosto 2014

El día 25 de Agosto de 2014, se llevó a cabo la reunión de la Plenaria de la Industria en la cual estuvo presente la representación de los países presentes en el CAMEVET.
Los principales puntos que se manifestaron durante el desarrollo de esta reunión fueron:
1. Mejorar la comunicación entre la Industria y las autoridades para establecer un diálogo abierto con nuestras autoridades para fortalecer el Plan Estratégico.

2. Rotulado: Se solicita que las autoridades atiendan las recomendaciones de internalizar documentos Camevet, con el fin que los rotulados no se conviertan en una barrera comercial.

3. Normas VICH: La Industria espera que se reconozca el VICH como una recomendación, más no que se apliquen en nuestros países, esperando que se internalicen prioritariamente las normas Camevet.

4. Fijar criterios en reuniones en conjunto, entre Industria y Sector Oficial para la aplicación de normas, Para evitar discrecionalidad y exceso de exigencias entre los diferentes funcionarios. Recomendación: Crear documentos respetando acuerdos alcanzables para fijar estos criterios.

5. La Industria sugiere no repetir localmente una serie de estudios que ya han sido hechos con anterioridad a las moléculas (residuos, eficacia, inocuidad etc.), siempre y cuando haya sustento técnico y/o que existan antecedentes con bases Científicas sobre las moléculas que se comercializan y que son ampliamente conocidas.

6. Combinaciones de medicamentos; Se solicita dar retroalimentación a la mesa de trabajo de VICH (principalmente en cuanto a la posición de China al respecto).

7. Creación de la Federación Latinoamericana de la Industria Veterinaria "FLAIVET": Se creó con el fin de unificar criterios y posiciones de las Industrias de Medicamentos Veterinarios Nacionales de la región ante las autoridades regulatorias.

8. Capacitación: Se solicita crear una comisión para generar programas de capacitación en temas regulatorios y de buen uso de los medicamentos veterinarios al sector oficial e industrial, con miras a alinear criterios y estandarizar el entrenamiento para ambas partes.

ANNEX II. i

September 2010
Revision: April 2013

TECHNICAL GUIDELINE TO CONDUCT METABOLISM AND RESIDUE KINETIC STUDIES OF VETERINARY PHARMACOLOGICAL AGENTS IN FOOD-PRODUCING ANIMALS

MARKER RESIDUE DEPLETION STUDIES TO ESTABLISH VETERINARY DRUG WITHDRAWAL PERIODS

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1. Introduction

As part of the approval process for veterinary medicinal products in food-producing animals, regulatory authorities require data from marker residue depletion studies in order to establish appropriate withdrawal periods in edible products, including meat, milk, eggs and honey. The objective of this guidance is to provide study design recommendations which will facilitate the universal acceptance of the generated residue depletion data to fulfill this requirement.

General recommendations here established apply for most situations in which a period of use restriction must be determined. Nevertheless, it should be born in mind that there may be situations in which these recommendations may result inadequate or not applicable. In those cases, the health authority will evaluate the specific proposed designs as long as they are appropriately justified.

1.1. Objective of the guidance

This document, based on recognized previous works, aims at proposing protocols for the conduction of residue depletion studies to establish withdrawal periods of veterinary drugs. These protocols have been adapted to meet the characteristics of the reality of the country members of CAMEVET and could be useful for other countries.

1.2. Background

VICH GL 48 (November 2009) – Marker Residue Depletion Studies.
EMEA/CVMP/036/95-Final (January 1997). Approach Towards Harmonization of Withdrawal Periods.

2. Scope

In order to register a veterinary drug intended for food-producing animals, marker residue depletion studies are recommended in the target species to:

- Demonstrate the depletion of the marker residue upon cessation of drug treatment to the regulatory safe level (*e.g.* maximum residues limit [MRL] or tolerance).
- Generate suitable data for the elaboration of appropriate withdrawal periods to warranty food safety.

The idea is that one residue depletion study (per species), conducted within any global region, be sufficient to satisfy the data requirements for the determination of appropriate withdrawal periods for a specific product in food-producing animals. The guideline encompasses the most common species, namely cattle, pig, sheep and chicken; however, the principles of this guideline allow sufficient flexibility for application to related species not mentioned in this core group (e.g. cattle vs. all ruminants, chickens vs. all poultry).

This guideline does not include considerations regarding aquiculture nor apiculture products.

Studies should be conducted in conformity with the applicable principles of Good Laboratory Practice (GLP) and Good Clinical Practices (GCP).

3. Glossary

Standard food basket: It is an estimate of the total amount of food of animal origin consumed daily by an adult weighting 60 kg. The basic food basket employs arbitrary figures of consumption, based on percentiles higher than the daily intake of products of animal origin.

The figures of daily consumption of food of animal origin are:

For mammals:

300 g. of muscle, 50 g. of fat or fat and skin, 100 g. of liver and 50 g. of kidney.

For birds:

300 g. of muscle, 90 g. of fat or fat and skin, 100 g. of liver and 10 g. of kidney.

For fish:

300 g. of muscle and skin in natural proportions.

Also an intake of 1.5 l. of milk, 100 g. of eggs and 20 g. of honey are considered.

The estimate of the risk of consumption of present residues in a food basket is calculated taking into account the ADI.

Active Pharmaceutical Ingredient –Drug substance (API): Any substance intended to cure, mitigate, treat, prevent or diagnose diseases in humans or animals.

Acceptable Daily Intake (ADI): It is the estimate of the residue, expressed in terms of weight per kilogram of body weight (kg/bw) that can be daily consumed during consumer´s whole life without noticeable risks to his health.

Confidence Interval: Range of values in which the value of a population parameter is expected to be found with certain degree of certainty.

Maximum Residue Limit (MRL): Maximum concentration of residues founded in an animal product or by product that not introduce any risk for the consumer safety, based on known facts at the time of its publication (CAMEVET definition).

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.

Tolerance limits: The extreme values of a series of values (interval) within which a determined percentage of the individuals of a determined population is expected to be found with certain degree of certainty.

Veterinary drug: Any chemical, biological, biotechnological substance or manufactured (elaborated) preparation administered either individually or collectively, directly or mixed with food, aimed at the prevention, cure or treatment of animal diseases.

Safety Period – Use restriction- Withdrawal Period – Elimination Period: Minimum period of time that must happen between the last application of a veterinary medicinal product to an animal, in the normal conditions of use, and the extraction of food products from that animal, to warranty that such food products do not contain residues in quantities that exceed the established maximum limits.

Veterinary Product (as defined by CAMEVET): A veterinary product is any chemical, biological, biotechnological substance or manufactured preparation administered either individually or collectively, directly or mixed with food or water, aimed at the prevention, cure or treatment of animal diseases, including additives, supplements, promoters, animal production enhancers, antiseptics, disinfectants for environmental use or for devices and ectoparasiticides and any other product that, used in animals and their habitat, protects, restores or modifies the organic and biological functions. It also encompasses products destined to enhancing animals' beauty.

Marker Residue: It is an analyte that is reliable to determine the presence of residues of a specific drug in the tissue. The marker residue can be a mother cell or any of its metabolites, degradation products or a combination of any of them. The marker residue can also be a chemical derivate of one or various components of the residue. The relationship between the marker residue and the concentration of the residues of interest in edible tissues must be known (marker residue/ residue of interest). The MRL reflects the maximum allowed concentration of the marker residue in the edible tissues.

Total Residue: The total residue of a drug in animal derived food consists of the parent drug together with all the metabolites and drug based products that remain in the food after administration of the drug to food producing animals.

The total sum of the residues normally includes all parent residues of the pharmacological agent (parent drug and its metabolites), and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies.

Residues of toxicological relevance: For the estimate of an exposure based on a toxicological ADI, the residue of relevance is the residue of toxicological relevance. This normally includes all the parent compounds and the molecule (parent drug and the metabolites) and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies. However, if a component of the residue or a fraction of the total residues is demonstrated to be toxicologically inactive, it is possible to deduct it from the total residue or any other fraction of residues that is not bioavailable by oral route or the metabolites known to be toxicologically inactive.

Residues of pharmacological relevance: For the estimate of an exposure based on a pharmacological ADI, the residue of relevance is the residue of pharmacological relevance. In general, the mother cell plus any other of its residues are considered. If there is lack of data on pharmacological activity of the total residue components, the total residues are presumed to present the same pharmacological activity as the parent drug.

Residues of microbiological relevance: For the estimate of an exposure based on a microbiological ADI, the residue of relevance is the residue of microbiological relevance. In most cases, it is identical to the residues determined in microbiological assays. In case of lack of such data, the total residue can be used, or alternatively the sum of the individual components known

to present microbiological activity. Therefore, the microbiological activity of the total residue or of the metabolites and/or products of degradation is the same as the parent drug.

Injection site: It is the area of tissue where the veterinary medicinal product has been injected. The samples of tissue obtained from the injection sites for the conduction of residue studies should be representative of the edible tissue that is feasible of being selected in the slaughter procedures. The tissue sample should include muscular tissue, connective tissue and subcutaneous fat in natural proportions (cutting off the samples to eliminate the connective tissue and the fat attached to muscle are considered artificial procedures that differ from the real situation). Injection site should not include the skin portion that covers it, since it is not required for the residues analysis.

Tissue: Any edible animal tissue, including muscles and sub-products (Definitions established and adopted by the Joint FAO/WHO Expert Committee on Food Additives – JECFA).

4. Marker residue depletion studies

4.1. Investigational veterinary drug

The investigational veterinary drug used must be representative of the commercial formulation. Preferably, it should come from material elaborated with the use of good manufacturing practices (GMP) (at pilot and commercial scale); however, duly documented preparations at laboratory scale are also acceptable.

4.2. Animals and animal husbandry

In general, a single marker residue depletion study (in tissues) can be conducted in pigs, horses and poultry. In the case of ruminants, a single study can be applied for meat and milk-producing animals. However, due to differences in the physiology of ruminants and pre-ruminants, separate studies are recommended when target species include both adults and pre-ruminants. A separate study should be performed to demonstrate the residue depletion profile in milk of dairy animals or in eggs produced by laying hen.

Animals should be healthy and, preferably, should not have been previously medicated. However, it is recognized that animals might have received vaccinations or prior treatment, for example with anthelmintics. In the latter case, an appropriate wash-out period should be observed for the animals prior to enrollment in the actual trial. Study animals should be representative of the commercial breeds and of the target animal population that will be treated. The source of the animals, their weights, health status, ages and sex should be informed.

Animals should be allowed adequate time to acclimatize to the trial conditions, and good clinical practices should be applied. The feed and water supplied to the animals should be free from other drugs and/or contaminants and adequate environmental conditions should be ensured, in accordance with animal welfare practices.

4.2.1. Intramammary studies

For studies with veterinary products of intramammary application, all animals should have healthy udders, free from the effects of mastitis. For pre-parturition studies, pregnant animals with a predicted parturition date should be introduced in study facilities in advance of study enrollment.

4.2.2. Other parameters

All factors that may contribute to the variability of the residue levels in the animal products should be taken into account in the planning and conduct of trials. The intent is that these factors (e.g. animal breeds, physical maturity) be considered without increasing the number of animals recommended in 4.3. For example, if a milk residue depletion study recommends 20 animals, any factor determining variability should be represented within the 20 initially selected animals (20 more animals representing "other factors" should not be added).

4.3. Number of animals for the study

The number of animals used should be large enough to allow a statistically significant assessment of data. From a statistical point of view, for meat residue studies, data from a minimum of 16 animals should be collected: 4 animals euthanized at 4 appropriately distributed time intervals. A larger number of animals can be considered if the biological variability is anticipated to be substantial, as an increase in the number can contribute to determine more precisely the withdrawal period. Control (non-treated) animals are not necessarily called for as part of the actual marker residue depletion study; however, sufficient amounts of target tissue should be available for the preparation of matrices in the assessment of related analytical methods. The following section provides general recommendations on the number of animals to be included in the study design.

4.3.1. Ruminants, pigs and horses for tissue residue studies

At least 4 (evenly mixed as per sex) per each slaughter time are recommended. The body weight should be consistent with the class for which the veterinary drug is indicated.
Según lo expuesto en la sección 4.2, las vacas lecheras también pueden ser utilizadas para estos estudios de residuos en tejidos.Following section 4.2., milking cows can also be used for these tissue residue studies.

4.3.2. Dairy animals for milk residue studies

For lactating studies, at least 20 animals, randomly selected from a herd where all lactating stages are represented, are recommended. High yielding animals at an early lactation stage and low yielding animals at a late lactating stage should be included.

For pre-parturition and dry cow therapy studies, a minimum of 20 animals is recommended. The study should include randomly selected cows, representative of commercial dairy practices.

4.3.3. Poultry

A sufficient number of birds should be used to obtain at least 6 samples at each slaughter time for tissue residue studies.

For egg residue studies, a sufficient number of birds should be used to collect 10 or more eggs at each interval time point.

4.4. Route of administration

4.4.1. General guidance

Animal treatment should be consistent with the dosage and indications intended for the investigational veterinary product and must include, for injectable products the location and injection method. For multiple treatments, injections should be given alternatively between left and right sides of the animal.

The highest intended treatment dose should be administered for the maximum intended duration. For extended treatments including various doses, if there is available data indicating that the API concentration reaches a steady stage (moment when the API concentration in the target tissue neither increases nor decreases because the absorption speed is the same as the depletion speed) before the end of treatment, sampling could start at that time. Veterinary products intended for intra-mammary administration should be given to all four quarters of each cow.

For dry cow and pre-parturition studies the test article should be administered after the last milking (dry-off) and observing the interval until calf birth (usually 60 days).

4.4.2. Considerations for veterinary drugs intended for multiple routes of administration

If the veterinary drug is intended to be administered via more than one parenteral route (intramuscular, subcutaneous or intravenous), a separate marker residue depletion study for each route of administration should be provided. Note: If the withdrawal period is clearly defined by depletion of residues from the injection site following subcutaneous (SC) or intramuscular (IM) dosing, a separate intravenous residue study (at the same dose) is not needed, provided the same withdrawal period than the one applied for IM or SC route can be applied for IV route.

4.4.3. Recommendations for the application of a spray veterinary drug:

Spraying veterinary products are widely used in many member countries, indicated for ectoparasites treatment. To establish the standard dose is a critical requirement and it has to be done before the beginning of a residue trial. The following protocol is recommended.

a- The spraying equipment should be filled with a previously measured amount of the veterinary drug, prepared and ready for application.
b- Once the backpack sprayer or spraying equipment is filled the system should be cleaned.
c- Start spraying the animal´s dorsal part, from head to tail and then back to cranial through the contiguous lower part, covering thus a route that ensures the correct drenching of the animal. This should be done in both sides of the animal, up to the dripping point and always avoiding product application at the animal`s eyes. The dripping point indicates that the animal has been correctly drenched, and that from that point on, all products applied will not stay on the animal, but it will drain to the floor. This is the parameter usually employed to treat animals in field conditions, and one of the most appropriate ones to reduce dosage variables in this kind of application.
d- If feasible, once the application is done, the remaining product should be collected from the sprayer in a measuring glass to calculate the applied volume. This information should be registered in the "treatment record".

Loaded volume - Remaining volume = Applied volume

The applied volume should be correlated to the animal weight in order to accurately know the applied dose per animal in terms of total milligrams and milligrams per kilograms.

4.5. Animal euthanasia

Animals should be euthanized following OIE regulations on animal health, using when possible commercially applicable procedures, making certain to observe appropriate exsanguination times. Chemical euthanasia should be avoided.

4.6. Sampling

4.6.1. General considerations

Following euthanasia, edible tissue samples in sufficient amounts should be collected, trimmed of extraneous tissue, weighed and divided into aliquots. If the analysis cannot be completed immediately, samples should be adequately stored pending analysis. If samples are stored after collection, residue stability should be demonstrated through the assay time.

Table 1 indicates samples recommended being collected during euthanasia.

Table 1. Sample collection from animals used in a marker residue depletion study (All regions).

Edible tissue type	Species/ Sample Description	
	Cattle/ Sheep/ Pigs / Horses	**Poultry**
Muscle	Muscle from the lumbar region	Breast
Injection site:	Core of muscle tissue ~0,5 kg. 10 cm. diameter x 6 cm. deep for IM 15 cm. diameter x 2,5 cm. deep for SC	---
Liver	Cross-Section of lobes	Entire
Kidney	Composite from combined kidneys	Composite from combined kidneys
Fat (excluding pigs)	Omental and/or peri-renal	---

Skin (pigs and poultry)	Skin with fat in natural proportions	Muscle with skin in natural proportions
Milk (sheep and cattle)	Whole milk	---
Eggs	---	Clean shell, break the egg, white and yolk can be combined.

{0>Following euthanasia, edible tissue samples in sufficient amounts should be collected, trimmed of extraneous tissue, weighed and divided into aliquots. If the analysis cannot be completed immediately, samples should be adequately stored pending analysis. If samples are stored after collection, residue stability should be demonstrated through the assay time.

However, VICH guideline to conduct "Studies to evaluate the metabolism and determine the quantity and identify the nature of residues" recommends the collection of additional tissues to quantify total residues in order to address specific regional concerns. {0Table 2 Additional tissues that can be collected to answer regional concerns in the marker residue depletion study.

The additional tissues suggested for samples are included in Table 2.

Table 2 Additional tissues that can be collected to answer regional concerns in the marker residue depletion study.

Edible tissue type	Species/ Sample Description		
	Cattle/ Sheep/ Pigs / Horses	Poultry	
Gizzard	---	Entire	
Heart	Cross-section	Entire	
Small Intestine	Composite, rinsed of content	---	
Other organs	Composite	Composite	

For the purposes of this guidance, if necessary, one of the additional tissues (per species) will be selected to evaluate the marker residue and address regional concerns. The selected additional tissue is based on the results of the total residue (TTR) study and it would typically be the additional tissue with the highest residue concentration or with the slowest residue depletion rate. For instance, if the TTR study indicates that cattle heart presents the slowest depletion rate, that additional tissue should be selected for assay in the marker residue study, but cattle small intestine marker residue data will not be necessary. Likewise, if poultry gizzard presents the highest residue concentration, assays of poultry heart are not recommended.

4.6.2. Injection sites

For parenteral preparations (IM or SC), residue depletion data from the injection site(s) should be included. Samples should be collected from the last injection site. In case of products requiring multiple injections, the study design should be such that the last injection site will occur on the side where the animal received the higher number of injections. A minimum 10-centimeters distance between injection sites is recommended in order to obtain a better sample quality. Collection of the injection site muscle tissue (from large animals) should be centered on the point of injection and consistent with the recommendations shown in Table 1.

During the conduction of residue studies, injection site should be permanently marked, so that it can be easily and correctly identified at slaughter time. The veterinary drug should be administered at the center of the subjacent tissue and the injection site should be extracted immediately after euthanasia.

Sample collection technique should include, whenever possible: The needle track, the site where drug was released and any site presenting tissue irritation.

With the aim of ensuring that the described sampling procedures are adequate to represent residues concentration, it is recommended, whenever possible, to take from every injection site a ring control sample of around 300 g. to the primary sample.

The collection of an additional ring sample or a circular sample surrounding the injection site during the conduction of tissue residue depletion studies is required for the EU, but generally not for other regions.

This second sample may not be always obtained, especially in injections applied in neck sites; therefore the sample size of peripheral tissue to the primary sample may be reduced to the possible extent. However, this sample needs to present an adequate size to allow analytical processing.

For injectable products in which the applied dose exceeds the recommended volume for the application site, injection should be applied in more than one site (e.g.: IM or SC 1 mL/10 kg dose for an animal of 360 kg. when maximum recommended volume is 20 mL). In those cases, the injection site sample should be taken from the injection site receiving the highest volume (the site injected with 20 mL. and not the second site injected with 16 mL).

In real practice, it is difficult to obtain samples of the exact recommended weight, so it is accepted for the real weight to vary within certain limits with respect to the proposed theoretical weight. The core injection site samples presenting a weight fluctuating between 400 and 500 g. (500 g ± 20%) are acceptable.

Following tissue removal, the injection site samples collected (core and peripheral) should be appropriately homogenized before the final sampling (300 g.) for the determination of residues, thus avoiding analytical processing of a potentially non homogenous material.

Proposed dimensions and weights of injection site tissue samples cannot be applied in small animals, whose size and anatomy do not allow samples of 500 g.

A general strategy cannot be applied here, instead a case-by-base strategy should be designed and the sampling technique chosen and the weight of the tissue to be analyzed should be conveniently justified. In this case, a sample of the injection site peripheral tissue should be taken, as long as possible, to corroborate the reliability of the analytical method used.

It should be born in mind that residues concentration determined on the basis of samples taken from small animals or samples of tissues with a weight or size smaller than the one proposed in this guidance should be used without any type of correction or dilution for the calculation of withdrawal time.

4.6.3. Other considerations

- For formulations that are able to leave local residues, such as dermal pour-on products, samples of relevant tissues (*e.g.*, muscle, subcutaneous fat or skin/fat from the application site) should be harvested for analysis (in addition to those specified in Table 1).
- For clarity, if two or more of the tissues are assayed as composite tissues such as skin plus fat in natural proportions (pig and poultry), it is not recommended to assay separate samples of skin and fat.
- Muscle samples can be obtained from skeletal muscles that include intramuscular fat in natural proportion.

- Collection of only one type of fat sample (omental for ruminants and horses) or skin with fat (pigs and poultry).

4.6.4. Milk sampling

Milk samples should be taken from all animals included in the assay. Collection should be done at every milking time, at evenly spaced intervals (12 hours approximately). Samples of each animal will be milk from four quarters.
For multiple dose treatments, samples should be taken after the last treatment, except when the product might qualify for 0-day withdrawal periods, in which case samples should also be collected during treatment.

Although beyond the scope of this guidance, sponsor may be asked to analyze the tissue residues in calves fed by milk (including colostrum) from treated adults (e.g. mothers), if these animals are intended for human consumption.

4.6.5. Egg sampling

Egg samples should be obtained from 10 or more laying hen at every laying time point during medication period and after the final medication. Samples should be collected after the period necessary to complete egg yolk development, which is usually up to12 days. Egg white and yolk can be combined for analysis.

5. Recommendations for veterinary drugs proposed for 0-day withdrawal periods (Single Time-Point Studies)

For veterinary drugs administered as one or several treatment (e.g. daily for 3-5 days) or for continuous use products in which residues have reached steady state, a single time point study should be sufficient to qualify for 0-day withdrawal period, provided the total residue depletion characteristics of the drug have been adequately described. If such data are available, then a single time point study conducted with the specified number of animals is recommended to demonstrate acceptability of 0-day withdrawal period.

- Poultry: 12 birds
- Ruminants, horses and pigs: 6 animals
- Milk 10 animals

The time chosen for euthanasia for this study should be consistent with the peak concentrations observed during the total residue depletion study, a minimum transit time (e.g., not less than 3 hr.) and a maximum time that would still qualify for 0-day withdrawal period (e.g., ≤ 12 h).

The increased number from that recommended in Section 4.3. is appropriate for the single time point. However, for females producing milk for consumption or manufacturing, a minimum of 10 animals is recommended, since that amount is enough to determine milk concentration in a single time point (0 days). The concentrations of active principle remaining below the

appropriate reference point (e.g. MRL, tolerance) will be considered to determine 0-day withdrawal time.

While a 0-day withdrawal designation is possible based on a single time point (i.e. 12 hours) sampling protocol, it is recommended that additional samples (e.g. 1-4 milking) be collected for full assessment of the residue profile, in case of lack of these data. As milk studies do not call for terminal euthanasia for sample collection, this recommendation is straightforward.

In case of egg producing hens, a 0-day withdrawal period could be determined based on consecutive samples below the reference point (maximum residue limit or tolerance) during the 12-days egg collection period for analysis, given the physiological conditions of the oogenesis process.

6. Withdrawal period confirmation studies.

The single confirmation point assay is a special test whose technical validity is restricted to the comparison of results of residue depletion of a veterinary drug widely known and used in the veterinary practice, a drug whose similar formulations have been previously registered for the country where the product is presented. In this study, fewer animals are used and the result only indicates if the same use restriction can be applied. It only serves one purpose: it determines if residues are the same or below the allowed MRL at the (time) point selected for the test. In practice, it does not calculate the withdrawal period, but it does confirm a specific withdrawal period compared to the already known veterinary drug.

6.1. Criteria to determine the need of a residue study or a withdrawal period confirmation study.

The criteria to determine which veterinary drugs should be submitted to a residue study will depend on the type of product (generic or similar), the present active principle(s) and the application criterion defined by each regulatory authority.

6.2. Interpretation of results in withdrawal period confirmation studies

- If an assay demonstrates that the residues of a veterinary drug submitted for trial are below the MRL, confirmation is accepted.
- If the study demonstrates the contrary, that is to say that residues exceed the MRL, then the trial is not accepted, and the period established for the reference product cannot be used and a complete residue depletion study should be conducted.

6.3. Study design

The species, age, breed, genetic line, sex and source of the animals should be informed. The assay should be conducted with the minimum number of animals informed in the following table:

Animal species/ product	Production status	Minimum number
Ruminants/pigs/horses	Any	6
Milking cows	Lactation/ dry-off/pre partum	10
Poultry	Any	10

Rabbits/guinea pigs	Any	10
Eggs	---	12

The trial should follow the indications stated at points 4.2; 4.4 and 4.5 of this guideline. The sampling (point 4.6) and the residue analysis have to be performed only using target tissue (the tissue that was used in previous residue trials to define the withdrawal).

7. Analytical method for marker residue assays

The sponsor is responsible for submitting validated analytical methods for the determination of the marker residue in samples generated from the residue depletion studies in edible tissues and where applicable in milk, eggs and honey. The method(s) should be capable of reliably determining the concentrations of marker residue which encompass the appropriate reference point (i.e. MRL/tolerance) for the respective tissues or products. (See: Guideline of recommendation for the validation of analytical methods).

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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.

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.
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),

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 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 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.

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.

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.

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
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.

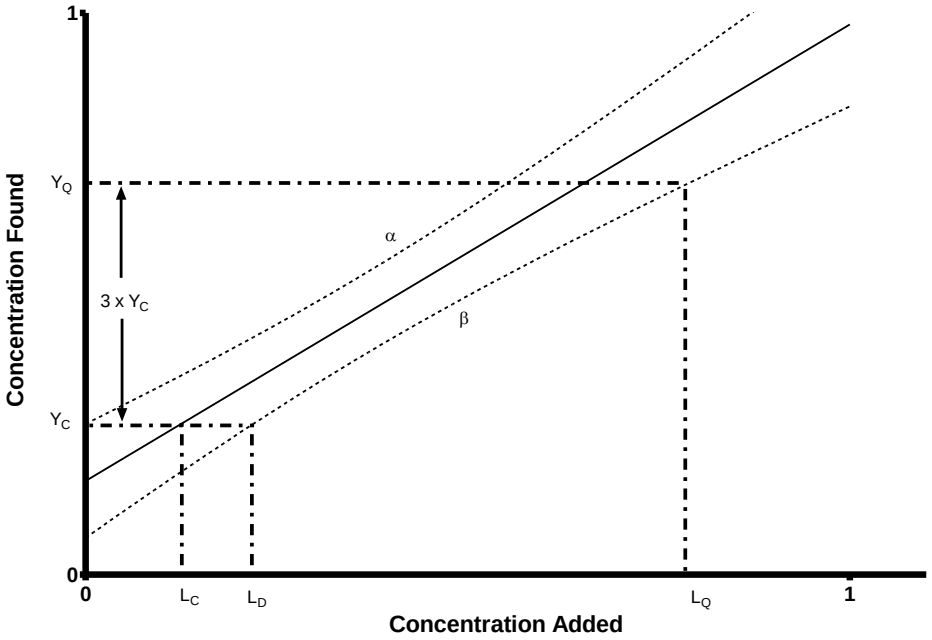
3. Assay the 18 samples each day and evaluate the results against a calibration standard curve.

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.



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	Results, ng/mL

	Level, 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).

- 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						
	50						
	150	9,2	97,3	9,4	**8,8**	**93,4**	**9,4**
	300	10,1	95,0	10,6			

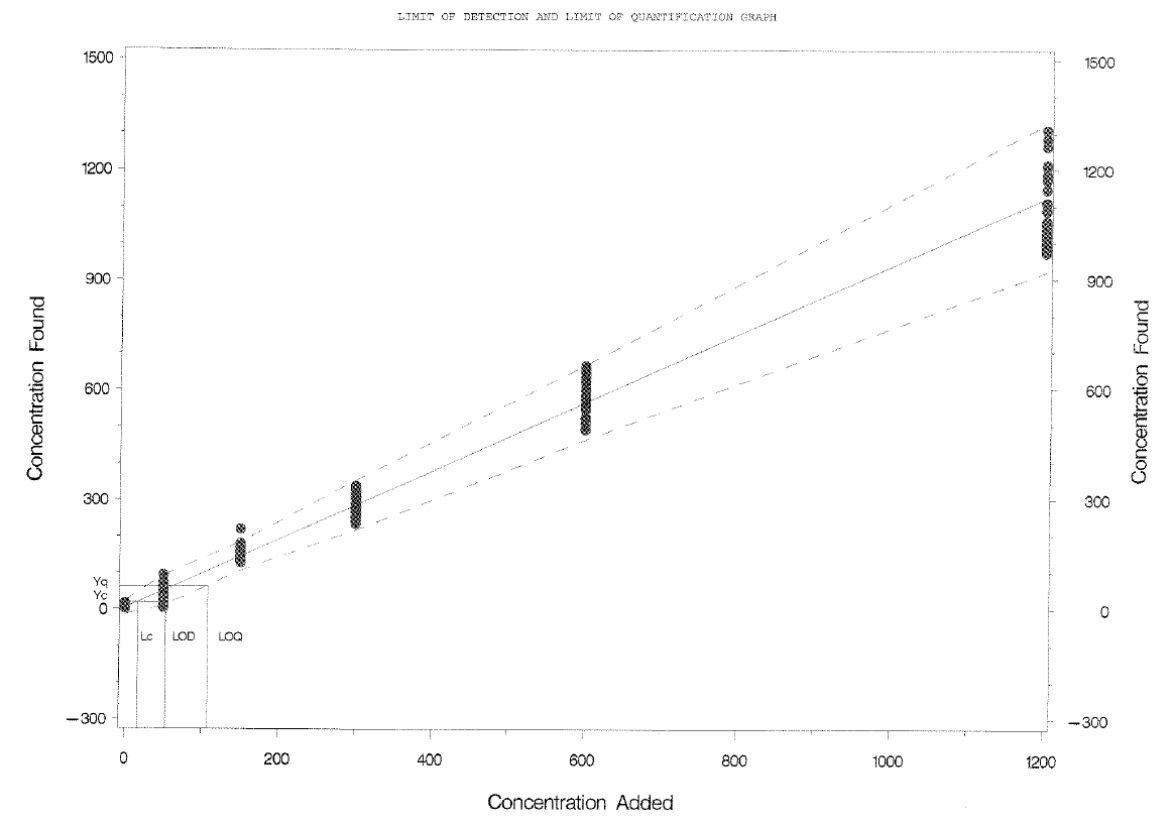
	600	7,5	91,8	8,1			
	1200	8,4	89,4	9,4			
2	0						
	50						
	150	11,6	101,9	11,4	**11,4**	**92,2**	**12,3**
	300	13,4	90,3	14,9			
	600	9,1	92,4	9,8			
	1200	1,7	84,3	2,1			
3	0						
	50						
	150	7,5	109,1	6,8	**7,6**	**101,8**	**7,4**
	300	7,8	99,9	7,9			
	600	5,2	98,9	5,2			
	1200	5,8	99,3	5,8			

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
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.

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.
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.

[2] 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. 42, CAC/GL 16-1993.

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[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.

ANNEX II. iii

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GUIDELINE FOR THE ESTIMATION OF THE WITHDRAWAL PERIOD IN EDIBLE TISSUES

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1. Introduction

Consumer´s safety needs to be safeguarded through the asessment of all pharmacologically active substances aimed at food-producing animals. Withdrawal periods are determined with the aim of ensuring that residues of such substances in edible tissues are reduced to authorized concentrations.

While the Maximum Residue Limit (MRL) for a specific tissue is applied to the active principle, the withdrawal period is determined individually for each veterinary drug as part of the commercialization approval process.

The active principle included in the veterinary drug administered to food-producing animals is not necessarily the substance that will be present in edible products. The enzymatic systems or physiological fluids of an animal can act on the active principle that has been administered and produce new substances such as metabolites that can be as or more damaging for the consumer than the original active principle. Concentration of these substances in edible products of animal origin will be in relation to the speed and degree of absorption of the original pharmacological agent, the speed of metabolism and the excretion rate both of the original active principle and its metabolites. Therefore, the total residue of the pharmacological agent administered to treated animals will be composed of the original active principle, free metabolites and metabolites united to endogenous molecules.

Since the different components of the total residues may differ in their toxicological potentials, information about the chemical nature, quantity and persistence of the total residues in edible tissues of treated animals should be provided.

The simplest and most practical way of determining the withdrawal period has been to identify the time when, in all monitored tissues of all animals in the trial, concentrations are found below the MRL. In some cases, and when there´s a marked variability within the depletion data, a period of time is usually added as security factor.

In other cases, statistical methods have been used that, if generally accepted, they would generate a great opportunity of harmonization.

The choice of the statistical method to be applied is responsibility of the applicant and, in any case, it should be properly justified with adequate documents.

This guidance describes standardized procedures to establish the appropriate withdrawal period for each active principle associated to a pharmaceutical form, dose and route of administration proposed for a specific animal species, in order to warranty consumer´s safety.

2. Glossary

Standard food basket: It is an estimate of the total amount of food of animal origin consumed daily by an adult weighting 60 kg. The basic food basket employs arbitrary figures of consumption, based on percentiles higher than the daily intake of products of animal origin.

The figures of daily consumption of food of animal origin are:

For mammals:

300 g. of muscle, 50 g. of fat or fat and skin, 100 g. of liver and 50 g. of kidney.

For birds:

300 g. of muscle, 90 g. of fat or fat and skin, 100 g. of liver and 10 g. of kidney.

For fish:

300 g. of muscle and skin in natural proportions.

Also an intake of 1.5 l. of milk, 100 g. of eggs and 20 g. of honey are considered.

The estimate of the risk of consumption of present residues in a food basket is calculated taking into account the ADI.

Active Pharmaceutical Ingredient –Drug substance (API): Any substance intended to cure, mitigate, treat, prevent or diagnose diseases in humans or animals.

Acceptable Daily Intake (ADI): It is the estimate of the residue, expressed in terms of weight per kilogram of body weight (kg/bw) that can be daily consumed during consumer´s whole life without noticeable risks to his health.

Confidence Interval: Range of values in which the value of a population parameter is expected to be found with certain degree of certainty.

Maximum Residue Limit (MRL): Maximum concentration of residues founded in an animal product or by product that not introduce any risk for the consumer safety, based on known facts at the time of its publication (CAMEVET definition).

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.

Tolerance limits: The extreme values of a series of values (interval) within which a determined percentage of the individuals of a determined population is expected to be found with certain degree of certainty.

Veterinary drug: Any chemical, biological, biotechnological substance or manufactured (elaborated) preparation administered either individually or collectively, directly or mixed with food, aimed at the prevention, cure or treatment of animal diseases.

Safety Period – Use restriction- Withdrawal Period – Elimination Period: Minimum period of time that must happen between the last application of a veterinary medicinal product to an animal, in the normal conditions of use, and the extraction of food products from that animal, to warranty that such food products do not contain residues in quantities that exceed the established maximum limits.

Veterinary Product (as defined by CAMEVET): A veterinary product is any chemical, biological, biotechnological substance or manufactured preparation administered either individually or collectively, directly or mixed with food or water, aimed at the prevention, cure or

treatment of animal diseases, including additives, supplements, promoters, animal production enhancers, antiseptics, disinfectants for environmental use or for devices and ectoparasiticides and any other product that, used in animals and their habitat, protects, restores or modifies the organic and biological functions. It also encompasses products destined to enhancing animals' beauty.

Marker Residue: It is an analyte that is reliable to determine the presence of residues of a specific drug in the tissue. The marker residue can be a mother cell or any of its metabolites, degradation products or a combination of any of them. The marker residue can also be a chemical derivate of one or various components of the residue. The relationship between the marker residue and the concentration of the residues of interest in edible tissues must be known (marker residue/ residue of interest). The MRL reflects the maximum allowed concentration of the marker residue in the edible tissues.

Total Residue: The total residue of a drug in animal derived food consists of the parent drug together with all the metabolites and drug based products that remain in the food after administration of the drug to food producing animals.

The total sum of the residues normally includes all parent residues of the pharmacological agent (parent drug and its metabolites), and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies.

Residues of toxicological relevance: For the estimate of an exposure based on a toxicological ADI, the residue of relevance is the residue of toxicological relevance. This normally includes all the parent compounds and the molecule (parent drug and the metabolites) and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies. However, if a component of the residue or a fraction of the total residues is demonstrated to be toxicologically inactive, it is possible to deduct it from the total residue or any other fraction of residues that is not bioavailable by oral route or the metabolites known to be toxicologically inactive.

Residues of pharmacological relevance: For the estimate of an exposure based on a pharmacological ADI, the residue of relevance is the residue of pharmacological relevance. In general, the mother cell plus any other of its residues are considered. If there is lack of data on pharmacological activity of the total residue components, the total residues are presumed to present the same pharmacological activity as the parent drug.

Residues of microbiological relevance: For the estimate of an exposure based on a microbiological ADI, the residue of relevance is the residue of microbiological relevance. In most cases, it is identical to the residues determined in microbiological assays. In case of lack of such data, the total residue can be used, or alternatively the sum of the individual components known to present microbiological activity. Therefore, the microbiological activity of the total residue or of the metabolites and/or products of degradation is the same as the parent drug.

Injection site: It is the area of tissue where the veterinary medicinal product has been injected. The samples of tissue obtained from the injection sites for the conduction of residue studies should be representative of the edible tissue that is feasible of being selected in the slaughter procedures. The tissue sample should include muscular tissue, connective tissue and subcutaneous fat in natural proportions (cutting off the samples to eliminate the connective tissue and the fat attached to muscle are considered artificial procedures that differ from the real situation). Injection site should not include the skin portion that covers it, since it is not required for the residues analysis.

Tissue: Any edible animal tissue, including muscles and sub-products (Definitions established and adopted by the Joint FAO/WHO Expert Committee on Food Additives – JECFA).

3. Scope of the guidance

This guidance is a recommendation for the estimation of appropriate withdrawal periods for edible tissues from food-producing animals receiving a specific veterinary medicinal product, in an attempt to harmonize the methodology internationally used for those purposes. This guideline also focuses on the determination of the withdrawal period through the statistical method based on the maximum residue limit (MRL), considered of first choice.
The evaluations of withdrawal periods in other animal products such as milk, eggs, honey and aquaculture products, which require a separate consideration, are not in the scope of this guidance.

The following procedures are described:

- Statistical procedure based on MRL
- Alternative procedure based on MRL
- Pre-slaughter withdrawal period estimated from the residues in the injection site

The residue studies mentioned above should include the description and validation of analytical methods (see Guideline 2: "Guideline for the validation of analytical methods for the estimation of residues in biological matrices").

For the experimental design of the animal phase, see Guideline 1 "Technical guideline to conduct metabolism and residue kinetic studies of veterinary pharmacological agents in food-producing animals".

4. Estimation of the withdrawal period

4.1. Statistical model

The calculation of the withdrawal period by the statistical method is based on accepted, basic pharmacokinetic principles. According to the pharmacokinetic compartment model, the relationship between active principle concentration and time through absorption, distribution and depletion phases can be described by multi-exponential exponential terms. However, depletion of the substance and/or its metabolites from tissues follows a curve of tissue concentrations that follows a first order exponential decay, which can be adequately described by a one-compartment model with only one exponential term. The first order equation that describes the tissue depletion kinetics is the following:

$$C_t = C_0 \, e^{-kt}$$

where C_t is the tissue concentration at a given time, C_0 is the pre-exponential term, e is the base of the natural logarithms, k is the first order elimination rate constant and t is the time.

The term C_0 represents the intersection point at the y-axis at zero time; actually it is only a theoretical concentration needed for the adjustment of experimental data to one exponential term

model. Being an equation that describes a decreasing exponential function, the constant takes a minus sign.

Linearity of the natural log plot of tissue concentrations versus time ($\log_e C_t$ vs t), provides evidence that the one exponential term model is applicable and that linear regression statistical analysis of the logarithmic transformed data can be considered a reliable method for the estimation of the withdrawal period. In that case, experimental data can be described by the first order equation, also known as general equation of a line given by the following expression:

$$y = a + bx$$

where y is the value at the ordinate or y- axis, x is the value at the abscissa or x-axis, a is the point where the line crosses the y-axis and b indicates the quantity at which y changes for each change unit at x. The value of a is known as the y-axis intercept in a chart and the value of b as slope intercept.

The procedure used to obtain the expected line is known as method of least squares, and the resulting line (best mean estimated values in each sampling point) is known as least square line.

4.1.1. Database

The analysis of linear regression requires experimental data which are independent from each other. Generally, tissue depletion data meet this requirement, since they originate from different individuals.

In case of counting with duplicated or triplicated measurements of tissue concentration from a single sample, the mean value will be used for the conduction of a linear regression analysis.

To avoid errors in the calculation of slope and intercept, each data point of tissue concentration should, if possible, originate from the same number of repeated sample measurements.

As a general recommendation and depending on the animal species, between 4 and 10 animals should be used per sampling point (slaughter day). A basic diagram would include the tissue concentration data from 16 (sixteen) animals, which would be slaughtered in groups of 4 (four) individuals in 4 (four) appropriately distributed slaughter days

Experimental data of tissue concentration falling above the limit of detection (LOD) and below the limit of quantitation (LOQ) do have informational value, if not strictly quantifiable value. Hence, if it is necessary to use them, they will be considered optional values and this fact should be properly justified.

Experimental data reported as below the LOD should be excluded from the analysis.

When all or some of the reported experimental data in a slaughter time are "optional" the possibility of excluding from the analysis the corresponding slaughter time should be considered. It should be borne in mind that three sampling points (slaughter time) taken during the terminal elimination phase and three samples (animals) per sampling point are required at least in order to perform a linear regression analysis.

Tissue concentration experimental data should be reported just like they were quantified, that is without any analytical method correction for recovery, and should be attached to supporting data involving recovery experiments and the correction value derived from them. In that case, prior to linear regression analysis, experimental data should be corrected with the value of correction for

recovery before being logarithmically transformed. When the test is done using a calibration curve obtained from fortified samples before the extraction process, there is no need of correction for recovery.

4.1.2. Linear regression analysis assumptions

In order to conduct a linear regression analysis, the following basic assumptions need to be met:

- Homogeneity of variances (homoscedasticity) of the $\log_e$ of the experimental data in each sampling point (slaughter day).
- Linearity of the $\log_e$ of the experimental data versus time.
- Normal (Gaussian) distribution of the errors.

4.1.2.1. Homogeneity of variances (homoscedasticity)

It should be confirmed that variances of log_e of experimental data from the different slaughter days are homogeneous. Different statistical tests, such as Bartlett´s Test[1], Hartley´s Test[9] and Cochran´s Test[16], may be used for this purpose

4.1.2.2. Linearity of log_e of experimental data

A visual inspection of the $\log_e$ plot of experimental data versus time is usually sufficient to assure that there is a linear relationship among experimental data.

A deviation from linearity of $\log_e$ of experimental data in the first sampling points may indicate that the distribution process of the marker residues has not been concluded yet and these points should therefore be excluded from the analysis.

Deviations from linearity in the late sampling points may be due to concentrations below the LOD, so the kinetic process of tissue depletion should not be considered in these sampling points, and exclusion of these values from the statistical analysis is justified.

It should be taken into account that all other experimental data from the remaining sampling points should be preserved, unless their exclusion is properly justified.

For statistical assurance of the linearity of the regression line, an analysis of variance should be performed. The procedure consists of comparing the variation between group means and the estimated line with the variation between animals within the groups.

4.1.2.3. Normality of errors

Normal distribution of errors can be observed through visual inspection of the ordered residuals versus their cumulative frequency distribution on a normal probability scale.

Residuals are the differences between the observed values and their corresponding estimated values (differences between the logarithmically transformed value and the values estimated by the regression line).

A straight line indicates that the observed distribution of residuals is consistent with the assumption of a normal distribution. To verify the results of the residuals plot, the Shapiro-Wilk Test[13] can be applied. This test has proved to be efficient even with small samples.

The plot of cumulative frequency distribution of the residuals can be used as a highly sensitive test. Deviations from the straigth line indicate a non-normal distribution of the residuals, which can be due to:

- Deviations from normality of logarithmically transformed marker residue tissue concentrations data within one or more slaughter groups.
- Deviations from values estimated by linear regression (regression line).
- Non homogeinity of variances (heteroscedasticity).
- Outliers

In the submission of the experimental data using the standardized residuals (residual divided by the residual error Sy, x), an outlier could present a value <-4 o > + 4, indicating that the residual is four standard deviations off the regression line. The use of this kind of data must be properly justified. The information of the animal that originated the sample should be taken into account.

4.1.3. Statistical procedure based on MRL

The withdrawal period should be calculated using data results estimated by the regression line. The withdrawal period is the time when the upper limit of a 95% tolerance interval estimated with a 95% confidence interval intercepts the value of the maximum residue limit (MRL).

If this time point does not correspond to a full day, the estimated withdrawal period will be rounded up to the following day. For example, if the estimated withdrawal period is 6.3 days, it will be fixed at 7 days.

The value of the upper limit of a 95% tolerance interval at a 95% confidence interval is calculated through the non-central *t* distribution method described in the annex.

It is not valid to estimate the withdrawal period based on the absence of data from tissue concentration below the MRL. Therefore, to calculate it, experimental data with values below the MRL in at least the last sampling time should be available. Given this perspective, the LOQ of the analytical method always needs to be less than the MRL used. Preferentially, the LOQ should be at least half the MRL.

The withdrawal period can be estimated using the software WT1.4, recommended by the EMA, The withdrawal period can also be estimated using the method developed by Stange[14], proposed by the EMA[5]. It is methodologically easier to perform and provides results comparable to the method that uses the non-central *t* distribution.

4.1.4 Alternative procedure based on the MRL

This procedure, also known as "decision rule" is an alternative method when the experimental data available do not allow the use of the statistical model based on the MRL.

It is not possible to propose general recommendations for this procedure, since results will depend on sample size, animal slaughter time, biological sampling, variability of the experimental data and factors related to the analytical methodology.

The method is based on establishing a withdrawal period, at the time point when tissue concentrations of all animals are below the MRL value[7]. However, once this time has been

estimated, a security span should be established to compensate for the biological uncertainty that represents the variability in tissue depletion kinetic.

The dimension of the security span will depend on various factors associated to the experimental design and the pharmacokinetic properties of the active principle under study.

Although it is not possible to give general recommendations applicable to all cases, an approximate guidance to calculate the duration of the security span is to increase in 10% - 30% the value of time in which all tissue concentrations are below the MRL. Another alternative is to increase the time mentioned by a value equivalent to 1-3- times the tissue depletion half-life.

4.2. Estimated withdrawal period based on injection site residues

In addition to the effect of the formulation, dose and frequency of administration on the duration of the withdrawal period, the latter is significantly influenced by the route of administration. Injectable formulations can present significantly slower residue elimination kinetics from the injection site than that evidenced with other edible tissues.

This phenomenon may be attributed to the design of slow-release systems, depot-forming formulations, physical and chemical properties of the molecule, use of the subcutaneous and/or intramuscular route of administration, or other factors related to the variability of the route of administration (eg: in the connective tissue between semitendinosus and semimembranosus muscles).

Unlike other tissues, the exact localization of injection site samples to be analyzed can have a significant impact on the residue concentration found. Additionally, the metabolism and/or degradation of active ingredients at the injection site may cause the composition of the total residue to vary significantly from values found in other tissues.

In view of all of the above, from a pharmacological viewpoint, the injection site is not directly comparable with muscle or other edible tissue. Similarly, withdrawal periods established for muscle tissue distant from the injection site are not suitable to guarantee that residues at the injection site will have dropped to safe concentrations within a given period. It must be taken into account that the procedure for calculating MRLs includes a risk analysis of veterinary drug residue in food based on the ADI which takes into consideration dietary exposure to residue throughout a lifetime.

Consequently, injection site residues need to be considered specifically in relation to the risk to the consumers of the treated animals.

4.2.1. Interpretation

It must be taken into account that the injection site withdrawal period estimated according to this guideline should not necessarily be considered the definitive withdrawal period for the veterinary drug analyzed.

The injection site withdrawal period must be considered in comparison with withdrawal periods based on residue depletion in other edible tissues. Lastly, the withdrawal period selected for the veterinary drug in question must be duly justified.

4.2.2. General principles

It is necessary to identify the injection site residues that are of interest. In the case of veterinary drugs that contain new active ingredients, residues relating to active molecules must be properly identified, including metabolites and degradation and/or conversion products with potential biological impact. This information is obtained from radiometric residue depletion studies (eg: total residue) or, where appropriate, from residue depletion studies directed at the toxicological, pharmacological and microbiological characterization of residues.

For veterinary drugs that contain known active ingredients whose injection site residue composition is known, radiometric residue depletion studies are not necessary. What is needed is an appraisal of the original active ingredient or of any other relevant component of the injection site residue (eg: marker residue). Information relating to the ratio of marker residue / total residue can be obtained from information available in literature.

4.2.3 Study design and sampling

We recommend using Guide nr. 1 G.F: "Technical Guide for conducting Metabolism and Kinetic Studies of Residues of Veterinary Pharmacological Ingredients in Food Producing Animals", item 4.6.2.

The injection site residue depletion study report must include a full description, detailed study design and experimental conditions, selection of the injection site for the product, injection technique used, instruments used, depth of injection (intramuscular), measures taken to allow the exact localization of the injection site at slaughter, detailed description of the sample taking technique, and sample conditioning.

4.2.4 Procedure

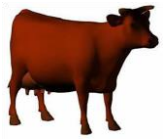
It must be taken into account that the ingestion of injection site residues, as mentioned above, is a sporadic event. Therefore, the determination of the withdrawal period must be based on parameters that allow the quantification of the short-term risk of dietary exposure. Depending on the characteristics of the active ingredient, the Acute Reference Dose (ARfD) is the most suitable concept. Nevertheless, in some cases it may be recommended to use a different reference parameter. Also, the ARfD is not available for many active ingredients. In these cases another reference limit must be proposed and justified, including: therapeutic human dose, maximum recommended intake (eg: vitamins), maximum tolerable intake (ej: minerals/trace elements), basal or natural levels for compounds that can be produced endogenously (eg: hormones). A security factor should be considered for any reference limit selected.

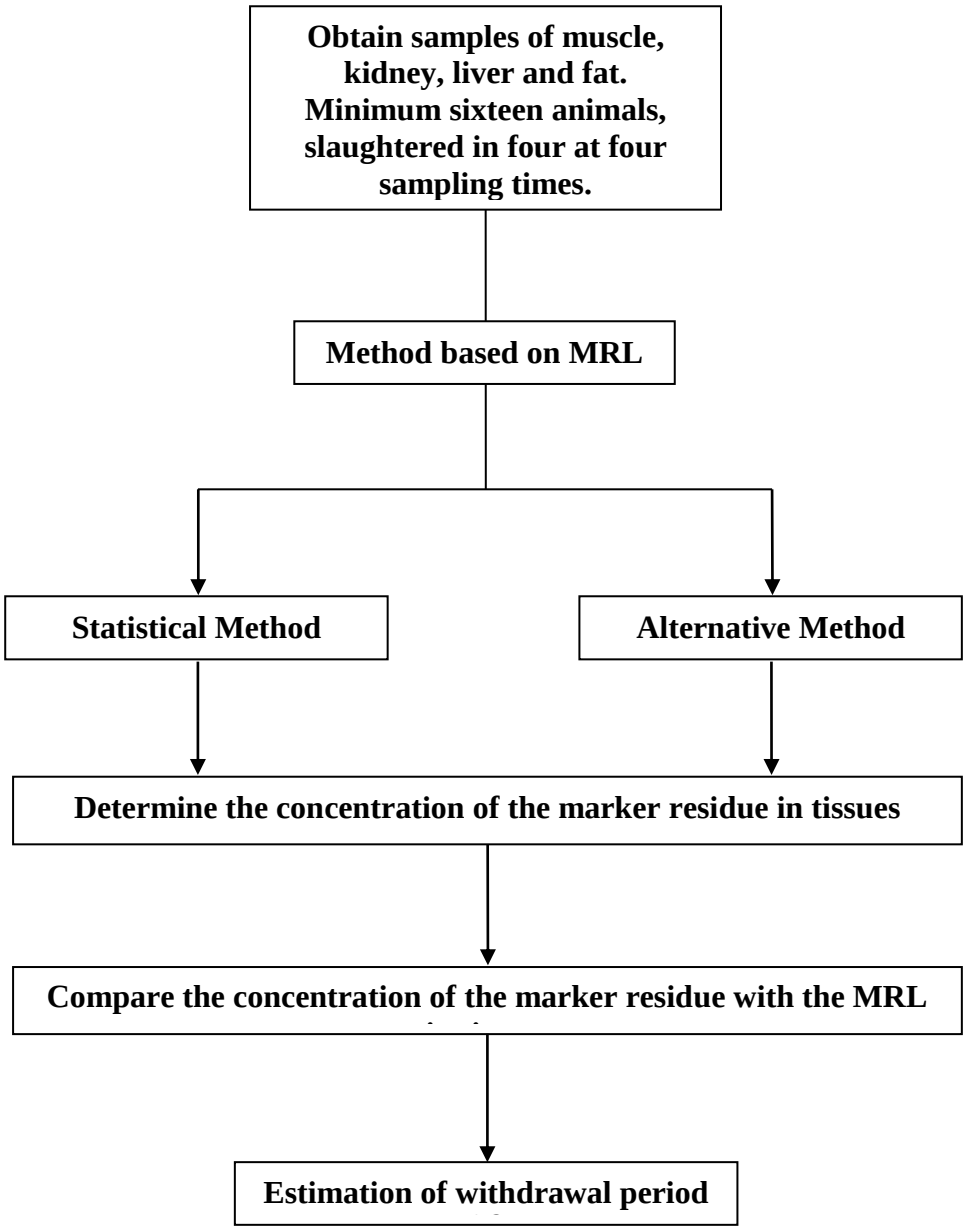
It is also possible to transform the MRL for muscle or fat (according to the animal species or the active ingredient in question) by applying a factor of 10 or more within an appropriate reference limit. The selection of any reference limit must be justified.

When this procedure is used, it is necessary to check that the marker residue is valid for predicting the target injection site residues.

Withdrawal periods must guarantee that the concentration of the residues has dropped below the chosen reference limit for the injection site within the specified period. It can be calculated as described in items 4.1.3 and 4.1.4 for edible tissues.

COMPARATIVE PLOT OF SAMPLING AND ANALYSIS OF EDIBLE TISSUES AND ESTIMATION OF THE WITHDRAWAL PERIOD USING THE METHOD BASED ON THE MRL.





1- The withdrawal period should be estimated for all edible tissues and will be calculated following the procedures proposed in this guideline. The longest withdrawal period will be considered the most appropriate one.
2- If the veterinary product is parenterally administered (IM – SC), the withdrawal period of the musle tissue will be replaced by the withdrawal period of tissues at the injection site.

5. References

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6. Annex

Statistical procedures

Bartlett´s Test

The Bartlett´s test[1] is used to check if a number of *k* samples come from populations presenting similar variances. Equality of variances across samples is called homogeneity of variances or homoscedasticity.

The use of Bartlett´s test is justified by the fact that many statistical tests, such as Student´s *t*-test of mean differences, or the variance analysis assume that variances across samples are equal.

The FDA[6, 7] recommends the use of this test due to its robustness, even though it is extremely sensitive to deviations from normality. On the other hand, this test can only be used when each group of data is equal to or higher than 5 (five). It presents the advantage of allowing the comparison of data groups of different sizes.

Bartlett´s test is used to check the nule hypothesis H_0 states that the population variances are equal, compared to the alternative hypothesis H_1 that states that at least two are different. If we count with *k* samples with a size of n_i samples and a S_i^2 variance, the statistical Bartlett test would be:

$$X^2 = \frac{(N-k)\ln(S_p^2) - \sum_{i=1}^{k}(n_i-1)\ln(S_i^2)}{1+\frac{1}{3(k-1)}\left(\sum_{i=1}^{k}\left(\frac{1}{n_i-1}\right)-\frac{1}{N-k}\right)}$$

where: $N = \sum_{i=1}^{k} n_i$ ar$S_p^2 = \frac{1}{N-k}\sum_i (n_i-1)S_i^2$ is the estimator of the total variance.

Bartlett`s statistical test has approximately a distribution X^2_{k-1}. The nule hypothesis is rejected when $X^2 > X^2_{k-1,\alpha}$, where $X^2_{k-1,\alpha}$, is the upper critical value for a distribution X^2_{k-1}.

Hartley´s Test

This test was developed in 1950 by Hartley[9] and is also known as the F_{max} test or Hartley´s F_{max} test. It is used in the analysis of variances to verify that different groups have similar variances, indispensable condition to make comparisons across groups through the application of parametric statistical test. The disadvantage of the test is that it can only be used to compare groups of data of the same size.

The test is based on the calculation of the relationship between the upper group variance (max s_j^2) and the lower group variance (min s_j^2). The resulting value is then compared to the critical value present in the $F_{max\ 3,\ 12}$ distribution table.

It is assumed that the groups present similar variances if the calculated value is lower than the critical value.

The Hartley test assumes that the data of each group presents normal distribution and that the groups present the same number of individuals. This test, though convenient, is not very sensitive to deviations from normal distribution[10].

Cochran Test

This test, developed by William Gemmell Cochran[16] is the analysis of two randomized block designs, where the result of the comparison can only have two results. The Cochran test is also known as Cochran´s Q Test, it is a non-parametric statistical test.

The EMEA[5] considers it the best test, since it is simpler than Bartlett´s test. On the other hand, it is less seinsitive to deviations from normality than the latter and it can also be used to analyze groups of data of different sizes.

The test assumes that the number of experimental treatments is higher than two (k>2) and that the observations are arranged in a certain number of (b) blocks, as shown below:

	Treatment 1 (k_1)	**Treatment 2 (k_2)**	**….**	**Treatment$_i$ (k_i)**
Block 1 (b_1)	X_{11}	X_{12}	….	X_{1k}
Block 2 (b_1)	X_{21}	X_{22}	….	X_{2k}
Block 3 (b_1)	X_{31}	X_{32}	….	X_{3k}
Block 4 (b_1)	….	….	….	…..
Block$_i$ (b_i)	X_{b1}	X_{b2}	…..	X_{bk}

Cochran´s test is based on the nule hypothesis (H_0) that states that the treatments are equal and on the alternative hypothesis (H_1) which states that there is a difference across treatments.

Cochran´s statistical test is:

$$T = k\,(k-1)\sum_{j=1}^{k}\left(X_{\bullet j}-\frac{N}{k}\right)^2 \Big/ \sum_{i=1}^{b} X_{i\bullet}\,(k-X_{i\bullet})$$

where:

k is the number of treatments

$X_{\cdot j}$ is the column total at j^{th} treatment

b is the number of blocks

$X_{i\cdot}$ is the total value of cells al $j^{ésimo}$ bloque$X_{\cdot j}$ is the total row at j^{th} block$N$ is the total value of all samples

The significance level of the critical region is given by:

$$T > \mathrm{X}^2_{1-\alpha,k-1}$$

where $X_{1-\alpha, k-1}^2$ is the quantile (1-α) of chi-square distribution with k-1degree of freedom. The nule hypothesis is rejected if the statistical falls in the critical region.

Shapiro-Wilk Test

The test was published in 1965 by Samuel Shapiro and Martin Wilk[13], and is used to check the null hypothesis (H_0) that a sample $X_1,\ldots\ldots,X_n$ comes from a normally distributed population.

The test starts with a null hypothesis (H_0) which affirms that the experimental data come from a normally distributed population, and an alternative hypothesis (H_1) that states that the experimental data come from a not normally distributed population. The statistical test is:

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\sum_{i=1}^{n}(x_i - \overline{x})^2}$$

where:

$x_{(i)}$ is the i^{th} order statistic, i.e.: the smallest number of the sample.

$\overline{x}$ is the sample mean.

The constants a_i are given by the following equation:

$$(a_1, \ldots, a_n) = \frac{m^\top V^{-1}}{(m^\top V^{-1} V^{-1} m)^{1/2}}$$

where:

$$m = (m_1, \ldots, m_n)^\top$$

$m_1,\ldots,m_n$ are the expected values of the order statistics of independent and identically distributed random variables, sampled from the standard normal distribution and V is the covariance matrix of those order statistics.

The null hypothesis is rejected when the value statistic (W) is lower than the chosen alpha level (0.05). In case the statistic (W) is higher than the chosen alpha level, then the null hypothesis

cannot be rejected and experimental data are assumed to be from a normally distributed population.

Calculation of the upper limit of the tolerance interval

The FDA proposes to estimate the upper limit of the tolerance interval at 99% with a 95% confidence interval. However, an objection to this criterion is the excessive extrapolation of the values of tolerance interval, since many times this intersects the MRL value posteriorly to the values of the last detected tissue concentrations. This excessive extrapolation can result in an inadequate estimation of the withdrawal time. The EMEA[5] proposes a 95% tolerance interval, which minimizes the extrapolation problem and provides a more realistic estimation of the withdrawal period.

Procedure of non-central *t* distribution (FDA)

The upper limit of the tolerance interval at any time is calculated with the following equation:

$$T(y) = a + b.t + k.s.\left[\frac{1}{n}\cdot\left(\frac{t-\bar{x}t}{\sum t_i - \bar{x}t}\right)^2\right]^{0.05}$$

where:

k = 95th percentile of a non- central"t" distribution with a non-central "d" parameter and a degree of freedom equal to S^2.

$$d = \frac{z}{\left[\frac{1}{n}\cdot\left(\frac{t-\bar{x}t}{\sum t_i - \bar{x}t}\right)^2\right]^{0.05}}$$

z = 95th percentile of a normalized standard distribution.

In order to calculate the "k" value, see D.B. Owen, *Handbook of Statistical Tables, Addison-Wesley, Reading, Massachusetts* (1962)11, and employ "d" value and the table of factors for the calculation of critical values for the non-central "t" distribution with a 95% percentile (0, 95) and "n" degree of freedom.

The upper limit of the tolerance interval is calculated as the antilogarithm of the calculated value. Check that the estimated value does not exceed the MRL value. If that happens, t value should be

increased and the calculation procedure should be repeated until the estimated value is lower than the MRL, in that case *t* value is consistent with the withdrawal period.

Stange equation (EMEA)

The calculation of the upper limit of a 95% tolerance interval with a 95% confidence interval can be also be performed following the procedure reported by Stange[14], as described below:

$$Ty = a + bt + k_T s_{y.x}$$

where:

Ty = upper limit of the tolerance interval at a determined sampling time.

a = point where the straight line crosses the ordinate- axis

b = slope of the straight line

t = time.

$$k_T = \frac{\sqrt{(2n-4)}}{(2n-4)^* - u_{1-\alpha}^2}\left[\sqrt{(2n-4)^* - u_{1-\gamma}^2 + u_{1-\alpha} W_n}\right]$$

$$W_n = \sqrt{u_{1-\gamma}^2 + \left[(2n-4)^* - u_{1-\alpha}^2\right]\left[\frac{1}{n} + \frac{(x-\bar{x})^2}{s_{xx}}\right]}$$

$$s_{xx} = \sum x_i^2 - \frac{1}{n}\left(\sum x_i\right)^2$$

The respective standard normal distribution statistical values are:

- For $1-\alpha = u_{1-\alpha} = 1{,}6449$
- For $1-\alpha = u_{1-\alpha} = 1{,}6449$

$S_{y,x}$ = residual error, ()* = (2n-5) according to Graf et al.[7].

The correction proposed by Graaf[8] (using the term (2n-5) instead of (2n-4), results in a slightly higher tolerance interval limit. According to Stange, the equation is valid for a value n ≈ 10, while Graf increases the validity of the estimation to a value n ≈ 20.

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