Guidance Document #08
ADME Evaluation in the Context of Risk Assessment of Feed Ingredients
October 2024
At Step 7: Steering Committee Endorsement

ADME EVALUATION IN THE CONTEXT OF RISK ASSESSMENT OF FEED INGREDIENTS

Endorsed by the Steering Committee
October 2024

It is recommended for the companies planning to submit applications/dossiers for pre-market authorization, to contact the jurisdictions of the countries to confirm their acceptance of the current guidance document.

The International Cooperation for Convergence of Technical Requirements for the Assessment of Feed Ingredients (ICCF) was launched in 2017 and aims to develop and establish common guidance documents to provide technical recommendations for the assessment of feed ingredients, including new uses of existing feed ingredients.

This guidance document has been developed by the appropriate ICCF Experts Working Group and was subject to consultation by the Parties, in accordance with the ICCF Process.

The founding members of the ICCF include the Canadian Food Inspection Agency (CFIA), the European Commission (DG SANTE), the U.S. Food and Drug Administration (FDA), as well as the American Feed Industry Association (AFIA), the Animal Nutrition Association of Canada (ANAC), the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA) and the International Feed Industry Federation (IFIF).

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ADME EVALUATION IN THE CONTEXT OF RISK ASSESSMENT OF FEED INGREDIENTS

1. INTRODUCTION

1.1 Objective of the guidance document

This guidance document addresses the evaluation of the absorption, distribution, metabolism, and excretion (ADME) of feed ingredients and/or their constituent entity(ies) as a critical component of the risk assessment for the consumer of edible products. The guidance document describes recommended approaches with corresponding endpoints and methods/procedures to support the ADME evaluation of feed ingredients, while minimizing use of animals.

1.2 Definitions

The definitions applicable in the context of this guidance document are provided in the ICCF Glossary of Terms.

1.3 Scope of the guidance document

The scope of this guidance document is the ADME evaluation used in the consumer risk assessment of edible products derived from animals fed the feed ingredient under investigation. Beyond this guidance document, the results of the ADME evaluation may also be used in the risk assessment of the feed ingredient for the target species, the environment (fate of the feed ingredient and its metabolites in the excreta), and/or the workers exposed to the feed ingredient, while handling it.

This guidance document generally applies to the risk assessment of new feed ingredients and of already marketed feed ingredients in case of new conditions of use or new target species.

2. GENERAL PRINCIPLES

Recommendations for the ADME evaluation (when required) of a feed ingredient in this guidance document can be applied to each constituent entity of the feed ingredient or of the ingredient market formulation. The test article should be selected using sound scientific judgement. For example, if the ingredient market formulation influences the absorption of the active substance(s) or the safety of the feed ingredient, it may be recommended to use the ingredient market formulation as a test article (e.g., encapsulated carotenoids). However, if the

ingredient market formulation does not influence the absorption of the constituent entity(ies) or the safety of the feed ingredient, the ADME evaluation could be conducted on the individual constituent entity(ies) of the feed ingredient, as needed.

An initial step of the ADME evaluation of feed ingredients to support consumer risk assessment is the review of information available on the ADME properties of the feed ingredient or its constituent entity(ies), and the identification of critical gaps in that information using the weight of evidence approach. Gaps in critical information needed for the ADME evaluation of feed ingredients should be assessed on a case-by-case basis. For example, strategies could involve a diversity of approaches (e.g., read-across, new studies). If ADME studies (e.g., *in vitro*, *in vivo*) are required to fulfil critical data gaps in information for the ADME evaluation of feed ingredients, the studies should be carefully designed and conducted using scientifically recognized methods (including validated novel technologies and methodologies). The type of study selected should depend on various factors, including but not limited to, the type of feed ingredient and potential metabolites (e.g., constituent entities with bioaccumulation potential).

3. ASPECTS TO CONSIDER IN THE ADME EVALUATION OF FEED INGREDIENTS

Several aspects should be considered when deciding if an ADME evaluation of a feed ingredient is needed for addressing consumer safety. These aspects are summarized in a decision tree included in Annex I. The decision tree also includes recommendations on which types of studies should be conducted for addressing critical data gaps in the ADME evaluation of the feed ingredient subject to a pre-market authorization or approval.

The initial review of available information to determine whether an ADME evaluation of a feed ingredient or its constituent entity(ies) is needed should consider the following assessments, as listed below and in the decision tree in Annex I:

1. Identity and Characterization of the feed ingredient: Information on the individual constituent entity(ies) and/or the ingredient market formulation(s) (recommendations on this topic can be found in the ICCF Guidance Document on 'Identification and Characterization of Feed Ingredient'). Constituent entity(ies) with known metabolism do not require ADME evaluation.
2. Intended target species: ADME evaluation of feed ingredient is not required when the feed ingredient is intended only for non-food producing target species.

3. Evaluation of toxicologically relevant information: The toxicity profile of the feed ingredient or its constituent entity(ies) and/or metabolites and its potential for bioaccumulation is evaluated using a weight of evidence approach (e.g., from *in vivo* or *in vitro* studies, read-across methods, Quantitative Structure Activity Relationship (QSAR) analysis).
4. Potential safety concerns for the consumer: Potential toxicity of the feed ingredient or its constituent entity(ies) and/or metabolite(s) for the consumer identified from the available information (e.g., from *in vitro* and *in vivo* studies) in the weight of evidence approach.

The outcome of the assessments listed above should allow:

1. Selecting an appropriate point of departure (POD), such as the no observed adverse effect level(s) (NOAEL[s]) for critical toxicological endpoints. These NOAEL(s) should be used to derive the acceptable daily intake (ADI) of the constituent entity(ies) of the feed ingredient for the consumer. If the available information does not allow selecting an appropriate POD, a gap analysis should determine the information needed for selecting a POD.
2. Evaluating the potential consumer exposure to the constituent entity(ies) of the feed ingredient based on its conditions of use, and/or tissue residue data. In the absence of tissue residue data in edible products and adequate safety data, ADME studies should be considered.
3. Comparing the estimated ADI or Tolerable Upper Intake Level (UL) with the potential consumer exposure. This comparison allows to determine if there are safety concerns for the consumer due to the exposure to the constituent entity(ies) and/or its(their) metabolites in edible products.

4. ADME EVALUATION

A tiered approach is recommended, if the ADME evaluation of the constituent entity(ies) of the feed ingredient is considered necessary based on the assessments that should be considered (as listed in Section 3 and in the decision tree of Annex I).

The first tier would be the evaluation of all information available (e.g., from read-across, *in silico* models and/or *in vitro* or *in vivo* studies) to conduct the safety assessment for the consumer using a weight of evidence approach.

A second tier is triggered, if critical data gaps exist in the available information that prevents conducting the ADME evaluation of the constituent entity(ies) of the feed ingredient using the weight of evidence approach. In this case, it is recommended to prioritize studies providing

information on the absorption of the constituent entity(ies), followed by studies on their metabolism and then, if required, their potential bioaccumulation. There are established models for absorption studies (including *in vitro* and *in vivo* studies, and *ex-vivo* absorption and bioavailability models). Demonstration of negligible absorption, either through experimental studies or from theoretical considerations, may provide a scientific justification for not undertaking higher tiered toxicological studies on a constituent entity.

4.1 Weight of Evidence Approach

The weight of evidence approach in scientific assessment[1] comprises three (3) basic steps:

- Assembling the evidence,
- Weighing the evidence, and
- Integrating the evidence.

At each step, the reliability, relevance, and consistency of the evidence used should be evaluated. Detailed information on the weight of evidence approach in scientific assessments can be found in the EFSA guidance (13). Although the guidance is not specific for the ADME evaluation of feed ingredient, the principles highlighted in the guidance can be applied to the weight of evidence approach for ADME evaluation of constituent entity(ies).

The weight of evidence approach aims at considering all information available on the ADME and toxicologically relevant characteristics of the constituent entity(ies) from relevant studies/information.

4.1.1 Read-across Assessment

If no information is available on the specific constituent entity, an alternative method that could be used, on a case-by-case basis, is the read-across assessment (also known as bridging assessment). Read-across assessment involves the use of information in the public domain (e.g., scientific literature) (the 'source information') from analogous substances (the 'reference substance') that have similar structure, physical and chemical characteristics to predict the properties and potential behavior of the constituent entity(ies) of the feed ingredient[2].

The read-across information should provide information on pivotal endpoints required for the ADME evaluation of the constituent entity(ies) of the feed ingredient, by

[1] Adapted from Guidance on the use of the weight of evidence approach in scientific assessments. EFSA Journal 2017;15(8):4971.
[2] Adapted from ECHA-17-R-01-EB. Read-across assessment framework (RAAF)

extrapolation/interpolation and prediction from available data on the reference substances. These typical endpoints should be selected using scientific judgement considering the feed ingredient under assessment.

The read-across should cover the evaluation of the substance or a group of substances used as reference. The reference substance(s) and the endpoint(s) of interest selected, as well as the approach taken for the read-across evaluation should be accurately described, and justified to show how the conclusion from the read-across properly supports the hypothesis considered.

If the read-across provides the information required for the ADME evaluation of the constituent entity, the applicant may not be required to provide additional data. Otherwise, further evaluation considering *in silico* models or *in vitro* studies (if appropriate) is recommended.

4.1.2 *In Silico Models*

In silico models such as QSAR(s), and physiologically based pharmacokinetics are computerized models to predict qualitatively and quantitatively the physical, chemical, and biological properties, and environmental fate of substances, based on the information available on their chemical structures. The models are built on relevant and available information from similar reference substances. However, it is important to consider that these models may not cover all the target species, and physiological status, as most of the models have been derived from studies on laboratory animals and/or humans.

For *in silico* models, it is important to evaluate the constituent entity against substance(s) within the same applicability domain. Reliability is improved with the use of established models. Examples of *in silico* models available are listed in Annex II.

The selection of an *in silico* model should be properly justified. Furthermore, the results of the evaluation of the *in silico* models should be carefully examined to ensure the information obtained from the model can fulfil the requirements for the ADME evaluation of the constituent entity. Uncertainties in *in silico* model results should be addressed and may be compensated by additional information on the constituent entity.

If the combination of read-across and *in silico* models provides enough information required for conducting the ADME evaluation, the applicant may not be required to provide additional information. However, if critical data gaps or uncertainties remain after conducting the read-across assessment and *in silico* models, additional *in vitro* studies (if appropriate) are recommended.

It is to be noted that, while read-across and *in silico* models may be used to replace *in vitro* and/or *in vivo* studies, they may also be used to provide supplementary information to assess the results from *in vitro* and/or *in vivo* studies.

4.1.3 *In Vitro* Studies

Various *in vitro* test systems (Annex III) have been published and could be used in the ADME evaluation of the constituent entity(ies) of the feed ingredient. These test systems may support the evaluation of

- the digestion of the constituent entity(ies) of the feed ingredient, such as:
  - the simulated gastric fluid study, simulating the physical and chemical properties of the fluid contained in the stomach of the animals, or
  - the simulated intestinal fluid study, simulating the microbiome, and physical and chemical conditions of the intestinal fluid, or
- the absorption of the constituent entity(ies) of the feed ingredient, such as the Caco2 permeability assay, using the Caco2 cells of the intestinal tract of the animals to simulate the absorption of the constituent entity(ies), or
- the metabolism of the constituent entity(ies) of the feed ingredient after absorption, such as the studies with primary hepatocytes, liver microsomes, S9 sub-cellular fraction, cytosol, liver slices, or whole cell lines.

The choice of the *In vitro* system(s) should be properly justified by the applicant for achieving the level of information necessary for the ADME evaluation.

However, the protocols for the above-mentioned *in vitro* studies have not yet been standardized (e.g., by regulatory bodies). Therefore, when used, it is recommended that good laboratory practices[3] relevant to the test system is used and the reference to the test system selected is properly justified.

The robustness and reliability of *in vitro* methods can accelerate their use for early screening testing.

If the results of the *in vitro* studies, in combination with read-across and *in silico* models (if appropriate) provide enough information required for conducting the ADME evaluation, the applicant may not be required to provide additional information. However, if critical data gaps or uncertainties remain, *in vivo* studies may be required.

[3] For further details see the OECD guidelines No. 286

4.2 *In-Vivo* Studies

In the tiered approach recommended in the guidance document, *in vivo* studies should be the last tier considered when critical information required for the ADME evaluation of the constituent entity(ies) of the feed ingredient cannot be sufficiently provided by read-across assessment, *in silico* models, or *in vitro* studies. The goal of *in vivo* ADME studies in the safety assessment of a feed ingredient in the context of consumer safety is to generate data on the quantity and nature of residues of toxicological concern in edible products of animals fed the feed ingredient or its constituent entity(ies).

Therefore, it is preferable to provide the feed ingredient or its constituent entity(ies) orally, to properly determine the ADME properties of the constituent entity(ies) in the relevant target species. Intravenous applications may provide information on the toxicokinetic of the constituent entity(ies) and may be used, if properly justified by the applicant.

The Veterinary International Commission on Harmonization (VICH) Guideline No. 46 provides a framework for metabolism and residue testing. However, to adequately characterize the residue(s) of concern, it is important that the design of studies remain flexible.

If the production of radiolabeled constituent entity is not feasible (e.g., plant extracts) and/or if scientifically justified (e.g., if the constituent entity is not metabolized or the metabolite(s) can be quantified otherwise) studies using no radiolabeled substances should be considered first.

In other cases, as needed, metabolism studies should be accomplished using properly radiolabeled[4] substances of the constituent entity(ies) of toxicological concern of the feed ingredient for the consumer.

In that case, the radiolabeled constituent entity(ies) are fed to the target species in feed or water, according to the maximum intended use level of the feed ingredient. As ADME studies are aimed to evaluate the fate of the radiolabeled substances and not their toxicokinetics, the use of a single oral administration is recommended. In addition, ADME studies may be envisaged in laboratory animals, if the physiological similarities of the laboratory animals with the target species can be justified based on the information available from lower tiers and *in vitro* metabolism studies.

[4] Radiolabel should allow the tracking of radioactivity in all relevant metabolites.

Edible products are collected at different time points of the study, to measure the concentration of the constituent entity(ies) and/or its metabolites with time, measured as total radioactivity in the relevant edible product or quantified using a validated method of analysis (ICCF Guidance Document on Analytical Methods[5]). Enough control and test edible products should be collected to enable the related analytical methods testing.

Although the excreta and blood are usually not collected in *in vivo* residue studies, the analysis of those samples may provide useful information, such as:

1. Estimating the mass-balance of the radioactivity, supporting the quality of the study,
2. Obtaining information on the metabolites present in excreta, for further use in the conduct of environmental risk assessment (ICCF guidance document on feed ingredients environmental risk assessment approach and ICCF guidance document on feed ingredient environmental risk assessment (Phase 2)[5]) and in blood.

Further to the evaluation of the concentration of metabolite(s) in the edible products, it is recommended to characterize and identify the structure of the major metabolites (i.e., with concentration greater than 100 µg / kg wet basis or representing more than 10 % of the total residue). An evaluation of the non-extractable residues may warrant discount of some of the residues, as non-extractable residues usually result from incorporation of small fragments of the constituent entity(ies) or its(their) metabolites in naturally occurring molecules and are not of significance.

The determination of a marker residue (i.e., a metabolite that can be analyzed and has a direct relationship with the total amount of metabolites) is recommended to allow the evaluation of the consumer's exposure to the metabolites from the constituent entity. The exposure of the marker residue is calculated based on results of a marker residue study conducted with the non-labeled constituent entity and can then be compared with the pre-determined POD.

5. INTERPRETATION OF THE INFORMATION

For each step of the ADME tiered approach, the aim of the information collected should be interpreted to allow:

- For read-across
  - The information gathered for the ADME of (an) analogous substance(s).
    - The applicability of this information for the feed ingredient.

[5] Under development at the time of writing this guidance document.

- For *in silico* models
  - The information provided by the *in silico* model(s) regarding the ADME of the constituent entity(ies) of the feed ingredient, considering the methodology used for the evaluation.
  - The potential presence and concentration of the constituent entity and its metabolites of toxicological concern when the feed ingredient is fed to the target species.
- For *in vitro* studies
  - The information provided on the characterization and concentration of the constituent entity and its metabolite(s) of the constituent entity(ies) of feed ingredient in the relevant *in vitro* studies used.
  - The potential for extrapolation of results obtained to laboratory animals and/or the target species.
- For *in vivo* studies
  - Identification, characterization, and concentration of the constituent entity and its metabolite(s) resulting from the proposed conditions of use of the feed ingredient.
  - Evaluation of the toxicological relevance of the metabolite(s), analyzed in the relevant edible products.

and interpreted to achieve the following conclusions:

- For read-across
  - The conclusion with regards to potential presence of the constituent entity and its metabolite(s) of toxicological concern in the edible products of the target species fed the feed ingredient:
    - If the conclusion allows the consideration of low level of exposure compared to the POD and/or limited toxicological concern, no further evaluation is required.
    - If not, it is recommended to go to the next step of the approach.
- For evaluation of *in silico* models
  - The conclusion with regards to potential presence of the constituent entity and its metabolite(s) of toxicological concern in the edible products of the target species fed the feed ingredient:
    - If low level of exposure and/or limited toxicological concern is found, no further evaluation is required.
    - If not, it is recommended to go to the next step of the approach.

- For *in vitro* studies
  - The conclusion with regards to potential presence of the constituent entity and its metabolite(s) of toxicological concerns in the edible products of the target species fed the feed ingredient:
    - If the conclusion allows the consideration of low level of exposure and/or limited toxicological concern, no further evaluation is required.
    - If not, it is recommended to go to the next step of the approach.
- For *in vivo* studies
  - Comparison of the concentration of the constituent entity and its metabolite(s) of toxicological concern with the relevant POD (reference point), to ensure the safety of the feed ingredient for the consumer:
    - If the concentration of the constituent entity and its metabolites is lower than the POD, when applying an appropriate uncertainty factor, continue with the risk assessment of the feed ingredient,
    - If the concentration of the constituent entity and its metabolites is higher than the POD, when applying an appropriate uncertainty factor:
      - Modify the conditions of use of the feed ingredient, if possible, to reduce the exposure of the consumer to achieve a safe amount of the toxicologically relevant metabolite(s) (e.g., reduce incorporation rate, propose a withdrawal period),
      - Otherwise, evaluate the possibility of stopping the use of the feed ingredient.

6. REPORTING THE INFORMATION

For each step of the ADME approach, data and results should be reported:

- For read-across:
  - Justification of the reference substance(s) used for the analysis, based on similarity,
  - Approach taken for the extensive literature search,
  - Information gathered and analyzed for the evaluation.
- For *in silico* models:
  - Justification of the methodology used for the evaluation,
  - Information gathered and analyzed for the evaluation.

- For *in vitro* studies:
  - Justification of the model(s) used for the evaluation,
  - Description and justification of the protocol used for the study(ies),
  - Characterization of the metabolites obtained with the study(ies) and evaluation of their safety,
  - Analytical methods and their validation
  - Number/quantity of metabolites obtained.
- For *in vivo* studies:
  - Description and justification of the protocol used for the study(ies),
  - Characterization and identification of the main metabolite(s),
  - Analytical methods and their validation
  - Concentration of the main metabolite(s) in the edible products,
  - Information on the toxicological properties of the metabolite(s).

7. ACRONYMS

ADME	Absorption, Distribution, Metabolism, and Excretion
ADI	Acceptable Daily Intake
LOAEL	Lowest Observe Adverse Effect Level
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
POD	Point of Departure
QSAR	Quantitative Structure Activity Relationship
UL	Tolerable Upper Intake Level

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8.3 European Union

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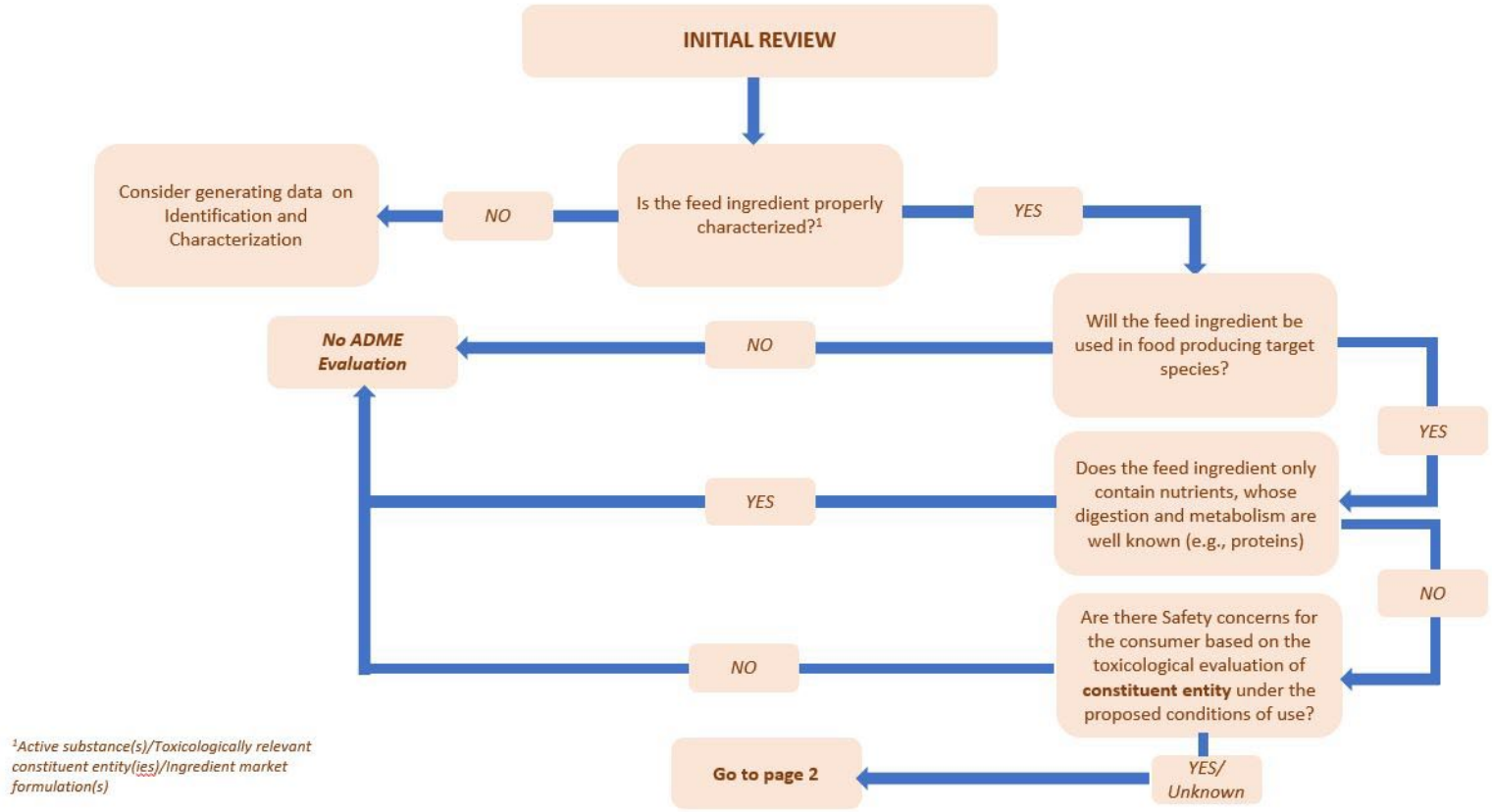
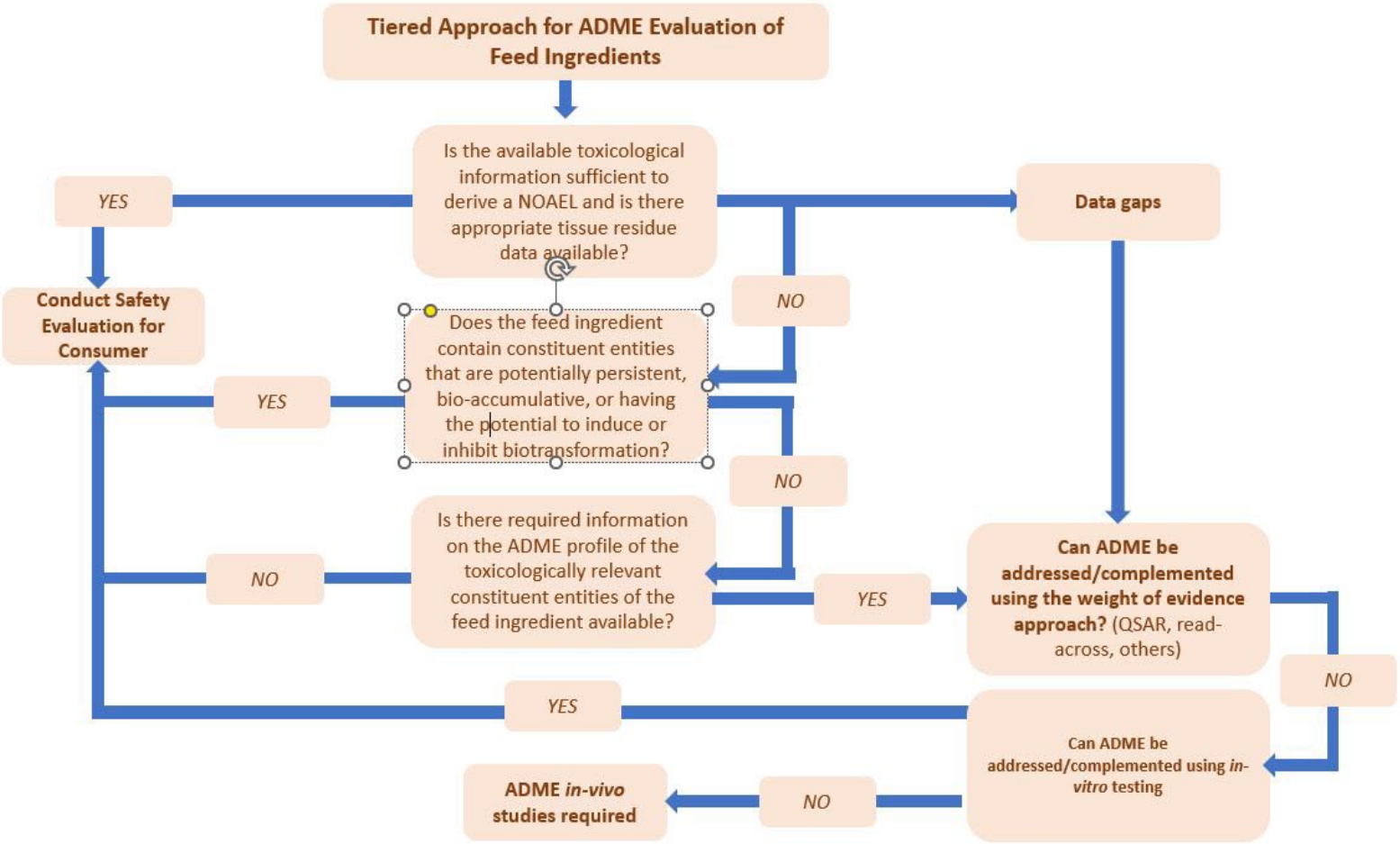
ANNEX I – DECISION TREE




Tiered Approach for ADME Evaluation of Feed Ingredients
Is the available toxicological information sufficient to derive a NOAEL and is there appropriate tissue residue data available?
YES
Data gaps
NO
Conduct Safety Evaluation for Consumer
Does the feed ingredient contain constituent entities that are potentially persistent, bio-accumulative, or having the potential to induce or inhibit biotransformation?
YES
NO
Is there required information on the ADME profile of the toxicologically relevant constituent entities of the feed ingredient available?
NO
YES
Can ADME be addressed/complemented using the weight of evidence approach? (QSAR, read-across, others)
NO
YES
Can ADME be addressed/complemented using in-vitro testing
ADME in-vivo studies required
NO

ANNEX II – *IN SILICO* MODELS

Table 1 provides summary information available at the time of the publication of this guidance document. Further models may be developed and used at the time of the preparation of the submission package.

Table 1 – Freely available databases for toxicological, physical, and chemical, and other relevant information for risk assessment

Database	Website details and further information
AMBIT	http://cefic-lri.org/ambit/ Developed by the European Chemical Industry Council's Long-Range Initiative (cefic-lri). It contains information on > 450.000 chemicals including the European Chemical Agency's (ECHA's) REACH data.
Chemspider	http://www.chemspider.com/ Developed by the Royal Society of Chemistry, it provides information on over 83 million chemicals, using 275 data sources: includes direct links to other relevant sources.
ChemIDplus	https://chem.nlm.nih.gov/chemidplus/ Developed by the US National Library of Medicine contains information relating to > 300.000 chemical structures including physical and-chemical property and toxicity data.
Computational Toxicology Dashboard	https://comptox.epa.gov/dashboard Hosted by the US Environmental Protection Agency (US EPA): a repository of data currently for 875.000 chemicals: links out to additional data sources: integrates data e.g., from ToxCast/Tox21 high-throughput screening initiatives.
eChemPortal	http://www.echemportal.org Developed in collaboration with the Organisation for Economic Cooperation and Development (Organisation for Economic Co-operation and Development (OECD)), provides links to information prepared for governmental chemical reviews at national and international levels, including submissions to the European Chemicals Agency (ECHA): provides exposure and use information.
EMBL-EBI/ChEMBL	https://www.ebi.ac.uk/ https://www.ebi.ac.uk/chemb/ European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI): source of biological and biomolecular data incorporating the ChEMBL database of bioactive molecules with druglike properties (>15 million values from >1.8 million chemicals).

Database	Website details and further information
OCHEM	https://lochem.eu/home/show.da Online chemical database with modelling environment: 2.9 million records for over 600 properties, based on the wiki principle.
QSAR Toolbox	https://www.qsartoolbox.org/ Developed to help fill data gaps in (eco)toxicity data: version 4.4 contains 57 databases with 2.6 million data points for 92.134 chemicals.
PubChem	https://pubchem.ncbi.nlm.nih.gov/ Open chemistry database from US National Institutes of Health (NIH) with data on over 102 million chemicals.
OpenFoodTox 2.0	https://www.efsa.europa.eu/en/data-report/chemical-hazards-database-openfoodtox Openfoodtox is a compilation of chemical and toxicological information on chemicals assessed by EFSA since its creation and included in already published scientific opinions. The database represents the data that was available to EFSA at the time of assessment and does not provide any reassurance on whether any of the chemicals are suitable or not for food applications in Europe. EFSA owns this database and its content.
Nexus Meteor	https://www.lhasalimited.org/solutions/metabolite-identification-and-analysis/

Source: adapted from Madden et al. 2020

PK-SIM: PK-SIM is a publicly available tool (https://www.open-systems-pharmacology.org/). This tool is using mathematical models for studying systems. The model used aggregates and integrates existing knowledge with an aim to systematically analyze systems behavior, test, generate hypothesis and plan experimental next steps, as appropriate. The approach taken by PK-SIM is selected and biased, as it focused on organisms and topics of broader relevance in pharmaceutical research and development, i.e., systems pharmacology. However, facets of the tool can be used beyond systems pharmacology.

TIMES (tissue metabolism simulator) is a heuristic algorithm used to generate plausible metabolic maps from a comprehensive library of biotransformation and abiotic reactions. The ability of TIMES to predict in the same interface the metabolism of chemicals and toxicity resulting from their metabolic activation is an important advantage of the method. The software is available online and requires a license fee (http://oasis-lmc.org/products/software.aspx).

OECD toolbox: The OECD toolbox is a software application intended to be used in filling gaps in (eco)toxicity data needed for assessing the hazards of chemicals. The seminal features of the toolbox are:

- Identification of relevant structural characteristics and potential mechanism or mode of action of a target chemical,
- Identification of other chemicals that have the same structural characteristics and/or mechanisms or mode of action,
- Use of existing experimental data to fill the data gap(s). The toolbox is publicly available. (https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm).

ANNEX III – *IN VITRO* STUDIES

This Annex provides summary information for well-established *in vitro* ADME studies at the time of the publication of this guidance document. Further studies may be developed and used at the time of the preparation of the submission package.

Absorption studies

In vitro digestion models simulate the conditions of the gastrointestinal tract in livestock (ruminants, non-ruminants) by adjusting the ionic strength and pH, as well as addition of enzymes, bile salts, mechanical stresses, and even fermentation reactions to simulate colon (hindgut) conditions. It includes simulated gastric fluid and simulated intestinal fluid studies.

Caco-2 permeability assays for membrane transfer: *In vitro* Caco-2 permeability assays provide a measure of the permeability of a substance across the intestinal barrier and its potential for interactions with transporters:

- The Caco-2 cell line is derived from a human colon carcinoma. The cells have characteristics that resemble intestinal epithelial cells such as the formation of a polarized monolayer, well defined brush border on the apical surface and intercellular junctions.
- These cells differentiate spontaneously after 14-21 days of incubation in a culture medium. The cell monolayer divides the apical and basolateral sides of absorption. With Hank's balanced salt solution as the carrier fluid, the analyte is introduced onto the apical side, and the absorbed moiety is collected on the basolateral side at the desired time intervals. Percent of analyte that is absorbed through the Caco-2 cells is determined by analytical methods.

Distribution studies

Blood/plasma stability: Blood/plasma stability assay measures the stability of molecules in mouse, rat, and human (and/or other species) blood/plasma. The molecule to be evaluated and controls (positive and negative) are incubated with blood/plasma for a defined period and the percent remaining and half-life determined.

Protein binding assays are used to measure distribution in tissue. Binding is evaluated by equilibrium dialysis and ultrafiltration methods, which determine the proportion or percentage of the substance that is bound to proteins and free in solution, as generally only the unbound substance is available for passive diffusion to extravascular or tissue sites. It is therefore an important factor for the efficacy of a substance. Several different binding tests are recognized

and used based on specific requirements, e.g., brain tissue binding, plasma protein binding, whole blood binding, microsomal binding, blood to plasma ratio.

Metabolism studies

Measuring the metabolic profile of a molecule *in vitro* gives an estimate concerning its stability and thus elimination rate by metabolism in the body. The liver is the most important site of metabolism in the body. Therefore, hepatic clearance is a critical parameter for the assessment of the metabolic stability. In *vitro* metabolism systems include primary hepatocytes, liver microsomes; liver slices, S9 sub cellular fraction, cytosol, whole cell lines, recombinant enzymes, or extrahepatic tissues, as described below.

Microsomes and hepatic assays aims to evaluate the metabolic stability of a chemical substance and aims at predicting the pharmacokinetic parameters underpinning the use of the substance. Microsomes are typically used as the enzyme source for the measurement of metabolic stability.

Primary (fresh or cryopreserved) hepatocytes contain functional biochemical pathways typical of the liver. Hence, the primary cultures of hepatocytes carry enzymes and cofactors at physiological concentrations and closely mimic the moiety metabolism *in vivo.*

Liver microsomes are subcellular fractions that are useful to model hepatic clearance *in vitro*. They contain many of the metabolizing enzymes found in the liver.

S9 sub-cellular fraction consists of both microsomal and cytosolic enzymes that help understanding the metabolism of chemical moiety *in vivo*. The system may be supplemented with co-factors such as Uridine Diphosphate Glucuronic Acid and 3'-PhosphoAdenosine-5'-PhosphoSulfate Phase II metabolic pathways, such as N-acetylation, methylation, cysteine and glucuronidation binding.

Cytosol is used to identify the soluble enzymes involved in metabolic pathways of chemical moiety. The assays complement microsomal studies for assessing chemical moiety metabolism pathways.

Other methods such as the liver slices and the whole cell lines may also be used.

ANNEX IV- *IN VIVO* STUDIES

The following guidance documents may be used when designing a ADME *in vivo* study:

- OECD Guidelines for the testing of chemicals. Section 4: Health effects. Test No. 417: Toxicokinetics.
- OECD Guidelines for the testing of chemicals. Section 5: Other Test Guidelines. Test No 503: Metabolism in Livestock.
- OECD Guidelines for the testing of chemicals. Section 5: Other Test Guidelines. Test No 505: Residues in Livestock.
- VICH Guidelines No. 46 Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food producing animals: metabolism study to determine quantity and identify the nature of residues.
- EFSA Guidance Document on the safety of feed additives for the consumers, European Food Safety Authority (EFSA) Journal 2017 :15(10) :5022 www.efsa.europea.eu/efsajournal
- Guidance For Industry #205. Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-producing Animals: Metabolism Study to Determine the Quantity and Identify the Nature of Residues (MRK).

Table 2 – Comparative list of the parameters of in vivo ADME studies mentioned in various guidelines

Parameters	OECD	VICH	EFSA	FDA
Types of animals	Young adult laboratory animals (usually, rats) 6-10 weeks Weight +/- 20%	Target species (representative) - swine - sheep - poultry (laying hens for evaluation egg concentration) - beef or dairy (dairy necessary for evaluation milk concentration)	Target species	Target species (representative) - swine (40-80 kg) - sheep (40-60 kg) - poultry (laying hens for evaluation egg concentration) - beef (250-400 kg) or dairy (dairy necessary for evaluating milk concentration)
Housing	Individual housing	Individual housing	Individual housing	Individual housing
Number of animals	≥ 4 animals per dose (one sex)	≥ 3 animals per euthanasia time (depending on withdrawal period) ≥ 8 cows with different milk production Laying hens to allow for the collection of at least 10 eggs	≥ 3 animals	≥ 3 animals per euthanasia time (depending on withdrawal period) ≥ 8 cows with different milk production Laying hens to allow for the collection of at least 10 eggs

Parameters	OECD	VICH	EFSA	FDA
Dose	Highest dose from toxicology and a fraction of this dose (2 treatments)	Intended maximum dose used (steady state)	Highest proposed dose (single dose)	Intended maximum dose used (steady state)