SCIENTIFIC OPINION

Guidance on the establishment of the residue definition for dietary risk assessment[1]

EFSA Panel on Plant Protection Products and their Residues (PPR)[2, 3]

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ABSTRACT

This guidance is intended to harmonise the process for the establishment of the residue definition for dietary risk assessment by inclusion/exclusion of residues on the basis of their toxicity and the potential for exposure in the diet. The guidance provides a practical instrument including a combination of scientific tools ((Q)SAR, read across, TTC) and criteria for identification of residues for which hazard identification and characterisation is needed, to characterise pesticide metabolites and to define compounds that should be included in the residue definition for dietary risk assessment. It is proposed to make use of all information available, including mechanistic understanding, in order to support the decision process and to enable the risk assessors to provide the risk manager with detailed information on toxicity and exposure of every single metabolite as well as on the uncertainties connected to the proposal. The guidance document is complemented by three practical case studies which are intended to demonstrate the applicability of the proposed decision scheme.

KEY WORDS

Pesticide, residue definition, dietary risk assessment, (Q)SAR, read across, TTC, metabolite

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Plant Protection Products and their Residues (PPR Panel) to prepare Guidance on the establishment of the residue definition to be used for dietary risk assessment.

This guidance should consist of a stepwise method helping the risk assessor, on the basis of factual information (derived from toxicological data, metabolism data) and non-testing methods, by weight of evidence, to:

- conclude for which of the terminal residues[4] of a pesticide on food and feed commodities a hazard identification and characterisation is needed;
- perform such a hazard identification and characterisation
- define the compounds present as terminal residues that should be included in the residue definition for risk assessment.

This guidance document aims at satisfying the needs of modern residue assessments and at harmonising the setting of residue definitions between active substances. It is the intention to guide the assessment per se rather than providing a simple decision scheme. Specifically it is proposed to make use of and apply weighing of all information available, including mechanistic evidence, in order to support an informed and transparent decision process and to enable the risk assessors to provide the risk manager with detailed information on toxicity and exposure of every single metabolite as well as on the uncertainties connected to a proposed residue definition.

The procedure of derivation of the residue definition for dietary risk assessment is a screening exercise, where the relevance of all individual metabolites or groups thereof is thoroughly assessed in a stepwise approach, starting with the compilation of an inventory of metabolites and the assessment of their genotoxicity endpoints (Module 1), followed by the assessment of other regulatory endpoints of toxicity (termed general toxicity; Module 2). For every single metabolite, this screening generates an inventory of toxicity and dietary exposure information for consumers and livestock. The exposure information includes sources and types of exposure, relevance of a particular exposure path and exposure from groundwater used as drinking water All together the hazard and exposure information forms the basis to estimate the contribution of each metabolite to the total toxicological burden for consumers and the final proposal of the residue definition (Module 3). The scenario-specific information is completed by a list of uncertainties identified that were considered in the final proposal and that are deemed relevant for decision making (risk management).

The guidance document is complemented by three practical case studies which are intended to demonstrate the applicability of the proposed decision scheme. Although the guidance document -and the examples- describe EFSA's current thinking on this topic, this has to be viewed as recommendation only. Thus, in the context of this guidance, the word "should" is used for something suggested or recommended rather than required.

[4] Terminal residues: Residues to which humans and livestock will be exposed, i.e. in crops at harvest, or in stored commodities at the time of out-loading, or in commodities upon processing, or in food of animal origin at collection/slaughter, respectively.

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Commission Regulation (EU) No 283/20135 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/20096 of the European Parliament and of the Council concerning the placing of plant protection products on the market, provides that the following elements shall be considered when judging which compounds are to be included in the residue definition for risk assessment:

- The toxicological significance of the compounds;
- The amounts likely to be present.

The general principles for the establishment of the residue definition to be used for dietary risk assessment are covered by the OECD guidance document on the residue definition (OECD, 2009a). The OECD guidance is considering the actual toxicological burden for consumers by inclusion/exclusion of not only the active substance but any residue such as metabolites, degradates, transformation products (herein after referred to only as metabolites) on the basis of their toxicity compared to that of the parent active substance and the potential for exposure in the diet.

In 2008, the Panel on Plant Protection Products and their Residues (PPR) has received a mandate to develop a scientific opinion on the assessment of the toxicological relevance of pesticide metabolites. This mandate also stated that a guidance document on the establishment of residue definition for dietary risk assessment would later be developed by using the opinion as a scientific basis for such a future guidance.

After adoption of the scientific opinion on the evaluation of the toxicological relevance of pesticide metabolites in 2012 (EFSA PPR Panel, 2012), it is now desirable that the PPR Panel prepares guidance on the residue definition for dietary risk assessment. This guidance should be a practical instrument, aimed at helping risk assessors to adopt such definitions based on a combination of scientific tools as described in the opinion. The guidance should also be used for identifying cases where further toxicological data are needed to characterise pesticide metabolites.

A public consultation of stakeholders on a draft of the guidance will be launched before finalising the guidance.

Any relevant opinions and guidance documents elaborated by the Scientific Committee of EFSA will be duly considered, as will on going work on mixture toxicity and cumulative risk assessments. Along the steps of progress in developing the guidance, the suggested approach will be validated using data on previously evaluated compounds. In case needed, the European Commission and Member States will be consulted on particular risk management elements contained in the guidance.

[5] Commission Regulation (EU) No 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.

[6] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EC and 91/414/EEC. Official Journal L 309, 1-50. 24 November 2009

TERMS OF REFERENCE AS PROVIDED BY EFSA

In application of Article 29 1(b) of Regulation (EC) No 178/2002[7], the Panel on Plant Protection Products and their Residues (PPR Panel) is requested to prepare Guidance on the establishment of the residue definition to be used for dietary risk assessment.

This guidance should consist of a stepwise method helping the risk assessor, on the basis of factual information (derived from toxicological data, metabolism data) and non-testing methods, by weight of evidence, to:

- conclude for which of the terminal residues[8] of a pesticide on food and feed commodities a hazard identification and characterisation is needed;
- perform such a hazard identification and characterisation
- define the compounds present as terminal residues that should be included in the residue definition for risk assessment.

In carrying out this mandate, the panel should consider that the components of the terminal residues of pesticide active substances will have been duly identified following the requirements of Commission Regulation (EU) No 283/2013 and in the context of this Regulation referred OECD test guidelines. As provided under point 1.11 of the introduction of the annex of the aforementioned Regulation, this includes information on the possible metabolic conversion of isomers for active substances consisting in a mixture of isomers.

A case study should be included in an appendix to the guidance document to demonstrate the practical application of the developed methodology.

ASSESSMENT

1. Introduction

This guidance document applies to chemical active substances ("pesticides") and their residues as defined in Regulation EC (No) 1107/2009.

The need for new guidance on how to establish the residue definition for risk assessment of pesticides has arisen as current regulatory requirements in this regards are not completely and explicitly addressed in available guidance documents like EC, 1997; FAO, 2009 or OECD, 2009a. In particular, Regulation (EC) 283/2013 states that "the risk assessment has to take into account the residue definition established for risk assessment", which requires considerations on the relevance of metabolites for the consumer risk assessment as to whether or not they can cause potential risks to the consumer. This, in turn, means that for all compounds not included in the residue definition a justification for their non-inclusion should be made.

In addition, the implications of recent scientific developments in the regulatory area, such as the issue of mixture toxicity and the agreement to apply the dose addition concept for compounds that produce common adverse outcomes on the same target organ / system (phenomenological effect (EFSA PPR Panel, 2013, 2014)) or the relevance of potential non-thresholded effects, are not discussed in the framework of setting the residue definition for risk assessment in the above mentioned guidance documents. Available guidance does also not consider the application of tools such as the TTC approach, (Q)SAR and read across (EFSA PPR Panel, 2012). Furthermore, improved analytical performance and the development of new analytical methods have led to an increase in number of

[7] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 01.02.2002, p.1-24.

[8] Terminal residues: Residues to which humans and livestock will be exposed, i.e. in crops at harvest, or in stored commodities at the time of out-loading, or in commodities upon processing, or in food of animal origin at collection/slaughter, respectively.

identified metabolites, including isomers, and thereby an increased demand for addressing their potential concern for consumers. This guidance document therefore aims at satisfying the needs of modern residue assessments and at harmonising the setting of residue definitions between the active substances. It had been the intention to guide the assessment per se rather than providing a simple decision scheme. Specifically it is proposed to make use of and apply careful weighing to all information available, including mechanistic understanding, in order to support a highly informed decision process and to enable the risk assessors to provide the risk manager with detailed information on toxicity and exposure of every single metabolite as well as on the uncertainties connected to the proposal.

The fate of pesticides after application onto the crop or soil may be affected by numerous biophysicochemical degradation processes resulting in a change of the chemical entity of the pesticide and occurrence of a mixture of compounds in harvestable commodities and the environment – parent substance, metabolites and degradates (in the following termed "metabolites"). The residue pattern in food and feed items is modulated by a set of different criteria like the substance properties, application scheme, crop, cultivation practices, harvesting or environmental factors, resulting in a divergent composition of residues over time and in different commodities of the harvested crop. For many pesticides, the soil acts as a sink and source for residues and the transfer between soil sphere and plants may play an important role in the formation of the residue profile in crops.

Metabolism studies are in general performed with the radiolabelled parent compound. Such studies in plants and livestock as well as studies simulating food processing practices, aim at identifying the nature and, to a certain extent, the quantity of individual residue compounds in commodities at stages of intermediate and commercial harvest, and in by-products. Metabolism studies form the basis for the proposal of the residue definitions while field studies with the non-radiolabelled active substance support quantitative metabolite assessments.

The residue definition for risk assessment is used by risk assessors to evaluate the potential risk of dietary intake of pesticide residues resulting from the application of a pesticide. The residue definition should consider all compounds that are of toxicological significance for human and livestock, taking into account the amounts likely to be present in food and feed. It is therefore necessary to consider aspects of both, toxicity and dietary exposure to residues and to account for the use specific residue pattern in food commodities of plant origin as well as in animal commodities that result from livestock exposure via feed.

The approach chosen in this guidance document recommends the combined use of relative exposures (in percentage of the total residues) and absolute exposures (in mg/kg bw/d) where necessary for a decision. In the context of this guidance it is possible to apply TTC triggers. However, potential simultaneous dietary exposure to multiple metabolites should be taken account of, and the possibility that all or part of the metabolites will cause the same adverse outcome should be considered. In such case, dose addition should be used, in consistency with earlier Scientific Opinions of the PPR Panel (EFSA PPR Panel, 2012, 2013, 2014), and consequently exposure should be calculated as the sum of the single metabolites. Where an exposure assessment is performed in the framework of setting the residue definition, the variety of potential exposure situations has to be considered by setting up a reasonable worst case scenario that takes into account the complexity of the temporal and spatial changes that can occur with the residues. The necessary robustness of a residue definition against future regulatory changes (e.g. extension of authorisations and increase of exposure) therefore depends on the completeness of the underlying data set in terms of the uses intended. It should be noted that a proposal of the residue definition for risk assessment made under a premise not reflecting critical conditions, even in the same crop category, does not necessarily apply to any other situation by default.

The procedure of derivation of the residue definition for dietary risk assessment is a screening exercise, where the relevance of all individual metabolites or groups thereof is thoroughly assessed in a stepwise approach, starting with the compilation of an inventory of metabolites and the assessment

of genotoxicity endpoints (Module 1),continuing with other regulatory endpoints of toxicity (termed general toxicity; Module 2). For every single metabolite, this screening generates an inventory of toxicity and dietary exposure information for consumers and livestock including sources and types of exposure, relevance of a particular exposure path and exposure from groundwater used as drinking water. This information forms the basis for the final proposal of the residue definition (Module 3). The scenario-specific information is completed by a list of uncertainties identified that were considered in the final proposal and that are deemed relevant for decision making (risk management).

Where exposure assessments are used to waive further data requirements, e.g. when applying the TTC approach or when major metabolites are excluded in the dietary exposure screening due to low absolute residue values, the boundary conditions of these estimations are transparently described and should be considered in future assessments.

The guidance document is complemented by three practical case studies which are intended to demonstrate the applicability of the proposed decision scheme. Although the guidance document -and the examples- describe EFSA's current thinking on this topic, this has to be viewed as recommendation only. Thus, in the context of this guidance, the word "should" is used for something suggested or recommended rather than required.

2. Module 1: Exclusion of genotoxicity (steps 1-9)

For all metabolites the genotoxic potential has to be assessed (Module 1, Fig. 1). The genotoxicity assessment should start with identification of the metabolites at any level in nature-of-residue studies (i.e. primary and rotational crops, livestock, fish, food processing). The assessment continues with the exclusion of metabolites of no appreciable concern, e.g. sugar or lignin (step 2 of the decision scheme). In step 3, screening for genotoxic compounds classified according to Regulation (EC) 1272/2008[9] should be done (see point 2.5). If no concern is identified, proceed with step 4.

[9] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 (Text with EEA relevance) OJ L 353, 31.12.2008, p. 1–1355 (BG, ES, CS, DA, DE, ET, EL, EN, FR, GA, IT, LV, LT, HU, MT, NL, PL, PT, RO, SK, SL, FI, SV) Special edition in Croatian: Chapter 13 Volume 020 P. 3 - 1357

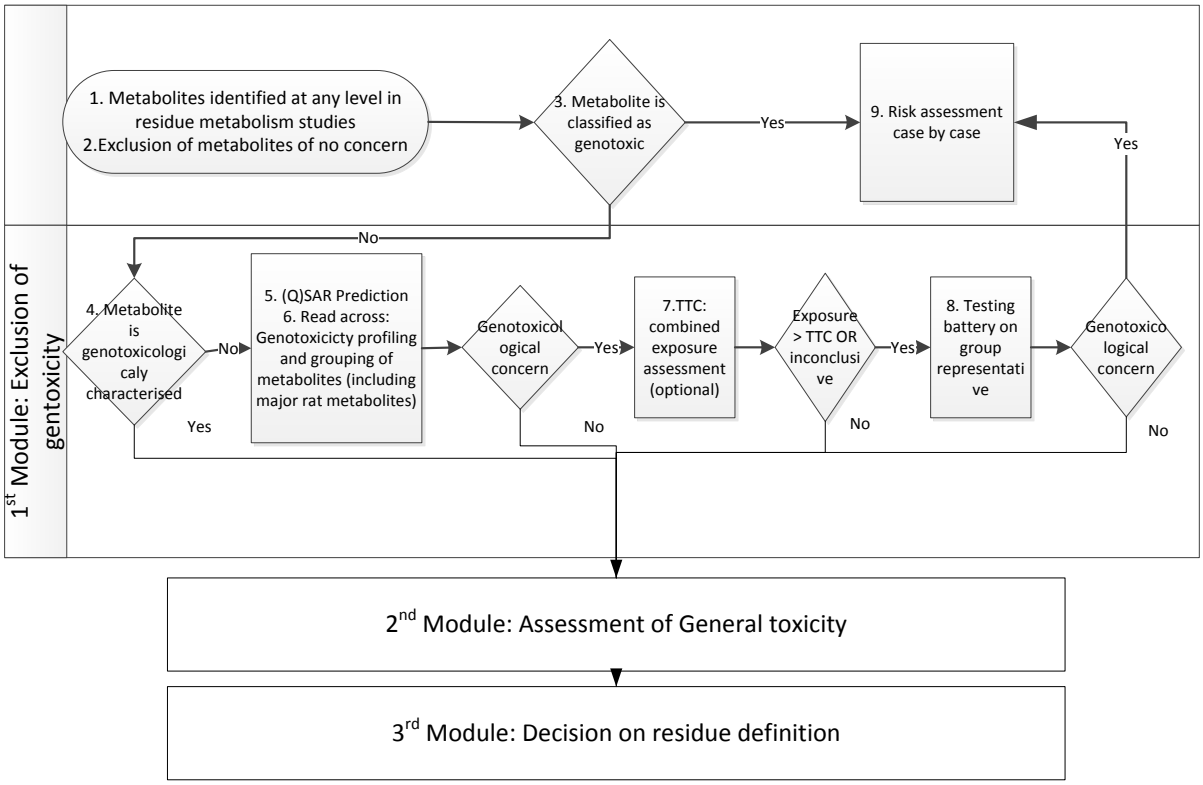


Figure 1: Module 1 exclusion of genotoxicity

2.1. Identification of metabolites characterised by the toxicological studies conducted with the parent compound (step 4)

Where no specific studies are available, the metabolites to have been studied in the toxicological studies conducted with the parent (i.e. active substance) are those contributing to 10% or more (as individual metabolite) of the administered dose in terms of total radioactive material recovered in the urine as detected in ADME studies. The study design and the dose selection of the ADME study should allow for a comparison with the general toxicity studies conducted with the parent.

As a general rule metabolites quantification would be based on the amount of metabolite considering the lowest available dose and the animal sex showing the lowest excreted amount from a repeat dose ADME study. ADME studies conducted in rat by repeated administration at doses similar to the one applied in the general toxicity studies should preferentially be used for the hazard characterisation of the metabolites. In case a different study design has been applied, e.g. single dose administration or doses much higher than those used in the general toxicity studies their use for the hazard characterisation of the metabolites should be justified.

For the metabolites considered to be evaluated by the toxicological studies conducted with the parent compound, the conclusions about the genotoxicity properties of the parent will apply to these metabolites as well and no further testing/data would be required (step 4 of the decision scheme). In addition if a metabolite is not characterized by the toxicological studies conducted with the parent, but found to be common to another active substance and covered by the toxicological properties of this active substance, the conclusion about the genotoxicity properties of this active substance can be used to characterize the metabolite.

However, in some cases a different approach can be taken:

i) When dealing with mixtures of isomers, the 10% value should be considered as the sum of the individual isomers (EFSA PPR Panel, 2012).

ii) A special case should be also considered for conjugated metabolites. Glucoside and glucuronide conjugates will be evaluated in terms of their aglycon moiety; all the remaining conjugated metabolites (e.g. sulfate, amino acid) will be assessed case-by-case.

iii) For poorly or limited absorbed active substances, the 10% of total radioactive material recovered in the urine from the ADME study can be referred to the absorbed dose rather than to the administered dose. In the absence of an agreed definition of poorly or limited absorbed substances a threshold of 80% or less in terms of calculated absorption or bioavailability is considered as indicative for limited absorbed substances, though a case-by-case consideration can be applied e.g. for potent active substances, see point 3.3.

iv) If a metabolite or degradate occurs ≤10% of the absorbed dose in rat urine from the ADME study, expert judgement may still conclude that the hazard has been characterised by testing with the parent; though criteria for such conclusion should be provided (e.g. the metabolite only differs from the parent by simple structural changes that are not expected to cause additional hazard).

2.2. Application of (Q)SAR and read across for the exclusion of genotoxicity (steps 5-6)

The genotoxicity assessment should be assisted by application of (Q)SAR (step 5 of the decision scheme) and read across of metabolites (step 6 of the decision scheme) and by considerations on exposure (step 7 of the decision scheme) against the threshold of toxicological concern (TTC) for genotoxicity (0.0025 µg/kg bw/day). For substances grouped according to their predicted effect it is considered appropriate to apply the dose addition approach, as would be done for compounds included in the same residue definition for dietary risk assessment (EFSA Scientific Committee, 2012; EFSA PPR Panel, 2013, 2014).

Step 5 of the decision scheme includes the use of scientifically valid (Q)SAR models (see 2.2.1). The use of computational models for predictions of genotoxicity should not be based on the use of any single model alone, but on a "weight of evidence" approach including all available information provided by the models (e.g. applicability domain, proposed mechanistic information, prediction for the similar substance). To maximise the sensitivity and specificity of the prediction, at least two independent (Q)SAR models, where possible, (e.g based on different training sets and/or algorithms) should be applied for each genotoxicity endpoint, including both knowledge based and statistical based models (Worth et al., 2010, 2011a).

To address the possibility of false negative and false positive (Q)SAR predictions, grouping and read across is proposed (Worth et al., 2011a,b, 2013) (step 6). Structural and functional similarity, grouping criteria and selection of representative metabolite(s) for potential testing have to be substantiated by appropriate and relevant information. For guidance on grouping and profiling see OECD (2014).

2.2.1. Quality criteria for the application of (Q)SAR analysis for genotoxicity assessment

A framework for assessing (Q)SAR applicability builds on guidance already adopted for the REACH regulation (ECHA, 2008), including international (OECD) guidance on the scientific validation and documentation of (Q)SAR models for regulatory purposes (OECD, 2007a).

In order a (Q)SAR prediction to be adequate for the assessment purpose i.e. genotoxicity assessment, the following conditions should be fulfilled (Gleeson et al., 2012):

i) The prediction should be generated by a scientifically valid (i.e. relevant and reliable) model;

ii) The model should be applicable to the chemical of interest with the necessary level of reliability;

iii) The model endpoint should be relevant for the purpose (i.e. genotoxicity assessment);

iv) The information should be well documented.

2.2.1.1. Scientific validity of the model

The first condition for using the (Q)SAR for regulatory purpose is the demonstration of the model validity. A set of five validation principles has been established by the OECD (OECD, 2007a) to guide regulatory agencies in the evaluation of the performance of (Q)SAR. According to them the model should be associated with:

i) A defined endpoint;

ii) An unambiguous algorithm;

iii) A defined domain of applicability;

iv) Appropriate measures of goodness-of-fit, robustness and predictivity;

v) A mechanistic interpretation, where possible.

Information which covers the above listed five principles should be available to the assessor as a part of the relevant documentation of the prediction. Information for some of the models may be available from the JRC QSAR model database http://qsardb.jrc.ec.europa.eu/qmrf/index.jsp.

2.2.1.2. Applicability domain

The concept of applicability domain was introduced to assess the probability of a chemical of interest being covered by the chemical space of the (Q)SAR model. When the substance to be predicted is within its applicability domain, the model is generally considered to give reliable results. If a substance is outside the applicability domain of the model, the reliability of the prediction is uncertain. In this case, the prediction itself can be only used as a part of the overall weight of evidence or as supporting information, though a positive prediction will be considered as alerting structure and deviations should be justified.

For statistically based and hybrid models (e.g. CAESAR), the training set is used to develop the applicability domain of the model.

For knowledge based models (e.g. DEREK), where no training set is available, the applicability domain cannot be defined as described above. However, knowledge based models usually provide multiple supporting information e.g. suggested mode of action, examples, references, that can be used to evaluate the reliability and adequacy of the prediction.

Some software tools do not give any information on the applicability domain for the chemical of interest. In this case, since the concept of the applicability domain is related to the reliability of the prediction, model predictions for similar substances with known experimental data can be used as an alternative. These analogues may be selected from the training set of the model (if available) and/ or from additional data sets. The selection of analogues and the consequent prediction and analyses of the results may be provided by the software used, or can, as an alternative, be done by the applicant.

Information on the applicability domain (reliability of the prediction) should be provided where applicable as a part of the documentation of the prediction.

Description, experimental data and predictions of the substances considered analogues of the chemical of interest (provided by the software or selected by the applicant) should be provided as part of the

supporting documentation for the prediction. If the information is not provided by the software itself, criteria for the selection of analogues should be provided by the applicant.

2.2.1.3. Relevant endpoints for genotoxicity

In the context of this guidance, (Q)SAR should be used as a scientific tool for the genotoxicity assessment of residues of pesticide active substances. The genotoxicity endpoints explored and assessed through the application of (Q)SAR should be described and the information provided to the assessor. Any additional information provided by the model e.g. suggested mechanism of action, uncertainties, should be included in the supporting documentation. The relevant genotoxicity endpoints that have to be explored are gene mutations, and structural and numerical chromosomal alterations.

2.2.1.4. Documentation

The following should be provided to support the quality of the prediction

i) Used model (title, name of authors, reference);

ii) Information about modelled endpoint (endpoint, experimental protocol);

iii) Used training set (number of the substances, information about the chemical diversity of the training set chemicals);

iv) Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection);

v) Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity (including specificity and sensitivity);

vi) External statistic, if available;

vii) Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain);

*viii)*Mechanistic interpretation of the model;

ix) Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant);

x) Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties;

Information mentioned in the points 1 to 8 can be substituted by referencing to the JRC QSAR model reporting format database (QMRF), if the model is included in the database. However, irrespective from the source of information, applicants should evaluate the validity of the model used (in relation to the application) as well as the adequacy of the individual model prediction.

2.2.1.5. Conclusions from the completed (Q)SAR predictions

As a final step, a conclusion on the (Q)SAR prediction should be done as a part of the assessment. The conclusive step includes analysis of the prediction and its reliability.

2.2.2. Quality criteria for the application of “read across” analysis for genotoxicity assessment

A framework for assessing “read across” applicability for genotoxicity assessment builds on guidance already adopted for the REACH regulation (ECHA, 2008), including the updated OECD guidance on grouping of chemicals (OECD, 2014) and ECHA Read across Assessment Framework (ECHA, 2015).

The term “read across” indicates an approach making use of endpoint information i.e. experimental data on genotoxicity for a chemical(s) (source chemical(s)), to make a prediction for the same

endpoint for a different chemical(s) (target chemical(s)). The source and target chemical(s) are considered to provoke similar effects related to the assessed endpoints, usually based on structural similarity, and therefore assumed to exhibit similar biological activity (OECD, 2007b).

The approach proposed in this guidance is an implementation of the read across based on analogues since it will be used for the analysis of a group generally composed of a limited number of substances. The simplest case will be consistent with the use of the experimental data on genotoxicity generated for the active substance (source chemical) for prediction of the genotoxic potential of its metabolite(s) (target chemical(s)). However, any other available experimental information, e.g. experimental data for other compounds, could be used and considered acceptable for performing the read across as long as they fulfil and comply with the relevant OECD guidelines.

Read across must be, in all cases, scientifically justified and thoroughly documented.

In accordance to ECHA (ECHA, 2008), the main steps for the read across adapted for genotoxicity assessment of metabolites of pesticide active substances are:

i) Define the endpoint(s) that is/are going to be evaluated by read across

The endpoint(s) that is/are going to be evaluated by read across should be clearly defined, e.g. in vitro Ames mutagenicity, with/without S9. This is critical in order to demonstrate the regulatory relevance of the selected endpoint, to justify the use of the read across working hypothesis and to assess the similarity between the analogues which are considered endpoint related.

ii) Make a clear working hypothesis and justification for the read across

It is recommended that the read across working hypothesis would be based on the molecular initiating events (knowledge on how the chemical is expected to interact with the biological system), e.g. covalent binding with DNA. This would facilitate the definition of similarity and would provide mechanistic evidence, enhancing the confidence in the read across prediction (Patlewicz, 2013).

Molecular initiating events of relevance for the genotoxicity assessment are well known and the chemical properties important for the interaction with the DNA and/or proteins have been encoded into structural alerts (Ashby and Tennant, 1988 and 1991; Bailey et al, 2005; Kazius et al 2005; Serafimova et al, 2007; Benigni et al 2008; Enoch and Cronin, 2012).

Some of these lists (called primary profilers) are included into OECD QSAR Toolbox (http://www.qsartoolbox.org/) and could be used for grouping. Primary profilers are mechanistic or endpoint specific. Mechanistic primary profilers contain structural alerts that have been developed around the chemistry related to a specific molecular initiating event (e.g. DNA binding by OASIS v1.2, DNA binding by OECD Toolbox). The structural alerts within this type of profiler are not necessarily supported by toxicological data. Endpoint specific primary profilers contain structural alerts that have been identified from the analysis of toxicological data (e.g. DNA alerts from the Ames, Micronucleus and Chromosome Assay tests by OASIS v 1.2, in vitro mutagenicity test (Ames test) alerts by ISS).

Mechanistic and endpoint specific primary profilers should be applied in a complementary way to the active substance and metabolites. The ideal profiling case will be when one (or both) of the mechanistic profilers identifies a single mechanism related to the predicted endpoint that is supported by appropriate endpoint specific profiler(s); in such a case the theoretically derived structural alert(s) is/are confirmed by the experimental data.

Based on the results of the profiling a specific group should be formed. The first intention is to use the active substance and (if available) its metabolites that have been tested for genotoxicity as source chemical(s). Therefore, all metabolites sharing the same alert(s) as the active substance or tested

metabolites, for the predicted endpoint, or lack of alert(s), should be placed in the same group. The remaining metabolites should be grouped depending on the resulting profile, e.g. to form a group including all metabolites sharing the same alert, but not present in the active substance. It is worth to note that read across can only be accepted in the framework of the current data requirements.

Provide information on substance identity for all the substances included in the read across

Detailed information on composition, including substance identity and purity, should be provided for all substances (source and target (s) chemicals) included in the chemical groups formed and used for read across. Information should be detailed enough to allow the assessor to unambiguously identify the substances and to assess the structural similarity on which the read across hypothesis is based. Lack of adequate information on structure and impurities could undermine the read across.

iii) Outline the structural similarity(ies) between substances

The structural similarity of the target and the source substances needs to be assessed. The impact of the structural differences between substances for the endpoint(s) under consideration also needs to be assessed.

The analysis on structural similarity should consider all the appropriate elements, namely:

- Presence of structural alerts;
- Presence, relevance and number of common functional groups;
- Presence and relevance of non-common functional groups;
- Similarity of the 'core structure' apart from the (non-)common functional groups;
- Potential differences due to differences in reactivity, metabolism and mode of action;
- Potential differences due to steric hindrance;

Secondary profilers (i.e. organic functional groups) in the OECD QSAR Toolbox could be used in this analysis. However, it should be mentioned that the software could only help in the first part of the analysis, in particular to identify common and non-common functional groups present in source and target chemical(s). The relevance of the similarities and dissimilarities identified for making use of the read across to evaluate the endpoint considered in the analysis should be discussed.

iv) Conclusions from the completed read across investigations

As a final step, a conclusion on the applicability of the read across should be done as a part of the assessment. The conclusive step includes a scientific justification on the applicability of the read across resulting in the following possibilities:

- A group of metabolites is proven to be similar to the source substance (i.e. the active substance or a compound tested for genotoxicity), if the existing experimental data allows concluding on a lack of genotoxicity concern for the source substance, then no genotoxicity concern would exist for the substances included in that group.
- A group of metabolites is proven to be dissimilar to the source substance, if e.g. a new structural alert has been identified and considered of genotoxicity concern. In this case, genotoxicity cannot be excluded and the substance will move to step 7 of the decision scheme.

In performing read across, a case should be made when positive in-vitro micronucleus test and negative in-vivo micronucleus test exist for the same substance. In this case, before discharging the positive concern by making use of the in-vivo micronucleus test, evidence of bone marrow exposure has to be proven.

v) Documentation

The documentation provided must be sufficient to allow an independent assessment of the adequacy and the scientific validity of the read across approach. The following elements are considered essential to adequately document a read across approach (adapted from ECHA 2008, OECD 2014, ECHA, 2013):

- Description of the endpoint(s) that is/are to be read across;
- A read across hypothesis;
- A justification for the read across hypothesis;
- A list of all the substances included in the approach with their detailed substance identity information;
- An analysis of the similarity/dissimilarity
- A conclusion on the applicability of the proposed read across approach.

2.2.3 Conclusion

A final conclusion on the genotoxic potential should be made for all metabolites based on the information of (Q)SAR predictions and read-across. In case of diverging results between QSAR predictions and read-across analysis, justification for the decision has to be provided (see case studies).

2.3. TTC assessment for evaluation of genotoxicity (step 7)

The Threshold of Toxicological Concern (TTC) approach is a method that can be applied to evaluate the toxicological relevance of metabolites (EFSA PPR Panel, 2012) when chemical-specific data are not available. The assessment is based on the known chemical structure of the substance and the estimated exposure.

In the context of this guidance, the TTC approach is not intended to supersede the evaluation of available toxicological data; including those cases where structural analogues can be assessed based on the toxicological data from the tested compounds i.e. parent and/or metabolites. For the genotoxicity assessment, the TTC approach is a subordinate screening tool to (Q)SAR and read across, where human exposure is estimated to be very low.

In addition, in case of dietary exposure to co-occurring pesticide metabolites, the application of the TTC should assume dose addition.

For the genotoxicity endpoints it is proposed that metabolites showing commonality in reaction mechanisms i.e. the same specific genotoxicity endpoint (i.e. point mutation or structural and numerical chromosome aberration) to be grouped and optionally assessed against the TTC value of 0.0025 µg/kg bw/day as a combined exposure (see chapter 5), or directly to be subjected to genotoxicity testing (step 8 of the decision scheme).

Substances considered to be of genotoxic concern following (Q)SAR prediction and read across, and exceeding the cumulative exposure of 0.0025 µg/kg bw/day will go to step 8 of the decision scheme to be tested.

2.4. Testing battery for assessment of genotoxicity (step 8)

After profiling and grouping of metabolites (if necessary), in vitro tests on at least one representative metabolite per group should be performed (step 8). The selection of the representative metabolite can be based on multiple aspects e.g. relevant exposure or technical factors, and should be justified. For one or more metabolites identified to be tested for their genotoxic potential, the testing battery should include as a minimum two in vitro tests, covering all three genetic endpoints, i.e. gene mutations, structural and numerical chromosomal alterations (EFSA Scientific Committee 2011; Kirkland et al., 2014a, b). The need for in vivo follow up testing should be considered on a case by case, through the evaluation of the spectrum of genotoxic events observed in vitro (if any), the data on toxicokinetics, on bioavailability and on the potential target organ. Applicants and assessors should refer to the Scientific

Opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011) for selection of the most appropriate assays and results interpretation.

Individual metabolites or group representatives that are negative in the genotoxicity testing battery will be considered of no genotoxicity concern and will go to the next step of the assessment decision tree (Module 2). If testing is conducted on one or more group representatives the negative outcome of the study will be applied to the full group. Metabolites or group representatives resulting positive in a test battery will be considered of genotoxicity concern. If testing is conducted on a group representative, the positive outcome of the study will be applied to the full group.

2.5. Genotoxicity concern (Step 9)

For all compounds identified as of genotoxic concern under steps 3 and all metabolites for which genotoxic properties cannot be excluded after testing and read-across (positive in step 4 or 8), a case-by-case assessment is required. These metabolites are not suitable candidates to be carried further through the process for inclusion into the residue definition in the remit of this guidance document. Instead, risk assessors and risk managers need to take further actions to exclude any unacceptable risk for consumers (e.g. in depth-assessment of exposure, proposal of mitigation measures, management decision on acceptability of known genotoxicants in regulated products).

3. Module 2: General Toxicity Assessment (steps 10 – 19)

In this guidance, the general acute and chronic toxicity assessment of the metabolite of interest is understood to enable a quantitative and qualitative comparison of the toxicity profile(s) of the metabolite(s) with the parent substance and to identify any specific hazard for the metabolite in order to derive respective health based limits for human exposure to the relevant metabolite(s), when appropriate.

The assessment scheme is proposing the combined use of the TTC approach, occurrence level of metabolites, elements of grouping and read across, and testing.

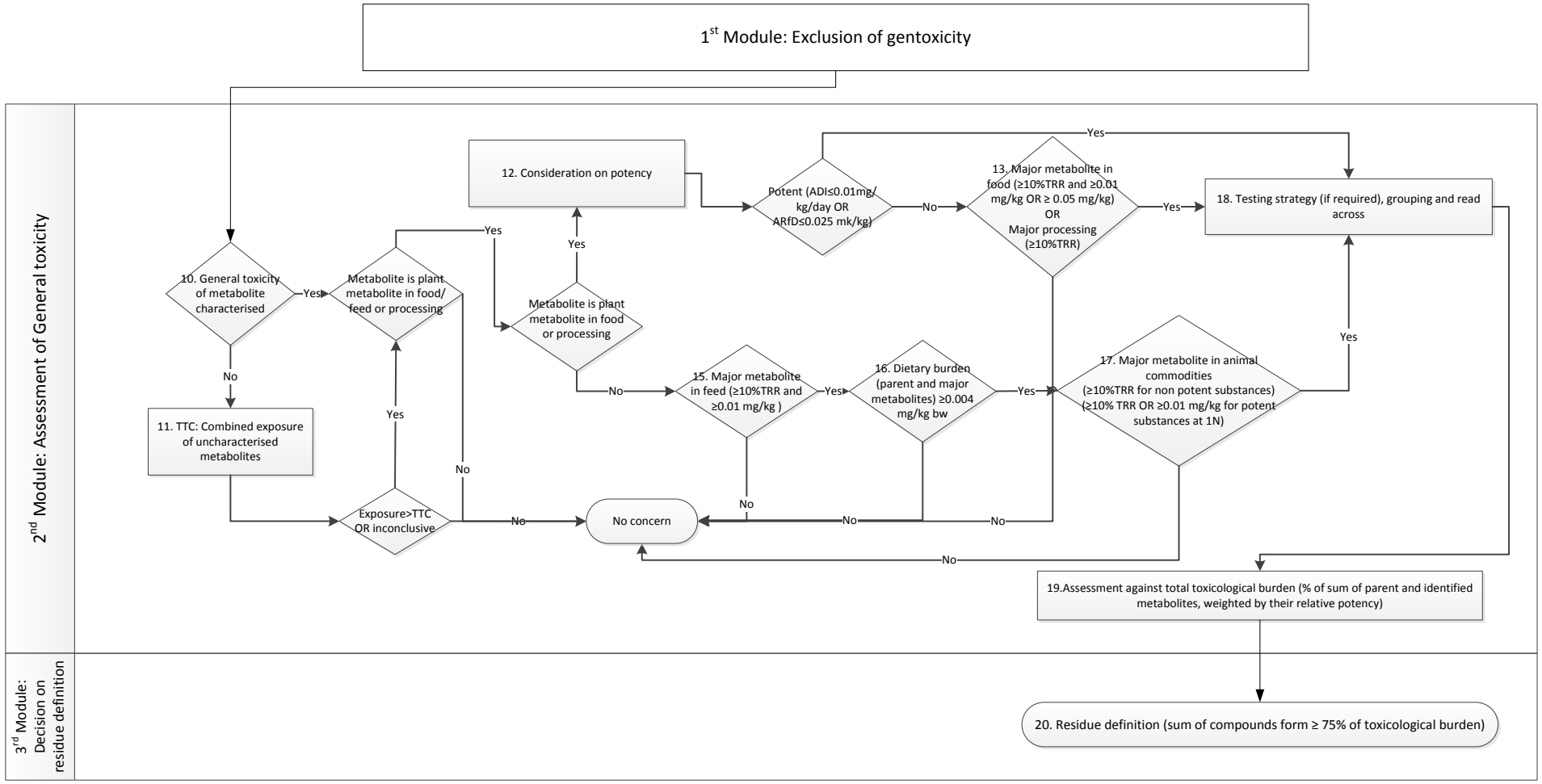


Figure 2: Assessment scheme for general toxicity and decision on residue definition

3.1. Identification of metabolites characterised by the toxicological studies conducted with the parent (step 10)

In line with the criteria described in chapter 2.1, no further toxicological testing will be necessary, if the metabolite is considered quantitatively covered by the mammalian metabolism studies (ADME studies; step 10). If the criteria described in the chapter 2.1 are met, the general toxicity assessment of the metabolite would be considered characterised by the studies conducted with the active substance and the reference values of parent compound apply.

3.2. TTC assessment for general toxicity (step 11)

Following the specific criteria described in chapter 2.3, the combined exposure of all metabolites not covered by the ADME study conducted in mammalian species or by specific studies, can be summed up and compared to the specific TTC value as an optional assessment step (step 11).

For the general toxicity assessment (see Module 2), the exposure is intended as a combined exposure of all identified, but toxicologically non-characterised metabolites (see step 11 of the decision scheme). The TTC approach in this module should be seen as a screening tool, which is optional and restricted to cases where the exposure can be reliably estimated and is not subject to large uncertainties due to foreseeable extensions of authorisations, or limited knowledge about the identity and/or magnitude of residues e.g. in case of transfer and metabolism of feed metabolites in livestock.

In order to apply the TTC in a cumulative way, the ratio between the exposure of each metabolite and the corresponding Cramer Class TTC will be summed up. If the sum is ≥ 1, specific hazard and/or comparative risk assessment will be conducted. If the resulting sum is ≤ 1, no further assessment is necessary.

The thresholds of 0.3 µg/kg bw/d (for organophosphate and carbamate with anti-cholinesterase activity) or 1.5 µg/kg bw/d (Cramer Class III and Cramer Class II) and 30 µg/kg bw/d (Cramer Class I) should be used (EFSA Scientific Committee, 2012).

Besides the standard chronic exposure assessment, an acute TTC assessment can be similarly conducted, where necessary. In line with EFSA Scientific Opinion (EFSA PPR Panel, 2012) ad hoc acute TTC values derived from short term exposure pesticide NOAELs can be adopted: 0.3 µg/kg body weight/day for substances with neurotoxicity alert and 5 µg/kg body weight/day for substances allocated to Cramer class II and III. The same TTC values as for chronic exposure is adopted for substances allocated in the Cramer class I (30 µg/kg body weight/day).

3.3. Potency considerations for metabolites (step 12)

In the context of this guidance, potency is defined by the ADI or the ARfD of the parent substance or the metabolite(s), if respective data exist. General criteria for definition of low ADIs and ARfDs were derived from the evaluation of the distribution of ADIs and ARfDs from a pesticide database comprising 270 and 195 active substances, respectively (see Appendix A). A conservative assumption was made by considering that all the substances included in the lowest 25th percentile of the distribution of ADIs or ARfDs were considered of potential concern, and it is expected that most of the neurotoxic substances will be included in this range. It was concluded by extrapolation of the corresponding ADI or ARfD values that active substances with an ADI <0.01 mg/kg/body weight per day, or an ARfD <0.025 mg/kg/body weight, as appropriate, should be considered "potent". In this case the values for orientation to categorise significant and insignificant residues (see chapter 3.4) should **not** be applied for the exclusion, by default, of any metabolite as of "no concern".

If a metabolite is more potent than the parent on the basis of data (dossier, data base on toxicology according to Annex I of OECD 2009a) and/or additional information (e.g. public literature) the relative potency is addressed by the application of a relative potency factor (RPF). This might need to

be reflected in the characterisation of a metabolite as being potent or not according to the criteria for the definition of potency.

Additional complementary elements can be considered at this point when estimating the relevance of metabolites to be included in further assessment (OECD, 2009a), like:

- number and level of identified minor metabolites
- uses considered for the active substance
- the metabolite is common to other active substances and already characterised
- quantitative relevance of the metabolite in the mammalian metabolism study (see chapter 2.1)

If the overall assessment would be inconclusive, and a safety concern cannot be dismissed, then the metabolites should be further assessed to define their toxicological relevance.

3.4. Toxicological assessment of plant metabolites in food and feed (steps 13-15)

Further assessment should be performed for major metabolites contributing at any point in time in the residue metabolism studies to ≥10% of the TRR and ≥0.01 mg/kg in food and in feed commodities, or (if <10%TRR) to ≥ 0.05 mg/kg in food commodities (whichever set of conditions is met). For nature of processing studies, 10% TRR applies as sole trigger for relevance. These thresholds are arbitrary and should be considered only as indicative for a metabolite having a potential for exposure that could significantly contribute to the dietary risk. Metabolites below 10% of the TRR in food and feed and less than 0.05 mg/kg in food (minor metabolites), or above 10% of TRR but <0.01 mg/kg (non-relevant major metabolites), are generally considered as unlikely to contribute significantly to the dietary risk, unless they are presumed as "potent" based on considerations described in 3.3.

If the conditions described in chapter 2.1 are not met, then additional testing should be considered (step 18) for all relevant major and potent minor plant metabolites in food. The testing strategy should take into account the toxicological profile of the parent and the possibility to explore specific hazards.

Toxicological testing of livestock or plant metabolites can be waived, if it can be demonstrated that an extension of uses or an increase of the application rate is unlikely to change the conclusion on the relevance of metabolites (e.g. non-detectable residues of a metabolite of a non-potent active substance in a feeding study at an exaggerated dose rate; a very limited number of target crops for a herbicide and observed phytotoxicity precluding higher application rates).

3.5. Livestock Dietary burden calculation (steps 16 and 17)

Livestock metabolism data are used to identify potential candidates for inclusion into the residue definition for plants (as for potential residue transfer into livestock matrices from feed) and into the residue definition for livestock commodities itself (step 17). For the livestock dietary burden calculation against the trigger of 0.004 mg/kg bw/d (step 16), parent and major plant metabolites (≥10% TRR and ≥0.01 mg/kg) observed in feed items are used as the sum expressed as parent unless information is available from animal studies that they belong to separate pathways in animals.

If a metabolism study in livestock is required (OECD 503 (2007c), all major livestock metabolites ≥10% TRR are selected (in step 17) for subsequent grouping and testing (step 18), if not yet toxicologically characterised. In case of substances of high potency (see chapter 3.3), metabolites <10 % TRR are relevant for toxicological grouping and testing, if their anticipated individual level in animal tissues or milk at 1N rate is ≥0.01 mg/kg.

3.6. Testing Strategy (step 18)

In general, a 28-day rat study according to OECD 407 enhanced (OECD, 2008) would be appropriate as a first step.

Grouping can be used for the selection of representative substance/s to be tested and read across according to the recommendations of OECD (2014); grouping criteria and/or selection of representative substance/s for testing should be at least substantiated by:

- identification of the critical effect(s)/endpoint(s) of the parent to be read across
- criteria for similarity (e.g. structural similarities and chemical reactivity which are assumed to trigger a similar toxicokinetic and toxicodynamic properties) and analogues selection.
- compile toxicity data for analogous chemicals
- support the proposed toxicity mechanism by comparative mechanistic data

In line with the general principles described above, the design of the 28 day rat toxicity study has to be considered carefully. The following considerations should be taken into account for the design of the study:

- The top dose of the metabolite should achieve the maximum tolerated dose (MTD) for repeated administration. Alternatively, the maximum administrable dose or the maximum dose of 1 g/kg body weight should be used in case the MTD cannot be determined.
- The range of doses selected in the study should allow for comparison with the toxicity of the parent; alternatively, a parallel group should be tested with the parent. The experimental conditions should be, as far as possible, close to the ones applied for the parent in terms of animal species, strain, number of animals, endpoints evaluated and general experimental conditions.

If a comparable 28-day rat study was not conducted with the parent, the choice of a 28-day study with the metabolite could still represent a valid option; though, an expert toxicology judgment and/or the use of an additional safety factor should be considered (see 3.7).

The enhanced OECD 407 (OECD, 2008) study has a number of optional endpoints in regard to endocrine-mediated effects; these endpoints are recommended in order to make a robust and comprehensive hazard characterisation of the metabolite.

Furthermore, the test should include an assessment of the male reproductive system by means of a detailed histopathological evaluation of the testes, i.e. a stage-dependent qualitative evaluation of spermatogenesis should be conducted on section of testes from all control and high dose terminal necropsy animals. A qualitative examination of spermatogenesis stages will be made for normal progression of the stages of spermatogenesis, cell associations and proportions expected to be present during spermatogenesis. If potential effects are identified, then other groups should be examined (Creasy, 2003 and Russell et al., 1990).

One important limitation of the extended 28 day rat toxicity study (OECD 407) is lack of exploration of developmental and reproductive toxicity (DART) endpoints after in utero exposure

To minimize the risk associated with potential DART effects, different options can be considered:

If the parent compound has no DART precedents and the tested metabolite is considered qualitatively similar to the parent in terms of toxicological profile, no further testing would be necessary and the DART profile of the metabolite will be considered based on the parent.

If the parent compound has no DART precedents and the tested metabolite is considered qualitatively different from the parent (i.e. different hazard profile or no hazard identified) the following options are available:

1) Apply an additional safety factor of 10 when establishing reference dose (s) of the metabolite (Blackburn et al., 2015).
2) Test the metabolite in a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test according to OECD 422 (OECD, 1996). This test would replace the necessity of a 28 day rat toxicity study.
3) Test the DART endpoints with the specific studies (developmental toxicity study (OECD TG 414 (2001a), 416 (2001b), 443 (2011).

If the parent compound has DART precedent and the tested metabolite is qualitatively similar to the parent, the same reference dose set for the parent can be applied to the metabolite. This should be applied irrespectively from the fact that the reference dose is triggered or not by the DART based effect. Alternatively, testing for the DART endpoint of interest is an option.

If the parent compound has DART precedents and the tested metabolite is considered qualitatively different from the parent (i.e. different hazardous profile or no hazard identified) the following options are available: 1) apply an additional safety factor of 10 when establishing reference dose(s), 2) test for the DART endpoint of interest.

Deviations from this approach should be scientifically justified and alternative, *ad-hoc* toxicity studies or additional toxicity studies should be considered on a case by case basis. The choice should take into account the toxicological and toxicokinetic profile and, if available, information on mode of action of the parent compound. The studies should be informative enough to characterize the toxicological profile of the metabolite, derive a reference value where necessary or provide mechanistic information to enable a comparative assessment to the parent.

If specific, unexpected alerts are detected in the 28-day rat study for the metabolite of interest, or if the studies conducted are not considered appropriate to characterize the hazard for the metabolite, (e.g. parent is carcinogenic or neurotoxic), targeted toxicity studies may be required, case by case, to establish the toxic profile of the metabolite and to enable establishment of reference values.

Targeted toxicity studies could be for example:

- acute neurotoxicity in rodents (OECD TG 424 (1997), 418 (1995a))
- repeated dose neurotoxicity in rodents (OECD TG 424 (1997), 419 (1995b))
- developmental toxicity study (OECD TG 414 (2001a), 426 (2007d) or 443 (2011) with DNT cohorts)
- 2-generation reproductive toxicity study in rats or extended one-generation study
- (OECD TG 416 (2001b), 443 (2011))
- Carcinogenicity, also combined with chronic toxicity, study (OECD TG 451 (2009b), 452 (2009c), 453 (2009d))

However, mechanistic evidences (e.g. absence of the proven mechanistic effect leading to carcinogenicity of the parent molecule) or a convincing toxicological assessment taking into consideration all available data, can be provided to establish reference doses.

Also in cases where an acute assessment is necessary, the hazard triggering the regulatory reference value i.e. the ARfD, should be explored with appropriate testing if not already characterised by testing

performed with the parent. Furthermore, the above mentioned consideration should also apply for the acute assessment, where appropriate.

3.7. Assessment of the toxicological burden and relevance of metabolites (step 19)

Metabolites that are considered as candidates for inclusion into the residue definition for risk assessment have to be screened for their individual impact on the dietary exposure and risk (step 19). The relative contribution of a metabolite (or group of metabolites with similar profile) to the overall toxicological burden (i.e. the sum of identified metabolites, weighted by their relative potency), is considered a suitable measure to assess the relevance of a metabolite in terms of dietary consumer safety. The toxicological burden is meant as the sum of those residue compounds that were not previously excluded from the assessment (e.g. due to low potency, minor quantitative relevance, non-significant transfer of major feed metabolites to food of animal origin).

Where an exposure assessment is performed within the decision process for the residue definition (TTC, livestock dietary burden), the individual metabolite exposure data should be derived from the representative uses or from an extended data set of intended uses (if submitted) according to the conditions set out in chapter 5.

The outcome of toxicological testing of metabolites should be followed by establishment of the toxicity profile and relative potencies for risk assessment. The possible outcomes could be:

- The toxicity of the metabolite is similar to or lower than that of the parent (the relative potency factor (RPF) is ≤ 1); in this case the risk assessment can be performed using the acute and chronic reference values of the parent or applying a RPF < 1 to the reference values based on an appropriate data set

- The toxicity of the metabolite is higher compared to parent i.e. has lower NOAEL/LOAEL referring to the critical endpoint (RPF >1); the same ADI or ARfD of the parent has to be used, though the potency of the metabolite should be considered for the residue definition.

- The metabolite has a toxicity profile different from the parent; in this case specific acute and chronic reference values should be established. If the assessment conducted was not including the establishment of an acute reference value, the worst case assumption will be that the same value should be applied to both the acute as well as the chronic reference values. To establish the reference doses in absence of a full data package, an additional safety factor of 10 should be applied, if the 28 day rat study is the only study available.

- A specific relationship with the parent toxicity cannot be established e.g. because the endpoint of reference for the parent was only observed in a study of longer duration, and it may represent an evolution of the finding observed in the study conducted with the metabolite; in this situation a case by case approach including expert judgement should be applied by considering e.g. expert evaluation of the observed toxicity/pathology or the use of an additional assessment factor.

4. Module 3: Decision making for residue definition for risk assessment (step 20)

The parent is considered as relevant for inclusion into the residue definition if present in at least one commodity of relevance for human consumption (either via food of plant or animal origin).

The residue definition should be proposed per crop or livestock category.

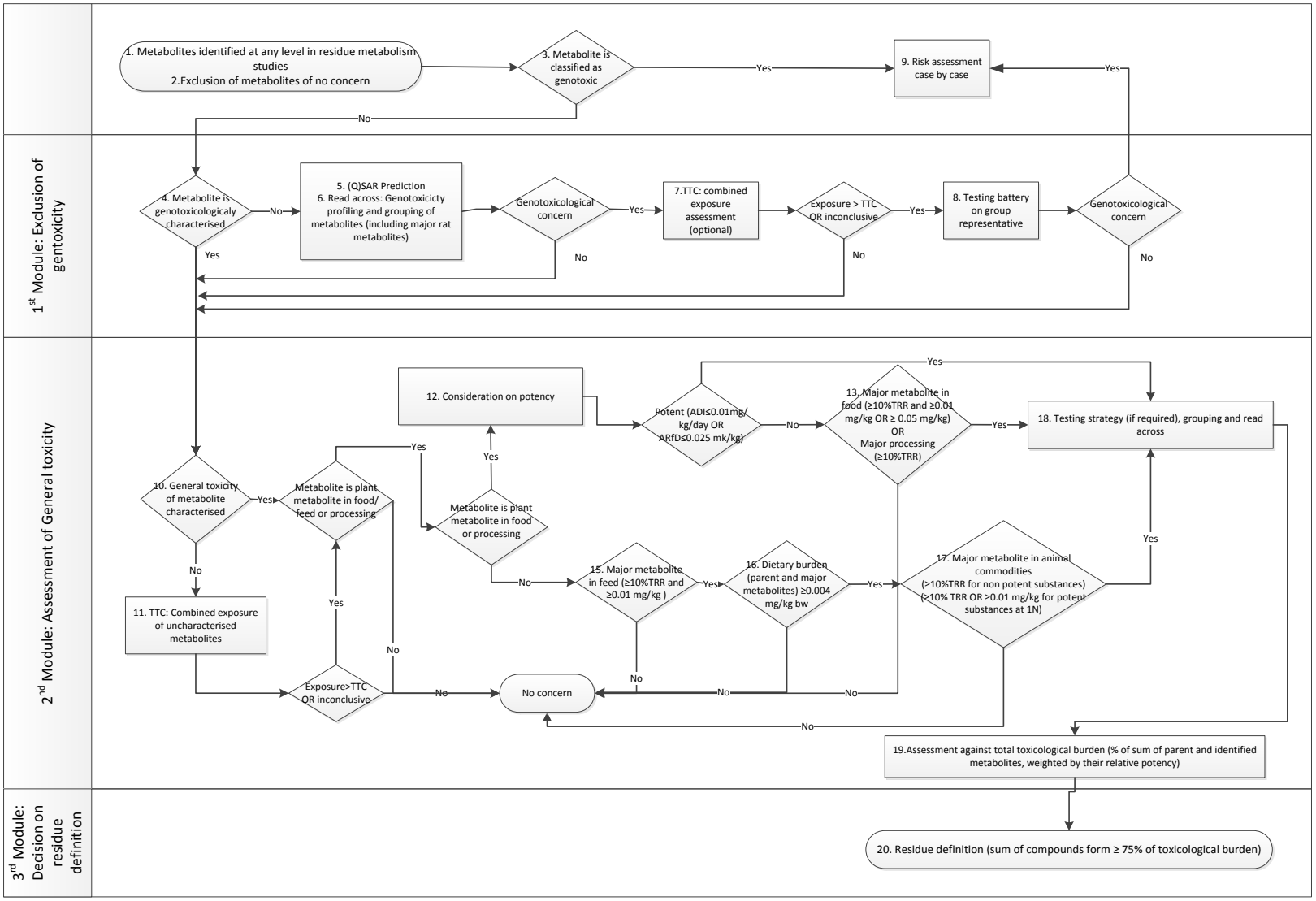
Within a crop category, a residue definition consisting of separate components should be proposed for metabolites bearing a toxicity profile different from parent and/or other relevant metabolites (see chapter 3.7).

After grouping and toxicological assessment, the toxicological burden of each metabolite in a group of metabolites with a comparable toxicity profile is expressed as percentage of the overall burden for the critical effect (i.e. the effect triggering the reference value). By default, metabolites or groups of them comprising ≥ 75% of the overall toxicological burden should be considered relevant for inclusion into the residue definition. Differences in the potency to parent should be balanced by accounting for a relative potency factor (RPF). If a metabolite or group of metabolites from a highly potent active substance is considered as significantly less toxic (falling in the group of non-potent substances), the criteria of non-potent active substances apply to these metabolites. The threshold of 75% should be considered as indicative and is not expected to cover all possible cases.

Where for derivation of the residue definition absolute exposure considerations[10] are applied in addition to the concept of relative contribution of metabolites to the dietary toxicological burden, these exposure considerations need to consider the full picture of possible dietary exposure, i.e. direct exposure via food of plant and indirect via food of animal origin, and where appropriate from groundwater used as drinking water. Where this condition cannot be met, i.e. reliable dietary exposure estimates cannot be provided, the assessment has either to be skipped (TTC), or a conservative approach has to be applied (e.g. covering uncertainty on residue uptake from soil by rotational crops), or a data gap is identified. In the latter case, the setting of a residue definition is either not possible or only possible on a provisional basis.

In case of relevant isomeric properties of a residue of concern (see chapter 6), additional uncertainty factors may be applied.

[10] Absolute exposure considerations may refer to e.g. exclusion of metabolites via TTC, refinement of input values from metabolism data by field studies supporting a specifc use



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746 **Figure 3: Overall assessment scheme**

5. Exposure assessments

5.1. General aspects

The significance of absolute quantities of residues in metabolism and field studies is limited as regards their use in exposure estimates. While for a very limited number of uses (e.g. the representative uses in the pesticide peer review) the regulatory relevant consumer exposure (in mg/kg bw/d) can in most instances be reliably estimated, this is not the case for uses intended in the future, which may create a higher exposure potential, e.g. by higher application rates or shorter PHIs. In order to set up a residue definition that is sufficiently robust against changes of authorisations of additional uses, the relative contribution of metabolites to the toxicological burden is preferred over absolute exposure estimates as a decision criteria for the residue definition.

However, within the decision process on the residue definitions, exposure calculations and assessment of metabolites against agreed triggers may be performed where appropriate (obligatory in case of potentially relevant metabolites in feed items, or on a facultative basis (TTC assessments of genotoxicity (module 1) and general toxicity (module 2).

This guidance will rely on the core criteria and principles set out in test methods, guidelines and guidance documents relevant to the submission of information to be used for the assessments for pesticides in Europe or by the pertinent regulations them-selves. In this context it is implied that the uncertainties and boundaries of assessments to inform regulatory decisions are acknowledged and accepted, and as they are considered to apply to a comparable extend to every assessment within the same framework, they are not further detailed and discussed in this guidance.

The following prerequisites and established principles are taken as given when applying the approach suggested in this guidance document:

- In plant metabolism studies, selection of crops and use patterns are representative and consistent with existing or intended GAPs and will reflect the situation where the highest amount of radioactivity resulting from metabolism would be expected in the consumable[11] parts of the crop at harvest (steps 13-15)

- Where metabolism data is intended to be directly used for exposure estimates (TTC, livestock dietary burden), scaling of overdosed metabolism studies is acceptable within an agreed range of application rates and where the latter is the only deviation from cGAP (proportionality approach EFSA, 2015).

- Only models and parameters agreed as applicable for assessments in the EU (variability and processing factors; consumption data, livestock feeding tables etc.) are used in line with the most current requirements and conventions.

- All residue data are expressed as equivalents of a reference compound (in most cases parent compound) for exposure estimates and subsequent comparison against relevant triggers (TTC, livestock dietary burden).

- Potential exposure from other possible sources related to the authorisation of a pesticide (including drinking water) will be taken into account in order to ensure that total exposure of consumers to a given metabolite is appropriately assessed. Metabolites that both occur in food and in groundwater should be considered with their full consumer exposure potential in the frame of TTC assessments for screening of dietary non-relevance and to inform risk managers on additional sources of exposure.

- Similarly, the simultaneous use of the active substance as a biocide or in veterinary medicine is reported and all available information submitted, to appropriately consider possible

[11] for livestock and consumers, respectively

cumulated exposure due to different uses of the same substance. Also, where the structure of a metabolite is identical to that of another registered active substance all accessible information will be provided, to take into account in the frame of TTC assessments for screening of dietary non-relevance, and to inform risk managers on additional sources of exposure.

In principle, where metabolites are excluded from the residue definition based on exposure estimates falling below the appropriate trigger (TTC, livestock dietary burden), the residue definitions are only applicable within the boundaries of this specific exposure assessment. If a more critical residue situation is created e.g. by extension of uses or number of applications, even within the assessed crop groups (e.g. root crops, cereals, fruit crops), affirmation of the established residue definition by an updated exposure assessment is required. However, if the metabolism data used for the initial assessment of the residue definition are truly corresponding to realistic worst case conditions and are covering an extensive range of uses[12], and any complementing relevant information with regard to other sources of exposure to the active substance or its metabolites is complete, the necessity for a soon revision is less probable.

Metabolites identified exclusively in plant feed items need to be considered for the livestock dietary burden calculation when detected at significant proportions and levels (≥10% TRR and ≥0.01 mg/kg). In addition, all metabolites deemed relevant for food items (≥10% TRR and ≥0.01 mg/kg, OR ≥0.05 mg/kg, respectively) that can be used as feed items have to be included for livestock dietary burden estimates. In case the exposure to livestock is comprised mostly of minor metabolites, the overall dietary burden should yet be assessed. In this case a case by case evaluation is required, and the scrutiny necessary in this evaluation will likely be driven by the structural and intrinsic properties of these minor metabolites (e.g. a metabolite with a structure indicating it might be highly fat-soluble or itself hardly metabolised may require more thorough considerations than a metabolite lacking such properties). If exposure is significant and there is the potential that measurable residues may be transferred into animal commodities, a best estimate of the levels (or a likely range of levels) of the metabolite residues should be provided. The applicability of the available livestock data with parent to the metabolites under assessment should be discussed.

5.2. Metabolite residue input levels for exposure calculation

Assessments should be made for the **target PHI**, even if this may not always represent the worst case residue situation for all metabolites individually. However, in case of suspected genotoxicity of metabolites (based on (Q)SAR, new alerts, *in vitro* tests), the maximum occurrence of metabolites at the target PHI or later should be calculated and exposure compared to the adequate trigger and highest consumption (equivalent to short-term consumer risk assessments). The reason is the underlying assumption of non-thresholded genotoxicity, where a single exposure event may already provoke a genotoxic effect. Where metabolites are grouped based on their common genotoxicity endpoint of concern (i.e. point mutation, structural and numerical chromosome aberrations), the occurrence of these metabolites should be assessed based on their critical occurrence level at a specific PHI (the "worst case PHI"). Combination of different PHIs for metabolites within one group is not considered adequate.

As a first step of exposure assessment, **median** and **maximum residue levels** for every single metabolite or for a group of metabolites as appropriate should be derived. These should be based on as much information as possible.

i) Best data would be **measured levels** from residue field trials (assisted by targeted processing studies, if applicable) or livestock feeding studies performed under realistic worst case conditions. If measured data are available, but not conforming GAP or anticipated dietary burden levels (parent and metabolites), approximation to more realistic conditions should be

[12] e.g. where a number of MRL applications are part of dossier submission

attempted to receive highest and mean metabolite levels (linear extra- or intrapolation to GAP rate according to the generally accepted rate range of between 0.3x and 4x the GAP rate, respecting the limitations of this approach; refer to EFSA, 2015). While scaling of residue field trial data has been investigated and confirmed as appropriate within the ranges established, for livestock, toxicokinetic information should be used to assess if interpolation can be made to adjust for a different exposure rate of the animal than investigated in tests and studies.

ii) If such data are not available, **conversion factors** (residue level of indicator compound divided by residue level of metabolite) should be derived from appropriate metabolism and/or field data and be applied to the set of field samples analysed for the indicator compound. This would allow determination of highest and mean metabolite levels. Often, parent compound is an appropriate indicator, however, another main residue compound (dominant metabolite analysed in metabolism and field trials or feeding studies) may provide more reliable estimates of exposure at the relevant sampling stage. If a targeted primary crop metabolism study is available that covers the intended use in terms of the type of crop or crop group, the number and type of applications and sampling, then this study should be preferred for use in exposure assessments over averaged data from a set of metabolism studies not exactly reflecting target conditions. If several metabolism studies cover the same intended use (e.g. by differing only in the radiolabel position), then mean conversion factors should be applied. If no targeted metabolism study is available, adequate mean conversion factors should be derived on base of available data, accompanied by a justification. Considerations should include the type of application (e.g. soil or foliar), number of applications, their interval and sampling stage (rate of metabolism), matrix type, active substance properties (systemic behaviour), differences between metabolism studies (crop groups; mammals). Conversion factors may also be based on intermediate or non-food/non-feed samples. In any case, attention should be given to observed differences between metabolism and field trials (e.g. reduced or enhanced metabolism in field trials compared to metabolism studies as observed by residue levels of the indicator compound).

iii) In the context of this guidance, conversion factors are only intended to be used for screening purposes for the relevance assessment of metabolites before setting the final residue definition. It is not intended to supersede data requirements for the generation of field trials according to the residue definition for risk assessment.

iv) It is only meaningful to apply conversion factors to field trials, where residues of the indicator compound can be reliably determined in the field (>LOQ in at least 25% of field trials). Where no adequate field data is available, the metabolite input level for exposure assessments can be derived by **normalising** the metabolism study values to 1N GAP conditions (if outside ±25% of application rate), thus resulting in a single residue value. This value, derived for one (or more) model crops in metabolism studies, may need to be extrapolated to all intended crops for exposure assessment. Where only one indicator residue value from field trials is available ≥ LOQ, the highest residue from field and (normalised) metabolism studies should be used.

v) The same principles for exposure assessments should apply to primary and **rotational crops** (conversion factors, selection of indicator compound, preference of field data over metabolism data, normalisation and extrapolation of crops).

Special consideration should be given to the effective N rate, at which rotational crop studies are performed. The N rate is understood as the ratio of actual residues in the soil under study conditions to the maximum likely residue soil situation comprising the background levels from long-term use as well as realistic seasonal applications (e.g. crop failure is likely to be relevant after early applications at growth stages, where crop damage cannot be excluded, while it is unlikely to be relevant after applications immediately prior to harvest).

Exposure estimates for rotational crops may be normalised to 1N rate. The following should be considered in the derivation of 1N rate:

- The expected maximum background levels of parent and metabolites after GAP compliant use are calculated based on empirically derived kinetic types, degradation half-lifes and specific boundary conditions. Such background levels for assessment should be adopted from the environmental fate assessment of parent and metabolites and expressed in terms of g as/ha for scaling purposes. Documented evidence of reduced bioavailability of soil residues over time ("aging") may be used for refinement.
- If soil residue data for parent and metabolites are - together with the plant residue data - available in rotational crop studies, these should be preferred for comparison with the predicted soil background levels to calculate the effective N rate, especially where metabolites show significant transfer from soil into the crops. Thereby, the soil-plant transfer of relevant metabolites in the rotational crop studies at the different plant-back intervals can be quantitatively assessed. Individual N rates may be derived for parent and metabolites.
- Where soil residue data are provided within a rotational crop study for one sampling point only, the time-dependent occurrence of metabolites might be calculated.
- Where no soil residue data are provided in the study reports and the active substance and/or metabolites are considered as persistent with accumulation over years of GAP compliant use, the transfer of soil residues into rotational crop has to be estimated based on the calculated mean concentration of residues in soil under study conditions and the maximum occurrence in the rotational field crops.

A case should be provided for the set-up of the scenario used for decision making.

Rotational crop studies are usually performed on a set of model crops (cereals, root and tuber vegetable, leafy crop). In case of accumulating compounds, where assumptions on the likely crop rotation can hardly be made for years, extrapolation to all potential field crops may be required.

6. Assessment of stereoisomers (enantiomers and diastereoisomers) for the parent and metabolites

The current data requirements for plant protection products indicate that the information provided must be sufficient to permit an evaluation to be made on the nature and extent of the risks for consumers from exposure to the active substance, its metabolites, degradation and reaction products, where they are of toxicological significance, and also that it is necessary to establish the isomeric composition and possible metabolic conversion of isomers when relevant. This does also include the case when metabolites are isomers of the active substance, i.e. when interconversion (induced enzymatically, photochemically, microbially, thermally, or in a different manner) leads to the generation of isomers of the active substance that do not match the technical specification of the latter.

The impact of stereochemistry on the toxicological relevance of pesticide metabolites for dietary risk assessments has previously been discussed in detail (EFSA PPR Panel, 2012). Since isomers may differ in their toxicological potency or profile, changes in stereoisomeric compositions need to be considered for the risk assessment. Therefore, the potential differences between the toxicologically tested isomeric mixture(s) and the stereoisomeric composition of the residues to which humans will be exposed need to be addressed.

Guidance regarding the technical aspects of addressing the aspects including basic chemical evaluations, approaches to study design, sampling and analysis strategies or similar aspects relevant for obtaining information on the stereochemical composition of the residues is considered out of scope of this document. For guidance on these matters, a "Guidance of EFSA on completing risk

assessments for active substances of plant protection products that have stereoisomers and for transformation products of any active substances where these transformation products may have stereoisomers" is currently under development (hereinafter referred to as EFSA Guidance on isomers). Moreover, the criteria to determine whether or not a change in the stereoisomer compositions is significant (in terms of residue analysis) will also be defined in the ESFA Guidance on isomers, taking into account the variability that can be reasonably expected in the analytical results obtained with stereo-selective methods used in radiolabelled metabolism studies and/or in field studies.

With regard to the dietary risk assessment considerations for isomers, a stepwise approach is proposed in this guidance document. The stepwise approach can be initiated with **either considerations on the exposure profile or on the hazard characterisation** of the different isomers whatever is deemed most suitable and adequate for the specific situation. A special case is derived in terms of the evaluation of the genotoxic potential of isomers.

6.1. Exclusion of genotoxicity for isomers or changed isomeric compositions

Since biological systems are chiral entities, in a chiral environment stereoisomers can show selective absorption, accumulation, enzyme interactions and metabolism, receptor interactions and DNA binding. Consequently each stereoisomer or isomeric mixture can have different kinetic, dynamic and toxicological profile. With the DNA 3D structure certain compounds could interact stereo selectively. Examples include Cis-platin (Boudvillain et al., 1995; Kasparkova et al., 2008; Marchan et al., 2004) and transformations leading to epoxide intermediates which are particularly prone to stereo selective mutagenicity and carcinogenicity i.e. Aflatoxin B1 (Stewart et al., 1996, Iyer et al., 1994) and styrene 7,8-epoxide.

For pesticide substances there is substantial evidence of stereo-selective metabolism, stereo-selective toxicity and also data on isomerisation in the environment, but no examples for stereo-selective genotoxicity of pesticides or their metabolites are currently known. Such conditions may not be completely excluded i.e. stereo-selective genotoxicity might not have been discovered by studies; however (Q)SAR analysis predict structural alerts independently from the stereochemical composition.

The low level of uncertainties linked to the potential genotoxicity of isomers or different isomer compositions of a compound leads to the conclusion that isomers and changes in the isomers composition is not anticipated to be a genotoxicity concern and will be not further addressed by the guidance.

6.2. Isomer assessment step 1 Exposure profile

The stereoisomeric ratio to which humans will be exposed has to be defined.
Different outcomes from the investigation of the isomeric composition of the residues in consumable crop parts/commodities are possible, making it difficult to suggest a generic strategy that will cover all situations; however, the most likely cases are expected as follows:

Case 1: The stereoisomeric ratio of the active substance and the pertinent metabolites (i.e. metabolites evaluated in module 2 for which the stereoisomeric composition should be known), found in samples from nature-of-residues studies across different crops (including rotational crops) and at different sampling times, as well as in livestock metabolism studies where appropriate, show no difference in stereoisomeric ratio compared to the parent and the metabolites addressed in module 2. In this case, it is important that the mixture composition used in the key study performed to assess the hazard in module 2 is reflecting the mixture composition of the residues studies. In the case of 'no difference' further investigation of isomer ratios is not required, and the magnitude of residue studies with analysis of residues as the sum of the respective stereoisomers are appropriate to be used for dietary exposure and risk assessments. No further isomer-specialised hazard assessment is required.

Case 2: The stereoisomeric compositon of residues found in the nature-of-residues studies show a significant difference compared to the stereoisomeric ratio of parent and compounds addressed in module 2, and these changes are consistently observed across crops / commodities and different sampling / harvesting intervals (and number of available studies satisfies the criteria set out in current guidance for establishing a global residue definition). Further investigation of isomer ratios in residue trials is not necessarily required. Hazard evaluation should be conducted.

Case 3: The stereoisomeric composition of residues found in samples from the nature-of-residue studies show a difference compared to parent and compounds addressed in module 2 or the composition / ratio of isomers found in the nature-of-residue studies is not coherent across the crops / commodities, in particular when showing a change of the isomeric ratio into different directions (for both the active substance and pertinent metabolites). Hazard evaluation should be conducted. In a case where a significant impact of the isomer ratio on the observed toxicity is expected, robust data for exposure assessment become necessary. Studies on the magnitude of residues (decline and at harvest trials, processing trials, rotational crop trials, feeding studies as appropriate) have to be conducted using stereoselective analytical methods for. This applies to all crops and commodities to be assessed in order to generate a representative number of results. With regard to representativeness the same standards should be applied as defined by current guidance on magnitude-of-residue studies.

6.3. Isomer assessment step 2: Hazard evaluation

The stereoisomeric composition established as the likely exposure profile should be compared with that of the material used in the toxicological studies conducted with parent or metabolites, if that is the case. If no significant change in composition (including ratio) is observed, the data for the toxicologically tested substance should be used for risk assessment.

Upon assessment of the study results, and where feasible, a case might be made for waiving further toxicological testing by deriving a factor to describe the change of ratio of the individual isomers in residue studies compared to the ratio initially tested in the toxicology studies for the parent and metabolites if is the case. This factor can be used as an equivalence/correction factor in the dietary risk assessment. This conservative approach might be meaningful mostly when the number of isomers is very limited, and the uncertainty added to the risk assessment by using such factors is noted.

This worst case approach may be taken for the derivation of the ADI by applying a factor of two when the mixture is a sample racemate of two isomers and is based on the assumption that the biological activity (i.e. target effect, toxicodynamic and toxicokinetic/metabolism properties) is due to one isomer which is representing all residue.

A larger factor can be applied for compounds with more than one chiral centre with the assumption that all biological activity is due to the isomer present in the smallest proportion and that all residues in food are present in this form. This approach is however considered to be very conservative though it could be used for the definition of "significant changes" in isomer composition and then trigger further considerations when exposure is above the ADI.

When significant change in isomer composition is detected in residue studies, the next step is the hazard evaluation of the isomeric mixture considering **all the available data** on isomers present in the mixture and the **nature and severity of the toxicological** effects observed with the mixture. The aim is to conclude if the stereoisomers will contribute qualitatively and quantitative to the hazard. This should be done by providing supporting evidences. Supporting evidences can be provided by additional investigations as described in the module 2 of this guidance and by making use of *in-vitro* and/or *in-vivo* studies to investigate initially the toxicological and metabolic properties of the mixture and, if is the case, of the single enantiomer.

If the hazard evaluation concludes that no quantitative and/or qualitative differences are likely and this is scientifically justifiable, the risk assessment based on total exposure to all stereoisomers is

appropriate. If conclusion cannot be made, the risk assessment will be made by considering the specific isomeric hazard characterisation to provide a specific ADI.

6.4. Isomer assessment step 3: Consumer risk assessment

Both acute and chronic risk assessments need to be considered. As for case 1 and 2, the consumer risk assessment is conducted against the toxicological reference values derived for the residue of concern from the data package deemed suitable.
As for case 2 and 3, if data on the toxicity of individual isomers and quantitative data on the isomeric composition of residues in food are available, calculation of the consumer intake can be carried out.

7. Uncertainties

This chapter is still under development and might be changed according to the Scientific Committee guidance on uncertainty in scientific assessment (pending adoption).

In its Scientific Opinion the PPR Panel (EFSA PPR Panel, 2012) proposed different levels of uncertainties analysis (i.e. qualitative, deterministic or probabilistic) for the uncertainties affecting the assessment. It is assumed that the uncertainty assessment will take into account case by case circumstances and that will be used to identify critical areas that need further refinement. PPR Panel (2012) recommended initially, all significant uncertainties to be evaluated qualitatively; however, if the outcome is not considered clear enough for a decision making, those critical uncertainties should be analysed quantitatively.

A tabular approach is recommended for evaluation and expression of uncertainties affecting the residue definition.

Table 1. Tabular approach for evaluation and expression of uncertainties affecting the residue definition. The +/– symbols indicate whether each source of uncertainty has the potential to make the true risk higher (+) or lower (–) than the indicated outcome. The number of symbols provides a subjective relative evaluation of the magnitude of the effect (e.g. +++ indicates an uncertainty that could make the true risk much higher). If the effect could vary over a range, lower and upper evaluations are given (e.g. + / ++). If possible, the user should indicate the meaning of different numbers of symbols (e.g. two symbols might be used to represent a factor of 5, and three symbols a factor of 10). Finally, the combined impact of all the uncertainties is evaluated subjectively. More detail on the rationale for these evaluations (especially for the more important uncertainties and the overall uncertainty) should be provided as separate text accompanying the table.

Source of uncertainty	**Magnitude and direction of influence**
Concise description of source of uncertainty	Symbols to show evaluation of influence (e.g.: +/++)
Insert one row for each source of uncertainty affecting the assessment	
Overall evaluation of uncertainty affecting the assessment outcome Add narrative text here, describing the assessor's subjective evaluation of the overall degree of uncertainty affecting the assessment outcome, taking account of all the uncertainties identified	**Evaluation of overall uncertainty (e.g., - - - /+)**

above.	

A number of uncertainties having a potential impact on the residue definition (and therefore ultimately on the dietary risk assessment) were noted across the different steps described in this guidance document and they were listed below. The list should be intended as indicative and a more thorough evaluation should be performed on a case by case basis as the uncertainties are largely dependent on the amount and quality of the available data.

- Exclusion of genotoxicity using of (Q)SAR and read across. In particular, the use of read across for further assessment of chemical structures of concern following the (Q)SAR is a potential source of uncertainties.
- Grouping and read across are applied as a tool to support the general toxicological assessment of metabolites and this is a potential source of uncertainties.
- The use of TTC as a screening tool in the toxicological risk assessment of residues was considered a source of uncertainties, particularly because of the uncertainties linked to the exposure scenario.
- Differential metabolism of the isomers may lead to a predominance of one of the isomers in animals or plants and this is considered a source of uncertainties in the toxicity evaluation of residues.
- The exclusion criteria based on simple structural changes is a source of uncertainties, particularly when dealing with endpoints of chronic toxicity. Metabolism of a chemical often comprises, among others, demethylation or hydroxylation of a ring structure. It is assumed that the simple demethylation or hydroxylation of a ring structure without opening the ring will not increase the toxicity of the metabolite. This assumption is based on a conclusion in an External Scientific Report to EFSA prepared by AGES (2010). It is noted that AGES based this conclusion on data obtained mostly from acute toxicity studies. AGES also noted that there are some compounds were hydroxylation of a ring structure may increase its toxicity (e.g. hydrochinon). Therefore, some uncertainty remains on the applicability to predict the toxicity after short-term or long-term exposure.
- In the context of this guidance thresholds are applied across multiple steps of the decision scheme. They are arbitrary in their nature and considered a source of uncertainties. The use of the ADME study conducted in rodent species is a relevant source of uncertainties, particularly when dealing with effects observed in different species, pregnant animals or in the foetus.
- A number of uncertainties are linked to the experimental conditions applied for the characterisation of the metabolic and toxicological profile of the parent substance and of the metabolites.
- The lack of information about the nature and quantity of unidentified residues needs to find due considerations in the uncertainty assessment.
- Metabolite exposure assessment has to rely not only on the available data, but on extrapolations and additional assumptions of varying degrees of uncertainty, whose inherent uncertainties need to be addressed. Risk managers should be informed about additional sources of exposure (e.g. groundwater, metabolites common to other active substances).
- The potential contribution of individual metabolites to adequate reference values is assessed under step 19; a detailed uncertainty analysis covering the overall level of conservatism for the chosen scenario can be provided upon request of risk management.

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APPENDICES

Appendix A. ADI and ARfD distribution for pesticide active substances

1. Introduction

In the context of this guidance, separation between major and minor metabolites is made using arbitrary thresholds. These thresholds have a limited scientific validity; though, they have been used in the regulatory field in multiple circumstances and for this reason they are generally accepted as a pragmatic and practical tools. However, because of this arbitrary nature, additional considerations on the toxicological properties of the parent substance are necessary to accomplish the aim of predicting whether a metabolite is toxicologically similar or different to the parent substance. In particular, and in line with the OECD guidance (OECD, 2009), it is important to take into account the potency and the relevant endpoints of toxicity of the parent substance. In the absence of toxicity data on the metabolites, the default assumption is that they possess the same toxicological profile as for the parent substance and the more toxic is the parent compound, the greater is the need for inclusion of the metabolites in the assessment. In order to propose a definition of potency that could be used to complement the arbitrary thresholds, an evaluation of the distribution of the ADIs and ARfDs for European approved active substances was performed, assuming that most of the active substances eliciting neurotoxic effects are the one with the lower reference values. The data used in performing this exercise were extracted from the external report on "Investigation of the state of the art on identification of appropriate reference point for the derivation of health-based guidance values (ADI, AOEL and AAOEL) for pesticides and on the derivation of uncertainty factors to be used in human risk assessment" (CRD-HSE, 2013). Additional data were added to the database for the most recently evaluated active substances by EFSA (until end of 2014) which were not included in the database at the time of publication.

The Assessment and Methodological Support Unit (AMU) of EFSA was requested to support the PRAS unit in identifying a data driven distribution of ADIs and ARfDs for pesticide active substances.

2. Material and methods

2.1 Data

Two sets of raw data were provided to the AMU unit. A first set (ADIclean.csv) listed 270 approved compounds and their related Acceptable Daily Intake (ADI) values. A second set (ARDclean.csv) listed 195 approved compounds and their related Acute Reference Dose (ARfD) values.

2.2 Methodologies

A simple descriptive statistics was first computed to understand the distribution of the data. The data were visualised using boxplots.

As a second step, a set of quantiles (from 10% to 50% with steps of 5%) were calculated based on the available raw data. The quantiles were calculated both for the ADI and the ARfD values. The results were then plotted in a density graph.

Finally, a set of tables were produced in order to list all the compounds with an ADI or an ARfD value lower than each threshold.

All analysis were performed in R[13] and the following packages were used:

- stats

[13] R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

- ggplot2

3. Results

3.1 ADI

The boxplots based on the available data showed a distribution of the data concentrated mainly around the median value (0.02 – see Figure 1, the white box around the zero value groups the data up the 75th percentile of the ADI data distribution) with some outliers relatively far from the median (the maximum value observed is equal to 10, not represented in Figure 1). Table 1 shows the summary statistics on the ADI values.

Table 1: Summary statistics on ADI values (in mg/kg bw/d)

Min.	1st Qu.	Median	Mean	3rd Qu	Max.
0.00015	0.01	0.02150	0.12970	0.08000	10

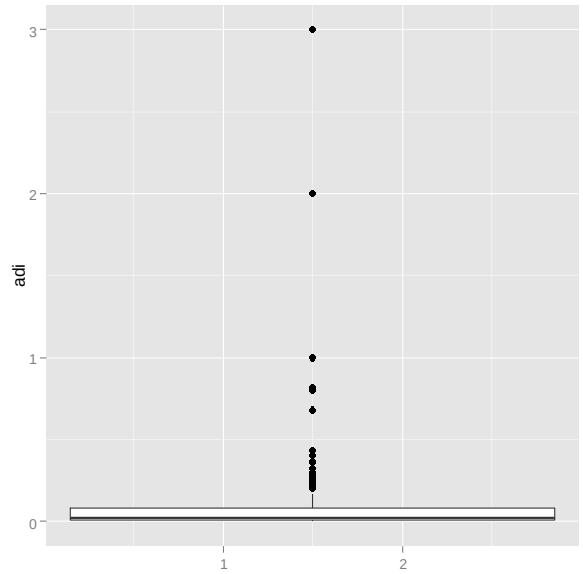


Figure 1: ADI boxplot. The observations with a value higher than 3 are excluded from the visualisation.

Figure 2 shows the density distribution of the ADI values. The coloured lines represent the location of the different quantiles on the distribution. The values are reported in the legend.

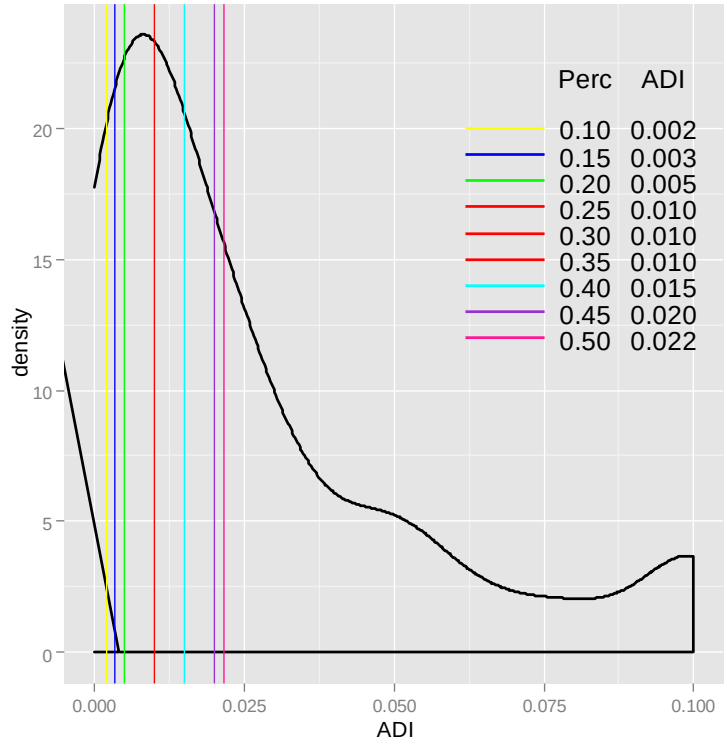


Figure 2: Density plot of the ADI values. The coloured lines show the different percentiles and the correspondent ADI value

In order to have information on the relevant endpoints triggering the distribution of the ADIs, the distribution of the NOAELs relative to the endpoints of interest was performed for the active substances included in the external report (n= 224 active substances) (CRD-HSE, 2013).

Results are summarized in Figure 3.

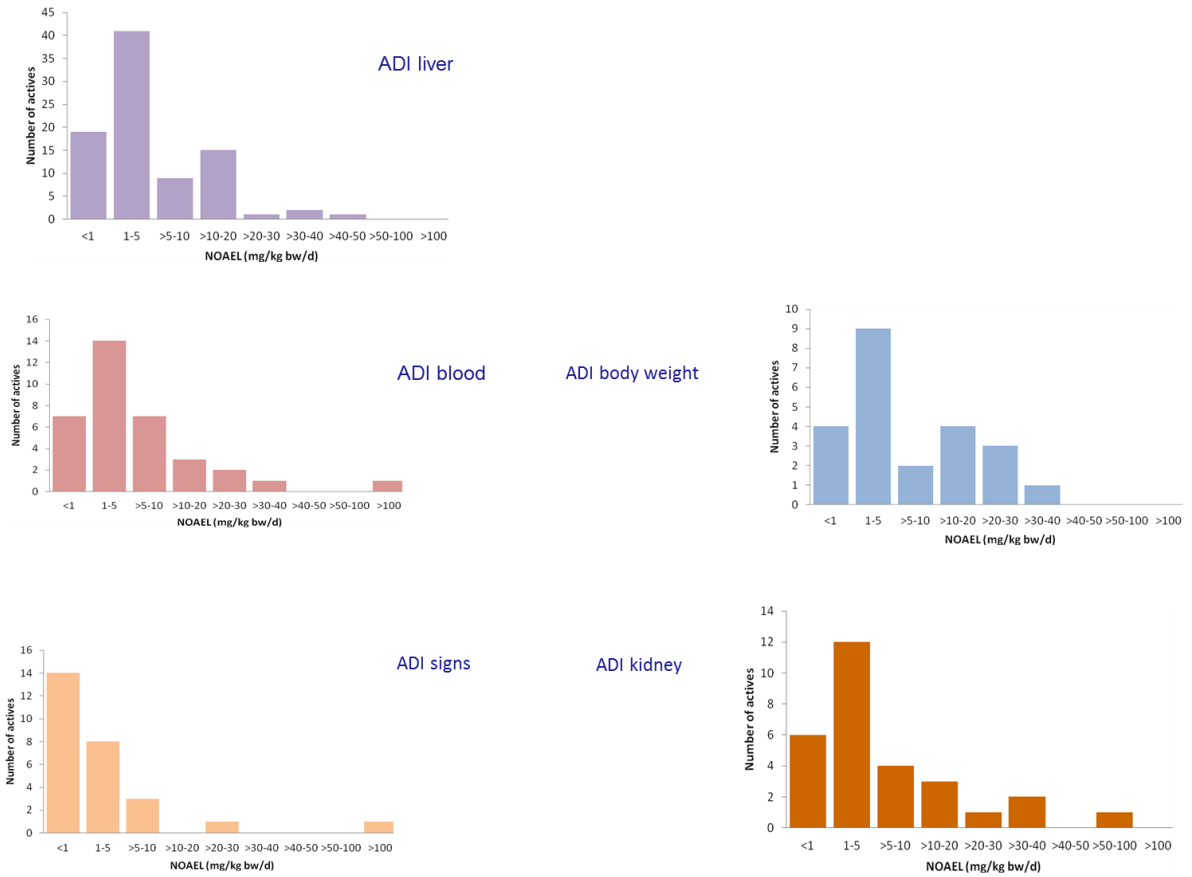


Figure 3 Plots of the different NOAELs used for the derivation of the ADI separated by target organ

3.2 ARfD

The boxplots based on the available ARfD data shows the distribution of the data concentrates mainly around the median value (0.1 mg/kg bw – see Figure 14, the white box around the zero value groups the data up the 75th percentile of the ARfD data distribution) with some outliers relatively far from the median. Table 2 shows the summary statistics on the ARfD values.

Table 2: Summary statistics on ARfD values

Min.	1st Qu.	Median	Mean	3rd Qu	Max.
0.0002	0.0275	0.1000	0.2428	0.3000	4.5000

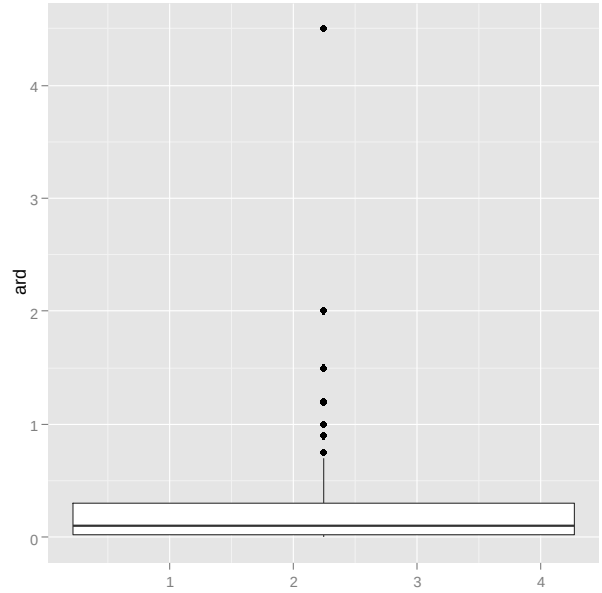


Figure 4: ARfD boxplot. All observations are included.

Figure 25 shows the density distribution of the ARfD values. The coloured lines represent the location of the different quantiles on the distribution. The values are reported in the legend.

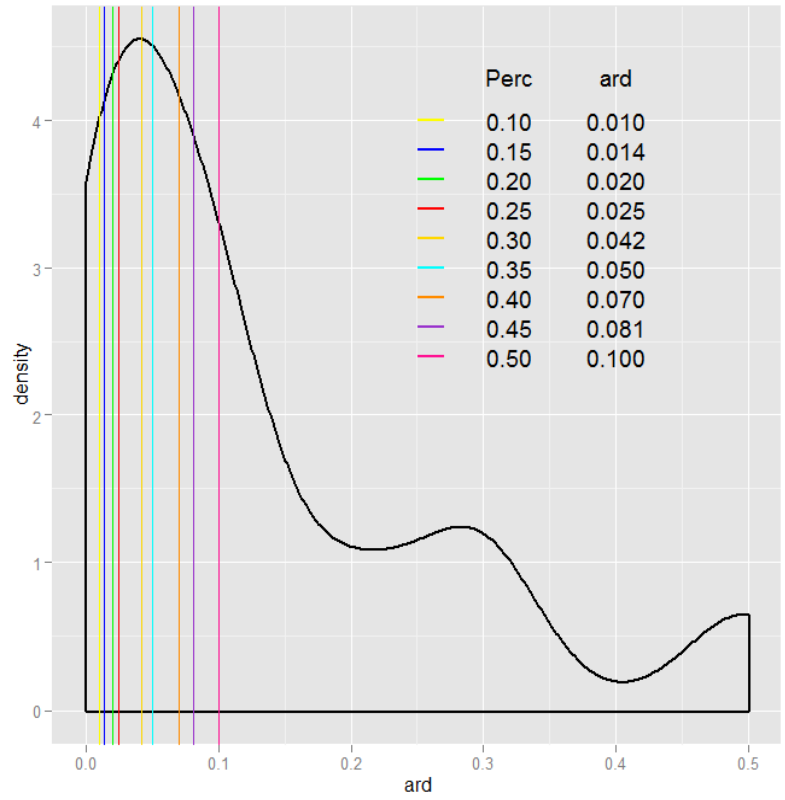


Figure 5 Density plot of the ARfD values. The coloured lines show the different percentiles and the correspondent ARfD value

In order to have information on the relevant endpoints triggering the distribution of the ARfDs, the distribution of the NOAELs relative to the endpoints of interest was performed for the active substances included in the external report (n= 224 active substances). Results are summarized in figure 6.

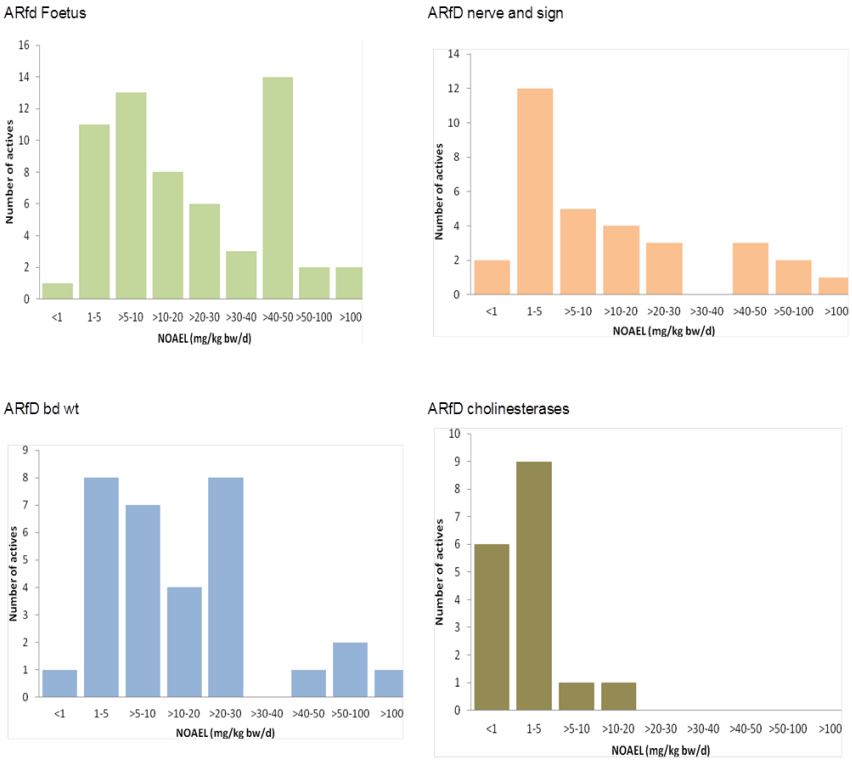


Figure 6 Plots of the different NOAELs used for the derivation of the ARfD separated by target organ

4. Discussion and Conclusions

The plots of NOAELs (Figure 3) used to derive the ADIs indicate that, other than for clinical signs, there is a broad range of potencies and none of the end-points is related to either relatively high or low dose levels. The liver is the most common target and clinical signs are the most frequent endpoint triggering relatively low ADIs values.

The plots of NOAELs (Figure 6) used to derive the ARfDs indicate that, other than cholinesterase inhibition, there is a broad range of potencies and none of the end points can be considered as prevalent. The effects on foetus (and maternal toxicity) and nervous system/clinical signs are the main targets/end points.

As expected the ADI is more conservative concerning the neurotoxic effects and provides a better estimation of the distribution of the effect (e.g. effects only observed after repeated dose).

The ADI of 0.01 mg/kg/bw/d is representing the 25th percentile of the ADIs distribution and includes most of the active substances (approx. 67%) for which neurotoxic effects are relevant and the most toxic substances for other target organs toxicity in general. The value of 0.01mg/kg/bw/d will only partially include active substances with developmental effects (maternal and foetal effects).

The ARfD of 0.025 mg/kg/bw is representing the 25th percentile and includes most of substances (approx. 50%) inducing acute clinical signs and/or neurotoxic effects. However, as for the ADI, this value only partially covers foetal effects.

Based on these considerations, the ADI dose of <0.01 mg/kg/bw/d and the ARfD of <0.025 mg/kg/bw are proposed as a threshold to define toxicologically potent substances.

5. References

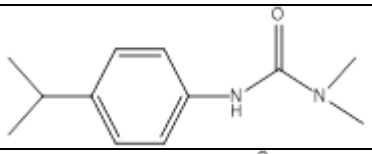
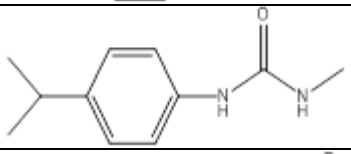
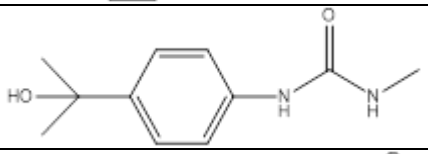
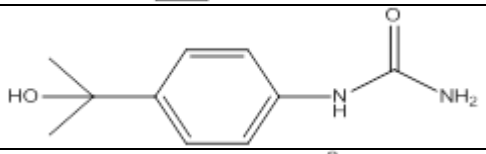
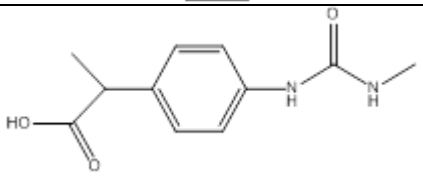
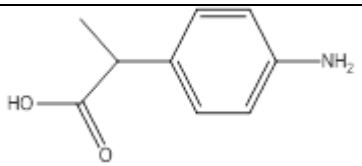
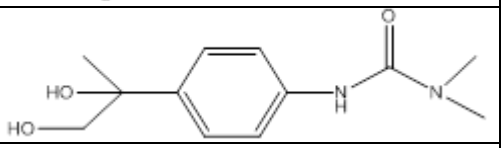
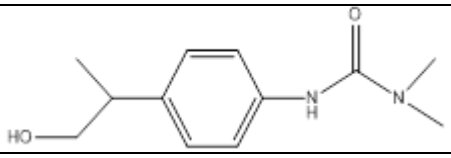
OECD (Organisation for Economic Co-operation and Development), 2009. Series on testing and assessment No. 63 and Series on pesticides No. 31 guidance document on the definition of residue; ENV/JM/MONO(2009)30; 28-Jul-2009

CRD-HSE (Chemicals Regulation Directorate, Health & Safety Executive, UK), 2013. Investigation of the state of the art on identification of appropriate reference points for the derivation of health-based guidance values (ADI, AOEL and AAOEL) for pesticides and on the derivation of uncertainty factors to be used in human risk assessment. Supporting Publications 2013:EN-413. [169 pp.]

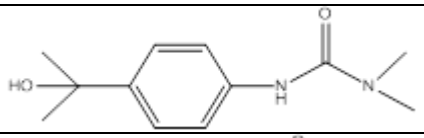
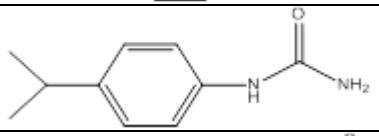
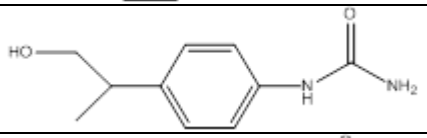
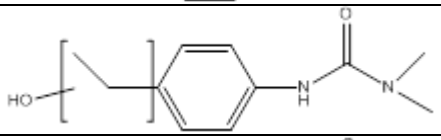
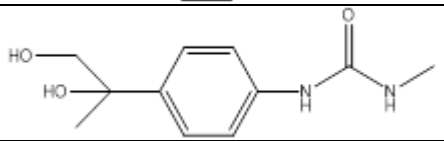
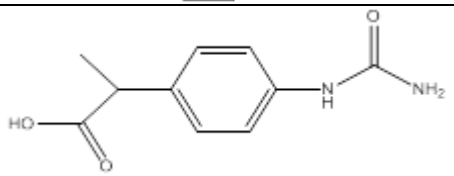
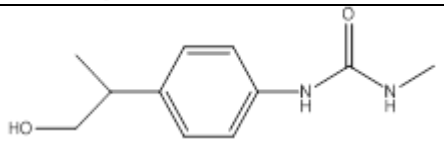
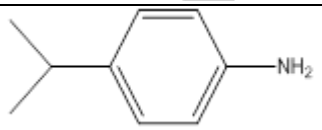
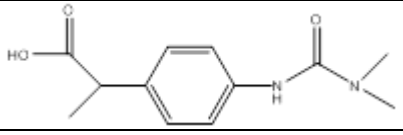
Appendix B. Case study – Isopoturon (Germany, 2014)[14]

Step 1: Metabolite identified at any level in residue metabolism (plant) and groundwater studies

Table 1 *Isoproturon metabolites*

Compound identifier	Name in Study and Assessment reports and SMILES	Structure
Parent	Isoproturon CC(C)c1ccc(NC(=O)N(C)C)cc1	
M02	AE F064145 Monodesmethyl isoproturon CC(C)c1ccc(NC(=O)NC)cc1	
M03	RPA 415044 Hydroxy-mono-desmethyl CC(C)(O)c1ccc(NC(=O)NC)cc1	
M04	RPA 410365 Hydroxy-didesmethyl CC(C)(O)c1ccc(NC(N)=O)cc1	
M05	RPA 409656 CC(c1ccc(NC(=O)NC)cc1)C(O)=O	
M06	RPA 410198 CC(C(O)=O)c1ccc(N)cc1	
M07	RPA 410226, sum of isomers CC(O)(CO)c1ccc(NC(=O)N(C)C)cc1	
M07a	BD4236D2 (Isomer 1), RPA 410226	
M07b	BD4236D2 (Isomer 2), RPA 410226	
M08	RPA 409658 1-OH-isoproturon CC(CO)c1ccc(NC(=O)N(C)C)cc1	

[14] Germany, 2014. Renewal Assessment Report (RAR) on the active substance isoproturon prepared by the rapporteur Member State Germany in the framework of Regulation (EU) No 1141/2010,February 2014. Available at http://dar.efsa.europa.eu/dar-web/provision

Compound identifier	Name in Study and Assessment reports and SMILES	Structure
M09	BD4236D7 Hydroxypropyl isoproturon CC(C)(O)c1ccc(NC(=O)N(C)C)cc1	
M10	LS 730334 Didesmethyl isoproturon CC(C)c1ccc(NC(N)=O)cc1	
M11	BD4236D3. RPA 409660 CC(CO)c1ccc(NC(N)=O)cc1	
M12	BD4236D4 CN(C)C(=O)Nc1ccc(CCO)cc1	
M13	BD4236D CC(O)(CO)c1ccc(NC(=O)NC)cc1	
M14	RPA 409657 CC(c1ccc(NC(N)=O)cc1)C(O)=O	
M15	RPA 409659 CC(CO)c1ccc(NC(=O)NC)cc1	
M16	RPA 710989 CC(C)c1ccc(N)cc1	
M18	RPA 409394 Propanoic acid isoproturon CC(c1ccc(NC(=O)N(C)C)cc1)C(O)=O	

Step 2: Exclusion of metabolites of no concern

None.

Step 3: Metabolite is known to be genotoxic

No specific information on genotoxicity of metabolites is available.

Step 4: Metabolites genotoxicologically characterised – yes/no

Step 4.1 Assessment of metabolites whether they are covered by studies with the parent (Table 2) or specific studies.

Step 4.2 Conclusion

Proceed with genotoxicity assessment (steps 5 to 9) for all metabolites whose toxicological properties are not covered by the parent compound (shaded in grey) or by specific studies.

Table 2 Assessment of occurrence of isoproturon residue metabolites in toxicological studies with parent compound (RAR Germany 2014)

Compound	Name in Study and Assessment reports	Occurrence in rat metabolism (% administered dose)	Toxicological properties covered by studies with parent compound
Parent	Isoproturon	<1	Yes
M02	AE F064145 Monodesmethyl isoproturon	1	No (specific bacterial mutagenicity study available)
M03	RPA 415044 Hydroxy-mono-desmethyl	24	Yes (>10% AD)
M04	RPA 410365 Hydroxy-didesmethyl	51	Yes (>10% AD)
M05	RPA 409656	8	No
M06	RPA 410198	-	No
M07	RPA 410226, sum of isomers	6	No
M08	RPA 409658 1-OH-isoproturon	3	No
M09	BD4236D7 Hydroxypropyl isoproturon	3	No
M10	LS 730334 Didesmethyl isoproturon	15	Yes (>10% AD)
M11	BD4236D3. RPA 409660	6	No
M12	BD4236D4	-	No
M13	BD4236D	11	Yes (>10% AD)
M14	RPA 409657	2	No
M15	RPA 409659	3	No
M16	RPA 710989	-	No
M18	RPA 409394 Propanoic acid isoproturon	2	No

AD: administered dose

Step 5: (Q)SAR prediction of genotoxicity

Step 5.1: Description of (Q)SAR strategy

In order to predict the genotoxic potential (gene mutation and chromosomal aberrations) of the minor rat and plant specific metabolites, four models have been applied: CAESAR Mutagenicity Model v 2.1.12 - implemented in the VEGA software (v 1.0.8) and DEREK Nexus Mutagenicity Model (v 4.0.6.) for prediction of gene mutation; and a rule base with the structural alerts for in vivo micronucleus - implemented in the Toxtree v.2.6.6. (R. Benigni, C., O. Tcheremenskaia and A. Worth, Development of structural alerts for the in vivo micronucleus assay in rodents", European Commission report EUR 23844) and DEREK Nexus Chromosome Damage model Model (v 4.0.6) for prediction of chromosomal aberrations

Independently from the predictions of the models, the metabolite(s) will be subject of read across analysis (step 6).

Step 5.2: Documentation of CAESAR Mutagenicity model

xi) Used model (title, name of authors, reference)

CAESAR Mutagenicity Model v 2.1.12, Ferrari T., Gini G.

An open source multistep model to predict mutagenicity from statistical analysis and relevant structural alerts.Ferrari T., Gini G.Chemistry Central Journal 2010, 4(Suppl 1):S2 (29 July 2010)

xii) Information about modelled endpoint (endpoint, experimental protocol)

Ames Mutagenicity essay.

xiii) Used training set (number of the substances, information about the chemical diversity of the training set chemicals)

4204 compounds from the Kazius-Bursi mutagenicity database (Kazius J, Mcguire R, Bursi R: Derivation and validation of toxicophores for mutagenicity prediction.J Med Chem 2005, 48(1):312-320.), 2348 classified as mutagenic and *1856* classified as non-mutagenic by Ames test. 80% of the entire data set (3367 compounds) was used for the development of the model, while the other 20% (837 compounds) was used as a test (validation set).

xiv) Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)

A mutagenicity classifier has been arranged integrating two different techniques: a machine learning algorithm from the Support Vector Machines (SVM) collection, to build an early model with the best statistical accuracy, then an ad hoc expert system based on known *structural alerts* (SAs)(Benigni-Bossa rule base), tailored to refine its predictions. The purpose is to prevent hazardous molecules misclassified in first instance (*false negatives*) from being labelled as safe. The resultant classifier can be presented as a cascading filters system: compounds evaluated as positive by SVM are immediately labelled *mutagenic*, whereas the presumed negatives are further shifted through two consecutive checkpoints for SAs with rising sensitivity. The first checkpoint (12 SAs) has the chance to enhance the prediction accuracy by attempting a precise isolation of potential *false negatives* (FNs); the second checkpoint (4 SAs) proceeds with a more drastic (but more prudent) FNs removal, as much as this doesn't noticeably downgrade the original accuracy by generating too many *false positives* (FPs) as well. To reinforce this distinction, compounds filtered out by the first checkpoint are labelled mutagenic while those filtered out by the second checkpoint are labelled suspicious: this label is a warning that denotes a candidate mutagen, since it has fired a SA with low specificity. Unaffected compounds that pass through both checkpoints are finally labelled non-mutagenic.

xv) Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity

The authors reported accuracy of around 92% for the training set and around 82% for the test set.

xvi) External statistic, if available

Not available

xvii) Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)

The model provides evaluation of the reliability of the prediction which is in three steps scale: Compound is in model Applicability Domain, Compound could be out of model Applicability Domain and Compound is out of model Applicability Domain.

The Applicability Domain evaluation is based of combination of 5 Applicability Domain scores:

Similarity index – measure for the similarity between the predicted substance and training set substances with known experimental value;

Concordance – the similar substances found in the training set have (or have not) experimental values that are in agreement with the predicted value;

Accuracy – accuracy of prediction for similar molecules found in the training set

Atom centred fragments similarity check – all atom centred fragments of the substance are (are not) found in the list of atom centred fragments of the training set substances.

Model descriptor range check – descriptors for the substance have (or have not) values inside the descriptor range of the training set substances.

xviii) Mechanistic interpretation of the model

Not available

xix) Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)

The software provides six most similar substances from the training set with their experimental and predicted values.

xx) Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties

Not available

Documentation of DEREK Nexus mutagenicity model

1. Used model (title, name of authors, reference)

DEREK Nexus Mutagenicity Model v 4.0.6.

Lhasa Ltd, Leeds, UK, http://www.lhasalimited.org/

Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. Human and Experimental Toxicology 10, 261-273.
Judson PN, Marchant CA & Vessey JD (2003). Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370.
Marchant CA, Briggs KA & Long A (2003). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177–187.
Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79.

2. *Information about modelled endpoint (endpoint, experimental protocol)*

The Derek Nexus model for mutagenicity is developed from Ames test data in both S.typh and E.coli. Supporting data from in vivo lacZ-transgenic assay, in vitro L5178Y TK+/- assay, in vitro HGPRT gene mutation assay, in vitro Na+/K+ ATPase gene mutation assay has also been considered for the development of a small number of alerts. Additionally, alert writers consider both mechanistic evidence and chemical properties (such as reactivity).

3. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The DEREK model for mutagenicity is base of rules which codified the knowledge about the relation between a structural features and a toxicological (i.e. mutagenic) effect. Although almost all alerts are related with mechanistic explanation and examples, these rules are not related with particular training set.

Recently a model for negative prediction (non-mutagenic) has been developed and added to the previous model. For it development a training set of above 10 000 substances has been used (the number of mutagenic and non-mutagenic substances is almost equal). The training set is a compilation of six public available data sets (e.g. Kirkland, ISSSTY, NTP data sets)

4. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Derek Nexus is a rule-based expert system for the prediction of toxicity. Its knowledge base is composed of alerts, examples and reasoning rules which may each contribute to the predictions made by the system. Each alert in Derek describes a chemical substructure believed to be responsible for inducing a specific toxicological outcome (often referred to as a toxicophore). Alerts are derived by experts, using toxicological data and information regarding the biological mechanism of action. Where relevant, metabolism data may be incorporated into an alert, enabling the prediction of compounds which are not directly toxicity but are metabolised to an active species. The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible. In addition a likelihood is provided (e.g. certain, probable, plausible) which takes into account the presence of a structural alert and a limited number of molecular descriptors.

5. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

Derek is a knowledge-based expert system containing mechanistically-based rules which are built using all the underlying evidence available to the SAR developer. Therefore, there is no defined training or test set, and therefore there are no internal validation statistics to report.

6. *External statistic, if available*

Not public available

7. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The scope of the structure-activity relationships describing the mutagenicity endpoint are defined by the developer to be the applicability domain for the model. Therefore, if a chemical matches an alert describing a structure-activity for mutagenicity it can be considered to be within the applicability domain. The applicability domain of each alert is defined by the alert developer on the basis of the

training set data and expert judgement on the chemical and biological factors which affect the mechanism of action for each alert.

8. *Mechanistic interpretation of the model*

All alerts describing structure-activity relationships for the mutagenicity endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target. The mechanistic basis of the model was developed a priori by examining the active and inactive structures before developing the structure-activity relationship. All references supporting the mechanistic basis of an alert are detailed and available for inspection within the software.

9. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible

10. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Described above

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/

Documentation of Toxtree model – structural alerts for in vivo micronucleus assay

1. *Used model (title, name of authors, reference)*

Structural alerts for in vivo micronucleus implemented in the Toxtree v.2.6.6

Structural analysis and predictive value of the rodent in vivo micronucleus assay results. Benigni R, Bossa C, Worth A, Mutagenesis.2010 Jul;25(4):335-41

2. *Information about modelled endpoint (endpoint, experimental protocol)*

A large majority of the data are based on the analysis of micronuclei in bone marrow cells for rationale of and details on the assay, see (Krishna, G. and Hayashi, M. (2000) In vivo rodent micronucleus assay: protocol, conduct and data interpretation. Mutat. Res., 455, 155–166.
Morita, T., Asano, N., Awogi.T et al. (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B) the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Collaborative Study of the Micronucleus Group Test. Mammalian Mutagenicity Study Group. Mutat. Res., 389, 3–122.
Hayashi, M., MacGregor, J. T., Gatehouse, D. G. et al. (2000) In vivo rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring. Environ. Mol. Mutagen., 35, 234–252.
Hayashi, M., MacGregor, J. T., Gatehouse, D. G. et al. (2007) In vivo erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, withdiscussion of non-hematopoietic target cells and a single dose-level limit test. Mutat. Res., 627, 10–30.)].

3. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

690 chemicals from 'FDA SAR Genetox Database'; Leadscope Inc. 178 are micronucleus positive and 512 are micronucleus negative.

4. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

The model is based on both existing hypotheses on the mechanisms of toxic action (by e.g. checking the relative influence on micronucleus induction of DNA reactivity and protein binding) and on a structural analysis of the chemicals tested in the assay.

The rulebase consists of the Benigni-Bossa mutagenicity-carcinogenicity alerts, with the exclusion of the alerts specific for non-genotoxic carcinogenicity – 30 alerts, and five additional alerts associated with a few suggested mechanisms related with in vivo micronucleus (e.g. mitotic spindle poisoning, topoisomerase II inhibition)

5. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

The authors reported an accuracy of prediction around 57%

6. *External statistic, if available*

Not available

7. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

Not available

8. *Mechanistic interpretation of the model*

The structural alerts included in the model relate with the mechanisms of action suggested by the authors: DNA damaging, mitotic spindle poisoning or topoisomerase II inhibition. The latter effects are likely related to interference with proteins.

9. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

Not available

10. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Not available

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/

Documentation of DEREK Nexus Chromosome damage model

1. *Used model (title, name of authors, reference)*

DEREK Nexus chromosome damage Model v 4.0.6.

Lhasa Ltd, Leeds, UK, http://www.lhasalimited.org/

Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. Human and Experimental Toxicology 10, 261-273.
Judson PN, Marchant CA & Vessey JD (2003) Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370.
Marchant CA, Briggs KA & Long A (2003). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177–187.
Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79.

2. *Information about modelled endpoint (endpoint, experimental protocol)*

The Derek Nexus model for chromosome damage is developed from several sources of data. Sources of primary data used for alert development include in vitro and in vivo chromosome aberration test, in vitro and in vivo micronucleus test, in vitro L5178Y TK+/- assay. Alert writers consider both mechanistic evidence and chemical properties (such as reactivity). Depending on evidence in vitro and/or in vivo prediction can be made.

3. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The DEREK model for chromosome damage is base of rules which codified the knowledge about the relation between a structural features and a toxicological (i.e. mutagenic) effect. Although almost all alerts are related with mechanistic explanation and examples, these rules are not related with particular training set.

4. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Derek Nexus is a rule-based expert system for the prediction of toxicity. Its knowledge base is composed of alerts, examples and reasoning rules which may each contribute to the predictions made by the system. Each alert in Derek describes a chemical substructure believed to be responsible for inducing a specific toxicological outcome (often referred to as a toxicophore). Alerts are derived by experts, using toxicological data and information regarding the biological mechanism of action. Where relevant, metabolism data may be incorporated into an alert, enabling the prediction of compounds which are not directly toxicity but are metabolised to an active species. The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible. In addition a likelihood is provided (ie certain, probable, plausible, equivocal and nothing to report) which takes into account the presence of a structural alert and a limited number of molecular descriptors.

5. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

Derek is a knowledge-based expert system containing mechanistically-based rules which are built using all the underlying evidence available to the SAR developer. Therefore, there is no defined training or test set, and therefore there are no internal validation statistics to report.

6. *External statistic, if available*

Not public available

7. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The scopes of the structure-activity relationships describing the chromosome damage endpoint are defined by the developer to be the applicability domain for the model. Therefore, if a chemical activates an alert describing a structure activity for chromosome damage it can be considered to be within the applicability domain. If a compound does not activate an alert or reasoning rule in Derek, a result of 'nothing to report' is presented to the user. This can be interpreted as a negative prediction or that the query compound is outside the domain of the model. Which of these is more appropriate may depend on the endpoint of interest.

8. *Mechanistic interpretation of the model*

All alerts describing structure-activity relationships for the chromosome damage endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target. The mechanistic basis of the model was developed a priori by examining the active and inactive structures before developing the structure-activity relationship. All references supporting the mechanistic basis of an alert are detailed and available for inspection within the software.

9. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible

10. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Described above

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/

Step 5.3: Description of results, toxicological analysis of predicted effects and applicability domain

Table 3 ***Prediction of genotoxicity (gene mutation - CAESAR and DEREK Nexus Mutagenicity models and chromosomal aberrations - Toxtree – in vivo micronucleus model and DEREK Nexus in vitro human and mammalian chromosomal damage models) of minor rat and plant specific metabolites by (Q)SAR***

Metabolite	**CAESAR prediction of gene mutation (Applicability Domain)**	**DEREK Nexus prediction of gene mutation (no Applicability Domain evaluation is available)**	**Toxtree prediction of *in vivo* micronucleus (no Applicability Domain evaluation is available)**	**DEREK Nexus prediction of *in vitro/in vivo* Chromosome damage (human and mammalian)**
Parent	Not applied	Not applied	Not applied	Not applied
M02	Negative (Could be out)	Negative	Negative	Nothing to report
M05	Negative (Could be out)	Negative	Negative	Nothing to report
M06	Negative (Out)	Negative	Positive alert	Equivocal
M07	Negative (In)	Negative	Positive alert	Nothing to report
M08	Negative (Could be out)	Negative	Negative	Nothing to report
M09	Negative (In)	Negative	Negative	Nothing to report
M11	Negative (In)	Negative	Negative	Nothing to report

Metabolite	CAESAR prediction of gene mutation (Applicability Domain)	DEREK Nexus prediction of gene mutation (no Applicability Domain evaluation is available)	Toxtree prediction of *in vivo* micronucleus (no Applicability Domain evaluation is available)	DEREK Nexus prediction of *in vitro/in vivo* Chromosome damage (human and mammalian)
M12	Positive (Could be out)	Negative	Negative	Nothing to report
M14	Negative (Could be out)	Negative	Negative	Nothing to report
M15	Negative (In)	Negative	Negative	Nothing to report
M16	Positive (Could be out)	Negative	Positive alert	Equivocal
M18	Negative (In)	Negative	Negative	Nothing to report

CAESAR Mutagenicity model predicts 10 out of 12 metabolites as negative (non-mutagenic): metabolites M02, M05, M06, M07, M08, M09, M11, M14, M15 and M18. One of the metabolites M06 is out of the model applicability domain, four of them M02, M05, M08 and M14 could be out of model applicability domain and five – M07, M09, M11, M15 and M18 are into model applicability domain.

Two of the metabolites – M12 and M16 are predicted as potential mutagenic. Both substances could be out of the model applicability domain. Additional analysis of the six most similar substances from the training set for the metabolite M16 shows that the similarity range is between 0.952 – 0.929. All of them are primary aromatic amines with a different numbers of small alkyl (methyl or ethyl) substituents in the aromatic ring. For all of them with one exception the experimental data are positive, which gives additional confidence of the positive prediction.

The same analyse for the metabolite M12 shows that in general similarity between the chemical of interest and the most similar substances from the training set is lower but still high enough (0.896 – 0.854), but experimental value for all substances (with one exception which is an aromatic amine) are non-mutagenic, which challenges the positive prediction for the metabolite M12.

DEREK Nexus Mutagenicity model predicts all 12 metabolites as negative (non-mutagenic).

Toxtree in vivo micronucleus model predicts 9 out of 12 metabolites as negative (there are not alerts for micronucleus) – M02, M05, M08, M09, M11, M12, M14, M15 and M18. No additional information is provided by the model.

Three of the metabolites are predicted as positive (at least one positive alert for micronucleus assay was found) – M06, M07 and M16. As a structural alert for the metabolite M06 and M16 is identified: Primary aromatic amine, hydroxyl amine and its derived esters (with restrictions). This is an alert related with potential DNA reactive agents who are known to be positive in the micronucleus assay. For the metabolite M07, the identified alerts is H-acceptor-path3-H-acceptor. According to the authors this alert represents a molecular framework that could account for non-covalent interactions with proteins or DNA. Such interactions, as in the case of DNA intercalation or groove binding, are potentially genotoxic. However, the positive prediction value of this alert reported by the authors (Benigni, 2010) is low 34%.

DEREK Nexus in vitro human and mammalian chromosomal damage models predict equivocal results for the metabolites M06 and M16 due to an aniline or alkylaniline moieties in the molecule.

Step 5.4: Conclusion

(Q)SAR assessment identified a potential of metabolites M06, M07, M12 and M16 to provoke genotoxic effects. Metabolites M02, M05, M08, M09, M11, M14, M15 and M18 are predicted as negative from all models. All metabolites are moved to the next step – read across analyses.

Step 6: Read across (OECD toolbox)[15]

Step 6.1: Read across

Both endpoints, gene mutation and chromosomal aberrations, should be evaluated by read across for all metabolites.

Molecular initiating events of relevance for this assessment are interaction with DNA and/or proteins. The profilers included in the OECD Toolbox which codified the structural alerts which are important for these two types of interactions are mechanistic profilers - DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers- DNA alerts for AMES, MN and CA by OASIS v1.3, In vitro mutagenicity (AMES test) alerts by ISS, In vivo mutagenicity (Micronucleus) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

Above mentioned profilers have been applied to metabolites M02, M05, M06, M07, M08, M09, M11, M12, M14, M15, M16 and M18 as chemicals of interest and to the parent substance and all majors rat metabolites, which are considered characterised by the provided genotoxicity studies, as substances with known experimental genotoxic activity.

In order to evaluate the structural similarity, in addition to the structural alerts related to the evaluated endpoints, organic functional group profiler has been applied. This additional step will provide information on the presence/ absence of other functional groups different to the structural alerts and will give indication for the potential influence of the remaining part of the molecule to the relevant structural alerts (i.e. electronic and structural influence).

No structural alerts in the parents substance and in all evaluated metabolites were found by Protein binding by OASIS v 1.3, Protein binding by OECD, Protein binding alerts for Chromosomal aberrations by OASIS v1.1. profilers.

The alerts found by DNA binding by OASIS v.1.3, DNA binding by OECD and endpoint specific profilers - DNA alerts for AMES, MN and CA by OASIS v1.3, In vitro mutagenicity (AMES test) alerts by ISS, In vivo mutagenicity (Micronucleus) alerts by ISS and organic functional group are presented in the Table 4.

[15] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm

Table 4 ***Genotoxicity profiling of isoproturon metabolites by OECD Toolbox***

	DNA binding by OASIS v1.3		DNA binding by OECD			DNA alerts for AMES, MN and CA by OASIS v1.3		In vitro mutagenicity (AMES test) by ISS	in vivo mutagenicity (MN) by ISS		Organic functional groups						
	alert 1	alert 2	alert 3	alert 4	alert 5	alert 6	alert7	alert 8	alert 9	alert 10	Alkyl arenes	Iso-propyl	Alco-hol	Carbo xylic acid	Aryl	Urea deri-vates (SA)	Aniline
Parent			**x**	**x**					**x**		**x**	**x**			**x**	**x**	
M02				x					x		x	x			x	x	
M03				**x**					**x**				**x**		**x**	**x**	
M04				**x**					**x**				**x**		**x**	**x**	
M05				x					x					x	x	x	
M06	x	x			x	x	x	x		x				x	x		x
M07			x	x					x				x		x	x	
M08			x	x					x				x		x	x	
M09			x	x					x				x		x	x	
M10				**x**					**x**		**x**	**x**			**x**	**x**	
M11				x					x				x		x	x	
M12			x	x					x				x		x	x	
M13				**x**					**x**				**x**		**x**	**x**	
M14				x					x					x	x	x	
M15				x					x				x		x	x	
M16	x	x			x	x	x	x		x	x	x			x		x
M18			x	x					x					x	x	x	

alert 1- Radical mechanism via ROS formation (indirect): Single-ring substituted primary aromatic amines
alert 2- Nucleophilic attack after metabolic activation: Single-ring substituted primary aromatic amines
alert 3 - SN1: Iminium Ion Formation, Aliphatic tertiary amines
alert 4 - SN1: Nitrenium Ion formation, Aromatic phenylureas
alert 5 –Nitrenium ion formation: Primary aromatic amines
alert 6 - Radical mechanism via ROS formation (indirect): Single-ring substituted primary aromatic amines
alert 7 - Nucleophilic attack after metabolic activation: Single-ring substituted primary aromatic amines
alert 8 - Primary aromatic amines, hydroxyl amines and derived esters (genotox)
alert 9 - H-acceptor-path3-H-acceptor
alert 10 - Primary aromatic amines, hydroxyl amines and derived esters
bold: compounds covered in their genotoxicological properties by studies with parent
grey shaded: predicted as a potential mutagen by one of the two Ames/(Q)SAR models or predicted as potentially positive for chromosomal aberration from one of the two (Q)SAR model.

Analyses of the results from the read across for the metabolites predicted as negative by the (Q)SAR models

Alerts 4 and 9 are present in all evaluated metabolites as well as in the parent substance and all major rat metabolites (bold in Table 4). Alert 3 is present in three of the metabolites of evaluation M08, M09 and M18 and in the parent substance. Therefore the alerts are considered covered by the experimental studies and not relevant for the metabolites in terms of the genotoxicity. No other alerts were identified

Secondary profiler gives no hints on additional organic functional groups of concern. The changes in the structural features are related with demethylation of the methylurea group (M02, M05, M11, M14 and M15) and/or hydroxylation (M02, M08, M09, M11 and M15) or carboxylation (M05, M14 and M18) of the side alkyl chain on the 4th position. Demethylation and hydroxylation are observed in the major rat metabolites and therefore the metabolites M02, M08, M09, M11 and M14 are considered similar to the parent substance and the major rat metabolites, thus not of genotoxic concern. The carboxyl group is not detected in any of the major rat metabolites but considering that the group is not recognised as a structural alert for genotoxicity and it is outlying from the structural alert for genotoxicity (methylurea group), it is considered that the group could not lead to activation of the structural alert, therefore M05, M14 and M18 are not considered of genotoxic concern.

Analyses of the results from the read across for the metabolites predicted as positive by the (Q)SAR models

Alerts 3, 4, and 9 present in the parent substance and the major rat metabolites are identified also in the metabolites M07 and M12. No new alerts were identified. In both metabolites OH group(s) appeared as a result of metabolism of the alkyl chain on the 4th position in comparison with the parent substance. The organic functional hydroxy group of metabolites M07 and M12, which is not present in the parent, is found in metabolites M03 and M04, both major in the rat (sum 75 % of AD). Based on the read across analysis, metabolites M07 and M12 could be regarded as very similar to the parent substance and its major rat metabolites. Metabolites M07 and M12 are concluded to be of no genotoxicity concern.

Alerts 3, 4, and 9 present in the parent substance and the major rat metabolites are not present in the metabolites M06 and M16, but almost all applied profilers identified aromatic primary amine present in their structure as a potential alert for genotoxicity (DNA binding by OASIS v 1.3, DNA binding by OECD, In vivo mutagenicity (Micronucleus) alerts by ISS, DNA alerts for AMES, MN and CA by OASIS v1.3). This alert is not present neither in the parent substance nor in the major rat metabolites and together with the positive prediction of the (Q)SAR models the possibility for genotoxic potential cannot be excluded. To exclude an unacceptable dietary risk by potentially genotoxic metabolites, either a combined exposure estimate and comparison against TTC can be performed (step 7) or metabolites M06 and M16 would need to be tested (step 8).

Step 6.2: Conclusion

Metabolites M02, M05, M08, M09, M11, M14, M15 and M18 are not predicted to be of concern for genotoxicity.

Metabolite M12 is predicted as a potential mutagen by one of the two Ames/(Q)SAR models. Metabolite M07 is predicted as potential positive for chromosomal aberration from one of the two (Q)SAR model. However, both metabolites (M12 and M07) are not considered of genotoxicity concern following read-across analysis.

Metabolite M06 was predicted positive for *in-vivo* micronucleus and “equivocal” following prediction for *in-vitro* chromosome aberration.

Metabolite M16 was predicted as a potential mutagen by one of the two Ames/(Q)SAR models and predicted positive for in-vivo micronucleus and "equivocal" following prediction for in-vitro chromosome aberration.

For **metabolites M06 and M16,** genotoxicity concern cannot be excluded following read-across analysis. In addition, both metabolites M06 and M16 are predicted to be of potential concern for the same end point of genotoxicity ie *in-vivo* micronucleus and *in-vitro* chromosome aberration; therefore, combined exposure for metabolites M06 and M16 should be calculated and assessed against TTC for genotoxicity (step7) and/or testing (step 8).

Step 7: Generation of input data and combined exposure assessment against $TTC_{genotoxicity}$

The uses in Table 5 are considered in the exposure estimate to be used in the TTC assessment. Regulatory decisions based on specific exposure estimates are therefore restricted to the particular GAP conditions considered.

Table 5 Uses considered for exposure estimates

Crop	Application			
	Growth stage	**Number**	**kg as/ha**	**PHI**
Cereals (wheat, barley, rye, triticale)	BBCH 00-32	1	1.5	not relevant

Step 7.1: Derivation of residue input data for metabolites16

a) Residue levels in primary crop (cereal) and in groundwater

- Metabolite identification was performed in winter wheat plant parts treated with ^{14}C-isoproturon at 1.875 kg/ha (ca. 1.25 N rate) post-emergence 5 months after planting.
- Residue situation for pre-emergence use in cereals has been deemed addressed by the 30 days plant-back interval investigated in the rotational crop study (see point b)
- Residues in grain are relevant for consumer and livestock exposure; residues in straw are relevant for livestock exposure calculation; residues in forage are not relevant for livestock exposure (restriction in the GAP for grazing livestock on the treated crop), chaff and stubble are neither relevant for quantitative consumer or livestock exposure calculation.
- Residue levels of metabolites in grain were adopted from the metabolism study, while values for straw were calculated by applying the parent/metabolite ratio of the metabolism study to field trial data for parent. A conversion factor can only be successfully applied if quantifiable parent residue values are available; this was not (or hardly) the case for the field trial data for grain, where in 82 out of 89 residue trials parent levels were below the LOQ. The untransformed metabolism data for grain were therefore considered more adequate for exposure estimation of metabolites, while for straw the field data were used to estimate exposure.
- Residues in groundwater were relevant for quantitative consumer exposure calculation and comparison against the TTC (if applicable)

[16] Based on metabolism and residue data in the Assessment Report (Germany, 2014)

b) Residue levels in rotational crops

- Rotational crop metabolism study was performed at 1N rate (bare soil application) and sowing of rotational crops at 30 day plant-back interval (PBI), 210 day PBI and 320 day PBI.
- No field study is available; no conversion was required to account for differences in application rate or accumulation (1N study). Thus, metabolite data were directly used for exposure estimates.
- Crop groups covered: Cereals (grain, straw), root crops (turnip root and leaf), leafy crops (Swiss chard; 210 and 320 d PBI only). Additional crop groups (oilseed; fruiting vegetables) are not considered relevant.
- 30 day PBI (cereals): Data are considered suitable for the evaluation of GAP compliant pre-emergence application due to the 1N application rate, bare soil application, and sowing shortly after application. It is therefore considered appropriate to evaluate a possible GAP residue situation, to derive the residue definition and to provide input values for dietary risk assessment and livestock burden calculation.
- 30 day PBI (root/tuber and leafy crops): The case of crop failure in cereals upon herbicide use could lead to a residue situation relevant for acute dietary exposure scenarios) and is therefore relevant for genotoxicity screening. For general toxicity assessment, only the chronic scenario is relevant for the consumer as no ARfD is proposed for isoproturon and its metabolites.[17] It is assumed that residues after crop failure in root/tuber and leafy crops do not contribute to a significant long-term dietary burden of livestock animals relevant for dietary risk assessment. Therefore, residues from 30 d PBI (root/tuber, leafy crops) do not need to be considered for risk assessment.
- 214/312 day PBI (all crops): The rotation crop residue data represent realistic replanting scenarios and are considered for exposure assessment.

[17] The status of the evaluation of Isoproturon at the time of 2014 was considered during the development of the case. It is noted that an ARfD was set for Isoproturon later in 2015.

Table 6 ***Residue levels of isoproturon and metabolites in a primary crop winter wheat metabolism study, in a field study and in groundwater for the relevant application rate***

	Wheat, primary crop									
	Straw				***Grain***				***Groundwater***	
	Metabolism study 1.25 N			Field data	Metabolism study 1.25 N			Field data	PECgw FOCUS [#]	
	TRR		CF	HR/HRc	TRR		CF	STMR/ HRc		
	%	**mg [equ]/kg**		**mg [equ]/kg**	**%**	**mg [equ]/kg**		**mg [equ]/kg**	**µg/L**	**µg [equ]/L**
		5.197				0.088				
Parent	17.8	0.923	1.00	0.15	3.3	0.003	1.00	0.010		
M02										
M03	3.9	0.203	0.22	0.033						
M04	6.3	0.329	0.36	0.053	1.2	0.001	0.33	N/A		
M05	1.9	0.101	0.11	0.016	19.3	0.017	5.67	N/A		
M06	3.4	0.179	0.19	0.029	0.6	0.001	0.33	N/A		
M07	4.3	0.225	0.24	0.037	5.4	0.005	1.67	N/A		
M08	3.3	0.170	0.18	0.028	2.2	0.002	0.00	N/A	11.682	10.841
M09									2.838	2.634
M10	3.1	0.160	0.17	0.026					1.712	1.981
M11										
M12										
M13										
M14										
M15										
M16										
M18									7.227	6.310

[equ] Isoproturon equivalents

PECgw - FOCUS modelling results (80th percentile annual average concentration at 1 m) – pre-emergence application in wheat of 1500 g a.s./ha; highest predicted concentration across all scenarios

Table 7 ***Residue levels of isoproturon metabolites in rotational crops following application of 1566 g a.s./ha (1N)***

	Wheat									
	30 days		213 days		30 days		213 days		324 days	
	Grain		Grain		Straw		Straw		Straw	
	TRR 0.106 mg/kg		TRR 0.019 mg/kg		TRR 2.089 mg/kg		TRR 0.187 mg/kg		TRR 0.127 mg/kg	
	%	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg
Parent					0.3	0.006				
M02										
M03	3.6	0.004	3.7	0.0007	15.5	0.323	9.9*	0.019	13.7	0.017
M04										
M05										
M06										
M07a			1.6	0.0003	4.9	0.102	6.5	0.012	4.9	0.006
M07b					2.6	0.055			2.5	0.003
M08					1.1	0.023				
M09	5.1	0.005	1.5	0.0003	24.2	0.505	2.4	0.005	13.8	0.018
M10										
M11	4.4	0.005	7.5	0.0014	7.1	0.149	11.2	0.021	9.4	0.012
M12	2.6	0.003	4.3	0.0008	4.7	0.098	4.7	0.009	5.6	0.007
M13			1.6	0.0003	2.9	0.061	6.2	0.012	5.1	0.006
M14										
M15										
M16										
M18										

* Re-calculated 10.2% TRR based on the available values

Table 7 (cont.)

	Swiss chard				Turnip									
	213 days		324 days		30 days **				213 days				324 days	
	mature		mature		roots		leaves		roots		leaves		leaves	
	TRR 0.045 mg/kg		TRR 0.027 mg/kg		TRR 0.116 mg/kg		TRR 0.948 mg/kg		TRR 0.012 mg/kg		TRR 0.029 mg/kg		TRR 0.025 mg/kg	
	%	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**	**%**	**mg/kg**
Parent					2.9	0.003	0.4	0.004						
M02					1.2	0.001	0.7	0.006						
M03	22.5	0.010	14.6	0.004	3.9	0.005	36.2	0.343	2.3	<0.001 (0.0003)*	25.7	0.008	26.8	0.007
M04														
M05														
M06														
M07a	3.8	0.002					3.3	0.032			3.8	0.001		
M07b	1.5	0.001			5.2	0.006	4.2	0.040	2.8	<0.001 (0.0003)*				
M08	3.7	0.002			1.4	0.002	1.2	0.011						
M09	15.7	0.007	24.5	0.007	28.9	0.034	36.8	0.348	12.2	0.002	16.1	0.005	29.2	0.007
M10														
M11	7.4	0.003	12.3	0.003	1.4	0.002	2.0	0.019	1.2	<0.001 (0.0001)*	10.8	0.003	6.6	0.002
M12	3.3	0.001					0.5	0.004			2.1	0.001		
M13	5.4	0.002			2.9	0.003	2.9	0.027	2.3	<0.001 (0.0003)*	5.2	0.002	3.7	0.001
M14														
M15														
M16														
M18														

* calculated based on TRR

** considered only relevant in case of acutely toxic compounds. Not relevant for isoproturon at the status of the evaluation considered in this case study.

Step 7.2: Combined exposure calculation for those metabolites, for which genotoxic effects cannot be excluded

Table 8 Exposure calculation[18] and TTC assessment of metabolites with potential genotoxicity concern

Metabolite	Root crops	Cereal grain	Leafy crops	Groundwater
	mg/kg	mg/kg	mg/kg	µg/L
M06	-	0.0010	-	-
M16	-	-	-	-
Sum of metabolites	**-**	**0.0010**	**-**	**-**

Chronic exposure (most critical diets)

Combined metabolite exposure for M06, M16

0.010 µg/kg bw/d (DK child)	= 413 % $TTC_{genotoxicity}$
0.009 µg/kg bw/d (WHO Cluster diet B)	= 356 % $TTC_{genotoxicity}$
0.007 µg/kg bw/d (WHO Cluster diet D)	= 288 % $TTC_{genotoxicity}$

Individual metabolite exposure

M06: 0.000-0.020 µg/kg bw/d	= 0-413 % $TTC_{genotoxicity}$
M16: No exposure.	= 0 % $TTC_{genotoxicity}$

Acute exposure

Combined metabolite exposure for M06, M16

0.0145 µg/kg bw (wheat, children)	= 578 % $TTC_{genotoxicity}$
0.0078 µg/kg bw (wheat, adults)	= 313 % $TTC_{genotoxicity}$
0.0072 µg/kg bw (barley, adults)	= 290 % $TTC_{genotoxicity}$
0.0049 µg/kg bw (rye, adults)	= 194 % $TTC_{genotoxicity}$

Individual max. metabolite exposure

M06: 0.0145 µg/kg bw (wheat, children)	= 578 % $TTC_{genotoxicity}$
M16: No exposure.	= 0 % $TTC_{genotoxicity}$

Step 7.3: Conclusion

The combined exposure assessment for all metabolites, for which potential genotoxic effects cannot be excluded *a priori*, reveals an exceedance of the applicable TTC for genotoxicity of 0.0025 µg/kg bw/d.

Metabolite M06 individually exceeds the genotoxicity threshold for chronic as well as acute consumption data. Testing is required (step 8).

Metabolite M16 does not contribute to the consumer exposure since its occurrence is limited to primary crop non-food intermediate commodities (cereal forage) that are also not destined for animal feed. No extrapolation to other crops is required. Testing of metabolite M16 is not required.

[18] Exposure assessment performed with EFSA PRIMo rev.2

Step 8: Genotoxicity testing

No tests on metabolite M06 is available. As the prediction indicate a potential concern for the *in-vivo* micronucleus and *in-vitro* chromosome aberration (equivocal), metabolite M06 is recommended to be tested *in-vitro* to investigate structural and numerical chromosome aberration (e.g. TG 487). In addition, although metabolite M06 was predicted as negative for point mutation the analysis was considered potentially out of the chemical domain and the read-across showed a positive outcome for some alerts of relevance for this end point; therefore an Ames test (TG471) is also recommended.

Step 9: Genotoxicity concern

Additional information on metabolite M06 is required.

Step 10: General toxicity of metabolites characterized by studies with parent or by specific studies

Step 10.1: Toxicological assessment of parent compound

The ADI for the parent compound, isoproturon, was set at 0.015 mg/kg bw/d based on liver tumours in the rat carcinogenicity study.
Based on the tumour effect observed in rat the parent substance is classified as carcinogen Cat.2
An ARfD was considered as not necessary.[19]

Step 10.2: Toxicological assessment of metabolites

Metabolites M03, M04, M10 and M13 are considered covered in their toxicological properties by the studies with the parent. No further toxicological assessment is needed.

Step 11: Combined exposure of all metabolites to assess general toxicity (optional)

The TTC assessment is not applicable to the representative uses of isoproturon due to significant residues in animal feed items that may create additional consumer intakes via food of animal origin. Therefore, no comprehensive and sufficiently precise consumer exposure assessment can be performed. Moreover, already the consumer exposure via groundwater that could be used as drinking water exceeds the TTC for Cramer Class III.

Step 12: Consideration on potency

The ADI for the parent compound, isoproturon, was set at 0.015 mg/kg bw/d based on liver tumours in the rat carcinogenicity study. Based on the ADI value (>0.01 mg/kg bw/d), isoproturon and its metabolites are not considered of concern in terms of potency.
No additional consideration of potency is required.

[19] The status of the evaluation of Isoproturon at the time of 2014 was considered during the development of the case. It is noted that an ARfD was set for Isoproturon later in 2015.

Step 13: Assessment of major plant metabolites in food (≥10 % TRR (and at least 0.01mg/kg) OR ≥0.05 mg/kg)

Metabolites M03 and M05 are candidates for inclusion into the residue definition from food (extracted from Table 6 and 7), while M09 is a candidate only due to its occurrence in feed (see Step 7.1, point b).

Metabolite M03 is covered in its toxicological properties by parent compound studies, because is above 10% of AD in terms of total radioactive material recovered in the urine as detected in ADME studies.

Metabolite M05 was only observed in primary crops and is present in the rat metabolism study at a level in the urine of 8% of the administered dose. Toxicological assessment is needed (step 18)

Step 14: Assessment of minor plant metabolites in food

Based on the ADI value for parent (>0.01 mg/kg bw/d), metabolites defined as minor by their insignificant presence in food commodities are not considered of concern. Minor metabolites of substances not falling under the 'high potency' definition are usually not expected to significantly contribute to the toxicological dietary burden at the levels observed and no further toxicological or exposure assessment is needed. Metabolite M07 is a racemic mixture of two isomers. The sum of the two isomers is <10% of the TRR and <0.01 mg/kg and thus M07 is considered a minor metabolite of no concern.

Step 15: Assessment of major plant metabolites in feed (≥10% TRR and ≥0.01 mg/kg)

For the dietary burden calculation, only those compounds are considered that occur in at least one relevant commodity at≥10 % TRR (and at least 0.01 mg/kg)

This is the case for parent and metabolites M03, M05, M09 and M11 (see Table 6 and 7)

For the purpose of livestock dietary burden calculation the converted residue levels will be used, i.e. the calculation is specific for the representative uses (see Table 5).

Step 16: Potential of residue transfer from feed to livestock

The dietary burden calculation as decision tool for the requirement of an animal metabolism study has to consider highest likely residues of the relevant compounds in feed items. This is also required for MRL setting in food of animal origin.

The residue situation for pre- and post-emergence use differs in terms of composition of the residue and the total amount. Both situations are considered equally relevant for the dietary burden calculation. Derivation of input data is described for primary crops in step 7.1 (incl. Table 6) and for rotational crops in step 7.2 (incl. Table 7).

Post-emergence use

The dietary burden calculation for the post-emergence scenario is summarised in Table 9a, consisting of field data (parent; grain/straw), values from the primary crop metabolism study (M05; grain) and converted field data (M05; straw). Rotational crop data are relevant for M03 (leafy crops; 213 d PBI).

Table 9a Dietary burden calculation for isoproturon and potentially relevant metabolites – post-emergence use scenario

Compound	Primary/Rotational crops		Rotational crops	Contribution to livestock burden		
	Cereal grain	Cereal straw	Leafy			
	mg/kg	mg/kg	mg/kg	Diet	mg/kg bw/d	%
Post-emergence scenario						
Isoproturon	0.010	0.150	-	Lamb Ewe Layer	0.004 0.004 0.002	80 81 55
M05	0.017	0.016	-	Lamb Ewe Layer	0.001 0.001 0.001	14 15 39
M03	-	-	0.010	Lamb Ewe Layer	<0.001 <0.001 <0.001	5 5 7
Sum	**0.027**	**0.166**	**0.010**	**Lamb** **Ewe** **Layer**	**0.005** **0.004** **0.003**	**100** **100** **100**

Pre-emergence use

The dietary burden calculation for pre-emergence scenario is summarised in Table 9b, consisting of wheat data from the rotational crop metabolism study, 30 day plant-back interval, as a surrogate for the assessed scenario (all compounds). Rotational crop data are relevant for M03 (leafy crops; 213 d PBI).

Table 9b ***Dietary burden calculation for isoproturon and potentially relevant metabolites – pre-emergence use scenario***

Compound	Primary/Rotational crops		Rotational crops	Contribution to livestock burden		
	Cereal grain	Cereal straw	Leafy			
	mg/kg	mg/kg	mg/kg	Diet	mg/kg bw/d	%
Pre-emergence scenario						
Isoproturon	-	0.006	-	Lamb Ewe Layer	<0.001 <0.001 <0.001	<1 <1 <1
M03	0.004	0.323	0.010	Lamb Ewe Layer	0.009 0.007 0.003	32 33 32
M09	0.005	0.505	0.007	Lamb Ewe Layer	0.015 0.011 0.004	51 51 50
M11	0.005	0.149	0.003	Lamb Ewe Layer	0.004 0.003 0.002	15 15 18
Sum	**0.014**	**0.983**	**0.020**	**Lamb** **Ewe** **Layer**	**0.028** **0.022** **0.009**	**100** **100** **100**

The trigger of 0.004 mg/kg bw/d for conduct of a livestock metabolism study is exceeded for ruminants (maximum 0.028 mg/kg bw/d) and poultry (maximum 0.009 mg/kg bw/d).

It is concluded based on all the information, that a new ruminant metabolism study with parent isoproturon is required. In case that the potentially relevant feed metabolites are not found in the metabolic pathway of isoproturon in animals in the livestock metabolism studies, further metabolism data might be required if the dietary burden of these metabolites is still significant.

Step 17: Major animal metabolites >10% TRR in food

No data available (data gap).

Step 18: Testing strategy, grouping and read-across

Metabolite M03 is covered in its toxicological properties by parent compound studies, because it is above 10% of AD in terms of total radioactive material recovered in the urine as detected in ADME studies.

Metabolite M05 is considered structurally similar to the parent and to the major rat metabolite - M03. The structural difference with M03 is the presence of a carboxyl group instead of the hydroxyl group in alkyl chain at 4th position. A carboxylic group can potentially produce idiosyncratic reactions through formation of acyl glucoronide reaction (Bailey M, Dickinson R, Acryl glucuronide reactivity in perspective: biological consequences, 2003, Chemico-Biological Interactions, 145, 117-137). Idiosyncratic reactions are unlikely to be captured by any additional testing and as M05 is occurring at the level of 8% in the rat urine, the toxicological data provided by the parent are considered to cover the toxicological assessment of M05.

Metabolite M09 is considered similar with the parent substance. The structural difference is a simple hydroxylation of the alkyl chain at 4th position; therefore parent reference values can be applied.

Metabolite M11 is considered covered by the toxicological data provided for the parent and occurrence of a major rat metabolite – M10. The structural difference with M10 is a simple hydroxylation of the alkyl chain at 4th position; therefore parent reference values can be applied.

Step 19: Assessment of consumer toxicological burden

Besides parent isoproturon, metabolites M03 (via leafy rotational crops) and M05 are candidates for inclusion into the residue definition for plants as they all occur at levels exceeding ≥10 % TRR (and at least 0.01 mg/kg) OR ≥0.05 mg/kg in terms of absolute levels in at least one food commodity (see Table 6 and 7, step 7).
Additional major metabolites in cereal straw (M09, M11) might become relevant pending finalisation of the assessment of their relevance in feed items for transfer into food of animal origin (livestock metabolism studies required).
Results of exposure assessment for the use in cereals and rotational crops and comparison against the overall toxicological burden in plant products are presented in Table 10.

Table 10 Residues of potential concern in food of plant and animal origin

		Cereals (pre-emergence)				Cereals (post-emergence)				Leafy RC (213 d)	
		Grain		Straw		Grain		Straw		Swiss chard	
Metabolite	RPF	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	1			0.3	0	3.3	10	17.8	40		
M03	1	3.6	23 [a]	15.5	24					22.5	36
M05	**1**					19.3	60	1.9	4		
M09	1	5.1	32 [a]	24.2	38					15.7	25 [a]
M11	1	4.4	28 [a]	7.1	11					7.4	12 [a]
Other (minor)	1 [b]	2.6	17	16.2	27	32.0	30	20.4	56	17.7	27
Sum of relevant metabolites	**1**	**Not relevant** [a]		47.1	**73**	22.6	**70**	19.7	**44**	22.5	**36**
Sum of non-considered metabolites	1 [b]			16.2	27	32.0	30	24.3	56	40.8	64

[a] Not relevant for the residue definition as metabolite is minor in food (<0.01 mg/kg in grain)
[b] Default assumption; no characterisation of general toxicological required for minor metabolites

The post-emergence scenario is considered relevant for setting the residue definition with parent and metabolite M05 as major contributors to the overall toxicological burden (70%, considering RPF 1). Metabolite M03 is relevant due to its occurrence in rotational leafy crops.

Additional metabolites (M03, M09, M11) might become relevant pending information on their potential transfer from feed to food of animal origin.

Step 20: Residue definitions

The proposed residue definition for risk assessment (expressed as isoproturon) in plants is

Cereals: **Parent, M05 (primary crops, provisional, pending closure of data gaps)**
Leafy crops: **M03 (rotational crops only)**

No residue definition for risk assessment in livestock can be proposed (data gap).

Data gaps

1. Genotoxic potential for metabolite M06 needs to be addressed by *in-vitro* test on structural and numerical chromosome aberration (e.g. OECD 487) and Ames test (OECD 471).
2. The applicability of parent reference values for M05 has to be evidenced. Alternatively, non-relevant exposure of M05 can be demonstrated by proven absence of residues under GAP conditions (residues in field trials below LOQ of 0.01 mg/kg).
3. Livestock exposure is significant under GAP conditions (ruminant, poultry). No acceptable livestock metabolism study with parent isoproturon is available to assess the potential for residues and their nature in food of animal origin. Unless the nature and quantity of residues in food of animal origin is known, no residue definition (animals) can be proposed.

Uncertainties of particular relevance for decision making

The finalisation of the evaluation of the uncertainties is underdevelopment pending adoption of the Scientific Committee guidance on uncertainty in scientific assessment.

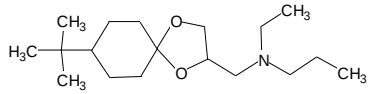
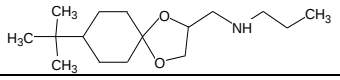
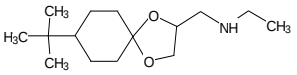
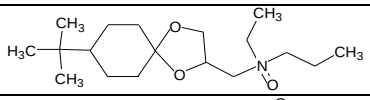
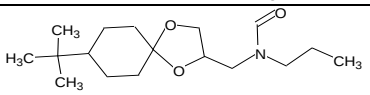
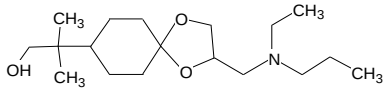
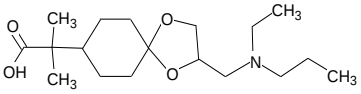
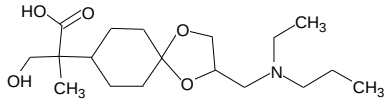
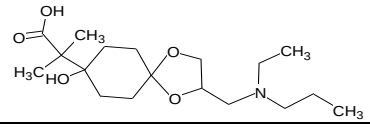
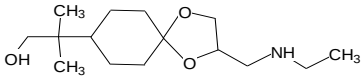
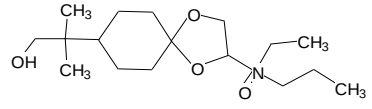
1. The residue definition in plants comprises 70 % of the total toxicological burden for consumer (only food of plant origin considered; pending information on the transfer from feed to food of animal origin). Although this is slightly below the target of 75% this has only a marginal impact on the calculated dietary consumer risk.
2. Grouping of metabolites is based on criteria for similarity. However, these criteria are not fully characterized. For genotoxicity endpoints, grouping on profiling and presence of functional groups was considered suitable for the purpose of risk assessment. Grouping of metabolites for section of representative substance for testing for general toxicity was based on common moiety and similarity in the chemical reactivity and this was considered appropriate for this purpose. However, some uncertainties still exist as no testing was performed.
3. Limitations in the assessment of metabolites (e.g. containing a carboxylic group in the structure) that can potentially produce reactive metabolites exist and this is recognized as an uncertainty.
4. Genotoxic alerts indicated by (Q)SAR for metabolites M07 and M12 are considered not relevant on the basis ofread-across. This bears a higher uncertainty compared to *in vitro* results according to the proposed testing scheme.
5. Groundwater exposure may increase the dietary intake of toxicological relevant residues M08, M09, M10 and M18. This is not considered to impact the proposal on which metabolites are relevant in food of plant and animal origin.

Appendix C. Case study – Spiroxamine (Germany, 2009)[20]

Step 1: Metabolite identified at any level in nature-of-residue studies

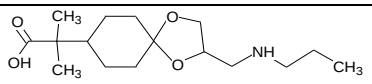
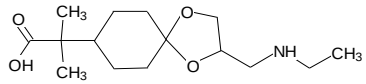
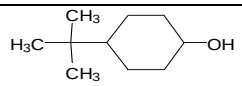
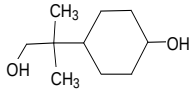
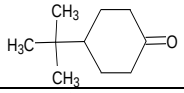
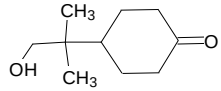
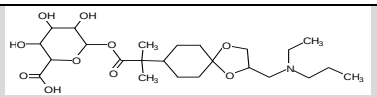
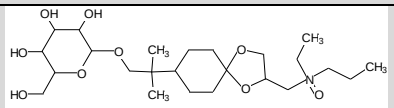
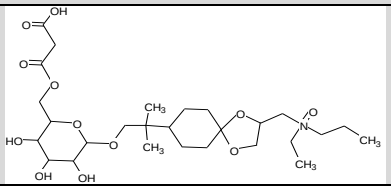
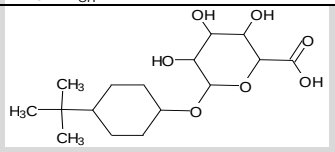
A list of metabolites detected in residue metabolism studies is given in Table 1. Conjugated metabolites (glycosides, glucuronides) are assumed to be covered in their toxicological properties by their respective aglycons[21].

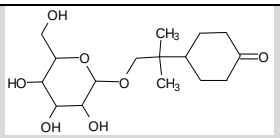
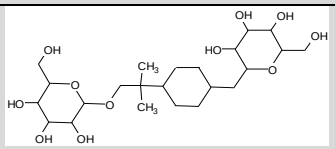
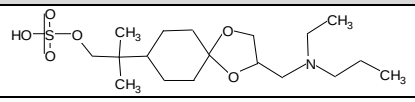
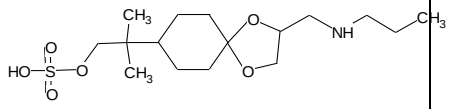
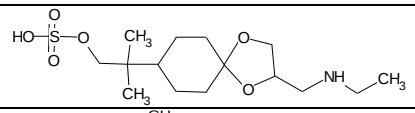
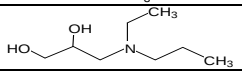
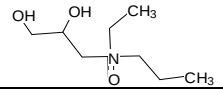
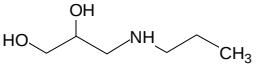
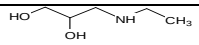
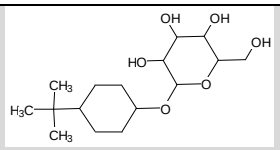
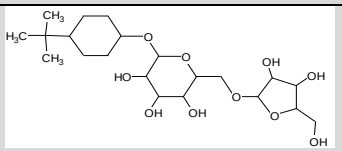
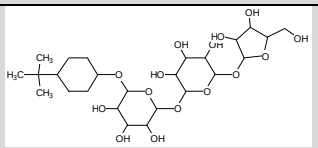
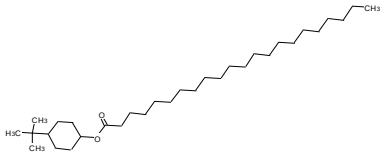
Table 1 Spiroxamine metabolites

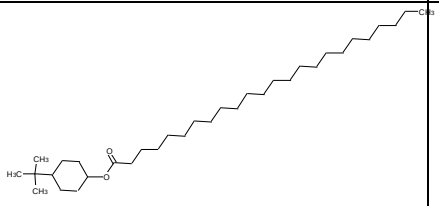
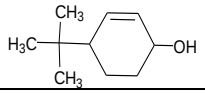
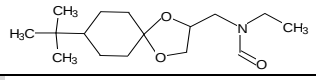
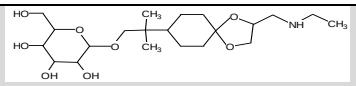
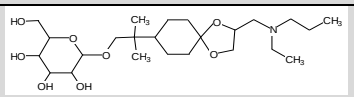
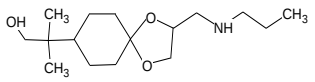
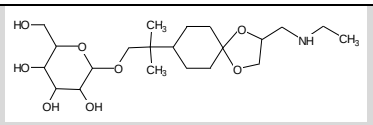
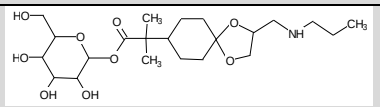
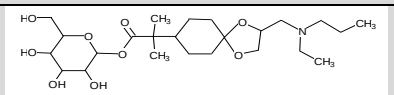
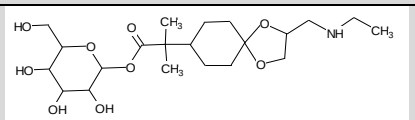
Compound identifier	Name in Study and Assessment reports and SMILES	Structure	Remark
Parent	Spiroxamine CCCN(CC)CC1COC2(CCC(C(C)(C)C)CC2)O1		
M01	Desethyl CCCNCC1COC2(CCC(C(C)(C)C)CC2)O1		
M02	Despropyl CCNCC1COC2(CCC(C(C)(C)C)CC2)O1		
M03	N-oxide CCCN(=O)(CC)CC1COC2(CCC(C(C)(C)C)CC2)O1		
M04	N-formyl-desethyl CCCN(CC1COC2(CCC(C(C)(C)C)CC2)O1)C=O		
M05	Hydroxyl CCCN(CC)CC1COC2(CCC(C(C)(C)C)CO)CC2)O1		M05 representative for conjugate M40, M25
M06	Acid CCCN(CC)CC1COC2(CCC(C(C)(C)C(O)=O)CC2)O1		M06 representative for conjugates M19, M44
M07	Hydroxy acid CCCN(CC)CC1COC2(CCC(C(C)(CO)C(O)=O)CC2)O1		
M08	8-hydroxy acid CCCN(CC)CC1COC2(CCC(O)(C(C)(C)C(O)=O)CC2)O1		
M09	Hydroxy-despropyl CCNCC1COC2(CCC(C(C)(C)C)CO)CC2)O1		M09 representative for conjugate M39, M27
M10	Hydroxy-N-oxide CCCN(=O)(CC)C1COC2(CCC(C(C)(C)C)CO)CC2)O1		M10 representative for conjugates M20, M21

[20] Germany 2009. Assessment Report on the active substance spiroxamine prepared by the rapporteur Member State Germany in consultation with Hungary in the framework of Commission Regulation (EC) No 737/2007, September 2009. Available at http://dar.efsa.europa.eu/dar-web/provision

[21] Greyed out in this table

Compound identifier	Name in Study and Assessment reports and SMILES	Structure	Remark
M11	Desethyl acid CCCNCC1COC2(CCC(C(C)(C)C(O)=O)CC2)O1		M11 representative for conjugate M43
M12	Despropyl acid CCNCC1COC2(CCC(C(C)(C)C(O)=O)CC2)O1		M12 representative for conjugate M45
M13	Cyclohexanol CC(C)(C)C1CCC(O)CC1		M13 representative for conjugates M22, M32, M33, M34, tentative for M35 and M36 (upon closing of data gap; step 20)
M14	Diol CC(C)(CO)C1CCC(O)CC1		M14 representative for conjugate M24
M15	Ketone CC(C)(C)C1CCC(=O)CC1		
M16	Hydroxy-ketone aglycon M23 CC(C)(CO)C1CCC(=O)CC1		M16 representative for conjugate M23
M19	Acid glucuronide CCCN(CC)CC1COC2(CCC(C(C)(C)C(=O)OC3C(O)C(O)C(O)C(C(O)=O)O3)CC2)O1		Toxicological assessment covered by M06
M20	Hydroxy-N-oxide glucoside CCCN(=O)(CC)CC1COC2(CCC(C(C)(C)COC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M10
M21	Hydroxy-N-oxide malonyl glucosid CCCN(=O)(CC)CC1COC2(CCC(C(C)(C)COC3C(O)C(O)C(O)C(COC(=O)CC(O)=O)O3)CC2)O1		Toxicological assessment covered by M10
M22	Cyclohexanol-glucuronide CC(C)(C)C1CCC(OC2C(O)C(O)C(O)C(C(O)=O)O2)CC1		Toxicological assessment covered by M13

Compound identifier	Name in Study and Assessment reports and SMILES	Structure	Remark
M23	Hydroxy-ketone-conjugate CC(C)(COC1C(O)C(O)C(O)C(CO)O1)C1CCC(=O)CC1		Toxicological assessment covered by M16
M24	Diol-diglycoside CC(C)(COC1C(O)C(O)C(O)C(CO)O1)C1CCC(CC2C(O)C(O)C(O)C(CO)O2)CC1		Toxicological assessment covered by M14
M25	Sulfate CCCN(CC)CC1COC2(CCC(C(C)(C)COS(O)(=O)=O)CC2)O1		
M26	Desethyl-sulfate CCCNCC1COC2(COC(C(C)(C)COS(O)(=O)=O)OC2)O1		
M27	Despropyl-sulfate CCNCC1COC2(CCC(C(C)(C)COS(O)(=O)=O)CC2)O1		
M28	Aminodiol CCCN(CC)CC(O)CO		
M29	Aminodiol-N-oxide CCCN(=O)(CC)CC(O)CO		
M30	Desethyl-aminodiol CCCNCC(O)CO		
M31	Despropyl-aminodiol CCNCC(O)CO		
M32	Cyclohexanol glucoside CC(C)(C)C1CCC(OC2C(O)C(O)C(O)C(CO)O2)CC1		Toxicological assessment covered by M13
M33	Cyclohexanol - glucopyranosyl-pentose CC(C)(C)C1CCC(OC2C(O)C(O)C(O)C(COC3C(O)C(O)C(CO)O3)O2)CC1		Toxicological assessment covered by M13
M34	Cyclohexanol-glucopyranosyl-glucopyranosyl-pentose CC(C)(C)C1CCC(OC2C(O)C(O)C(O)C(OC3C(O)C(O)C(O)C(COC4C(O)C(O)C(CO)O4)O3)O2)CC1		Toxicological assessment covered by M13
M35	Docosanoic acid ester CCCCCCCCCCCCCCCCCCCCCC(=O)OC1CCC(C(C)(C)C)CC1		

Compound identifier	Name in Study and Assessment reports and SMILES	Structure	Remark
M36	Tetracosanoic acid ester CCCCCCCCCCCCCCCCCCCCCCCC(=O)OC1CCC(C(C)(C)C)CC1		
M37	Cyclohexenol CC(C)(C)C1CCC(O)C=C1		
M38	N-formyl-despropyl CCN(CC1COC2(CCC(C(C)(C)C)CC2)O1)C=O		
M39	Hydroxy despropyl glycoside CCNCC1COC2(CCC(C(C)(C)COC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M09
M40	Hydroxy glycoside CCCN(CC)CC1COC2(CCC(C(C)(C)COC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M05
M41	Hydroxy-desethyl CCCNCC1COC2(CCC(C(C)(C)CO)CC2)O1		M41 representative for conjugate M42, M26
M42	Hydroxy-desethyl glycoside CCNCC1COC2(CCC(C(C)(C)COC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M41
M43	Desethyl acid glycoside CCNCC1COC2(CCC(C(C)(C)C(=O)OC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M11
M44	Acid glycoside CCCN(CC)CC1COC2(CCC(C(C)(C)C(=O)OC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M06
M45	Despropyl acid glycoside CCNCC1COC2(CCC(C(C)(C)C(=O)OC3C(O)C(O)C(O)C(CO)O3)CC2)O1		Toxicological assessment covered by M12

Step 2: Exclusion of metabolites of no concern

None.

Step 3: Metabolite is known to be genotoxic

No specific information on genotoxicity of metabolites is available.

Step 4: Metabolites genotoxicologically characterised

Step 4.1: Assessment of metabolites whether they are covered by studies with parent (Table 2) or by specific studies.

Genotoxicity studies on metabolite M03 indicated no genotoxic concern in the conditions described for *in vitro* testing conducted to explore genototoxicity endpoints i.e. point mutations and numerical and structural chromosome aberrations (DE, 2009).

Step 4.2: Conclusion

Proceed with metabolite genotoxicity assessment (steps 5 to 8) for all metabolites whose toxicological properties are not covered by parent compound (shaded in grey) or by specific studies.

Table 2 ***Assessment of occurrence of spiroxamine metabolites in toxicological studies with parent compound – major and minor rat metabolites***

		Occurrence in rat metabolism (% administered dose)	**Toxicological properties covered by studies with parent compound or by specific studies**
Parent	Spiroxamine		Yes
M01	Desethyl		No
M02	Despropyl		No
M03	N-oxide		Yes (specific studies)
M04	N-formyl-desethyl		No
M05	Hydroxyl		No
M06	Acid	24.3	Yes
M07	Hydroxy acid		No
M08	8-hydroxy acid	3.6	No
M09	Hydroxy-despropyl		No
M10	Hydroxy-N-oxide		No
M11	Desethyl acid	6.1	No
M12	Despropyl acid	4	No
M13	Cyclohexanol		No
M14	Diol		No
M15	Ketone		No
M16	Hydroxy-ketone		No
M25	Sulfate	1.4	No
M26	Desethyl-sulfate	3.2	No
M27	Despropyl-sulfate	3.1	No
M28	Aminodiol		No
M29	Aminodiol-N-oxide		No
M30	Desethyl-aminodiol		No
M31	Despropyl-aminodiol		No
M35	Docosanoic acid ester		No
M36	Tetracosanoic acid ester		No
M37	Cyclohexenol	0.8	No
M38	N-formyl-despropyl		No
M41	Hydroxy-desethyl		No

Step 5: (Q)SAR prediction of genotoxicity

Step 5.1: Description of (Q)SAR strategy

To predict the genotoxic potential (gene mutation and chromosomal aberrations) of the minor rat and plant specific metabolites four models have been applied. The CAESAR Mutagenicity Model v 2.1.12 - implemented in the VEGA software (v 1.0.8) and OASIS AMES Mutagenicity model (v08.08) implemented in the TIMES software (v2.27.13) for prediction of gene mutation; and a rule base model with the structural alerts for in vivo micronucleus- implemented in the Toxtree v.2.6.6. (Romualdo Benigni, Cecilia Bossa, Olga Tcheremenskaia and Andrew Worth, Development of structural alerts for the in vivo micronucleus assay in rodents", European Commission report EUR 23844) and OASIS

Chromosomal Aberration model (v08.08) implemented in the TIMES software (v2.27.13) for prediction of chromosomal aberrations.

Independently of the predictions from (Q)SAR models, the metabolite(s) will be subject of read across analysis (step 6).

Step 5.2: Documentation of prediction models

Documentation of CAESAR Mutagenicity model (VEGA software)

xxi) Used model (title, name of authors, reference)

CAESAR Mutagenicity Model v 2.1.12, Ferrari T., Gini G.

An open source multistep model to predict mutagenicity from statistical analysis and relevant structural alerts.Ferrari T., Gini G.Chemistry Central Journal 2010, 4(Suppl 1):S2 (29 July 2010)

xxii) Information about modelled endpoint (endpoint, experimental protocol)

Ames Mutagenicity essay.

xxiii) Used training set (number of the substances, information about the chemical diversity of the training set chemicals)

4204 compounds from the Kazius-Bursi mutagenicity database (Kazius J, Mcguire R, Bursi R: Derivation and validation of toxicophores for mutagenicity prediction.J Med Chem 2005, 48(1):312-320.), 2348 classified as mutagenic and *1856* classified as non-mutagenic by Ames test. 80% of the entire data set (3367 compounds) was used for the development of the model, while the other 20% (837 compounds) was used as a test (validation set).

xxiv) Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)

A mutagenicity classifier has been arranged integrating two different techniques: a machine learning algorithm from the Support Vector Machines (SVM) collection, to build an early model with the best statistical accuracy, then an ad hoc expert system based on known *structural alerts* (SAs)(Benigni-Bossa rule base), tailored to refine its predictions. The purpose is to prevent hazardous molecules misclassified in first instance (*false negatives*) from being labelled as safe. The resultant classifier can be presented as a cascading filters system: compounds evaluated as positive by SVM are immediately labelled *mutagenic*, whereas the presumed negatives are further shifted through two consecutive checkpoints for SAs with rising sensitivity. The first checkpoint (12 SAs) has the chance to enhance the prediction accuracy by attempting a precise isolation of potential *false negatives* (FNs); the second checkpoint (4 SAs) proceeds with a more drastic (but more prudent) FNs removal, as much as this doesn't noticeably downgrade the original accuracy by generating too many *false positives* (FPs) as well. To reinforce this distinction, compounds filtered out by the first checkpoint are labelled mutagenic while those filtered out by the second checkpoint are labelled suspicious: this label is a warning that denotes a candidate mutagen, since it has fired a SA with low specificity. Unaffected compounds that pass through both checkpoints are finally labelled non-mutagenic.

xxv) Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity

The authors reported accuracy of around 92% for the training set and around 82% for the test set.

xxvi) External statistic, if available

Not available

xxvii) Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)

The model provides evaluation of the reliability of the prediction which is in three steps scale: Compound is in model Applicability Domain, Compound could be out of model Applicability Domain and Compound is out of model Applicability Domain.

The Applicability Domain evaluation is based of combination of 5 Applicability Domain scores:

Similarity index – measure for the similarity between the predicted substance and training set substances with known experimental value;

Concordance – the similar substances found in the training set have (or have not) experimental values that are in agreement with the predicted value;

Accuracy – accuracy of prediction for similar molecules found in the training set

Atom centred fragments similarity check – all atom centred fragments of the substance are (are not) found in the list of atom centred fragments of the training set substances.

Model descriptor range check – descriptors for the substance have (or have not) values inside the descriptor range of the training set substances.

xxviii) Mechanistic interpretation of the model

Not available

xxix) Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)

The software provides six most similar substances from the training set with their experimental and predicted values.

xxx) Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties

Not available

Documentation of OASIS Ames Mutagenicity model (TIMES software)

1. Used model (title, name of authors, reference)

OASIS AMES mutagenicity model v08.08, Laboratory of mathematical chemistry, Burgas University

R. Serafimova, M. Todorov, T. Pavlov, S. Kotov, E. Jacob,A. Aptula, O. Mekenyan, Identification of the structural requirements for mutagencitiy by incorporating molecular flexibility and metabolic activation of chemicals. II. General Ames mutagenicity model. *Chem. Res. Toxicol*, 20, (2007), pp. 662−676.

2. Information about modelled endpoint (endpoint, experimental protocol)

Ames Mutagenicity essay.

3. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The training set consists of 3489 chemicals (NTP database) separated in three groups: 641 mutagenic chemicals as parents, 418 chemicals mutagenic after S9 metabolic activation (non mutagens as parents), and 2430 non mutagenic chemicals. These three classes of chemicals were considered as biologically dissimilar in the modeling process; i.e., chemicals being mutagenic as parents are distinguished from chemicals, which were metabolically activated

4. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

The TIMES system combines in the same modelling platform metabolic activation of chemicals and their interaction with target macromolecules. The reactivity Ames model (-S9) describing interactions of chemicals with DNA was based on an alerting group approach. Only those toxicophores having clear interpretation for the molecular mechanism causing the ultimate effect were included in the model. The alerts were classified as direct acting and metabolically activated. The mechanistic interrelation between alerts and related parametric ranges generalizing the effect of the rest of the molecules on the alert is also considered. In the Ames model (+S9), the reactivity component was combined with a metabolic simulator, which was trained to reproduce documented maps for mammalian (mainly rat) liver metabolism for 260 chemicals. Parent chemicals and each of the generated metabolites were submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals were predicted to be mutagenic as parents only, parents and metabolites, and metabolites only.

5. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

For 3489 chemicals, the Ames model (-S9) was able to predict correctly 82% of the Ames positive and 91% of the Ames negative training set chemicals. When metabolic activation is taken into account, the Ames model (+S9) predicts 76% of the Ames positive and 76% of the Ames negative training set chemicals.

6. *External statistic, if available*

Not available

7. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The stepwise approach was used to define the applicability domain of the model. It consists of the following sub-domain levels:

- General parametric requirements - includes ranges of variation log K_{OW} and MW,
- Structural domain - based on atom-centered fragments (ACFs).
- Interpolation space - estimates the population density of the parametric space defined by the explanatory variables of the QSAR models by making use the training set chemicals.
- Domain of simulator of metabolism - determines the reliability of the simulated metabolism.

A chemical is considered In Domain if its log K_{OW} and MW are within the specified ranges and if its ACFs are presented in the training chemicals. The information implemented in the applicability

domain is extracted from the correctly predicted training chemicals used to build the model and in this respect the applicability domain determines practically the interpolation space of the model.

S. Dimitrov, G. Dimitrova, T. Pavlov, N. Dimitrova, G. Patlevisz, J. Niemela and O. Mekenyan, *J. Chem. Inf. Model.* Vol. 45 (2005), pp. 839-849.

8. Mechanistic interpretation of the model

Each structural alert in the model is related with a suggested mechanism of action which is reported together with the prediction.

9. Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)

Not available

10. Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties

The model provided suggested mechanism of action, examples of the substances documented to have the mechanism of action, generation of metabolites and prediction for them, information for experimental observed metabolites (if available).

Documentation of rule based model on structural alerts for in vivo micronucleus assay (Toxtree software)

11. Used model (title, name of authors, reference)

Structural alerts for in vivo micronucleus implemented in the Toxtree v.2.6.6

Structural analysis and predictive value of the rodent in vivo micronucleus assay results. Benigni R, Bossa C, Worth A, Mutagenesis.2010 Jul;25(4):335-41

12. Information about modelled endpoint (endpoint, experimental protocol)

A large majority of the data are based on the analysis of micronuclei in bone marrow cells [for rationale of and details on the assay, see (Krishna, G. and Hayashi, M. (2000) In vivo rodent micronucleus assay: protocol, conduct and data interpretation. Mutat. Res., 455, 155–166.
Morita, T., Asano, N., Awogi.T et al. (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (groups 1, 2A and 2B) the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Collaborative Study of the Micronucleus Group Test. Mammalian Mutagenicity Study Group. Mutat. Res., 389, 3–122.
Hayashi, M., MacGregor, J. T., Gatehouse, D. G. et al. (2000) In vivo rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring. Environ. Mol. Mutagen., 35, 234–252.
Hayashi, M., MacGregor, J. T., Gatehouse, D. G. et al. (2007) in vivo erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test. Mutat. Res., 627, 10–30.)].

13. Used training set (number of the substances, information about the chemical diversity of the training set chemicals)

690 chemicals from 'FDA SAR Genetox Database'; Leadscope Inc. 178 are micronucleus positive and 512 are micronucleus negative.

14. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

The model is based on both existing hypotheses on the mechanisms of toxic action (by e.g. checking the relative influence on micronucleus induction of DNA reactivity and protein binding) and on a structural analysis of the chemicals tested in the assay.

The rulebase consists of the Benigni-Bossa mutagenicity-carcinogenicity alerts, with the exclusion of the alerts specific for non-genotoxic carcinogenicity – 30 alerts, and five additional alerts associated with a few suggested mechanisms related with in vivo micronucleus (e.g. mitotic spindle poisoning, topoisomerase II inhibition)

15. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

The authors reported sensitivity 66%, specificity 54% and concordance (overall accuracy) around 57%

16. *External statistic, if available*

Not available

17. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

Not available

18. *Mechanistic interpretation of the model*

The structural alerts included in the model are related with suggested by the authors mechanisms of action: DNA damaging, mitotic spindle poisoning or topoisomerase II inhibition. The latter effects are likely related to interference with proteins.

19. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

Not available

20. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Not available

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/

Documentation of OASIS *in vitro* chromosomal aberration model (TIMES software)

1. *Used model (title, name of authors, reference)*

OASIS in vitro chromosomal aberration model v08.08, Laboratory of mathematical chemistry, Burgas University

O. Mekenyan, M. Todorov, R. Serafimova, S. Stoeva, A. Aptula, R. Finking, E. Jacob, Identifying the structural requirements for chromosomal aberration by incorporating molecular flexibility and metabolic activation of chemicals. *Chem. Res. Toxicol.* Vol. 20, (2007), pp. 1927−1941.

2. *Information about modelled endpoint (endpoint, experimental protocol)*

In vitro structural chromosomal aberrations

3. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The training set consists of 506 chemicals separated in three groups: 243 mutagenic chemicals as parents, 77 chemicals mutagenic after S9 metabolic activation (non mutagens as parents), and 186 non mutagenic chemicals

Sofuni, T., Ed. (1998). Data Book of Chromosomal Aberration Test in vitro, Revised Edition. Life-Science Information Center, Tokyo, Japan.

4. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Modeling the potential of chemicals to induce chromosomal damage has been hampered by the diversity of mechanisms which condition this biological effect. The direct binding of a chemical to DNA is one of the underlying mechanisms that is also responsible for bacterial mutagenicity. Disturbance of DNA synthesis due to inhibition of topoisomerases and interaction of chemicals with nuclear proteins associated with DNA (e.g., histone proteins) were identified as additional mechanisms leading to CA. Reactivity component of the CA model (-S9) describing interactions of chemicals with DNA and/or proteins was based on an alerting group approach. Only those toxicophores having clear interpretation for the molecular mechanism causing the ultimate effect were included in the model. Some of the specified alerts interact directly with DNA or nuclear proteins, whereas others are applied in a combination of two-dimensional QSAR models assessing the degree of activation of the alerts from the rest of the molecules. In the CA model (+S9), the reactivity component was combined with a metabolic simulator, which was trained to reproduce documented maps for mammalian (mainly rat) liver metabolism for 260 chemicals. Parent chemicals and each of the generated metabolites were submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals were predicted to be mutagenic as parents only, parents and metabolites, and metabolites only.

5. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

For 506 chemicals, the CA model (-S9) was able to predict correctly 79% of the CA positive and 87% of the CA negative training set chemicals. When metabolic activation is taken into account, the CA model (+S9) predicts 81% of the CA positive and 75% of the CA negative training set chemicals.

6. *External statistic, if available*

Not available

7. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The stepwise approach was used to define the applicability domain of the model. It consists of the following sub-domain levels:

- General parametric requirements - includes ranges of variation log KOW and MW,
- Structural domain - based on atom-centered fragments (ACFs).
- Interpolation space - estimates the population density of the parametric space defined by the explanatory variables of the QSAR models by making use the training set chemicals.
- Domain of simulator of metabolism - determines the reliability of the simulated metabolism.

A chemical is considered In Domain if its log K_{OW} and MW are within the specified ranges and if its ACFs are presented in the training chemicals. The information implemented in the applicability domain is extracted from the correctly predicted training chemicals used to build the model and in this respect the applicability domain determines practically the interpolation space of the model.

S. Dimitrov, G. Dimitrova, T. Pavlov, N. Dimitrova, G. Patlevisz, J. Niemela and O. Mekenyan, *J. Chem. Inf. Model.* Vol. 45 (2005), pp. 839-849.

8. Mechanistic interpretation of the model

Each structural alert in the model is related with a suggested mechanism of action which is reported together with the prediction.

9. Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)

Not available

10. Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties

The model provided suggested mechanism of action, examples of the substances documented to have the mechanism of action, generation of metabolites and prediction for them, information for experimental observed metabolites (if available).

Step 5.3: Description of results, toxicological analysis of predicted effects and applicability domain

Table 3. Prediction of genotoxicity (gene mutation - CAESAR and OASIS AMES model; chromosomal aberrations – rule based in vivo micronucleus and OASIS CA model) of minor rat and plant specific metabolites by (Q)SAR

		CAESAR prediction of gene mutation (Applicability Domain)	**OASIS prediction of gene mutation (Applicability Domain)**	**Rule based model for prediction of *in vivo* CA (Toxtree) (no Applicability Domain evaluation is available)**	**OASIS prediction of CA (Applicability Domain)**
M01	Desethyl	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M02	Despropyl	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M04	N-formyl-desethyl	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M05	Hydroxyl	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)

		CAESAR prediction of gene mutation (Applicability Domain)	**OASIS prediction of gene mutation (Applicability Domain)**	**Rule based model for prediction of *in vivo* CA (Toxtree) (no Applicability Domain evaluation is available)**	**OASIS prediction of CA (Applicability Domain)**
M07	Hydroxy acid	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M08	8-hydroxy acid	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M09	Hydroxy-despropyl	Positive (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M10	Hydroxy-N-oxide	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M11	Desethyl acid	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M12	Despropyl acid	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M13	Cyclohexanol	Negative (In)	Negative (In)	Negative	Negative (out)
M14	Diol	Negative (In)	Negative (In)	Negative	Negative (In)
M15	Ketone	Negative (Could be out)	Negative (In)	Negative	Negative (out)
M16	Hydroxy-ketone	Negative (In)	Negative (In)	Negative	Negative (out)
M25	Sulfate	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M26	Desethyl-sulfate	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M27	Despropyl-sulfate	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M28	Aminodiol	Negative (In)	Negative (In)	Positive alert for CA	Negative (In)
M29	Aminodiol-N-oxide	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)
M30	Desethyl-aminodiol	Negative (Could be out)	Negative (In)	Positive alert for CA	Negative (out)
M31	Despropyl-aminodiol	Negative (In)	Negative (In)	Positive alert for CA	Negative (out)
M35	Docosanoic acid ester	Negative (Could be out)	Negative (In)	Negative	Negative (out)
M36	Tetracosanoic acid ester	Negative (Could be out)	Negative (In)	Negative	Negative (out)
M37	Cyclohexenol	Negative (In)	Negative (out)	Negative	Positive (In)
M38	N-formyl-despropyl	Negative (Could be out)	Negative (out)	Positive alert for CA	Negative (out)
M41	Hydroxy-desethyl	Negative (Out)	Negative (out)	Positive alert for CA	Negative (out)

CAESAR Mutagenicity model predicts 25 out of 26 metabolites as negative (non-mutagenic): metabolites M01, M02, M04, M05, M07, M08, M10, M11, M12, M13, M14, M15, M16, M25, M26, M27, M28, M29, M30, M31, M35, M36, M37, M38 and M41. Seven of the metabolites, M07, M10, M11, M12, M25, M29 and M41 are out of the model applicability domain, twelve M01, M02, M04, M05, M08, M15, M26, M27, M30, M35, M36 and M38 could be out of model applicability domain and six – M13, M14, M16, M28, M31 and M37 are into model applicability domain.

One metabolite M09 is predicted as potential mutagenic. The substance could be out of the model applicability domain. Additional analysis of the 6 most similar substances from the training set shows that the similarity is low between 0.795 – 0.773. All of them do not share the same functional groups as the predicted substance. The most similar substance from the training set is mutagenic but it is an epoxide..

OASIS Ames mutagenicity model predicts all 26 metabolites as negative (non-mutagenic). Seventeen of the metabolites are out of the model applicability domain M01, M02, M04, M05, M07, M08, M09, M10, M11, M12, M25, M26, M27, M29, M37, M38 and M41). Nine of the metabolites are in the model applicability domain M13, M14, M15, M16, M28, M30, M31, M35 and M36.
Toxtree in vivo micronucleus model predicts 19 metabolites as positive (at least one positive alert for micronucleus assay was found) – M01, M02, M04, M05, M07, M08, M09, M10, M11, M12, M25, M26, M27, M28, M29, M30, M31, M38 and M41. H-acceptor-path3-H-acceptor is identified as a structural alert. According to the authors this alert represents a molecular framework that could account for non-covalent interactions with proteins or DNA. Such interactions, as in the case of DNA intercalation or groove binding, are potentially genotoxic. However, the positive prediction value of this alert reported by the authors (Benigni, 2010) is low 34%.
OASIS Chromosomal aberration model predicts 25 out of 26 metabolites as negative. Two of them (M14 and M28) are in the model applicability domain and the rest are out of the model applicability domain.
One metabolite (M37) is predicted as positive (could cause chromosomal aberrations). The analyses of the prediction shows that the positive prediction is after metabolic activation and the predicted as a positive metabolite is in the model applicability domain and the reliability of the prediction reported by the model is high (more or equal to 60%). The identified alert is an alpha/beta-unsaturated carbonyls and related compounds and it is related with the mechanism of action: Interactions with topoisomerases / proteins.
Step 5.4: Conclusion

(Q)SAR assessment identified a potential of metabolites M01, M02, M04, M05, M07, M08, M09, M10, M11, M12, M25, M26, M27, M28, M29, M30, M31 M37, M38 and M41 to provoke genotoxic effects. Metabolites M13, M14, M15; M16, M35, M36 are predicted as negative from all models. All metabolites are moved to the next step – read across analysis.

Step 6: Read across (OECD toolbox)[22]
Step 6.1: Read across

Both endpoints gene mutation and chromosomal aberrations should be evaluated by read across for all metabolites.

Molecular initiating events of relevance for this assessment are interaction with DNA and/or proteins. The profilers included in the OECD Toolbox which codified the structural alerts which are important for these two types of interactions are mechanistic profilers - DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers- DNA alerts for AMES, MN and CA by OASIS v1.3, In vitro mutagenicity (AMES test) alerts by ISS, In vivo mutagenicity (Micronucleus) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

Above mentioned profilers have been applied to metabolites M01, M02, M04, M05, M07, M08, M09, M10, M11, M12, M13, M14, M15, M16, M25, M26, M27, M28, M29, M30, M31, M35, M36, M37, M38 and M41 as chemicals of interest and to the parent substance, the major rat metabolite, which is considered characterised by the provided genotoxicity studies and M03 for which studies were provided, as substances with known experimental genotoxic activity.

[22] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm

In order to be evaluated, the structural similarity in addition to the structural alerts related with the evaluated endpoints, an organic functional group profiler has been applied. This additional step provides information of the presence/ absence of other functional groups different than the structural alerts and gives indication for the potential influence of the rest part of the molecule to the relevant structural alerts (i.e. electronic and structural influence).

No structural alerts in the parent substance and in all evaluated metabolites were found by the profilers: DNA binding by OASIS v.1.3, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers- DNA alerts for AMES, MN and CA by OASIS v1.3, In vitro mutagenicity (AMES test) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

The alerts found by DNA binding by OECD, Protein binding by OECD and endpoint specific profiler - In vivo mutagenicity (Micronucleus) alerts by ISS and Organic functional group are presented in the Table 4.

Table 4 Genotoxicity profiling of Spiroxamine metabolites by OECD Toolbox

		DNA binding by OECD	in vivo mutagenicity (MN) by ISS	Protein binding by OECD	**Organic functional groups**															
		SN1: Iminium Ion Formation, Aliphatic tertiary amines	Hacceptor-path3-Hacceptor	Acetates	Aliphatic amine, tertiary	Aliphatic amine, secondary	N-Oxide	Formyl amino	Alcohol	Carboxylic acid	Alkane branched with quaternary carbon	Alkane branched with tertiary carbon	Cycloalkane	Dioxolane	Ether, cyclic	Saturated heterocyclic fragment	Tert-butyl	Cyclo keton	Sulfate	Carboxylic acid ester
Parent*	Spiroxamine	x	x		x						x	x	x	x	x	x	x			
M01	desethyl		x			x					x	x	x	x	x	x	x			
M02	despropyl		x			x					x	x	x	x	x	x	x			
M03	N-oxide		x				x				x	x	x	x	x	x	x			
M04	N-formyl-desethyl	x	x					x			x	x	x	x	x	x	x			
M05	hydroxyl	x	x		x				x		x	x	x	x	x	x				
M06*	acid	x	x		x					x		x	x	x	x	x				
M07	hydroxy acid	x	x		x				x	x		x	x	x	x	x				
M08	8-hydroxy acid	x	x		x				x	x			x	x	x	x				
M09	hydroxy-despropyl		x			x			x		x	x	x	x	x	x				
M10	hydroxy-N-oxide		x				x		x		x	x	x	x	x	x				
M11	desethyl acid		x			x				x		x	x	x	x	x				
M12	despropyl acid		x			x				x		x	x	x	x	x				
M13	Cyclohexanol								x		x	x	x				x			
M14	Diol								x		x	x	x							
M15	ketone										x	x	x				x	x		
M16	hydroxy-ketone								x		x	x	x					x		
M25	sulfate	x	x		x						x	x	x	x	x	x			x	
M26	desethyl-sulfate		x			x					x	x	x	x	x	x			x	
M27	despropyl-sulfate		x			x					x	x	x	x	x	x			x	
M28	aminodiol	x	x		x				x											
M29	aminodiol-N-oxide		x		x		x		x											
M30	desethyl-		x			x			x											

		DNA binding by OECD	in vivo mutagenicity (MN) by ISS	Protein binding by OECD	**Organic functional groups**															
		SN1: Iminium Ion Formation, Aliphatic tertiary amines	Hacceptor-path3-Hacceptor	Acetates	Aliphatic amine, tertiary	Aliphatic amine, secondary	N-Oxide	Formyl amino	Alcohol	Carboxylic acid	Alkane branched with quaternary carbon	Alkane branched with tertiary carbon	Cycloalkane	Dioxolane	Ether, cyclic	Saturated heterocyclic fragment	Tert-butyl	Cyclo keton	Sulfate	Carboxylic acid ester
	aminodiol																			
M31	despropyl-aminodiol		x			x			x											
M35			x	x							x	x	x				x			x
M36			x	x							x	x	x				x			x
M37	cyclohexenol								x		x		x				x			
M38	N-formyl-despropyl	x	x					x			x	x	x	x	x	x	x			
M41	hydroxy-desethyl		x			x			x		x	x	x	x	x	x				

* Compounds covered in their genotoxicological properties by studies with parent

Grey shaded: predicted as a potential mutagen by one of the two Ames/(Q)SAR models or predicted as potentially positive by one of the two chromosomal aberration/(Q)SAR model

Both alerts aliphatic tertiary amine and Hacceptor-path3-H-acceptor present in the parent substance and in the major rat metabolite (M06) are identified also in the metabolites M04, M05, M07, M08, M25 and M38. No new alerts were identified. In metabolites M04 and M38 the N-formyl amino group appeared as a result of metabolism of ethyl or propyl chain of the tertiary amine. OH group (in metabolites M05, M07 and M08), a carboxylic group (M07 and M08) and a sulphate group (M25) appeared as a result of the metabolism of t-butyl group. Therefore, based on the read across analysis metabolites M04, M05, M07, M08, M25 and M38 could be considered very similar to the parent substance and metabolite M06 and are not of genotoxicity concern.

Aliphatic tertiary amine as an alert has disappeared in metabolites M01, M02, M03, M09, M10, M11, M12, M26, M27 and M41, the second alert - Hacceptor-path3-Hacceptor, is present in all of them. No new alerts were identified. The changes in the rest part of the molecules compare with the parent substance and are related with metabolism of the ethyl or propyl chain of the tertiary amine with forming of N-oxide (M03 and M10) and secondary amine (M01 M02, M9, M11, M12, M26, M27 and M41); and oxidation or sulphation of the t-butyl group with forming: an alcohol (M09, M10 and M41) a carboxylic acid (M11 and M12) and a sulphate (M26 and M27). Based on the read across analysis metabolites M01, M02, M09, M11, M12, M26, M27 and M41 could be considered similar to the parent substance and the major rat metabolite; and are not of genotoxicity concern. Experimental data for the metabolite M03 –N-oxide are available. No evidence for a genotoxic potential was identified in the submitted in vitro studies (Ames test, HPRT gene mutation assay, chromosome aberration assay; Ref: Spiroxamine_AR_09_Vol3_B6_16-09-2009). Metabolite M10 is also an N-oxide with oxidised t-butyl group, therefore could be considered very similar to metabolite M03 and the genotoxic potential for it could be excluded.

Metabolite M28 has both structural alerts - aliphatic tertiary amine and Hacceptor-path3-Hacceptor but in general the substance is significantly different than the parent substance and the major rat metabolite. It is a smaller aliphatic molecule, and a different behaviour could be expected. Structurally similar to metabolite M28 are also metabolites M29, M30 and M31. The difference is that in them the structural alert aliphatic tertiary amine disappears forming N-oxide, and secondary amine. Hacceptor-path3-Hacceptor is present in three of them. No new alerts were identified. Taken into account the positive prediction from the (Q)SAR models for these metabolites and since they are rather different from the parent substance and the major rat metabolite,concern of genotoxicity cannot be excluded. To exclude an unacceptable dietary risk by potentially genotoxic metabolites, either a combined exposure estimate and comparison against TTC can be performed (step 7) or metabolites M28, M29, M30 and M31 would need to be tested (step 8). The metabolite M28 contents both structural alerts and could be tested as representative for the other three metabolites.

In metabolites M35 and M36 the structural alert aliphatic tertiary amine has disappeared, H-acceptor-path3-H-acceptor is still present and a new alert is identified (direct acylation involving leaving group – acetates; protein binding by OECD). Based on the new alert, the concern of genotoxicity of metabolites M35 and M36 cannot be excluded. To exclude an unacceptable dietary risk by potentially genotoxic metabolites, both a combined exposure estimate and comparison against TTC can be performed (step 8), or metabolites M35 and/or M36 would need to be tested for genotoxicity (step 9). As the genotoxic concern for metabolites M35 and M36 is due to the presence of the ester bond, they should be grouped as stand-alone. Alternatively, hydrolysis data across a range of physiological conditions (pH 3 to pH 6) could be provided. Should these data be indicative of a fast hydrolysis resulting in the metabolite M13 and carboxylic acid, the carboxylic acid should be assessed through, at least initially, (Q)SAR and read across (see data gap).

In metabolites M13, M14, M15, M16 and M37 the structural alerts aliphatic tertiary amine and Hacceptor-path3-Hacceptor have disappeared. The substances are different than the parent substance and the major rat metabolite. They are cyclic aliphatic alcohols (M13, M14 and M37) and cyclic aliphatic ketons (M15 and M16). In metabolites M14 and M16 additional OH group is appeared in the t-butyl group. No new alerts were identified.

Although no new alerts were identified for the metabolite M37 based on the high reliable positive (Q)SAR prediction (TIMES model for chromosomal aberration) the concern of genotoxicity for metabolite M37 cannot be excluded.

Metabolites M13, M14, M15 and M16 are predicted as negative by all (Q)SAR models though the prediction was not reliable for one model for CA, while the applicability domain was not defined for the second CA model leading to uncertainty on the prediction for CA. They are very similar to metabolite M37, however in all of them there is no a double bond in the cycle which leads to different chemical reactivity and it is crucial for forming of the structural features (alpha, beta-unsaturated carbonyl substance) which has a potential to interact with topoisomerases / proteins (suggested mechanism of action by the authors of the (Q)SAR model).

Step 6.2: Conclusion

Metabolites M13, M14, M15 and M16 are predicted as negative by all (Q)SAR models and no new alerts are identified by read-across, hence they are not of concern for genotoxicity.

Metabolites M01, M02, M04, M05, M07, M08, M09, M10, M11, M12, M38 and M41, although predicted as potential genotoxicant by (Q)SAR models, analysis are not of concern for genotoxicity after read across.

For metabolites M28, M29, M30 and M31, a genotoxicity concern cannot be excluded, therefore they should be subject of exposure assessment and comparison against TTC (step 8) and/or testing (step 9). Metabolite M28 could potentially be tested as a representative for all of them.

For metabolites M35 and M36 genotoxicity concerns cannot be excluded, therefore they should be subject to exposure assessment and comparison against TTC (step 8) and/or testing (step 9). Alternatively, hydrolysis data across a range of physiological conditions (pH 3 to pH 6) could be provided. Should these data indicate a fast hydrolysis resulting in the metabolite M13 and carboxylic acid, the carboxylic acid should be assessed through, at least initially, (Q)SAR and read across.

For metabolite M37 genotoxicity concern cannot be excluded, therefore it should to be subject of exposure assessment and comparison against TTC (step 8) and/or testing (step 9).

Step 7: Combined exposure assessment (optional)

The representative uses in Table 5 are considered in the exposure estimate. Regulatory decisions based on exposure estimates are therefore restricted to these GAP conditions.

Table 5 Uses considered for exposure estimates

Crop	**Application**			
	Growth stage	**Number**	**kg as/ha**	**PHI**
Cereals (wheat, rye, triticale)	BBCH 30-69	2	0.375	not relevant
Cereals (barley, oats)	BBCH 30-61	2	0.375	not relevant
Grape	BBCH 13-85	5	0.200-0.400	14 (table) 35 (wine)
Banana	-	12	0.320	0

PHI pre-harvest interval

Step 7.1: Derivation of residue input data for metabolites[23]

a) Residue levels of in primary crops

[23] Based on metabolism and residue data in Assessment Report (2009)

Residue levels of metabolites in primary crops wheat, grapes (wine, table) and banana were derived from metabolism studies and attributed to parent spiroxamine values from field trials where appropriate via conversion factors. Samples analysed for a common moiety were not used for the recalculation of individual metabolite levels.

Residues in cereal grain, grapes and banana (fruit) are relevant for consumer exposure; residues in cereal grain and straw are relevant for livestock burden calculation; residues in cereal forage, chaff and stubble and banana (peel) are neither relevant for quantitative consumer nor livestock exposure calculation (no feed items).

Residues in grain are listed in Table 6; metabolite residue levels are adopted from the metabolism study with cyclohexyl label since in the vast majority of field trials no quantifiable residues were detected (<LOQ).

Residues in table grapes were calculated by applying the maximum conversion factor for every metabolite from grape metabolism (on day 35) to the respective worst case field data (PHI 14 d or 35 d).

Residues in banana were calculated for pulp as edible commodity and as metabolite analysis was performed for peel/pulp separately.

b) Residue levels in rotational crops

A rotational crop metabolism and a field study are available. In the field rotational crop study, no significant residues (LOQ 0.05 mg/kg) were detected with a total residue method covering large parts of total residues (validated for parent and metabolites M01, M02 and M03). However, although appreciable exposure from metabolites in rotational crops can be largely excluded, the LOQ level and the limited number of analytes of the analytical method do not allow to expand the conclusions to the very low residue levels required to provoke unacceptable genotoxic effects ($TTC_{genotox}$ 0.0025 µg/kg bw/d).

A genotoxicity assessment by (Q)SAR and read-across revealed that a potential for such effects could not be excluded for the group of metabolites M28-M31, M35, M36 and M37. Therefore, residue levels in rotational crops from metabolism studies are summarised in Table 7 for those compounds that require further genotoxicity assessment (exposure; TTC). Further residue data on metabolites in food and feed items are listed to assess the relevance for consumer and livestock exposure.

Table 6 Residue levels of spiroxamine metabolites in primary crop metabolism and converted field data

Grape, 35 day PHI	***Cyclohexyl-1-^{14}C label (1N)***			***1,3-dioxolane-4-^{14}C label (1N)***			***Overall CF***		***Measured field data (table/wine grape)*** [a]		***Converted field data*** [b]		
											Table grape		***Wine grape***
	% TRR	mg/kg	CF	% TRR	mg/kg	CF	mean	max	STMR	HR	STMR (mg/kg)	HR (mg/kg)	STMR (mg/kg)
Spiroxamine, parent compound	24.6	0.84	1.00	45.6	5.96	1.00	1	1	0.19/0.13	0.33/n.r.	0.190	0.330	0.100
Desethyl (M01)	1.1	0.04	0.05	2.1	0.27	0.05	0.05	0.05			0.009	0.015	0.005
Despropyl (M02)	0.5	0.02	0.02	1.5	0.20	0.03	0.03	0.03			0.006	0.011	0.003
N-oxide (M03)	2.9	0.10	0.12	4.7	0.61	0.10	0.11	0.12			0.022	0.039	0.012
Hydroxy (M05)	n.d.	n.d.	n.d.	0.3	0.04	0.007	-	0.007			0.001	0.002	0.001
Diol (M14)	13	0.44	0.53	n.d.	n.d.	n.d.	-	0.53			0.100	0.174	0.053
Tert.butylketone (M15)	1.3	0.04	0.05	n.d.	n.d.	n.d.	-	0.05			0.010	0.017	0.005
Hydroxyketone (M23)	0.5	0.02	0.02	n.d.	n.d.	n.d.	-	0.02			0.004	0.007	0.002
Aminodiol (M28)*	n.d.	n.d.	n.d.	37.5	4.91	0.82	-	0.82			0.156	0.271	0.082
Aminodiol-N-oxide (M29)*	n.d.	n.d.	n.d.	0.1	0.01	0.002	-	0.002			<0.001	<0.001	<0.001
Desethyl-aminodiol (M30)*	n.d.	n.d.	n.d.	1.1	0.14	0.02	-	0.02			0.005	0.008	0.002
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	1.2	0.16	0.03	-	0.03			0.005	0.009	0.003
Cyclohexanol conj. (M33, M34)	25.3	0.86	1.03	n.d.	n.d.	n.d.	-	1.03			0.195	0.339	0.103
Docosanoic acid ester (M35) *	13	0.44	0.53	n.d.	n.d.	n.d.	-	0.53			0.100	0.174	0.053
Tetracosanoic acid ester (M36) *	4.2	0.14	0.17	n.d.	n.d.	n.d.	-	0.17			0.032	0.056	0.017
Cyclohexenol (M37)*	3.2	0.11	0.13	n.d.	n.d.	n.d.	-	0.13			0.025	0.043	0.013

[a] based on untransformed field data (Southern-EU data for table and wine grapes (STMR) and Northern-EU data for table grapes (HR) as reported in RAR 2009

[b] based on max CF x parent (measured residues only; no recalculation from total residues)

CF: Conversion factor

** Metabolites of potential genotoxic concern

Banana pulp, 35 day PHI (no further metabolites identified in peel)	***Cyclohexyl-1-^{14}C label (1N)***			***1,3-dioxolane-4-^{14}C label (1N)***			***Overall CF***		***Measured field data*** [a]		***Converted field data*** [b]	
	% TRR	**mg/kg**	**CF**	**% TRR**	**mg/kg**	**CF**	**mean**	**max**	**STMR**	**HR**	**STMR**	**HR**
Spiroxamine, parent compound	44.9	0.20	1.00	60.0	0.333	1.00	1	1	0.07	0.08	0.07	0.08
Desethyl (M01)	1.1	0.005	0.02	0.9	0.005	0.02	0.02	0.02			0.002	0.002
Despropyl (M02)	0.5	0.002	0.01	0.4	0.002	0.007	0.009	0.01			0.001	0.001
N-oxide (M03)	0.8	0.003	0.02	1.2	0.007	0.02	0.02	0.02			0.001	0.002
Diol-[hexose-pentose] (M24)	9.2	0.041	0.21	n.d.	n.d.	n.d.	-	0.21			0.014	0.016
Aminodiol (M28)*	n.d.	n.d.	n.d.	31.2	0.173	0.52	-	0.52			0.036	0.042
Desethyl-aminodiol (M30)*	n.d.	n.d.	n.d.	0.6	0.003	0.01	-	0.01			0.001	0.001
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	0.6	0.003	0.01	-	0.01			0.001	0.001
Cyclohexanol-[hexose-hexose] (M33a)	10.4	0.046	0.23	n.d.	n.d.	n.d.	-	0.23			0.016	0.018
Cyclohexanol-[hexose-pentose] (M33)	3.2	0.014	0.07	n.d.	n.d.	n.d.	-	0.07			0.005	0.006

[a] pulp data
[b] based on max CF x parent (measured residue)
* Metabolites of potential genotoxic concern

Wheat, 56-61 day PHI	***Cyclohexyl-1-^{14}C label (1.1N)***			***1,3-dioxolane-4-^{14}C label (2.2N)***			***CF***		***Measured field data*** [a]		***Converted field data*** [b]	
	% TRR	mg/kg	CF	% TRR	mg/kg	CF	mean	max	STMR	HR	STMR	HR
Grain												
Spiroxamine, parent compound	14.3	0.010	1.00	2.8	0.013	1.00	1	1	0.05	0.05	n.a. [d]	n.a. [d]
Desethyl (M01)	0.5	<0.001	0.03	n.d.	n.d.	-	-	0.03				
Despropyl (M02)	3.0	0.002	0.21	n.d.	n.d.	-	-	0.21				
N-oxide (M03)	17.8	0.012	1.24	1.2	0.005	0.43	0.84	1.24				
N-formyl-desethyl (M04)	6.9	0.005	0.48	n.d.	n.d.	-	-	0.48				
Hydroxy (M05)	1.6	0.001	0.11	n.d.	n.d.	-	-	0.11				
Straw												
Spiroxamine, parent compound	25.1	8.76	1.00	20.6	17.01	1.00	1	1	0.53	2.0	0.53	2.0
Desethyl (M01)	2.0	0.70	0.08	n.d.	n.d.		-	0.08			0.04	0.16
Despropyl (M02)	3.2	1.12	0.13	4.2	3.48	0.20	0.17	0.20			0.11	0.41
N-oxide (M03)	22.0	7.68	0.88	20.9	17.26	1.01	0.95	1.01			0.54	2.03
N-formyl-desethyl (M04)	7.5	2.62	0.30	9.7	8.06	0.47	0.38	0.47			0.25	0.94
Hydroxy (M05)	2.4	0.84	0.10	n.d.	n.d.	-	-	0.10			0.05	0.19
Hydroxy-despropyl (M09)	0.3	0.11	0.01	0.4	0.35	0.02	0.02	0.02			0.01	0.04
Hydroxy-N-oxide glucoside (M20)	2.0	0.70	0.08	n.d.	n.d.	-	-	0.08			0.04	0.16
Malonic acid glucoside (M21)	1.9	0.67	0.08	3.1	2.57	0.15	0.11	0.15			0.08	0.30
Hydroxy-ketone conj (M23)	1.8	0.63	0.07	n.d.	n.d.	-	-	0.07			0.04	0.14
Desethyl (M01)+ Hydroxy (M05)	-	-	-	5.2	4.32	- [c]	-	-			-	-

[a] based on untransformed field data (wheat, S-EU as critical case for cereal straw; wheat grain data applicable to barley)
[b] based on max CF x parent
[c] not used; individual values for M01 and M05 available
[d] not used; number of non-detects too high (10/12)

Table 7 Residue levels of spiroxamine metabolites in rotational crop metabolism (food and feed)

	Cyclohexyl-1-^{14}C label (2N rate)				*1,3-dioxolane-4-^{14}C label (2N rate)*					
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	30 days		**161 days**		**30 days**		**193 days**		**294 days**	
Swiss chard (immature)	**No data**				**TRR = 0.846 mg/kg**		**TRR = 0.410 mg/kg**		**TRR = 0.204 mg/kg**	
Despropyl-aminodiol (M31)*					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aminodiol-N-oxide (M29)*					1.0	0.008	5.2	0.021	n.d.	n.d.
Aminodiol (M28)*					1.8	0.016	2.4	0.010	n.d.	n.d.
Desethyl-aminodiol (30)*					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyclohexenol (M37)*					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Parent					9.4	0.080	2.4	0.010	8.2	0.017
M01					6.6	0.056	3.1	0.012	12.6	0.026
M02					15.0	0.127	11.0	0.045	50.0	0.102
M03					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M04					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M05					17.2	0.146	1.9	0.008	2.7	0.005
M38					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M39					2.0	0.017	n.d.	n.d.	n.d.	n.d.
M40					4.7	0.040	0.8	0.003	n.d.	n.d.
M42					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M43					1.8	0.015	n.d.	n.d.	n.d.	n.d.
M44					2.2	0.019	4.6	0.019	3.3	0.007
M45					3.5	0.029	6.0	0.025	2.6	0.005
Swiss chard (mature)	**TRR = 0.150 mg/kg**		**TRR = 0.07 mg/kg**		**TRR = 0.676 mg/kg**		**TRR = 0.348 mg/kg**		**TRR = 0.104 mg/kg**	
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aminodiol-N-oxide (M29)*	n.d.	n.d.	n.d.	n.d.	0.8	0.006	n.d.	n.d.	n.d.	n.d.
Aminodiol (M28)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.9	0.014	n.d.	n.d.
Desethyl-aminodiol (30)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyclohexenol (M37)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Parent	40.8	0.061	8.8	0.006	9.4	0.064	3.3	0.011	10.0	0.010
M01					9.0	0.061	4.0	0.014	12.3	0.013
M02	7.5	0.011	14.2	0.010	19.7	0.133	19.0	0.066	51.2	0.053
M03	6.1	0.009	14.2	0.010	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M05					12.8	0.086	1.5	0.005	n.d.	n.d.
M05/M01 [a]	11.6	0.017	12.1	0.008						
M20	2.5	0.004	n.d.	n.d.						
M21	1.6	0.002	n.d.	n.d.						
M23	2.2	0.003	n.d.	n.d.						

	Cyclohexyl-1-^{14}C label (2N rate)				*1,3-dioxolane-4-^{14}C label (2N rate)*					
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	30 days		**161 days**		**30 days**		**193 days**		**294 days**	
M24	3.0	0.005	n.d.	n.d.						
M38					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M39					2.8	0.019	n.d.	n.d.	n.d.	n.d.
M40					3.8	0.025	0.8	0.003	n.d.	n.d.
M42					0.4	0.003	1.6	0.005	n.d.	n.d.
M43					0.6	0.004	n.d.	n.d.	n.d.	n.d.
M44					3.1	0.021	2.4	0.008	n.d.	n.d.
M45					1.8	0.012	5.5	0.019	2.8	0.003
Turnip roots	**TRR = 0.040 mg/kg**		**TRR = 0.020 mg/kg**		**TRR = 0.101 mg/kg**		**TRR = 0.026 mg/kg**		**TRR = 0.012 mg/kg**	
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	n.d.	6.1	0.006	n.d.	n.d.	No data	
Aminodiol-N-oxide (M29)*	n.d.	n.d.	n.d.	n.d.	4.8	0.005	4.7	0.001		
Aminodiol (M28)*	n.d.	n.d.	n.d.	n.d.	4.9	0.005	n.d.	n.d.		
Desethyl-aminodiol (30)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
Cyclohexenol (M37)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
Parent	45.8	0.018	27.4	0.005	3.5	0.003	n.d.	n.d.		
M01					1.5	0.001	n.d.	n.d.		
M02	2.6	0.002	3.3	0.001	2.9	0.003	n.d.	n.d.		
M03	2.8	0.001	3.5	0.001	n.d.	n.d.	n.d.	n.d.		
M04	n.d.	n.d.	n.d.	0.001	n.d.	n.d.	n.d.	n.d.		
M05					0.8	0.001	n.d.	n.d.		
M05/M01 [a]	4.4	0.002	3.7	0.001						
M20	n.d.	n.d.	n.d.	n.d.						
M21	n.d.	n.d.	n.d.	n.d.						
M23	8.1	0.003	n.d.	n.d.						
M24	7.8	0.003	n.d.	n.d.						
M38					n.d.	n.d.	n.d.	n.d.		
M39					n.d.	n.d.	n.d.	n.d.		
M40					n.d.	n.d.	n.d.	n.d.		
M42					2.8	0.003	n.d.	n.d.		
M43					2.6	0.003	n.d.	n.d.		
M44					1.9	0.002	n.d.	n.d.		
M45					3.7	0.004	9.1	0.002		
Wheat straw	**TRR = 1.070 mg/kg**		**TRR = 1.270 mg/kg**		**TRR = 3.178 mg/kg**		**TRR = 2.631 mg/kg**		**TRR = 0.986 mg/kg**	
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.4	0.037	1.8	0.018
Aminodiol-N-oxide (M29)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aminodiol (M28)*	n.d.	n.d.	n.d.	n.d.	0.2	0.008	n.d.	n.d.	n.d.	n.d.
Desethyl-aminodiol (30)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyclohexenol (M37)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

	Cyclohexyl-1-^{14}C label (2N rate)				*1,3-dioxolane-4-^{14}C label (2N rate)*					
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	30 days		**161 days**		**30 days**		**193 days**		**294 days**	
Parent	6.8	0.073	4.0	0.051	15.2	0.485	3.0	0.078	4.2	0.041
M01					15.1	0.479	6.2	0.163	5.6	0.055
M02	3.5	0.037	2.9	0.037	17.4	0.553	14.3	0.376	17.4	0.172
M03	12.7	0.136	12.1	0.154	7.4	0.235	5.0	0.132	1.4	0.014
M04	9.2	0.098	7,5	0.095	6.4	0.204	2.8	0.075	n.d.	n.d.
M05					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M05/M01[a]	4.5	0.048	2.4	0.030						
M20	2.1	0.022	2.6	0.033						
M21	1.5	0.016	2.4	0.030						
M23	2.2	0.024	1.9	0.024						
M24	n.d.	n.d.	4.4	0.056						
M38					7.6	0.243	6.9	0.181	3.4	0.034
M39					0.5	0.015	1.6	0.043	n.d.	n.d.
M40					2.8	0.088	0.9	0.024	12.6	0.124
M42					0.8	0.025	4.7	0.124	n.d.	n.d.
M43					0.5	0.017	3.4	0.088	n.d.	n.d.
M44					1.0	0.032	3.9	0.104	10.5	0.103
M45					0.6	0.018	2.8	0.075	2.8	0.027
Wheat grain	**TRR = 0.060 mg/kg**		**TRR = 0.050 mg/kg**		**TRR = 0.131 mg/kg**		**TRR = 0.223 mg/kg**		**TRR = 0.092 mg/kg**	
Despropyl-aminodiol (M31)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aminodiol-N-oxide (M29)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Aminodiol (M28)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Desethyl-aminodiol (30)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyclohexenol (M37)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Parent	n.d.	n.d.	n.d.	n.d.	2.7	0.004	n.d.	n.d.	n.d.	n.d.
M01					2.9	0.004	n.d.	n.d.	n.d.	n.d.
M02	n.d.	n.d.	n.d.	n.d.	4.8	0.006	n.d.	n.d.	n.d.	n.d.
M03	n.d.	n.d.	n.d.	n.d.	4.3	0.006	n.d.	n.d.	n.d.	n.d.
M04	n.d.	n.d.	n.d.	n.d.	1.5	0.002	n.d.	n.d.	n.d.	n.d.
M05					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M05/M01[a]	n.d.	n.d.	n.d.	n.d.						
M20	n.d.	n.d.	n.d.	n.d.						
M21	n.d.	n.d.	n.d.	n.d.						
M23	n.d.	n.d.	n.d.	n.d.						
M24	n.d.	n.d.	n.d.	n.d.						
M38					2.7	0.003	n.d.	n.d.	n.d.	n.d.
M39					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M40					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M42					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

	Cyclohexyl-1-^{14}C label (2N rate)				*1,3-dioxolane-4-^{14}C label (2N rate)*					
	% TRR	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**
	30 days		**161 days**		**30 days**		**193 days**		**294 days**	
M43					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M44					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M45					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* Metabolites of potential genotoxic concern

[a] not used for assessment; individual values for M01 and M05 available

Step 7.2: Exposure calculations for those metabolites, for which genotoxic effects cannot be excluded

Table 8 Exposure calculation[24] and TTC assessment of metabolites with potential genotoxicity concern

	Wine grape	Table grape		Banana		Cereal grain	Root crops	Leafy crops
	STMR	STMR	HR	STMR	HR	Metabolism data		
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Metabolite M28	0.082	0.156	0.271	0.036	0.042	nd	0.005	0.010
Metabolite M29	0.001	0.0004	0.001	nd	nd	nd	0.005	0.021
Metabolite M30	0.002	0.005	0.008	0.001	0.001	nd	nd	nd
Metabolite M31	0.003	0.005	0.009	0.001	0.001	nd	0.006	nd
Sum of metabolites	**0.088**	**0.166**	**0.289**	**0.038**	**0.044**	-	**0.016**	**0.031**
Metabolite M35	0.053	0.100	0.174	nd	nd	nd	nd	nd
Metabolite M36	0.017	0.032	0.056	nd	nd	nd	nd	nd
Sum of metabolites	**0.070**	**0.132**	**0.230**	-	-	-	-	-
Metabolite M37	0.013	0.025	0.043	nd	nd	nd	nd	nd
Sum of metabolites	**0.013**	**0.025**	**0.043**	-	-	-	-	-

Chronic exposure (most critical; metabolite groups)

M28-M31: 0.419 µg/kg bw/d (FR all population) = >10000 % $TTC_{genotoxicity}$
M35-M36: 0.294 µg/kg bw/d (FR all population) = >10000 % $TTC_{genotoxicity}$
M37: 0.087 µg/kg bw/d (FR all population) = 3480 % $TTC_{genotoxicity}$

Acute exposure (most critical; metabolite groups)

M28-M31: 18.9 µg/kg bw/d (table grapes, children) = >10000 % $TTC_{genotoxicity}$
M35-M36: 15.1 µg/kg bw/d (table grapes, children) = >10000 % $TTC_{genotoxicity}$
M37: 2.82 µg/kg bw (table grapes, children) = >10000 % $TTC_{genotoxicity}$

Step 7.3: Conclusion

The combined as well as the individual exposure assessment for all metabolites, for which an unacceptable risk of genotoxic effects cannot be excluded *a priori* (M28-31; M35-36; M37), reveals an exceedance of the acceptable TTC threshold for genotoxicity of 0.0025 µg/kg bw/d, see table 8.

Additional exposure by food of animal origin via rotational crops cannot be excluded for M28-M31. The reliability of the exposure estimate is, although limited with regard to the upper bound, reliable for the lower bound high exposure (exceedance of TTC).

Metabolite M28 has the highest exposure potential within the group of metabolites M28-M31. It is in this respect a suitable candidate for genotoxicity testing.

Potential genotoxic effects (indicated during genotoxicity hazard assessment) cannot be excluded for metabolite group M35/M36 and for M37 based on exposure estimates, which do not provide indications of dietary non-relevance. Genotoxicity concerns should be addressed for M35/M36 and M37 either by testing according to step 9 or by hydrolysis experiments under physiological conditions combined with QSAR assessment (M35/M36 only).

[24] Exposure assessment performed with EFSA PRIMo rev.2

Step 8: Genotoxicity testing

Metabolite M28, selected as representative for metabolites M28 - M31, should be tested for the exclusion of genotoxicity (data gap; see step 20).

Metabolite M37 should be tested for the exclusion of genotoxicity (data gap; see step 20).

Genotoxicity endpoints (point mutations, structural and numerical chromosome aberrations) should be investigated. In-vitro studies (e.g. Ames test (TG 471) and in vitro micronucleus assay (TG 487)) are considered suitable for the exploration of the above mentioned genotoxicity endpoints.

Similar testing strategy should be applied for the selected group representative for metabolites M35 and M36 or by hydrolysis experiments under physiological conditions combined with QSAR assessment (data gap; see step 20).

Step 9: Genotoxicity concern

None (pending additional information on metabolites M28, M35 and M36, M37).

Step 10: General toxicity of metabolites characterized by studies with parent or by specific studies

Step 10.1: Toxicological assessment of parent compound

The ADI for the parent compound, spiroxamine, was set at 0.025 mg/kg bw per day based on the effects observed on the liver and the eye in the dog toxicity studies (NOAEL of 2.5 mg/kg bw per day, UF 100; EFSA, 2010).

The ARfD for the parent compound was set at 0.1 mg/kg/bw based on unspecific toxicity in the rat neurotoxicity study (NOAEL 10 mg/kg bw; UF of 100; EFSA, 2010).

Malformations (i.e. cleft palate) were observed at 100 mg/kg bw per day in the developmental toxicity in rats leading to a proposal for classification with R63 "Possible risk of harm to the unborn child" (EFSA, 2010). The developmental NOAEL in rats was 30 mg/kg bw per day.

The liver and gastrointestinal tract were the target organs of toxicity in the 28-day and 90-day toxicity studies conducted with the parent in rats with an established NOAEL of 3.4 and 1.9 mg/kg bw per day respectively (DE, 2009).

Step 10.2: Toxicological assessment of metabolites

Metabolites M06 is considered covered in its toxicological properties by the studies with the parent (i.e. above the threshold of 10% of the administered dose in terms of total radioactive material recovered in the urine as detected in ADME studies; see Table 2). No further toxicological assessment is needed.

Toxicological studies on M03 showed that M03 has an acute oral toxicity to rats (LD50 oral: ~707 mg/kg bw). The liver and gastrointestinal tract were the target organs of toxicity in the 28-day and 90-day toxicity studies in rats. The NOAEL were 12.9 and 8.8 mg/kg bw/day respectively (DE, 2009). Further toxicological assessment is not needed (step 18).

Step 11: Combined exposure of all metabolites to assess general toxicity (optional)

The TTC assessment is only of limited applicability to the representative uses of spiroxamine due to the level of uncertainties linked to the multiple uses, the number of metabolites and their grouping as well as possible exposure scenarios considering residues from treated plant commodities as well as livestock animals. Therefore, TTC assessment is not an adequate assessment tool and the exposure assessment is not conducted.

Step 12: Consideration on potency

The ADI for the parent compound, spiroxamine, was set at 0.025 mg/kg bw per day based on the effects observed on the liver and the eye in the dog toxicity studies. Based on the ADI value (>0.01 mg/kg bw/d), spiroxamine is not considered of concern in terms of potency.

Step 13: Assessment of major plant metabolites in food (≥10% TRR and ≥0.01 mg/kg OR ≥0.05 mg/kg)

The metabolite spectra for cereals and fruits are considered dissimilar and justify separate residue definitions, if necessary (pending closure of data gaps).

For cereals and rotational crops (food items only), parent, metabolites M01, M02, M03 and M05 are candidates for inclusion into the residue definition for plants (Table 6 and 7).

For fruits, parent, metabolites M01, M02, M03, M14, M28, M30, M31, M33 (=M13 conj.), M34 (=M13 conj.), M35, M36 and 37 are candidates for inclusion into the residue definition for plants (Table 6 and 7).

Metabolite M03 is considered toxicologically characterised by specific studies (see step 10.2).

Metabolites M01, M02, M05, M14, M28, M30, M31, M33 (=M13 conj.), M34 (=M13 conj.), M35,M36 and M37 are below the threshold of 10% of the AD in terms of total radioactive material recovered in the urine as detected in ADME studies. Consequently, further toxicological and exposure considerations are needed (step 18).

Metabolites M33 and M34 are sugar conjugates and they likely result in metabolite M13 after hydrolysis; therefore, the assessment will be conducted on M13.

Metabolites M(35) and M(36) are esters of M13 and docosanoic and tetracosanoic acid and they need to be assessed.

Step 14: Assessment of minor plant metabolites in food (<10% TRR AND <0.05 mg/kg)

Based on the ADI value for parent (>0.01 mg/kg bw/d) the minor metabolites are not considered of concern in terms of potency in relationship to parent. Minor metabolites are not expected to significantly contribute to the toxicity burden and no further toxicological or exposure assessment is needed.

Step 15: Assessment of major plant metabolites in feed (≥10% TRR and ≥0.01 mg/kg)

For dietary burden calculation, those compounds are considered that occur at 1N rate in at least one feed commodity at ≥10 % TRR (and at least 0.01 mg/kg).

These are parent, metabolites M01, M02, M03, M05, M40 (conjugate of M05), and M44 (conjugate of M06).

Grape and banana are not considered as feed items.

Step 16: Potential of residue transfer from feed to livestock

The dietary burden calculation for requiring an animal metabolism study and further define the relevance of metabolites, has to consider the highest likely residues of major residues in feed items, if they exceed the triggers in at least one food commodity (Table 9).

Using input data of parent and all major feed metabolites for the dietary burden calculation, the trigger of 0.004 mg/kg bw/d for requirement of a livestock metabolism study is exceeded for ruminants and poultry.

Parent (including the group of related metabolites) and metabolite M03 are considered separately due to structural dissimilarity that may lead to a different kinetic ADME behaviour in livestock (assessment based on structural similarities; see step 18).

Metabolism of spiroxamine is addressed by radiolabelled studies in goats and laying hens (step 17).

Metabolism of lead compound M03 in ruminants and poultry is not characterised. The dietary risk associated with the potential transfer of metabolite M03 into animal commodities cannot be assessed by means of the parent residue profile and levels. Since M03 exceeds the trigger of 0.004 mg/kg bw/d, it is concluded on base of all information, that a new ruminant and poultry metabolism study with lead compound M03 is required (data gap).

Table 9 Input data and dietary burden calculation for spiroxamine and potentially relevant metabolites[25]

Compound	Primary/Rotational crops		Rotational crops		Contribution to livestock burden		
	Cereal grain	Cereal straw	Leafy	Root			
	mg/kg	mg/kg	mg/kg	mg/kg	Diet	mg/kg bw/d	%
Spiroxamine group							
Spiroxamine	0.010	2.0	0.061	0.018	Lamb Ewe Layer	0.060 0.047 0.017	41 41 38
M01	0.001	0.16	0.031	n.d.	Lamb Ewe Layer	0.005 0.004 0.001	3 3 3
M02	0.002	0.41	0.067	0.002	Lamb Ewe Layer	0.012 0.009 0.003	8 8 6
M05	0.001	0.19	0.073	n.d.	Lamb Ewe Layer	0.005 0.004 0.002	4 4 4
M40 (conjugated M5)	n.d.	0.062	0.020	n.d.	Lamb Ewe Layer	0.002 0.001 0.000	1 1 1
M44 (conjugated M06)	n.d.	0.052	0.010	0.001	Lamb Ewe Layer	0.002 0.001 0.000	1 1 1
M03	0.012	2.0	0.005	0.001	Lamb Ewe Layer	0.060 0.047 0.022	41 41 47
Sum					**Lamb** **Ewe** **Layer**	**0.120** **0.094** **0.039**	**100** **100** **100**

Step 17: Major animal metabolites ≥10% TRR in food

Ruminants

Radiolabelled metabolism studies with spiroxamine as lead compound serve as basis for the proposal of a residue definition for risk assessment (applicable for feed metabolites of the spiroxamine group; Table 9). After administration of spiroxamine to lactating ruminants, the identified metabolite spectra in goat contains 10 metabolites (Table 10). Metabolite M06, its glucuronide M19 and M07 occur as major metabolites of parent compound at levels exceeding each 10% of TRR and need further assessment.

Parent, M06, M07 and M19 make up between 40-67% of TRR and 59-84% of identified residues.

Other significant feed metabolites included in the dietary burden calculation within the parent group are considered assessable by available metabolism and feeding data, even if they were not observed in livestock metabolism (e.g. M01, M02 in ruminant feed are covered by their sulfate conjugates M26 and M27) (DE, 2009).

[25] EFSA livestock burden calculator considering OECD feeding table.

146 ***Table 10: Metabolite levels in a ruminant metabolism study after administration of spiroxamine (85N rate)***

Residue component	**Kidney**		**Liver**		**Muscle**	
	mg/kg	**% of TRR**	**mg/kg**	**% of TRR**	**mg/kg**	**% of TRR**
Parent	0.028	0.2	1.10	5.0	nd	nd
M06	1.48	10.4	4.33	19.6	0.500	48.3
M07	2.27	16.0	0.38	1.7	0.106	10.3
M08	0.33	2.3	0.26	1.2	nd	nd
M11	0.82	5.8	0.84	3.8	0.066	6.4
M12	1.28	9.0	0.7	3.5	0.070	6.8
M19	1.89	13.3	7.22	32.7	0.082	7.9
M22	0.06	0.4	nd	nd	nd	nd
M25	0.23	1.6	0.49	2.2	nd	nd
M26	0.46	3.2	0.42	1.9	nd	nd
M27	0.82	5.8	1.04	4.7	nd	nd

Residue component	**Fat**		**Milk**	
	mg/kg	**% of TRR**	**mg/kg**	**% of TRR**
Parent	nd	nd	nd	nd
M06	0.199	30.5	0.496	53.3
M07	0.063	9.7	0.101	10.9
M08	nd	nd	nd	nd
M11	0.028	4.3	0.051	5.5
M12	0.041	6.2	nd	nd
M19	0.101	15.4	nd	nd
M22	nd	nd	nd	nd
M25	nd	nd	0.076	8.2
M26	0.023	3.5	nd	nd
M27	0.023	3.5	nd	nd

147

148 No final conclusion can be made for metabolites following administration of the second lead
149 compound M03 to ruminants.

150 **Poultry**

151 A radiolabelled metabolism study is available. Residues found in the metabolism study comprise the
152 major compounds parent, M06, M01 and M02 (all >10% TRR in at least one edible matrix; no further
153 metabolites identified). Quantitative transfer into animal matrices at 1N cannot be excluded *a priori*.

154 ***Table 11 Metabolite levels in a poultry metabolism study after administration of spiroxamine (300N rate)***

Residue component	**Liver**		**Muscle**		**Fat**		**Eggs**	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
Spiroxamine	2.324	13.3	0.430	17.8	9.562	77.4	0.100	11.8
M06	1.486	8.5	0.901	37.3	0.210	1.7	0.317	37.4
M02	3.793	21.7	0.273	11.3	0.420	3.4	0.086	10.2
M01	3.723	21.3	0.225	9.3	1.038	8.4	0.097	11.5
Identification rate	11.326	64.8	1.829	75.7	11.230	90.9	0.600	70.9

155

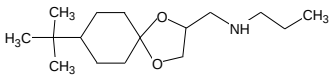
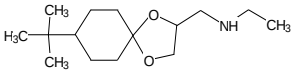
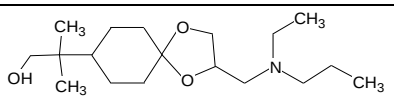
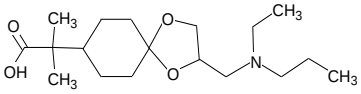
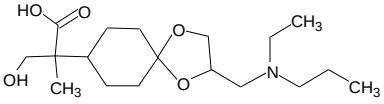
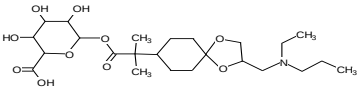
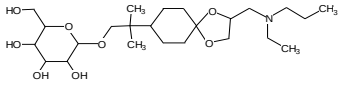
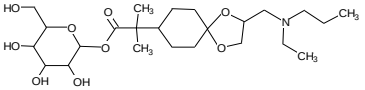
156 No conclusion can be made for metabolites following administration of metabolite M03 (data gap).
157 Other significant feed metabolites, although not observed in livestock metabolism, are considered as
158 covered by available data. M44 is a conjugate of M06, and M05 is considered an intermediate in
159 parent metabolism to M06 (DE, 2009).

Step 18: Testing strategy, grouping and read-across

Proposal for grouping based on structural similarities:

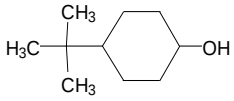
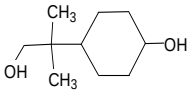
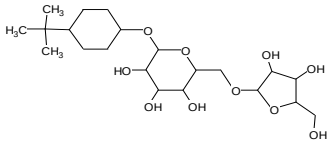
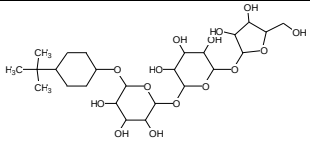
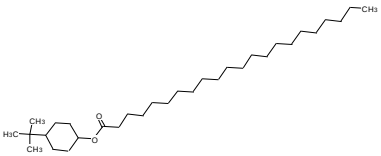
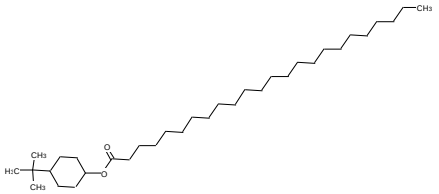
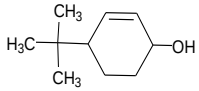
Group A (parent similar metabolites):

M01, M02, M05, M06, M07, M19, M40, M44

 M1	 M2	 M5
 M6	 M7	 M19
 M40	 M44	

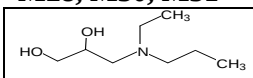
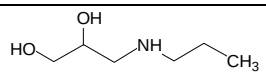
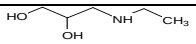
Group B

M14, M33 (conjugate M13), M34 (conjugate M13), M35, M36, M37,

 M13	 M14	 M33
 M34	 M35	 M35
 M37		

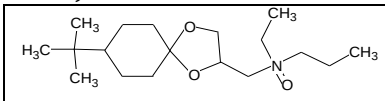
Group C - aminodiol

M28, M30, M31

 **M28**	 **M30**	 **M31**

Group D – oxide

M03,

 **M3**		

Group A: (parent similar metabolites)

Toxicological assessment of metabolites M01 and M02

Metabolites M01 and M02 at first instance are considered as similar to the parent substance, the difference is that they are secondary amines while the parent is a tertiary amine, therefore parent reference values can be applied.

Toxicological assessment of metabolite M05 and its conjugate M40

Metabolite M05 is considered similar to the parent substance. The structural difference is a simple hydroxylation of the t-butyl group, therefore parent reference values can be applied.

Toxicological assessment of metabolite M06 and its conjugates M19 and M44

Metabolite M06 is covered in its toxicological properties by parent compound studies, because it is above 10% of AD in terms of total radioactive material recovered in the urine as detected in ADME studies.

Toxicological assessment of metabolite M07

Metabolite M07 is very similar with M06. The structural difference is an additional hydroxyl group in the t-butyl group, therefore no further toxicological consideration is needed.

Group B:

Toxicological assessment of metabolite M13 and its conjugates M33, M34

Metabolite M13 is identified in conjugated form (M33 and M34) as a major plant metabolite in fruits. Additional testing is recommended in order to establish adequate toxicological reference values (relevant for representative uses grapes and banana). M13 is lead compound for M33, M34 and M14. Metabolites M33 and M34 are considered sugar conjugates and they likely result in metabolite M13 after hydrolysis. The 28 days rat toxicity study should be performed following the study design as recommended in the guidance document (chapter 3.1). In addition, as spiroxamine is proposed for classification (cat.2) due to the concern on developmental toxicity, a tiered approach should be considered by first addressing the hazard characterization and the reference potency factor for potential waiving of testing for developmental toxicity. Alternatively, as recommended in section 3.4 of the guidance, if the metabolite M13 is common to other active substances and already characterised, these data could be considered, if relevant, for the risk assessment.

Toxicological assessment of metabolite M14

Metabolite M14 is very similar with metabolite M13, the difference is a hydroxyl group in the t-butyl group, therefore the metabolite could be grouped with M13.

Toxicological assessment of metabolites M35 and M36

Metabolites M35 and M36 are esters of M13 and docosanoic and tetracosanoic acid data on hydrolysis are not available. Should hydrolysis data demonstrate hydrolysis of the ester bond, the two resulting alcohols can be grouped based on chemical similarity of the moiety and represented by the lead compound (M13). However, the resulting acids need to be assessed separately. Similarly, if hydrolysis cannot be demonstrated, the two esters (M35 and M36) should be assessed as such.

Toxicological assessment of metabolite M37

Metabolite M37 contains the same general structural moiety as M13, however there is a double bound in the cycle which could lead to a different chemical reactivity and similarity in the toxicological properties cannot be assumed.

The 28 days rat toxicity study should be performed following the study design as recommended in the guidance document (chapter 3.1). In addition, as spiroxamine is proposed for classification (cat.2) due to the concern on developmental toxicity, a tiered approach should be considered by first addressing the hazard characterization and the reference potency factor for potential waiving of testing for developmental toxicity. Alternatively, as recommended in section 3.4 of the guidance, if the metabolite M37 is common to other active substances and already characterised, these data could be considered, if relevant, for the risk assessment.

Group C (aminodiols**)**

Toxicological assessment of metabolites M28, M30 and M31

Additional testing is recommended for metabolite M28 (e.g. selection criteria based on the relevant exposure) to establish adequate toxicological reference values. Initially, the 28 days rat toxicity study should be performed. In addition, as spiroxamine is proposed for classification (cat.2) due to the concern on developmental toxicity, a tiered approach should be considered by first addressing the hazard characterization and the reference potency factor for potential waiving of testing for developmental toxicity.

Group D - oxide

Toxicological assessment of metabolite M03

In principle based on the results above, additional testing would be needed for metabolite M03 in order to establish adequate toxicological reference. However, adequate 28 day and 90-day rat toxicity studies are available (DE, 2009). As spiroxamine is proposed for classification (cat.2) due to the concern on developmental toxicity, this hazard needs to be assessed for metabolite M03. With the parent compound, spiroxamine, developmental toxicity effect was observed at doses higher than the one used as a point of departure for the establishment of the reference values. Additionally, in the 28 and 90 day rat toxicity studies the metabolite M03 was less potent than the parent. For these reasons testing for developmental toxicity with the metabolite M03 can be waived. In this case the development hazard characterisation will be the same as for the parent.

No further toxicological testing required.

Step 19: Assessment of toxicogical burden

The following major compounds of (qualitative) toxicological relevance were identified in food and feed of plant origin and require further assessment: Parent, M01, M02, M03, M05 (including its conjugate M40), M44 (conjugate of M6), M14, M28, M30, M31, M33 (conjugate of M13), M34 (conjugate of M13), M35, M36 and M37.

Their quantitative occurrence is expressed in Table 12 and Table 13 in terms of %TRR (as determined in metabolism studies) and in % of toxicological burden, which is meant as percentage of identified residue compounds on the total identified compounds of toxicological relevance.

The following compounds are quantitatively relevant for risk assessment:

Cereals:	Parent, M03 and additionally (for rotational crops only) M01, M02 and M05 (free and conjugated)
Root crops:	Parent; rotational crops only
Leafy crops:	Parent, M01, M02, M05 (free and conjugated); rotational crops only
Fruit crops:	Parent, M14, M28, M33 and M34 (conjugates of M13), M35 (provisionally; open data requirements)

In primary crops, the coverage of the toxicological burden is between 85-97% for fruit crops (provisionally) and 70-73% for cereals.

Table 12 Residue input data for residues of potential concern in food and feed of plant origin (primary crops)

		Cereals				Fruits							
		Grain (C)[a]		Straw (C)[a]		Grapes (C)		Grapes (D)		Banana (C)		Banana (D)	
Metabolite	**RPF**	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	**1**	**14.3**	**32.4**	**25.1**	**36.8**	**24.6**	**27.5**	**45.6**	**48.5**	**44.9**	**64.1**	**60.0**	**60.9**
M01	1	0.5	1.1	2.0	3.8	1.1	1.2	2.1	2.2	1.1	1.6	0.9	0.9
M02	**1**	3.0	6.8	3.2	6.1	0.5	0.6	1.5	1.6	0.5	0.7	0.4	0.4
M03	1	17.8	**40.4**	22.0	**33.6**	2.9	3.2	4.7	5.0	0.8	1.1	1.2	1.3
M14 (incl. conj. M24)	1[b]					13.0	**14.5**			9.2	**13.1**		
M28	1[b]							37.5	**39.9**			31.2	**31.6**
M30	1[b]							1.1	1.2			0.6	
M31	1[b]							1.2	1.3			0.6	
M13 (incl. conj. M33, M34)	1[b]					25.3	**28.2**			13.6	**19.4**		
M35	1[b]					13.0	**14.5**						
M36	1[b]					4.2	4.7						
M37	1[b]					3.2	3.6						
Other (minor)		8.5		15.9		1.8		0.4		0		0	
Sum of relevant metabolites		32.1	**72.8**	47.1	**70.4**	75.9	**84.7**[b]	83.1	**88.4**[b]	67.7	**96.6**[b]	91.2	**92.5**[b]
Sum of non-considered metabolites		12.0		21.1		3.4		11.0		2.4		3.7	

[a] The residue situation in cereal D-label is covered by assessment of C-label
[b] Provisional; toxicological characterisation not finalised

Table 13 Residue input data for major residues of potential concern in food of plant origin (rotational crops)

Metabolite	RPF	30 d PBI				294d [a]		30 d PBI		30 d PBI		161 d PBI [a]	
		Straw (C)		Straw (D)		Straw (D)		Turnip roots (C)		Swiss chard (D)		Swiss chard (C)	
		%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	**1**	**6.8**	**16.0**	**15.2**	**20.0**	**4.2**	**7.0**	**45.8**	**64.1**	**9.4**	**14.4**	**8.8**	**15.0**
M01	1	**n.d.**	**n.d.**	**15.1**	**19.8**	**5.6**	**9.4**	**4.4**	**6.2**	**9.0**	**14.0**	**12.1**	**20.6**
M02	**1**	**3.5**	**8.2**	**17.4**	**22.9**	**17.4**	**29.1**	**2.6**	**3.6**	**19.7**	**30.7**	**14.2**	**24.2**
M03	1	**12.7**	**29.9**	**7.4**	**9.7**	**1.4**	**2.3**	**2.8**	**3.9**			**14.2**	**24.2**
M05 (+conj. M40)	1	**4.5**	**10.6**	**2.8**	**3.7**	**12.6**	**21.1**			**16.6**	**25.8**	**-**	**-**
M06 (+ conj. M44)	1			**1.0**	**1.3**	**10.5**	**17.6**			**3.1**	**4.8**		
Sum of relevant metabolites		**27.5**	**64.7**	**58.9**	**78.0**	**51.7**	**86.6**	**45.8**	**64.1**	**54.7**	**84.9**	**49.3**	**84.1**
Sum of non-considered metabolites		**15.0**	**35.3**	**16.6**	**22.0**	**8.0**	**13.4**	**25.7**	**35.9**	**9.5**	**15.1**	**9.3**	**15.9**

[a] 30d PBI is critical due to higher exposure potential; no higher toxicity is assumed for compounds identified as major at later PBIs (therefore non-consideration of M06 (free+conjugated) for cereals and M03 for Swiss chard)

Livestock

The following major residue compounds are considered as candidates for inclusion into the residue definition for livestock: Parent spiroxamine, M01 (poultry), M02 (poultry), M06 (including its glucuronide conjugate M19; goat and poultry), M07 (goat).

Their quantitative occurrence is expressed in Table 14 (for ruminants) and Table 15 (for poultry) in terms of %TRR (as determined in metabolism studies) and in % of toxicological burden, which is meant as percentage of identified residue compounds.

Table 14 Residues of concern for food of animal origin following administration of spiroxamine: Ruminants (≥10 % TRR and at least 0.01 mg/kg)

		Kidney		**Liver**		**Muscle**		**Fat**		**Milk**	
Metabolite	**RPF**	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	1	0.2	0.3	5.0	6.6	nd	-	nd	-	nd	-
M06 (incl conjug. M19)	1	23.7	34.9	52.3	68.6	56.2	70.5	45.9	62.8	53.3	68.4
M07	1	16.0	23.5	1.7	2.2	10.3	10.3	9.7	9.7	10.9	14.0
Sum of relevant metabolites		39.9	58.7	59.0	77.4	66.5	70.8	55.6	72.5	64.2	84.4
Sum of non-considered metabolites (all minor)		28.1		17.3		13.2		17.5		77.9	

Table 15 Residues of concern for food of animal origin following administration of spiroxamine: Poultry (≥10 % TRR and at least 0.01 mg/kg)

		Liver		**Muscle**		**Fat**		**Eggs**	
Metabolite	**RPF**	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	1	13.3	20.5	17.8	23.5	77.4	85.1	11.8	16.6
M01	1	21.3	32.9	9.3	12.3	8.4	9.2	11.5	16.2
M02	1	21.7	33.5	11.3	14.9	3.4	3.7	10.2	14.4
M06	1	8.5	13.1	37.3	49.3	1.7	1.9	37.4	52.8
Sum of relevant metabolites		64.8	100	75.7	100	90.9	100	70.9	100
Sum of non-considered metabolites		0	0	0	0	0	0	0	0

The fate of parent and feed metabolites M01, M02 and M05 in ruminants and poultry is considered as covered by available studies with parent.

The following compounds in food of animal origin are relevant for risk assessment:

Ruminants: Parent, M06 (free and conjugated), M07

Poultry: Parent, M01, M02, M06

No final conclusion is possible unless information on the metabolic fate of feed metabolite M03 in ruminants and poultry is available (data gap).

Step 20: Residue definition for risk assessment

Plants

Due to the different metabolism of spiroxamine in cereals, grapes and rotational crops, the following separate residue definitions are proposed (all expressed as spiroxamine).

Cereals: **Parent, M03 (primary crops)**
Parent, M03, M01, M02, M05 (free and conjugated); rotational crops only

Root crops: **Parent; rotational crops only**

Leafy crops: **Parent, M01, M02, M05 (free and conjugated); rotational crops only**

Fruit crops: **Parent, M14, M28, M33 and M34 (conjugates of M13), M35 (open data requirements)**

The residue definition for fruit crops is provisional pending full toxicological assessment of metabolites of group B and C (see step 18; data requirement). Separate risk assessments or the application of RPFs might be indicated for the different metabolite groups.

Although the relevance of M03 in food for direct human consumption is low, it is proposed to include M03 into the residue definition based on the toxicological properties of M03, the exposure potential for livestock and human exposure via food of animal origin and uncertainties for isomers composition.

Animals

The following residue definition is proposed for ruminants, pigs and horses (provisional pending addressing of the data gap in livestock animals):

Ruminants: **Parent, M06 (free and conjugated), M07**

Poultry: **Parent, M01, M02, M06**

Data gaps

- Genotoxicity studies for M28 and M37 should be provided. The testing battery should as a minimum include two in vitro tests, covering all three genetic endpoints, i.e. gene mutations, structural and numerical chromosomal alterations.

- Adequate toxicological references should be provided for M28 or other representative substance for Group C (M28, M30 and M31), M13 (as a group representative metabolite for M14, M33 and M34) and M37. The 28 day rat study is recommended as a first tier approach.

- Hydrolysis study demonstrating cleavage of M35 and M36 under physiological conditions, followed by a(Q)SAR/ Read across for the exclusion of genotoxicity and followed by the general toxicological assessment or testing for the ester compounds..

- Ruminant and poultry metabolism of metabolite M03 in feed has to be addressed.

Uncertainties of particular relevance for decision making

The finalisation of the evaluation of the uncertainties is underdevelopment pending adoption of the Scientific Committee guidance on uncertainty in scientific assessment.

The quantitative relevance of all identified metabolites in food and feed as well as their toxicological assessment is discussed in this case study under conditions considered as reasonable worst case by the assessors. A detailed uncertainty assessment for particular elements of toxicity and exposure calculation can be provided on request of risk managers.

The following describes those steps in the decision scheme where alternative, more conservative, decisions could have been made and provides the justification for the approach taken.

- For metabolites M13, M15 and M16 the prediction for genotoxicity is negative, but not considered reliable because they were out of the applicability domain in one CA model and the applicability domain was not defined in the second CA model. This was considered unlikely to be of concern based on expert judgment on the absence of reactive chemical groups in the structure.

- Grouping of metabolites is based on criteria for similarity. However, these criteria are not fully characterized. For genotoxicity endpoints, grouping on profiling and presence of functional groups was considered suitable for the purpose of risk assessment. Grouping of metabolites for section of representative substance for testing for general toxicity was based on common moiety and similarity in the chemical reactivity and this was considered appropriate for this purpose. However, uncertainties still exist as no testing against the toxicological endpoint/s was performed (e.g. the difference between secondary and tertiary amines or hydroxylation of a butyl-group can call for a different reactivity. In absence of testing this is still considered an uncertainty).

- Genotoxic alerts indicated by (Q)SAR for 12 metabolites are considered not relevant on the basis of grouping and read-across. This bears a higher uncertainty compared to *in vitro* results according to the proposed testing scheme.

- Minor rat and plant metabolites were assessed for their genotoxicity potential through (Q)SAR, grouping and read across. . However, minor plant metabolites were not assessed for general toxicity endpoints based on the assumption that the parent is a low potency substance. The uncertainty with regard to non-consideration of minor metabolites is therefore based on the assumption that their toxicological burden will be limited and then refers only to the exposure part.

- The toxicological burden covered by the residue definition is slightly below the target of 75% of the total toxicological burden for cereals, some rotational crops and ruminant matrices. This has only a marginal impact on the calculated dietary consumer risk

- No data are provided to assess the impact of the possible preferential metabolism/degradation of each enantiomer in animals, plants and the environment. As spiroxamine has diastereoisomers the risk assessment should consider the highest intake, assuming that all the toxic activities are due to a single isomer which is representing the residue and a factor of two to the ADI and ARfD should be applied.

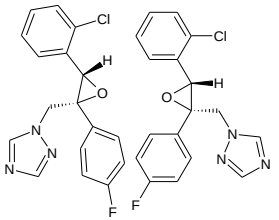
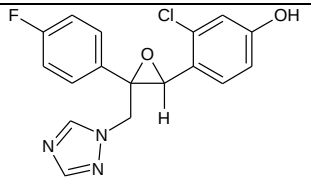
Appendix D. Case study – Epoxiconazole (Germany, 2005[26], 2008[27] & 2015[28])

Step 1: Metabolite identified at any level in residue metabolism (plant)

A list of metabolites detected in residue metabolism studies is given in Table 1. Conjugated metabolites (i.e. glucosides and glucoronides) are assumed to be covered in their toxicological properties by their respective aglycons. For these metabolites, the results of the aglycon assessment can be adopted; the assessment for the conjugates is restricted to exposure estimates.

For the assessment of genotoxicity, position isomers are considered as individual entities, while enantiomers are considered as one entity; since the majority of the applied (Q)SAR models and profilers base their evaluation on a part of the molecule (the structural alerts) and not on the whole molecule, the concept of enantiomers is not relevant in regard to genotoxicity.

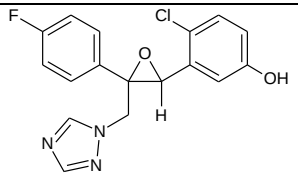
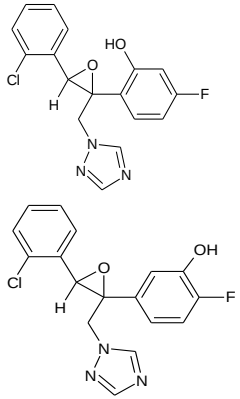
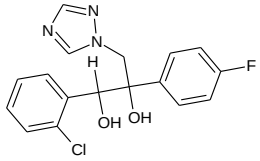
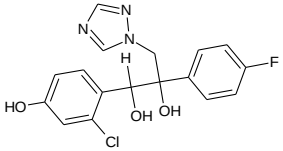
Table 1. Epoxiconazole metabolites

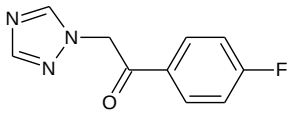
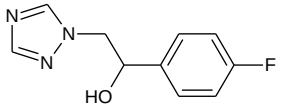
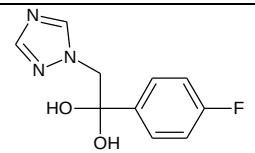
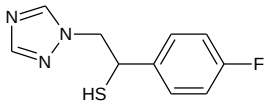
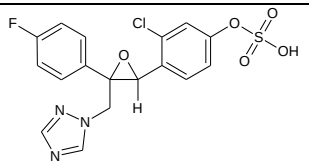
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
Parent	BAS 480 F Epoxiconazole (2RS,3SR)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)propyl]-1H-1,2,4-triazole Fc1ccc(cc1)[C@]4(Cn2cncn2)O[C@H]4c3ccccc3Cl Fc1ccc(cc1)[C@@]4(Cn2cncn2)O[C@@H]4c3ccccc3Cl		
M01	480M1 3-chloro-4-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenol Fc1ccc(cc1)C4(Cn2cncn2)OC4c3ccc(O)cc3Cl		M01 representative for conjugate M11, M61, M67, M68

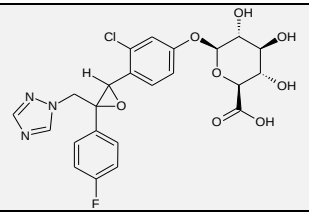
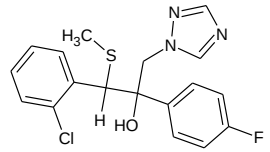
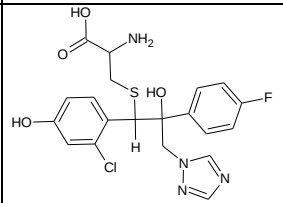
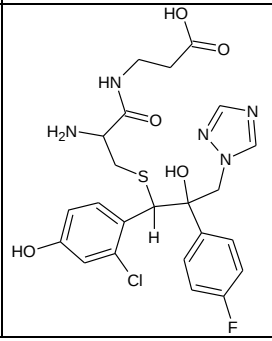
[26] Germany, 2005. Draft Assessment Report (DAR) on the active substance epoxiconazole prepared by the rapporteur Member State Germany in the framework of Directive 91/414/EEC, April 2005. Available at http://dar.efsa.europa.eu/dar-web/provision

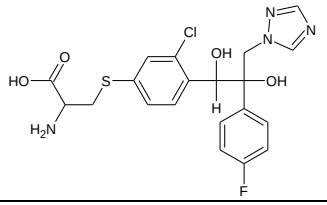
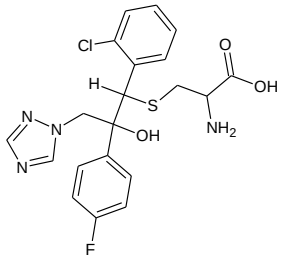
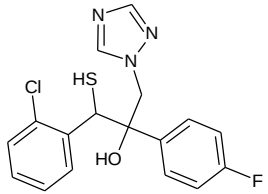
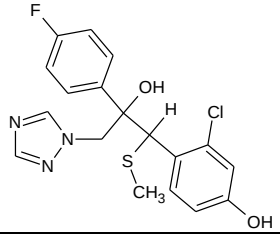
[27] Germany, 2008. Final addendum to the Draft Assessment Report (DAR) on epoxiconazole, compiled by EFSA, February 2008. Available online: www.efsa.europa.eu

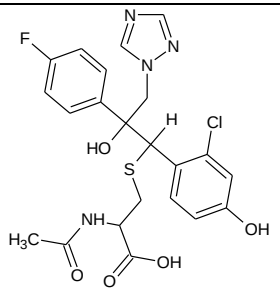
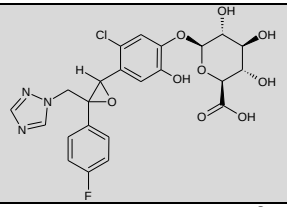
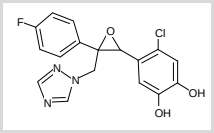
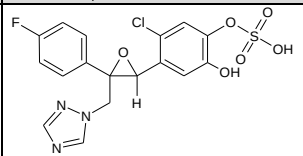
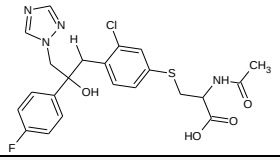
[28] Germany, 2015. Final addendum to the addendum to the draft assessment report (DAR) on epoxiconazole, compiled by EFSA, April 2015. Available online: www.efsa.europa.eu

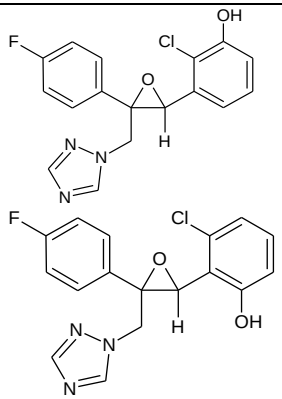
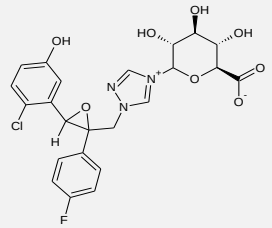
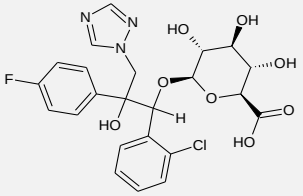
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M02	480M2 BF 480-2, II (Chloro-hydroxy-metabolite) 4-chloro-3-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenol Fc1ccc(cc1)C4(Cn2cncn2)OC4c3cc(O)ccc3Cl		M02 representative for conjugate M27, M32, M61, M67, M68
M03	480M3 (XXXIV) 2-[3-(2-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]-5-fluorophenol Fc1ccc(c(O)c1)C4(Cn2cncn2)OC4c3ccccc3Cl 5-[3-(2-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]-2-fluorophenol Fc1ccc(cc1O)C4(Cn2cncn2)OC4c3ccccc3Cl		Both isomers are used for ananlysis M03 representative for conjugate M66
M04	480M4 BF 480-11 1-(2-chlorophenyl)-2-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propane-1,2-diol OC(Cn1cncn1)(c2ccc(F)cc2)C(O)c3ccccc3Cl		M04 representative for conjugate M28, M29
M05	480M5 1-(2-chloro-4-hydroxyphenyl)-2-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propane-1,2-diol OC(Cn1cncn1)(c2ccc(F)cc2)C(O)c3ccc(O)cc3Cl		Used for ananlysis M05 representative for conjugate M60

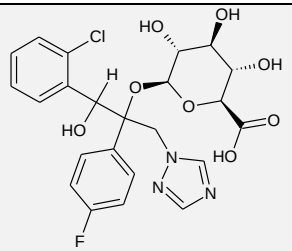
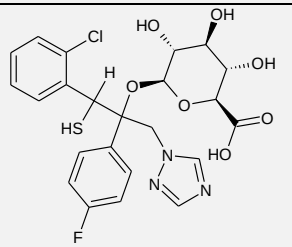
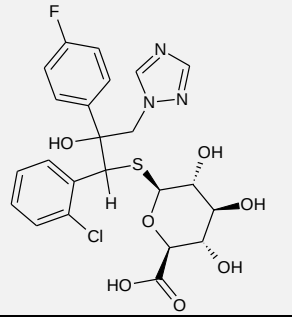
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M06	480M6 1-(4-fluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanone O=C(Cn1cncn1)c2ccc(F)cc2		
M07	480M07 BAS 480-F-alcohol 1-(4-fluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanol OC(Cn1cncn1)c2ccc(F)cc2		
M08	480M08 1-(4-fluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethane-1,1-diol OC(O)(Cn1cncn1)c2ccc(F)cc2		
M09	480M09 1-(4-fluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanethiol SC(Cn1cncn1)c2ccc(F)cc2		
M10	480M10 3-chloro-4-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenyl hydrogen sulfate O=S(=O)(O)Oc1ccc(c(Cl)c1)C4OC4(Cn2cncn2)c3ccc(F)cc3		

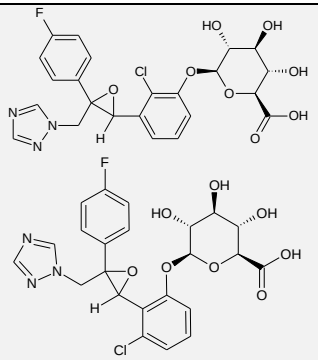
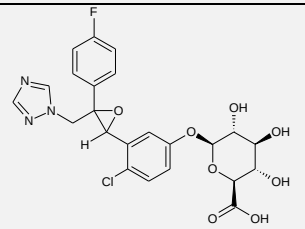
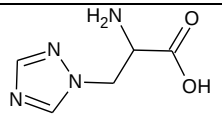
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M11	480M11 3-chloro-4-[3-(4-fluorophenyl)-3-(1H-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C5(Cn2cncn2)OC5c3ccc(cc3Cl)O[C@@H]4O[C@@H]([C@@H](O)[C@H](O)[C@H]4O)C(=O)O		Toxicological assessment covered by M01
M12	480M12 1-(2-chlorophenyl)-2-(4-fluorophenyl)-1-(methylthio)-3-(1*H*-1,2,4-triazol-1-yl)propan-2-ol OC(Cn1cncn1)(c2ccc(F)cc2)C(SC)c3ccccc3Cl		
M13	480M13 *S*-[1-(2-chloro-4-hydroxyphenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]cysteine OC(Cn1cncn1)(c2ccc(F)cc2)C(SCC(N)C(=O)O)c3ccc(O)cc3Cl		M13 representative for conjugate M25
M15	480M15 *S*-[1-(2-chloro-4-hydroxyphenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]cysteinyl-b-alanine OC(Cn1cncn1)(c2ccc(F)cc2)C(SCC(N)C(=O)NCCC(=O)O)c3ccc(O)cc3Cl		M15 representative for conjugate M25

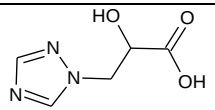
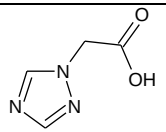
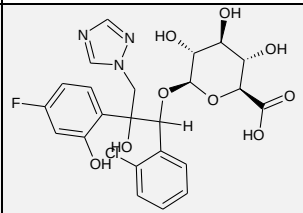
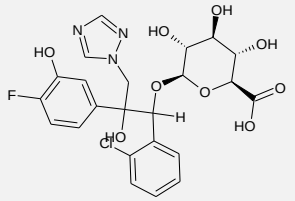
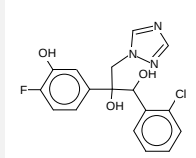
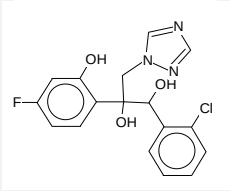
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M16	480M16 *S*-{3-chloro-4-[2-(4-fluorophenyl)-1,2-dihydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]phenyl}cysteine OC(Cn1cncn1)(c2ccc(F)cc2)C(O)c3ccc(SCC(N)C(=O)O)cc3Cl		
M17	480M17 *S*-[1-(2-chlorophenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]cysteine OC(Cn1cncn1)(c2ccc(F)cc2)C(SCC(N)C(=O)O)c3ccccc3Cl		
M18	480M18 Thio-BF 480-11, V 1-(2-chlorophenyl)-2-(4-fluorophenyl)-1-mercapto-3-(1*H*- 1,2,4-triazol-1-yl)propan-2-ol OC(Cn1cncn1)(C(S)c2ccccc2Cl)c3ccc(F)cc3		M18 representative for conjugate M30
M19	480M19 3-chloro-4-[2-(4-fluorophenyl)-2-hydroxy-1-(methylthio)-3-(1*H*-1,2,4-triazol-1-yl)propyl]phenol OC(Cn1cncn1)(c2ccc(F)cc2)C(SC)c3ccc(O)cc3Cl		M19 representative for conjugate M54

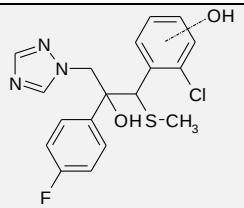
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M20	480M20 *N*-acetyl-*S*-[1-(2-chloro-4-hydroxyphenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]cysteine OC(Cn1cncn1)(c2ccc(F)cc2)C(SCC(NC(C)=O)C(=O)O)c3ccc(O)cc3Cl		
M21	480M21 5-chloro-4-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]-2-hydroxyphenyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C5(Cn2cncn2)OC5c4cc(O)c(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)cc4Cl		Toxicological assessment is done on 
M22	480M22 5-chloro-4-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]-2-hydroxyphenyl hydrogen sulfate O=S(=O)(O)Oc1cc(Cl)c(cc1O)C4OC4(Cn2cncn2)c3ccc(F)cc3		
M23	480M23 *N*-acetyl-*S*-{3-chloro-4-[2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]phenyl}cysteine OC(Cn1cncn1)(Cc2ccc(SCC(NC(C)=O)C(=O)O)cc2Cl)c3ccc(F)cc3		
M25	480M25 Unknown conjugate of MW=57 with x-chloro-y-[2-(4-fluoro-phenyl)-2-hydroxy-1-sulfanyl-3-(1H-1,2,4-triazol-1-yl)propyl] phenol	Structure to be covered by 480M13 / 480M15, Uncertainty assessment	Toxicological assessment covered by M13, M15

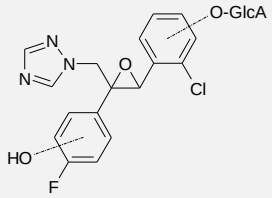
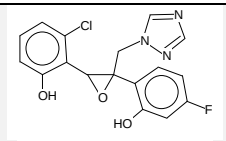
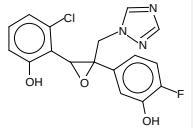
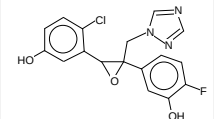
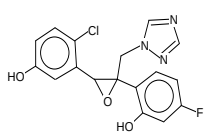
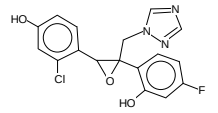
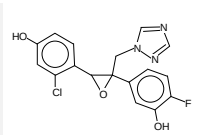
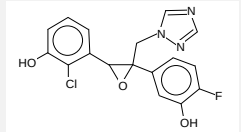
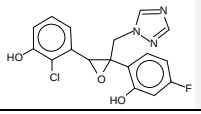
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M26	480M26 2-chloro-3-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)-2-oxiranyl]phenol Fc1ccc(cc1)C4(Cn2cncn2)OC4c3cccc(O)c3Cl 3-chloro-2-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)-2-oxiranyl]phenol Fc1ccc(cc1)C4(Cn2cncn2)OC4c3c(O)cccc3Cl		Both isomers are used for ananlysis M26 representative for conjugate M31, M65, M67, M68
M27	480M27 Parent glucuronide, VI (1-{[3-(2-chloro-5-hydroxy-phenyl)-2-(4-fluorophenyl)-2-oxiranyl]methyl}-1,2,4-triazoliumyl) D-1-deoxy-glucopyranosiduronate (site of conjugation is nitrogen atom of the trizole ring requ. zwitter ionic strcuture) Oc1cc(c(Cl)cc1)C5OC5(Cn2nc[n+](c2)C3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C([O-])=O)c4ccc(F)cc4		Toxicological assessment covered by M02
M28	480M28 BF 480-11-glucuronide, VII 1-(2-chlorophenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C(O)(Cn2cncn2)C(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccccc4Cl		Toxicological assessment covered by M04

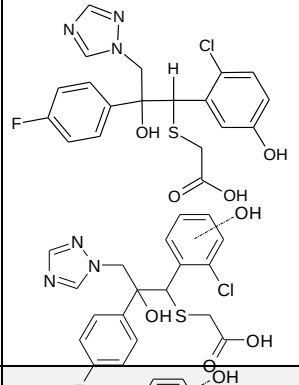
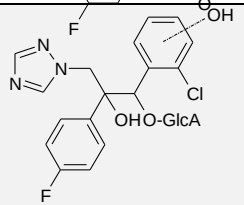
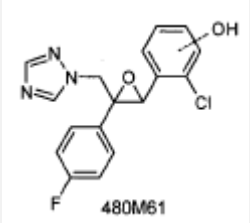
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M29	480M29 BF 480-11-conjugate 1-(2-chlorophenyl)-2-(4-fluorophenyl)-1-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propan-2-yl b-D-glucopyranosiduronic acid Clc1ccccc1C(O)C(Cn2cncn2)(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccc(F)cc4		Toxicological assessment covered by M04
M30	480M30 Thio-BF 480-11-conjugate 1-(2-chlorophenyl)-2-(4-fluorophenyl)-1-mercapto-3-(1*H*-1,2,4-triazol-1-yl)propan-2-yl b-D-glucopyranosiduronic acid Clc1ccccc1C(S)C(Cn2cncn2)(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccc(F)cc4 1-(2-chlorophenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl 1-thio-b-D-glucopyranosiduronic acidFc1ccc(cc1)C(O)(Cn2cncn2)C(S[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccccc4Cl	 	Toxicological assessment covered by M18

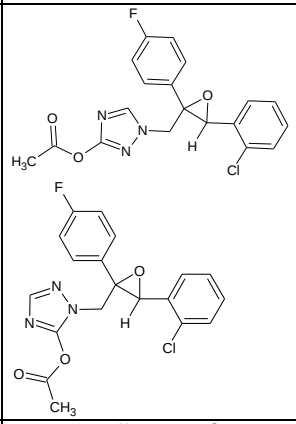
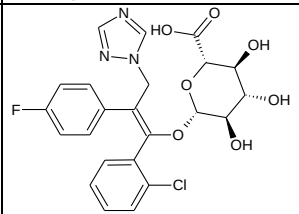
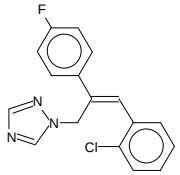
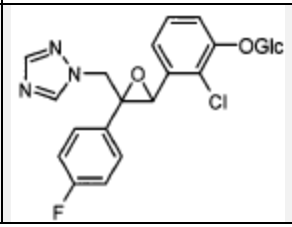
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M31	480M31 Conjugate of III 2-chloro-3-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C5(Cn2cncn2)OC5c4cccc(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4Cl 3-chloro-2-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C5(Cn2cncn2)OC5c4c(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)cccc4Cl		Toxicological assessment covered by M26 (both isomers)
M32	480M32 Conjugate of II 4-chloro-3-[3-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-ylmethyl)oxiran-2-yl]phenyl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C5(Cn2cncn2)OC5c3cc(ccc3Cl)O[C@@H]4O[C@@H]([C@@H](O)[C@H](O)[C@H]4O)C(=O)O		Toxicological assessment covered by M02
M52	480M52 1,2,4-Triazole BF 480-16 (87 085)(CGA 71019)(CGA 98032) 1*H*-1,2,4-triazole c1nncn1		
M49	480M49 Triazolyl alanine 3-(1*H*-1,2,4-triazol-1-yl)alanine NC(Cn1cncn1)C(=O)O		

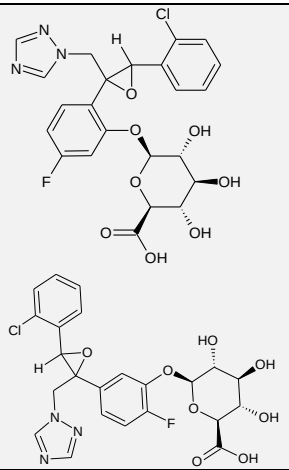
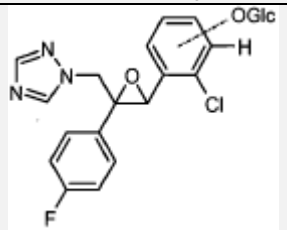
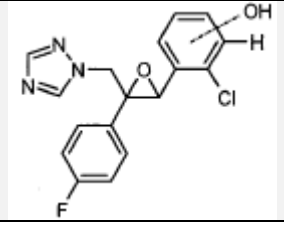
Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M50	480M50 Triazolyl hydroxy propionic acid 2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propanoic acid OC(Cn1cncn1)C(=O)O		
M51	480M51 BF 480-17 Triazolyl acetic acid 1*H*-1,2,4-triazol-1-ylacetic acid O=C(O)Cn1cncn1		
M53	480M53 (and/or isomers) 1-(2-chlorophenyl)-2-(4-fluoro-2-hydroxyphenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl b-D-glucopyranosiduronic acid Fc1ccc(c(O)c1)C(O)(Cn2cncn2)C(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccccc4Cl 1-(2-chlorophenyl)-2-(4-fluoro-3-hydroxyphenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl b-D-glucopyranosiduronic acid Fc1ccc(cc1O)C(O)(Cn2cncn2)C(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccccc4Cl	 	Toxicological assessment is done on  

Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M54	480M54 (and/or isomers) Exact position of OH not known, unresolved mixture (?) To be covered by 480M19, 480M53, and uncertainty to be discussed		Toxicological assessment covered by M19

Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M55	480M55 (and/or isomers)		       

Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M56	480M56 (and/or isomers) Exact position of OH not known, assumed to be at 5- position, and uncertainty to be discussed {[1-(2-chloro-5-hydroxyphenyl)-2-(4-fluorophenyl)-2-hydroxy-3-(1*H*-1,2,4-triazol-1-yl)propyl]sulfanyl}acetic acid OC(Cn1cncn1)(c2ccc(F)cc2)C(SCC(=O)O)c3cc(O)ccc3Cl		
M60	480M60 (and/or isomers) Exact position of OH not known (mixture?), structure coverd by ?		Toxicological assessment covered by M5
M61	480M61 Exact position of OH not known (mixture?), covered by either480M1,480M2, or 480M26		Toxicological assessment covered by M1, M2, M26

Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M62	480M62 1-{[3-(2-chlorophenyl)-2-(4-fluorophenyl)-2-oxiranyl]methyl}-1*H*-1,2,4-triazol-3-yl acetate CC(=O)Oc1ncn(n1)CC3(OC3c2ccccc2Cl)c4ccc(F)cc4 1-{[3-(2-chlorophenyl)-2-(4-fluorophenyl)-2-oxiranyl]methyl}-1*H*-1,2,4-triazol-5-yl acetate CC(=O)Oc4ncnn4CC2(OC2c1ccccc1Cl)c3ccc(F)cc3		
M63	480M63 (1*Z*)-1-(2-chlorophenyl)-2-(4-fluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)-1-propen-1-yl b-D-glucopyranosiduronic acid Fc1ccc(cc1)C(/Cn2cncn2)=C(/O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c4ccccc4Cl		Toxicological assessment is done on 
M65	480M65 Identical to 480M31 first structure		Toxicological assessment covered by M26 (the first structure)

Compound identifier	Name in Study snd Assessment reports and SMILES	Structure	Remark
M66	480M66 2-[3-(2-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-2-oxiranyl]-5-fluorophenyl b-D-glucopyranosiduronic acid Clc1ccccc1C5OC5(Cn2cncn2)c4ccc(F)cc4O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O 5-[3-(2-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-2-oxiranyl]-2-fluorophenyl b-D-glucopyranosiduronic acid Clc1ccccc1C5OC5(Cn2cncn2)c4cc(O[C@@H]3O[C@@H]([C@@H](O)[C@H](O)[C@H]3O)C(=O)O)c(F)cc4		Toxicological assessment covered by M3
M67	480M67 Covered by 480M31 480M32		Toxicological assessment covered by M1, M2, M26_2
M68	480M68 Exact position of OH not known (mixture?), covered by either480M1,480M2, or 480M26		Toxicological assessment covered by M1, M2, M26_2

Step 2: Exclusion of metabolites of no concern

None.

Step 3: Metabolite is known to be genotoxic

No specific information on genotoxicity of metabolites is available.

Step 4/Step 5: Metabolite is covered by rat metabolism

No major rat metabolites (>10% of AD in rat urine from the ADME study) were identified.

Proceed with the genotoxicity assessment (steps 5 to 9) for all metabolites.

Step 5: (Q)SAR prediction of Ames genotoxicity

Step 5.1: Description of (Q)SAR strategy

In order to predict the genotoxic potential (gene mutation and chromosomal aberrations) of the minor rat and plant specific metabolites, four models have been applied: OASIS AMES Mutagenicity and Chromosomal Aberration models (v08.08) implemented in the TIMES software (v2.27.13) and DEREK Nexus Mutagenicity and Chromosome Damage Models v 4.0.6. Lhasa Ltd, Leeds.

Independently of the predictions from (Q)SAR models, the metabolite(s) will be subject of read across analysis (step 6).

Step 5.2: Documentation of OASIS Ames Mutagenicity model (TIMES software)

11. *Used model (title, name of authors, reference)*

OASIS AMES mutagenicity model v08.08, Laboratory of mathematical chemistry, Burgas University

R. Serafimova, M. Todorov, T. Pavlov, S. Kotov, E. Jacob,A. Aptula, O. Mekenyan, Identification of the structural requirements for mutagencitiy by incorporating molecular flexibility and metabolic activation of chemicals. II. General Ames mutagenicity model. *Chem. Res. Toxicol*, 20, (2007), pp. 662−676.

12. *Information about modelled endpoint (endpoint, experimental protocol)*

Ames Mutagenicity essay.

13. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The training set consists of 3489 chemicals (NTP database) separated in three groups: 641 mutagenic chemicals as parents, 418 chemicals mutagenic after S9 metabolic activation (non mutagens as parents), and 2430 non mutagenic chemicals. These three classes of chemicals were considered as biologically dissimilar in the modeling process; i.e., chemicals being mutagenic as parents are distinguished from chemicals, which were metabolically activated

14. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

The TIMES system combines in the same modeling platform metabolic activation of chemicals and their interaction with target macromolecules. The reactivity Ames model (-S9) describing interactions of chemicals with DNA was based on an alerting group approach. Only those toxicophores having clear interpretation for the molecular mechanism causing the ultimate effect were included in the model. The alerts were classified as direct acting and metabolically activated. The mechanistic interrelation between alerts and related parametric ranges generalizing the effect of the rest of the molecules on the alert is also considered. In the Ames model (+S9), the reactivity component was combined with a metabolic simulator, which was trained to reproduce documented maps for mammalian (mainly rat) liver metabolism for 260 chemicals. Parent chemicals and each of the generated metabolites were submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals were predicted to be mutagenic as parents only, parents and metabolites, and metabolites only.

15. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

For 3489 chemicals, the Ames model (-S9) was able to predict correctly 82% of the Ames positive and 91% of the Ames negative training set chemicals. When metabolic activation is taken into account, the Ames model (+S9) predicts 76% of the Ames positive and 76% of the Ames negative training set chemicals.

16. *External statistic, if available*

Not available

17. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The stepwise approach was used to define the applicability domain of the model. It consists of the following sub-domain levels:

- General parametric requirements - includes ranges of variation log KOW and MW,
- Structural domain - based on atom-centered fragments (ACFs).
- Interpolation space - estimates the population density of the parametric space defined by the explanatory variables of the QSAR models by making use the training set chemicals.
- Domain of simulator of metabolism - determines the reliability of the simulated metabolism.

A chemical is considered In Domain if its log K_{OW} and MW are within the specified ranges and if its ACFs are presented in the training chemicals. The information implemented in the applicability domain is extracted from the correctly predicted training chemicals used to build the model and in this respect the applicability domain determines practically the interpolation space of the model.

S. Dimitrov, G. Dimitrova, T. Pavlov, N. Dimitrova, G. Patlevisz, J. Niemela and O. Mekenyan, *J. Chem. Inf. Model.* Vol. 45 (2005), pp. 839-849.

18. *Mechanistic interpretation of the model*

Each structural alert in the model is related with a suggested mechanism of action which is reported together with the prediction.

19. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

Not available

20. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

The model provided suggested mechanism of action, examples of the substances documented to have the mechanism of action, generation of metabolites and prediction for them, information for experimental observed metabolites (if available).

Documentation of DEREK Nexus mutagenicity model

11. Used model (title, name of authors, reference)

DEREK Nexus Mutagenicity Model v 4.0.6.

Lhasa Ltd, Leeds, UK, http://www.lhasalimited.org/

Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. Human and Experimental Toxicology 10, 261-273.

Judson PN, Marchant CA & Vessey JD (2003). Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370.

Marchant CA, Briggs KA & Long A (2003). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177–187.

Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79.

12. Information about modelled endpoint (endpoint, experimental protocol)

The Derek Nexus model for mutagenicity is developed from Ames test data in both S.typh and E.coli. Supporting data from in vivo lacZ-transgenic assay, in vitro L5178Y TK+/- assay, in vitro HGPRT gene mutation assay, in vitro Na+/K+ ATPase gene mutation assay has also been considered for the development of a small number of alerts. Additionally, alert writers consider both mechanistic evidence and chemical properties (such as reactivity).

13. Used training set (number of the substances, information about the chemical diversity of the training set chemicals)

The DEREK model for mutagenicity is a base of rules which codified the knowledge about the relation between a structural features and a toxicological (i.e. mutagenic) effect. Although almost all alerts are related with mechanistic explanation and examples, these rules are not related with particular training set.

Recently, a model for negative prediction (non-mutagenic) has been developed and added to the previous model. For its development a training set of above 10 000 substances has been used (the number of mutagenic and non-mutagenic substances is almost equal). The training set is a compilation of six public available data sets (e.g. Kirkland, ISSSTY, NTP data sets).

14. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Derek Nexus is a rule-based expert system for the prediction of toxicity. Its knowledge base is composed of alerts, examples and reasoning rules which may each contribute to the predictions made by the system. Each alert in Derek describes a chemical substructure believed to be responsible for inducing a specific toxicological outcome (often referred to as a toxicophore). Alerts are derived by experts, using toxicological data and information regarding the biological mechanism of action. Where relevant, metabolism data may be incorporated into an alert, enabling the prediction of compounds which are not directly toxic but are metabolised to an active species. The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible. In addition, a likelihood is provided (e.g. certain, probable, plausible) which takes into account the presence of a structural alert and a limited number of molecular descriptors.

Derek Nexus contains new expert-derived functionality to provide negative predictions for bacterial in vitro mutagenicity. Non alerting compounds are evaluated to identify unclassified and misclassified features (from a data set of $>10^4$ compounds).

• Misclassified features in the molecule are derived from non alerting mutagens in the Lhasa reference set.

• Features in the molecule that are not found in the Lhasa reference set are considered unclassified.

In compounds where all features in the molecule are found in accurately classified compounds from the reference set, a negative prediction is displayed. Predictions for compounds with misclassified or unclassified features remain negative, and these features are highlighted to the user to enable expert assessment of the prediction. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

Derek is a knowledge-based expert system containing mechanistically-based rules which are built using all the underlying evidence available to the SAR developer. Therefore, there is no defined training or test set, and therefore there are no internal validation statistics to report.

15. *External statistic, if available*

Not public available for positive predictions.

Performance against three external, proprietary data sets highlights that negative predictivity for all outcomes is good (generally > 80%) with the presence of unclassified or misclassified features slightly reducing accuracy.

16. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The scope of the structure-activity relationships describing the mutagenicity endpoint is defined by the developer to be the applicability domain for the model. Therefore, if a chemical matches an alert describing a structure-activity for mutagenicity it can be considered to be within the applicability domain. The applicability domain of each alert is defined by the alert developer on the basis of the training set data and expert judgement on the chemical and biological factors which affect the mechanism of action for each alert.

If a compound does not activate an alert or reasoning rule then Derek makes a negative prediction. The applicability of the negative prediction to the query compounds can be determined by an expert, if required, by investigating the presence (or absence) of misclassified and/or unclassified features.

17. *Mechanistic interpretation of the model*

All alerts describing structure-activity relationships for the mutagenicity endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target. The mechanistic basis of the model was developed a priori by examining the active and inactive structures before developing the structure-activity relationship. All references supporting the mechanistic basis of an alert are detailed and available for inspection within the software.

18. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible.

19. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Described above.

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/.

Documentation of OASIS *in vitro* chromosomal aberration model (TIMES software)

11. *Used model (title, name of authors, reference)*

OASIS in vitro chromosomal aberration model v08.08, Laboratory of mathematical chemistry, Burgas University

O. Mekenyan, M. Todorov, R. Serafimova, S. Stoeva, A. Aptula, R. Finking, E. Jacob, Identifying the structural requirements for chromosomal aberration by incorporating molecular flexibility and metabolic activation of chemicals. *Chem. Res. Toxicol.* Vol. 20, (2007), pp. 1927−1941.

12. *Information about modelled endpoint (endpoint, experimental protocol)*

In vitro structural chromosomal aberrations

13. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The training set consists of 506 chemicals separated in three groups: 243 mutagenic chemicals as parents, 77 chemicals mutagenic after S9 metabolic activation (non mutagens as parents), and 186 non mutagenic chemicals

Sofuni, T., Ed. (1998). Data Book of Chromosomal Aberration Test in vitro, Revised Edition. Life-Science Information Center, Tokyo, Japan.

14. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Modeling the potential of chemicals to induce chromosomal damage has been hampered by the diversity of mechanisms which condition this biological effect. The direct binding of a chemical to DNA is one of the underlying mechanisms that is also responsible for bacterial mutagenicity. Disturbance of DNA synthesis due to inhibition of topoisomerases and interaction of chemicals with nuclear proteins associated with DNA (e.g., histone proteins) were identified as additional mechanisms leading to CA. Reactivity component of the CA model (-S9) describing interactions of chemicals with DNA and/or proteins was based on an alerting group approach. Only those toxicophores having clear interpretation for the molecular mechanism causing the ultimate effect were included in the model. Some of the specified alerts interact directly with DNA or nuclear proteins, whereas others are applied in a combination of two-dimensional QSAR models assessing the degree of activation of the alerts from the rest of the molecules. In the CA model (+S9), the reactivity component was combined with a metabolic simulator, which was trained to reproduce documented maps for mammalian (mainly rat) liver metabolism for 260 chemicals.

Parent chemicals and each of the generated metabolites were submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals were predicted to be mutagenic as parents only, parents and metabolites, and metabolites only.

15. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

For 506 chemicals, the CA model (-S9) was able to predict correctly 79% of the CA positive and 87% of the CA negative training set chemicals. When metabolic activation is taken into account, the CA model (+S9) predicts 81% of the CA positive and 75% of the CA negative training set chemicals.

16. *External statistic, if available*

Not available

17. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The stepwise approach was used to define the applicability domain of the model. It consists of the following sub-domain levels:

- General parametric requirements - includes ranges of variation log KOW and MW,
- Structural domain - based on atom-centered fragments (ACFs).
- Interpolation space - estimates the population density of the parametric space defined by the explanatory variables of the QSAR models by making use the training set chemicals.
- Domain of simulator of metabolism - determines the reliability of the simulated metabolism.

A chemical is considered In Domain if its log K_{OW} and MW are within the specified ranges and if its ACFs are presented in the training chemicals. The information implemented in the applicability domain is extracted from the correctly predicted training chemicals used to build the model and in this respect the applicability domain determines practically the interpolation space of the model.

S. Dimitrov, G. Dimitrova, T. Pavlov, N. Dimitrova, G. Patlevisz, J. Niemela and O. Mekenyan, *J. Chem. Inf. Model.* Vol. 45 (2005), pp. 839-849.

18. *Mechanistic interpretation of the model*

Each structural alert in the model is related with a suggested mechanism of action which is reported together with the prediction.

19. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

Not available

20. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

The model provided suggested mechanism of action, examples of the substances documented to have the mechanism of action, generation of metabolites and prediction for them, information for experimental observed metabolites (if available).

Documentation of DEREK Nexus Chromosome damage model

11. *Used model (title, name of authors, reference)*

DEREK Nexus Mutagenicity Model v 4.0.6.

Lhasa Ltd, Leeds, UK, http://www.lhasalimited.org/

Sanderson DM & Earnshaw CG (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. Human and Experimental Toxicology 10, 261-273.

Judson PN, Marchant CA & Vessey JD (2003) Using argumentation for absolute reasoning about the potential toxicity of chemicals. Journal of Chemical Information and Computer Sciences 43, 1364-1370.

Marchant CA, Briggs KA & Long A (2003). In silico tools for sharing data and knowledge on toxicity and metabolism: Derek for Windows, Meteor, and Vitic. Toxicology Mechanisms and Methods 18, 177–187.

Judson PN, Stalford SA & Vessey J (2013). Assessing confidence in predictions made by knowledge-based systems. Toxicology Research 2, 70-79.

12. *Information about modelled endpoint (endpoint, experimental protocol)*

The Derek Nexus model for chromosome damage is developed from several sources of data. Sources of primary data used for alert development include in vitro and in vivo chromosome aberration test, in vitro and in vivo micronucleus test, in vitro L5178Y TK+/- assay. Alert writers consider both mechanistic evidence and chemical properties (such as reactivity). Depending on evidence in vitro and/or in vivo prediction can be made.

13. *Used training set (number of the substances, information about the chemical diversity of the training set chemicals)*

The DEREK model for chromosome damage is a base of rules which codified the knowledge about the relation between a structural features and a toxicological (i.e. chromosome damage) effect. Although almost all alerts are related with mechanistic explanation and examples, these rules are not related with particular training set.

14. *Information on the algorithm used for deriving the model and the molecular descriptors (name and type of the descriptors used, software used for descriptor generation and descriptor selection)*

Derek Nexus is a rule-based expert system for the prediction of toxicity. Its knowledge base is composed of alerts, examples and reasoning rules which may each contribute to the predictions made by the system. Each alert in Derek describes a chemical substructure believed to be responsible for inducing a specific toxicological outcome (often referred to as a toxicophore). Alerts are derived by experts, using toxicological data and information regarding the biological mechanism of action. Where relevant, metabolism data may be incorporated into an alert, enabling the prediction of compounds which are not directly toxicity but are metabolised to an active species. The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible. In addition likelihood is provided (ie certain, probable, plausible, equivocal and nothing to report) which takes into account the presence of a structural alert and a limited number of molecular descriptors.

15. *Internal statistics (performance of the model to the training set chemicals)- goodness-of-fit, robustness and predictivity*

Derek is a knowledge-based expert system containing mechanistically-based rules which are built using all the underlying evidence available to the SAR developer. Therefore, there is no defined training or test set, and therefore there are no internal validation statistics to report.

16. *External statistic, if available*

Not public available.

17. *Information about the applicability domain (description of the applicability domain of the model and method used to assess the applicability domain)*

The scope of the structure-activity relationships describing the chromosome damage endpoint is defined by the developer to be the applicability domain for the model. Therefore, if a chemical matches an alert describing a structure-activity for mutagenicity it can be considered to be within the applicability domain. The applicability domain of each alert is defined by the alert developer on the basis of the training set data and expert judgement on the chemical and biological factors which affect the mechanism of action for each alert. If a compound does not activate an alert or reasoning rule in Derek, a result of 'nothing to report' is presented to the user. This can be interpreted as a negative prediction or that the query compound is outside the domain of the model. Which of these is more appropriate may depend on the endpoint of interest.

18. *Mechanistic interpretation of the model*

All alerts describing structure-activity relationships for the chromosome damage endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target. The mechanistic basis of the model was developed a priori by examining the active and inactive structures before developing the structure-activity relationship. All references supporting the mechanistic basis of an alert are detailed and available for inspection within the software.

19. *Description, experimental data and predictions of possible structural analogues of the substance (provided by the software or selected by the applicant)*

The derivation of each alert is described in the alert comments along with supporting references and example compounds where possible

20. *Any additional information provided by the model, e.g. suggested mechanism of action, uncertainties*

Described above

The model is published in the QMRF JRC Database: http://qsardb.jrc.it/qmrf/

Step 5.3: Description of results. Analysis of genotoxicity prediction and applicability domain

Table 3 ***Prediction of genotoxicity (gene mutation - OASIS and DEREK Nexus models and chromosomal aberrations - OASIS chromosomal aberration model and DEREK Nexus in vitro human and mammalian chromosomal damage models) of rat and plant specific metabolites by (Q)SAR***

	OASIS gene mutation model (Applicability Domain)	**DEREK Ames model**	**DEREK Chromosome damages model**	**OASIS Chromosomal aberration model (Applicability Domain)**
M01	Negative (Out)	Negative	Nothing to report	Positive with MA* (Out)
M02	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M03_1	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M03_2	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M04	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M05	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M06	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M07	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M08	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M09	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M10	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M12	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M13	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M15	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M16	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M17	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)

M18	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M19	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M20	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M21	Negative (Out)	Negative	Plausible	Positive with MA(Out)
M22	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M23	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M26_1	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M26_2	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M49	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M50	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M51	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M52	Negative (Out)	Negative	Nothing to report	Negative with/without MA (Out)
M53_1	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M53_2	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_1	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_2	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_3	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M55_4	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)

M55_5	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_6	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_7	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M55_8	Negative (Out)	Negative	Nothing to report	Positive with MA(Out)
M56_1	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M56_2	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M56_3	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M56_4	Negative (Out)	Negative	Nothing to report	Positive with/without MA (Out)
M62_1	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M62_2	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)
M63	Negative (Out)	Negative	Nothing to report	Positive with MA (Out)

*MA – metabolic activation

OASIS Mutagenicity model predicts all 45 metabolites as negative, out of the applicability domain.

DEREK Nexus Mutagenicity model predicts all 45 metabolites as negative (non-mutagenic).

OASIS Model for chromosomal aberrations, predicts nine metabolites M05, M13, M15, M19, M20 and M56 (all isomers) as positive with and without metabolic activation. In all cases a phenol formed as a result of hydroxylation of benzene halogenated ring is recognised as an alert for interactions with topoisomerases/proteins. Thirty metabolites, M01, M02, M03 (all isomers), M04, M07, M09, M10, M12, M16, M17, M18, M21, M22, M23, M26 (all isomers), M53 (all isomers), M55 (all isomers), M62 (all isomers) and M63, are predicted to be positive with metabolic activation. In all cases mono or bi hydroxylation is predicted to occur in benzene halogenated rings, and formed phenol(s) is recognised as an alert for interactions with topoisomerases/proteins.

All predictions are out of the model applicability domain. Six metabolites are predicted as negative with and without metabolic activation M06, M08, M49, M50, M51 and M52. They are out of the model applicability domain.

DEREK Nexus in vitro human and mammalian chromosomal damage models predict metabolite M21 as plausible to cause chromosomal damages due to a catechol alert in the molecule. For all other metabolites the outputs are "nothing to report".

Step 5.4: Conclusion on (Q)SAR

Metabolites M06, M08, M49, M50, M51 and M52 are predicted as negative from all models.

(Q)SAR assessment identified a potential of metabolite M21 to induce genotoxicity hazard. Both models for chromosomal damages predicted the metabolite as positive.

Metabolites M04, M05, M06, M07, M08, M09, M12, M13, M15, M16, M17, M18, M19, M20, M23, M49, M50, M51, M52, M53 (all isomers), M56 (all isomers) and M63 are predicted as negative from three model, though they are predicted as positive by the OASIS chromosomal aberration model.

Step 6: Read across (OECD toolbox)[29]

Step 6.1:

Both endpoints, gene mutation and chromosomal aberrations, should be evaluated by read across for all metabolites.

Molecular initiating events of relevance for this assessment are interaction with DNA and/or proteins. The profilers included in the OECD Toolbox which codified the structural alerts that are important for these two types of interactions are the mechanistic profilers - DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers- DNA alerts for AMES, MN and CA by OASIS v1.3, In vitro mutagenicity (AMES test) alerts by ISS, In vivo mutagenicity (Micronucleus) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1.

The above mentioned profilers have been applied to all metabolites of interest and to the parent substance as a substance with known experimental genotoxic activity.

[29] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm

In order to evaluate the structural similarity, in addition to the structural alerts related to the evaluated endpoints, organic functional group profiler has been applied. This additional step will provide information on the presence/absence of other functional groups different to the structural alerts and will give indication for the potential influence of the remaining part of the molecule to the relevant structural alerts (i.e. electronic and structural influence).

No structural alerts were reported for the parent substance and for all the metabolites evaluated for the profilers DNA alerts for AMES, MN and CA following the application of OASIS v1.3.

The alerts found for DNA binding following the profilers for DNA binding by OASIS v.1.3, DNA binding by OECD, Protein binding by OASIS v 1.3, Protein binding by OECD and endpoint specific profilers, In vitro mutagenicity (AMES test) alerts by ISS, In vivo mutagenicity (Micronucleus) alerts by ISS, Protein binding alerts for Chromosomal aberrations by OASIS v1.1 and organic functional group are presented in the Table 4.

Table 4 Genotoxicity profiling of isoproturon metabolites by OECD Toolbox

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
parent	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Saturated heterocyclic fragment Triazole
M01	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M02	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M03_1	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Phenol Saturated heterocyclic fragment Triazole
M03_2	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M04					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Dihydroxyl group Triazole
M05					Alert6	Alert 8 Alert 9	Alert 10	Alcohol Amidine Aryl Aryl halide Dihydroxyl group Phenol Triazole
M06					Alert6	Alert 8 Alert 9		Amidine Aryl

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Aryl halide Ketone Triazole
M07					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Triazole
M08					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Triazole
M09	Alert 11		Alert 12	Alert 13	Alert6	Alert 8 Alert 9		Amidine Aryl Aryl halide Thioalcohol Triazole
M10	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Saturated heterocyclic fragment Sulfate Triazole
M12					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Sulfide Triazole
M13					Alert6	Alert 8 Alert 9	Alert10	Alcohol Aliphatic Amine, primary Alpha amino acid Amidine Aryl Aryl halide Carboxylic acid Phenol Sulfide Triazole
M15			Alert 14		Alert6	Alert 8 Alert 9	Alert10	Alcohol Aliphatic Amine, primary Amidine Aryl Aryl halide Carboxamide Carboxylic acid Phenol Sulfide Triazole
M16					Alert6	Alert 8 Alert 9		Alcohol Aliphatic

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Amine, primary Alpha amino acid Amidine Aryl Aryl halide Carboxylic acid Dihydroxyl group Sulfide Triazole
M17					Alert6	Alert 8 Alert 9		Alcohol Aliphatic Amine, primary Alpha amino acid Amidine Aryl Aryl halide Carboxylic acid Sulfide Triazole
M18	Alert 11		Alert 12	Alert 13	Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Thioalcohol

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Triazole
M19					Alert6	Alert 8 Alert 9	Alert 10	Alcohol Amidine Aryl Aryl halide Phenol Sulfide Triazole
M20			Alert 14		Alert6	Alert 8 Alert 9	Alert 10	Alcohol Amidine Aryl Aryl halide Carboxamide Carboxylic acid Phenol Sarcosine Sulfide Triazole
M21	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M22	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Epoxide Phenol Saturated heterocyclic fragment Sulfate Triazole
M23			Alert 14		Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Carboxamide Carboxylic acid Sarcosine Sulfide Triazole
M26_1	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M26_2	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Saturated heterocyclic fragment Triazole
M49					Alert6	Alert 8 Alert 9		Aliphatic Amine, primary Alpha amino acid Amidine Aryl Carboxylic acid Triazole
M50					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Carboxylic acid Triazole
M51					Alert6	Alert 8 Alert 9		Amidine Aryl Carboxylic acid Triazole
M52					Alert6	Alert 8 Alert 9		Amidine Aryl Triazole
M53_1					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
								Aryl halide Dihydroxyl group Phenol Triazole
M53_2					Alert6	Alert 8 Alert 9		Alcohol Amidine Aryl Aryl halide Dihydroxyl group Phenol Triazole
M55_1 to M55_8	Alert 1	Alert2	Alert 3	Alert 4	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Amidine Aryl Aryl halide Epoxide Phenol Saturated heterocyclic fragment Triazole
M56_1 to M56_4					Alert6	Alert 8 Alert 9	Alert 10	Alcohol Amidine Aryl Aryl halide Carboxylic acid Phenol Sulfide Triazole

	DNA Binding by OASIS v1.3	DNA Binding by OECD	Protein binding by OASIS v 1.3	Protein binding by OECD	In vitro mutagenicity (AMES) alerts by ISS	In vivo mutagenicity (MN) by Iss	Protein binding alerts for CA by OASIS v 1.1	Organic functional groups
M62_1 and M62_2	Alert 1 Alert 15	Alert2	Alert 3	Alert 4 Alert 16	Alert 5 Alert6	Alert 7 Alert 8 Alert 9		Acetoxy Amidine Aryl Aryl halide Carboxylic acid ester Epoxide Saturated heterocyclic fragment Triazole
M63					Alert6	Alert 8 Alert 9		Alkene Allyl Amidine Aryl Aryl halide Triazole

Alert 1: SN2 > Alkylation, direct acting epoxides and related > Epoxides and Aziridines
Alert 2:SN2 > Direct Acting Epoxides and related > Epoxides
Alert 3: SN2 > Ring opening SN2 reaction > Epoxides, Aziridines and Sulfuranes
Alert 4: SN2 > Epoxides and Related Chemicals > Epoxides
Alert 5: Epoxides and aziridines
Alert 6: Hydrazine
Alert 7: Epoxides and aziridines
Alert 8: H-acceptor-path3-H-acceptor
Alert 9: Hydrazine
Alert 10: AN2 > Michael-type addition to quinoid structures > Phenols
Alert 11: Radical > Generation of reactive oxygen species > Thiols
Alert 12: SN2 > Interchange reaction with sulphur containing compounds > Thiols and disulfide compounds
Alert 13: SN2 > SN2 reaction at a sulphur atom > Thiols
Alert 14: Acylation > Ester aminolysis > Amides
Alert 15: Specific Acetate Esters (different mechanisms e.g. Nucleophilic attack after cerbenium ion formation; Acylation)
Alert 16: Acylation > Direct Acylation Involving a Leaving group > Acetates

Read across results:

All profilers (except DNA alerts for AMES, MN and CA following OASIS v1.3 analysis) recognized in the parent molecule epoxide ring, as a potential alert for binding with DNA and/or proteins (alerts 1, 2, 3, 4, 5, and 7). The profilers developed by ISS for Ames mutagenicity and in vivo MN identified additional two alerts - Hydrazine (alerts 6 and alert 8) and H-acceptor-path3-H-acceptor (alert 9).

The three alerts present in the parent substance are also present in metabolites M01, M02, M03, M10, M21, M22, M26, M55 (all isomers). No new alerts were identified. One or more hydroxyl groups (in metabolites M01, M02, M03, M21, M26 and M55) and a sulphate group (in metabolite M10 and M22) are considered consequent to the metabolism of the halogenated rings. Metabolites M01, M02, M03, M10, M22, M26, M55 (all isomers) could be considered very similar to the parent substance and therefore of not genotoxicity concern. Although the read across analysis show that the metabolite M21 is also very similar to the parent substance and the OECD Toolbox profilers didn't identified any new alert, both models for chromosomal damages (DEREK Nexus and OASIS) predicted the metabolite as positive. Therefore the concern of genotoxicity cannot be excluded.

Metabolite M62 contains all alerts present in the parent substance but a new alert – Acetates is identified by two profilers (DNA binding by OASIS and Protein binding by OECD). Therefore the concern of genotoxicity cannot be excluded.

The alert linked to the epoxide ring disappeared for metabolites M04, M06, M07, M08, M12, M16, M17, M49, M50, M51, M52, M53 and M63; the two additional alerts - Hydrazine and Hacceptor-path3-Hacceptor, are present in all of them. No new alerts were identified. Differences in the remaining part of the molecules, compared to the parent substance are related with opening of the epoxide ring and formation of OH group in metabolites M04, M53 and M17. For the metabolite M53, an additional OH group in the fluorinated aromatic ring is present while the metabolite M17 is a cysteine conjugate of metabolite M04. Therefore, based on the read across analysis, metabolites M04 and M53 should be considered very similar to the parent substance and therefore of no genotoxicity concern. If hydrolysis can be justified for metabolite M17, its toxicological assessment is covered by metabolite M04 and therefore the genotoxicity concern for this metabolite could also be excluded.

For metabolite M12, after opening of the epoxide ring, a methylthiol group is formed, and for metabolite M63 a double bond is present. Although these two new functional groups are not recognized as structural alerts for genotoxicity, they could change the molecular reactivity and consequently the biological behaviour of these two metabolites when compared to the parent substance and therefore genotoxic concern for them could not be excluded.

For metabolites M06, M07 and M08, the epoxide ring and the halogenated aromatic ring are not present and they should be considered structurally different from the parent substance, two of them are predicted (by OASIS CA model) as potentially causing chromosomal aberrations (M07 and M09), therefore their genotoxic potential cannot be excluded.

Metabolites M49, M50, M51 and M52, for which no new alerts were identified, belong to the triazole class M52 containing alanine M49, propanoic M50 and ylacetic M51 acids groups; therefore they cannot be considered structurally similar to the parents substance. They are predicted as negative by all 4 models but the predictions are out of the model applicability model, therefore and their genotoxic potential should be evaluated.

A special case is the metabolite M16, a cysteine conjugate of metabolite M05 (see the analyses done for the metabolite M05 below).

The OH group, present in the chlorinated aromatic ring, is recognized as an alert for interaction with proteins (Protein binding alerts for CA by OASIS) in metabolites M05, M13, M15, M19, M20, M56 (all isomers). Metabolites M13, M15, M16, M19, M20 and M56 are all conjugates of metabolite M05. They are hydrolysis products of metabolite M05 for which genotoxic potential cannot be excluded since a new alert is present. It should be noted that for metabolites M15 and M20 a new alert – amides was recognized by Protein binding by OASIS but this alert is a part of the cysteine molecule and therefore it was not considered of genotoxicity concern.

Similarly, the new alert – amides (Protein binding by OASIS) was reported for metabolite M23. Also in this case, the alert was linked to the cysteine molecule. If hydrolyses is demonstrated the metabolite could be considered similar to metabolite M05, although has one OH group less.

For metabolites M09 and M18 a new alert – thiols, for DNA and protein binding (DNA binding by OASIS, Protein binding by OASIS and Protein binding by OECD) was reported. Therefore their genotoxic potential cannot be excluded. Considering the remaining part of the molecule the metabolite M09 should be considered similar to metabolites M06, M07 and M08 and grouped together. Metabolite M18 is similar to metabolite M12 and they could be grouped together.

Summary:

1. Metabolites M01, M02, M03, M10, M22, M26, M55 (all isomers) are very similar to the parent substance and therefore considered of no genotoxicity concern.
2. Metabolites M04 and M53 should be also considered very similar to the parent substance and therefore of no genotoxicity concern. If hydrolysis can be considered as a likely event for metabolite M17 its toxicological assessment is covered by metabolite M04 and therefore the genotoxicity concern for this metabolite could also be excluded.
3. **Metabolite M21** the genotoxicity concern cannot be excluded therefore it should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9).
4. **Metabolite M62** the genotoxicity concern cannot be excluded therefore it should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9).
5. **Metabolite M63** the genotoxicity concern cannot be excluded therefore it should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9).
6. **Metabolites M12 and M18** the genotoxicity concern cannot be excluded therefore they should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9). Metabolite M18 could be potentially tested as a representative, since an alert was identified for it.
7. **Metabolites M06, M07, M08 and M09** the genotoxicity concern cannot be excluded therefore they should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9). Metabolite M06 (a ketone) and M09 (a thiol) could be tested as group representatives.
8. Metabolites M49, M50, M51 and M52 the genotoxicity concern cannot be excluded therefore they should to be subject of exposure assessment and comparison against TTC (step8) and/or testing (step 9).
9. **M05, M13, M15, M16, M19, M20, M23,** M56 (all isomers) the genotoxicity concern cannot be excluded therefore they should to be subject of exposure assessment and comparison

against TTC (step 8) and/or testing (step 9). Metabolite M05 could be tested as representative in case hydrolysis is considered as likely.

Step 7: Generation of input data and combined exposure assessment against $TTC_{genotoxicity}$

For following uses an exposure assessment was attempted.

Table 5 Uses considered for exposure estimates

Crop	Application			
	Growth stage	Number	kg as/ha #	PHI
Cereals (wheat, rye, barley, oat, spelt, triticale)	BBCH 25-69	2	0.125	35
Sugar beets	BBCH 39-49	2	0.125	28
Banana	Not specified	Not specified	0.098	0

PHI pre-harvest interval
per treatment

Step 7.1. Derivation of residue input data for metabolites

a) Residue levels in primary crop (cereal, sugar beet, banana) and in groundwater

Metabolite identification was attempted in the following commodities of crops treated post-emergence with ^{14}C-epoxiconazole:

- spring wheat plant parts, treated
  - at growth stages BBCH 37 and 47-49 with 0.12 kg as/ha at a time (ca. 0.96 N rate) – triazole label
  - at growth stage BBCH 29 with 0.25 kg as/ha (1 N rate) – oxirane label
  - at growth stage BBCH 38 and 69 with 0.125 kg as/ha at a time (1 N rate) for each label, oxirane and triazole
- sugar beet roots and tops, treated twice with 0.15 kg as/ha (1.2 N), growth stages not reported
- protected and unprotected bunches of bananas, treated post-emergence with 4 x 0.15 kg as/ha, growth stages not reported
- coffee beans of plants treated post-emergence with 0.15 and 0.10 kg as/ha, growth stages at treatment not reported
- Residues in banana and coffee beans are relevant for consumers only. Residues in grain and sugar beet root are relevant for consumer and livestock exposure; residues in straw and beat tops are relevant for livestock exposure calculation; residues in forage are not deemed relevant for livestock exposure (GAP is on cereals for grain production).
- FOCUS groundwater level predictions are available for epoxiconazole and 1,2,4-triazole. The PECgw values for both substances are far below the 0.1 µg/L level in all 9 FOCUS scenarios. Information is not available for any other metabolite. (assessed for cereals and sugar beet uses)
- Where necessary for the assessment residue data from field trials (HR, STMR) as reported in the DAR and addenda to the DAR were used.

b) Residue levels in rotational crops

- Rotational crop metabolism study was simulating realistic worst case situations in terms of soil residue concentrations, considering the soil accumulation potential of epoxiconazole, and sowing of rotational crops at 30 day plant-back interval (PBI), 120 day PBI and 356 day PBI. Field trials are available but cannot be used for conversion. Parent residues were <LOQ. Soil concentrations in the field trials upon a single use of epoxiconazole were significantly lower than the predicted plateau concentration in soil. Thus, metabolism data could be used for exposure estimates for metabolites, where suitable. Crop groups studied: Cereals (grain, straw), root crops (radish root and leaf), and leafy crops (lettuce). Data on additional crop groups (oilseed; fruiting vegetables) are not available. However, metabolite identification in rotational crops was limited to cereals (triazole label) only.
- 30 day PBI (root/tuber and leafy crops): Upon regular harvest with the intended PHI, ploughing and fallowing will precede replanting, that however may occur earlier than after 120 days. Therefore, residue data of the 30 day PBI should be considered when deriving highest and median residues in rotational crops across the three plant back intervals.

Wheat:

Study 1

Oxirane label (1 N): 6 additional - not identified - components were found in the organic phases of the straw samples (<0.001-0.007 mg/kg <0.1-0.4% TRR). The aqueous phase was composed of 31 distinct peaks (0.001-0.04 mg/kg, 0.1-2.2% TRR, not identified). In grains, greater parts of radioactivity were associated with or incorporated into the starch fraction; no identification of metabolites was performed.

Triazole label (0.96 N): 12 additional - not identified - components were found in the organic phases of the straw samples (0.001-0.017 mg/kg, ≤0.1% TRR). The aqueous phase was composed of 20 distinct peaks, 18 of them unidentified (0.001-0.015 mg/kg, ≤0.1% TRR). In grains, greater parts of radioactivity were associated with or incorporated into the starch fraction; no identification of metabolites was performed.

Study 2

Generally, the identification and characterisation rate was high in all matrices accounting for around 90% of the radioactivity present. In grain (oxirane label), the identification and characterisation rates were slightly lower, however the residue concentration was low (TRR 0.049 mg/kg) which resulted in a higher uncertainty of the values measured. Due to high matrix load only some structures could be elucidated: metabolites 480M61 and 480M63. Some unidentified peaks were present in the medium polar region in amounts.

From the two cereal metabolism studies, the identity of additional cereal metabolites was proposed as displayed in column 5 of Table 1; however concentrations were only determined for metabolites listed in Table 6.

Table 6 Wheat metabolism summary

	Wheat, primary crop - Study 1								**Wheat, primary crop - Study 2**															
	Metabolism study 1 N (oxirane)				*Metabolism study 0.96 N (triazole)*				*Metabolism study 1 N (oxirane)*				*Metabolism study 1N (triazole)*				*Metabolism study 1 N (oxirane)*				*Metabolism study 1N (triazole)*			
	Straw				***Straw***				***Straw***				***Straw***				***Grain***				***Grain***			
	TRR		CF	HRc	TRR		CF	HRc	TRR		CF	HRc	TRR		CF	HRc	TRR		CF	STMRc	TRR		CF	STMRc
	%	mg /kg		mg /kg	%	mg /kg		mg /kg	%	mg /kg		mg /kg	%	mg /kg		mg /kg	%	mg /kg		mg /kg	%	mg /kg		mg /kg
TRR		*1.98*				*13.71*				*13.99*				*15.23*				*0.049*				*0.324*		
Parent	**42.7**	**0.84**	**1**	**15.4**	**63.4**	**8.70**	**1**	**15.4**	**89.2**	**12.47**	**1**	**15.4**	**92.1**	**14.02**	**1**	**15.4**	**53.4**	**0.026**	**1**	**0.14**	**4.5**	**0.015**	**1**	**0.14**
M02	1.2	0.02	0.024	0.367																				
M04	0.1	0.002	0.002	0.037																				
M06					0.4	0.061	0.007	0.108																
M07	1.5	0.03	0.036	0.550																				
M26 (M61)	1.8	0.04	0.048	0.733																				
M61 conj.					1.1	0.157	0.018	0.278																
M61/ M63 **									3.1	0.432	0.035	0.53	1.9	0.295	0.021	0.324	**2.6**	**0.001**	**0.039**	**0.005**				
M49*													0.7	0.053	0.004	0.058					**78.6**	**0.121**	**8.07**	**1.13**

* Concentration [mg/kg] of M49 was calculated using the molecular mass of triazole alanine
** "Medium polar" with retention times between 40 and 62 minutes using HPLC method LCO1, containing metabolites M61/M63

- **Sugar beet (1.2 N):**

No identification of any compound in roots and leaves attempted except parent compound.

Sugar beet roots: Parent 0.032-0.034 mg/kg, corresponding to 57-64% TRR, up to 6 additional compounds at 0.001– 0.003 mg/kg;

Sugar beet tops: Parent 4.09-6.0 mg/kg corresponding to 92-98% TRR, no metabolites determined.

- **Coffee** (0.15 + 0.1 kg as/ha; N rate factor unknown):

Coffee beans: No identification of residues due to low absolute levels (TRR 0.008 – 0.009 mg/kg), only presence of parent (0.001 mg/kg) could be confirmed.

Coffee leaves: Identified compounds are summarised in Table 7.

The data have limited relevance to support the metabolism in the pulses/oilseed crop category to which coffee beans have been allocated.

Table 7 Coffee metabolism summary

Designation	**Coffee leaves 0 DAT**		**Coffee leaves 57/62 DAT**		**Coffee leaves 77/82 DAT**	
	mg/kg	%TRR	mg/kg	%TRR	mg/kg	%TRR
Oxirane-2-^{14}C label						
TRR	30.348	100	39.154	100	22.946	100
480M65/480M66	0.242	0.8	0.732	1.9	0.509	2.2
480M67	0.469	1.5	1.551	4.0	0.970	4.2
480M68	0.459	1.5	0.621	1.6	0.278	1.2
Parent	26.274	86.6	28.406	72.5	18.339	79.9
Triazole-3(5)-^{14}C label						
TRR	28.921	100	36.497	100	26.795	100
480M65/480M66	-	-	0.808	2.2	0.433	1.6
480M67	-	-	0.608	1.7	1.110	4.1
480M68	-	-	-	-	-	-
Parent	28.243	97.7	31.397	86.0	20.457	76.3

- **Banana** (4 x 0.15 kg as/ha; N rate factor unknown):

Table 8 ***Banana metabolism summary***

	Unprotected banana				**Protected banana**			
	C Phenyl label		F Phenyl label		C Phenyl label		F Phenyl label	
	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg
Parent	**64.2**	**0.029**	**61.4**	**0.012**	**72.8**	**0.025**	**79.4**	**0.17**
unknown	7.3	0.003						
unknown	6.5	0.003	1.7	0.001	2.1	0.001		
unknown	6.1	0.003	0.9	<0.001	1.0	<0.001		
unknown	1.6	0.001					4.0	0.002
unknowns*	(3) 1.1-1.8	≤0.001	(5) 0.9-2.1	≤0.001				

* Number of metabolites in parentheses and range of concentration

- **Rotational crops:**

Identification of residues was only made in cereals in the triazole label study, and results are summarised in Table 9.

Table 9 ***Rotational crop metabolism***

	Grain		**Straw**	
	% TRR	mg/kg	% TRR	mg/kg
Parent epoxiconazole	-	-	**39.4**	**0.458**
M49 (Triazolyl alanine)	**54.1**	**0.612**	-	-
M50 Triazolyl hydroxy propionic acid	-	-	**16.0**	**0.186**
M51 Triazolyl acetic acid	**25.8**	**0.292**	**10.1**	**0.118**
Unknown	-	-	4.0	0.047
Unknown	-	-	2.4	0.028
Unknown	-	-	3.4	0.040
Unknown	-	-	3.4	0.040
Fraction containing M61 conj.[1)]	**-**	**-**	**3.3**	**0.038**
Fraction containing M61 conj. [2)]	**-**	**-**	**6.8**	**0.079**
M61[1)]	**-**	**-**	**2.7**	**0.032**
Isomer of M26[3)]	**-**	**-**	**1.5**	**0.018**

[1)] Enzyme treatment afforded 53.6 % metabolite M61
[2)] Enzyme treatment afforded 71.1 % metabolite M61
[3)] Metabolite hydroxylated at the chloroaromatic ring at position 5.

Step 7.2 Combined exposure calculation for those metabolites, for which genotoxic effects cannot be excluded

According to the outcome of Module 1 (exclusion of genotoxicity), metabolites M05, M06, M07, M08, M09, M12, M13, M15, M16, M18, M19, M20, M21, M23, M62, M63, should be further addressed for their relevance in dietary exposure.

For metabolites M49, M50, M51, M52 (triazole derivative metabolites, "TDMs") a genotoxicity concern could not be ruled out after the screening in Module 1, however TDMs are common metabolites to a number of active substances, and separate toxicological data are available. It is therefore not appropriate to conduct a TTC assessment for these metabolites.

M62 was reported as identified in wheat but was concluded as an artefact of work-up with ethyl acetate.

Further, metabolites M08, M09, M16, M19, M20, M21 and M23 were reported in the DAR as poultry metabolites. From the original study report it appears that identification of the said metabolites was based on hen excreta, and they were not identified in commodities relevant for consumers, though a large number of peaks in the hen edible commodities remained unidentified. Therefore, their presence in hen edible commodities cannot be ruled out in general, but they will have to be disregarded in the consumer exposure estimates.

Metabolites, for which dietary exposure finally should be assessed to use the $TTC_{genotox}$:

M06	Poultry liver & eggs; Ruminant milk, liver, kidney, fat; Cereal straw
M07	Ruminant liver, Poultry liver, muscle, skin, fat & eggs; Cereal straw
M13	Ruminant milk, liver, kidney; Poultry liver & eggs
M5	Ruminant milk & liver
M56	Ruminant milk, liver & kidney
M18	Ruminant muscle & fat
M12	Poultry liver
M15	Poultry liver & eggs
M63	Cereal grain & straw

Step 7.3 Conclusion

With the data and information available it is not possible to conduct reliable quantitative dietary exposure assessments with regard to metabolites for assessment of genotoxicity against the TTC.

For most of the identified or tentatively identified metabolites the residue levels were either not or insufficiently reported, or cannot be precisely calculated as they can arise in animal commodities upon livestock exposure to epoxiconazole in feed items. Hence, a conclusive calculation of reliable dietary exposure, necessary for a TTC assessment, is not possible.

However, only from the contribution of M63 in a dietary exposure assessment for cereal grain with the available information of potential levels, the TTCgenotox is already exceeded.

Metabolites M49, M50, M51 and M52 (the triazole derivative metabolites aka TDMs) were identified in grain and straw. A triazole label study is only available in ruminants; nothing can be stated with regard on TDM occurrence in poultry commodities. Studies were only conducted with epoxiconazole, and not with TDMs that are major residues in cereal grain and rotational commodities. However, TDMs are common metabolites to a number of substances and have got separate reference values allocated, therefore, this is considered a special case where a separate risk assessment is highly

recommended, and Metabolites M49, M50, M51 and M52 are therefore not dealt with in the subsequent assessment, but the suggestion as candidates for risk assessment is taken forward.

Step 8: Genotoxicity testing

1. **Metabolite (21)** the genotoxicity concern cannot be excluded therefore it should be subject to testing (step 9).
2. **Metabolite M63** the genotoxicity concern cannot be excluded therefore it should be subject to testing (step 9).
3. **Metabolites M12 and M18** the genotoxicity concern cannot be excluded therefore metabolite M18 could be potentially tested as a representative, since an alert was identified for it.
4. **Metabolites M06, M07, M08 and M09** the genotoxicity concern cannot be excluded therefore metabolite M06 (a ketone) and (9) (a thiol) could be tested as group representatives.
5. **M05, M13, M15, M56 (all isomers)** the genotoxicity concern cannot be excluded therefore metabolite M05 could be tested as representative in case hydrolysis of the other metabolites into metabolite M5 is demonstrated. If this cannot be demonstrated, all metabolites should be subject to individual testing. Livestock metabolites M16, M19, M20, M23 may be considered covered by this group.

Genotoxicity endpoints (point mutations, structural and numerical chromosome aberrations) should be investigated. In-vitro studies (e.g. Ames test (TG 471) and in vitro micronucleus assay (TG 487)) are considered suitable for the exploration of the above mentioned genotoxicity endpoints.

Step 9: Genotoxicity concern

For several metabolites a genotoxicity concern can only be ruled out upon further investigations (See step 8 above).

Step 10 Assessment of toxicological properties of parent compound and metabolites

Step 10.1 Toxicological assessment of parent compound

Introduction: Summary of the toxicity of epoxiconazole

The most sensitive effects of epoxiconazole were reduced body weight gain and liver toxicity, as observed in a 18-month study in mice, a 2-year study in rats and a 1-year study in dogs. In addition anemia was observed.

Epoxiconazole is considered to induce liver tumours in mice and rats through a phenobarbitone-like mechanism, i.e. induction of liver enzymes and hepatic growth. Increased incidences of adrenal gland cortex neoplasms, ovarian cysts, ovarian theca granulosa cell tumours, and decreased incidences of neoplasms in the testes (Leydig cell tumours), in the adrenal gland medulla (phaeochromocytomas) and in the pituitary gland (adenomas) in rats were considered indicative of an effect on the synthesis or availability of steroid hormones. Hormonal changes were detected after 4 days of administration supporting the conclusion that hormonal imbalances were induced within the first week of exposure at least in females. Specific steroid hormones affected by epoxiconazole in male and female rats included androgens, oestradiol, corticosterone and aldosterone. LH, FSH, ACTH were however generally increased, indicating intact feed-back mechanisms.

In reproductive toxicity studies epoxiconazole increased precoital intervals, prolonged or abolished oestrus cycles and decreased levels of relevant steroid hormones. Duration of pregnancy was prolonged, probably due to interference with parturition-inducing signals. This resulted in an increased number of pups either being born dead or dying in the early postnatal period.

In several prenatal developmental toxicity studies in rats, among others reductions in oestradiol, progesterone and prolactin levels and increased placental weights were observed. The increase in placental weight may be related to the hormonal changes induced in the dams and indicate an increased placental metabolic function (synthesis of steroids, detoxification of epoxiconazole). Embryofoetal toxicity consisted of increased embryo- or foetolethality and higher incidence of skeletal variations cleft palate malformations and increased post implantation loss.

In a prenatal developmental toxicity study in rabbits, dose-dependent maternal toxicity (reduced food consumption, impairments in body weight) marked increase in post implantation loss and reduced uterine weights) was observed.

Epoxiconazole was not neurotoxic.

The current classification of epoxiconazole is:

Category 1B for developmental and reproductive toxicity, Category 2 for carcinogenicity

In conclusion, many of the effects of epoxiconazole appear to be the result of liver enzyme induction or effects on hormone levels (androgens, oestradiol, corticosterone and aldosterone, LH, FSH, ACTH), including the ones observed in the developmental toxicity study. Some studies are showing that the teratogenic effect is likely due to retinoic metabolism linked to liver enzyme induction ie CYP 26 induction (Menegola et al. 2005[30] and 2006[31]) It is noted that foetal effects (NOAEL 20 mg/kg bw/day) occurred at doses well above the overall NOAEL of 0.8 mg/kg bw/day that formed the basis for the ADI. However, an ARfD was also set based on the reproductive effects of epoxiconazole.

Step 10.2 Toxicological assessment of metabolites

None of the metabolites was present in rat urine above 10%AR and no individual studies on metabolites exist except for the TDMs. It is known that M49, M50, M51 and M52 - belonging to the TDMs - are common metabolites to a number of active substances and have got separate reference values allocated, based on a separate dossier with toxicological studies. Therefore, for this special case a separate risk assessment is highly recommended, and Metabolites M49, M50, M51 and M52 are not dealt with further in this case study, as the case is for demonstration purposes only. However, the suggestion to consider TDMs as candidates for risk assessment is taken forward.

It is further noted, that the assessment of expoxiconazole in this case study is using the assumption that expoxiconazole and TDMs have separate reference values and do not share common effects. However, it should be born mind that there probably are effects shared between TDMs and epoxiconazole, and therefore, in reality, this needs to be considered for a proper assessment.

Step 11: Combined exposure of all metabolites to assess general toxicity (optional)

[30] Menegola, E., Broccia, M.L., Di Renzo, F., Massa, V. and Giavini, E., 2005. Study on the common teratogenic pathway elicited by the fungicides triazole-derivatives. Toxicology in Vitro 19, 737– 748.

[31] Menegola, E., Broccia, M.L., Di Renzo, F., Massa, V. and Giavini, E., 2006. Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. Reproductive Toxicology 22,

For the rest TTC is not applicable due to uncertainty caused by significant livestock exposure and residue transfer in animal commodities, and the knowledge of existence of several non identified metabolites in the edible plant matrices.

Step 12 Consideration on potency

The ADI was set at 0.008 mg/kg bw/d derived from the NOAEL of the 18-month carcinogenicity study in mice (0.8 mg/kg bw/d) and using as safety factor of 100.

ARfD 0.023 mg/kg bw based on two generation reproduction study in rat applying a safety factor of 100.

The substance is considered potent.

Step 13

Based on its potency all metabolites in food meeting the criteria for potent substance metabolites in food commodities of plant and animal origin should be toxicologically assessed (Refer to listing in step 18.)

Step 15

Major plant residues in feed are the parent compound and the TDMs. As has been indicated already earlier (see Step 7.3), the assessment of Metabolites M49, M50, M51 and M52 is not dealt with further in this case study; however, the suggestion to consider the TDMs as candidates for risk assessment is taken forward by default.

In rotational cereal straw metabolites hydroxylated at the chloroaromatic ring were tentatively identified as free compounds and as conjugates and the sum considered together in the different fractions would exceed 10% TRR. However definite confirmation of the identity of these residues and their total levels is missing. Further it appears that the potential contribution to the livestock burden of residues in rotate cereals might be marginal in view of the residue concentrations observed in the primary cereal commodity which is driving the livestock dietary burden. Hence the livestock dietary burden calculation is conducted for parent residues only for primary cereal and sugar beet commodities. Banana is not considered relevant for livestock feeding.

The trigger of 0.004 mg/kg bw/d for requirement of a livestock metabolism study is exceeded for ruminants and poultry. Metabolism of expoxiconzole is addressed by radiolabelled studies in goats and laying hens (step 17).

Epoxiconazole are considered fat soluble and having a potential for accumulation.

Step 16

Table 13 Input data and dietary burden calculation for epoxiconazole[32]

Commodity	Median dietary burden		Maximum dietary burden	
	Input value (mg/kg)	**Comment**	**Input value** (mg/kg)	**Comment**
Epoxiconazole				
Sugar beet pulp (dry)	0.9	Median residue* default PF 18	0.9	Highest residue * default PF 18
Sugar beet tops	0.68	Median residue	1.44	Highest residue
Wheat, rye, spelt, triticale grain	0.03	Median residue	0.03	Median residue
Wheat, rye, spelt, triticale bran	0.126	Median residue *PF 4.2	0.126	Median residue *PF 4.2
Barley, oat grain	0.14	Median residue	0.14	Median residue
Barley, oat bran	0.588	Median residue *PF 4.2	0.588	Median residue *PF 4.2
Cereal straw	2.42	Median residue	15.4	Highest residue

Contribution to livestock burden

Epoxiconazole - Maximum intakes		
Diet	**mg/kg bw/d**	**%**
Lamb	0.459	100
Ram/ Ewe	0.360	100
Dairy cattle	0.210	100
Beef cattle	0.131	100
Poultry(Layer)	0.130	100

Step 17

10% TRR (red)	0.01 mg/kg at N rate for critical diet (blue)

Livestock Studies

Table 14 Goat metabolism (oxirane label, 10 mg/kg bw)

Code	Milk		Muscle		Fat		Liver		Kidney	
	mg/kg	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**	**mg/kg**	**% TRR**
Parent	0.260	52.9	0.470	58.5	4.999	90.9	8.545	32.8	1.815	22.1
M02	0.008	1.9	0.033	4.1	0.071	1.3	0.395	1.5	0.191	2.3
M02 conj. (M32)	0.011	3.5								
M04	0.011	2.7	0.048	5.9	0.084	1.5	1.441	5.5	0.405	4.9

[32] EFSA livestock burden calculator considering OECD feeding table.

Code	Milk		Muscle		Fat		Liver		Kidney	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
M04 conj. (M28)	0.004	0.9	0.040	5.0			3.355	12.9	0.052	0.6
M04 conj. (M29)									0.186	2.3
M18			0.033	4.2	0.089	1.6				
M18 conj. (M30)									0.491	6.0
M26	0.003	0.9					0.316	1.2	0.121	1.5
M26 conj. (M31)	0.016	4.0								
M27	0.005	1.1					3.019	11.6	2.080	25.3

186 ***Table 15*** ***Goat metabolism (triazole label, 0.35 mg/kg bw)***

Code	Milk		Liver		Kidney		Muscle		Fat	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
Parent			0.027	1.6	0.004	1.7			0.005	7.2
M02 / M26 and/or isomers			0.035	2.1						
M04	<0.0005	1.0	0.017	1.0						
M04 conj. (M28 or isomeric gluc.)	<0.0005	0.6	0.015	0.9						
M05 and/or isomers	<0.0005	1.3	0.033	2.0						
M05 / M54 and/or isomers	0.001	1.6	0.012	0.7						
M05 / M54 / M55 / M56 and/or isomers	<0.0005	1.1	0.009	0.6	0.003	1.3				
M13 /M53 / M05 conj. (M60) and/or isomers	0.001	4.0	0.019	1.2	0.009	3.8				
M06	<0.0005	0.9	0.122	7.2	0.008	3.1			0.009	12.5
M07			0.011	0.7						
M52	0.022	63.8	0.036	2.2	0.042	17.2	0.029	69.7	0.026	37.1

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188 ***Table 16*** ***Poultry metabolism (oxirane label – Laying hens 14.75 mg/kg bw)***

Code	Liver		Muscle		Skin		Fat		Eggs	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
Parent	3.201	14.0	0.349	47.6	4.521	84.7	10.866	98.7	1.245	54.6
M01	0.167	0.7								
M01 conj. (M11)	1.267	5.5	0.035	4.6						
M02	0.312	1.4	0.006	0.9						
M06	1.087	4.7							0.096	4.2
M07	1.096	4.8	0.144	19.6	0.608	11.4	0.039	0.4	0.163	7.1

Code	Liver		Muscle		Skin		Fat		Eggs	
	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR	mg/kg	% TRR
M10	0.515	2.2	0.016	2.2						
M12	0.049	0.2								
M13	1.107	4.8							0.213	9.3
M13 conj. (M25)	0.375	1.6								
M15	0.554	2.4							0.263	11.5

Step 18 Testing strategy, grouping and read-across

The following metabolites are to be included on considerations of their toxicity (as identified in step 7 and 17):

M02, M04, M05, M06, M07, M11, M13, M15, M26, M27, M28, M30, M53, M54, M55, M56, M60, M61/ M63 (and the TDMs: M52, M49, M50, M51)

In specific cases, for conjugated metabolites their aglycons will be referenced.

Toxicological information is only available for the parent compound. Therefore, the grouping proposal is based only on the structural similarity. Substances belonging to the same group are expected to have a similar chemical reactivity.

Group A

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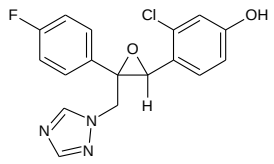
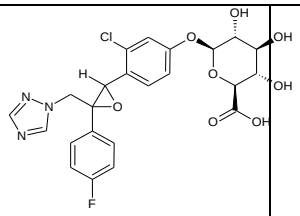
<tr><td> 
M01</td><td> 
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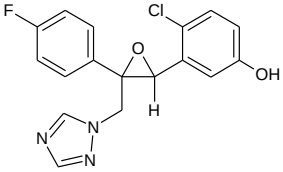
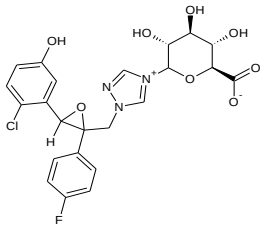
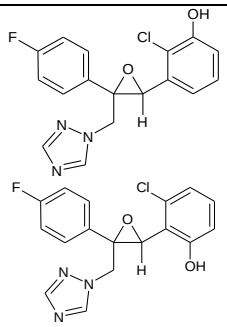
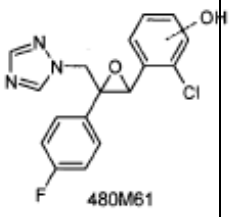
<tr><td> 
M02</td><td> 
M27</td><td> 
M26</td><td> 
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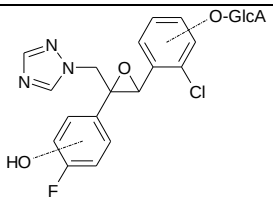
<tr><td> 
M55</td><td></td><td></td><td></td></tr>
</table>

The metabolites identified in group A mainly differ from the parent by the addition of a hydroxyl group in one or two halogenated benzene rings, or conjugates thereof.

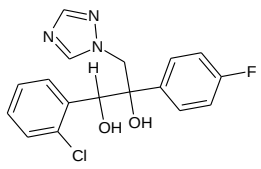
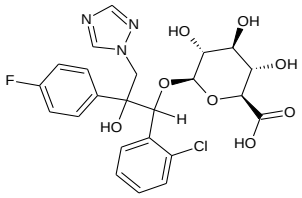
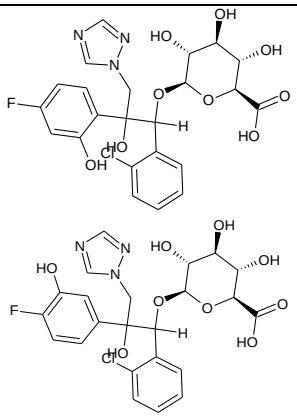
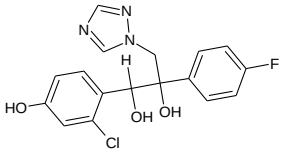
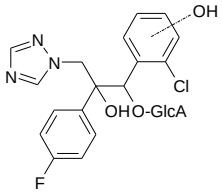
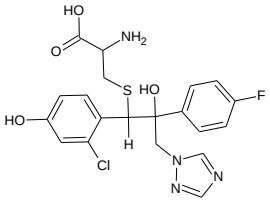
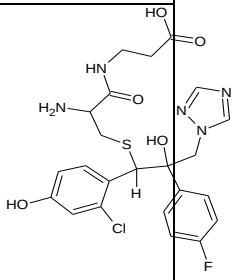
The following subgroups may be considered:

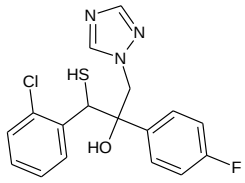
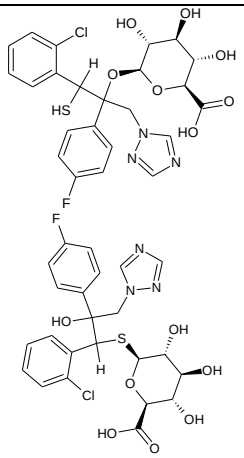
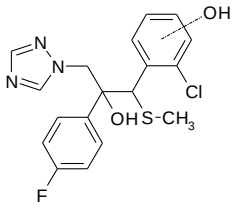
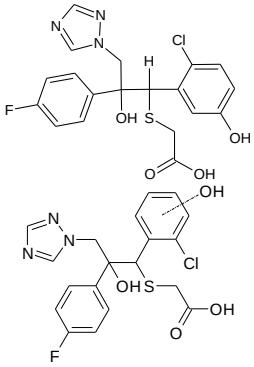
- Metabolites M01 (aglycon of M11), M02 (aglycon of M27) and M26, M61, the difference with the parent molecule is due to the presence of a hydroxyl group in different positions in one of the halogenated benzene rings.
- M55 is also conjugated metabolites, however their aglycons are unique (two hydroxyl groups are presented either in one of benzene halogenated ring or in both benzene halogenated rings).

Strategy for M01, M02, M11, M26, M27, M55 and M61:

- The hydroxylation on a ring system without cleavage of the ring is not expected to cause additional hazard or increase the toxicity of the compound.
- M11, M27 are conjugated metabolites (i.e. glucosides and glucoronides) and the toxicology of the glucosides or glucuronides are considered to be covered by their respective aglycons: M1 as representative for M11; M2 as representative for M27.
- Based on the observations above it is concluded that the toxicological properties of metabolites M01, M02, M11, M26, M27, M55 and M61 are covered by the toxicology of the parent. For these metabolites no further testing is required.
-

Group B

 M04	 M28	 M53	
 M05	 M60	 M13	 M15

 M18	 M30	 M54	
 M56			

220

221 In metabolites belonging to group B the epoxide ring is opened to form diols which maybe
222 subsequently metabolized further. Although it is likely that this will render the compounds less
223 reactive it is not clear how this may affect their toxicity.

224 The following subgroups may be considered:

225 • In metabolites M04 (aglycon of M28) and M05 (aglycon of M60) the epoxide ring is open and
226 an additional hydroxyl group appears in the chlorinated benzene ring in metabolite M05.
227 • M53 is also a conjugated metabolite; its aglycon is unique as it is monohydroxylated on the
228 fluorinated ring.
229 • Metabolites M13, M15 are conjugates (cysteine or cysteinyl-beta-alanine) of metabolite M05
230 and therefore, if their hydrolysis is demonstrated, the toxicological assessment might rely on
231 the assessment of the respective aglycon or its representative.
232 • In the metabolites M18 (aglycon of M30) and M54 one of the OH groups is is replaced by a
233 methylthiol group (M54) or sulfhydryl group (M18) and therefore they might have different
234 reactivity.
235 • M56 as acetate should be evaluated as such (or its hydrolysis product if the hydrolysis is
236 demonstrated)
237 *Strategy for M04, M05, M13, M15, M28, M53, M60:*

238 The toxicity of the glucoside and glucuronide conjugates is considered to be covered by their
239 respective aglycons, i.e. M04 as representative for M28 and M53; M05 as representative for M60

Metabolites M13, M15 are conjugates (cysteine or cysteinyl-beta-alanine) of metabolite M05 and therefore if hydrolysis to M05 is demonstrated the toxicological assessment might rely on the assessment of M05. As a first step, it should be demonstrated whether the compounds M13, M15 are converted to M05. If this occurs the toxicological properties of these compounds are considered covered by the toxicology of M05.

The toxicity of M05 is considered to be covered by that of M04.

M04 can be considered a representative of this group of metabolites. The potency of this compound to induce liver enzymes and to induce endocrine disruption should be tested in vitro (e.g. according to Kjaerstad et al., 2010)[33]. Epoxiconazole should be included in these studies and the relative potency of these metabolites as compared to epoxiconazole should be assessed.

In view of the structural similarities between epoxiconazole and the metabolites of group B, and since developmental effects of epoxiconazole were observed at doses well above the NOAEL that formed the basis of the ADI, no further testing of the developmental potency of the metabolites in group B is required.

For those compounds that are not hydrolysed to M05 one representative metabolite should be tested according to the strategy as described above for M04.

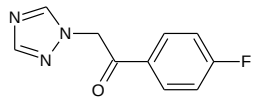
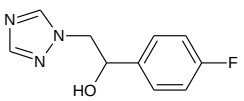
Strategy for M18, M54 and M56

M56 is an acetate. If hydrolysis is demonstrated its toxicity can be considered covered by that of M18, since the hydroxylation of the rings is not expected to increase the toxicity. As a first step, it should be demonstrated whether the acetate group in M56 is hydrolysed.

M18 can be considered a representative of this group of metabolites. The potency of this compound to induce liver enzymes and to induce endocrine disruption should be tested in vitro (e.g. according to Kjaerstad et al., 2010)[34]. Epoxiconazole should be included in these studies and the relative potency of these metabolites as compared to epoxiconazole should be assessed.

If the acetate group in M56 is not hydrolysed it should be tested according to the strategy as described above for M18.

Group C:

 M06	 M07		

Strategy for M06 and M07

The metabolites M06 and M07 lack the chlorobenzene ring. No metabolites lacking the chlorobenzene ring were identified in the metabolism study in rats. It is therefore possible that these metabolites have a toxicity profile that differs from that of epoxiconazole.

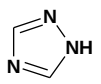
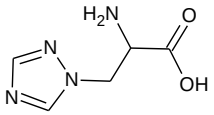
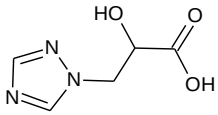
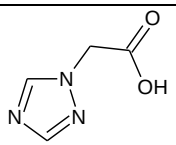
[33] Kjaerstad et al., Reproductive Toxicology 30 (2010) 573-582
[34] Kjaerstad et al., Reproductive Toxicology 30 (2010) 573-582

M6 is probably the most reactive of these metabolites and therefore toxicologically most relevant. Thus, M6 can be considered representatives for the metabolites in group C. It is not clear whether the metabolites of group C also affect liver enzymes and steroid hormones in a similar way as epoxiconazole. Therefore, the potency of M06 to induce liver enzymes and to induce endocrine disruption should be tested in vitro (e.g. according to Kjaerstad et al., 2010)[35]. Epoxiconazole should be included in these studies and the relative potency of these metabolites as compared to epoxiconazole should be assessed.

Since the toxicity profile may differ from that of epoxiconazole the toxicity of M6 should be assessed in the enhanced OECD 407.

Secondly, the potential developmental toxicity effects of M06 should be tested in a developmental toxicity study in rats (OECD 414).

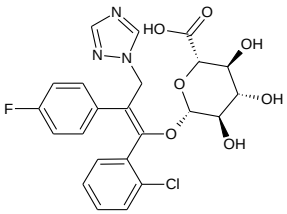
Group D:

			
M52	M49	M50	M51

Strategy for M49, M50, M51 and M52

This group consists of the triazole derivative metabolites (TDMs). The toxicology of triazole (M52), triazole acetic acid (M51) and triazole alanine (M49) has been assessed and reference values have been established (EFSA, 2008) [36]. The toxicity of M50 (triazole hydroxypropionic acid) is considered covered by that of M49 and M51. As for the existence of data that were deemed sufficient to set toxicological reference values, no further considerations on toxicity assessment for these metabolites will be made in this case study.

Group E: Metabolite M63 is a glucopyranosiduronic acid conjugate. It is aglycon is unique and rather different than other metabolites (with double bond between the three rings) and should be kept in separate group.



First Tier: For all the metabolites with an alert for genotoxicity in module 1, it should be investigated whether or not there is a genotoxicity concern by appropriate genotoxicity tests in vitro and, when necessary, in vivo.

[35] Kjaerstad et al., Reproductive Toxicology 30 (2010) 573-582
[36] EFSA (European Food Safety Authority), 2008. Conclusion regarding the peer review of the pesticide risk assessment of the active substance penconazole. 104 pp. doi:10.2903/j.efsa.2008.175r

Group E

Metabolite M63 is a glucopyranosiduronic acid conjugate. Its aglycon is unique and rather different than other metabolites (with a double bond between the three rings).

Strategy for M63

Firstly, the potency of this compound to induce liver enzymes and to induce endocrine disruption should be tested in vitro (e.g. according to Kjaerstad et al, 2010)[37]. Epoxiconazole should be included in these studies and the relative potency of this metabolite as compared to epoxiconazole should be assessed. Depending on results, an additional safety factor of 10x can be applied in case of negative outcome for endocrine effect. If the results are indicative that the metabolite has a similar qualitative profile of the parent, the same reference dose of the parent can be applied. Testing for DART endpoints can be an option.

Step 19 Assessment against total toxicological burden

The following compounds of relevance were identified in food and feed of plant origin and require further assessment:

Cereals: According to the findings in Tables 6 and 9, parent compound, M49, M50 and M51 (TDMs), and M61/63 pending the finalisation of the toxicological relevance assessment (in particular genotoxicity for M63).

The TDMs should be subject to a separate assessment considering all sources, which is not conducted here as this would go beyond the scope of this case study.

Root crops: Parent

Fruit crops: Parent

The following compounds of relevance were identified in food of animal origin and require further assessment (According to the findings in Tables 14 to 16):

Parent, M02, M04, M05, M06, M07, M11, M13, M15, M26, M27, M28, M30, M52, M53, M54, M55, M56, M60.

Their quantitative occurrence is expressed in Table 17 (for poultry) and Tables 17 and 18 (for ruminants) and in terms of %TRR (as determined in metabolism studies) and in % of toxicological burden, which is meant as percentage of identified residue compounds.

In the absence of toxicological data on the metabolites a RPF of 1 is assumed for all metabolites except TDMs. It is again noted that the assessment in this case study is using the assumption that epoxiconazole and TDMs have separate reference values and do not share common effects. However, it should be born mind that there probably are effects shared between TDMs and epoxiconazole, and therefore, in reality, this needs to be considered for a proper assessment.

[37] Kjaerstad et al., Reproductive Toxicology 30 (2010) 573-582

334 ***Table 17 Residues of concern for food of animal origin(≥10 % TRR and at least 0.01 mg/kg)- Poultry***
335 ***(oxirane label)***

Residue component		**Liver**		**Muscle**		**Skin**		**Fat**		**Eggs**	
	RPF	% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden
Parent	1	14	33.1	47.6	63.6	84.7	88.1	98.7	99.6	54.6	63.0
M01		*0.7*									
M01 conj. (M11)		*5.5*		*4.6*							
M01 (sum)	1	6.2	14.7	4.6	6.1						
M02	1	1.4	3.3	0.9	1.2						
M06	1	4.7	11.1							4.2	4.8
M07	1	4.8	11.3	19.6	26.2	11.4	11.9	0.4	0.4	7.1	8.2
M10	1	2.2	5.2	2.2	2.9						
M12	1	0.2	0.5								
M13		*4.8*								*9.3*	
M13 conj. (M25)		*1.6*									
M13 (sum)	1	6.4	15.1							9.3	10.7
M15	1	2.4	5.7							11.5	13.3
Total identified		42.3	100	74.9	100	96.1	100	99.1	100	86.7	100
Sum of relevant compounds		**31.4**	**74.2**	**71.8**	**95.9**	**96.1**	**100.0**	**99.1**	**100.0**	**71.0**	**81.9**
Sum of non-considered compounds		10.9	25.8	3.1	4.1	0	0	0	0	15.7	18.1

336

337 ***Table 18 Residues of concern for food of animal origin(≥10 % TRR and at least 0.01 mg/kg)-***
338 ***Ruminant metabolism low dose (triazole label, 0.35 mg/kg bw)***

Residue component	**RPF**	**Milk**		**Liver**		**Kidney**		**Muscle**		**Fat**	
		% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden	% TRR	% tox burden
Parent	1			1.6	8.9	1.7	17.2			7.2	36.5
M02 / M26	1			2.1	11.7						
M04		1		1.0							
M04 conj.		0.6		0.9							
M04 (sum)	1	1.6	15.2	1.9	10.6						
M05	1	1.3	12.4	2.0	11.1						
M05 / M54	1	1.6	15.2	0.7	3.9						
M05 / M54 / M55 / M56	1	1.1	10.5	0.6	3.3	1.3	13.1				
M13 /M53 / M05 conj.	1	4.0	38.1	1.2	6.7	3.8	38.4				
M06	1	0.9	8.6	7.2	40.0	3.1	31.3			12.5	63.5
M07	1			0.7	3.9						
M52 (TDM)	n/a	63.8	100.0	2.2	100.0	17.2	100.0	69.7	100	37.1	100.0
Total identified - Parent group	1	10.5	100.0	18.0	100.0	9.9	100.0	-	-	19.7	100.0

Sum of relevant compounds - Parent group		**2.5**	**23.8**	**10.7**	**59.4**	**4.8**	**48.5**	**n/a**	**n/a**	**19.7**	**100.0**
Sum of non-considered compounds - Parent group		8.0	76.2	7.3	40.6	5.1	51.5	n/a	n/a	0	0
Total identified –TDM group	n/a	63.8	100.0	2.2	100.0	17.2	100.0	69.7	100	37.1	100.0
Sum of relevant compounds – TDM group		**63.8**	**100.0**	**2.2**	**100.0**	**17.2**	**100.0**	**69.7**	**100.0**	**37.1**	**100.0**
Sum of non-considered compounds TDM group		0	0	0	0	0	0	0	0	0	0

339

340 ***Table 19 Residues of concern for food of animal origin(≥10 % TRR and at least 0.01 mg/kg)-***
341 ***Ruminant metabolism high dose (oxirane label, 10 mg/kg bw)***

Residue component		**Milk**		**Muscle**		**Fat**		**Liver**		**Kidney**	
	RPF	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden	%TRR	% tox burden
Parent	1	52.9	77.9	58.5	75.3	90.9	95.4	32.8	50.1	22.1	34.0
M02		1.9	2.8	4.1	5.3	1.3	1.4	1.5	2.3	2.3	3.5
M02 conj.		3.5	5.2		0.0		0.0		0.0		0.0
M02 (sum)	1	5.4	8.0	4.1	5.3	1.3	1.4	1.5	2.3	2.3	3.5
M04		2.7	4.0	5.9	7.6	1.5	1.6	5.5	8.4	4.9	7.5
M04 conj. (M28)		0.9	1.3	5	6.4		0.0	12.9	19.7	0.6	0.9
M04 conj. (M29)	1		0.0		0.0		0.0		0.0	2.3	3.5
M04 (sum)	1	3.6	5.3	10.9	14.0	1.5	1.6	18.4	28.1	7.8	12.0
M18			0.0	4.2	5.4	1.6	1.7		0.0		0.0
M18 conj. (M30)			0.0		0.0		0.0		0.0	6.0	9.2
Sum M18	1	0	0.0	4.2	5.4	1.6	1.7		0.0	6.0	9.2
M26		0.9	1.3		0.0		0.0	1.2	1.8	1.5	2.3
M26 conj. (M31)		4	5.9		0.0		0.0		0.0		0.0
Sum M26	1	4.9	7.2	0	0.0	0	0.0	1.2	1.8	1.5	2.3
M27	1	1.1	1.6		0.0		0.0	11.6	17.7	25.3	38.9
Total identified		67.9	100.0	77.7	100.0	95.3	100.0	65.5	100.0	65.0	100.0
Sum of relevant compounds		**57.6**	**84.8**	**69.4**	**89.3**	**92.4**	**97.0**	**62.8**	**95.9**	**55.2**	**84.9**

Sum of non-considered compounds		10.3	15.2	8.3	10.7	2.9	3.0	2.7	4.1	9.8	15.1

Step 20

Residue definition for plants

The following compounds are relevant for risk assessment:

Cereals (primary and rotational crops):

Parent, and provisionally M61/63 pending the finalisation of the toxicological relevance assessment (in particular genotoxicity for M63)

Separately M49, M50 and M51 (TDMs)

Root crops: **Parent (default)**

Fruit crops: **Parent (default)**

Residue definition for livestock

With regard to the ruminant studies the following is noted: In the high dose goat metabolism study primarily the parent compound was recovered. From the rat metabolism data it has been shown that the excretion of radioactivity is dose related (low excretion of parent at higher dose rates and more intensive excretion at lower dose rates). Moreover, the high dosed goat study used a shorter slaughter interval than the low dosed study. It was therefore assumed that these are the reasons why the residue pattern differs significantly between the two studies so that at the high dosed metabolism study M06 was not observed while there was significant occurrence in animal matrices in the low dosed study.

Poultry: **Parent + M07 + M01 and M13, including their conjugates**

Ruminants: **Parent + M04 (free & conj.) + M06 +M27**

Separately M52 (TDM)

The residue definitions are provisional pending full toxicological assessment of metabolites in the "parent group" as appropriate (refer to steo 18) or investigation of their occurrence in a ruminant feeding study.

Assessment of stereoisomers (enantiomers and diastereoisomers) for the parent and metabolites

The toxicological studies are performed with a racemic mixture of parent compound.

Enantiomer ratio in commodities of plant origin (tables X-X9) (Final addendum to the Draft Assessment Report (DAR) in the context of confirmatory data peer review, 2012)

The enantiomer ration in plant samples was determined in methanol extracts (and concentrated by SPE (solid-phase extraction) fractionation) and chromatography using a chiral column.

Table X Enantiomer ration in wheat grain samples

Proportion		Residue in wheat grain					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
20	80	0.004	0.017	0.021	42	89	EU-N
20	80	0.003	0.013	0.016	29	85	EU-N
21	79	0.004	0.014	0.018	34	87	EU-N
27	73	0.003	0.008	0.010	42	89	EU-S
29	71	0.008	0.019	0.026	42	89	EU-N
31	69	0.010	0.022	0.032	41	89	EU-N
32	68	0.003	0.006	0.009	35	87	EU-S
32	68	0.010	0.021	0.031	35	89	EU-N
33	67	0.012	0.024	0.036	38	83	EU-N
34	66	0.007	0.013	0.020	43	85	EU-N
35	65	0.006	0.011	0.017	35	87	EU-N
37	63	0.008	0.013	0.021	49	92	EU-S
38	62	0.004	0.007	0.012	42	89	EU-N
42	58	0.033	0.045	0.077	28	85	EU-N
46	54	0.006	0.007	0.012	41	89	EU-S
47	53	0.020	0.022	0.042	48	89	EU-S
49	51	0.038	0.039	0.077	42	89	EU-S

*DALA – interval after last application

Table X2 Enantiomer ration in barley grain samples

Proportion		Residue in barley grain					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
16	84	0.014	0.072	0.086	42	89	EU-S
16	84	0.015	0.080	0.096	49	92	EU-S
18	82	0.022	0.103	0.126	34	87	EU-S
22	78	0.009	0.032	0.041	35	87	EU-S
26	74	0.022	0.064	0.086	56	89	EU-N
26	74	0.011	0.032	0.042	49	92	EU-S
27	73	0.011	0.031	0.042	42	89	EU-S
28	72	0.017	0.045	0.063	42	89	EU-S
30	70	0.022	0.053	0.075	49	89	EU-S

31	69	0.024	0.051	0.075	50	87	EU-N
32	68	0.009	0.019	0.028	56	89	EU-N
33	67	0.012	0.024	0.036	48	87-89	EU-N
33	67	0.020	0.042	0.063	49	89	EU-S
34	66	0.015	0.029	0.044	42	89	EU-S
38	62	0.027	0.043	0.069	42	89	EU-S
38	62	0.050	0.084	0.134	41	89	EU-S
38	62	0.013	0.021	0.034	42	89	EU-S
39	61	0.014	0.022	0.036	48	89	EU-S
40	60	0.039	0.058	0.096	36	87-89	EU-S
40	60	0.036	0.055	0.091	43	89	EU-S
40	60	0.030	0.045	0.075	49	89	EU-S
41	59	0.066	0.095	0.161	48	89	EU-S
42	58	0.066	0.092	0.157	35	89	EU-S
44	56	0.061	0.076	0.137	50	89	EU-S

Table X3 Enantiomer ration in pea seed samples

Proportion		Residue in pea seed					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
38	62	0.09	0.15	0.24	35	89	EU-S
40	60	0.10	0.15	0.25	20	83	EU-S
43	57	0.12	0.16	0.28	28	85	EU-N
45	55	0.09	0.11	0.21	21	83	EU-N

Table X4 Enantiomer ration in wheat plant samples

Proportion		Residue in wheat plant					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
36	64	0.085	0.150	0.235	42	87	EU-S
39	61	2.190	3.408	5.597	27	85	EU-N
41	59	1.290	1.894	3.184	44	87	EU-S
43	57	1.068	1.391	2.459	34	85	EU-S
44	56	0.410	0.525	0.935	42	87	EU-S
44	56	0.457	0.577	1.034	35	78-80	EU-N
44	56	1.030	1.319	2.349	36	85	EU-S
46	54	0.128	0.151	0.278	36	83	EU-N
46	54	0.768	0.895	1.663	27	75	EU-N
47	53	0.390	0.441	0.830	35	83	EU-S
48	52	2.402	2.593	4.995	34	87	EU-S
48	52	0.386	0.411	0.797	35	85	EU-S
49	51	0.411	0.423	0.834	35	87	EU-N
50	50	0.565	0.556	1.121	29	77	EU-S
51	49	0.358	0.342	0.701	29	83	EU-N
57	43	1.164	0.883	2.047	28	85	EU-N

386 **Table X5** Enantiomer ration in barley plant samples

Proportion		Residue in barley plant					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
34	66	0.237	0.460	0.697	42	83	EU-N
36	64	0.331	0.598	0.929	42	83-87	EU-N
37	63	0.402	0.690	1.092	35	77	EU-N
38	62	0.345	0.559	0.904	35	75-81	EU-N
38	62	0.741	1.216	1.958	35	87-89	EU-S
41	59	0.380	0.544	0.925	34	87	EU-S
44	56	1.237	1.559	2.796	35	87	EU-S
43	57	0.258	0.347	0.605	34	85	EU-S

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388 **Table X6** Enantiomer ration in maize plant samples

Proportion		Residue in maize plant					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
18	82	0.033	0.145	0.177	19	75	EU-N
18	82	0.019	0.087	0.106	46	85	EU-N
20	80	0.004	0.014	0.018	86	89	EU-N
21	79	0.061	0.232	0.293	12	71	EU-N
23	77	0.007	0.023	0.030	71	89	EU-N
25	75	0.007	0.021	0.028	64	85	EU-N
25	75	0.013	0.039	0.053	28	75	EU-N
29	71	0.010	0.023	0.033	57	85	EU-N
30	70	0.138	0.315	0.453	69	89	EU-S
31	69	0.020	0.043	0.063	17	71	EU-N
31	69	0.182	0.413	0.595	49	85	EU-S
31	69	0.082	0.186	0.269	21	75	EU-S
31	69	0.080	0.180	0.260	49	89	EU-S
32	68	0.016	0.033	0.049	29	75-77	EU-N
32	68	0.045	0.097	0.142	77	89	EU-S
33	67	0.200	0.399	0.599	14	71	EU-S
34	66	0.076	0.146	0.222	16	71	EU-S
35	65	0.046	0.084	0.130	49	85	EU-S
35	65	0.170	0.321	0.491	35	85	EU-S
35	65	0.006	0.011	0.016	91	89	EU-N
36	64	0.030	0.053	0.083	13	71	EU-N
36	64	0.051	0.090	0.142	27	77	EU-S
40	60	0.293	0.435	0.728	21	75-77	EU-S
45	55	0.255	0.311	0.566	6	71	EU-S

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392 **Table X7** Enantiomer ration in pea plants

Proportion		Residue in pea plants					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
50	50	0.64	0.63	1.27			EU-N
50	50	1.67	1.66	3.33			EU-S
52	48	1.15	1.05	2.20			EU-S

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394 **Table X8** Enantiomer ration in wheat ear samples

Proportion		Residue in wheat ears					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
48	52	0.076	0.081	0.156	36	83	EU-N
48	52	0.133	0.143	0.276	35	78-80	EU-N
48	52	0.085	0.091	0.177	36	85	EU-S
48	52	0.293	0.313	0.606	44	87	EU-S
48	52	0.747	0.799	1.546	34	87	EU-N
48	52	0.123	0.132	0.256	34	85	EU-S
48	52	0.121	0.130	0.251	35	83-85	EU-S
48	52	0.101	0.108	0.209	42	87	EU-S
48	52	0.124	0.133	0.256	35	85	EU-S
48	52	0.117	0.125	0.243	27	75	EU-N
48	52	0.581	0.622	1.203	29	83	EU-N
48	52	0.187	0.200	0.387	29	77	EU-S
48	52	0.289	0.309	0.598	35	83	EU-S
48	52	0.358	0.383	0.741	42	87	EU-S
48	52	0.595	0.636	1.231	28	87	EU-S
48	52	0.502	0.537	1.038	28	87	EU-S
48	52	0.647	0.692	1.338	34	87	EU-S

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396 **Table X9** Enantiomer ration in maize cob with husk samples

Proportion		Residue in cob with husk					
(-)-BAS 480 F	(+)-BAS 480 F	(-)-BAS 480 F	(+)-BAS 480 F	Sum	DALA*	Growth stage	Region
(%)	(%)	(mg/kg)	(mg/kg)	(mg/kg)		BBCH	
25	75	0.001	0.004	0.006	64	85	
35	65	0.004	0.007	0.011	17	71	
37	63	0.009	0.015	0.025	28	75	
40	60	0.004	0.006	0.009	13	71	
45	55	0.007	0.009	0.016	30	85	
47	53	0.010	0.011	0.021	9	71	
49	51	0.010	0.010	0.019	20	75-77	
54	46	0.009	0.008	0.017	6	71	

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Results of analyses of the plant samples, present in the Tables X-X8, show that the applied ratio (51:49) is not maintained. A significant and reproducible increase of the (+) - enantiomer is observed for cereal grains (wheat - 33:6, barley - 32:68 and pea - 42:58) and other parts of the plants (maize plant - 30:70 and cob - 42:48, barley plant - 39:61, wheat plant – 46:54). The ratio is changed slightly in the same direction also for wheat ear (48:52) and kept not changed for pea plants (but only 3 samples are analysed). Slightly change in opposite direction was observed in coffee, both in beans and leaves the proportion of (+)- enantiomer decreased to 41.6% and 45.4%, respectively.

Enantiomer ratio in samples of animal origin (goat, rat)(tables X10, X11)

The enantiomer ratio in animal tissue and milk samples was determined by chromatography using a chiral column.

Table X10. Enantiomer ration in samples from BAS 480 F metabolism studies conducted in goat (re-extraction extracts)

Origin	Matrix	Previous studies Methanol extract		Present Study Methanol exctract	
		BAS 480 F		(-)-BAS 480 F	(+)-BAS 480 F
		(mg/kg)	(%TRR)	(% ROI)	(% ROI)
Goat (Study 151738)	Urine (Day 7)	0.042	1.4	43.1	56.9
	Liver	0.027	1.6	16.7	83.3
	Kidney	0.004	1.7	44.0	56.0
	Fat	0.005	7.2	47.0	53.0

Table X11. Enantiomer ration in samples from BAS 480 F metabolism studies conducted in rat

Matrix/Experiment/Sampling Time	Animal	BAS 480 F			Enantiomer ratios	
					(-)-BAS 480 F	(+)-BAS 480 F
		(mg/kg)	(% TRR)	(% Dose)	(%)	(%)
Application solution					51.2	48.8
Plasma	F01	4.146	64.1	0.03	29.6	70.4
WxF	F02	5.731	71.0	0.07	34.2	65.8
(1h)	Mean				31.9	68.1
Liver	F01	31.812	65.5	0.98	45.8	54.2
WxF	F02	41.557	71.2	1.33	46.4	53.7
(1h)	Mean				46.1	53.9
Liver	F03	0.918	3.7	0.04	23.9	76.1
DxF	F04	0.426	2.2	0.02	19.4	80.6
(48 h)	Mean				21.7	78.3
Faeces	F03	106.564	20.2	3.20	53.9	46.1
DxF	F04	482.697	74.8	6.65	50.1	50.0
(0-24 h)	Mean				52.0	48.0
Faeces	F03	46.980	3.8	1.91	60.0	40.0
DxF	F04	229.748	14.4	9.39	51.3	48.7
(24-48h)	mean				55.6	44.4

Results of analyses of the goat matrices (table X10) after administration of a racemic mixture show significant increase of the proportion of (+)-enantiomer to 83% in liver. Observed isomer change in kidney, fat and urine was in the same direction but not so significant, the proportion of (+)- enantiomer was 56%, 53% and 57%, respectively. The increase of the proportion of the positive enantiomer in goat liver was in accordance with the results for liver in rat study – 78% (Table X11).

Stereoisomeric composition of residues found in the samples (plant and animal origin) shows a difference compared to the sample used in the toxicological studies (a racemic mixture). In the absence of additional sterioselective toxicological studies, correction factors of 1.3 and 1.7 were derived for cereal grain and liver, respectively in order to address the relevance of stereoisomeric composition change on the consumer dietary risk assessment. These factors take into account the reduction of the (-/+) enantiomer ratio and assuming that the toxicity is attributed to the (+)-enantiomer.

Data gaps

Uncertainties of particular relevance for decision making

- A large number of metabolites contain a hydroxyl group in either the chlorinated or the fluorinated ring structure or in both structures. The position of the hydroxyl group in the ring structure varies between metabolites. For some metabolites the position of the hydroxyl group has not been identified. It is assumed that for the epoxiconazole metabolites the hydroxylation of a ring structure without opening the ring will not increase the toxicity of the metabolite. This assumption is based on a conclusion in an External Scientific Report to EFSA prepared by AGES (2010). It is noted that AGES based this conclusion on data obtained mostly from acute toxicity studies. AGES also noted that there are some compounds were hydroxylation of a ring structure may increase its toxicity (e.g. hydrochinon). Therefore, some uncertainty remains on the applicability to predict the toxicity after short-term or long-term exposure
- QSAR/RA
- Exposure, coverage of tox burden, rate of identification in metabolism studies, variability between studies, testing strategy

ABBREVIATIONS

1N GAP	Application rate according to Good Agricultural Practice
AAOEL	Acute Acceptable Operator Exposure Level
ACF	atom-centered fragments
AD	administered dose
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, and excretion
AOEL	Acceptable operator exposure levels
ARfD	acute reference dose
BBCH	Scale describing the phenomenological growth stage of plants
CA	Chromosomal aberration
CF	Conversion factor
cGAP	critical GAP
FN	false negative
FP	false positive
GAP	Good Agricultural Practice
HR	highest residue
HRc	Highest residues converted
ISS	Istituto Superiore di Sanità
K_{OW}	Octanol-Water Partition Coefficient
LOAEL	lowest observed adverse effect level
LOQ	Limit of Quantitation
MN	Micronucleus
MTD	maximum tolerated dose
MW	Molecular weight
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
PBI	plant-back interval

PHI	pre-harvest interval
QSAR	(Quantitative) structure–activity relationship
RPF	relative potency factor
SA	structural alerts
STMR	supervised trials median residue
SVM	Support Vector Machines
TRR	total radioactive residue
TTC	Threshold of Toxicological Concern