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Statement on genotoxicity assessment of chemical mixtures

EFSA Scientific Committee

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Abstract

The EFSA Scientific Committee addressed in this document the peculiarities related to the genotoxicity assessment of chemical mixtures, as this aspect could not be considered in sufficient detail in its guidance document on harmonised risk assessment methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. The EFSA Scientific Committee stresses that first a mixture should be chemically characterised as far as possible. Although the characterisation of mixtures is relevant also for other toxicity aspects, it is particularly important for the assessment of genotoxicity. If a mixture contains one or more substances that are assessed to be genotoxic *in vivo* via a relevant route of administration, the mixture is considered as genotoxic. If a fully characterised mixture does not contain genotoxic substances, the mixture is of no concern with respect to genotoxicity. If a mixture contains a fraction of substances that are not chemically identified, experimental testing of the unidentified fraction should be considered as first option or, if this is not feasible, testing of the whole mixture should be undertaken. If testing of the whole mixture or of these fraction(s) in an adequately performed set of *in vitro* assays provides clearly negative results, the mixture should be considered non-genotoxic. If *in vitro* testing provides one or more positive results, an *in vivo* follow-up study should be considered. In case of negative results in the *in vivo* follow-up test(s), the possible limitations of *in vivo* testing should be weighed in an uncertainty analysis before reaching a conclusion of no concern with respect to genotoxicity. In case of positive results in the *in vivo* follow-up test(s), it can be concluded that the mixture does raise a concern regarding genotoxicity.

Keywords: genotoxicity assessment, chemical mixtures, uncertainty analysis

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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

Human and ecological risk assessment of combined exposure to multiple chemicals ('chemical mixtures') poses a number of challenges to scientists, risk assessors and risk managers, particularly because of the complexity of the problem formulation, the almost infinite number of possible combinations of chemicals and the large amount of data needed to describe the toxicological profiles and exposure patterns of these chemicals in humans and species present in the environment. The development of harmonised methodologies for combined exposure to multiple chemicals in all areas of EFSA's remit has been identified by EFSA's Scientific Committee as a key priority area. Some EFSA panels and units have initiated activities to support harmonisation of risk assessment methods for both human health and ecology. In particular, the Scientific Committee initiated in 2016 an activity to develop guidance on harmonised risk assessment methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. Work is ongoing and a draft guidance will be published for public consultation in summer 2018. Finalisation of the guidance is expected by the end of 2018.

At present, information on the genotoxicity of a chemical drives the type of the assessment in human risk assessment: If the chemical is not genotoxic, a health-based guidance value is usually established while in case of a genotoxic and unavoidable chemical, the margin of exposure approach is usually applied (EFSA 2005). With respect to assessing the genotoxicity of mixtures, specific additional considerations might be needed, e.g. when it is not possible to fully characterise a complex mixture due to analytical problems.

Different areas within EFSA's remit have different data requirements in relation to the assessment of mixtures:

- Plant protection products:
  - Regulation (EC) No. 1107/2009 on the placing of plant protection products on the market requires that 'interaction between the active substance, safeners, synergists and co-formulants shall be taken into account' in the evaluation and authorisation of plant protection products (Article 29).
  - Commission Regulation (EU) No. 283/2013, setting out the data requirements for active substances in plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament further requests:
    - a risk assessment of consumer exposure, including, when relevant, a cumulative risk assessment deriving from exposure to more than one active substance;
    - an estimation of the exposure to operators, workers, residents and bystanders including, when relevant, the cumulative exposure to more than one active substance.
  - Commission Regulation (EU) No. 284/2013, setting out the data requirements for plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament further requests 'any information on potentially unacceptable effects of the plant protection product on the environment, on plants and plant products shall be included as well as known and expected cumulative and synergistic effects'.
  - Regulation (EC) No. 396/2005 on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin requires cumulative risk assessment for pesticides to be performed. Recital 6 states: 'It is also important to carry out further work to develop a methodology to take into account cumulative and synergistic effects'. It further specifies that MRLs should be set in 'view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health'.
- Feed additives: Regulation (EC) No. 429/2008 on the assessment and authorisation of feed additives explicitly addresses risks that may arise from combined exposures if feed additives

placed on the market contain more than one (active) ingredient. Annex II lists the requirement that 'where an additive has multiple components, each one may be separately assessed for consumer safety and then consideration given to the cumulative effect (where it can be shown that there are no interactions between the components). Alternatively, the complete mixture shall be assessed'.

- Smoke flavourings: Regulation (EC) No. 2065/2003 on the assessment and authorisation of smoke flavourings used or intended for use in or on food. Annex II includes the information necessary for the scientific evaluation of primary products, i.e. primary smoke condensates and primary tar fractions produced by controlled thermal degradation of wood in a limited supply of oxygen (pyrolysis), all of these being complex chemical mixtures. In accordance with this Annex, the toxicological data requirements should follow the advice of the Scientific Committee on Food, given in its report of 25 June 1993, according to which relevant data should be generated on the whole mixture.
- Legislation in relation to food additives, food contact materials and food contaminants does not have specific provisions requiring risk assessment of mixtures. However, this does not imply that mixtures are never addressed. For example, in Regulation (EC) No. 1881/2006, the setting of maximum levels for certain contaminants in foodstuffs (e.g. dioxins, polycyclic aromatic hydrocarbons and a number of mycotoxins) are underpinned by a mixtures risk assessment.

Given this background, the SC discussed and agreed to develop a statement clarifying how to perform genotoxicity assessment of chemical mixtures with cross-reference to previous EFSA guidance documents.

1.1.1. Terms of Reference

Starting from the basic definition of chemical mixtures as presented in the draft 'Guidance on harmonised risk assessment methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals', the SC should develop a statement that:

- clarifies the peculiarities related to genotoxicity assessment of mixtures, i.e. identification of specific additional considerations and their triggers (cross- reference to previous guidance);
- addresses both component-based and whole mixture approaches.

Consideration from the different areas within EFSA's remit should be given to explore the feasibility and spectrum of applications of the proposed approaches for human health risk assessment.

1.2. Interpretation of the Terms of Reference

Based on the draft guidance on mixtures under development, a differentiation is made between mixtures that are fully characterised (simple mixtures) and mixtures in which not all the components have been characterised (complex mixtures). These two situations are considered separately in this statement.

Definition of the terms used in this statement are given in the glossary in the end of this document.

1.3. Additional information

A general guidance document addressing harmonised methods for risk assessment of combined exposure to multiple chemicals for all relevant areas within EFSA's remit, including human health and environmental aspects, has been developed in parallel to this statement. These risk assessment principles are not repeated in this document. This statement is addressing only specific issues related to the genotoxicity assessment of mixtures. For all other aspects of risk assessment of mixtures, the reader is referred to the draft guidance document on chemical mixtures (EFSA 2018 under development).

2. Assessment

2.1. Chemical characterisation of simple and complex mixtures

The demonstration of the identity and stability (batch-to-batch variability as well as stability over time) of a mixture is always required to ensure that the mixture tested is representative of the mixture to be placed on the market (e.g. for regulated products) or representative for mixtures present in the environment or food (e.g. contaminants).

2.1.1. Qualitative and quantitative analysis of the composition of a mixture

The first step must be to characterise the mixture as fully as possible. Compositional data are required for qualitative and quantitative analysis of a mixture. Although the characterisation of mixtures is relevant also for other toxicity aspects, it is particularly important for the assessment of genotoxicity.

2.1.1.1. Fully characterised mixtures (simple mixtures)

For mixtures whose components are fully chemically characterised (mixtures of chemically defined substances), information on the identities and the relative ratios should be provided. This should allow the elaboration of a mass balance. In the case of mixtures prepared by adding individual substances, the decision on which degree of 'purity' of the individual components can be considered sufficient does not differ from the decision to be taken for individual substances. This may also depend on specific sources and the production process of the mixture.

2.1.1.2. Mixtures containing a substantial fraction of unidentified components (complex mixture)

For mixtures for which not all components have been fully chemically identified, a quantitative characterisation of the main constituents should be performed, at least via sum parameters (e.g. total phenols, total acids, total protein or reducing sugars). On the basis of these data, a mass balance should be calculated. The percentage of unidentified components should be indicated and should be as low as possible. Therefore, the analytical methods employed to characterise the mixture should at least be able to cover the type and the expected analytes (i.e. compounds that, based on the knowledge on the source and the production/formation of the mixtures, are expected to be potentially present).

2.2. Genotoxicity assessment of fully characterised mixtures

For fully characterised mixtures, the Scientific Committee recommends applying a component-based approach, i.e. assessing all components individually using all available information including structure–activity relationships regarding their genotoxic potential, following the Scientific Committee guidance (EFSA 2011/2017). This means that for regulated products, conclusions regarding genotoxicity will be required for all components or at least for representative substances for mixtures containing structurally related substances. If such a mixture contains one or more substances that are assessed to be genotoxic *in vivo* via a relevant route of administration (i.e. in most cases after oral exposure), the whole mixture has to be considered as genotoxic.

For mixtures that contain individual components that may indicate a concern for genotoxicity but for which the data available are not sufficient to conclude on genotoxicity, e.g. only positive results in *in vitro* genotoxicity tests of an individual component, additional data would be needed to complete an assessment.

For a mixture that contains a large number of substances with positive results in *in vitro* genotoxicity tests, the whole mixture could be considered as genotoxic as *in vivo* follow-up testing of a large number of substances is likely to result in one or more positive outcomes. If nevertheless further *in vivo* follow-up testing is considered, testing of a specific fraction of the mixture containing these substances may be considered on a case-by-case basis. When the mixture contains structurally related substances, a representative substance (ideally having the highest expected DNA reactivity among the structurally related substances), could be further tested and used as an indicator substance for all structurally related substances. This should be carried out as for individual substances following the Scientific Committee guidance (EFSA 2011/2017).

If in certain incidences, i.e. unavoidable contaminants and impurities, human exposure to the known genotoxic substance(s) identified in such a mixture is very low [when considering consumption data and the respective concentration(s) in the mixture] it might be concluded that such an exposure is likely to be of low concern from the public health perspective. For unavoidable contaminants and impurities, such a conclusion may be reached based on a Margin of Exposure approach (EFSA 2005) when respective carcinogenicity data are available, either for the genotoxicant itself or for a structurally closely related substance. There is, as yet, no consensus on applying a MOE approach for genotoxicity data.

If no relevant carcinogenicity data are available and the estimated exposure to the substance is very low, it might be possible to apply the Threshold of Toxicological Concern (TTC) (EFSA 2012b). Provided that the genotoxicant in question is not a member of one of the TTC exclusion categories and estimated high level exposure is less than 0.0025 µg/kg bw/day, then a substance with structural alerts for genotoxicity would be considered to have a low probability of a health effect (EFSA, 2012b). Circumstances under which these criteria are met are expected to be rare.

If a mixture is known to contain more than one genotoxicant, both the Margin of Exposure (EFSA 2005, 2012a) and TTC approaches could potentially be applied, using the default assumption of dose addition as they would share the same mode of action (e.g. DNA reactivity). If a benchmark dose lower confidence limit for a 10% extra risk of cancer ($BMDL_{10}$), derived from a rodent carcinogenicity assay is available for only one of the substances of the mixture, the MOE can be calculated by dividing this $BMDL_{10}$ by the combined exposure, which is then interpreted in the same way as for a single substance. If $BMDL_{10}$ values are available for more substances, then the combined (total) Margin of Exposure (MOET, WHO 2017) can be calculated as the reciprocal of the sum of the reciprocals of the MOE of the individual substances. If the MOET is higher than 10,000, then the exposure to the mixture would be of low concern from a public health point of view:

$$MOE = \frac{BMDL_{10}}{Exposure}.$$

$$MOET = \frac{1}{(1/MOE_1) + (1/MOE_2) + (1/MOE_3)} \text{ etc.}$$

If the TTC approach is considered applicable, and if the combined exposure to all genotoxic components of the mixture is less than 0.0025 µg/kg bw/day, the mixture would be considered to have a low probability of a health effect. However, circumstances under which these criteria would be met are again likely to be rare.

The Scientific Committee, however, reiterates its earlier statement that substances that are both genotoxic and carcinogenic should not be deliberately added to foods or used earlier in the food chain. Therefore, the MOE approach for genotoxic carcinogens have only been used for unavoidable contaminants and impurities in food or feed additives or in food contact materials (EFSA, 2005; 2012a).

2.3. Genotoxicity assessment of mixtures containing a substantial fraction of unidentified components

If a mixture contains, besides chemically identified substances, a substantial[1] fraction of compounds that have not been chemically identified, the Scientific Committee recommends that first the chemically identified substances be assessed individually for their potential genotoxicity, using all available information, following the Scientific Committee guidance (EFSA 2011, 2017). This means that for regulated products, any conclusion regarding genotoxicity will be required for all identified components or at least for these representative substances for mixtures containing structurally related

[1] A general definition of 'substantial' is not possible as it depends on several factors such as the nature of the source and the production/formation process.

substances. As described in Chapter 2.2, if the mixture contains one or more substances that are evaluated to be genotoxic *in vivo* via a relevant route of administration, the whole mixture raises concern regarding genotoxicity.

If none of the identified substances in a mixture is genotoxic, the genotoxic potential of the unidentified fraction should also be evaluated to complete the assessment of the mixture. Experimental testing of the unidentified fraction should be considered as the first option or, if this is not feasible, testing of the whole mixture should be undertaken. Further fractionation of the test material could be considered case by case to remove inert, toxicologically irrelevant components from the mixture (e.g. high-molecular-weight polymers) to minimise the dilution of the components of interest in the tested sample, or to remove highly toxic components (e.g. surface active substances) that may prevent testing adequately high doses of the mixture because of overt toxicity. Moreover, if either the starting material used or the production process indicate the possible presence of genotoxicants in the unidentified fraction of the mixture, an attempt should be made to isolate and test the fraction of concern as such, if it is not possible to chemically identify and quantify the substance.

The testing strategy for whole mixture or its fraction(s) should follow the Scientific Committee testing strategy guidance for individual compounds (EFSA 2011, 2017). However, as mentioned in the *in vitro* OECD TGs 473, 476, 487, 490:

'When the test chemical is not of defined composition e.g. substance of unknown or variable composition, complex reaction products or biological materials (i.e. UVCBs) (42), environmental extracts etc., the top concentration may need to be higher (e.g. 5 mg/ml), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components'.'

If testing of the whole mixture or its fraction(s) in an adequately performed set of *in vitro* assays (e.g. OECD 471 and 479) following the Scientific Committee testing strategy (EFSA 2011, 2017) provides clearly negative results, the mixture should be considered as of no concern with respect to genotoxicity and no further testing (e.g. by *in vivo* assays) is recommended.

If testing of the whole mixture or its fraction(s) in an adequately performed set of *in vitro* assays provides one or more positive results, *in vivo* follow-up testing should be considered to assess the relevance of these findings for risk assessment. The follow-up study should be tailored case by case based on the activity profile/mode of action observed *in vitro*, following the Scientific Committee genotoxicity testing strategy (EFSA 2011, 2017), and taking into account any other relevant information (e.g. on source and chemical characteristics of the mixture).

If the *in vivo* testing of an *in vitro* positive mixture provides negative results, the relevance of the findings obtained in the *in vivo* follow-up tests will depend on the relevance of the genetic effect assessed, the test system applied and expert judgement regarding the reliability of the results obtained.

In some instances, it can be anticipated that negative results in the follow-up tests can support, with adequate confidence, a lack of concern regarding the *in vivo* genotoxicity of the mixture: for example, for a mixture that is directly clastogenic *in vitro*, a robust assessment *in vivo* could be performed by applying a mammalian alkaline comet assay (OECD TG 489) to several tissues, including the site of first contact, to animals in which the mixture was administered orally. For other effects, such as induction of gene mutations and/or clastogenicity following *in vitro* metabolic activation, the assessment of systemic genotoxic effects (e.g. in the liver or bone marrow) may be limited by the fact that target tissue exposure cannot be demonstrated, as any toxic effect elicited in the target tissue by the mixture cannot be unequivocally attributed to the (*in vitro*) genotoxic component. In this scenario, the conclusion drawn would have a higher uncertainty.

Another relevant concern is the follow-up testing of *in vitro* aneugens. At present the only validated methodology is the rodent bone marrow micronucleus assay (OECD TG 474). In this scenario, the lack of information on target tissue exposure may be a critical limitation, also because of the possibility of effects at the site of first contact, where local concentrations may be higher than in the bone marrow and aneugenic effects cannot be directly evaluated.

So, for negative results in the *in vivo* follow-up study, the possible limitations of *in vivo* testing should be weighed in an uncertainty analysis before reaching a conclusion of no concern with respect to genotoxicity of complex mixtures that provided positive *in vitro* results.

Conversely, for positive results in the *in vivo* follow-up tests, it can be concluded that the mixture does raise a concern regarding genotoxicity. In this scenario it may be prudent to consider in the overall assessment also positive test results *in vivo* that are obtained under conditions associated with overt toxicity, which are usually considered of limited relevance, as it cannot be decided upon whether cytotoxic and genotoxic effects are elicited by the same or different components.

3. Conclusions

- The mixture should be chemically characterised as far as possible, using state of the art analytical methods.
- If a mixture contains one or more substances that are assessed to be genotoxic *in vivo* via a relevant route of administration, the mixture is considered as genotoxic.
- If the assessment of all components of a fully characterised mixture results in the conclusion that none of these raises a concern with respect to genotoxicity, the mixture is also considered of no concern with respect to genotoxicity.
- If a mixture contains a fraction of substances that have not been chemically identified, experimental testing of the unidentified fraction should be considered as the first option or, if this is not feasible, testing of the whole mixture should be undertaken, following the Scientific Committee guidance for individual substances (EFSA, 2011, 2017):
  - If testing in an adequately performed set of *in vitro* assays provides clearly negative results, the mixture should be considered as of no concern with respect to genotoxicity.
  - If testing in an adequately performed battery of *in vitro* assays provides one or more positive results, an *in vivo* follow-up study should be considered.
  - For negative results in the *in vivo* follow-up tests, the possible limitations of *in vivo* testing should be weighed in an uncertainty analysis before reaching a conclusion of no concern with respect to genotoxicity.
  - For positive results in the *in vivo* follow-up tests, the mixture does raise a concern regarding genotoxicity.

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Glossary and Abbreviations

Term	Explanation
Benchmark dose (BMD)	The BMD is a dose level, derived from the estimated dose–response curve, associated with a specified change in response (e.g. a 10% increase in the incidence cancer)
$BMDL_{10}$	Benchmark dose lower confidence limit for e.g. a 10% increased incidence of cancer, usually derived from a rodent carcinogenicity assay
Component-based approach	An approach in which the risk of a mixture is assessed based on exposure and effect data of its individual components
Complex mixture	A mixture e.g. extracts, protein hydrolysates, smoke flavourings, in which not all constituents are known or can be fully characterised
Dose addition	A component-based model in which the components are treated as having a similar mode of action. The components may vary in toxic potency. Components contribute to the mixture effect relative to the ratio between dose and toxic potency
Margin of exposure (MOE)	Ratio of the no-observed-adverse-effect level or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted or estimated exposure dose or concentration
Mass balance	Mass balance is the percentage compilation of individual constituents or classes of constituents, in the ideal case summing up to 100%
Mixture	Any combination of two or more chemicals, regardless of source and spatial or temporal proximity that may jointly contribute to actual or potential effects.
Mode of action	Biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. It refers to the major steps leading to an adverse health effect following interaction of the compound with biological targets. It does not imply full understanding of mechanism of action at the molecular level
Simple mixture	Mixture whose components are fully chemically characterised, e.g. a group of defined substances
Sum parameters	Parameters determining the content of classes of compound with common structural aspects (e.g. phenols, proteins or reducing sugars) rather than individual constituents
UVCBs	Substances of Unknown or Variable composition, Complex reaction products or Biological materials
Whole mixture approach	A risk assessment approach in which the mixture is treated as a single entity, similar to single chemicals and so requires dose–response information for the whole mixture of concern