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MixTox

Assessment of the potential toxicity of mixtures

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GUIDANCE

EFSA Journal

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Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals

EFSA Scientific Committee,
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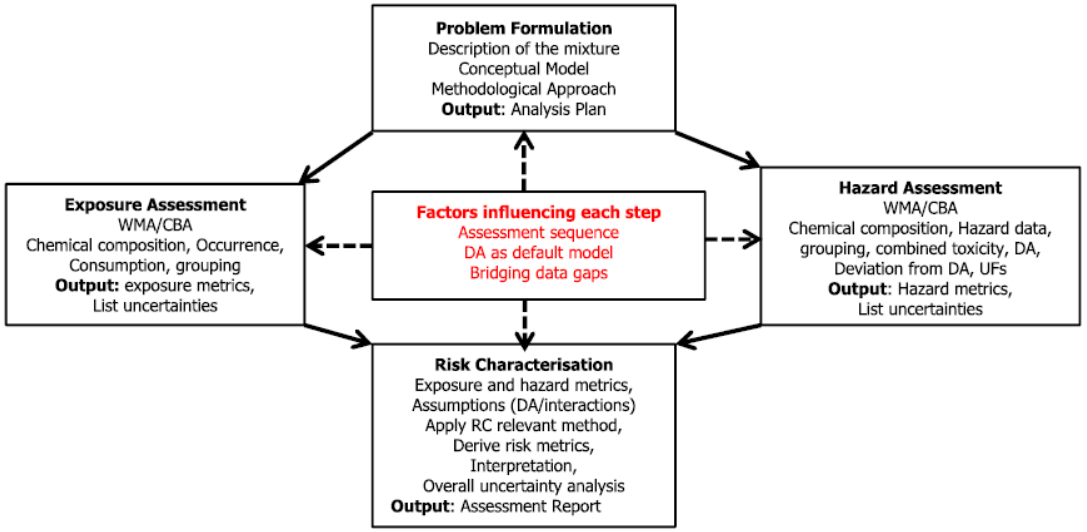
General principles

- Whole mixture (WMA) *vs* components-based approach (CBA)
- Tiering: purpose of the assessment, data availability
- Harmonised overarching framework for human, animal and ecological risk assessment
  - Problem formulation
  - Exposure assessment
  - Hazard assessment
  - Risk characterisation
- Grouping of chemicals
  - Dose addition
  - Bridging data gaps



Whole mixture approach (WMA)

- Applied to assess complex mixtures (with *unknowns*?)
- Mixture treated as a *single chemical*
- The components and concentrations in the mixture do not vary significantly
- Assessment based on toxicity data on the mixture itself
  - ⇧ No need of specific guidance
  - ⇧ It accounts for unidentified compounds or interactions among mixture components, *'holistic'* approach
  - ⇩ It cannot identify substances responsible for toxicity or interactions: *limited mechanistic understanding*
  - ⇩ Conclusions limited to the mixture under assessment: *limited possibility of extrapolation* to *'similar'* mixtures

Component-based approach (CBA)

- The risk of a mixture is assessed based on exposure and effect data of its individual components
- Applicable to well characterised mixtures
- The components and concentrations in the mixture may vary significantly
- Prediction of combined toxicity from the toxicity of the individual components
  - ⇧ Preferred approach, *mechanistic understanding*
  - ⇧ More general applicability and *generalisable conclusions* (assessment based on the max value of the variability range)
  - ⇩ High data requirement
  - ⇩ Guidance is needed

Tiering in practice

	Tier 0	Tier 1	Tier 2	Tier 3
Occurrence	Default values, permitted levels	Modelled and experimental data	Monitoring survey	Individual co-occurrence data
Consumption	Default values, portion size	Food basket	Summary statistics	Individual data
Exposure	Semi-quantitative point estimate	Deterministic	Semi-probabilistic	Probabilistic
Hazard	Read across *In silico,* EKE One group	NOAEL BMDL	Index chemical, Relative Potency Factors (RPF) Toxic equivalents	MoA, AOP PB-TK, TK-TD CSAF
Risk	Hazard Index	Combined MOET	Corrected by RPF, TEq	Corrected by internal dose

CBA: grouping of chemicals

- **Grouping chemicals into assessment groups**
  - definition of assessment groups in **problem formulation**
    - regulatory requirements, exposure
    - physicochemical characteristics, biological and toxicological properties
  - refinement of assessment groups in **hazard characterisation**
    - using weight of evidence, dosimetry, mode of action
  - collection of hazard data to derive reference values for the individual components or for the group
    - handling data gaps within an assessment group (read across, *in silico*)
    - in the absence of interactions, response/*dose addition* as default
  - application of dose addition within assessment groups in **risk characterisation**
    - e.g. hazard index (HI), combined margin of exposure (MOET)

Which mixtures in FEEDAP

- Preparations with different additives of chemical and botanical origin
  - mostly as zootechnical additives
- Fermentation products
  - e.g. vitamins, amino acids, enzymes containing fermentation solubles, carotenoids
- Smoke flavourings
- Plant extracts
  - as sensory additives, flavourings: **>200 preparations** from 166 plant species (re-evaluation, since 2010)

FEEDAP: safety for target species

Whole mixture approach in FEEDAP

WMA is the *common approach* for feed additives

- It is applied for well characterised additives
- FEEDAP Guidance documents apply
- Studies performed with the mixture under assessment
  - tolerance studies in the target animal species (residues)
  - 90-day toxicological studies in laboratory animals

(FEEDAP guidance on safety of feed additives for the target species, 2017)

Issues deserving discussion

- Which validity of genotoxicity testing with the whole mixture? (*dilution effects, interpretation the outcome?*)
- Which applicability to generic applications, when the composition of the additive is *highly variable*?

WMA of an essential oil

SCIENTIFIC OPINION

EFSA Journal

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Safety and efficacy of an essential oil from *Origanum vulgare* subsp. *hirtum* (Link) letsw. var. Vulkan when used as a sensory additive in feed for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Paul Brantom, Birgit Dusemund, Patrick Van Beelen, Johannes Westendorf, Lucilla Gregoretti, Paola Manini and Andrew Chesson

'*Similar*' mixtures

3.3.3.1. Toxicology

A subchronic 90-day oral toxicity rat study with the essential oil of *O. vulgare* has been published (Llana-Ruiz-Cabello et al., 2017). Although the test item derived from another subspecies *O. vulgare* subsp. *virens* (Hoffmanns & Link) letsw., analysis shows that it is similar in composition and content to the essential oil under application (Table 5).

Table 5: Comparison of the test item used in the subchronic oral toxicity study (A) and the essential oil under application (B)

Compound	Essential oil A (%)	Essential oil B (%)
Carvacrol	55.82	60.80
Thymol	5.14	2.26
γ-Terpinene	4.71	7.62
p-Cymene	16.31	8.40
Linalool	nr	3.82
β-Caryophyllene	2.40	3.56
α-Terpinene	1.62	0.60
Terpinen-4-ol	1.33	0.80
trans-Sabinene hydrate	nr	0.42
Total	87.3	88.3

Nr: not reported.

Rationale for extrapolating:

- Differences accounted by **structurally related** compounds with similar toxicological profile (e.g. thymol and carvacrol; α-terpinene and γ-terpinene)
- The essential olil under assessment is **well characterised** (up to 99.1%)
- No **substances of concern** or **genotoxic substances** were detected in the characterised fraction and are not expected extracts from in *Origanum vulgare*

No Observed Adverse Effect Level (NOAEL) of 200 mg/kg bw per day, the top dose tested

Component-based approach in FEEDAP

Data requirement for CBA of botanical preparations

- The mixture should be well characterised (quali/quantitative)
- Identification of the components by name, synonyms, CAS number, FLAVIS number (if available), presence of isomers
- Range of variation (batch to batch, different sources/origin)
- Consolidated specifications
- Presence of genotoxic compounds identified (from screening *in silico*, literature searches, generation of experimental data)
- Reference values for the individual components (from literature searches, generation of experimental data)
- Allocation of the components to the relevant Cramer Class

Some considerations

- The full (quali/Quantitative) characterisation of the mixture can be achieved for essential oils by GC-MS-(FID) analysis
- The full (q/Q) characterisation cannot be achieved for other botanical preparations (e.g. oleoresins, tinctures or extracts), as the analysis of water-soluble components needs HPLC
- The absence of substances of concern or genotoxic compounds (< limit of detection/quantification) is linked to the sensitivity of the analytical method used
- How to take into account the intrinsic variability of botanical preparations?
- How to consider the presence of unidentified components?
- How to handle data gaps? Read across? Threshold of Toxicological Concern (TTC)?

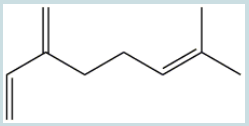
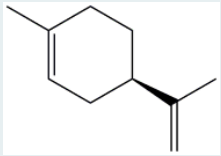
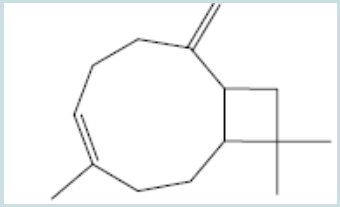
Grouping of compounds: flavourings

The FEEDAP approach for grouping is based on the Chemical Groups (CGs) established for flavouring substances, as defined in Annex I of Reg. (EC) 1565/2000

- CGs are defined in a very broad manner
  - e.g. CG 31 '*Aliphatic and aromatic hydrocarbons*'
- Subgroups can be identified based on *structural criteria*
  - Flavouring Group Evaluation (FGE) 25, 8 subgroups:

    I) acyclic alkanes, II) acyclic alkenes, III) cyclohexene hydrocarbons, IVa) benzene hydrocarbons, IVb) napthalene hydrocarbons, IVc) diphenylmethane, V) bi-and tricyclic, non-aromatic hydrocarbons, VI) macrocyclic, non-aromatic hydrocarbons
  - Representative compounds for testing
  - *Read-across* within a (sub)group

Grouping of compounds: CG 31, FGE 25

Subgroup	Representative substance	NOAEL mg/kg bw	Read across
II: Acyclic alkenes	Myrcene 	44	β-Ocimene α-Farnesene Undeca-1,3,5-triene 1-Octene
III: Cyclohexene hydrocarbons	Limonene 	250	β-Bisabolene δ-Elemene α-Phellandrene Terpinolene γ-Terpinene
V: Bi- and tricyclic non-aromatic hydrocarbons	β-Caryophyllene 	222	Pin-2(10)-ene Pin-2(3)-ene Camphene, Valencene β-Bourbonene δ-3-Carene 4(10)-Thujene

Risk characterisation: dose addition

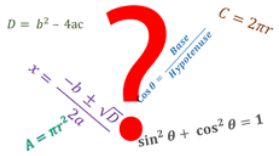
Different tools available for mixtures, to address dose addition within a (sub)group

The FEEDAP approach for essential oils is based on the

- Combined (total) margin of exposure (MOET)

MOE = Reference Value/Exposure

$MOET_{(1\text{-}n)} = 1/[(1/MOE_1)+\ldots+(1/MOE_n)]$



	Intake	NOAEL	MOE
C1	0.569	150	264
C1	0.158	300	1,899
C3	0.010	3	300

MOET=1/[(1/264)+(1/1899)+(1/300)]=
=1/[0.003792+0.000527+0.00333]=
=1/0.007652=
=131

Risk characterisation: MOET

- The uncertainty factor is ***not applied*** to the individual reference values
- Magnitude of the MOET, usually >100
  - A MOET > 100 corresponds to the application of an UF of 100 (10 x 10, for intra- and inter-species extrapolation)
  - *Depending on the nature of the effect and the target population a different value can be considered acceptable*
  - Applicable to *genotoxic carcinogens*, with a different magnitude (MOET >10,000, when calculated based on a $BMDL_{10}$ from a rodent carcinogenicity study)
- **Refinement and iteration** if a risk is identified (tiering)

CBA of an essential oil

SCIENTIFIC OPINION

EFSA Journal

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Safety and efficacy of an essential oil from *Elettaria cardamomum* (L.) Maton when used as a sensory additive in feed for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Maryline Kouba, Mojca Kos Durjava, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Villa, Ruud Woutersen, Paul Brantom, Andrew Chesson, Boris Kolar, Patrick Van Beelen, Johannes Westendorf, Lucilla Gregoretti, Paola Manini and Birgit Dusemund

Cardamom oil:characterisation

Cardamom essential oil is well characterised

- 48 identified compounds accounting for 99.4% (99.2-100%) of the composition of the oil (GC-MS and GC-FID analysis)
- 42 of them are authorised as food flavourings
- almost all components are terpenoids, belonging to chemical groups 31, 6, 16, 3, 4, 8 and 1 (as defined in Regulation (EC) 1565/2000)
- unidentified compounds (<0.1%) treated as Cramer Class III compounds
- the presence of potential genotoxic compounds excluded, i.e. methyleugenol <LOD (0.0002%)

Cardamom oil: safety for target species

Table 3: Compositional data, intake values, reference points and margin of exposure (MOE) for the individual components of cardamom oil classified according to assessment groups based on chemical groups (CGs) as defined in Annex I of Regulation (EC) No 1565/2000[a]

Essential oil composition			Exposure		Hazard characterisation		Risk characterisation	
Assessment group	**FLAVIS No**	**Max conc. in the oil**	**Max Feed conc.**	**Daily Intake**	**Cramer Class**	**NOAEL[b]**	**MOE**	**MOET**
Constituent	–	%	mg/kg	mg/kg bw per day	–	mg/kg bw per day	–	–
CG 6								
Terpineol acetate	09.830	37.3	1.865	0.1473	I	250	1,697	
α-Terpineol		2.03	0.118	0.0093	I	**250**	26,932	
Linalyl acetate	09.013	7.01	0.351	0.0277	I	117	4,225	
Linalool	02.013	4.62	0.231	0.0182	I	**117**	6,411	
Nerolidol	02.018	1.56	0.078	0.0062	I	117	18,987	
Terpinen-4-ol	02.072	2.03	0.102	0.0080	I	250	31,178	
β-Terpineol	02.097	[redacted]	0.043	0.0034	I	250	73,594	
Dihydroterpinyl acetate	n.a.	[redacted]	0.035	0.0027	I	250	91,726	
CG 6				0.2229				886
CG 16								
1,8-Cineole	03.001	24.13	1.207	0.0953	II	**562.5**	5,902	

Exposure

Max occurrence in feed
Highest use level in feed (5 mg/kg) x
max concentration in the oil

Daily feed intake calculated for chicken for fattening, the species with the highest ratio feed intake/body weight (worst case scenario)
Intake = feed conc x (FI)/BW

Conclusive considerations

- The overarching framework described in the SC guidance is flexible and applicable to the relevant areas of EFSA's work
  - tiered and step-wise approach for both WMA and CBA
  - several options for refinement in all the four steps of risk assessment
- Discussion is needed to tailor the general framework to the specific needs of a Panel
- Case studies are presented, but more experience will be gained with the application of the guidance

Thank you for your attention