Food and Chemical Toxicology 188 (2024) 114654

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

Risk assessment of feed components of botanical origin – Approaches taken in the European Union

Birgit Dusemund [a], Gabriele Aquilina [b], Maria de Lourdes Bastos [c], Francesca Marcon [d,*], Fabiola Pizzo [e], Ruud Woutersen [f], Paola Manini [e]

[a] German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 10, 10589, Berlin, Germany
[b] National Center for Chemical Substances, Italian Institute of Health, Viale Regina Elena 299, 00161, Rome, Italy
[c] UCIBIO/REQUIMTE Unit – Laboratory of Toxicology, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313, Porto, Portugal
[d] Dept. Environment and Health, Italian Institute of Health, Viale Regina Elena 299, 00161, Rome, Italy
[e] Feed and Contaminants Unit, European Food Safety Authority, via Carlo Mango 1A, 43126, Parma, Italy
[f] TNO Quality of Life, Utrecht and Wageningen University & Research, Wageningen, the Netherlands

A R T I C L E I N F O

Handling Editor: Dr. Bryan Delaney

Keywords:
Feed
Risk assessment
Botanicals
Additives
Contaminants
Mixtures

A B S T R A C T

In view of a continuous trend in replacing synthetic feed additives and especially flavouring compounds by botanical preparations, different aspects of the safety evaluations of plants and plant-derived preparations and components in feed are discussed. This includes risk assessment approaches developed by the European Food Safety Authority (EFSA) for phytotoxins regarding unintentional exposure of target animals and of consumers to animal derived food via carry-over from feed. Relevant regulatory frameworks for feed additives and feed contaminants in the European Union are summarised and the essentials of existing guidelines used in the safety evaluation of botanicals and their preparations and components in feed are outlined. The examples presented illustrate how the safety of the botanicals, their preparations and components present in feed is assessed. An outlook on possible future developments in risk assessment by applying new *in vitro* and *in silico* methodologies is given.

Abbreviations, ADI, acceptable daily intake; ADME, absorption, distribution, metabolism and excretion; ARfD, Acute Reference Dose; BMD, benchmark dose; $BMDL_{10}$, the lower confidence limit of the benchmark dose associated with a 10% response; bw, body weight; CBA, component-based approach; CLP, Classification, Labelling and Packaging (of substances and mixtures); EC, European Commission; EFSA, European Food Safety Authority; EGCG, (−)-epigallocatechin-3-O-gallate; EMA, European Medicines Agency; EU, European Union; GLP, Good Laboratory Practice; HBGV, Health-Based Guidance Values; IARC, International Agency for Research on Cancer; IATA, Integrated Approaches to Testing and Assessment; JECFA, the Joint FAO/WHO Expert Committee on Food Additives; MOE, Margin of Exposure; MOET, combined (total) margin of exposure; NAMs, new approach methodologies; NOAEL, no observed adverse effect level; NTP, National Toxicology Program; OECD, Organization for Economic Cooperation and Development; PBK model,physiologically based kinetic model; PECsoil, predicted environmental concentration in soil; QPS, qualified presumption of safety; (Q)SAR, (quantitative) structure–activity relationship; TDI, tolerable daily intake; TTC, Threshold of Toxicological Concern; UF, uncertainty factor; WMA, whole mixture approach.

1. Introduction

Plants form the natural basis of herbivores‘ and omnivores‘ diets. Furthermore, the use of plant-derived feed additives for technological, zootechnical or sensory (e.g. colouring or flavouring) purposes leads to the exposure of livestock and companion animals to substances of botanical origin. Plant materials, botanicals[1] and botanical preparations[2] being part of the animal diet may contain phytotoxins either as a natural intrinsic secondary metabolite or as a contaminant and, therefore, have to undergo safety evaluations to avoid unintentional exposure of target animals and of consumers of animal derived food products via

* Corresponding author.
E-mail address: francesca.marcon@iss.it (F. Marcon).

[1] This term refers to plants, including algae, fungi, and lichens, and parts of plants as a whole or cut.
[2] This term refers to preparations obtained by all kinds of processing, e.g., pressing, extraction, fractionation, concentration, drying, and/or fermentation.

https://doi.org/10.1016/j.fct.2024.114654
Received 29 November 2023; Received in revised form 29 March 2024; Accepted 7 April 2024
Available online 10 April 2024

carry-over from feed, which may lead to health impairments. Contaminations with toxic plant material may be reduced by suitable cultivation methods and controlled harvesting, processing and storage conditions, as well as appropriate cleaning measures of the harvested products.

2. Legal framework

Directive 2002/32/EC[3] (Annex I) defines maximum levels "on undesirable substances in animal feed" including also botanicals and their components. The Directive aims to ensure target animal safety and consumer protection from carry-over of undesired substances into food from animal origin. It addresses i.a.

(i) mycotoxins and their sources (e.g. aflatoxin B_1 and rye ergot from *Claviceps purpurea*)
(ii) phytotoxins (e.g. gossypol, hydrocyanic acid, theobromine)
(iii) harmful botanical impurities, such as poisonous plants as a whole, their seeds, fruits or other parts, which contain alkaloids, glycosides or other toxic substances, (e.g. *Datura* spp., *Crotalaria* spp., *Croton tiglium, Ricinus communis, Ambrosia* spp., certain *Brassica* species).

Feed additives of plant origin, e.g. for technological, zootechnical or sensory purposes, underly an authorisation in the frame of Regulation (EC) No 1831/2003[4] on additives for use in animal nutrition, taking into account aspects of animal welfare, consumer and user safety and the protection of the environment.

3. Specifics in the risk assessment of botanicals and their preparations

Independent from the function of the botanical or botanical preparation, being either added for sensory, zootechnical or technological purposes to feed, or occurring as an impurity in feed, risk assessments of botanicals and their preparations differ from that of clearly defined single substances as a component of feed.

3.1. Occurrence of secondary plant metabolites with pharmacological and toxicological activities

Plants produce secondary metabolites, such as alkaloids, terpenoids, glucosinolates or cyanogenic glycosides, inducing toxic effects in animals as a defence mechanism to protect against herbivores (e.g. Mithoefer and Boland, 2012).

Human and veterinary medicine benefits from the pharmacological potential of botanicals, their preparations and components and therapeutical effects although associated adverse reactions are well described in literature and in the assessment reports published by the European Medicines Agency (EMA). Consequently, the existing knowledge from animals and humans on plant poisonings and on the use of medicinal plants and their components is taken into account in the safety evaluation of feed components of botanical origin.

Substances of concern in botanicals are not only known from cases of intoxications or use of medicinal plants but also from experimental *in vivo* and *in vitro* studies. Especially the genotoxicity and carcinogenicity shown by *p*-allylalkoxybenzenes (e.g. methyleugenol, estragole and safrole), which occur across different plant families in culinary herbs and botanicals used as spices, need consideration (SCF, 2001a,b,c; JECFA, 2009; van den Berg et al., 2011; EMA, 2019; Eisenreich et al., 2021; Götz et al., 2022). While exposure of animals via unprocessed feed material which contains only low concentrations of *p*-allylalkoxybenzenes is expected to be unlikely of safety concern, these components may be enriched in preparations obtained from certain botanicals, such as nutmeg (*Myristica fragrans* Houtt.), star anise fruit (*Illicium verum* Hook.f.), bitter fennel fruit (*Foeniculum vulgare* Mill. ssp. *vulgare* var. *vulgare*), basil leaves (*Ocimum basilicum* L.), laurel leaves (*Laurus nobilis* L.) (see next section). In these cases, also cumulative exposure via use of different botanical preparations containing these substances of concern has to be taken into account.

3.2. Variation in the composition of a botanical and its preparations

Under different aspects, it has to be considered that botanicals and their preparations generally show a high variation in their composition.

(i) Botanical preparations generally consist of a complex mixture of phytochemicals, the composition of which is often chemically not sufficiently characterised. Regarding the active substances of interest for technological or sensory reasons, their concentrations in the preparation may be rather low and even if their toxicological profile is known, that of the remaining substances is often unknown or incomplete.
(ii) A botanical preparation of a specific botanical species may be represented by different extracts varying in their chemical composition, due to the use of different source materials (e.g. different botanical chemotypes, different geographical origin and conditions of growth and harvesting, different parts of a plant) and different extraction methods and solvents. In consequence, for the safety evaluation read-across between different preparations of the same botanical species which differ in composition or for which information on the chemical profile is lacking may be problematic.
(iii) In case of selective extractions, enrichments of natural ingredients of concern can occur. For example, the genotoxic and carcinogenic *p*-allylalkoxybenzenes are enriched and manifold concentrated in the essential oils from the seeds of *Myristica fragrans* Houtt. (nutmeg oil) or in essential oils from the leaves of *Laurus nobilis* L. (laurel leaf oil) compared to the source materials (EFSA FEEDAP Panel, 2023a,b). Fractional distillation of essential oils may lead to further concentration of *p*-allylalkoxybenzenes as illustrated by the example of "star anise terpenes" obtained from the essential oil of the fruit of *Illicium verum* Hook. f. (star anise oil) (EFSA FEEDAP Panel, 2023c).
(iv) Pharmacological and toxicological effects of botanical products are usually associated with their contents of secondary plant metabolites, which can vary among plants belonging to the same species or variety as a consequence of multiple biotic and abiotic factors. In the risk assessment it has to be ascertained that specifications of the products of trade and those investigated in the toxicity testing are the same or allow read-across.
(v) It also becomes evident that breeding methods, which influence biosynthesis pathways, will change the pattern in which chemically related bioactive substances occur in a botanical and thus affect its toxicity profile. For example, among the opium alkaloids contaminating poppy seeds derived from *Papaver somniferum,* morphine is usually the major component accompanied by codeine, both alkaloids acting as agonists binding to the μ-opioid receptors (EFSA CONTAM Panel, 2011a). An altered pattern of alkaloids was observed in Australian poppy seeds originating from poppy cultivars developed by genetic regulation of certain enzymatic biosynthesis processes to give a high yield of thebaine and oripavine. These compounds are used as precursors in drug synthesis and show only partial agonistic activity at the

[3] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, OJ (EC) 2002 No. L140, p.10.

[4] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

μ-receptor, thebaine acting even as an antagonist at higher dosages (EFSA CONTAM Panel, 2011a).

3.3. Interactions of secondary plant components in mixtures, grouping, potency factors, and matrix effects

Botanicals and botanical preparations represent mixtures of active substances in which combined exposures and possible interactions have to be considered in risk assessments (see sections 4.2 and 5.2). In a botanical the main biologically active substance or substance of concern usually occurs accompanied by chemically related compounds formed, e.g. as precursors or by-products of biosynthesis pathways. Therefore, a variety of components of a botanical may show the same structural alerts for specific toxicity endpoints, may react as agonists or antagonists regarding receptor-mediated effects, or may compete for binding sites of metabolising enzymes responsible for their activation, deactivation, or elimination. Consequently, interactions with accompanying ingredients which may weaken or enhance the toxic effects of a known substance of concern have to be considered in the risk assessment of plant materials (matrix effects). Thus, in general, basing the safety evaluation of botanical preparations or botanical contaminants on the exposure and toxicity data of only one active component and ignoring the accompanying ones is inappropriate.

Possible interactions including cumulative effects have been considered for example in grouping of phytochemicals into common assessment groups when applying a component-based approach in several recent European Food Safety Authority (EFSA) safety evaluations of feed additives (see section 5.2). The consideration of structure-activity relationships and underlying mode of actions are important preconditions, when defining assessment groups. For example, in the case of propenylalkoxybenzenes, the mechanism of genotoxic carcinogenicity leading to induction of hepatic tumours has only been established for 2-propenylalkoxybenzenes, namely *p*-allylalkoxybenzenes, such as safrole, methyleugenol and estragole (for reviews see e.g. JECFA, 2009; Dusemund et al., 2017). For 1-propenylalkoxybenzenes, such as *trans*-anethole or alpha- and beta-asarone, the mode of action of liver tumour formation is less clear and may also be based on other hepatotoxic effects (Rietjens et al., 2014; Cartus and Schrenk, 2016; Berg et al., 2016).

Also for several alkaloids, such as ergot alkaloids, pyrrolizidine alkaloids, tropane alkaloids, opium alkaloids, glycoalkaloids, occurring in food and feed, the reference points used for acute or chronic toxicity are based on grouping (EFSA CONTAM Panel, 2011b, 2012, 2013, 2017, 2018, 2020). For example, in the risk assessment of ergot alkaloids present in the sclerotia of *Claviceps purpurea*, which are contaminating grain, reference is made to a sum of ergot alkaloids including, i.a., ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine, which have in common the tetracyclic ergoline ring system which is associated with their activity as ligands for adrenergic, serotonergic and dopaminergic receptors (EFSA CONTAM Panel, 2012). A cumulative assessment approach is also carried out for all 1,2-unsaturated pyrrolizidine alkaloids, occurring mainly in plants of the Boraginaceae, Asteraceae and Fabaceae families, the double bond being a prerequisite for metabolic activation to genotoxic and carcinogenic pyrrolic esters (COT, 2008; EFSA CONTAM Panel, 2011b, 2017; Dusemund et al., 2018).

Group approaches may consider toxicity differences of individual substances by using relative potency factors or equivalency factors, provided that adequate data from toxicity studies on relevant endpoints (see section 4.1) are available. In this way, Raquet and Schrenk (2014) proposed relative potency factors for furocoumarins for photocytotoxic, photomutagenic, and photoclastogenic potencies setting the value for 5-methoxypsoralen at 1.00. Different potencies were also considered in the risk assessment of opium alkaloids in poppy seeds by using a group ARfD (Acute Reference Dose) of 10 μg morphine equivalents/kg body weight (bw) for morphine and codeine and by converting codeine concentration in poppy seed samples to morphine equivalents, using an equivalence factor of 0.2. This was based on the assumption that at maximum 20% of codeine is metabolically activated to morphine in ultra-rapid metabolisers of codeine (EFSA CONTAM Panel, 2011a, 2018).

Interactions may also play a role in the toxicokinetics and toxicodynamic of polyphenols present in green tea extracts originating from the leaves of *Camellia sinensis* which are inter alia used in food supplements. It has been hypothesised that the principal ingredient, (−)-epigallocatechin-3-O-gallate (EGCG), consumed as part of a green tea extract, shows slower elimination than when ingested as an isolated compound due to competition with other accompanying polyphenols in the extract for binding sites of metabolising enzymes (EFSA ESCO, 2009).

4. Risk assessment approaches and EFSA guidance documents with relevance for the safety evaluation of botanicals and their preparations to be used in feed

4.1. General guidances on the safety assessment of botanicals and botanical preparations to be used in food and feed

For the assessment of feed additives of botanical origin, two EFSA documents are considered relevant which have been developed for food: the "**Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements**" (EFSA, 2012) and the **"Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements**" (EFSA Scientific Committee, 2009).

The Compendium (EFSA, 2012; EFSA, 2021 - online version) is a tool in which EFSA listed more than 1200 plant genus, species, and varieties with the purpose to draw the attention of manufacturers and food safety authorities to possible safety issues when botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern are used in the food chain. Similar lists, albeit less extensive, have also been published by national authorities in EU Member States (e.g. BVL, 2020).

The intention of the Guidance (EFSA Scientific Committee, 2009) is to support manufacturers and risk assessors in preparing dossiers and evaluating the safety of a given botanical preparation. EFSA emphasises that the principles of the approach developed, even though focusing on the use in food supplements, are applicable also to other uses of botanical preparations in the food and feed areas. The information considered as mandatory for the safety assessment of a botanical preparation consists of technical, exposure, and toxicological data. The technical data comprise details on (i) the identity of the source material, (ii) the manufacturing process of the botanical preparation, (iii) the chemical composition, (iv) its specifications, (v) the stability in the matrix (food or feed), (vi) the proposed uses and use levels, and (vii) the information on existing assessments. Proposed specifications should be modelled considering existing European or other internationally accepted specifications (e.g. pharmacopoeia monographs). Regarding exposure data information is required on (i) the anticipated exposure, (ii) the cumulative exposure via different products including medicinal products, (iii) the modality of use and (iv) the information on historical uses. Regarding the toxicological data of the botanical preparation, studies on toxicity and toxicokinetics should be available using internationally agreed protocols, the test methods described by the Organization for Economic Cooperation and Development (OECD) being recommended. Studies should be carried out according to the principles of Good Laboratory Practice (GLP).

Depending on the available knowledge, the Guidance foresees a two-level tiered approach for the safety evaluation of the botanical preparation (EFSA Scientific Committee, 2009). At level A of the risk assessment, a decision is derived with respect to (i) a safety concern, (ii) no safety concern, or (iii) a need for additional data. Only in the latter case, the requirement for further testing is seen and has to be specified on the subsequent level B.

At level A, the decision "no safety concern" may be based on the principle of a "presumption of safety." Requirements for a "presumption of safety" are that (i) exposure to known levels of the botanical preparation has occurred in large population groups for many years without reported adverse effects, (ii) not only use levels but also chemotypes of the botanicals and the chemical composition of the botanical preparations should be in line with historically used ones and (iii) intakes due to the intended use levels are within the range of intake levels derived from the European Member States' average diets (see sections 4.4. and 5.2 for analogue approach in feed).

In case specific compounds of concern can be well defined at level A, the evaluations can focus on them. For a botanical preparation with a potential to contain toxic substances that may be of concern, the "presumption of safety" approach can be applied only if there is convincing evidence that these undesirable substances are either absent or significantly reduced or inactivated during processing. In these cases, a "presumption of safety" of the botanical preparation is only justified when the overall exposure to the substances of concern is acceptable compared to existing Health-Based Guidance Values (HBGVs) such as the acceptable/tolerable daily intake (ADI/TDI). Comparison of exposure to the substance of concern with the Threshold of Toxicological Concern (TTC) values (EFSA Scientific Committee, 2019c, see section 4.2) may be used as an alternative. When the botanical (preparation) contains substances that are both genotoxic and carcinogenic, the "Margin of Exposure" (MOE) approach (EFSA, 2005, see section 4.2) could be applied. Alternatively, it could be evaluated if the estimated exposure to the genotoxic and carcinogenic components is likely to be increased, compared to the intake from other sources.

At "level B″, decision is taken which additional studies are needed for botanical preparations for which a "presumption of safety" was not justified at "level A". The spectrum of toxicological data asked for comprises primarily studies on toxicokinetics including metabolism, genotoxicity, and subchronic toxicity. Depending on the outcome of these studies, further studies, e.g., on reproductive toxicity, developmental toxicity, neurotoxicity, immunotoxicity, or chronic toxicity/carcinogenicity, may be required. The specifications of the botanical preparation used for the toxicity studies in comparison to the final product to be marketed should be described in detail.

The adequacy of the two-level tiered approach outlined in the guidance (EFSA Scientific Committee, 2009) was tested with selected botanicals and botanical preparations in order to address various safety issues, such as adulteration, hepatotoxicity and possible presence of genotoxic and carcinogenic compounds. The results were published in the report **"Advice on the EFSA guidance document for the safety assessment of botanicals and botanical preparations intended for use as food supplements"** (EFSA ESCO, 2009; Speijers et al., 2010).

In 2014, EFSA examined the suitability of a **"Qualified Presumption of Safety (QPS) approach"** initially developed for the assessment of microorganisms, as a method for assessing botanicals and botanical preparations for which an adequate body of knowledge exists. In view of the high variability, especially in chemical composition, it was concluded that only limited possibilities exist to establish a QPS status at high taxonomic levels for botanicals. However, the use of a developed structured assessment scheme was recommended as an extension of the 2009 EFSA guidance document (EFSA Scientific Committee, 2014).

4.2. General guidances on risk assessment methodologies of relevance for the safety evaluation of botanicals and their preparations to be used in feed

Of the many guidance documents published by EFSA on risk assessment methodologies in general, those relevant for the assessment of mixtures and for substances occurring in low concentrations, and those dealing with the assessment of genotoxic and/or carcinogenic substances are of particular interest for the approaches used in the risk evaluations of botanicals, their preparations and components.

The principles and the methodologies applicable to the risk assessment of chemical mixtures are described in the **"Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals"** (EFSA Scientific Committee, 2019a). The document describes a harmonised framework, which covers all the risk assessment steps, namely problem formulation, exposure assessment, hazard assessment and risk characterisation. Tiered and stepwise approaches are described for both, whole mixture approach (WMA) and the component-based approach (CBA). In the WMA, a mixture is evaluated applying the same methodology as for single chemicals assuming in general that an MOE of at least 100 calculated for a reference point, such as a no observed adverse effect level (NOAEL) identified for a non-neoplastic effect, is not associated with health risks. For the CBA, the guidance develops specific considerations on the grouping of chemicals into assessment groups, the use of dose (or concentration) addition within an assessment group as a default assumption unless evidence for response addition or interactions (antagonism, synergism) is available. In the CBA a "combined (total) Margin of Exposure (MOET)" is calculated and in general acceptable when it is equal to or greater than 100. The guidance provides case studies with examples of application in the different areas under EFSA's remit, including the assessment of target animal safety of feed additives of botanical origin (for example essential oils).

In the "**Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment**" (EFSA Scientific Committee, 2011) a step-wise approach is recommended for the evaluation of the genotoxic potential of substances and preparations, including first a basic *in vitro* test battery[5] for the assessment of the complementary endpoints gene mutations and chromosomal damage, followed, if necessary, by *in vivo* studies. Clarification of the mechanism of action of a genotoxic substance is an additional fundamental step since genotoxicants acting by non-DNA-reactive mechanisms may exhibit a thresholded, non-linear dose–response (Elhajouji et al., 2011). In this respect, the **"Scientific Opinion on the guidance on aneugenicity assessment"** published in 2021 (EFSA Scientific Committee, 2021) describes the approach for the assessment of aneugenicity, which is the potential of a compound to induce changes in the number of chromosomes through interactions with cellular targets other than DNA (e.g., mitotic spindle, centromeric proteins). A major challenge in the genotoxicity assessment of botanical preparations comes from their nature of complex mixtures, in which a relevant fraction may consist of uncharacterised components. This problem has been addressed by the "**Statement on the genotoxicity assessment of chemical mixtures"** (EFSA Scientific Committee, 2019b), aimed to provide indications for a harmonised approach in the area of food and feed safety.

In botanical preparations, individual components may be present at low concentrations. While a genotoxic substance can represent a concern for human and animal health even at a low exposure level, the sensitivity of the experimental systems is strongly affected by the concentration of the genotoxic component. Testing a whole mixture in which one or more genotoxic components are present at low concentrations could prevent the detection of its genotoxic activity in the standard testing battery. On the other hand, an accurate chemical analysis of the individual components could reveal the presence of known genotoxic substances.

For the above reasons, EFSA's Scientific Committee recommends, as first step in the genotoxicity assessment of a complex mixture, the best feasible characterisation in order to identify as many individual components as possible. If one or more of the identified components is

[5] Bacterial reverse mutation test (OECD TG 471) and *In vitro* mammalian cell micronucleus test (OECD TG 487).

recognised as genotoxic in a reliable *in vivo* assay, the whole mixture will be considered to raise concern for genotoxicity. The analytical approach can also identify substances suspected to have genotoxic activity, e.g. chemicals found to be positive in *in vitro* tests or whose molecular structure shows alerts for genotoxicity. In these cases, further experimental investigation is recommended to clarify the concern.

If the chemical analysis identifies no genotoxic substances, the whole mixture can be considered not genotoxic only if the mixture is fully chemically defined, an unlikely occurrence in botanical preparations. More often, complex mixtures contain a substantial fraction of substances that have not been chemically identified; therefore, further investigation is necessary to rule out the possibility that genotoxic components are present in the uncharacterised fraction. To this aim, the optimal option would be to test separately the uncharacterised fraction. In most cases, this approach is not feasible, therefore, the whole mixture is tested. In case of negative outcome in the recommended basic battery of *in vitro* tests, the mixture is considered safe regarding genotoxicity. If one or more *in vitro* tests give positive results, an adequate follow-up has to be performed to verify whether the genotoxic potential observed *in vitro* is expressed also *in vivo*. If the *in vivo* follow-up shows positive results, it is concluded that the mixture raises a concern about genotoxicity.

The scenario is more complex in the case of a negative outcome. For example, if an *in vivo* micronucleus assay (performed as a follow-up of an *in vitro* positive outcome) provides a negative outcome, this result can lead to the conclusion that the substance is not genotoxic only if exposure of the target tissue (e.g., bone marrow) to the substance is demonstrated. The lines of evidence of target exposure and the criteria to consider a negative *in vivo* micronucleus test sufficient to conclude on the safety of a tested substance are described in (EFSA Scientific Committee, 2017). In particular, bone marrow toxicity is considered in itself a sufficient evidence of target exposure, while the measurement of the plasmatic level of the test item can provide relevant information on target exposure, to be considered in a weight-of-evidence approach. However, these criteria are hardly applicable when the tested material is a mixture. Any toxic effect elicited in the target tissue by the mixture cannot be unequivocally attributed to the genotoxic component. Also, the measurement of plasma levels cannot be applied to a mixture, as the genotoxic activity reported *in vitro* cannot be attributed to a specific component. Therefore, if the *in vivo* follow-up is negative, the possible limitations associated with the *in vivo* testing of mixtures have to be weighed in an uncertainty analysis before concluding on the safety of the material under assessment.

In case one or more characterised botanical components have no or only incomplete genotoxicity data the evaluation is considered as inconclusive or follows the TTC approach.

It is generally assumed that genotoxic and carcinogenic substances, even at low doses, can cause an adverse effect. This implies that the exposure to these substances has to be kept at the lowest level possible in food and feed when their presence cannot be readily eliminated or avoided. In the opinion of the EFSA Scientific Committee on **"A harmonised approach for the Risk Assessment of Substances which are both Genotoxic and Carcinogenic"** (EFSA, 2005), it is recommended to apply the MOE approach for assessing the risk of genotoxic and carcinogenic substances found in food and feed. The MOE approach uses the benchmark dose (BMD) as standardised reference point derived from animal data by mathematical modelling and compares it with dietary intake estimates in humans. In particular the use of the BMDL10 (benchmark dose lower confidence limit 10%) which is an estimate of the lowest dose causing with 95% certainty no more than a 10% cancer incidence in rodents is recommended. To interpret the MOE, inter-species differences (differences between animals and humans), intra-species differences (differences between human individuals), the nature of the carcinogenic process and the reference point on the dose-response curve have to be taken into consideration. In general, an MOE >10,000, when based on animal data, is considered of low concern for human health. It was clarified that the MOE should not be used to evaluate the safety of regulated substances deliberately added to the food chain (e.g. to feed additives).

However, in the **"Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities which are both genotoxic and carcinogenic in substances added to food/feed"** published in March 2012 EFSA's Scientific Committee advised that the MOE could be useful in assessing the safety of any genotoxic and carcinogenic impurities present in regulated substances at very low levels (EFSA Scientific Committee, 2012).

The **„Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment“** (EFSA Scientific Committee, 2019c) has been developed as a pragmatic tool for screening and prioritisation purposes applicable to substances whose toxicological profile is not adequately characterised. The TTC approach uses the classification scheme originally proposed by Cramer considering non-cancer endpoints (Cramer et al., 1978). Based on their chemical structure and reactivity, chemicals are categorised into three structural classes indicating a low (Class I), medium (Class II) or high (Class III) level of concern. Additional classes have been included for organophosphates and for substances that have the potential to be DNA-reactive mutagens and/or carcinogens. In the TTC approach, each class is associated with a specified human exposure level (a TTC value), below which there is a low probability of adverse effects.

Although the TTC approach should not be used for substances for which EU food/feed legislation requires the submission of toxicity data, it has been widely applied to the assessment of flavouring substances in food and feed in a conservative manner (EFSA Scientific Committee, 2019c). The components of complex mixtures such as botanical preparations can be screened by applying the TTC to identify substances needing further toxicological characterisation. Conservatism in the application of the TTC to chemical mixtures is granted by the application of dose addition in line with the EFSA Guidance on risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a). However, the applicability of the TTC approach as a tool for the evaluation of mixtures depends on the nature and the level of characterisation of the mixture and should, therefore, be considered on a case–by-case basis.

4.3. Guidances on feed safety evaluations considering aspects of animal and human health and of the environment

The adequate information on the composition, specifications, purity and use levels of a (botanical) feed additive is the essential basis for the risk assessment. The **EFSA „Guidance on the identity, characterisation and conditions of use of feed additives“** (EFSA FEEDAP Panel, 2017a) describes the details on these data requirements addressing also the need for information on physicochemical properties, manufacturing process, stability, and available analytical methods and the technical particularities for additives of botanical origin.

An assessment of risk to users/workers is part of the evaluation of feed additives including botanical preparations and follows the **"Guidance on studies concerning the safety of use of the additive for users/workers"** (EFSA FEEDAP Panel, 2012), which is under revision and will be replaced by the **"Guidance on the assessment of the safety of feed additives for the users"** (EFSA FEEDAP Panel, 2023d). Information according to the Classification, Labelling and Packaging of Substances and Mixtures Regulation (EC) 1272/2008[6] and from the Material Data Sheets e.g., on hazard for skin and eye contact and

[6] Regulation (EC) No 1271/2008 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. OJ L 353, 31.12.2008, p. 1–1355.

respiratory exposure may be applicable. In case the botanical preparation contains unavoidable low levels of compounds that are genotoxic and/or carcinogenic, the exposure of unprotected users should be addressed.

In particular, if a botanical preparation contains ≥0.1% of a compound which is classified as carcinogenic (category 1B), e.g. safrole in cinnamon bark oil and/or leaf oil (EFSA FEEDAP Panel, 2022a), according to the criteria in Annex I of the CLP Regulation (1272/2008/EC)[7] the mixture is classified as carcinogenic and should be handled accordingly.[8]

As described in the "**Guidance on the assessment of the safety of feed additives for the target species"** (EFSA FEEDAP Panel, 2017b), the safety for the target animals can be demonstrated by tolerance studies in the relevant target species/categories or can be derived from toxicological studies in laboratory animals by applying an uncertainty factor (UF) of 100 (to cover intra- and interspecies variation, each with a factor of 10) which would be equivalent to an MOE of 100. In the absence of toxicological data, the TTC could be applied resulting in maximum acceptable concentration in complete feed.

The **"Guidance on the assessment of the safety of feed additives for the consumer"** (EFSA FEEDAP Panel, 2017c) provides advice on how to evaluate possible health risks associated with the consumption of food products derived from animals exposed to the additive via feed.

The safety assessment of botanical preparations represents a great challenge in what refers to consumer safety that ideally should be supported by data on the non-detectability of residues of the parent compounds and/or their metabolites in animal food products. Some botanical preparations contain over hundreds of compounds and data on the absorption, distribution, metabolism and excretion (ADME) in target animal species are rarely available. The approach developed for botanical flavourings is based on the assumption that ADME in the target species is similar to that in experimental animals, for which data are available for representative compounds. Importantly, it is assumed that in food producing animals, expressing the same enzymes as the experimental models, the metabolism of compounds follows similar pathways as in experimental animals. The approach for the assumption of ADME of terpenes belonging to Chemical Group 31 in food producing animals was based on the existence in these animals of enzymes that mediate Phase I and Phase II metabolic reactions favouring the formation of metabolites and subsequent excretion (EFSA FEEDAP Panel, 2015a, 2016). Examples of the safety evaluation of feed additives for consumers are presented in a review by Woutersen et al. (2019).

The principles for the safety assessment of feed additives for the environment are described in the "**Guidance on the assessment of the safety of feed additives for the environment"** (EFSA FEEDAP Panel, 2019a). An assessment of the safety of feed additives for the environment is not required for botanical preparations intended only for non-food producing animals or obtained from plants which are native to Europe or widely distributed in the EU environment (EFSA FEEDAP Panel, 2019a). For those preparations which are not excluded at the level of the plant, an assessment of the safety for the environment is based on the individual components (component-based approach). For the individual components, an environmental risk assessment is not needed if the component (i) is naturally occurring at concentrations above the application rate, (ii) is extensively metabolised in the target animals, (iii) has been already evaluated for use in feed as flavouring compound and considered safe at concentrations in feed higher than those resulting from the use of the mixture in feed; (iv) its use in feed would result in concentrations <0.5 mg/kg complete feed, a threshold which ensures that the trigger value for the predicted environmental concentration in soil (PEC_{soil}) of 10 μg/kg is not exceeded.

[7] Regulation (EC) No 1271/2008 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. OJ L 353, 31.12.2008, p. 1–1355.

[8] Directive 2004/37/EC of the European Parliament and of the Council of 29 April 2004 on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Sixth individual Directive within the meaning of Article 16(1) of Council Directive 89/391/EEC). OJ L 158, 30.4.2004, p. 50.

4.4. Specific approach in the assessment of target animal safety of botanical preparations which contain compounds that are genotoxic and/or carcinogenic

Compounds which are both genotoxic and carcinogenic should not be deliberately added to food or feed, as repeatedly indicated by EFSA bodies (EFSA, 2005; EFSA Scientific Committee, 2012, EFSA FEEDAP Panel, 2015b). However, since genotoxic and carcinogenic compounds occur naturally in plants, their presence at low concentrations in plant preparations intended for use as feed additives cannot be fully avoided. Against this background, the FEEDAP Panel developed in 2021, at the request of the European Commission (EC), a **"General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic when used as feed additives"** (EFSA FEEDAP Panel, 2021a). It is based on three possible scenarios. For substances for which carcinogenicity studies in rodents are available, from which a $BMDL_{10}$ can be derived, the MOE approach similar to human risk assessment is applied (EFSA, 2005; EFSA Scientific Committee, 2012). If adequate carcinogenicity studies in rodents are not available, either the TTC concept for genotoxic substances is used (EFSA Scientific Committee, 2019c) or a comparison on the increase in exposure to the substances of concern relative to intake from other dietary sources (for examples see section 5.2).

Preconditions for the applicability of the approach are that the manufacturing processes of botanical feed additives avoid selective extraction and enrichment of genotoxic and/or carcinogenic substances and that it aims at the reduction of these substances. Furthermore, ADME/residue data should be available to consider possible carry-over of genotoxic and/or carcinogenic substances to the animal-derived food products.

The approach also considers that genotoxicity and carcinogenicity endpoints are biologically relevant for "long-living" animals (pets and animals for reproduction), whereas for "short-living" animals (animals for fattening), non-neoplastic endpoints are considered more appropriate.

It should be noted that MOE values do not quantify risk but only indicate priority for risk management measures (EFSA Scientific Committee, 2012). An MOE(T) < 10,000 is indicative of concern and an MOE (T) > 10,000 of low concern. Also for the latter, the recommendation of the FEEDAP Panel is that the botanical feed additives should contain the lowest possible concentration of genotoxic and carcinogenic substances. In these cases, limits for genotoxic carcinogens are set when the feed additives are authorised.

5. Some examples for safety evaluations of feed contaminants and feed additives of botanical origin

5.1. Feed contaminants of botanical origin

Contamination of feed with botanicals occurs through wild weeds or fungal infestation.

Risk assessment of botanical contaminants in feed and in food of animal origin, has gained importance in the recent years (Dusemund, 2019). Due to their effect as genotoxic carcinogens, the main focus has been on the occurrence of pyrrolizidine alkaloids in the food chain (EFSA CONTAM Panel, 2017). For most other botanical contaminants in the food chain which have been assessed, such as ergot alkaloids (EFSA CONTAM Panel, 2012; EFSA, 2017) or tropane alkaloids (EFSA, 2008;

EFSA CONTAM Panel, 2013; Mulder et al. (2016); FAO and WHO, 2020; EFSA, 2022), the underlying toxicity mechanisms are thresholded.

The International Agency for Research on Cancer has evaluated several plant materials containing 1,2-unsaturated pyrrolizidine alkaloids, as well as the substances themselves (IARC, 1976, 1983, 1987, 2002). For example, for lasiocarpine (e.g. in *Heliotropium* spp.), riddelliine (e.g. in *Senecio* spp.) and monocrotaline (e.g. in *Crotalaria* spp.) the available data gave sufficient evidence for carcinogenicity in experimental animals and they have been classified by IARC as being possibly carcinogenic to humans – group 2B (IARC, 1983, 1987, 2002). In 2011, the EFSA CONTAM Panel published an opinion on pyrrolizidine alkaloids (see section 3.3) in food and feed (EFSA CONTAM Panel, 2011b) which was updated for food in 2017 (EFSA CONTAM Panel, 2017). For the incidence of liver haemangiosarcomas in female rats exposed to riddelliine, a $BMDL_{10}$ of 237 μg/kg bw per day was established for the sum of pyrrolizidine alkaloids. This was used as reference point to assess carcinogenic risks for humans by the MOE approach, an MOE of 10,000 or higher being considered of low concern from a public health point of view (EFSA, 2005). A possible concern for human health was seen particularly for frequent and high consumers of tea and herbal infusions. Livestock and domestic animals may be exposed to pyrrolizidine alkaloids by the consumption of forage, roughage or herbal mixtures used as feed, which are contaminated with parts e.g. of *Senecio* spp. or of plants of the Boraginaceae family. Available data were too limited to quantify these risks for target animals. However, it was concluded that the risks of pyrrolizidine alkaloids poisoning for target animals in the EU appeared to be low, apart from accidental exposure (EFSA CONTAM Panel, 2011b). Overall, based on reports on available occurrence and exposure data (Mulder et al., 2015; EFSA, 2016), it can be concluded that for consumers, contaminated herbal teas, honey and certain food supplements are the main sources of exposures to pyrrolizidine alkaloids. They could not be detected in meat and their carry-over from animal feed to milk and eggs seems to be limited. Therefore, animal-derived food products were not considered to be major sources of pyrrolizidine alkaloids intake (Dusemund et al., 2018).

5.2. Feed additives of botanical origin

Assessing the safety of feed additives requires a multidisciplinary approach (Woutersen et al., 2019).

In the context of the evaluation of feed additives that are already on the market, as foreseen by Regulation (EU) 1831/2003,[9] the FEEDAP Panel is assessing more than 200 preparations of botanical origin (e.g. extracts, tinctures, essential oils, oleoresins) intended for use in animal feed mainly for flavouring purposes. Both approaches described in the SC guidance (EFSA Scientific Committee, 2019a), the WMA and the CBA (see section 4.2), have been extensively applied.

The safety assessment of essential oils from *Origanum vulgare* ssp. *hirtum* (Link) Iestw. has been performed by applying the WMA (see section 4.2), either via tolerance studies performed with the additive under assessment (EFSA FEEDAP Panel, 2017d, 2019b) or based on read-across from a sufficiently similar mixture tested in a 90-day study in rat, from which a NOAEL of 200 mg/kg bw per day could be derived (EFSA FEEDAP Panel, 2019c). The concept of referring to chemically similar mixtures introduces uncertainty in the assessment and should be properly justified. The rationale for extrapolating in the assessment (EFSA FEEDAP Panel, 2019c) from the oil tested in the 90-day study to the oil under assessment was based on the following arguments: (i) the essential oil under assessment is well characterised (up to 99.1%); (ii) no genotoxic substances or other substances of concern were detected in the characterised fraction of the oil and were not expected to occur in preparations from *Origanum vulgare* (based on extensive literature search); (iii) differences in the composition were accounted by structurally related compounds characterised by similar toxicological profile (e.g. thymol and carvacrol, α terpinene and γ-terpinene). The same approach, read-across from a sufficiently similar mixture tested in a 90-day study in rat, was applied for ginger oil from *Zingiber officinale* Roscoe (EFSA FEEDAP Panel, 2020a) and turmeric oil from *Curcuma longa* L. (EFSA FEEDAP Panel, 2020b).

The safety assessment of cardamom oil from *Elettaria cardamomum* (L.) Maton is the first example of the application of the CBA to an essential oil (EFSA FEEDAP Panel, 2019d). Based on considerations on the structural and metabolic similarity, the 38 identified compounds (mostly terpenoids) were grouped into eleven (sub)-assessment groups. For all the assessment groups, the calculated MOET was above 100, indicating no safety concern for the target species at the proposed use level in complete feed (5 mg/kg).

For the botanical preparations originating from plants of the order of Sapindales (e.g. expressed lemon oil from fruit peels of *Citrus limon* (L.) Osbeck and its fractions) the risk assessment was driven by the major component (d-limonene in most cases) and by the presence of substances of concern, i.e. furocoumarins present in expressed oils and perillaldehyde. For these substances of concern, the approach to the safety assessment was based on the comparative intake of the same substances via feed of plant origin. Evidence was available that citrus peels are used as feed material for several target species (ruminants, pigs, poultry, salmon). However, this approach was not applicable to draw conclusions for those species for which there was no evidence of the use of citrus peel in natural feed, i.e. dogs, cats and ornamental fish (EFSA FEEDAP Panel, 2021b).

In particular, plants belonging to the botanical orders of the Laurales (e.g. *Laurus nobilis* L.), Magnoliales (e.g. *Myristica fragrans* Houtt.), Piperales (e.g. *Piper nigrum* L.), Apiales (e.g. *Pimpinella anisum* L., *Foeniculum vulgare* Mill. ssp. *vulgare*) and Austrobaileyales (e.g. *Illicium verum* Hook.f.) are characterised by the presence of *p*-allylalkoxybenzenes, e.g. estragole, methyleugenol, safrole, myristicin, elemicin, apiole and dillapiole. The occurrence of *p*-allylalkoxybenzenes is largely variable and ranges from trace amounts, e.g. estragole in tinctures prepared from Apiaceae fruit up to 6% for estragole in star anise oil from the fruit of *Illicium verum*. As these genotoxic and carcinogenic compounds share the same mode of action, they are assessed as a group and dose addition is applied. For *p*-allylalkoxybenzenes, the FEEDAP Panel identified the $BMDL_{10}$ of 22.2 mg/kg bw per day derived from rodent carcinogenicity studies with methyleugenol (NTP, 2000; Suparmi et al., 2019), as the reference point for the entire group of *p*-allylalkoxybenzenes regardless the relative potency (EFSA FEEDAP Panel, 2022b). In the safety evaluation for the target animals MOE(T)s are calculated based on these reference points. Similar to human risk assessments an MOE(T) $>$ 10,000 is considered indicative of low concern and an MOE (T) $<$ 10,000 indicative of concern for risk management perspectives (see section 4.4).

For Apiaceae tinctures, the presence of *p*-allylalkoxybenzenes at concentrations near the limit of detection resulted in an MOE(T) in the order of 10^5-10^8 when the exposure of the target animals at the maximum proposed use level in feed (50–200 mg/kg complete feed) was compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day derived for methyleugenol (see e.g. EFSA FEEDAP Panel, 2022c).

The risk assessment of essential oils containing higher concentrations of *p*-allylalkoxybenzenes required a refined approach, with different reference points for "long-living animals" and for "short-living animals" (see section 4.4). For non-neoplastic lesions, a NOAEL of 10 mg/kg bw per day was identified from a 90-day study in mice with methyleugenol (NTP, 2000) and selected as reference point for all the group of *p*-allylalkoxybenzenes.

For example, for star anise oil when the estimated exposures of long-living animals were compared to the $BMDL_{10}$ for carcinogenic endpoints, an MOE >10,000, which is indicative of low concern, was

[9] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

obtained for "long-living animals" at the proposed use levels in feed (0.6–6.5 mg/kg). For "short-living animals", when comparing the exposure to the reference point for non-neoplastic endpoints, the magnitude of the MOE was >100 and was considered of no concern (EFSA FEEDAP Panel, 2023e).

6. *In silico* methods and new approach methodologies (NAMs)

The development of new approach methodologies (NAMs) and their use in risk assessment follows the 3R principles of replacing, reducing, and refining standard animal experiments. The term NAMs encompasses *in silico*, *in chemico*, *in vitro* and *ex vivo* approaches. A wide range of methods falls under the umbrella of NAMs, such as computational modelling, -omics applications, high throughput screening and imaging bioassays, cell cultures and tissue/organ engineering (Schmeisser et al., 2023). The integration of NAMs in risk assessment seems particularly promising in the case of complex mixtures containing hundreds of components, like botanical preparations.

New methodologies have been developed for the characterisation and quantification of botanical constituents to facilitate *in silico* safety assessment (Baker and Regg, 2018; Little et al., 2017). To add to ref. list.

In silico models can be applied to fill data gaps in the absence of information on one or more components contained in complex mixtures (EFSA Scientific Committee, 2019a). *In silico* models, also called "non-testing methods" comprise: (i) (quantitative) structure–activity relationship ((Q)SAR), (ii) grouping and read-across approaches and (iii) expert systems. (Q)SAR are mathematical descriptions of the biological/toxicological activity of a substance based on its physical-chemical structure. Read-across approaches are based on the extrapolation of the biological/toxicological activity of a substance, using data available on similar compound(s) and finally expert systems are typically a combination of more than one approach/model (Benfenati et al., 2019).

In particular, for the genotoxicity assessment of chemically fully defined mixtures, as is the case for certain botanical feed additives, the EFSA Scientific Committee recommends applying a CBA, using all available information, including those deriving from read-across and (Q)SAR (EFSA Scientific Committee, 2019b). In the assessment of essential oils as feed additives, the OECD QSAR toolbox[10] is extensively applied to screen all the components for their genotoxic potential and identify those needing further consideration and, eventually, further testing. Among the different genotoxic endpoints, the models predicting gene mutations, based on the experimental data obtained with *in vitro* bacterial mutagenicity assay (Ames test) and available on different platforms, seem to be those with higher accuracy contrarily to those predicting other genotoxic endpoints, which present lower reliability (Benigni et al., 2020).

When grouping is done in the safety assessment of botanical mixtures by the CBA, read-across is applied to fill data gaps, e.g. to derive a reference point for components without toxicity data. For grouping and read-across approaches, the similarity between chemicals can be defined on the basis of the expert judgement and/or can be computed through combinations of similarity metrics (e.g. water solubility, chemical structure features, etc.), making the read-across a more reproducible approach (QSAR OECD toolbox, ToxRead[11]).

In silico models for prediction of well-defined toxicity endpoints (e.g. gene mutation) are more reliable than those for other endpoints (e.g. chronic toxicity), which are affected by higher uncertainty. This is mainly due to the different biological/toxicological effects and mechanisms of action involved in the experimental trials (e.g. repeated-dose toxicity tests) (Mazzatorta et al., 2008), reducing the routinely applicability of these methods for regulatory purpose.

The new -omics technologies, including metabolomics, can provide comprehensive information on the toxicological mode of action of compounds. The use of data obtained by –omics tools was already considered by EFSA in 2014 in its scientific report "Modern methodologies and tools for human hazard assessment of chemicals" (EFSA, 2014). This would provide assessors to proceed changing the assessment of the safety of compounds from an observational paradigm (apical data from animals) to a more mechanistic and scientific based approach, thus contributing to a weight of evidence approach, i.e. as a component of Integrated Approaches to Testing and Assessment (OECD, 2020).

The use of NAMs in the assessment of consumer safety of essential oils used as feed additives is under investigation to fill the data gaps particularly focusing on interspecies difference in the metabolism of *p*-allylalkoxybenzenes.[12] Comparative *in vitro* metabolism data are being generated for several *p*-allylalkoxybenzenes (estragole, methyleugenol, safrole, myristicin and elemicin) in selected target species (pig, chicken, bovine and cat) and compared with available data in rodents and humans. These data will be used to perform quantitative *in vitro* to *in vivo* extrapolations using physiologically based kinetic (PBK) models to predict the metabolic fate of these compounds in the target animals. The results will be used to refine the risk assessment with respect to (i) species differences in kinetics, (ii) differences in the bioactivation between different *p*-allylalkoxybenzenes, (iii) the potential of transfer of the compounds from feed to food.

Disclaimer

Prof. Bastos, Drs. Dusemund, Marcon and Woutersen are members of the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Dr. Aquilina is member of the EFSA Panel on Food additives and Flavourings (FAF). Drs. Manini and Pizzo are EFSA staff in the Feed and Contaminants (FEEDCO) Unit. The present article is published under the sole responsibility of the authors, and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the authors alone and do not represent the views or any official position or scientific works of EFSA.

Funding

This review article was written without funding. The publication of the open-access manuscript was funded by the Italian Institute of Health.

CRediT authorship contribution statement

Birgit Dusemund: Writing – review & editing, Conceptualization. **Gabriele Aquilina:** Writing – review & editing. **Maria de Lourdes Bastos:** Writing – review & editing. **Francesca Marcon:** Writing – review & editing. **Fabiola Pizzo:** Writing – review & editing. **Ruud Woutersen:** Writing – review & editing. **Paola Manini:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Disclaimer Prof. Bastos, Drs. Dusemund, Marcon and Woutersen are members of the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Dr. Aquilina is member of the EFSA Panel on Food additives and Flavourings (FAF). Drs. Manini and Pizzo are EFSA staff in the Feed and Contaminants (FEEDCO) Unit. The

[10] Available at: https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm. Last access: October 2023.

[11] Available at: https://www.vegahub.eu/portfolio-item/toxread/. Last access: October 2023.

[12] Case Studies NAMs Essential Oils as Feed Additives. OC/EFSA/SCER/2021/14. https://etendering.ted.europa.eu/cft/cft-display.html?cftId=9112.

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If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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