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SCIENTIFIC OPINION

Safety and efficacy of a feed additive consisting of a tincture derived from the flowers of *Syzygium aromaticum* (L.) Merr. & L.M. Perry (clove tincture) for all animal species (FEFANA asbl)

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) | Vasileios Bampidis | Giovanna Azimonti | Maria de Lourdes Bastos | Henrik Christensen | Birgit Dusemund | Mojca Durjava | Maryline Kouba | Marta López-Alonso | Secundino López Puente | Francesca Marcon | Baltasar Mayo | Alena Pechová | Mariana Petkova | Fernando Ramos | Roberto Edoardo Villa | Ruud Woutersen | Paul Brantom | Andrew Chesson | Josef Schlatter | Johannes Westendorf | Yvette Dirven | Paola Manini | Birgit Dusemund

Correspondence: feedap@efsa.europa.eu

Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of a tincture from the dried flower bud of *Syzygium aromaticum* (L.) Merr. & L.M. Perry (clove tincture) when used as a sensory additive in feed and water for drinking for all animal species. The product is a [redacted]) solution, with a dry matter content of ~1.66%. The product contains on average 0.511% phenolic acids (of which 0.0344% were flavonoids), 0.039% eugenol, 0.00019% methyleugenol and 0.00008% estragole. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that the use of clove tincture is very unlikely to be of safety concern for the target species up to the maximum proposed use level of 50 mg clove tincture/kg complete feed for all animal species, except for horses, for which the proposed use level is 200 mg/kg complete feed. The FEEDAP Panel considers that the use in water for drinking alone or in combination with use in feed should not exceed the daily amount that is considered very unlikely to be of safety concern when consumed via feed alone. No safety concern would arise for the consumer and the environment from the use of clove tincture up to the maximum proposed use levels in feed. The additive under assessment should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser. When handling the additive, exposure of unprotected users to methyleugenol and estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised. Since the flower buds of *S. aromaticum* and their preparations were recognised to flavour food and their function in feed would be essentially the same, no demonstration of efficacy was considered necessary.

KEYWORDS

clove tincture, estragole, eugenol, flavouring compounds, methyleugenol, sensory additives, *Syzygium aromaticum*

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1 | INTRODUCTION

1.1 | Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In addition, Article 10(2) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of 7 years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping[2] for authorisation/re-evaluation of 18 additives (namely geranium oil, geranium rose oil, eucalyptus oil, eucalyptus tincture, clove oil, clove tincture, broom teatree oil, purple loosestrife tincture, tea tree oil, melaleuca cajuputi oil, niaouli oil, allspice oil, bay oil, pomegranate bark extract, bambusa tincture, citronella oil, lemongrass oil and vetiveria oil) belonging to botanically defined group (BDG) 07 – Geraniales, Myrtales, Poales when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). During the assessment, the applicant withdrew the application for six additives.[3] These additives were deleted from the register of feed additives.[4] During the course of the assessment, this application was split and the present opinion covers only one out of the remaining 12 addtitives under application: clove tincture from *Syzygium aromaticum*[5] for all animal species.

The remaining 11 additives belonging to botanically defined group (BDG) 07 – Geraniales, Myrtales, Poales under application are assessed in separate opinions.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 21 December 2010.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product clove tincture (*S. aromaticum*), when used under the proposed conditions of use (see Section 3.3.3).

1.2 | Additional information

A tincture from *Syzygium aromaticum* L. (clove tincture) is currently authorised as a feed additive according to the entry in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 (2b natural products – botanically defined) and foreseen for re-evaluation. It has not been assessed as a feed additive in the EU.

2 | DATA AND METHODOLOGIES

2.1 | Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier in support of the authorisation request for the use of clove tincture from *S. aromaticum* as a feed additive. The dossier was received on 26 March 2024 and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2024-00191.[6]

The FEEDAP Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

Some of the components of the tincture under assessment have been already evaluated by the FEEDAP Panel as chemically defined flavourings. The applicant submitted a written agreement to use the data submitted for the assessment of chemically defined flavourings (dossiers, publications and unpublished reports) for the risk assessment of preparations belonging to BDG 07, including the current one under assessment.[7]

[1]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.
[2]On 13/3/2013, EFSA was informed by the applicant that the applicant company changed to FEFANA asbl, Avenue Louise 130 A, Box 1, 1050 Brussels, Belgium.
[3]Broom teatree oil, geranium oil, bay oil and vetiveria oil (27 February 2019); bambusa tincture and allspice oil (18 November 2022).
[4]Register of feed additives, Annex II, withdrawn by OJ L162, 10.5.2021, p. 5.
[5]Accepted name: *Syzygium aromaticum* (L.) Merr. & L.M. Perry; synonyms: *Eugenia caryophyllata* Thunb.
[6]The original application EFSA-Q-2010-01282 was split on 26 March 2024 and a new EFSA-Q-2024-00191 was generated.
[7]Technical dossier/Supplementary information February 2023/Letter dated 31 January 2023.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the phytochemical markers in botanically defined flavourings from Group 07 – Geraniales, Myrtales and Poales. During the assessment, upon request from EFSA, the EURL issued two amendments[8] of the original report. The additive under assessment, clove tincture, is included in the second amendment. In particular, for the characterisation of clove tincture the EURL recommended methods based on (i) spectrophotometry for the determination of total polyphenols and flavonoids, (ii) gas chromatography–flame ionisation detection (GC–FID) for the determination of *eugenol* (the phytochemical marker), estragole, methyleugenol and eugenol acetate and (iii) high performance thin layer chromatography (HPTLC) for the determination of gallic acid in *clove tincture*.[9]

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of clove tincture from *S. aromaticum* is in line with the principles laid down in Regulation (EC) No 429/2008[10] and the relevant guidance documents: Opinion of the Scientific Committee on harmonised approach for risk assessment of substances that are both genotoxic and carcinogenic (EFSA, 2005), Statement on the applicability of the margin of exposure (MOE) approach for the safety assessment of impurities that are both genotoxic and carcinogenic in substances added to food/feed (EFSA Scientific Committee, 2012), Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern (EFSA, 2012), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance on the assessment of the safety of feed additives for the users (EFSA FEEDAP Panel, 2023a), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019), Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019), Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee, 2019) and General approach to assess the safety for the target species of botanical preparations, which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021).[11]

3 | ASSESSMENT

The additive under assessment, clove tincture, is derived from the dried flower buds of *Syzygium aromaticum* (L.) Merr. & L.M. Perry and is intended for use as a sensory additive (functional group: flavouring compounds) in feed and water for drinking for all animal species.

3.1 | Origin and extraction

Syzygium aromaticum (L.) Merr. & L.M. Perry is a small evergreen tree belonging to the myrtle (Myrtaceae) family. It is native to the Maluki islands (Moluccas or spice islands) of Indonesia but, because of its commercial importance, it is now widely cultivated in other parts of the world with similar climatic conditions. The tree is the source of 'cloves', the immature flower buds harvested just before flowering and dried, which are widely used as a spice. The essential oil is obtained either from the cloves (clove bud oil) or from the leaves (clove leaf oil).

The tincture is produced from the dried flower buds by extended extraction for [redacted] under ambient conditions with a [redacted]. The tincture is recovered by pressing to separate solid and liquid phases, and the solution obtained is then clarified by filtration.

[8]Additives included in the first amendment: geranium rose oil, eucalyptus oil, lemongrass oil and clove oil; additives included in the second amendment: citronella oil, melaleuca cajuputi oil, tea tree oil, clove tincture and eucalyptus tincture.

[9]The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2010-0219_en.

[10]Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

[11]https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf.

3.2 | Uses other than feed flavouring

While there is no specific EU authorisation for any *S. aromaticum* preparation when used to provide flavour in food, according to Regulation (EC) No 1334/2008[12] flavouring preparations produced from food, may be used without an evaluation and approval.

'Clove (Caryophylli flos)' and 'Clove oil (Caryophylli floris aetheroleum)' from *Syzygium aromaticum* (L.) Merr. et L.M.Perry are described in monographs of the European Pharmacopoeia 11.0 (PhEur, 2022a, 2022b) and of the European Medicines Agency (EMA, 2011a, 2011b, 2020) for medicinal uses. Clove oil has been also evaluated for veterinarian uses (EMA, 1998).

3.3 | Characterisation

3.3.1 | Characterisation of clove tincture

Clove tincture is a brown liquid, with a characteristic odour (characteristic of clove). It has an average density of 977 kg/m^3 (range: 975–979 kg/m^3) and a pH of 4.60 (4.52–4.65).[13]

Table 1 summarises the results of the proximate analysis of five batches of the additive.[14] The solvent represents about 98.3% of the additive leaving a dry matter (DM) content of about 1.7%. The DM consists of inorganic material measured as ash (13.8%) and a plant-derived organic fraction of 86.2%, which includes protein, lipids and 'carbohydrates', described as the fraction of organic matter remaining after subtraction of the values for protein and lipids. It contains a variety of plant-derived compounds including phenolic compounds, in addition to any carbohydrate present.

TABLE 1 Proximate analysis of a tincture derived from the flower buds of *Syzygium aromaticum* (L.) Merr. & L.M. Perry based on the analysis of five batches.

Constituent	Mean % (w/w)	Range % (w/w)
Dry matter	1.66	1.57–1.77
Ash	0.23	0.21–0.24
Organic fraction	1.43	1.33–1.56
Proteins	0.38	0.34–0.50
Lipids	< 0.5	< 0.5
'Carbohydrates+ fibre'[a]	0.56	0.47–0.72
Solvent	98.34	98.23–98.23

[a]'Carbohydrates+fibre' (by difference) include secondary plant metabolites, such as phenolic compounds.

The fraction of secondary metabolites was characterised in the same batches of the tincture, and the results expressed as % of the tincture (w/w)[15] are summarised in Table 2. The tincture was shown to contain polyphenols (≤ 0.541%) determined by spectrophotometry (at 760 nm) and expressed as gallic acid equivalent (GAE).[16] Six unidentified polyphenols were detected by HPTLC and also expressed as GAE.[17] The concentration of flavonoids (≤ 0.0418% expressed as quercetin equivalents) was determined by spectrophotometry at 415 nm.[18] The concentrations of eugenol [04.003] and eugenyl acetate [09.020] were determined by GC–FID in the same five batches of the tincture.[19] Eugenyl acetate [09.020] was below the limit of detection (0.00017 mg/mL) in all batches.

[12]Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Regulation (EC) No 1601/91 of the Council, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34.

[13]Technical dossier/Supplementary information September 2022/Annex_II_3_Results of analysis.

[14]Technical dossier/Supplementary information September 2022/Section II_Identity and Annex_II_3_Results of analysis.

[15]For each batch, the values analysed in each individual batch and expressed as mg/mL were converted into % (w/w) considering the value of the density determined for each individual batch.

[16]Technical dossier/Supplementary information September 2022/Section_II_Identity and Annex_II_3_Results of analysis.

[17]Technical dossier/Supplementary information September 2022/Annex II_9_HPTLC Profile_clove tincture_polyphenols.

[18]Technical dossier/Supplementary information May 2023.

[19]Technical dossier/Supplementary information September 2022/Annex II_10_Certificate of analysis of eugenol, methyleugenol, acetate eugenol and estragole in Clove tincture.

TABLE 2 Characterisation of the fraction of secondary metabolites of a tincture derived from the flower buds of *Syzygium aromaticum* (L.) Merr. & L.M. Perry based on the analysis of five batches (mean and range).

Constituent	Method	Mean % (w/w)	Range % (w/w)
Total polyphenols[a]	Folin–Ciocalteu[b]	0.511	0.476–0.541
Flavonoids[c]	Spectrophotometry	0.0344	0.0284–0.0418
Eugenol	GC–FID	0.0387	0.0296–0.0497
Methyleugenol	GC–FID	0.00019	0.00016–0.00022
Estragole	GC–FID	0.00008	0.00006–0.00009

Abbreviation: GC–FID, gas chromatography–flame ionisation detector.

[a]Expressed as gallic acid equivalent (GAE).

[b]Determined by an internal method based on European Pharmacopoeia (PhEur, 2022c): chapter 2.8.14, Determination of tannins in herbal drugs.

[c]Expressed as quercetin equivalents.

The identified secondary metabolites account on average for 33.2% of the DM content of the tincture (range: 32.1%–34.9%).

According to existing monographs (PhEur, 2022a; PhEur Commentary, 2020; EMA, 2011b), cloves from *S. aromaticum* are known to contain a fraction of phenolic compounds in addition to 14%–26% essential oil with the main components being eugenol (75%–88%), eugenyl acetate (4%–15%) and β-caryophyllene (5%–14%). Apart from the volatile phenolic components specified in Table 2, according to the literature, the phenolic fraction consists of (i) about 12% hydrolysable tannins, such as ellagitannins and gallotannins, (ii) about 0.4% flavonoids, mainly quercetin, kaempferol and their glycosides apart from eugenyl-β-rutinoside and myricetin, (iii) phenolic acids, such as gallic- and ellagic acid, 3- and 4-caffeoyl-, 3-*p*-cumaroyl- and 3-feruloylquinic acid, ferulic acid, *p*-hydroxybenzoic acid, caffeic acid, salicylic acid, syringic acid, protocatechuic acid and *p*-coumaric acid and (iv) acetophenones, such as 2,4,6-trihydroxy-3-methyl acetophenone-2-O-β-D-glucoside and 2,4,6-trihydroxy acetophenone-3-C-β-D-glucoside.

3.3.1.1 | *Substances of concern*

The applicant performed a literature search to identify substances of concern in *S. aromaticum* and its botanical preparations, essential oils and aqueous and hydroalcoholic extracts.[20] Among the compounds identified in the essential oil from the flower buds of *S. aromaticum*, eugenol (up to 90%) and eugenyl acetate (up to 17%) are reported in the EFSA Compendium of botanicals (EFSA, 2012).[21] Eugenol and eugenyl acetate are the main constituents of clove oil and are authorised flavourings. One reference reported the occurrence of methyleugenol (up to 0.08%) in clove bud oil from Madagascar and Indonesia (Razafimamonjison et al., 2014). The literature search did not retrieve publications reporting on the occurrence of substances of concern in aqueous alcoholic preparations.

The applicant provided analytical data by GC–FID on the content of methyleugenol (1.64–2.25 mg/kg) and estragole (0.62–0.92 mg/kg) in five batches of the additive.[22] There is no specification defining limit values for undesirable compounds in the tincture.

3.3.1.2 | *Impurities*

The applicant controls contamination at the level of the raw material, including knowledge of the cultivation conditions and pesticides applied. Specifications are set with suppliers covering cadmium, mercury, lead and arsenic, mycotoxins, pesticides, dioxins and polychlorinated biphenyls, polycyclic aromatic hydrocarbons (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluorantene and chrysene) and microbial contamination.[23] Three certificates of analysis of the raw material showing compliance with specifications were provided.[24] Analysis of impurities in the tincture is made on irregular basis and does not form part of the Hazard Analysis and Critical Control Points plan.

[20]Technical dossier/Supplementary information September 2022/Annex II_4_Bibliographic data concerning chemical composition of *Syzygium aromaticum* and *Syzygium aromaticum* extracts.

[21]Online version: https://www.efsa.europa.eu/en/data-report/compendium-botanicals.

[22]Technical dossier/Supplementary information September 2022/Annex II_10_Certificate of analysis of eugenol, methyleugenol, acetate eugenol and estragole in Clove tincture.

[23]Technical dossier/Supplementary information September 2022/Annex II_6_Product description from clove supplier.

[24]Technical dossier/Supplementary information September 2022/Annex_II_7_ Certificate of analysis for plant raw material.

3.3.2 | Stability

The shelf-life of the tincture is declared by the applicant to be at least 36 months when stored in tightly closed containers under standard conditions. No evidence was provided to support this claim.

3.3.3 | Conditions of use

The additive is intended for use in feed and in water for drinking for all animal species. The applicant proposes a maximum concentration of 50 mg clove tincture/kg complete feed for all animal species, except for horses, for which the proposed use level is 200 mg/kg complete feed. No use level has been proposed by the applicant for the use in water for drinking.

3.4 | Safety

The safety assessment is based on the maximum use levels proposed by the applicant in complete feed, which are 50 mg eucalyptus tincture/kg complete feed for all animal species, except for horses, for which the proposed use level is 200 mg/kg complete feed.

No studies to support the safety for target animals, consumers or users were performed with the additive under assessment.

Clove tincture contains 1.7% (w/w) plant-derived material, which includes ash, protein, lipids and carbohydrates (other than secondary metabolites), which are not of concern, and are not further considered.

The main identified individual component of clove tincture is eugenol, a compound identified with the EU Flavour Information System number [04.003]. Eugenol has been assessed for use in food and feed by the EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) and the FEEDAP Panel, respectively. (EFSA AFC Panel, 2009; EFSA FEEDAP Panel, 2011). In its assessment of allylhydroxybenzenes (chemical group 18), the FEEDAP Panel concluded that eugenol [04.003] was safe at the maximum proposed use level of 25 mg/kg complete feed for all animal species, except for fish. The assessment was based on an a no observed adverse effect level (NOAEL) of 300 mg/kg body weight (bw) per day identified from a 103-week study in rats (NTP, 1983) based on bw reduction observed in females at 625 mg/kg bw per day (WHO, 2006). Eugenol was considered safe for the consumer. However, the lack of data on metabolism and residues in poultry precluded an assessment of consumer exposure from this source. Eugenol was considered safe for the environment, whereas hazards for skin and eye contact and respiratory exposure were recognised for all compounds belonging to chemical group 18 (EFSA FEEDAP Panel, 2011). Eugenol is currently authorised for use in feed for all animal species (except poultry and fish) with a recommended maximum content of 25 mg/kg complete feed[25] and for use in food[26] without limitations.

Among the secondary plant metabolites, total phenolic compounds including flavonoids were quantified but not identified. They will be assessed based on considerations at the level of the assessment group (see Section 3.4.3.2). These compounds are readily metabolised and excreted, and are not expected to accumulate in animal tissues and products.

Trace concentrations of methyleugenol (1.64–2.25 mg/kg) and estragole (0.62–0.92 mg/kg) were detected in all batches of the additive. For the absorption, distribution, metabolism and excretion (ADME) and on the toxicology of these compounds reference is made to the safety evaluation made by the FEEDAP Panel in the EFSA opinions on laurel oil and star anise oil (EFSA FEEDAP Panel, 2023b, 2023c).

3.4.1 | Genotoxicity and carcinogenicity

3.4.1.1 | *Methyleugenol and estragole*

Clove tincture contains trace amounts of methyleugenol (1.64–2.25 mg/kg) and estragole (0.62–0.92 mg/kg), two compounds with experimentally proven genotoxic and carcinogenic activities in rodents (as reviewed in European Commission, 2001; EMA, 2005, 2021; IARC, 2018). The carcinogenicity of methyleugenol, estragole and other structurally related *p*-allylalkoxybenzenes has been reviewed by the FEEDAP Panel in the opinion on olibanum extract (EFSA FEEDAP Panel, 2022).

For *p*-allylalkoxybenzenes, the FEEDAP Panel identified a reference point for neoplastic endpoints derived from a carcinogenicity study in rat with methyleugenol (NTP, 2000) by applying the benchmark dose (BMD) approach with model averaging. Dose–response modelling using hepatocellular carcinomas in male rats as a response yielded a BMD lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 22.2 mg/kg bw per day (Suparmi et al., 2019). In the current

[25]Commission Implementing Regulation (EU) 2022/1452 of 1 September 2022 concerning the authorisation of 3-ethylcyclopentan-1,2-dione, 4-hydroxy-2,5-dimethylfuran-3(2H)-one, 4,5-dihydro-2-methylfuran-3(2H)-one, eugenol, 1-ethoxy-4-(prop-1(*trans*)-enyl)benzene, α-pentylcinnamaldehyde, α-hexylcinnamaldehyde and 2-acetylpyridine as feed additives for certain animal species. OJ L 228, 2.9.2022, pp. 17–29.

[26]Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1.

assessment this reference point is applied to assess the combined exposure of target animal species to methyleugenol and estragole (EFSA FEEDAP Panel, 2022).

3.4.1.2 | *Genotoxicity studies with clove preparations*

EMA (2011b) reports an increase in gene mutations induced by the dry residue of aqueous and methanolic extracts (100 mg/mL) in *Bacillus subtilis* (recA assay) (Morimoto et al., 1982, as referenced in EMA, 2011b), no genotoxic effects in *Drosophila melanogaster* after administration of a decoction (1:100) of cloves (Schulz & Herrmann, 1980, as referenced in EMA, 2011b) and negative results for the induction of chromosomal damages in bone marrow from mice treated with 0.5% and 2% of cloves (purchased from the local market, New Delhi, India) in the diet for 10 days (Kumari, 1991, as referenced in EMA, 2011b).

A bacterial gene mutation assay was performed in *Salmonella* Typhimurium strains TA 98, TA 100 and TA 102 to evaluate the mutagenic potential of a standardised hydro-ethanolic dry extract of clove buds (Clovinol[27]) containing 41.2% GAE of polyphenols. Four concentrations were tested (0.5, 1, 2.5, 5 mg/plate) in the presence and absence of metabolic activation. No increase in the number of revertant colonies was observed in any tester strain (Vijayasteltar et al., 2016).

3.4.2 | Toxicological studies

Twenty rats of each sex were given Clovinol (a standardised hydro-ethanolic dry extract of clove buds) by gavage at doses of 0.25, 0.5 and 1 g/kg bw per day for 90 days (Vijayasteltar et al., 2016). Observations included many of those normally expected from a 90-day study (clinical observations, ophthalmoscopy, bw, food and water intake, haematology, urinalysis, clinical chemistry, necropsy, organ weights [liver, kidney, heart, spleen and brain] and histopathology [same tissues as organ weights]). However, the organ weights and haematology reported are for only five animals per sex per group. Although the results are slightly more limited than would be expected from a fully Good Laboratory Practice and Organisation for Economic Co-operation and Development test guideline compliant 90-day study, there are no indications of adverse effects in any of the parameters reported and a NOAEL of 1 g Clovinol/kg bw per day, corresponding to 412 GAE mg/kg bw per day was identified.

Despite the differences in the manufacturing process and in the DM content of the test item compared to the additive under assessment, the FEEDAP Panel notes that for both products polyphenols were quantified using the same analytical method (Folin–Ciolcalteu) and were expressed in the same units (as GAE). Therefore, the FEEDAP Panel considers the NOAEL of 412 mg/kg bw per day for GAE can be used to assess the phenolic fraction of clove tincture.

3.4.3 | Safety for the target species

Tolerance studies in the target species and/or toxicological studies in laboratory animals made with the tincture under application were not submitted.

In the absence of these data, the approach to the safety assessment of the mixture is based on the safety assessment of its individual components (when individually identified and quantified) or groups of components. For eugenol, subchronic studies are available, from which a NOAEL can be derived (EFSA FEEDAP Panel, 2011). For the group assessment of polyphenols expressed as GAE, a NOAEL has been identified from a 90-day study in rats (see Section 3.4.2). For *p*-allylalkoxybenzenes, rodent carcinogenicity studies with methyleugenol are available from which a $BMDL_{10}$ can be derived (EFSA FEEDAP Panel, 2023b).

3.4.3.1 | *Eugenol*

The feed concentrations of eugenol calculated at the highest proposed use levels in complete feed and considering the highest analysed concentration of eugenol in the tincture (0.0497%, w/w) are reported in Table 3. Applying an UF of 100 to the NOAEL of 300 mg/kg bw per day identified from a 103-week study in rats (NTP, 1983) based on bw reduction observed in females at 625 mg/kg bw per day (WHO, 2006), the maximum safe concentrations of eugenol in complete feed for the target species were calculated according to the FEEDAP Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b). For cats an additional UF of 5 is applied, considering their unusually low capacity for glucuronidation of compounds (Court and Greenblatt, 1997; Lautz et al., 2021). The calculated safe concentrations of 1,8-cineole in feed for the target species are shown in Table 3.

[27]Clovinol contained 41.2% GAE (Folin-Ciocalteau test). Gallic acid, ellagic acid, chlorogenic acid, quercetin, luteolin, eugenol and eugenol acetate were identified and confirmed in Clovinol by comparing the liquid chromatography–mass spectrometry details either with reference compounds or with literature data. Gas chromatography–mass spectrometry analysis confirmed the absence of methyleugenol in Clovinol (No limit of detection cited).

TABLE 3 Highest feed concentration of **eugenol** from clove tincture (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) calculated at the highest proposed use levels in complete feed and maximum safe concentrations in feed for the target species calculated by using the NOAEL of 300 mg/kg bw per day.

Animal category	Daily feed intake (g DM/kg bw)	Proposed use level in feed (mg additive/kg complete feed)[a]	Highest feed concentration eugenol (mg/kg complete feed)[a]	Maximum safe concentration eugenol (mg/kg complete feed)[a]
Chickens for fattening	79	50	0.025	33
Laying hens	53	50	0.025	50
Turkeys for fattening	59	50	0.025	45
Piglets	44	50	0.025	60
Pigs for fattening	37	50	0.025	72
Sows lactating	30	50	0.025	94
Veal calves (milk replacer)	19	50	0.025	150
Cattle for fattening	20	50	0.025	132
Dairy cows	31	50	0.025	86
Sheep/goats	20	50	0.025	132
Horses	20	200	0.099	132
Rabbits	50	50	0.025	53
Salmonids	18	50	0.025	–
Dogs	17	50	0.025	158
Cats[b]	20	50	0.025	26
Ornamental fish	5	50	0.025	–

[a]Complete feed containing 88% DM, milk replacer 94.5% DM.
[b]The uncertainty factor for cats is increased by an additional factor of 5 because of the reduced capacity of glucuronidation.

The FEEDAP Panel notes that fish were excluded from the former assessment owing to the anaesthetic properties of eugenol (EFSA FEEDAP Panel, 2011); however, at the concentration of 0.025 mg/kg complete feed in fish, anaesthetic effects of eugenol are not expected. The calculated safe concentrations of eugenol for the target species are at least three orders of magnitude higher than the concentrations in feed resulting from the use of clove tincture at the proposed use levels in feed. Therefore, the presence of eugenol in clove tincture is not considered of concern for all animal species.

3.4.3.2 | *Phenolic compounds including flavonoids*

Among the secondary metabolites, up to 0.541% are polyphenols including up to 0.042% flavonoids. Other non-volatile phenolic compounds were not identified in the tincture; however, the occurrence of phenolic acids, hydrolysable tannins and acetophenones has been described in literature for the flower buds of *S. aromaticum* (see Section 3.3.1).

At the maximum proposed use level of 50 mg clove tincture/kg complete feed, the highest concentration of the fraction of polyphenols (≤ 0.541% of the tincture, measured by the Folin–Ciocalteu method and expressed as GAE) would be 0.27 mg/kg complete feed. For horses, at the maximum proposed use level of 200 mg/kg complete feed, the highest concentration of polyphenols would be 1.08 mg GAE/kg feed.

When the intake values of the target species are compared to the NOAEL of 412 mg/kg bw per day derived for polyphenols expressed as GAE from a 90-day study in rats, a MOE of at least 17,337 is calculated (see Table 4). Therefore, the presence of polyphenols in clove tincture is not considered of concern for the target species.

TABLE 4 Target animal intake of **polyphenols** as gallic acid equivalent (GAE, in µg/kg bw per day) and MOE calculated at the maximum proposed use level of the additive in feed.

Animal categories	Daily feed intake (g DM/kg bw)	Use level (mg additive/kg complete feed)[a]	GAE intake[b] (µg/kg bw per day)	MOE[c]
Chickens for fattening	79	50	24.28	17,337
Laying hens	53	50	16.29	25,842
Turkeys for fattening	59	50	18.03	23,346
Piglets	44	50	13.53	31,128
Pigs for fattening	37	50	11.27	37,353
Sows lactating	30	50	9.27	45,394
Veal calves (milk replacer)	19	50	5.41	77,819
Cattle for fattening	20	50	5.72	73,539

(Continues)

TABLE 4 (Continued)

Animal categories	Daily feed intake (g DM/kg bw)	Use level (mg additive/kg complete feed)[a]	GAE intake[b] (μg/kg bw per day)	MOE[c]
Dairy cows	31	50	9.46	44,512
Sheep/goats	20	50	6.15	68,481
Horses	20	200	24.59	17,120
Rabbits	50	50	15.37	27,392
Salmonids	18	50	5.38	78,264
Dogs	17	50	5.12	82,177
Cats	20	50	6.15	68,481
Ornamental fish	5	50	1.38	304,358

Abbreviations: bw, body weight; DM, dry matter; GAE, gallic acid equivalent; MOE, margin of exposure.

[a]Complete feed containing 88% DM, milk replacer 94.5% DM.

[b]The intake values of GAE are calculated based on the estimated concentration of 0.541% for polyphenols expressed as GAE.

[c]The MOE for GAE is calculated as the ratio of the reference point to the intake: the NOAEL of 412 mg/kg bw per day for polyphenols expressed as GAE derived from a 90-day study in rats with a standardised polyphenolic extract of clove buds (Vijayasteltar et al., 2016).

Overall, no concern for the target species arises from the phenolic compounds including flavonoids.

3.4.3.3 | *Methyleugenol and estragole*

Trace concentrations of methyleugenol (≤ 0.00022%) and estragole (≤ 0.00009%), were detected in all five batches of the additive.

Methyleugenol and estragole belong to the group of *p*-allylalkoxybenzenes and are genotoxic carcinogens. For this kind of compounds, different reference points and a different magnitude of the MOE would be applied for long-living and reproductive animals (including those animals reared for laying/breeding/reproduction) and for short-living animals (animal for fattening).

In the current assessment, considering the very low concentrations of methyleugenol and estragole in feed resulting from the use of clove tincture at the proposed use levels, the FEEDAP Panel did not consider it necessary to distinguish between long-living and reproductive animals and short-living animals.

For all animals, a combined margin of exposure (MOET) with a magnitude > 10,000 when comparing estimated exposure to genotoxic and/or carcinogenic substances with a $BMDL_{10}$ from a rodent carcinogenicity study is considered indicative of low concern. 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 (EFSA FEEDAP Panel, 2022). In the current assessment, this reference point is applied to assess the exposure of all animal species to methyleugenol and estragole.

At the maximum proposed use level of 50 mg tincture/kg complete feed, the highest concentration of methyeugenol in the additive (0.00022%) would lead to a concentration of 0.11 μg/kg complete feed (0.44 μg/kg for horses at the use level of 200 mg tincture/kg complete feed). For estragole, present in the additive at the highest analysed concentration (≤ 0.00009%), the corresponding highest concentrations in feed would be 0.045 μg/kg complete feed (0.18 μg/kg for horses at the use level of 200 mg tincture/kg complete feed).

The individual intake for methyleugenol and estragole, their combined intake calculated for the target species at the maximum proposed use levels in complete feed, and the MOET are reported in Table 5.

TABLE 5 Individual intake of methyleugenol and estragole, their combined intake and combined margin of exposure (MOET) calculated for the target animal categories at the maximum proposed use level of the additive in feed.

Target species	Daily feed intake g DM/kg bw	Use level in feed mg/kg	Methyl eugenol intake[a]	Estragole intake[a]	Combined Intake[a]	MOET[b]
			μg/kg bw per day			
Chickens for fattening	79	50	0.0099	0.0040	0.0139	1,595,427
Laying hens	53	50	0.0066	0.0027	0.0093	2,378,089
Turkeys for fattening	59	50	0.0073	0.0030	0.0103	2,148,387
Piglets	44	50	0.0055	0.0023	0.0078	2,864,516
Pigs for fattening	37	50	0.0046	0.0019	0.0065	3,437,419
Sows lactating	30	50	0.0038	0.0015	0.0053	4,177,419
Veal calves (milk replacer)	19	50	0.0022	0.0010	0.0031	7,161,290
Cattle for fattening	20	50	0.0023	0.0010	0.0033	6,767,419

TABLE 5 (Continued)

Target species	Daily feed intake g DM/kg bw	Use level in feed mg/kg	Methyl eugenol intake[a]	Estragole intake[a]	Combined Intake[a]	MOET[b]
			μg/kg bw per day			
Dairy cows	31	50	0.0038	0.0016	0.0054	4,096,258
Sheep/goats	20	50	0.0025	0.0010	0.0035	6,301,935
Horses	20	200	0.0100	0.0041	0.0141	1,575,484
Rabbits	50	50	0.0063	0.0026	0.0088	2,520,774
Salmonids	18	50	0.0022	0.0009	0.0031	7,202,212
Dogs	17	50	0.0021	0.0009	0.0029	7,562,323
Cats	20	50	0.0025	0.0010	0.0035	6,301,935
Ornamental fish	5	50	0.0006	0.0002	0.0008	28,008,602

[a]The values of methyleugenol and estragole intake are calculated considering that both are present at a concentration corresponding to the highest analysed value in the additive (methyleugenol, 0.0002% w/w and estragole 0.0009% w/w).

[b]The combined margin of exposure (MOET) for methyleugenol and estragole is calculated as the ratio of the reference point ($BMDL_{10}$) to the intake. The MOET is calculated for each assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances.

When the estimated exposures to methyleugenol and estragole for the different animal categories are compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day (Suparmi et al., 2019) a MOET of at least 1,500,000 is calculated (Table 5). The magnitude of this MOET indicated that the presence of methyleugenol and estragole in clove tincture is very unlikely to be of safety concern for the target species.

3.4.3.4 | *Use in water for drinking*

The FEEDAP Panel considers that the use in water for drinking alone or in combination with use in feed should not exceed the daily amount that is considered very unlikely to be of safety concern when consumed via feed alone.

3.4.3.5 | *Conclusions on safety for the target species*

The use of clove tincture is very unlikely to be of safety concern for the target species up to the maximum proposed use levels of 50 mg clove tincture/kg complete feed for all animal species, except for horses, for which the proposed use level is 200 mg/kg complete feed.

The FEEDAP Panel considers that the use in water for drinking alone or in combination with use in feed should not exceed the daily amount that is considered very unlikely to be of safety concern when consumed via feed alone.

3.4.4 | Safety for the consumer

Cloves and their preparations including ethanolic extracts are added to a wide range of food categories as a spice or for flavouring purposes. Although individual consumption figures for the EU are not available, the Fenaroli's handbook of flavour ingredients (Burdock, 2009) cites values of 0.43 mg/kg bw per day for cloves and 0.045 mg/kg bw per day for clove bud oil.

No data on residues in products of animal origin were made available for any of the constituents of the tincture. When considering the ADME of the individual components, the data available for eugenol indicate that it is absorbed, metabolised and rapidly excreted, and is not expected to accumulate in animal tissues and product (EFSA FEEDAP Panel, 2011). Similarly, the phenolic compounds, including flavonoids, will be readily metabolised and excreted, and are not expected to accumulate in animal tissues and products. Methyleugenol and estragole, occurring in the additive at trace concentrations, are also not expected to accumulate in animal tissues and products (see Section 3.3.1).

Considering the above and the reported human exposure due to direct use of cloves and their preparations in food (Burdock, 2009), it is unlikely that consumption of products from animals given clove tincture at the proposed maximum use level would significantly increase human background exposure.

No safety concern would be expected for the consumer from the use of clove tincture up to the maximum proposed use level in feed.

3.4.5 | Safety for the user

No specific data were provided by the applicant regarding the safety of the additive for users.

The applicant provided information according to Classification, Labelling and Packaging Regulation (EC) 1272/2008[28] concerning the presence of ethanol in the tincture.[29]

The additive contains eugenol, a compound for which hazards for skin and eye contact and respiratory exposure were recognised (EFSA FEEDAP Panel, 2011).

The additive under assessment should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser.

When handling the essential oil, exposure of unprotected users to methyleugenol and estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised.

3.4.6 | Safety for the environment

Although *S. aromaticum* L. is not a native species to Europe, the most abundant components in the tincture, that is eugenol and polyphenols including flavonoids, are naturally occurring in European plants. Therefore, the use of the tincture under the proposed conditions of use in animal feed is not expected to pose a risk to the environment.

3.5 | Efficacy

Clove and its preparations are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009), by the Flavour and Extract Manufactures Association with the reference numbers 2327 (cloves), 2322 (clove bud extract) and 2323 (clove bud oil).

Since clove and its preparations are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary.

4 | CONCLUSIONS

Clove tincture from *Syzygium aromaticum* L. may be produced from plants of different origins and by various processes, resulting in preparations with different composition and toxicological profiles. Thus, the following conclusions apply only to clove tincture, which contains ≤ 2.25 mg/kg (0.00022%) methyleugenol and ≤ 0.92 mg/kg (0.00009%) estragole and is produced by ethanol/water extraction from the immature flower buds of *S. aromaticum* L.

The FEEDAP Panel concludes that the use of clove tincture is very unlikely to be of safety concern for the target species up to the maximum proposed use levels of 50 mg clove tincture/kg complete feed for all animal species, except for horses, for which the proposed use level is 200 mg/kg complete feed. The FEEDAP Panel considers that the use in water for drinking alone or in combination with use in feed should not exceed the daily amount that is considered very unlikely to be of safety concern when consumed via feed alone.

No safety concern would arise for the consumer from the use of clove tincture in animal nutrition up to the maximum proposed use levels in feed.

The additive under assessment should be considered as irritant to skin and eyes, and as a skin and respiratory sensitiser. When handling the additive, exposure of unprotected users to methyleugenol and estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised.

The use of clove tincture at the maximum proposed use level is not considered to be a risk to the environment.

Since the buds of *S. aromaticum* and their preparations are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary for the tincture under assessment.

5 | RECOMMENDATION

The specification should ensure that the methyleugenol and estragole concentrations should not exceed 2.25 and 0.92 mg/kg clove tincture, respectively, corresponding to 0.00022% and 0.00009%.

[28]Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC and amending Regulation (EC) No 1907/2006. *OJ L 353, 31.12.2008, p. 1–1355*.

[29]H319: causes serious eye irritation (relevant for dermal exposure).

6 | DOCUMENTATION PROVIDED TO EFSA/CHRONOLOGY

Date	Event
28/10/2010	Dossier received by EFSA. Botanically defined flavourings from Botanical Group 07 – Geraniales, Myrtales, Poales for all animal species and categories. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)
09/11/2010	Reception mandate from the European Commission
21/12/2010	Application validated by EFSA – Start of the scientific assessment
22/03/2011	Comments received from Member States
01/04/2011	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: analytical methods*
08/01/2013	Reception of supplementary information from the applicant
26/02/2013	EFSA informed the applicant (EFSA ref. 7,150,727) that, in view of the workload, the evaluation of applications on feed flavourings would be re-organised by giving priority to the assessment of the chemically defined feed flavourings, as agreed with the European Commission
20/01/2014	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
24/06/2015	Technical hearing during risk assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products": data requirement for the risk assessment of botanicals
27/02/2019	Partial withdrawal by applicant (EC was informed) for the following additives: broom teatree oil, geranium oil, bay oil and vetiveria oil
12/12/2019	EFSA informed the applicant that the evaluation process restarted
18/12/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization, safety for the target species, safety for the consumer, safety for the user, safety for the environment*
18/11/2022	Partial withdrawal by applicant (EC was informed) for the following additives: bambusa tincture and allspice oil
06/12/2022	Reception of supplementary information from the applicant (partial dataset on clove tincture) - Scientific assessment remains suspended
17/02/2023	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. Issues: characterization
11/05/2023	Reception of supplementary information from the applicant (partial dataset on clove tincture) - Scientific assessment remains suspended
06/06/2023	Reception of an amendment of the Evaluation report of the European Union Reference Laboratory for Feed Additives related to geranium rose oil, eucalyptus oil, lemongrass oil and clove oil
01/03/2024	Reception of an amendment of the Evaluation report of the European Union Reference Laboratory for Feed Additives related to citronella oil, melaleuca oil, tea tree oil, eucalyptus tincture, clove tincture
26/03/2024	The application was split and a new EFSA-Q-2024-00191 was assigned to the additive included in the present assessment. Scientific assessment re-started
17/04/2024	Opinion adopted by the FEEDAP Panel on clove tincture (EFSA-Q-2024-00191). End of the Scientific assessment for the additive included in the present assessment. The assessment of other additives in BGD 07 is still ongoing

ABBREVIATIONS

ADME	absorption, distribution, metabolism and excretion
AFC	EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
BDG	botanically defined group
BMD	Benchmark dose
$BMDL_{10}$	benchmark dose (BMD) lower confidence limit for a benchmark response of 10%
BW	body weight
DM	dry matter
EMA	European Medicines Agency
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GAE	gallic acid equivalent
GC–FID	gas chromatography–flame ionisation detection
HPTLC	high performance thin layer chromatography
MOE	margin of exposure
MOET	combined margin of exposure (total)
NOAEL	no observed adverse effect level
PhEur	European Pharmacopoeia
SC	EFSA Scientific Committee

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CONFLICT OF INTEREST
If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR
European Commission

QUESTION NUMBER
EFSA-Q-2010-01282 New EFSA-Q-2024-00191

PANEL MEMBERS
Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa and Ruud Woutersen.

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