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# Safety and efficacy of feed additives obtained from the fruit of *Pimpinella anisum* L.: anise oil for use in poultry and horses and anise tincture for use in poultry, dogs, cats and horses (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, 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, Ruud Woutersen, Paul Brantom, Andrew Chesson, Johannes Westendorf, Paola Manini, Fabiola Pizzo and Birgit Dusemund

## Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of anise oil and anise tincture obtained from the fruit of *Pimpinella anisum* L., when used as sensory additives. The use of the anise oil at the proposed use level in complete feed of 1.9 mg/kg for laying hens and 5 mg/kg for horses was considered of low concern. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) had no safety concern when anise oil is used at the proposed use levels of 1.5 mg/kg for chickens for fattening and at 1.7 mg/kg for turkeys for fattening. The use of anise tincture at the proposed conditions of use was considered of low concern in dogs, cats, horses and laying hens, and of no concern in chickens for fattening. The use of the additives up to the highest level in feed which was considered of low or no concern for target animals was also expected to be of no concern for consumers. The additives under assessment should be considered as irritants to skin and eyes, and as dermal and respiratory sensitisers. Due to the high concentration of estragole (≥ 1%), anise oil is classified as suspected of causing genetic defects and of causing cancer and should be handled accordingly. When handling anise tincture, exposure of unprotected users to estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised. The use of these additives in animal feed was not expected to pose a risk to the environment. Since the fruit of *P. anisum* and its preparations are recognised to flavour food and their function in feed would be the same, no further demonstration of efficacy was considered necessary.



**Keywords:** sensory additives, flavouring compounds, *Pimpinella anisum* L., anise oil, anise tincture, estragole, myristicin

**Requestor:** European Commission

**Question number:** EFSA-Q-2010-01286 (new EFSA-Q-2023-00180)

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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 seven years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)[2] for authorisation/re-evaluation of 29 preparations (namely dill herb oil, dill seed extract, dill tincture, dong quai tincture, celery seed oil, celery seed extract (oleoresin), celery tincture, hares ear tincture, caraway seed oil, caraway oleoresin/extract, coriander oil, cumin oil, taiga root extract (solvent-based, sb), taiga root tincture, fennel oil, fennel tincture, common ivy extract (sb), opoponax oil, ginseng tincture, parsley oil, parsley tincture, anise oil, anise tincture, ajowan oil, Ferula Assa-foetida oil, anise star oil, anise star tincture, anise star terpenes and omicha tincture) belonging to botanically defined group (BDG) 02 – *Apiales/Austrobaileyales* when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). During the assessment, the applicant withdrew the application for nine preparations (dill seed extract, celery seed extract (oleoresin), caraway oleoresin/extract, opoponax oil,[3] parsley oil, hares ear tincture, taiga root extract (sb), ajowan oil[4] and parsley tincture[5]). These preparations were deleted from the register of feed additives.[6] During the course of the assessment, this application was split and the present opinion covers two out of the 20 preparations under application: an essential oil and a tincture derived from the fruit (or seeds) of *Pimpinella anisum* L. (anise tincture) for all animal species. During the assessment, the applicant requested a change in the species limiting the application for authorisation to poultry and horses (anise oil)[7] and to poultry, dogs, cats and horses (anise tincture).[8] Another anise tincture for use in all animal species will be assessed in a separate opinion.

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 24 June 2019.

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 products anise oil and anise tincture (*P. anisum*), when used under the proposed conditions of use (see Sections 3.2.1.4 and 3.3.1.4).

The remaining 19 preparations belonging to botanically defined group (BDG) 02 – *Apiales/ Austrobaileyales* under application are assessed in separate opinions.

## 1.2. Additional information

Anise oil and anise tincture from *Pimpinella anisum* L. are currently authorised as feed additives according to the entry in the European Union Register of Feed Additives pursuant to Regulation (EC)

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, pp. 29.
[2] On 13/03/2013, EFSA was informed by the applicant that the applicant company changed to FEFANA asbl, Avenue Louise 130 A, Box 1, 1050 Brussels, Belgium.
[3] On 27 February 2019, EFSA was informed by the applicant about the withdrawal of the applications on dill seed extract, celery seed extract (oleoresin), caraway oleoresin/extract, and opoponax oil.
[4] On 2 April 2020, EFSA was informed by the applicant about the withdrawal of the applications on parsey oil, hares ear tincture, taiga root extract (sb), ajowan oil.
[5] On 9 December 2020, the applicant informed EFSA about the withdrawal of the application on celery tincture.
[6] Register of feed additives, Annex II, withdrawn by OJ L162, 10.05.2021, pp. 5.
[7] Technical dossier/Supplementary information February 2021/SIn_reply_anise_oil and Supplementary information February 2023/Sin reply_anise_oil_revision.
[8] Technical dossier/Supplementary information December 2022/SIn_reply_anise_tincture.

No 1831/2003 (2b natural products – botanically defined). They have not been assessed as feed additives in the EU.

There is no specific EU authorisation for any *P. anisum* L. preparation when used to provide flavour in food. However, according to Regulation (EC) No 1334/2008[9] flavouring preparations produced from food, may be used without an evaluation and approval as long as 'they do not, on the basis of the scientific evidence available, pose a safety risk to the health of the consumer, and their use does not mislead the consumer'.

'Aniseed (Anisi fructus)' is described in a monograph of the European Pharmacopoeia 10.0 (PhEur, 2020a). They are defined as the whole dry cremocarp of *Pimpinella anisum* L. with a minimum content of 20 ml/kg of essential oil in the anhydrous drug.

'Anise oil (Anisi aetheroleum)' is described in a monograph of the European Pharmacopoeia 10.0 (PhEur, 2020b). It is defined as the essential oil obtained by steam distillation from the dry ripe fruit of *Pimpinella anisum* L.

For *Pimpinella anisum* L., fructus and *P. anisum* L., aetheroleum the European Medicines Agency (EMA) issued individual monographs for human medicinal use (EMA, 2014a, 2014b) and a common assessment report (EMA, 2014c).

The World Health Organization (WHO) issued a monograph on 'Fructus anisi' (WHO, 2007), described as the dried fruits of *Pimpinella anisum* L. Estragole (0.5–6.0%) is mentioned among the major components of the essential oil.

Many of the individual components of the essential oil have been already assessed as chemically defined flavourings for use in feed and food by the FEEDAP Panel, the EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) and the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). The list of flavouring compounds currently authorised for feed[10] and/or food[11] use, together with the EU Flavour Information System (FLAVIS) number, the chemical group as defined in Commission Regulation (EC) No 1565/2000[12] and the corresponding EFSA opinion are listed in Table 1.

**Table 1:** Flavouring compounds already assessed by EFSA as chemically defined flavourings, grouped according to the chemical group (CG) as defined in Commission Regulation (EC) No 1565/2000, with indication of the EU Flavour Information System (FLAVIS) number and the corresponding EFSA opinion

| CG | Chemical Group | Product (EU register name) | FLAVIS No | EFSA opinion(*), Year |
|---|---|---|---|---|
| 01 | Straight-chain primary aliphatic alcohols/ aldehydes/acids, acetals and esters with esters containing saturated alcohols and acetals containing saturated aldehydes | Nonanal | 05.025 | 2013a |
| 02 | Branched-chain primary aliphatic alcohols/ aldehydes/acids, acetals and esters with esters containing branched-chain alcohols and acetals containing branched-chain aldehydes | 3-Methylbutanal | 05.006 | 2012a |
| | | 2-Methylbutyraldehyde | 05.049 | |
| 06 | Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols and esters with esters containing tertiary alcohols ethers | Linalool | 02.013 | 2012b |
| | | 2-(4-Methylphenyl)propan-2-ol | 02.042 | |

[9] 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, pp. 34.

[10] European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003. Available online: https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-eu-reg-comm_register_feed_additives_1831-03.pdf

[11] 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, pp. 1.

[12] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. OJ L 180, 19.7.2000, pp. 8.

| CG | Chemical Group | Product (EU register name) | FLAVIS No | EFSA opinion(*), Year |
|---|---|---|---|---|
| | | 4-Terpinenol | 02.072 | |
| | | (l)-α-Bisabolol | 02.129 | 2011a, CEF |
| 08 | Secondary alicyclic saturated and unsaturated alcohols, ketones, ketals and esters with ketals containing alicyclic alcohols or ketones and esters containing secondary alicyclic alcohols | Carvone[a],[b] | 07.012 | 2014, SC 2016a |
| | | d-Camphor[c] | 07.215 | 2016a |
| 18 | Allylhydroxybenzenes | 1-Methoxy-4-(prop-1(trans)-enyl)benzene (*trans*-anethole) | 04.010 | 2011 |
| 21 | Aromatic ketones, secondary alcohols and related esters | 4-Methoxyphenylacetone[a] (anisyl methyl ketone) | 07.087 | 2008, EFSA (AFC) |
| 23 | Benzyl alcohols, aldehydes, acids, esters and acetals | Anisyl alcohol | 02.018 | 2012c |
| | | 4-Methoxybenzaldehyde (anisaldehyde) | 05.015 | |
| | | 4-Isopropylbenzaldehyde | 05.022 | |
| | | Anisyl acetate | 09.019 | |
| 26 | Aromatic ethers including anisole derivatives | 1,2-Dimethoxy-4-(prop-1-enyl)benzene[d] (methyl isoeugenol) | 04.013 | 2012d |
| 31 | Aliphatic and aromatic hydrocarbons and acetals containing saturated aldehydes | 1-Isopropyl-4-methylbenzene (p-cymene) | 01.002 | 2015 |
| | | Terpinolene | 01.005 | |
| | | α-Phellandrene | 01.006 | |
| | | 1-Isopropenyl-4-methylbenzene | 01.010 | |
| | | γ-Terpinene | 01.020 | |
| | | d-Limonene | 01.045 | |
| | | Pin-2(10)-ene (β-pinene) | 01.003 | 2016b |
| | | Pin-2(3)-ene (α-pinene) | 01.004 | |
| | | β-Caryophyllene | 01.007 | |
| | | trans-β-Ocimene | 01.018 | |
| | | β-Bisabolene[a] | 01.028 | 2011b, CEF |
| | | 1,2-Dihydro-1,1,6-trimethylnaphthalene[a],[e] | 01.031 | |
| | | δ-Elemene[a] | 01.039 | |
| | | 3,7,10-Humulatriene[a],[e] | 01.043 | |
| | | 4(10)-Thujene (sabinene)[a] | 01.059 | 2015a, CEF |

(*): FEEDAP opinion unless otherwise indicated.
(a): Evaluated for use in food. According to Regulation (EC) 1565/2000, flavourings evaluated by JECFA before 2000 are not required to be re-evaluated by EFSA.
(b): The FEEDAP Panel evaluated d-carvone [07.146] and l-carvone [07.147] for use as feed flavourings (EFSA FEEDAP Panel, 2016a).
(c): EFSA evaluated d-camphor [07.215] for use in food (EFSA, 2008) and feed (EFSA FEEDAP Panel, 2016a).
(d): EFSA evaluated 1,2-dimethoxy-4-(prop-1-enyl)benzene [04.013] or methyl isoeugenol, a mixture of *(Z)*- and *(E)*-isomers (EFSA FEEDAP Panel, 2012d).
(e): Evaluated applying the 'Procedure' described in the Guidance on the data required for the risk assessment of flavourings to be used in or on food (EFSA CEF Panel, 2010). No longer authorised for use as flavours in food, as the additional toxicity data requested (EFSA CEF Panel, 2011b) were not submitted and the CEF Panel was unable to complete its assessment (EFSA CEF Panel, 2015a).

# 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[13] in support of the authorisation request for the use of anise oil and anise tincture from *P. anisum* L. as feed additives. The dossier was received on 3 February 2023 and the general information and supporting documentation is available at https://open.efsa.europa.eu/questions/EFSA-Q-2023-00180.[14]

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.

Many of the components of the essential oil 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 2, including the current one under assessment.[15]

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 animal feed. The evaluation report is related to the methods of analysis for each feed additive included the group BDG 02 (Apiales and Austrobaileyales). During the assessment, the EURL issued a partial report[16] and an addendum of the report.[17] In particular, for the characterisation of *anise oil*, the EURL recommended methods based on gas chromatography with flame ionisation detection (GC-FID) for the quantification of the phytochemical marker 1-methoxy-4-(prop-1(trans)-enyl)benzene (hereinafter referred as to *trans*-anethole) in *anise oil*. For anise tincture, the evaluation of the method of analysis is included in the partial report. In particular, for the characterisation of anise tincture, the EURL recommended methods based on spectrophotometry (for the determination of total polyphenols in the feed additive) and high-performance thin layer chromatography (HPTLC) (for the determination of the content of total flavonoids and of the phytochemical markers anethole and anisaldehyde in the feed additive).[18]

## 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of anise oil and anise tincture from *P. anisum* L. is in line with the principles laid down in Regulation (EC) No 429/2008[19] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA SC, 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, 2012e), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012f), 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 safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA SC, 2019a), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Statement on the genotoxicity assessment of chemical mixtures (EFSA SC, 2019b), Guidance on the use of the Threshold of Toxicological Concern approach in food safety

[13] FEED dossier reference: FAD-2010-0221.
[14] The original application EFSA-Q-2010-01286 was split on 3 February 2023 and a new EFSA-Q-2023-00180 was generated.
[15] Technical dossier/Supplementary information/Letter dated 29/04/2021.
[16] Preparations included in the partial report: dill herb oil, dill tincture, dong quai tincture, cumin oil, fennel tincture, parsley tincture, anise tincture, star anise tincture and ferula assa-foetida oil.
[17] Preparations included in the addendum: celery seed oil, caraway seed oil, coriander oil, taiga root tincture, fennel oil, common ivy extract (sb), ginseng tincture, anise oil, anise star oil, anise star terpenes and omicha tincture.
[18] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2010-0221_en
[19] 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, pp. 1.

assessment (EFSA SC, 2019), 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, 2021a).[20]

# 3. Assessment

The additives under assessment, anise oil and anise tincture, are derived from the dry fruit of *Pimpinella anisum* L.

Anise oil is intended for use as a sensory additive (functional group: flavouring compounds) in feed for poultry and horses.

Anise tincture is intended for use as a sensory additive (functional group: flavouring compounds) in feed for dogs, cats and horses and in feed and in water for drinking for poultry.

## 3.1. Origin and extraction

*Pimpinella anisum* L. (commonly referred to as aniseed or anise) is an annual herbaceous plant belonging to the Apiaceae family. It was first cultivated in the Middle East but was early introduced into Europe because of its medicinal properties. It is thought to no longer exist in the wild. Like other members of the family, its small yellow–white flowers form loose umbels which give rise to multiple small fruits (2–3 mm). The term 'anise' refers to the ground or whole dried fruit used as a spice for culinary purposes or to flavour alcoholic drinks. The term 'anise' is also used to describe the plant itself. The young leaves may also be consumed raw or cooked. *P. anisum* is botanically unrelated to 'star anise' which is obtained from the seed pods of another genus (*Illicium* spp.), although both share a similar flavour profile.

The essential oil is extracted from the dry fruit by steam distillation. The volatile constituents are condensed and then separated from the aqueous phase by decantation.

The tincture is produced from the dry fruit by extended extraction for 3 weeks under ambient conditions with a water/ethanol (55:45, v/v) solvent mixture and a plant to solvent ratio of 1:5 (w/v). The tincture is then recovered by pressing to separate solid and liquid phases and the extracted solution is then clarified by filtration.

## 3.2. Anise oil

### 3.2.1. Characterisation of anise oil

Anise oil is a colourless to pale yellow (or amber to yellowish green) clear mobile liquid, with a characteristic aroma. In five recent batches of the additive (all originating from Spain), the refractive index (20°C) ranged between 1.556 and 1.558 (average: 1.557), the density (20°C) between 983 and 987 kg/m$^3$ (average: 985 kg/m$^3$), the optical rotation (20°C) between 0° and 0.6° (three batches only).[21] Anise oil is identified with the single Chemical Abstracts Service (CAS) number 84775-42-8, the European Inventory of Existing Commercial Chemical Substances (EINECS) number 283-872-7, the Flavour Extract Manufacturers Association (FEMA) number 2094 and the Council of Europe (CoE) number 336.

For anise oil, the product specifications used by the applicant are based on those developed by the International Organisation for Standardization (ISO) 3475:2020 for essential oil of aniseed (*P. anisum* L.),[22] adapted to reflect the concentrations of the main volatile components of the essential oil (Table 2). Four components contribute to the specifications as shown in Table 2, with *trans*-anethole selected as the phytochemical marker. Analysis of five batches of the additive showed compliance with these specifications when analysed by GC-FID and expressed as percentage of gas chromatographic peak area (% GC area).[23] The applicant provided the full characterisation of the volatile constituents in five batches obtained by gas chromatography coupled with mass spectrometry (GC–MS).[24] The four compounds account for about 94.3% on average (range 92.1–96.3%) of % GC area (Table 2).[21]

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

[21] Technical dossier/Supplementary information February 2021/Annex_II_anise_oil_CoA_chrom.

[22] Technical dossier/Supplementary information February 2021/Annex_III_SIn_reply_anise_oil_ISO.

[23] Technical dossier/Supplementary information February 2021/Sin_reply_anise oil/GC-FID analysis: trans-anethole (90.3–94.7%), γ-himalachene (1.0–3.1%), pseudoisoeugenyl 2-methylbutyrate (0.3–1.0%) and estragole (0.5–1.0%).

[24] Technical dossier/Supplementary information February 2021/Annex_II_ anise_oil_CoA_chromatogram.

**Table 2:** Major constituents of the essential oil from the fruit of *Pimpinella anisum* L. as defined by specifications: batch to batch variation based on the analysis of five batches. The content of each constituent is expressed as the area per cent of the corresponding chromatographic peak (% GC area), assuming the sum of chromatographic areas of all detected peaks as 100%

| Constituent<br>EU register name | CAS No | FLAVIS No | % GC area | | |
|---|---|---|---|---|---|
| | | | Specification | Mean | Range[(a)] |
| *trans*-Anethole | 4180-23-8 | 04.010 | 85–96 | 90.0 | 85.0–93.9 |
| γ-Himachalene | 53111-25-4 | – | 0.5–5.0 | 2.29 | 1.28–4.28 |
| Pseudoisoeugenyl 2-methylbutyrate | 58989-20-1 | – | 0.1–2.0 | 1.24 | 0.53–1.79 |
| Estragole[(b)] | 140-67-0 | 04.011 | 0.1–3.0 | 0.82 | 0.52–1.08 |
| Total | | | | 94.3 | 92.1–96.3 |

EU: European Union; CAS No: Chemical Abstracts Service number; FLAVIS No: EU Flavour Information System numbers.
(a): The values given for the total (range) are the lowest and the highest values of the sum of the components in the five batches analysed.
(b): Substance which shall not be added as such to food (Annex III), maximum level in food is set by Regulation (EC) No 1334/2008, including dairy products (50 mg/kg), processed fruits, vegetables (incl. mushrooms, fungi, roots, tubers, pulses and legumes), nuts and seeds (50 mg/kg), fish products (50 mg/kg) and non-alcoholic beverages (10 mg/kg).

In total, up to 106 constituents were detected, 59 of which were identified and accounted on average for 99.3% (99.1–99.5%) of the GC area. Besides the four compounds indicated in the product specifications, 22 other compounds were detected at individual levels > 0.05% and are listed in Table 3. These 26 compounds > 0.05% together accounted on average for 98.7% (98.3–99.1%) of the GC area. The remaining 33 compounds (ranging between 0.002% and 0.05%) and accounting for 0.6% are listed in the footnote.[25] Based on the available data on the characterisation, anise oil is considered a fully defined mixture (EFSA SC, 2019a).

The FEEDAP Panel notes that the maximum level proposed for estragole in the specification (3%) exceeds the highest level measured in the five batches described above. According to the applicant, batches with higher concentrations of estragole (3%), methyleugenol (0.03%), myristicin (1.0%) and dillapiole (0.3%) could occasionally reach the market.

**Table 3:** Other constituents of the essential oil from the fruits of *Pimpinella anisum* L. accounting for > 0.05% of the composition (based on the analysis of five batches) not included in the specifications. The content of each constituent is expressed as the area per cent of the corresponding chromatographic peak (% GC area), assuming the sum of chromatographic areas of all detected peaks as 100%

| Constituent<br>EU register name | CAS No | FLAVIS No | % GC area | |
|---|---|---|---|---|
| | | | Mean | Range[(a)] |
| 4-Methoxybenzaldehyde | 123-11-5 | 05.015 | 0.86 | 0.37–1.44 |
| α-Curcumene | 644-30-4 | – | 0.44 | 0.27–0.59 |
| Myristicin | 607-91-0 | – | 0.34 | 0.16–0.63 |
| α-Zingiberene | 495-60-3 | – | 0.32 | 0.13–0.46 |
| β-Bisabolene | 495-61-4 | 01.028 | 0.32 | 0.19–0.48 |
| *(Z)*-Anethole | 25679-28-1 | – | 0.29 | 0.22–0.38 |
| Epoxyanethole | 51410-46-9 | – | 0.24 | 0.05–0.56 |
| α-Himachalene | 3853-83-6 | – | 0.22 | 0.08–0.41 |
| 4-Methoxyphenylacetone | 122-84-9 | 07.087 | 0.20 | 0.07–0.47 |

[25] Additional constituents: (33 components < 0.05% and > 0.002%): carvone, α-ylangene, γ-dehydro-ar-himachalene, pin-2(10)-ene (β-pinene), pin-2(3)-ene (α-pinene), terpinolene, α-calacorene, α-copaene, *(Z)*-methyl isoeugenol, 1,2-dihydro-1,1,6-trimethylnaphthalene, 3,7,10-humulatriene, spathulenol, geijerene, β-himachalene oxide, trans-para-1(7),5-menthadien-2-ol, himachalol, *(Z)*-α-bergamotene, *(Z)*-β-farnesene, camphor, (l)-α-bisabolol, 3-methylbutanal, 2-methylbutyraldehyde, methyleugenol, 2-(4-methylphenyl)propan-2-ol, γ-terpinene, 1-isopropenyl-4-methylbenzene, 4-terpinenol, 4-isopropylbenzaldehyde, β-ocimene, 4(10)-thujene (sabinene), nonanal, mint sulfide and α-thujene.

| Constituent<br>EU register name | CAS No | FLAVIS No | % GC area | |
|---|---|---|---|---|
| | | | Mean | Range[(a)] |
| β-Himachalene | 1461-03-6 | – | 0.16 | 0.09–0.33 |
| Epoxypseudoisoeugenyl 2-methylbutyrate | – | – | 0.14 | 0.01–0.26 |
| Dillapiole | 484-31-1 | – | 0.12 | 0.03–0.21 |
| *(E)*-Methyl isoeugenol | 6379-72-2 | – | 0.12 | 0.06–0.16 |
| Thymol | 89-83-8 | 04.006 | 0.08 | 0–0.18 |
| p-Cymene | 99-87-6 | 01.002 | 0.07 | 0.03–0.13 |
| β-Caryophyllene | 87-44-5 | 01.007 | 0.07 | 0.05–0.10 |
| *(E)*-α-bergamotene | 13474-59-4 | – | 0.07 | 0.06–0.08 |
| β-Elemene | 33880-83-0 | – | 0.06 | 0–0.11 |
| d-Limonene | 5989-27-5 | 01.045 | 0.06 | 0.01–0.10 |
| δ-Elemene | 20307-84-0 | 01.039 | 0.06 | 0.02–0.11 |
| Linalool | 78-70-6 | 02.013 | 0.06 | 0.02–0.11 |
| α-Phellandrene | 99-83-2 | 01.006 | 0.05 | 0.01–0.08 |
| Total | | | 4.35 | 2.82–6.36 |

EU: European Union; CAS No: Chemical Abstracts Service number; FLAVIS No: EU Flavour Information System numbers.
(a): The values given for the total (range) are the lowest and the highest values of the sum of the components in the five batches analysed.

#### 3.2.1.1. Substances of concern

The applicant performed a literature search regarding substances of concern and chemical composition of the plant species *P. anisum* and its preparations.[26] Among the compounds identified, furocoumarins in trace concentrations in fruit and estragole (methylchavicol, 1–5%) in the essential oil from the fruit are reported in the EFSA Compendium of botanicals as substances of concern (EFSA, 2012).[27] As furocoumarins are not volatile, they are not expected to occur in the essential oil. Several publications retrieved by the applicant reported the occurrence of estragole in essential oils from the fruit of *P. anisum* (e.g. Tisserand and Young, 2014; Abdel-Rehem and Oraby, 2015; Anastasopoulou et al., 2020; Boumahdi et al., 2000; Mohammed and Ebraheem, 2020). Two publications (Abdel-Rehem and Oraby, 2015; Anastasopoulou et al., 2020) also reported the presence of methyleugenol (range: 0.14–1.5%) in the same essential oils albeit in lower concentrations (at least twofold less) compared to estragole.

An analysis of the five batches of anise oil under assessment confirmed the presence of estragole in all batches (0.52–1.08%) and methyleugenol (0.002–0.015%). Although not reported in the literature, the presence of myristicin (0.164–0.627%) and dillapiole (0.032–0.207%) was detected in the oil under assessment.

#### 3.2.1.2. Impurities

The applicant referred to the 'periodic testing' of some representative flavourings premixtures for mercury, cadmium, lead, arsenic, fluoride, dioxins and polychlorinated biphenyls (PCBs), organo-chloride pesticides, organo-phosphorous pesticides, aflatoxins (B1, B2, G1, G2) and ochratoxin A. However, no data have been provided on the presence of these impurities. Since anise oil is produced by steam distillation, the likelihood of any measurable carry-over of all the above-mentioned elements is considered low, except for mercury.

#### 3.2.1.3. Shelf-life

The typical shelf-life of anise oil is stated to be at least 12 months, when stored in tightly closed containers under standard conditions (in a cool, dry place protected from light).[28] However, no data supporting this statement were provided.

[26] Technical dossier/Supplementary information February 2021/Literature search_anise_oil.
[27] Online version: https://www.efsa.europa.eu/en/data-report/compendium-botanicals
[28] Technical dossier/Section II.

#### 3.2.1.4. Conditions of use

Anise oil is intended to be added to feed for poultry and horses. The maximum proposed use level in complete feed is 1.5 mg/kg for chickens for fattening and game birds, 1.9 mg/kg for laying hens, 1.7 mg/kg for turkeys for fattening and 5 mg/kg for horses. The additive is not intended for use in water for drinking.

### 3.2.2. Safety

The assessment of safety of anise oil is based on the maximum use levels proposed by the applicant for the species listed above.

Many of the components of anise oil, accounting for about 88% of the % GC peak areas, have been previously assessed and considered safe for use as flavourings, and are currently authorised for use in food[11] without limitations and for use in feed[10] at individual use levels higher than those resulting from the intended use of the essential oil in feed. The list of the compounds already evaluated by the EFSA Panels is given in Table 1 (see Section 1.2). The FEEDAP Panel notes that for the major component of anise oil, *trans*-anethole [04.010], the lack of data on metabolism and residues in poultry precluded an assessment of consumer exposure from this source (EFSA FEEDAP Panel, 2011).

Two compounds listed in Table 1, 1,2-dihydro-1,1,6-trimethylnaphthalene [01.031] and 3,7,10-humulatriene [01.043], were evaluated in FGE25.Rev2 (EFSA CEF Panel, 2011b) by applying the procedure described in the Guidance on the data required for the risk assessment of flavourings to be used in or on food (EFSA CEF Panel, 2010). For these compounds, for which there is no concern for genotoxicity, EFSA requested additional toxicity data (EFSA CEF Panel, 2011b). In the absence of such toxicological data, the EFSA CEF Panel was unable to complete its assessment (EFSA CEF Panel, 2015a). As a result, these compounds are not authorised for use as flavours in food. For these compounds, in the absence of toxicity data, the FEEDAP Panel applies the threshold of toxicological concern (TTC) approach or read-across from structurally related substances, as recommended in the Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA SC, 2019a).

γ-Himachalene (2.3%), pseudoisoeugenyl 2-methylbutyrate (1.24%) and 25 additional compounds, each accounting for < 0.5% of the % GC area, have not been previously assessed for use as flavourings. The FEEDAP Panel notes that 15 of them[29] are aliphatic mono- or sesquiterpenes structurally related to flavourings already assessed in CG 31 and a similar metabolic and toxicological profile is expected. These lipophilic compounds are expected to be rapidly absorbed from the gastro-intestinal tract, oxidised to polar oxygenated metabolites, conjugated and excreted (EFSA FEEDAP Panel, 2015, 2016b). Four additional components (camphor, *cis*-anethole, *(Z)*- and *(E)*-methyl isoeugenol) are structurally related to compounds that have been evaluated for use in food and/or feed and one component (epoxyanethole) is a metabolite of the major compound *trans*-anethole.

The following sections focus on the *p*-allylalkoxybenzenes estragole, methyleugenol, myristicin and dillapiole and on the other seven compounds[30] not previously assessed or not structurally related to flavourings previously assessed, based on the evidence provided by the applicant in the form of literature searches and quantitative structure–activity relationship (QSAR) analysis. For the absorption, distribution, metabolism and excretion (ADME) and the toxicology of methyleugenol, reference is made to the safety evaluation made by the FEEDAP Panel in the EFSA opinion on buchu leaf oil (EFSA FEEDAP Panel, 2022a). An update of the ADME of *trans*-anethole in poultry is also presented in the next section.

#### 3.2.2.1. Absorption, distribution, metabolism and excretion

*Estragole, methyleugenol, myristicin and dillapiole*

Estragole is a lipophilic compound and, as such, readily and completely absorbed from the gastrointestinal tract in laboratory animals. Phase I metabolism is catalysed by cytochrome P450 (CYP450) enzymes mainly in the liver. Demethylation of the 4-methoxygroup with formation of 4-allylphenol is followed by conjugation with glucuronic acid or sulfate and renal excretion. Oxidation of the double bond

[29] *(Z)*-β-Farnesene, geijerene, β-elemene, α-zingiberene, α-chalacorene, γ-dehydro-ar-himachalene, α-curcumene, α-thujene, *(Z)*-α-bergamotene, α-copaene, α-yanglene, *(E)*-α-bergamotene, β-himachalene, α-himachalene and γ-himachalene (CG 31).

[30] Spathulenol and himachalol (CG 6); trans-para-1(7),5-menthadien-2-ol (CG 8); trans-pseudoisoeugenyl 2-methylbutyrate (CG 17); mint sulfide (CG 20); β-himachalene oxide, and epoxypseudoisoeugenyl 2-methylbutyrate (CG 32).

of the allyl-side chain leads to estragole-2′,3′-epoxide, which is hydrolysed to the corresponding diol with subsequent glucuronidation and excretion. Both metabolic pathways result in the detoxification of estragole. The formation of genotoxic metabolites is initiated by oxidation of the side chain with formation of 1′-hydroxy-estragole. Sulfate-conjugation of the hydroxyl group leads to 1′-sulfooxyestragole, which is unstable and breaks down to form a highly reactive carbonium ion, which can react covalently with DNA (as reviewed in EMA, 2021).

The metabolism of estragole was evaluated in experimental animals with special focus on the formation of its proximate metabolite, 1′-hydroxyestragole, and the influence of the dose administered on the quantity excreted in urine (Zangouras et al., 1981; Anthony et al., 1987, as referenced in EMA, 2021). When $^{14}$C-estragole (4-[$^{14}$C-methoxyl]-allylbenzene) was given in low doses to rodents, the radioactivity was mainly excreted as $^{14}CO_2$ in exhaled air as a result of demethylation and only a minor portion in urine in the form of several metabolites resulting from hydroxylation in 1′-C and epoxidation at 2′,3′-C followed by ring hydrolysis. In a single study performed in two volunteers orally given 100 μg of methoxy-$^{14}$C-estragole, 1′-hydroxyestragole quantified in urine of both individuals was 0.2% and 0.4% of the dose; the majority of the radioactivity was excreted in expired air as $^{14}CO_2$ in the first 8 h (Sangster et al., 1987, as referenced in EMA, 2021). Metabolites identified in urine indicate that estragole follows a similar biotransformation profile in rats, mice and humans. There are no studies in human volunteers with high doses of estragole, but in rats and in mice, it is consistently shown that as doses increase the urinary levels of 1′-estragole as glucuronide significantly increase (Zangouras et al., 1981; Anthony et al., 1987, as referenced in EMA, 2021).

Similar metabolic pathways as for estragole have been described for methyleugenol (EFSA FEEDAP Panel, 2022a) and the other *p*-allylalkoxybenzenes including myristicin (as reviewed in WHO, 2009).

No data on the ADME of dillapiole are available. However, considering the structural similarity with the other p-allylalkoxybenzenes, a similar ADME is expected for dillapiole, including the formation of the 1′-sulfoxymetabolite.

Apiole, elemicin and dillapiole have more functional groups (*methoxy and methylendioxy substituents*) at the aromatic ring as compared to estragole, methyleugenol and safrole. The higher substitution increases the sterically hindrance and the likelihood that demethylation of the methoxy group(s) followed by conjugation would occur, modulating the relative formation of the 1′-sulfoxymetabolite. The application of physiologically based kinetic (PBK) models predicted that in rat liver the formation of the 1′-sulfoxymetabolite is about three times lower for highly ring-substituted substances such as apiole than for safrole (Alajlouni et al., 2016). Similarly, for elemicin, the formation of the DNA reactive 1′-sulfoxymetabolite was predicted to be 11- and 2-fold lower compared to the formation of the 1′-sulfoxymetabolites of estragole and methyleugenol, respectively (van den Berg et al., 2012). Based on considerations on the structure of dillapiole, a similar reduced formation of 1′-hydroxydillapiole is expected.

*trans-Anethole in poultry*

In laboratory animals, *trans*-anethole is rapidly absorbed, metabolised and excreted, the main route of excretion (> 90%) being the urine. *trans*-Anethole is mainly metabolised by three primary oxidation pathways: *O*-demethylation, omega side-chain oxidation and epoxidation of the side chain, followed by subsequent oxidation and hydration. The resulting products are extensively conjugated with sulfate, glucuronic acid, glycine and glutathione (WHO, 2000a,b). In its former assessment on CG 18, the Panel noted that the metabolic pathways involved in the biotransformation of *trans*-anethole are common to mammalian species, but no data were available concerning its metabolic fate in poultry. Therefore, the FEEDAP Panel concluded that the efficient metabolism of *trans*-anethole in mammals and the subsequent rapid excretion of the metabolites preclude their accumulation in tissues and transfer to products. However, the lack of data on metabolism and residues in poultry precluded an assessment of consumer exposure from this source (EFSA FEEDAP Panel, 2011). Subsequently, the FEEDAP Panel reviewed the literature available on the enzymes involved in the biotransformation of several classes of compounds and found that both Phase I (CYP450 monooxygenase families, epoxide hydrolases) and Phase II enzymes (glucuronide- sulfate- and glutathione-transferases) are also expressed in birds (EFSA FEEDAP Panel, 2013b, 2016b,c). Therefore, birds can also be assumed to have the ability to metabolise and excrete *trans*-anethole as mammals and there is no evidence that they or their metabolites would accumulate in tissues and cause a concern for consumer safety.

### 3.2.2.2. Genotoxicity and carcinogenicity

For fully defined mixtures, the EFSA Scientific Committee (EFSA SC) recommends applying a component-based approach, i.e. assessing all components individually for their genotoxic potential using all available information, including read-across and QSAR considerations about their genotoxic potential (EFSA SC, 2019b). Therefore, the potential genotoxicity of identified constituents is first considered. Then, *in vitro* genotoxicity studies performed with anise oils similar to the additive under assessment are described.

The genotoxic potential for seven substances (spathulenol, himachalol, trans-para-1(7),5-menthadien-2-ol, mint sulfide, trans-pseudoisoeugenyl 2-methylbutyrate, β-himachalene oxide and epoxypseudoisoeugenyl 2-methylbutyrate) was predicted by the applicant using the Organization for Economic Co-operation and Development (OECD) QSAR Toolbox. No alerts were identified for *in vitro* mutagenicity (Ames test), for genotoxic and non-genotoxic carcinogenicity and for other endpoints for spathulenol, himachalol and mint sulfide. Structural alerts for mutagenicity for trans-para-1(7),5-menthadien-2-ol were due to the presence of the vinyl/allyl alcohol group, for trans-pseudoisoeugenyl 2-methylbutyrate were due to the presence of esters and acylations, for β-himachalene oxide to the presence of epoxides and aziridines and for epoxypseudoisoeugenyl 2-methylbutyrate were due to the presence of acetates, epoxides and aziridines. For these substances, predictions of Ames mutagenicity (with and without S9) were made by 'read-across' analyses of data available for similar substances to the target compounds (i.e. analogues obtained by categorisation). Read-across-based predictions were found consistently negative for all categories of analogues. On this basis, the alerts raised were discounted.[31]

*Estragole, methyleugenol, myristicin and dillapiole*

Anise oil contains estragole (up to 1.08%) and trace amounts of methyleugenol, two compounds with experimentally proven genotoxicity and carcinogenicity in rodents (as reviewed in EC, 2001; EMA, 2021; IARC, 2018). Anise oil also contains myristicin and dillapiole, two compounds which belong to the class of *p*-allylalkoxybenzenes. They are structurally related to compounds with experimentally proven genotoxicity and carcinogenicity in rodents like safrole, estragole and methyleugenol.

The carcinogenicity of estragole, methyleugenol and other structurally related *p*-allylalkoxybenzenes (including myristicin and dillapiole) has been reviewed by the FEEDAP Panel in the opinion on olibanum extract (EFSA FEEDAP Panel, 2022b).

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 body weight (bw) per day (Suparmi et al., 2019). This $BMDL_{10}$ value was selected as reference point for the assessment group of *p*-allylalkoxybenzenes irrespective of their relative potency (EFSA FEEDAP Panel, 2022b).

*Genotoxicity studies with anise oils*

The applicant provided a literature search on the genotoxicity of preparations obtained from the fruit of *P. anisum*. Anise oil was not mutagenic in bacteria when tested in several *Salmonella* Typhimurium tester strains (Sivaswamy et al., 1991; NTP, 2018[32]). However, in all the studies submitted, the composition of the test item was unknown. These studies were not considered relevant for the current assessment.

### 3.2.2.3. Subchronic toxicity studies

The FEEDAP Panel identified a no observed adverse effect level (NOAEL) of 10 mg/kg bw per day for non-neoplastic lesions (effect on liver and the glandular stomach) from a 90-day study in mice with methyleugenol (NTP, 2000). Considering the structural similarity and the similar mode of action of *p*-allylalkoxybenzenes, the FEEDAP Panel selected the NOAEL of 10 mg/kg bw per day as reference point for the assessment group *p*-allylalkoxybenzenes for non-neoplastic endpoints (EFSA FEEDAP Panel, 2023).

[31] Technical dossier/Supplementary information February 2021/Annex_VI_Sin_reply anise_oil_QSAR.

[32] Technical dossier/Supplementary information/January 2023/Additional references. NTP (National Toxicology Program), 2018. G06: Ames Summary data. Test compound anise oil CAS Number 8007-70-3. NTP Study Number 502259.

### 3.2.2.4. Safety for the target species

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

In the absence of these data, the approach to the safety assessment of a mixture whose individual components are known is based on the safety assessment of each individual component (component-based approach). This approach requires that the mixture is sufficiently characterised and that the individual components can be grouped into assessment groups, based on structural and metabolic similarity. The combined toxicity can be predicted using the dose addition assumption within an assessment group, taking into account the relative toxic potency of each component.

As the additive under assessment is a fully defined mixture (the identified components represent > 99% of the % GC area, see Section 3.2.1), the FEEDAP Panel applied a component-based approach to assess the safety for target species of the essential oil. Substances for which a concern for genotoxicity has been identified (estragole, methyleugenol, myristicin and diallpiole) are assessed separately.

*Components other than estragole, methyleugenol myristicin and dillapiole*

Based on considerations related to structural and metabolic similarities, the components were allocated to nine assessment groups, corresponding to the chemical groups (CGs) 1, 2, 6, 8, 16, 18, 21, 23, 25, 26 and 31, as defined in Annex I of Regulation (EC) No 1565/2000. For CG 31 ('aliphatic and aromatic hydrocarbons'), subassessment groups as defined in Flavouring Group Evaluation 25 (FGE.25) and FGE.78 were established (EFSA CEF Panel, 2015a,b). The allocation of the components to the (sub-)assessment groups is shown in Table 4 and in the corresponding footnote.

For each component in the assessment group, exposure of target animals was estimated considering the use levels in feed, the percentage of the component in the oil and the default values for feed intake according to the guidance on the safety of feed additives for target species (EFSA FEEDAP Panel, 2017b). Default values on body weight (bw) are used to express exposure in terms of mg/kg bw per day. The intake levels of the individual components calculated for chickens for fattening, the species with the highest ratio of feed intake/body weight per day, are shown in Table 4.

For hazard characterisation, each component of an assessment group was first assigned to the structural class according to Cramer classification (Cramer et al., 1978). For some components in the assessment group, toxicological data were available to derive NOAEL values. Structural and metabolic similarity among the components in the assessment groups was assessed to explore the application of read-across allowing extrapolation from a known NOAEL of a component of an assessment group to the other components of the group with no available NOAEL or, if sufficient evidence were available for members of a (sub-)assessment group, to derive a (sub-)assessment group NOAEL.

Toxicological data of subchronic studies, from which NOAEL values could be derived, were available for acetaldehyde [05.001] the representative compound in CG 1 (EFSA FEEDAP Panel, 2013a), 2-ethylhexan-1-ol [02.082] the representative compound in CG 2 (EFSA FEEDAP Panel, 2012a), linalool [02.013] and terpineol[33] [02.230] in CG 6 (EFSA FEEDAP Panel, 2012b), carvone in CG 8 (EFSA SC, 2014), *trans*-anethole [04.010] in CG 18 (EFSA FEEDAP Panel, 2011), 4-(4-hydroxyphenyl)butan-2-one [07.055] in CG 21 (EFSA FEEDAP Panel, 2016c), anisaldehyde [05.015] in CG 23 (EFSA FEEDAP Panel, 2012c), methyl isoeugenol [04.013] in CG 26 (EFSA FEEDAP Panel, 2012d), myrcene [01.008], d-limonene [01.045], p-cymene [01.002] and β-caryophyllene [01.007] in CG 31 (EFSA FEEDAP Panel, 2015, 2016b).

The NOAEL of 120 mg/kg bw per day for acetaldehyde [05.001] was selected as reference point for CG 1 compounds and the NOAEL of 50 mg/kg bw per day for 2-ethylhexan-1-ol [02.082] was used as a group NOAEL for all compounds belonging to CG 2.

Considering the structural and metabolic similarities, for the subgroup of terpinyl derivatives in CG 6, i.e. α-terpineol [02.014], 4-terpinenol [02.072], α-terpinyl acetate [09.015] and other terpinyl derivatives, the reference point was selected based on the NOAEL of 250 mg/kg bw per day available for terpineol [02.230] and d-limonene [01.045].

[33] Terpineol is a mixture of four isomers: α-terpineol [02.014], a mixture of *(R)*-(+)-α-terpineol and *(S)*-(−)-α-terpineol, β-terpineol, γ-terpineol and 4-terpinenol [02.072] (or δ-terpineol). The specification for terpineol [02.230] covers α-, β-, γ and δ-terpineol. Composition of mixture: 55–75% α-terpineol, 16–23% γ-terpineol, 1–10% cis-β-terpineol, 1–13% trans-β-terpineol and 0–1% δ-terpineol (EFSA CEF Panel, 2015c) FGE.18Rev 3.

The NOAEL of 300 mg/kg bw per day for *trans*-anethole [04.010] was applied to *cis*-anethole in CG 18 and the NOAEL 100 mg/kg bw per day for methyl isoeugenol [04.013] was applied to *(Z)*-methyl isoeugenol in CG 26.

Similarly, the NOAELs of 44, 250, 154 and 222 mg/kg bw per day for the representative compounds of CG 31, myrcene [01.008], d-limonene [01.045], p-cymene [01.002] and β-caryophyllene [01.007] were applied, respectively, using read-across to the compounds within subassessment group II (β-ocimene and *(Z)*-β-farnesene), group III (β-bisabolene, α-phellandrene, terpinolene and γ-terpinene), group IVe (α-curcumene) and group V (α-thujene, sabinene, α-copaene, α-pinene and β-pinene)[34] (EFSA CEF Panel, 2015a,b). The NOAEL of 155 mg/kg bw per day for α-zingiberene was applied to α-curcumene in CG 31, VIe (EFSA FEEDAP Panel, 2020).

For the remaining compounds,[35] toxicity studies were not available and read-across was not possible. Therefore, the TTC approach was applied (EFSA FEEDAP Panel, 2017b).

As the result of the hazard characterisation, a reference point was identified for each component in the assessment group based on the toxicity data available (NOAEL from *in vivo* toxicity study or read across) or from the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class (i.e. 3, 0.91 and 0.15 mg/kg bw per day, respectively, for Cramer Class I, II and III compounds, Munro et al., 1996). Reference points selected for each compound are shown in Table 4.

For risk characterisation, the margin of exposure (MOE) was calculated for each component as the ratio between the reference point and the exposure. For each assessment group, the combined (total) margin of exposure (MOET) was calculated as the reciprocal of the sum of the reciprocals of the MOE of the individual substances (EFSA SC, 2019a). A MOET > 100 allowed for interspecies and intra-individual variability (as in the default 10 × 10 uncertainty factor). The compounds resulting individually in an MOE > 50,000 were not further considered in the assessment group as their contribution to the MOE(T) is negligible. They are listed in the footnote.[36]

The approach to the safety assessment of anise oil for the target species is summarised in Table 4. The calculations were done for chickens for fattening, the species with the highest ratio of feed intake/ body weight and represent the worst-case scenario at the use level of 1.5 mg/kg.

**Table 4:** Compositional data, intake values, reference points and margin of exposure (MOE) for the individual components of anise oil classified according to assessment groups

| **Essential oil composition** | | | **Exposure** | | **Hazard characterisation** | | **Risk characterisation** | |
|---|---|---|---|---|---|---|---|---|
| **Assessment group** | **FLAVIS-No** | **Highest conc. in the oil** | **Highest feed conc.** | **Daily intake[a]** | **Cramer class[b]** | **NOAEL[c]** | **MOE** | **MOET** |
| Constituent | – | % | mg/kg | mg/kg bw/day | – | mg/kg bw/day | – | – |
| **CG 6** | | | | | | | | |
| Himachalol | – | 0.026 | 0.0007 | 0.00001 | III | *0.15* | 4,284 | |
| Spathulenol | – | 0.049 | 0.0004 | 0.00001 | I | *3* | 45,466 | |
| MOET CG 6 | | | | | | | | 3,915 |
| **CG 8** | | | | | | | | |
| Camphor | – | 0.032 | 0.0005 | 0.00004 | II | *0.91* | 21,118 | |

[34] Some of these compounds are not listed in Table 5 because their individual margin of exposure (MOE) was > 50,000.

[35] Spathulenol, himachalol, 2-(4-methylphenyl)propan-2-ol, camphor, *trans*-para-1(7),5-menthadien-2-ol, trans-pseudoisoeugenyl 2-methylbutyrate, mint sulfide, δ-elemene, β-elemene, geijerene, 1,2-dihydro-1,1,6-trimethylnaphthalene, α-chalacorene, γ-dehydro-Ar-himachalene, (Z)-α-bergamotene, α-yanglene, *(E)*-α-bergamotene, γ-himachalene, α-himachalene, β-himachalene, 3,7,10-humulatriene, epoxyanethole, epoxypseudoisoeugenyl 2-methylbutyrate and β-himachalene oxide.

[36] Compounds included in the assessment groups but not reported in the table: nonanal (CG 1); 2-methylbutyraldehyde and 3-methylbutanal (CG 2); linalool, (l)-α-bisabolol, 2-(4-methyulphenyl)propan-2-ol and 4-terpinenol (CG 6); *trans*-para-1(7),5-menthadien-2-ol and carvone (CG 8); (Z)-anethole (CG 18); isopropylbenzaldehyde (CG 23); thymol (CG 25); *(Z)*-methyl isoeugenol and *(E)*-methyl isoeugenol (CG 26); β-ocimene and (Z)-β-farnesene (CG 31, II); β-bisabolene, d-limonene, α-phellandrene, terpinolene, geijerene and γ-terpinene (CG 31, III); 1-isopropenyl-4-methylbenzene, 1-isopropyl-4-methylbenzene and α-curcumene, (CG 31, IVe); α-thujene, sabinene, *(Z)*-α-bergamotene, α-copaene,α-pinene, β-pinene and β-caryophyllene (CG 31, V); 3,7,10-humulatriene (CG 31, VI).

| Essential oil composition | | | Exposure | | Hazard characterisation | | Risk characterisation | |
|---|---|---|---|---|---|---|---|---|
| **Assessment group** | **FLAVIS-No** | **Highest conc. in the oil** | **Highest feed conc.** | **Daily intake[a]** | **Cramer class[b]** | **NOAEL[c]** | **MOE** | **MOET** |
| **CG 17** | | | | | | | | |
| trans-Pseudoisoeugenyl 2-methylbutyrate | – | 1.79 | 0.0269 | 0.00241 | I | *3* | 1,245 | |
| **CG 18** | | | | | | | | |
| *trans*-Anethole | 04.010 | 93.9 | 1.409 | 0.1264 | (I) | **300** | 2,373 | |
| **CG 21** | | | | | | | | |
| 4-Methoxyphenylacetone | 07.087 | 0.47 | 0.007 | 0.0006 | I | *3* | 4,710 | |
| **CG 23** | | | | | | | | |
| Anisaldehyde | 05.015 | 1.44 | 0.022 | 0.0019 | (I) | **20** | 10,314 | |
| **CG 30** | | | | | | | | |
| Mint sulfide | – | 0.006 | 0.0001 | 0.00001 | III | *0.15* | 18,565 | |
| **CG 31, III** | | | | | | | | |
| δ-Elemene | 01.039 | 0.114 | 0.0017 | 0.00015 | I | *3* | 19,543 | |
| β-Elemene | – | 0.107 | 0.0016 | 0.00014 | I | *3* | 20,821 | |
| MOET CG 31, III | | | | | | | | 10,081 |
| **CG 31, IVe** | | | | | | | | |
| γ-Dehydro-Ar-himachalene | – | 0.080 | 0.0012 | 0.00011 | I | *3* | 27,848 | |
| α-Calacorene | – | 0.057 | 0.0009 | 0.00008 | I | *3* | 39,085 | |
| 1,2-Dihydro-1,1,6-trimethylnaphthalene | – | 0.038 | 0.0006 | 0.00005 | II | *0.91* | 17,588 | |
| MOET CG 31, IVe | | | | | | | | 8,449 |
| **CG 31, V** (Bi-, tricyclic, non-aromatic hydrocarbons) | | | | | | | | |
| γ-Himachalene | – | 4.280 | 0.064 | 0.0058 | I | *3* | 521 | |
| α-Himachalene | – | 0.412 | 0.006 | 0.0006 | I | *3* | 5,407 | |
| β-Himachalene | – | 0.329 | 0.005 | 0.0004 | I | *3* | 6,772 | |
| α-Ylanglene | – | 0.076 | 0.001 | 0.0001 | I | *3* | 29,314 | |
| (E)-α-Bergamotene | – | 0.076 | 0.001 | 0.0001 | I | *3* | 29,314 | |
| CG 31, V | | | | | | | | 431 |
| **CG 32** | | | | | | | | |
| Epoxyanethole | – | 0.24 | 0.008 | 0.0008 | I | *3* | 4,000 | |
| Epoxypseudoisoeugenyl 2-methylbutyrate | – | 0.14 | 0.004 | 0.0003 | III | *0.15* | 432 | |
| β-Himachalene oxide | – | 0.02 | 0.001 | 0.0001 | III | *0.15* | 2,931 | |
| | | | | | | | | 344 |

(a): Intake calculations for the individual components are based on the use level of 1.5 mg/kg in feed for chickens for fattening, the species with the highest ratio of feed intake/body weight. The MOE for each component is calculated as the ratio of the reference point (no observed adverse effect level, NOAEL) to the intake. The combined margin of exposure (MOET) is calculated for each assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances.

(b): When a NOAEL value is available or read-across is applied, the allocation to the Cramer class is put into parentheses.

(c): Values **in bold** refer to those components for which the NOAEL value was available, values *in italics* are the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class, other values (plain text) are NOAELs extrapolated by using read-across.

As shown in Table 4, for all the assessment groups, the MOET was higher than 100 (≥ 344). Therefore, no safety concern was identified for the anise oil when used as a feed additive for chickens for fattening at the proposed use level (1.5 mg/kg) without considering the presence of estragole, methyleugenol, myristicin dillapiole.

From the lowest MOET of 344 for chickens for fattening, the MOET for the assessment group 'epoxides' (CG 32) was calculated for the other target species considering the respective daily feed intake and conditions of use. The results are summarised in Table 5.

**Table 5:** Combined margin of exposure (MOET) for the assessment group 'epoxides' (CG 32) calculated for the other target species at the proposed use level

| Animal category | Body weight (kg) | Daily feed intake (g DM/kg bw) | Feed intake (g DM/day) | Use level (mg/kg feed) | Lowest MOET |
|---|---|---|---|---|---|
| Chicken for fattening | 2 | 79 | 158 | 1.5 | 344 |
| Laying hen | 2 | 53 | 106 | 1.9 | 405 |
| Turkey for fattening | 3 | 59 | 176 | 1.7 | 406 |
| Horse | 400 | 20 | 8,000 | 5 | 408 |

Table 5 shows that when the additive was used at the proposed use levels in complete feed, the MOET is above the value of 100 for all species. Therefore, for all species, no safety concern (without considering the presence of estragole, myristicin, dillapiole and elemicin methyleugenol) is identified for anise oil, when used as a feed additive at the proposed use levels.

*p-Allylalkoxybenzenes: Estragole, myristicin, dillapiole and methyleugenol*

Estragole, methyleugenol, myristicin and dillapiole belong to the same structural group (*p*-allylalkoxybenzenes) and share the same metabolic pathways, particularly the formation of the reactive 1′-sulfoxymetabolite (see Section 3.2.2.1) and the same mode of action. They are allocated to the same assessment group (*p*-allylalkoxybenzenes) and an assessment of the combined exposure is performed as described in the Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA SC, 2019a). According to the 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, 2021a), different reference points and a different magnitude of the MOET are applied for long-living and reproductive animals (including those animals reared for laying/breeding/reproduction) and for short-living animals. Short-living animals are defined as those animals raised for fattening whose lifespan under farming conditions makes it very unlikely that they develop cancer as a result of the exposure to genotoxic and/or carcinogenic substances in the diet.

For long-living and reproductive animals, a MOE(T) with a magnitude > 10,000, when comparing the estimated exposure to genotoxic and/or carcinogenic substances with a $BMDL_{10}$ from a rodent carcinogenicity study, is considered 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, 2022b). In the current assessment, this reference point is applied to assess the combined exposure of long-living and reproductive animals (laying hens and horses) to estragole, methyleugenol, myristicin and dillapiole.

For short-living animals, genotoxicity and carcinogenicity endpoints are not considered relevant; therefore, a lower magnitude of the MOET (> 100) when comparing estimated exposure with a reference point based on non-neoplastic endpoints is considered adequate (EFSA FEEDAP Panel, 2021b). The FEEDAP Panel identified a NOAEL of 10 mg/kg bw per day for non-neoplastic lesions (effect on liver and the glandular stomach) from a 90-day study in mice with methyleugenol (NTP, 2000). In the current assessment, this reference point is applied to assess the combined exposure of short-living animals (chickens for fattening and turkeys for fattening) to estragole, methyleugenol, myristicin and dillapiole.

Estragole (0.52–1.08%), myristicin (0.164–0.627%), dillapiole (0.032–0.207%) and methyleugenol (0.002–0.015%) were detected in all batches of the oil under assessment (see Section 3.2.1). The FEEDAP Panel notes that anise oils with higher concentrations of estragole and other *p*-allylalkoxybenzenes may occasionally reach the market. In particular, the applicant set a specification for estragole up to 3%. For other *p*-allylalkoxybenzenes, the applicant provided maximum expected concentrations for myristicin (1.0%), dillapiole (0.3%) and methyleugenol (0.03%).

For the assessment of *p*-allylalkoxybenzenes for the target species, the FEEDAP Panel identified two possible scenarios.

a) An essential oil with a content of estragole corresponding to the highest specification of 3%, and other *p*-allylalkoxybenzenes present at the maximum expected concentration (1.0% myristicin, 0.3% dillapiole and 0.03% methyleugenol).

The highest daily intake of estragole and the highest combined intake of *p*-allylalkoxybenzenes (estragole, myristicin, dillapiole and methyleugenol) were calculated considering the maximum proposed use level of the additive in feed for the different animal categories and the maximum expected concentration in the additive (3.0%, 1.0%, 0.3% and 0.03% for estragole, myristicin, dillapiole and methyleugenol, respectively). The intake values are reported in Table 6, together with the corresponding MOET for the combined intake calculated considering the relevant reference point for long-living and reproductive animals and for short-living animals (target species for fattening).

**Table 6:** Target animal intake of estragole and total *p*-allylalkoxybenzenes and combined margin of exposure (MOET) calculated at the maximum proposed use level of the additive in feed for an essential oil with a content of estragole of 3%, and other *p*-allylalkoxybenzenes present at the maximum expected concentration (1.0% myristicin, 0.3% dillapiole and 0.03% methyleugenol)

| Animal category | Daily feed intake | Body weight | Use level in feed | Estragole intake[(a)] | Combined intake[(a)] | MOET |
|---|---|---|---|---|---|---|
| **Long-living and reproductive animals** | kg DM/day | kg | mg/kg feed | μg/kg bw per day | | |
| Laying hen | 0.106 | 2 | 1.9 | 3.433 | 4.480 | 4,480[(b)] |
| Horse | 8 | 400 | 5 | 3.409 | 4.515 | 4,512[(b)] |
| **Target species for fattening** | | | | | | |
| Chicken for fattening | 0.158 | 2 | 1.5 | 4.040 | 5.831 | 1,715[(c)] |
| Turkey for fattening | 0.176 | 3 | 1.7 | 3.400 | 4.424 | 2,038[(c)] |

DM: dry matter.
(a): The intake value of estragole is calculated at the specification of 3%, the intake values of methyleugenol, myristicin and dillapiole in feed are calculated considering the maximum expected concentration in the additive for each compound.
(b): The MOET is calculated as the ratio of the reference point ($BMDL_{10}$ of 22.2 mg/kg bw per day) to the combined intake.
(c): The MOET is calculated as the ratio of the reference point (NOAEL of 10 mg/kg bw per day) to the combined intake.

When the estimated exposures of long-living animals are compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day derived for methyleugenol by Suparmi et al. (2019) from a rodent carcinogenicity study (NTP, 2000), a MOET < 10,000, which is indicative of concern, is obtained for laying hens and horses (Table 6).

For short-living animals, the magnitude of the MOET is > 100 and is of no safety concern, when comparing the exposure to the reference point for non-neoplastic endpoints.

Considering that analysed values of *p*-allylalkoxybenzenes provided for the batches under assessment are well below the highest specification for estragole or the maximum expected concentrations for the other compounds, the FEEDAP Panel proposed a scenario based on the analytical values provided for the five batches described in Section 3.2.1.

b) An essential oil with a content of estragole, myristicin, dillapiole and methyleugenol corresponding to the highest analysed concentration (1.08% estragole, 0.67% myristicin, 0.21% dillapiole and 0.015% methyleugenol).

The use of anise oil at the proposed use levels (ranging from 1.5 to 5 mg/kg complete feed) would result in concentrations in complete feed in the ranges 16.2–54 μg estragole/kg, 9.41–31 μg myristicin/kg, 3.11–10 μg dillapiole/kg and 0.225–0.75 μg methyleugenol/kg.

The highest daily intake of estragole and the highest combined intake of *p*-allylalkoxybenzenes (estragole, myristicin, dillapiole and methyleugenol) were calculated considering the maximum proposed use level of the additive in feed for the different animal categories and the highest analysed value of these substances detected in the additive (1.08%, 0.627%, 0.207% and 0.015% for estragole, myristicin, dillapiole and methyleugenol, respectively). The intake values are reported in Table 7 together with the corresponding MOET for the combined intake calculated considering the relevant reference point for long-living and reproductive animals and for short-living animals (target species for fattening).

**Table 7:** Target animal intake of estragole and total *p*-allylalkoxybenzenes and combined margin of exposure (MOET) calculated at the maximum proposed use level of the additive in feed for an essential oil with a content of estragole, myristicin, dillapiole and methyleugenol at the highest analysed concentration (1.08%, 0.627%, 0.207% and 0.015%, respectively)

| Animal category: | Daily feed intake | Body weight | Use level in feed | Estragole intake[(a)] | Combined intake[(a)] | MOET |
|---|---|---|---|---|---|---|
| **Long-living and reproductive animals** | kg DM/day | kg | mg/kg feed | μg/kg bw per day | | |
| Laying hen | 0.106 | 2 | 1.9 | 1.236 | 2.209 | 10,052[(b)] |
| Horse | 8 | 400 | 5 | 1.227 | 2.193 | 10,122[(b)] |
| **Target species for fattening** | | | | | | |
| Chicken for fattening | 0.158 | 2 | 1.5 | 1.454 | 2.599 | 3,848[(c)] |
| Turkey for fattening | 0.176 | 3 | 1.7 | 1.224 | 2.187 | 4,572[(c)] |

DM: dry matter.
(a): The intake values of estragole, methyleugenol, myristicin and dillapiole in feed are calculated considering the highest analysed value in the additive for each compound.
(b): The MOET is calculated as the ratio of the reference point ($BMDL_{10}$ of 22.2 mg/kg bw per day) to the combined intake.
(c): The MOET is calculated as the ratio of the reference point (NOAEL of 10 mg/kg bw per day) to the combined intake.

When the estimated exposures of long-living animals are compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day derived for methyleugenol by Suparmi et al. (2019) from a rodent carcinogenicity study (NTP, 2000), a MOET > 10,000, which is indicative of low concern, is obtained for laying hens and horses (Table 6).

For short-living animals, the magnitude of the MOET is > 100 and is of no safety concern, when comparing the exposure to the reference point for non-neoplastic endpoints.

*Conclusions on safety for the target species*

Based on the magnitude of the MOET calculated considering the presence of estragole, myristicin, dillapiole and methyleugenol in anise oil and the conditions of use in the different target species, the FEEDAP Panel concludes that:

*a) For anise oil with a content of estragole at the highest proposed specification of 3.0% and the other p-allylakoxybenzenes at maximum expected concentration* (1.0% myristicin, 0.3% dillapiole and 0.03% methyleugenol)

- The use of the additive at the proposed level of 1.9 mg/kg complete feed in laying hens and 5 mg/kg in horses is considered of concern (MOET < 10,000). The conclusion for laying hens is extrapolated to breeding birds, birds reared for laying/breeding/reproduction and ornamental birds (including game birds).
- The Panel has no safety concern when the additive is used at the proposed use level of 1.7 mg/kg for turkeys for fattening and at 1.5 mg/kg complete feed for chickens for fattening and other poultry species for fattening.

*b)* For anise oil essential oil with a content of estragole, myristicin, dillapiole and methyleugenol corresponding to the highest analysed concentration (1.08% estragole, 0.67% myristicin, 0.21% dillapiole and 0.015% methyleugenol)

- The use of the additive at the proposed use level of 1.9 mg/kg complete feed for laying hens and at 5 mg/kg complete feed for horses is of low concern (MOET > 10,000). The conclusion for laying hens is extrapolated to breeding birds, birds reared for laying/breeding/reproduction and ornamental birds (including game birds).
- The Panel has no safety concern when the additive is used at the proposed use level of 1.7 mg/kg for turkeys for fattening and at 1.5 mg/kg complete feed for chickens for fattening and other poultry for fattening.

### 3.2.2.5. Safety for the consumer

Anise oil obtained by steam distillation of the dried, ripe fruit of *P. anisum* is added to a wide range of food 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 0.033 mg/kg bw per day for anise oil (FEMA 2094) and of 0.22 mg/kg bw per day for anise fruit (FEMA 2093).

The majority of the individual constituents of the essential oil under assessment are currently authorised as food flavourings without limitations and have been already assessed for consumer safety when used as feed additives in animal production (see Table 1, Section 1.2).

No data on residues in products of animal origin were made available for any of the constituents of the essential oil. However, the Panel recognises that the constituents of anise oil are expected to be extensively metabolised and excreted by the target species. Also, for estragole, methyleugenol, myristicin and dillapiole, the available data indicate that they are absorbed, metabolised and rapidly excreted, and are not expected to accumulate in animal tissues and products (see Section 3.2.2.1).

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

No safety concern would be expected for the consumer from the use of anise oil up to the maximum proposed use level in feed for the target animals.

#### 3.2.2.6. Safety for the user

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

The applicant made a literature search aimed at retrieving studies related to the safety of preparations obtained from *P. anisum* for the users.[37] The only paper retrieved (Opdyke, 1973) did not provide data on endpoints relevant to user safety and is considered of limited value.

The applicant produced a safety data sheet[38] for anise oil, where hazards for users have been identified.

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

Due to the high level of estragole (> 1%), the applicant also proposes to classify the additive according to Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation)[39] as suspected of causing genetic defects (category 2 mutagen) and of causing cancer (category 2 carcinogen).

For preparations with these classifications, precautionary statements as indicated in the Regulation (EC) No 1272/2008 have to be followed, and the additive should be handled accordingly.[40]

#### 3.2.2.7. Safety for the environment

*P. anisum* is a native species to Europe, where it is widely grown both for commercial and decorative purposes. Use of the essential oil under the proposed conditions of use in animal production is not expected to pose a risk to the environment.

## 3.3. Anise tincture

### 3.3.1. Characterisation of the tincture

The tincture under assessment is soluble in water and has an average density of 987 kg/m$^3$ (range: 951–1,002 kg/m$^3$, five batches).[41] By specification, the product is a water/ethanol (55/45, v/v) solution, with a dry matter (DM) content of 1–2%, which contains 50–400 μg/mL *trans*-anethole.

Table 8 summarises the results of the proximate analysis of five batches of the additive (origin: Turkey and Egypt) expressed as % (w/w).[42] The solvent represents about 98.5% of the additive

[37] Technical dossier/ Supplementary information February 2021/Literature_search_Anise_oil.

[38] Technical dossier/ Supplementary Information February 2021/Annex_VIII_SIn reply_anise_oil_MSDS. For the oil: May cause allergic skin reactions (H317), suspected to cause genetic defects (H341), suspect of causing cancer (H351). For the main component trans-anethole: hazards for skin irritation (H315, category 2), skin sensitisation (H317, category 1B).

[39] 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, pp. 1–1355.

[40] 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, pp. 50.

[41] Technical dossier/Supplementary information December 2022/Annex_I_Dry matter and density.

[42] Technical dossier/Supplementary information December 2022/Annex_II_SIn_reply_anise_tincture_Nutritional anal+Microbiol+Dioxins.

leaving a DM content of about 1.5%.[41] The DM consists of inorganic material measured as ash (18.5% on average) and a plant-derived organic fraction, which includes lipids, proteins, fibre and sugars. About 36.5% of the DM content in the tincture was not identified.

**Table 8:** Proximate analysis of anise tincture derived from the fruit of *Pimpinella anisum* L. based on the analysis of five batches. The results are expressed as % of the tincture (w/w)

| Constituent | Mean | Range |
|---|---|---|
| | % (w/w) | % (w/w) |
| Dry matter | 1.51 | 1.36–1.68 |
| Ash | 0.28 | 0.2–0.4 |
| Organic fraction | | |
| Lipids | 0.12 | 0.1–0.2 |
| Protein | 0.34 | 0.2–0.6 |
| Fibre | < 0.5 | < 0.5 |
| Sugars | 0.28 | 0.2–0.3 |
| Unknown | 0.55 | 0.45–0.68 |
| Solvent (water/ethanol, 55/45, v/v) | 98.49 | 98.32–98.64 |

The fraction of secondary metabolites was characterised in the same batches of the tincture and the results are summarised in Table 9. The tincture was shown to contain polyphenols determined by spectrophotometry (at 760 nm) and expressed as gallic acid equivalents.[43] Individual compounds were determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detector: phenolic compounds (at 320 nm) including flavonoids, caffeoylquinic and other quinic derivatives, other phenolic acids and non-phenolic organic acids (detected at 210 nm).[44] The tincture also contained volatiles[45] identified and quantified by GC–MS.

**Table 9:** Characterisation of the fraction of secondary metabolites of anise tincture derived from the fruit of *Pimpinella anisum* L. based on the analysis of five batches. The results are expressed as μg/mL of anise tincture

| Constituent | CAS No | FLAVIS No | Mean | Range[(a)] |
|---|---|---|---|---|
| | | | μg/mL | μg/mL |
| Phenols (total, by photometry) | – | – | 474 | 391.2–538.3 |
| Flavonoids (HPLC, 320 nm) | | | | |
| Luteolin 2''-O-pentosyl-6-C-hexoside | – | – | 20.8 | 14.8–30.9 |
| Luteolin 2''-O-hexosyl-6-C-glucoside | – | – | 49.2 | 34.9–65.8 |
| Isoorientin (luteolin-6-C-glucoside) | 4261-42-1 | – | 96.0 | 76.7–124.1 |
| Apigenin 2''-O-pentosyl-6-C-hexoside | – | – | 23.1 | 17.6–27.8 |
| Apigenin 2''-O-pentosyl-6-C-glucoside | – | – | 106.2 | 83.4–136.8 |
| Isovitexin (apigenin-6-C-glucoside) | 38953-85-4 | – | 32.2 | 21.3–41.1 |
| Luteolin-derivative A (luteolin-7-O-glycoside derivative) | 5373-11-5 | – | 26.7 | 15.8–35.2 |
| Luteolin-derivative B (luteolin-7-O-glycoside derivative) | 5373-11-5 | – | 20.6 | 14.9–30.1 |
| Flavanone derivative (hesperidin) | 520-26-3 | – | 10.2 | 7.2–16.3 |
| Apigenin-7-O-glucoside | 578-74-5 | – | 12.0 | 5.8–16.0 |
| Total flavonoids | | | 397.0 | 319.0–492.9 |

[43] Technical dossier/Supplementary information December 2022/Annex_VI_Sin_reply_anise_tincture_Total phenols.
[44] Technical dossier/Supplementary information December 2022/Annex_III_Sin_reply_anise_tincture_Organic acids.
[45] Technical dossier/Supplementary information December 2022/Annex_VII_SIn_reply_anise_tincture_Essential oi compounds.

| Constituent | CAS No | FLAVIS No | Mean µg/mL | Range[a] µg/mL |
|---|---|---|---|---|
| Caffeoylquinic and other quinic acid derivatives (HPLC, 320 nm) | | | | |
| Feruloylquinic acid (3-O-, 4-O- or 5-O-)[b] | | – | 3.8 | 3.0-4.8 |
| Neochlorogenic acid | 906-33-2 | – | 19.2 | 16.4–22.5 |
| p-Coumaroylquinic acid derivative A (3-p-, 4-p- or 5-p-)[c] | | – | 8.6 | 4.6-13.3 |
| p-Coumaroylquinic acid derivative B (3-p-, 4-p- or 5-p-)[c] | | – | 6.2 | 5.1-7.6 |
| Chlorogenic acid | 327-97-9 | – | 84.2 | 65.2–107.4 |
| 4-O-Caffeoylquinic acid | 905-99-7 | – | 6.6 | 4.1–8.4 |
| 3,5-Dicaffeoylquinic acid | 89919-62-0 | – | 27.1 | 20.3–38.1 |
| 4,5-Dicaffeoylquinic acid | 57378-72-0 | – | 29.9 | 17.0–41.7 |
| Total caffeoylquinic acids | | | 185.6 | 144.4–236.9 |
| Other phenolic acids (HPLC, 320 nm) | | | | |
| Caffeic acid | 331-39-5 | – | 19.7 | 19.7–19.7 |
| Caffeic acid-/Ferulic acid derivative A | – | – | 2.36 | 2.2–2.7 |
| Caffeic acid-/Ferulic acid derivative B | – | – | 6.92 | 4.7–9.1 |
| Caffeic acid-/Ferulic acid derivative C | – | – | 14.7 | 6.5–29.9 |
| Total other phenols | | | 27.9 | 15.3–46.6 |
| Non-phenolic organic acids (HPLC, 220 nm) | | | | |
| Malic acid | 6915-15-7 | 08.017 | 2,829 | 2,076-3,654 |
| Fumaric acid | 110-17-8 | 08.025 | 79.7 | 79.7–79.7 |
| Shikimic acid | 138-59-0 | | 16.2 | 13.4–19.3 |
| Total organic acids | | | 2,861 | 2,089-3,672 |
| Total identified[d] | | | 3,472 | 2,607-4,379 |
| Volatiles (GC–MS) | | | | |
| Linalool (CG 6) | 78-70-6 | 02.013 | 1.86 | 0.5–3.2 |
| trans-Pseudoisoeugenyl 2-methylbutyrate (CG 17) | 58989-20-1 | – | 6.89 | 5.1–9.4 |
| trans-Anethole (1-methoxy-4-(prop-1(trans)-enyl)benzene, CG 18) | 4180-23-8 | 04.010 | 203.6 | 149.4–270.0 |
| 4-Methoxyphenylacetone (CG 21) | 122-84-9 | 07.087 | 4.63 | 1.8–8.5 |
| 1-(4-Methoxy-phenyl)-1-propanol (CG 21) | 5349-60-0 | – | 3.20 | 3.2–3.2 |
| Anisaldehyde (4-methoxybenzaldehyde, CG 23) | 123-11-5 | 05.015 | 43.56 | 17.6–115.3 |
| Anisyl alcohol (CG 23) | 105-13-5 | 02.128 | 25.66 | 1.4–63.8 |
| Anisyl acetate (CG 23) | 104-21-2 | 09.019 | 6.47 | 3.2–17.9 |
| 4-tert-Butylanisole (CG 26) | 5396-38-3 | – | 9.67 | 3.3–20.8 |
| Butanoic acid, 2-methyl-, 4-methoxy-2-(3-methyloxiranyl)phenyl ester (CG 32) | 97180-28-4 | – | 6.00 | 4.5–8.8 |
| Total volatiles identified | | | 291.5 | 223.2–374.7 |
| Estragole | 140-67-0 | 04.011 | 3.43 | 0.7–7.7 |
| Other volatile compounds (GC–MS) | | | | |
| Unknown (phenylpropanoid) | – | – | 20.65 | 7.4–50.0 |
| Unknown (phenylpropanoid) | – | – | 8.95 | 2.8–22.6 |
| Unknown (3-methoxymandelic acid) | – | – | 61.76 | 20.7–198.5 |
| Unknown (2-methoxymandelic acid) | – | – | 20.19 | 9.7–56.6 |
| Unknown (phenylpropanoid) | – | – | 6.11 | 1.9–10.6 |
| Unknown | – | – | 4.32 | 1.2–12.7 |
| Unknown | – | – | 1.76 | 0.9–2.6 |

| Constituent | CAS No | FLAVIS No | Mean | Range[(a)] |
|---|---|---|---|---|
| | | | µg/mL | µg/mL |
| Total other volatiles | | | 121.3 | 53.7–348.9 |
| Total volatiles[(e)] | | | 415.6 | 277.6–731.3 |

EU: European Union; CAS No: Chemical Abstracts Service number; FLAVIS No: EU Flavour Information System numbers.
(a): The values given for the total are the lowest and the highest values of the sum of the components in the five batches analysed.
(b): CAS numbers: 62929-69-5 (3-O-feruloylquinic acid) and 1899-29-2 (5-O-feruloylquinic acid).
(c): CAS numbers: 87099-71-6 8 (3-p-coumaroylquinic acid), 1108200-72-1 (4-p-coumaroylquinic acid) and 1899-30-5 (5-p-coumaroylquinic acid).
(d): considering the sum of flavonoids, caffeoylquinic acids, other phenolic acids and organic acids.
(e): considering the sum of volatiles, estragole and other compounds.

The identified secondary metabolites (3,586 µg/mL) account on average for 21.9% of the DM content of the tincture (range: 18.6–26.8%).

#### 3.3.1.1. Substances of concern

The literature search made by the applicant[46] identified furocoumarins in trace concentrations in anise fruit and estragole (methylchavicol, 1–5%) and methyleugenol (0.14–1.5%) in the essential oil from the fruit (see Section 3.2.1.1).

The applicant submitted a certificate of analysis for the screening of coumarins and furocoumarins in anise tincture by HPLC with diode array detection (DAD) following a method developed by the international fragrance association (IFRA). Coumarins and furocoumarins were not detected in the tincture, even at 20-fold concentrates of the lipophilic fraction (limit of detection 0.2 µg/mL).[47] A GC–MS analysis of the five batches of the tincture under assessment (see Table 3) confirmed the presence of estragole in four batches. The concentration of estragole was on average 3.43 µg/mL (range: 0.73–7.72 µg/mL).[45] Methyleugenol, myristicin and dillapiole were not detected in the tincture under assessment.

#### 3.3.1.2. Impurities

Data on impurities were provided for three batches of anise tincture. Mercury was below the corresponding limit of quantification (LOQ) in all batches. Arsenic was below the LOQ in one batch and was 0.012 and 0.019 mg/kg in the other two batches. Cadmium ranged between 0.0009 and 0.0012 mg/kg in all batches. The concentrations of lead were in the range 0.0012–0.0025 mg/kg. In all batches, the mycotoxins were below the corresponding LOQ and pesticides were not detected in a multiresidue analysis.[48] Polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) were below the corresponding LOQ. The calculated upper bound for the sum of dioxins was 31.6 ng WHO PCDD/F-TEQ (World Health Organisation polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) toxic equivalents (TEQ))/kg, the sum of dioxin and dioxin like PCBs was 33 ng WHO PCCD/F + PCB TEQ/kg.[42]

Analysis of microbial contamination of five batches of anise tincture indicated that *Salmonella* spp. was not detected in 25 g, and *E. coli* was < 1 colony-forming unit (CFU)/g.[49]

The FEEDAP Panel considers that the microbial contamination and the amounts of the detected impurities do not raise safety concerns.

#### 3.3.1.3. Shelf-life

The shelf-life of the tincture is declared by the applicant to be at least 12 months when stored in tightly closed containers under standard conditions. No evidence was provided to support this claim.

[46] Technical dossier/Supplementary information December 2022/ Literature search_anise_tincture.
[47] Technical dossier/Supplementary information December 2022/Annex_V_SIn_reply_anise_tincture_Coumarins-Furocoumarins. Compounds analysed (n = 15): biakangelicin, oxypeucedanin, oxypeucedanin hydrate, psoralen, xanthotoxin, isopimpinellin, bergapten, heraclenin, biakangelicol, imperatorin, phellopterin, isoimperatorin, epoxybergamottin, 8-geranyloxypsoralen and bergamottin.
[48] Technical dossier/Supplementary information December 2022/Annex_VIII_Sin_reply_anise_tincture_Heavy Metals, Mycotoxins, Pesticides. LOQ for heavy metals and arsenic: < 0.005 mg/kg for arsenic, < 0.002 mg/kg for mercury; LOQ for individual pesticides: 0.001–0.005 mg/L; LOQ for mycotoxins: < 0.1 µg/kg for aflatoxins B1, B2, G1 and G2, < 1 µg/kg for ochratoxin A, < 2 µg/kg for zearalenone, α- and β-zearalenone, HT2-toxin, T2-toxin cytrochalasin E and sterigmatocystin, < 5 µg/kg for nivalenol, fusarenon X and diacetoxyscirpenol, and < 10 for deoxynivalenol, deoxynivalenol-3-glycoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, citrinin, patulin and fumonisins B1, B2 and B3.
[49] Technical dossier/Supplementary information/ Annex_II_SIn_reply_anise_tincture_Nutritional Anal+Microbiol+Dioxins.

#### 3.3.1.4. Conditions of use

Anise tincture intended for use in complete feed for horses, dogs and cats at maximum proposed use levels of 5.0, 0.4 and 0.06 mL tincture/head and day, respectively, corresponding to 617, 1,548.5 and 987.2 mg tincture/kg complete feed. The tincture is also intended for use in feed and water for drinking in poultry species at the maximum proposed use levels of 0.5 mL/kg complete feed or water for drinking (corresponding to 493.6 mg tincture/kg feed or water for drinking).

### 3.3.2. Safety

The safety assessment of the additive is based on the highest proposed use levels.

No studies to support the safety for target animals, consumers and users were performed with the additive under assessment. The applicant provided a literature search on the ADME and on the toxicology of preparations obtained from *P. anisum*.[50]

The additive under assessment, anise tincture, consists of 98.5% (w/w) of a water/ethanol mixture. The concentration of plant-derived compounds is about 1.5% (w/w) of the tincture. The dry matter included minerals (expressed as ash), proteins, lipids and carbohydrates, which are not of concern and are not further considered.

Among the identified secondary plant metabolites, up to 0.049% (w/w) of the tincture is constituted by flavonoids (10 compounds identified), up to 0.029% (w/w) by simple phenols (12 compounds were identified), up to 0.37% (w/w) by non-phenolic organic acids (malic acid, fumaric acid and shikimic acid) and up to 0.074% (w/w) by volatile compounds (10 compounds identified plus seven unidentified or tentatively identified).

Simple phenols and non-phenolic organic acids, including malic acid, fumaric acid and shikimic acid, are ubiquitous in food and feeds of plant origin and are not expected to raise concern for genotoxicity. They will be readily metabolised and excreted and are not expected to accumulate in animal tissues and products. These compounds are not of concern at concentrations resulting from the use of the additive at the maximum proposed use level in feed and are not further considered in the assessment.

The tincture contains flavonoids, mainly glucosides and hexosides of luteolin and apigenin. For the ADME and the toxicology of flavonoids, reference is made to the safety evaluation made by the FEEDAP Panel in the EFSA opinion on bitter orange extract (EFSA FEEDAP Panel, 2021b).

Several volatile constituents of anise tincture have been previously assessed and considered safe for use as flavourings. They are currently authorised for use in food[11] without limitations and for use in feed[10] at individual use levels higher than those resulting from the intended use of the tincture in feed. The list of the compounds already evaluated by the EFSA Panels is given in Table 1 (see Section 1.2).

Estragole (0.4–1.2 mg/kg), a compound with experimentally proven genotoxicity and carcinogenicity in rodents (as reviewed in EMA, 2021), was detected in all batches of the additive. The ADME and the toxicology of estragole have been already addressed in Sections 3.2.2.1 and 3.2.2.2.

#### 3.3.2.1. Safety for the target species

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

In the absence of these data, the approach to the safety assessment of the mixture is based on its individual components or groups of components (assessment groups). The combined toxicity can be predicted using the dose addition assumption within an assessment group (EFSA SC, 2019a).

The safety assessment is based on flavonoids derivatives, which are allocated to the same assessment group based on considerations related to structural and metabolic similarities, and on the volatile compounds present in tincture. A separate risk assessment is performed for the presence of estragole in the tincture.

*Components other than estragole*

Based on considerations related to structural and metabolic similarities, flavonoids were allocated to the same assessment group. The volatile compounds present in the tincture were allocated to seven assessment groups, corresponding to the chemical groups (CGs) 6, 17, 18, 21, 23, 26 and 32, as defined in Annex I of Regulation (EC) No 1565/2000. The allocation of the components to the (sub-) assessment groups is shown in Table 10 and in the corresponding footnote.

[50] Technical dossier/Supplementary information December 2022/Anise_tincture_literature_search.

For hazard characterisation, each component of an assessment group was first assigned to the structural class according to the Cramer classification (Cramer et al., 1978). For some components in the assessment group, toxicological data were available to derive NOAEL values. Structural and metabolic similarity among the components in the assessment groups were assessed to explore the application of read-across. If justified, extrapolation from a known NOAEL of a component of an assessment group to the other components of the group with no available NOAEL was made. If sufficient evidence was available for members of a (sub-)assessment group, a (sub-)assessment group NOAEL was derived.

For flavonoids, the FEEDAP Panel identified a NOAEL of 500 mg/kg bw per day for hesperidin, which was applied as a group NOAEL to all the flavanones and flavones present in bitter orange extract (EFSA FEEDAP Panel, 2021b). Considering the structural and metabolic similarities within the assessment group of flavonoids, the FEEDAP Panel applies the same NOAEL to all the flavonoids present in anise tincture at very low concentrations.

For the volatile components of the tincture, toxicological data of subchronic studies, from which NOAEL values could be derived, were available for linalool [02.013] in CG 6 (EFSA FEEDAP Panel, 2012b), anisaldehyde [05.015] in CG 23 (EFSA FEEDAP Panel, 2012c) and *trans*-anethole [04.010] in CG 18 (EFSA FEEDAP Panel, 2011).

Considering the structural and metabolic similarities, the NOAEL of 20 mg/kg bw per day for anisaldehyde [05.015] was applied to anisyl alcohol [02.128] and anisyl acetate [09.019] in CG 23.

For the remaining compounds, trans-pseudoisoeugenyl 2-methylbutyrate (CG 17), 4-methoxyphenylacetone (CG 21), 4-tert butylanisole (CG 26) and butanoic acid, 2-methyl-, 4-methoxy-2-(3-methyloxiranyl)phenyl ester (CG 32), toxicity studies were not available and read-across was not possible. Therefore, the TTC approach was applied (EFSA FEEDAP Panel, 2017b). All these compounds belong to Cramer class I except 1-(4-methoxy-phenyl)-1-propanol which belongs to Cramer class II.

As the result of the hazard characterisation, a reference point was identified for each component in the assessment group based on the toxicity data available (NOAEL from *in vivo* toxicity study or read-across) or from the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class (i.e. 3 mg/kg bw per day for Cramer Class I compounds, Munro et al., 1996). Reference points selected for each compound are shown in Table 9.

For risk characterisation, the margin of exposure (MOE) was calculated for each component as the ratio between the reference point and the exposure. For each assessment group, the combined (total) margin of exposure (MOET) was calculated as the reciprocal of the sum of the reciprocals of the MOE of the individual substances (EFSA SC, 2019a). A MOET > 100 allowed for interspecies differences and intra-individual variability (as in the default 10 × 10 uncertainty factor).

The approach to the safety assessment of anise tincture for the target species is shown in Table 10. As the water intake for poultry would be two to three times higher than feed intake (EFSA FEEDAP Panel, 2010), the calculations shown in Table 9 were made for chickens for fattening at the proposed use level of 494 mg tincture/kg water for drinking.

**Table 10:** Compositional data, intake values (calculated for chickens for fattening when supplemented at 494 mg/kg water for drinking), reference points and margin of exposure (MOE) for the individual components of anise tincture classified according to assessment group

| Tincture composition | | | Exposure | | Hazard characterisation | | Risk characterisation | |
|---|---|---|---|---|---|---|---|---|
| **Assessment group** | **FLAVIS-No** | **Highest conc. in the tincture** | **Highest conc. water** | **Intake[a]** | **Cramer class[b]** | **NOAEL[c]** | **MOE** | **MOET** |
| Constituent | – | (μg/mL) | mg/L | mg/kg bw | – | mg/kg bw | – | – |
| **Flavonoids** | | | | | | | | |
| Luteolin 2′′-O-pentosyl-6-C-hexoside | – | 30.9 | 0.015 | 0.0041 | (III) | 500 | 121,966 | |
| Luteolin 2′′-O-hexosyl-6-C-glucoside | – | 65.8 | 0.032 | 0.0087 | (III) | 500 | 57,276 | |

| Tincture composition | | | Exposure | | Hazard characterisation | | Risk characterisation | |
|---|---|---|---|---|---|---|---|---|
| **Assessment group** | **FLAVIS-No** | **Highest conc. in the tincture** | **Highest conc. water** | **Intake[(a)]** | **Cramer class[(b)]** | **NOAEL[(c)]** | **MOE** | **MOET** |
| Isoorientin (luteolin 6-C-glucoside) | – | 124.1 | 0.061 | 0.0165 | (III) | 500 | 30,369 | |
| Apigenin 2''-O-pentosyl-6-C-hexoside | – | 27.8 | 0.014 | 0.0037 | (III) | 500 | 134,078 | |
| Apigenin 2''-O-pentosyl-6-C-glucoside | – | 136.8 | 0.067 | 0.0181 | (III) | 500 | 27,549 | |
| Isovitexin (apigenin-6-C-glucoside) | | 41.1 | 0.020 | 0.0055 | (III) | 500 | 90,690 | |
| Luteolin-derivative A (luteolin-7-O-glycoside derivative) | | 35.2 | 0.017 | 0.0047 | (III) | 500 | 107,067 | |
| Luteolin-derivative B (luteolin-7-O-glycoside derivative) | | 30.1 | 0.015 | 0.0040 | (III) | 500 | 124,083 | |
| Flavanone derivative (hesperidin) | – | 16.3 | 0.008 | 0.0022 | (III) | **500** | 228,673 | |
| Apigenin-7-O-glucoside | – | 16.0 | 0.008 | 0.0021 | (III) | 500 | 233,431 | |
| MOET | | | | | | | | 7,172 |
| **Volatile constituents** | | | | | | | | |
| **CG 6** | | | | | | | | |
| Linalool | 02.013 | 3.18 | 0.002 | 0.0008 | (I) | **117** | 277,323 | |
| **CG 17** | | | | | | | | |
| trans-Pseudoisoeugenyl 2-methylbutyrate | – | 9.42 | 0.005 | 0.0013 | I | *3* | 2,392 | |
| **CG 18** | | | | | | | | |
| *trans*-Anethole | 04.010 | 270 | 0.133 | 0.0358 | (I) | **300** | 8,375 | |
| **CG 21** | | | | | | | | |
| 4-Methoxyphenylacetone | 07.087 | 8.45 | 0.004 | 0.0011 | I | *3* | 2,647 | |
| 1-(4-Methoxy-phenyl)-1-propanol | – | 3.20 | 0.002 | 0.0004 | II | *0.91* | 2,120 | |
| MOET CG 21 | | | | | | | | 1,177 |
| **CG 23** | | | | | | | | |
| Anisaldehyde | 05.015 | 115.3 | 0.057 | 0.0155 | (I) | **10**[(d)] | 647 | |
| Anisyl alcohol | 02.128 | 63.8 | 0.032 | 0.0086 | (I) | 10 | 1,168 | |
| Anisyl acetate | 09.019 | 17.9 | 0.009 | 0.0024 | (I) | 10 | 4,165 | |
| MOET CG 23 | | | | | | | | 378 |
| **CG 26** | | | | | | | | |
| 4-tert-Butylanisole | – | 20.8 | 0.010 | 0.0028 | I | *3* | 1,087 | |
| **CG 32** | | | | | | | | |
| Butanoic acid, 2-methyl-, 4-methoxy-2-(3-methyloxiranyl)phenyl ester | – | 8.8 | 0.004 | 0.0012 | I | *3* | 2,562 | |

(a): Intake calculations for the individual components are based on the use level of 494 mg tincture/kg water for drinking for chickens for fattening, assuming a water intake threefold higher than feed intake. The MOE for each component is calculated as the ratio of the reference point (NOAEL) to the intake. The combined margin of exposure (MOET) is calculated for each assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances.

(b): When a NOAEL value is available or read-across is applied, the allocation to the Cramer class is put into parentheses.

(c): Values **in bold** refer to those components for which the NOAEL value was available, values *in italics* are the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class, other values (plain text) are NOAELs extrapolated by using read-across.
(d): The NOAEL for anisaldehyde [05.015] was halved because of the short duration of the study (EFSA FEEDAP Panel, 2012c).

As shown in Table 10, for poultry species at the proposed use levels in water for drinking, a MOET ≥ 378 was calculated for all assessment groups assuming that the water intake is up to threefold higher than feed intake for all the assessment groups. Therefore, no safety concern was identified for the anise tincture (without considering the presence of estragole) when used as a feed additive for chickens for fattening at the proposed use level in feed or water for drinking (494 mg/kg). From the lowest MOE of 378 for chickens for fattening, the MOE for CG 23 compounds (benzyl alcohols, aldehydes, acids, esters and acetals) was calculated for the other target species considering the respective daily feed intake and conditions of use. The results are summarised in Table 10.

**Table 11:** The combined margin of exposure (MOET) for CG 23 (benzyl alcohols, aldehydes, acids, esters and acetales) calculated for the different target animal categories at the proposed use level of the additive in water for drinking or in feed

| Animal category | Body weight (kg) | Water intake[(a)] (g/day) | Proposed use level (mg additive/kg water) | MOET |
|---|---|---|---|---|
| Chicken for fattening | 2 | 474 | 494 | 378 |
| | | **Feed intake** | **(mg additive/kg feed)** | |
| Horse | 400 | 8,000 | 617 | 3,573 |
| Dog | 15 | 250 | 1,548.5 | 1,708 |
| Cat | 3 | 60 | 987.5 | 2,232 |

(a): Calculated from the default values for feed intake (EFSA FEEDAP Panel, 2017b) assuming a water intake threefold higher than feed intake (EFSA FEEDAP Panel, 2010).

Table 11 shows that for all species, the MOET exceeds the value of 100. Because glucuronidation is an important metabolic reaction to facilitate the excretion of the components of the essential oil and considering that cats have an unusually low capacity for glucuronidation (Court and Greenblatt, 1997; Lautz et al., 2021), the use of anise tincture as additive in cat feed needs a wider margin of exposure. A MOET of 500 is considered adequate. Therefore, for all species, no safety concern (without considering the presence of estragole) was identified for anise tincture, when used as a feed additive at the proposed use levels.

*Estragole*

Estragole was detected in all five batches of the additive (0.73–7.72 μg/mL).

At the maximum proposed use level of anise tincture, the highest concentration of estragole (0.0008%, measured by GC–MS method) would be 0.0039 mg/kg complete feed/water for drinking for poultry, 0.0048 mg/kg complete feed for horses, 0.0121 mg/kg complete feed for dogs and 0.0077 mg/kg complete feed for cats. The corresponding highest intake of estragole for the target species is shown in Table 12.

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, 2022b). In the current assessment, this reference point is applied to assess the exposure of long-living and reproductive animals (laying hens, dogs, cats and horses) to estragole.

**Table 12:** Target animal intake of estragole (as μg/kg bw per day) and margin of exposure (MOE) calculated at the maximum proposed use level of the additive in feed for each target animal category

| Target species | Daily feed intake | Body weight | Use level | Estragole intake | MOE |
|---|---|---|---|---|---|
| **Long-living and reproductive animals** | kg DM/day | kg | mg/kg | μg/kg bw per day | |
| Laying hen | 0.106 | 2 | 494 | 0.698[(a)] | 31,824[(b)] |

| Target species | Daily feed intake | Body weight | Use level | Estragole intake | MOE |
|---|---|---|---|---|---|
| Horse | 8 | 400 | 617 | 0.110 | 202,405[(b)] |
| Dog | 0.25 | 15 | 1,548.5 | 0.229 | 96,778[(b)] |
| Cat | 0.06 | 3 | 987.5 | 0.176 | 126,464[(b)] |
| **Target species for fattening** | | | | | |
| Chicken for fattening | 0.158 | 2 | 494 | 1.040[(a)] | 9,617[(c)] |

(a): Calculated from the default values for feed intake (EFSA FEEDAP Panel, 2017b) assuming a water intake threefold higher than feed intake (EFSA FEEDAP Panel, 2010).
(b): The MOET is calculated as the ratio of the reference point ($BMDL_{10}$ of 22.2 mg/kg bw per day) to the combined intake.
(c): The MOET is calculated as the ratio of the reference point (NOAEL of 10 mg/kg bw per day) to the combined intake.

The FEEDAP Panel also identified a NOAEL of 10 mg/kg bw per day for non-neoplastic lesions (NTP, 2000) as the reference point for the entire group of *p*-allylalkoxybenzenes (EFSA FEEDAP Panel, 2023). In the current assessment, this reference point is applied to assess the exposure of short-living animals (chickens for fattening) to estragole.

When the estimated exposures of long-living animals are compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day, a MOE > 31,000 is calculated (Table 12). The magnitude of this MOE is indicative of a low concern for the target species.

For chickens for fattening, the magnitude of the MOET is > 100 and is of no safety concern, when comparing the estimated exposure to the reference point for non-neoplastic endpoints.

#### 3.3.2.2. Conclusions on safety for the target species

The use of the additive at the proposed levels of 1,548.5, 987.2 and 617 mg/kg complete feed for dogs, cats and horses is of low concern (MOET > 10,000). For laying hens, breeding birds and birds reared for laying/breeding/reproduction, the use of the additive at 494 mg/kg complete feed or mg/kg water for drinking is considered of low concern.

The Panel has no safety concern when the additive is used at the proposed use level of 494 mg/kg complete feed or mg/kg water for drinking for chickens for fattening and all poultry species for fattening.

#### 3.3.2.3. Safety for the consumer

Anise fruit and its preparations, including ethanolic extracts, are added to a wide range of food categories as 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 0.22 mg/kg bw per day for anise (fruit) and 0.033 mg/kg bw per day for anise oil obtained from the fruit.

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 phenolic compounds, including flavonoids, present in the additive at concentrations below the thresholds for Cramer Class I compounds or Cramer Class III compounds, respectively, will be readily metabolised and excreted and are not expected to accumulate in animal tissues and products. Similarly, for the volatile compounds present in the tincture, the available data indicate that they are absorbed, metabolised and rapidly excreted and are not expected to accumulate in animal tissues and products. For estragole, occurring at low concentration, the available data indicate that it is absorbed, metabolised and rapidly excreted and is not expected to accumulate in animal tissues and products (see Section 3.2.2.1).

Considering the above and the reported human exposure due to direct use of anise and its preparations in food (Burdock, 2009), it is unlikely that consumption of products from animals given anise 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 anise tincture up to the maximum proposed use levels in feed.

#### 3.3.2.4. 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 (CLP) Regulation (EC) 1272/2008[51] concerning the presence of ethanol in the tincture.[52]

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 estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised.

#### 3.3.2.5. Safety for the environment

*P. anisum* L. is a native species to Europe where it is widely grown both for commercial and decorative purposes. 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.

### 3.4. Efficacy of anise oil and anise tincture

Anise fruit (*P. anisum*) and its oil are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009), by the Flavour and Extract Manufactures Association (FEMA) with the reference numbers 2093 (anise) and 2094 (anise oil).

Since anise 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 for anise oil and anise tincture.

## 4. Conclusions

Anise oil obtained by steam distillation of the fruit of *Pimpinella anisum* L. may be produced from plants of different geographical origins and by various processes, resulting in preparations with different composition and toxicological profiles, particularly concerning the presence of estragole and other *p*-allylalkoxybenzenes.

Based on the magnitude of the MOET calculated considering the presence of estragole, myristicin, dillapiole and methyleugenol in anise oil and the conditions of use in the different animal species, the FEEDAP Panel concludes that:

a) *For anise oil with a content of estragole at the highest proposed specification of 3.0% and the other p-allylakoxybenzenes at maximum expected concentration (1.0% myristicin, 0.3% dillapiole and 0.03% methyleugenol)*
   - The use of the additive at the proposed level of 1.9 mg/kg complete feed in laying hens and 5 mg/kg in horses is considered of concern (MOET < 10,000). The conclusion for laying hens is extrapolated to breeding birds, birds reared for laying/breeding/reproduction and ornamental birds (including game birds).
   - The Panel has no safety concern when the additive is used at the proposed use level of 1.7 mg/kg for turkeys for fattening and at 1.5 mg/kg complete feed for chickens for fattening and other poultry species for fattening.

b) *For anise oil which contains ≤ 1.08% estragole, ≤ 0.67% myristicin, ≤ 0.21% dillapiole and ≤ 0.015% methyleugenol*
   - The use of the additive at the proposed use level of 1.9 mg/kg complete feed for laying hens and at 5 mg/kg complete feed for horses is of low concern. The conclusion for laying hens is extrapolated to breeding birds, birds reared for laying/breeding/reproduction and ornamental birds (including game birds).
   - The Panel has no safety concern when the additive is used at the proposed use level of 1.7 mg/kg for turkeys for fattening and at 1.5 mg/kg complete feed for chickens for fattening and other poultry for fattening.

The use of anise oil up to the highest level in feed which is considered of no concern for target animals is also expected to be of no concern for consumers.

[51] 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, pp. 1–1355*.

[52] Technical dossier/Supplementary information September 2021/Annex_XV_SIn reply_dong_quai_tincture_MSDS. H319: moderate eye irritation.

Anise oil should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser. Due to the high concentration of estragole (≥ 1%), the additive is classified as suspected of causing genetic defects and of causing cancer and should be handled accordingly.

The use of anise oil under the proposed conditions in animal feed is not expected to pose a risk to the environment.

Since the fruit of *P. anisum* and its preparations are recognised to flavour food and its function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary for anise oil.

*Anise tincture*

Anise tincture from *Pimpinella anisum* 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 anise tincture which contains ≤ 7.4 mg/kg estragole and ≤ 0.2 mg/L furocoumarins (limit of detection 0.2 mg/L) and is produced from the fruit of *P. anisum* L.

The use of the additive at the proposed levels of 1,548.5, 987.2 and 617 mg/kg complete feed for dogs, cats and horses is of low concern (MOET > 10,000). For laying hens, breeding birds and birds reared for laying/breeding/reproduction, the use of the additive at 494 mg/kg complete feed or mg/kg water for drinking is considered of low concern.

The Panel has no safety concern when the additive is used at the proposed use level of 494 mg/kg complete feed or mg/kg water for drinking for chickens for fattening and other poultry for fattening.

No safety concern would arise for the consumer from the use of anise tincture up to the highest proposed use level 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 estragole may occur. Therefore, to reduce the risk, the exposure of the users should be minimised.

The use of anise tincture as a flavour in animal feed is not considered to be a risk to the environment.

Since the fruit of *P. anisum* 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 for the tincture under assessment.

## 5. Recommendation

Although the FEEDAP Panel is aware that anise oil with estragole 3% could be present on the market, the analytical data provided by the applicant demonstrate that anise oil with reduced contents of genotoxic and carcinogenic substances can be produced.

In line with the principles of the 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), that 'manufacturing processes of botanical feed additives should avoid selective extraction and enrichment of genotoxic and/or carcinogenic substances and should aim at the reduction of these substances,' the FEEDAP Panel recommends that anise oil intended to be used as feed additive should contain the lowest possible concentrations of estragole and other *p*-allylalkoxybenzenes.

The specification for anise tincture should ensure that the concentration of estragole and furocoumarins should be as low as possible and should not exceed 7.4 mg/kg estragole and 0.2 mg/L furocoumarin, respectively.

## 6. Documentation provided to EFSA/chronology

| Date | Event |
|---|---|
| **28/10/2010** | Dossier received by EFSA. Botanically defined flavourings from Botanical Group 02 - Apiales and Austrobaileyales 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 |
| **26/02/2013** | EFSA informed the applicant (EFSA ref. 7150727) 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 |

| Date | Event |
|---|---|
| **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: dill seed extract, celery seed extract (oleoresin), caraway oleoresin/extract, and opoponax oil |
| **24/06/2019** | Application validated by EFSA – Start of the scientific assessment |
| **03/07/2019** | 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* |
| **30/09/2019** | Comments received from Member States |
| **28/10/2020** | Reception of supplementary information from the applicant (partial submission: anise tincture included in another assessment) - Scientific assessment remains suspended |
| **18/02/2021** | Reception of supplementary information from the applicant (partial dataset on anise oil) - Scientific assessment remains suspended |
| **31/10/2022** | Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives – partial report related to nine additives (*dill herb oil, dill tincture, dong quai tincture, cumin oil, fennel tincture, parsley tincture, anise tincture, star anise tincture and ferula assa-foetida oil*) |
| **13/12/2022** | Reception of supplementary information from the applicant (partial submission: anise tincture included in the present assessment) |
| **16/12/2022** | Reception of an addendum of the Evaluation report of the European Union Reference Laboratory for Feed Additives – final report related to 11 additives (*celery seed oil, caraway seed oil, coriander oil, taiga root tincture, fennel oil, common ivy extract (sb), ginseng tincture, anise oil, anise star oil, anise star terpenes and omicha tincture*) |
| **02/03/2023** | The application was split and a new EFSA-Q-2023-00180 was assigned to the preparations included in the present assessment |
| **09/03/2023** | Scientific assessment re-started |
| **22/03/2023** | Opinion adopted by the FEEDAP Panel on anise oil and anise tincture (EFSA-Q-2023-00180). End of the Scientific assessment for the preparations included in the present assessment. The assessment of other preparations belonging to BDG 02 is still ongoing |

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## Abbreviations

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|---|---|
| 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 |
| CAS | Chemical Abstracts Service |
| CEF | EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids |
| CFU | colony-forming unit |
| CG | chemical group |
| CLP | Classification, Labelling and Packaging |
| CoE | Council of Europe |
| CYP450 | cytochrome P450 |
| DAD | diode array detection |
| DM | dry matter |
| EEIG | European economic interest grouping |
| EINECS | European Inventory of Existing Chemical Substances |
| EMA | European Medicines Agency |
| EURL | European Union Reference Laboratory |
| FEEDAP | EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed |
| FEMA | Flavour Extract Manufacturers Association |
| FFAC | Feed Flavourings authorisation Consortium of (FEFANA) the EU Association of Specialty Feed Ingredients and their Mixtures |
| FGE | Flavouring Group Evaluation |
| FLAVIS | the EU Flavour Information System |
| FLAVIS-No | FLAVIS number |
| GC | gas chromatography |
| GC-FID | gas chromatography with flame ionisation detector |
| GC–MS | gas chromatography–mass spectrometry |
| HPLC | High-performance liquid chromatography |
| HPTLC | high-performance thin layer chromatography |
| IFRA | international fragrance association |
| ISO | International standard organisation |
| LOQ | Limit of quantification |
| JECFA | The Joint FAO/WHO Expert Committee on Food Additives |
| MOE | margin of exposure |
| MOET | combined margin of exposure (total) |
| NOAEL | no observed adverse effect level |
| NTP | national toxicology program |
| OECD | Organization for Economic Co-operation and Development |
| PBK | physiologically based kinetic |
| PCB | polychlorinated biphenyl |
| PCDD | polychlorinated dibenzo-p-dioxin |
| PCDF | polychlorinated dibenzofuran |
| PhEur | European Pharmacopoeia |
| sb | Solvent-based |
| SC | EFSA Scientific Committee |
| TEQ | toxic equivalent |
| TTC | threshold of toxicological concern |
| UV | ultraviolet |
| WHO | World Health Organization |