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SCIENTIFIC OPINION

Safety and efficacy of a feed additive consisting of a dry extract obtained from the leaves of *Ginkgo biloba* L. (ginkgo extract) for horses, dogs, cats, rabbits and guinea pigs (FEFANA asbl)

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of a feed additive obtained from the dried leaves of *Ginkgo biloba* L. (ginkgo extract) when used as a sensory additive in feed for horses, dogs, cats, rabbits and guinea pigs. Ginkgo extract contains ≥ 24% total flavonoids, ≥ 6% total terpene lactones and ≤ 1 mg/kg ginkgolic acids. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that ginkgo extract is safe for the target species at the following concentrations in complete feed: 2.8 mg/kg for horses and cats, 1.1 mg/kg for rabbits and guinea pigs, and 3.3 mg/kg for dogs. No safety concern would arise for the consumers from the use of ginkgo extract up to the highest level in feed which is considered safe for food-producing species (horses and rabbits). The additive should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser. The use of the additive at the proposed level in feed for the target species is not considered to be a risk to the environment. While the available data indicate that *Ginkgo* preparations have a distinctive flavour profile, there is no evidence that the ginkgo extract would impart flavour to a food or feed matrix. Therefore, the FEEDAP Panel cannot conclude on the efficacy of the additive.

KEYWORDS

efficacy, flavouring compounds, *Ginkgo biloba* L., ginkgo extract, safety, sensory additives

CONTENTS

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 feed additive shall submit an application in accordance with Article 7 and in addition, Article 10(2) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of 7 years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)[2] for the authorisation/re-evaluation of ten additives (namely juniper oil, juniper berry extract (wb) and juniper tincture from *Juniper communis* L., cedarwood Texas oil from *Juniperus mexicana* Schiede, pine oil and pine tincture from *Pinus pinaster* Soland., pine oil white from *Pinus* spp., e.g. *P. sylvestris* L., pine needle oil from *Abies alba* Mill., *Abies sibirica* Ledeb., ginkgo extract (water-based, wb) and ginkgo tincture from *Ginkgo biloba* L.) belonging to botanically defined group (BDG) 18 – *Gymnosperms (Coniferales, Ginkgoales)* when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). During the assessment, the applicant withdrew the application for three additives.[3] These additives were deleted from the register of feed additives.[4] During the course of the assessment, this application was split and the present opinion covers one out of the seven remaining additives under application: ginkgo extract (*Ginkgo biloba* L.) for all animal species. During the assessment, the applicant requested a change in the species limiting the application for authorisation of ginkgo extract to horses, dogs and cats, rabbits and guinea pigs.[5]

The remaining six additives belonging to botanically defined group (BDG) 18 – Gymnosperms (Coniferales, Ginkgoales) under application are assessed in separate opinions.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 11 February 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 the feed additives consisting of the products ginkgo extract (*G. biloba* L.), when used under the proposed conditions of use (see Section 3.2.1.3).

1.2 | Additional information

Ginkgo extract from *Ginkgo biloba* L. is currently authorised as a feed additive according to the entry in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 (2b natural products – botanically defined). It has not been previously assessed by EFSA as a feed additive.

For human medicinal uses, the European Medicines Agency issued an assessment report and a herbal monograph on *G. biloba* L., folium (EMA, 2015a, 2015b).

Ginkgo leaf (*Ginkgonis folium*) is described as whole or fragmented, dried leaf of *Ginkgo biloba* L. in a monograph of the European Pharmacopoeia 11 (PhEur, 2022a).

Ginkgo dry extract, refined and quantified (*Ginkgonis extractum siccum raffinatum et quantificatum*), is described as refined and quantified dry extract produced from Ginkgo leaf in a monograph of the European Pharmacopoeia 11 (PhEur, 2022b).

2 | DATA AND METHODOLOGIES

2.1 | Data

The present assessment is based on the data submitted by the applicant in the form of a technical dossier[6] in support of the authorisation request for the use of ginkgo extract as a feed additive. The dossier was received on 02 February 2024, and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2024-00063.[7]

[1]Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2]On 13 March 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]Juniper berry extract (wb), pine oil (27 February 2019); pine needle oil (18 March 2021).
[4]Register of feed additives, Annex II, withdrawn by OJ L162, 10.5.2021, p. 5.
[5]On 29 January 2024, the applicant withdrew the application for certain animal species.
[6]FEED dossier reference: FAD-2010-0320.
[7]The original application EFSA-Q-2010-01516 was split on 2 February 2024 and a new EFSA-Q-2024-00063 was generated.

The EFSA Scientific 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.

EFSA has verified the European Union Reference Laboratory report as it relates to the methods used for the control of the phytochemical marker in the additive. In particular, for the characterisation of ginkgo extract the EURL recommended a method based on high-performance liquid chromatography with ultraviolet detection (HPLC-UV) for the determination of the phytochemical marker *total flavone glycosides* in *ginkgo extract*.[8]

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of ginkgo extract is in line with the principles laid down in Regulation (EC) No 429/2008[9] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern (EFSA, 2012), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance on the assessment of the safety of feed additives for the users (EFSA FEEDAP Panel, 2023), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a), Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b) and Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee 2019c).

3 | ASSESSMENT

The additive under assessment, ginkgo extract, is a dry extract obtained from the dried leaves of *Ginkgo biloba* L. It is intended for use as a sensory additive (functional group: flavouring compounds) in feed for horses, dogs, cats, rabbits and guinea pigs.

3.1 | Origin and extraction

Ginkgo biloba L. is a dioecious tree species commonly called the maidenhair tree or simply ginkgo. It is the only living member of the Ginkgoaceae, a family of gymnosperms now mostly extinct and, for this reason, it is often referred to as a 'living fossil'. The fossil records of the Ginkgoaceae indicate a worldwide distribution, but it seems likely that the modern relatives of *G. biloba* originated in China. It is now widely cultivated as an ornamental species in temperate parts of the world. The seeds of Ginkgo are consumed as food, particularly in China, and Ginkgo leaf extracts have a history of use in medicine worldwide.

The additive ginkgo extract under assessment is obtained by soaking the dried leaves of *G. biloba* L. (originating from China) with 60% ethanol/water. After the removal of the ethanol, the solution is adsorbed on a resin that is selected to bind ginkgo flavonol glycosides and terpene lactones. The bound components are eluted from the resin with ethanol, the collected liquid fraction is concentrated by removing the ethanol and then spray dried.[10]

3.2 | Characterisation

3.2.1 | Characterisation of ginkgo extract

The extract under assessment is a yellow (light yellow to brownish yellow) powder, soluble in water (min. 50 g/L) with a bulk density of 550–680 kg/m^3.

The product contains by specification ≥ 24% total flavonoids (selected as the phytochemical marker, expressed as flavonol glycosides), ≥ 6% total terpene lactones and ≤ 1 mg/kg ginkgolic acids. Analysis of five batches showed compliance

[8]The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2010-0320_en.

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

[10]Technical dossier/Supplementary information March 2023.

with the specifications: total flavonoids were on average 28.31% (26.20%–29.92%), total terpene lactones 8.18% (7.12%–8.79%) and ginkgolic acids 0.47 mg/kg (0.19–0.70 mg/kg, analysed in three batches).[11]

Table 1 summarises the results of the proximate analysis of five batches of the additive (of Chinese origin) expressed as % (w/w). The dry matter (DM) represents on average 97.0% of the additive and consists of inorganic material measured as ash (1.25%, on average) and a plant-derived organic fraction, which includes lipids, protein, fibre and sugars.

TABLE 1 Proximate analysis of the additive under assessment, ginkgo extract (*Ginkgo biloba* L.), based on the analysis of five batches. The results are expressed as % (w/w).

Constituent	Mean % (w/w)	Range % (w/w)
Dry matter	97.04	94.7–99.6
Lipids	< 0.1	< 0.1
Protein	3.6	2.8–4.3
Ash	1.25	0.6–2.5
Fibre	< 1.0	< 1.0
Total sugars (as sucrose)[a]	12.2	11.5–13.1
Moisture	2.96	0.40–5.30

[a]Based on the analysis of three batches.

The fraction of secondary metabolites was characterised in the same batches of the additive and the results are summarised in Table 2. Individual compounds were determined by HPLC-UV as described in the European Pharmacopoeia (PhEur, 2022a, 2022b) or by HPLC with refractive index detctor: flavonol glycosides (using quercetin as reference, calculated as flavonol glycoside equivalents), ginkgolic acids and terpene lactones.[12] Organic acids (malic acid, quinic acid, fumaric acid and shikimic acid) were determined by HPLC-UV.[13] Analytical results are expressed as % (w/w), except for ginkgolic acids, which are expressed as mg/kg.

The fraction of secondary metabolites accounts on average for 37.7% of the DM fraction of the extract (range: 33%–40%). Considering the components identified by proximate analysis (sugars accounting on average for 12.2%), about 56% of the DM of the extract (two batches) was characterised. Other plant constituents would account for the remaining 44%.

TABLE 2 Characterisation of the fraction of secondary metabolites of the additive under assessment, ginkgo extract (*Ginkgo biloba* L.), based on the analysis of five batches (mean and range). The results are expressed as % (w/w) of ginkgo extract except ginkgolic acids (as mg/kg).

Constituent	CAS no.	Mean	Range
Ginkgolic acid[a] (mg/kg)	22910-60-7	0.47	0.19–0.70
		% (w/w)	% (w/w)
Flavonol and other flavonoid glycosides		28.31	26.20–29.92
Quercetin glycoside	–	12.77	12.12–13.49
Kaempferol glycoside	–	10.99	10.39–11.47
Isorhamnetin glycoside	–	3.37	3.18–3.72
Other flavonoid glycosides	–	1.71	1.43–5.82
Ginkgolides and derivatives (terpene lactones)		8.18	7.12–8.79
Ginkgolides A, B and C		3.78	2.75–4.71
Ginkgolide A[b]	15291-75-5		2.28
Ginkgolide B[b]	15291-77-7		1.22
Ginkgolide C[b]	15291-76-6		1.21
Bilobalide	33570-04-6	4.40	2.41–5.82
Organic acids other than ginkgolic acids[a]		1.56	0.22–4.21
Malic acid	6915-15-7	0.04	0.02–0.09
Quinic acid	77-95-2	0.03	0.003–0.10
Fumaric acid	110-17-8	0.01	0.004–0.02
Shikimic acid	138-59-0	1.03	0.03–3.77
Vanillic acid	121-24-6	< 0.00001	< 0.00001

(Continues)

[11]Technical dossier/Supplementary information March 2023/Annex_I_Batch_to_batch.
[12]Technical dossier/Supplementary information March 2023/Annex_I_Batch_to_batch.
[13]Technical dossier/Supplementary information March 2023/Annex_II_Organic acids.

TABLE 2 (Continued)

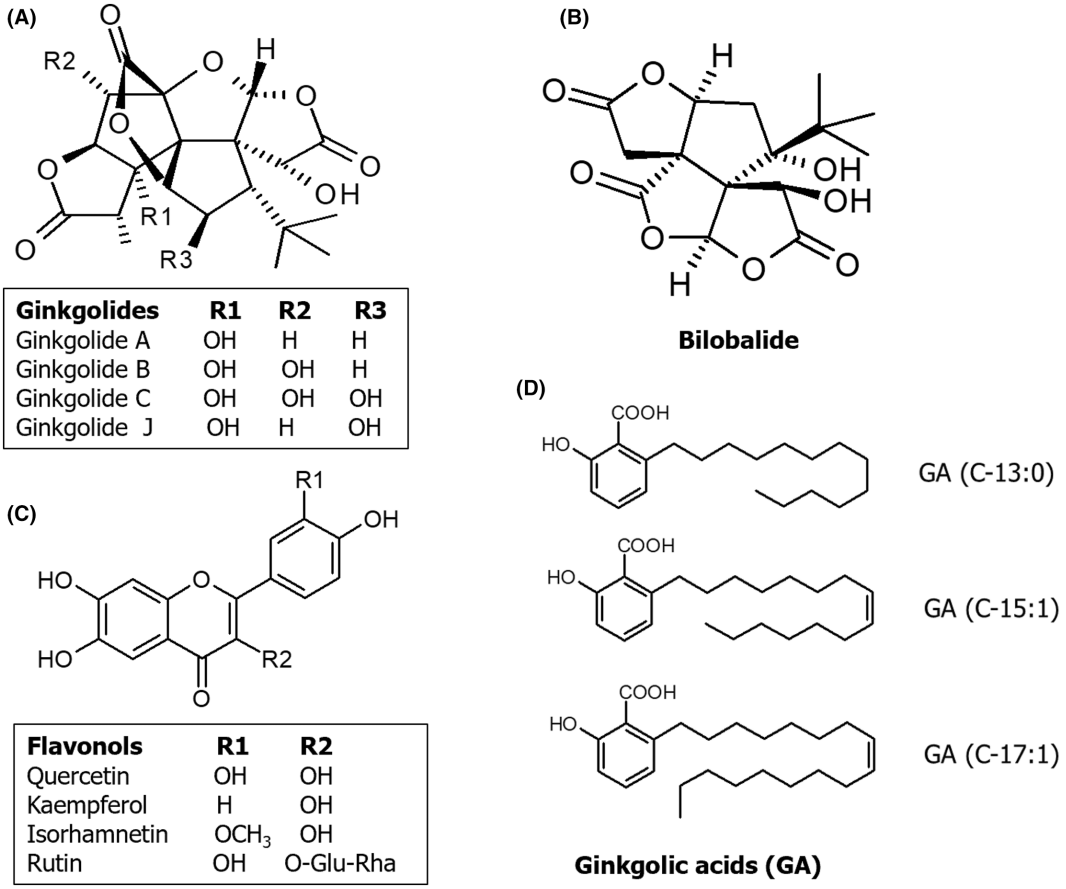
Constituent	CAS no.	Mean	Range
4-Hydroxybenzoic acid	99-96-7	< 0.00001	< 0.00001
Total identified		37.66	33.32–40.36

Abbreviation: CAS, chemical abstracts service.

[a]Determined in three batches.

[b]Separately analysed in one batch.

The structures of the main secondary metabolites, the ginkgolides A, B and C, bilobalide and the flavonols quercetin, kaempferol, isorhamnetin and the flavonol glycoside rutin are shown in Figure 1.



Ginkgolides	R1	R2	R3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH
Ginkgolide J	OH	H	OH

Flavonols	R1	R2
Quercetin	OH	OH
Kaempferol	H	OH
Isorhamnetin	OCH_3	OH
Rutin	OH	O-Glu-Rha

FIGURE 1 Molecular structures of (A) ginkgolides A, B, C and J, (B) bilobalide, (C) flavonols/flavonol glycosides (quercetin, kaempferol, isorhamnetin and rutin) and (D) ginkgolic acids identified in ginkgo extract.

The applicant further investigated minor constituents by HPLC with electrospray ionisation quadrupole time of flight mass spectrometry.[14] The analysis showed that the bisflavones ginkgetin, sciadopitysin and isoginkgetin were below the limit of quantification (LOQ, 0.01%) in three batches of the extract under assessment. Bilobetin was found in one batch at 0.015%.

A detailed analysis of flavonoids and polyphenols using normal phase and reversed phase HPLC with different detectors (UV or UV/VIS-MS/MS or UV-MS/MS) identified the presence of ellagic acid, quercetin, rutin and various catechins (epicatechin and catechin gallate). Anthocyanins and related components were all below the corresponding limit of detection (0.0005 mg/g for anthocyanins and 0.20 mg/g for proanthocyanidin polymers).[15]

A detailed analysis of the additive by liquid chromatography/high-resolution mass spectrometry (LC/HRMS) found 27 different compounds, for which a molecular formula could be deduced from the mass spectrum.[16] A subsequent tiered

[14]Technical dossier/Supplementary information_Annex_III_Minor consitutents.

[15]Technical dossier/Supplementary information/Annex_V_Polyphenols. Limit of detection (LOD): for < 0.20 mg/kg for proanthocyanidin monomer e and polymers ($n = 2–9$); < 0.0005 mg/kg for individual anthocyanes; < 0.016 mg/kg for neohespeiridin, < 0.02 mg/kg for other flavonoids (hesperidin, narirutin, hesperetin, naringin, apigenin-di-C-glycioside and hesperetin 5-O-glycoside), < 0.04 mg/kg for gallocatechin, gallocatechigallate, epicatechingallate, epigallocatechingallate and methyl-epigaloocatechingallate.

[16]Technical dossier/Supplementary information/Annex_VI_LC_HRMS.

search for known compounds reported to occur in ginkgo leaves, allowed to identify the likely presence of several ginkgolides (A, B, J, M, P and Q), argaminolic acid A, urolignoside and several flavonol glycosides.[17]

3.2.1.1 | *Impurities*

Data on impurities were provided for three batches of ginkgo extract. Lead (0.15–0.21 mg/kg) was detected in all batches, arsenic, mercury and cadmium were below the corresponding LOQ in all batches.[18] In the same batches, pesticides were not detected in a multiresidue analysis. When specifically analysed, carbendazim was detected in two batches (0.25 and 0.04 mg/kg), imidacloprid (0.011 mg/kg) and propamocarb (0.186 mg/kg) were detected in one batch. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans, dioxin-like polychlorinated biphenyls (DL-PCBs) and non-DL-PCBs were analysed in the same batches and were below the corresponding LOQs. The calculated upper bound (UB) concentration was 0.115 ng World Health Organization $(WHO)_{2005}$-PCDD/F-toxic equivalent (TEQ)/kg for the sum of PCCD/F, 0.148 ng WHO_{2005}-PCCD/F + PCB TEQ/kg for the sum of PCCD/F and DL-PCBs, and was 1.63 µg/kg for the sum of non DL-PCBs (all values are expressed based on 88% DM).[22] Mycotoxins (aflatoxin B1, deoxynivalenol, zearalenone, T2-toxin, HT2-toxin, ochratoxin A, and fumonisins B1 and B2) were below the corresponding LOQ.[19]

Analysis of microbial contamination of five batches of ginkgo extract indicated that *Salmonella* spp. was absent in 25 g. *Escherichia coli* was < 10 colony forming units (CFUs)/g and *Enterobacteriaceae* was not detected.[20]

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

3.2.1.2 | *Shelf life*

The shelf life of the additive is stated to be at least 12 months, when stored in tightly closed containers under standard conditions. However, no data supporting this statement were provided.

3.2.1.3 | *Conditions of use*

Ginkgo extract is intended for use in complete feed for horses at 25 mg/kg, dogs at 30 mg/kg, and cats, rabbits and guinea pigs at 60 mg/kg.

3.2.2 | Safety

Ginkgo biloba leaf extracts (GBEs) are complex mixtures containing different classes of compounds, such as flavonoids, diterpene trilactones, sesquiterpenes, ginkgolic acids, simple organic acids and others (Chen et al., 2013; Ude et al., 2013).

The applicant carried out structured database searches to identify data related to the safety of *G. biloba* leaves and on their constituents, in particular quercetin and kaempferol.[21] Four cumulative databases (LIVIVO, NCBI, OVID and ToxInfo), 13 single databases including PubMed and Web of Science and 12 publishers' search facilities including Elsevier, Ingenta, Springer and Wiley were used. The literature search (from 1980) was conducted in September 2023. The keywords used covered different aspects of safety and the inclusion and exclusion criteria were provided by the applicant.

Several extracts with similar composition to the additive under assessment were tested in absorption, distribution, metabolism and excretion (ADME) and toxicological studies. The majority of the studies (Chen et al., 2013, 2015; Lin et al., 2014; Maeda et al., 2014; NTP, 2013) were done with different lots of a standardised GBE (GBE50) available on the market for medicinal use and whose composition is very similar to the additive under assessment (Table 3). For other commercial ginkgo extracts tested in ADME studies, the composition is given in the text.

[17]Quercetin-3-(2-glucosylrhamnoside), mearnsetin-3-rutinoside, kaempferol-3-O-[β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranoside, isorhamnetin 3-O-[β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranoside, quercetin-3-O-[4-hydroxy-E-cinnamoyl-(→6)-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranoside], quercetin-3-O-[4-hydroxy-E-cinnamoyl-(→6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranoside], kaempferol-3-O-[4-hydroxy-E-cinnamoyl-(→6)-β-D-glucopyranosyl-(1 → 4)-α-L-rhamnopyranoside] and kaempferol-3-O-[4-hydroxy-E-cinnamoyl-(→6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranoside.

[18]Technical dossier/Supplementary information March 2023/Annex_I_Batch_to_batch. LOQ: < 0.20 mg/kg arsenic, < 0.01 mg/kg for mercury, and < 0.05 mg/kg cadmium. LOQ for individual pesticides: 0.01–0.05 mg/kg. Upper bound concentrations are calculated on the assumption that all values of the different congeners below the limit of quantification are equal to the limit of quantification. TEQ = toxic equivalency factors for dioxins, furans and dioxin-like PCBs established by WHO in 2005 (van den Berg et al., 2006).

[19]Technical dossier/Supplementary information March 2023/Annex_XII_Mycotoxins. LOQ for mycotoxins: < 0.0003 mg/kg for aflatoxin B1, < 0.050 mg/kg for deoxynivalenol and fumonisins B1 and B2, < 0.010 mg/kg for zearalenone, HT2-toxin, T2-toxin, < 0.0005 mg/kg ochratoxin A.

[20]Technical dossier/Supplementary information March 2023/Annex_IX_ Microbiology.

[21]Technical dossier/Supplementary information/December 2023/Annex_2_ELS.

TABLE 3 Comparison between the composition of the standardised *Ginkgo biloba* leaf extract (GBE50) tested in toxicological studies by the National Toxicology Program report (NTP, 2013) and that of the additive under assessment.

Compound	GBE50[a] % (w/w)	Additive under assessment % (w/w)
Ginkgolic acid	0.001045	0.000019–0.000070
Quercetin glycosides	16.71	12.77
Kaempferol glycosides	12.20	10.99
Isorhamnetin glycosides	2.37	3.37
Ginkgolide A	3.74	2.28
Ginkgolide B	1.62	1.22
Ginkgolide C	3.06	1.21
Bilobalide	6.94	4.40

[a]Lot 020703 (NTP, 2013).

The sum of flavonol glycosides and terpene lactones (ginkgolides and bilobalide) represents 31.3% and 15.4% of GBE50, respectively. The standardised extract GBE50 and the additive under assessment were considered similar despite the different concentrations of ginkgolic acids (15- to 55-fold lower in the additive under assessment compared to GBE50).

The biological properties of *GBE*s are dominated by terpene lactones and flavonols (occurring mainly in the form of their glycosides), which make up the majority of the fraction of secondary metabolites. Although ginkgolic acids (salicylic acid derivatives with an unsaturated aliphatic side chain) are highly toxic to liver and kidneys (Ahlemeyer et al., 2001; Baron-Ruppert & Luepke, 2001), their relevance for the toxicity of the additive under assessment is limited because the maximum concentration in the additive is in compliance with the monograph of the PhEur, 2022a, 2022b, which restricts the content of ginkgolic acids in Ginkgo leaf extracts to a maximum of 5 mg/kg. The concentration of ginkgolic acids in three batches of the additive is 0.19–0.70 mg/kg and thus well below the maximum limit of 5 mg/kg set for medicinal uses.

3.2.2.1 | *Absorption, distribution, metabolism and excretion*

The literature search provided by the applicant[22] (see Section 3.2.2) identified pharmacokinetic data in laboratory animals and humans for the main components of GBEs. Although GBEs contain a great variety of compounds belonging to different chemical classes, the biological activity of standardised GBEs (e.g. GBE50 and EGb 761®[23]) is associated with diterpene trilactones, bilobalide and flavonols mainly present as glycosides. The following text will therefore focus on the ADME of these compounds.

The ADME data of *Ginkgo biloba* flavonol glycosides and terpene lactones in experimental animals and humans have been reviewed in the report by the National Toxicology Program (NTP, 2013). Rats were orally administered 30, 55 or 100 mg/kg of a commercial *Ginkgo biloba* extract (EGb 761®) containing 24% flavonol glycosides, 3.1% ginkgolides and 2.9% bilobalide The results showed that bilobalide and ginkgolides A and B were bioavailable with linear pharmacokinetics with half-lives of 2.2, 1.7 and 2.0 h, respectively, for the lowest dose administered (Biber & Koch, 1999, as referenced in NTP, 2013). The metabolism of flavonol glycosides in rat has been investigated by analysing blood, faeces, or urine 24 h after oral administration of a commercial *Ginkgo biloba* extract (composition not given, Pietta et al., 1995). No intact flavonol glycosides were present in urine, faeces or blood samples. The metabolites identified in urine samples were 3,4-dihydroxyphenylacetic acid, benzoic acid, its glycine conjugate (hippuric acid), 3-hydroxyphenylacetic acid, homovanillic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(3-hydroxyphenyl)propionic acid.

The studies described in humans given orally *Ginkgo biloba* extracts (EGb 761®) containing 24% flavonol glycosides, 3.1% ginkgolides and 2.9% bilobalide showed a high bioavailability of bilobalide (approximately 70%), the half-life values of bilobalide and ginkgolides A and B were 4, 6 and 3 h, respectively. These compounds were excreted unchanged in the urine at approximately 30%, 70% and 50%, respectively (unpublished data, as reported in NTP, 2013). After the administration of 9.6-mg terpene lactones from Ginkgoselect® or Ginkgoselect® phytosome (containing 24% flavonol glycosides and 6% total terpenes either as free formulation or phospholipid complex),[24] the maximum plasma concentrations of total terpene lactones (ginkgolides A, B and bilobalide) were 85 and 181.8 µg/mL, respectively, attained at 120 min and 180–240 min (Mauri et al., 2001; as referenced in NTP, 2013).

In 2015, EMA published an assessment report on *Ginkgo biloba* L. describing the pharmacokinetics of a GBE in humans (EMA, 2015a). After the oral administration of 120 mg of a Ginkgo extract (GBE741, containing 22%–27% flavonol glycosides

[22]Technical dossier/Supplementary information/December 2023/References_Request_2.

[23]EGb 761® is standardised to contain 24% 'flavone glycosides' (glycosides of quercetin, kaempferol and isorhamnetin), 6% 'terpene lactones' [3.1% ginkgolides (A, B, C, J) and 2.9% bilobalide], various organic acids (5%–10%) and other constituents (as described in NTP, 2013).

[24]*Ginkgo biloba* extracts containing 24% flavonol glycosides and 6% total terpenes, Ginkgoselect® (free formulation) and Ginkgoselect® phytosome (phospholipid complex).

and 5%–7% terpene lactones),[25] dissolved in water, the mean absolute bioavailability of the lactones was: ginkgolide A (80%), ginkgolide B (88%) and bilobalide (79%). The peak plasma concentrations were 25–33 ng/mL, 9–17 ng/mL and 19–35 ng/mL for ginkgolides A and B and bilobalide, respectively. The related half-life for ginkgolide A was 5 h, for ginkgolide B, 9–11 h and for bilobalide, 3–4 h. When administered in tablets, the peak plasma concentrations were in the range of 16–22 ng/mL for ginkgolide A, 8–10 ng/mL for ginkgolide B and 27–54 ng/mL for bilobalide. The corresponding half-lives of ginkgolides A and B and bilobalide were 3–4, 4–6 and 2–3 h, respectively.

Beyond the studies reviewed in the NTP report (2013) and in the EMA assessment report (EMA, 2015a), a pharmacokinetic study of a GBE in rats was published (Chen et al., 2013). This study was carried out by administering via per os (p.o.) a GBE (GBE50), whose composition is similar to the additive under assessment.[26]

The full pharmacokinetic parameters of **flavonols** (quercetin, kaempferol and isorhamnetin), **diterpene trilactones** (ginkgolide A, B, C and J) and **bilobalide** as the main secondary metabolites of GBE50 were described. After application by gavage a dose of 10, 30 and 90 mg/kg bw of GBE50, the three flavonols showed a biphasic plasma concentration. The first peak appeared after 15 min and was followed by a rapid decrease in the plasma concentration. This occurrence can be attributed to the hydrolysation of the flavonol glycosides in the small intestine and the absorption of the aglycones, subsequently conjugated with glucuronic acid and/or sulfate and excreted. The second peak that appears between 4 and 6 h was followed by a slow decrease in plasma concentration and probably results from the hydrolysis of non-absorbed glycosides by colonic bacteria and absorption of the aglycones with subsequent conjugation and excretion. Repeated oral exposure (7 days, 30 mg/kg bw per day) causes an accumulation of the flavonols in rat plasma. Because of the bimodal concentration curve of the flavonols in plasma after p.o. application, half-life values could not be calculated. Maximum plasma concentrations of total flavonols (free and conjugated) increased linearly with the dose. After the oral application of 10 mg GBE50/kg bw, equivalent to 65.0-µg quercetin, 68.1-µg kaempferol and 14.4-µg isorhamnetin, their plasma concentrations were 1.6, 14.1 and 1.2 µg/mL, respectively. The plasma levels after the doses of 30 and 90 mg GBE50/kg bw were 11.9, 34.8, 4.3 µg/mL and 36.0, 90.7 and 11.7 µg/mL, respectively. Although quercetin and kaempferol were present in equal concentrations in the extract, kaempferol was the dominating flavonol in rat plasma after the oral application of GBE50. This is also reflected by the absorption rates after the application of 10 mg GBE50/kg bw, which were 0.48% for quercetin and 6.58% for kaempferol. Higher doses of GBE50 resulted in an increase of the absorption rate to 2.1% and 9.5% for quercetin and kaempferol at 90 mg GBE50/kg bw. The absorption rate for isorhamnetin was 9.4% and thus equal to that of kaempferol. After the application of 90 mg GBE50/kg bw, flavonol concentrations in tissues were highest in kidney, followed by liver, lung and heart. For all three flavonols, the concentration in liver was equal to the plasma concentration, whereas a higher concentration was observed in kidney (Table 4). Quercetin showed nearly constant kidney/plasma ratios around 2 in kidney at 1 and 4 h, whereas an increase from 1.25 to 2.85 and a decrease from 3.6 to 1.14 was observed for kaempferol and isorhamnetin, respectively. Quercetin and kaempferol were present in blood mainly as their glucuronide derivatives.

TABLE 4 Concentrations of flavonoids, ginkgolides and bilobalide in tissues (kidney and liver), plasma concentrations and tissue/plasma ratio after an application of an oral dose of 90 mg GBE50/kg bw in rats.

	Kidney (mg/kg)	Liver (mg/kg)	Plasma (mg/L)	Kidney/plasma	Liver/plasma
Quercetin, 30 min	0.052	0.024	0.024	2.167	1.000
Quercetin, 4 h	0.071	0.032	0.030	2.333	1.067
Kaempferol, 30 min	0.186	0.149	0.149	1.250	1.000
Kaempferol, 4 h	0.326	0.114	0.114	2.850	1.000
Isorhamnetin, 30 min	0.094	0.026	0.026	3.611	1.000
Isorhamnetin, 4 h	0.071	0.062	0.062	1.140	1.140
Ginkgolide A, 30 min	2.165	3.512	0.327	6.625	10.750
Ginkgolide B, 30 min	0.849	2.433	0.141	6.000	17.200
Ginkgolide C, 30 min	0.145	0.361	0.031	4.714	11.714
Ginkgolide J, 30 min	0.283	2.121	0.113	2.500	18.750
Bilobalide, 30 min	2.806	2.806	0.718	3.909	3.909

Urine and faeces samples were collected before and at 0–8, 8–24 and 24–32 h after a p.o. dose of GBE50 at 90 mg/kg bw and analysed by liquid chromatography–tandem mass spectrometry. Several flavonol glycosides and flavonol aglycone

[25]The extract GBE741 contained several chemical constituents, among which the two main constituents were 'flavone glycosides' (total 22.0%–27.0%), represented by the glycosides of quercetin, kaempferol and isorhamnetin, and 'terpene lactones' (total 5.0–7.0), represented by ginkgolides A, B, C (2.8%–3.4%) and bilobalide (2.6%–3.2%). The content of ginkgolic acids is limited with max. 5 ppm.

[26]In the publication by Chen et al. (2013), the test item was described to contain the following concentrations of flavonols and ginkgolides expressed as µmol/10 mg: 2.149 quercetin, 2.378 kaempferol and 0.665 isorhamnetin (as aglycones); 0.551 ginkgolide A, 0.325 ginkgolide B, 0.316, ginkgolide C, 0.105 ginkgolide J and 0.945 bilobalide. The concentrations of the flavonol glycosides were calculated from the flavonol aglycons according to the methods described in the PhEur (PhEur, 2022a, 2022b) and USP (US-Pharmacopoeia, 2015). The concentrations of flavonol glycosides and ginkgolides were converted as % (w/w). The test item contained 36.6% flavonol glycosides (16.3%, 16.6% and 3.7% as glycosides of quercetin, kaempferol and isorhamnetin) and 8.58% ginkgolides (2.25% ginkgolide A, 1.38% ginkgolide B 1.39%, ginkgolide C, 0.45% ginkgolide J and 3.11% bilobalide).

conjugates as well as the sulfated kaempferol glycoside were recovered in rat bile. Flavonol glycosides and glucuronides were recovered in urine but few were found in the faeces.

The **diterpene trilactones** ginkgolide A, B, C, J and **bilobalide** showed a rapid absorption after oral application to rats of 10 mg GBE50/kg bw with a single maximum plasma concentration peak after 15–30 min. The half-life value of bilobalide and ginkgolide B was 0.9 h. The corresponding values for ginkgolide A and ginkgolide C were 1.4 and 3 h, respectively. Higher doses did not change the elimination kinetics significantly. The bioavailability of bilobalide and ginkgolide A was about 60%, whereas the corresponding values for the other ginkgolides were lower (ginkgolide B = 33%–40%, ginkgolide C = 5%–10%, ginkgolide J = 33%–34%). A linear increase in the plasma concentrations of the ginkgolides and bilobalide was observed after the application of 10, 30 or 90 mg GBE50/kg bw. The highest plasma concentrations were obtained for bilobalide (50 μg/mL at 10 mg GBE50/kg bw, 139 μg/mL at 30 mg GBE50/kg bw and 442 μg/mL at 90 mg GBE50/kg bw). The maximum plasma concentrations of ginkgolide C were only 2.8 μg/mL and 21 μg/mL after the application of 10 or 90 mg GBE50/kg bw, respectively. The corresponding values for ginkgolide A were 20 μg/mL and 194 μg/mL, and 8 μg/mL and 75 μg/mL for ginkgolide B. The ginkgolides showed liver/plasma ratios between 3.9 (bilobalide) and 18.7 (ginkgolide J) at 30 min. The corresponding maximum and minimum values for kidneys were 2.5 (ginkgolide J) and 6.6 (ginkgolide A) at 30 min. Ginkgolides are not metabolised in vivo in rats, except for the methylation of ginkgolide C, and most of the absorbed compounds are excreted unchanged via the kidneys. The values in muscle tissues (monitored in heart) were considerably lower. After the p.o. administration of 90 mg/kg GBE50, about 30% of intact bilobalide was excreted in urine. The biliary excretion was very low. Terpene lactones were found to be eliminated from the body very rapidly. Therefore, no accumulation in tissues is expected.

A study in Beagle dogs receiving, by oral administration, ginkgolide extracts (6.0 mg/kg) containing 28% (w/w) ginkgolide A and 63% (w/w) ginkgolide B showed that the bioavailability of the ginkgolides A and B increased significantly when the extracts were given together with food (Aa et al., 2018). The absorption rate for ginkgolide A and ginkgolide B increased from 34.8% and 5.2% in fasted dogs to 78.6% and 17.0% in dogs receiving the compounds together with a meal. Also, the area under the curve (AUC) of the compounds was significantly higher in fed animals. The acidification of the solution of ginkgolides prior to application reduced the bioavailability. Reduction in the pH-value shifts the equilibrium from the prototype lactones to the open acid form, which is more hydrophilic and thus less permeable for biological membranes. As the acidity of the stomach is higher in carnivorous than in herbivorous animals, it can be expected that horses, rabbits and guinea pigs have a higher systemic exposure to ginkgolides than cats and dogs at the same feed concentration.

The influence of feed on the absorption of ginkgolides present in ginkgo extracts was also demonstrated in rats given orally 6 mg/kg bw of a standardised ginkgo product, containing 98.54% of ginkgo terpenoids (27.69% of bilobalide, 37.15% of ginkgolide A, 22.04% of ginkgolide B and 11.66% of ginkgolide C; Huang et al., 2014). Compared to the fasted group, the $t_{1/2}$ values and AUC_{0-t} and $AUC_{0-\infty}$ for bilobalide and ginkgolides A, B and C in fed rats were significantly higher ($p < 0.05$), while the C_{max} values were significantly lower ($p < 0.05$). Compared to the fasted group, the $t_{1/2}$ values and AUC_{0-t} and $AUC_{0-\infty}$ for bilobalide and ginkgolides A, B and C in fed rats were significantly higher ($p < 0.05$), while the C_{max} values were significantly lower ($p < 0.05$).

3.2.2.2 | *Toxicological studies*

The literature search provided by the applicant[27] (see Section 3.2.2) identified in vitro and in vivo genotoxicity studies and toxicological studies with GBEs, as well as with single compounds present in the extract under assessment. The studies considered relevant were evaluated by the FEEDAP Panel and reported below.

Genotoxicity and carcinogenicity

For fully defined mixtures, the EFSA Scientific Committee (EFSA SC) recommends applying a component-based approach, that is, assessing all components individually for their genotoxic potential using all available information, including read-across and quantitative structure–activity relationship (QSAR) considerations (EFSA SC, 2019b). Therefore, the potential genotoxicity of identified constituents is first considered. Then, in vitro genotoxicity studies performed with GBEs similar in composition to the additive under assessment are considered.

Quercetin

In vitro studies showed a genotoxic activity of quercetin in bacterial and mammalian cells where gene mutations (Lin et al., 2014; Meltz & MacGregor, 1981; Resende et al., 2012) and structural chromosomal aberrations were induced (Caria et al., 1995; Carver et al., 1983) at concentrations not increasing the level of reactive oxygen species (ROS; Lin et al., 2014). These effects were possibly related to the intercalating potential of the planar molecule of quercetin (Solimani, 1996) and the induction of double strand breaks (Lin et al., 2014) resulting from the inhibition of topoisomerase II activity (Chen et al., 2015).

Inconsistent results were observed in vivo after oral and intraperitoneal (i.p.) treatment with quercetin. No increase in the frequency of micronuclei was observed in the bone marrow after the oral administration of quercetin over the dose

[27]Technical dossier/Supplementary information/December 2023/References_Request_2.

range of 10–1000 mg/kg bw (MacGregor et al., 1983). The micronucleus test in rodent erythrocytes gave positive results following i.p. treatment at doses up to 1250 mg/kg bw (da Silva et al., 2002; Sahu et al., 1981). The FEEDAP Panel notes that the studies by i.p. administration are of limited relevance.

The complex picture observed in vivo is possibly related to the rapid metabolism of quercetin leading to different results after oral and i.p. treatments. After oral application, the absorption rate is low, and the absorbed quercetin will be rapidly transformed to the glucuronide and excreted or metabolised to smaller molecules. Because of the high hydrophilicity, the conjugated quercetin cannot enter the cells and does not express the genotoxic activity. On the other hand, the clastogenic effect observed after i.p. treatment clearly shows that the genotoxic activity of quercetin is due to the aglycone, which could reach the target site (i.e. bone marrow) unconjugated after i.p. injection. Therefore, only unmetabolised free quercetin could have a genotoxic activity. Crebelli et al. (1987) demonstrated that only about 0.6% quercetin is not metabolised and appears in the excreta (urine and faeces) in rats. In this study, quercetin was extracted from urine and showed mutagenic activity in an in vitro test in bacterial cells. However, in another study no DNA damage was observed by an in vivo comet assay in the spleen and bone marrow from mice treated by gavage with quercetin at 8 or 80 mg/kg bw, once a day, for 40 consecutive days (Papiez et al., 2008). These negative results showed that the genotoxic effects observed in vitro are not expressed in vivo after systemic exposure. This is also in agreement with the absence of observed carcinogenic activity of this compound in several chronic feeding experiments in rats and mice (Ito et al., 1989; Saito et al., 1980; Takanashi et al., 1983).

Kaempferol

Kaempferol induced gene mutations in a bacterial mutagenicity test in the presence of metabolic activation (Resende et al., 2012) and in a mouse lymphoma assay performed with L5178Y cells (Carver et al., 1983; Lin et al., 2014). A statistically significant increase in the level of DNA strand breaks was observed in vitro in the HepG2 cell line together with the activation of the DNA damage response pathways and inhibition of topoisomerase II activity (Chen et al., 2015) at concentrations of kaempferol not influencing the level of ROS production (Lin et al., 2014). The induction of structural chromosomal aberrations was reported by Carver et al. (1983). Mice treated i.p. with kaempferol up to 800 mg/kg bw showed a statistically significant increase in the frequency of micronuclei in bone marrow erythrocytes at 200 and 400 mg kaempferol/kg bw. The dose of 800 mg kaempferol/kg bw was lethal to the animals (Sahu et al., 1981). Like quercetin, kaempferol did not induce malignant tumours in ACI rats after chronic application of 0.04% in the diet (Takanashi et al., 1983). The requirement of metabolic activation of kaempferol in order to induce mutagenicity suggests that the compound is not a DNA intercalator itself but has to be transformed to quercetin by oxidation of the B-ring of the flavonol structure.

Ginkgolic acids

The genotoxic potential of three ginkgolic acids (C13:0, C15:1, C17:1) was investigated in vitro by the Ames fluctuation assay and with the hypoxanthine phosphoribosyl transferase assay in rodent V79 cells by Berg et al. (2015). No mutagenic effects were detected in the Ames test in a concentration range between 0.16 and 5 mM (corresponding to 36 µg/mL and 1.73 mg/mL for GA-C15:1). An additional in vitro study showed that ginkgolic acids induced DNA strand breaks in primary rat hepatocytes at 1 µg/mL and above but no DNA repair synthesis. The DNA strand breaks were possibly induced via the production of oxygen radicals as suggested by the increase of DNA strand breaks observed when applying the formamidopyrimidine-DNA glycosylase (fpg)-modified comet assay (Westendorf & Regan, 2000). In this respect, it was shown that ginkgolic acid treatment induced mitochondrial fragmentation, decreased the mtDNA copy number and the levels of proteins involved in the maintenance of mitochondrial function, impaired the production of mitochondrial adenosine 5′-triphosphate and oxygen consumption (Wang et al., 2019). Since mitochondrial dysfunctions are associated with an increased production of ROS, the results obtained in this study support the hypothesis that the genotoxicity of ginkgolic acids is based on oxidative stress.

Ginkgolides and bilobalide

The main ginkgolides A, B, C, J and bilobalide present in the additive under assessment were tested for their genotoxic potential in the mouse lymphoma assay with L5178Y cells and a comet assay detecting DNA strand breaks (Lin et al., 2014). None of the compounds showed an effect in either assay. Oxidative stress was observed after treatment of the cells with bilobalide at 1 and 2 mM (corresponding to 326.3 and 652.5 µg/mL) during 4 h, whereas the ginkgolides reduced the ROS formation at the same concentration and time. ROS induced apoptosis was also observed in 3T3-L1 mature adipocytes in culture at treatment with bilobalide (Bu et al., 2023).

Ginkgo biloba leaf extracts

GBEs have been tested in various studies. The composition of GBE50, the test item used in the mutagenicity, carcinogenicity and 90-day studies described in NTP (2013) and in other studies described below (Chen et al., 2015; Lin et al., 2014; Maeda et al., 2014) is described in Table 3.

GBE50 induced gene mutations in a bacterial mutagenicity test performed in Salmonella Typhimurium (TA98 and TA100) and *E. coli* (WP2 *uvrA*/pKM101). The concentrations ranged from 1.0 to 10 mg/plate in the presence and the absence of metabolic activation (NTP, 2013).

The positive mutagenic response was confirmed in vitro in a mouse lymphoma assay performed with L5178Y cells, showing a concentration-dependent increase in the mutant frequency induced by GBE50 in the absence of metabolic activation (Lin et al., 2014). In the same study, eight single components of GBE50 (quercetin, kaempferol, isorhamnetin, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J and bilobalide) were also investigated. From the single compounds, only quercetin and kaempferol showed positive effects at concentrations ≥ 9 and 14 µg/mL, respectively. As 1 mg/mL GBE50 contains 7 µg/mL quercetin and 6 µg/mL kaempferol, it is likely that the two flavonols are responsible for the genotoxicity observed. In the frame of this study, GBE50 induced a statistically significant increase in DNA double strand breaks at 1.0 mg/mL, as measured in vitro by the neutral comet assay. Chromosomal damage was observed by the loss of heterozygosity (LOH) analysis of mutants. GBE50 triggered the activation of the DNA damage response associated with the increased levels of γ-H2AX and phosphorylated Chk2 and Chk1. In addition, treatment with GBE50 significantly increased the production of ROS and decreased glutathione levels in L5178Y cells at 0.4 mg/mL and above, suggesting a key role of oxidative stress in the genotoxic effects observed at pro-oxidant concentrations of GBE50. Consistent results were reported by Silva et al. (2019) showing the major involvement of ROS in the increase of DNA strand breaks induced by GBE treatment in vitro through the use of the fpg-modified comet assay detecting oxidised DNA bases. An additional mode of action was identified in a study performed by Chen et al. (2015) showing in vitro an inhibitory effect on topoisomerase II activity induced by GBE50.

In a NTP study (NTP, 2013), GBE50 was tested for carcinogenesis in 2-year studies in rats and mice. Groups of 50 male and 50 female rats received 0, 100, 300 or 1000 mg GBE50/kg bw per day by oral gavage. Groups of 50 male and 50 female mice received by gavage 0, 200, 600 or 2000 mg GBE50/kg bw per day. At the end of the studies, tissues from more than 40 sites were examined for every animal. Rodents dosed with GBE50 showed increased rates of a variety of lesions in the liver, thyroid gland and nose. These lesions included hypertrophy in the liver and thyroid gland in rats and mice, liver hyperplasia in male and female rats, hyperplasia, atrophy and metaplasia of the epithelium in the nose of male and female rats.[28] Increased incidences of cancers of the thyroid gland were seen in female rats and liver cancers in male and female mice. Mononuclear cell leukaemia was observed in male rats at 300 and 1000 mg/kg bw per day.[29]

The increased incidence of mononuclear cell leukaemia in Fisher rats is probably a background finding, as this is a strain of rats with a highly variable background rate in historical controls for this tumour. No malignant tumours were observed in the nose, but benign respiratory epithelium tumours (adenoma) were observed in two female rats at the intermediate dose level (300 mg/kg bw per day). These findings were considered equivocal as the occurrence of this rare adenoma in rats was not dose related and occurred only in females. In addition, dose-dependent morphological changes were observed in the nose. It should be noted that these non-neoplastic lesions (transitional epithelium hyperplasia, respiratory epithelium hyperplasia, olfactory epithelium atrophy, respiratory metaplasia, nerve atrophy and pigmentation) may have been secondary effects, following, for example, enzyme induction via systemic exposure (EMA, 2015a).

As discussed in the NTP report, 'The thyroid gland was another important target for Ginkgo biloba extract toxicity and the pathology at this site is consistent with a hepatic microsomal enzyme induction mechanism of toxicity. Increased thyroid stimulating hormone (TSH) levels were observed in all male dosed groups and the high dose female special study rats following 14 weeks of Ginkgo biloba extract administration. In the 3-month rat study, follicular cell hypertrophy was observed in dosed male and female rats' (NTP, 2013).

As pointed out by EMA (2015a), the relation of thyroid lesions to increased metabolic activity in liver is well known in rodents, and rats are especially sensitive to that mechanism. This is in line with study results where hepatic effects were more severe in mice than in rats, while thyroid effects were more pronounced in rats (Hernandez et al., 2009; Li et al., 2009; Silva Lima & Van der Laan, 2000).

The in vivo genotoxicity of *Ginkgo biloba* leaf extract (GBE50, same lot tested in the above mentioned NTP study) was investigated applying several methods, including a gene mutation assay using *gpt* (guanine phosphoribosyltransferase) delta transgenic mice, and a combined liver comet assay and bone marrow micronucleus assay using constitutive androstane receptor (CAR) knockout and wild-type mice (Maeda et al., 2014). The results of the transgenic mice study showed no biologically relevant increases in *gpt* or *Spi* – mutation frequencies in the liver DNA of *gpt* delta mice following exposure to GBE50 up to 2000 mg/kg bw per day. The results of the comet and micronucleus assays showed no statistically significant increases in the percentage of DNA tail (% Tail DNA) and in the number of micronucleated polychromatic erythrocytes at

[28]Male rats: hepatocyte hypertrophy (1/50, 17/50, 26/50, 27/50); bile duct hyperplasia (32/50, 43/50, 46/50, 46/50); thyroid gland hypertrophy (13/50, 37/50, 41/49, 41/45); respiratory epithelium hyperplasia (14/50, 28/49, 45/49, 35/50), respiratory epithelium atrophy (1/50, 26/49, 37/50, 31/50); transitional epithelium hyperplasia (2/50, 18/49, 43/49, 31/50); olfactory epithelium, respiratory metaplasia (9/50, 30/49, 40/49, 32/50).
Female rats: hepatocytes hypertrophy (7/50, 15/50, 27/50, 33/50); bile duct hyperplasia (11/50, 31/50, 31/50, 33/50); thyroid gland hypertrophy (15/49, 41/50, 45/49, 48/49); respiratory epithelium hyperplasia (9/49, 9/49, 19/50, 34/46), olfactory epithelium atrophy (0/49, 18/49, 25/50, 37/46); transitional epithelium hyperplasia (0/49, 6/49, 32/50, 36/46); olfactory epithelium, respiratory metaplasia (8/49, 4/49, 32/50, 37/46). Male mice: liver hypertrophy (3/50, 19/50, 35/50, 23/50); thyroid gland hypertrophy (2/49, 0/49, 2/50, 38/50). Female mice: liver hypertrophy (1/50, 18/50, 37/50, 37/50); thyroid gland hypertrophy (1/49, 5/48, 9/49, 39/48).

[29]Male rats: mononuclear cell leukaemia (9/50, 12/50, 22/50, 21/50). Female rats: thyroid gland, follicular cell adenoma (1/49, 0/50, 3/49, 1/49), follicular cell carcinoma (0/49, 0/50, 1/49, 1/49), follicular cell adenoma or carcinoma combined (1/49, 0/50, 4/49, 2/49). Male mice: hepatocellular adenoma (31/50, 46/50, 33/50, 33/50); hepatocellular carcinonoma (22/50, 31/50, 41/50, 47/50); hepatoblastoma (3/50, 28/50, 36/50, 38/50); hepatocellular adenoma, hepatocellular carcinonoma or hepatoblastoma (39/50, 48/50, 48/50, 49/50). Female mice: hepatocellular adenoma (17/50, 37/50, 41/50, 48/50); hepatocellular carcinonoma (9/50, 10/50, 15/50, 44/50); hepatoblastoma (1/50, 1/50, 8/50, 11/50); hepatocellular adenoma, hepatocellular carcinonoma or hepatoblastoma (20/50, 39/50, 41/50, 49/50).

doses up to 2000 mg/kg bw per day of GBE50 in either mouse genotype. The authors concluded that GBE50-induced hepatocarcinogenesis in mice is most likely not due to a direct DNA reactive mode of action.

Supporting evidence comes from studies investigating the involvement of enzyme induction via CARs in GBE-induced hepatocarcinogenesis and liver hypertrophy. These studies show a relevant role of this nuclear receptor for these effects (Maeda et al., 2015; Ueda et al., 2002). These mechanisms may also contribute to toxic effects seen in the thyroid gland via increased glucuronidation and excretion of thyroid hormones and stimulation of the hypothalamus-pituitary-thyroid axis (Maeda et al., 2015; Silva Lima & Van der Laan, 2000). In contrast to rats, humans are less sensitive to this mechanism (Shizu & Yoshinari, 2020). However, the sensitivity of most other species is unknown and other target species may be sensitive.

Overall conclusion on genotoxicity and carcinogenicity

Genotoxic effects of Gingko leaf extracts were observed in bacteria and cultured mammalian cells. Two main mechanisms could be elucidated to be responsible for the effects: oxidative stress and DNA intercalation of flavonols, mainly quercetin. Ginkgo leaf extract is demonstrated to have antioxidative properties at low concentrations and pro-oxidative properties at higher concentrations. Although ginkgolic acids are able to produce oxygen radicals, it is questionable whether these compounds are involved in the toxicity of GBE50 and of the additive, because their concentration in the extracts used for the investigations was very low. Thus, oxidative stress is more likely due to bilobalide and flavonols, such as quercetin, which may also function as pro-oxidative agents at higher concentrations via its catechol moiety.

Investigations with flavonols confirmed the genotoxic potential of these compounds. Quercetin, kaempferol and galangin showed mutagenicity in bacterial systems. The mutagenicity of quercetin did not require metabolic activation, whereas the two other compounds were only mutagenic with metabolic activation. The reason for the difference in activity is most probably the enzymatic oxidation of the B-ring, which transforms kaempferol and galangin into quercetin. Quercetin is known to intercalate into DNA and to cause the formation of DNA double strand breaks by interfering with topoisomerase II. While genotoxic effects of quercetin could be consistently demonstrated in several in vitro systems, this was not observed in vivo after oral administration. The reason may be the rapid conjugation of quercetin to the glucuronide and excretion or further metabolism to smaller molecules. As the conjugation of quercetin and other flavonoids is important for the detoxification, animals with a genetic lack of glucuronyl transferases, such as Felidae, may have an enhanced sensitivity to the toxic properties of flavonols.

The 2-year study in mice and rats (NTP, 2013) has shown that oral treatment of the animals with a *Ginkgo biloba* leaf extract resulted in the formation of liver and thyroid tumours.

The FEEDAP Panel concluded, based on the available literature, that the genotoxicity of *Ginkgo biloba* leaf extracts observed in vitro is not expressed in vivo. The tumours observed in the long-term study with GBE50 similar to the additive under assessment are most probably the result of a thresholded non genotoxic carcinogenic mode of action. They are most likely not due to a direct DNA reactive mode of action but rather caused via oxidative stress or/and enzyme induction via CARs.

Subchronic toxicity studies with GBEs

No toxicity study with the additive was provided by the applicant; however, studies with extracts similar to the additive (e.g. GBE50) are available in the literature.

A 90-day study was made as part of the NTP of toxicity studies in rats and mice. Groups of 10 male and 10 female rats and mice received GBE50 in corn oil by gavage at doses of 0, 62.5 (rats only), 125, 250, 500, 1000 or 2000 (mice only) mg/kg bw 5 days per week for 14 weeks. In male rats, slight but significant increases in total protein and albumin, and a decrease in alkaline phosphatase (ALP) were observed at doses of 125 mg/kg bw and higher, and a decrease in alanine aminotransferase (ALT) was observed for all doses. In female rats, a decrease in ALP at all doses and an increase in ALT at doses of 125 mg/kg bw and higher were observed. Hypertrophy and fatty change[30] of the liver were observed in male rats at all doses and in females at 500 and 1000 mg/kg bw. Follicular hypertrophy was observed in male rats at doses ≥ 250 mg/kg bw and in females at 1000 mg/kg bw. Because of the liver changes in male rats at 62.5 mg/kg bw, a no observed adverse effect level (NOAEL) could not be established.

Toxic effects of *Ginkgo biloba* leaf extract on the thyroid gland were described by Abdellah et al. (2007) using a standardised extract from the Egyptian market (composition not available). Groups of 20 rats received 40 mg/kg or 500 mg/kg of the extract in corn oil for 90 days. Thyroid hormones (T3 and T4) and TSH were measured at the beginning and the end of the study and histopathology of the thyroid gland was performed at the end of the study. The application of the extract had no influence on the level of the hormones. In the low-dose group, thyroid glands showed disorganised follicles of varying diameters with little amount of the colloid in some follicles while others demonstrated absent colloid with desquamated epithelial cells in their lumens. In the high-dose group, thyroid glands were composed of very small follicles. Some follicles had no apparent lumina. Follicular cells were found in more than one layer (adenoma) with the infiltration of interfollicular spaces by fatty cells.

The data described above indicate that the lesions in thyroid and liver are due to induction of liver enzymes.

[30]Fatty change consisted of small numbers of scattered midzonal hepatocytes having small to a few large, clear, discrete intracytoplasmic vacuoles that had the typical appearance of lipid droplets filling most or all of the cytoplasm (NTP, 2013).

Conclusions on toxicology

Based on the available studies, a NOAEL could not be identified. The FEEDAP Panel considered 62.5 mg GBE50/kg bw per day, derived from the 90-day study, as the lowest observed adverse effect level (LOAEL) for male rats, and applied an additional uncertainty factor (UF) of 3 to extrapolate from the LOAEL to a NOAEL of 21 mg/kg bw per day. The magnitude of the UF was selected considering (i) that liver changes were detected in male rats at all doses but were observed in females only at doses ≥ 500 mg/kg bw per day and (ii) the involvement of enzyme induction in liver hypertrophy. In such circumstances, a factor of 3 is considered by the FEEDAP Panel as sufficient for deriving a NOAEL. In addition, a UF of 3 was considered necessary to account for the effects seen in the long-term studies and the uncertainty in the relevance of mode of action (liver and thyroid enzyme induction) in the target species. Therefore, an overall UF of 10 is applied to derive a reference point of 6.25 mg/kg bw per day to be used in the assessment.

3.2.2.3 | *Safety for the target species*

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

Because of the similarity of the *Ginkgo biloba* extract (GBE50) tested by NTP with the ginkgo extract under assessment (see Table 3), the FEEDAP Panel applied the reference point of 6.25 mg/kg bw per day to derive maximum safe feed concentrations of the additive for the target species.

Following the EFSA Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), a UF of 100 was applied to the reference point. The glucuronidation of the hydroxylated or oxygenated metabolites of the individual constituents of ginkgo extract is an important metabolic pathway facilitating the excretion of these compounds (see Section 3.2.2.1). Generally, for cats, an additional UF of 5 is applied, considering their unusually low capacity for glucuronidation of compounds (Court & Greenblatt, 1997; Lautz et al., 2021). Because the reference point was derived applying an additional UF of 10 to the LOAEL, the resulting UF of 1000 was considered adequate. For guinea pigs, the applicant proposed to apply the same default values as for rabbits. The calculated maximum safe feed concentrations of ginkgo extract for the target species are shown in Table 5.

TABLE 5 Maximum safe concentration of ginkgo extract (*Ginkgo biloba* L.) in feed for target animal species and categories calculated using the reference point of 6.25 mg/kg bw per day derived from a 90-day toxicity study in rats (NTP, 2013).

Animal category	Daily feed intake (g DM/kg bw)	Proposed use level in feed (mg/kg complete feed)[a]	Maximum safe concentration (mg/kg complete feed)[a]
Horses	20	25	2.8
Rabbits	50	60	1.1
Guinea pigs	50	60	1.1
Dogs	17	30	3.3
Cats	20	60	2.8

[a]Complete feed containing 88% DM.

Based on the available evidence, the FEEDAP Panel cannot conclude on the safety of the maximum use levels proposed by the applicant. The calculated safe concentrations in complete feed for the target species are 2.8 mg/kg for horses and cats, 1.1 mg/kg for rabbits and guinea pigs and 3.3 mg/kg for dogs.

3.2.2.4 | *Safety for the consumer*

No data on residues in products of animal origin (horse and rabbit meat) were made available for any of the constituents of ginkgo extract.

The pharmacokinetic study in rat orally exposed to 90 mg GBE50/kg bw per day (see Section 3.2.2.1) showed that the absorbed flavonoids were metabolised and excreted. Similarly, the terpene lactones (ginkgolides and bilobalide) were found to be eliminated from the body very rapidly. No accumulation in tissues is expected for the main components of ginkgo extract. Therefore, the FEEDAP Panel considers that it is unlikely that the use of the additive would result in a relevant intake of the individual constituents by humans consuming horse and rabbit meat.

No safety concern would arise for the consumer from the use of ginkgo extract up to the highest level in feed considered safe for the target species (horses and rabbits).

3.2.2.5 | *Safety for the user*

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

The additive contains low concentrations (< 1 mg/kg) of ginkgolic acids, which are contact allergens (Hori et al., 2004). In addition, other potent allergens, the urushiols may be present in extracts of *Ginkgo biloba* leaves if not efficiently removed in the manufacturing process (Schötz, 2004).

The applicant provided information according to Classification, Labelling and Packaging Regulation (EC) 1272/2008[31] concerning the presence of ginkgolic acids in the extract.[32]

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

3.2.2.6 | *Safety for the environment*

Although *Ginkgo biloba* is not native to Europe, the plant was introduced into Europe centuries ago.

The expected accumulation into the environment of *Ginkgo biloba* constituents from excreta from horses and rabbits given the additive is very low.

Among the main constituents of the extract, flavonols (quercetin, kaempferol, isorhamnetin) and the corresponding flavonol glycosides, as well as organic acids other than ginkgolic acids (malic acid, quinic aid, fumaric acid, shikimic acid, etc.) are ubiquitous compounds naturally present in feed and food and therefore are not expected to be of concern for the environment.

The extract under assessment also contains terpene lactones (ginkgolides and bilobalide). Because of the degradation of terpene lactones by the metabolism in the target animals and environmental processes and considering the limited environmental exposure of excreta from horses and rabbits, the use of the additive in feed for horses and rabbits at the proposed conditions of use is not expected to pose a risk to the environment.

Therefore, the FEEDAP Panel considers ginkgo extract added to horse and rabbit feed as safe for the environment.

3.3 | Efficacy

Ginkgo biloba L. or its extracts are not listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009) or by the Flavour and Extract Manufactures Association. However, infusions of Ginkgo leaves are widely consumed as a tea and other extracts may be used in food supplements. The applicant provided data in the form of sensory evaluations describing the taste and aroma characteristics of such infusions.

In a sensory evaluation performed by trained panellists (Biernacka et al., 2023), the sensory profiling of the flavour and smell of *G. biloba* leaf infusion was described to have a distinct herbal flavour and a bitter smell. The applicant also provided a sensory analysis of the extract under assessment based on a triangle test performed according to DIN EN ISO 4120:2021-06.[33] Five panellists identified the following aroma profile: chamomile/herbal, tobacco, hay/dry grass, jute sack and sweet/caramel. Aqueous solutions of the additive ginkgo extract at concentrations of 0.3% and 0.5% were described as having an intense aroma, significantly different from plain water.

While the available data indicate that *Ginkgo* preparations have a distinctive flavour profile, there is no evidence that the ginkgo extract would impart flavour to a food or feed matrix. Therefore, the FEEDAP Panel cannot conclude on the efficacy of the additive.

4 | CONCLUSIONS

The following conclusions apply to ginkgo extract, which contains < 1 mg/kg ginkgolic acid and is obtained from the dried leaves of *Ginkgo biloba* L.

The additive is safe for the target species at the following concentrations in complete feed: 2.8 mg/kg for horses and cats, 1.1 mg/kg for rabbits and guinea pigs, and 3.3 mg/kg for dogs.

The use of the additive up to the highest level in feed which is considered of no concern for target animals (horses and rabbits) is also expected to be of no concern for consumers.

Ginkgo extract should be considered as an irritant to skin and eyes, and as a dermal and respiratory sensitiser.

The use of the additive at the proposed use level in feed for horses and rabbits is not considered to be a risk to the environment.

While the available data indicate that *Ginkgo* preparations have a distinctive flavour profile, there is no evidence that the ginkgo extract would impart flavour to a food or feed matrix. Therefore, the FEEDAP Panel cannot conclude on the efficacy of the additive.

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

[32]Ginkgolic acids: skin irritation 2 (H315), eye irritation 2 (H319), STOT SE 3 (H335), skin sensitisation 1 (H317).

[33]Technical dossier/Supplementary information December 2023/Annex_8_Sensory analysis.

5 | DOCUMENTATION PROVIDED TO EFSA/CHRONOLOGY

Date	Event
05/11/2010	Dossier received by EFSA. Botanically defined flavourings from Botanical Group 18 – Gymnosperms (Coniferales, Ginkgoales) for all animal species and categories. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)
14/12/2010	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
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
11/02/2019	Application validated by EFSA – Start of the scientific assessment
20/02/2019	Request of supplementary information to the applicant in line with Article 8(1) (2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety for target species, safety for the consumer, safety for the user and environment*
13/05/2019	Comments received from Member States
18/03/2021	Partial withdrawal by applicant (EC was informed) for the following additive: pine needle oil
10/03/2023	Reception of supplementary information from the applicant (partial dataset: ginkgo extract) – Scientific assessment remains suspended
14/03/2023	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives- Scientific assessment restarted
25/07/2023	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization, safety for the consumer, efficacy*
04/12/2023	Reception of supplementary information from the applicant (partial dataset: ginkgo extract and ginkgo tincture). Scientific assessment restarted
02/02/2024	The application was split and a new EFSA-Q-2024-00063 was assigned to the additive included in the present assessment. Scientific assessment re-started for the additives included in the present assessment
14/03/2023	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment for the additive included in the present assessment.

ABBREVIATIONS

ADME	absorption, distribution, metabolism and excretion
AUC	area under the curve
bw	body weight
CAR	constitutive androstane receptor
CAS	chemical abstracts service
CFU	colony forming unit
DL	dioxin-like
DM	dry matter
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
fpg	formamidopyrimidine-DNA glycosylase
FL-no	FLAVIS number
GBE	*Ginkgo biloba* leaf extract
GBE50	Standardised *Ginkgo biloba* leaf extract
HPLC	high-performance liquid chromatography
i.p.	intraperitoneal
LC/HRMS	liquid chromatography/high-resolution mass spectrometry
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
NOAEL	no observed adverse effect level
NTP	National Toxicology Program
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PhEur	European Pharmacopoeia
p.o.	per os
ROS	reactive oxygen species
TEQ	toxic equivalent
UB	upper bound
UF	uncertainty factor

UV	ultraviolet
WHO	World Health Organization

CONFLICT OF INTEREST

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

REQUESTOR

European Commission

QUESTION NUMBER

EFSA-Q-2010-01516 (new EFSA-Q-2024-00063)

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