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SCIENTIFIC OPINION

Modification of the terms of authorisation of the feed additive consisting of canthaxanthin for chickens for fattening, minor poultry species for fattening, laying poultry and poultry reared for laying, ornamental fish and ornamental birds and ornamental breeder hens to include canthaxanthin produced with *Yarrowia lipolytica* CBS 146148 (DSM Nutritional Products Ltd.)

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

Following a request from the European Commission, the European Food Safety Authority was asked to deliver a scientific opinion on the proposed modification of the terms of the authorisation of canthaxanthin, regarding the addition of a new production route, by the yeast *Yarrowia lipolytica* CBS 146148. The additive is already authorised as sensory feed additive for use in feed for chickens for fattening, minor poultry species for fattening, laying poultry, poultry reared for laying, ornamental fish, ornamental birds and ornamental breeder hens. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concludes that canthaxanthin produced with *Yarrowia lipolytica* CBS 146148 is considered safe for the target species, the consumer and the environment under the current authorised conditions of use. Canthaxanthin is not an irritant to skin or eyes and it is unlikely to be a skin sensitiser. No conclusion can be reached on the respiratory sensitisation of canthaxanthin. In the absence of data, no conclusions on the safety for the user can be reached for any preparation produced with canthaxanthin. Canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is efficacious as a colouring agent in feed for chickens for fattening and minor poultry species for fattening, laying poultry and poultry reared for laying, ornamental fish and ornamental birds.

KEYWORDS

canthaxanthin, colourants, efficacy, safety, sensory additives, *Yarrowia lipolytica* CBS 146148

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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 13(3) of that Regulation lays down that if the holder of an authorisation proposes changing the terms of the authorisation by submitting an application to the Commission, accompanied by the relevant data supporting the request for the change, the Authority shall transmit its opinion on the proposal to the Commission and the Member States.

The European Commission received a request from DSM Nutritional Products Ltd.,[2] represented in the EU by DSM Nutritional Products Sp. Z o.o, for modification of the terms of authorisation of the additive consisting of canthaxanthin (produced by chemical synthesis) when used as a feed additive for chickens for fattening and minor poultry species for fattening, laying poultry and poultry reared for laying (category: sensory additives; functional group: colourants (ii) substances which, when fed to animals, add colours to food of animal origin) and for ornamental fish and ornamental birds except ornamental breeders hens, ornamental breeder hens (category: sensory additives; functional group: colourants (iii) substances which favourably affect the colour of ornamental fish or birds). The modification consists in the change of the manufacturing process, to include canthaxanthin produced by fermentation with *Yarrowia lipolytica* CBS 146148.

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 13(3) (modification of the authorisation of a feed additive). The dossier was received on 15 March 2021 and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2021-00149. The particulars and documents in support of the application were considered valid by EFSA as of 19 May 2022.

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 additive consisting of canthaxanthin produced by *Y. lipolytica* CBS 146148, when used under the proposed conditions of use (see Section 3.1.6).

1.2 | Additional information

Canthaxanthin is currently authorised as a sensory feed additive (functional group: colourants) for use in feed for chickens for fattening, minor poultry species for fattening, laying poultry, poultry reared for laying, ornamental fish, ornamental birds and ornamental breeder hens (2a161g).[3] A preparation of canthaxanthin (CAROPHYLL® Red 10%) is also authorised as zootechnical additive for use in feed for breeder hens (4d161g).[4] Both authorisations refer to canthaxanthin produced via chemical synthesis, while canthaxanthin produced by fermentation is currently not authorised.

The FEEDAP Panel adopted two opinions on the use of canthaxanthin, one as colourings (EFSA FEEDAP Panel, 2014) and one as zootechnical feed additive (EFSA FEEDAP Panel, 2013).

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[5] in support of the authorisation request for the use of canthaxanthin produced by *Y. lipolytica* CBS 146148, as a feed additive.

The confidential version of the technical dossier was subject to a target consultation of the interested Member States from 26 May 2021 to 26 August 2021; the comments received were considered for the assessment.

The FEEDAP Panel 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.

The European Union Reference Laboratory (EURL) considered that the conclusions and recommendations reached in the previous assessment regarding the methods used for the control of the canthaxanthin in animal feed are valid and applicable for the current application.[6]

[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]DSM Nutritional Products Ltd., represented in the EU by DSM Nutritional Products Sp. Z o.o., Tarczyńska 113, 96–320 Poland.
[3]COMMISSION IMPLEMENTING REGULATION (EU) 2015/1486 of 2 September 2015 concerning the authorisation of canthaxanthin as feed additive for certain categories of poultry, ornamental fish and ornamental birds, OJ L 229, 3.9.2015, p. 5–8.
[4]COMMISSION IMPLEMENTING REGULATION (EU) No 684/2014 of 20 June 2014 concerning the authorisation of canthaxanthin as a feed additive for breeder hens (holder of the authorisation DSM Nutritional products Ltd). OJ L 182, 21.6.2014, p. 20.
[5]FEED dossier reference FAD-2021-0014.
[6]The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/reports-and-technical-documentation/fad-2008-0048_en.

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess modification of the terms of the authorisation of the additive consisting of canthaxanthin produced with *Y. lipolytica* CBS 146148 is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018) and Guidance on the assessment of the safety of feed additives for the users (EFSA FEEDAP Panel, 2023).

3 | ASSESSMENT

Canthaxanthin produced by chemical synthesis is currently authorised, for use as a sensory additive (functional group: colourant (ii) substances which, when fed to animals, add colours to food of animal origin in feed), for chickens for fattening and minor poultry species for fattening and laying poultry and poultry reared for laying. It is also authorised under the functional group: colourant (iii) substances which favourably affect the colour of ornamental fish or birds in feed for ornamental fish and ornamental birds and ornamental breeder hens. The applicant is requesting a modification of the current authorisation, to include canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148.

3.1 | Characterisation

3.1.1 | Manufacturing process

The additional manufacturing process for the active substance canthaxanthin includes the fermentation step, the pasteurisation of the fermentation broth, the spray-drying of the biomass, the extraction of canthaxanthin from the biomass and the final crystallisation.[8]

The current authorisation for canthaxanthin states that synthetic canthaxanthin may be placed on the market and used as an additive consisting of a preparation. For the current application, the applicant stated that canthaxanthin, either synthetic or produced by fermentation, will not be placed in the market as such but only in the form of a preparation and provided an example of a commercial preparation (see Section 3.1.4).

3.1.2 | Characterisation of the production organism

Canthaxanthin is produced by a genetically modified strain of *Y. lipolytica* which is deposited [redacted] with deposit number CBS 146148.[9]

The taxonomic identification of the production strain as *Y. lipolytica* was confirmed using whole genome sequence (WGS) data [redacted].[10]

3.1.2.1 | *Characteristics of the parental microorganisms*

[redacted].[11]

3.1.2.2 | *Characteristics of the introduced sequences*

[redacted].[12]

[7]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.

[8]Technical dossier/Section II/ Sect_II_Identity.

[9]Technical dossier/Section II/Annex II.4.

[10]Technical dossier/Section II/Annex II.4.

[11]Technical dossier/Section II/Annex II.4.

[12]Technical dossier/Section II/Annex II.4.

3.1.2.3 | *Structure of the genetic modification*

The structure of the genetic modification has been determined by comparing the WGS data of the production strain [redacted]

- [redacted]
- [redacted]
- [redacted]
- [redacted]

[redacted]. The absence of antimicrobial resistance genes or vector sequences in the production strain was demonstrated by the WGS analysis.

3.1.3 | Characterisation of the active substance

Canthaxanthin produced by fermentation is a crystalline powder of violet-brown colour and has a very low solubility in water (0.053 mg/L). The applicant proposed the same specifications for purity as for synthetic canthaxanthin i.e. minimum 96% and content of carotenoids other than canthaxanthin as 'not more than 5 % of total colouring matters'.

Compliance with the purity as defined in the specifications was shown in five batches with an average of 99% canthaxanthin (98%–100%).[13]

The same batches were analysed[14] for the content of carotenoids other than canthaxanthin. Results showed compliance with the specifications resulting in an average of 4% (range 4%–5%). Further chromatographic analysis indicated that the main carotenoids identified are metabolic precursors of canthaxanthin.[15] The most abundant are echinenone (around 2.5%) and 4-keto-gamma carotene (4-KGC; around 1.5%), followed by minor amounts of beta-carotene and gamma-carotene (below 0.4%), and traces of lycopene (around 0.05% or below). The sum of unknown carotenoid impurities (HPLC-UV/VIS) represents 0.1–.3% area, individually all below 0.2% area.

The comparison of the carotenoids profile of the active substance produced by fermentation and by chemical synthesis (EFSA FEEDAP Panel, 2014) showed that the two canthaxanthins are equivalent. The FEEDAP Panel concludes that the data submitted support the read across of the toxicological profile obtained with the synthetic canthaxanthin to the one produced by fermentation.

Levels of dichloromethane and [redacted] were measured in five batches and were on average [redacted] the applicant proposes to set a limit for dichloromethane in the preparation (i.e. 80 mg/kg in 10% canthaxanthin preparation; see Section 3.1.4). [redacted]

The applicant further investigated the presence of impurities other than carotenoids that could derive from the fermentation ingredients, such as amino acids, lipids and residues of the antifoaming agent. Proximate analyses on five batches indicate very low residual amounts of amino acids (0.01%–0.08%) and free fatty acids (0.9%–1.1%); mono-, di- or triglycerides of fatty acids were not detected. No residues of the anti-foaming agent were detected.[17]

Five batches of the active substance were analysed for cadmium, mercury, lead and arsenic which were below their respective limit of quantification (LOQ).[18]

No data were provided on the levels of mycotoxins and microbial contaminants. However, considering the raw materials and the manufacturing process, the FEEDAP Panel considers that microbial contamination/presence of mycotoxins is unlikely.

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

The presence of viable cells of the production strain was investigated in three batches of an intermediate concentrate from the pilot scale production in triplicate. [redacted]

[13]Technical dossier/Section II/Annex_II_1. Sum of total carotenoids expressed as canthaxanthin – UV spectrophotometry.

[14]Analysed by high performance liquid chromatography (HPLC) method using spectrophotometric detection.

[15]Technical dossier/Section II/Annex_II_3.

[16]Technical dossier/Section II/Annex_II_1.

[17]Technical dossier/Section II/Annex II_8. The limit of detection (LOD) was not indicated.

[18]Technical dossier/Section II/Annex_II_1, and Supplementary information December 2022. LOQs in mg/kg were: 0.1 for lead cadmium and arsenic and 0.01 for mercury.

[redacted] No viable cells of the production strain were detected in the analysed samples.[19]

The presence of DNA from the production strain was investigated in three batches of the intermediate concentrate from the pilot scale. Samples of 1 g were analysed in triplicate. [redacted] No DNA was detected. The limit of detection was 10 ng/g of product.[20]

3.1.4 | Characterisation of the additive/final preparation

The current authorisation of the additive containing synthetic canthaxanthin specifies limit for triphenylphosphine oxide (TPPO) of ≤ 100 mg/kg and dichloromethane of 600 mg/kg.

The specification of TPPO is considered relevant only for canthaxanthin produced by chemical synthesis but not for canthaxanthin produced by fermentation.

The current authorisation states that canthaxanthin may be placed on the market and used as an additive consisting of a preparation. The applicant stated that canthaxanthin will not be placed in the market as such but only in the form of a preparation and provided an example of a preparation containing 10% canthaxanthin, 4.4% butylated hydroxytoluene (BHT), 10% dextrin (yellow), 15% corn starch and 60.6% lignosulfonate.[21]

The analysis of five batches of this preparation containing canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 showed an average value of 10.9% canthaxanthin (range: 10.3%–11.5%).[22] The loss on drying was in the range of 5.3%–7.4%.[23]

The applicant proposed to set the specification for dichloromethane of ≤80 mg/kg in this preparation. Dichloromethane levels (five batches) were in the range of 25–66 mg/kg.[24] These concentrations are well below the level of set in the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) GL18(R2) (VICH, 2023) and the current limit in the authorisation (600 mg/kg), and are therefore, considered to be of no concern.

The preparation appears as a red-violet free-flowing granulated powder ('beadlet'). Three batches of the preparation were tested for its physical properties. The average loose density resulted to be 640 kg/m^3, the average tapped density 750 kg/m^3. The particle size distribution was analysed by laser diffraction method; the results showed that the average particle size ranged from 333 to 381 μm and no particles below 150 μm were detected in any of the batches.[25] The dusting potential was determined using the Stauber-Heubach method and resulted an average of 0.88 g/m^3 (range: 0.45–1.43 g/m^3) (mg airborne dust per m^3 of air).

Further investigation was performed by the applicant on the presence of small/nanoparticles providing data on particle size/solubility in line with the Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA SC, 2021). [redacted]

3.1.5 | Stability and homogeneity of the commercial preparation

The applicant provided stability and homogeneity studies with the preparation described above formulated with canthaxanthin from fermentation with *Y. lipolytica* CBS 146148. In addition, the shelf-life of the additive containing synthetic canthaxanthin was also studied in the same experiment.

3.1.5.1 | *Shelf-life*

Stability testing was carried out at 25°C/60% relative humidity (RH) and at 40°C/75% RH with the preparation formulated with either synthetic (one batch) or fermentation-derived canthaxanthin (three batches). The recovery of canthaxanthin after 12-month storage was 90% (synthetic) and 95% (average of three batches, fermentation based) at 25°C/60% RH and 90% (synthetic) and 92% (average of three batches, fermentation based) at 40°C/75% RH.[26]

[19]Technical dossier/Section II/Annex II_4.
[20]Technical dossier/Supplementary Information October 2021/DSM Canthaxanthin 0014 SIn – Annex.
[21]The applicant markets this preparation with the trade name CAROPHYLL® Red 10%.
[22]Technical dossier/Section II/Annex II.2.
[23]Technical dossier/Section II/Annex II.2.
[24]Technical dossier/Section II/Annex II.2.
[25]Technical dossier/Section II/Annex_II_11.
[26]Technical dossier/Section II/Annex_12.

3.1.5.2 | *Stability in premixtures and feedingstuffs*

The stability of the preparation (three batches) in a vitamin and mineral premixture (containing 5.4% choline chloride) was studied when supplemented at 1 g canthaxanthin/kg and stored at 25°C for 1, 3 and 6 months and at 30°C for 1 month. Recoveries of canthaxanthin after 3 months were on average 81% (range: 79%–85%) and after 6 months were on average 67% (range: 63%–70%) when stored at 25°C. Recoveries of canthaxanthin after 1 month were 91% in all batches when stored at 30°C.[27]

The stability of the preparation (three batches) in two wheat-based compound feeds pelleted at 90°C, was studied when supplemented at 20 mg canthaxanthin/kg feed, stored in plastic bags at 20–25°C with 50%–60% RH for 3 months. Recoveries of canthaxanthin after the end of storage period ranged from 82% to 102% in one feed and from 82% to 86% in the other feed. The pelleting process did not result in a loss of canthaxanthin content.[28]

3.1.5.3 | *Homogeneity*

The capacity for homogeneous distribution of the preparation was studied in 10 subsamples of the mash feed (one batch) and in 10 subsamples of the pelleted feed (two batches). The supplementation level was 20 mg canthaxanthin/kg feed. The coefficient of variation of the analysis of the canthaxanthin concentration resulted in 5.3% for the mash feed and ranged from 6.2 to 7.0% for the pelleted feed.[29]

3.1.6 | Conditions of use

The applicant is proposing to keep the same conditions of use as the ones for synthetic canthaxanthin (min.96%) currently authorised.[30]

- chickens for fattening and minor poultry species for fattening at a maximum content of 25 mg/kg complete feed,
- laying poultry, poultry reared for laying and ornamental breeder hens at a maximum content of 8 mg/kg complete feed
- ornamental fish and ornamental birds except ornamental breeder hens at a maximum content of 100 mg/kg complete feed.

Maximum residue limits (MRLs) are also set in the authorising regulation as follows:

- Poultry 15 mg canthaxanthin/kg liver (wet tissue) and 2.5 mg canthaxanthin/kg skin/fat (wet tissue)
- Laying poultry 30 mg canthaxanthin/kg egg yolk (wet tissue)

3.2 | Safety

The safety of the chemically synthetised canthaxanthin as a colouring agent for poultry and for ornamental birds and ornamental fish was previously evaluated (EFSA FEEDAP Panel, 2014). In that opinion, the FEEDAP Panel concluded that synthetic canthaxanthin was considered safe under the proposed conditions of use for the target species, the consumer and the environment. In the absence of data, canthaxanthin was considered an irritant to skin and eyes, a skin sensitiser and hazardous by inhalation.

For the present assessment, the applicant made reference to the previous evaluations (EFSA FEEDAP Panel, 2013, 2014), provided a literature search covering the safety aspects of the additive related to the safety of canthaxanthin for the target animal and toxicology, submitted a new tolerance study in chickens for fattening and new genotoxicity studies performed with the preparation described in Section 3.1.4 containing 10% canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148. For the user safety, the applicant made reference to the studies evaluated in the opinion adopted by EFSA FEEDAP Panel (2013).

The FEEDAP Panel notes that, in the commercial preparation described above, BHT is included, which is at present authorised for all animal species and it is currently under re-evaluation.

[27]Technical dossier/Section II/Annex_11 and Supplementary information January 2023/Annex 2.

[28]Technical dossier/Section II/Annex_11 and Supplementary information January 2023/Annex 2.

[29]Technical dossier/Section II/Annex_11.

[30]The authorising regulation states under 'other provisions': (1) Canthaxanthin may be placed on the market and used as an additive consisting of a preparation. (2) The mixture of canthaxanthin with other carotenoids and xanthopylls shall not exceed 80 mg/kg of complete feed. (3) For safety: breathing protection, safety glasses and gloves should be worn during handling.

3.2.1 | Safety of the production strain

The production strain belongs to a species, *Y. lipolytica*, considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach when used for production purposes (EFSA BIOHAZ Panel, 2024). The identity of the strain has been confirmed and the genetic modifications do not introduce safety concerns. In addition, no viable cells or DNA of the production strain were detected in an intermediate concentrate representing the additive. Therefore, the use of *Y. lipolytica* CBS 146148 in the production of canthaxanthin is considered of no concern with regard to the production strain and/or potential fermentation residues that might be present in the final additive.

3.2.2 | Literature search

The applicant provided two literature searches covering the period 2014–2020. The first one was carried out in SCOPUS and PubMed and focused on the safety of canthaxanthin for the target species. A total of 58 papers were identified, of which 17 were considered relevant by the applicant.[31] The second one was conducted on MEDLINE, FSTA and TOXCENTER and focused on the toxicology of canthaxanthin. The safety for the environment was not included in the literature search. A total of 288 hits were retrieved of which 15 were considered relevant by the applicant.[32]

The FEEDAP Panel reviewed the relevant papers and concluded that none of them identified safety concerns of canthaxanthin for the target species or the consumer under the current conditions of authorization.

3.2.3 | Tolerance study in chickens for fattening

A total of 576 1-day-old male chickens (Ross 308) were distributed to 48 pens and randomly allocated to four experimental groups (12 birds/pen; 12 pens/treatment).[33,34,35] The study followed a three-phase feeding programme with starter (from day 1 to day 14), grower (from day 15 to day 28) and finisher (from day 29 to day 35) diets. Diets were based on wheat, maize and soya bean meal. The diets were either not supplemented (control) or supplemented with the preparation to provide 15 (0.6× maximum authorised level), 30 (1.2×) or 90 (3.6×) mg of canthaxanthin/kg complete feed. Analytical values of canthaxanthin in the diets (see Table 1) confirmed that the concentrations were below the intended values leading to 0.5×, 0.9× and 2.8× relative to the maximum authorised use level. Feed in pelleted form and water were offered ad libitum. General bird health status and mortality were monitored daily. Birds' weight and feed consumption were determined at 1, 14, 28 and 35 days per pen and average daily weight gain, daily feed intake and feed to gain ratio were calculated. Blood samples for haematology[36] and clinical chemistry[37] were taken on day 35 from two preselected birds per pen. At the end of the study, two chickens per pen were killed and necropsied for gross pathology[38] examination. Tissue samples from selected organs[39] were taken for histopathology analysis.

The experimental unit was the pen for performance parameters whereas for blood biomarkers, organ necropsy and histopathology parameters, the bird was the experimental unit. The non-inferiority of treated groups relative to the control group was tested by one-sided parametric *t*-tests in normally distributed parameters and by non-parametric Wilcoxon tests instead of *t*-tests in non-normally distributed parameters. Organ weights were tested with a parametric linear model with treatment as fixed factor (normality distributed variables) or by a non-parametric Kruskal–Wallis test (not normally distributed variables). Blood parameters were assessed by linear mixed effect model with treatment group as fixed effect and floor pen as random effect, using a compound-symmetric variance–covariance structure. Significance was set at $p < 0.05$. The main results are shown in Table 1.

[31]Technical dossier/Section III/Annex III-1 and Annex III-1bis.

[32]Technical dossier/Section III/Annex-III-4_literature-toxicology_FIZ33996.

[33]Technical dossier/supplementary information September 2023/DSM Canthaxanthin SIn x2 (FAD-2021-0014 and FAD-2021-0015) – Appendix.

[34]Technical dossier/supplementary information September 2023/DSM Canthaxanthin SIn x2 (FAD-2021-0014 and FAD-2021-0015) – Annex.

[35]Technical dossier/supplementary information January 2024/ DSM Canthaxanthin SIn x2 - Safety 2024 (FAD-2021-0014 and FAD-2021-0015) – Annex.

[36]Total count for red blood cells, packed cell volume, haematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total counts for leucocytes and differential counts for leucocytes and platelet counts as thrombocytes for birds.

[37]Sodium, potassium, chloride, calcium, phosphate, magnesium, total protein, albumin, globulin, glucose, urea, uric acid, cholesterol, creatinine, bilirubin, acute phase protein, amylase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, alkaline phosphatase and creatine kinase.

[38]Lungs, liver, kidney, heart, spleen and different parts of the gastro-intestinal tract.

[39]Spleen, bursa of Fabricius, liver, duodenum, jejunum, ileum, Peyer's patches, colon and caecum.

TABLE 1 Results of the tolerance study with diets supplemented with canthaxanthin in chickens for fattening (35 days).

	Canthaxanthin (mg/kg complete feed, intended)			
	0	**15**	**30**	**90**
Canthaxanthin (mg/kg feed)[1]				
Analysed, starter (1–14 days)	ND	11.1 ± 0.4	20.6 ± 2.1	64.2 ± 4.9
Analysed, grower (15–28 days)	ND	12.7 ± 0.9	23.6 ± 2.3	73.7 ± 0.4
Analysed, finisher (28–35 days)	ND	11.9 ± 0.4	23.7 ± 1.0	69.5 ± 0.1
Performance parameters				
Mortality (%)/(n/144 birds)	2.8(4)	0.7(1)	1.4(2)	1.4(2)
Final body weight (g/bird)	2028	2047	2022	2018
Feed intake (g/bird)	3038	3102	3060	2960
Average weight gain (g/bird)	1984	2003	1978	1974
Feed to gain	1.53	1.55	1.55	1.50
Blood haematology and clinical chemistry				
Total bilirubin (μmol//L)	0.9	1.9	6.6*	13.4*
Total protein (g/L)	28	28	30*	33*
Albumin (g/L)	12.3	12.7	13.7	15.1*
Albumin/Total protein (%)	44.8	44.6	45.3	45.8

Abbreviation: ND, Not detected.

*Means with an asterisk differed statistically from the control group ($p < 0.05$).

[1]Limit of detection (LOD) was 10 mg/kg.

Mortality was low and not affected by treatment. None of the performance, haematological or gross pathology parameters were affected by the supplementation with canthaxanthin at any levels.

Some increases in the total bilirubin, total protein and albumin in blood were found in the animals that received canthaxanthin compared to control. Such increases could be attributed to interferences in the colorimetric analytical methods used, that used wavelengths close to the canthaxanthin maximum absorbance. The Panel further considers that the diagnostic value of bilirubin in poultry is low as birds have little biliverdin reductase activity in liver, and therefore, biliverdin is the main biliary pigment in birds. Nevertheless, the bilirubin values remain in the physiological range (Okpogba et al., 2019; Ukpanukpong et al., 2019). The absence of liver lesions in gross pathology and histology confirmed further the absence of possible negative effects of these increases seen in the levels of several serum parameters.

Overall, the 35-day tolerance study provided no adverse effects at any dose level of the additive.

3.2.4 | Genotoxicity studies

In the previous evaluations, no concern for genotoxicity was identified for the synthetic canthaxanthin (EFSA FEEDAP Panel, 2013, 2014; EFSA ANS Panel, 2010).

Considering that the purity of canthaxanthin from *Y. lipolytica* CBS 146148 is comparable with the purity of synthetic canthaxanthin (Section 3.1.3), the conclusions reached for synthetic canthaxanthin in previous FEEDAP Panel assessments can be extended to the canthaxanthin produced by *Y. lipolytica* CBS 146148. Moreover, the strain under assessment qualifies for the QPS approach and is considered safe.

In addition, the applicant submitted genotoxicity studies performed with the preparation CAROPHYLL® Red 10% containing 10% canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148, which are reported below.

Bacterial reverse mutation test

In order to investigate the potential of the test item (above-mentioned example of preparation of CAROPHYLL® Red 10%) to induce gene mutations in bacteria, an Ames test was performed in Salmonella Typhimurium strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA in accordance with OECD Guideline 471 in a study claimed to be compliant with good laboratory practices (GLP).[40] Two independent experiments were performed applying the plate incorporation and preincubation methods both in the presence and in the absence of metabolic activation. Six concentrations were tested ranging from 33 to 5000 μg/plate (corresponding to 3.3–500 μg canthaxanthin/plate). No toxicity was observed in any experimental condition. No increase in the mean number of revertant colonies was observed in any tested condition in any

[40]Technical dossier/Section III/Annex III_6.

tester strain. The FEEDAP Panel concludes that the test item did not induce gene mutations in bacteria under the experimental conditions applied in the study.

In vitro mammalian cell micronucleus test

To evaluate the potential of the test item (above-mentioned example of preparation CAROPHYLL® Red 10%) to induce chromosomal damage, an in vitro micronucleus test was carried out in human peripheral blood lymphocytes according to OECD Test Guideline 487 (2016), in a study claimed to be compliant with GLP.[41] The highest concentration tested was limited by the occurrence of precipitation at 213 μg/mL after short treatment (4 h + 16 h of recovery) and 107 μg/mL after continuous treatment (20 h + 20 h of recovery), corresponding to 21.3 and 10.7 μg/mL of canthaxanthin, respectively. Cytochalasin B (Cyt B) was added 20 hours after the beginning of treatment and the cells were cultured for additional 20 hours (in total 88 h of culturing). Therefore, in the short-term treatment, a 16-hour recovery period in the absence of CytB was applied. The Panel noted that this is a deviation from the OECD TG 487, recommending the addition of CytB at the end of treatment. The long-term test was performed in line with OECD TG 487. No cytotoxicity and no increase of the frequency of micronuclei were observed in binucleated cells after treatment with the test item in any experimental condition. The FEEDAP Panel concludes that the test item did not induce structural and numerical chromosomal aberrations in human peripheral blood lymphocytes under the experimental conditions applied in this study.

Conclusions on genotoxicity

The FEEDAP Panel noted that the two in vitro studies were performed with the formulated additive containing only 10% of the active substance canthaxanthin and, thus, considered the results of limited relevance.

In addition, the FEEDAP Panel took into account that the conclusions reached for synthetic canthaxanthin in previous opinions raising no concern for genotoxicity (EFSA FEEDAP Panel, 2013, 2014; EFSA ANS Panel, 2010) could be extended to canthaxanthin produced by *Y. lipolytica* CBS 146148 since the purity of canthaxanthin from *Y. lipolytica* CBS 146148 is comparable with the purity of synthetic canthaxanthin (Section 3.1.3). Moreover, the FEEDAP Panel noted that the strain under assessment qualifies for the QPS approach and is considered safe. Based on all the available data, the FEEDAP Panel concludes that canthaxanthin produced with *Y. lipolytica* CBS 146148 does not raise concern for genotoxicity.

3.2.5 | Safety for the user

With regard to user safety, the applicant made reference to the studies evaluated in the opinion adopted on synthetic canthaxanthin as zootechnical additive (EFSA FEEDAP Panel, 2013). In that opinion, the FEEDAP Panel concluded that canthaxanthin was not an irritant to skin or eyes and it is unlikely to be a skin sensitiser.

Considering the dusting potential of the commercial preparation, exposure by inhalation is likely. No data on the safety for the user for this preparation were provided; therefore, no conclusions on the safety for the user can be reached for any preparation produced with canthaxanthin.

3.2.6 | Assessment of the safety of the additive

The use of *Y. lipolytica* CBS 146148 in the production of canthaxanthin is considered of no safety concern.

Considering that canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is equivalent to the canthaxanthin produced by chemical synthesis, the safety of canthaxanthin produced by fermentation can be based on the read across of the metabolic/residues and toxicological profile obtained with the synthetic canthaxanthin without the need of additional studies. The new tolerance study conducted with an example of preparation containing canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 provided supporting evidence that it is safe for the target species.

No new studies were found in the literature that would change the previous conclusions on the safety of the canthaxanthin for the target species and consumer.

The FEEDAP Panel considers that the conclusions previously reached for synthetic canthaxanthin with regard to target animals, consumer and environment, also apply to canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148.

3.2.7 | Conclusions on the safety

The FEEDAP Panel concludes that canthaxanthin produced with *Y. lipolytica* CBS 146148 is considered safe for the target species, the consumer and the environment under the current authorised conditions of use for synthetic canthaxanthin.

[41] Technical dossier/Section III/Annex III_7.

Canthaxanthin is not an irritant to skin or eyes and it is unlikely to be a skin sensitiser. No conclusion can be reached on the respiratory sensitisation of canthaxanthin. In the absence of data, no conclusions on the safety for the user can be reached for any preparation produced with canthaxanthin.

3.3 | Efficacy

In 2014, the FEEDAP Panel concluded that: 'Independent of the route of administration, via feed or water, canthaxanthin is efficacious in pigmenting egg yolk and skin/fat of poultry. Canthaxanthin also has the potential to enhance plumage pigmentation of ornamental birds and the skin pigmentation of ornamental fish.' (EFSA FEEDAP Panel, 2014). Considering that canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is equivalent to the canthaxanthin produced by chemical synthesis, the FEEDAP Panel considers that the conclusions on the efficacy of the synthetic canthaxanthin reached in 2014 would apply to the present assessment.

The applicant submitted an in vivo study in laying hens to support the bioequivalence between the canthaxanthin produced by chemical synthesis and that produced by fermentation with *Y. lipolytica* CBS 146148.[42]

A total of 84 Hy-Line Brown hens were allocated in battery cages and kept under environmentally controlled conditions.[43] Prior to the experimental period, birds received a conventional diet low in xanthophylls for at least 28 days. Thereafter, cages were randomly allocated to one of the seven experimental groups with four replicates of three hens each. The basal diets were either not supplemented (control) or supplemented with 2, 4 or 8 mg canthaxanthin/kg of complete feed from two different sources (synthetic canthaxanthin or canthaxanthin from *Y. lipolytica*). The content of canthaxanthin in the experimental diets was confirmed by analysis (Table 2). The basal diet was based on wheat, rice and soybean meal. Birds had ad libitum access to feed (mash) and water. Feed intake, egg production, egg weight and egg mass were determined for the whole duration of the study (21 days). In the last week of the study (i.e. 14–21 days), a total of 24 eggs per treatment were randomly selected for egg yolk weight and colour determination and canthaxanthin content analysis.

All parameters were analysed by a one-way ANOVA per replicate followed by pairwise comparisons (i.e. within a product comparing doses vs. each other and between products comparing to the same dose of the other product respectively). The deposition of canthaxanthin in egg yolk was analysed by linear regression and the slope for each of the two tested products compared (non-inferiority). Statistical significance was set at $p < 0.05$. The main results are reported in Table 2.

TABLE 2 Bioequivalence results between two different sources of canthaxanthin in laying hens on the analytical content of canthaxanthin (CTX) in the yolk and yolk colour.

Canthaxanthin concentration feed (mg/kg feed)		Source of Canthaxanthin	Yolk weight (g)	Canthaxanthin in yolk (mg/kg)	YolkFan™ colour[1]
Intended	Analysed				
0	< LOD		16.9	0.00	1
2	1.9	Synthetic	17.8	2.7	8
	1.6	Fermentation	16.8	3.1*	10*
4	3.6	Synthetic	17.1	7.2	12
	4.2	Fermentation	16.9	7.0	12
8	8.2	Synthetic	16.7	14.5	14
	7.1	Fermentation	17.0	14.4	14

[1]YolkFan™: commercial scoring system in a scale from 1 (pale) to 16 (dark orange).

*Means with asterisks indicate significant differences (pairwise comparison, $p < 0.05$) between the different sources of canthaxanthin at the same supplementation level (2, 4 or 8 mg/kg).

No differences between treatments were observed on the 21-day performance of the hens nor on egg yolk weight.

A dose–response linear increase in yolk canthaxanthin content was observed. Linear regression analysis revealed a slope of 1.86 mg/kg and 1.98 mg/kg for synthetic canthaxanthin and canthaxanthin from *Y. lipolytica* CBS 146148, respectively. No significant difference in the dose–response slopes between the two tested products was found. The results showed that the increase in the canthaxanthin in yolk with the diet supplementation happened at a similar rate between the sources. Thus, the supplementation of the canthaxanthin from *Y. lipoltyica* CBS 146148 led to similar yolk deposition as that observed from canthaxanthin from chemical synthesis.

It is therefore concluded that canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is bioequivalent to synthetic canthaxanthin in terms of egg yolk pigmentation in laying hens.

[42]Technical dossier/Section IV/Annex-IV_1_RD-00063040-bioequivalence.

[43]Cages were stacked by three, so spillage of feed from the top to the bottom cages could not be avoided and thus, the statistical unit was each replicate of three hens in individual cages.

Conclusions on efficacy

Considering the specifications/purity of the canthaxanthin under assessment and the supporting data showing the bio-equivalence with synthetic canthaxanthin, the FEEDAP Panel concludes that canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is efficacious as a colouring agent in feed for chickens for fattening and minor poultry species for fattening, laying poultry and poultry reared for laying, ornamental fish and ornamental birds.

4 | CONCLUSIONS

The FEEDAP Panel concludes that canthaxanthin produced with *Yarrowia lipolytica* CBS 146148 is considered safe for the target species, the consumer and the environment under the current authorised conditions of use.

Canthaxanthin is not an irritant to skin or eyes and it is unlikely to be a skin sensitiser. No conclusion can be reached on the respiratory sensitisation of canthaxanthin. In the absence of data, no conclusions on the safety for the user can be reached for any preparation produced with canthaxanthin.

Canthaxanthin produced by fermentation with *Y. lipolytica* CBS 146148 is efficacious as a colouring agent in feed for chickens for fattening and minor poultry species for fattening, laying poultry and poultry reared for laying, ornamental fish and ornamental birds.

5 | RECOMMENDATIONS

Considering that xanthophylls are a subgroup of carotenoids, the FEEDAP Panel recommends that the provision 'The mixture of canthaxanthin with other carotenoids and xanthopylls shall not exceed 80 mg/kg of complete feed' should be corrected in the current authorisation of synthetic canthaxanthin and in the eventual authorization of the product under assessment as follows: 'The mixture of this additive with other additives containing canthaxanthin and other carotenoids is allowed provided that the total concentration of the mixture does not exceed 80 mg total carotenoids/kg of complete feed.'

ABBREVIATIONS

ANS	EFSA Scientific Panel on Additives and Nutrient Sources added to Food
CAS	Chemical Abstracts Service
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
LOQ	limit of quantification
MRL	maximum residue limit
QPS	Qualified Presumption of Safety
RH	relative humidity

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