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Safety and efficacy of lutein and lutein/zeaxanthin extracts from *Tagetes erecta* for poultry for fattening and laying (except turkeys)

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Abstract

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) evaluated (i) lutein from a saponified extract from *Tagetes erecta* obtained via extraction and saponification (lutein not less than 85% of total carotenoids (TC)) and (ii) lutein/zeaxanthin extract from *Tagetes erecta* obtained via extraction, saponification and isomerisation (lutein not less than 45% and zeaxanthin not less than 35% of TC). The maximum proposed use level of 80 mg TC from saponified *Tagetes* extract/kg complete feed for chickens for fattening and laying hens is safe for these animal categories. This conclusion can be extrapolated to minor poultry species for fattening and laying. The conclusions on saponified *Tagetes* extract for poultry for fattening and laying are extended to the saponified/isomerised *Tagetes* extract. The maximum use level of the saponified/isomerised *Tagetes* extract in breeding minor poultry should not exceed 50 mg TC/kg feed, considering the toxicological potential of zeaxanthin on reproduction. The saponified *Tagetes* extract is not genotoxic. This conclusion is extended to the saponified/isomerised *Tagetes* extract. Consumer exposure related to the consumption of animal products is very low compared to the exposure from other sources. The active substance is a viscous paste and may be irritant to skin and eyes; no exposure by inhalation is expected. In the absence of data, the Panel cannot conclude on the safety for the user of commercial preparations. The use of *Tagetes* extracts in poultry feed raised no concern for the environment. *Tagetes* extracts at levels up to the proposed maximum use level of 80 mg TC/kg complete feed have the potential to colour the egg yolk of laying hens and the skin of chickens for fattening. This conclusion is extended to minor poultry species for laying and for fattening. The use of the additive in feed and water for drinking is considered bioequivalent.

Keywords: lutein, zeaxanthin, extracts of *Tagetes erecta*, sensory additives, colouring agents, poultry for fattening, poultry for laying

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Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of lutein and lutein/zeaxanthin extracts from *T. erecta* for poultry for fattening and laying (except turkeys).

The application is for two different additives: (i) lutein from a saponified extract from *T. erecta* obtained via extraction and saponification (lutein not less than 85% of total carotenoids (TC)) and (ii) lutein/zeaxanthin extract from *T. erecta* obtained via extraction, saponification and isomerisation (lutein not less than 45% and zeaxanthin not less than 35% of TC).

The maximum proposed use level of 80 mg TC from saponified *Tagetes* extract/kg complete feed for chickens for fattening and laying hens is safe for these animal categories. Whereas the margin of safety for chickens for fattening is probably greater than 2.5, no margin of safety could be derived from the laying hen study. The conclusion on the safe level of *Tagetes* extracts can be extrapolated to minor poultry species kept for fattening and laying. The conclusions on the safety of saponified *Tagetes* extract for poultry for fattening and laying are extended to the saponified/isomerised *Tagetes* extract. The maximum use level of the saponified/isomerised *Tagetes* extract in breeding minor poultry should not exceed 50 mg TC/kg feed, considering the toxicological potential of zeaxanthin on reproduction.

Lutein and zeaxanthin are absorbed in the small intestine and enter the blood stream in their free form; they are esterified when entering the target cells. No cleavage of the isoprenoid chain occurs. Metabolisation is characterised by the oxidation of the hydroxyl groups to metabolites which are readily excreted.

The saponified *Tagetes* extract is not genotoxic. This conclusion is extended to the saponified/isomerised *Tagetes* extract. Since no specific studies with the *Tagetes* extracts under application are available, the FEEDAP Panel bases its assessment on the conclusions of the EFSA Panel on Additives and Nutrient Sources added to Food (ANS) and Panel on Dietetic Products, Nutrition and Allergies (NDA) made for preparations intended to be used in humans. The derived health-based guidance values (acceptable daily intake, ADI) are 1 mg lutein/kg body weight (bw) and 0.75 mg zeaxanthin/kg bw.

When administering *Tagetes* extracts to poultry, the consumer is exposed essentially to lutein and zeaxanthin deposited in tissues and products. Therefore, the health-based guidance values established by the ANS and the NDA Panels are used in the assessment of consumer safety of these xanthophylls deposited after the use of *Tagetes* extract as feed additive.

Consumer exposure related to the consumption of animal products from treated animals is very low compared to the exposure from other sources.

The active substance is a viscous paste and as such users will not be exposed by inhalation. The applicant recognises that the active substance may be irritant to skin and eyes. The FEEDAP Panel cannot conclude on the potential of any preparation to be toxic by inhalation, skin/eye irritant or skin sensitizer since no data were submitted.

The use of *Tagetes* extracts in poultry feed as a source of lutein and zeaxanthin would not alter the concentration or distribution of these carotenoids in the environment given their natural occurrence and oxidative susceptibility.

Tagetes extracts, either saponified or saponified/isomerised, at levels up to the proposed maximum use level of 80 mg TC/kg complete feed have the potential to colour the egg yolk of laying hens and the skin of chickens for fattening. This conclusion is extended to minor poultry species for laying and for fattening.

The use of the additive in feed and water for drinking is considered bioequivalent.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] OJ L 268, 18.10.2003, p. 29. establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In particular, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest one year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of 7 years after the entry into force of this Regulation for additives authorised without a time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from CARAC EEIG (Carotenoids Authorisation Consortium European Economic Interest Grouping)[2] for authorisation/re-evaluation of the product lutein, when used as a feed additive for crustaceans, tilapia, pet animals, ornamental fish and birds, and poultry (category: sensory additives; functional group: colourants). During the assessment it was clarified with the applicant that there are two additives under assessment; a saponified *T. erecta* extract containing lutein as the main carotenoid source and a saponified and isomerised extract containing lutein and zeaxanthin as the main carotenoid sources. The applicant requested a change in the species limiting the application to poultry for fattening (chickens for fattening and minor poultry for fattening) and poultry for laying (laying hens, ducks, partridges, and quails).[3]

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 23 July 2012.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product lutein, when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The data submitted in support of the application were obtained with (i) a saponified and (ii) a saponified and isomerised extract of *T. erecta* containing as main carotenoid source lutein and lutein/zeaxanthin, respectively.

The xanthophylls lutein (E161b) and zeaxanthin (E161h) are included in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003. They are authorised without a time limit in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs (2004/C 50/01) for its use in poultry as colourant (carotenoids and xanthophylls) with a maximum concentration of 80 mg/kg of complete feedingstuffs (alone or with the other carotenoids and xanthophylls).

The common use of the terms 'carotenoids' and 'xanthophylls' is often misleading and needs therefore clarification.

The term 'carotenoids' is the generic name for a class of hydrocarbons formally derived from the acyclic $C_{40}H_{56}$ structure, comprising 'carotenes' (non-oxygenated hydrocarbon forms) and 'xanthophylls' (oxygenated hydrocarbon forms, i.e. hydroxy and epoxy functions). They consist typically of a chain of eight isoprenoid units joined in such a manner that their arrangement is reversed at the centre (carbons 9 and 10) of the chain. Xanthophylls are yellow to red compounds,

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition.

[2] On 19/12/2012, the rights of CARAC/EEIG were transferred to FEFANA asbl, Rue de Trèves 45, 1040 Brussels, Belgium. Companies: NOVUS Spain, S.A.; Industrial Tecnica Pecuaria S.A. (ITPSA), Spain; Sinotrade Technology UK Ltd. United Kingdom (representing Alcosa S.A.).

[3] Technical dossier/Supplemetary information July 2015 and Supplemetary information July 2017.

more polar than carotenes.[4] Although the polyene chain double bonds present in lutein and zeaxanthin exist in a *cis* (*Z*) or *trans* (*E*) conformation, giving rise to a large number of isomers, the vast majority of carotenoids is in the *all-trans* configurations.

Lutein is authorised as food colourant in the EU in accordance with Annex II and Annex III to Regulation (EC) No 1333/2008.[5] The specific purity criteria concerning the use of lutein (E 161b) (synonyms: Mixed Carotenoids and Xanthophylls) in foodstuffs are included in Regulation (EC) No 231/2012.[6]

The placing on the market of synthetic zeaxanthin as a novel food ingredient was granted by Regulation (EC) No 258/97.[7]

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) published an opinion in 2009 on the safety of use of colouring agents in animal nutrition (including lutein and zeaxanthin) (EFSA, 2009). The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) evaluated the safety of synthetic zeaxanthin as an ingredient in food supplements (EFSA, 2008a). The NDA EFSA Panel in 2012 updated its opinion on the safety of synthetic zeaxanthin as a novel food ingredient in food supplements in the light of additional information made available (EFSA NDA Panel, 2012). The re-evaluation of lutein (E 161b) as a food additive was carried by the EFSA Panel on Food Additives and Nutrient Sources added to Food (EFSA ANS Panel, 2010, 2011). The same EFSA Panel published a statement on the safety assessment of the exposure to lutein preparations based on new data on the use levels of lutein (EFSA ANS Panel, 2012).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated lutein from *T. erecta* and synthetic zeaxanthin in 2004 (JECFA, 2004/WHO 2006) and 2018 (JECFA, 2018) and lutein esters from *T. erecta* in 2014 (JECFA, 2014), 2016 (JECFA, 2016) and 2018 (JECFA, 2018).

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[8] in support of the authorisation request for the use of lutein and zeaxanthin as feed additives.

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, to deliver the present output.

EFSA has verified the European Union Reference laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.[9]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of lutein and zeaxanthin is in line with the principles laid down in Regulation (EC) No 429/2008[10] and the relevant guidance documents: Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance for the preparation of dossiers for the re-evaluation of certain additives already authorised under Directive 70/524/EEC (EFSA, 2008b), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c), Guidance on studies concerning

[4] https://www.qmul.ac.uk/sbcs/iupac/carot/car1t7.html
[5] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 345, 31.12.2008, p.16.
[6] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p.1.
[7] Commission Implementing Decision of 22 January 2013 authorising the placing on the market of synthetic zeaxanthin as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament an of the Council. OJ L 21, 24.1.2013, p. 33.
[8] FEED dossier reference: FAD-2010-0372.
[9] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0372.pdf
[10] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d), Technical Guidance: Extrapolation of data from major species to minor species regarding the assessment of additives for use in animal nutrition (EFSA, 2008c).

3. Assessment

The applicant asks for the re-evaluation of the use of lutein and lutein/zeaxanthin for poultry for fattening (chickens for fattening and minor poultry for fattening) and poultry for laying (laying hens, ducks, partridges and quails) and proposes a maximum content of 80 mg/kg of complete feedingstuffs (alone or with the other carotenoids and xanthophylls). Furthermore, the applicant applies for the use in water; the proposed content in water is 40 mg/L (alone or with the other carotenoids and xanthophylls).

During the assessment, it was clarified with the applicant that the additives under assessment are the saponified *T. erecta* extract containing lutein as the main carotenoid source and a saponified and isomerised extract containing lutein and zeaxanthin as the main carotenoid source. Data were provided by a consortium of three companies.

It should be recognised that these data cover only a fraction of existing additives containing lutein and zeaxanthin.

The active substances in the extracts need protection against oxidation and are therefore marketed as preparations only.

3.1. Characterisation

According to the specification proposed, there are two extracts under assessment depending on the main carotenoid source: (i) lutein from a saponified extract from *T. erecta* obtained via extraction and saponification (lutein not less than 85% of total carotenoids (TC)) and (ii) lutein/zeaxanthin extract from *T. erecta* obtained via extraction, saponification and isomerisation (lutein not less than 45% and zeaxanthin not less than 35% of TC).[11] The TC content of the extracts is specified to be > 60 mg/kg.

3.1.1. Manufacturing of the active substances

The extracts are manufactured by a two-step procedure. The oleoresin of *T. erecta* is prepared by evaporation of the hexane extract of the dried flower petals. The oleoresin contains lutein, lutein esters, other carotenoids including zeaxanthin, triglycerides, other lipid-soluble esters and waxes. The oleoresin is subject to saponification (using potassium or sodium hydroxide) [redacted], yielding the lutein extract (saponified) from *T. erecta*. This extract will be further named as lutein-rich extract in order to differentiate from the lutein/zeaxanthin extract which is obtained by the isomerisation of lutein during the saponification process using different conditions of pH, temperature and reaction time. Antioxidants are added several times during manufacturing to protect carotenoids against oxidation.

3.1.2. Composition

3.1.2.1. Lutein-rich extract (saponified) from *T. erecta*

Data were provided from two companies.[12] The analysis of two batches per company showed a nearly complete saponification (> 98%).[13] Based on the analysis of three batches per company, the extracts contained an average concentration of TC of 115 g/kg (range 96–134 g/kg), of which 81–96% was lutein and 6–8% zeaxanthin.[14] These values are in compliance with the specifications. The applicant provided information on the distribution of the different lutein and zeaxanthin isomers (one batch per company) confirming as most abundant the *all-trans* isomers.[15]

[11] Technical dossier/Supplementary information June 2015.
[12] The two companies that provided data for the saponified extract are designated as Company A and C.
[13] Technical dossier/Supplementary information January 2015/Annex 9.
[14] Technical dossier/Supplementary information January 2015/Annex 5 and 9.
[15] Technical dossier/Supplementary information January 2015/Annex 5.

A second data set (three batches per company) confirmed the above data on the contents of TC, lutein and zeaxanthin (Table 1).[16] Moreover, a detailed composition of the extracts in terms of dry matter, crude ash, fibre, protein, fat (Table 1) and fatty acids (Table 2) was given.

Table 1: Approximate composition (%) of lutein-rich *Tagetes* extracts(1) (mean values of three batches)

Component	Company A	Company C
Dry matter	89.9	88.7
Crude protein	0.98	0.40
Crude ash	18.2	13.7
Crude fibre	1.26	1.13
Crude fat	45.5	54.7
Free fatty acids	13.1	20.3
Total carotenoids	10.9	13.1
Lutein(2)	8.5	10.4
Zeaxanthin	0.68	1.03
N-free extracts(3)	24.0	18.7

(1) Approximate composition due to the use of different analytical methods.
(2) *All-trans* isomer only.
(3) N-free extracts, consisting of carbohydrates, sugars, starches and hemicellulose, were calculated by subtracting all other components of the sample from the total: 100% − (% crude protein + %crude fat + %crude fibre + %crude ash + %moisture).

The major fatty acids in the extracts were palmitic acid (C16:0), linoleic acid (C18:2), myristic acid (C14:0) and stearic acid (C18:0) (Table 2).

Table 2: Fatty acid distribution (%) in crude fat (mean values of three batches)

Component	Company A	Company C
Octanoic acid (C8:0)	0.2	0.2
Decanoic acid (C10:0)	0.1	0.1
Dodecanoic acid (C12:0)	1.2	1.1
Tetradecanoic acid (C14:0)	13.3	12.3
Hexadecanoic acid (C16:0)	39.2	39.7
Octodecanoic acid (C18:0)	11.7	12.2
Octadecenoic acid (C18:1)	–	0.7
Octadecadienoic acid (C18:2)	16.1	17.4
Octadecatrienoic acid (C18:3)	10.4	9.2
Eicosanoic acid (C20:0)	0.9	0.8
Docosanoic acid (C22:0)	0.8	0.7
Tetracosanoic acid (C24:0)	1.0	0.9
Hexacosanoic acid (C26:0)	0.5	0.5
Fatty acid (total identified)	95.4	95.8

One of the three batches from each company was further analysed using various analytical techniques.[17] In particular, gas chromatography-mass spectrometry (GC-MS) identified and quantified saturated hydrocarbons, phytosterols, triterpenes and very limited amounts of glycerol (1.0–2.1%) (Table 3).

[16] Technical dossier/Supplementary information July 2017/Annex_02.
[17] Technical dossier/Supplementary information July 2017/Annex_02.

Table 3: Constituents in the different saponified *Tagetes* extracts expressed as area % of the peaks detected by gas chromatography-mass spectrometry (GC-MS) analysis.

Component	Company A	Company C
Fatty acids	77.4	73.7
Hydrocarbons (nonacosane, hentriacontane, eicosane)	3.8	5.9
Phytosterols (stigmasterol, β-sitosterol)	3.3	3.4
Triterpenes (β-amyrin, α-amyrin)	4.9	4.4
Glycerol	2.1	1.0
Ethoxyquin	2.8	–

Ethoxyquin[18] was analysed in one batch per company and amounted to 16 (Company A) and 3,700 (Company C) mg/kg.[19] Data for an additional batch per company were submitted (Table 3); ethoxyquin was present in the batch of company A with 2.8%.[20]

[redacted]

The mineral fraction was characterised by high amounts of potassium 103.5 g/kg (Company A) and 65.7 g/kg (Company C) due to the use of potassium hydroxide in the saponification.[23]

3.1.2.2. Lutein/zeaxanthin extract (saponified/isomerised) from *T. erecta*

Data were provided from one company.[24] The analysis of two batches showed a nearly complete saponification (> 98%).[25] Based on the analysis of three batches, the extracts contained 100–129 g/kg TC, of which 48–56% was lutein and 44–52% zeaxanthin.[26] These values are in compliance with the specifications. The applicant provided information on the distribution of the different lutein and zeaxanthin isomers (from one batch) confirming as most abundant the *all-trans* isomers.[27]

A second data set (three batches) confirmed the above data on the contents of TC, lutein and zeaxanthin (Table 4).[28] Moreover, a detailed composition of the extracts in terms of dry matter, crude ash, fibre, protein, fat (Table 4) and fatty acids (Table 5) was given.

Table 4: Approximate composition (%) of lutein/zeaxanthin *Tagetes* extracts(1) (mean values of three batches)

Component	Company E
Dry matter	94.7
Crude protein	0.32
Crude ash	17.6
Crude fibre	1.52
Crude fat	55.2
Free fatty acids	22.2
Total carotenoids	12.9

[18] The FEEDAP Panel notes that authorisation of ethoxiquin as a feed additive for all animal species and categories has been suspended by COMMISSION IMPLEMENTING REGULATION (EU) 2017/962 of 7 June 2017. OJ L 145, 8.6.2017 , p.13.
[19] Technical dossier/Supplementary information January 2015/Annex 6.
[20] Technical dossier/Supplementary information July 2017/Annex_02.
[redacted]
[23] Technical dossier/Supplementary information January 2015/Annex 6.
[24] The company that provided data for the saponified/isomerised extract is designated as Company E.
[25] Technical dossier/Supplementary information January 2015/Annex 9.
[26] Technical dossier/Supplementary information January 2015/Annex 5 and 9.
[27] Technical dossier/Supplementary information January 2015/Annex 5.
[28] Technical dossier/Supplementary information July 2017/Annex_02.

Component	Company E
Lutein(2)	5.59
Zeaxanthin	5.52
N-free extracts(3)	20.1

(1): Approximate composition due to the use of different analytical methods.
(2): *All-trans* isomer only.
(3): N-free extracts, consisting of carbohydrates, sugars, starches and hemicellulose, were calculated by subtracting all other components of the sample from the total: 100% − (%crude protein + %crude fat + %crude fibre + %crude ash + % moisture).

The major fatty acids in the extracts were palmitic acid (C16:0), linoleic acid (C18:2), myristic acid (C14:0) and stearic acid (C18:0) (Table 5).

Table 5: Fatty acid distribution (%) in crude fat (mean values of three batches)

Component	Company E
Octanoic acid (C8:0)	0.2
Decanoic acid (C10:0)	0.1
Dodecanoic acid (C12:0)	1.0
Tetradecanoic acid (C14:0)	12.0
Hexadecanoic acid (C16:0)	38.4
Octodecanoic acid (C18:0)	12.7
Octadecenoic acid (C18:1)	8.1
Octadecadienoic acid (C18:2)	18.0
Octadecatrienoic acid (C18:3)	–
Eicosanoic acid (C20:0)	0.8
Docosanoic acid (C22:0)	0.7
Tetracosanoic acid (C24:0)	0.8
Hexacosanoic acid (C26:0)	0.6
Fatty acid (total identified)	93.4

One of the three batches was further analysed using various analytical techniques. In particular, GC-MS identified and quantified saturated hydrocarbons, phytosterols, triterpenes and very limited amounts of glycerol (0.5%) (Table 6).

Table 6: Constituents (other than carotenoids and fatty acids) in the different saponified *Tagetes* extracts expressed as area % of the peaks detected by gas chromatography-mass spectrometry (GC-MS) analysis

Component	Company E
Fatty acids	73.7
Hydrocarbons (nonacosane, hentriacontane, eicosane)	6.0
Phytosterols (stigmasterol, β-sitosterol)	3.7
Triterpenes (β-amyrin, α-amyrin)	4.9
Glycerol	0.5
Ethoxyquin	–

Ethoxyquin was also analysed and was not present. Analysis of a former batch resulted in 1.6 mg/kg.[29]

[29] Technical dossier/Supplementary information January 2015/Annex 6.

[redacted]

The mineral fraction was characterised by high amounts of potassium (67.9 g/kg) due to the use of potassium hydroxide in the saponification.[32]

3.1.3. Purity

3.1.3.1. Lutein-rich extract (saponified) from *T. erecta*

During the manufacturing process, hexane is used in the extraction. The content of hexane was analysed in three batches per company in the saponified *Tagetes* extracts.[33] In one batch from company C, the quantity of hexane was found to be higher than the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline limit (290 mg/kg, Class 2 solvent)[34] with value up to 1,600 mg/kg. This limit was not exceeded when the analysis was performed on the final preparation.[35] In one preparation from company C, hexane residues were < 5 mg/kg (three batches); in three preparations from company A, the values ranged between 32 and 175 mg/kg.

Data on residues of other solvents were submitted.[36] Overall, the data did not raise any concern except one batch from company C in which the concentration of benzene was 150 mg/kg which exceeds the VICH limit for this Class 1 solvent (2 mg/kg) and according to VICH guidelines, substances of Class 1 should be avoided in active substances, excipients and preparations. The company involved in the production confirmed that benzene is not used in any step of the manufacturing and HACCP procedure is in place to control solvent levels.[37] In addition, analytical data for three additional batches of the saponified *Tagetes* extract were submitted that showed values of benzene 0.1, 0.09 and 0.08 mg/kg.[38]

Heavy metals (lead, cadmium and mercury) and arsenic were measured in one batch per company of saponified *Tagetes* extracts. Values were lead < 0.05 mg/kg, cadmium < 0.01 mg/kg, mercury < 0.005 mg/kg and arsenic < 0.1 mg/kg.[39]

Aflatoxins (B1, B2, G1, G2) were measured in one batch per company. All values were < 0.1 μg/kg.[40] The applicant reported that mycotoxins are regularly monitored in the starting material (unsaponified oleoresin).

The applicant stated that dioxins (polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)), dioxin-like polychlorinated biphenyls (PCBs) and non dioxin-like PCBs are regularly monitored in the starting material (unsaponified oleoresin). Data were provided in three batches of company A and four batches of Company C; values were in the range of 0.46–1.5 ng WHO-PCDD/F-TEQ/kg and 0.60–2.1 ng WHO-PCDD/F-PCBs-TEQ/kg. Non dioxin-like PCBs ranged from 2.4 to 3.2 μg/kg.[41]

3.1.3.2. Lutein/zeaxanthin extract (saponified/isomerised) from *T. erecta*

During the manufacturing process, hexane is used in the extraction. The content of hexane was analysed in three batches.[42] In one batch, the quantity of hexane was found to be higher than the VICH limit (290 mg/kg, Class 2 solvent) with values up to 610 mg/kg. This limit was not exceeded when the analysis was performed on the final preparation (< 5 mg/kg).[43]

Data on residues of other solvents were submitted.[44] The data did not raise any concern.

Heavy metals and arsenic were measured in one batch of saponified/isomerised *Tagetes* extracts. Values were lead < 0.05 mg/kg, cadmium < 0.01 mg/kg, mercury < 0.005 mg/kg and arsenic < 0.1 mg/kg.[45]

[redacted]

[32] Technical dossier/Supplementary information January 2015/Annex 6.
[33] Technical dossier/Supplementary information January 2015/Annex 6 and 9.
[34] https://www.ema.europa.eu/en/vich-gl18-residual-solvents-new-veterinary-medicinal-products-active-substances-excipients
[35] Technical dossier/Supplementary information July 2015/Annex 6 and 7.
[36] Technical dossier/Supplementary information January 2015/Annex 6.
[37] Technical dossier/Supplementary information July 2015/Annex 4.
[38] Technical dossier/Supplementary information July 2015/Annex 5.
[39] Technical dossier/Supplementary information January 2015/Annex 6.
[40] Technical dossier/Supplementary information January 2015/Annex 6.
[41] Technical dossier/Supplementary information January 2015/Annex 17 and 52.
[42] Technical dossier/Supplementary information January 2015/Annex 6 and 9.
[43] Technical dossier/Supplementary information July 2015/Annex 8.
[44] Technical dossier/Supplementary information January 2015/Annex 6.
[45] Technical dossier/Supplementary information January 2015/Annex 6 and 9.

Aflatoxins (B1, B2, G1, G2) were measured in one batch of saponified/isomerised extract. All values were < 0.1 μg/kg.[46] The applicant reported that mycotoxins are regularly monitored in the starting material (unsaponified oleoresin).

The applicant stated that dioxins and dioxin-like PCBs are regularly monitored in the starting material (unsaponified oleoresin). Data were provided in one batch; values were 0.69 ng WHO-PCDD/F-TEQ/kg and 0.84 ng WHO-PCDD/F-PCBs-TEQ/kg.[47]

Pesticides (178 substances) were analysed in one batch of *Tagetes* oleoresin.[48] All results were below the limit of detection of 0.01 mg/kg.

3.1.4. Physico-chemical properties of lutein and zeaxanthin

Lutein ((3*R*,3′*R*,6′*R*)-beta,epsilon-carotene-3,3′-diol, Chemical Abstracts Service (CAS) number 127–40–2) has the molecular formula $C_{40}H_{56}O_2$ and the molecular weight 568.88. Its structural formula is given in Figure 1.

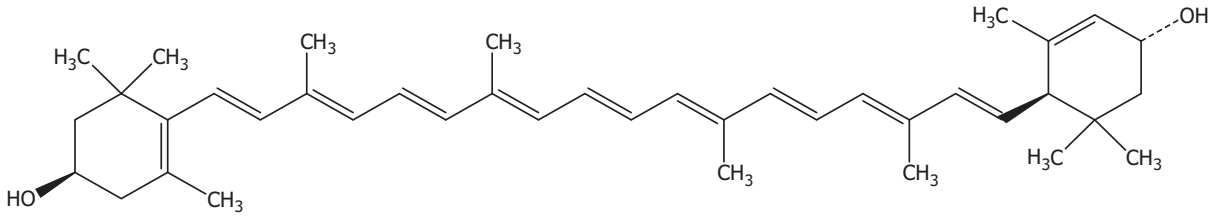

Figure 1: Structural formula of lutein

Zeaxanthin, a structural isomer of lutein, ((3*R*,3′*R*)-beta,beta-carotene-3,3′-diol, CAS number 144-68-3) has the same molecular formula and molecular weight. Its structural formula is given in Figure 2.

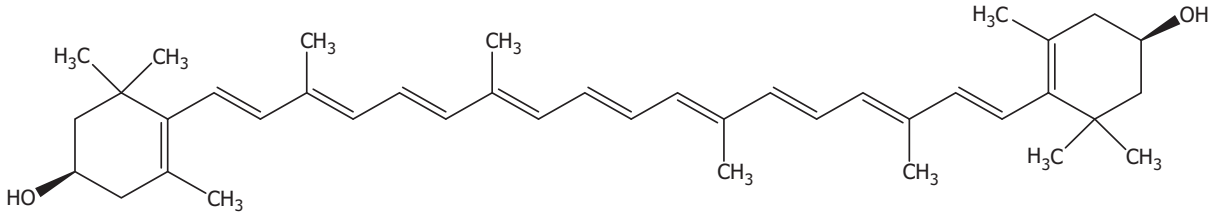

Figure 2: Structural formula of zeaxanthin

Physico-chemical data of lutein and zeaxanthin were provided by the applicant and were also described in the EFSA opinion on the use of colouring agents in animal nutrition (EFSA, 2009).

3.1.5. Feed additive preparations based on lutein and lutein/zeaxanthin extracts of *T. erecta*

3.1.5.1. Typical composition

The extracts described above are not used directly as feed additives. They are diluted, standardised and stabilised before placing on the market. The applicant reported that such products typically contain 0.5–7.0% TC, 0.3–6.7% lutein and 0.02–2.1% zeaxanthin.[49] The preparations may be solid or liquid and may contain carriers (e.g. silicon dioxide, calcium carbonate, colloidal silica, kaolin, *Tagetes* meal bagasse, rice hulls, water), antioxidants (e.g. ethoxyquin[50]), anticaking agents (silicic acid) and emulsifying agents (polyoxyethylene sorbitan monooleate).

[46] Technical dossier/Supplementary information January 2015/Annex 6.
[47] Technical dossier/Supplementary information January 2015/Annex 18.
[48] Technical dossier/Supplementary information January 2015/Annex 12.
[49] Technical dossier/Supplementary information July 2015.
[50] The FEEDAP Panel notes that authorisation of ethoxiquin as a feed additive for all animal species and categories has been suspended by COMMISSION IMPLEMENTING REGULATION (EU) 2017/962 of 7 June 2017. OJ L 145, 8.6.2017 , p.13.

3.1.5.2. Physical state

Two companies submitted the results of triplicate sieve analysis of three solid preparations covering both extracts (two saponified and one saponified/isomerised). The amount of particles with a diameter < 50 μm ranged between 24% and 26% (w/w) for one company, no particles < 10 μm were found. The amount of particles < 63 μm for the preparations of another company was 8% and 11%.

The dusting potential was determined in one preparation per company using the Stauber–Heubach method.[51] Preparations containing 1.5%, 2% and 3% TC showed a dusting potential of 0.5, 1.68 and 22.3 g/m^3, respectively.

3.1.5.3. Stability and homogeneity

The sensitivity of carotenoids to temperature, light and oxygen requires the inclusion of antioxidants at the different steps of the manufacturing process of the active substance and of the preparations of the additive to assure the stability of lutein/zeaxanthin.

Shelf-life

Samples of three batches of a solid lutein preparation from company A, containing 10% TC were kept at 25°C and 60% of relative humidity (RH)) in sealed opaque plastic bags over a period of 36 months.[52] TC, *trans*-lutein and *trans*-zeaxanthin were analysed. After 24 months, the recovery of carotenoids was 98%, 97% and 100%, respectively. After 36 months, only one batch was tested with 97% recovery.

Samples of two liquid lutein preparations (both containing 1.5% TC) from the same company were stored at 25°C and 60% RH in sealed plastic bags over a period of 36 months.[53] TCs were analysed. Recoveries after 24 months (one batch) and 36 months (two batches) were 100 and 95–99%, respectively.

Samples of 20 batches of a solid lutein/zeaxanthin preparation from company E, containing 1.5% TC, were kept at 22±2°C protected from light in high density polyethylene bags.[54] Lutein, zeaxanthin and TC contents were determined at time points different for all 20 batches ranging from 40 to 545 days. The sample stored for the longest period (545 days) showed recoveries of 101% and 98% for lutein and zeaxanthin, respectively. Stability data after 21 days under accelerated conditions (40°C and 75% RH) showed recovery rate of 90% and 95% for lutein and zeaxanthin, respectively.[55]

Stability in premixture and feedingstuffs

The stability of three batches of a solid lutein preparation from company A containing 10% TC in a vitamin mineral premix (without choline chloride) was assessed over period of 6 months (at 25°C and 60% RH) at inclusion levels providing 2, 8 and 10 mg TC/g premix.[56] Samples were stored in open bags. After 3 months, recoveries of TC were 80, 96 and 90% for the batches with inclusion levels of 2, 8 and 10 mg/g, respectively. The corresponding figures after 6 months were 75, 89 and 85%.

The stability of TC in complete feed for poultry species was assessed for two different preparations from company A containing 10% and 0.4% TC, respectively, at intended levels in feed corresponding to 300 (one batch), 400 (one batch) and 70–90 mg TC/kg complete feed (four batches).[57] The content of TC was analysed. The batches with 300–400 mg TC/kg were not considered since the carotenoid content exceeded the legal provisions. TC levels in batches with 70–90 mg TC/kg complete feed after 1, 2 and 3 months of storage (at 25°C and 60% RH) showed mean losses of 10, 15 and 18%, respectively.

The stability of a lutein preparation from company C before and after pelleting (75°C) was assessed in one batch of a broiler feed (two samples) with a supplementation of 30 mg TC/kg.[58] Pelleting did not affect the final concentration of TC and lutein.

[51] Technical dossier/Supplementary information July 2015/Annex 9.
[52] Technical dossier/Supplementary information January 2015/Annex_36.
[53] Technical dossier/Supplementary information January 2015/Annex_39.
[54] Technical dossier/Supplementary information January 2015/Annex_37.
[55] Technical dossier/Supplementary information January 2015/Annex_40.
[56] Technical dossier/Supplementary information January 2015/Annex_41.
[57] Technical dossier/Supplementary information January 2015/Annex_42.
[58] Technical dossier/Supplementary information January 2015/Annex_43.

Stability in water for drinking

The stability of a liquid lutein preparation from company E containing 1.1% TC added to water for drinking at an inclusion level of 40 mg/L was determined after 48 h.[59] The contents of lutein and zeaxanthin were 96.4 and 96.3% of the initial concentrations respectively.

Homogeneity

The homogeneous distribution of TC and lutein from a lutein preparation with 0.1% TC content (from company C) was assessed by TC and lutein analysis of 15 mash samples of one batch of a feed for chickens for fattening with a supplementation of 30 mg TC/kg.[60] The coefficients of variation were 4.64% for TC and 4.49% for lutein.

A second study, performed with a lutein/zeaxanthin preparation containing 1.5% TC (from company E) followed the same design as above, but with a higher supplementation rate (66 mg TC/kg). The coefficients of variation were 5.43% for TC, 5.63% for lutein and 5.39% for zeaxanthin.[61]

No data on the homogenous distribution in water for drinking were provided.

3.1.6. Conditions of use

The applicant proposes the use of saponified (lutein ≥ 85% of TC) and saponified/isomerised (lutein ≥ 45% and zeaxanthin ≥ 35%) extracts of *T. erecta* in feed for chickens for fattening and minor poultry species for fattening and laying hens, ducks, partridges and quails for laying to a maximum content of 80 mg TCs (alone or with the other carotenoids)/kg of complete feed. The applicant proposes a maximum of 40 mg TC/L to be used in water for drinking.

3.2. Safety

3.2.1. Absorption, distribution, metabolism and excretion

3.2.1.1. Metabolic studies

The absorption, distribution, metabolism and excretion (ADME) of lutein and zeaxanthin has already been assessed by the FEEDAP Panel in 2009 (EFSA, 2009). A summary of the relevant information is given below.

In the chicken, the absorption and distribution of lutein have been established following administration of free lutein or its diacylester(s) from *Tagetes* petals. Lutein esters are hydrolysed in the intestinal tract and free lutein is absorbed at the duodenum and jejunum levels. A linear increase of total lutein (free plus esters) with dietary lutein is observed in blood serum, liver and toe web. Lutein occurs in blood mainly in its free form (96%) and to a minor extent (4%) as monoester(s). In the liver, the distribution is similar (80% and 20%, respectively, plus traces of diester). Biotransformation occurs mainly in the liver where two major metabolites that correspond to the oxidation (dehydrogenation) of one hydroxyl group (3 or 3′-oxolutein) followed by a second reaction on a second hydroxyl group (lutein-3,3′-dione) have been identified. No cleavage of the lutein molecule occurred, which is consistent with the fact that lutein does not exhibit vitamin A activity. In the laying hen, lutein/metabolites deposition in the egg following free lutein administration showed that the ratio 3 or 3′-oxolutein vs lutein reached a plateau at 14 days that amounted to 0.12.

Limited information is available concerning the absorption, distribution and fate of zeaxanthin in poultry. Zeaxanthin is predominantly absorbed in the ileum, but the absorption rate has not been established. The metabolic fate in the laying hen indicated: (i) its biotransformation in the liver, where esterified zeaxanthin was found, (ii) a deposition rate in the egg yolk of 25% of the administered dose (multidose for 4 weeks, 16 mg/kg in the diet), (iii) that free zeaxanthin represented 90% of the whole residues in the egg, a metabolite identified as 3 and 3′-oxozeaxanthin amounting to 5–10% and a minor oxidised metabolite identified as (6S,6′S)-ε,ε-caroten-3,3′-dione, 3% (iv) that the isomeric composition of zeaxanthin deposited in the egg yolk reflected that of the compound administered.

In humans, about 45 and 10% of a single dose of [^{14}C]-lutein administered orally were excreted the first 48 h in the faeces and urine, respectively (de Moura et al., 2005). The metabolic pathways of both compounds are common to those in the poultry (Khachik et al., 2002); moreover, the oxidation at

[59] Technical dossier/Supplementary information January 2015/Annex_44.
[60] Technical dossier/Supplementary information January 2015/Annex_43.
[61] Technical dossier/Supplementary information January 2015/Annex_45.

3 followed by reduction and epimerisation (3′-epilutein) and the non-enzymatic dehydration at 3 giving rise to two didehydro metabolites have been identified. The conversion of lutein to zeaxanthin and the reverse have been shown to occur *in vivo* in humans, through an equilibrium involving oxidation/reduction and double bond isomerisation reactions involving the intermediate 3′-epilutein (Khachik et al., 1995, 2002).

Lutein and zeaxanthin are released from the food matrix during digestion, emulsified with lipids and biliary salts, incorporated and solubilised into micelles which are selectively absorbed at the duodenum and jejunum levels, mainly by facilitated diffusion mediated by enterocyte Scavenger Receptors (SR-B1, CD36) and cholesterol transporter NPC1L1, then secreted into lymph as chylomicrons. These mechanisms are largely affected by the food matrix, dietary components (lipids) and carotenoid competition (structure dependent), explaining a very wide range of absorption values (Reboul, 2013).

3.2.1.2. Deposition of lutein and zeaxanthin in the egg yolk

No new studies performed with the extracts under assessment were provided.

The studies concerning the deposition of lutein and zeaxanthin from *Tagetes* extracts in eggs have been assessed formerly by the FEEDAP Panel (EFSA, 2009). In the current assessment, the same studies have been reconsidered together with published studies submitted by the applicant not available in the previous assessment. The FEEDAP Panel noted that among the studies assessed in 2009 and those newly submitted, only two publications (Steinberg et al., 2000 and Pizzey, 2005) were performed with a supplementation level comparable to the highest supplementation rate proposed by the applicant.

The study from Steinberg et al. was performed in laying hens with 15, 30, 60 and 120 mg total xanthophylls supplemented/kg feed for 26 days. The total xanthophyll concentration measured in the feed, due to the presence of dietary background xanthophylls (maize-based diet), resulted to be 30.7, 48.5, 75.9 and 141.0 mg/kg. This background may vary depending on the diet composition from near 0 to 24 mg/kg (Leeson and Caston, 2004). Based on the above, the Panel notes that the supplementation level of 60 mg/kg feed would comply with current legal provisions (maximum content of TC: 80 mg/kg feed). At such inclusion level, following a supplementation period of 26 days, Steinberg et al. measured lutein concentration in the egg yolk of 34.5 mg/kg and the zeaxanthin concentration 8.8 mg/kg.

The above results were confirmed by the study from Pizzey which was performed in laying hens given a diet supplemented with 0, 30, 60, 90, 120, 150 mg lutein/kg feed (dietary background lutein concentration was not provided). The analysis of egg yolk showed a concentration of 32.7 mg lutein/kg yolk from eggs collected after 61 days administration of the experimental diet at inclusion level of 60 mg lutein/kg.

The Panel also noted that zeaxanthin in the yolk reached a plateau at about 10 mg/kg when dietary zeaxanthin varied from 5 to 21 mg/kg in the diet and lutein from 14 to 141 mg/kg (Steinberg et al., 2000). For lutein, such a plateau could be observed at higher dietary concentrations (> 400 mg/kg) (Leeson and Caston, 2004).

3.2.1.3. Deposition of lutein and zeaxanthin in poultry tissues

Tissue deposition data for lutein and zeaxanthin are very scarce. Pérez-Vendrell et al. (2001) fed a diet containing 25–34 mg lutein/kg to chickens for fattening for 6 weeks. The deposition ratios of total dietary lutein to skin and abdominal fat were 0.11 and 0.09, respectively, corresponding to mean values of 3.3 and 2.4 mg lutein/kg skin and abdominal fat. Deposition data for the control group given an unsupplemented diet were not provided.

In the same study, Pérez-Vendrell et al. (2001) also analysed the zeaxanthin content of skin and abdominal fat for diets with zeaxanthin concentrations of between 6.8 and 14.2 mg/kg. The deposition ratios to skin and to abdominal fat were about 0.09 and 0.05, respectively, corresponding to mean values of 0.85 and 0.45 mg zeaxanthin/kg.

Toyoda et al. (2002) published a study performed with quails administered feed containing about 36 mg supplemental zeaxanthin (derived from *Sphingobacterium multivorum*)/kg and 0.2–2.8 mg lutein/kg for 3 weeks. Zeaxanthin deposition was analysed in liver and fat amounting to 0.88 and 0.34 mg/kg, respectively, for the group with 36 mg zeaxanthin/kg. Lutein in liver ranged from 0.03 to 0.13 mg/kg and from 0.02 to 0.04 mg/kg in fat.

3.2.2. Toxicology

3.2.2.1. Genotoxicity studies

The genotoxicity of several lutein/zeaxanthin products, obtained by extraction from different plants, including *T. erecta*, has been evaluated by JEFCA (JECFA, 2004/WHO, 2006, 2014) and EFSA (EFSA, 2008a; EFSA ANS Panel, 2010, 2011; EFSA NDA Panel, 2012). All the available studies showed the absence of genotoxic potential of lutein and zeaxanthin *per se* and of several plant extracts.

The genotoxicity of the saponified *Tagetes* extract under assessment has been investigated in two new *in vitro* tests (a bacterial reverse mutation assay and a micronucleus assay) provided by the applicant. The TC content in the batch tested was determined to be 104.12 g/kg, in line with the proposed specifications. The content of lutein and zeaxanthin was not provided.

The saponified *Tagetes* extract was tested in the bacterial reverse mutation assay in Salmonella Typhimurium strains TA1535, TA1537, TA100 and TA98 and in *Escherichia coli* strain WP2uvrA.[62] The test was performed in two independent experiments (plate incorporation assay and pre-incubation assay) both in the absence and presence of S9-mix (rat liver S9-mix induced Aroclor 1254) in compliance with OECD test guideline 471. The vehicle was hexane.

In the first experiment (plate incorporation assay), the test item was tested up to the concentrations of 512 μg/plate, with and without metabolic activation. In the second experiment (pre-incubation assay), the top concentration was 512 μg/plate in the presence of S9-mix and 17 μg/plate in its absence. The highest concentration used was limited by the cytotoxicity of the test item. Precipitate was observed at the highest concentration in the plate incorporation assay. The test item did not induce significant dose-related increases in the number of revertants in any experimental condition. The positive controls performed as expected.

The clastogenic and aneugenic potential of the saponified *Tagetes* extract was tested in an *in vitro* micronucleus assay in cultured peripheral human lymphocytes.[63] Two independent experiments were conducted in the presence and absence of a metabolic activation system (phenobarbital and β-naphthoflavone induced rat liver S9-mix) in compliance with OECD test guideline 487. The test item was soluble in hexane at concentrations of 1.25 mg/mL and below but formed a suspension at concentrations of 2.5 mg/mL and upwards. In the first cytogenetic assay, the test item was tested up to 50 μg/mL for a 3 h exposure time with a 27 h harvest time in the absence and presence of S9-fraction. The test item precipitated in the culture medium at this dose level. In the second cytogenetic assay, the test item was again tested up to 50 μg/mL for a 24 h exposure time with a 24 h harvest time in the absence of S9-mix. The test item precipitated in the culture medium at this dose level. The test item did not induce any biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the absence and presence of S9-mix, in two independent experiments. The positive control chemicals produced a statistically significant increase in the number of binucleated cells with micronuclei, showing the adequacy of the test system.

Based on the results of the *in vitro* genotoxicity tests, it is concluded that the saponified *Tagetes* extract is not genotoxic. This conclusion is extended to the saponified/isomerised *Tagetes* extract.

3.2.2.2. General toxicology

The FEEDAP Panel noted that the toxicological profile of saponified and saponified/isomerised carotenoid extracts of *T. erecta* with similar specification has not been previously evaluated. However, the toxicological profile of lutein from *Tagetes* extracts and synthetic zeaxanthin has been evaluated by various committees (JECFA, 2004/WHO, 2006, 2014, 2016, 2018; EFSA, 2008; EFSA ANS Panel, 2010, 2011; EFSA NDA Panel, 2012).

In 2004, the JECFA set a group acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) for lutein from *T. erecta* (purified and saponified extract of xanthophylls obtained from marigold oleoresin with not less than 80% TC and not less than 70% lutein) and synthetic zeaxanthin on the basis of applying a 100-fold uncertainity factor to the no observed adverse effect level (NOAEL) of 200 mg lutein/kg bw per day (the highest dose administered) observed in a 13-week rat study, (JECFA, 2004/WHO, 2006). In 2014, JECFA evaluated lutein esters from *T. erecta* (carotenoid esters > 60%) and established a temporary ADI 'not specified' for lutein esters from *T. erecta*. The ADI was made temporary because the specifications for lutein esters from *T. erecta* were tentative (JECFA, 2014). In 2016, the Committee removed the temporary designation from the ADI 'not specified'

[62] Technical dossier/Supplementary information July 2017/Annex_06.
[63] Technical dossier/Supplementary information July 2017/Annex_07.

because the tentative status of the specifications was removed and established an ADI 'not specified' for lutein esters from *T. erecta* (lutein not less than 75% TC esters (as lutein esters) and zeaxanthin not more than 10% of TCs) (JECFA, 2016). In 2018, the Committee, based on the absence of toxicity in a wide range of studies, established a group ADI 'not specified' for lutein from *T. erecta*, lutein esters from *T. erecta* and synthetic zeaxanthin (JECFA, 2018).

The ANS Panel in 2010 established an ADI of 1 mg/kg bw based on the NOAEL of 200 mg/kg bw per day (the highest dose level tested) in a 90-day rat study, applying an uncertainty factor of 200 given the absence of a multigeneration reproductive toxicity study and of chronic toxicity/carcinogenicity studies. The ANS Panel noted that this ADI refers to lutein derived from *T. erecta* containing at least 80% carotenoids consisting of lutein and zeaxanthin (79 and 5%, respectively). In 2011, the ANS Panel adopted an opinion on the re-evaluation of lutein preparations other than lutein with high concentrations of total saponified carotenoids at levels of at least 80%. The additional data were performed with a specific lutein ester preparation extracted from *T. erecta* containing > 60% carotenoid esters (> 93% lutein esters, remainder zeaxanthin esters) and included a 90-day toxicity study and a reproductive and developmental toxicity study. The Panel established for both studies a NOAEL of 1,000 mg/kg bw per day, the highest dose level tested (equivalent to 538 mg lutein equivalents/kg bw per day). The Panel noted that this NOAEL of 538 mg lutein equivalents/kg bw per day is higher than the NOAEL of 200 mg/kg bw per day (the highest dose level tested) in the 90-day rat study with lutein from which the ADI has been derived. Based on these results, the Panel concluded that the additional database supports the conclusion that the ADI of 1 mg/kg bw per day also refers to lutein with high concentrations of TCs extracted from *T. erecta* and present as esters at levels of ≥ 60%. The ANS Panel concluded that the toxicological database available is too limited to conclude that the ADI also applies to lutein preparations of lower purity or from other sources.

The NDA Panel (EFSA, 2008a; EFSA NDA Panel, 2012) identified a NOAEL for synthetic zeaxanthin (*all-trans*-zeaxanthin (not less than 96%)) of 150 mg/kg bw per day for adverse effects on reproduction at a dose of 508 mg/kg bw per day in a two-generation rat reproduction study performed with a zeaxanthin product ('Zeaxanthin 10% WS beadlets').[64] The NDA Panel applied a 200-fold safety factor to the NOAEL of 150 mg/kg bw per day to identify 0.75 mg/kg bw per day (53 mg for a 70 kg person per day) as a dose of synthetic zeaxanthin at which there was no safety concern.

3.2.2.3. Conclusion on the toxicology

The saponified *Tagetes* extract is not genotoxic. This conclusion is extended to the saponified/isomerised *Tagetes* extract.

No new toxicology studies with saponified and saponified/isomerised carotenoid extracts of *T. erecta* with similar specification were available for the current assessment. However, the toxicological profile of lutein from *Tagetes* extracts and synthetic zeaxanthin has been evaluated by various committees. The FEEDAP Panel bases its assessment on the conclusions of the EFSA ANS and NDA Panels made for preparations intended to be used in humans. Lutein and zeaxanthin are of low acute toxicity.

When administering *Tagetes* extracts to poultry, the consumer is exposed essentially to lutein and zeaxanthin deposited in tissues and products. Therefore, the health-based guidance values established by the ANS and the NDA Panels were used in the assessment of consumer safety of these xanthophylls deposited after the use of *Tagetes* extract as feed additive.

The derived health-based guidance values (ADI) are 1 mg lutein/kg bw and 0.75 mg zeaxanthin/kg bw.

3.2.3. Safety for the target species

The applicant provided target animal studies from the literature performed with saponified (n = 13), saponified/isomerised extracts (n = 2) and non-saponified (n = 5) *Tagetes* extracts. Most of the studies (15) are performed with laying hens, one with breeder hens, one with roosters and three with chickens for fattening. Since egg pigmentation studies are usually done for a time period not longer than 4 weeks, 12 of the 15 studies performed with laying hens are short-term studies and not suitable for a safety assessment. Four long-term studies (duration 67–308 days) were provided, one

[64] A dose of approximately 500 mg/kg bw per day administered to rats for two successive generations induced slightly lower post-implantation survival index in the high dose group in the P generation, slightly lower body weight gain during the gestation period of the F1 generation; adverse effect on fertility of the F1 generation (statistically significantly reduced mating index), slightly fewer pups were born and the post-implantation survival index was also slightly lower.

with breeder hens and three with laying hens, the maximum doses tested corresponding to about the two- to threefold use level. In such a case, haematological and clinical chemistry parameters are required (EFSA, 2008a, 2011). None of the studies submitted provided these endpoints. No trial was made available with the saponified/isomerised extract in breeders.

Two tolerance studies (one in chickens for fattening and one in laying hens) were provided with lutein-rich saponified *Tagetes* extracts that are described below in detail.

3.2.3.1. Tolerance studies with a lutein-rich saponified *Tagetes* extract in chickens for fattening

A total of 1,200 1-day-old male chickens for fattening (Ross 308, 46 g bw) were distributed in pens of 50 birds and allocated to three treatment groups (representing eight replicates per treatment).[65] The groups were fed diets supplemented with 0 (control), 80 or 240 mg TC/kg complete feed for 35 days. The test item, a preparation of saponified *Tagetes* extract contained by analysis 62 g TC/kg, lutein and zeaxanthin were not determined (specification: ≥ 60 g TC, ≥ 48 g lutein and ≥ 3 g zeaxanthin per kg). The pelleted corn soybean-based diets were given as starter (day 1–21; 19.9% crude protein (CP), 5.0% ether extract (EE) and 5.2% ash analysed) and grower (day 22 until study completion; 18.0% CP, 6.6% EE and 5.1% ash analysed). The analysed TC levels in starter diets were 6, 70 and 197, in the grower diets 7, 67, 198 mg TC/kg for the control and the diets with nominal 80 and 240 mg TC/kg feed.

Health status including mortality was monitored daily, body weight (per replicates) was measured on day 21 and 35 and feed intake and feed to gain ratio were calculated for the corresponding periods. Blood samples for haematology[66] and clinical chemistry[67] were taken at the end of the study from two randomly selected birds/pen (16 per treatment). The same birds were killed for necropsy, organ weight determination (liver, spleen, kidneys) and histological examination of liver, kidney, muscle and skin/fat samples.

Statistical evaluation was made by analysis of variance (ANOVA) of data from a randomized complete block design (with block (three adjacent pens) as random effects); mean groups were compared with Tukey test. Mortality was assessed by chi-square from Kruskal–Wallis test.

No significant differences between the three groups were found for final body weight, feed intake and feed to gain ratio. The average performance obtained met the Ross 308 standards for final body weight (mean 2,297 g) and for feed to gain ratio (mean 1.47). Mortality was low (average about 1%) and not treatment related. No significant differences between the groups were found for the haematological and the clinical chemical parameters except alkaline phosphatase (ALP), which showed a treatment-related increase from 11.0 U/mL in the control group to 18.5 and 19.2 U/mL for the groups with nominal 80 and 240 mg TC/kg feed.

No statistically significant differences were found in organ weights, either expressed in actual weight or in percentage of body weight.

The pathological assessment identified, as expected, a dose-dependent yellowish discoloration in liver, skin/fat and muscle indicating carotenoid deposition. Discoloration of moderate to intense intensity was not found in the control group, but found in the use level and the overdose groups for 3 and 11 of 16 livers, for 12 and 16 of 16 skin/fat samples and for 3 and 11 of 16 muscle samples, respectively.

Several animals shown vacuolation of hepatocytes (13/16, 14/16 and 10/16 in treatments with 0, 80 and 240 mg TC/kg feed, respectively); the distribution of animals with these lesions in all experimental groups suggests that no relationship with the treatment did exist. Mononuclear hepatitis was found in several animals (14/16, 9/16 and 8/16 in treatments with 0, 80 and 240 mg TC/kg feed, respectively); these lesions were mainly of slight intensity and present in all experimental groups. A few chickens had mononuclear nephritis (7/16, 3/16 and 1/16 in treatments with 0, 80 and 240 mg TC/kg feed, respectively); these lesions were mainly of slight intensity, present in all experimental groups and without real pathological relevance. Mild hyperkeratosis and/or epithelial erosion (superficial desquamation) of skin/fat were observed in several animals from all treatments

[65] Technical dossier/Supplementary information July 2017/Annex_04.

[66] Haematocrit (HT), haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), erythrocytes count (ERY), and white blood cell count (WBC) and differentials as well (eosinophils, basophils, etc.).

[67] Alkaline phosphatase (ALP), gamma glutamine transpeptidase (GGT), aspartate amino transferase (AST), alanine aminotransferase (ALT), uric acid (URIC), total proteins (PROT), albumin (ALB), cholesterol, free fatty acids, total glycerides, Mg, Na, K, Cl and P.

(7/16, 3/16 and 9/16 in treatments with 0, 80 and 240 mg TC/kg feed, respectively). No microscopic lesions were found in spleen and muscle tissue.

3.2.3.2. Tolerance studies with a lutein-rich saponified *Tagetes* extract in laying hens

A total of 192 laying hens (Hy-Line Brown, 26 weeks old, 1.8 kg bw) was allocated to three treatment groups fed diets supplemented with 0 (control), 80 or 240 mg TC/kg complete feed for 56 days according to a randomised complete block design.[68] Blocking factor for the eight blocks with three replicates, each was the laying rate measured the week before the allocation. Group size was eight replicates (enriched laying hen cages) with eight hens each.

The test item, a preparation of saponified *Tagetes* extract, contained by analysis 40.7 g TC/kg, lutein and zeaxanthin were not determined (specification: $\geq$ 40 g TC, $\geq$ 32 g lutein and $\geq$ 1.6 g zeaxanthin per kg). The mash corn soybean-based diet contained by analysis 14.7% CP, 3.0% EE and 12.7% ash. The analysed TC levels in the diets were 3, 94 and 253 mg TC/kg, lutein levels 1.4, 74.4 and 198.0 mg and zeaxanthin levels 0.8, 7.8 and 19.7 mg/kg for the control and the diets with nominal 80 and 240 mg TC/kg feed.[69]

Health status including mortality was monitored daily. Animals were weighed by replicate at the beginning and end of the study. Feed consumption was checked every 28 days. All eggs laid were counted and weighed per replicate every second day on weekly days. Performance variables (laying rate, average egg weight and mass, average daily feed intake and average feed to egg mass ratio) were calculated. At the end of the study, one bird/replicate (eight per treatment) was selected for blood samples (haematology[66] and clinical chemistry[67]) and killed thereafter for necropsy (gross pathology examination); organ weight determination (liver, spleen, kidneys) and histological examination of liver, kidney, muscle and skin/fat. Egg quality (yolk colour and Haugh Units, shell and yolk percentages, shell index and egg classification) was assessed at the end of each period.

Statistical evaluation (comparison of Tukey-adjusted means) was made by ANOVA of data from a randomized complete block design (with block as random effects). Mortality was assessed by chi-square from Kruskal–Wallis test.

No mortality occurred during the study. The main zootechnical parameters were not significantly different between the groups, average laying rate was 93.0%, daily egg mass 56.6 g and feed to egg mass ratio 2.16. No significant differences between the groups were detected concerning egg quality (except egg yolk colour), blood biochemistry and haematology (except for mean corpuscular haemoglobin (MCH), MCH concentration (MCHC) which were lower in the high dose group compared to the control), and organ weight. During the pathological assessment, no macroscopic lesions of kidneys and spleens were observed. Yellowish discolouration of fat was detected in 7/8 hens from the overdose group. Microscopically, no lesions were found in these adipose tissue samples. For many of the animals from the use-level and overdose treatment groups, a yellowish-brownish discolouration and friability of the liver were recorded during necropsy, but this was not seen in any of the animals in the control group. Liver friability was reported in 5/8 birds from the use-level and 4/8 from the overdose group. One bird from the overdose group also had discolouration and friability in the kidneys without microscopic lesions. The results reported in the conclusion of the pathology report showed incidences of lesions in control, use-level and overdose groups, respectively, as hepatic steatosis: 0/8, 2/8 and 2/8 and hydropic hepatic degeneration: in 2/8, 1/8 and 5/8. However, necropsy and histopathology results for individual animals showed a slightly different incidence of 4/8 for hydropic hepatic degeneration in the overdose group. Four birds in the overdose group having hydropic hepatic degeneration were the same ones from that group that had friable livers and the one bird from the use-level group that had hydropic hepatic degeneration also had a friable liver. Thus, the two findings seem to be different aspects of a common pathology (hepatotoxicity). Although a dose without adverse effects was not identified that it is noted that the incidence of hydropic hepatic degeneration in the use-level group was less than in the control group. Hydropic hepatic degeneration and liver friability were of low severity (described as 'mild diffuse' and 'low liver friability' to 'liver friability', respectively) in all birds apart from one bird in the overdose group which had a more severe expression of hydropic hepatic degeneration ('moderate to intense') and of liver friability ('intense'). Although hepatic steatosis was found only in treated groups (not in controls), the incidence was low

[68] Technical dossier/Supplementary information July 2017/Annex_05.

[69] The supplementation rate of the test item was 10 % higher than nominally necessary to reduce losses before analysis and feeding.

for both treated groups and there was no dose–response relationship. Consequently, it may have been an incidental finding, unrelated to the treatments. It seems likely that the liver friability and hydropic hepatic degeneration were adverse effects caused by the treatments, but the effects at 80 mg TC/kg complete feed were considered by FEEDAP Panel to have minimal impact on the overall health of the birds. The higher incidence of hydropic hepatic degeneration and the more intense effects in one bird suggest a higher toxicity at 240 mg TC/kg complete feed that might impact on health.

3.2.3.3. Safety of zeaxanthin to reproductive target species

A multigeneration study with chemically synthesised zeaxanthin (Section 3.2.2.2) showed a NOAEL of 155 mg/kg bw per day for the absence of a variety of effects on reproduction. None of the parameters tested in the tolerance studies above covered such endpoints. The application is also for laying poultry including minor poultry species, breeders are not mentioned. Since the breeding husbandry in minor poultry species does not differentiate between laying and breeding, it cannot be excluded that the additive contained in a layer diet would be given to minor breeding poultry.

The FEEDAP guidance for assessing target species safety indicates different means to assess the safety of an additive when no applicable tolerance studies are submitted. A safe level for target species can be derived from a NOAEL of a subchronic or chronic study with laboratory animals, applying a safety factor of at least 100 and the default values for body weight and feed intake of the target species (EFSA FEEDAP Panel, 2011, 2017a). Such a calculation resulted in a safe level of 26 mg zeaxanthin/kg feed.

The incorporation of saponified *Tagetes* extracts is limited to a maximum of 80 mg TC/kg feed. In case this quantity is provided by a *Tagetes* extract with analysed concentrations of lutein between 90 and 96% and zeaxanthin $\leq$ 8%, the highest zeaxanthin addition to complete feed would not exceed 7 mg/kg. In case the same TC quantity is provided by a saponified/isomerised *Tagetes* extract with an analysed zeaxanthin concentration between 39 and 52%, the zeaxanthin addition to feed would be about 32 to 40 mg/kg.

Considering the toxicological potential of zeaxanthin on reproduction and the calculated safe level of zeaxanthin in feed (26 mg/kg), the concentration of TC in feed for minor breeding poultry should not exceed 50–65 mg/kg if it is provided from saponified/isomerised *Tagetes* extract.

3.2.3.4. Conclusions on safety for the target species

The results of tolerance studies with chickens for fattening and laying hens performed with saponified *Tagetes* extracts (lutein $\geq$ 85% of TC) indicate that the proposed level of 80 mg TC/kg complete feed is safe for these animal categories. Whereas the margin of safety for chickens for fattening is probably greater than 2.5, no margin of safety could be derived from the laying hen study. The conclusion can be extrapolated to minor poultry species kept for fattening and laying (e.g. ducks, partridges and quails).

The safety of saponified/isomerised *Tagetes* extract ($\geq$ 45% lutein and $\geq$ 35% zeaxanthin) for poultry for fattening and laying can be extrapolated from the data obtained with saponified *Tagetes* extract.

Considering the toxicological potential of zeaxanthin on reproduction, and the use of the saponified/isomerised *Tagetes* extract with higher zeaxanthin content in breeding minor poultry, the supplementation with the saponified/isomerised *Tagetes* extract for minor breeding poultry should not exceed 50 mg TC/kg feed.

3.2.4. Safety for the consumer

The consumer is exposed essentially to lutein and zeaxanthin deposited in animal tissues and products and not to the feed additive containing these xanthophylls. The derived health-based guidance values (ADI) are 1 mg lutein/kg bw (corresponding to 60 mg lutein per 60-kg person) and 0.75 mg zeaxanthin/kg bw (corresponding to 45 mg zeaxanthin per 60-kg person).

Exposure to lutein

The FEEDAP Panel estimated the potential exposure of consumers to lutein in eggs and poultry edible tissues under the conditions of a maximum use of the additive under assessment. Considering the maximum content for TC of 80 mg/kg complete diet, the lutein content of the egg yolk would be about 35 mg/kg (see Section 3.2.1.2) which corresponds to 9.5 mg/kg whole egg.[70] Applying the food

[70] Calculating with 27% of yolk content in whole egg.

basket of Regulation (EC) No 429/2008, in which the daily egg consumption is 100 g, the lutein intake from the consumption of eggs would not exceed 0.95 mg/person per day (corresponding to 0.0158 mg/kg bw per day).

For exposure to lutein by poultry tissues, only skin (likely associated to natural proportion of fat) data are available (see Section 3.2.1.3). Applying the deposition ratio (0.11) obtained by Pérez-Vendrell et al. (2001) for the skin of chickens for fattening at dietary total lutein between 25–34 mg/kg, and assuming that this ratio would be unchanged at higher lutein levels in feed, the maximum lutein concentration of skin would be about 9 mg/kg for diets containing 80 mg total lutein/kg. Applying the food basket of Regulation (EC) No 429/2008, consumer exposure from consumption of 90 g skin would be 0.81 mg lutein/person per day (corresponding to 0.0135 mg/kg bw per day). Data obtained with quails indicated that lutein may also deposit to some extent in the liver (Toyoda et al., 2002); however, deposition data were not available at supplementation rate in the range of the proposed conditions of use.

Considering the intake of lutein from the consumption of eggs and skin/fat calculated with the food basket of Regulation (EC) No 429/2008 of 1.76 mg/person per day (corresponding to 0.0293 mg/kg bw per day), consumer exposure (adults only) corresponds to about 3% of the ADI.

Using the European food consumption data of different age classes from EFSA's Comprehensive European Food Consumption Database as detailed in the Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017b), exposure of adult consumers was calculated to be 0.0149 mg/kg bw per day (1.5% of the ADI). The highest exposure using this calculation was for the age class 'other children' with 0.0398 mg/kg bw per day (4% of the ADI). These data confirm that the exposure of consumer to residues of lutein is well below the ADI.

The ANS Panel (EFSA ANS Panel, 2012) estimated consumer exposure to lutein of European adults and children from the intakes by its natural occurrence in food and from the use of lutein as food colour. Combining these intakes would result in an overall mean exposure of 0.14 mg/kg bw per day for adults and of 0.16–0.53 mg/kg bw per day for children. Both values (and 0.39 mg/kg bw per day, the 95th percentile exposure of adults) do not exceed the ADI of 1 mg/kg bw. For children, this ADI would be reached or exceeded at the 95th/97.5th percentile (0.42–1.32 mg/kg bw/day) in some European countries (The Netherlands, UK).

Exposure to zeaxanthin

The FEEDAP Panel made an estimation on the potential exposure of consumers to zeaxanthin in eggs and poultry edible tissue under the conditions of a maximum use of the additive under assessment. Considering the plateau value in egg yolk of 10 mg zeaxanthin/kg (correspondingly 2.7 mg zeaxanthin/kg whole egg) at low and maximum dietary levels of the additive (Steinberg et al., 2000) (see Section 3.2.1.2), zeaxanthin intake from the consumption of 100 g eggs would not exceed 0.3 mg/person (corresponding to 0.005 mg/kg bw per day) applying the food basket of Regulation (EC) No 429/2008.

Taking the deposition ratio of zeaxanthin to broiler skin of 0.09 (likely associated to natural proportion of fat) (Pérez-Vendrell et al., 2001) (see Section 3.2.1.3) and the highest dietary zeaxanthin derived from the use of saponified/isomerised *Tagetes* extracts at a concentration of 80 mg TC/kg (corresponding to a maximum of 42 mg zeaxanthin/kg), a maximum zeaxanthin concentration in skin of 3.8 mg/kg could be expected, corresponding to a consumer exposure of 0.34 mg/person per day (corresponding to 0.006 mg/kg bw per day) applying the food basket of Regulation (EC) No 429/2008. For liver, only quail data after feeding a diet with 36 mg zeaxanthin/kg were available (see Section 3.2.1.3, Toyoda et al., 2002) resulting in 0.88 mg zeaxanthin/kg liver. Assuming a constant deposition ratio at higher dietary zeaxanthin and a similar metabolic behaviour of zeaxanthin in quail and chicken, it can be reasonably expected that chicken liver would contain no more than 1.0 mg zeaxanthin/kg. The consumer exposure from liver consumption (food basket of Regulation (EC) No 429/2008) would be about 0.10 mg/person per day (corresponding to 0.002 mg/kg bw per day). No substantial deposition of zeaxanthin in meat is expected.

Considering the intake of zeaxanthin from the consumption of eggs, skin/fat and liver calculated with the food basket of Regulation (EC) No 429/2008 of 0.74 mg/person per day (corresponding to 0.012 mg/kg bw per day), consumer exposure (adults only) corresponds to about 2% of the ADI.

Using the European food consumption data of different age classes from EFSA's Comprehensive European Food Consumption Database as detailed in the Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017b), exposure of adult consumers was calculated to be 0.004 mg/kg bw per day (0.5% of the ADI). The highest exposure using this

calculation was for the age class 'other children' with 0.0113 mg/kg bw per day (1.5% of the ADI). These data confirm that the exposure of consumer to residues of zeaxanthin is well below the ADI.

The NDA Panel reviewed the zeaxanthin intake from natural sources of the European population on the basis of limited data from national food consumption surveys (France and Germany) and several other studies (EFSA, 2008a). In European countries, the average intake level of zeaxanthin via food was estimated to be between 0.2 and 0.9 mg/day and for people with a high intake of zeaxanthin-rich vegetables and fruits this could result in a level of 1.8 mg/day (95th percentile). The average intake would meet 0.3–1.7%, the 95th percentile intake 3.5% of the health-based guidance value of synthetic zeaxanthin (0.75 mg kg bw per day; 53 mg/70 kg person, EFSA NDA Panel, 2012). Also food supplements with a proposed use level of 20 mg/person per day would not raise concern (EFSA NDA Panel, 2012).

3.2.4.1. Conclusions

The FEEDAP Panel noted that the exposure to lutein and zeaxanthin related to the consumption of animal products from treated animals is very low compared to the exposure from other sources. Therefore, the Panel concludes that the use of *Tagetes* extract, either saponified (lutein ≥ 85% of TC) or saponified/isomerised (≥ 45% lutein and ≥ 35% zeaxanthin), for poultry for fattening and for laying/breeding, would not be of concern for the consumer.

No maximum residue limits (MRL) are considered necessary, provided that the maximum content set for TCs in complete feed is respected.

3.2.5. Safety for the user

The active substance is a viscous paste and as such users will not be exposed by inhalation. The applicant recognises that the active substance may be irritant to skin and eyes.

Due to the susceptibility of the active substance to oxidation (Section 3.1.5.3), the additive will be placed in the market only in the form of preparations either in solid or in liquid form. The applicant provided data in the dossier on the physical properties of some of these preparations which showed dusting potential ranging from 0.5 to 22.3 g/m^3 and the safety data sheet of a preparation.[71]

The FEEDAP Panel recognises that once authorised, multiple formulations of the additive can be placed in the market, and consequently, not all preparations can be directly tested for user safety. No information has been provided on the inhalation toxicity or the irritation/sensitisation potential of any preparation. In the absence of these data, the FEEDAP Panel cannot conclude on the potential of the preparations to be toxic by inhalation or on their potential as skin/eye irritant or skin sensitiser.

3.2.6. Safety for the environment

The additive is extracted from a natural source (*T. erecta*) and contains compounds which occur in nature: free carotenoids, mainly lutein and zeaxanthin, mono- and diglycerides, fatty acids, glycerol, waxes, potassium/sodium, tocopherol and water. The use of *Tagetes* extracts in poultry feed as a source of lutein and zeaxanthin would not alter the concentration or distribution of these compounds in the environment given the natural occurrence of these carotenoids and their oxidative susceptibility. Therefore, the FEEDAP Panel considers that use of extracts from *T. erecta* in poultry feed will not adversely affect the environment.

3.3. Efficacy

The additive is used to provide colour to the egg yolk and skin of poultry. In practice, the so-called yellow colouring pigments are used together in an appropriate proportion with red colouring pigments to achieve the desired golden yellow tone of egg yolk and skin.

In layer feeding, it is common practice to provide a basic dietary yellow colour (e.g. by xanthophylls as lutein and zeaxanthin) by including yellow corn, plata corn, alfalfa meal or *T. erecta* extracts in diets and to adjust the yolk colour desired by supplementing other yellow and/or red pigments. The need for additional red or yellow carotenoids in any particular producing system will also depend on the content of natural endogenous pigments in feed components and on the relative deposition rates of

[71] Technical dossier/Section III/Annex 30.

the various carotenoids. Thus, there is no standard approach to egg pigmentation which can vary considerably between egg producers.

3.3.1. Egg yolk pigmentation

The FEEDAP Panel reviewed eight studies in laying hens in which the effect of supplementing diets with *Tagetes* extract on egg yolk pigmentation was investigated (EFSA, 2009). The extracts were often used together with sources of red pigments. It was noted that lutein supplementation is only seldom calculated as such but rather as total xanthophylls. Poultry diets rich in corn and corn products may contain about 10 mg lutein and 5 mg zeaxanthin while supplemented diets contain about 20–30 mg lutein/kg and about 8–12 mg zeaxanthin/kg. Those dietary levels may lead to egg yolk concentrations of 13–25 mg lutein/kg and 8–10 mg zeaxanthin/kg. Lutein and zeaxanthin are effective in colouring yolk. Lutein alone would allow Roche egg yolk colour fan (RYCF) values of 9–10, zeaxanthin allowing more intensive yolk pigmentation than lutein.

Since the studies were performed with *Tagetes* extracts, the FEEDAP Panel considers *Tagetes* extract as an effective additive in colouring egg yolk of laying hens.

The applicant submitted five studies in laying hens and one study in breeder hens not already reviewed in the 2009 FEEDAP opinion examining the effect of dietary *Tagetes* extract on egg yolk colour.

Three studies (Galobart et al., 2004; Santos-Bocanegra et al., 2004; Leeson et al., 2007) confirmed higher egg yolk colour when *Tagetes* extracts (7.5 to 250 mg TC/kg feed) were added to the diet. Three studies (Pizzey, 2005; Leeson et al., 2007; Sirri et al., 2007) found increasing egg yolk xanthophylls with higher dietary xanthophylls (12 to 240 mg TC/kg feed). The study of Baiao et al. (1999) confirmed that zeaxanthin leads to a more intensive egg yolk colour than lutein as also concluded by the FEEDAP Panel in 2009 (EFSA, 2009).

Egg yolk colour was also measured (by the RYCF) in the tolerance study with laying hens (see Section 3.2.3.2) after 28 and 56 days of experiment. Egg yolk colour was significantly increased at increasing levels of saponified *Tagetes* extract in feed, values being higher in the second (RYCF 7.6, 10.5 and 12.3 for the groups with 0, 80 and 240 mg supplemented TC/kg, respectively) than in the first period of the study (RYCF 5.6, 9.8 and 11.3) for all treatments.

Tagetes extracts, saponified and saponified/isomerised, have the potential to colour egg yolk of laying hens. This conclusion is extended to minor laying poultry.

3.3.2. Skin pigmentation

One study was submitted on the pigmentation property of *Tagetes* extracts. Castañeda et al. (2005) studied the effect of supplementing a broiler diet (sorghum-soybean type, low in carotenoids) with different quantities of xanthophylls from *Tagetes* extracts (yellow pigments) and from *Capsicum annuum* (red pigments) on skin pigmentation. Grower diet (days 29–35) was supplemented with 25 + 2 and 45 + 3 (yellow + red pigment) mg TC/kg feed and finisher diet (days 36–49) with 65 + 2 and 85 + 3 mg TC/kg feed. The addition of xanthophylls from *T. erecta* to the diet resulted in a dose-dependent increase of b* values (yellowness according to CIE-Lab System (Commission Internationale de l'Eclairage) measured by a Minolta CR 300 colorimeter) of the skin from week 4 on and could also be confirmed in measurements of the carcass. The a* value (redness) decreased with increasing xanthophyll supplementation. The plasma levels of yellow and red pigments reflected the different dietary concentration.

The tolerance study (Section 3.2.3.1) provided also data on the skin pigmentation capacity of a saponified *T. erecta* extract. On day 39, three birds per replicate (24 birds per treatment) were randomly selected for colour measurements (on the following day after cooling of carcasses) at the foot pad and pectoral pterilium. In the pectoral pterilium, redness (a*) was significantly lower in the marigold supplemented groups whereas yellowness (b*) significantly increased in a dose-dependent manner. In the foot pad, both redness and yellowness increased significantly by xanthophyll supplementation in a dose-dependent manner. The effect can be interpreted as a result of the higher deposition of xanthophylls in the skin.

In the study of Pérez-Vendrell et al. (2001), already mentioned under Section 3.2.1.3 (deposition of lutein and zeaxanthin in poultry tissues), the pigmentation of broiler shanks and breast skin was measured by the Roche Color Fan (RCF) and by colour reflectance (Minolta CR-300) according to the CIE-Lab color system. After feeding a saponified/isomerised *Tagetes* extract providing 25 mg lutein and 14 mg zeaxanthin/kg feed for 42 days, the RCF value in shanks was 7.36, and significantly higher than the control value (2.16) obtained after feeding an unsupplemented diet containing 4.4 mg lutein

and 3.4 mg zeaxanthin/kg. The a* (redness) and b* values (yellowness) of shanks were 3.46 and 60.82, respectively, and significantly higher than those of the control group (1.75 and 35.93). The a* and b* values of breast skin behaved correspondingly.

In the EFSA opinion on the use of colouring agents in animal nutrition (EFSA, 2009), four studies are described showing a positive influence of *T. erecta* extract supplementation on shank pigmentation.

In summary, the data confirm that the carotenoids present in *Tagetes* extract have the potential to colour skin of poultry for fattening in doses below and up to the proposed use level of 80 mg TC/kg complete feed. This conclusion is extended to minor poultry species for fattening.

3.3.3. Product quality

The pathological assessment of muscle tissue in the tolerance study with a saponified *Tagetes* extract (with ≥ 80% lutein in TC) in chickens for fattening identified a dose-dependent yellowish discolouration. The frequency of these findings (moderate to intensive discolouration) was 3/16 animals at the use level (80 mg TC/kg) and 11/16 animals at the threefold use level (240 mg TC/kg).

The FEEDAP Panel considers the yellowish discolouration of poultry meat as an undesired effect. Nevertheless, it is noted that, under practical feeding conditions, the supplementation rate of *Tagetes* extracts would not exceed 80 mg TC/kg diet or it could be even lower when *Tagetes* extracts are used in combination with red pigments.

3.3.4. Conclusions on efficacy for the target species

Tagetes extracts, either saponified (lutein ≥ 85% of TC) or saponified/isomerised (≥ 45% lutein and ≥ 35% zeaxanthin), have the potential to colour the egg yolk of laying hens and the skin of chickens for fattening at the proposed conditions of use. This conclusion is extended to minor poultry species for fattening and for laying.

The use of *Tagetes* extracts in water for drinking is considered bioequivalent to that in feed, the corresponding maximum concentration would be 40 mg TC/L water for drinking.

4. Conclusions

The application is for two different *Tagetes* extracts: (i) lutein from a saponified extract from *T. erecta* obtained via extraction and saponification (lutein not less than 85% of TCs) and (ii) lutein/zeaxanthin extract from *T. erecta* obtained via extraction, saponification and isomerisation (lutein not less than 45% and zeaxanthin not less than 35% of TC).

The maximum proposed use level of 80 mg TC from saponified *Tagetes* extract/kg complete feed for chickens for fattening and laying hens is safe for these animal categories. Whereas the margin of safety for chickens for fattening is probably greater than 2.5, no margin of safety could be derived from the laying hen study. The conclusion on the safe level of *Tagetes* extracts can be extrapolated to minor poultry species kept for fattening and laying. The conclusions on the safety of saponified *Tagetes* extract for poultry for fattening and laying are extended to the saponified/isomerised *Tagetes* extract. The maximum use level of the saponified/isomerised *Tagetes* extract in breeding minor poultry should not exceed 50 mg TC/kg feed, considering the toxicological potential of zeaxanthin on reproduction.

Lutein and zeaxanthin are absorbed in the small intestine and enter the blood stream in their free form; they are esterified when entering the target cells. No cleavage of the isoprenoid chain occurs. Metabolisation is characterised by the oxidation of the hydroxyl groups to metabolites which are readily excreted.

The saponified *Tagetes* extract is not genotoxic. This conclusion is extended to the saponified/isomerised *Tagetes* extract. Since no specific studies with the *Tagetes* extracts under application are available, the FEEDAP Panel bases its assessment on the conclusions of the EFSA ANS and NDA Panels made for preparations intended to be used in humans. The derived health-based guidance values (ADI) are 1 mg lutein/kg bw and 0.75 mg zeaxanthin/kg bw.

When administering *Tagetes* extracts to poultry, the consumer is exposed essentially to lutein and zeaxanthin deposited in tissues and products. Therefore, the health-based guidance values established by the ANS and the NDA Panels are used in the assessment of consumer safety of these xanthophylls deposited after the use of *Tagetes* extract as feed additive.

Consumer exposure related to the consumption of animal products from treated animals is very low compared to the exposure from other sources.

The active substance is a viscous paste and as such users will not be exposed by inhalation. The applicant recognises that the active substance may be irritant to skin and eyes. The FEEDAP Panel cannot conclude on the potential of any preparation to be toxic by inhalation, skin/eye irritant or skin sensitiser since no data were submitted.

The use of *Tagetes* extracts in poultry feed as a source of lutein and zeaxanthin would not alter the concentration or distribution of these carotenoids in the environment given their natural occurrence and oxidative susceptibility.

Tagetes extracts, either saponified or saponified/isomerised, at levels up to the proposed maximum use level of 80 mg TC/kg complete feed have the potential to colour the egg yolk of laying hens and the skin of chickens for fattening. This conclusion is extended to minor poultry species for laying and for fattening.

The use of *Tagetes* extracts in water for drinking is considered bioequivalent to that in feed, the corresponding maximum concentration would be 40 mg TC/L water for drinking.

5. Recommendations

The name lutein does not provide sufficient clarity to the feed business operator due to the potential different ratio of lutein and zeaxanthin in the additive. This ratio would indicate differences in efficacy when the additive is used to colour food from treated animals (subgroup ii of the functional group, colourants).

The FEEDAP Panel recommends consideration of two different additives to provide better clarity to the additive market:

- saponified extract of *T. erecta,* lutein not less than 85% of TCs
- saponified/isomerised extract of *T. erecta,* lutein not less than 45% and zeaxanthin not less than 35% of TCs.

The FEEDAP Panel recommends that the provision 'alone or with other carotenoids and xanthophylls' with regard the maximum content should be maintained and corrected as follows: alone or with other carotenoids.

The active substances in the extracts need protection against oxidation; therefore, the extracts should be marketed as preparations only, containing antioxidants authorised for use in feed.

Appropriate measures should be taken by the additive's producer to prevent benzene in the *Tagetes* oleoresin exceeding 2 mg/kg.

Documentation provided to EFSA/Chronology

Date	Event
8/11/2010	Dossier received by EFSA. Lutein for poultry, fish/tilapias and pet animals. Submitted by FEFANA asbl.
3/4/2012	Reception mandate from the European Commission
23/7/2012	Application validated by EFSA – Start of the scientific assessment
11/10/2012	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
23/10/2012	Comments received from Member States
16/11/2012	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 and conditions of use*
8/1/2015	Reception of supplementary information from the applicant - Scientific assessment re-started
3/2/2015	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 and conditions of use*
30/7/2015	Reception of supplementary information from the applicant - Scientific assessment re-started
3/5/2016	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 the target species and genotoxicity*
28/7/2017	Reception of supplementary information from the applicant - Scientific assessment re-started
3/4/2019	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADI	acceptable daily intake
ANOVA	analysis of variance
ANS	EFSA Scientific Panel on Additives and Nutrient Sources added to Food
bw	body weight
EURL	European Union Reference Laboratory
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MRL	maximum residue limit
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
NOAEL	no observed adverse effect level
TC	total carotenoid

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for lutein

In the current application authorisation is sought under articles 4(1) and 10(2) for *lutein (E161b)*, under the category/functional group 2(a) 'sensory additives'/'colourants', for the following subgroups: - (i) substances that add or restore colour in *feedingstuffs*; - (ii) substances which, when fed to animals, add colours to food of animal origin; and - (iii) substances which favourably affect the colour of ornamental fish or birds, according to the classification system of Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the *feed additive* (*lutein, E161b*) is a deep brownish-orange viscous paste with a characteristic odour – obtained by saponification of the extract of *Tagetes* dried petals – containing levels of *total carotenoids & xanthophylls* ranging from 40 to 160 g/kg, which corresponds to a minimum *lutein* (*all-trans-lutein* isomer) content in the *feed additive* ranging from 24 to 96 g/kg and a maximum *zeaxanthin* content in the *feed additive* ranging from 12 to 48 g/kg.

As indicated by the Applicant, the typical formulations of the *feed additive* to be marketed are in liquid or solid forms with a minimum *total carotenoids & xanthophylls* concentrations ranging from 0.5% to 7%.

Specifically, authorisation is sought for the use of the *feed additive* for all poultry species, cats and dogs, crustaceans, fish/tilapias, ornamental fish and birds. The *feed additive* is intended to be incorporated in *premixtures*, *feedingstuffs* and *water*.

While no maximum and minimum levels were proposed for cats, dogs, ornamental fish and birds, the Applicant proposed for all poultry species, crustaceans and fish/tilapias a maximum concentration of *lutein* (*all-trans-lutein* isomer only) or *total carotenoids & xanthophylls* of 80 mg/kg in *feedingstuffs* and 40 mg/L in *water* (except for crustaceans and fish/tilapias).

For the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive* the Applicant proposed the internationally recognised FAO JECFA monograph for food additives, as recommended by Commission Directive 2008/128/EC, where identification is based on several tests, including: - solubility; - spectrophotometry; - test for carotenoids, while quantification is achieved by spectrophotometry with or without separation by High Performance Liquid Chromatography (HPLC). Even though no performance characteristics are provided, the EURL recommends for official control the methods recommended by Commission Directive 2008/128/EC and described in the JECFA monograph mentioned above for the determination of *lutein* (*all-trans-lutein* isomer only), *zeaxanthin* and *total carotenoids & xanthophylls* in the *feed additive*.

For the determination of *total carotenoids & xanthophylls* (including all forms of *lutein* and other relevant colouring matters) in *feedingstuffs* the Applicant submitted the official method of the Association of Analytical Communities (AOAC, 970.64) based on saponification, chromatographic separation of carotenes & xanthophylls and further detection of the different fractions by spectrophometry at 436 nm and 474 nm. This method can also be applied to *premixtures* samples after dilution with blank feed. This AOAC method does not distinguish between added and endogenous *carotenoids & xanthophylls*. Even though no performance characteristics are provided, the EURL recommends for official control the above mentioned AOAC official method for the determination of *total carotenoids & xanthophylls* in *premixtures* and *feedingstuffs*.

For the determination of lutein (*all-trans-lutein* isomer only) in premixtures and feedingstuffs the Applicant submitted a single laboratory validated and further verified method, based on normal phase High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis). This HPLC-Vis method does not distinguish between added and endogenous lutein. The following performance characteristics were reported for lutein (*all-trans-lutein* isomer):

- a relative standard deviation for repeatability (RSDr) ranging from 0.1 to 11.2%;
- a relative standard deviation for intermediate precision (RSDip) ranging from 0.9 to 11.2%;
- a recovery rate ranging from 78 to 101%; and
- a limit of quantification of 0.7 mg/kg feedingstuffs

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified method, based on High Performance Liquid Chromatography with visible wavelength detection (HPLC-Vis) to determine *lutein* (*all-trans-lutein* isomer only) in *premixtures* and *feedingstuffs*. As the Applicant did not provide any analytical method

or experimental data for the determination of *lutein* (*all-trans-lutein* isomer) and *total carotenoids & xanthophylls* in *water*, the EURL cannot evaluate nor recommend any method for official control for their determination in *water*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.