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Safety and efficacy of *cis*-norbixin di-potassium salt (annatto F) for cats and dogs

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Abstract

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 annatto F (alkali-processed norbixin, acid-precipitated) for cats and dogs. Norbixin is considered safe for cats at a maximum concentration of 13 mg/kg complete feed and for dogs at 16 mg/kg complete feed. Annatto 2.5% is a strong alkaline solution of potassium or sodium hydroxide that makes it corrosive and therefore harmful for the user. Although the ability of the additive to colour feed has generally been demonstrated, no conclusion on the effective concentration can be drawn.

Keywords: annatto F, norbixin, colourant, cats and dogs, safety, efficacy

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In 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 the company Sensient Food Colors Germany GmbH[2] for re-evaluation of the product annatto F (alkali-processed norbixin, acid-precipitated),[3] when used as a feed additive for cats and dogs[4] (category: 2. sensory additive; functional group: (a) colourants/substances that add or restore colour in feedingstuff).

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 6 April 2011.

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 annatto F (alkali-processed norbixin, acid-precipitated), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

Annatto F (alkali-processed norbixin, acid-precipitated) is currently not authorised as a feed or food additive.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) assessed annatto extracts in 1974, 1982, and 2006 (JECFA, 1974, 1982, 2007) and the EU Scientific Committee for Food (SCF) in 1975 and 1979 (EC, 1975, 1979). In 2016, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) adopted an opinion on the safety of annatto extracts as food additives (EFSA ANS Panel, 2016). This last opinion deals with the re-evaluation of the safety of annatto extracts (E 160b), when used as food additives and permitted in the European Union (EU) before 2009. Furthermore, at the request of the European Commission, the opinion evaluated the safety of five annatto extracts categorised as bixin-based (annatto E and B) or norbixin-based (annatto C, F and G), with the view to replace the currently authorised annatto extracts (E 160b) by the latter five.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[5] in support of the authorisation request for the use of annatto F (alkali-processed norbixin,

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2] Sensient Food Colors Germany GmbH, Geesthachter Strasse 103-105, 21502, Geesthacht, Germany. On 10/5/2016, EFSA was informed by the applicant that the applicant company changed to Sensient Colors Europe GmbH.

[3] Initially, the application was presented for the re-evaluation of norbixin potassium (bixin, annatto, norbixin, E 160b). During the assessment, the applicant clarified that that the additive under assessment is the *alkali-processed norbixin, acid-precipitated*, also called annatto F (Technical dossier/Supplementary information May 2016/Answer to question 1).

[4] Initially, the application was presented for all animal species. During the assessment, the applicant limited the application to cats and dogs (Technical dossier/Supplementary information May 2016/Answer to question 2).

[5] FEED dossier reference: FAD-2010-0267.

acid-precipitated) as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[6] and the applicable EFSA guidance documents.

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.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of norbixin potassium in animal feed. The Executive Summary of the EURL report can be found in Annex A.[7]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of annatto F (alkali-processed norbixin, acid-precipitated) is in line with the principles laid down in Regulation (EC) No 429/2008 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, 2011a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), 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 on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), and Guidance on the assessment of additives intended to be used in pets and other non-food-producing animals (EFSA FEEDAP Panel, 2011b).

3. Assessment

The applicant requests the authorisation of the use of the colourant annatto extract F in feed for cats and dogs.

3.1. Characterisation

3.1.1. Characterisation of the active substance

The active substance, alkali-processed norbixin, acid-precipitated (annatto F), is a carotenoid derivative prepared by removal of the outer coating of the seeds of the annatto tree (*Bixa orellana* L) with aqueous alkali (potassium or sodium hydroxide). The obtained solution containing bixin is hydrolysed to norbixin in hot alkaline solution (de-esterification) and is acidified to precipitate the norbixin. The precipitate is filtered, dried and milled to give a granular powder. The main colouring principle of annatto F is described as the potassium salt of norbixin (common chemical name dipotassium 6,6′-diapo-psi,psi-carotenedioate; chemical formula $C_{24}H_{26}K_2O_4$; Chemical Abstract Services (CAS) number 33261-80-2; molecular weight 456.66), under its *cis*-form. The structural formula of *cis*-norbixin is given in Figure 1.

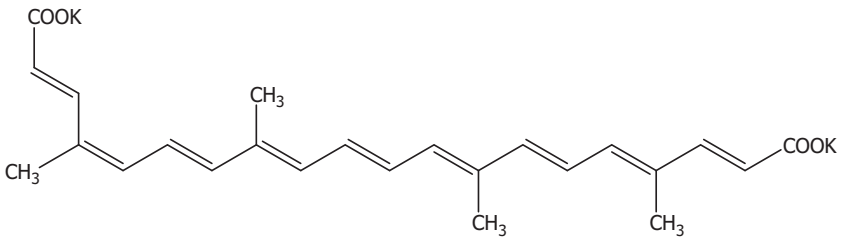

Figure 1: Structural formula of *cis*-norbixin dipotassium

Annatto F is specified to contain not less than 35% *cis*-norbixin as colouring matter and not more than 10% moisture. Analytical data was provided on annatto F from one batch indicating contents of

[6] 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.

[7] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0267.pdf

35.6% norbixin and 5.5% moisture.[8] Additional analytical data on annatto F composition was provided on three batches are given in Table 1.[9]

Table 1: Approximate composition of annatto F (%)[(a)]

Component	Batch 1	Batch 2	Batch 3
cis-Norbixin	43.4	48.9	51.9
Norbixin isomers	2.8	4.3	4.5
Water	3.2	3.2	2.6
Ash	2.1	1.9	1.6
Protein	2.5	7.8	12.5
Cellulose	31.3	12.5	11
Hydrocarbons	11.3	12.9	13.7
Geranyl geraniol	6.7	2.3	4.3

(a): Approximate composition due to the use of different analytical methods.

Heavy metals content determined in the same batches amounted to 0.08–1.22 mg lead/kg, 0.017–0.020 mg mercury/kg and was below the limit of quantification (LOQ) of 0.01 mg/kg for cadmium. Arsenic content was 0.05–0.12 mg/kg. Aflatoxins B1, B2, G1 and G2 were all < LOQ of 0.001 mg/kg, ochratoxin was < LOQ of 0.003 mg/kg in two batches and amounted 0.057 mg/kg in one batch. Pesticides were all < LOQ.

3.1.2. Characterisation of the additive[10]

The applicant stated that annatto F will be used only in form of a liquid additive called Annatto 2.5%. To obtain the additive, annatto F is dissolved in an aqueous alkaline solution which is then standardised to the desirable colour concentration and filtrated. The final product is an orange-red liquid with a pH between 10 and 12, specified to contain 2.3–2.7% of the colouring matter norbixin potassium salt, < 95% demineralised water and approximately 1.9% of potassium hydroxide.[11]

Analytical data from five batches of Annatto 2.5% were provided indicating contents of 2.5–2.7% norbixin, 81.3–90.1% water, < 3 mg/kg arsenic, < 10 mg/kg lead.[12] In the same batches, microbial impurities were measured giving the following results: total plate count: < 1,000 colony-forming units (CFU)/g, yeast and mould: < 100 CFU/g, enterobacteriaceae: < 1 CFU/g, *Salmonella*: negative/25 g.

3.1.3. Stability and homogeneity

No data on stability were submitted. The applicant recommends storing the additive at cool temperatures (5–15°C) in a tightly closed container protected from light for not more than nine months. This recommendation was supported by 9-month stability data in one batch of the additive.[13] A review was provided reporting data on stability in food colouring formulations of annatto extracts (Scotter, 2009) which confirmed that similarly to other carotenoids, annatto extracts are susceptible to oxidative degradation and *cis–trans* isomerisation.

Data on the capacity of the additive to homogenously distribute in different feedingstuffs is not required for additives which are used to add or restore colour to feedingstuffs.

3.1.4. Conditions of use

Annatto F is intended to be used in complete and complementary feed for cats and dogs without maximum content. Upon request, the applicant provided data indicating that the use dose is in the range of 50–100 mg annatto F/kg complete feed (corresponding to the addition of 2,000–4,000 mg Annatto 2.5%/kg feed).[14]

[8] Technical Dossier/Section II/Annex II 9.
[9] Technical dossier/Supplementary information March 2017.
[10] This section has been amended following the confidentiality claims made by the applicant.
[11] Technical dossier/Section II/Annex 1 and Annex 3.
[12] Technical dossier/Section II/Annex 2.
[13] Letter 10/3/2017 – Annatto 2.5% storage supervision.
[14] Technical dossier/Supplementary information May 2016.

3.2. Safety

3.2.1. Toxicological studies

JECFA assessed annatto extracts in 1974, 1982, and 2006 (JECFA, 1974, 1982, 2007) and the EU Scientific Committee for Food (SCF) in 1975 and 1979 (EC, 1975, 1979). In 2016, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) adopted an opinion on the safety of annatto extracts as food additives (EFSA ANS Panel, 2016). This last opinion deals with the evaluation of the safety of five annatto extracts, including annatto F.

Reports of the same toxicological and genotoxicity studies on annatto F assessed in the ANS Panel opinion (EFSA ANS Panel, 2016) were submitted for the current application.

3.2.1.1. Genotoxicity

Bacterial reverse mutation assay

The mutagenic effects of annatto F were investigated in two bacterial reverse mutation studies.

In the first study, annatto F was tested in *Salmonella* Typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 and in *Escherichia coli* strain WP2uvrA with and without the addition of a metabolic activation system (S9 mix from liver of Sprague–Dawley rats pretreated with phenobarbital and 5,6-benzoflavone), in compliance with OECD Guideline 471 (1997).[15] Annatto F was dissolved in dimethyl sulfoxide (DMSO) and tested up to 10 mg/plate. A mutagenic response was reported in *Salmonella* Typhimurium strain TA 100, without and (to a lesser degree) with metabolic activation. No mutagenic activity was observed in the other bacterial strains in any experimental condition.

In the second study,[16] annatto F was tested in *Salmonella* Typhimurium strains TA 1535, TA 1537, TA 98, TA 100, TA 102 and TA 104 and in *Escherichia coli* strain WP2uvrA (pKM101) with and without metabolic activation system (S9 mix from liver of Sprague–Dawley rats pretreated with Aroclor 1254), in compliance with OECD Guideline 471 (1997).[14] Weak mutagenic activity was reported in two independent experiments in strain TA 100 only in the absence of S9 mix (2.4- and 2.7-fold increase, respectively). No other significant effect was observed in any other strain.

In vitro mammalian cell gene mutation test

Annatto F was assayed for mutagenic potential in the mouse lymphoma L5178Y cell line, scoring for forward mutations at the thymidine kinase locus, in the presence of a post-mitochondrial supernatant fraction obtained from Aroclor 1254-induced livers of adult, male rats (S9), in compliance with OECD Guideline 476 (1997).[17] The maximum tested concentrations were established with a preliminary cytotoxicity experiment. Five independent mutation experiments were conducted: three in the absence and two in the presence of S9 mix. In the first experiment in the absence of S9 mix, and in both experiments in the presence of S9 mix, exposure to the cells was for 4 h. In the second and third experiments in the absence of S9 mix, exposure to the cells was for 24 h. A statistically significant but weak mutagenic effect was reported in two independent experiments conducted with a 24-h exposure in the absence of S9 mix. A weak positive result was also observed in one of the two experiments in the presence of S9 mix after 4-h exposure, at a dose level near the acceptable limit of toxicity (60 μg/mL, 13% of survival), while the second experiment conducted in the same conditions resulted negative. No evidence of mutagenicity was detected after 4-h exposure without S9 mix.

In vitro mammalian chromosome aberration test

Annatto F was assayed for clastogenic potential in a Chinese hamster ovary (CHO) cell line, according to the OECD Guideline 473 (1997).[18] The maximum tested concentrations were established with a preliminary cytotoxicity experiment. Tests were conducted both in the presence and absence of a postmitochondrial supernatant fraction obtained from the livers of adult, male rats treated with Aroclor 1254 (S9). Cultures, established approximately 20 h before testing, were treated for 6 h with Annatto F, dissolved in DMSO, in the presence and 6 or 22 h in the absence of S9 mix. Cultures were harvested at 24 h (Test 1 and 2) or 48 h (Test 2) from the beginning of treatment period. There was

[15] Technical dossier/Section III/Annex 7.
[16] Technical dossier/Supplementary information May 2016/Annex 15_Riach and Stevenson 2001a.
[17] Technical dossier/Supplementary information May 2016/Annex 15_Riach and Stevenson 2001b.
[18] Technical dossier/Section III/Annex 8.

no evidence that Annatto F induced structural chromosomal aberrations in either the presence or absence of S9 mix, up to cytotoxic doses. The positive controls performed as expected.

Mammalian erythrocyte micronucleus test

The *in vivo* genotoxic potential of annatto F was evaluated in a micronucleus test in bone marrow erythrocytes of mice, in compliance with OECD Guideline 474 (1997).[19] Three groups of CD-1 mice (10/sex in the highest dose group; 5 males/group in the other groups) were dosed orally by gavage at 0 and 24 h at three exposure levels up to the maximum tolerated dose of 1,200 mg/kg body weight per day, established by a previous toxicity study. Mice were killed 24 h after the last treatment by cervical dislocation, one femur of each mouse was dissected and smears prepared for microscopic examination. Clinical signs of systemic toxicity were observed in treated animals, but no alteration of polychromatic erythrocytes (PCE)/normochromatic erythrocytes (NCE) ratio, indicating local toxicity at bone marrow, was reported. No micronucleus induction was detected in bone marrow erythrocytes. Mice treated with the vehicle alone showed normal background levels of micronuclei, while mice dosed with cyclophosphamide responded with substantial increases in the numbers of bone marrow micronuclei.

Comet assay in vivo

The potential of annatto F to induce DNA damage in the liver and stomach of rats was tested in the alkaline comet assay.[20] The test item was a commercial batch compliant with the JECFA specifications.

Annatto F was administered by gavage in high viscosity 0.5% carboxymethyl cellulose to male Sprague–Dawley rats (six rats/group) at dose levels of 500, 1,000 and 2,000 mg/kg body weight (bw) per day at 0 and 21 h. The study design included a vehicle control (three male rats) and a positive control group (three male rats) that received 150 mg/kg ethylmethanesulfonate in a single oral administration at 21 h. Three hours after the second administration, the rats were anaesthetised with isoflurane and killed. No signs of toxicity were observed in any animal. All animals dosed at 2,000 mg/kg bw per day showed discoloured (yellow) skin on their ears, snout and paws. Histological analysis revealed orange and yellow discoloration in several organs and tissues. On microscopic examination, increased hepatocyte mitosis and decreased glycogen vacuolation related to administration of annatto F were present in the liver from all treated animals. Detectable levels of norbixin were observed in the plasma (range: 18–32 μg/mL) and liver (range: 8–14 μg/mL) of all animals dosed at 2,000 mg/kg bw per day.

Following treatment with annatto F at all dose levels, group mean % tail intensity and tail moment values for both liver and stomach were comparable with the concurrent vehicle control group; therefore, under the experimental conditions used, the test item did not cause any DNA damage in the analysed organs.

Conclusions on genotoxicity

Annatto F showed mutagenic effect in two bacterial reverse mutation studies in the *Salmonella* Typhimurium only in strain TA 100 in the absence of metabolic activation; positive effects were reported in one of the studies also in the presence of S9 mix. The substance showed a positive response in the forward mutation test in the mouse lymphoma L5178Y cell line, but resulted negative in the *in vitro* mammalian chromosomal aberration test. No induction of micronuclei was observed in mouse bone marrow erythrocytes up to the maximum tolerated dose, but without evidence of target cell exposure (alteration of the PCE/NCE ratio). In an *in vivo* comet assay, annatto F did not induce DNA damage in the site of contact (stomach) and in the liver of rats treated the maximum dose recommended by OECD for this test, in conditions of evident systemic exposure. The FEEDAP Panel concludes that the mutagenicity observed in some *in vitro* experiments is not expressed *in vivo* and therefore annatto F is to be considered not genotoxic.

3.2.1.2. Subacute toxicity

The 28-day range-finding toxicity study[16] provided was not performed according to Good Laboratory Practice (GLP). Five groups of five male and five female Sprague–Dawley rats (including a control group) were used. As the test material (annatto F) was considered to be unpalatable, the concentrations administered to all the test groups of rats were initially 7,000 mg/kg diet and increased in steps throughout the study to achieve the higher doses administered (20,000 mg/kg).

[19] Technical dossier/Section III/Annex 9.
[20] Technical dossier/Supplementary information June 2016/Annex 01, 02, 03.

The test material complied with the specifications for annatto F (41.5% norbixin). The results for all of the test groups were similar to those of the control group with regard to mortality, body weight gain, feed intake and feed conversion. Absolute and relative liver weights were markedly increased, compared to the controls, in all treated groups. Histopathological examination of the livers of male and female animals from the control and Group 2 (7,000 mg annatto F/kg diet) revealed diffuse hepatocellular hypertrophy in all treated animals examined, except in one female. A no observed adverse effect level (NOAEL) could not be established from this study as adverse effects on the liver were seen at all doses tested.

3.2.1.3. Subchronic toxicity

A 90-day rat toxicity study[17] was performed in accordance with OECD Guideline 408. The test material complied with the specifications for annatto F (38.4% norbixin).

Four groups of 20 male and 20 female Sprague-Dawley rats, were given annatto F at dietary concentrations of 0 (control), 1,000, 3,000 or 9,000 mg/kg diet for 13 weeks (equal to 0, 79, 240 and 753 mg annatto F/kg bw per day for males and 0, 86, 275 and 816 mg annatto F/kg bw per day for females). Since the test material contained 38.4% norbixin, these dosages corresponded to 0, 30, 92 and 289 mg norbixin/kg bw per day in males, and 0, 33, 106 and 313 mg norbixin/kg bw per day in females.

Blood and urine were collected at the end of the treatment period. Animals were killed and subjected to necropsy. A histopathological examination of tissues was carried out on all animals in the controls and the high-dose group, and of the kidney, liver and lungs of all other groups, and all abnormal appearing tissues observed at necropsy. No adverse effects on mortality, general condition and behaviour were seen in any of the test groups. Terminal blood samples showed increased activities of alkaline phosphatase and alanine aminotransferase in high-dose males and increased concentrations of glucose and triglycerides in high-dose rats. Plasma creatinine concentrations were increased in mid- and high-dose rats, and urea concentrations were increased in high-dose males and in treated females at all dose levels. Urinary analysis showed slightly reduced urinary output in mid- and high-dose males, and slightly low pH values were recorded for high-dose males. Increased liver weights were found for high-dose males and for mid- and high-dose females, and this was associated in many animals with centrilobular hepatocellular hypertrophy. Analyses of liver tissue for cytochrome-P450 activity showed a specific induction of hepatic CYP4A. The ANS Panel regarded these liver changes as adaptive and not indicative of toxicity. Increased kidney weights were found in mid- and high-dose males and in high-dose females. The FEEDAP Panel, in agreement with the ANS Panel (EFSA ANS Panel, 2016), considered that increased kidney weights and changes in renal function without histopathological changes were due to the high mineral content (approximately 2% sodium and 5% sulfate) of the test material. The NOAEL for this study was 1,000 mg annatto F/kg diet (79 and 86 mg annatto F/kg bw per day for males and females, respectively; 30 and 33 mg norbixin/kg bw per day) based on the renal changes seen at dietary concentrations of 3,000 mg annatto F/kg or more.

3.2.1.4. Reproductive and developmental toxicity

At the 61st JECFA Meeting (JECFA, 2004), the Committee noted that, whereas the reproductive toxicity of bixin has been studied, such data for a norbixin-rich extract were not available. In response to this remark, a prenatal developmental toxicity study[18] was performed according to the OECD Guideline 414 (2001) with annatto F. Groups of 22 pregnant female Sprague–Dawley rats were administered annatto F (with a norbixin content of 42.5%) by oral gavage at doses of 0, 20, 40, 80 or 160 mg/kg bw per day (equivalent to 8.5, 17, 34 and 68 mg norbixin/kg bw per day) on gestation days 6–19. Animals were killed on gestation day 20 for reproductive assessment and foetal examination.

In the dams, there were no deaths, no treatment-related clinical findings, and no effects on body weight gain or food consumption. There was no increase in embryo lethality and no reduction in fetal or placental weight. Annatto F did not induce any increase in the incidence of externally visible, visceral or skeletal abnormalities in the exposed offspring at any dose tested. The NOAEL for this study was 160 mg annatto F/kg bw per day (68 mg norbixin/kg bw per day), which was the highest dose tested.

Apart from the above developmental toxicity study, no studies on the reproductive toxicity of annatto F have been performed. However, the ANS Panel reported on a multigeneration study in rats that was part of long-term toxicological investigations of other annatto extracts (Van Esch et al., 1959). Essentially, 5% fat-soluble annatto extract (equivalent to 2,500 mg/kg bw per day) or 5%

water-soluble annatto extract (equivalent to 2,500 mg/kg bw per day) was administered in the diet to groups of 10 male and 10 female rats, and then to two further generations for 7 and 8.5 months. No adverse effects on mortality, growth or reproduction were seen. In another study, in which animals (10 rats/sex) were fed 0.5% fat-soluble (FL10) annatto extract in the diet (equivalent to 250 mg/kg bw per day) for 32 months, and administration continued in a further generation to five rats/sex for 7 months, also no treatment-related findings were observed. It was concluded that no evidence of reproductive toxicity of annatto extracts (E 160b) were observed in the multigeneration reproduction toxicity studies in rats with these fat-soluble or water-soluble annatto extracts (E 160b).

3.2.1.5. Chronic toxicity and (short term) carcinogenicity

The results from two mouse and three rat studies of annatto extracts were previously submitted to JECFA, and re-assessed in the ANS Panel opinion (EFSA ANS Panel, 2016). Although annatto F was not the test material used in any of the studies, the ANS Panel was sufficiently reassured by the results of these studies of other annatto extracts to conclude: 'Based on the absence of evidence for carcinogenic response, the Panel considered that annatto B, C, E, F and G were of no concern with respect to carcinogenicity'.

3.2.1.6. Conclusions on toxicology

Annatto F has no genotoxic potential *in vivo*. Toxicological studies in laboratory animals showed no alerts for particular toxicological effects that need to be taken into consideration when assessing target species safety. Annatto F did not cause developmental toxicity. No multigeneration reproduction studies of annatto F were available but it is noted that no adverse effects were observed when high doses of other annatto extracts were tested in such studies. Based on this and on reassuring results from carcinogenicity studies of other annatto extracts, dietary annatto F is not considered carcinogenic. The lowest dose of annatto F to cause adverse effects in toxicological studies was 240 mg/kg bw per day in the 90-day rat toxicity study, which corresponded to a NOAEL of 79 mg annatto F/kg bw per day (30 mg norbixin/kg bw per day). The most sensitive effects were altered renal function and enlarged kidneys. These conclusions are in agreement with the ANS Panel evaluation.

3.2.2. Safety for the target species

No tolerance studies were submitted with dogs or cats. Therefore, the FEEDAP Panel applied the procedure described in the FEEDAP Panel guidance for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b) to derive safe feed concentrations for these species (Table 2). The NOAEL used in the calculation was 30 mg norbixin/kg bw per day and an uncertainty factor (UF) of 100 was applied.

Norbixin is considered safe for cats at a concentration of 13 mg/kg complete feed (corresponding to 520 mg of the formulated additive Annatto 2.5%) and for dogs at 16 mg/kg complete feed (corresponding to 640 mg of the formulated additive Annatto 2.5%).

Table 2: Calculated maximum safe dietary levels of norbixin in complete feed for cats and dogs

Species	Body weight (kg)	Feed intake (g dry matter/day)	Safe intake (mg/day)	Maximum safe dietary level (mg/kg complete feed)[(a)]
Cat	3	60	0.9	13
Dog	15	250	4.5	16

(a): Complete feed containing 88% DM.

3.2.3. Safety for the user

No studies have been submitted by the applicant. The FEEDAP Panel notes that the alkaline nature of the additive (pH 10–12) indicates the corrosive potential towards skin, eyes and other tissues. However, considering that the additive is in a liquid form the exposure by inhalation is considered to be minimal.

3.2.4. Safety for the environment

Following the provision of the Regulation (EC) No 429/2008, there is no requirement for the assessment of the environmental impact of the use of a feed additive when used in pets and other non food-producing animals. This is the case for annatto F.

3.3. Efficacy

Annatto F is intended to be used to add or restore colour in feedingstuffs for cats and dogs. It is an authorised colourant for use in food. Where the function requested for feed is the same as that used in food, no further demonstration of efficacy might be necessary (Regulation (EC) No 429/2008).[21] However, considering the wide variety of feedingstuffs used in complete and complementary feed for cats and dogs and the uncertainty which concentration of annatto F would result in a visible effect, the FEEDAP Panel normally requires an effect demonstration. The applicant provided pictures of extruded feed pellets coloured with annatto F indicating that the additive is effective in colouring a typical complementary feed for pets.[13] However, the exact supplementation level was not given.

4. Conclusions

Norbixin is considered safe for cats at a maximum concentration of 13 mg/kg complete feed and for dogs at 16 mg/kg complete feed.

Annatto 2.5% is a strong alkaline solution of potassium or sodium hydroxide that makes it corrosive and therefore harmful for the user.

Although the ability of the additive to colour feed has generally been demonstrated, no conclusion on the effective concentration can be drawn.

Documentation provided to EFSA

1) Bixin, Annatto, Norbixin (Norbixin Potassium). November 2010. Sensient Food Colors Germany GmbH.
2) Annatto F (alkali-processed norbixin, acid-precipitated). May 2016. Submitted by Sensient Colors Europe GmbH.
3) Annatto F (alkali-processed norbixin, acid-precipitated). June 2016. Submitted by Sensient Colors Europe GmbH.
4) Annatto F (alkali-processed norbixin, acid-precipitated). March 2017. Submitted by Sensient Colors Europe GmbH.
5) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for norbixin potassium.
6) Comments from Member States.

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[21] OJ L 133, 22.5.2008, p.1.

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Abbreviations

ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
Bw	body weight
CAS	Chemical Abstract Services
CFU	colony-forming unit
CHO	Chinese hamster ovary
DMSO	dimethyl sulfoxide
EURL	European Union Reference Laboratory
FAO	Food and Agriculture Organization
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LOQ	limit of quantification
NCE	normochromatic erythrocytes
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
SCF	Scientific Committee for Food
UF	uncertainty factor
WHO	World Health Organization

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

In the current application authorisation is sought under articles 4(1) and 10(2) under the category/ functional group 2(a) "sensory additives"/"colourants", subgroup (i) substances that add or restore colour in feedingstuffs, according to the classification system of Annex I of Regulation (EC) No 1831/ 2003. The *feed additive* was previously authorized under provisions of Council Directive 70/524/EEC for Bixin (E 160b). However, according to the Applicant's request, the subject of this authorization concerns only *norbixin potassium*. Authorisation is sought for the use of the *feed additive* for all species and categories. The *feed additive* is intended to be incorporated in dry, moist and liquid *feedingstuffs*, with no minimum or maximum levels.

For the identification and quantification of *norbixin potassium* in the *feed additive*, the Applicant proposed the internationally recognised FAO JECFA monograph for *Annatto extracts* (*alkali processed norbixin, acid-precipitated*) in food additives. Identification is based on UV/VIS Absorption and Thin Layer Chromatography, while quantification of the *norbixin potassium* in the *feed additive* is based on spectrophotometry at 482 nm. Even though no performance characteristics are provided, the EURL recommends for official control the JECFA monograph based on spectrophotometry for the quantification of the *norbixin potassium* in the *feed additive*.

The Applicant did not submit any experimental data for the quantification of *norbixin potassium* in *feedingstuffs*. Therefore, the EURL could not evaluate nor recommend any method for official control to determine *norbixin potassium* in *feedingstuffs*.

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.