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The safety of annatto extracts (E 160b) as a food additive

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)

Abstract

Following a request from the European Commission to EFSA, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to re-evaluate the safety of annatto extracts, bixin-based and norbixin-based, when used as a food additive and to evaluate the safety of aqueous-processed bixin (Annatto E); solvent-extracted bixin (Annatto B); alkali-processed norbixin, acid-precipitated (Annatto F); alkali-processed norbixin, not acid-precipitated (Annatto G) and solvent-extracted norbixin (Annatto C) with the view to replace the currently authorised annatto extracts (E 160b). Given: (a) that read-across among the five bixin- and norbixin-based annatto extracts was feasible; (b) the availability of adequate 90-day toxicity studies with Annatto B, C, E and F; (c) the absence of concern for mutagenicity, carcinogenicity, reproductive and developmental toxicity of Annatto B, C, F and G, whereas the mutagenicity of Annatto E is equivocal, the Panel concluded that the safety of the currently authorised solvent-extracted bixin and norbixin (E 160b(i)), alkali-extracted annatto (E 160b(ii)) and oil-extracted annatto (E 160b(iii)), with the specifications defined in Commission Regulation (EU) No 231/2012, could not be assessed due to the lack of data, both in terms of identification and toxicological studies; solvent-extracted bixin (Annatto B), solvent-extracted norbixin (Annatto C), alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G and its norbixin salts) should comply with the specifications as recommended by the Panel. The toxicological database is sufficient to derive an acceptable daily intake (ADI) of 6 mg bixin/kg body weight (bw) per day and an ADI of 0.3 mg norbixin/kg bw per day. Exposure estimates for bixin were below the ADI for all population groups and for all refined exposure scenarios, including for the extension of use. For norbixin, exceedance was observed for the extension of use at the 95th percentile for some population groups.

Keywords: Annatto, *cis*-bixin, *trans*-bixin, *cis*-norbixin, *trans*-norbixin, *trans*-norbixin dipotassium salt, *trans*-norbixin disodium salt

Requestor: European Commission

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Summary

Following a request from the European Commission to the European Food Safety Authority (EFSA), the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion re-evaluating the safety of annatto extracts (E 160b(i), (ii), (iii)) when used as a food additive.

Furthermore, the ANS Panel was requested to evaluate the safety of five other annatto extracts, following the request of the Annatto Interest Group of the Natural Food Colours Association (NATCOL) for authorisation of these extracts, with the view to replace the currently authorised food additive (E 160b). These include the two bixin-based annatto extracts: aqueous-processed bixin (Annatto E) (≥ 25% colouring matter) and solvent-extracted bixin (Annatto B) (≥ 85% colouring matter), and the three norbixin-based annatto extracts: alkali-processed, acid-precipitated norbixin (Annatto F) (≥ 35% colouring matter), alkali-processed, not acid-precipitated norbixin (Annatto G) (≥ 15% colouring matter) and solvent-extracted norbixin (Annatto C) (≥ 85% colouring matter).

The qualitative and quantitative composition of the non-pigment fraction of the annatto extracts proposed by the applicant revealed that the non-pigment fraction contains several well-known plant constituents, including proteins (≤ 6%), lignocelluloses (< 16%), fatty acids (≤ 4%; probably as oil), polyphenols (≤ 4%) and ash (0.1–12%). Unidentified terpenoids (up to 13.4%) were only present in Annatto E. The applicant indicated that results for the solvent-extracted bixin (Annatto B) and the alkali-processed (acid-precipitated) norbixin (Annatto F) would be applicable also to the solvent-extracted norbixin (Annatto C).

Based on studies in rats and humans, the Panel concluded that bixin and norbixin are absorbed. In all studies, norbixin was the major component present in the plasma and urine, even following administration of bixin-based extracts, most likely derived from both the norbixin present in the extract, as well as metabolism of bixin to norbixin.

Four of the annatto extracts (Annatto B, C, E and F) evaluated in the present opinion were tested in 90-day studies in rats. The administration of all the four test materials to rats for 13 weeks did not result in any treatment-related deaths, and the general condition and behaviour of the animals were not affected by treatment. Some toxicological effects were observed. The effects observed related generally to increases in liver and kidney weights, with some indications of impaired function at high-dose levels. No evidence of histopathological damage was observed in any tissue, except for hepatocellular necrosis at the two highest dose levels of solvent-extracted norbixin (Annatto C). With the alkali-processed, acid-precipitated norbixin (Annatto F), haematological changes were also observed. The studies with the solvent-extracted bixin (Annatto B) revealed an effect on the kidney, with raised protein concentrations noted in urine samples obtained from males receiving 50,000 mg/kg diet. Blood plasma phosphorus concentrations were also increased in these animals, indicating a possible reduction in the glomerular filtration rate. Studies with the aqueous-processed bixin (Annatto E) revealed increased thyroid and kidney weights. No-observed-adverse-effect level (NOAEL) values were identified by the Panel from these 90-day studies. These NOAEL values were in accordance with the NOEL values identified in these studies by the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) in 2006.

No conclusion can be drawn from published genotoxicity studies with water-soluble annatto extracts, which used not validated test methods and/or limited protocols. However, a set of unpublished properly performed *in vitro* and *in vivo* genotoxicity studies on Annatto B, E and F were also available to the Panel for evaluation. In these studies, all the three annatto extracts produced weakly positive or equivocal results *in vitro*, especially at high doses, with no consistent pattern of activity. As an *in vivo* follow-up, a micronucleus test in mouse bone marrow and a comet assay in rat stomach and liver, the main target tissues following oral exposure, were performed. In these studies, the oral administration of Annatto B, E and F up to the maximum recommended dose did not induce any detectable genetic damage in mouse bone marrow, in which, however, no evidence of exposure was obtained; clearly negative results were, however, also obtained with comet assays in rat stomach and, for Annatto B and F, in rat liver. An unexplained heterogenicity in response was instead observed in the liver of animals treated with Annatto E. Negative results were obtained for Annatto C (solvent-extracted norbixin) in a separate bacterial mutation assay. Overall, based on the available experimental results, and according to the EFSA Scientific Committee Recommendations on Genotoxicity Testing Strategy (2011), Annatto B and F (and following read-across, also Annatto C and G) were evaluated by the Panel as not genotoxic, whereas more information on the identity of the components of the extract and further testing were considered necessary to clarify the equivocal response elicited by Annatto E in rat liver.

Annatto extracts (E 160b) of lower purity than those evaluated in the present opinion were without significant toxicity when administered in long-term studies to mice and rats. The applicant considered it appropriate to extrapolate the conclusions from these old carcinogenicity studies to the annatto extracts Annatto B, C, E, F and G. The Panel agreed with this assumption. Based on the absence of evidence for carcinogenic response, the Panel concluded that the annatto extracts were of no concern with respect to carcinogenicity.

A prenatal developmental toxicity study revealed that oral gavage administration of the alkali-processed, acid-precipitated norbixin (Annatto F) during gestation from the time of implantation until just before delivery resulted in no treatment-related effects on the progress or outcome of pregnancy for female CD (Sprague–Dawley) rats. No adverse effects on development were observed after treatment of rats with the aqueous-processed bixin (Annatto E). Moreover, in a long-term study with the annatto extracts of lower purity, no adverse effects on reproduction and no teratogenic effects were seen in rats. Overall, based on the read-across from Annatto F to Annatto G and from Annatto E to Annatto B and Annatto C, and considering the results of older studies with annatto preparations of low purity, the Panel concluded that no adverse effects on reproduction or development were to be expected from the five annatto extracts described in the present opinion.

To assess the dietary exposure to annatto (E 160b) from its use as a food additive, the exposure was calculated based on (1) maximum permitted levels (MPL) set out in the European Union (EU) legislation (defined as the *regulatory maximum level exposure assessment scenario*); (2) the reported use levels (defined as the *refined exposure assessment scenario*); and (3) use levels proposed by the applicant for the extension of use (defined as *extension of use scenario*). For the refined exposure scenarios, the Panel calculated exposure estimates separately for bixin and norbixin, based on the information provided by industry.

From the *refined estimated exposure scenarios*, mean exposure to bixin ranged from 0.001 mg/kg body weight (bw) per day in adults to 0.10 mg/kg bw per day in toddlers. The 95th percentile exposure to bixin ranged from 0.01 mg/kg bw per day in infants, children, adolescents, adults and the elderly to 0.32 mg/kg bw per day in toddlers. For norbixin, mean exposure ranged from 0.002 mg/kg bw per day in infants and the elderly to 0.11 mg/kg bw per day in toddlers. The 95th percentile exposure to norbixin ranged from 0.01 mg/kg bw per day in infants, adults and the elderly to 0.24 mg/kg bw per day in toddlers. The applicant has requested the extension of use of bixin- and norbixin-based annatto extracts in 16 additional food categories, of which 15 uses for bixin-based and seven for norbixin-based annatto extracts. For bixin, from the *extension of use scenario* considering additional exposure from food categories and levels proposed by the applicant, mean exposure ranged from 0.004 mg/kg bw per day for infants to 0.33 mg/kg bw per day for toddlers. The 95th percentile ranged from 0.01 mg/kg bw per day in the elderly to 0.65 mg/kg bw per day in toddlers. For norbixin, from the *extension of use scenario* considering additional exposure from food categories and levels proposed by the applicant, mean exposure ranged from 0.003 mg/kg bw per day for infants to 0.24 mg/kg bw per day for toddlers. The 95th percentile ranged from 0.02 mg/kg bw per day for infants, adults and the elderly to 0.46 mg/kg bw per day in infants and toddlers.

The Panel noted that raising the acceptable level for norbixin in the bixin-based annatto extract Annatto B from 2.5% to 5%, as proposed by the applicant, would result in an additional exposure to norbixin of up to 0.017 mg/kg bw per day (considering the extension of use scenario, 95th percentile in toddlers).

Given:

- that read-across among the five bixin- and norbixin-based annatto extracts was feasible;
- the availability of adequate 90-day toxicity studies with annatto extracts B, C, E and F;
- the absence of concern for mutagenicity, carcinogenicity, reproductive and developmental toxicity of annatto extracts B, C, F and G, whereas the mutagenicity of Annatto E is equivocal,

the Panel concluded that:

- the safety of the currently authorised solvent-extracted bixin and norbixin (E 160b(i)), alkali-extracted annatto (E 160b(ii)) and oil-extracted annatto (E 160b(iii)), with the specifications defined in Commission Regulation (EU) No 231/2012, could not be assessed due to the lack of data, both in terms of identification and toxicological studies;
- as regards the new annatto extracts: solvent-extracted bixin (Annatto B), solvent-extracted norbixin (Annatto C), alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G) and its salts:

— they should comply with the specifications as recommended by the Panel;
— the toxicological database is sufficient to derive an acceptable daily intake (ADI) of 6 mg bixin/kg bw per day and an ADI of 0.3 mg norbixin/kg bw per day, applying an uncertainty factor of 200 to the NOAEL values derived from the 90-day studies (1,206 mg/kg bw and 63 mg/kg bw, respectively).

Based on the reported current use levels provided by the industry, the Panel concluded that exposure estimates were below the ADI of 6 mg/kg bw per day for bixin and below the ADI of 0.3 mg/kg bw per day for norbixin for all population groups and for all refined exposure scenarios.

Considering the extension of use for the additional 16 food categories, all refined exposure estimates for bixin were below the ADI of 6 mg/kg bw per day for all populations. For norbixin, the ADI of 0.3 mg/kg bw per day was not exceeded in the non-brand-loyal scenario and in the brand-loyal scenario at the mean. The only exceedance observed for norbixin was in the brand-loyal scenario at the 95th percentile for infants (in one country), toddlers (in three countries) and children (in one country). However, the Panel noted that this exceedance results from the overestimation of the contribution from at least one food category (i.e. unripened cheese).

- as regards Annatto E, due to the equivocal results obtained with the *in vivo* comet assay, the Panel could not conclude on its safety.

The Panel recommended that the alkali-extracted annatto (E 160b(ii)), the oil-extracted annatto (E 160b(iii)) and the solvent-extracted bixin and norbixin (E 160b(i)), currently authorised in the EU, should be replaced by the solvent-extracted bixin (Annatto B); solvent-extracted norbixin (Annatto C); alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G). In addition, the Panel recommended that the specifications for bixin- and norbixin-based annatto extracts (E 160b) according to the Commission Regulation (EU) No 231/2012 should be replaced by the specifications for the annatto extracts (Annatto B, C, F and G) as given by JECFA (2007, 2015). However, the maximum limits for the impurities of toxic elements (arsenic, lead, mercury) should be revised in order to ascertain that the annatto extracts as food additives will not be a significant source of exposure to these toxic elements in foods. Moreover, the Panel recommended that a maximum limit for cadmium should also be included in the specifications.

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Background as provided by the European Commission

Regulation (EC) No 1333/2008[1] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union (EU). In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the EU before 20 January 2009 has been set up under Regulation (EU) No 257/2010[2]. This Regulation also foresees that food additives are re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[3] of 2001. The report 'Food additives in Europe 2000' submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with the highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of adoption of Regulation (EU) 257/2010, the 2003 Terms of References are replaced by those below.

Annex II to Regulation (EC) No 1333/2008 authorises the use of annatto, bixin, norbixin (E 160b) in certain food categories only. Commission Regulation (EU) No 231/2012 laying down specifications for food additives specifies three annatto products (i) *solvent-extracted bixin and norbixin*, (ii) *alkali-extracted annatto* and (iii) *oil-extracted annatto*.

The SCF has previously evaluated the annatto extracts, the last time, in 1979 when the SCF allocated an acceptable daily intake (ADI) of 2.5 mg/kg body weight (bw) for an annatto extract (containing 2.6% carotenoid expressed as bixin) equivalent to 0–0.065 mg/kg bw for carotenoids of annatto expressed as bixin.

The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) re-evaluated six annatto extracts on the basis of new data in 2003 and 2006. An ADI for bixin of 0–12 mg/kg bw and a group ADI for norbixin and its disodium and dipotassium salts of 0–0.6 mg/kg bw (expressed as norbixin) were established at the 67th JECFA (2006). Five specifications have been established for the annatto extracts covered by the established ADIs, but only tentative specifications were established for the oil-processed bixin for which no ADI has been established. JECFA requested data on the non-colouring fraction of the oil-processed bixin extract by the end of 2008.

The Natural Food Colours Association (NATCOL)/Annatto Interest Group requested the authorisation of five annatto extracts categorised as bixin- or norbixin-based, with the view to replacing the currently authorised annatto extracts (E 160b). NATCOL's application covers the five annatto extracts for which JECFA has established full ADIs: (i) aqueous-processed annatto extracts, bixin-based, (ii) solvent-processed annatto extracts, bixin-based, (iii) alkali-processed, acid-precipitated annatto extracts, norbixin-based, (iv) alkali-processed, not acid-precipitated annatto extracts, norbixin-based and (v) solvent-processed annatto extracts, norbixin-based.

Terms of Reference as provided by the European Commission

The Commission asks EFSA to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the

[1] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.

[2] Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19–27.

[3] Report from the Commission on Dietary Food Additive Intake in the European Union, Brussels, 1.10.2001, COM (2001) 542 final.

priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Commission asks EFSA to provide a scientific opinion on the safety of the annatto extracts covered by this application as a food colour for the uses proposed by the applicant.

Interpretation of Terms of Reference

The Panel noted that the Terms of Reference include raising the acceptable level for norbixin in the bixin-based annatto extract Annatto B from 2.5% to 5%, and the extension of use in 16 additional food categories.

Assessment

1. Introduction

The present opinion deals with the re-evaluation of the safety of annatto extracts (E 160b), when used as a food additive and permitted in the EU before 2009. Furthermore, at the request of the European Commission, the present 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.

In 1974, JECFA established a temporary ADI of 1.25 mg/kg bw for the sum of bixin and norbixin (expressed as bixin) (JECFA, 1975), which was endorsed by the EU SCF in 1975 (SCF, 1975).

In 1978, the SCF assessed the results of pharmacokinetic and metabolism studies of annatto extracts in rats following short- and long-time exposure and reviewed the results of acute metabolic studies in man. The Committee allocated an ADI of 0–2.5 mg/kg bw per day for annatto extracts containing 2.6% carotenoids expressed as bixin, equivalent to 0–0.065 mg/kg bw per day of carotenoids, expressed as bixin (SCF, 1979). At its 26th meeting in 1982, JECFA allocated an ADI of 0–0.065 mg/kg bw 'in terms of the carotenoid content expressed as bixin' (JECFA, 1982). The ADIs of the SCF (1979) and JECFA (1982) were both based on a long-term rat study performed with well-defined annatto extracts, which varied in total bixin content from 0.2% to 2.6%.

Within the food colour legislation of the EU (Commission Regulation (EU) No 231/2012[4] laying down specifications for food additives), three different annatto extracts are described: (i) solvent-extracted bixin and norbixin (E 160b(i)); (ii) alkali-extracted annatto (E 160b(ii)); and (iii) oil-extracted annatto (E 160b(iii)).

In 2006, JECFA agreed upon two separate ADIs for bixin- and norbixin-containing annatto extracts: an ADI of 12 mg/kg bw for bixin (92% bixin pure) and a group ADI of 0.6 mg/kg bw for norbixin (91.6% norbixin pure) and its sodium and potassium salts (JECFA, 2007).

The request of the NATCOL/Annatto Interest Group to evaluate five annatto extracts categorised as bixin- or norbixin-based, with the view to replacing the currently authorised annatto extracts (E 160b) is included in the present opinion. The application by NATCOL covers the five annatto extracts for which JECFA has established full ADIs (JECFA, 2007): (i) aqueous-processed bixin (Annatto E); (ii) solvent-extracted bixin (Annatto B); (iii) alkali-processed norbixin, acid-precipitated (Annatto F); (iv) alkali-processed norbixin, not acid-precipitated (Annatto G); and (v) solvent-extracted norbixin (Annatto C).

The Panel based its evaluation on the dossier submitted by the applicant, previous evaluations and reviews, additional literature that came available since then and the data available following public calls for data.[5–7] The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

[4] Commission Regulation (EU) No 231/2012 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.

[5] Call for scientific data on food colours to support re-evaluation of all food colours authorised under the EU legislation. Published 7 December 2006. Available online: http://www.efsa.europa.eu/en/dataclosed/call/afc061208

[6] Call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Published 27 March 2013. Available online: http://www.efsa.europa.eu/en/dataclosed/call/130327

[7] Call for scientific data on selected food additives permitted in the EU. Published 23 March 2014. Available online: http://www.efsa.europa.eu/en/dataclosed/call/140324

2. Technical data

2.1. Identity of the substance

The food colour annatto extracts (E 160b) is obtained from the outer layer (pericarp) – specifically, the outer coating – of the seeds of the tropical tree *Bixa orellana*. The tree is indigenous to Central and South America, where its seeds are used as a spice in traditional cooking. Yearly world production of annatto seeds was estimated to be 14,500 metric tonnes (dry weight) in the late 1990s: important producers of the seeds are Brazil, Guatemala, India, Peru, the Philippines and a few African countries (Smith and Wallin, 2006; Raghavan, 2007; Giridhar and Parimalan, 2010). In Brazil, Mexico and other Latin American countries, as well as in Asia (the Philippines), substantial quantities of processed annatto seeds are sold in retail outlets, often blended with other ingredients for addition to soups and meat dishes similar to the use of paprika seasonings in Europe. These condiments are known locally under various names; like paprika preparations, they impart not only colour, but also a distinct flavour.

The principle pigment in the seeds is the carotenoid *cis*-bixin; processing may involve aqueous alkaline hydrolysis with simultaneous production of *cis*-norbixin (Figure 1). Annatto seeds contain *cis*-bixin (> 80% of the total carotenoid content), mainly in the 9′-*cis* configuration, with small quantities of *trans*-bixin and norbixin. In annatto seeds, the amount of norbixin is typically below 5% of the total colouring matter (Doc. provided to EFSA n. 3). Under specific conditions of temperature and pH, bixin can be hydrolysed into norbixin – the dicarboxylic acid – which can readily be turned into its sodium or potassium salt (Figure 1).

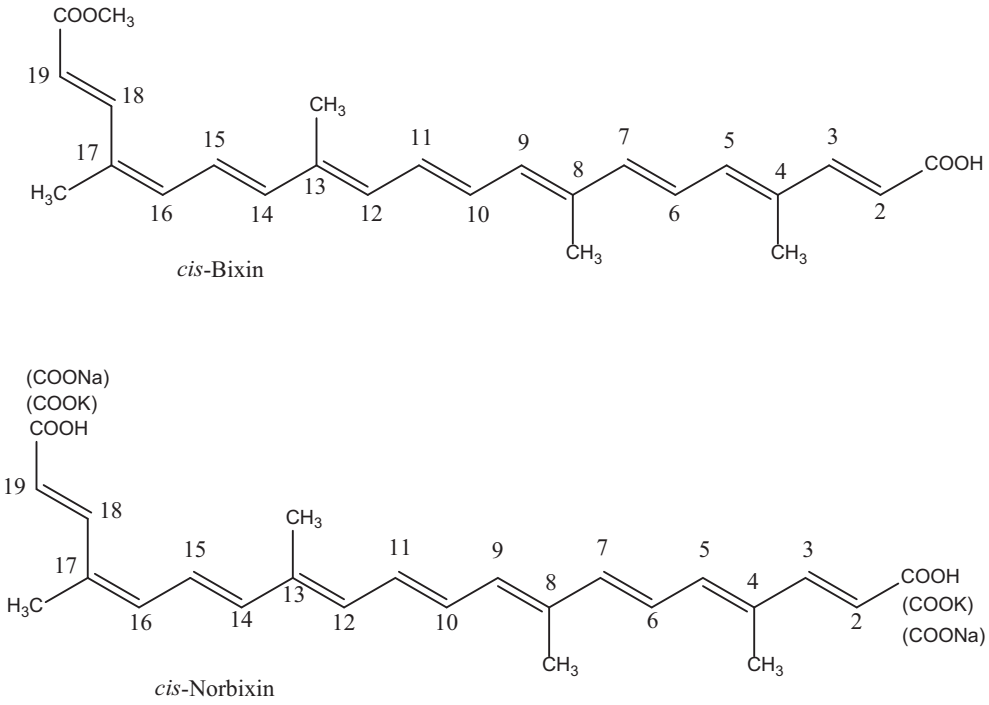

Figure 1: Structural formulas of *cis*-bixin and *cis*-norbixin, the latter also being used as the disodium or dipotassium salt. Position C-9′, eventually referred to in the text and coming from a different carbon atom numbering of the apocarotenoid structure, corresponds to position C-17 in the illustration above

A common chemical name for *cis*-bixin is methyl (9′-*cis*)-hydrogen-6,6′-diapo-ψ,ψ-carotenedioate; its molecular formula and molecular weight are $C_{25}H_{30}O_4$ and 394.50 g/mol. Similarly, a common chemical name for *cis*-norbixin is (9′-*cis*)-6,6′-diapo-ψ,ψ-carotenedioic acid; *cis*-norbixin also occurs as the dipotassium or disodium salt (dipotassium or disodium 6,6′-diapo-ψ,ψ-carotenedioate). Their molecular formulas and molecular weights (g/mol) are: $C_{24}H_{28}O_4$ and 380.48 (acid); $C_{24}H_{26}K_2O_4$ and 456.66 (dipotassium salt); $C_{24}H_{26}Na_2O_4$ and 424.44 (disodium salt). The pertinent Chemical Abstracts Service (CAS) and European Inventory of Existing Commercial chemical Substances (EINECS) (EC) number identifiers for the *cis* and *trans* configurations of bixin and norbixin are presented in Table 1.

Table 1: CAS Registry and EINECS list numbers identifying the annatto extract principles of interest in the present opinion

Substance	CAS registry no.	EINECS list no.
cis-Bixin	6983-79-5	230-248-7
trans-Bixin	39937-23-0	–
cis-Norbixin (free acid)	626-76-6	–
cis-Norbixin, dipotassium salt	–	–
cis-Norbixin, disodium salt	–	–
trans-Norbixin (free acid)	542-40-5	208-810-8
trans-Norbixin, dipotassium salt	33261-80-2	251-431-8
trans-Norbixin, disodium salt	33261-81-3	251-432-3

CAS: Chemical Abstracts Service; EINECS: European Inventory of Existing Commercial chemical Substances.

The CAS Registry and EINECS Nos for annatto are 1393-63-1 and 215-735-4, respectively. Annatto seed extracts are identified by CAS Registry and EINECS Nos 89957-43-7 and 289-561-2, respectively.

A few synonyms, trade names and abbreviations for annatto extracts are: Achiote; Annatto; Annatto colour; Annatto extract; Annatto pigment; C.I. 75120; C.I. Natural Orange 4; FEMA No 2103; FEMA No 2103 (annatto extract); FEMA No 2104 (annatto seed); Orlean; Rocou or Roucou; Terre orellana; INS 160b; Urucum (JECFA, 2007, 2015; Doc. provided to EFSA n. 3; Smith and Wallin, 2006).

The structural difference between bixin and norbixin – namely, an esterified carboxyl group – leads to appreciable differences in the physicochemical properties of the two molecules. Bixin- and norbixin-based annatto extracts are substantially insoluble in pure water and slightly soluble in ethanol; norbixin-based annatto extracts are soluble in alkaline water, but norbixin will precipitate in acidic solutions. Both products are soluble in ethyl ether and oils.

Bixin and norbixin are the two colouring principles present in the annatto extracts, dark red-brown to red-purple in appearance. Due to their different chemical and toxicological properties, commercially available annatto extracts are categorised as either bixin- or norbixin-based (CAC, 2007; JECFA, 2007). Table 2 presents the annatto extracts evaluated in the present opinion.

Table 2: Annatto extracts proposed by the applicant and evaluated in the present opinion

Product names (for labelling)	Laboratory code	Manufacturing principle	Substance identifier in this Opinion	INS no.	Specified content of colouring matter (%)
Annatto extracts, bixin-based	Annatto E	Aqueous-processed	Aqueous-processed bixin	160b(i)	≥ 25
	Annatto B	Solvent-extracted	Solvent-extracted bixin		≥ 85
Annatto extracts, norbixin-based	Annatto F	Alkali-processed, acid-precipitated	Alkali-processed, acid-precipitated norbixin	160b(ii)	≥ 35
	Annatto G	Alkali-processed, not acid-precipitated	Alkali-processed, not acid-precipitated norbixin		≥ 15
	Annatto C	Solvent-extracted	Solvent-extracted norbixin		≥ 85

The product names and International Numbering System (INS) numbers used in this table were adopted by the Codex Alimentarius Commission (CAC, 2007).

It should be noted that there are also annatto extracts processed from seeds using vegetable oil (namely, Annatto D), which contain mainly bixin; Annatto D was not included by JECFA under the ADI established for bixin and is not covered in the present opinion.

When dry, annatto extracts are fine or granular powders of dark red-brown to red-purple colour (JECFA, 2007, 2015). The alkali-processed norbixin, not acid-precipitated (Annatto G) is also used directly in liquid form, since drying leads to a highly alkaline product (Doc. provided to EFSA n. 3).

2.2. Specifications

Specifications for annatto extracts (E 160b) have been defined in Commission Regulation (EU) No 231/2012 laying down specifications for food additives (Table 3). In the Regulation, the substances of

interest are identified with the following EINECS numbers: 215-735-4 (annatto), 289-561-2 (annatto seed extracts) and 230-248-7 (bixin). They are described as reddish-brown powder, suspension or solution.

Table 3: Specifications for E 160b according to Commission Regulation (EU) No 231/2012[(a)]

Compound	Alkali-extracted annatto (E 160b(ii))	Oil-extracted annatto (E 160b(iii))	Solvent-extracted bixin and norbixin (E 160b(i))
Assay	Contains not less than 0.1% of total carotenoids expressed as norbixin	Contains not less than 0.1% of total carotenoids expressed as bixin	Content of bixin powders not less than 75% total carotenoids calculated as bixin Content of norbixin powders not less than 25% total carotenoids calculated as norbixin
Purity			
Solvent residues			
Acetone Methanol Hexane	– – –	– – –	≤ 50 mg/kg, singly or in combination
Dichloromethane	–	–	≤ 10 mg/kg
Heavy metals			
Arsenic	≤ 3 mg/kg	≤ 3 mg/kg	≤ 3 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	≤ 1 mg/kg	≤ 1 mg/kg
Cadmium	≤ 1 mg/kg	≤ 1 mg/kg	≤ 1 mg/kg

(a): In the Regulation, bixin and norbixin are also identified as 6′-methylhydrogen-9′-[*cis* or *trans*]-6,6′-diapocarotene-6,6′-dioate and 9′-[*cis* or *trans*]-6,6′-diapocarotene-6,6′-dioic acid, respectively.

Specifications for annatto extracts have also been defined by JECFA (Table 4).

Table 4: Specifications for annatto extracts according to JECFA

Compound	Aqueous-processed bixin (Annatto E) (JECFA, 2007)	Solvent-extracted bixin (Annatto B) (JECFA, 2015)	Solvent-extracted norbixin (Annatto C) (JECFA, 2015)	Alkali-processed norbixin, acid-precipitated (Annatto F) (JECFA, 2007)	Alkali-processed norbixin, not acid-precipitated (Annatto G) (JECFA, 2007)
Definition	Contains several coloured components; the major colouring principle is *cis*-bixin, a minor colouring principle is *trans*-bixin; thermal degradation products of bixin may also be present as a result of processing	Contains several coloured components; the major colouring principle is *cis*-bixin, a minor colouring principle is *trans*-bixin; thermal degradation products of bixin may also be present as a result of processing	Contains several coloured components; the major colouring principle is *cis*-norbixin, a minor colouring principle is *trans*-norbixin; thermal degradation products of norbixin may also be present as a result of processing	Contains several coloured components; the major colouring principle is *cis*-norbixin, a minor colouring principle is *trans*-norbixin; thermal degradation products of norbixin may also be present as a result of processing	Contains several coloured components; the major colouring principle is *cis*-norbixin, a minor colouring principle is *trans*-norbixin; thermal degradation products of norbixin may also be present as a result of processing
Assay	≥ 25% colouring matter (expressed as bixin)	≥ 85% colouring matter (expressed as bixin)	≥ 85% colouring matter (expressed as norbixin)	≥ 35% colouring matter (expressed as norbixin)	≥ 15% colouring matter (expressed as norbixin)
Description	Dark red-brown to red-purple powder				
Solubility	Insoluble in water, slightly soluble in ethanol		Soluble in alkaline water, slightly soluble in ethanol		
Purity					
Residual solvents					
Ethanol Isopropyl alcohol Ethyl acetate	– – –	≤ 50 mg/kg, singly or in combination	≤ 50 mg/kg, singly or in combination	– – –	– – –

Compound	Aqueous-processed bixin (Annatto E) (JECFA, 2007)	Solvent-extracted bixin (Annatto B) (JECFA, 2015)	Solvent-extracted norbixin (Annatto C) (JECFA, 2015)	Alkali-processed norbixin, acid-precipitated (Annatto F) (JECFA, 2007)	Alkali-processed norbixin, not acid-precipitated (Annatto G) (JECFA, 2007)
Acetone	–	≤ 30 mg/kg	≤ 30 mg/kg	–	–
Methanol	–	≤ 50 mg/kg	≤ 50 mg/kg	–	–
Hexane	–	≤ 25 mg/kg	≤ 25 mg/kg	–	–
Norbixin	≤ 7% of total colouring matters	≤ 2.5% of total colouring matters	–	–	–
Heavy metals					
Arsenic	≤ 3 mg/kg	≤ 3 mg/kg	≤ 3 mg/kg	≤ 3 mg/kg	≤ 3 mg/kg
Lead	≤ 2 mg/kg	≤ 2 mg/kg	≤ 2 mg/kg	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	≤ 1 mg/kg	≤ 1 mg/kg	≤ 1 mg/kg	≤ 1 mg/kg

JECFA: Joint FAO/WHO Expert Committee on Food Additives.

The applicant stated that 'in order to simplify specifications and harmonise their structure with the INS numbers as adopted by the Codex Alimentarius Commission for the two major types of annatto extracts in 2007 (after the JECFA meeting), two separate specifications are proposed, which relate to the JECFA specifications as outlined in Table 4' (Doc. provided to EFSA n. 3).

The Panel noted that the specifications in Commission Regulation (EU) No 231/2012 have only a limited relationship with those of the annatto extracts evaluated by JECFA. It was also noted that various extraction solvents can be used, but information on how this affects the characteristics and specifications of the final preparations is not adequately provided.

The Panel noted that, in the JECFA specifications which refer to the annatto extracts dealt with in the present Opinion, the level of bixin in Annatto E (≥ 25%) and B (≥ 85%), and the level of norbixin in Annatto C (≥ 85%), F (≥ 35%) and G (≥ 15%), are not defined more precisely.

The Panel noted that according to the EU specifications for the food additive annatto, bixin, norbixin, (E 160b), impurities of the toxic elements arsenic, lead, mercury and cadmium are accepted up to a concentration of 3, 2, 1 and 1 mg/kg, respectively. Contamination at these levels would have a significant impact on the exposure to these metals, for which exposures are already close to the health-based guidance values established by EFSA (EFSA, 2009a; EFSA CONTAM Panel, 2009, 2010, 2012). The Panel considered that the maximum limits for the impurities of toxic elements (arsenic, lead, mercury and cadmium) in the EC specifications should be revised in order to ascertain that E 160b as a food additive will not be a significant source of exposure to these toxic elements in foods.

The specifications for impurities proposed by the applicant are: arsenic, ≤ 3 mg/kg; lead, ≤ 2 mg/kg; mercury, ≤ 1 mg/kg; level of norbixin in Annatto E, ≤ 7% of total colouring matter; level of norbixin in Annatto B, ≤ 5% of total colouring matter. Likewise, the specifications for residual solvents (applicable to solvent-extracted bixin and norbixin only, i.e. Annatto B and Annatto C) are: acetone, ≤ 30 mg/kg; methanol, ≤ 50 mg/kg; hexane, ≤ 25 mg/kg; ethanol, isopropyl alcohol and ethyl acetate, ≤ 50 mg/kg, single or in combination. The Panel noted that a maximum limit for cadmium is not included in the JECFA specifications.

Bixin-based products also contain norbixin (JECFA, 2004a). Since JECFA's ADI for norbixin is 20-fold lower than the ADI for bixin, JECFA agreed to establish maximum limits for norbixin in bixin-based products as follows: ≤ 7% of total colouring matter for the aqueous-processed bixin (Annatto E), and ≤ 2.5% of total colouring matter for the solvent-extracted bixin (Annatto B).

The Panel noted that the norbixin levels in the specifications proposed by the applicant for the solvent-extracted bixin (Annatto B) are up to 5%. The applicant indicated that the proposed norbixin levels were based on data from the material that was tested in the 90-day study in rats (Section 3.2.2.3), and that JECFA did not take into account natural variations of norbixin levels in annatto seeds from which bixin-based extracts are prepared. The applicant argued that representative data from different sources show that natural levels of norbixin in unprocessed annatto seeds range between 1.3% and 5.1% (Giridhar and Parimalan, 2010; Doc. provided to EFSA n. 3), and that since the solvent extraction of bixin does not lead to a decrease in norbixin levels, it is proposed to raise the acceptable level for norbixin in the bixin-based annatto extract Annatto B from 2.5% to 5% (Doc. provided to EFSA n. 3).

Based on the origin of the annatto extracts (i.e. obtained from annatto seeds), the Panel noted that data on pesticides, mycotoxins and other components with biological activity (e.g. phytoestrogens, phytotoxins and allergens), possibly present in the food additive as used, may be relevant for the specifications.

2.2.1. Colouring principles

The major pigment present in the bixin-based annatto extracts – aqueous-processed Annatto E and solvent-extracted Annatto B – is 9′-*cis*-bixin; also present, as minor constituents, are bixin isomers, norbixin, and possibly bixin's thermal degradation products from processing. The two annatto extracts contain at least 25% and 85% of colouring matter expressed as bixin, respectively (Doc. provided to EFSA n. 5).

Norbixin-based annatto extracts – alkali-processed, acid-precipitated (Annatto F), alkali-processed, not acid-precipitated (Annatto G) and solvent-extracted (Annatto C) – respectively, contain at least 35%, 15% and 85% of colouring matter expressed as norbixin.

2.2.2. Other constituents

The qualitative and quantitative compositions of the non-pigment fraction of annatto extracts were investigated in the attempt to achieve a complete mass balance (> 95%) for representative batches of Annatto B, E, F and G products (Lea, 2005; Sheridan, 2005) (Table 5).

The applicant considered that the solvent-extracted norbixin (Annatto C) is derived from the solvent-extracted bixin (Annatto B) through a hot alkaline hydrolysis, and that they share the small quantity of non-pigment material. The applicant also stated that it is unlikely that the alkaline hydrolysis of bixin at moderate temperatures would be accompanied by reactions between non-pigment constituents (Doc. provided to EFSA n. 5). However, upon request of EFSA, the applicant provided further information on the analytical composition of solvent-extracted norbixin (Annatto C) (Doc. provided to EFSA n. 6).

Table 5: Mass balances for four annatto extracts (Doc. provided to EFSA n. 5 and n. 6)

Fraction/component	Solvent-extracted bixin (Annatto B)	Aqueous-processed bixin (Annatto E)	Alkali-processed norbixin, acid-precipitated (Annatto F)	Alkali-processed norbixin, not acid-precipitated (Annatto G)	
	Per cent w/w	Per cent w/w	Per cent w/w	Per cent w/w[(a)]	Calculated: per cent w/dry w[(b)]
Hexane solubles	2.4	26.8	12.6	0.75	
Geranyl geraniol	ND	8.4	10.0	0.75	7.7
Aliphatic hydrocarbons (wax)	NQ	1.6	Present	–	–
Tocotrienols	< 0.01	3.4	0.02	< 0.01	< 0.1
Other terpenoids by difference including geranyl geranene	ND	13.4	ND	–	–
Aromatic component	NQ	Traces	Present	–	–
Acetone solubles					
cis-Bixin	87 (9′-*cis*)	29.2 (9′-*cis*)	–	–	–
cis-Norbixin	–	0.9 (9′-*cis*)	39.0 (9′-*cis*)	1.8 (9′-*cis*)	18
Norbixins[(c)]	2.0 (others)		8.0 (others)	0.5 (others)	5
Bixin isomers	4.0	–	–	–	–
Unknown bixins	–	1.1	–	–	–
Fatty acid esters	< 0.01	4.1	1.9	< 0.01	< 0.1
Polyphenols	ND	4.0	ND	ND	–
Moisture	0.1	9.4	4.1	90.6	–
Acetone insolubles	< 1	20.4	34.5	7.4	
Protein	0.9	5.6	6.2	0.5	5
Ash	0.1	4.9	12.1	3.2	33

Fraction/component	Solvent-extracted bixin (Annatto B)	Aqueous-processed bixin (Annatto E)	Alkali-processed norbixin, acid-precipitated (Annatto F)	Alkali-processed norbixin, not acid-precipitated (Annatto G)	
	Per cent w/w	Per cent w/w	Per cent w/w	Per cent w/w[(a)]	Calculated: per cent w/dry w[(b)]
Carbohydrate	0.1	0.3	0.7	1.5	15
Lignocellulose	< 0.1	9.6	15.5	1.5	15
Total	95	95.9	> 95	> 99	> 99

ND: not detected; NQ: not quantifiable.
(a): Alkaline solution.
(b): Calculated on dry weight from the results of the alkaline solution.
(c): Norbixin isomers: di-*cis*-norbixin, *trans*-norbixin, 13′-*cis*-norbixin.

The non-pigment fractions of the less pure extracts – aqueous-processed bixin (Annatto E), alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G) – were found to contain, besides bixin and norbixin, several well-known plant constituents: proteins (≤ 6%), lignocelluloses (< 16%), fatty acid esters (≤ 4%), polyphenols (≤ 4%) and ash (≤ 12%). Unidentified terpenoids (up to 13.4%) were only present in Annatto E. Moisture content was less than 10% in Annatto E and F samples. The Annatto G extract was analysed as an alkaline solution containing approximately 91% of water (w/w) based on the analytical report, norbixin and norbixin isomers could make up to approximately 24% of the original solute.

Annatto E contained significant amounts of terpenoids (mainly geranyl geraniol and related diterpenoids) at levels almost equal to the amount of bixin. Also, Annatto F and Annatto G contained geranyl geraniol at rather relevant levels.

Until now, very limited data on the composition of the non-pigment material of annatto extracts were available. The presence of several carotenoids, fatty acids esters and resins with a bitter taste was reported in the 1970s by Hager (Hager, 1972). The quantitative/qualitative data for the four different annatto extracts presented by the applicant (Annatto B, E, F and G) show that those components which probably form the coating around the annatto seeds can still be detected in commercial extracts (Lea, 2005; Sheridan, 2005).

The detection and partial identification of 107 volatile compounds present in the four annatto extracts obtained from commercial sources – two water-extracts and two oil-extracts, not clearly related to the annatto extracts of this Opinion – were reported by Galindo-Cuspinera et al. (2002) using dynamic headspace-solvent desorption sampling and gas chromatography–mass spectrometry (GC–MS).

According to JECFA (2007), the non-colouring fractions of the annatto extracts do not raise any safety concerns under the intended conditions of use.

2.2.3. Relationship between annatto extracts evaluated and read-across as presented by the applicant

Based on the manufacturing processes described for bixin-based and norbixin-based annatto extracts (Figure 2), the following read-across approaches were proposed by the applicant (NATCOL, 2015).

The solvent-extracted bixin (Annatto B) is considered by the applicant as a proper subset of aqueous-processed bixin (Annatto E); it contains the enriched carotenoid fraction and some minor accompanying material from the raw material. Biological data on Annatto B will apply to the bixin fraction of Annatto E; biological data on Annatto E may be relevant to the non-bixin part of Annatto B (although content is much lower and the fraction containing terpenoids is removed almost quantitatively).

The solvent-extracted norbixin (Annatto C) is derived from the solvent-extracted bixin (Annatto B); they share the small quantity of non-pigment material.

The non-pigment parts of the alkali-processed norbixin, acid-precipitated (Annatto F) and alkali-processed norbixin, not acid-precipitated (Annatto G) are proper subsets of the non-pigment part of the aqueous-processed bixin (Annatto E) – as discussed above the alkaline hydrolysis is not expected

to have an impact on the non-pigment fraction, except for the oxidation of tocotrienols (which were not detected in the samples of Annatto F and G tested). Biological data on the non-pigment part of Annatto E may be relevant to the non-pigment parts of Annatto F and G, with the exception of tocotrienols, which should not be present anymore in an active form in both products after alkaline treatment.

Considering that the different annatto extracts share several steps in the manufacturing route (Figure 2), the applicant considered that data from the solvent-extracted bixin (Annatto B) and the alkali-processed norbixin, acid-precipitated (Annatto F) are also applicable to the solvent-extracted norbixin (Annatto C).

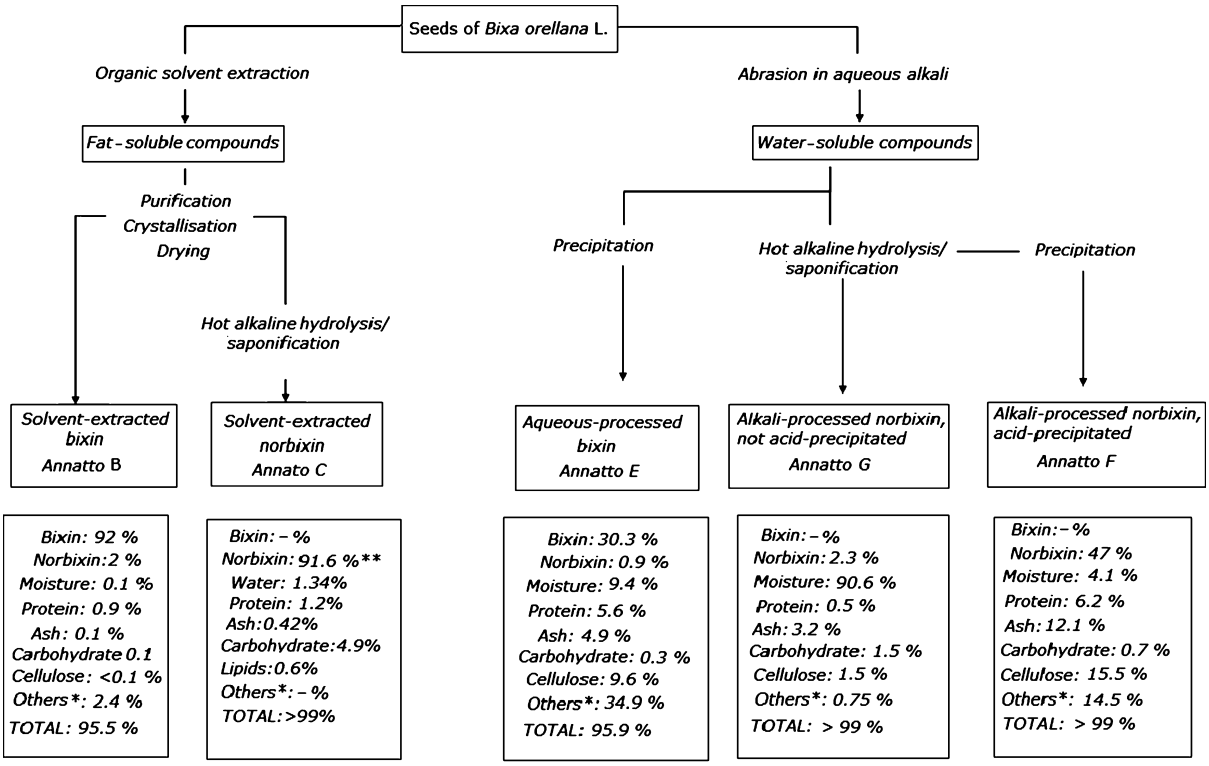

Figure 2: Overview of the processing techniques used in the manufacturing of annatto extracts (Doc. provided to EFSA n. 5 and n. 6). The Panel noted that Annatto E and Annatto F were obtained by precipitation following acidification of the alkaline extraction mixture

2.3. Manufacturing process

Annatto extracts are obtained from the outer coating of the seeds of the tropical tree *B. orellana* L. Of the total amount of seeds gathered yearly worldwide, it may be estimated that some 7,500 metric tonnes are used as a food colour source: assuming an average colour content of 2%, it may be calculated that around 150 metric tonnes of bixin are yearly available for extraction. The amount of seeds not used as a food colour source is consumed locally, mainly for seasoning (Smith and Wallin, 2006).

The first processing step is mechanical abrasion, which involves primarily abrading or raspelling (mechanical removal of the aril from the seed) of the pigment in an appropriate suspending agent, followed by removal of the seeds from this suspension. For the purpose of removing the pigment, it is not necessary to grind the seed. Processing methods may be aimed at the production of the native bixin from the seeds, which requires a neutral liquid such as water or organic solvents, or may involve aqueous alkaline hydrolysis with the simultaneous production of norbixin.

In addition, solvent processing techniques are used as a means of production of more concentrated extracts.

Acid precipitation is another step that increases the purity by separating the annatto pigment from those components that remain soluble at a low pH.

All the three techniques are used in several combinations and can lead to different products that are either rich in bixin or norbixin. There were also other manufacturing methods used previously (e.g. oil-processing); an overview of these processes is provided by Preston and Rickard (1980).

2.3.1. Manufacturing of annatto extracts, bixin-based

Bixin-based extracts are prepared by two different processes (JECFA, 2007, 2015; Doc. provided to EFSA n. 3; Smith and Wallin, 2006).

Aqueous-processed bixin (Annatto E) is prepared by removal of the outer coating of the seeds of the annatto tree by abrading them in the presence of cold, mildly alkaline water (alkalised with potassium or sodium hydroxide). The resultant suspension is acidified to precipitate bixin, which is then filtered, washed, dried and milled. The resulting product is a granular powder.

Solvent-extracted bixin (Annatto B) is obtained from the outer coating of the seeds of the annatto tree with one or more of the following food grade solvents: acetone, alkaline alcohol, ethanol, ethyl acetate, hexane, methanol, isopropyl alcohol or supercritical carbon dioxide. The crude extract is filtered to remove insoluble material. Subsequent processing involves removal of fats and wax-like substances. The resulting preparation may be acidified, followed by the removal of the solvent, drying and milling.

2.3.2. Manufacturing of annatto extracts, norbixin-based

Norbixin-based extracts are prepared by three different processes (JECFA, 2007, 2015; Doc. provided to EFSA n. 3; Smith and Wallin, 2006).

Alkali-processed, acid-precipitated norbixin (Annatto F) is prepared by removal of the outer coating of the seeds of the annatto tree with aqueous alkali (potassium or sodium hydroxide). The bixin is hydrolysed to norbixin in hot alkaline solution and acidified to precipitate the norbixin. The precipitate is filtered, dried and milled to give a granular powder.

Alkali-processed, not acid-precipitated norbixin (Annatto G) is prepared by removal of the outer coating of the seeds of the annatto tree with aqueous alkali (potassium or sodium hydroxide). The bixin is hydrolysed to norbixin in hot alkaline solution. The solution is filtered and can subsequently be dried and milled to give a granular powder. Extracts contain mainly the potassium or sodium salt of norbixin, as the major colouring matter.

Solvent-extracted norbixin (Annatto C) is obtained from the outer coating of the seeds of the annatto tree with one or more of the following food grade solvents: acetone, alkaline alcohol, ethanol, ethyl acetate, hexane, methanol, isopropyl alcohol or supercritical carbon dioxide. Extraction is followed by solvent removal, crystallisation and drying. Aqueous alkali is added to the resultant powder, which is then heated to hydrolyse bixin, and cooled. The aqueous solution is filtered and acidified to precipitate the norbixin. The precipitate is filtered, washed, dried and milled to give a granular powder.

2.4. Methods of analysis in food

Several methods and techniques for the qualitative identification and quantitative determination of bixin, norbixin and their degradation products are described in the reviews of Giridhar et al. (2013) and Scotter (2009). There are a number of validated methods available for the analysis of the individual colour principles of annatto extracts in a variety of food matrices, as the following examples illustrate.

Scotter (1998) analysed samples of commercial annatto formulations for bixin or norbixin by applying a developed method utilising high-performance liquid chromatography (HPLC) and photodiode array detection (HPLC-PDA); results were compared with those obtained from using UV-visible spectrophotometric methods. HPLC-PDA provided superior qualitative and quantitative data, particularly with respect to the determination of coloured degradation compounds. Two samples of norbixin of known production history were subjected to detailed HPLC analysis to identify possible differences in their coloured and degradation component profiles. The samples differed significantly in their all-*trans*- and di-*cis*-norbixin isomer contents, which was indicative of their respective production histories.

Chisté et al. (2011) designed a study to identify and quantify carotenoid and phenolic compounds from annatto seeds using HPLC equipped with a diode array detector (DAD) connected in series to a mass spectrometer performing multiple mass spectrometry (MS/MS). In addition to bixin, hypolaetin and a caffeoyl derivative were identified as the main phenolic compounds: HPLC-DAD quantification was carried out at 459 nm for bixin and at 320 nm for the other two compounds. The optimised procedure involved numerous extractions using a mixture of acetone, methanol and water (50:40:10, v/v/v), and an extraction time of 5 min. Based on validation data, the HPLC method proposed appeared to have a good reliability for the simultaneous analysis of phenolic compounds and carotenoids in annatto seeds. Tracers, including bixin, added to annatto seeds, exhibited recovery rates in the extracts higher than 90%, while the limits of quantification and detection (LOQ and LOD, respectively) of bixin were estimated to be (rounded) 0.6 and 0.2 mg/L, respectively.

Lancaster and Lawrence (1995) determined annatto pigments – (bixin and norbixin) – in high-fat dairy products (cheese and butter), margarine and hard candy by solvent extraction followed by HPLC and spectrophotometric determination at 500 nm. Average recovery rates around 90% were attained in all cases over the bixin and norbixin concentration ranges explored, while the LOD for both principles in the fatty foods analysed was 0.02 mg/kg.

A number of publications (Scotter, 1995, 1998, 2009; Scotter et al., 2002) describe a selection of suitable solvent extraction systems depending on food matrix, followed by HPLC-PDA analysis, whose response was optimised at 455 nm with UV-visible PAD, extensively investigated by Scotter (2009) and Scotter et al. (2002). This approach enabled identification and quantification of the major colour principles – 9′-*cis*-bixin, 9′-*cis*-norbixin and *trans*-bixin – and semiquantitative determination of, among others, *trans*-norbixin and the minor mono-*cis*-(other than the 9′-*cis* forms) and di-*cis*-bixin, and norbixin isomers mono-*cis*-(other than the 9′-*cis* forms) and di-*cis*-. Mean recoveries obtained from spiked food samples of 12 different food matrices spiked with annatto at levels up to 27.7 mg/kg and ranged from 61% to 96%; the LOQ was reported to be 0.1 mg/kg (LOD, 0.01 mg/kg). Noppe et al. (2009) developed a specific method for routine analysis of bixin and norbixin in meat tissue. Liquid-solid extraction was carried out using acetonitrile, while quantification was achieved by HPLC-PDA, using two wavelengths (458 and 486 nm). The possibilities of ion trap (IT) MS were also assessed. Method recoveries were comprised between 99% and 102%; the LOQ was 0.5 mg/kg. In addition, MS detection was evaluated using an IT instrument equipped with an electrospray ionisation interface (HPLC–MS/MS). In the review by Scotter (2009), several other methods are also mentioned.

For the analysis of several samples of extruded corn snack food, De Oliveira and Mercadante (2004) described a method involving enzymatic digestion with α-amylase at room temperature of the ground sample (pretreatment), followed by multiple extractions of the aqueous phase with ethyl acetate. Lipids were removed by alkaline saponification (50–70°C) – the latter also causing a 100% conversion of extracted bixin to norbixin with an energic alkaline treatment – and analyte determination was carried out by HPLC equipped with a spectrophotometric detector (450 nm). A mean recovery rate, in the 96–98% range, of 97% was obtained; LOQ and LOD were estimated to be (rounded) 0.04 and 0.01 mg/kg, respectively. Using this method, annatto pigments could be analysed as norbixin only; thus, no information was provided on the presence of bixin.

2.5. Reaction and fate in food

Bixin and norbixin, being carotenoids, are susceptible to oxidation in foods by various agents. In particular, oxidation can be exacerbated by the presence of light and heat, especially above 70°C. However, for practical purposes, annatto principles appear to be sufficiently stable under the common conditions of food storage and use (Levy and Rivadeneira, 2000; Scotter, 2009; Balaswamy et al., 2012; Gallardo-Cabrera and Rojas-Barahona, 2015).

The involved degradation mechanisms were studied extensively, and some of the degradation products were identified, such as toluene, *m*-xylene and a C_{17}-isoprenoid (Scotter, 1995; Scotter et al., 2000). Degradation may occur in annatto extracts exposed to heat resulting in the isomerisation of bixin and norbixin, a process described in more detail by Scotter (1998).

In his 2009 review, Scotter (2009) summarised the key aspects of the structural determination of bixin and norbixin, paying special attention to *cis*–*trans* isomerisation and how this is linked with their chemical structure, spectroscopic properties, and oxidative, thermal and photochemical stability. The latter and the subsequent implications for its use in the colouring of foods, food processing, and the analysis of foods and beverages were discussed, along with important mechanistic, thermodynamic and kinetic aspects.

Norbixin will precipitate at low pH and can react with metallic salts in water to give a haze. Norbixin can complex with proteins to give a product more stable to light that is redder in colour (Smith and Wallin, 2006).

No effects on nutrients have been reported. Due to its distinct structural features leading to amphoteric properties, no interaction with the absorption of fat-soluble nutrients such as retinol or β-carotene is to be expected.

2.6. Case of need and proposed uses

Annatto extracts are food colours of natural origin that provide yellow, orange and orange-red colour shades. Annatto seeds and extracts have been used for over 200 years in Europe and North America to impart a yellow to red colour to foods, especially dairy products, such as cheese (Doc. provided to EFSA n. 3).

The chemical properties of the colouring principles bixin and norbixin, lead to a solubility profile that distinguishes annatto extracts from other carotenoids (Freund et al., 1988; Collins, 1992; Levy and Rivadeneira, 2000).

Bixin-based annatto extracts are used to impart colour to foods such as margarine, shortenings and processed cheese. They are also used extensively in bakery products, biscuit fillings, popcorn and snack foods, sauces, dressings and cream desserts. For application in aqueous products, emulsions are the more appropriate product forms (Doc. provided to EFSA n. 3).

Norbixin-based annatto extracts have traditionally been used for the colouring of cheese, but are now used in many other applications. As norbixin is not acid proof (it precipitates below pH 7), it is normally not used in clear acidic solutions, such as clear soft drinks, but it is claimed to be suitable in acidic foodstuffs having a matrix or solid structure. The amphoteric nature of norbixin makes it suitable in products where the pigment is absorbed by protein and/or starch, stabilising it thereby and making it less soluble in water. These characteristics make norbixin-based extracts suitable for products like cheese, smoked fish and breakfast cereals (Doc. provided to EFSA n. 3).

The present applications of the water-soluble annatto extracts include sausage casing, sausages, puddings, tomato sauce, breakfast cereals, milk desserts, chocolate fillings, smoked fish and pet food. As the water-soluble preparations also exist in powdered form, their applications include also powdered foods. These products will become yellow or orange yellow when reconstituted (Doc. provided to EFSA n. 3).

Maximum levels of E 160b have been defined in Annex II to Regulation (EC) No 1333/2008 on food additives, as amended. These levels are defined by the Panel as maximum permitted levels (MPLs) in this document.

Currently, E 160b is an authorised food colour in the EU with MPLs ranging from 10 to 50 mg/kg in foods. Table 6 summarises foods that are permitted to contain annatto, bixin, norbixin (E 160b) and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

Table 6: MPLs of E 160b in foods according to the Annex II to Regulation (EC) No 1333/2008

FCS category no.	Food category name	Restrictions/exceptions	MPL[(a)] (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat-treated products		10
01.7.2	Ripened cheese	Only ripened orange, yellow and broken-white cheese and red and green pesto cheese	15
01.7.2	Ripened cheese	Only red Leicester cheese	50
01.7.2	Ripened cheese	Only Mimolette cheese	35
01.7.3	Edible cheese rind		20
01.7.5	Processed cheese		15
01.7.6	Cheese products (excluding products falling in category 16)	Only ripened orange, yellow and broken-white products	15
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	Only fats	10

FCS category no.	Food category name	Restrictions/exceptions	MPL[(a)] (mg/L or mg/kg as appropriate)
02.2.2	Other fat and oil emulsions including spreads as defined by Council Regulation (EC) No 1234/2007 and liquid emulsions	Excluding reduced fat butter	10
03	Edible ices		20
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Only decorations and coatings	20
06.3	Breakfast cereals	Only extruded puffed and or fruit-flavoured breakfast cereals	25
06.6	Batters	Only batters for coating	20
07.2	Fine bakery wares		10
08.3.3	Casings and coatings and decorations for meat		20
09.2	Processed fish and fishery products including molluscs and crustaceans	Only smoked fish	10
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Only liqueurs	10
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	Only alcoholic drinks with less than 15% of alcohol	10
15.1	Potato-, cereal-, flour- or starch-based snacks	Excluding extruded or expanded savoury snack products	10
15.1	Potato-, cereal-, flour- or starch-based snacks	Only extruded or expanded savoury snack products	20
15.2	Processed nuts	Only savoury-coated nuts	10
16	Desserts excluding products covered in categories 1, 3 and 4		10

MPL: maximum permitted level; FCS: Food Categorisation System presented in Annex II to Regulation (EC) No 1333/2008.
(a): Use levels apply to the colouring principles bixin/norbixin.

The applicant has requested the extension of use of bixin- and norbixin-based annatto extracts in 16 food categories, in which 15 uses were proposed for bixin-based and 7 for norbixin-based annatto extracts (Doc. provided to EFSA n. 4). The uses and use levels of bixin- and norbixin-based annatto extracts proposed by the applicant are presented in Table 7. It is important to note that the proposed use levels apply to the colouring principles bixin and norbixin and not to the extracts.

For FCS category 14.2.6 (liqueurs), the use level proposed by the applicant for bixin is higher than the currently authorised MPL (Table 6). For FCS category 15.1, already regulated in the legislation, an additional use is proposed by the applicant for bixin and norbixin in cereal-based snacks. For FCS category 15.2, in which the use of E 160b is currently restricted to savoury-coated nuts only, the applicant requested the authorisation for the use of bixin and norbixin in the entire food category 15.2.

According to the applicant, bixin and norbixin differ significantly in their physicochemical properties. The amphophilic bixin molecule is rather fat-soluble, but its free carboxyl group may also interact with matrices that contain both lipids and polar substances (e.g. emulsions). The two carboxyl groups of norbixin render the molecule water-soluble. However, its hydrocarbon central chain allows interaction with molecules that have partially hydrophobic properties; binding to proteins is for norbixin a typical mode of colouring action (Doc. provided to EFSA n. 3). As a result, the areas of application partially overlap, as can be seen from the proposed uses and use levels presented in Table 7.

Table 7: Typical and maximum use levels of bixin- and norbixin-based annatto extracts (mg/kg) as proposed by the applicant for the extension of uses. Use levels presented apply to the colouring principles bixin and norbixin

FCS category no.	Food category name	Food description	Bixin/ norbixin	Bixin use levels			Norbixin use levels		
				Min	Typical	Max	Min	Typical	Max
01.7.1	Unripened cheese excluding products falling in category 16	Other cheese	Norbixin	–	–	–	10	–	50
04.2.4.1	Fruit and vegetable preparations excluding compote	Fruit preparations for use in desserts/yogurts	Bixin	10	50	100	–	–	–
04.2.5	Jam, jellies and marmalades and similar products		Bixin	1	8	20	–	–	–
04.2.6	Processed potato products	Only dried potato granules and flakes	Bixin	0.5	10	20	–	–	–
05.2	Other confectionery including breath refreshening microsweets		Bixin	1	30	100	–	–	–
06.5	Noodles		Bixin	–	–	20	–	–	–
08.2	Meat preparations as defined by Regulation (EC) No 853/ 2004	Breakfast sausages with a minimum cereal content of 6%; burger meat with a minimum vegetable and/or cereal content of 4%	Bixin	0.5	10	20	–	–	–
08.3.1	Non-heat-treated meat products	*Chorizo sausage*; *Salchichon*; *Pasturmas* (edible external coating); *Sobrasada*; *luncheon meat*	Bixin	0.5	10	20	–	–	–
08.3.2	Heat-treated meat products	Sausages, pâtés and terrines	Bixin	0.5	10	20	–	–	–
09.2	Processed fish and fishery products including molluscs and crustaceans	Only surimi and similar products and salmon substitutes	Bixin, norbixin	–	–	30	–	–	30
12.2.2	Seasonings and condiments	Seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney, piccalilli	Bixin, norbixin	1	–	30	1	–	30
12.5	Soups and broths	Soups	Bixin, norbixin	0.5	10	15	0.5	10	15
12.6	Sauces		Bixin, norbixin	1	15	30	1	15	30
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Only liqueurs	Bixin	1	5	20	–	–	–
15.1	Potato-, cereal-, flour- or starch-based snacks	Only cereal-based snacks	Bixin, norbixin	1	5	10	1	5	10
15.2	Processed nuts		Bixin, norbixin	1	5	10	1	5	10

FCS: Food Categorisation System.

2.7. Reported use levels or data on analytical levels of E 160b in food

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a lower level than the MPL. Therefore, information on actual use levels is required for performing a more realistic exposure assessment, especially for those additives for which no MPL is set and which are authorised according to quantum satis (QS).

In the framework of Regulation (EC) No 1333/2008 on food additives and of Regulation (EU) No 257/2010[8] regarding the re-evaluation of approved food additives, EFSA issued a public call[5] for scientific data on food additives, including present use and use patterns (i.e. which food categories and subcategories, proportion of food within categories/subcategories in which it is used, actual use levels (typical and maximum use levels), especially for those uses which are only limited by QS).

In March 2013, a public call[6] for food additive usage level and/or concentration data in food and beverages intended for human consumption, including annatto extracts (E 160b) was launched, with a deadline in November 2013. In response to this public call, updated information on the actual use levels of E 160b in foods was made available to EFSA by industry and the Member States.

2.7.1. Summarised data on reported use levels of E 160b in foods provided by industry

Industry provided EFSA with data on use levels (n = 99) of E 160b in foods for all 22 food categories in which it is authorised.

Updated information on the actual use levels of E 160b in foods was made available to EFSA by the FoodDrinkEurope (FDE) (Doc. provided to EFSA n. 1) (n = 48), the Natural Food Colours Association (NATCOL) (Doc. provided to EFSA n. 4) (n = 47) and a private[9] company (Doc. provided to EFSA n. 7) (n = 4) for several food categories of finished products. Some information became available from industry (i.e. NATCOL), differentiating between the uses and use levels of bixin- and norbixin-based extracts (in 8 and 17 food categories, respectively), whereas the remaining data referred to annatto (E 160b).

Appendix A provides data on the use levels of annatto, bixin and norbixin (E 160b) in foods as reported by industry.

2.7.2. Summarised data on concentration levels of E 160b in foods from the Member States

Additionally, analytical results from the Member States were collected through the EFSA call for concentration data. The Panel noted that complete information on the methods of analysis (e.g. validation) was not made available to EFSA. The analytical results provided referred to E 160b and did not differentiate between bixin- and norbixin-based extracts.

In total, 377 analytical results were reported to EFSA by 3 countries: the Czech Republic (n = 14), Germany (n = 317) and Ireland (n = 46). Foods were sampled between 2004 and 2014. Out of this data set, analytical results of E 160b were not detected (< LOD) in 173 samples, not quantified (< LOQ) in 72 samples and were numerical values (quantified) in 132 samples. All samples were analysed in accredited laboratories.

Data (n = 2) above the MPL set for authorised uses of E 160b as food additive were reported in ripened cheese (FCS 01.7.2; 17.85 and 20.90 mg/kg in Gouda cheese). For the exposure assessment, the Panel considered only analytical data resulting from authorised uses at levels not exceeding the MPLs. Exposure resulting from the presence of food additives in food at levels above the MPL is part of risk management (e.g. non-compliance controls). For this reason, such analytical results are not considered in the exposure assessment. It should, however, be noted that the analytical results reported in non-authorised food categories were below the LOD or the LOQ, except for seven samples where annatto (E 160b) was quantified: 'unripened cheese excluding products falling in category 16' (FCS 01.7.1) (n = 3), 'seasonings and condiments' (FCS 12.2.2) (n = 1) and 'butter and concentrated butter and butter oil and anhydrous milkfat' (FCS 02.2.1) (n = 3).

Overall, 169 out of the 377 total analytical results reported for E 160b in foods were considered by the Panel for the exposure estimates after discarding the following: the analytical results in foods in which the direct addition of E 160b is not authorised according to Annex II to Regulation (EC) No 1333/2008 (n = 206) and the samples exceeding the MPL (n = 2).

Appendix B shows the analytical results of E 160b in foods as reported by the Member States.

[8] Commission Regulation (EU) 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19.

[9] The data provider requested anonymity.

2.8. Summarised data extracted from the Mintel GNPD database

The Mintel's Global New Products Database (GNPD) is an online database which monitors product introductions in consumer packaged goods markets worldwide. It contains information on over 2 million food and beverage products, of which more than 800,000 are or have been available in the European food market. Mintel started covering European food markets in 1996, currently having 20 out of the 28 EU member countries presented in the GNPD.[10]

For the purpose of this Scientific Opinion, the GNPD[11] was used for checking the labelling of products containing E 160b within the EU's food products, as the GNPD shows the compulsory ingredient information presented on the labelling of products. According to Mintel, E 160b was labelled in more than 4,500 food, drink and supplement products between 2011 and 2015.

Appendix C presents the percentage of the food products labelled with E 160b between 2011 and 2015, out of the total number of food products, per food subcategories according to the Mintel food classification. Overall, around 1.4% of the food products available on the Mintel GNPD for this period reported E 160b on the label.

2.9. Information on existing authorisations and evaluations

The SCF expressed for the first time in 1975 an opinion on the use of annatto extracts as food colours, where the committee endorsed the temporary ADI of 0–1.25 mg/kg bw (for the sum of bixin and norbixin, expressed as bixin), established by JECFA 1 year earlier (JECFA, 1975). The SCF requested that the results of metabolic studies be presented for evaluation, and stressed that metabolic and other biological data must relate to the main pigment present in extracts, and not to another geometrical isomer.

The SCF assessed, 4 years later, the results of pharmacokinetic and metabolism studies of annatto extracts in rats following short- and long-time exposure; it also reviewed the results of acute metabolic studies in man. The Committee allocated an ADI of 0–2.5 mg/kg bw for annatto extracts containing 2.6% carotenoids expressed as bixin, equivalent to 0–0.065 mg/kg bw per day of annatto extract carotenoids, expressed as bixin (SCF, 1979).

At its 26th meeting in 1982, JECFA allocated an ADI of 0–0.065 mg/kg bw 'in terms of the carotenoid content expressed as bixin' (JECFA, 1982).

The ADI established by the SCF (1979) and by JECFA (1982) was based on a long-term rat study performed with well-defined annatto extracts in which the total bixin content varied from 0.2% to 2.6%. The level causing no toxicological effect in rats was determined as 0.5% annatto extracts in the diet, equivalent to 250 mg annatto extracts/kg bw, the highest level administered, which corresponds to 6.5 mg of carotenoids (expressed as bixin) per kg bw.

This ADI applied to both bixin- and norbixin-rich extracts, which were described in the specifications prepared at the 26th meeting of JECFA defining 'Annatto extracts' as either annatto extracts in oil (containing mainly bixin) or water-soluble annatto extracts (containing mainly norbixin). Different routes of manufacturing were described for both main categories, reflecting a wider range of commercial extracts. In line with the extracts tested toxicologically, a minimum assay value of 0.2% total carotenoids was set.

JECFA agreed at its 46th meeting (JECFA, 1997) to revise the specifications for annatto extracts and split them according to the methods of manufacturing into two: one to cover oil- and alkali-extracted products, the other to cover solvent-extracted products. For solvent-extracted products, higher minimum carotenoid content (75% for bixin-rich extracts and 25% for norbixin rich extracts) was requested, whereas, for the other products, the minimum limit of 0.2% was replaced by 'as stated by the vendor'. Within the food colour legislation of the EU, three different annatto extracts were described by the specifications laid down in 1995[12]: (i) solvent-extracted bixin and norbixin; (ii) alkali-extracted annatto; and (iii) oil-extracted annatto. Although the first solvent-extracted bixin and norbixin corresponded to JECFA's category of products with higher minimum carotenoid content (75% for bixin-rich extracts and 25% for norbixin-rich extracts), the two other entries corresponded to the description of JECFA's annatto extracts (oil- and alkali-extracted) with one small difference: the EC specification requires a minimum pigment content of 0.1%.

[10] Missing Bulgaria, Cyprus, Estonia, Latvia, Lithuania, Luxembourg, Malta and Slovenia.
[11] http://www.mintel.com/global-new-products-database; accessed on 1 June 2016.
[12] Commission Directive 95/45/EC of 26 July 1995 laying down specific purity criteria concerning colours for use in foodstuffs. OJ L 226, 22.9.1995, p. 1.

JECFA decided at its 61st meeting in 2003 to consider all six substances for which data had been submitted (Annatto B, C, D, E, F and G) as separate entities, developed correspondingly six tentative specifications, and adopted temporary ADIs for the four compounds (Annatto B, C, E and F) for which 90-day studies in rats had been made available. The differences observed in the rat studies were attributed to the non-colouring material present in the extracts. Consequently, the ADIs were based on the materials as tested biologically, and did not relate to their content of the active colouring principles bixin or norbixin (JECFA, 2004a). Based on the assumption that the non-pigment fraction may explain the different toxicological patterns observed, JECFA requested 'additional information to clarify the role that the non-pigment components of the extract play in the expression of the qualitative and quantitative differences in toxicity of the various extracts. In addition, the Committee requested data on the reproductive toxicity of an extract, such as Annatto F, that contains norbixin'.

Three years later, JECFA re-considered at its 67th meeting its opinion and agreed that the observed toxicological effects were rather attributed to bixin and norbixin than to the non-colouring material (JECFA, 2007). Two separate ADIs for bixin- and norbixin-containing annatto extracts were agreed upon. For norbixin, a group ADI that included also salts of norbixin was adopted. These ADIs differ by an order of magnitude; the ADI for bixin is in the range 0–12 mg/kg bw, the group ADI for norbixin and its salts is 0.6 mg/kg bw. Five specifications were adopted as final, with the exception of the oil-extracted annatto (Annatto D), for which no ADI had been established, and continued to be described by a tentative specification. As no no-observed-effect level (NOEL) could be identified for the oil-processed bixin and no compositional data were available, the Committee decided that the above evaluation could not be applied to this extract. Data on the non-colouring fraction of this product have been requested in 2008.

2.10. Exposure

2.10.1. Food consumption data used for exposure assessment

2.10.1.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with data from national food consumption surveys at a detailed level. Competent authorities in the European countries provide EFSA with food consumption data at the level of the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a). New consumption surveys added in 2015 in the Comprehensive Database were also taken into account in this assessment.[13,14]

Food consumption data included in the Comprehensive Database were collected through different methodologies and thus direct country-to-country comparisons should be interpreted with caution. Depending on the food category and the level of detail used in the exposure calculations, uncertainties can be introduced because of possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless, the EFSA Comprehensive Database represents the best available source of food consumption data across Europe at present.

The Panel estimated the chronic exposure to different forms of annatto extracts (E 160b; i.e. annatto, bixin and norbixin) for the following population groups: infants, toddlers, children, adolescents, adults and the elderly. For these population groups, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Table 8).

Table 8: Population groups considered for the exposure estimates to E 160b

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, United Kingdom
Toddlers	From 12 up to and including 35 months of age	Belgium, Bulgaria, Denmark, Germany, Spain, Finland, United Kingdom, Italy, Netherlands

[13] Available online: http://www.efsa.europa.eu/en/press/news/150428
[14] Available online: https://www.efsa.europa.eu/en/food-consumption/comprehensive-database

Population	Age range	Countries with food consumption surveys covering more than 1 day
Children[(a)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Germany, Denmark, Spain, Finland, France, United Kingdom, Greece, Italy, Latvia, Netherlands, Sweden
Adolescents	From 10 up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Germany, Denmark, Spain, Finland, France, United Kingdom, Italy, Latvia, Netherlands, Sweden
Adults	From 18 up to and including 64 years of age	Austria, Belgium, Czech Republic, Germany, Denmark, Spain, Finland, France, United Kingdom, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Sweden
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Germany, Denmark, Finland, France, United Kingdom, Hungary, Ireland, Italy, Netherlands, Romania, Sweden

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011b).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the food categorisation system (FCS) as presented in the Annex II to Regulation (EC) No 1333/2008, part D, to perform exposure calculations. In practice, FoodEx food codes were matched to the FCS food categories.

2.10.1.2. Food categories considered for the exposure assessment of E 160b

The food categories in which the use of annatto extracts (E 160b) is authorised were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system food codes) at the most detailed level possible (up to FoodEx Level 4) (EFSA, 2011b).

Some food categories and/or their relative restrictions/exceptions are not referenced in the EFSA Comprehensive Database and could therefore not be taken into account in the present exposure assessment. This may have resulted in an underestimation of the exposure. The food categories which were not taken into account are described below (in ascending order of the FCS codes):

- 01.7.3 Edible cheese rind;
- 01.7.6 Cheese products (excluding products falling in category 16), only ripened orange, yellow and broken-white products;
- 02.2.2 Other fat and oil emulsions including spreads as defined by Council Regulation (EC) No 1234/2007 and liquid emulsions, excluding reduced fat butter;
- 05.4 Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4, only decorations and coatings;
- 06.6 Batters, only batters for coating;
- 08.3.3 Casings and coatings and decorations for meat.

For the following food categories, the restrictions which apply to the use of E 160b could not be taken into account, and therefore the whole food category was considered in the exposure estimates. This may have resulted in an overestimation of the exposure:

- 01.7.2 Ripened cheese, only ripened orange, yellow and broken-white cheese, and red and green pesto cheese;
- 02.1 Fats and oils essentially free from water (excluding anhydrous milkfat), only fats;
- 15.1 Potato-, cereal-, flour- or starch-based snacks, only extruded or expanded savoury snack products/excluding extruded or expanded savoury snack products;
- 15.2 Processed nuts, only savoury-coated nuts.

Overall, 16 out of 22 food categories were considered in the present exposure assessment for the current permitted uses of the annatto extracts (E 160b): six were not taken into account in the exposure assessment because these and/or their relative restrictions/exceptions are not referenced in the EFSA Comprehensive Database; four food categories were included in the exposure assessment without considering their restrictions/exceptions.

For the proposed extension of use (15 categories for bixin and seven for norbixin), all the food categories proposed either for bixin or norbixin were taken into account in the respective scenarios. For the following food categories, the proposed restrictions could not be taken into account, and therefore the whole food category was considered in the exposure estimates. This may have resulted in an overestimation of the exposure:

- 04.2.4.1. Fruit and vegetable preparations excluding compote;
- 15.1 Potato-, cereal-, flour- or starch-based snacks, only cereal-based.

The concentration levels used in the refined exposure scenarios of E 160b are presented in Appendices D and E.

2.10.2. Exposure to bixin and norbixin from the use of annatto extracts (E 160b) as food additive

The Panel estimated chronic exposure to E 160b for the following population groups: infants; toddlers, children, adolescents, adults and the elderly. Dietary exposure to E 160b was calculated by multiplying the concentration levels (Appendices D and E) per food category with their respective consumption amount per kg body weight for each individual in the Comprehensive Database. The exposure per food category was subsequently added to derive an individual total exposure per day. These exposure estimates were averaged over the number of survey days, resulting in an individual average exposure per day for the survey period. Dietary surveys with only 1 day per subject were excluded as they are considered as not adequate to assess repeated exposure.

These calculations were carried out for all individuals per survey and per population group, resulting in distributions of individual exposure per survey and population group (Table 8). Based on these distributions, the mean and 95th percentile of exposure were calculated per survey and per population group. The 95th percentile of exposure was only calculated for those population groups where the sample size was sufficiently large to allow this calculation (EFSA, 2011a). Therefore, in the present assessment, the 95th percentiles of exposure for infants from Italy and for toddlers from Belgium, Italy and Spain were not included.

Concentration data used to assess the exposure to annatto, bixin and norbixin (E 160b) were: (1) MPLs as set down in the EU legislation, expressed as annatto (E 160b) (defined as the *regulatory maximum level exposure assessment scenario*); (2) bixin and norbixin usage data provided by industry (defined as the *refined exposure assessment scenario*); and (3) use levels for bixin and norbixin as proposed by the applicant for the extension of use (defined as *extension of use scenario*).

Based on the information made available by industry, separate exposure estimates for bixin- and norbixin-based extracts were calculated (except for the *regulatory maximum level exposure assessment scenario* based on the MPLs).

The different exposure assessment scenarios are discussed in detail below.

2.10.2.1. Regulatory maximum level exposure assessment scenario

The regulatory maximum level exposure assessment scenario for annatto (E 160b) is based on the MPLs as set in Annex II to Regulation (EC) No 1333/2008 and listed in Table 6.

The exposure estimates derived from this scenario can be considered as conservative since it is assumed that the consumer will be continuously (over a lifetime) exposed to annatto (E 160b) present in food at the MPL.

2.10.2.2. Refined exposure assessment scenarios

The refined exposure assessment scenarios were calculated separately for bixin and norbixin. With regard to the analytical results provided, the Panel noted that they referred to E 160b and did not differentiate between bixin- and norbixin-based extracts. Therefore, the concentration levels considered by the Panel for bixin and for norbixin were extracted only from the reported use levels provided by industry. The Panel noted that for some food categories (06.5, 09.2, 12.2.2 and 15.1) no typical, but only the maximum use level was reported by industry. For these food categories, half of the maximum use level was used as the typical use level in the refined exposure scenarios (Appendices D and E).

For bixin and norbixin, based on the available data set, the Panel calculated two estimates based on different model populations:

- *The brand-loyal consumer scenario*: It was assumed that a consumer is exposed long-term to the food additive present at the maximum reported use level for one food category. This exposure estimate is calculated as follows:
  - combining food consumption with the maximum of the reported use levels for the main contributing food category at the individual level;
  - using the mean of the typical reported use levels for the remaining food categories.
- *The non-brand-loyal consumer scenario*: It was assumed that the population is exposed long-term to the food additive present at the mean reported use levels in food. This exposure estimate is calculated using the mean of the typical reported use levels for all food categories.

For the extension of use in the additional 16 food categories, the brand-loyal consumer scenario and the non-brand-loyal consumer scenario were calculated considering the use levels proposed by the applicant for bixin and norbixin.

2.10.2.3. Dietary exposure to annatto extracts (E 160b)

Table 9 summarises the estimated exposure to E 160b (MPL scenario, expressed as annatto), the refined exposure estimates from the current uses of E 160b and the refined exposure estimates considering the proposed extension of use (for bixin and norbixin) in six population groups. Detailed results per population group and survey are presented in Appendices F, G and H.

Table 9: Summary of dietary exposure to annatto, bixin and norbixin (E 160b) from their use as food additive in the regulatory maximum level exposure assessment scenario and in the refined exposure assessment scenarios, in six population groups (minimum–maximum across the dietary surveys in mg/kg bw per day)

	Infants (12 weeks to 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Annatto extracts (bixin and/or norbixin)						
Regulatory maximum level exposure assessment scenario						
Mean	0.01–0.04	0.06–0.16	0.04–0.14	0.02–0.06	0.01–0.03	0.01–0.03
95th percentile	0.02–0.15	0.16–0.38	0.10–0.34	0.04–0.14	0.03–0.08	0.02–0.06
Bixin						
Refined estimated exposure scenario – brand-loyal scenario						
Mean	0.003–0.03	0.01–0.10	0.004–0.09	0.002–0.03	0.002–0.02	0.004–0.02
95th percentile	0.01–0.13	0.05–0.32	0.02–0.26	0.02–0.09	0.01–0.05	0.01–0.04
Refined estimated exposure scenario – non-brand-loyal scenario						
Mean	0.002–0.02	0.01–0.08	0.003–0.07	0.002–0.02	0.001–0.01	0.003–0.01
95th percentile	0.01–0.11	0.04–0.26	0.01–0.21	0.01–0.07	0.01–0.04	0.01–0.04
Extended use scenario – brand-loyal scenario						
Mean	0.01–0.05	0.04–0.33	0.04–0.24	0.01–0.09	0.01–0.06	0.01–0.05
95th percentile	0.03–0.14	0.17–0.65	0.13–0.51	0.04–0.24	0.03–0.13	0.02–0.12
Extended use scenario – non-brand-loyal scenario						
Mean	0.004–0.04	0.02–0.22	0.02–0.16	0.01–0.06	0.01–0.04	0.01–0.04
95th percentile	0.02–0.11	0.08–0.40	0.06–0.36	0.03–0.13	0.02–0.09	0.01–0.08
Norbixin						
Refined estimated exposure scenario – brand-loyal scenario						
Mean	0.003–0.02	0.04–0.11	0.03–0.10	0.02–0.04	0.01–0.02	0.003–0.02
95th percentile	0.01–0.09	0.11–0.24	0.06–0.21	0.03–0.09	0.02–0.05	0.01–0.04

	Infants (12 weeks to 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Refined estimated exposure scenario – non-brand-loyal scenario						
Mean	0.002–0.02	0.03–0.09	0.02–0.08	0.01–0.03	0.003–0.02	0.002–0.02
95th percentile	0.01–0.07	0.08–0.18	0.05–0.17	0.02–0.07	0.01–0.04	0.01–0.03
Extended use scenario – brand-loyal scenario						
Mean	0.005–0.14	0.05–0.24	0.04–0.17	0.02–0.08	0.01–0.05	0.01–0.06
95th percentile	0.03–0.46	0.12–0.46	0.08–0.37	0.04–0.18	0.03–0.13	0.02–0.13
Extended use scenario – non-brand-loyal scenario						
Mean	0.003–0.08	0.04–0.18	0.03–0.13	0.01–0.06	0.01–0.04	0.01–0.04
95th percentile	0.02–0.25	0.09–0.27	0.06–0.27	0.03–0.13	0.02–0.09	0.02–0.09

2.10.3. Main food categories contributing to exposure to bixin and norbixin from the use of annatto extracts (E 160b)

The main food categories contributing to exposure to annatto, bixin, norbixin (E 160b) and the number of surveys in which each food category is contributing are reported in Appendices I–Q.

For the regulatory maximum level exposure assessment scenario, the main food categories contributing to the total mean exposure to annatto (E 160b) were fine bakery wares, flavoured fermented milk products, and fats and oils, and for infants, fine bakery wares and flavoured fermented milk products for toddlers, children and adolescents, and fine bakery wares for adults and the elderly.

The main food categories contributing to the total mean exposure to bixin from the currently authorised uses in the brand-loyal scenario were fats and oils and flavoured fermented milk products for infants, toddlers, children, flavoured fermented milk products and ripened cheese for adolescents, and fats and oils and ripened cheese for adults and the elderly. For the non-brand-loyal scenario, the main food categories were fats and oils and flavoured fermented milk products for infants, toddlers, children, flavoured fermented milk products and ripened cheese for adolescents, and ripened cheese and fats and oils for adults and the elderly.

Considering the proposed extension of use, the main food categories contributing to the total mean exposure to bixin in the brand-loyal scenario were flavoured fermented milk products for infants, flavoured fermented milk products and other confectionary for toddlers, other confectionary and soups and broths for children, adolescents, and adults, and soups and broths and ripened cheese for the elderly. For the non-brand-loyal scenario, the main food categories were flavoured fermented milk products for infants and toddlers, flavoured fermented milk products and soups and broths for children, and soups and broths and ripened cheese for adolescents, adults and the elderly.

The main food categories contributing to the total mean exposure to norbixin from the currently authorised uses in the brand-loyal scenario were fine bakery wares and flavoured fermented milk products for toddlers, children and adolescents, and fine bakery wares and ripened cheese for infants, adults and the elderly. For the non-brand-loyal scenario, the main food categories were fine bakery wares for all population groups together with flavoured fermented milk products for infants, toddlers, children and adolescents.

Considering the proposed extension of use, the main food categories contributing to the total mean exposure to norbixin in the brand-loyal scenario were unripened cheese, soups and broths and fine bakery wares for infants, children and adolescents, unripened cheese, fine bakery wares and flavoured fermented milk products for toddlers, unripened cheese, soups and broths and sauces for adults, and unripened cheese and soups and broths for the elderly. For the non-brand-loyal scenario, the main categories were fine bakery wares, soups and broths, and unripened cheese for infants and adolescents, fine bakery wares and unripened cheese for toddlers, fine bakery wares and soups and broths for children and unripened cheese and soups and broths for adults and the elderly.

The Panel noted that raising the acceptable level for norbixin in the bixin-based annatto extract Annatto B from 2.5% to 5%, as proposed by the applicant, would result in an additional exposure to norbixin of up to 0.017 mg/kg bw per day (considering the extension of use scenario, 95th percentile in toddlers).

2.10.4. Uncertainty analysis

Uncertainties in the exposure assessment of annatto, bixin and norbixin (E 160b) have been discussed above. In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 10.

Table 10: Qualitative evaluation of influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction[(a)]
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Correspondence of reported use levels to the food items in the EFSA Comprehensive Database: uncertainties to which types of food the levels refer to	+/–
Food categories selected for the exposure assessment: exclusion of food categories due to missing FoodEx linkage (n = 6)	–
Food categories selected for the exposure assessment: inclusion of food categories without considering the restrictions/exceptions (n = 4 for currently authorised uses and n =2 for the extension of use)	+
Concentration data: levels considered applicable to all food items within the entire food category	+/–
Regulatory maximum level exposure assessment scenario: – Exposure calculations based on the MPL	+
Refined exposure assessment scenarios: – Exposure calculations based on the maximum or mean levels (use levels reported by industry)	+
Uncertainty in possible national differences in use levels of food categories	+/–

MPL: maximum permitted level.
(a): +, uncertainty with potential to cause over-estimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

Overall, the Panel considered that the uncertainties identified would lead to an overestimation of the exposure to annatto (E 160b) as food additive in Europe considering the regulatory maximum level exposure scenario, and the refined exposure scenarios for bixin and norbixin in European countries for the food categories considered in this opinion.

3. Biological and toxicological data

The applicant stated that, like many natural products, annatto seeds originate from many different countries and are extracted for commercial use by a variety of procedures, involving oil, water, alkali, heat, etc. While bixin and norbixin are recognised as the main agents contributing to the colour of annatto extracts, the other materials present in the extracts had been largely uncharacterised. For this reason, a number of extracts (rather than bixin or norbixin) have been used for the toxicological investigations. However, these preparations are broadly similar to the main types of extracts prepared commercially. The applicant indicated that all five preparations, on which data are reported, apply to the proposed specifications (Doc. provided to EFSA n. 3).

The applicant also stated that it should be noted that one product, bixin-based annatto extracts (oil-processed), for which the internal code 'Annatto D' had been used, was not included in this submission, since JECFA did not finalise the safety evaluation of this extract because neither a 90-day study nor a comprehensive quantitative/qualitative description was available to the committee (JECFA, 2007). This product is also not evaluated in the present opinion.

The Panel based its evaluation on the dossier submitted by the applicant, previous evaluations and reviews, additional literature that became available since then and the data available following public calls for data.[5–7] The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

3.1. Absorption, distribution, metabolism and excretion (ADME)

3.1.1. Animal studies

3.1.1.1. Animal studies reported by JECFA (1982)

As reported by JECFA (1982), 'the metabolism of three annatto preparations has been studied (Philp, 1981, report non-available). The materials were:

1) OSB: a vegetable oil solution which contains bixin (0.22%) and the thermal degradation pigment;
2) R10: a vegetable oil suspension which contains mainly bixin (1.84%);
3) WSA: a water-soluble preparation which contains mainly norbixin (0.27%)'.

The Panel noted that these annatto preparations are similar, respectively, to Annatto FL, Annatto FL10 and Annatto WL, which were used by Van Esch et al. (1959) in their long-term feeding tests.

In a first study, as reported by JECFA 'groups of four male and four female rats were fed 0% or 5% of OSB, R10 and WSA annatto extracts for a 4-week period. Rats were fed annatto extracts in the diet for the first 2 weeks only, or the last 2 weeks prior to killing. Direct evidence of the intestinal absorption of annatto pigments is provided by the presence of these pigments in blood (serum) in the same proportion as they are to be found in the commercial products. This is consistent with the finding that the pigments excreted in faeces were also in the same proportions as they are to be found in the commercial products. Thin-layer chromatography of the sera of rats fed OSB, R10 and WSA also revealed components which are possibly metabolites of annatto pigments. There is no evidence that the major components of annatto extracts colour the adipose tissue of rats. The fact that WSA-fed rats do not have coloured fat also suggests that the major annatto pigments are not involved in the coloration. The yellow pigment which colours the adipose tissue of rats fed OSB and R10 is possibly identical to a minor component which occurs in OSB and R10. The marked difference in the colouration of adipose tissue from rats fed OSB or R10 in weeks 3 and 4, from those fed during weeks 1 and 2, indicates that the adipose tissue store of pigment is rapidly depleted when ingestion of annatto ceases'.

In a second study, as also reported by JECFA 'five groups of four male and four female rats were given by stomach tube a single dose of undiluted WSA (10 mL/kg), R10 (2 mL/kg), OSB (2 mL/kg) annatto, sunflower oil (2 mL/kg) or water (10 mL/kg). The annatto pigments in rat's blood were determined 3 h and 24 h after treatment. In rats dosed with WSA, the blood level was 270 mg/100 mL after 3 h and 10 mg/100 mL after 24 h. The results suggest that, within 24 h, WSA was almost completely absorbed and metabolised. In rats dosed with OSB, the blood level was 62 mg/100 mL after 3 h and 19 mg/100 mL after 24 h. Thus, OSB was metabolised less rapidly than WSA, presumably because the OSB pigments would need to be metabolised to water-soluble pigments, similar to those of WSA, before being metabolised further. Likewise, R10 was metabolised more slowly than WSA. The relatively low blood levels of annatto preparation 24 h after large, oral doses provide evidence of the ability of the rat to metabolise both water-soluble and fat-soluble annatto pigments'.

3.1.1.2. Additional animal studies provided by the applicant

The applicant provided results from a study in Sprague–Dawley rats on the absorption, distribution and excretion of bixin and norbixin from the annatto extracts evaluated in the present opinion (Hughes, 2002). This study was designed to provide pharmacokinetic data following oral doses of two bixin-based (Annatto B and E: solvent-extracted bixin and aqueous-processed bixin) and one norbixin-based annatto extract (Annatto F: alkali-processed, acid-precipitated norbixin). Prior analysis of the annatto extracts showed that the solvent-extracted bixin (Annatto B) had a colour content of 92%, of which 97% was *cis*-bixin and 1.7% was *cis*-norbixin, 0.45% *trans*-bixin and 0.36% others; the aqueous-processed bixin (Annatto E) had a colour content of 26%, of which 90% was *cis*-bixin, 4% *cis*-norbixin, 2.41% *trans*-bixin and 3.4% others; the alkali-processed, acid-precipitated norbixin (Annatto F) had a colour content of 38%, of which 87% was *cis*-norbixin, 9.5% *trans*- and di-*cis*-norbixin and 3.5% others, but no *cis*-bixin was present.

Sprague–Dawley rats (108 males and 108 females, strain Crl:CD(SD)IGS BR VAF/plus) in the weight range of 180–240 g were used. Food was withheld overnight, and restored 2 h after dosing; water was available *ad libitum* throughout the study. The bixin-based extracts (Annatto B and E) were suspended in corn oil, and the norbixin-based extract (Annatto F) was suspended in sterile water with

0.5% carboxymethyl cellulose containing approximately 25 or 250 mg of Annatto F/g for the two dose levels tested. Rats were given a single oral dose of each extract by gavage, at doses of 100 or 1,000 mg/kg bw (n = 3 males and n = 3 females per dose level and extract), and sacrificed at the following times: predose, 2, 4, 8, 12 and 24 h postdose. Plasma samples were analysed for bixin and norbixin (*cis*- and *trans*-isomers of both compounds). In addition, urine was collected from the 24-h groups prior to dosing and for the subsequent 24 h, and analysed for bixin and norbixin. Bone marrow and livers were excised from rats sacrificed at 4 and 12 h and analysed for bixin and norbixin.

Upon oral dosing of the solvent-extracted bixin (Annatto B) at both 100 and 1,000 mg/kg bw, the following species were present in the plasma of male and female rats: 9′-*cis*-bixin, *trans*-bixin, 9′-*cis*-norbixin, *trans*-norbixin, di-*cis*-norbixin and a norbixin isomer identified by the retention time (R_t) 6.8 min. After dosing of the aqueous-processed bixin (Annatto E), the above isomers were detected in plasma of both genders plus an additional *trans*-bixin species. By contrast, following administration of the alkali-processed, acid-precipitated norbixin (Annatto F), only the norbixin isomers (9′-*cis*-norbixin, *trans*-norbixin, di-*cis* norbixin and the norbixin isomer at R_t 6.8 min) were present in the plasma of both male and female rats. Plasma concentrations of 9′-*cis*-norbixin were higher than 9′-*cis*-bixin after administration of all the three annatto extracts, although the bixin-based extracts contained more than 90% 9′-*cis*-bixin. The T_{max} for the major component of each test material in plasma, in males and females at both dose levels was 2–4 h, and, within 12 h, only trace amounts of bixin remained, although norbixin levels were still measurable at 24 h. Although the plasma concentrations of the main components were increased when the oral dose was increased from 100 to 1,000 mg/kg bw for all the three extracts, these increases were less than 10-fold.

The applicant indicated that the data suggested that the absorption of norbixin from the gut is significantly greater than that of bixin. Although the pigment fraction of bixin extracts contained > 90% 9′-*cis*-bixin and only 1.7% and 4% 9′-*cis*-norbixin, the plasma concentrations of 9′-*cis*-norbixin were higher than those of 9′-*cis*-bixin. Since, despite the relatively low percentage of 9′-*cis*-norbixin in Annatto B and E (1.73% and 4.16%, respectively), there was sufficient norbixin to account for the plasma levels of 9′-*cis*-norbixin at these relatively high doses of 100 and 1,000 mg/kg bw, the results provide no evidence for the conversion of *cis*-bixin into norbixin. The very small difference in mean plasma peak concentrations (C_{max}) and area under curve (AUC) of *cis*-bixin between the 100 and 1,000 mg/kg bw dose levels also suggested non-linear kinetics of bixin-based annatto extracts. The Panel noted that the absorption of the aqueous-processed bixin (Annatto E) would be higher than that of the solvent-extracted bixin (Annatto B). Following administration of alkali-processed, acid-precipitated norbixin (Annatto F), 87% of which is 9′-*cis*-norbixin, the plasma concentrations of 9′-*cis*-norbixin were more than an order of magnitude higher than that of 9′-*cis*-bixin, following administration of bixin-rich extracts (Annatto B or E). Some 9′-*cis*-norbixin was present in the urine of male rats following dosing with Annatto E at 1,000 mg/kg bw, but this accounted for less than 0.1% of the dose. After dosing with the norbixin-based extract Annatto F at 100 and 1,000 mg/kg bw, there were quantifiable amounts of 9′-*cis*-norbixin in urine, but these accounted for less than 3% of the dose. The uptake of bixin isomers in liver and bone marrow was examined, and the isomers present in these tissues were consistent with those seen in the plasma at 4 and 12 h for each extract; however, the assay method was not validated for these tissues, therefore quantitative conclusions cannot be drawn.

By considering the plasma levels of bixin and norbixin obtained in this study at different times before and after the oral administration of the various annatto extracts, the Panel noted that these two compounds are absorbed in rats and that the more polar free acid form of norbixin appears to be better absorbed than the less polar bixin. The Panel considered that in the absence of measurement of volume of distribution and of biliary excretion of both compounds, the absorption cannot be adequately determined. The Panel also considered that the residual level of norbixin present in Annatto B and Annatto E cannot only explain the high plasma levels of norbixin found in rats given a single dose of these extracts and that metabolic conversion of *cis*-bixin into norbixin should occur in rats.

3.1.2. Human studies

As reported in JECFA (1982), single oral doses of E 160b as OSB (7 mg/kg; containing 0.22% bixin), R10 (7 mg/kg; containing 1.84% bixin) and WSA (14 mg/kg; containing 0.27% norbixin) 'were given to adult males and the blood and excreta were analysed for annatto pigments (Philp, 1981; report not available). Blood samples were taken between 2 and 12 h after treatment, urine was collected during the 7 h after the dose and faeces over the 2 days following the day of treatment.

WSA (14 mg/kg) produced a blood level of 12 μg/mL after 2.25 h, which corresponds to 6% of the dose. OSB (7 mg/kg) produced a blood level of 2.4 μg/mL after 3 h, which corresponds to 2.4% of the dose. R10 (7 mg/kg) produced a blood level of 0.44 μg/mL after 3.25 h, which corresponds to 0.32% of the dose. Blood levels had returned to zero 6 h after WSA (14 mg/kg), OSB (7 mg/kg) and R10 (7 mg/kg), respectively. No annatto pigments were detected in the urine samples and none were detected in faeces samples collected the next day. The faeces collected on the second day after treatment contained 0.17 mg R10 (0.03% of the dose) and 0.44 mg WSA (0.06% of the dose), but no pigments associated with the consumption of OSB were detected. Thus, as in the rat, the annatto pigments were absorbed and rapidly cleared from the blood'.

Absorption of annatto pigments in human volunteers was reported by Levy et al. (1997). Seven volunteers of both sexes ingested a single dose of 1 mL of a commercial annatto food colour containing 16 mg of *cis*-bixin and about 0.5 mg *cis*-norbixin in soybean oil. Blood samples were taken 2, 4, 6, 8, 24 and 48 h after ingestion. The authors noted that low levels of bixin and norbixin appeared to be normal, but occasional, constituents of human plasma in countries where annatto is a frequent constituent of the traditional diet. These levels were within the same general range as those of other carotenoids.

With regard to the kinetics of the administered compounds, the peak plasma level of bixin was shown to occur at 2 h (11.6 ng/mL), and that of norbixin at 4 h (57.8 ng/mL) after administration, reaching about five times the peak levels of bixin in plasma. While bixin levels had fallen to zero by 8 h, norbixin levels fell more slowly (28.7 ng/mL at 8 h), and norbixin was still detected in plasma after 24 h (16.1 ng/mL). Even after 48 h, some norbixin was found in the plasma of some participants. Thus, humans also showed similar low, but disproportionate absorption of norbixin over bixin. The increase in plasma norbixin suggested a conversion of bixin to norbixin. It was also observed that although natural annatto extracts mainly contain *cis*-bixin, *trans*-isomers appeared in the plasma, suggesting isomerisation at some stage of the uptake. The authors noted that similar conversion occurred with other carotenoids, such as β-carotene and lycopene.

By considering the plasma levels of bixin and norbixin obtained in this study at different times before and after the oral administration, the Panel considered that these two compounds are absorbed in humans. The Panel noted that the intake of norbixin (0.5 mg, 7% of the dose) was much lower than that of bixin (16 mg, 93% of the dose). As the major metabolite recovered in plasma was norbixin (more than 90%), the Panel considered that this is due to the *in vivo* conversion of bixin to norbixin, as suggested by the authors. In consequence, the Panel considered that norbixin is a metabolite of toxicological relevance following the oral administration of bixin in humans.

3.1.3. Summary of ADME studies and conclusions

Studies in rats using three annatto extracts of the present opinion revealed that the more polar free acid form norbixin was better absorbed than the less polar bixin. Norbixin was the major component present in plasma and urine, even following oral administration of bixin-based extracts, most likely derived from both the norbixin present in the extract, as well as metabolism of bixin to norbixin. The total percentage of the dose of bixin and norbixin absorbed could not be assessed from these studies without data on biliary excretion.

In humans, a first study using the same water-soluble (WSA) and oil-soluble preparations (OSB and R10) as in rats, reported that the annatto pigments were absorbed and rapidly cleared from the blood. An additional study using a commercial annatto food colour containing 16 mg of *cis*-bixin and about 0.5 mg of *cis*-norbixin demonstrated a major presence of norbixin in plasma. This would suggest that in humans as in the rat, bixin and norbixin are absorbed and that bixin is converted to norbixin. Although the database was limited, the overall pattern of absorption and metabolism appeared to be similar in the rat and humans and, therefore, the Panel considered the rat an adequate model for assessing the toxicity of annatto extracts in humans. Additional studies, preferably with pure bixin and norbixin, on their metabolism in rats and humans may reduce the uncertainty.

3.2. Toxicological data

3.2.1. Acute oral toxicity

Single dose studies in rats and mice were previously reported (JECFA, 1975, 1982). LD_{50} values amounted to 700 mg/kg bw for a water-soluble annatto extract (no further details given) upon intraperitoneal (i.p.) administration in mice (Dunham and Allard, 1960), and > 50 g/kg bw for an

oil-soluble annatto extract, and > 35 g/kg bw for a water-soluble annatto extract, upon oral administration in rats (Van Esch et al., 1959).

The studies by Dunham and Allard (1960) were not relevant for the annatto extracts used as a food colour, as they refer to extracts obtained from the roots of *B. orellana*, rather than from the outer seed case, which is the source of the food colour, and since dosing was not via the oral route.

No signs of toxicity (on gross or tissue examination) were observed in the studies of Van Esch et al. (1959).

3.2.2. Short-term and subchronic toxicity

3.2.2.1. Animal studies already included in previous evaluations

Studies have been reported in the literature in the mouse, rat and dog, and the results for all the three species were discussed in previous evaluations of the SCF and JECFA. Studies by Fernandes et al. (2002) essentially confirmed the absence of toxicity of annatto extracts in the rat, although some changes in plasma chemistry were observed in mouse studies.

Mice

In a study in mice (Fernandes et al., 2002), the toxicity of norbixin, purified or not (annatto extract containing 50% norbixin along with many other unidentified molecules), was investigated after 21 days of ingestion through drinking water. Male Swiss mice were exposed to doses of 25.5 mg% annatto extract (corresponding to 56 mg annatto extract/kg bw per day; n = 7) or 133 mg% annatto extract (corresponding to 351 mg annatto extract/kg bw per day; n = 6) or four doses of purified norbixin: 0.3 mg% (0.8 mg norbixin/kg bw per day; n = 10), 2.7 mg% (7.6 mg norbixin/kg bw per day; n = 16), 26.5 mg% (66 mg norbixin/kg bw per day; n = 17) and 56.7 mg% (274 mg norbixin/kg bw per day; n = 7).

Norbixin induced an increase in plasma alanine aminotransferase (ALT) activity, while both norbixin and annatto extract induced a decrease in plasma total protein and globulins ($p < 0.05$). However, no signs of toxicity were detected in liver upon histopathological examination. No enhancement in DNA breakage was detected in the liver or kidney of mice treated with annatto pigments, as evaluated by the comet assay. Norbixin induced hypoglycaemia that ranged from 14.4% (0.8 mg/kg norbixin per day, $p < 0.05$) to 21.5% (66 mg/kg norbixin per day, $p < 0.001$) below control levels. Mice treated with annatto pigments showed hypoinsulinaemia.

Rats

In studies performed in rats (Fernandes et al., 2002), a similar absence of toxicity was observed when either an annatto extract (containing 50% norbixin along with many other unidentified molecules) or purified norbixin was administered to female Wistar rats for 21 days in drinking water. Rats were exposed to three doses of annatto extract (each group consisted of five animals): 0.6 mg% (0.8 mg annatto extract/kg bw per day), 5.7 mg% (7.5 mg annatto extract/kg bw per day) and 56.7 mg% (68 mg annatto extract/kg bw per day), or three doses of purified norbixin: 0.6 mg% (0.8 mg norbixin/kg bw per day), 5.7 mg% (8.5 mg norbixin/kg bw per day) or 56.7 mg% (74 mg norbixin/kg bw per day). No toxicity was detected by plasma chemistry. In rats, norbixin induced hyperglycaemia that ranged from 26.9% (at 8.5 mg norbixin/kg bw per day) to 52.6% (at 74 mg norbixin/kg bw per day, $p < 0.01$) above control levels. Rats treated with annatto pigments showed hyperinsulinaemia (c.f. hypoglycaemia in mice).

A short-term toxicity study in rats has been published by Bautista et al. (2004), using annatto extract powder (bixin 27%, not further specified) suspended in corn oil and administered by gavage for 4 weeks (once a day, 5 days each week) to 15 male and 15 female Wistar rats. As no adverse effects had been noted in a preliminary experiment with 1,000 mg/kg bw per day, the annatto extract was administered by gavage at a single dose level of 2,000 mg/kg bw per day. Haematological and biochemical examination and necropsy were performed on 10 rats of each sex on day 29, and on the remaining five rats of each sex after recovery at day 43. Decreased body weight gain was observed in males, but there was no effect on either food intake or food conversion efficiency. Decreased red cell counts and haemoglobin concentrations were observed in males, and decreased white cell counts in males and females on day 29, but not in the recovery groups. At necropsy on day 29, no lesions were observed, but, in the kidneys of two out of the 10 treated female rats examined, apoptosis occurred in restricted areas without proliferation or tubular segments modification. The precise nature of apoptosis was not investigated in the study. The authors suggested that the findings show that annatto extract was not toxic in the rat.

Dogs

In an unpublished report submitted to JECFA in 1974, Kay and Calandra (1961a) demonstrated that the fat-soluble annatto extract, given over a year, produced no adverse effects on growth, food intake, mortality, liver and kidney function, haematology or histopathology. One treated female dog died with hepatocellular degeneration, which was thought to be due to an intercurrent infection.

In a second study submitted to JECFA in the same year, Kay and Calandra (1961b) administered aqueous annatto extracts. Growth inhibition and reduced food intake occurred at the 20% dietary level, but the mortality rate, liver and kidney function, haematology and histopathology of all major tissues showed no abnormalities attributable to the test substance at any level.

3.2.2.2. Additional 28-day rat studies provided by the applicant

In addition to the studies included in previous evaluations described above, the applicant provided results from 28-day dietary feeding studies in rats fed with different annatto extracts, representative of the annatto extracts of the present evaluation.

Solvent-extracted bixin (Annatto B) 28-day range-finding study

A 28-day range-finding study (non-good laboratory practice (GLP)) is described in HLS Report ATE010 (HLS, 2000). Five groups of five male and five female Crl:CD BR rats were used. The controls (Group 1) received untreated diet for 4 weeks. The test material complied with the specifications for solvent-extracted bixin (Annatto B) (actual content of the tested batch: 84.7%). Group mean achieved dosages in the treated rat groups (Groups 2–5) were 572, 1,650, 1,722 and 3,733 mg/kg bw per day for males (receiving 5,000, 10,000/20,000, 15,000, or 20,000/50,000 mg/kg diet, respectively), and 557, 1,590, 1,653 and 3,751 mg/kg bw per day for the equivalent female groups.

No deaths occurred. Orange staining of the coat and orange staining of the cage tray paper and faeces were reported for all animals that received the annatto extract, due to the colour of the test material. There were no dose-related effects of treatment on body weight gain, food intake and organ weights. Macroscopic examination revealed orange discolouration of the tongue and the gastrointestinal tract in all treated animals. Orange discolouration of the mesentery was reported in a few animals which had received 20,000/50,000 mg/kg diet (Group 5), due to the intense orange colour of the annatto extract. Histopathological examination did not reveal treatment-related effects. It was concluded that dietary concentrations up to, and including, 50,000 mg Annatto B/kg diet were well tolerated by the animals.

Aqueous-processed bixin (Annatto E) 28-day range-finding study

A 28-day range-finding study (non-GLP) is described in HLS Report ATE012 (HLS, 2001a). Because of the relative unpalatability of this annatto extract, observed in a preliminary palatability study, it was decided to use an escalating dose protocol.

Four groups of five male and five female Crl:CDBR rats were used. Three groups received the test material by dietary administration for a 4-week period, and one control group received untreated diet alone. The test material complied with the specifications for aqueous-processed bixin (Annatto E) (actual content of the tested batch: 27.2%). Mean group achieved dosages during the fourth week for Group 2 receiving 30,000 mg/kg diet were 2,872 mg/kg bw per day for males and 2,886 mg/kg bw per day for females. For Groups 3 and 4, which both received 40,000 mg/kg diet during week 4, mean group achieved dosages were 3,339 and 4,570 mg/kg bw per day for males, and 3,234 and 4,526 mg/kg bw per day for females, respectively.

No deaths occurred. The only signs observed were orange staining of the coat, faeces and cage tray paper, which were reported for all animals receiving the test material. These were due to the intense colour of the annatto extract. Overall body weight gains were decreased for all treated groups, but the effect was more marked in females than in males. When compared with the controls, reduced food intake was seen in all treated groups throughout, but most markedly in the first week of the study. Food conversion efficiency values were not significantly reduced in any of the groups. When compared with the controls, increased, but not in a dose-related manner, absolute and relative liver weights were recorded in all male and female groups. Orange discolouration of the tongue and the gastrointestinal tract was reported for all animals given the test material. Orange staining of the mesentery was also observed in the majority of treated animals. Histopathological examination revealed diffuse hepatocellular hypertrophy in the livers of the Group 2 females and Group 3 males.

Alkali-processed, acid-precipitated norbixin (Annatto F) 28-day range-finding study

A 28-day range-finding study (non-GLP) is described in HLS Report ATE013 (HLS, 2001b). Because of the relative unpalatability of this annatto extract, observed in a preliminary palatability study, it was decided to use an escalating dose protocol.

Five groups of five male and five female Crl:CDBR rats were used. Four groups received the test material by dietary administration for a 4-week period, and one control group received untreated diet alone. The treatment table is presented below:

Group	1	2	3	4	5
Test material	Control	Alkali-processed, acid-precipitated norbixin (Annatto F)			
		Level (mg/kg diet)			
Week 1	0	7,000	7,000	7,000	7,000
Week 2	0	7,000	10,000	10,000	10,000
Week 3	0	7,000	15,000	15,000	20,000
Week 4	0	7,000	15,000	20,000	20,000

The test material complied with the specifications for alkali-processed, acid-precipitated norbixin (Annatto F) (actual content of the tested batch: 41.5%).

No deaths occurred. Orange staining of the coat, orange-stained faeces and brown-stained tails were observed in all treated animals. Orange staining of the tray paper was also evident. These were due to the intense colour of the annatto extract. The body weight gains of treated animals were not significantly affected by the treatment in any of the groups. The food intake and food conversion efficiency in all treated groups were not significantly different from that of the controls. Absolute and relative liver weights were markedly increased, compared with the controls, in all treated groups. Findings reported at necropsy comprised orange discolouration of the tongue and the gastrointestinal tract in the majority of animals from Groups 3, 4 and 5, and in several animals from Group 2. Orange discolouration of the mesentery tissue was observed in few animals from Groups 4 and 5, and in one female from Group 2.

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.

Alkali-processed, not acid-precipitated norbixin (Annatto G) 28-day range-finding study

A 28-day range-finding study (non-GLP) is described in HLS Report ATE014 (HLS, 2001c). Because of the relative unpalatability of this annatto extract observed in a preliminary study, it was decided, in addition to the *ad libitum* fed controls, to include a 'pair-fed' control group to match the food intake of the highest dose group. This would indicate whether the effects on body weight gain were mainly due to the expected reduction in food intake, or were more specifically related to toxicity.

Five groups of five male and five female Crl:CDBR rats were used. Groups 3 and 4 received, by dietary administration, escalating concentrations of the test material for a 4-week period, whereas control Group 1 received untreated diet alone *ad libitum*, and control Group 2 was pair-fed with Group 4.

The test material complied with the specifications for alkali-processed, not acid-precipitated norbixin (Annatto G) (actual content of the tested batch: 17.1%).

When compared with the controls, there was a clear effect of treatment on food intake in treated males. The food intake of the 'pair-fed' animals was slightly reduced when compared with the Group 4 animals. Reduced food conversion efficiency values were noted in the Group 4 males in weeks 3 and 4 (30,000 mg/kg diet), but these were not affected in females. Group mean achieved dosages during the fourth week of treatment were 1,579 and 3,146 mg/kg bw per day for males of Groups 3 and 4 respectively, and 1,631 and 3,127 mg/kg bw per day for the respective female groups. Overall body weight gains were lower in the treated male groups. In the treated females, the overall body weight gains were similar to the controls.

3.2.2.3. Additional 90-day rat studies provided by the applicant

In subsequent studies provided by the applicant, four annatto extracts, representing four (Annatto B, E, F and C) of the extracts evaluated in the present opinion, were subjected to a 90-day repeated dose toxicity testing.

The studies followed essentially identical protocols, with the exception that the Organization for Economic Co-operation and Development (OECD) guideline changed slightly during the performance of the studies and a functional observational battery of tests was performed only on the animals treated with the solvent-extracted bixin (Annatto B) and the alkali-processed, acid-precipitated norbixin (Annatto F), but not with the aqueous-processed bixin (Annatto E).

Solvent-extracted bixin (Annatto B) 90-day study

The authors stated that the test material complied with the specifications for solvent-extracted bixin (Annatto B) (actual content of the tested batch: 92%) (HLS, 2002a). Dietary concentrations used were based on the results from the preliminary 4-week range-finding study discussed in the previous subsection (HLS, 2000).

Four groups of 20 male and 20 female (Crl:CD (SD)IGS BR VAF/Plus) rats, 35–39 days of age at the start of the treatment, were used for the study. Rats received Annatto B, by dietary administration, at levels of 5,000, 16,000 or 50,000 mg/kg diet for 13 weeks. A similarly constituted control group received the vehicle diet (Rat and Mouse No 1 Maintenance Diet) alone.

Mean group achieved dosages over the 13-week treatment were 407, 1,311 and 4,201 mg/kg bw per day for the males receiving 5,000, 16,000 or 50,000 mg Annatto B/kg diet, respectively, and 449, 1,446 and 4,507 mg/kg bw per day for the equivalent female groups. Since the test material contained 92% bixin, these achieved doses corresponded to 374, 1,206 and 3,865 mg bixin/kg bw per day in males, and 413, 1,330 and 4,146 mg bixin/kg bw per day in females.

At the end of the treatment period, animals were killed by carbon dioxide inhalation and all animals were subjected to a detailed necropsy. Full histopathological examination of all tissues was carried out on all animals in Groups 1 and 4, and of the kidneys, liver and lungs of all other groups, and all abnormal appearing tissues observed at necropsy.

Administration of the test material to CD rats for 13 weeks at dietary concentrations of 5,000, 16,000 and 50,000 mg/kg diet was well tolerated. At a dietary concentration of 50,000 mg/kg diet, changes were seen in relative liver weight, blood chemistry parameters and the composition of the urine, but all these changes were minor in degree and, with the exception of the relative liver weight effect, were not consistent between the sexes. No treatment-related histopathological lesions were seen in any of the organs examined. An increase in liver weight is a common finding in rodents which have been given high levels of a xenobiotic, and was considered by the applicant to be an adaptive response rather than a toxic effect of treatment. The increased ALT and aspartate aminotransferase (AST) activities seen in individual females receiving 16,000 or 50,000 mg Annatto B/kg diet, were not accompanied by histopathological evidence of liver damage. Because these changes were minor and were seen in one sex only, they were considered by the authors to be of no toxicological significance. The Panel agreed with this conclusion.

A marginal effect was seen in the kidney, with raised protein concentrations noted in urine samples obtained from males receiving 50,000 mg Annatto B/kg diet. Blood plasma phosphorus concentrations were also increased in these animals, indicating a possible reduction in the glomerular filtration rate. The Panel considered these kidney effects adverse and concluded that the no-observed-adverse-effect level (NOAEL) in this study is the dietary concentration of 16,000 mg Annatto B/kg diet (equivalent to 1,311 mg Annatto B/kg bw per day, or 1,206 mg bixin/kg bw per day in males, and to 1,446 mg Annatto B/kg bw per day or 1,330 mg bixin/kg bw per day in females).

Aqueous-processed bixin (Annatto E) 90-day study

The authors of this study stated that the test material complied with the specifications for aqueous-processed bixin (Annatto E) (actual content of the tested batch: 26%) (HLS, 2002b). Dietary concentrations used in this study were based on the results from the preliminary 4-week range-finding study discussed above (HLS, 2001a).

Four groups of 20 male and 20 female (Crl:CD (SD)IGS BR VAF/Plus) rats, 40–44 days of age at the start of the treatment, were used for the study. Rats received Annatto E by dietary administration at levels of 3,000, 10,000 or 30,000 mg/kg diet for 13 weeks. A similarly constituted control group received the vehicle diet (Rat and Mouse No 1 Maintenance Diet) alone. Group mean achieved dosages over the 13-week treatment were 224, 734 and 2,204 mg Annatto E/kg bw per day for males receiving 3,000, 10,000 or 30,000 mg Annatto E/kg diet, respectively, and 238, 801 and 2,398 mg Annatto E/kg bw per day for the corresponding female groups. Since the test material contained 26% bixin, these dosages corresponded to 58, 191 and 573 mg bixin/kg bw per day in males, and 62, 208 and 623 mg bixin/kg bw per day in females.

At the end of the treatment period, animals were killed by carbon dioxide inhalation and all animals were subjected to a detailed necropsy. Full histopathological examination of all tissues was carried out on all animals in Groups 1 and 4 and of the kidneys, liver and lungs of all other groups, and all abnormal appearing tissues observed at necropsy.

Administration of the test compound to CD rats for 13 weeks at dietary concentrations of 3,000, 10,000 or 30,000 mg/kg diet was well tolerated.

Decreased overall body weight gains were noted for males receiving 30,000 mg Annatto E/kg diet and in females receiving 30,000 and 10,000 mg Annatto E/kg diet. The authors indicated that the reduced body weight gain in the absence of any effect on food conversion efficiency observed in the females fed 10,000 mg Annatto E/kg diet was most likely due to a palatability effect on food intake. The Panel agreed with this conclusion.

Histopathological examination revealed treatment-related findings in the liver and thyroid. Centrilobular hepatocellular hypertrophy was seen in the livers of two of 20 males and one of 20 females which had received 3,000 mg/kg diet, seven of 20 males and two of 20 females which had received 10,000 mg/kg diet, and in 11/19 males and 10/20 females which had received 30,000 mg/kg diet. The severity of this finding was graded as 'slight' in all animals. Thyroid follicular cell hypertrophy was present in five of 19 males and four of 20 females which had received 30,000 mg/kg diet.

The increased liver weights seen in many of the treated animals, with the exception of females which had received 3,000 mg/kg diet, were associated with centrilobular hepatocyte hypertrophy, but not with signs of hepatocellular damage. This histopathological change in the livers of rodents which have been given high levels of a xenobiotic, as such, was considered by the authors to be an adaptive change rather than a toxic effect. The slight changes in plasma proteins (in treated females and males receiving 30,000 mg/kg diet) and cholesterol concentrations (animals receiving 30,000 mg/kg diet), and the longer activated partial thromboplastin times (males receiving 30,000 mg/kg diet) may all be indicative of altered liver function. Increased relative thyroid weights were seen in females which had received Annatto E at 30,000 mg/kg diet, and microscopic examination of the thyroid tissue of males and females receiving this dietary concentration revealed follicular cell hypertrophy. The Panel agreed with the authors that this finding was related to the treatment-induced increase in the metabolic activity of the liver and, hence, increased metabolism of thyroid hormones.

A slight effect was seen in the kidney, with increased relative weights recorded for females which had received Annatto E at 30,000 mg/kg diet, and raised plasma creatinine concentrations were seen in treated females and in males receiving 30,000 mg/kg diet. The slightly raised plasma phosphorus concentrations seen in males and females receiving the highest concentration of aqueous-processed bixin (Annatto E) indicated a reduction in the glomerular filtration rate. There was also reduced urinary output in animals receiving 30,000 mg/kg diet, with urinary pH and specific gravity increased in the males, and a slight effect noted on urinary electrolytes in both sexes, which was probably a simple reflection of the reduced volumes of urine produced by these animals. In the absence of any macroscopic or histopathological changes noted in the kidneys, the Panel considered these findings to be of only minor, if any toxicological importance.

Overall, considering the number of effects observed at the top-dose level of 30,000 mg Annatto E/kg diet, this dose level may be regarded as a lowest-observed-adverse-effect level (LOAEL). Therefore, the Panel concluded that the NOAEL in this study for both males and females is 10,000 mg Annatto E/kg diet. This is equivalent to an intake of aqueous-processed bixin (Annatto E) of 734 mg/kg bw per day for males and 801 mg/kg bw per day for females. Taking into account that Annatto E contained 26% bixin, these NOAELs correspond to 191 and 208 mg bixin/kg bw per day in males and females, respectively.

Alkali-processed, acid-precipitated norbixin (Annatto F) 90-day study

The authors of the study stated that the test material complied with the specifications for alkali-processed, acid-precipitated norbixin (Annatto F) (actual content of the tested batch: 38.4%) (HLS, 2002c). Dietary concentrations used in this study were based on the results from the preliminary 4-week range-finding study discussed in the previous subsection (HLS, 2001b).

Four groups of 20 male and 20 female (Crl:CD (SD)IGS BR VAF/Plus) rats, 38–42 days of age at the start of the treatment, were used for the study. Rats received alkali-processed, acid-precipitated norbixin (Annatto F) by dietary administration at levels of 1,000, 3,000 or 9,000 mg/kg diet for 13 weeks. A similarly constituted control group received the vehicle diet (Rat and Mouse No 1 Maintenance Diet) alone.

Group mean achieved dosages over the 13 weeks of treatment were 79, 240 and 753 mg Annatto F/kg bw per day for the males receiving 1,000, 3,000 or 9,000 mg Annatto F/kg diet, respectively, and

86, 275 and 816 mg Annatto F/kg bw per day for the equivalent female groups. Since the test material contained 38.4% norbixin, these dosages corresponded to 30, 92 and 289 mg norbixin/kg bw per day in males, and 33, 106 and 313 mg norbixin/kg bw per day in females.

At the end of the treatment period, animals were killed by carbon dioxide inhalation and all animals were subjected to a detailed necropsy. A full histopathological examination of all tissues was carried out on all animals in Groups 1 and 4, and of the kidney, liver and lungs of all other groups, and all abnormal appearing tissues observed at necropsy.

Administration of the test material to CD rats at dietary concentrations up to, and including 9,000 mg/kg diet for 13 weeks, did not result in any treatment-related deaths, and the general condition and behaviour of the animals were not affected by treatment. Orange and/or yellow staining was seen in all animals dosed at 9,000 mg/kg diet and in many animals dosed at 3,000 mg/kg diet. This was considered to be a direct effect of the highly coloured diets.

A statistically significant increase in liver weights was seen in males treated with 9,000 mg Annatto F/kg diet and in females treated with 3,000 and 9,000 mg Annatto F/kg diet, and this was associated in many animals with centrilobular hepatocellular hypertrophy. Increase in liver weight and centrilobular hepatocellular hypertrophy are common findings in rodents which have been given high levels of a xenobiotic, and are generally considered to be an adaptive response and not a toxic effect of treatment. However, the magnitude of the effect seen at 9,000 mg/kg diet in both sexes (+ 50% in the males and + 67% in the females) and at 3,000 mg/kg diet in the females (+ 44%) was large. Based on the results of the P450 enzymes analyses, the authors suggested that the increase in liver weights observed with the annatto extracts was due to a pleiotropic response mediated by peroxisome proliferator-activated receptor-α (PPARα), and that the annatto extracts are peroxisomal proliferators. The authors indicated that the specific induction of CYP4A and the absence of histopathological changes in the liver, were all compatible with an interpretation of metabolically induced liver enlargement of no toxicological relevance for humans. The Panel agreed with this conclusion.

Further evidence of an effect on hepatic metabolism was seen in the increased alkaline phosphatase and ALT activities recorded in males receiving 9,000 mg/kg diet, together with the increased glucose and triglyceride concentrations seen in animals receiving 9,000 mg/kg diet, and the changes in plasma proteins, noted in animals receiving 3,000 or 9,000 mg/kg diet.

Slightly increased kidney weights were recorded for males and females which had received 9,000 mg/kg diet, and for males which had received 3,000 mg/kg diet. Plasma creatinine concentrations were increased in animals receiving 3,000 or 9,000 mg/kg diet, and urea concentrations were increased in males receiving 9,000 mg/kg diet and in treated females. In addition, slightly reduced urinary output was seen in males receiving 3,000 or 9,000 mg/kg diet, and slightly low pH values were recorded for males receiving 9,000 mg/kg diet. Chemical analysis of the Annatto F test material indicated a sodium content of approximately 2% and a very high sulfate content (approximately 5%). The applicant indicated that this high mineral content of the diet may have resulted in an increased workload for the kidneys, with subsequent increase in kidney weight and altered renal function, but with no evidence of histopathological changes in the kidney. The Panel agreed with this conclusion.

The Panel concluded that the changes in renal function observed at the two highest dose levels, point to a NOAEL of 1,000 mg Annatto F/kg diet, equivalent to 79 mg Annatto F/kg bw per day in males and 86 mg Annatto F/kg bw per day in females, corresponding to 30 mg norbixin/kg bw per day in males and 33 mg norbixin/kg bw per day in females.

Solvent-extracted norbixin (Annatto C) 90-day study

This study (Hagiwara, 2002) was performed in compliance with the Japanese Ministry of Health and Welfare Guidelines for Designation of Food Additives and for Revision of Standards for use of Food Additives. The authors stated that the test material complied with the specifications for solvent-extracted norbixin (Annatto C) (actual content of the tested batch: 91.6%). Preliminary testing confirmed the homogeneity and stability of the Annatto C in the powder diet MF. A report of this study has been published by Hagiwara et al. (2003a).

Four groups of 10 male and 10 female (Crj:CD (SD)IGS) rats, aged 6 weeks at the start of treatment, were used for the study. Rats received solvent-extracted norbixin (Annatto C) by dietary administration, at levels of 0, 1,000, 3,000 or 9,000 mg/kg diet for 13 weeks. Dose levels were chosen based on a preliminary 2-week range-finding study (Aoki, 2001).

At the end of the treatment period, animals were fasted overnight and killed by exsanguination under ether anaesthesia, and all of them were subjected to a detailed necropsy. A wide range of

organs was removed and weighed, and these, with all other major tissues and any abnormal tissues, were preserved for histopathology. Full histopathological examination of all tissues was carried out on all animals of Groups 1 and 4 and of the skin and liver of all other groups, and all abnormal appearing tissues observed at necropsy.

The average intakes of the test material in the 1,000 mg/kg diet group for males and females were 69 and 76 mg Annatto C/kg bw per day, respectively, in the 3,000 mg/kg diet group, 204 and 242 mg Annatto C/kg bw per day, respectively, and in the 9,000 mg/kg diet group, 598 and 735 mg Annatto C/kg bw per day, respectively. Considering that the extract contained 91.6% norbixin, these intakes correspond to 63, 187 and 548 mg norbixin/kg bw per day for the males, and 70, 222 and 673 mg norbixin/kg bw per day for the females in the low- to high-dose groups, respectively.

No mortalities or clinical signs of toxicity were observed. There was no effect on body weight or food intake. There were minor changes in haematology and clinical chemistry. Gross examination revealed discolouration of the whole body in both sexes of the 9,000 mg/kg diet group. A discoloured area in the liver was found in one female administered 9,000 mg/kg diet. However, no other treatment-related macroscopic changes were found in treated animals at autopsy. A slight increase in kidney weight was found at the top-dose levels in both sexes, but was not accompanied by histopathological changes. Marked elevation of absolute and relative liver weight was observed in both sexes receiving 3,000 and 9,000 mg/kg diet. The liver weight in animals treated with 1,000 mg Annatto C/kg diet was not significantly different from controls. Hepatocellular hypertrophy was found in all rats fed 9,000 mg Annatto C/kg diet, and six males and five females in the 3,000 mg/kg diet group; one male and one female in the top-dose group had focal necrosis in the liver. No other treatment-related histopathological changes were observed in any of the treated groups.

The Panel concluded that the lowest dose level of 1,000 mg/kg diet, equivalent to an intake for males and females of 69 and 76 mg/kg bw per day, respectively, of the test material, or 63 and 70 mg norbixin/kg bw per day, respectively, was the NOAEL in this study.

Furthermore, the Panel noted that for Annatto B, Annatto E and Annatto C tested in these 90-day studies, the specified bixin and norbixin contents generally matched those in the proposed specifications, mounting to not less than 85% bixin, not less than 25% bixin and not less than 85% norbixin, respectively.

3.2.3. Genotoxicity

3.2.3.1. Bacteria

The rec assay for DNA damage in the bacterium *Bacillus subtilis* was conducted with a water-soluble annatto extract, but no experimental details were given. No significant response was obtained (Kawachi et al., 1980). In a second rec assay study (Haveland–Smith, 1981), a food grade annatto extract was dissolved in water and tested to a concentration of 1 mg/mL against *B. subtilis* without the addition of exogenous metabolic activation, or in the presence of liver postmitochondrial fraction (S9) from phenobarbital-treated rats. Testing was also conducted by incubating the annatto extract solution under anaerobic conditions with a rat caecal preparation, before conducting the DNA repair test in the absence or presence of the rat liver S9. The caecal preparation completely decolourised the annatto extract. No significant response was obtained. The Panel noted that the test method used in this study was not validated and not recommended for genotoxic hazard identification. Thus, the results of this study could not be considered for risk assessment.

In studies reported by Kawachi et al. (1980), an equivocal mutagenic response was obtained in a bacterial mutation assay using *Salmonella* Typhimurium TA100, but there was no significant response when using *S.* Typhimurium TA98. It was not clear from the study report whether an exogenous activation system was included, and there was no information on the doses used. The Panel noted that, due to the lack of information on the experimental conditions and the test material, the results of this study could not be used for risk assessment.

Using the same series of exogenous activation conditions as described above (Haveland–Smith, 1981), a water-soluble annatto extract was tested for mutagenicity in fluctuation assays against *S.* Typhimurium TA1538 and *Escherichia coli* WP2 uvrA, at a concentration of 1 mg/mL. No significant responses were obtained under any of the four activation conditions. The Panel noted that the protocol of this study was severely limited – in terms of tester strains and doses tested – compared to current guideline requirements, and considered that this study had a limited value.

Sodium and potassium salts of annatto extract (i.e. water-soluble) were tested for mutagenic activity in pre-incubation assays against *S.* Typhimurium strains TA92, TA1535, TA100, TA1537, TA94

and TA98 in both the absence and presence of liver S9 preparations from Fischer rats, treated 5 days previously with a polychlorobiphenyl mixture (Ishidate et al., 1984). The purities of the samples tested were not stated. At dose levels up to 0.4 mg/plate (sodium salt) or 10 mg/plate (potassium salt), no significant responses were obtained.

3.2.3.2. Mammalian cells in culture

In a poorly reported study, water-soluble annatto extracts did not produce chromosomal damage in human or hamster cells (not specified) in the absence of exogenous metabolic activation (Kawachi et al., 1980; Sasaki et al., 1980). A similar lack of effect was reported using sodium or potassium salts of annatto extracts (i.e. water-soluble) in tests for chromosomal aberration induction in Chinese hamster lung cells, after treatment for 48 h (in the absence of any exogenous activation system) with 10 mg/mL of the potassium salt and 25 mg/mL of the sodium salt (Ishidate et al., 1984). An equivocal result was reported, however, for the sodium salt of annatto extracts after treatment with 25 mg/mL for 24 h in the absence of an exogenous metabolic activation system. Polyploidy was 2.5%, while 7% of cells had structural aberrations, compared to $\leq$ 3% in untreated and solvent-treated cultures. No significant response was observed after treatment with the potassium salt of annatto extracts (Ishidate et al., 1984). The Panel noted that the equivocal result reported was associated with an exceedingly high dose, higher than recommended, and considered the overall result of the study negative.

Kovary et al. (2001) did not demonstrate any DNA damaging activity of norbixin (purity > 98% by HPLC analysis) at concentrations up to 450 μM for 2 h in cultures of mouse fibroblasts, using the single-cell alkaline gel elution (or comet) assay.

3.2.3.3. *In vivo* studies

In summaries of a Japanese co-operative programme on short-term assays for carcinogenicity, it was reported by Kawachi et al. (1980), when referring to earlier work from the same laboratory, that there was no increase in chromosomal damage in the bone marrow of rats treated with water-soluble annatto extract.

In an *in vivo* comet assay, no increase in DNA breakage was detected in the liver and kidney of mice treated with an annatto extract containing 50% norbixin (56 and 351 mg annatto extract/kg bw per day) or purified norbixin (0.8, 7.6, 66 and 274 mg norbixin/kg bw per day) in drinking water for 21 days (Fernandes et al., 2002).

Positive results were reported in a series of studies from another laboratory (Banzon and Aranez, 1984; Aranez and Bayot, 1997) using atsuete (annatto extract) dye extracted from the seed coat of *B. orellana.* In an *in vivo* micronucleus test in mouse bone marrow, the chloroform extract of dried seed coat (CE) or the residue from CE after extraction with petroleum ether (BE) were dissolved in dimethyl sulfoxide (DMSO) and given to mice at doses of 125 or 300 mg/kg bw. According to the study authors, treatment with CE produced a significant increase in the proportion of micronucleated polychromatic erythrocytes. The BE preparation from the crude material produced similar results, whereas the residue from the BE preparation did not (Banzon and Aranez, 1984). Positive results were also reported from a mouse dominant lethal test using the same test materials (CE and BE) (Aranez and Bayot, 1997). The Panel noted several methodological shortcomings in the above described studies, such as the insufficient number of treated animals and inadequate mating time. Moreover, the tested material was considered not representative of the annatto extracts used as food additive. Therefore, these studies were considered not relevant for the present safety assessment of annatto extracts.

In a study on the modifying effect of annatto on diethylnitrosamine genotoxicity, an annatto preparation containing 5% bixin was administered to male Wistar rats at 1,000 mg/kg diet, corresponding, according to the study authors, to 4.23 mg bixin/kg bw (Agner et al., 2004). At the end of the treatment period, no increase in DNA damage was observed by the comet assay in the liver of treated rats compared to control animals fed with standard diet, and no modifying effect on DNA damage induced by diethylnitrosamine (20 mg/kg bw, i.p. injection).

3.2.3.4. Other test systems

No mutagenic activity was reported in silk worms treated with a water-soluble annatto extract (Kawachi et al., 1980, 1981). The Panel noted that this study is based on a test system not validated, nor used for risk assessment, and did not consider this work relevant for safety assessment.

Using extracts similar to those used in the dominant lethal test reported above, Aranez and Rubio (1996) reported that the CE and BE of the ripe seeds of *B. orellana* were genotoxic in dividing onion cells as shown by a significantly lower mitotic index value, when compared with DMSO-treated

controls, while the petroleum ether extract of the residue of chloroform extraction was inactive. The Panel noted that this study used a non-validated test system, and that the effect reported (decreased mitotic index) pertains to cytotoxicity rather than genotoxicity.

The chloroform extract (CE) of dried seed coat or the residue from petroleum extraction of the CE residue (BE) tested in the mouse micronucleus assay was also evaluated in a mutation assay in *Drosophila* (SMART; somatic wing mutation and recombination test) (Banzon and Aranez, 1984). In this test, the crude CE extract and the BE preparation produced significant increases in the number of flies with wing abnormalities, whereas the residue from the BE preparation did not. The Panel noted that the material tested was considered not representative of the annatto extracts used as food additive.

3.2.3.5. Genotoxicity studies on the extracts of the present opinion

Additional studies using the annatto extracts evaluated in the present opinion were provided by the applicant and are described below.

The solvent-extracted bixin (Annatto B), aqueous-processed bixin (Annatto E) and alkali-processed, acid-precipitated norbixin (Annatto F) were subjected to four *in vitro*/*in vivo* test systems for assessing their mutagenic and clastogenic potential. Analytical grade samples of bixin and norbixin were also tested in the bacterial mutation assay only. The solvent-extracted norbixin (Annatto C) was tested separately in one bacterial mutation assay. The results of these genotoxicity studies are summarised in Table 11, and described in more detail below.

Table 11: Summary of genotoxicity studies with the annatto extracts evaluated in the present opinion

Test material	Mutagenicity assay	Reference	Result without S9 mix	Result with S9 mix	Overall result
Solvent-extracted bixin (Annatto B)	Bacterial mutation	Riach and Stevenson (2001a)	Weakly positive in TA100	Negative	Weakly mutagenic
	Mammalian cell mutation	Riach (2001a)	Negative	Negative	Negative
	Mammalian cell chromosomal aberrations	Murie (2001a)	Negative	Weakly positive at toxic concentration	Weakly clastogenic
	Mouse bone marrow micronucleus test	Innes (2002a)	Negative	Not applicable	Negative
	In vivo comet assay in rat stomach and liver	Beevers (2015a)	Negative	Not applicable	Negative
Aqueous-processed bixin (Annatto E)	Bacterial mutation	Riach and Stevenson (2001b)	Weakly positive in TA100	Equivocal in *E. coli* and TA104	Weakly mutagenic
	Mammalian cell mutation	Riach (2001b)	Weakly positive, 4 and 24 h	Negative	Weakly mutagenic
	Mammalian cell chromosomal aberrations	Murie (2001b)	Negative	Negative	Negative
	Mouse bone marrow micronucleus test	Innes (2002b)	Negative	Not applicable	Negative
	In vivo comet assay in rat stomach and liver	Beevers (2015c)	Negative	Not applicable	Negative in stomach; equivocal in liver
Alkali-processed, acid-precipitated norbixin (Annatto F)	Bacterial mutation	Riach and Stevenson (2001c)	Weakly positive in TA100 and *E. coli*	Equivocal in TA104 and *E. coli*	Weakly mutagenic
		Aoki (2002)	Weakly positive in TA100	Weakly positive in TA100	Weakly mutagenic
	Mammalian cell mutation	Riach (2001c)	Negative, 4 h; weakly positive, 24 h	One negative, one inconclusive	Weakly mutagenic
	Mammalian cell chromosomal aberrations	Murie (2001c)	Negative	Weakly positive	Weakly clastogenic
	Mouse bone marrow micronucleus test	Innes (2002c)	Negative	Not applicable	Negative
	In vivo comet assay in rat stomach and liver	Beevers (2015b)	Negative	Not applicable	Negative

Test material	Mutagenicity assay	Reference	Result without S9 mix	Result with S9 mix	Overall result
Solvent-extracted norbixin (Annatto C)	Bacterial mutation	Aoki (2002)	Negative	Negative	Negative
Bixin	Bacterial mutation	Riach and Stevenson (2001d)	Negative	Negative	Negative
Norbixin	Bacterial mutation	Riach and Stevenson (2001e)	Negative	Negative	Negative

Bacterial mutation test

The bacterial mutation assays on Annatto B, Annatto E and Annatto F, bixin and norbixin performed by Riach and Stevenson (2001a–e) used the *S.* Typhimurium strains TA1535, TA1537, TA98, TA100, TA102 and TA104 and *E. coli* WP2uvrA (pKM101). The assays were conducted in the presence and in the absence of an exogenous metabolic activation system based upon a 9,000 g supernatant fraction of liver from Aroclor 1,254-treated male rats (S9 mix). All substances were dissolved in DMSO and tested at 125, 250, 500, 1,000, 2,500 and 5,000 μg/plate using triplicate plates. Two independent experiments were performed using the preincubation method. The studies were performed under GLP, following a protocol basically compliant with the OECD guideline for bacterial mutation assays. It is noted that the predetermined limit of 5,000 μg/plate exceeded the limits of solubility in agar medium for all extracts. Even though, normally, the highest dose would have been the one where precipitation was first observed, the applicant decided to modify the standard procedure including doses with visible precipitation, because an earlier study conducted by the US National Toxicology Program (NTP), had demonstrated a significant, positive response for an annatto extract at doses above the solubility limit (NTP, 1999/2000). The Panel agreed with this modification of the protocol, which is considered appropriate in case of testing complex mixtures.

In experiments without exogenous metabolic activation, all three annatto extracts (Annatto B, E and F) were weakly mutagenic in *S.* Typhimurium TA100 (and also in *E. coli* in the case of Annatto F), inducing approximately twofold increases in revertants at dose levels where precipitation occurred (from 1,000 μg/plate onwards). In the presence of S9 mix, no mutagenic effect was observed in strain TA100, while borderline (30–50%) increases in revertants were observed in *E. coli* WP2 and in the *S.* Typhimurium strain TA104. Analytical grade bixin and norbixin samples did not produce any significant mutagenic effect in any tester strain, either with or without metabolic activation.

Another study (Aoki, 2002) was performed to compare the genotoxicity of solvent-extracted norbixin (Annatto C, norbixin content 91.6%) and alkali-processed, acid-precipitated norbixin (Annatto F, norbixin content 38.6%) in the standard Ames battery. A range of doses of annatto extracts (0, 10, 50, 100, 500, 1,000, 5,000 and 10,000 μg/plate) were tested in a battery of *Salmonella* (TA1535, TA1537, TA1538, TA98, TA100) and *E. coli* (WP2 uvrA) strains, using the preincubation method, with and without metabolic activation. The solvent-extracted norbixin (Annatto C) did not increase the number of revertants at any concentration in any strain, whereas the alkali-processed, acid-precipitated norbixin (Annatto F) was positive against *S.* Typhimurium TA100, with and without activation, inducing at the highest tested dose approximately twofold and fourfold increases in revertant counts in the presence and absence of S9, respectively. The Panel noted that this study was not performed under GLP; however, the protocol was considered acceptable and the results were adequately reported.

Mouse lymphoma mutation test

The mutagenicity of Annatto B, Annatto E and Annatto F in mammalian cells was evaluated in the mouse lymphoma L5178Y cell tk+/− forward mutation assay (Riach, 2001a,b,c). The microwell version of the assay was performed in the presence and absence of an exogenous metabolic activation system by Aroclor-induced rat liver S9. All substances were dissolved in DMSO and tested up to a concentration in the culture medium that caused some toxicity, but permitting at least 10% relative total growth. The studies were performed under GLP. The Panel noted that, even though a specific OECD guideline for the tk+/− forward mutation assay in mammalian cells was not available at the time the study was performed, the study protocol could be considered acceptable.

The solvent-extracted bixin (Annatto B) was not mutagenic in mouse lymphoma L5178Y cells. Some mutagenic activity was observed with the other two extracts in the absence of S9 mix: statistically significant increases in mutant frequencies were observed in the presence of S9 mix with Annatto E, while a non-reproducible increase was observed with Annatto F. The Panel noted that, albeit statistically significant, the increases in mutant frequencies were small, approaching the threshold for biological significance of 126 mutants/million (based on the Global Evaluation Factor approach established by the International Workshops on Genotoxicity Testing – IWGT in 2007) only with the highest dose of Annatto B (241×10^{-6} vs 126×10^{-6} mutants in treated and control cultures, respectively).

Chinese hamster ovary cell chromosomal aberration test

The potential of the test substances to induce structural and numerical chromosomal aberrations was evaluated in *in vitro* cytogenetic assays with Chinese hamster ovary cells performed in compliance with GLP (Murie, 2001a–c). The assays were conducted in the presence and in the absence of exogenous metabolic activation by Aroclor-induced rat liver S9. All substances were dissolved in DMSO and tested up to a concentration that included some toxicity (the viable cell population after treatment was reduced to < 50% of the vehicle control), but permitted the microscopic examination of at least 100 metaphases for each concentration. Cultures were treated for 6 h in the presence, and 6 or 22 h in the absence of S9 mix. Cells were harvested at 24 h (Tests 1 and 2) or 48 h (Test 2) from the beginning of treatment. Each test was performed with duplicate cell cultures, and each experiment was performed twice on independent occasions. Cyclophosphamide and methyl methanesulfonate were used as positive controls.

The solvent-extracted bixin (Annatto B) induced structural chromosomal aberrations in the presence of S9 mix at concentrations of 75 and 100 μg/mL and in the absence of S9 mix (48 h harvest) at 15 and 30 μg/mL. An increase in endoreduplication was observed in cultures treated with the highest dose in the absence of S9 mix and harvested 48 h post-treatment (Murie, 2001a). The Panel noted that endoreduplication is related to cell cycle disturbance rather than to genotoxicity.

The aqueous-processed bixin (Annatto E) induced structural chromosomal aberrations in the presence, but not in the absence, of S9 mix at the highest tested dose (100 μg/mL) (Murie, 2001b). The Panel noted that the treatment associated with clastogenicity was remarkably toxic, with 21% surviving cells. This level of toxicity is not considered acceptable according to the OECD Guideline 473 (2014), and thus the clastogenic effect reported is regarded as biologically not relevant. Neither endoreduplication, nor any other numerical chromosomal aberration was observed.

The alkali-processed, acid-precipitated norbixin (Annatto F) induced a marginal increase in chromosomal aberrations, just above the 95th percentile of the historical control range, only in the presence of S9 at the two highest tested doses (8 and 12 μg/mL). Neither endoreduplication nor any other numerical chromosomal aberration was observed (Murie, 2001c).

Mouse bone marrow micronucleus test

The *in vivo* clastogenic and aneugenic potential of the annatto extracts was evaluated with the bone marrow micronucleus test in CD-1 mice (Innes, 2002a–c). The three annatto extracts (Annatto B, Annatto E and Annatto F) were administered by gavage to five male and five female mice at three exposure levels (the maximum tolerated dose and two lower ones) twice, 24 h apart, and mice sacrificed 24 h after the last treatment. Water was used as the vehicle for Annatto B, and 0.5% carboxymethyl cellulose for Annatto E and F. Cyclophosphamide was used as a positive control.

No increase in micronucleated polychromatic erythrocytes, and no change in the proportion of polychromatic erythrocytes/normochromatic erythrocytes was observed after administration of Annatto B, Annatto E (both at 500, 1,000 and 2,000 kg/kg) and Annatto F (at 300, 600 and 1,200 mg/kg). The Panel noted that no evidence of bone marrow exposure, highlighted by the altered proportion of young to mature erythrocytes, was achieved in these studies.

Comet assay in vivo

The potential of Annatto B, E and F to induce DNA damage in the liver and stomach of rats was tested in the alkaline comet assay (Beevers, 2015a: Annatto B; Beevers, 2015c: Annatto E; Beevers, 2015b and Kobbelgaard, 2015: Annatto F).

Annatto B was a blend of two representative commercial products and complied with the JECFA specification for the solvent-extracted bixin, with the exception of the purity criteria for acetone (50.9 mg/kg instead of ≤ 30 mg/kg) and methanol (645 mg/kg instead of ≤ 50 mg/kg). Annatto E and

F were commercial batches representative of the products on the market and in compliance with the JECFA specifications for the aqueous-processed bixin (Annatto E) and alkali-processed norbixin, acid-precipitated (Annatto F).

Annatto extracts were administered by gavage in corn oil (Annatto B and E) or high viscosity 0.5% carboxymethyl cellulose (Annatto F) to male Sprague–Dawley rats (six rats/group) at dose levels of 500, 1,000 and 2,000 mg/kg per day at 0 h (day 1) and 21 h (day 2). 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 (day 2). At the end of the main experiment (24 h), the rats were anaesthetised with isoflurane and sacrificed.

Following treatment with Annatto B and 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. Annatto B and F did not induce DNA damage in the liver and stomach of rats treated with 2,000 mg/kg per day (the maximum recommended dose for the *in vivo* comet assay).

The livers of rats administered Annatto E showed a dose-related increase in group mean intensity and tail moment values for groups of animals treated with 1,000 or 2,000 mg/kg per day. Although there was no statistically significant difference in tail intensity between the groups treated with 1,000 or 2,000 mg/kg per day, a significant linear trend was observed. Annatto E-treated animals had tail intensities in the range of 0.17–6.32% compared with the vehicle control range of 0.07–8.13% and the historical control 95% reference range of 0.01–8.50%. However, it was noted that the range of tail intensities in the vehicle control group was skewed by a single animal (Animal 6M, tail intensity of 8.13%); the majority of vehicle control animals fell within a range of 0.07–1.84%. If Animal 6M was excluded from the data analysis, five out of six animals treated at 1,000 mg/kg per day and five out of six animals treated at 2,000 mg/kg per day exceeded the range of tail intensity values seen in the vehicle control group. It was concluded that Annatto E did not clearly induce DNA damage in the liver or stomach of rats treated with up to 2,000 mg Annatto E/kg per day. However, due to the unexplained heterogenicity in responses between animals, the applicant considered an additional dosing experiment to obtain a clear result on the genotoxicity of aqueous-processed bixin (Annatto E).

3.2.3.6. Summary of genotoxicity studies and conclusions

No reliable conclusion on the genotoxic potential of annatto extracts (E 160b) can be drawn from the available published studies, which use non-validated test methods or suffer from methodological shortcomings and inadequate reporting.

A set of adequately performed, GLP-compliant studies was made available on solvent-extracted bixin (Annatto B), aqueous-processed bixin (Annatto E), and alkali-processed, acid-precipitated norbixin (Annatto F). In these studies, all the three annatto extracts were weakly mutagenic in bacteria, especially towards the *S.* Typhimurium strain TA100. The mutagenicity was decreased or even abolished in presence of S9, and was observed only at high doses, which were associated with visible precipitation of the dye. The latter observation suggested the presence of minor genotoxic component(s) in the annatto extracts, different from the colour, as also indicated by the negative results obtained in similar studies with analytical grade bixin and norbixin. In a separate bacterial mutation assay, the solvent-extracted norbixin (Annatto C) tested negative.

The positive results obtained in bacteria were only partially replicated in tests in mammalian cells, which provided limited evidence of mutagenicity, with borderline increases of mutant frequencies with two of the extracts (Annatto E and F), and some evidence of *in vitro* clastogenicity with Annatto B and F. Overall, although the effects reported were small, the results indicated that these annatto extracts were weakly genotoxic *in vitro*.

As an *in vivo* follow-up, a bone marrow micronucleus test was performed. Although the results obtained were clearly negative, the Panel noted that this kind of study only detects effects on chromosome integrity and segregation, but not gene mutations which were the prevailing effect detected *in vitro*. Moreover, no evidence of the exposure of the bone marrow to the test substances was obtained. Thus, according to the EFSA Scientific Committee guidance criteria on genotoxicity testing strategies (EFSA, 2011c), the available *in vivo* studies were considered not sufficient to rule out a genotoxic concern. To this aim, an *in vivo* comet assay, which is an adequate follow-up test for gene mutagens and clastogenes, has been performed on the main target tissues of oral exposure (i.e. the stomach and the liver). Neither Annatto B nor Annatto F induced DNA damage in the liver or stomach of rats treated with up to 2,000 mg/kg bw per day of the test compounds, whereas Annatto E induced an unexplained heterogenicity in responses among animals. Therefore, the applicant considered an

additional dosing experiment to get a clear result of the genotoxicity of aqueous-processed bixin (Annatto E).

Overall, based on the available experimental data, the Panel concluded that Annatto B and F do not raise concern for genotoxicity, whereas for Annatto E an additional *in vivo* study is needed to clarify the genotoxic potential in rat liver.

For Annatto C, only a negative bacterial mutation assay was available, whereas no data were available for Annatto G. However, the Panel considered that, as Annatto C is derived from Annatto B, and Annatto G has a similar composition (on a dry weight basis) to Annatto F, read-across from Annatto B and Annatto F to Annatto C and Annatto G, respectively, was possible, and that the latter could be considered not to raise a genotoxic concern either.

3.2.4. Chronic toxicity and (short-term) carcinogenicity

The results from two mouse and three rat studies were previously submitted to JECFA, and are described in some more detail below.

3.2.4.1. Mice

In a 2-year oral study conducted by Engelbreth-Holm and Iversen (1955), body weights and lifetimes of treated mice were similar to those of the control group, there were no pathological differences between the groups at *post mortem* and, in particular, there was no evidence that the annatto extract administered (not further specified) was carcinogenic, although it was not specified whether this was concluded based on gross examination or detailed microscopy.

Similarly, when given in the diet and supplemented by subcutaneous injection, fat-soluble annatto extracts (no further details given in this limitedly reported study) did not cause a significant increase in tumour incidence. Necrosis was observed at the site of injection, and most animals died between 15 and 21 months from intercurrent infections (Van Esch et al., 1959).

3.2.4.2. Rats

Engelbreth-Holm and Iversen (1955) reported that body weights of animals treated with the annatto extract (not further specified) for 26 months were similar to those of the controls, that treated animals did not turn yellow, and that no toxic or carcinogenic effects occurred.

Similarly, in life-time studies (Van Esch et al., 1959), no significant toxicity was reported when animals were fed with either the fat-soluble or the water-soluble annatto extract containing between 0.2% and 2.6% bixin (no further details given in this limitedly reported study). When treatment was continued in two further generations for 7 and 8.5 months, there was no adverse effect on growth or reproduction, and no teratogenic effects, or effects on mortality or tumour incidence were seen (Van Esch et al., 1959). In another study, where treatment was given for 32 months and continued in a further generation for 7 months, similar negative findings were observed (Van Esch et al., 1959).

Five groups of six male and six female Wistar rats were treated with three annatto extracts (E 160b) in the diet, either alone or in combination, as follows (Philp, 1981):

Control	Purified diet	0% bixin
OSB	0.1% solution of annatto extract in vegetable oil	0.22% bixin
R10	0.02% suspension of annatto extract in vegetable oil	1.84% bixin
WSA	0.1% water soluble preparation of annatto extract	0.27% norbixin
Combined	0.1% OSB, 0.02% R10 and 0.1% WSA	2.33% norbixin/bixin

The rats were killed during weeks 48–52 of treatment and were fed control diet during the 24 h prior to their death. Liver, kidneys and abdominal adipose tissue were removed at *post mortem*, weighed, examined (gross appearance only) and subjected to an extraction procedure to identify the presence of annatto pigments. Body weights and food intakes were similar in all treatment groups and not significantly different from the control group. The organ and tissue appearance and weights showed that the animals were in good health, with no differences between the groups. Analysis of the extracts of liver, kidney, adipose tissue and carcass failed to reveal the presence of annatto extract pigments. The Panel noted that this study could not be used for risk assessment.

Agner et al. (2004) investigated the carcinogenic and anticarcinogenic properties of the dietary annatto extract (not further specified) in male Wistar rat liver using the preneoplastic glutathione

S-transferase (GST-P) foci and the comet assay as parameters. An oil extract of annatto, containing 5% bixin, was administered mixed in the diet at concentrations of 20, 200 and 1,000 mg/kg diet (1.5, 16.45 and 84.8 mg annatto extract/kg bw per day; 0.07, 0.8 and 4.23 mg bixin/kg bw per day, respectively), to groups of 14 male Wistar rats continuously during 2 weeks before (pre-initiation protocol), and 8 weeks after *N*-nitrosodiethylamine (DEN) treatment (200 mg/kg bw, i.p.) (post-initiation protocol), with corresponding control groups, to evaluate its effect on the liver-carcinogenesis medium-term bioassay. Groups of 10 male Wistar rats were dosed with the annatto extract for 2 weeks prior to DEN injection (20 mg/kg bw, i.p.) and sacrificed 4 h later, along with the corresponding control groups. The results showed that the annatto extract was not carcinogenic up to the highest concentration tested (1,000 mg/kg diet). No protective effects were observed in either GST-P foci development. The authors concluded that the annatto extract showed no hepatocarcinogenic effect or modifying potential against DEN-induced preneoplastic foci in the rat liver.

Solvent-extracted norbixin (Annatto C)

The modifying potential of solvent-extracted norbixin (Annatto C) on liver carcinogenesis was investigated in groups of 18 male F344/DuCrj rats initially treated with DEN (Hagiwara et al., 2003b). Two weeks after a single dose of DEN (200 mg/kg, i.p.), rats were given the annatto extract at dietary levels of 0%, 0.03%, 0.1% and 0.3%, or phenobarbital sodium at 0.05% as a positive control for 6 weeks. All animals were subjected to partial hepatectomy at week 3, and were killed at week 8. In contrast to the positive control, Annatto C did not significantly increase the quantitative values for GST placental form positive liver cell foci observed after DEN initiation. The results demonstrated that Annatto C at a dietary level of up to 0.3% (200 mg/kg bw per day) did not possess promoting potential in this rat medium-term bioassay system.

3.2.4.3. Conclusions on chronic toxicity and carcinogenicity

The applicant indicated that the fat-soluble annatto preparation (E 160b) used in the studies of Engelbreth-Holm and Iversen (1955) and Van Esch et al. (1959) was obtained by extraction of annatto seeds with chloroform (Iversen and Lam, 1953). The applicant assumed that the extraction with chloroform is comparable with the organic solvent extraction technique used for the solvent-extracted bixin (Annatto B). The water-soluble annatto preparation described in the life-time study of Van Esch et al. (1959) was obtained by the water extraction (Diemair and Zacharias, 1951), a processing technique that was comparable with the method used for aqueous-processed bixin (Annatto E). Considering that the raw material for the preparations described by Iversen and Lam (1953) and Diemair and Zacharias (1951) was the annatto seed (i.e. outer coat or aril), it seemed likely that the non-colouring constituents of the test materials used in the old carcinogenicity studies possessed similar chemical properties as the non-colouring fractions of solvent-extracted bixin (Annatto B) and aqueous-processed bixin (Annatto E). Taking into account the low purity of the fat- and water-soluble annatto preparations tested for carcinogenicity, the applicant considered it appropriate to extrapolate the conclusions from those old carcinogenicity studies to Annatto B, C, E, F and G. The Panel agreed with this assumption.

In a study by Hagiwara et al. (2003b), Annatto C (solvent-extracted norbixin), tested up to a dietary level of up to 0.3% did not possess promoting potential.

Since submission of the dossier for the annatto extracts (Doc. provided to EFSA n.3), several systematic reviews on annatto have been published (Lim, 2012; Ulbricht et al., 2012; Vilar et al., 2014). The toxicological activity of annatto was addressed in all three reviews. The authors did not find any evidence of possible carcinogenic risks related to the use of annatto extracts.

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.

3.2.5. Reproductive and developmental toxicity

A two- and three-generation study in rats has been reported as part of long-term toxicological investigations (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. The applicant concluded that no evidence of reproductive toxicity of annatto extracts (E 160b) has been observed in the multigeneration and developmental toxicity studies in rats with fat-soluble or water-soluble annatto extracts (E 160b).

The Panel noted that the number of animals used in these studies was low and the reporting of the reproduction parameters limited.

3.2.5.1. Aqueous-processed bixin (Annatto E)

Paumgartten et al. (2002) have published a developmental toxicity study in rats using an annatto extract that is the same as aqueous-processed bixin (Annatto E). They reported that the annatto extract (containing 28% bixin) suspended in corn oil, and administered by gavage to groups of 16–27 female Wistar rats in doses of 0, 31.2, 62.5, 125, 250 and 500 mg/kg bw per day on gestational day (GD) 6–15, was neither maternally toxic nor embryotoxic. Caesarean sections were performed on GD 21; implantations, living and dead fetuses and resorptions were recorded. Fetuses were weighed and examined for externally visible abnormalities. One-third of the fetuses in each litter were examined for visceral abnormalities by a microsectioning technique. The remaining fetuses were cleared and stained with Alizarin Red S for skeletal evaluation. No adverse effects of the annatto extract on the dams were noted, there was no increase in embryolethality and no reduction in fetal body weight. Furthermore, the annatto extract did not induce any increase in the incidence of externally visible, visceral or skeletal abnormalities in the exposed offspring. The NOAEL in this study was the highest dose tested, 500 mg/kg bw per day annatto extract, which was equivalent to 140 mg/kg bw per day of bixin.

3.2.5.2. Alkali-processed, acid-precipitated norbixin (Annatto F)

At the 61st JECFA Meeting (2003), 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 was performed according to the OECD Guideline 414 (2001) with the alkali-processed, acid-precipitated norbixin (Annatto F) (Armour, 2005).

The study was performed in compliance with GLP guidelines (Armour, 2005). The dose levels of the test material were chosen based on the results obtained in a previous 90-day subchronic toxicity study with this compound performed in the same laboratory (see Section 3.2.2.3). In that study, the norbixin-rich extract was administered in the diet to Sprague–Dawley rats at concentrations of 1,000, 3,000 and 9,000 mg/kg diet, giving mean achieved doses over 13 weeks in the female rats of 86, 275 and 816 mg/kg bw per day. The NOAEL was determined by the study authors to be 86 mg/kg bw per day (equivalent to 33 mg norbixin/kg bw per day) based on the changes in renal function observed at the two highest dose levels. The high-dose level for this reproductive toxicity study was set at 160 mg/kg bw per day, which was expected to be mildly toxic to the dams, and a low dose of 20 mg/kg bw per day was chosen in the expectation that this would be a clear NOAEL.

Groups of 22 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 GD 6–19. Controls were dosed with 0.5% aqueous sodium carboxymethyl cellulose. Animals were killed on GD 20 after mating for reproductive assessment and fetal examination. Adult females were examined macroscopically at necropsy on day 20 after mating, the uterus and contents weighed, corpora lutea counted, and living and dead fetuses and resorptions were recorded. Fetuses were weighed and examined for externally visible abnormalities, and all fetuses were examined macroscopically at necropsy; subsequently, half the fetuses had detailed internal visceral examination followed by skeletal examination, and the other half were fixed and serially sectioned for examination for visceral abnormalities.

In the dams, there were no deaths, no clinical findings considered to be related to treatment, and no adverse effects of treatment on body weight gain or food consumption. There was no increase in embryolethality and no reduction in fetal or placental weight. The annatto extract (Annatto F) did not induce any increase in the incidence of externally visible, visceral or skeletal abnormalities in the exposed offspring at doses up to 160 mg/kg bw per day. The amniotic sacs of the majority of litters in the 80 and 160 mg/kg bw per day groups were stained yellow/orange, showing that norbixin had been well absorbed.

The authors concluded that oral gavage administration of the alkali-processed, acid-precipitated norbixin (Annatto F) at dosages of up to 160 mg/kg bw per day (equivalent to 68 mg norbixin/kg bw per day), the highest dose tested, when administered during gestation from the time of implantation

until just before delivery, resulted in no treatment-related effects on the progress or outcome of pregnancy for female CD (Sprague–Dawley) rats. The Panel agreed with this conclusion.

3.2.5.3. Read-across from Annatto F to Annatto G and from Annatto E to Annatto B and Annatto C

As discussed in Section 2.2.3, the composition of the alkali-processed norbixin, not acid-precipitated (Annatto G) is similar to that of the alkali-processed, acid-precipitated norbixin (Annatto F), when compared on a calculated dry weight basis, as the liquid Annatto G contains 90% water. Therefore, the applicant concluded that, if Annatto F did not cause treatment-related effects on the progress or outcome of pregnancy for female rats (Armour, 2005), no adverse effects were to be expected from a treatment with Annatto G.

Furthermore, all components present in the non-colouring fraction of the solvent-extracted bixin (Annatto B) were also detected in the non-colouring part of the aqueous-processed bixin (Annatto E), which was the less pure of the two extracts and which was the one tested by Paumgartten et al. (2002). No adverse effects on development were observed after treatment of rats with the aqueous-processed bixin (Annatto E). Based on the similarity of the non-colouring fraction, the applicant expected that the solvent-extracted bixin (Annatto B) would not have any negative effects on development and reproduction.

The solvent-extracted norbixin (Annatto C) shares the same manufacturing route as the solvent-extracted bixin (Annatto B) but undergoes, in addition, a saponification step that converts bixin into norbixin. However, saponification is not expected to alter the non-colouring components of the extract. Under this assumption, the applicant concluded that the findings of the developmental toxicity study with the aqueous-processed bixin (Annatto E) performed by Paumgartten et al. (2002) were not only applicable to the solvent-extracted bixin (Annatto B), but also to the solvent-extracted norbixin (Annatto C).

The applicant also noted that, in the life-time study of Van Esch et al. (1959), no adverse effects on reproduction and no teratogenic effects were seen in rats fed with either the fat-soluble annatto preparation containing between 0.2% and 2.6% bixin or the water-soluble annatto preparation containing 0.2% norbixin. Considering the low purity of these annatto preparations used by Van Esch et al. (1959), no adverse effect on reproduction or development were to be expected from the five annatto extracts described in the present opinion, which all show higher concentrations of bixin or norbixin.

3.2.6. Other studies

3.2.6.1. Allergenicity, hypersensitivity and intolerance

Animal study

Cis-bixin and norbixin were purified from an annatto extract (ANT) and their contact allergenic effects were studied in female BALB/c mice using the local lymph node assay (LLNA) and the mouse ear swelling test (MEST) (Auttachoat et al., 2011). Positive responses in both the LLNA and MEST were observed in mice treated with *cis*-bixin at concentrations as low as 0.1–0.5%, but not with norbixin. These results demonstrated that *cis*-bixin, but not norbixin, is likely a contact sensitiser and contributes to the contact hypersensitivity effects observed following dermal exposure to ANT in mice.

Human studies

In humans, there are a limited number of studies that reported that the annatto extracts might be a possible cause of angiooedema, urticaria or eczema. However, as the annatto extracts were given in combination with other food colours, it was not always possible to know whether the observed effects were due to the annatto extracts or to one of the other colours present in the challenging material (Mikkelsen et al., 1978; Juhlin, 1981; Vien et al., 1987; Young et al., 1987; Fuglsang et al., 1993, 1994).

An anaphylactic reaction attributed to the annatto extract has been reported in a 62-year-old man (Nish et al., 1991) after consumption of a type of cereal that he had never eaten before. Skin prick tests to milk, corn and wheat were all negative, but a positive reaction to the annatto extract was observed. The patient's serum immunoglobulin E (IgE) recognised a 50-kDa protein in the annatto extract, considered by the authors to be a contaminant from the pericarp of the seed in the preparation of the annatto extract.

In a patient without other apparent food allergies, a close relationship between ingestion of annatto-containing cheeses and immediate anaphylactic reaction was reported as highly indicative of an IgE-mediated reaction to the natural food colour additive (Ebo et al., 2009). This presumption was supported by the positive skin tests, the basophil activation test and IgE-immunoblot to annatto.

The Panel noted that, whereas the annatto extracts (E 160b) are widely present in foods, only two cases of an anaphylactic reaction were reported, and therefore this should be regarded as a very rare event. The allergenic potential of bixin seems higher than that of norbixin. In addition, IgE-mediated allergy from this natural dye appeared to be rather anecdotal. In general, when investigated in well-designed double-blind trials, the incidence of reactions to the annatto extracts alone was very small (Lucas et al., 2001). The Panel considered that the available database did not indicate a high allergenic potential of the annatto extracts (E 160b) used as food additive in humans. However, the Panel noted that this conclusion might not be correct for the new extracts. This is because the amount of contaminating proteins, which may also be responsible for an allergic response, is not specified in the current specifications for E 160b, whereas it is variable in the five new extracts Annatto B, E, C, F, G, and may be as high as 62 g/kg for Annatto F.

3.2.6.2. Cytochrome P450 induction

Bixin has been investigated for its effect on drug metabolising enzymes (Jewell and O'Brien, 1999). A variety of carotenoids were administered to male Wistar rats at a dose of 300 mg/kg in the diet over a period of 16 days, and the activities of a range of enzymes were assessed in the liver, lung, kidney and small intestine. Control animals received diet alone, and positive controls received the enzyme-inducing agent, 3-methylcholanthrene. Cytochrome P450 activity, GST and reduced glutathione status and carotenoid uptake into the tissues were assessed. Bixin was shown to induce the 1A1 isoenzyme of cytochrome P450 in the liver, lung and kidney and, to a lesser extent, the 2B1/2 enzyme in the liver. None of the carotenoids produced detectable effects on intestinal enzymes or glutathione status.

At the end of the 90-day toxicity studies (Section 3.2.2.3) on the solvent-extracted bixin (Annatto B), aqueous-processed bixin (Annatto E) and alkali-processed, acid-precipitated norbixin (Annatto F) in rats, liver samples were taken from 10 males and 10 females. The effects of Annatto B, E and F on the specific hepatic microsomal content of total P450 and of the P450 apoproteins CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, CYP3A1, CYP3A2 and CYP4A (primarily CYP4A2 and/or CYP4A3) were determined (Boobis, 2002). The annatto extracts did not increase total P450 content. All test materials induced CYP4A (primarily CYP4A2 and CYP4A3). In general, the response was greater in males than in females. Overall, the Panel concluded that these observations suggested that the increase in liver weight observed in rats following treatment with the annatto extracts might be due to peroxisome proliferation via an induction of the PPARα receptor. This hypothesis was, however, at variance with some of the other available data. For example, the increase in liver weight was most marked with the alkali-processed, acid-precipitated norbixin (Annatto F), less with the aqueous-processed bixin (Annatto E) and hardly present with the solvent-extracted bixin (Annatto B), whereas the response of the aqueous-processed bixin (Annatto E) was greater than that of the solvent-extracted bixin (Annatto B). In the absence of electron microscopic evidence in the liver of animals treated with the aqueous-processed bixin (Annatto E) and alkali-processed, not acid-precipitated norbixin (Annatto F), no definite conclusion could be reached from the present data on the mechanism of the increased liver weight.

Subsequently, De Oliveira et al. (2003) reported on the effects of an annatto extract (containing 28% bixin) and bixin (95% pure) on rat liver monooxygenases. Adult female Wistar rats were treated by gavage with daily doses of the annatto extract (250 mg/kg bw per day, equivalent to approximately 70 mg bixin/kg bw per day), bixin (95% pure) (250 mg/kg bw per day) or the vehicle only (corn oil, 3.75 g/kg bw per day) for five consecutive days, or were not treated (untreated control). The authors concluded that the enzyme-inducing effects, even at these high doses of the annatto extract, were weak, and that it was unlikely that any effects would occur at normal dietary intake levels of annatto extract. The Panel agreed with this conclusion.

3.2.6.3. Pharmacological activity

The pharmacological activity of extracts of the root of *B. orellana* has been addressed in previous evaluations by JECFA (JECFA, 2004a,b). Since then, a few observations have been made on the effects of annatto extracts (obtained from the seeds of *B. orellana*) on the physiological and biochemical functioning of the body, following administration to animals.

An oil suspension of the seeds of the annatto tree, *B. orellana,* is used in the West Indies as a folk remedy known as 'Bush tea' for the treatment of diabetes mellitus, and studies by Morrison and West (1982, 1985) and Morrison et al. (1987) investigated and demonstrated the hypoglycaemic activity of chloroform extract residues of *B. orellana* dissolved in either oil (peanut or olive) or 95% ethanol in the dog.

In a subsequent study, Morrison et al. (1991) purified the extract by column chromatography and monitored the biological activity using the oral glucose tolerance test in anaesthetised mongrel dogs. They revealed that the active constituent responsible for the hyperglycaemic action was the methyl ester *trans*-bixin (MW 394). As little as 0.8 g of the purified *trans*-bixin produced a sustained hyperglycaemia similar to that seen with 15 g of the crude extract. Mitochondrial damage in the liver and pancreas was produced with the purified material. The authors noted that a minimum of 0.06 g pure *trans*-bixin/kg bw was required to cause toxicity (a diabetogenic effect) in the dog, which was equivalent to 4.2 g of pure *trans*-bixin (or 63 g of the crude extract) in a 70-kg (possibly undernourished) man.

This hyperglycaemic action has been confirmed in rats, but a hypoglycaemic effect has been reported in mice (Fernandes et al., 2002). The authors showed that, when rats were given norbixin in drinking water for 21 days, hyperglycaemia ranging from 26.9% (8.5 mg norbixin/kg bw per day) to 52.6% (74 mg norbixin/kg bw per day, $p < 0.01$) above control values was observed. However, in mice, norbixin induced hypoglycaemia ranging from 14.4% (0.8 mg norbixin/kg bw per day) to 21.5% (66 mg norbixin/kg bw per day) below control values. The rats and mice, when treated in this way, demonstrated hyperinsulinaemia and hypoinsulinaemia, respectively, indicating that the pancreatic β-cells were functional.

4. Discussion

In the present opinion, the safety of alkali-extracted annatto, oil-extracted annatto and solvent-extracted bixin and norbixin (E 160b(i), (ii), (iii)) was re-evaluated, when used as a food additive.

Furthermore, at the request of the European Commission, the present opinion included the evaluation of the safety of five other annatto extracts categorised as bixin- or norbixin-based, following the request of the NATCOL/Annatto Interest Group for authorisation of these extracts, with the view to replace the currently authorised food additive (E 160b). The application by NATCOL covered the five annatto extracts for which JECFA (2007) has established ADIs: (i) aqueous-processed bixin (Annatto E); (ii) solvent-extracted bixin (Annatto B); (iii) alkali-processed norbixin, acid-precipitated (Annatto F); (iv) alkali-processed norbixin, not acid-precipitated (Annatto G); and (v) solvent-extracted norbixin (Annatto C). In 2006, JECFA agreed upon two separate ADIs for bixin- and norbixin-containing annatto extracts: an ADI of 12 mg/kg bw for bixin (92% bixin pure) and a group ADI of 0.6 mg/kg bw for norbixin (91.6% norbixin pure) and its sodium and potassium salts (JECFA, 2007).

The Panel based its evaluation of the annatto extracts on the dossier submitted by the applicant, previous evaluations and reviews, additional literature that came available since then and the data available following public calls for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

The qualitative and quantitative composition of the non-pigment fraction of the annatto extracts proposed by the applicant has been determined attempting to achieve a complete mass balance (> 95%) of representative batches (Lea, 2005; Sheridan, 2005). The applicant indicated that results for the solvent-extracted bixin (Annatto B) and the alkali-processed (acid-precipitated) norbixin (Annatto F) would be applicable also to the solvent-extracted norbixin (Annatto C). The non-pigment fraction of the less pure extracts (aqueous-processed bixin, alkali-processed, not acid-precipitated norbixin, and alkali-processed, acid-precipitated norbixin) (Annatto extracts E, G and F) contained several well-known plant constituents: proteins (1–6%), lignocelluloses (up to 16%), fatty acids (up to 4%; probably as oil), polyphenols (up to 4%) and ash (0.1–12%). Unidentified terpenoids (up to 13.4%) were only present in Annatto E.

The major identified components of the non-pigmented fraction were geranyl geraniol and related terpenoids. The safety of geraniol, geranyl acetate and geranial was reviewed by JECFA (JECFA, 1997, 2004b) and EFSA (EFSA, 2009b, 2012). Acute-, intermediate- and long-term studies have been carried out on these and related terpenoid compounds at high-dose levels, including studies by the NTP, and no specific toxic or carcinogenic effects have been identified.

Studies in rats suggested that within 24 h, water-soluble annatto extracts (WSA: a water-soluble preparation containing 0.27% norbixin) were almost completely absorbed and metabolised, whereas

oil-soluble preparations (OSB: vegetable oil solution containing 0.22% bixin and R10: a vegetable oil suspension containing 1.84% bixin) were metabolised less rapidly than WSA, presumably because the OSB pigments would need to be metabolised to water-soluble derivatives. Additional studies in rats using Annatto B, Annatto E and Annatto F revealed that the more polar norbixin was better absorbed than the less polar bixin. Norbixin was the major component present in plasma and urine, even following oral administration of bixin-based extracts, most likely derived from both the norbixin present in the extract, as well as metabolism of bixin to norbixin. The total percentage of the dose of bixin and norbixin absorbed could not be assessed from these studies without data on biliary excretion. In humans, a study using a commercial annatto food colour containing 16 mg of *cis*-bixin and about 0.5 mg of *cis*-norbixin demonstrated the major presence of norbixin in plasma. This would suggest that in humans as in the rat, bixin and norbixin are absorbed, and that bixin is converted to norbixin

Although the database was limited, the overall pattern of absorption and metabolism appeared to be similar in rat and human and, therefore, the Panel considered that the rat would be an adequate model for assessing the toxicity of annatto extracts in humans. Additional studies, preferably with pure bixin and norbixin, on their metabolism in rats and humans may reduce the uncertainty.

Four of the annatto extracts (Annatto B, C, E and F) evaluated in the present opinion were tested in 90-day studies in rats. The administration of all four test materials to rats for 13 weeks did not result in any treatment-related deaths, and the general condition and behaviour of the animals were not affected by treatment. Some toxicological effects were observed. The effects observed related, generally, to increases in liver and kidney weight, with some indications of impaired function at high-dose levels. No evidence of histopathological damage was observed in any tissue, except for hepatocellular necrosis at the two highest dose levels of the solvent-extracted norbixin (Annatto C). With the alkali-processed, acid-precipitated norbixin (Annatto F), haematological changes were also observed. The studies with solvent-extracted bixin (Annatto B) revealed an effect on the kidney, with raised protein concentrations noted in urine samples obtained from males receiving 50,000 mg/kg diet. Blood plasma phosphorus concentrations were also increased in these animals, indicating a possible reduction in the glomerular filtration rate. Studies with aqueous-processed bixin (Annatto E) revealed increased thyroid and kidney weights.

Table 12: Results of 90-day toxicity studies with annatto extracts in rats

Annatto extract	Pigment in extract tested (%)		Extract NOAEL (mg/kg bw)		Pigment NOAEL (mg/kg bw)	
	Bixin	Norbixin	Male	Female	Male	Female
Solvent-extracted bixin (Annatto B)	92	1.7	1,311	1,446	1,206	1,330
Solvent-extracted norbixin (Annatto C)	NR	91.6	69	76	63	70
Aqueous-processed bixin (Annatto E)	26	4.2	734	801	191	208
Alkali-processed norbixin (acid-precipitated) (Annatto F)	NA	38.4	79	86	30	33

NOAEL: no-observed-adverse-effect-level; NA: not applicable; NR: not reported.

Table 12 presents an overview of the NOAEL values identified by the Panel from these 90-day studies. The NOAEL values were in accordance with the NOEL values identified in these studies by JECFA (JECFA, 2007).

No conclusion can be drawn from published genotoxicity studies with water-soluble annatto extracts, which used not validated test methods and/or limited protocols. However, a set of unpublished properly performed *in vitro* and *in vivo* genotoxicity studies on Annatto B, E, and F were also available to the Panel for evaluation. In these studies, all the three annatto extracts produced weakly positive or equivocal results *in vitro*, especially at high doses, with no consistent pattern of activity. As an *in vivo* follow-up, a micronucleus test in mouse bone marrow and a comet assay in rat stomach and liver, the main target tissues following oral exposure, were performed. In these studies, the oral administration of Annatto B, E, and F up to the maximum recommended dose did not induce any detectable genetic damage in mouse bone marrow, in which, however, no evidence of exposure was obtained; clearly negative results were, however, also obtained with comet assays in rat stomach and, for Annatto B and F, in rat liver. An unexplained heterogenicity in response was instead observed in the liver of animals treated with Annatto E. Negative results were obtained for Annatto C (solvent-extracted norbixin) in a separate bacterial mutation assay.

Overall, based on the available experimental results, and according to the EFSA Scientific Committee Recommendations on Genotoxicity Testing Strategy (EFSA, 2011c), Annatto B and F (and following read-across, also Annatto C and G, see Section 3.2.3.6) were evaluated by the Panel as not genotoxic, whereas more information on the identity of the components of the extract and further testing were considered necessary to clarify the equivocal response elicited by Annatto E in rat liver. For Annatto C, only a negative bacterial mutation assay was available, whereas no data were available for Annatto G. However, the Panel considered that, as Annatto C is derived from Annatto B, and Annatto G has a similar composition (on dry weight basis) to Annatto F, read-across from Annatto B and Annatto F to Annatto C and Annatto G, respectively, was possible, and that the latter could be considered not to raise a genotoxic concern either.

Annatto extracts (E 160b) of lower purity than the ones evaluated in the present opinion were without significant toxicity when administered in long-term studies to mice and rats. According to the applicant, considering that the raw material for the preparations described by Iversen and Lam (1953) and Diemair and Zacharias (1951) was the annatto seed (i.e. outer coat or aril), it seemed very likely that the non-colouring constituents of the test materials used in old carcinogenicity studies (Engelbreth-Holm and Iversen, 1955; Van Esch et al., 1959) possessed similar chemical properties as the non-colouring fractions of solvent-extracted bixin (Annatto B) and aqueous-processed bixin (Annatto E). Taking into account the low purity of the fat- and water-soluble annatto preparations tested for carcinogenicity in the studies performed by Engelbreth-Holm and Iversen (1955) and Van Esch et al. (1959), the applicant considered it appropriate to extrapolate the conclusions from these old carcinogenicity studies to the annatto extracts Annatto B, C, E, F and G. The Panel agreed with this assumption.

Based on the absence of evidence for carcinogenic response, the Panel concluded that the annatto extracts were of no concern with respect to carcinogenicity.

The composition of the alkali-processed, not acid-precipitated norbixin (Annatto G) is similar to composition of the alkali-processed, acid-precipitated norbixin (Annatto F), when compared on a calculated dry weight basis, as the liquid Annatto G contains 90% water. Therefore, the Panel agreed with the applicant that since Annatto F did not cause treatment-related effects on the progress or outcome of pregnancy for female rats (Armour, 2005), no adverse effects were to be expected from a treatment with Annatto G.

Furthermore, all components present in the non-colouring fraction of the solvent-extracted bixin (Annatto B) were also detected in the non-colouring part of the aqueous-processed bixin (Annatto E), which was the less pure of the two extracts and the one tested by Paumgartten et al. (2002). No adverse effects on development were observed after treatment of rats with the aqueous-processed bixin (Annatto E). Based on the similarity of the non-colouring fraction, the Panel agreed with the applicant that the solvent-extracted bixin (Annatto B) would also not have adverse effects on development and reproduction.

The solvent-extracted norbixin (Annatto C) shares the same manufacturing route as the solvent-extracted bixin (Annatto B) but undergoes, in addition, a saponification step that converts bixin into norbixin. However, saponification is not expected to alter the non-colouring components of the extract. Under this assumption, the applicant concluded that the findings of the developmental toxicity study with the aqueous-processed bixin (Annatto E) performed by Paumgartten et al. (2002) are not only applicable to the solvent-extracted bixin (Annatto B), but also to the solvent-extracted norbixin (Annatto C). The Panel agreed with this read-across approach.

Moreover, in the life-time study of Van Esch et al. (1959), no adverse effects on reproduction and no teratogenic effects were seen in rats fed with either fat-soluble annatto preparation containing between 0.2% and 2.6% bixin or water-soluble annatto preparation containing 0.2% norbixin. Considering the low purity of these annatto preparations used by Van Esch et al. (1959), the Panel agreed with the applicant that no adverse effects on reproduction or development were to be expected from the five annatto extracts described in the present opinion, which all showed higher concentrations of bixin or norbixin than those used in previous studies.

To assess the dietary exposure to annatto (E 160b) from its use as a food additive, the exposure was calculated based on (1) the MPLs set out in the EU legislation (defined as the *regulatory maximum level exposure assessment scenario*); (2) the reported use levels (defined as the *refined exposure assessment scenario*); and (3) the use levels proposed by the applicant for the requested extension of use (defined as *extension of use scenario*). Based on the information made available by industry, separate exposure estimates for bixin- and norbixin-based extracts were calculated (except for the *regulatory maximum level exposure assessment scenario* based on the MPLs). With regard to the

analytical results provided, the Panel noted that they referred to E 160b and did not differentiate between bixin- and norbixin-based extracts. Therefore, the concentration levels considered by the Panel for bixin and for norbixin were extracted only from the reported use levels provided by industry.

The Panel calculated two refined exposure estimates based on different assumptions: a *brand-loyal consumer scenario*, where it is assumed that the population is exposed over a long period of time to the food additive present at the maximum reported use levels for one food category and to the mean reported use levels for the remaining food categories; and a *non-brand-loyal scenario*, where it is assumed that the population is exposed over a long period of time to the food additive present at the mean reported use levels in all relevant food categories. These scenarios were calculated for the current uses of annatto, bixin and norbixin (E 160b), as well as for the extension of use scenario.

For bixin, from the *refined estimated exposure scenario*, in the *brand-loyal scenario*, mean exposure ranged from 0.002 mg/kg bw per day in adolescents and adults to 0.10 mg/kg bw per day in toddlers. The 95th percentile exposure ranged from 0.01 mg/kg bw per day in infants, adults and the elderly to 0.32 mg/kg bw per day in toddlers. In the *non-brand-loyal scenario*, mean exposure ranged from 0.001 mg/kg bw per day in adults to 0.08 mg/kg bw per day in toddlers. The 95th percentile exposure ranged from 0.01 mg/kg bw per day in infants, children, adolescents, adults and the elderly to 0.26 mg/kg bw per day in toddlers. Both in the *brand-loyal* and the *non-brand-loyal scenario,* the main food categories contributing to the total mean exposure to bixin were fats and oils for infants and the elderly, flavoured fermented milk products for toddlers and children, and ripened cheese and fats and oils for adults. For adolescents, the main food categories contributing to the total mean exposure to bixin were flavoured fermented milk products and ripened cheese both in the *brand-loyal scenario* and the *non-brand-loyal scenario.*

For norbixin, from the *refined estimated exposure scenario*, in the *brand-loyal scenario*, mean exposure ranged from 0.003 mg/kg bw per day in infants and the elderly to 0.11 mg/kg bw per day in toddlers. The 95th percentile exposure ranged from 0.01 mg/kg bw per day in infants and the elderly to 0.24 mg/kg bw per day in toddlers. In the *non-brand-loyal scenario*, mean exposure ranged from 0.002 mg/kg bw per day in infants and the elderly to 0.09 mg/kg bw per day in toddlers. The 95th percentile exposure ranged from 0.01 mg/kg bw per day in infants, adults and the elderly to 0.18 mg/kg bw per day in toddlers. Both in the *brand-loyal* and the *non-brand-loyal scenario*, the main food categories contributing to the total mean exposure to norbixin were fine bakery wares for all population groups.

The applicant has requested the extension of use of bixin- and norbixin-based annatto extracts in 16 additional food categories. For bixin, from the *extension of use scenario* considering additional exposure from food categories and levels proposed by the applicant, in the *brand-loyal scenario*, mean exposure ranged from 0.01 mg/kg bw per day for infants, adolescents, adults and the elderly to 0.33 mg/kg bw per day for toddlers. The 95th percentile ranged from 0.02 mg/kg bw per day in the elderly to 0.65 mg/kg bw per day in toddlers. In the *non-brand-loyal scenario*, mean exposure ranged from 0.004 mg/kg bw per day for infants to 0.22 mg/kg bw per day for toddlers. The 95th percentile exposure ranged from 0.01 mg/kg bw per day in the elderly to 0.40 mg/kg bw per day in toddlers. Both in the *brand-loyal* and the *non-brand-loyal scenario*, the main contributing food categories to the total mean exposure to bixin were flavoured fermented milk products for infants and toddlers, and soups and broths for children, adolescents, adults and the elderly.

For norbixin, from the *extension of use scenario* considering additional exposure from food categories and levels proposed by the applicant, in the *brand-loyal scenario*, mean exposure ranged from 0.005 mg/kg bw per day for infants to 0.24 mg/kg bw per day for toddlers. The 95th percentile ranged from 0.02 mg/kg bw per day for the elderly to 0.46 mg/kg bw per day in infants and toddlers. In the *non-brand-loyal scenario*, mean exposure ranged from 0.003 mg/kg bw per day for infants to 0.18 mg/kg bw per day for toddlers. The 95th percentile exposure ranged from 0.02 mg/kg bw per day in infants, adults and the elderly to 0.27 mg/kg bw per day in toddlers and children. Both in the *brand-loyal* and the *non-brand-loyal scenario*, the main contributing food categories to the total mean exposure to norbixin were unripened cheese, soups and broths and fine bakery wares for infants, unripened cheese and fine bakery wares for toddlers, unripened cheese, fine bakery wares and soups and broths for children and adolescents, and unripened cheese and soups and broths for adults and the elderly.

The Panel noted that raising the acceptable level for norbixin in the bixin-based annatto extract Annatto B from 2.5% to 5%, as proposed by the applicant, would result in an additional exposure to norbixin of up to 0.017 mg/kg bw per day (considering the extension of use scenario, 95th percentile in toddlers).

Conclusions

Given:

- that read-across among the five bixin- and norbixin-based annatto extracts was feasible;
- the availability of adequate 90-day toxicity studies with the annatto extracts B, C, E, F;
- the absence of concern for mutagenicity, carcinogenicity, reproductive and developmental toxicity of annatto extracts B, C, F and G, whereas the mutagenicity of Annatto E is equivocal,

the Panel concluded that:

- the safety of the currently authorised solvent-extracted bixin and norbixin (E 160b(i)), alkali-extracted annatto (E 160b(ii)) and oil-extracted annatto (E 160b(iii)), with the specifications defined in Commission Regulation (EU) No 231/2012, could not be assessed due to the lack of data, both in terms of identification and toxicological studies;
- as regards the new annatto extracts: solvent-extracted bixin (Annatto B), solvent-extracted norbixin (Annatto C), alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G) and its salts:
  - they should comply with the specifications as recommended by the Panel;
  - the toxicological database is sufficient to derive an ADI of 6 mg bixin/kg bw per day and an ADI of 0.3 mg norbixin/kg bw per day, applying an uncertainty factor of 200 to the NOAEL values derived from the 90-day studies (1,206 mg/kg bw and 63 mg/kg bw, respectively);

  Based on the reported current use levels provided by industry, the Panel concluded that exposure estimates were below the ADI of 6 mg/kg bw per day for bixin and below the ADI of 0.3 mg/kg bw per day for norbixin for all population groups and for all refined exposure scenarios.
  Considering the extension of use for the additional 16 food categories, all refined exposure estimates for bixin were below the ADI of 6 mg/kg bw per day for all populations. For norbixin, the ADI of 0.3 mg/kg bw per day was not exceeded in the non-brand-loyal scenario, and in the brand-loyal scenario at the mean. The only exceedance observed for norbixin was at the 95th percentile in the brand-loyal scenario for infants (in one country), toddlers (in three countries) and children (in one country). However, the Panel noted that this exceedance results from the overestimation of the contribution from at least one food category (i.e. unripened cheese).
- as regards Annatto E, due to the equivocal results obtained with the *in vivo* comet assay, the Panel could not conclude on its safety.

Recommendations

The Panel recommended that:

- the alkali-extracted annatto (E 160b(ii)), the oil-extracted annatto (E 160b(iii)), and the solvent-extracted bixin and norbixin (E 160b(i)), currently authorised in the EU, should be replaced by the solvent-extracted bixin (Annatto B), solvent-extracted norbixin (Annatto C), alkali-processed, acid-precipitated norbixin (Annatto F) and alkali-processed, not acid-precipitated norbixin (Annatto G).
- the specifications for bixin- and norbixin-based annatto extracts (E 160b) according to the Commission Regulation (EU) No 231/2012 should be replaced by the specifications for the annatto extracts (Annatto B, C, F and G) as given by JECFA (2007, 2015). However, the maximum limits for the impurities of toxic elements (arsenic, lead, mercury) should be revised in order to ascertain that the annatto extracts as food additives will not be a significant source of exposure to these toxic elements in foods. Moreover, the Panel recommended that a maximum limit for cadmium should also be included in the specifications.

Documentation provided to EFSA

1) FDE (FoodDrinkEurope), 2013. Data on usage levels of annatto extracts (E 160b) in foods in response to the EFSA call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Submitted on 29 November 2013.

2) ICGA (International Chewing Gum Association), 2013. Data on usage levels of annatto extracts (E 160b) in foods in response to the EFSA call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Submitted on 29 November 2013.
3) NATCOL (Natural Food Colours Association), 2008. Annatto extracts. Submission to EFSA by the Natural Food Colours Association/Annatto Interest Group. 4 April 2008.
4) NATCOL (Natural Food Colours Association), 2014. Current and proposed uses and use levels of annatto extracts. Submitted on 15 January 2014. Clarifications on requested extension of use provided in April 2016.
5) NATCOL (Natural Food Colours Association), 2015. Additional data on annatto extracts (E 160b). Submitted on 30 October 2015.
6) NATCOL (Natural Food Colours Association), 2016. Reply to request for clarification, 29 February 2016.
7) Private company, 2014. Data on usage levels of annatto extracts (E 160b) in foods in response to the EFSA call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Submitted on 4 July 2014.

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Abbreviations

ADI	acceptable daily intake
ALT	alanine aminotransferase
ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
AST	aspartate aminotransferase
AUC	area under curve
C_{max}	mean plasma peak concentrations
CAC	Codex Alimentarius Commission

CAS	Chemical Abstracts Service
CONTAM	EFSA Panel on Contaminants in the Food Chain
DAD	diode array detector
DEN	*N*-nitrosodiethylamine
DMSO	dimethyl sulfoxide
EINECS	European Inventory of Existing Commercial chemical Substances
FAO	Food and Agriculture Organization
FCS	Food Categorisation System
FDE	FoodDrinkEurope
FEMA	Flavor and Extract Manufacturers Association
GC	gas chromatography
GD	gestational day
GLP	good laboratory practice
GNPD	Mintel's Global New Products Database
GST	glutathione *S*-transferase
HPLC	high-performance liquid chromatography
ICGA	International Chewing Gum Association
INS	International Numbering System
i.p.	intraperitoneal
IgE	immunoglobulin E
IT	ion trap
IWGT	International Workshops on Genotoxicity Testing
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
MEST	mouse ear swelling test
MPL	maximum permitted level
MS	mass spectrometry
NATCOL	Natural Food Colours Association
NDNS	National Dietary and Nutrition Survey
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PPARα	peroxisome proliferator-activated receptor-α
QS	quantum satis
R_t	retention time
PDA	photodiode array detection
SCF	Scientific Committee on Food
SMART	somatic wing mutation and recombination test
UV	ultraviolet
WHO	World Health Organization

Appendix A – Summary of reported use levels of different forms of annatto extracts (E 160b) added to food provided by industry (mg/kg)

FCS category no.	Substance	FCS Food category	MPL[a]	Restrictions/ exceptions	Provided by	N values	Mean of typical usage levels	Maximum usage level
01.4	Bixin	Flavoured fermented milk products including heat-treated products	10		NATCOL	1	8	10
01.4	Norbixin	Flavoured fermented milk products including heat-treated products	10		NATCOL	1	4	6
01.7.2	Annatto	Ripened cheese	15	Only ripened orange, yellow and broken-white cheese and red and green pesto cheese	FDE	1	0.2	0.2
01.7.2	Bixin	Ripened cheese	15		NATCOL	1	15	15
01.7.2	Norbixin	Ripened cheese	15		NATCOL	6	6.4	15
01.7.2	Norbixin	Ripened cheese	50	Only red Leicester cheese	NATCOL	1	50	50
01.7.2	Norbixin	Ripened cheese	35	Only Mimolette cheese	NATCOL	1	35	35
01.7.3	Norbixin	Edible cheese rind	20		NATCOL	1	0	0
01.7.5	Bixin	Processed cheese	15		NATCOL	2	7.5	15
01.7.5	Norbixin	Processed cheese	15		NATCOL	1	5	8
01.7.6	Norbixin	Cheese products (excluding products falling in category 16)	15	Only ripened orange, yellow and broken-white products	NATCOL	1	5	8
02.1	Bixin	Fats and oils essentially free from water (excluding anhydrous milkfat)	10	Only fats	NATCOL	1	5	7
02.2.2	Annatto	Other fat and oil emulsions including spreads as defined by Council Regulation (EC) No 1234/ 2007 and liquid emulsions	10	Excluding reduced fat butter	FDE	2	4.2	4.9
02.2.2	Bixin	Other fat and oil emulsions including spreads as defined by Council Regulation (EC) No 1234/ 2007 and liquid emulsions	10		NATCOL	2	6.5	10
03	Annatto	Edible ices	20		FDE	30	2.4	16.5
03	Annatto	Edible ices	20		Private company	2	5	15
03	Norbixin	Edible ices	20		NATCOL	5	10.2	20
05.4	Annatto	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	20	Only decorations and coatings	Private company	1	10	15
05.4	Bixin	Decorations, coatings and fillings, except fruit-Based fillings covered by category 4.2.4	20	Only decorations and coatings	NATCOL	2	16	20
05.4	Norbixin	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	20		NATCOL	1	15	20

FCS category no.	Substance	FCS Food category	MPL[a]	Restrictions/ exceptions	Provided by	N values	Mean of typical usage levels	Maximum usage level
06.3	Annatto	Breakfast cereals	25	Only extruded puffed and or fruit-flavoured breakfast cereals	FDE	1	20	25
06.3	Norbixin	Breakfast cereals	25		NATCOL	2	12.5	20
06.6	Norbixin	Batters	20	Only batters for coating	NATCOL	2	17.5	20
07.2	Annatto	Fine bakery wares	10		FDE	2	5.0	10
07.2	Annatto	Fine bakery wares	10		Private company	1	1.2	4
07.2	Norbixin	Fine bakery wares	10		NATCOL	2	7.3	10
08.3.3	Annatto	Casings and coatings and decorations for meat	20		FDE	1	0	20
08.3.3	Annatto	Casings and coatings and decorations for meat	20	Only edible casings	FDE	1	0	0.02
09.2	Annatto	Processed fish and fishery products including molluscs and crustaceans	10	Only smoked fish	NATCOL	1	10	10
14.2.6	Bixin	Spirit drinks as defined in Regulation (EC) No 110/ 2008	10	Only liqueurs	NATCOL	1	10	10
14.2.8	Norbixin	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	10	Only alcoholic drinks with less than 15% of alcohol	NATCOL	1	10	10
15.1	Norbixin	Potato-, cereal-, flour- or starch-based snacks	20	Only extruded or expanded savoury snack products	NATCOL	1	15	20
15.1	Norbixin	Potato-, cereal-, flour- or starch-based snacks	10	Excluding extruded or expanded savoury snack products	NATCOL	1	10	10
15.2	Annatto	Processed nuts	10		NATCOL	1	10	10
15.2	Norbixin	Processed nuts	10	Only savoury-coated nuts	NATCOL	1	5	10
16	Annatto	Desserts excluding products covered in category 1, 3 and 4	10		FDE	10	2.0	9
16	Bixin	Desserts excluding products covered in category 1, 3 and 4	10		NATCOL	1	5	10
16	Norbixin	Desserts excluding products covered in category 1, 3 and 4	10		NATCOL	4	7.5	10

FCS: Food Categorisation System; NATCOL: Natural Food Colours Association; FDE: FoodDrinkEurope.
(a): Maximum permitted level (MPL) for annatto (E 160b).

Appendix B – Summary of analytical results of annatto extracts (middle bound mg/kg or mg/L as appropriate) as provided by the Member States

FCS category no.	FCS food category	Restrictions/ exceptions	MPL[a]	N	LC%	Range				All data[b]				
						LOD		LOQ		Min	Median	Mean	P95[c]	Max
01.4	Flavoured fermented milk products including heat-treated products		10	4	50	0.20	0.20	0.50	0.50	0.25	0.43	0.60	–	1.30
01.7.1	Unripened cheese excluding products falling in category 16		–	14	78.6	0.00	0.20	0.01	0.50	0.00	0.25	0.66	–	3.20
01.7.2	Ripened cheese	Only ripened orange, yellow and broken-white cheese and red and green pesto cheese	15	125	20	0.00	0.05	0.00	0.10	0.00	0.49	2.05	11.2	20.9
01.7.5	Processed cheese		15	13	46.2	0.20	0.20	0.50	0.50	0.01	0.25	1.15	–	3.60
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	Only fats	10	2	100	2	3.30	10	10	1	1	1	–	1
02.2.1	Butter and concentrated butter and butter oil and anhydrous milkfat		–	14	78.6	0.20	0.20	0.50	0.50	0.25	0.25	0.51	–	2
03	Edible ices		20	5	60	0.20	3.30	0.50	10	0.14	0.25	1.16	–	5
04.2	Processed fruit and vegetables		–	1	3.30	3.30	10	5	10	5	5	5	–	5
04.2.4.1	Fruit and vegetable preparations excluding compote		–	4	100	2	2	10	10	1	1	1	–	1
06	Cereals and cereal products		–	1	100	2	2	10	10	1	1	1	–	1
06.4	Pasta		–	15	100	2	2	10	10	1	1	1	–	1
08.2	Meat preparations as defined by Regulation (EC) No 853/2004		–	11	100	2	2	10	10	1	1	1	–	1
09.1	Unprocessed fish and fisheries products		–	1	100	2	2	10	10	1	1	1	–	1
12.2.1	Herbs and spices		–	53	100	0.25	3.30	0.50	10	0.13	0.13	1.33	–	5
12.2.2	Seasonings and condiments		–	17	94.1	0.25	2	0.50	10	0.13	1	3.78	–	49.8
12.3	Vinegars		–	1	100	2	2	10	10	1	1	1	–	1
12.5	Soups and broths		–	4	100	2	2	10	10	1	1	1	–	1
12.6	Sauces		–	21	100	2	2	10	10	1	1	1	–	1

FCS category no.	FCS food category	Restrictions/ exceptions	MPL[a]	N	LC%	Range				All data[b]				
						LOD		LOQ		Min	Median	Mean	P95[c]	Max
12.7	Salads and savoury-based sandwich spreads		–	45	100	2	2	10	10	1	1	1	–	1
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)		–	1	100	2	2	10	10	1	1	1	–	1
15.1	Potato-, cereal-, flour- or starch-based snacks		10	8	100	2	2	10	10	1	1	1	–	1
16	Desserts excluding products covered in category 1, 3 and 4		10	14	0	0.20	0.20	0.50	0.50	0.70	4.74	4.10	–	6.48
18	Processed foods not covered by categories 1–17, excluding foods for infants and young children		–	3	100	0.20	2	0.50	10	0.25	1	0.75	–	1

FCS: Food Categorisation System; LC: left-censored data; %LC: percentage of left-censored data; LOD: limit of detection; LOQ: limit of quantification; Max: maximum; Min: minimum; N: number of analytical results; P95: 95th percentile.
(a): Maximum permitted level (MPL) for annatto (E 160b).
(b): Under the middle bound assumption.
(c): The 95th percentiles based on occurrence data with fewer than 60 analytical results are not reported in the table (EFSA, 2011a).

Appendix C – Number and percentage of food products labelled with E 160b out of the total number of food products present in the Mintel GNPD per food subcategory between 2011 and 2015

Mintel subcategory[a]	Total number of products	Products labelled with E 160b	
		Number	%
Hard cheese and semi-hard cheese	6,792	1,107	16.3
Dairy-based frozen products	7,485	400	5.3
Chilled desserts	5,834	228	3.9
Sweet biscuits/cookies	16,792	212	1.3
Cakes, pastries and sweet goods	12,423	206	1.7
Soft cheese and semisoft cheese	5,531	161	2.9
Shelf-stable desserts	3,152	142	4.5
Savoury biscuits/crackers	4,558	140	3.1
Prepared meals	10,138	129	1.3
Cold cereals	5,866	123	2.1
Sandwiches/wraps	2,501	121	4.8
Pizzas	4,062	112	2.8
Corn-based snacks	2,091	100	4.8
Wheat and other grain-based snacks	1,846	87	4.7
Hors d'œuvres/canapés	3,767	85	2.3
Potato snacks	4,680	85	1.8
Bread and bread products	9,465	82	0.9
Processed cheese	2,069	82	4.0
Pastry dishes	1,802	80	4.4
Baking ingredients and mixes	8,593	79	0.9
Meat products	15,384	79	0.5
Water-based frozen desserts	1,121	62	5.5
Fish products	12,066	61	0.5
Other frozen desserts	1,751	56	3.2
Poultry products	5,819	54	0.9
Margarine and other blends	955	51	5.3
Meal kits	1,859	31	1.7
Popcorn	1,059	31	2.9
Snack mixes	1,376	30	2.2
Snack/cereal/energy bars	4,449	22	0.5
Non-individually wrapped chocolate pieces	5,195	19	0.4
Potato products	2,976	19	0.6
Salads	2,443	19	0.8
Eggs and egg products	1,579	18	1.1
Rice snacks	372	18	4.8
Seasonal chocolate	5,307	18	0.3
Meat substitutes	1,962	12	0.6
Seasonings	9,045	12	0.1
Sandwich fillers/spreads	923	11	1.2
Spoonable yogurt	9,409	10	0.1
Stuffing, polenta and other side dishes	2,479	9	0.4
Table sauces	5,804	9	0.2
Dessert toppings	604	8	1.3
Dips	1,322	8	0.6

Mintel subcategory[a]	Total number of products	Products labelled with E 160b	
		Number	%
Nuts	4,636	8	0.2
Meat pastes and pâtés	2,904	7	0.2
Vitamins and dietary supplements	5,996	7	0.1
Other sugar confectionery	1,175	6	0.5
Pastilles, gums, jellies and chews	3,659	6	0.2
Savoury vegetable pastes/spreads	1,493	6	0.4
Toffees, caramels and nougat	1,829	6	0.3
Chocolate tablets	7,994	5	0.1
Cooking sauces	4,650	5	0.1
Drinking yogurt and liquid cultured milk	3,516	5	0.1
Mayonnaise	904	5	0.6
Other snacks	121	5	4.1
Soft cheese desserts	1,548	5	0.3
Dressings and vinegar	3,201	4	0.1
Flavoured milk	1,372	4	0.3
Fruit snacks	3,156	3	0.1
Pasta	9,610	3	0.0
Soy-based frozen products	73	3	4.1
Vegetables	10,031	3	0.0
Baby biscuits and rusks	300	2	0.7
Beverage mixes	830	2	0.2
Chocolate countlines	2,323	2	0.1
Dry soup	1,634	2	0.1
Fresh cheese and cream cheese	2,698	2	0.1
Instant noodles	1,041	2	0.2
Medicated confectionery	946	2	0.2
Beverage concentrates	2,157	1	0.0
Cream	1,725	1	0.1
Flavoured alcoholic beverages	1,866	1	0.1
Fruit	2,656	1	0.0
Fruit/flavoured still drinks	2,824	1	0.0
Gum	1,466	1	0.1
Individually wrapped chocolate pieces	2,660	1	0.0
Liqueur	1,512	1	0.1
Meat snacks	943	1	0.1
Mixed assortments	281	1	0.4
Other sauces and seasonings	888	1	0.1
Pasta sauces	3,558	1	0.0
Rice	3,190	1	0.0
Rice/nut/grain- and seed-based drinks	974	1	0.1
Soy yogurt	363	1	0.3
Standard and power mints	842	1	0.1
Vegetable snacks	572	1	0.2
Wet soup	3,842	1	0.0
Total samples	324,665	4,556	1.4

Mintel's GNPD: Mintel's Global New Products Database.
(a): According to Mintel food categorisation.

Appendix D – Concentration levels of bixin used in the refined exposure scenarios (mg/kg or mg/L as appropriate)

FCS category no.	FCS Food category/description	Restrictions/ exceptions	MPL[a]	Concentration levels used in the refined exposure assessment		Data source/ comments
				Mean	Max	
01.4	Flavoured fermented milk products including heat-treated products		10	8	10	Use levels
01.7.2	Ripened cheese	Only ripened orange, yellow and broken-white cheese and red and green pesto cheese	15	15.0	15.0	Use levels
01.7.2	Ripened cheese	Only red Leicester cheese	50	–	–	No use levels reported
01.7.2	Ripened cheese	Only Mimolette cheese	35	–	–	No use levels reported
01.7.3	Edible cheese rind		20	–	–	Not taken into account (no FoodEx code)
01.7.5	Processed cheese		15	7.5	15	Use levels
01.7.6	Cheese products (excluding products falling in category 16)	Only ripened orange, yellow and broken-white products	15	–	–	No use levels reported (no FoodEx code)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	Only fats	10	5	7	Use levels
02.2.2	Other fat and oil emulsions including spreads as defined by Regulation (EC) No 1234/2007 and liquid emulsions	Excluding reduced fat butter	10	–	–	Not taken into account (no FoodEx code)
03	Edible ices		20	–	–	No use levels reported
04.2.4.1[b]	Fruit and vegetable preparations excluding compote (fruit preparations for use in desserts/yogurts)		–	50	100	Proposed levels
04.2.5[b]	Jam, jellies and marmalades and similar products		–	8	20	Proposed levels
04.2.6[b]	Processed potato products	Only dried potato granules and flakes	–	10	20	Proposed levels
05.2[b]	Other confectionery including breath refreshening microsweets		–	30	100	Proposed levels
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Only decorations and coatings	20	–	–	Not taken into account (no FoodEx code)
06.3	Breakfast cereals		25	–	–	No use levels reported
06.5[b]	Noodles		–	10[c]	20	Proposed levels
06.6	Batters		20	–	–	No use levels reported (no FoodEx code)
07.2	Fine bakery wares		10	–	–	No use levels reported
08.2[b]	Meat preparations as defined by Regulation (EC) No 853/2004 (breakfast sausages with a minimum cereal content of 6%; burger meat with a minimum vegetable and/or cereal content of 4%)		–	10	20	Proposed levels

FCS category no.	FCS Food category/description	Restrictions/ exceptions	MPL[a]	Concentration levels used in the refined exposure assessment		Data source/ comments
				Mean	Max	
08.3.1[b]	Non-heat-treated processed meat (*Chorizo sausage*; *Salchichon*; *Pasturmas* (edible external coating); *Sobrasada*; *luncheon meat*)		–	10	20	Proposed levels
08.3.2[b]	Heat-treated processed meat (sausages, pâtés and terrines)		–	10	20	Proposed levels
08.3.3	Casings and coatings and decorations for meat		20	–	–	Not taken into account (no FoodEx code)
09.2	Processed fish and fishery products including molluscs and crustaceans	Only smoked fish	10	–	–	No use levels reported
09.2[b]	Processed fish and fishery products including molluscs and crustaceans – only surimi and similar products and salmon substitutes		–	15[c]	30	Proposed levels
12.2.2[b]	Seasonings and condiments (seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney, piccalilli)		–	15[c]	30	Proposed levels
12.5[b]	Soups and broths (soups)		–	10	15	Proposed levels
12.6[b]	Sauces		–	15	30	Proposed levels
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Only liqueurs	10	10	10	Use levels
14.2.6[b]	Spirit drinks as defined in Regulation (EC) No 110/2008	Only liqueurs	–	5	20	Proposed levels
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	Only alcoholic drinks with less than 15% of alcohol	10	–	–	No use levels reported
15.1	Potato-, cereal-, flour- or starch-based snacks	Excluding extruded or expanded savoury snack products	10	–	–	No use levels reported
15.1	Potato-, cereal-, flour- or starch-based snacks	Only extruded or expanded savoury snack products	20	–	–	No use levels reported
15.1[b]	Potato-, cereal-, flour- or starch-based snacks	Only cereal-based snacks	–	5	10	Proposed levels
15.2	Processed nuts	Only savoury-coated nuts	10	–	–	No use levels reported
15.2[b]	Processed nuts		–	5	10	Proposed levels
16	Desserts excluding products covered in category 1, 3 and 4		10	5	10	Use levels

FCS: Food Categorisation System.
(a): Maximum permitted level (MPL) for annatto extracts (E 160b).
(b): Proposed level for the extension of use.
(c): No levels were proposed at the mean; therefore half of the maximum proposed level was used in the exposure assessment.

Appendix E – Concentration levels of norbixin used in the refined exposure scenarios (mg/kg or mg/L as appropriate)

FCS category no	FCS food category	Restrictions/ exceptions	MPL[a]	Concentration levels used in the refined exposure assessment		Data source/ comments
				Mean	Max	
01.4	Flavoured fermented milk products including heat-treated products		10	4	6	Use levels
01.7.1[b]	Unripened cheese excluding products falling in category 16 (other cheese)		–	25[c]	50	Proposed levels
01.7.2	Ripened cheese	Only ripened orange, yellow and broken-white cheese and red and green pesto cheese	15	6.4	15	Use levels
01.7.2	Ripened cheese	Only red Leicester cheese	50	50	50	Use levels
01.7.2	Ripened cheese	Only Mimolette cheese	35	35	35	Use levels
01.7.3	Edible cheese rind		20	–	–	Not taken into account (no FoodEx code)
01.7.5	Processed cheese		15	5	8	Use levels
01.7.6	Cheese products (excluding products falling in category 16)	Only ripened orange, yellow and broken-white products	15	–	–	Not taken into account (no FoodEx code)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	Only fats	10	–	–	No use levels reported
02.2.2	Other fat and oil emulsions including spreads as defined by Regulation (EC) No 1234/2007 and liquid emulsions	Excluding reduced fat butter	10	–	–	Not taken into account (no FoodEx code)
03	Edible ices		20	10.2	20	Use levels
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Only decorations and coatings	20	–	–	Not taken into account (no FoodEx code)
06.3	Breakfast cereals	Only extruded puffed and or fruit-flavoured breakfast cereals	25	12.5	20	Use levels
06.6	Batters	Only batters for coating	20	–	–	Not taken into account (no FoodEx code)
07.2	Fine bakery wares		10	7.3	10	Use levels
08.3.3	Casings and coatings and decorations for meat		20	–	–	Not taken into account (no FoodEx code)
09.2	Processed fish and fishery products including molluscs and crustaceans	Only smoked fish	10	–	–	No use levels reported
09.2[b]	Processed fish and fishery products including molluscs and crustaceans – only surimi and similar products and salmon substitutes		–	15[c]	30	Proposed levels
12.2.2[b]	Seasonings and condiments (seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney, piccalilli)		–	15[c]	30	Proposed levels
12.5[b]	Soups and broths (soups)		–	10	15	Proposed levels
12.6[b]	Sauces		–	15	30	Proposed levels

FCS category no	FCS food category	Restrictions/ exceptions	MPL[a]	Concentration levels used in the refined exposure assessment		Data source/ comments
				Mean	Max	
14.2.6	Spirits drinks as defined in Regulation (EC) No 110/2008	Only liqueurs	10	–	–	No use levels reported
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	Only alcoholic drinks with less than 15% of alcohol	10	10	10	Use levels
15.1	Potato-, cereal-, flour- or starch-based snacks	Excluding extruded or expanded savoury snack products	10	12.5	20	Use levels
15.1	Potato-, cereal-, flour- or starch-based snacks	Only extruded or expanded savoury snack products	20			
15.1[b]	Potato-, cereal-, flour- or starch-based snacks	Only cereal-based snacks	–	5	10	Proposed levels
15.2	Processed nuts	Only savoured-coated nuts	10	5	10	Use levels
15.2[b]	Processed nuts		–	5	10	Proposed levels
16	Desserts excluding products covered in category 1, 3 and 4		10	7.5	10	Use levels

FCS: Food Categorisation System.
(a): Maximum permitted level (MPL) for annatto extracts (E 160b).
(b): Proposed level for the extension of use.
(c): No levels were proposed at the mean; therefore half of the maximum proposed level was used in the exposure assessment.

Appendix F – Summary of total estimated exposure to annatto (E 160b) from its use as a food additive for the regulatory maximum level exposure scenario (MPL scenario) per population group and survey: mean and 95th percentile (mg/kg bw per day)

Population group	Country (survey)	Number of subjects	Regulatory maximum level scenario (annatto)	
			Mean	P95
Infants				
Bulgaria	NUTRICHILD	659	0.02	0.08
Denmark	IAT 2006_07	826	0.04	0.15
Finland	DIPP_2001_2009	500	0.01	0.02
Germany	VELS	159	0.03	0.11
Italy	INRAN_SCAI_2005_06	12	0.03	–
United Kingdom	DNSIYC_2011	1,369	0.03	0.11
Toddlers				
Belgium	Regional_Flanders	36	0.13	–
Bulgaria	NUTRICHILD	428	0.06	0.17
Denmark	IAT 2006_07	917	0.07	0.18
Finland	DIPP_2001_2009	500	0.07	0.22
Germany	VELS	348	0.09	0.18
Italy	INRAN_SCAI_2005_06	36	0.09	–
Netherlands	VCP_kids	322	0.16	0.38
Spain	enKid	17	0.10	–
United Kingdom	DNSIYC_2011	1,314	0.06	0.16
United Kingdom	NDNS-RollingProgrammeYears1-3	185	0.08	0.16
Children				
Austria	ASNS_Children	128	0.07	0.15
Belgium	Regional_Flanders	625	0.12	0.30
Bulgaria	NUTRICHILD	433	0.07	0.16
Czech Republic	SISP04	389	0.08	0.16
Denmark	DANSDA 2005-08	298	0.04	0.10
Finland	DIPP_2001_2009	750	0.08	0.17
France	INCA2	482	0.09	0.16
Germany	EsKiMo	835	0.05	0.11
Germany	VELS	293	0.09	0.17
Greece	Regional_Crete	838	0.07	0.16
Italy	INRAN_SCAI_2005_06	193	0.07	0.16
Latvia	EFSA_TEST	187	0.04	0.11
Netherlands	VCPBasis_AVL2007_2010	447	0.11	0.23
Netherlands	VCP_kids	957	0.14	0.34
Spain	NUT_INK05	399	0.09	0.18
Spain	enKid	156	0.08	0.19
Sweden	NFA	1,473	0.09	0.19
United Kingdom	NDNS-RollingProgrammeYears1-3	651	0.08	0.15
Adolescents				
Austria	ASNS_Children	237	0.03	0.08
Belgium	Diet_National_2004	576	0.03	0.07
Cyprus	Childhealth	303	0.02	0.04
Czech Republic	SISP04	298	0.05	0.11

Population group	Country (survey)	Number of subjects	Regulatory maximum level scenario (annatto)	
			Mean	P95
Denmark	DANSDA 2005-08	377	0.02	0.04
Finland	NWSSP07_08	306	0.03	0.06
France	INCA2	973	0.04	0.09
Germany	EsKiMo	393	0.03	0.08
Germany	National_Nutrition_Survey_II	1,011	0.02	0.08
Italy	INRAN_SCAI_2005_06	247	0.04	0.09
Latvia	EFSA_TEST	453	0.03	0.08
Netherlands	VCPBasis_AVL2007_2010	1,142	0.06	0.14
Spain	AESAN_FIAB	86	0.03	0.08
Spain	NUT_INK05	651	0.04	0.09
Spain	enKid	209	0.04	0.11
Sweden	NFA	1,018	0.05	0.11
United Kingdom	NDNS-RollingProgrammeYears1-3	666	0.04	0.09
Adults				
Austria	ASNS_Adults	308	0.03	0.07
Belgium	Diet_National_2004	1,292	0.02	0.06
Czech Republic	SISP04	1,666	0.03	0.06
Denmark	DANSDA 2005-08	1,739	0.01	0.03
Finland	FINDIET2012	1,295	0.03	0.07
France	INCA2	2,276	0.03	0.06
Germany	National_Nutrition_Survey_II	10,419	0.02	0.06
Hungary	National_Repr_Surv	1,074	0.01	0.03
Ireland	NANS_2012	1,274	0.02	0.04
Italy	INRAN_SCAI_2005_06	2,313	0.02	0.05
Latvia	EFSA_TEST	1,271	0.01	0.04
Netherlands	VCPBasis_AVL2007_2010	2,057	0.03	0.08
Romania	Dieta_Pilot_Adults	1,254	0.01	0.03
Spain	AESAN	410	0.03	0.06
Spain	AESAN_FIAB	981	0.02	0.05
Sweden	Riksmaten 2010	1,430	0.03	0.06
United Kingdom	NDNS-RollingProgrammeYears1-3	1,266	0.02	0.05
Elderly and very elderly				
Austria	ASNS_Adults	92	0.02	0.05
Belgium	Diet_National_2004	1,215	0.02	0.05
Denmark	DANSDA 2005-08	286	0.01	0.03
Finland	FINDIET2012	413	0.02	0.06
France	INCA2	348	0.02	0.05
Germany	National_Nutrition_Survey_II	2,496	0.02	0.05
Hungary	National_Repr_Surv	286	0.01	0.03
Ireland	NANS_2012	226	0.02	0.04
Italy	INRAN_SCAI_2005_06	518	0.02	0.05
Netherlands	VCP-Elderly	739	0.03	0.05
Netherlands	VCPBasis_AVL2007_2010	173	0.03	0.06
Romania	Dieta_Pilot_Adults	128	0.01	0.02
Sweden	Riksmaten 2010	367	0.03	0.06
United Kingdom	NDNS-RollingProgrammeYears1-3	305	0.02	0.05

P95: 95th percentile.

Appendix G – Summary of total estimated exposure to bixin from the use of annatto extracts (E 160b) as food additive (refined exposure assessment scenarios) per population group and survey: mean and 95th percentile (mg/kg bw per day)

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Infants										
Bulgaria	NUTRICHILD	659	0.003	0.01	0.004	0.01	0.004	0.02	0.01	0.03
Denmark	IAT 2006_07	826	0.02	0.11	0.03	0.13	0.03	0.11	0.03	0.14
Finland	DIPP_2001_2009	500	0.002	0.01	0.003	0.01	0.01	0.02	0.01	0.06
Germany	VELS	159	0.01	0.04	0.01	0.06	0.01	0.05	0.02	0.08
Italy	INRAN_SCAI_2005_06	12	0.02	–	0.02	–	0.04	–	0.05	–
United Kingdom	DNSIYC_2011	1,369	0.02	0.07	0.02	0.09	0.03	0.10	0.04	0.14
Toddlers										
Belgium	Regional_Flanders	36	0.07	–	0.09	–	0.22	–	0.33	–
Bulgaria	NUTRICHILD	428	0.01	0.04	0.01	0.05	0.02	0.08	0.04	0.17
Denmark	IAT 2006_07	917	0.04	0.12	0.05	0.15	0.06	0.14	0.08	0.20
Finland	DIPP_2001_2009	500	0.04	0.15	0.05	0.19	0.05	0.17	0.06	0.20
Germany	VELS	348	0.03	0.08	0.04	0.10	0.06	0.13	0.09	0.18
Italy	INRAN_SCAI_2005_06	36	0.05	–	0.06	–	0.06	–	0.08	–
Netherlands	VCP_kids	322	0.08	0.26	0.10	0.32	0.17	0.40	0.26	0.65
Spain	enKid	17	0.06	–	0.07	–	0.07	–	0.09	–
United Kingdom	DNSIYC_2011	1,314	0.02	0.08	0.03	0.10	0.05	0.14	0.08	0.19
United Kingdom	NDNS-RollingProgramme Years1-3	185	0.02	0.06	0.03	0.09	0.06	0.14	0.09	0.23
Children										
Austria	ASNS_Children	128	0.01	0.04	0.02	0.05	0.05	0.13	0.09	0.21
Belgium	Regional_Flanders	625	0.06	0.19	0.08	0.24	0.16	0.36	0.24	0.51
Bulgaria	NUTRICHILD	433	0.01	0.01	0.01	0.02	0.02	0.06	0.04	0.16
Czech Republic	SISP04	389	0.03	0.08	0.04	0.10	0.05	0.12	0.08	0.22
Denmark	DANSDA 2005-08	298	0.02	0.06	0.02	0.07	0.04	0.10	0.08	0.17
Finland	DIPP_2001_2009	750	0.04	0.11	0.05	0.13	0.07	0.15	0.10	0.24
France	INCA2	482	0.03	0.07	0.04	0.08	0.05	0.10	0.07	0.13
Germany	EsKiMo	835	0.01	0.04	0.02	0.05	0.06	0.13	0.12	0.30
Germany	VELS	293	0.03	0.07	0.03	0.08	0.06	0.11	0.09	0.17
Greece	Regional_Crete	838	0.01	0.05	0.02	0.06	0.03	0.10	0.05	0.16
Italy	INRAN_SCAI_2005_06	193	0.02	0.06	0.03	0.07	0.03	0.09	0.05	0.15
Latvia	EFSA_TEST	187	0.003	0.02	0.004	0.03	0.06	0.14	0.09	0.20
Netherlands	VCPBasis_AVL2007_2010	447	0.04	0.14	0.06	0.17	0.09	0.21	0.15	0.32
Netherlands	VCP_kids	957	0.07	0.21	0.09	0.26	0.14	0.34	0.19	0.41
Spain	NUT_INK05	399	0.04	0.11	0.05	0.14	0.08	0.17	0.10	0.22
Spain	enKid	156	0.03	0.11	0.04	0.13	0.05	0.14	0.08	0.22
Sweden	NFA	1,473	0.03	0.10	0.04	0.12	0.09	0.18	0.14	0.31
United Kingdom	NDNS-RollingProgramme Years1-3	651	0.01	0.05	0.02	0.06	0.05	0.11	0.09	0.21

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Adolescents										
Austria	ASNS_Children	237	0.01	0.02	0.01	0.03	0.03	0.07	0.04	0.11
Belgium	Diet_National_2004	576	0.01	0.02	0.01	0.03	0.04	0.10	0.08	0.17
Cyprus	Childhealth	303	0.004	0.02	0.005	0.02	0.01	0.03	0.01	0.04
Czech Republic	SISP04	298	0.02	0.05	0.02	0.06	0.03	0.08	0.05	0.15
Denmark	DANSDA 2005-08	377	0.01	0.03	0.01	0.03	0.02	0.05	0.04	0.11
Finland	NWSSP07_08	306	0.02	0.04	0.02	0.05	0.03	0.07	0.05	0.16
France	INCA2	973	0.01	0.03	0.02	0.04	0.02	0.05	0.03	0.08
Germany	EsKiMo	393	0.01	0.02	0.01	0.03	0.04	0.10	0.08	0.20
Germany	National_Nutrition_Survey_II	1,011	0.01	0.03	0.01	0.03	0.03	0.08	0.05	0.16
Italy	INRAN_SCAI_2005_06	247	0.01	0.03	0.01	0.03	0.02	0.04	0.02	0.07
Latvia	EFSA_TEST	453	0.002	0.01	0.002	0.02	0.04	0.10	0.06	0.14
Netherlands	VCPBasis_AVL2007_2010	1,142	0.02	0.07	0.03	0.09	0.06	0.13	0.09	0.21
Spain	AESAN_FIAB	86	0.01	0.03	0.01	0.04	0.02	0.04	0.03	0.07
Spain	NUT_INK05	651	0.01	0.04	0.02	0.06	0.04	0.08	0.05	0.11
Spain	enKid	209	0.02	0.05	0.02	0.05	0.03	0.07	0.05	0.12
Sweden	NFA	1,018	0.02	0.06	0.02	0.07	0.05	0.12	0.09	0.24
United Kingdom	NDNS-RollingProgramme Years1-3	666	0.01	0.02	0.01	0.03	0.03	0.07	0.05	0.13
Adults										
Austria	ASNS_Adults	308	0.01	0.02	0.01	0.03	0.03	0.08	0.05	0.11
Belgium	Diet_National_2004	1,292	0.01	0.03	0.01	0.03	0.04	0.09	0.06	0.13
Czech Republic	SISP04	1,666	0.01	0.03	0.01	0.04	0.01	0.04	0.02	0.05
Denmark	DANSDA 2005-08	1,739	0.01	0.02	0.01	0.02	0.02	0.03	0.02	0.06
Finland	FINDIET2012	1,295	0.01	0.04	0.01	0.05	0.02	0.06	0.04	0.10
France	INCA2	2,276	0.01	0.03	0.01	0.03	0.02	0.04	0.02	0.05
Germany	National_Nutrition_Survey_II	10,419	0.01	0.02	0.01	0.03	0.03	0.07	0.04	0.11
Hungary	National_Repr_Surv	1,074	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.03
Ireland	NANS_2012	1,274	0.01	0.02	0.01	0.02	0.02	0.04	0.03	0.07
Italy	INRAN_SCAI_2005_06	2,313	0.01	0.02	0.01	0.03	0.01	0.03	0.01	0.04
Latvia	EFSA_TEST	1,271	0.001	0.01	0.002	0.01	0.03	0.07	0.04	0.10
Netherlands	VCPBasis_AVL2007_2010	2,057	0.01	0.04	0.02	0.05	0.04	0.08	0.05	0.12
Romania	Dieta_Pilot_Adults	1,254	0.004	0.01	0.005	0.01	0.01	0.02	0.01	0.03
Spain	AESAN	410	0.01	0.03	0.01	0.04	0.01	0.04	0.02	0.05
Spain	AESAN_FIAB	981	0.01	0.02	0.01	0.03	0.01	0.03	0.02	0.04
Sweden	Riksmaten 2010	1,430	0.01	0.02	0.01	0.03	0.03	0.06	0.04	0.10
United Kingdom	NDNS-RollingProgramme Years1-3	1,266	0.01	0.02	0.01	0.02	0.02	0.05	0.03	0.07
Elderly and very elderly										
Austria	ASNS_Adults	92	0.004	0.01	0.01	0.02	0.02	0.06	0.04	0.08
Belgium	Diet_National_2004	1,215	0.01	0.02	0.01	0.03	0.04	0.08	0.05	0.12
Denmark	DANSDA 2005-08	286	0.01	0.02	0.01	0.02	0.01	0.03	0.02	0.04
Finland	FINDIET2012	413	0.01	0.03	0.01	0.03	0.01	0.04	0.02	0.06
France	INCA2	348	0.01	0.02	0.01	0.03	0.02	0.04	0.02	0.06
Germany	National_Nutrition_Survey_II	2,496	0.01	0.02	0.01	0.02	0.02	0.06	0.03	0.08

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Hungary	National_Repr_Surv	286	0.004	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Ireland	NANS_2012	226	0.01	0.02	0.01	0.03	0.01	0.03	0.02	0.05
Italy	INRAN_SCAI_2005_06	518	0.01	0.02	0.01	0.02	0.01	0.03	0.01	0.03
Netherlands	VCP-Elderly	739	0.01	0.03	0.02	0.04	0.03	0.07	0.04	0.09
Netherlands	VCPBasis_AVL2007_2010	173	0.01	0.04	0.02	0.04	0.03	0.07	0.04	0.08
Romania	Dieta_Pilot_Adults	128	0.003	0.01	0.004	0.01	0.01	0.02	0.01	0.03
Sweden	Riksmaten 2010	367	0.01	0.02	0.01	0.03	0.02	0.06	0.03	0.08
United Kingdom	NDNS-RollingProgramme Years1-3	305	0.01	0.02	0.01	0.03	0.02	0.04	0.03	0.06

P95: 95th percentile.

Appendix H – Summary of total estimated exposure to norbixin from the use of annatto extracts (E 160b) as food additive (refined exposure assessment scenarios) per population group and survey: mean and 95th percentile (mg/kg bw per day)

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Infants										
Bulgaria	NUTRICHILD	659	0.01	0.05	0.02	0.07	0.01	0.05	0.02	0.08
Denmark	IAT 2006_07	826	0.02	0.07	0.02	0.09	0.02	0.07	0.02	0.10
Finland	DIPP_2001_2009	500	0.002	0.01	0.003	0.01	0.003	0.02	0.005	0.03
Germany	VELS	159	0.01	0.07	0.02	0.09	0.03	0.11	0.05	0.17
Italy	INRAN_SCAI_2005_06	12	0.01	–	0.01	–	0.03	–	0.04	–
United Kingdom	DNSIYC_2011	1,369	0.02	0.06	0.02	0.08	0.08	0.25	0.14	0.46
Toddlers										
Belgium	Regional_Flanders	36	0.07	–	0.09	–	0.18	–	0.24	–
Bulgaria	NUTRICHILD	428	0.04	0.10	0.05	0.13	0.05	0.12	0.06	0.16
Denmark	IAT 2006_07	917	0.03	0.08	0.04	0.11	0.04	0.09	0.05	0.12
Finland	DIPP_2001_2009	500	0.03	0.09	0.04	0.13	0.04	0.12	0.05	0.16
Germany	VELS	348	0.05	0.10	0.06	0.13	0.09	0.19	0.13	0.29
Italy	INRAN_SCAI_2005_06	36	0.04	–	0.05	–	0.06	–	0.09	–
Netherlands	VCP_kids	322	0.09	0.18	0.11	0.24	0.13	0.25	0.17	0.34
Spain	enKid	17	0.05	–	0.06	–	0.05	–	0.07	–
United Kingdom	DNSIYC_2011	1,314	0.03	0.09	0.04	0.11	0.11	0.27	0.18	0.46
United Kingdom	NDNS-Rolling ProgrammeYears1-3	185	0.04	0.09	0.05	0.11	0.11	0.25	0.15	0.38
Children										
Austria	ASNS_Children	128	0.04	0.09	0.05	0.12	0.06	0.13	0.08	0.17
Belgium	Regional_Flanders	625	0.07	0.14	0.09	0.19	0.13	0.27	0.17	0.37
Bulgaria	NUTRICHILD	433	0.04	0.10	0.05	0.13	0.05	0.11	0.07	0.16
Czech Republic	SISP04	389	0.04	0.09	0.05	0.11	0.05	0.11	0.06	0.15
Denmark	DANSDA 2005-08	298	0.02	0.05	0.03	0.06	0.03	0.06	0.04	0.08
Finland	DIPP_2001_2009	750	0.03	0.07	0.05	0.10	0.05	0.10	0.07	0.15
France	INCA2	482	0.05	0.09	0.06	0.11	0.07	0.14	0.09	0.20
Germany	EsKiMo	835	0.02	0.06	0.03	0.08	0.04	0.09	0.06	0.13
Germany	VELS	293	0.05	0.10	0.06	0.12	0.09	0.16	0.11	0.23
Greece	Regional_Crete	838	0.04	0.09	0.05	0.12	0.06	0.13	0.08	0.18
Italy	INRAN_SCAI_2005_06	193	0.03	0.08	0.04	0.10	0.06	0.13	0.09	0.19
Latvia	EFSA_TEST	187	0.02	0.07	0.03	0.10	0.09	0.18	0.12	0.25
Netherlands	VCPBasis_AVL2007_2010	447	0.06	0.12	0.07	0.15	0.08	0.15	0.10	0.25
Netherlands	VCP_kids	957	0.08	0.17	0.10	0.21	0.11	0.22	0.14	0.29
Spain	NUT_INK05	399	0.04	0.09	0.05	0.13	0.08	0.20	0.10	0.25
Spain	enKid	156	0.04	0.11	0.05	0.12	0.05	0.13	0.07	0.16
Sweden	NFA	1,473	0.05	0.10	0.06	0.13	0.07	0.14	0.09	0.18
United Kingdom	NDNS-RollingProgramme Years1-3	651	0.04	0.08	0.05	0.10	0.07	0.14	0.09	0.21

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Adolescents										
Austria	ASNS_Children	237	0.02	0.05	0.02	0.06	0.03	0.07	0.04	0.10
Belgium	Diet_National_2004	576	0.02	0.04	0.02	0.05	0.04	0.10	0.06	0.14
Cyprus	Childhealth	303	0.01	0.03	0.02	0.04	0.02	0.05	0.03	0.08
Czech Republic	SISP04	298	0.03	0.07	0.04	0.08	0.03	0.07	0.04	0.09
Denmark	DANSDA 2005-08	377	0.01	0.02	0.02	0.03	0.01	0.03	0.02	0.04
Finland	NWSSP07_08	306	0.01	0.03	0.02	0.04	0.02	0.03	0.02	0.05
France	INCA2	973	0.02	0.05	0.03	0.07	0.03	0.07	0.04	0.09
Germany	EsKiMo	393	0.02	0.04	0.02	0.05	0.03	0.07	0.04	0.10
Germany	National_Nutrition_Survey_II	1,011	0.01	0.05	0.02	0.06	0.03	0.08	0.05	0.11
Italy	INRAN_SCAI_2005_06	247	0.02	0.05	0.03	0.06	0.04	0.07	0.06	0.11
Latvia	EFSA_TEST	453	0.02	0.05	0.02	0.07	0.06	0.13	0.08	0.18
Netherlands	VCPBasis_AVL2007_2010	1,142	0.03	0.07	0.04	0.09	0.05	0.11	0.07	0.14
Spain	AESAN_FIAB	86	0.02	0.04	0.02	0.05	0.02	0.04	0.03	0.05
Spain	NUT_INK05	651	0.02	0.05	0.03	0.06	0.04	0.08	0.05	0.11
Spain	enKid	209	0.02	0.06	0.03	0.08	0.03	0.06	0.04	0.08
Sweden	NFA	1,018	0.03	0.06	0.03	0.07	0.04	0.08	0.05	0.11
United Kingdom	NDNS-RollingProgramme Years1-3	666	0.02	0.05	0.03	0.06	0.03	0.07	0.04	0.10
Adults										
Austria	ASNS_Adults	308	0.02	0.04	0.02	0.05	0.04	0.08	0.05	0.12
Belgium	Diet_National_2004	1,292	0.01	0.03	0.02	0.04	0.04	0.09	0.05	0.13
Czech Republic	SISP04	1,666	0.01	0.03	0.02	0.04	0.02	0.04	0.02	0.06
Denmark	DANSDA 2005-08	1,739	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.03
Finland	FINDIET2012	1,295	0.01	0.04	0.02	0.05	0.03	0.06	0.04	0.10
France	INCA2	2,276	0.02	0.03	0.02	0.04	0.02	0.05	0.03	0.07
Germany	National_Nutrition_Survey_II	10,419	0.01	0.03	0.02	0.04	0.03	0.08	0.04	0.11
Hungary	National_Repr_Surv	1,074	0.003	0.01	0.01	0.02	0.01	0.02	0.01	0.04
Ireland	NANS_2012	1,274	0.01	0.02	0.01	0.03	0.02	0.04	0.03	0.06
Italy	INRAN_SCAI_2005_06	2,313	0.01	0.03	0.01	0.04	0.02	0.05	0.04	0.09
Latvia	EFSA_TEST	1,271	0.01	0.03	0.01	0.04	0.04	0.08	0.05	0.12
Netherlands	VCPBasis_AVL2007_2010	2,057	0.02	0.04	0.02	0.05	0.04	0.08	0.05	0.10
Romania	Dieta_Pilot_Adults	1,254	0.004	0.02	0.01	0.02	0.01	0.03	0.02	0.05
Spain	AESAN	410	0.01	0.03	0.02	0.04	0.02	0.05	0.03	0.07
Spain	AESAN_FIAB	981	0.01	0.03	0.01	0.04	0.01	0.03	0.02	0.05
Sweden	Riksmaten 2010	1,430	0.01	0.03	0.02	0.04	0.03	0.06	0.04	0.08
United Kingdom	NDNS-RollingProgramme Years1-3	1,266	0.01	0.03	0.01	0.04	0.02	0.05	0.03	0.07
Elderly and very elderly										
Austria	ASNS_Adults	92	0.01	0.03	0.02	0.04	0.03	0.06	0.04	0.09
Belgium	Diet_National_2004	1,215	0.01	0.03	0.01	0.04	0.04	0.09	0.06	0.13
Denmark	DANSDA 2005-08	286	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02
Finland	FINDIET2012	413	0.01	0.03	0.02	0.04	0.02	0.04	0.02	0.06
France	INCA2	348	0.01	0.03	0.02	0.03	0.02	0.05	0.03	0.08
Germany	National_Nutrition_Survey_II	2,496	0.01	0.03	0.01	0.04	0.03	0.07	0.04	0.10

Population group	Country (survey)	Number of subjects	Current uses				Extension of use			
			Non-brand-loyal scenario		Brand-loyal scenario		Non-brand-loyal scenario		Brand-loyal scenario	
			Mean	P95	Mean	P95	Mean	P95	Mean	P95
Hungary	National_Repr_Surv	286	0.003	0.01	0.005	0.02	0.01	0.02	0.01	0.04
Ireland	NANS_2012	226	0.01	0.02	0.01	0.03	0.01	0.03	0.02	0.05
Italy	INRAN_SCAI_2005_06	518	0.01	0.02	0.01	0.03	0.02	0.04	0.03	0.07
Netherlands	VCP-Elderly	739	0.01	0.03	0.02	0.04	0.03	0.08	0.05	0.12
Netherlands	VCPBasis_AVL2007_2010	173	0.02	0.03	0.02	0.04	0.03	0.07	0.04	0.09
Romania	Dieta_Pilot_Adults	128	0.002	0.01	0.003	0.01	0.01	0.04	0.03	0.07
Sweden	Riksmaten 2010	367	0.01	0.03	0.02	0.04	0.03	0.06	0.04	0.08
United Kingdom	NDNS-RollingProgramme Years1-3	305	0.01	0.03	0.02	0.04	0.02	0.05	0.03	0.06

P95: 95th percentile.

Appendix I – Main food categories contributing to exposure to annatto (E 160b) using the regulatory maximum level exposure assessment scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	10.6–50.1 (5)	7.7–64.3 (10)	7.9–50.5 (16)	6.2–46.5 (15)	5.5–29.6 (16)	5.4–24.1 (13)
01.7.2	Ripened cheese	5.9–26.8 (4)	8.1–14.7 (6)	5.5–20.4 (12)	5.7–33.9 (15)	8.3–40.5 (16)	6.3–34.6 (14)
01.7.5	Processed cheese	6.2–28.0 (2)	6.2–14.8 (2)	5.5–6.0 (2)	8.1 (1)	6.7–7.7 (2)	6.0–7.2 (4)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	7.8–52.5 (4)	6.2–14.4 (4)	6.0–17.0 (6)	7.7–17.6 (6)	6.0–41.5 (7)	8.6–57.8 (4)
03	Edible ices	5.1–6.0 (2)	6.1–16.0 (7)	6.6–25.0 (17)	7.2–21.2 (16)	6.0–15.4 (13)	5.6–15.4 (10)
06.3	Breakfast cereals	35.6 (1)	8.6–12.4 (3)	5.4–13.0 (3)	7.1–11.8 (3)	5.5–8.8 (4)	–
07.2	Fine bakery wares	5.9–64.4 (4)	7.9–56.5 (9)	8.9–53.8 (17)	7.8–53.3 (16)	11.4–64.3 (17)	14.0–59.0 (14)
09.2	Processed fish and fishery products including molluscs and crustaceans	5.1–6.1 (2)	–	–	–	5.3–6.9 (2)	6.5–11.1 (3)
15.1	Potato-, cereal-, flour- or starch-based snacks	5.0–16.3 (3)	6.6–13.0 (5)	6.5–18.5 (11)	6.5–20.9 (13)	5.7–25.3 (10)	12.5 (1)
16	Desserts excluding products covered in category 1, 3 and 4	9.7–20.3 (3)	5.6–15.2 (7)	5.6–13.1 (10)	5.6–11.8 (7)	5.8–12.3 (6)	5.1–19.4 (7)

FCS: Food Categorisation System.

(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix J – Main food categories contributing to exposure to bixin (E 160b) from currently authorised uses for the brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[(a)]					
01.4	Flavoured fermented milk products including heat-treated products	5.9–66.2 (6)	37.4–88.3 (10)	13.8–83.2 (18)	6.3–76.4 (17)	19.8–56.4 (16)	12.5–56.2 (13)
01.7.2	Ripened cheese	6.4–34.6 (5)	5.4–26.7 (9)	7.9–40.1 (15)	14.7–68.1 (15)	19.3–70.5 (16)	13.3–61.0 (14)
01.7.5	Processed cheese	6.0–30.7 (2)	6.0–18.8 (2)	7.6–17.0 (4)	5.6–26.8 (5)	5.5–22.1 (5)	5.6–25.7 (6)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	12.4–87.7 (4)	5.5–54.1 (3)	5.3–77.9 (10)	6.0–32.7 (10)	6.9–70.8 (8)	7.5–84.9 (5)
16	Desserts excluding products covered in category 1, 3 and 4	7.7–29.3 (3)	11.2–31.0 (6)	6.5–41.1 (13)	9.1–30.9 (12)	5.2–23.9 (10)	6.5–30.5 (10)

FCS: Food Categorisation System.

(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix K – Main food categories contributing to exposure to bixin (E 160b) from currently authorised uses for the non-brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	7.5–64.2 (6)	39.5–87.1 (10)	15.0–81.3 (18)	6.0–73.7 (17)	18.1–54.4 (16)	11.8–54.5 (13)
01.7.2	Ripened cheese	8.6–41.9 (5)	6.6–34.5 (9)	5.3–44.6 (16)	6.0–76.1 (16)	23.9–73.1 (16)	17.6–64.9 (14)
01.7.5	Processed cheese	21.9 (1)	5.5–14.5 (2)	7.1–13.1 (4)	5.4–19.5 (5)	8.4–16.6 (3)	5.1–17.8 (6)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	12.2–84.0 (4)	5.5–52.2 (4)	5.9–76.4 (10)	6.8–30.9 (10)	7.5–65.4 (8)	8.1–81.0 (5)
16	Desserts excluding products covered in category 1, 3 and 4	7.7–20.3 (3)	9.9–22.0 (6)	5.8–31.4 (12)	6.6–24.3 (12)	6.6–16.1 (10)	5.2–20.4 (9)

FCS: Food Categorisation System.

(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix L – Main food categories contributing to exposure to norbixin (E 160b) from currently authorised uses for the brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	7.6–49.4 (5)	5.0–67.8 (10)	6.6–46.6 (15)	5.3–45.0 (14)	6.3–24.0 (15)	5.8–19.4 (12)
01.7.2	Ripened cheese	5.4–50.1 (5)	6.8–17.4 (6)	5.5–22.8 (11)	5.7–39.9 (13)	6.7–51.9 (16)	9.1–44.9 (13)
01.7.5	Processed cheese	32.8 (1)	10.3 (1)	–	–	–	6.0 (1)
03	Edible ices	5.9–8.2 (2)	5.4–20.7 (8)	8.3–36.2 (17)	8.0–28.0 (16)	7.5–18.6 (13)	5.4–18.2 (12)
06.3	Breakfast cereals	84.6 (1)	5.0–15.2 (2)	6.1 (1)	5.9–7.4 (2)	5.4–6.1 (3)	–
07.2	Fine bakery wares	8.5–80.4 (4)	9.9–73.3 (9)	10.5–67.1 (17)	8.9–57.7 (16)	12.8–75.3 (17)	17.4–72.5 (14)
14.2.8	Other alcoholic drinks	–	–	–	–	6.1 (1)	–
15.1	Potato-, cereal-, flour- or starch-based snacks	5.5–21.7 (3)	7.0–13.9 (5)	7.1–23.2 (11)	5.4–27.4 (15)	5.6–49.2 (12)	7.2–32.2 (2)
16	Desserts excluding products covered in category 1, 3 and 4	13.3–27.7 (3)	7.0–24.0 (7)	5.1–20.7 (11)	5.7–15.2 (9)	5.6–18.8 (9)	5.0–26.3 (8)

FCS: Food Categorisation System.
(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix M – Main food categories contributing to exposure to norbixin (E 160b) from currently authorised uses for the non-brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	5.6–47.2 (6)	5.5–66.4 (10)	5.8–49.1 (16)	5.3–47.1 (15)	6.6–23.3 (15)	6.9–19.2 (12)
01.7.2	Ripened cheese	5.5–44.7 (4)	6.2–14.5 (6)	5.3–18.5 (11)	5.6–31.0 (13)	6.1–38.5 (16)	7.6–34.2 (14)
01.7.5	Processed cheese	36.5 (1)	11.4 (1)	–	–	5.3 (1)	6.2–6.9 (2)
03	Edible ices	7.3 (1)	5.8–17.7 (7)	6.4–31.0 (17)	7.0–23.1 (16)	6.0–17.5 (13)	5.4–18.2 (11)
06.3	Breakfast cereals	85.7 (1)	5.5–15.8 (2)	6.4 (1)	6.2–7.0 (2)	5.1–6.0 (3)	–
07.2	Fine bakery wares	10.2–81.2 (4)	12.4–73.8 (9)	13.8–70.4 (17)	12.2–60.7 (16)	18.5–76.4 (17)	23.6–70.8 (14)
14.2.8	Other alcoholic drinks	–	–	–	–	5.5–8.0 (2)	–
15.1	Potato-, cereal-, flour- or starch-based snacks	6.0–19.2 (3)	7.8–14.5 (5)	5.1–22.2 (12)	5.4–25.7 (15)	5.0–47.1 (14)	6.9–29.5 (2)
16	Desserts excluding products covered in category 1, 3 and 4	16.1–29.3 (3)	9.2–25.8 (7)	5.8–22.3 (11)	5.5–16.2 (10)	6.1–20.2 (9)	5.5–27.0 (9)

FCS: Food Categorisation System.
(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix N – Main food categories contributing to exposure to bixin (E 160b) from the proposed extension of use for the brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	6.5–59.7 (5)	11.7–67.6 (10)	5.1–38.6 (15)	5.3–26.0 (13)	5.9–26.4 (14)	8.8–27.2 (11)
01.7.2	Ripened cheese	7.7–14.5 (4)	7.5–17.3 (5)	5.1–17.1 (10)	5.6–23.2 (13)	7.2–32.5 (15)	7.9–37.9 (12)
01.7.5	Processed cheese	5.3–12.8 (2)	13.3 (1)	–	–	6.7 (1)	7.4–8.9 (2)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	6.7–25.8 (4)	7.8–16.0 (2)	12.6–17.3 (2)	8.9–16.3 (2)	7.3–27.3 (6)	21.7–32.6 (3)
04.2.4.1	Fruit and vegetable preparations excluding compote	69.1 (1)	11.7 (1)	16.9 (1)	6.5–17.1 (2)	11.7–18.0 (2)	5.5–24.9 (10)
05.2	Other confectionery including breath refreshening microsweets	5.3–12.9 (2)	8.3–54.9 (8)	7.1–64.5 (18)	9.7–59.9 (16)	5.8–50.0 (15)	5.1–29.7 (9)
06.5	Noodles	–	–	5.0–19.2 (3)	6.8–21.2 (3)	5.2–10.8 (2)	6.7–8.0 (2)
08.2	Meat preparations as defined by Regulation (EC) No 853/2004	–	5.2 (1)	6.3 (1)	40.9 (1)	–	5.2 (1)
09.2	Processed fish and fishery products including molluscs and crustaceans	5.6 (1)	6.7–9.5 (3)	5.6–16.4 (3)	7.5 (1)	–	
12.5	Soups and broths	7.3–56.4 (3)	6.8–19.3 (5)	5.9–65.8 (8)	5.7–61.5 (9)	9.7–68.3 (9)	5.0–59.2 (8)
12.6	Sauces	16.7–21.6 (2)	6.5–20.6 (4)	6.1–19.6 (10)	5.4–30.0 (12)	6.2–41.2 (15)	7.0–34.1 (11)
15.1	Potato-, cereal-, flour- or starch-based snacks	8.8–12.2 (2)	9.0 (1)	6 (1)	5.7–9.1 (2)	8.3 (1)	–
16	Desserts excluding products covered in category 1, 3 and 4	6.9–14.0 (3)	7.5–29.8 (6)	5.6–30.9 (7)	5.0–10.1 (6)	5.2–14.2 (4)	5.7–11.3 (6)

FCS: Food Categorisation System.

(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix O – Main food categories contributing to exposure to bixin (E 160b) from the proposed extension of use for the non-brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	7.1–59.0 (5)	15.4–70.3 (10)	6.0–46.0 (17)	5.5–33.6 (14)	6.7–29.7 (15)	6.0–33.6 (12)
01.7.2	Ripened cheese	11.0–19.6 (4)	5.3–22.3 (8)	5.8–25.7 (14)	7.1–34.8 (15)	7.3–39.8 (16)	6.4–45.0 (14)
01.7.5	Processed cheese	10.2 (1)	11.2 (1)	5.0 (1)	–	6.3 (1)	6.0–6.7 (2)
02.1	Fats and oils essentially free from water (excluding anhydrous milkfat)	7.4–32.5 (4)	9.9–25.7 (2)	5.1–30.6 (4)	6.2–21.2 (5)	9.5–29.2 (6)	5.7–35.4 (4)
04.2.4.1	Fruit and vegetable preparations excluding compote	53.6 (1)	7.4 (1)	5.8–14.6 (2)	8.4–14.9 (2)	5.1–14.3 (4)	6.0–17.9 (9)
05.2	Other confectionery including breath refreshening microsweets	5.8 (1)	6.1–32.9 (7)	8.6–42.2 (17)	5.1–33.2 (16)	5.6–25.5 (12)	5.0–14.2 (5)
06.5	Noodles	–	–	10.1–24.1 (2)	7.9–25.8 (3)	6.7–11.1 (2)	8.0–9.0 (2)
08.2	Meat preparations as defined by Regulation (EC) No 853/2004	–	5.6 (1)	6.8 (1)	5.4–32.3 (4)	–	–
09.2	Processed fish and fishery products including molluscs and crustaceans	–	7.7–10 (2)	5.2–13.8 (13)	6.4 (1)	–	–
12.5	Soups and broths	7.8–50.9 (3)	5.6–23.1 (7)	5.6–67.0 (11)	7.7–62.6 (9)	8.9–69.2 (9)	26.4–55.6 (8)
12.6	Sauces	6.1–17.4 (3)	5.0–18.1 (6)	5.2–21.4 (13)	5.2–28.4 (14)	5.8–37.8 (15)	8.1–28.0 (11)
15.1	Potato-, cereal-, flour- or starch-based snacks	7.7–10.5 (2)	11.6 (1)	8.6 (1)	6.0–9.7 (2)	7.3 (1)	–
16	Desserts excluding products covered in category 1, 3 and 4	7.1–11.6 (3)	8.0–26.5 (6)	5.4–24.6 (8)	6.0–10.2 (4)	5.9–11.8 (4)	5.5–9.3 (6)

FCS: Food Categorisation System.

(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix P – Main food categories contributing to exposure to norbixin (E 160b) from the proposed extension of use for the brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	5.8–45.6 (3)	7.5–46.2 (7)	5.0–31.4 (12)	5.3–30.4 (9)	5.3–11.9 (10)	5.6–11.9 (5)
01.7.1	Unripened cheese excluding products falling in category 16	5.1–77.9 (5)	23.0–62.3 (8)	6.3–47.2 (16)	6.2–55.1 (14)	7.8–64.3 (16)	5.4–76.9 (13)
01.7.2	Ripened cheese	11.1–13.5 (2)	9.3–11.7 (2)	5.2–16.2 (5)	5.1–30.8 (7)	5.0–35.0 (11)	5.2–31.5 (9)
01.7.5	Processed cheese	9.5 (1)	5.8 (1)	–	–	–	–
03	Edible ices	7.5 (1)	5.6–17.1 (4)	5.8–24.5 (15)	5.8–18.5 (11)	5.8–11.4 (2)	5.1–13.4 (3)
06.3	Breakfast cereals	45.4 (1)	10.2 (1)	–	5.7 (1)	–	–
07.2	Fine bakery wares	7.8–68.9 (3)	7.2–53.9 (9)	8.1–48.2 (17)	7.1–40.7 (16)	5.0–38.7 (17)	8.9–33.3 (13)
09.2	Processed fish and fishery products including molluscs and crustaceans	–	5.2 (1)	5.1–8.7 (4)	5.8 (1)	–	–
12.5	Soups and broths	71.5 (1)	5.6–27.8 (6)	5.5–50.6 (10)	6.2–45.7 (10)	7.3–50.9 (9)	12.6–51.8 (8)
12.6	Sauces	6.7–15.0 (2)	5.9–12.7 (6)	5.8–22.9 (14)	6.4–33.5 (13)	5.6–46.0 (14)	9.9–30.2 (11)
15.1	Potato-, cereal-, flour- or starch-based snacks	8.0–8.1 (2)	6.0–10.4 (3)	5.5–12.9 (7)	5.2–14.4 (9)	5.9–11.9 (3)	–
16	Desserts excluding products covered in categories 1, 3 and 4	6.0–12.3 (2)	5.3–18.2 (6)	6.8–14.1 (7)	5.3–10.4 (5)	6.7–12.7 (4)	5.8–9.1 (5)

FCS: Food Categorisation System.
(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.

Appendix Q – Main food categories contributing to exposure to norbixin (E 160b) from the proposed extension of use for the non-brand-loyal scenario (> 5% to the total mean exposure) and number of surveys in which each food category is contributing

FCS category no.	FCS food category	Infants	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (number of surveys)[a]					
01.4	Flavoured fermented milk products including heat-treated products	6.5–43.4 (3)	9.2–46.8 (7)	5.5–33.7 (13)	6.2–32.1 (9)	5.3–13.3 (11)	5.4–13.5 (6)
01.7.1	Unripened cheese excluding products falling in category 16	10.2–68.1 (4)	18.0–49.7 (8)	5.1–37.4 (16)	5.6–43.5 (12)	7.9–52.9 (16)	5.2–66.7 (12)
01.7.2	Ripened cheese	11.1–11.5 (2)	9.1–10.8 (2)	5.6–14.1 (5)	5.0–23.4 (9)	5.4–26.1 (14)	5.4–24.0 (10)
01.7.5	Processed cheese	9.4 (1)	7.2 (1)	–	–	–	–
03	Edible ices	6.7 (1)	5.4–14.9 (3)	5.5–21.3 (15)	5.5–17.5 (11)	6.6–11.8 (2)	5.2–12.8 (3)
06.3	Breakfast cereals	49.6 (1)	11.1 (1)	–	5.5 (1)	–	–
07.2	Fine bakery wares	9.3–71.8 (3)	10.5–58.1 (9)	10.5–53.6 (17)	9.2–42.1 (16)	7.3–41.3 (17)	7.2–36.2 (14)
09.2	Processed fish and fishery products including molluscs and crustaceans	–	6.0 (1)	7.9 (1)	–	–	–
12.5	Soups and broths	5.0–70.0 (2)	7.2–28.1 (6)	5.7–48.4 (10)	6.6–44.1 (10)	7.2–50.7 (9)	17.8–49.9 (8)
12.6	Sauces	5.8–13.6 (3)	5.6–11.8 (7)	6.0–19.3 (14)	6.0–27.1 (13)	5.7–35.8 (14)	9.9–23.7 (11)
15.1	Potato-, cereal-, flour- or starch-based snacks	8.5–9.0 (2)	6.8–11.4 (3)	5.3–13.3 (9)	5.1–14.4 (10)	5.3–13.1 (6)	–
16	Desserts excluding products covered in category 1, 3 and 4	5.7–15.7 (3)	6.6–19.9 (7)	5.9–16.0 (9)	6.2–11.6 (5)	8.4–14.7 (4)	6.9–11.1 (5)

FCS: Food Categorisation System.
(a): The total number of surveys may be greater than the total number of countries as listed in Table 8, as some countries submitted more than one survey for a specific population.