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SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of Indigo Carmine (E 132) as a food additive[1]

EFSA Panel on Food additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The ANS Panel provides a scientific opinion re-evaluating the safety of Indigo Carmine (E132). The Panel observed that Indigo Carmine was poorly absorbed and does not raise concern for genotoxicity. No adverse effects in subacute, chronic, reproduction and developmental toxicity studies, and no modifications of haematological and biological parameters in chronic toxicity studies have been identified at doses less than or equal to 500 mg/kg bw/day. The only report of an adverse effect was in testis with a LOAEL of 17 mg/kg bw/day which would give rise to a safety concern if confirmed. The Panel considered that this study has shortcomings since it is not clear to the Panel whether the adverse effects observed were due to the food additive itself or to impurities and/or contaminants present in the material tested and/or to the conduct of the study. The Panel considered that the current ADI of 5 mg/kg bw/day for Indigo Carmine was applicable to a material with the same purity and manufacturing process as material used in studies without adverse effects on testis (93% pure colouring and 7% volatile matter) and concluded that any extension of this ADI to Indigo Carmine of lower purity and/or manufactured using a different process would require new data which would need to address the adverse effects on testis. The Panel noted that at the MPL, exposure estimates of Indigo Carmine would exceed the ADI for toddlers and children at the high level. Exposure estimates using the available usage and analytical data did not show an exceedance of the ADI for any population groups.

KEY WORDS

Indigo Carmine, Indigotine, E 132, disodium 3,3'dioxo-2,2'-bi-indolylidene-5,5'-disulphonate, food additive, EINECS 212-728-8, FD&C Blue No. 2

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SUMMARY

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to deliver a scientific opinion re-evaluating the safety of Indigo Carmine (E 132) when used as a food additive. In this re-evaluation, only studies with Indigo Carmine and Indigotine were taken into consideration, provided that the identity of the test material was clear. However, only the term Indigo Carmine is used in this opinion.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a temporary Acceptable Daily Intake (ADI) of 0-2.5 mg/kg bw/day in 1969 that was increased to a full ADI of 0-5 mg/kg bw/day in 1975. These ADIs were probably based on the long-term rat study by Hansen et al. (1966) in which a significantly inhibited growth of male rats was observed at dietary doses of 1000 and 2500 mg/kg bw/day. In this study, the no observed adverse effect level (NOAEL) was determined to be equivalent to 500 mg/kg bw/day. JEFCA established a temporary ADI in 1969 probably by applying an uncertainty factor of 200 to the above NOAEL. In 1975, JECFA applied an uncertainty factor of 100 to the NOAEL of 500 mg/kg bw/day to derive an ADI of 0-5 mg/kg bw/day for Indigo Carmine. In 1975, the EU Scientific Committee for Food (SCF) endorsed the ADI of 5 mg/kg bw/day as established by JECFA. In the latest evaluation of 1984, taking into consideration more recent studies, the SCF agreed to retain this ADI. JECFA and the SCF in their evaluations used the term of Indigotine to design Indigo Carmine.

In vivo studies demonstrated that radioactivity from ^{35}S-Indigo Carmine was poorly detected in urine (1.6 to 2.1 % of the radioactivity) after oral administration in rats. Moreover, incubation of ^{35}S-Indigo Carmine with intestinal contents of rats for 48 hours suggested that isatin-5-sulphonic acid and 5-sulphoanthranilic acid might be metabolites formed by intestinal bacteria. By comparison to the data obtained after intravenous administration, the Panel considered that the data available on the absorption, metabolism and excretion indicated that Indigo Carmine or its metabolites were poorly absorbed. However, the Panel noted that both identification of faecal metabolites and tissue distribution of radioactivity were not investigated in these studies.

Overall the Panel considered that based on the available data including a newly performed *in vivo* study on micronuclei induction, Indigo Carmine does not raise concern for genotoxicity.

In a subacute toxicity study (45-day) performed with adult male Swiss albino mice of B-6 strain (5 animals/group) at oral doses of 0, 17 and 39 mg Indigo Carmine/kg bw/day, statistically significant severe adverse effects on the testis were characterised by Dixit and Goyal, 2013. The Panel noted that no NOAEL could be identified in this study and the lowest observed adverse effect level (LOAEL) was 17 mg/kg bw/day, the lowest dose level tested. The Panel noted limitations in the design of the study and lack of information on the specifications of the test material used in the study.

Several chronic toxicity studies were available when JECFA allocated the ADI (1975). Two chronic and carcinogenicity studies, one in rats and one in mice, were published since the latest evaluation of Indigo Carmine by JECFA in 1975.

In a long-term toxicity study in mice, microscopical evaluation of selected tissues revealed a variety of randomly distributed neoplastic, degenerative, hyperplastic and inflammatory changes usually encountered in ageing mice (Borzelleca and Hogan, 1985). These changes occurred without relationship to dose and were considered to be unrelated to the dietary administration of Indigo Carmine. It was, therefore, concluded that this lifetime exposure of mice to Indigo Carmine did not demonstrate carcinogenic or toxic effects. The Panel agreed with the authors that the NOAEL identified in this study was 5 % in the diet providing an average intake of 8259 mg Indigo Carmine/kg bw/day for males and 9456 mg Indigo Carmine/kg bw/day for females.

In a chronic/carcinogenicity study, a statistically significant increased incidence of gliomas and malignant mammary glands tumours was seen in the male rats at the highest dose level of 1282 mg Indigo Carmine/kg bw/day. Overall, the Panel identified a NOAEL in this study of 632 mg/kg bw/day for male rats, the mid-dose, based on these carcinogenic effects. In the absence of any genotoxic activity, the Panel considered that Indigo carmine was acting as a non-genotoxic carcinogen and therefore, that there would be a threshold for this effect.

In a multigeneration reproductive toxicity study no test substance related effects were observed up to doses of 250 mg/kg bw/day. No adverse effects have been detected in developmental studies in rats and rabbits at tested doses (0, 25, 75 or 250 mg/kg bw/day). The Panel noted that the concentrations tested in the developmental toxicity studies were lower than the dose levels up to 2000 mg/kg bw/day used in several subacute or long-term toxicity studies but close to the NOAEL of 500 mg/kg bw/day on which JECFA and SCF based the ADI.

No cases of Indigo Carmine intolerance or allergy have been reported after ingestion.

Overall, the Panel noted that no adverse effects in subacute, chronic, reproduction and developmental toxicity studies, and no modifications of haematological and biological parameters in chronic toxicity studies have been identified at doses less than or equal to 500 mg/kg bw/day. The only report of an adverse effect was in testis with a LOAEL of 17 mg/kg bw/day which would give rise to a safety concern if confirmed. The Panel considered that this study has shortcomings since it is not clear to the Panel whether the adverse effects observed were due to the food additive itself or to impurities and/or contaminants present in the material tested and/or to the conduct of the study. However no effects on the testes or reproductive function were observed in chronic toxicity studies and in a 3-generation reproduction toxicity study undertaken with Indigo Carmine (FD&C Blue No 2) containing approximately 93% pure colouring and 7% volatile matter (Borzelleca et al., 1985, 1986; Borzelleca and Hogan 1985). The Panel noted that the material as described in the Dixit and Goyal study (2013) is presumed to meet the current EU specifications and it cannot be excluded that such material could be legally used as a food additive in the EU market.

The Panel confirmed the ADI of 5 mg/kg bw/day for Indigo Carmine. The Panel considered that the current ADI was applicable to a material with a purity of 93% pure colouring and manufactured using processes resulting in comparable residuals as material used in the Borzelleca et al. studies (1985, 1986) and Borzelleca and Hogan (1985). Given the uncertainties in the database, the Panel was not able to conclude whether this ADI should apply to Indigo Carmine with lower purity manufactured using these same processes or material manufactured using a different but not equivalent process.

The Panel considered that any extension of this ADI to Indigo Carmine of lower purity and/or manufactured using a different process would require new data which would need to address the adverse effects on testis observed in the Dixit and Goyal (2013) study. The chemical identity of the tested food additive, including the presence of possible impurities and/or contaminants, should be investigated and reported. The EFSA Guidance for submission for food additives evaluation (EFSA ANS Panel, 2012) requires information on the manufacturing process to identify hazards which may need to be controlled in the specifications.

Furthermore, the Panel considered that the current specifications should be revised in order to restrict the Indigo Carmine (E 132) permitted as food additive to that for which the ADI is applicable.

Exposure assessment for food additives under re-evaluation was carried out by the ANS Panel based on (1) MPLs set down in the EU legislation (defined as the *regulatory maximum level exposure assessment* scenario) and (2) the availability of adequate usage or analytical data (defined as the *refined exposure assessment* scenario).

To date, the ANS Panel has used the maximum concentration value (maximum reported use level or maximum value from the analytical results) available for each authorised food category. However,

given the extensive range of analytical data that have been made available through the most recent calls, the ANS Panel considered that this should also be used in additional scenarios of the exposure assessment approach intended to provide more realistic exposure estimates.

Overall, the Panel considered the regulatory maximum level exposure assessment scenario as being conservative since it assumes that all processed foods and beverages contain the food additive Indigo Carmine at the MPLs. The Panel considered that exposure assessment approach that uses reported use levels and analytical data was a more realistic scenario, since it was based on the extensive range of analytical data and assumes that the processed foods and beverages contain the additive at the mean concentration level for all products (non-brand-loyal consumer scenario) and considers one product containing Indigo Carmine at the maximum concentration level (brand-loyal consumer scenario).

Using the regulatory maximum level exposure assessment scenario, exposure estimates of Indigo Carmine (E 132) exceed the ADI for toddlers and children at the high level (95th percentile). The main contributing food categories to the total mean exposure estimates for adolescents, adults and the elderly in this scenario were flavoured drinks and fine bakery wares. For children, the main contributing food categories were flavoured drinks, fine bakery wares and flavoured fermented milk products, while for toddlers, the main contributing food categories were flavoured fermented milk products and fine bakery wares.

For the refined exposure assessment scenarios (brand-loyal and non-brand-loyal), none of the population groups exceeded the ADI of 5 mg/kg bw/day, neither at the mean nor at the high level (95th percentile). However, the Panel noted that this scenario was calculated using limited data provided by industry. The Panel further noted that for the food categories where both usage levels and analytical results were available, the analytical results were much lower than the use levels reported by industry.

The Panel noted that both reported use levels and quantified analytical results were reported only for the food categories of chewing gum, flavoured drinks and desserts. For several food products for which the use of Indigo Carmine is authorised e.g. flavoured fermented milk products, seasonings and condiments, mustard, soups and broths, sauces, cider and perry, aromatised wine based-products and other alcoholic drinks, no usage data nor quantified positive analytical results were reported. In addition, the Panel further noted that for some other authorised food categories such as edible ices, fine bakery wares, fish roe, spirit drinks, potato-, cereal, flour-, or starch-based snacks and processed nuts, no usage data had been reported while positive analytical results were obtained from analytical data.

The Panel concluded that at the maximum permitted level of use, exposure estimates of Indigo Carmine (E 132) would exceed the ADI for toddlers and children at the high level. Exposure estimates using the available usage and analytical data did not show an exceedance of the ADI for any population groups.

The Panel noted that the three main contributing food categories for age-groups where MPL scenario estimates exceeded the ADI showed high number of analytical data far below MPL or had no use level reported combined with no detection in limited analytical data. The Panel therefore considered that it is not likely that the ADI will be exceeded.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 1333/2008[4] 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. 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 European Union before 20 January 2009 has been set up under Regulation (EU) No 257/2010[5]. This Regulation also foresees that food additives are re-evaluated whenever necessary in 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[6] of 2001. The report "Food additives in Europe 2000[7]" 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 a 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.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority 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 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 the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

[4] OJ L 354, 31.12.2008, p.16.
[5] OJ L 80, 26.03.2010, p. 19
[6] COM(2001) 542 final.
[7] Food Additives in Europe 2000, Status of safety assessments of food additives presently permitted in the EU, Nordic Council of Ministers, TemaNord 2002:560.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of Indigo Carmine/Indigotine (E 132) when used as a food additive. The Panel noted that the term Indigotin is sometimes used to indicate Indigo while Indigotine is a synonym of Indigo Carmine (Regulation (EC) No 1333/2008[8]). In this re-evaluation, only studies with Indigo Carmine and Indigotine were taken into consideration, provided that the identity of the test material was clear. However, only the term Indigo Carmine is used in this opinion.

Indigo Carmine (E 132) is an indigoid colour authorised as a food additive in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 and has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969 and 1975, and the EU Scientific Committee for Food (SCF) in 1975 and 1984.

The Panel on Food Additives and Nutrient Sources added to Food (ANS) was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following an EFSA public call for data.[9,10,11] The Panel noted that not all original studies on which previous evaluations were based were available to the Panel.

2. Technical data

2.1. Identity of the substance

Indigo Carmine is an indigoid colouring substance. Its systematic name (IUPAC) is disodium (2*E*)-3-oxo-2-(3-oxo-5-sulphonato-2,3-dihydro-1*H*-indol-2-ylidene)-2,3-dihydro-1*H*-indole-5-sulphonate, the CAS Registry Number is 860-22-0, the EINECS number is 212-728-8, the Colour Index number is 73015. The molecular formula is $C_{16}H_8Na_2N_2O_8S_2$ and the molecular weight is 466.36 g/mol. The structural formula is given in Figure 1.

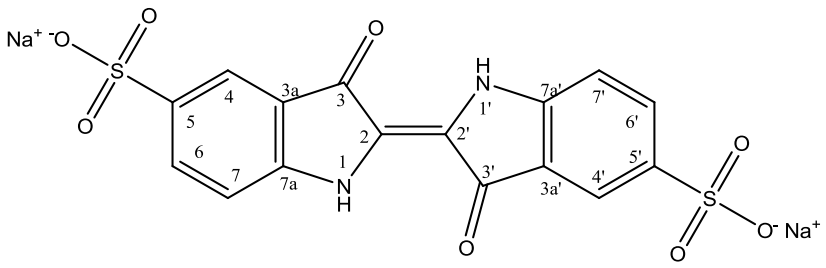


Figure 1: Structural formula of Indigo Carmine (disodium 3,3′-dioxo-2,2′-bi-indo-lylidene-5,5′-disulphonate)

Indigo Carmine takes the form of a dark-blue powder or granules, is soluble in water (1.6 % at 20 ^{0}C in water (Sensiet Colours Group, 2011)) and sparingly soluble in ethanol (JECFA, 2010).

8 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.

9 Call for scientific data on food colours to support re-evaluation of all food colours authorised under the EU legislation. Published: 8 December 2006. Available online: http://www.efsa.europa.eu/en/dataclosed/call/afc061208.htm

10 Call for scientific data on Indigo Carmine (E 132). Published: 26 June 2011. Available online: http://www.efsa.europa.eu/en/data/call/ans110126.htm .

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

At least more than 100 synonyms are known (ChemIdplusAdvances, online); the most commonly used names are: CI natural blue II, FD&C Blue No. 2, Indigotine and Acid blue 74.

2.2. Specifications

Specifications for Indigo Carmine (E 132) have been defined in Commission Regulation (EU) No 231/2012[12] and by JECFA (JECFA, 2010) (Table 1).

According to Commission Regulation (EU) No 231/2012, Indigo Carmine consists essentially of a mixture of disodium 3,3′-dioxo-2,2′-bi-indo-lylidene-5,5′-disulphonate and disodium 3,3′-dioxo-2,2′-bi-indolylidene-5,7′-disulphonate and subsidiary colouring matters together with sodium chloride and/or sodium sulphate as the principal uncoloured components. Indigo Carmine is described as the sodium salt.

The purity of Indigo Carmine is specified as to be not less than 85 % total colouring matters, calculated as the sodium salt, with disodium 3,3'-dioxo-2,2'-bi-indolylidene-5,7'-disulphonate not more than 18 %.

The remainder is ≤ 0.2 % water insoluble matter, ≤ 1.0 % subsidiary colouring matters (excluding disodium 3,3′-dioxo-2,2′-bi-indolylidene-5,7′-disulphonate), a total of ≤ 0.5 % organic compounds other than colouring matters (e.g. of isatin-5-sulphonic acid or 5-sulphoanthranilic acid or anthranilic acid), ≤ 0.01 % unsulphonated primary aromatic amines (calculated as aniline) and ≤ 0.2 % of ether extractable matter under neutral conditions (Commission Regulation (EU) No 231/2012).

The Panel noted that if the existing specifications were extended to include < 15 % of sodium chloride and/or sodium sulphate as the principal uncoloured components, most of the material would be accounted for.

Table 1: Specifications for Indigo Carmine according to Commission Regulation (EU) No 231/2012 and JECFA (2010)

Purity	**Commission Regulation (EU) No 231/2012**	**JECFA (2010)**
Assay	≥ 85 % (calculated as sodium salt) ≤ 18 % disodium 3,3'-dioxo-2,2'-bi-indolylidene-5,7'-disulphonate	≥ 85 % total colouring matters. ≤ 18 % of disodium 3,3' dioxo-[delta-2,2'-biindoline]-5,7'-disulphonate
Water insoluble matter	≤ 0.2 %	≤ 0.2 %
Subsidiary colouring matters	≤ 1.0 % (excluding disodium 3,3'-dioxo-2,2'-bi-indolylidene-5,7'-disulphonate)	≤ 1.0 % (except disodium 3,3'-dioxo-[delta-2,2'-biindoline]- 5,7'-disulphonate)
Organic compounds other than colouring matters - Isatin-5-sulphonic acid - 5-sulphoanthranilic acid - Anthranilic acid	≤ 0.5 %	≤ 0.5 %
Unsulphonated primary aromatic amines	≤ 0.01 % (calculated as aniline)	≤ 0.01 % (calculated as aniline)
Ether extractable matter	≤ 0.2 % (under neutral conditions)	≤ 0.2 %
Arsenic	≤ 3 mg/kg	-
Lead	≤ 2 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-

[12] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012.

The Panel noted that the specifications for the purity of Indigo Carmine would permit concentrations of unsulphonated aromatic amines to be present in concentrations of up to 100 mg/kg Indigo Carmine (calculated as aniline). Given the maximum allowed concentration of Indigo Carmine that can be added to food (500 mg/kg food), the concentration of these unidentified unsulphonated primary aromatic amines in food could be up to 50 µg/kg food.

According to Commission Regulation (EU) No 231/2012, the above purity criteria also apply to the raw material from which the aluminium lake is produced. In addition, under neutral conditions, the aluminium lake should contain ≤ 0.5 % HCl-insoluble material and ≤ 0.2 % ether-extractable material. There are no additional specification requirements for the aluminium lake.

The Panel noted that the aluminium lake of the food additive could add to the daily intake of aluminium for which a Tolerable Weekly Intake (TWI) of 1 mg aluminium/kg bw/week has been established (EFSA, 2008) and that therefore specifications for the maximum level of aluminium in the lakes may be required.

2.3. Manufacturing process

Indigo Carmine is prepared by the sulphonation of Indigo (2,2'-bindoline-3,3'-dione; CAS No: 482-89-3) that is one of the earliest known naturally occurring dyes. Originally, it was obtained from the leaves of plants *Indigofera tinctoria*, *Indigofera suifruticosa* or *Isatis tinctoria* where it occurs as indican, a glycoside of indoxyl. An enzyme known as indimulsin is added to hydrolyze the indican into indoxyl and glucose. Oxidation by exposure to air converts indoxyl to indigo (Steingruber, 2004). At the beginning of the 20th century the production by chemical synthesis superseded the extraction from plants. Reactants such as *N*-phenylglycine or *N*-(2-carboxyphenyl)glycine are heated with alkali under controlled conditions to produce indoxyl which is subsequently oxidized in air to form Indigo (Steingruber, 2004).

According to US Code of Federal Regulations (CFR, 1983) and information provided by industry (Sensiet Colours Group, 2011), the indigo (or indigo paste) is manufactured by the fusion of *N*-phenylglycine (prepared from aniline and formaldehyde) in a molten mixture of sodamide and sodium and potassium hydroxides under ammonia pressure. The indigo is isolated and subjected to purification procedures. Indigo Carmine is obtained by sulphonation of indigo. This is accomplished by heating indigo (or indigo paste) in the presence of sulphuric acid. The dye is isolated and subjected to purification procedures.

Indigo Carmine may be converted to the corresponding aluminium lake under aqueous conditions by reacting aluminium oxide with the colouring matter. Aluminium oxide is usually freshly prepared by reacting aluminium sulphate or aluminium chloride with sodium carbonate or sodium bicarbonate or aqueous ammonia. Following lake formation, the product is filtered, washed with water and dried (JECFA, 2004).

2.4. Methods of analysis in food

Various analytical methods for the determination of synthetic food colours have been reported in the literature. Thin layer chromatography (TLC) permits a qualitative analysis (IRMM, 2013). International Standard ISO 13469[13] is a TLC method for the detection of 12 synthetic colours including Indigo Carmine in meat and meat products. Methods for the determination of Indigo Carmine together with other synthetic colours in alcoholic beverages (Prado et al., 2006) or milk beverage (Huang et al., 2002) have been developed using capillary electrophoresis. Other methods proposed for the analysis of Indigo Carmine and other synthetic colours in commercial products are based on high-performance liquid chromatography (HPLC) combined with multivariate analysis (Chen et al., 1998; Berzas et al., 1999, Nevado et al., 1999; Kiseleva et al., 2003; Minioti et al., 2007; Yoshioka and Ichihashi, 2008; Mahnaz et al., 2012; Suh and Choi, 2012) and in the recent years more

[13] Available online: https://www.iso.org/obp/ui/#iso:std:iso:13496:ed-1:v1:en

sophisticated analytical techniques combining liquid chromatography with mass spectroscopy have been reported (Chao et al., 2011; Feng et al., 2011). Recently, a method based on liquid chromatography (LC) with photodiode array (PDA) detection have been used for the determination of seven food colours including Indigo Carmine in a small survey of 44 food products (Harp et al., 2013).

2.5. Reaction and fate in food

An aqueous 0.1 % solution of radiochemically pure ^{35}S-labelled Indigo Carmine was allowed to stand at room temperature for periods ranging from one day to six months. After one week 5 % of the radioactivity was identified as isatin-5-sulphonic acid. After six months 50 % of the radioactivity was present as the parent compound, 12 % was isatin-5-sulphonic acid and 38 % was 5-sulphoanthranilic acid (Lethco and Webb, 1966).

In general, the majority of colour additives are unstable in combination with oxidising and reducing agents in food. Since colour depends on the existence of a conjugated unsaturated system within the dye molecule, any substance which modifies this system (e.g. oxidising or reducing agents, sugars, acids, and salts) will affect the colour (Scotter and Castle, 2004).

According to industry (Sensiet Colours Group, 2011), the conjugated unsaturated system which forms part of the structure of Indigo Carmine is responsible for the dye colour; any substance which interacts or alters this unit of the molecule will affect the colour. As a result, Indigo Carmine will generally be unstable in the presence of oxidising or reducing agents, sugars, acids, salts etc. The recommended shelf-life of Indigo Carmine by industry is 4 to 6 years, depending on the manufacturer, stored in its original container in a dry, cool and well ventilated place (Sensiet Colours Group, 2011).

2.6. Case of need and proposed uses

Maximum permitted levels (MPLs) of Indigo Carmine (E 132) have been defined in Annex II to Regulation (EC) No 1333/2008 on food additives.

Currently, Indigo Carmine (E 132) is an authorised food colour in the EU with MPLs ranging from 50 to 500 mg/kg in foods. Indigo Carmine is included in Group III of food colours with a combined maximum limit.

Table 2 summarises foods that are permitted to contain Indigo Carmine (E 132) and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

Table 2: MPLs of Indigo Carmine (E 132) in foods according to the Annex II to Regulation (EC) No 1333/2008

FCS[(a)] Category number	Food category	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat-treated products		150
01.6.3	Other creams	only flavoured creams	150
01.7.1	Unripened cheese excluding products falling in category 16	only flavoured unripened cheese	150
01.7.3	Edible cheese rind		*quantum satis*
01.7.6	Cheese products (excluding products falling in category 16)	only flavoured unripened products	100
03	Edible ices		150
04.2.4.1	Fruit and vegetable preparations excluding compote	only *mostarda di frutta*	200
05.2	Other confectionery including breath freshening microsweets	except candied fruit and vegetables	300

FCS[a] Category number	Food category	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
05.2	Other confectionery including breath freshening microsweets	only candied fruit and vegetables	200
05.3	Chewing gum		300
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4	only decorations, coatings and sauces, except fillings	500
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4	only fillings	300
06.6	Batters	only batters for coating	500
07.2	Fine bakery wares		200
08.2.3	Casings and coatings and decorations for meat	only decorations and coatings except edible external coating of *pasturmas*	500
08.2.3	Casings and coatings and decorations for meat	only edible casings	*quantum satis*
09.2	Processed fish and fishery products including molluscs and crustaceans	only surimi and similar products and salmon substitutes	500
09.3	Fish roe	except Sturgeons´ eggs (Caviar)	300
12.2.2	Seasonings and condiments	Only seasonings, for example curry powder, tandoori	500
12.4	Mustard		300
12.5	Soups and broths		50
12.6	Sauces	including pickles, relishes, chutney and piccalilli; excluding tomato-based sauces	500
12.9	Protein products, excluding products covered in category 01.8	only meat and fish analogues based on vegetable proteins	100
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)		50
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)		50
14.1.4	Flavoured drinks	excluding chocolate milk and malt products	100
14.2.3	Cider and perry	excluding *cidre bouché*	200
14.2.4	Fruit wine and made wine	excluding *wino owocowe markowe*	200
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	except: spirit drinks as defined in Article 5(1) and sales denominations listed in Annex II, paragraphs 1-14 of Regulation (EC) No 110/2008 and spirits (preceded by the name of the fruit) obtained by maceration and distillation, Geist (with the name of the fruit or the raw material used), London Gin, Sambuca, Maraschino, Marrasquino or Maraskino and Mistrà	200
14.2.7.1	Aromatised wines	except *americano, bitter vino*	200
14.2.7.2	Aromatised wine-based drinks	except *bitter soda, sangria, claria, zurra*	200
14.2.7.3	Aromatised wine-product cocktails		200
14.2.8	Other alcoholic drinks including	only alcoholic drinks with less than	200

FCS[(a)] Category number	Food category	Restrictions/exceptions	MPL (mg/L or mg/kg as appropriate)
	mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol	15% of alcohol and *nalewka na winie owocowym, aromatyzowana nalewka na winie owocowym, nalewka na winie z soku winogronowego, aromatyzowana nalewka na winie z soku winogronowego, napój winny owocowy lub miodowy, aromatyzowany napój winny owocowy lub miodowy, wino owocowe niskoalkoholowe and aromatyzowane wino owocowe niskoalkoholowe*	
15.1	Potato-, cereal-, flour- or starch-based snacks	excluding extruded or expanded savoury snack products	100
15.1	Potato-, cereal-, flour- or starch-based snacks	only extruded or expanded savoury snack products	200
15.2	Processed nuts	only savoury-coated nuts	100
16	Desserts excluding products covered in categories 01, 03 and 04		150
17.1	Food supplements supplied in a solid form including capsules and tablets and similar forms, excluding chewable forms		300
17.2	Food supplements supplied in a liquid form		100
17.3	Food supplements supplied in a syrup-type or chewable form	only solid food supplements	300
17.3	Food supplements supplied in a syrup-type or chewable form	only liquid food supplements	100

[(a)] FCS : Food Categorisation System (food nomenclature) presented in the Annex II to Regulation (EC) No 1333/2008.

2.7. Reported use levels or data on analytical levels of Indigo Carmine (E 132) 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 food 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 Commission Regulation (EU) No 257/2010[14] regarding the re-evaluation of approved food additives, EFSA issued a public call[15] for concentration data (usage and/or analytical data) on Indigo Carmine (E 132).

In response to this public call, updated information on the actual use levels of Indigo Carmine (E 132) in foods was made available to EFSA by industry and Member States.

2.7.1. Summarised data on reported use levels in foods provided by industry

Industry provided EFSA with data on use levels (n = 7) of Indigo Carmine (E 132) in foods for five out of the 35 food categories in which Indigo Carmine is authorised.

[14] 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.

[15] 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/data/call/130327.htm

Updated information on the actual use levels of Indigo Carmine in foods was made available to EFSA by FoodDrinkEurope (FDE), the International Chewing Gum Association (ICGA), the Association of the European Self-Medication Industry (AESGP) and Capsugel for the following food categories of finished products: chewing gum (FCS Category 05.3), decorations, coatings of confectionary (FCS Category 05.4), non-alcoholic beverages (FCS Category 14.1.4), desserts (FCS Category 16) and food supplements (FCS Category 17).

Appendix A provides data on the use levels of Indigo Carmine (E 132) in foods as reported by industry.

2.7.2. Summarised data on concentration levels in foods from Member States

Additionally, analytical results from Member States were collected through the EFSA call for concentration data. The Panel noted that complete information on the methods of analysis was not made available to EFSA. In total, 4634 analytical results were reported to EFSA by 6 countries: Germany (n = 2843), Austria (n = 994), Czech Republic (n = 316), Slovakia (n = 289), Hungary (n = 155) and Ireland (n = 37). The data were mainly on flavoured drinks (FCS Category 14.1.4), fine bakery wares (FCS Category 07.2) and other confectionary[16] including breath freshening microsweets (FCS Category 05.1). Foods were sampled between 2002 and 2013 and analysed the same year of collection. Out of this dataset, analytical results of Indigo Carmine were not quantified (< LOQ) in 740 samples, not detected (< LOD) in 2926 samples, 247 were numerical values (quantified) and 721 were expressed as qualitative results. Only 22 of these samples are coming from a non-accredited laboratory.

Data resulting from non-authorised uses[17] (n = 593, in fruit and vegetables (FCS 04) other than *mostarda di frutta*, cocoa and chocolate products (FCS 05.1), cereal and cereal products (FCS 06) other than batters, non-alcoholic beverages (FCS 14.1) others than flavoured drinks) and above the MPL set for authorised uses of Indigo Carmine (E 132) (one sample of confectionary (FCS 05.2) above the MPL of 300 mg/kg allocated for this food group) were not considered in the exposure assessment. The 721 samples expressed as qualitative results could also not be used in the exposure assessment as they only give binary results (i.e. indication of presence or absence of the food additive in the food analysed).

Overall, 3319 out of the 4634 total analytical results reported for Indigo Carmine in foods were included by Panel for use in the exposure estimates after discarding: the provided analytical results on foods in which Indigo Carmine is not authorised; a sample exceeding the MPL of authorised use; and values expressed as qualitative results.

Appendix B shows the analytical results of Indigo Carmine (E 132) in foods as reported by Member States (whole set of analytical data reported and positive samples only).

2.8. Information on existing authorisations and evaluations

Indigo Carmine (E 132) is authorised as a food additive in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 on food additives. Specific purity criteria on Indigo Carmine (E 132) have been defined in the Commission Regulation (EU) No 231/2012. The calcium and the potassium salts are also permitted.

JECFA established a temporary Acceptable Daily Intake (tADI) of 0-2.5 mg/kg bw/day in 1969 that was increased to a full ADI of 0-5 mg/kg bw/day in 1975.

[16] Confectionary (FCS category 05) except cocoa and chocolate products (FCS category 05.1), chewing gum (FCS category 05.3) and decorations, coatings and fillings (FCS category 05.4).

[17] Such result might be due to the use of multi-screen methods covering a large range of compounds from food control laboratories analysing the food samples and/or due to errors in the classification of the foods sampled.

In 1975 the SCF established an ADI of 5 mg/kg bw/day. In its latest evaluation in 1984, the SCF agreed to retain this ADI after taking into consideration more recent studies.

JECFA and the SCF in their evaluations used the term Indigotine in place of Indigo Carmine.

Indigo Carmine has also been reviewed by TemaNord (2002).

2.9. Exposure assessment

2.9.1. Food consumption data used for exposure assessment

2.9.1.1. EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with data from national information on food consumption at a detailed level. Competent authorities in the European countries provide EFSA with data on the level of food consumption by 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).

The food consumption data gathered at EFSA were collected by 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 for exposure calculations uncertainties could be introduced due to possible subjects' underreporting and/or misreporting of the consumption amounts.

The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights. For calculation of chronic exposure, intake statistics have been calculated based on individual average consumption over the total survey period excluding surveys with only one day per subject considered as not adequate to assess chronic dietary exposure. High percentile exposure was only calculated for those foods and population groups where the sample size was sufficiently large to allow calculation of the 95th percentile of exposure (EFSA, 2011a). Therefore, in the present assessment, high levels of exposure for toddlers from Belgium, Italy and Spain were not included.

Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 European countries, as mentioned in Table 3:

Table 3: Population groups considered for the exposure estimates of Indigo Carmine (E 132)

Population	Age range	Countries with food consumption surveys covering more than one day
Toddlers	from 12 up to and including 35 months of age	Belgium, Bulgaria, Finland, Germany, Italy, the Netherlands, Spain
Children[18]	from 36 months up to and including 9 years of age	Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden
Adolescents	from 10 up to and including 17 years of age	Belgium, Cyprus, Czech Republic, Denmark, France, Germany, Italy, Latvia, Spain, Sweden
Adults	from 18 up to and including 64 years of age	Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Spain, Sweden, UK
The elderly[18]	from 65 years of age and older	Belgium, Denmark, Finland, France, Germany, Hungary, Italy

[18] 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, 2011a).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the Food Classification System (FCS) as presented in the Annex II to Regulation (EC) No 1333/2008, part D, to perform exposure estimates. In practice, FoodEx food codes were matched to the FCS food categories and the exposure was calculated by multiplying MPLs and values reported in Appendix C for each food group with their respective consumption amount per kg bw separately for each individual in the database, calculating the sum of exposure for each survey day for the individual and then deriving the daily mean for the survey period. Based on the individual exposures, the mean and 95th percentile exposure was calculated for the total survey population separately for each survey and for the five population groups described in Table 3.

2.9.1.2. Food categories selected for the exposure assessment of Indigo Carmine

The food categories in which the use of indigo carmine (E 132) is authorised were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system food codes), at a detailed level (up to FoodEx Level 4) (EFSA, 2011b).

Some food categories are not referenced in the EFSA Comprehensive Database and therefore could not be taken into account in the present estimate. This might result 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 flavoured unripened products
- 04.2.4.1. Fruit and vegetable preparations excluding compote, only mostarda di frutta
- 05.4. Decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4, only decorations, coatings and sauces, except fillings and only fillings
- 06.6. Batters
- 08.2.3. Casings and coatings and decorations for meat
- 14.2.4. Fruit wine and made wine

For the following food categories, the restrictions which apply to the use of Indigo Carmine could not be taken into account, and therefore the whole food category was considered for the exposure estimates. This results in an overestimation of the exposure:

- 01.7.1 Unripened cheese excluding products falling in category 16, only flavoured unripened products
- 09.3. Fish roe, except Sturgeons' eggs (Caviar)
- 14.2.3. Cider and perry, excluding *cidre bouché*
- 14.2.7.1. Aromatised wines, except *americano, bitter vino*
- 14.2.7.2. Aromatised wine-based drinks, except *bitter soda*, *sangria*, *claria*, *zurra*
- 15.1. Potato-, cereal-, flour-, starch-based snacks, excluding extruded or expanded savoury snack products or only extruded or expanded savoury snack products

- 17.1/17.2/17.3. Food supplements, in solid or liquid form.

The food category 01.6.3 (other creams[19] - only flavoured creams) cannot be not distinguished from the food category 01.6 (Cream and cream powder); the same applies in differentiating flavoured cream from plain cream. Therefore, taking into account that the whole food group 01.6 (Cream and cream powder) would result in a large overestimate, this food category (01.6.3) was not considered in the present estimate.

Overall, 8 food categories were not taken into account in the exposure assessment because are not referenced in the EFSA Comprehensive Database and 7 food categories were included in the exposure assessment without considering the restrictions as set in Annex II to Regulation No 1333/2008.

2.9.2. Exposure to Indigo Carmine (E 132) from its use as a food additive

Exposure assessment for food additives under re-evaluation is carried out by the ANS Panel based on: (1) MPLs set down in the EU legislation (defined as the *regulatory maximum level exposure assessment* scenario); and (2) the availability of adequate use levels or analytical data (defined as the *refined exposure assessment* scenario).

2.9.2.1. Regulatory maximum level exposure assessment scenario

The regulatory maximum level exposure assessment scenario is based on the MPLs as set in Annex II to Regulation No 1333/2008 and listed in Table 2.

The exposure estimates derived following this scenario should be considered as the most conservative since it assumes that the consumer will be continuously (over a lifetime) exposed to Indigo Carmine present in the food at the MPLs.

2.9.2.2. Refined exposure assessment scenario

The refined exposure assessment scenario is based on information on reported use levels by industry and analytical results submitted to EFSA by Member States. This exposure scenario can only consider food categories where the above data were available to the Panel.

Appendix C summarises the concentration levels of Indigo Carmine used in the refined exposure assessment scenario. Based on the available dataset, the Panel calculated two estimates based on different model populations:

(1) 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/analytical levels for one food category. This exposure estimate is calculated as follows:

- Combining food consumption with the maximum of the maximum reported use levels or the maximum of the analytical results for the main contributing food category at the individual level.
- Using the mean of the typical reported use levels or the mean of analytical results for the remaining food categories.

(2) 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/analytical levels in food. This exposure estimate is calculated using the mean of the typical reported use levels or the mean of analytical results for all food categories.

[19] Within the FCS category 01.6 cream and cream powder, 'other creams' are creams other than unflavoured pasteurised creams (FCS category 01.6.1) and unflavoured live fermented cream products and substitute products with a fat content of less than 20 % (FCS category 01.6.2).

In the refined exposure assessment scenarios, the concentration levels considered by the Panel were extracted from the whole dataset received (i.e. reported use levels and analytical results). For analytical results, for each food category the mean medium-bound (<LOD/LOQ = ½ LOD/LOQ) is used, which takes into consideration left censored data (i.e. analytical results < LOD or < LOQ). For the reported use levels, the mean typical reported use level for each food category is used.

If both reported use levels and analytical results were available for the same food category, the most reliable value was used.

Food categories for which none or inadequate reported use/analytical levels were available were not considered in the exposure assessment. This concerns the following food categories:

- Processed fish and fishery products, only surimi and similar products and salmon substitutes (FCS 09.2),
- Protein products, only meat and fish analogues based on vegetables proteins (FCS 12.9)
- Dietary foods for special medical purposes (FCS 13.2)
- Dietary foods for weight control diets (FCS 13.3)

The Panel noted that if Indigo Carmine is nevertheless used in those food categories for which reported use/analytical levels were not available, the calculated refined exposure assessment might result in underestimation of exposure to Indigo Carmine.

2.9.2.3. Anticipated exposure to Indigo Carmine (E 132)

Table 4 summarises the estimated exposure to Indigo Carmine (E 132) from its use as a food additive of all five population groups (Table 3). Detailed results by population group and survey are presented in Appendix D.

Table 4: Summary of anticipated exposure to Indigo Carmine (E 132) from its use as a food additive using the regulatory maximum level exposure assessment scenario and refined exposure scenarios, in five population groups (min-max across the dietary surveys in mg/kg bw/day)

	Toddlers (12-35 months)	**Children (3-9 years)**	**Adolescents (10-17 years)**	**Adults (18-64 years)**	**The elderly (>65 years)**
Regulatory maximum level exposure assessment scenario					
• Mean	0.9-3.8	0.8-3.2	0.3-1.5	0.3-1.0	0.1-0.6
• High level[20]	2.8-7.1	1.8-6.7	1.0-3.2	0.7-2.3	0.5-1.4
Refined estimated exposure scenario					
Brand-loyal scenario					
• Mean	0.1-0.4	0.1-0.3	0.04-0.2	0.02-0.1	0.02-0.1
• High level[20]	0.3-0.8	0.2-0.8	0.1-0.5	0.1-0.3	0.1-0.2
Non-brand-loyal scenario					
• Mean	0.02-0.2	0.02-0.1	0.01-0.1	0.01-0.04	0.003-0.03
• High level[20]	0.1-0.3	0.1-0.3	0.03-0.1	0.02-0.1	0.01-0.1

[20] typically 95th percentile of consumers only

2.9.3. Main food categories contributing to exposure to Indigo Carmine (E 132) using the regulatory maximum level exposure assessment scenario

Table 5: Main food categories contributing to exposure to Indigo Carmine (E 132) using MPLs (> 5 % to the total mean exposure) and number of surveys in which each food category is contributing

FCS Category number	Foods	Toddlers	Children	Adolescents	Adults	The elderly
		Range of % contribution to the total exposure (Number of surveys)[(a)]				
01.4	Flavoured fermented milk products	6.6-75.8 (7)	7.8 – 37.7 (13)	6.2 – 14.9 (9)	5.4 – 34.5 (12)	6.8 – 34.1 (6)
01.7.1	Unripened cheese excluding products falling in category 16	5.1-9.2 (4)	5.8-18.8 (3)	22.4 (1)	5.3-29.8 (6)	5.7-29.1 (5)
03	Edible ices	5.0-15.7 (6)	5.8-13.3 (9)	5.6-13.0 (4)	7.7-9.1 (3)	5.3-7.4 (2)
05.2	Other confectionery including breath freshening microsweets		5.2-10.7 (5)	7.5-10.2 (2)	9.2-9.8 (2)	5.9-6.4 (2)
07.2.	Fine bakery wares	11.5 – 55.2 (6)	12.4 – 48.7 (13)	13.9 – 46.2 (11)	5.7 - 45.2 (14)	11.8-38.9 (6)
12.5	Soups and broths	6.6 (1)	5.2 – 15.1 (3)	13.7 (1)	7.7 – 18.8 (2)	8.0-17.4 (2)
12.6	Sauces	7.0 – 12.3 (4)	6.0 – 26.0 (12)	7.7 – 35.8 (10)	8.1-34.5 (14)	5.9-28.5 (6)
14.1.4	Flavoured drinks	5.2-28.1 (5)	8.9 – 50.2 (14)	6.6 – 62.9 (12)	5.4 – 53.8 (15)	7.1-48.1 (4)
14.2	Alcoholic beverages, including alcohol-free and low-alcohol counterparts				8.5-18.5 (4)	6.3-14.4 (3)
15.1	Potato-, cereal-, flour- or starch-based snacks	5.5-7.5 (2)	5.1 (1)	5.0 - 10.5 (3)		
16	Desserts	12.1 – 14.9 (2)	5.2-10.4 (5)	8.4 (1)	5.4-6.2 (2)	7.6 (1)
17	Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children					5.4 (1)

(a): The total number of surveys may be greater than the total number of countries as listed in Table 3, as some countries submitted more than one survey for a specific population.

2.9.4. Main food categories contributing to exposure to Indigo Carmine (E 132) using the refined exposure assessment scenarios

Table 6: Main food categories contributing to exposure to Indigo Carmine (E 132) using the brand-loyal refined exposure scenario (> 5 % to the total mean exposure) and number of surveys in which each food category is contributing

FCS Category number	Foods	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	23.5 - 78 (6)	6.8 - 28.8 (10)	6.7 - 9 (5)	5.4 - 23 (8)	5.7 - 24.2 (5)
03	Edible ices	9.6 (1)	7.8 - 7.8 (1)	10.9 (1)		
05.2	Other confectionery including breath freshening microsweets	8.3 - 18.1 (5)	6 - 58.7 (13)	7.5 - 45.2 (9)	5.4 - 49.3 (10)	6.7 - 37.9 (3)
07.2	Fine bakery wares	7.6 - 54.1 (6)	8 - 71.5 (13)	7.7 - 67.3 (11)	10.2 - 58.9 (13)	8.5 - 59.5 (6)
12.5	Soups and broths	10.6 - 15.7 (2)	9.3 - 36 (4)	8.7 - 37.9 (4)	5.2 - 50.3 (6)	24.5 - 49.4 (2)
12.6	Sauces			5.2 - 7.2 (3)	5 - 9.7 (4)	5.9 - 6.4 (2)
14.1.4	Flavoured drinks	6.7 - 43.1 (5)	6.9 - 47.2 (14)	7 - 53.2 (12)	9.1 - 75.4 (15)	6.1 - 68.9 (6)
14.2	Alcoholic beverages, including alcohol-free and low-alcohol counterparts				6.5 (1)	5.1 (1)
15.1	Potato-, cereal-, flour- or starch-based snacks	7.9 (1)		6.5 - 8.9 (2)		
16	Desserts excluding products covered in category 01, 03 and 04	5.9 - 19.7 (3)	5.7 - 18.8 (6)	6 - 13.2 (2)	8.3 - 11.9 (2)	6.7 - 16.8 (2)
17	Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children				5.8 (1)	6.1 - 24.5 (2)

(a): The total number of surveys may be greater than the total number of countries as listed in Table 3, as some countries submitted more than one survey for a specific population.

Table 7: Main food categories contributing to exposure to Indigo Carmine (E 132) following the non-brand-loyal exposure scenario (> 5 % to the total mean exposure) and number of surveys in which each food category is contributing

FCS Category number	Foods	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	13.4 - 76.5 (6)	5.5 - 37.2 (12)	5.1 - 10.6 (6)	5.2 - 34.1 (9)	9 - 28.7 (3)
03	Edible ices	8.4 (1)	5.7 - 8.6 (3)	6.9 (1)		
05.2	Other confectionery including breath freshening microsweets		8.6 - 10.3 (2)	5.3 - 7.7 (2)	7.7 - 8.9 (2)	5.5 (1)
07.2	Fine bakery wares	5.6 - 67.9 (7)	6.3 - 74.2 (15)	5.3 - 70.8 (12)	9.5 - 63.3 (14)	16.6 - 63.6 (6)
12.5	Soups and broths	6.7 - 30.4 (3)	6.5 - 46.4 (7)	5.7 - 45.6 (6)	9.3 - 57 (7)	8.2 - 55.1 (3)
12.6	Sauces	5.6 (1)	5.5 - 12 (9)	7.1 - 17.1 (7)	5 - 21 (12)	6.7 - 12.8 (5)
14.1.4	Flavoured drinks	7.6 - 21.8 (5)	6.4 - 60.1 (14)	7.7 - 70.3 (12)	5.5 - 62.2 (15)	5.7 - 52.1 (4)
14.2	Alcoholic beverages, including alcohol-free and low-alcohol counterparts				5.1 - 5.2 (2)	5.3 - 5.6 (2)
15.1	Potato-, cereal-, flour- or starch-based snacks	5.2 - 8.4 (2)		5.5 - 11.7 (3)	5.5 - 5.7 (2)	
16	Desserts excluding products covered in category 1, 3 and 4	13.8 - 46.2 (3)	6.1 - 35.3 (9)	5.7 - 28.6 (7)	6 - 25.1 (8)	8.5 - 30.8 (3)
17	Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children				11.4 (1)	32.1 (1)

(a) The total number of surveys may be greater than the total number of countries as listed in Table 3, as some countries submitted more than one survey for a specific population.

The Panel noted that, considering all the authorised uses of Indigo Carmine (E 132) as a food additive, both reported use levels and quantified analytical results were reported only for the food categories of chewing gum, flavoured drinks and desserts. For several food products for which the use of Indigo Carmine is authorised e.g. flavoured fermented milk products, seasonings and condiments, mustard, soups and broths, sauces, cider and perry, aromatised wine based-products and other alcoholic drinks, no usage were reported and also reported analytical data showed only limited sample sizes (n<20). In addition, the Panel further noted that for some other authorised food categories such as edible ices, fine bakery wares, fish roe, spirit drinks, potato-, cereal, flour-, or starch-based snacks and processed nuts, no usages had been reported by industry, while positive analytical results (> LOQ) were obtained from the analytical data.

For the main contributing food categories of fine bakery wares and flavoured drinks, the analytical data comprised around 1000 samples and levels were found to be far below the MPL. Moreover, for the food category of flavoured fermented milk products (FCS 01.4), although being a major contributor to the total exposure estimated using the regulatory maximum level exposure assessment scenario, no usage was reported by industry, and none of the 10 analytical results reported for this food category (FCS 01.4) were quantified. For the additional food categories (i.e. seasonings and condiments, mustard, soups and broths, sauces, cider and perry, aromatised wine based-products, other

alcoholic drinks) for which no usage data or positive analytical values were reported and thus not available for use in the refined assessment, their contribution to the total exposure to Indigo Carmine in the MPL scenario is low.

2.9.5. Uncertainty analysis

Uncertainties in the exposure assessment of Indigo Carmine (E 132) have been discussed above. According to 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 summarised below:

Table 8: Qualitative evaluation of influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction[(a)]
Consumption data: different methodologies/representativeness/under reporting/misreporting/no portion size standard	+/-
Use of data from food consumption survey of few days to estimate long-term (chronic) exposure	+
Correspondence of reported use levels to the food items in the EFSA Comprehensive Food Consumption Database: uncertainties on which precise types of food the levels refer to	+/-
Concentration data: levels considered applicable for all items within the entire food category, exposure calculations based on the maximum or mean levels (reported use from industries or analytical data from MS)	+
Concentration data: levels above the MPLs or on non authorised food categories not considered	-
Uncertainty in possible national differences in use levels of food categories, concentration data not fully representative of foods on the EU market	+/-

(a): + = uncertainty with potential to cause over-estimation of exposure; - = uncertainty with potential to cause underestimation of exposure.

3. Biological and toxicological data

The biological properties of Indigo Carmine have been previously evaluated by JECFA in 1969 and 1975 (JECFA, 1969, 1975). The present opinion briefly reports the major studies evaluated in these reports. Additional information has been identified from the literature and the calls for data.

3.1. Absorption, distribution, metabolism and excretion

3.1.1. In vitro

Incubation of ^{35}S-labelled Indigo Carmine with intestinal contents of rats for 48 hours was investigated by Lethco and Webb (1966). This study resulted in loss of colour and formation of isatin-5-sulphonic acid and 5-sulphoanthranilic acid (further details were not available). The authors suggested that, by the action of intestinal bacteria, isatin-5-sulphonic acid and 5-sulphoanthranilic acid might be formed which could be absorbed after formation.

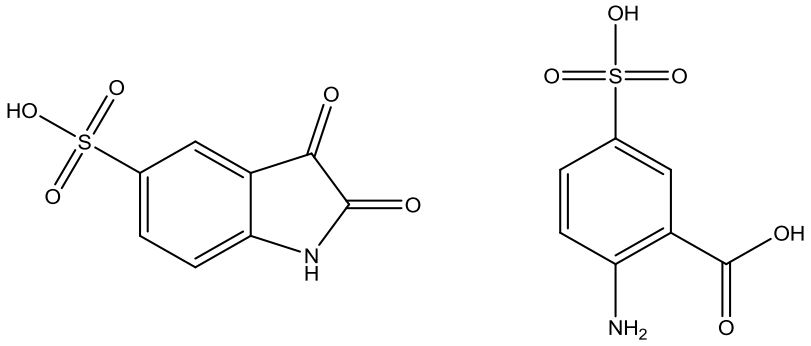
Singh et al. (1993) measured the degradation of Indigo Carmine (1 mg) in the caecal microflora of Wistar rats (0.2 ml caecal extract). The time period required to degrade 50 % of the original colour concentration was 54 minutes. This study indicated also that Indigo Carmine was transformed by caecal microflora to four unidentified fluorescent metabolites.

3.1.2. In vivo

A series of studies by Lethco and Webb (1966) used radiochemically pure ^{35}S-labelled Indigo Carmine (FD&C Blue No 2) to investigate the stability of the radiolabel, biliary excretion of intravenously

administered ^{35}S-labelled Indigo Carmine and excretion of orally administered ^{35}S-labelled Indigo Carmine.

After intravenous injections of 1.4 mg/kg bw ^{35}S-labelled Indigo Carmine to bile-duct-cannulated rats with ligated urethras, the animals were maintained under anaesthesia for 6 hours and urine was taken directly from the bladder. Within 6 hours 63 % of the radioactivity appeared in the urine and about 10 % in the bile, almost all the biliary excretion occurred in the first 30 minutes. The break-down products isatin-5-sulphonic acid and 5-sulphoanthranilic acid (Figure 2) were identified in the bile samples from 2 hours onwards and in the urine from 1 and 2 hours after dosing. In urine, 71 % of the excreted radioactivity was the parent compound with 19 % as isatin-5-sulphonic acid and 10 % as 5-sulphoanthranilic acid. Neither isatin-5-sulphonic acid nor 5-sulphoanthranilic acid were found in urine spiked with ^{35}S-labelled Indigo Carmine after 6 hours standing at room temperature. This indicates that their formation did not occur after the radioactivity was excreted. Small amounts of these two metabolites appeared in bile after 4 hours.



Isatin-5-sulphonic acid 5-sulphoanthranilic acid

Figure 2: Structural formulae of isatin-5-sulphonic acid and 5-sulphoanthranilic acid

Following oral administration of 5, 25 and 50 mg/rat ^{35}S-labelled Indigo Carmine to groups of 4 male rats (based on the cited body weight range for the rats these are approximately equivalent to 10.4-14.5, 52-72 and 104-145 mg/kg bw respectively), only 1.6 to 2.1 % of the radioactivity appeared in urine and between 61 % and 80 % was recovered in faeces within 3 days. Urinary and faecal excretion was essentially identical at all three doses. Since only 0.004 % of an oral dose of 20 mg/rat was excreted via bile in an additional experiment, the authors concluded that the oral experiment suggested a poor absorption of Indigo Carmine from the gastrointestinal tract. Following oral administration, the urine contained low amounts of radioactivity as Indigo Carmine, isatin-5-sulphonic acid and 5-sulphoanthranilic acid (0.53, 0.63 and 0.28 % of the radioactivity, respectively).

In an additional experiment, the oral administration of 5-sulphoanthranilic acid at doses of 1, 10 and 100 mg/animal resulted in the excretion of 19.8 % to 27.7 % of the dose in urine in 48 hours. From these data, the authors concluded that "*it is surprising that relatively large percentages of 5-sulfoanthranilic acid were found in the urine following its oral administration, indicating that 5-sulfoanthranilic acid is absorbed to a greater extent than FD&C blue no. 2 (Indigo Carmine)*".

Overall, in vivo studies demonstrated that radioactivity from ^{35}S-Indigo Carmine was poorly detected in urine (1.6 to 2.1 % of the radioactivity) after oral administration in rats. Moreover, incubation of ^{35}S-Indigo Carmine with intestinal contents of rats for 48 hours suggested that isatin-5-sulphonic acid and 5-sulphoanthranilic acid are metabolites formed by intestinal bacteria. By comparison to the data obtained after intravenous administration, the Panel considered that the data available on the absorption, metabolism and excretion of Indigo Carmine indicated that Indigo Carmine or its metabolites were poorly absorbed. However, the Panel noted that both identification of faecal metabolites and tissue distribution of radioactivity were not investigated in these studies.

3.2. Toxicological data

3.2.1. Acute oral toxicity

The JECFA evaluation (1975) briefly mentioned some studies on the acute oral toxicity of Indigo Carmine. LD_{50} values were 2500 mg/kg bw in mice (US FDA, 1969, as referred to by JECFA, 1975) and 2000 mg/kg bw in rats (Lu and Lavalleé, 1964, as referred to by JECFA, 1975).

3.2.2. Short-term and subchronic toxicity

3.2.2.1. Short-term toxicity studies

In a study by Aboel-Zahab et al. (1997) male Albino rats (strain not specified) received 800 mg/kg bw/day in the diet of a mixture that contained Brown HT and Indigo Carmine. The composition of the mixture was not specified as it was stated to be a company secret and, therefore, the concentration of each colour was not reported. The Panel considered that the results of this study cannot be used for the hazard characterisation, since the experimental animals have been exposed to a mixture of food colours in which the dose level of each colour has not been specified.

Weanling large white pigs (3/sex/group) were given Indigo Carmine at dietary doses of 0, 150, 450 and 1350 mg/kg bw/day for 90 days. Haemoglobin levels and red cell counts were slightly reduced after 45 and 90 days in males at the highest dose tested. There was no effect of treatment on growth, urine and serum analysis or organ weights. Histological examination revealed liver abscesses in one male in the low-dose group but this was considered not to be treatment-related. No other abnormalities were seen (Gaunt et al., 1969).

Adult male Swiss albino (B-6 strain) mice (5 animals/group) were fed with 0, 17 and 39 mg Indigo Carmine/kg bw/day for a period of 42 days (Dixit and Goyal, 2013). Indigo Carmine was given orally mixed with the standard diet. The test material (E 132; FD&C Blue # 2; CI 73015), used in the present study was procured from a local market[21]. Whether the test material met the EU specifications for the food additive E 132 or contained contaminants was not determined. Only the following parameters were studied: body weights, organ weights, histopathology of the testes, sperm density and sperm motility. The animals fed with the dye showed a statistically significant increase in their body weights at both dose levels. In addition, a significant decrease was observed in the average weight of the testes at both dose levels. Histopathological examination of the testes demonstrated a significant reduction in the average diameter of the seminiferous tubules at both dose levels. A marked reduction in testicular sperm density was found to be statistically non-significant at the low dose but significant at the high dose, when compared to the control group. According to the authors, Indigo Carmine caused a significant decrease in the sperm motility at both doses. Severe histopathological changes in the testis architecture at both the doses were described. At low dose, Indigo Carmine caused thickening of the tubular basement membrane, arrest of spermatogenesis at the spermatid stage and the debris from necrotic sperm was evident within the tubular lumen. At high dose, Indigo Carmine caused dissolution of the tubular basement membrane and exfoliation of cells in the lumen, leading to complete testicular blockage. Cytoplasmic vacuolation and pycnosis were also prominent. The Panel noted limitations in the design of the study and the lack of information on the identity of the test material used in the study. The authors stated that the dose range was based on the LD_{50} of the test substance. However, the percentage of the LD_{50} used was not stated. The Panel noted that the doses of 17 and 39 mg/kg bw/day were low compared to the LD_{50} of Indigo carmine (E132) of 2500 mg/kg bw. The Panel considered that a mouse between the age of 4-5 weeks (as used in this study) should grow more than 1.6 g as indicated for the control group. Normal feed intake in mice is 3-4 g/day. It was indicated by the authors that the control mice consumed a diet of 10 g/day. The authors reported that the mice were fed 5 g of diet containing Indigo Carmine and 5 g normal diet. The Panel noted that, the authors did

[21] The test material was reported by the authors to have been manufactured and packed by ASES Chemical Works Laboratory chemical division, Jodhpur.

not state whether the mice of the control and test groups received diet from the same batch. This method of feeding deviates from commonly used methods and also from the method described in the OECD guidelines. In addition, the Panel noted that the authors did not state whether the diet was analysed for the concentration of the test substance and contaminants.

The Panel noted that no NOAEL could be identified in this study and the lowest observed adverse effect level (LOAEL) was 17 mg/kg bw/day, the lowest dose level tested.

3.2.2.2. Subchronic toxicity

No subchronic toxicity studies were available with Indigo Carmine.

The JECFA evaluation described a short-term study conducted with isatin-5-sulphonic acid, one of the metabolites of Indigo Carmine. Isatin-5-sulphonic acid was fed to groups of 3-week-old rats (10/sex/group), at levels of 0, 0.25, 0.5, 1 and 2 % (equivalent to 0, 125, 250, 500 or 1000 mg/kg bw/day) for 13 weeks. Gross and histopathology examinations were conducted. The "no-effect" level of the compound was considered by JECFA to be 2 % equivalent to 1000 mg isatin-5-sulphonic acid/kg bw/day (US FDA, 1969, as referred to by JECFA, 1975).

3.2.3. Genotoxicity

3.2.3.1. In vitro

Bacterial mutagenicity of Indigo Carmine was studied using *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 with and without metabolic activation (Auletta et al., 1977; Bonin et al., 1980; Brown et al., 1978; Cameron et al., 1987; Ishidate et al., 1984). No mutations were observed. The Panel noted, however, that neither the TA102 strain nor any *Escherichia coli* strain was used in the standard plate or preincubation assays. Lück and Rickerl (1960, as referred to by JECFA, 1975) reported that Indigo Carmine did not induce reverse mutations in two *Escherichia coli* strains. However, the study only used a single concentration of Indigo Carmine (5 g/L), it was performed only in the absence of metabolic activation and was not compliant with current standard protocols . Indigo Carmine (10 mg/mL and liver S9 fraction) did not damage DNA in a *rec* and *pol* assay using strains of *Escherichia coli* (Haveland-Smith and Combes, 1980).

In the study by Ishidate et al. (1984), Indigo Carmine was tested in a chromosomal aberration test *in vitro* using the Chinese hamster fibroblast CHL cell line. The test results were reported only for the maximum concentration and only for one sampling time. At an Indigo Carmine concentration of 12 mg/mL the incidence of polyploid cells was 10 % and the frequency of cells with structural aberrations was 3 % at 24 hours after treatment. The frequency of chromosomal aberrations in the control cultures was not presented but the frequency of cells with polyploidy was largely in excess when compared to the results obtained with other compounds which were considered negative by the authors. Accordingly, the authors considered the result obtained with Indigo Carmine as a positive result. The Panel noted that the effect was observed at an excessive concentration of 12 mg/mL (equal to 26 mmol/L) and that no information on cytotoxicity was reported. Therefore, the Panel did not agree with the authors and considered that the result was inconclusive.

The Panel noted that the following four in vitro studies have been published after the JECFA and the SCF evaluations.

In a mouse lymphoma assay performed with Indigo Carmine by Cameron et al. (1987), there was an increase in mutation frequency (up to 277 mutants/10^6 cells compared to 53 mutants/10^6 cells in the negative control) which exceeded the “global evaluation factor” of 126 mutants/10^6 cells with no clear dose-relationship in the presence of metabolic activation, and no response without metabolic activation. However, in the presence of S9 only cytotoxic concentrations were tested ranging from 92 to 556 µg/mL (equal to 0.2-1.2 mmol/L). All concentrations tested induced a marked cytotoxicity expressed as relative total growth (RTG) ranging from 23 % at the lowest concentration to 10-13 % at

the highest concentration. The authors considered the result as indeterminate and noted that re-testing would be required in order to determine whether this result can be interpreted as a mutagenic response. The Panel agreed with the authors and noted that the absence of a dose response relationship in mutant frequency may be related to inadequate selection of concentrations since the lowest concentration already caused cytotoxicity.

Kornbrust and Barfknecht (1985) tested a range of food-dyes in an in vitro rat Hepatocyte Primary Culture/DNA Repair (HPC/DR) assay and, to a limited extent, in an in vivo/in vitro HPC/DR assay. It was found that Indigo Carmine (Acid blue 74) did not produce any detectable DNA repair increase.

The DNA damaging potential of Indigo Carmine (purity not reported) was investigated in an in vitro Comet assay using MCF-7 breast cancer cells in order to assess the safety of the use of this dye for clinical purposes (Masannat et al., 2009). The cells were exposed for five minutes at concentrations from 0.001 to 0.4 % (equal to 0.02-8.6 mmol/L). Formamidopyrimidine DNA glycosylase (FPG) was incorporated into the Comet assay in order to detect purine oxidation products such as 8-oxoguanine. At the concentration of 0.4 %, Indigo Carmine caused a slight increase in DNA damage, expressed as a percentage of tail DNA, compared with untreated control cells (6.81 % *vs* 4.04 %). The difference, however, did not reach statistical significance.

To investigate the effect of Indigo Carmine on the induction of DNA damage, Davies et al. (2006) conducted a Comet assay without and with FPG in order to demonstrate DNA oxidative damage in a human colonic adenocarcinoma cell line (CaCo2 cells) and before and after dye-spraying in human colonic cells taken at biopsy from 10 patients undergoing colonoscopic examination. The CaCo2 cells were exposed for two minutes at a concentration of 0.1 % (equal to 2 mmol/L, purity not reported). The mucosal biopsy samples were taken from the same area of the colon before and after the application of 2 mL of 0.1 % Indigo Carmine dye onto the colonic mucosa. In both the CaCo2 cell line and the human colonic cells (obtained from patients before and after administration of Indigo Carmine) no increase in DNA strand breaks was observed. The Panel noted that the exposure and sampling times were not reported for the biopsy samples, therefore the reliability of the negative result is limited.

3.2.3.2. In vivo

The Panel noted that none of the *in vivo* studies were available for the JEFCA and SCF evaluations.

Male Swiss albino mice (10 animals per group) received daily doses of Indigo Carmine alone (2 mg/kg bw/day) or Indigo Carmine (1 mg/kg bw/day) and nitrite (1 mg/kg bw/day) in combination for 30 days in order to investigate bone-marrow chromosomal aberrations (Giri et al., 1986). A total of 600 metaphases were scanned from 10 animals. When Indigo Carmine was administered alone, a statistically significant increase in the frequency of chromosomal aberrations (mainly breaks) was reported by the authors. However, the study shows significant shortcomings which include presence in the vehicle control of two cells with more than 10 aberrations per cell out of 600 cells scored. This outcome could have been caused either by an unhealthy status of animals or the use of an unusual method for preparation of slides ("flame dried preparation"), which could affect the integrity of chromatin thus generating artifactual breaks. The Panel also noted that - considering the limited absorption of test compound in vivo - the dose administered was very low (2 mg/kg bw/day) although no rationale for its selection was supplied by the authors. Furthermore, no historical control data were provided to assess the biological relevance of the results obtained. On this basis, the Panel considered that the study was not reliable for risk assessment.

Groups of male mice were administered a single i.p. injection with Indigo Carmine alone at dose levels of 5, 10, 25, 50 and 100 mg/kg bw and in combination with nitrite at concentrations equivalent to one-half of the concentrations of Indigo Carmine when administered alone, to evaluate induction of Sister Chromatid Exchanges (SCEs) in bone-marrow cells (Giri and Mukherjee, 1990). Statistically significant increases in the frequency of SCEs were observed at dose levels of 25, 50 and 100 mg/kg

bw of Indigo Carmine when administered alone. Increases over control were small (3.4 – 4.6 SCE per cell), and within the range normally found in negative control animals for this assay. The Panel also noted limitations in the selection of dose-levels and - for the evaluation of data - no historical control data were supplied to assess biological relevance of results obtained. On this basis, the Panel considered the outcome of this study not biologically relevant.

Durnev et al. (1995) studied the mutagenic activity of 6 food colours, including Indigo Carmine (E 132) in an in vivo chromosomal aberration assay. The substances were given orally to C57BL/6 mice (5 animals/group) for a period of 5 days, in daily doses of 1.4 or 14 mg/kg bw. The animals were sacrificed 6 hours after the last dose. The concurrent control group also consisted of 5 animals. Analysis of metaphase spreads (500/group) from bone-marrow from treated animals revealed no increase in the number of cells bearing chromosomal damage compared to the concurrent control. The Panel noted that the study was not performed according to OECD guidelines (e.g. with respect to the number of dose levels and sampling time), therefore the Panel considered that the reliability of this negative result is limited.

Sasaki et al. (2002) studied the genotoxicity of 39 chemicals currently in use as food additives including Indigo Carmine in an in vivo Comet assay. Groups of 4 male ddY mice were treated once orally with Indigo Carmine at the limit dose of 2000 mg/kg bw. The glandular stomach, colon, liver, kidney, urinary bladder, lung, brain and bone-marrow were analysed for DNA damage 3 and 24 hours after treatment. Indigo Carmine did not yield a statistically significant increase in DNA damage in any organ.

Following a call for scientific data on Indigo Carmine launched by EFSA for new genotoxicity data, an in vivo study on induction of micronuclei in the bone marrow of treated rats was performed (Whitwell, 2013). The study was conducted in compliance with the OECD guideline 474[22]. Groups of six rats were treated by oral gavage twice, with a 24 hour interval between each dose. The animals received the vehicle only (1 % methylcellulose) or Indigo Carmine[23] (E132) at 300, 2000 and 3000 mg/kg bw/day. Cyclophosphamide was used as positive control substance at 20 mg/kg bw/day. Animals were sacrificed at 48 hours. Results obtained indicate that no significant increases in the group mean frequencies of micronucleated polychromatic erythrocytes (MN PCE) were observed in any Indigo Carmine treatment group compared to the relevant concurrent negative control group. A slight increase in group mean frequency of MN PCE compared to the untreated control group was observed at 300 mg/kg bw/day (0.12 % - 0.24 %) which was mainly attributable to a single animal. It should be noted here that the number of PCE scored per animal for analysis of micronuclei was 4000 instead of 2000 PCE as recommended by OECD Guideline 474. The author justified this action as an "aid in data interpretation". The Panel did not consider this extra scoring a shortcoming since a greater number of cells was analysed thus increasing the statistical power. The Panel noted that there was no proof of target tissue exposure since there was no evidence of test article related bone marrow toxicity and toxicokinetic data indicated only a minor absorption from the gastrointestinal tract. However, the Panel considered that Indigo Carmine at the concentration of 2000 mg/kg bw/day did not induce DNA damage at first site of contact (i.e. the glandular stomach) in an in vivo Comet assay performed by Sasaki et al. (2002).

Overall the Panel concluded that based on the available data, Indigo Carmine does not raise concern for genotoxicity.

[22] OECD (1997). "Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test". In OECD Guidelines for the testing of chemicals. OECD Paris, Test Guideline 474.

[23] FD &blue No 2 powder. PD% 91.

3.2.4. Chronic toxicity and carcinogenicity

3.2.4.1. Mice

Two long term/carcinogenicity studies in mice were available.

Groups of Charles River CD1 mice (30/sex/group; 60/sex/controls) received diets containing 0, 0.2, 0.4, 0.8 or 1.6 % Indigo Carmine (equivalent to approximately 0, 300, 600, 1200, or 2400 mg/kg bw/day according to the authors) for 80 weeks (Hooson et al., 1975). The only effect noted was a slight anaemia in the 0.8 and 1.6 % groups. Furthermore, no effects were observed with regard to bodyweight gain, organ weights, deaths, histopathological examination, or tumour incidence. Indigo Carmine did not exert any carcinogenic effect. Based on these results the authors identified a NOAEL of approximately 600 mg/kg bw/day. The Panel agreed with this NOAEL.

In a long-term toxicity/carcinogenicity study by Borzelleca and Hogan (1985), 42-day old mice (Charles Rivers CD1 COBS ISC derived) (60/sex/group, 2 control groups) were fed Indigo Carmine (FD&C blue No 2[24]) at dietary concentrations of 0, 0.5, 1.5 or 5.0 % (equal to 0, 825, 2477 and 8259 mg/kg bw/day in male and 0, 945, 2836 and 9456 mg/kg bw/day in female) for a maximum of 23 months. Observations of deaths, morbidity and gross signs of toxicological effects were conducted twice daily. Detailed physical examinations for signs of toxicity and palpation for masses were conducted weekly. Body weight and food consumption were determined weekly through the first 14 weeks, biweekly for weeks 16-26 and monthly thereafter. Ten animals of each group were randomly selected for haematology tests at months 3, 6, 12 and 18 of the study. The parameters evaluated were haemoglobin, erythrocyte and total and differential leucocyte counts and erythrocyte morphology. Necropsies were conducted on all animals dying spontaneously, sacrificed in a moribund condition, or on schedule. A complete histopathology study was conducted on all animals from the 2 control groups and from the highest dose group (5 %). Histopathological examination was only carried out on tissues from any low-dose or mid-dose animal with a gross lesion or mass. Organ weights were recorded for brain, gonads, kidneys, liver, spleen and thyroid, and relative organ weights were calculated. In the treated animals at all doses, there was a moderate incidence of blue-green discolouration of the gastrointestinal tract with occasional concomitant involvement of the liver, gall-bladder and urine. This discolouration was attributed to the Indigo Carmine (Borzelleca and Hogan, 1985).

Other grossly observable abnormalities occurred randomly without relation to treatment or dose group. The most frequent were uterine enlargement by cystic and/or polypoid change as well as the presence of ovarian cysts. These changes are considered to be associated with ageing. Focal discolouration (red, tan, gray, brown) occurred at a moderate incidence in the heart, lungs, kidneys, 1iver, uterus and spleen. Irregularities of shape (noted in the kidneys and liver), enlargements of the spleen and mesenteric nodes occurred less frequently. Other observed changes occurred sporadically.

The changes thus mentioned were considered to be incidental findings as expected in ageing mice and were not associated with the test compound.

Histopathological examination revealed several non-neoplastic changes including amyloidosis of the kidney, ileum, duodenum, adrenal glands, ovaries, spleen and liver. Myocardial degeneration with frequent concomitant atrial thrombosis was noted, as were hyperplastic changes of the uterus and lymphoreticular tissue. These changes were of the type and incidence common in populations of ageing laboratory mice and were considered by the authors unrelated to the treatment.

Neoplastic lesions were found in the lungs, liver, lymph nodes and uteri of mice from all groups. The lymphoid neoplasms were heteromorphic and generally multicentric, and involved the spleen and mesenteric and mediastinal lymph nodes. Neither the incidence of neoplasms nor their primary

[24] According to the authors, the test material (FD&C Blue N°. 2) used in this study was manufactured by the Hilton-Davis Chemical Co., Cincinnati. It was certificated by the FDA prior to its use in this study and it was found to contain approximately 93% pure colouring and 7% volatile matter.

locations and histopathological characteristics differed significantly between control and treated animals. There were no statistically significant differences for time-to-tumour analyses between the control and treated animals.

In conclusion, microscopical evaluation of selected tissues revealed a variety of randomly distributed neoplastic, degenerative, hyperplastic and inflammatory changes usually encountered in ageing mice. These changes occurred without relationship to dose and were considered by the study authors to be unrelated to the dietary administration of Indigo Carmine.

The authors concluded that there were no consistent biologically significant compound- or dose-related effects on behaviour, morbidity, haematology or general physical observations and that all of the neoplasms were of types common in ageing mice. It was therefore concluded that this lifetime exposure of mice to Indigo Carmine did not demonstrate carcinogenic or toxic effects. According to the authors, the NOAEL in this study was 5 % in the diet (the highest dose tested) providing an average intake of 8259 mg/kg bw/day for males and 9456 mg/kg bw/day for females. The Panel agreed with this conclusion.

3.2.4.2. Rats

Four long term/carcinogenicity studies in the rat were available (Oettel et al., 1965; Hansen et al., 1966; Borzelleca et al., 1985).

Twenty six female and fifteen male Wistar rats with a body weight of 50 g at the start of the experiment (P-generation) were fed a diet containing 1 % Indigo Carmine, which, according to the authors, corresponded to an intake of 0.1-0.2 g Indigo Carmine/animal/day (equivalent[25] to 500 mg/kg bw/day) (Oettel et al., 1965). The control group for the P-generation consisted of 20 females and 15 males. After six months, some animals (no specification of numbers) of the P-generation treatment group or control group respectively, were mated. During lactation dams were fed a diet without Indigo Carmine. Rats were weaned 2–3 weeks after birth at a body weight of 25–35 g and fed a diet containing 1 % Indigo Carmine (treatment group of F_1 generation: 20 females, 20 males) or no colouring material (control group for the F_1 generation: 5 females, 5 males). To investigate spontaneous tumour development an additional control group of 100 female and 100 male Wistar rats was fed the control diet. Administration of Indigo Carmine to the rats of the P and F_1 generation was stopped after 2 years of treatment. All animals were observed until they died spontaneously or were killed when moribund. After macroscopical inspection neoplasms and conspicuous alterations of tissues were examined microscopically.

The authors combined the results of the treated animals of the P and F_1 generation and of the control animals of the two generations, since there were no differences observed in behaviour, growth, and survival time. No adverse effects were observed in the animals treated with Indigo Carmine. Body weight gains in treated and control animals were reported to be equivalent.

In the treated groups of the P or F_1 generation no malignant tumours were detected. Two out of 125 males and 4 out of 125 females of the control group died due to sarcomas at different locations. Mammary gland fibroadenomas were detected in one male and three females of the treated animals of the P and F_1 generation and in one female of the control animals of the P and F_1 generation. In the additional control group 10 mammary gland fibroadenomas were found in 9 out of 100 females.

The authors concluded that rats fed at a concentration of 1 % Indigo Carmine in the diet did not show any treatment-related pathological damage or tumour development (Oettel et al., 1965).

In a study by Hansen et al. (1966), ten groups of 24 Osborne-Mendel weanling rats equally divided by sex, received either 0.0, 0.5, 1, 2 or 5 % Indigo Carmine (equivalent to 0, 250, 500, 1000 and 2000 mg/kg bw/day) in their diets for 2 years. Animals were weighed weekly, and mortalities and

[25] Calculated by the Panel according to EFSA Scientific Committee (2012)

abnormalities were recorded. Blood counts (white blood cell count, haemoglobin, haematocrit and differential cell count) were performed on 10 animals at each dose at 3, 11, 17 and 22 months. Survivors were autopsied and organ weights recorded for the heart, liver, spleen, kidney and testes. Detailed microscopical examination of the heart, lung, liver, small intestine, colon, bone marrow, leg bone and muscle, urinary bladder, kidney, pancreas, thyroid, adrenal, testis and prostate or ovary and uterus was conducted. No effects were observed on mortality, haematology or on weights of the heart, liver, kidney, spleen and testis of the animals that received Indigo Carmine at dietary levels up to 5 %. The male animals of the two higher doses showed statistically significant growth inhibition. Slight but statistically non-significant growth depression was observed in the females at the same dosages. There was no treatment-related change in mortality, organ weights, haematology, or gross and microscopical examination. The NOAEL of this study was considered by the Panel to be 1 % in the diet, equivalent to 500 mg Indigo Carmine/kg bw/day based on growth inhibition at the highest dose level.

Indigo Carmine was fed to rats in the diet in a long-term toxicity/carcinogenicity study performed in F_1 animals of a reproductive toxicity study (Borzelleca et al., 1985). Indigo Carmine (FD&C Blue No 2[26]) was administered to groups of male and female Charles River CD albino rats (70 animals per sex group) at levels of 0.5, 1.0 and 2.0 % (equal to 304, 632 and 1282 mg/kg bw/day in male rats and 363, 775 and 1592 mg/kg bw/day in female rats). Two control groups, each containing 70 rats (F_1) of each sex, received a basal diet. The maximum duration was 30 months. A complete histopathological analysis was conducted on all animals from the two control groups and from the highest dose group (2 %). The following tissues were examined: adrenals, aorta, bone and marrow (femur), blood smear, brain, oesophagus, eyes, heart, intestine (caecum, colon, duodenum and ileum), kidneys, liver, lung and mainstem bronchi, lymph nodes, mammary gland, nerve, ovaries, pancreas, pituitary, prostate, salivary gland, seminal vesicles, skeletal muscle, spleen, stomach, testes, thymus, thyroid with parathyroids, trachea, urinary bladder, uterus, any tissue with gross changes of uncertain nature and any tissue masses or suspect tumours. If a potential effect was noted in a tissue, then that tissue was examined histologically in all animals. No consistent compound-related biologically adverse effects were noted. There were random statistically significant differences from the controls with respect to body weight, food consumption and clinical chemistry tests. Food consumption by the test groups showed a dose-related increase, probably due to the non-nutritive character of the colour. Age-related testicular atrophy of the seminiferous tubules, accompanied by oligospermia or aspermia, was present in male rats of all groups. These lesions were randomly distributed in treated and control animals and were not dose- related. Increased incidences of pituitary neoplasms (female and male), transitional-cell neoplasms of the urinary bladder (male) and malignant mammary-gland neoplasm (carcinoma and adenocarcinoma, female) were not statistically significantly different from controls.

A statistically significant ($p < 0.05$) increase in the incidence of malignant mammary gland tumours was noted in the high-dose male rats. The highest dose group had an incidence of 7.8 % (5/51 animals) of carcinomas/adenocarcinomas while the combined control groups had an incidence of 3.5 % (4/114 animals).

Male rats that received the highest level of Indigo Carmine showed an increased incidence of gliomas. Since each control group had a glioma incidence of 2.9 % (2/70 animals), the groups were combined for statistical analysis (4/140 animals). The low-dose and mid-dose had an incidence of 2.9 % (2/70 animals). The high-dose had an incidence of 9.9 % (7/71). The difference in incidence of gliomas between the control groups and the high-dose male rats was statistically significant $p < 0.05$. There was no increased incidence of gliomas in female rats. Two gliomas were observed in one female control group and one glioma was observed in the high-dose females. The authors observed that for the gliomas observed in treated animals, there was no clear dose-effect relationship, nor was there a concurrent decrease in survival time. The authors noted that the historical control data suggested that the total 4.4 % incidence of gliomas in all groups of male rats, and the 2.8 % incidence in males and

[26] According to the authors, the test material (FD&C Blue N°. 2) used in this study was manufactured by the Hilton-Davis Chemical Co., Cincinnati. It was certificated by the FDA prior to its use in this study and it was found to contain approximately 93% pure colouring and 7% volatile matter.

females exposed to Indigo Carmine are within the limits of biological variation. The Panel noted, however, that Bernishke et al. (1978) mentioned an incidence of 0.4 % (37/7803) gliomas in controls rats (Sprague Dawleys). Therefore, the Panel considered the high incidence of gliomas in males of the highest dose group cannot be ignored.

The Panel noted that statistically significant increased incidences of gliomas and malignant mammary gland tumours were seen in the male rats at the highest dose level of 1282 mg Indigo Carmine/kg bw/day.

Overall, the Panel considered that the NOAEL of this study is 632 mg/kg bw/day and 1592 mg/kg bw/day for male and female rats, respectively.

3.2.4.3. Dogs

Groups of Beagle dogs (2/sex/group; 1/sex/controls) were given diets containing 1 or 2 % (equivalent to 250 or 500 mg/kg bw/day) Indigo Carmine for up to two years (Hansen et al., 1966). Histopathological examinations were performed on the following organs: heart, lung, liver, gall bladder, kidney, spleen, pancreas, adrenal thyroid, mesenteric lymph node, intestine, bone, brain, testis and prostate or ovary and uterus, urinary bladder, salivary gland, sub-maxillary lymph node and pituitary. In addition, bone-marrow smears from all dogs were studied. Four out of the six dogs treated with 2 % Indigo Carmine died during the two years, and one out of the four treated with 1 % Indigo Carmine were sacrificed in a moribund condition. All deaths were attributed to intercurrent virus infections. No dye-related clinical signs, gross lesions, or microscopic pathology were noted because of deaths from intercurrent virus infections, a no-effect level was not established for Indigo Carmine. The Panel noted that due to the design deficiencies and infections no conclusion could be derived from this study.

3.2.5. Reproductive and developmental toxicity

3.2.5.1. Reproductive toxicity studies

A 2-generation study with continued Indigo Carmine exposure (except during lactation) has been conducted in Wistar rats (Oettel et al., 1965). Both the P generation (15 males and 26 females) and the F_1 generation (20/sex/group; 5/sex/controls) were given diets containing 0 or 1 % Indigo Carmine (equivalent to 0 or 500 mg/kg bw/day) for 2 years and observed over their lifespan. No adverse effects were observed on fertility, growth or survival. Reproductive and developmental parameters were poorly reported. The Panel identified a NOAEL of 500 mg/kg bw/day in this study.

In a 3-generation reproduction study, groups of ten males and twenty female Charles River CD rats were fed Indigo Carmine (FD&C Blue No 2[27]) at dietary levels providing intakes of 0, 2.5, 25, 75 and 250 mg/kg bw/day (Borzelleca et al., 1986). Fur and faeces were bluish-coloured in 75 mg/kg bw/day and 250 mg/kg bw/day groups. Gestation, viability and lactation indices of all litters from exposed animals did not differ from controls. Fertility indices were statistically significantly lower for F_2 female rats in the 2.5 and 25 mg/kg bw/day groups only and consequently considered as not being dose-related. Fertility indices were also reduced in F_{2b} and F_{2c} group of male rats. No statistically significant changes in the fertility index were observed in the F_3 generation. As effects on fertility indices in the F_2 generation were not dose-related and effects were not identified in the F_1 and F_3 generation this effect was not considered to be compound-related. Examination of the ovaries and uteri of F_1 dams killed on gestation day (GD) 19 revealed no gross anatomical abnormalities. No unusual changes were observed in the stillborn pups or in pups that died during the study. No compound-related gross or microscopic pathological lesions were noted in any of the F_1 or F_{3a} rats that were

[27] According to the authors, the test material (FD&C Blue No 2) used in this study was manufactured by the Hilton-Davis Chemical Co., Cincinnati. It was certificated by the FDA prior to its use in this study and it was found to contain approximately 93% pure colouring and 7% volatile matter.

sacrificed and necropsied. Finally, no compound-related organ-weight variations were recorded in the F_1 rats.

According to the authors the NOAEL for this study was 250 mg Indigo Carmine/kg bw/day, the highest dose tested. The Panel agreed with this NOAEL.

3.2.5.2. Developmental toxicity studies

Rats

Charles Rivers CD rats (20 pregnant rats/group) received by gavage on GD 6-15 0.5 % methocel (controls) or Indigo Carmine (FD&C Blue No 2[28]) at doses of 0, 25, 75 or 250 mg/kg bw/day (Borzelleca et al., 1987). Indigo Carmine was dissolved in 0.5 % methocel and administered by gavage. Animals were observed twice daily during the gestation period for signs of overt toxicity. Body weights were recorded on GD 0, 6, 12, 15 and 20. The animals were killed one day before term. No significant compound-related adverse effects were demonstrated on any maternal parameters (appearance or behaviour, body weight or mortality) or on any fetal parameters (body weight, viability or abnormalities) in rats treated with Indigo Carmine.

According to the authors the NOAEL of this study is 250 mg Indigo Carmine/kg bw/day, the highest dose tested. The Panel agreed with this NOAEL.

Rabbits

Pregnant Dutch belted rabbits (10 pregnant does/group) received on GD 6-18 Indigo Carmine (FD&C Blue No 2[29]) in doses of 0, 25, 75 or 250 mg/kg bw/day by gavage (Borzelleca et al., 1987). Indigo Carmine was dissolved in 0.5 % methocel and administered by gavage. Animals were observed twice daily during the gestation period for signs of overt toxicity. Body weights were recorded on GD 0, 6, 13, and 29. The animals were killed 1 day before term. No statistically significant compound-related adverse effects were demonstrated on any maternal parameters (appearance or behaviour, body weight or mortality) or on any fetal parameters (body weight, viability or abnormalities) in rabbits treated with Indigo Carmine.

According to the authors the NOAEL of this study was 250 mg Indigo Carmine/kg bw/day, the highest dose tested. The Panel agreed with this NOAEL.

3.2.6. Hypersensitivity, allergenicity and intolerance

No cases of Indigo Carmine intolerance or allergy have been reported after absorption by the oral route.

4. Discussion

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that became available since then and the data available following EFSA public calls for data. The Panel noted that not all original studies on which previous evaluations were based were available for the re-evaluation.

The Panel noted that the term Indigotin is sometimes used to indicate Indigo while Indigotine is a synonym of Indigo Carmine (Regulation (EC) No 1333/2008). In this re-evaluation, only studies with Indigo Carmine and Indigotine were taken into consideration, provided that the identity of the test material was clear. However, only the term Indigo Carmine is used in this opinion.

[28] According to the authors, the test material (FD&C Blue No 2) used in this study was manufactured by the Waner Jenkinson Co. (St. Louis, MO). It was certificated by the FDA prior to its use in this study and it was found to contain approximately 91% pure colouring and 9% volatile matter.

Indigo Carmine (E 132) is an indigoid colour authorised as a food additive in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 which has been previously evaluated by JECFA in 1969 and 1975 and the SCF in 1975 and 1984.

JECFA established a temporary ADI of 0-2.5 mg/kg bw/day in 1969 that was increased to a full ADI of 0-5 mg/kg bw/day in 1975. These ADIs were probably based on the long-term rat study by Hansen et al. (1966) in which a significantly inhibited growth of male rats was observed at dietary doses of 1000 and 2500 mg/kg bw/day. In this study, the NOAEL was determined to be equivalent to 500 mg/kg bw/day. JEFCA established a temporary ADI in 1969 probably by applying an uncertainty factor of 200 to the above NOAEL. In 1975, JECFA applied an uncertainty factor of 100 to the NOAEL of 500 mg/kg bw/day to derive an ADI of 0-5 mg/kg bw/day for Indigo Carmine.

In 1975 the SCF endorsed an ADI of 0-5 mg/kg bw/day established by JECFA. In the latest evaluation of 1984, taking into consideration more recent studies, the SCF agreed to retain this ADI.

Overall, in vivo studies demonstrated that radioactivity from ^{35}S-Indigo Carmine was poorly detected in urine (1.6 to 2.1 % of the radioactivity) after oral administration in rats. Moreover, incubation of ^{35}S-Indigo Carmine with intestinal contents of rats for 48 hours suggested that isatin-5-sulphonic acid and 5-sulphoanthranilic acid might be metabolites formed by intestinal bacteria. By comparison to the data obtained after intravenous administration, the Panel considered that the data available on the absorption, metabolism and excretion indicated that Indigo Carmine or its metabolites were poorly absorbed. However, the Panel noted that both identification of faecal metabolites and tissue distribution of radioactivity were not investigated in these studies.

Overall, the Panel considered that based on the available data, including a newly performed in vivo study on micronuclei induction, Indigo Carmine does not raise concern for genotoxicity.

In a subacute toxicity study (45-day) performed on adult male Swiss albino mice of B-6 strain (5 animals/group) at oral doses of 0, 17 and 39 mg Indigo Carmine/kg bw/day, statistically significant severe adverse effects on the testis were described (Dixit and Goyal, 2013). The Panel noted that no NOAEL could be identified in this study and the LOAEL was 17 mg/kg bw/day, the lowest dose level tested. The Panel noted limitations in the design of the study and lack of information on the specifications of the test material used in the study (E 132; FD&C Blue # 2; CI 73015) was procured from the local market in India).

Several chronic toxicity studies were available when JECFA allocated the ADI (1975). Two chronic and carcinogenicity studies, one in rats and one in mice were published since the latest evaluation of Indigo Carmine by JECFA in 1975.

In the mouse study, microscopic evaluation of selected tissues revealed a variety of randomly distributed neoplastic, degenerative, hyperplastic and inflammatory changes usually encountered in ageing mice (Borzelleca and Hogan, 1985). These changes occurred without relationship to dose and are considered to be unrelated to the dietary administration of Indigo Carmine. It was therefore concluded that this lifetime exposure of mice to Indigo Carmine did not demonstrate carcinogenic or toxic effects. The Panel identified a NOAEL in this study equal to 5 % in the diet providing an average intake of 8259 mg/kg bw/day for males and 9456 mg/kg bw/day for females.

In a chronic/carcinogenicity study, a statistically significant increased incidence of gliomas and malignant mammary gland tumours was seen in the male rats at the highest dose level of 1282 mg Indigo Carmine/kg bw/day. Overall, the Panel identified a NOAEL in this study of 632 mg/kg bw/day for male rats, the mid-dose, based on these carcinogenic effects. In the absence of any genotoxic activity, the Panel considered that Indigo carmine was acting as a non-genotoxic carcinogen and therefore, that there would be a threshold for this effect.

In a multigeneration reproductive toxicity study no test substance related effects were observed up to doses of 250 mg/kg bw/day.

No adverse effects have been detected in developmental studies in rats and rabbits at tested doses (0, 25, 75 or 250 mg/kg bw/day). The Panel noted that the concentrations tested in the developmental toxicity studies were lower than the dose levels up to 2000 mg/kg bw/day used in several subacute or long-term toxicity studies but close to the NOAEL of 500 mg/kg bw/day on which JECFA based its ADI.

No cases of Indigo Carmine intolerance or allergy have been reported after ingestion.

No adverse effects in subacute, chronic, reproduction and developmental toxicity studies, and no modifications of haematological and biological parameters in chronic toxicity studies have been identified at doses less than or equal to 500 mg/kg bw/day. The only report of an adverse effect was in testis with a LOAEL of 17 mg/kg bw/day by Dixit and Goya (2013). No such adverse effects have been described on testis in long-term toxicity studies in mice and rats (weight of testis, histopathology examination) at higher doses. Age-related testicular atrophy of the seminiferous tubules, accompanied by oligospermia or aspermia, was present in male rats of all groups in the 2-year chronic toxicity/carcinogenicity study (Borzelleca et al., 1985) but these lesions were randomly distributed in treated and control animals and were not dose-related. The Panel noted also that fertility indices were reduced in F_{2b} and F_{2c} group of male rats, but these changes were not considered to be compound-related because they were not dose-related in the 3-generation reproductive toxicity study (Borcella et al, 1986). Overall, the Panel noted that no effects on the testes or reproductive function were observed in chronic toxicity studies and in a 3-generation reproduction toxicity study undertaken with Indigo Carmine (FD&C Blue No 2) containing approximately 93% pure colouring and 7% volatile matter (Borzelleca et al., 1985, 1986,; Borzelleca and Hogan 1985). The Panel considered that the Dixit and Goyal (2013) study has shortcomings since it is not clear to the Panel whether the adverse effects observed were due to the food additive itself or to impurities and/or contaminants present in the material tested and/or to the conduct of the study.

The Panel considered that the current ADI of 5 mg/kg bw/day was applicable to a material with a purity of 93% pure colouring matter and manufactured using processes resulting in comparable residuals as material used in the Borzelleca et al. studies (1985, 1986) and Borzelleca and Hogan (1985).

Exposure assessment for food additives under re-evaluation was carried out by the ANS Panel based on (1) MPLs set down in the EU legislation (defined as the *regulatory maximum level exposure assessment* scenario) and (2) the availability of adequate usage or analytical data (defined as the *refined exposure assessment* scenario).

To date, the ANS Panel has used the maximum concentration value (maximum reported use level or maximum value from the analytical results) available for each authorised food category. However, given the extensive range of analytical data that have been made available through the most recent calls, the ANS Panel considered this should also be used in additional scenarios of the exposure assessment approach intended to provide more realistic exposure estimates.

Based on the available dataset, the Panel calculated two estimates based on different assumptions: a brand-loyal consumer scenario, where it was assumed that the population is exposed long-term to the food additive present at the maximum reported use/analytical levels for one food category; and a non-brand-loyal scenario, where it was assumed that the population is exposed long-term to the food additive present at the mean reported use/analytical levels in the food.

Overall, the Panel considered the regulatory maximum level exposure assessment scenario as being conservative since it assumes that all processed foods and beverages contain the food additive Indigo Carmine at the MPLs. The Panel considered that the refined exposure assessment approach was a

more realistic scenario, since it was based on the extensive range of analytical data and assumes that the processed foods and beverages contain the additive at the mean concentration level for all products (non-brand-loyal consumer scenario) and considers one product containing Indigo Carmine at the maximum concentration level (brand-loyal consumer scenario). For this exposure assessment scenario, food categories for which none or inadequate reported use/analytical levels were available were not considered in the exposure assessment. Therefore, the Panel noted that if Indigo Carmine is nevertheless used in those food categories not considered in the exposure estimate, the calculated refined exposure assessment might result in underestimation of exposure to Indigo Carmine. The Panel also noted that the refined exposure estimates will not cover future changes in the level of use of Indigo Carmine.

Using the regulatory maximum level exposure assessment scenario, mean exposure to Indigo Carmine from its use as a food additive ranged from 0.9-3.8 mg/kg bw/day in toddlers, 0.8-3.2 mg/kg bw/day in children, 0.3-1.5 mg/kg bw/day in adolescents, 0.3-1.0 mg/kg bw/day in adults and 0.1-0.6 mg/kg bw/day in the elderly. The high exposure to Indigo Carmine using this scenario ranged from 2.8-7.1 mg/kg bw/day in toddlers, 1.8-6.7 mg/kg bw/day in children, 1.0-3.2 mg/kg bw/day in adolescents, 0.7-2.3 mg/kg bw/day in adults and 0.5-1.4 mg/kg bw/day in the elderly. The Panel noted that exposure estimates of Indigo Carmine (E 132) exceed the ADI for toddlers and children at the high level (95th percentile). The main contributing food categories to the total mean exposure estimates for adolescents, adults and the elderly in this scenario were flavoured drinks and fine bakery wares. For children, the main contributing food categories were flavoured drinks, fine bakery wares and flavoured fermented milk products, while for toddlers, the main contributing food categories were flavoured fermented milk products and fine bakery wares.

Using the refined brand-loyal assessment exposure scenario, mean exposure to Indigo Carmine from its use as a food additive ranged from 0.02 mg/kg bw/day in adults and the elderly to 0.4 mg/kg bw/day in toddlers. The high exposure to Indigo Carmine using this scenario ranged from 0.1 mg/kg bw/day in children, adults and the elderly to 0.8 mg/kg bw/day in toddlers and children. The main contributing food categories for adolescents, adults and the elderly were flavoured drinks and fine bakery wares. For children, the main contributing food categories were fine bakery wares, flavoured drinks and other confectionary including breath freshening microsweets. For toddlers, in addition to fine bakery wares, flavoured drinks and flavoured fermented milk products were also main contributing food categories.

Using the refined non-brand-loyal assessment exposure scenario, mean exposure to Indigo Carmine from its use as a food additive ranged from 0.003 mg/kg bw/day in the elderly to 0.2 mg/kg bw/day in toddlers. The high exposure to Indigo Carmine using this scenario ranged from 0.01 mg/kg bw/day in the elderly to 0.3 mg/kg bw/day in children. The main contributing food categories for adolescents, adults and the elderly were also flavoured drinks and fine bakery wares. For children, the main contributing food categories were fine bakery wares and flavoured drinks. For toddlers, fine bakery wares and flavoured fermented milk products were the main contributing food categories.

For the refined exposure assessment scenarios (brand-loyal and non-brand-loyal), none of the population groups exceeded the ADI of 5 mg/kg bw/day, neither at the mean nor at the high level (95th percentile). However, the Panel noted that this scenario was calculated using limited data provided by industry and analytical data made available by EFSA call of data. The Panel further noted that for the food categories where both usage levels and analytical results were available, the analytical results were much lower than the use levels reported by industry.

The Panel noted that both reported use levels and quantified analytical results were reported only for the food categories of chewing gum, flavoured drinks and desserts. For several food products for which the use of Indigo Carmine is authorised e.g. flavoured fermented milk products, seasonings and condiments, mustard, soups and broths, sauces, cider and perry, aromatised wine based-products and other alcoholic drinks, no usage were reported and also reported analytical data showed only limited sample sizes (n<20). In addition, the Panel further noted that for some other authorised food categories

such as edible ices, fine bakery wares, fish roe, spirit drinks, potato-, cereal, flour-, or starch-based snacks and processed nuts, no usages had been reported by industry, while positive analytical results (> LOQ) were obtained from the analytical data.

The Panel noted that the three main contributing food categories for age-groups where MPL scenario estimates exceeded the ADI showed high number of analytical data far below MPL or had no use level reported combined with no detection in limited analytical data. The Panel therefore considered that it is not likely that the ADI will be exceeded.

CONCLUSIONS

The Panel noted that no effects on the testes or reproductive function were observed in chronic toxicity studies and in a 3-generation reproduction toxicity study undertaken with Indigo Carmine (FD&C Blue No 2) containing approximately 93% pure colouring and 7% volatile matter (Borzelleca et al., 1985, 1986; Borzelleca and Hogan 1985). The EFSA Guidance for submission for food additives evaluation requires information on the manufacturing process to identify hazards which may need to be controlled in the specifications. The Panel considered that the current ADI of 5 mg/kg bw/day was applicable to a material with this purity manufactured using processes resulting in comparable residuals.

The Panel also noted that a new study with Indigo Carmine (E 132; FD&C Blue # 2; CI 73015) showing adverse effects on testis (Dixit and Goyal, 2013) would give rise to a safety concern if confirmed. The Panel considered that the Dixit and Goyal (2013) study has shortcomings since it is not clear to the Panel whether the adverse effects observed were due to the food additive itself or to impurities and/or contaminants present in the material tested and/or to the conduct of the study. However, the Panel noted that the material as described in the publication is presumed to meet the current EU specifications and it cannot be excluded that such material could be legally used as a food additive in the EU market. Whilst there is no supporting evidence for these effects in the toxicological database, the Panel cannot confirm the applicability of the existing ADI for all materials (Indigo Carmine) meeting the current EU specifications (total colouring matter > 85%).

The Panel concluded that any extension of this ADI to Indigo Carmine of lower purity and/or manufactured using a different process would require new data which would need to address the adverse effects on testis observed in the Dixit and Goyal (2013) study. The chemical identity of the tested food additive, including the presence of possible impurities and/or contaminants, should be investigated and reported.

The Panel confirmed the ADI of 5 mg/kg bw/day for Indigo Carmine of at least 93% purity manufactured using the same or equivalent manufacturing process resulting in material tested in Borzelleca et al. studies. Given the uncertainties in the database, the Panel was not able to conclude whether this ADI should apply to Indigo Carmine with lower purity manufactured using these same processes or material manufactured using a different but not equivalent process.

Furthermore, the Panel concluded that the current specifications should be revised in order to restrict the Indigo Carmine (E 132) permitted as food additive to that for which the ADI is applicable.

The Panel concluded that at the maximum permitted level of use, exposure estimates of Indigo Carmine (E 132) would exceed the ADI for toddlers and children at the high level. Exposure estimates using the available usage and analytical data did not show an exceedance of the ADI for any population groups.

The Panel noted that the three main contributing food categories for age-groups where MPL scenario estimates exceeded the ADI showed high number of analytical data far below MPL or had no use level reported combined with no detection in limited analytical data. The Panel therefore considered that it is not likely that the ADI will be exceeded.

DOCUMENTATION PROVIDED TO EFSA

1. Pre-evaluation document prepared by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. February, 2008.
2. Reply to EFSA: Re-evaluation of food colours: call for data (7.12.06). Final submission of data_E132 Indigotine. Provided by The Robert Group, LLC, January, 2008.
3. Biodynamics Inc. 1981a. A long term oral carcinogenicity study of FD&C Blue # 2 in mice. Final report. Pathology report-volume I. Unpublished report provided by International Association of Color Manufacturers (IACM), June 2010.
4. Biodynamics Inc. 1981b. A long term oral toxicity/carcinogenicity study of FD&C Blue # 2 in rats. Final report, Volume I, toxicology report. Unpublished report provided by the FDA, November 2011.
5. Association of the European Self-Medication Industry (AESGP). Data on use levels of Indigo Carmine (E 132) in foods. Submitted on 31 July 2013.
6. Capsugel. Data on use levels of Indigo Carmine (E 132) in foods. Submitted on 9 September 2013.
7. FoodDrinkEurope (FDE). Data on use levels of Indigo Carmine (E 132) in foods. Submitted on 13 September 2013.
8. International Chewing Gum Association (ICGA). Data on use levels of Indigo Carmine (E 132) in foods. Submitted on 26 September 2013.
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APPENDICES

Appendix A. Summary of reported use levels (mg/kg) of Indigo Carmine (E 132) provided by industry

FCS Category No	FCS Food category	MPL	Restrictions/ exceptions	Number of data	Reported use levels from industry		Information provided by	Comments
					Typical mean (Range)	Highest maximum level		
05.3	Chewing gum	300		1	24	290	ICGA (Only a proportion of chewing gum contains this food additive and even less contains it at the maximum reported level.)	
05.4	Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	500	only decorations, coatings and sauces, except fillings	1	9	9	FDE	
14.1.4	Flavoured drinks	100	excluding chocolate milk and malt products	1 NP	20	20	FDE	As the level is on a niche product (NP), the mean value used for the exposure assessment was taken from the analytical results from MSs
16	Desserts excluding products covered in category 01, 03 and 04	150		1	20	35	FDE	
17	Food supplements as defined in Directive 2002/46/EC of the European Parliament and of the Council excluding food supplements for infants and young children	300		2	45 (10-80)	280	AESGP	
				1	0.48	0.96	Capsugel (data on empty capsules, gelatine or HPMC based)	Data considered as not relevant for the exposure assessment

Appendix B. Summary of analytical results (mg/kg) of Indigo Carmine (E 132) provided by Members States

FCS Category No	FCS food category	MPL	Total number of data	Number of left-censored data			All data										Positive values					
					Range		Middle bound					Upper bound					Number of positive values					
					LOD	LOQ	min	median	mean	p95	max	min	median	mean	p95	max		min	median	mean	p95	max
01.4	Flavoured fermented milk products including heat treated products	150	10	10	0.17-20	0.5-60	0.3	4.0	3.2	10.0	10.0	0.5	8.0	6.4	20	20						
1.6.3	Other creams	150	2	1	0.17-0.2	0.5	0.3	0.5	0.5	0.8	0.8	0.5	0.7	0.7	0.8	0.8	1	0.8	0.8	0.8	0.8	0.8
1.7.1	Unripened cheese excluding products falling in category 16	150	3	3	0.17	0.5-2	0.3	1.0	0.8	1.0	1.0	0.5	2.0	1.5	2	2						
3	Edible ices	150	136	128	0.04-20	0.1-60	0.1	2.5	2.1	2.5	16.0	0.1	5.0	3.9	5	20	8	0.1	0.3	5.0	16.0	16.0
5.2	Other confectionery including breath refreshening microsweets – except candied fruit and vegetables	300	586	457	0.04-20	0.1-60	0.1	2.5	6.3	10.0	281.0	0.1	3.1	10.0	20	281	129	0.1	1.7	11.6	66.8	281.0
5.2	Other confectionery including breath refreshening microsweets – only candied fruit and vegetables	200	51	51	0.03-20	0.1-60	0.1	0.3	1.9	10.0	10.0	0.1	0.5	3.8	20	20						
5.3	Chewing gum	300	26	20	0.04-20	0.1-60	0.3	1.5	4.3	10.0	10.0	0.3	3.0	8.3	20	20	6	0.3	0.6	1.6	4.5	4.5

FCS Category No	FCS food category	MPL	Total number of data	Number of left-censored data			All data										Positive values					
					Range		Middle bound					Upper bound					Number of positive values					
					LOD	LOQ	min	median	mean	p95	max	min	median	mean	p95	max		min	median	mean	p95	max
5.4	Decorations, coatings and fillings, except fruit based fillings covered by category 04.2.4	500	40	38	0.03-20	0.2-60	0.4	1.5	6.5	18.2	67.7	0.8	3.0	10.6	23.15	67.7	2	26.3	47.0	47.0	67.7	67.7
6.6	Batters	500	1	1	20	60	10.0	10.0	10.0	10.0	10.0	20.0	20.0	20.0	20	20						
7.2	Fine bakery wares	200	907	892	0.04-20	0.1-60	0.1	10.0	8.9	10.0	34.2	0.1	20.0	17.7	20	34.2	15	0.1	1.5	4.8	34.2	34.2
9.3	Fish roe	300	6	2	0.04-0.2	0.1-2	0.3	1.0	8.8	48.0	48.0	0.3	1.7	9.1	48	48	4	0.3	1.2	12.7	48.0	48.0
12.2.2	Seasonings and condiments	500	15	15	3-20	10-60	1.5	10.0	7.8	10.0	10.0	3.0	20.0	15.6	20	20						
12.4	Mustard	300	6	6	0.17	0.5-5	0.3	2.5	1.8	2.5	2.5	0.5	5.0	3.5	5	5						
12.5	Soups and broths	50	10	10	3-20	10-60	1.5	10.0	9.2	10.0	10.0	3.0	20.0	18.3	20	20						
12.6	Sauces	500	30	30	3-20	2.75-60	1.4	10.0	8.1	10.0	10.0	2.8	20.0	16.2	20	20						
14.1.4	Flavoured drinks	100	1056	1052	0.04-20	0.1-60	0.0	1.5	3.1	10.0	10.0	0.1	3.0	6.3	20	20	4	0.2	0.3	0.3	0.3	0.3
14.2.3	Cider and perry	200	1	1	3	10	1.5	1.5	1.5	1.5	1.5	3.0	3.0	3.0	3	3						
14.2.4	Fruit wine and made wine	200	33	33	0.12-20	0.42-60	0.1	1.5	3.8	10.0	10.0	0.1	3.0	7.5	20	20						
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	200	65	38	0.04-20	0.1-60	0.0	0.3	1.8	10.0	10.0	0.1	0.5	3.4	20	20	27	0.1	0.2	0.6	2.1	4.1
14.2.7.2	Aromatised wine-based drinks	200	1	1	20	60	10.0	10.0	10.0	10.0	10.0	20.0	20.0	20.0	20	20						
14.2.7.3	Aromatised wine-product cocktails	200	12	12	0.12-20	0.42-60	0.1	1.5	3.7	10.0	10.0	0.1	3.0	7.5	20	20						
14.2.8	Other alcoholic drinks including mixtures of	200	19	19	0.06-20	0.14-60	0.0	1.5	2.5	10.0	10.0	0.1	3.0	5.0	20	20						

FCS Category No	FCS food category	MPL	Total number of data	Number of left-censored data	Range		All data									Positive values					
							Middle bound					Upper bound					Number of positive values					
					LOD	LOQ	min	median	mean	p95	max	min	median	mean	p95	max		min	median	mean	p95	max
	alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol																				
15.1	Potato-, cereal-, flour- or starch-based snacks	100	25	5	0.04-20	0.1-60	0.1	2.5	8.1	35.0	38.5	0.1	3.0	9.0	34.95	38.5	20	0.1	2.5	9.0	36.7	38.5
	Potato-, cereal-, flour- or starch-based snacks	200																				
15.2	Processed nuts	100	3	2	0.17-5	0.5-20	0.1	2.5	4.0	9.3	9.3	0.2	5.0	4.8	9.3	9.3	1	9.3	9.3	9.3	9.3	9.3
16	Desserts excluding products covered in categories 01, 03 and 04	150	39	38	0.8-20	2-60	0.4	10.0	7.8	10.0	10.0	0.8	20.0	15.5	20	20	1	6.1	6.1	6.1	6.1	6.1
17	Food supplements supplied in a solid form including capsules and tablets and similar forms excluding chewable forms	300	36	36	0.17-20	0.5-60	0.3	10.0	6.8	10.0	10.0	0.5	20.0	13.6	20	20						
	Food supplements supplied in a liquid form	100																				

FCS Category No	FCS food category	MPL	Total number of data	Number of left-censored data			All data										Positive values					
					Range		Middle bound					Upper bound					Number of positive values					
					LOD	LOQ	min	median	mean	p95	max	min	median	mean	p95	max		min	median	mean	p95	max
	Food supplements supplied in a syrup-type or chewable form	300																				
	Food supplements supplied in a syrup-type or chewable form	100																				

Appendix C. Concentration levels of Indigo Carmine (E 132) used in the refined exposure scenarios (mg/kg)

FCS Category No	FCS Food category	MPL	Concentration levels used in the refined exposure assessment		Data source / Comments
			mean	max	
01.4	Flavoured fermented milk products	150	3.2	20	Analytical results
01.6.3	Other creams	150	-	-	Not taken into account (food category too broad in FoodEx)
01.7.1	Unripened cheese excluding products falling in category 16	150	0.8	2	Analytical results
01.7.3	Edible cheese rind	QS	-	-	Not taken into account (no corresponding FoodEx code)
01.7.6	Cheese products (excluding products falling in category 16)	100	-	-	Not taken into account (no corresponding FoodEx code)
03	Edible ices	150	2.1	20	Analytical results
04.2.4.1	Fruit and vegetable preparations excluding compote - only *mostarda di frutta*	200	-	-	Not taken into account (no corresponding FoodEx code)
05.2	Other confectionery including breath freshening microsweets – except candied fruit and vegetables	300	6.3	281	Analytical results
05.2	Other confectionery including breath freshening microsweets – only candied fruit and vegetables	200	1.9	20	Analytical results
05.3	Chewing gum	300	4.3	290	Analytical results/ Reported use levels
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4 - only decorations, coatings and sauces, except fillings	500	-	-	Not taken into account (no corresponding FoodEx code)
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 04.2.4 - only fillings	300	-	-	Not taken into account (no corresponding FoodEx code)
06.6	Batters - only batters for coating	500	-	-	Not taken into account (no corresponding FoodEx code)
07.2	Fine bakery wares	200	8.9	34.2	Analytical results
08.2.3	Casings and coatings and decorations for meat - only decorations and coatings except edible external coating of *pasturmas*	500	-	-	Not taken into account (no corresponding FoodEx code)
08.2.3	Casings and coatings and decorations for meat - only edible casings	QS	-	-	Not taken into account (no corresponding FoodEx code)
09.2	Processed fish and fishery products including molluscs and crustaceans - only surimi and similar products and salmon substitutes	500	-	-	Not taken into account (no analytical results or reported use levels)
09.3	Fish roe	300	8.8	48	Analytical results
12.2.2	Seasonings and condiments	500	7.8	20	Analytical results
12.4	Mustard	300	1.8	5	Analytical results

FCS Category No	FCS Food category	MPL	Concentration levels used in the refined exposure assessment		Data source / Comments
			mean	max	
12.5	Soups and broths	50	9.2	20	Analytical results
12.6	Sauces - including pickles, relishes, chutney and piccalilli; excluding tomato-based sauces	500	8.1	20	Analytical results
12.9	Protein products, excluding products covered in category 01.8 - only meat and fish analogues based on vegetable proteins	100	-	-	Not taken into account (no analytical results or reported use levels)
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)	50	-	-	Not taken into account (no analytical results or reported use levels)
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	50	-	-	Not taken into account (no analytical results or reported use levels)
14.1.4	Flavoured drinks - excluding chocolate milk and malt products	100	3.1	20	Analytical results/ Reported use levels
14.2.3	Cider and perry	200	1.5	3	Analytical results
14.2.4	Fruit wine and made wine	200	-	-	Not taken into account (no corresponding FoodEx code)
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	200	1.8	4.1	Analytical results
14.2.7	Aromatised wine-based products	200	4.2	20	Analytical results
14.2.8	Other alcoholic drinks incl. mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	200	2.5	20	Analytical results
15.1	Potato-, cereal-, flour- or starch-based snacks	100/200	8.1	38.5	Analytical results
15.2	Processed nuts	100	4.0	9.3	Analytical results
16	Desserts	150	20	35	Reported use levels
17	Food supplements	100/300	45	280	Reported use levels

Appendix D. Summary of total estimated exposure of indigo carmine (E 132) from its use as a food additive for MPL scenario and refined exposure scenarios per population group and survey: mean and high level (mg/kg bw/day)

	Number of subjects	MPL scenario		Brand-loyal scenario		Non-brand-loyal scenario	
		Mean	High level	Mean	High level	Mean	High level
Toddlers							
Belgium (Regional_Flanders)	36	3.8	-	0.4	-	0.2	-
Bulgaria (NUTRICHILD)	428	1.1	2.8	0.2	0.4	0.0	0.1
Germany (DONALD_2006_2008)	261	1.1	3.0	0.1	0.3	0.0	0.1
Spain (enKid)	17	1.5	-	0.2	-	0.1	-
Finland (DIPP_2003_2006)	497	0.9	3.1	0.1	0.4	0.0	0.1
Italy (INRAN_SCAI_2005_06)	36	0.9	-	0.1	-	0.0	-
Netherlands (VCP_kids)	322	3.3	7.1	0.4	0.8	0.1	0.3
Children							
Belgium (Regional_Flanders)	625	3.2	6.7	0.3	0.8	0.1	0.3
Bulgaria (NUTRICHILD)	433	1.4	3.5	0.2	0.5	0.0	0.1
Czech Republic (SISP04)	389	1.8	4.1	0.2	0.7	0.1	0.1
Germany (DONALD_2006_2008)	660	1.8	3.9	0.2	0.5	0.1	0.1
Denmark (Danish_Dietary_Survey)	490	1.5	2.7	0.2	0.5	0.0	0.1
Spain (enKid)	156	1.5	3.5	0.2	0.5	0.1	0.1
Spain (NUT_INK05)	399	1.5	3.2	0.1	0.3	0.1	0.1
Finland (DIPP_2003_2006)	933	1.2	2.9	0.2	0.6	0.0	0.1
Finland (STRIP)	250	2.4	4.1	0.3	0.7	0.1	0.2
France (INCA2)	482	1.6	3.1	0.2	0.3	0.1	0.1
Greece (Regional_Crete)	839	1.0	2.2	0.1	0.3	0.0	0.1
Italy (INRAN_SCAI_2005_06)	193	0.8	1.8	0.1	0.2	0.0	0.1
Latvia (EFSA_TEST)	189	1.4	3.3	0.2	0.5	0.1	0.2
Netherlands (VCP_kids)	957	3.0	6.1	0.3	0.7	0.1	0.3
Sweden (NFA)	1473	2.8	5.5	0.3	0.7	0.1	0.2
Adolescents							
Belgium (Diet_National_2004)	584	1.1	2.4	0.1	0.3	0.0	0.1
Cyprus (Childhealth)	303	0.3	1.0	0.0	0.1	0.0	0.0
Czech Republic (SISP04)	298	1.3	3.2	0.2	0.5	0.0	0.1
Germany (National_Nutrition_Survey_II)	1011	0.9	2.3	0.1	0.3	0.0	0.1
Denmark (Danish_Dietary_Survey)	479	1.1	2.4	0.2	0.5	0.0	0.1
Spain (AESAN_FIAB)	86	0.4	1.0	0.1	0.1	0.0	0.0
Spain (enKid)	209	1.0	2.3	0.1	0.3	0.0	0.1
Spain (NUT_INK05)	651	0.8	1.8	0.1	0.2	0.0	0.1
France (INCA2)	973	0.8	1.7	0.1	0.2	0.0	0.1
Italy (INRAN_SCAI_2005_06)	247	0.5	1.1	0.0	0.1	0.0	0.0
Latvia (EFSA_TEST)	470	0.9	2.3	0.1	0.3	0.1	0.1
Sweden (NFA)	1018	1.5	3.2	0.2	0.5	0.0	0.1
Adults							

	Number of subjects	MPL scenario		Brand-loyal scenario		Non-brand-loyal scenario	
		Mean	High level	Mean	High level	Mean	High level
Belgium (Diet_National_2004)	1304	0.8	2.1	0.1	0.2	0.0	0.1
Czech Republic (SISP04)	1666	0.5	1.4	0.1	0.2	0.0	0.0
Germany (National_Nutrition_Survey_II)	10419	0.7	1.7	0.1	0.2	0.0	0.1
Denmark (Danish_Dietary_Survey)	2822	0.5	1.3	0.1	0.2	0.0	0.0
Spain (AESAN)	410	0.4	1.0	0.0	0.1	0.0	0.0
Spain (AESAN_FIAB)	981	0.3	0.8	0.0	0.1	0.0	0.0
Finland (FINDIET_2007)	1575	0.3	1.1	0.1	0.2	0.0	0.0
France (INCA2)	2276	0.5	1.2	0.0	0.1	0.0	0.0
United Kingdom (NDNS)	1724	0.7	1.4	0.1	0.1	0.0	0.0
Hungary (National_Repr_Surv)	1074	0.3	0.8	0.0	0.1	0.0	0.0
Ireland (NSIFCS)	958	0.5	1.1	0.0	0.1	0.0	0.0
Italy (INRAN_SCAI_2005_06)	2313	0.3	0.7	0.0	0.1	0.0	0.0
Latvia (EFSA_TEST)	1306	0.5	1.2	0.1	0.1	0.0	0.1
Netherlands (DNFCS_2003)	750	1.0	2.3	0.1	0.3	0.0	0.1
Sweden (Riksmaten_1997_98)	1210	0.6	1.5	0.1	0.2	0.0	0.0
Elderly and very elderly							
Belgium (Diet_National_2004)	1230	0.6	1.4	0.1	0.2	0.0	0.1
Germany (National_Nutrition_Survey_II)	2496	0.5	1.2	0.1	0.1	0.0	0.1
Denmark (Danish_Dietary_Survey)	329	0.2	0.7	0.0	0.1	0.0	0.0
Finland (FINDIET_2007)	463	0.1	0.6	0.0	0.1	0.0	0.0
France (INCA2)	348	0.4	0.9	0.0	0.1	0.0	0.0
Hungary (National_Repr_Surv)	286	0.2	0.7	0.0	0.1	0.0	0.0
Italy (INRAN_SCAI_2005_06)	518	0.2	0.5	0.0	0.1	0.0	0.0

GLOSSARY AND ABBREVIATIONS

ADI	Acceptable daily intake
AESGP	Association of the European Self-Medication Industry
Aluminium lakes	Aluminium lakes are produced by the absorption of water soluble dyes onto a hydrated aluminium substrate rendering the colour insoluble in water. The end product is coloured either by dispersion of the lake into the product or by coating onto the surface of the product
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
bw	Body weight
CAS	Chemical abstracts service
DNA	Deoxyribonucleic acid
EC	European Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Chemical Substances
EU	European Union
FAO/WHO	Food and Agriculture Organization/World Health Organization
FDA	Food and Drug Administration
FDE	FoodDrinkEurope
FSC	Food Categorisation System
GD	Gestation day
GLP	Good laboratory practices
HPC/DR	Hepatocyte Primary Culture/DNA Repair
HPLC	High-performance liquid chromatography
ICGA	International Chewing Gum Association
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization.
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid chromatography
LD_{50}	Lethal Dose, 50 % i.e. dose that causes death among 50 % of treated animals
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOQ	Limit of quantification
MN PCE	Micronucleated polychromatic erythrocytes
MPL	Maximum Permitted Level
NDNS	UK National Diet and Nutrition Survey
NOAEL	No Observed Adverse Effect Level

NP	Niche products
OECD	Organisation for Economic Co-operation and Development
PDA	Diode array detector
QS	*Quantum Satis*
SCE	Sister Chromatid Exchange
SCF	EU Scientific Committee on Food
SGOT	Serum glutamic oxaloacetic transaminase
SGTP	Serum glutamic pyruvic transaminase
T4	Thyroxine hormone
tADI	Temporary acceptable daily intake
TLC	Thin layer chromatography
TWI	Tolerable Weekly Intake
UNESDA	Union of European Beverage Associations