SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of Brown HT (E 155) as a food additive[1]

EFSA Panel on Food Additives and Nutrient Sources (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

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ABSTRACT

The Panel on Food Additives and Nutrient Sources added to Food provides a scientific opinion re-evaluating the safety of Brown HT (E 155). Brown HT has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and 1984 and the EU Scientific Committee for Food (SCF) in 1975 and 1984. JECFA established an Acceptable Daily Intake (ADI) of 0-1.5 mg/kg body weight (bw)/day, while the SCF established an ADI of 0-3 mg/kg bw/day. 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 a public call for data. The Panel concluded that based on a long-term carcinogenicity and toxicity study in mice an ADI of 1.5 mg/kg bw/day can be derived. The Panel concludes that at both the maximum permitted level of use (Tier 2) and at the maximum reported levels of use of Brown HT (Tier 3), mean intake estimates are generally below the ADI of 1.5 mg/kg bw/day. However, in both adults and 1-10 years old children, the high percentile of exposure for both Tiers can be higher than the ADI at the upper end of the range.

KEY WORDS

Brown HT, E 155, CAS Registry Number 4553-89-3, disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylene bis-azo) di-(naphthalene-1-sulfonate), synthetic food colorant.

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SUMMARY

Following a request from the European Commission to the European Food Safety Authority (EFSA), the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion re-evaluating the safety of Brown HT (E 155) when used as a food colouring substance.

Brown HT (E 155) is a synthetic bis-azo dye authorised as a food additive in the EU and previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and the EU Scientific Committee for Food (SCF) in 1984. In 1984 JECFA established an Acceptable Daily Intake (ADI) of 0-1.5 mg/kg bw/day, while the SCF established an ADI of 0-3 mg/kg bw/day.

Based on studies with radioactive Brown HT, the Panel concluded that Brown HT or its metabolites are absorbed to a limited extent in mice, rats and guinea pigs and are excreted predominantly in faeces (up to 90%) and urine (13-16%). Faecal extracts of mice and rats contained only small amounts of unchanged Brown HT, naphthionic acid and two unidentified metabolites. Urine contained naphthionic acid and one unidentified metabolite. These findings indicate that the azo-bonds of Brown HT are reductively cleaved by intestinal bacteria as is the case with other azo-dyes. It is unclear if the central ring structure (2,4-dihydroxy-3,5-diamino benzyl alcohol; an aromatic amine) is released. The radiolabel was distributed limitedly and mostly associated with the gastrointestinal tract and to a minor extent with the liver and kidney in both rats and male mice. Brown HT and/or its metabolites deposited only in the kidney and mesenteric lymph nodes.

In a short-term toxicity studies of Brown HT in rats, effects generally appeared compound-related but not dose-related, or were observed at doses that would not lead to a lowering of the current ADI.

Bacterial genotoxicity tests with Brown HT have been negative. Because the activation process of these bis-azo dyes in animals is complex, bacterial tests with S9 might not be suitable to detect mammalian genotoxicity.

The Panel noted that Prival and Mitchell (1982) demonstrated that the metabolic conditions of the standard Ames test protocol were not appropriate for testing azo dyes for mutagenic activity in *Salmonella typhimurium* and developed a specific protocol including use of flavin mononucleotide (FMN) rather than riboflavin to reduce the azo compounds to free amines, and hamster liver S9 rather than rat liver S9 for metabolic activation. The Panel therefore noted that a final conclusion from negative Ames test results obtained under standard conditions cannot be drawn. The conversion of the parent compound by azo-reduction *in vivo* results in the formation of sulphonated naphthylamines as well as unsulphonated aromatic amines that may not be formed in the standard *in vitro* genotoxicity tests. Previously, a range of sulphonated aromatic amines, including the ones formed from Brown HT upon azo-reduction such as naphthionic acid, was shown to be in general not associated with genotoxicity *in vitro* and *in vivo*. In contrast with their unsulphonated analogues they have no or very low genotoxic potential. Hence it was concluded that exposure to sulphonated aromatic amines present in colourings, are unlikely to induce any significant genotoxic risk

The Panel also noted that the specifications on the purity of Brown HT permit concentrations of unidentified unsulphonated aromatic amines to be present in concentrations of up to 100 mg/kg Brown HT. Although some aromatic amines may be associated with genotoxicity or even carcinogenicity, the Panel notes that Brown HT was negative in *in vitro* genotoxicity as well as in long-term carcinogenicity studies.

Long term toxicity and carcinogenicity studies with Brown HT are available with rats and mice. No carcinogenic effects were observed in either species. No adverse effects were reported in rats at dietary dose levels up to 425 mg/kg bw/day (highest dose tested). Several effects were observed in the long-term mouse study at the highest dose tested (715 mg 85% pure Brown HT/kg bw/day). The NOAEL in the mouse study was 0.1% in the diet equivalent to 143 mg Brown HT/kg bw/day.

The negative outcome of the carcinogenicity studies is considered by the Panel to rule out the concern on potential genotoxicity of the unsulphonated aromatic amine which may result from azoreduction of Brown HT by intestinal bacteria. The Panel considered that in the light of the results of the available carcinogenicity studies there is no need for additional genotoxicity studies.

Conceivably the same study was used by SCF and JECFA for calculation of the ADI. No further details on both the SCF and JECFA evaluations are available.

The Panel notes that no cases of intolerance/allergenicity/hypersensitivity have been reported after oral exposure to Brown HT and that no data on sensitivity to Brown HT are available.

The ADI of 0-1.5 mg/kg bw/day defined by JECFA was based on a NOAEL of 143 mg/kg bw/day derived from the long-term feeding study in mice. Although it appears that both JECFA and SCF have derived a NOAEL from the same study, their respective ADIs for 85% pure Brown HT have been set at 0-1.5 and 0-3 mg/kg bw/day respectively. No further details on both the SCF and JECFA evaluations are available.

Brown HT was tested for reproduction toxicity in a dietary three-generation study in rats revealing a NOAEL of 250 mg Brown HT/kg bw/day. In a developmental toxicity study in rats no teratogenic and embryotoxic effects were observed up to a dose level of 1000 mg/kg bw/day.

The Panel noted that although no new studies are available since previous evaluations, at present these old studies have been reviewed and published in scientific journals.

The Panel concluded that an ADI of 1.5 mg Brown HT/kg bw/day can be established based on the NOAEL in a long-term mouse study of 143 mg/kg bw/day and an uncertainty factor of 100 and rounding off the ADI of 1.43 mg/kg bw/day.

The dietary exposure to Brown HT was estimated by the Panel based on the maximum permitted levels (MPLs) of use, by applying the Budget method (Tier 1) with the assumptions described in the report of the Scientific Cooperation (SCOOP) Task 4.2. The Panel calculated a theoretical maximum daily exposure of 8.1 mg/kg bw/day for adults, and 8.1 mg/kg bw/day for a typical 3 year-old child.

Refined exposure estimates have been performed both for children and the adult population according to the Tier 2 and Tier 3 approaches described in the SCOOP Task 4.2, which combines, respectively, detailed individual food consumption information from the population with the MPLs of use as specified in the Directive 94/36/EC on food colours (Tier 2), and with the maximum reported use levels of Brown HT listed in Table 3 (Tier 3), as identified by the Panel from the data made available by the FSA and UNESDA For children (1-10 years old), estimates have been calculated for nine European countries (Belgium, France, the Netherlands, Spain, Italy, Finland, Greece, Sweden and Germany) and for UK children separately. For the adult population, the Panel has selected the UK population as representative of the EU consumers for Brown HT exposure estimates.

When considering MPLs (Tier 2), the mean dietary exposure to Brown HT for European children, (aged 1-10 years), ranged from 0.3 to 2.2 mg/kg bw/day, and from 0.8 to 5.9 mg/kg bw/day at the 95th percentile. Estimates reported for the UK adult population give a mean dietary exposure of 0.5 mg/kg bw/day and of 2.9 mg/kg bw/day for high level (97.5th percentile) consumers of 'aromatized fruit wines, cider and perry'.

When considering the maximum reported use levels (Tier 3), the mean dietary exposure to Brown HT for European children (aged 1-10 years) ranged from 0.3 to 2.0 mg/kg bw/day, and from 0.7 to 5.8 mg/kg bw/day at the 95th percentile. Estimates reported for the UK adult population give a mean dietary exposure to Brown HT of 0.4 mg/kg bw/day, and of 2.8 mg/kg bw/day for high level (97.5th percentile) consumers of soft drinks.

The Panel concludes that at both the maximum permitted level of use (Tier 2) and at the maximum reported levels of use of Brown HT (Tier 3), mean intake estimates are generally below the ADI of 1.5 mg/kg bw/day. However, in both adults and children, the high percentile of exposure (97.5th) for both tiers can be higher than the ADI established by the Panel.

The Panel further notes that the specifications for Brown HT need to be updated with respect to the percentage of material not accounted. Thus, if the existing specifications would be extended to include < 30% of sodium or calcium chloride and/or sodium or calcium sulphate as the principal uncoloured components, most of the material would be accounted for.

The Panel noted that the JECFA specification for lead is ≤ 2 mg/kg whereas the EC specification is ≤ 10 mg/kg.

The Panel noted that the aluminium lake of the colour could add to the daily intake of aluminium for which a 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.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to the Framework Directive 89/107/EEC[5] on food additives, the Scientific Committee on Food (SCF) should be consulted before the adoption of provisions likely to affect public health, such as the drawing up of lists of additives and the conditions for their use. Accordingly, all food additives, prior to their authorization, have been evaluated for their safety by the SCF or by its successor, the European Food Safety Authority (EFSA).

Directive 89/107/EEC as well as Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives[6] which will apply as from 20 January 2010, require that food additives must be kept under continuous observation and must be re-evaluated whenever necessary in the light of changing conditions of use and new scientific information. In addition Regulation (EC) No 1333/2008 requires that all food additives which were permitted before 20 January 2009 shall be subject to a new risk assessment carried out by EFSA.

In accordance with Regulation (EC) No 1333/2008, the Commission should, after consultation with EFSA, set up by 20 January 2010 an evaluation programme for EFSA to re-evaluate the safety of the permitted food additives. That programme will define the needs and the order of priorities according to which the approved food additives are to be examined.

Food colours were among the first additives to be evaluated therefore, many of the evaluations are old. For some of these colours new studies have become available and the results of these studies should be included in the evaluation. Therefore, food colours should be evaluated with priority. The order of priorities for the re-evaluation of the remaining permitted food additives will be set in the Regulation for the re-evaluation program.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission asks the European Food Safety Authority to start a systematic re-evaluation of all authorised food additives and to issue scientific opinions on these additives, taking into account that colours as a group should be given the highest priority for the reasons outlined above.

[5] OJ L 40, 11.2.1989, p. 27

[6] OJ L 354, 31.12.2008, p. 16.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of Brown HT (E 155) when used as a food colouring substance.

Brown HT (E 155) is a synthetic bis-azo dye authorised as a food additive in the EU and previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and 1984, and the EU Scientific Committee for Food (SCF) in 1975 and 1984.

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 a public call for data. The Panel noted that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

2. Technical data

2.1. Identity of the substance

Brown HT (E 155) is a bis azo-dye food colouring consisting of reddish-brown powder or granules with the molecular formula $C_{27}H_{18}N_4Na_2O_9S_2$. It has a molecular weight of 652.56 g/mol and its CAS Registry Number is 4553-89-3. Its full chemical name is disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylene bis-azo) di- (naphthalene-1-sulfonate). Its structural formula is given in Figure 1:

Figure 1: Structural formula of Brown HT

At least 10 synonyms are in use (ChemIDplus advanced, via internet, 2007). The most commonly used synonyms in published literature are Brown HT, Chocolate Brown HT and CI Food Brown 3.

Brown HT is soluble in water and insoluble in ethanol.

2.2. Specifications

Specifications have been defined in the Directive 2008/128/EC[7] and by JECFA (JECFA, 2006) (Table 1).

Brown HT consists essentially of disodium 4,4'-(2,4-dihydroxy-5-hydroxy-methyl-1,3-phenylene bis-azo) di- (naphthalene-1-sulfonate) and subsidiary colouring matters, together with sodium chloride and/or sodium sulphate as the principal uncoloured components. Brown HT is described as the sodium salt. The calcium and the potassium salts are also permitted (Directive 2008/128/EC).

The purity is specified as not less than 70% of total colouring matters, calculated as the sodium salt. The remaining 30% may be accounted for by sodium or calcium chloride or sodium or calcium sulphate (but this is never mentioned explicitly), 10% subsidiary colouring matters, ≤ 0.7% 4 - aminonaphthalene-1-sulphonic acid, ≤ 0.01% unsulphonated primary aromatic amines and ≤ 0.2% ether extractable matter, originating from the manufacturing process.

Thus, if the existing specifications would be extended to include < 30% of sodium or calcium chloride and/or sodium or calcium sulphate as the principal uncoloured components, most of the material would be accounted for.

Table 1: Specifications for Brown HT according to Commission Directive 2008/128/EC and JECFA (JECFA, 2006)

Purity	Commission Directive 2008/128/ EC	JECFA (2006)
Water insoluble matter	≤ 0.2%	≤ 0.2%
Subsidiary colouring matters	≤ 10% (TLC method)	≤ 10%
Organic compounds other than colouring matters:		
- 4-aminonaphthalene-1-sulfonic acid	≤ 0.7%	≤ 0.7%
- unsulphonated primary aromatic amines	≤ 0.01% (calculated as aniline)	≤ 0.01% (calculated as aniline)
Ether extractable matter	≤ 0.2% in a solution of pH 7	≤ 0.2%
Arsenic	≤ 3 mg/kg	-
Lead	≤ 10 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb)	≤ 40 mg/kg	-

The Panel notes that the specifications on the purity of Brown HT would permit concentrations of unsulphonated aromatic amines to be present in concentrations of up to 100 mg/kg Brown HT. Given the maximal allowed concentration of Brown HT that can be added to food (500 mg/kg food), the concentration of these unidentified unsulphonated primary aromatic amines in food could be 50 µg/kg food.

The Panel noted that the JECFA specification for lead is ≤ 2 mg/kg, whereas the EC specification is ≤ 10 mg/kg.

According to Directive 2008/128/EC, the above purity criteria for the pure substance also apply to the raw material from which the aluminium lake is produced. In addition, the aluminium lake should contain no more than 0.5% HCl-insoluble material, and no more than 0.2% ether-extractable material under neutral conditions. There are no additional specification requirements for the aluminium lake.

[7] Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. OJ L 6, 10.1.2009, p. 20-63.

JECFA does not give specifications for aluminium lakes of Brown HT other than reference to the General Specifications for Aluminium Lakes of Colouring Matters (JECFA, 2006). The Brown HT used in the production process should comply with the specifications as given above, and the aluminium lake should contain not more than 2% water-soluble chlorides and sulphates calculated as sodium salts, not more than 0.5% HCl-insoluble matter, 0.2% ether-extractable matter, 3 mg arsenic/kg and 5 mg lead/kg. Unreacted aluminium oxide may also be present in the final product (not specified).

The Panel notes that the aluminium lake of the colour 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

No data on the manufacture of Brown HT are available. Brown HT may be converted to the corresponding aluminium lake under aqueous conditions by reacting aluminium oxide with the colouring matter. Undried 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 foods

Several methods for the determination of Brown HT in foods are described in published literature, of which variations of High Pressure Liquid Chromatography (HPLC) appear to be most generally employed.

2.5. Reaction and fate in foods

Very little information on the reaction and/or fate of Brown HT in foods appears to be available. The only data found originate from a study by Nursten and Williams (1969) who have investigated the stability of several coal tar food colours. Model systems under severe conditions (high temperature, high concentration of ascorbic acid) were used, allowing for estimation of colour break-down semi-quantitatively (by spectrophotometry). It was found that Brown HT is greatly affected by ascorbic acid at pH 3 (no further details were provided).

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).

2.6. Case of need and proposed use levels

Permitted use levels have been defined in Directive 94/36/EC[8] on colours for use in foodstuffs.

Currently, Brown HT is an authorised synthetic food colouring substance in the EU, with a maximal allowed use level of 50 to 500 mg/kg food for various foodstuffs. Brown HT is also allowed in

[8] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. OJ L 237, 10.09.1994, p.13-29.

alcoholic beverages at levels up to 200 mg/L. Table 2 summarizes those beverages and foodstuffs that are permitted to contain Brown HT up to specified maximum permitted levels (MPLs) set by Directive 94/36/EC.

Table 2: Maximum permitted levels of use of Brown HT in beverages and foodstuffs according to European Parliament and Council Directive 94/36/EC and maximum reported use levels of Brown HT in beverages and foodstuffs used for the refined exposure assessment

Beverages	**Maximum permitted level** (mg/L)	**Maximum reported use level** (mg/L)
Non-alcoholic flavoured drinks	50	20[1]
Liquid food supplements/dietary integrators	100	100[2]
Spirituous beverages Aromatized wines, aromatized wine-based drinks and aromatized wine-product cocktails Fruit wines, cider and perry	200	200[2]
Foodstuffs	**Maximum permitted level** (mg/kg)	**Maximum reported use level** (mg/kg)
Confectionery Fine bakery wares Edible ices Desserts including flavoured milk products Complete formulae for weight control intended to replace total daily food intake or an individual meal Complete formulae and nutritional supplements for use under medical supervision Soups	50	50[2]
Flavoured processed cheese Fish paste and crustaceans paste Smoked fish Savoury snack products and savoury coated nuts Meat and fish analogues based on vegetable proteins	100	100[2]
Candied fruit and vegetables, Mostarda di frutta Preserves of red fruits Extruded or expanded savoury snack products	200	200[2]
Pre-cooked crustaceans	250	250[2]
Mustard Fish roe Solid food supplements/dietary integrators	300	300[2]
Decorations and coatings Sauces, seasonings, pickles, relishes, chutney and piccalilli Salmon substitutes Surimi	500	500[2]
Edible cheese rind Edible casings	*Quantum satis* *Quantum satis*	100[3] 500[3]

1 Maximum use level or maximum level determined by analysis.
2 Maximum permitted level.
3 *quantum satis* data.

2.6.1 Actual levels of use of Brown HT

More information on current use levels was made available to the Panel for several food categories in finished products.

2.6.1.1 Beverages and foodstuffs

For non-alcoholic flavoured drinks, a usage survey conducted by the Union of European Beverage Associations (UNESDA) in 2005 suggested that the highest current use level of Brown HT in beverages was 6 mg/L (Tennant, 2006).

The UK Food Standards Agency (FSA) conducted an ad hoc survey in which artificial colours were analytically determined in 201 retail ready-to-drink soft drinks selected for being distinctly coloured (FSA, 2003). Brown HT was found to be present at a level higher than 0.1 mg/L (Limit of Detection - LOD) in 2 products, with levels ranging from 2 to 18 mg/L.

For all other food groups where Brown HT is legally permitted, it was not found to be above the LOD or Limit of Quantification (LOQ) in any of the survey data provided to the Panel.

In order to refine the exposure assessment for children and adults to food colours, the Panel has defined some rules to identify maximum reported use levels based either on maximum actual usage or maximum analytical data or quantum satis rules for Brown HT from food uses. The rules followed in order to deal with quantum satis authorisation, with usage data or observed analytical data, for all regulated colours re-evaluated by the Panel, are given in Annex A.

2.7. Information on existing authorisations and evaluations

Brown HT is permitted as a food additive in the EU under Directive 94/36/EC.

Brown HT has been previously evaluated by JECFA in 1977 and 1984 and SCF in 1975 and 1984. A temporary ADI of 0-0.25 mg/kg bw/day was allocated by JECFA in 1977, pending additional data on the metabolism of Brown HT and multigeneration reproduction and teratology studies. These data, and in addition, a special study on pigment deposition, became available in 1984. Subsequently, an ADI of 0-1.5 mg/kg bw/day was established on the basis of a no-effect level of 150 mg/kg bw/day derived from the long-term feeding study in mice by Drake *et al*. (1978). Obviously an uncertainty factor of 100 was applied.

In 1975, the SCF allocated to Brown HT a temporary ADI of 0-2.5 mg/kg bw/day, pending additional data on the metabolism of Brown HT and multigeneration reproduction and teratology studies. In 1984, the SCF established a full ADI of 0-3 mg/kg bw/day, based on a not further specified long-term mouse study. As the long-term mouse study by Drake *et al.* (1978) appears to be the only long-term mouse study available, conceivably this study was used. However, as in this study no dose of 300 mg/kg bw/day was used, this would imply that the ADI established was based on the No-Observed-Adverse-Effect Level (NOAEL) of 150 mg/kg bw/day, applying a safety factor of 50 instead of the more common safety factor of 100. The reason why an aberrant safety factor was used is unknown, as no further explanation were given.

2.8. Dietary exposure assessment

The Panel agreed to follow the principles of the stepwise approach, which were used in the report of the Scientific Cooperation (SCOOP) Task 4.2 (EC, 1998), to estimate additives' intakes. For each successive Tier, this involved a further refinement of intakes. The approach goes from the conservative estimates that form the first Tier (Tier 1) of screening, to progressively more realistic estimates that form the second (Tier 2) and third (Tier 3) Tier.

2.8.1. Crude estimates (Budget Method)

The dietary exposure to Brown HT from the maximum permitted use levels was estimated using the Budget method (Tier 1), with the assumptions described in the report of the SCOOP Task 4.2 (EC, 1998).

In the case of Brown HT, the maximum permitted use level in beverages was 200 mg/L (Directive 94/36/EC). The maximum permitted level in solid foods was 500 mg/kg (Table 2).

The default proportion (25%) of beverages and solid food that could contain the additive was considered adequate. In fact, even though Brown HT may be used in a variety of solid foods and beverages that could represent more than 25% of processed foods, it is unlikely that a person would systematically choose all processed foods with the same colour added even considering brand loyalty. The theoretical maximum daily exposure for adults would therefore be:

(200 x 0.1 x 0.25) + (500 x 0.025 x 0.25) = 5 + 3.12 = 8.1 mg/kg bw/day.

For children, the level of Brown HT considered in beverages was 50 mg/L (after exclusion of alcoholic drinks), and in solid food 300 mg/kg. As recommended by SCOOP task 4.2 (EC, 1998) for children, it is assumed that 100% of beverages contain the additive. This conclusion was derived from UK data on consumption of soft drinks by children aged under 5 years, where the 97.5th percentile of consumption was between 70 and 80 mL/kg bw/day.). This assumes that a typical 3 year-old child weighing 15 kg consumes daily 1.5 litres of beverages and 94 g of solid foods containing Brown HT. The overall theoretical maximum daily exposure to Brown HT in children would therefore be:

(50 x 0.1 x 1) + (500 x 0.025 x 0.25) = 5 + 3.12 = 8.1 mg/kg bw/day.

It was noted that Brown HT may be used *quantum satis* in edible cheese rinds and edible casings. As this is a very specific food category, which is unlikely to be consumed in high amounts on a daily basis, if at all, it was excluded from the Budget calculation, since it is not expected to influence the outcome of this exposure calculation to any relevant extent.

2.8.2. Refined estimates

Refined exposure estimates have been performed for Tier 2 using maximum permitted use levels, presented in Table 2, and for Tier 3 using the maximum reported use levels reported for non-alcoholic beverages for children and for the adult population.

For adults, the Panel calculated the exposure based in the UK consumption survey as the UK population is considered to be one of the highest consumers of soft drinks in Europe and also because detailed individual food consumption data (UK NDNS, 2000-2001) are available from the UNESDA report (Tennant *et al*., 2006) and the NATCOL reports (Tennant, 2007a,b). The maximum permitted levels (MPL's) of use as specified in the Directive 94/36/EC (EC, 1994) were used for the Tier 2 approach and the maximum reported use levels were used for the Tier 3 approach (Table 2) (see Annex A).

Exposure estimates for children (1-10 years old) have been performed by the EXPOCHI consortium, based on detailed individual food consumption data from nine European countries (Belgium, France, the Netherlands, Spain, Italy, Finland, Greece, Sweden and Germany) for Tier 2 and Tier 3. As the UK is not part of the EXPOCHI consortium, estimates for UK children (aged 1.5 - 4.5 years) were made by the Panel with the use of the detailed individual food consumption data (UK NDNS, 1992-1993) available from the UNESDA (Tennant *et al*., 2006) and the NATCOL reports (Tennant, 2007a,b) and

with the MPLs of use as specified in Directive 94/36/EC on food colours from Table 2 (Tier 2 approach), and with the maximum reported use levels (Tier 3 approach).

Table 3 summarises the anticipated exposure of adults and children to Brown HT.

2.8.2.1. Tier 2

In the case of Brown HT, when considering MPLs of use (Tier 2), estimates reported for the UK adult population give a mean dietary exposure to Brown HT of 0.5 mg/kg bw/day and 2.9 mg/kg bw/day for high level (97.5th percentile) consumers of spirituous beverages. The main contributors to the total anticipated mean exposure to Brown HT (>10%) were non-alcoholic flavoured drinks (41%), sauces and seasonings (14%) and aromatized fruit wines, cider and perry (13%).

The mean dietary exposure of European children (aged 1-10 years and weighing 25-30 kg) considered by the EXPOCHI consortium ranged from 0.3 to 2.2 mg/kg bw/day, and from 0.8 to 5.9 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to Brown HT (>10% in different countries), were soft drinks (up to 34%), fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (up to 28%), desserts, including flavoured milk products (up to 50%) and sauces, seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney and piccalilli (up to 72%).

For UK children aged 1.5 to 4.5 years and weighing 15 kg, the mean dietary exposure was 1.4 mg/kg bw/day and 3.4 mg/kg bw/day for high level (97.5th percentile) consumers of soft drinks. The main contributors to the total anticipated mean exposure (>10%) for UK pre-school children were non-alcoholic flavoured drinks (61%).

2.8.2.2. Tier 3

Further data suggest that current use levels of Brown HT in some food categories are lower than the MPLs. Therefore, it was decided that concentration data made available to the Panel by the UNESDA, FSA and the FSAI, would be used to refine the estimate of dietary exposure to Brown HT (Tier 3).

When considering the maximum reported use levels, estimates reported for the UK adult population give a mean dietary exposure to Brown HT of 0.4 mg/kg bw/day and 2.8 mg/kg bw/day for high level (97.5th percentile) consumers of spirituous beverages. The main contributors to the total anticipated mean exposure to Brown HT (>10%) were non-alcoholic beverages (22%), desserts (18%) and aromatized fruit wines, cider and perry (17%).

The mean dietary exposure of European children (aged 1-10 years and weighing 25-30 kg), considered by the EXPOCHI consortium, ranged from 0.3 to 2.0 mg/kg bw/day, and from 0.7 to 5.8 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to Brown HT (>10% in all countries), were soft drinks (up to 17%), fine bakery wares (e.g. Viennoiserie, biscuits, cakes, wafer) (up to 31%), sauces, seasonings (e.g. curry powder, tandoori), pickles, relishes, chutney and piccalilli (up to 34%) and desserts, including flavoured milk products (up to 64%).

For UK children, aged 1.5 to 4.5 years and weighing 15 kg, the mean dietary exposure to Brown HT was 0.9 mg/kg bw/day and 1.7 mg/kg bw/day for high level (97.5th percentile) consumers of non-alcoholic beverages. The main contributors to the total anticipated mean exposure (>10%) for UK pre-school children were non-alcoholic flavoured drinks (39%), desserts (14%) and snacks (extruded and expanded savoury snack products) (13%).

Table 3: Summary of anticipated exposure to Brown HT using the tiered approach (EC, 2001) in UK children and adult populations

	Adult UK population (>18 years old)	**Pre-school UK child** (1.5-4.5 years old, 15 kg body weight)	**Children EXPOCHI population** (1-10 years old, 25-30 kg body weight
	mg/kg bw/day		
Tier 1. Budget method	8.1	8.1	
Tier 2. Maximum Permitted Level of use			
• Mean exposure	0.5	1.4	0.3 – 2.2
• Exposure 95th*or 97.5th percentile**	2.9	3.4	0.8 – 5.9
Tier 3. Maximum reported use levels			
• Mean exposure	0.4	0.9	0.3 – 2.0
• Exposure 95th*or 97.5th percentile**	2.8	1.7	0.7 – 5.8

*For EU children, estimates are based on the EXPOCHI report, which gives the 95th percentile intake.
**For UK, estimates are based on the UNESDA report which gives the 97.5th percentile intake from beverages plus *per capita* average from the rest of diet (Tennant, 2006).

3. Biological and toxicological data

Brown HT has been evaluated previously by the JECFA (1984) and by the SCF (1984). It was also evaluated by TemaNord (2002). The present opinion briefly reports the major studies evaluated in these opinions and describes the additionally reported new literature data in some more detail.

3.1. Absorption, distribution, metabolism and excretion

The data describing the absorption, metabolism and excretion of ^{14}C-labelled Brown HT in male mice, male and female rats and male guinea pigs (Philips *et al.*, 1987) confirm that azo reduction to naphthionic acid does occur. Two samples of Brown HT labelled with ^{14}C were used in these studies, published by Philips *et al.* (1987). One sample (sample A) which was used for most of the investigations was labelled in the naphthionic acid moiety (1,4,5-8-^{14}C) and in the 2,4-dihydroxybenzyl alcohol moiety (uniform-^{14}C), and the other sample (sample B) was labelled in the 2,4-dihydroxybenzyl alcohol moiety (uniform-^{14}C). The specific activity was respectively 41.5 mCi/mmol and 9.75 m Ci/mmol.

Male mice were given single oral doses of ^{14}C-labelled Brown HT (70 or 250 mg/kg bw). Substantially the entire amount of radioactivity was excreted within 72 hours in the faeces (80-90%) and urine (7-16.5%); only traces of activity (< 0.2%) appeared in expired air. With regard to distribution, in male mice, the major part of the radioactivity remaining in the tissues after 72 hour was associated with the gastrointestinal tract (0.16%) (Phillips *et al.*, 1987). Use of isolated loops of small intestine revealed no significant absorption of radioactivity over a one-hour period at concentrations up to 5000 mg/L.

In a study in rats, male animals were given ^{14}C-labelled Brown HT repeatedly to examine tissue specific or irreversible accumulation of the colour or its metabolites. Animals were either pre-treated with unlabelled- or ^{14}C-Brown HT (250 mg/kg bw/day) for 21 days. Urinary and faecal excretion and tissue levels of radioactivity were monitored at 24, 48, 72 and 168 hours after dosing (Philips *et al.*, 1987). The recovery of the total administered radioactivity was close to 100%. Following administration of a single oral dose of ^{14}C-Brown HT (250 mg/kg bw) to non-pre-treated rats, the majority of the dose was excreted rapidly (mainly within 48 hours) in urine and faeces (not

quantified). After 24 hours, substantial amounts of radioactivity were found in tissues with the highest levels in the gastrointestinal tract, lymph nodes and kidney. The concentration of radioactivity remained similar from 48 hours to 168 hours after dosing. After 168 hours the concentration of radioactivity was similar in all tissues (less than 0.001% per gram tissue) except for the kidneys (0.009%) and mesenteric lymph nodes (0.003%). Distribution and excretion in animals pre-treated with unlabelled- or ^{14}C-labelled Brown HT was not significantly different than that observed in non-pre-treated animals. However, at the earlier times, in animals pre-treated with unlabeled Brown HT, radioactivity in the liver, kidney and lymph nodes was somewhat greater than in the non-pre-treated animals, and the concentration in the kidney fell from 0.02% (per gram tissue) at 48 hour to 0.0075% after 168 hours. JECFA states that the researchers concluded that Brown HT and/or its metabolites accumulated in most tissues of male rats during repeated daily administration (of 250 mg/kg bw), but that accumulation was tissue-specific, only in the kidney and mesenteric lymph nodes; in other organs the accumulated colouring was cleared rapidly after cessation of the treatment (Philips *et al,* 1982).

In another study, rats (both sexes) given single oral doses of ^{14}C-labelled Brown HT (50 or 250 mg/kg bw) showed no significant absorption in isolated small intestinal loops. Both sexes showed excretion similar to that observed in mice. In male rats, 0.6% of the dose was excreted in the bile (after 7 hours). No difference in elimination was seen between pregnant vs. non-pregnant rats (given Brown HT on gestational day 8). Distribution of radioactivity was also similar in male rats and mice, with the only exception that activity was somewhat lower in the gastrointestinal tract (0.04% compared to 0.16%). The liver and kidney contained 0.005% and 0.006% of the activity respectively (Philips *et al,* 1987). With regard to distribution in female rats, approximately 0.25% of the administered radioactivity was retained in the tissues after 72 hours, mainly in the gastrointestinal tract (0.17%); the liver and kidney contained 0.04% and 0.014% of the radioactivity, respectively. After 96 hours, approximately 0.30% of the dose was retained in the tissues in both pregnant and non-pregnant animals. In non-pregnant animals, most of the residual activity was associated with the gastrointestinal tract, liver, kidney and brain, while in the pregnant animals the highest levels were found in kidney, heart, and lung. In the pregnant animals a further 0.23% of the dose (no further detail) was found in foetuses. After repeated daily doses of ^{14}C-labelled Brown HT for 3 weeks, the radioactivity in the organs was low and associated mainly with the gastrointestinal tract (0.275% of total dose). The radioactivity in liver, kidney spleen and mesenteric lymph nodes was 0.015%, 0.009%, 0.004% and 0.001% of the total radioactivity administered (Phillips *et al.*, 1987).

In guinea pigs given ^{14}C-labelled Brown HT (50 or 250 mg/kg bw), no significant absorption took place in small intestinal loops. Faecal excretion appeared somewhat slower (possibly due to coprophagy), and approximately 90% of total ^{14}C-Brown HT was eliminated in 72 hours in faeces (74-75.5%) and urine (13-16%) with traces in expired air (Phillips *et al.*, 1987). Urine and faecal samples of mice, rats and guinea pigs were examined by thin-layer chromatography. Two metabolites were detected in the urine; the major component (65-90% of the urinary radioactivity) was identified as naphthionic acid, and the minor component was not identified. Faecal extracts contained small amounts of unchanged Brown HT (1.5-6.5% of the dose), together with naphthionic acid and two unidentified metabolites (Phillips *et al.*, 1987).

Since previous safety assessments no new data on toxicokinetics and metabolism have been submitted or published except for the study by Philips *et al*. (1987).

3.2. Toxicological data

Many of the studies used for the JECFA and SCF evaluations were performed before or around the mid-seventies. At this time the first GLP guidelines were implemented. OECD GLP guidelines were not promulgated before 1981. It is unclear whether studies described in the previous evaluations comply with the (OECD) GLP guidelines. Although the design of the various (older) studies may not

be in full compliance with current regulatory requirements, from the study descriptions available, and the studies that were revisited for this pre-evaluation, it appears that the available studies in themselves have been conducted adequately. The Panel noted that although no new studies are available since previous evaluations, at present these old studies have been reviewed and published in scientific journals.

3.2.1. Acute oral toxicity

Acute oral toxicity tests have been conducted in rats (both sexes) and mice (female only). LD_{50} values were in all instances >2000 mg/kg bw (Hall *et al.*, 1966). No new studies have been published or submitted since the previous evaluations.

3.2.2. Short-term and subchronic toxicity

In a study with rats (strain Porton; 12/sex/group), animals were given Brown HT (purity ≥ 85%) at levels of 0, 0.5, 1 and 2% (equivalent to 0, 250, 500 or 1000 mg/kg bw/day) for a period of 12 weeks. Males displayed growth retardation not associated with a diminished food intake at 500 and 1000 mg/kg bw/day. At 1000 mg/kg bw/day significant increases in the relative weights of the kidneys and spleen of both sexes, the brain and adrenals of males, and ovaries of females were found. In a kidney function test (week 12), increased urinary aspartate transferase (ASAT) activity was seen. A dose-related pigment deposition was evident in the proximal convoluted tubules of the kidney, Kupffer cells of the liver, and lymph nodes (especially the Patches of Peyer in the small intestine). Liver function was normal. Haematological examinations (weeks 6 and 12) revealed non-statistically significant reductions of red cell counts and haematocrit in males at 1000 mg/kg bw/day. No adverse effects were seen regarding the appearance or condition of the animals (Hall *et al.*, 1966). No pathological changes were seen. The NOAEL is established at 500 mg/kg bw/day.

In a rat study, animals (Carworth Farm) received Brown HT at dietary levels of 0, 0.02, 0.06, 0.2, 0.6, 1 or 2% (equivalent to 0, 10, 30, 100, 300, 500, 1000 mg/kg bw/day) during 90 days. A slight but significant growth retardation was observed at 500 mg/kg bw/day (males) and 1000 mg/kg bw/day (both sexes), but only after adjustment for total food intake. At a dose of 1000 mg/kg bw/day a slight yet significant decrease in haemoglobin, red cell counts and haematocrit was seen in male rats. In addition, males given 300 and 500 mg/kg bw/day displayed increased total serum protein, and both sexes showed significant reductions in blood urea levels (all groups except for the ones receiving 30 and 300 mg/kg bw/day). Finally, at 500 and 1000 mg/kg bw/day pigment deposition was seen in certain intestinal cells, lymph nodes and cells of the proximal convoluted tubules of the kidney. No adverse effects were observed with regard to appearance, behaviour, survival, or absolute organ weights (heart, kidneys, liver, spleen and testes). There was no evidence of any pathological changes (Chambers *et al.*, 1966). The NOAEL was established at 300 mg/kg bw/day.

In a limited toxicity study with pigs (Large White strain), 10 weeks-old animals (3/sex/group) were given Brown HT at dose levels of 0, 5, 20 or 100 mg/kg bw/day for 13 weeks. The only finding was that haemoglobin levels in male pigs were significantly below the control values at all doses. These findings were however inconsistent with other blood parameters and tissue pathology. No adverse effects were noted regarding mortality, growth, organ weights, urine composition or the incidence of histopathological lesions (no further details) (Hendy *et al.*, 1978). The NOAEL would be established at 100 mg/kg bw/day, which was the highest dose tested.

In a study by Aboel-Zahab *et al.* (1997) healthy adult male albino rats were given a synthetic chocolate colorant agent containing both Brown HT and Indigo carmine for 30- and 60-day periods

(no detail on Brown HT intake). Administration of the mixture significantly decreased rat body weight, serum cholesterol and HDL-cholesterol fraction, while T_4 hormone, liver RNA content, liver enzymes (ASAT, ALAT and alkaline phosphatase), total protein and globulin fractions were significantly elevated. Haematological investigations demonstrated selective neutropenia and lymphocytosis with no significant alterations of total white blood cell counts. Lastly, congested blood vessels and areas of haemorrhage in both liver and renal sections were seen.

The Panel concludes that the results of this study (Aboel-Zahab *et al.,* 1997) cannot be used as the basis for a re-assessment of the ADI of Brown HT, as the exposure of the experimental animals has been to a mixture of food colours in which the individual dose level has not been specified and it is not clear what were the amounts/percentage of the individual colours added to the diet to achieve the cited level of 0.8 g of mixture/kg bw/day.

3.2.3. Genotoxicity

In a genotoxicity screening test, 25 food dyes, currently or previously used in foods, were studied for mutations in bacterial assay systems *(Salmonella typhimurium* strain TA1535 and TA1538 and *Escherichia coli* strains WP2 trp, WP2trp uvrA, WP67 trp uvr Pol A and WP100 trp uvr A rec A), with and without rat-liver metabolic activation. Brown HT did not show mutagenic effects (Haveland-Smith and Combes, 1980).

In another mutagenicity screening test, 13 approved food dyes were assayed in the *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with or without metabolic activation. Brown HT was not mutagenic in this assay (Bonin and Baker, 1980).

BIBRA (1989) in a toxicity profile on Brown HT mentioned a number of bacterial assays, including the Ames' mutagenicity test, in which no evidence of genotoxicity was found.

The Panel noted that the genotoxicity data on Brown HT are limited to bacterial tests.

The Panel noted that Prival and Mitchell (1982) demonstrated that the metabolic conditions of the standard Ames test protocol were not appropriate for testing azo dyes for mutagenic activity in *Salmonella typhimurium* and developed a specific protocol including use of flavin mononucleotide (FMN) rather than riboflavin to reduce the azo compounds to free amines, and hamster liver S9 rather than rat liver S9 for metabolic activation. The Panel therefore noted that a final conclusion from negative Ames test results obtained under standard conditions cannot be drawn.

Azo-reduction of Brown HT may produce sulphonated aromatic amines as well as an unsulphonated aromatic amine. Jung *et al.* (1992) have reviewed the genotoxicity data of a range of sulphonated aromatic amines. To provide insight in the effect of sulphonation on the genotoxic potential of phenyl- and naphthylamines, the genotoxicity of sulphonated aromatic amines was compared with their unsulphonated analogues. It was found that in general sulphonated phenyl- and naphthylamines, including the sulphonated azo-reduction products of Brown HT such as naphthionic acid are non-mutagenic to *Salmonella* in Ames tests. Some other sulphonated aromatic amines the absence of genotoxicity was demonstrated with a variety of other test systems *in vitro* and *in vivo* (no details given). Based on the available data, the authors concluded that sulphonated aromatic amines, in contrast with their unsulphonated analogues, have no or very low genotoxic potential. Hence, the authors concluded that exposure to sulphonated aromatic amines, derived from metabolic cleavage or present as contaminants in colourings, are unlikely to induce any significant genotoxic risk.

No new studies have been published since the previous evaluations.

3.2.4. Chronic toxicity and carcinogenicity

In a BIBRA study, groups of mice (TF1; 48/sex/group) were given diets containing 0, 0.01, 0.1 or 0.5% Brown HT (equivalent to approximately 0, 14, 143 or 715 mg/kg bw/day) for 80 weeks. The purity was ≥ 80%. At a dose of 715 mg/kg bw/day a brown coloration of the internal organs was seen and considered due to feeding of Brown HT. Furthermore, at the same dose, male mice displayed a slightly reduced body weight gain and a lower heart weight. In females, at the same dose, the packed cell volume and total leucocyte count values (at week 77) were lower than those of controls (treatment relatedness was however considered questionable). In addition, in females at 715 mg/kg bw/day an increased incidence of leucocyte infiltration in the liver and an increase in cystic ovaries was seen. There was no difference in survival between the groups and distribution of tumours was comparable in all groups (Drake *et al.*, 1978). No carcinogenic effects were observed for Brown HT-treated groups. The NOAEL was considered to be 143 mg Brown HT/kg bw/day.

Groups of rats (Wistar; 48/sex/group) received diets containing 0, 500, 2000 or 10 000 mg Brown HT/kg diet for 2 years (equivalent to 0, 25, 100 or 500 mg Brown HT/kg bw/day). The purity was ≥ 80%. At a dose of 500 mg/kg bw/day, mortality was significantly increased in the male rats of the top dose group of the at week 72, but at the end of the study survival was comparable in treated and control rats with approximately 20 males and 15 females in each group surviving after two years. Histopathological examination revealed no adverse effects. No abnormalities were seen regarding body weight gain, food or water consumption, organ weights, haematology, renal function or serum constituents. The incidence of tumours in treated animals did not differ from controls (Carpanini *et al.*, 1978). It was concluded by the Panel that Brown HT did not exert carcinogenic effects and that the NOAEL was 500 mg/kg bw/day.

No new studies have been published since these previous evaluations.

3.2.5. Reproduction and developmental toxicity

3.2.5.1. Developmental toxicity studies

In the Grant and Gaunt 1987 study, groups of 30 female rats were given by oral intubation aqueous solutions of Brown HT at a dose volume of 5 ml/kg bw and concentrations to provide daily doses of 0(controls), 250,500 or 1000 mg/kg bw from days 0 to 19 of pregnancy. The day of positive for spermatozoa vaginal smear was designated as day 0 of pregnancy. On day 20 the animals were killed by cervical dislocation and the numbers of corpora lutea and uterine implantation sites were recorded as well as were the numbers and positions of the uterine sites with living, dead or resorbed foetuses. The foetuses were examined for gross abnormalities, weighed and preserved in either alcohol for skeletal abnormalities examination after staining with alizarin red or in Bouin's for internal organs examination. The rates of maternal body-weight gain were described as similar in all groups (no figures presented). In a preliminary study on females treated for 19 days with the same dose levels also no effect of treatment on body weight gain and no signs of toxicity were observed. The faeces of the treated animals were brown and at autopsy a brown discoloration of lymph nodes, colon and caecum was seen. There were no statistically significant differences in the mean numbers of corpora lutea, implantation sites, pre- and post-implantation losses, resorptions, live foetuses, foetal weight, litter weight and sex ratio. In the alizarin red stained foetuses of the highest dose group there was a significantly ($p<0.05$) higher incidence of those with five ossified sternebrae, ossified proximal phalanges and ossified fourth metacarpals. In the two lower dose groups but not at the top group the number of foetuses with incomplete or bipartite ossification of first and second sternebrae was lower

than those of controls. The fact that the above finding indicating certain advanced ossification were not dose related and were not accompanied by an advanced ossification of supra-occipitals but on the contrary incomplete ossification of it was with higher incidence in the two lower dose groups compared to controls, is in favour of a conclusion that these variants in ossifications should not to be regarded as treatment related. The Panel agrees with the authors' conclusion that at doses up to the highest one of 1000 mg/kg bw/day Brown HT given throughout pregnancy had no adverse effects on the dams or on foetal development and survival and 1000 mg/kg bw/day can be suggested for NOAEL.

No major changes in skeletal development were encountered in the teratogenicity studies on F0, F1 and F2 generations, nested in a three generation study with dietary exposure to Brown HT at 0, 50, 250, and 500 mg/kg bw/day (Mangham *et al*., 1987; further details see below). The finding of a slight dose-related reduction in ossification of the third sternebrae is at variance with the observation of Grant and Gaunt 1987 for slightly more advanced stage of skeletal development. These minor differences in degree of skeletal ossification encountered in both studies in the absence of clear signs of embryotoxicity are considered as acceptable variation of norm. The autopsy findings with detailed examination of the uterus and its content of the time-mated females (24 from the control group and 12 from each of the three dose groups) from F0, F1 and F2 generations were presented in a summary table. With exception of a significantly fewer corpora lutea and higher mean foetal body weight in the F0 high dose group and slightly but not significantly lower than control values of average No of implantation sites and live foetuses, there were no differences in autopsy findings in F0 teratogenicity test. There were no significant differences in any variables studied as indicators of intrauterine development in the F1 and F2 generations (No of corpora lutea, No of implantations, No of live foetuses, mean litter and fetal weight, Pre-and Post-implantation loss). There were no external, visceral or skeletal abnormalities of the foetuses.

3.2.5.2. Reproductive toxicity study

In the three generation study of Mangham *et al.*, 1987 weanling rats(F0) of a Wistar derived outbred strain were randomized into four groups for each strain/sex and fed diets providing daily intake of Brown HT at 0 (controls), 50, 250 or 500 mg/kg bw/day. Mating of the F0 animals to provide F1 litters commenced after 60 days on the test diet.

The first 24 females from the control group and 12 females from each of the treated groups to conceive were timed-mated and were killed on gestation day 20 in order to investigate the prenatal development (the results discussed above under subheading developmental toxicity studies). The remaining males and females were housed in pares for 12 days to allow mating. The pregnant females were allowed to deliver and rear their young. The survival, growth and development as well as several indicators of physical and neurological development of the offspring were monitored over the first 21 day of life. The criteria for the positive physical development were as follows: eye opening, ears uncurled, pinna unfolded, tooth eruption, incisors visible above the gums, hair growth. The tests used to evaluate auditory and neuromuscular development were: righting response, startle response, clinging ability. After weaning, young males and females were randomly selected to constitute the F1 generation or for autopsy. The above procedure was followed with F1 rats to provide F2 litters (however because of low litter survival in all F2a groups incl. control one, the F1 adults were mated a second time to provide F2b) and the procedure was repeated with the F2 rats to provide F3 litters. An autopsy with measurements of organ weights and gross examination of all the internal organs was carried out on the selected for the purpose weaning rats and on the adult rats from all the treatment groups and all the generations. A histological examination was carried out on the tissues taken from the F3 control rats and those given the highest dose, 500 mg/kg bw/day.

There were no adverse treatment-related trends in either food or water consumption or in body-weight gain. The fertility rate (proportion of paired females that became pregnant) was similar in the control

and treatment groups and the number of non viable litters did not increase with treatment. Although the authors declared a generally good health of the animals several death occurred at the time of parturition and a number of females from F1 and F2 generation failed to nurse their litters resulting in pup death mainly in the first four days *post partum*. As litter losses occurred in controls and in all treated groups and they were without a dose-related increase, the Panel agrees with the statement of the authors that they were not related to treatment with Brown HT. However a litter loss around 50% raises questions about the quality of the experimental animals. There were no statistically significant differences between the control and treated groups in average litter size, or growth, weight gain and development of the young or in the sex ratio throughout lactation period and weaning. No data were presented however about the results from declared parameters in the part materials and methods monitoring of physical, auditory and neuromuscular development of progeny.

In post-mortem examination the finding that was consistently associated with treatment at the adult stage of each generation was a statistically significant increase in absolute kidney weight. This was apparent at 500 mg/kg bw dose level of males in all generations and in females of F1 and F2, in the male and female rats at 250 mg/kg bw in F0 generation and in females at 50 mg/kg bw in the F2b generation only. The differences remained statistically significant for relative kidney weight in all cases except in female rats in the 50 mg/kg bw group. A statistically significant increase in relative kidney weight was found also in young top dose level rats of both sexes of the F2b and in the males of F3 generation. A slight caecal enlargement was seen on several occasions but not as a consistent finding. In all treatment groups the mesenteric lymph nodes were frequently reported to be brown, with the greatest coloration at the highest dose. At this dose a brown coloration was recorded also of the caecum and molars for some animals.

Microscopic examination of tissues from F3 control and top dose animals revealed no changes that were consistently associated with treatment (statement of the authors not supported by details).

The Panel agrees with the authors' statement that there was no evidence for Brown HT adverse effect on male and female fertility and pre- and postnatal development. The increased incidence of post-partum death was not treatment-related as it was similar for both control and treated animals. The NOAEL of 250 mg/kg bw proposed by the authors is on the basis indications of nephrotoxicity evidenced by increased kidney weight, caecum enlargement and brown coloration of the lymph nodes and some areas of gastro-intestinal tract in the highest dose group. The Panel agrees with this proposal.

To further examine the observed pigment deposition in the mesenteric lymph nodes and possibly the kidneys, an independent evaluation of histopathological material from the F3 generation of the above described three-generation study was performed (tissues examined: thyroid, heart, liver, kidney, voluntary muscle, caecum, thymus, mesenteric lymph node (an extra 10 males and 5 females examined) and cervical lymph node) (Roe, 1983). In animals given Brown HT at levels of 500 mg/kg bw/day (5/sex) degree of pigment deposition did not exceed that of controls (2/sex). Specifically, pigment deposition did not differ in follicular cells of the thymus, Kupffer cells of the liver, proximal convoluted tubules and other sites in the kidney, cardiac or voluntary muscle, or in the caecal wall. In mesenteric lymph nodes no pigment-laden macrophages in the sinuses or other evidence of pigment deposition was seen. The pigment observed in some tissues in these studies after administration of high doses of Brown HT did not survive normal histological tissue preparation and no accompanying histopathological changes could be detected in mesenteric lymph nodes or kidney. No other histopathological changes were seen (Roe, 1983).

No new studies have been published since the previous evaluations.

3.2.6. Hypersensitivity

Reactions to food colourings, including those triggered by immune (immediate and delayed type hypersensitivity) and non-immune (intolerance) mechanisms are assumed to be infrequent in the

population, and prevalence of 0.14 to around 2% has been reported (Young *et al.,* 1987; Hannuksela and Haahtela, 1987; Fuglsang *et al.,* 1993, Fuglsang *et al.*, 1994).

No data on sensitivity to Brown HT are available. Additionally, no cases of intolerance/allergenicity have been reported after oral exposure to Brown HT, and it appears therefore that at the current levels of exposure the incidence is very low if any. On the other hand, the low/absent reports of adverse clinical reactions after internal Brown HT exposure could be accentuated by the lack of clinical awareness of this possibility.

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 a public call for data. The Panel notes that new data were limited. The Panel also notes that not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

Brown HT (E 155) is a synthetic bis-azo dye authorised as a food additive in the EU and previously evaluated by JECFA in 1977 and 1984 and the SCF in 1975 and 1984. The JECFA established an ADI of 0-1.5 mg Brown HT/kg bw/day and the SCF an ADI of 0-3 mg Brown HT/kg bw/day.

The ADI of 0-1.5 mg/kg bw/day defined by JECFA was based on a NOAEL of 150 mg/kg bw/day derived from the long-term feeding study in mice (Drake *et al*., 1978).

Specifications have been defined in the EU legislation directives 2008/128/EC and by JECFA (JECFA, 2006). The purity is specified as not less than 70% of total colouring matters, calculated as the sodium salt. The remaining 30% may be accounted for by sodium or calcium chloride or sodium or calcium sulphate (but this is never mentioned explicitly), ≤ 10% subsidiary colouring matters, ≤ 0.7% 4-aminonaphthalene-1-sulphonic acid (naphthionic acid), ≤ 0.01% unsulphonated primary aromatic amines and ≤ 0.2% ether extractable matter, originating from the manufacturing process. Thus, if the existing specifications would be extended to include < 30% of sodium or calcium chloride and/or sodium or calcium sulphate as the principal uncoloured components, most of the material would be accounted for.

Based on studies with radiolabelled Brown HT, it may be concluded that Brown HT or its metabolites are absorbed to a limited extent in mice, rats and guinea pigs and are excreted predominantly in faeces (up to 90%) and urine. Faecal extracts of mice and rats contained only small amounts of unchanged Brown HT, naphthionic acid and two unidentified metabolites. Urine contained naphthionic acid and one unidentified metabolite. These findings indicate that the azo-bonds of Brown HT are reductively cleaved by intestinal bacteria as is the case with other azo-dyes. However, it is unclear if the central ring structure (2,4-dihydroxy-3,5-diamino benzyl alcohol; an aromatic amine) is released.

Radiolabelled Brown HT was mostly associated with the gastrointestinal tract and to a minor extent with the liver and kidney in both rats and male mice. Brown HT and/or its metabolites deposited only in the kidney and mesenteric lymph nodes. Tissue retention of Brown HT or its metabolites was further studied in a three-generation study in animals given Brown HT at 500 mg/kg bw/day. Pigment deposition was seen in intestinal cells, lymph nodes and cells of the kidney. The concentration of radioactivity was also higher in the mesenteric lymph nodes and kidneys compared to other tissues which could indicate that the pigment consists of Brown HT, or more likely, one of its metabolites. As pigment deposition does not appear to be accompanied by any pathological changes (also in long-term/developmental studies) these findings may be discarded for the toxicological evaluation of Brown HT.

After a single oral dose of Brown HT in pregnant rats, differences in distribution but not elimination were observed in non-pregnant rats vs. pregnant rats. In non-pregnant rats, most of the residual activity was associated with the gastrointestinal tract, liver, kidney and brain, while in the pregnant animals the highest levels were found in kidney, heart, and lung.

No sign of acute toxicity was seen after exposure to single doses of up to 2000 mg/kg bw, the highest dose tested.

A number of *in vitro* genotoxicity studies appear to have been conducted in the past in which no signs of genotoxicity were observed. However, the Panel notes that the database is limited to bacterial test systems.

The Panel noted that Prival and Mitchell (1982) demonstrated that the metabolic conditions of the standard Ames test protocol were not appropriate for testing azo dyes for mutagenic activity in *Salmonella typhimurium* and developed a specific protocol including use of flavin mononucleotide (FMN) rather than riboflavin to reduce the azo compounds to free amines, and hamster liver S9 rather than rat liver S9 for metabolic activation. The Panel therefore noted that a final conclusion from negative Ames test results obtained under standard conditions cannot be drawn.

The conversion of Brown HT by azo-reduction *in vivo* results in the formation of sulphonated naphthyl amines as well as in an unsulphonated aromatic amine that may not be formed in the *in vitro* genotoxicity test. In a review by Jung *et al.* (1992), a range of sulphonated aromatic amines was shown, in general, not to be associated with genotoxicity *in vitro* and *in vivo.* Since all the sulphonated aromatic amine metabolites that could in theory be formed by azo-reduction of Brown HT were included in the study, the Panel concludes that the data reviewed by Jung *et al.* (1992) are sufficiently re-assuring to support the conclusion that the sulphonated aromatic amines formed from Brown HT by azo-reduction such as naphthionic acid do not give reason for concern with respect to genotoxicity. In contrast with their unsulphonated analogues, have no or very low genotoxic potential. Hence, the authors concluded that exposure to sulphonated aromatic amines, derived from metabolic cleavage or present as contaminants in colourings, are unlikely to induce any significant genotoxic risk.

Long-term toxicity and carcinogenicity studies with Brown HT are available with rats and mice. No carcinogenic effects were observed in either species. No adverse effects were reported in rats at dietary dose levels up to 500 mg/kg bw/day (the highest dose tested). Several effects were observed in the long-term mouse study at the highest dose tested (715 mg, 80% pure Brown HT/kg bw/day). Although the effects observed were dissimilar in the opposite sexes, based on these findings, a NOAEL of 0.1% Brown HT in the diet determined to be equivalent to 143 mg/kg bw/day (80% pure was established.

The negative outcome of the carcinogenicity studies is considered by the Panel to rule out the concern on potential genotoxicity of the unsulphonated aromatic amine which may result from azoreduction of Brown HT by intestinal bacteria. The Panel considered that in the light of the results of the available carcinogenicity studies there is no need for additional genotoxicity studies.

Conceivably the same study was used by SCF and JECFA for calculation of the ADI. No further details on both the SCF and JECFA evaluations are available.

In the Grant and Gaunt (1987) developmental toxicity study, groups of 30 female rats were given by oral intubation daily doses of 0 (controls), 250,500 or 1000 mg/kg bw Brown HT from days 0 to 19 of pregnancy. There were no statistically significant differences in the mean numbers of corpora lutea, implantation sites, pre- and post-implantation losses, resorptions, live foetuses, foetal weight, litter weight and sex ratio or major abnormalities. The findings of certain advanced ossification were not dose related and were not seen in other parts of skeleton than metacarpals, phalangae and sternum. These variants of ossification should not be regarded as treatment related and a NOAEL of 1000 mg/kg bw/day may be derived. In the nested in the three generation study of Mangham *et al.* 1987

developmental study there were no consistent significant differences in number of corpora lutea, number of implantations, number of live foetuses, mean litter and foetal weight, pre-and post-implantation loss and no external, visceral or skeletal abnormalities of foetuses were found.

In the three generation study of Mangham *et al.* (1987) weanling rats (F0) of a Wistar derived outbred strain were randomized into four groups for each strain/sex and fed diets providing daily intake of Brown HT at 0 (controls), 50, 250 or 500 mg/kg bw/day. There was no evidence for Brown HT adverse effect on male and female fertility and pre- and postnatal development. The increased incidence of post-partum death was not treatment related as it was similar for both control and treated animals. The NOAEL of 250 mg/kg bw proposed by the authors on the basis indications of nephrotoxicity evidenced by increased kidney weight (but without histopathological changes), caecum enlargement and brown coloration of the lymph nodes and some areas of gastro-intestinal tract. The Panel agrees with this proposal.

The exposure assessment approach goes from the conservative estimates that form the First Tier of screening, to progressively more realistic estimates that form the Second and Third Tier. The dietary exposure to Brown HT from the MPLs of use was estimated by the Panel using the Budget method (Tier 1) with the assumptions described in the report of the SCOOP Task 4.2. The Panel calculated a theoretical maximum daily exposure of 8.1 mg/kg bw/day for adults, and 8.1 mg/kg bw/day for a 3 year-old child.

Refined exposure estimates have been performed both for children and the adult population according to the Tier 2 and Tier 3 approaches described in the SCOOP Task 4.2, which combines, respectively, detailed individual food consumption information from the population with the MPLs of use as specified in the Directive 94/36/EC on food colours (Tier 2), and with the maximum reported use levels of Brown HT, as identified by the Panel from the data by the FSA and UNESDA (Tier 3).

For children (1-10 years old), estimates have been calculated for nine European countries as part of the Expochi study (Belgium, France, the Netherlands, Spain, Italy, Finland, Greece, Sweden and Germany) and also for UK children separately. For the adult population, the Panel has selected the UK population as representative of the EU consumers for Brown HT exposure estimates.

When considering MPLs (Tier 2), the mean dietary exposure to Brown HT for European children, (aged 1-10 years), ranged from 0.3 to 2.2 mg/kg bw/day, and from 0.8 to 5.9 mg/kg bw/day at the 95th percentile. Estimates reported for the UK adult population give a mean dietary exposure of 0.5 mg/kg bw/day and of 2.9 mg/kg bw/day for high level (97.5th percentile) consumers of 'aromatized fruit wines, cider and perry'.

When considering the maximum reported use levels (Tier 3), the mean dietary exposure to Brown HT for European children (aged 1-10 years) ranged from 0.3 to 2.0 mg/kg bw/day, and from 0.7 to 5.8 mg/kg bw/day at the 95th percentile. Estimates reported for the UK adult population give a mean dietary exposure to Brown HT of 0.4 mg/kg bw/day, and of 2.8 mg/kg bw/day for high level (97.5th percentile) consumers of soft drinks.

The Panel further notes that the specifications of Brown HT need to be updated with respect to the percentage of material not accounted for that may represent sodium or calcium chloride and/or sodium or calcium sulphate as the principal uncoloured components.

The Panel notes that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$10 mg/kg.

CONCLUSIONS

Brown HT (E 155) is a synthetic bis-azo dye authorised as a food additive in the EU and previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and 1984 and the EU Scientific Committee for Food (SCF) in 1975 and 1984. The JECFA established an ADI of 0-1.5 mg Brown HT/kg bw/day and the SCF an ADI of 0-3 mg Brown HT/kg bw/day.

The Panel concluded that an ADI of 1.5 mg Brown HT/kg bw/day can be established based on the NOAEL in a long-term mouse study of 143 mg/kg bw/day and an uncertainty factor of 100 and rounding off the ADI of 1.43 mg/kg bw/day.

The Panel concludes that at both the maximum permitted level of use (Tier 2) and at the maximum reported levels of use of Brown HT (Tier 3), mean intake estimates are generally below the ADI of 1.5 mg/kg bw/day. However, in both adults and 1-10 years old children, the high percentile of exposure for both tiers can be higher than the ADI at the upper end of the range.

The Panel notes that no cases of intolerance/allergenicity have been reported after oral exposure to Brown HT and that no data on sensitivity to Brown HT are available.

The Panel further notes that the specifications for Brown HT need to be updated with respect to the percentage of material not accounted. Thus, if the existing specifications would be extended to include < 30% of sodium or calcium chloride and/or sodium or calcium sulphate as the principal uncoloured components, most of the material would be accounted for.

The Panel notes that the JECFA specification for lead is $\leq$ 2 mg/kg whereas the EC specification is $\leq$10 mg/kg.

The Panel notes that the aluminium lake of the colour could add to the daily intake of aluminium for which a TWI of 1 mg aluminium/kg bw/week has been established and that therefore specifications for the maximum level of aluminium in the lakes may be required.

DOCUMENTATION PROVIDED TO EFSA

Pre-evaluation document on Brown HT (E 155) prepared by the Dutch National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands.

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ANNEX A

Rules defined by the Panel to deal with *quantum satis* (QS) authorisation, usage data or observed analytical data for all regulated colours to be re-evaluated (30 July 09) and intake estimates

1. Decision rules taken to deal with QS authorisations:

a. In the category 'All other foodstuffs, the value of 500 mg/kg (the highest MPL) is used

b. At the food category level: if a colour is authorised QS in a food category for one or more colours

   i. If a value is available for only one colour, this value is used for all the colours (except if this value is available only for annatto-cf point c)

   ii. If many values are available for more than one colour, the highest value is used

c. At the colour level: if there is no available value or if there is just a single value for annatto, the available value for a similar food group for the same colour is used. If there is no similar food group, the highest MPL of 500 mg/kg is used.

Particular cases:

-**Edible casings**: if available use the pork-based products use level; if not available, the highest MPL of 500 mg/kg is used.

-**Edible cheese rinds:** 100 mg/kg (as the flavoured processed cheese category) is used, except for the E 120 (Cochineal) colour whose level is 125 mg/kg for red marbled cheese.

2. Rules defined to identify maximum reported use levels from maximum current usages or maximum observed analytical values:

a. If the identified maximum reported use level, adjusted for the highest current usage data or the highest analytical value, is lower than or equal to the actual MPL, then the actual MPL is used by default.

b. If analytical and current use level data are available, priority is given to the use level data, even if analytical values are higher; the figure is rounded up to the nearest integer.

c. If no use level data are available because no uses were reported (use level = 0) or industry was not asked, the choice is made between the highest analytical value or the MPL:

   i. if more than 10 analytical data are available, the highest value is used;

   ii. if less than 10 analytical data are available, the MPL is used.

d. If no data were reported by the industry, the MPL is used by default.

e. If the highest use level or the highest analytical data are higher than the proposed adjusted QS values, priority is given to the highest use level/analytical data

3. Tiered approach to intake estimation.

The basic principles of the stepwise approach for estimates of additives' intakes involve, for each successive Tier, further refinement of intakes from the conservative estimates that form the First Tier of screening until more realistic estimates that form the Second and Third Tiers (EC, 2001).

The three screening tiers performed both for children and adult population are:

a. Tier 1: Estimates are based MPLs of use, as specified in the Directive 94/36/EC on food colours and the principles of the Budget method.

b. Tier 2: Estimates are based on MPLs of use, as specified in the Directive 94/36/EC on food colours, adjusted for *quantum satis* usages, and national individual food consumption data.

c. Tier 3: Estimates are based on maximum reported use levels and national individual food consumption data.

GLOSSARY AND ABBREVIATIONS

ADI	Acceptable Daily Intake
AFC	Scientific Panel on Additives, Flavourings, Processing Aids and Materials in Contact with Food
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
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
ALT	Alanine transferase activity
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
AST	Aspartate transferase activity
BIBRA	British Industrial Biological Research Association
CAS	Chemical Abstracts Service
CIAA	Confederation of the Food and Drink Industries of the EU
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EXPOCHI	Refers to EFSA Article 36 2008 call for Proposals Focused on Children and Food Consumption
FAO/WHO	Food and Agriculture Organization/World Health Organization
FSA	UK Food Standard Agency
FSAI	Food Safety Authority of Ireland
GLP	Good Laboratory Practice
HPLC	High Pressure Liquid Chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of Detection
NATCOL	Natural Food Colours Association
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organisation for Economic Co-operation and Development

RNA	Ribonucleic acid
Y-GT	y-Gammaglutathion transferase activity
SCF	Scientific Committee on Food
SCOOP	A scientific cooperation (SCOOP) task involves coordination amongst Member States to provide pooled data from across the EU on particular issues of concern regarding food safety
TWI	Tolerable Weekly Intake
UNESDA	Union of European Beverage Associations