SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of Litholrubine BK (E 180) 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 Panel on Food Additives and Nutrient Sources added to Food provides a scientific opinion re-evaluating the safety of Litholrubine BK (E 180). Litholrubine BK has been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), with the latest evaluation in 1987 and by the EU Scientific Committee for Food (SCF) in 1983. JECFA was unable to establish an Acceptable Daily Intake (ADI), whereas the SCF established an ADI of 0-1.5 mg/kg bw/day. The Panel notes that SCF established the ADI of 0-1.5 mg/kg bw/day based on a reported NOAEL of 150 mg/kg bw/day identified in a long-term rat study. Overall, the Panel considers that the present database is too limited to continue supporting the ADI for Litholrubine BK set previously by the SCF or to establish a new ADI. The Panel was unable to identify a suitable NOAEL, LOAEL or BMD to establish an ADI from a combined repeated-dose and reproductive/developmental toxicity study with Litholrubine BK in rats; males were exposed for 42 days and females exposed for 17 days, in accordance with OECD Test Guideline 422 and GLP conditions. The Panel thus concludes that the existing SCF ADI of 0-1.5 mg/kg bw/day should be withdrawn. However, the Panel notes that the highest anticipated exposure to Litholrubine BK is 1700-fold lower than the identified effect level in female rats (100 mg/kg bw/day). Therefore, the Panel considers that it is unlikely there would be a significant safety concern for humans from the current single authorised use of Litholrubine BK in edible cheese rinds. No conclusion on the induction of hypersensitivity by Litholrubine BK could be drawn from the limited scientific evidence available, although after Litholrubine BK exposure from cosmetics, cheilitis has been documented in one case report.

KEY WORDS

Litholrubine BK, CI Pigment Red 57, Rubinpigment, Carmine 6B, D&C Red No. 7, E 180, CAS Registry Number 5281-04-9, calcium 3-hydroxy-4-[(4-methyl-2-sulphonatophenyl)azo]-2-naphthalenecarboxylate, food colouring substance, EINECS 226-109-5.

---

[1] On request from the European Commission, Question No EFSA-Q-2008-257, adopted on 15 April 2010.

[2] Panel members : F. Aguilar, B. Dusemund, P. Galtier, J. Gilbert, D.M. Gott, S. Grilli, R. Gürtler, J. König, C. Lambré, J-C. Larsen, J-C. Leblanc, A. Mortensen, D. Parent-Massin, I. Pratt, I.M.C.M. Rietjens, I. Stankovic, P. Tobback, T. Verguieva, R.A. Woutersen. Correspondence: ans@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank the members of the Working Group A on Food Additives and Nutrient Sources of the ANS Panel for the preparation of this opinion: F. Aguilar, N. Bemrah, P. Galtier, J. Gilbert, S. Grilli, R. Gürtler, NG. Ilback, C. Lambré, J.C. Larsen, J-C. Leblanc, A. Mortensen, I. Pratt, Ch. Tlustos, I. Stankovic.

Suggested citation: EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Scientific Opinion on the re-evaluation of Litholrubine BK (E 180) as a food additive on request of the European Commission. EFSA Journal 2010;8(5):1586. [26 pp.]. doi:10.2903/j.efsa.2010.1586. Available online: www.efsa.europa.eu

SUMMARY

Following a request from the European Commission to the European Food Safety Authority, the Scientific Panel on Food Additives and Nutrient Sources added to Food has been asked to deliver a scientific opinion re-evaluating the safety of Litholrubine BK (E 180) when used as a food colouring substance. The Panel notes that currently permitted use of Litholrubine BK is restricted to cheese rind.

Litholrubine BK (E 180) is a red mono-azo dye authorised as a food additive in the EU and previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), with the latest evaluation in 1987 and by the EU Scientific Committee on Food (SCF) in 1983. JECFA was unable to establish an Acceptable Daily Intake (ADI) (JECFA, 1987), whereas the SCF established an ADI of 0-1.5 mg/kg bw/day (SCF, 1983), based on a No-Observed-Adverse-Effect-Level (NOAEL) of 150 mg/kg bw/day identified in a long-term rat study, the details of which were not described in its report.

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.

The JECFA evaluation of D&C Red No. 6 (Litholrubine B) considered that there were limited histopathological examinations in two long-term studies in mice and rats that did not allow an unequivocal no-effect level to be determined. In order to comprehensively evaluate this dye, this evaluation requested results from a complete histopathological examination of all dose groups in the long-term mouse study, results of a new long-term study in rats, and an adequate reproductive and developmental toxicity study.

The Panel notes that the only toxicokinetic data available on Litholrubine BK, suggest that there is very limited absorption in animals; most of the orally administered dose being excreted via the faeces and not via the urine. No information was available on Litholrubine B.

The summarised data available for genotoxicity suggest that Litholrubine BK is not mutagenic or clastogenic *in vitro*.

The conversion of the parent compound by azo reduction *in vivo*, results in the formation of a sulphonated aromatic amine and a carboxylated amino-naphthol that may not be formed in the standard *in vitro* genotoxicity tests. Previously, a range of sulphonated aromatic amines was shown in general not to be associated with genotoxicity *in vitro* and *in vivo* and the Panel considered that 4-amino-3-hydroxy-2-naphthoic acid would be expected to behave similarly as the closely structurally related amino-naphthol-sulphonic acids which have been shown to be negative.

The Panel also notes that the specifications on the purity of Litholrubine BK permit concentrations of unidentified unsulphonated aromatic amines to be present in concentrations of up to 100 mg/kg Litholrubine BK. Although some aromatic amines may be associated with genotoxicity or even carcinogenicity, the Panel notes that Litholrubine BK was negative in *in vitro* genotoxicity assays.

A study done in rats for an exposure period of 42 days in males and 17 days in females, in accordance with OECD Test Guideline 422 and GLP conditions, reports decreased general biochemistry parameters in serum and Glutamic Oxaloacetic acid Transaminase (GOT) levels as well as increased kidney weights and histopathological lesions in renal tubular epithelium, for males receiving 1000 mg/kg bw/day Litholrubine BK (OECD SIDS, 1994). No effects were reported on reproductive/developmental toxicity parameters measured in this study. Female rats that received 100 or 1000 mg/kg bw/day showed statistically significant decreases in thymus weights in comparison to the controls. Decreases in thymus weight were not statistically significant in females of the mid-dose group. Relative thymus weight was statistically significantly decreased in females of the low-dose

group only. No further statistically significant differences in organ weights were observed in males or females.

Histopathological examinations showed alterations predominantly occurring in the kidney. The lesions included regenerated renal tubular epithelium in male rats receiving 300 mg/kg bw/day or higher. In all treated female groups, the incidence of foamy tubular epithelial cells was increased compared to controls. Although the incidence was similar in all dose groups, the severity of this lesion was slightly increased in the high-dose group only. In addition, increased incidences of necrotic tubular epithelium were seen in all treated groups compared to controls, however no dose-effect relationship either in incidence or severity was observed.

The Panel, having consulted the tables in the original Japanese and the English abstract of this study, concludes that there was a NOAEL of 100 mg/kg bw/day for males and that the NOAEL for females was below 100 mg/kg bw/day (the lowest dose tested). Based on the lack of a clear dose-response relationship in this study the Panel was unable to identify a suitable NOAEL, Low-Observed-Adverse-Effect-Level (LOAEL) or Bench Mark Dose (BMD) to establish an ADI and concludes that the existing SCF ADI of 0-1.5 mg/kg bw/day should be withdrawn.

The Panel concludes that while one case report of cheilitis after exposure from cosmetics containing Litholrubine BK has been documented, no conclusion on the induction of hypersensitivity could be drawn from the limited scientific evidence available.

The Panel notes that the anticipated exposure to Litholrubine BK from an assumed maximum level of use of 100 mg/kg in edible cheese rinds, based on exposure estimates in high consumers of edible cheese rinds, were from 0.02 to 0.06 mg/kg bw/day in children and from 0.01 to 0.03 mg/kg bw/day in adults. The highest level of anticipated exposure (95^{th} / 97.5^{th}) is 1700-fold lower than the identified effect level of 100 mg/kg bw/day in female rats. The Panel further notes that Litholrubine BK can be used following the *Quantum Satis* (QS) principle and information on actual use levels in edible cheese rinds is not available. The Panel also notes that from the call of usage data on additives, members of the Confederation of the Food and Drink Industries of the EU (CIAA) did not report using Litholrubine BK in cheese products.

Therefore, the Panel considers that it is unlikely there would be a significant safety concern for humans from the current single authorised use of Litholrubine BK in edible cheese rinds.

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

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

TABLE OF CONTENTS

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

According to the framework Directive 89/107/EEC[4] 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 89/107EEC as well as Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives[5] 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 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.

[4] OJ L 40, 11.2.1989, p. 27
[5] OJ L 354, 31.12.2008, p. 16.

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of Litholrubine BK (E 180) when used as a food colouring substance. The Panel notes that currently permitted use of Litholrubine BK is restricted to cheese rind.

Litholrubine BK (E 180) is a red mono-azo dye authorised as a food additive in the EU and previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), with the latest evaluation in 1987 and by the EU Scientific Committee for Food (SCF) in 1983. JECFA was unable to establish an Acceptable Daily Intake (ADI), whereas the SCF established an ADI of 0-1.5 mg/kg bw/day. It was also reviewed 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.

The Panel notes that according to Commission Directive 2008/128/EC[6] only the calcium salt of Litholrubine, called Litholrubine BK (CAS 5281-04-9) is an authorised food colour. Although the sodium salt of Litholrubine, called Litholrubine B (CAS 5858-81-1) is often mentioned in published literature and has been considered in previous evaluations of Litholrubine BK, it is not permitted as a food additive in the EU under Directive 94/36/EC[7] and therefore is considered to be outside the terms of reference of this evaluation. However, data on Litholrubine B has been used to support the present assessment of Litholrubine BK.

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 an EFSA 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

Litholrubine BK (E 180) is a red (powder) mono-azo dye with CAS Registry Number 5281-04-9, EINECS 226-109-5 and the following structural formula (Figure 1):

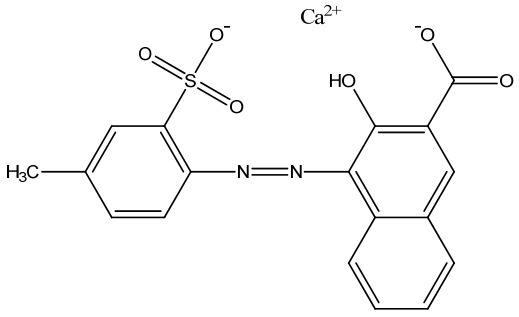

Figure 1. Structural formula of Litholrubine BK

[6] Commission Directive 2008/128/EC of 22 December 2008 laying down specific purity criteria concerning colours for use in foodstuffs. OJ L 6, 10.01.2009, p. 20-63.
[7] European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs. OJ L 237, 10.9.94. p. 13-29.

The full chemical name is calcium 3-hydroxy-4-[(4-methyl-2-sulphonatophenyl)azo]-2-naphthalenecarboxylate and it has the molecular formula $C_{18}H_{12}CaN_2O_6S$ and a molecular weight of 424.45 g/mol.

At least nine different synonyms are in use. The most commonly used synonyms in published literature are: Lithol Rubine, Litholrubine, CI Pigment Red 57, Rubinpigment, Carmine 6B, FD&C Red No. 7 and Brilliant Carmine 6B.

Litholrubine BK is described as slightly soluble in hot water (at 90 °C), insoluble in cold water and insoluble in ethanol (JECFA, 2006).

2.2. Specifications

Specifications for Litholrubine BK have been defined in Commission Directive 2008/128/EC and by JECFA (2006) (Table 1).

Litholrubine BK is stated as consisting essentially of the calcium salt and subsidiary colouring matters together with water, calcium chloride and/or calcium sulphate as the principal uncoloured components. The purity is specified as not less than 90% of total colouring matters. The remaining 10% may be accounted for by calcium chloride and/or calcium sulphate as the principal uncoloured components but this is never mentioned explicitly.

Table 1. Specifications established for Litholrubine BK by the Commission Directive 2008/128/EC and JECFA (2006).

Purity	**Commission Directive 2008/128/EC**	**JECFA (2006)**
Assay	Not less than 90% total colouring matter	Not less than 90% total colouring matter
Subsidiary colouring matters	≤ 0.5%	≤ 0.5%
Organic compounds other than colouring matters: - 2-amino-5-methylbenzenesulfonic acid, calcium salt - 3-hydroxy-2-naphthalenecarboxylic acid, calcium salt	 ≤ 0.2% ≤ 0.4%	 ≤ 0.2% ≤ 0.4%
Unsulphonated primary aromatic amines	≤ 0.01% (expressed as aniline)	≤ 0.01% (calculated as aniline)
Ether extractable matter	≤ 0.2% (from a solution of pH 7)	≤ 0.2%
Loss on drying at 135 °C	-	≤ 10% (together with chloride and sulphate calculated as calcium salts)
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, as with other azo dyes, that the specifications on the purity of Litholrubine BK permit concentrations of unidentified unsulphonated aromatic amines to be present in concentrations of up to 100 mg/kg Litholrubine BK.

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

No information was available to suggest that Litholrubine BK could be found as an aluminium lake. However, according to EU legislation (Directive 2008/128/EC), generally 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.

2.3. Manufacturing process

Litholrubine BK has been described as being manufactured from 2-amino-5-methylbenzenesulphonic acid, which is diazotized with hydrochloric acid and sodium nitrate (Federal Regulations, 1982). The diazo compound is coupled in alkaline medium with 3-hydroxy-2-naphthalenecarboxylic acid and the resulting dye is converted to the calcium salt with calcium chloride.

2.4. Methods of analysis in food

There are no published methods for the determination of Litholrubine BK in edible cheese rinds or in foods.

2.5. Reaction and fate in food

No data specifically on the reaction and fate in food of Litholrubine BK were available in the published literature. However, 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) may affect the colour (Scotter and Castle, 2004).

2.6. Case of need and proposed uses

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

The use of Litholrubine BK is only authorised for use in edible cheese rind, following the *Quantum Satis* (QS) principle.

No usage data on Litholrubine BK were reported by the members of the Confederation of the Food and Drink Industries of the EU (CIAA, 2009).

2.7. Information on existing authorisations and evaluations

Litholrubine BK has been evaluated previously by the SCF in 1979 and 1983. The former evaluation accepted the temporary use of Litholrubine BK for external colouring of cheese rind (SCF, 1979). In 1983 the SCF established an ADI of 0-1.5 mg/kg bw/day based apparently, on a No-Observed-Adverse-Effect-Level (NOAEL) of 150 mg/kg bw/day from a long-term rat study, done with the calcium salt of Litholrubine (Litholrubine BK) (SCF, 1983). However, it is not clear from the report which study was used for setting this ADI.

Litholrubine BK has also been evaluated by JECFA in 1987. JECFA was unable to establish an ADI (JECFA, 1987). The JECFA evaluation considered that there were limited histopathological examinations in two long-term studies in mice and rats that did not allow an unequivocal no-effect level to be determined. In order to comprehensively evaluate this dye, this evaluation requested results

from a complete histopathological examination of all dose groups in the long-term mouse study, results of a new long-term study in rats, and an adequate reproduction/teratology study. The Panel notes that according to the references in the rapport, this evaluation might have been done on D&C Red No. 6 (Litholrubine B).

The Organisation for Economic Co-operation and Development Screening Information DataSet (OECD SIDS) evaluated Litholrubine BK as part of the OECD High Production Volume (HPV) Chemicals Programme (OECD SIDS, 1994).

In 1993, the British Industrial Biological Research Association (BIBRA) issued two Toxicity Profiles on Litholrubine BK and Litholrubine B (BIBRA, 1993a; 1993b).

The safety of use of Litholrubine BK as a food colour has been reviewed by the Nordic Council of Ministers who concluded that its use was very limited and that the basis for the SCF ADI was unclear (Tema Nord, 2002).

2.8. Dietary exposure

Since Litholrubine BK is only allowed to be present in a single specified foodstuff at QS, estimation of exposure based on the Budget method was considered inappropriate by the Panel. As no actual levels of use or observed analytical data were provided to the Panel, only the Tier 2 approach has been assessed. Dietary exposure estimates have been performed for child and adult populations based on the maximum level of use of 100 mg/kg for the food category 'edible cheese rinds' in accordance with the decision rules defined by the Panel to deal with QS authorisations (Appendix A).

In the absence of specific food consumption data for the food category 'edible cheese rinds' as such, in the European database (except for UK data), the Panel decided to apply a general correction factor of 10% to the food consumption data for cheese in order to express food consumption data for edible cheese rinds for all members states. The percentage of edible cheese rinds taken is corresponding to the maximum value reported by Souci et al. (2000) for the quantity of edible cheese rinds in cheese products.

Exposure estimates for children (1-10 years old) have been done by the Panel for 12 European countries (Belgium, UK, France, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Denmark, Cyprus, Greece) based on detailed individual food consumption data for the cheese products category provided by the EXPOCHI consortium. As the UK is not part of the EXPOCHI consortium, exposure estimates for UK children aged 1.5-4.5 years were made by the Panel based on the UK detailed individual food consumption data on edible cheese rinds (UK National Diet and Nutrition Survey, 1992-1993) available from the reports of the Union of European Beverages Associations (UNESDA) and the Natural Food Colours Association (NATCOL) (Tennant et al., 2006 and 2007).

Exposure estimates for the adult population (>18 years old) have been made by the Panel with the use of data available from the EFSA concise food consumption database, which provides aggregate data on the cheese product category consumed by 15 European countries (EFSA. Parma, 17 March 2008 EFSA/DATEX/2008/01).

Table 2 summarises the anticipated dietary exposures to Litholrubine BK for child and adult consumers.

Based on the maximum level of use of 100 mg/kg defined by the Panel (Tier 2), for European children (aged from 1 to 10 years old and weighing 15-30 kg) mean dietary exposure was estimated to range from 0.01 mg/kg bw/day to 0.02 mg/kg bw/day and at the 95th percentile was estimated to range from 0.02 mg/kg bw/day to 0.06 mg/kg bw/day.

For European adults (aged >18 years old and weighing 60 kg), mean dietary exposure was estimated to range from 0.003 mg/kg bw/day to 0.015 mg/kg bw/day and at the 95th percentile was estimated to range from 0.01 mg/kg bw/day to 0.03 mg/kg bw/day.

Table 2. Summary of anticipated exposure to Litholrubine BK in child and adult consumers.

	Adult population (>18 years old, mg/kg bw/day)	**Child population (1-10 years old, mg/kg bw/day)**
Tier 2. Maximum Permitted Level* adjusted for *Quantum Satis*		
• Mean exposure ranges	0.003-0.015	0.01-0.02
• Exposure 95th or 97.5th percentile	0.01-0.03	0.02-0.06

* Maximum level of use of 100 mg/kg on edible cheese rind has been used for E 180 as defined by the Panel to deal with QS usage according to decision rules mentioned in Appendix A.

3. Biological and toxicological data

No toxicological or biological information was submitted for the re-evaluation of Litholrubine BK following an EFSA public call for data, prior to the start of this re-evaluation. The Panel notes that not all of the original studies on which previous evaluations were based were available for re-evaluation by the Panel.

A literature search was conducted on the most commonly available online databases for toxicological and biological information (PubMed, Science Direct, Toxline and Web of Knowledge), to cover recent published literature on Litholrubine BK. However, very little new information was identified.

The present opinion briefly summarises the major toxicological studies on Litholrubine BK evaluated previously by JECFA, with the latest evaluation in 1987, the SCF (SCF, 1983) and TemaNord (2002) and describes the additionally reported new literature data in some more detail.

3.1. Absorption, distribution, metabolism and excretion

The only available data in rats suggest that the parent compound Litholrubine BK is not absorbed, being excreted via the faeces and not the urine (Leist, 1982).

From the chemical point of view, the conversion of Litholrubine BK by azo reduction *in vivo* could result in the formation of a sulphonated amine (2-amino-5-methylbenzenesulphonic acid) and a carboxylated (4-amino-3-hydroxy-2-naphthoic acid) aromatic aminonaphthol. No additional information was available on Litholrubine B.

3.2. Toxicological data

3.2.1. Acute oral toxicity

For Litholrubine BK, BIBRA (1993a) referred to two experiments showing that exposure to 2 and 5 g/kg bw produced no signs of overt toxicity in rats (Leist, 1982).

The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP, 2004) evaluation reported LD_{50} values > 9.8 g/kg bw for Litholrubine BK in rats and dogs.

The OECD evaluation reported an LD_{50} value > 5 g/kg bw for rats and an LD_{50} value > 16 g/kg bw for the mouse for Litholrubine BK (OECD SIDS, 1994).

Overall, previous evaluations had concluded that the acute oral toxicity of Litholrubine BK is low.

3.2.2. Short-term and subchronic toxicity

The BIBRA (1993a) toxicity profile briefly referred to several short-term studies that are described below.

Groups of rats (5 animals/sex/dose) were fed diets containing 0.25, 0.5, 1 or 2% Litholrubine BK (approximately 125, 250, 500 or 1000 mg/kg bw/day, respectively) for 18 weeks. No effects were reported on food intake, body weight, blood composition, (unspecified) organ weights, and during microscopic evaluation of (unspecified) tissues (Hansen et al., 1958).

Groups of rats (20 animals/sex) were administered Litholrubine BK at a dose of 1 g/kg bw/day by stomach tube, for 5 days a week over 30 days (a total of 22 doses) (Leist, 1982). Growth was reported to be slightly reduced (without affecting food consumption), kidney weight was increased and kidney "damage" was evident on microscopic examination (no further details provided). These effects were reported as reversible over a 2-week recovery period. No effects were reported on blood and urine biochemistry or on weight and microscopic appearance of the liver, adrenals and spleen after treatment. The presence of red coloration in faeces (but not urine) allowed the authors to consider that Litholrubine BK is not absorbed through the gastrointestinal tract in the intact form (Leist, 1982).

In male dogs (unspecified number and strain) diets containing 1% Litholrubine BK (approximately 250 mg/kg bw/day) were reported to affect thyroid weights but were reported not to produce associated changes on gross examination of the organs (Vettorazzi, 1981). No further details were provided.

In addition to the studies referred to by BIBRA (1993a), the SCCNFP (2004) refers to an additional 13-week study in dogs in which 1 male and 1 female were fed diets containing 0.5% Litholrubine BK (weeks 1 and 2), 1.0% Litholrubine BK (weeks 2 and 3), 1.5% Litholrubine BK (weeks 5-10) and 2% Litholrubine BK (weeks 11-13). Assuming a dry laboratory chow diet, these doses would be equivalent to 125, 250, 375 and 500 mg/kg bw/day. The only reported effects were diarrhoea (observed a few days before the dose was increased to 1.5%) and vomiting (observed a few days after the dose was increased to 2%) (SCCNFP, 2004).

No new short-term studies have been published since these previous evaluations.

3.2.3. Genotoxicity

The SCF (1983) evaluation only referred to *in vitro* mutagenicity studies with Litholrubine BK as not showing genotoxic potential but no details were provided.

The BIBRA (1993a) toxicity profile on Litholrubine BK referred to several *in vitro* genotoxicity studies which are described briefly below.

No evidence of mutagenicity for Litholrubine BK was reported in an Ames test using *Salmonella typhimurium* TA1587, TA98, TA1535 or TA100 strains in the absence and presence of a S9 liver metabolic activation system (Muzzall and Cook, 1979; Green and Pastewka, 1980).

The BIBRA (1993b) toxicity profile on Litholrubine B referred to several *in vitro* genotoxicity studies which are described briefly below.

Negative results were reported in Ames tests using *Salmonella typhimurium* TA 98, TA1535, TA 100 or TA1537 strains in the absence or presence of a S9 rat liver metabolic activation system (Brown et

al., 1979; Green and Pastewka, 1980; Milvy and Kay, 1978; Muzzall and Cook, 1979). Two out of 9 commercial samples of Litholrubine B showed weak dose-related activity in 1of 2 strains of *Salmonella typhimurium*, when tested with or without a liver metabolic activation system (Miyagoshi et al., 1983). However, it was reported that a purified sample of Litholrubine B gave negative results in the same test.

The SCCNFP (2004) evaluation briefly described one negative *in vitro* bacterial reverse mutation assay with Litholrubine B tested at doses of up to 5000 µg/plate in accordance with OECD Test Guideline 471, as well as one *in vitro* mammalian cell gene mutation assay (OECD Test Guideline 476) and one *in vivo* mammalian erythrocyte micronucleus assay (OECD Test Guideline 474) both done on RED 201 WR 21176, which according to information available to the Panel is Litholrubine BK. The *in vitro* assay with RED 201 WR 21176 was reported to be negative at concentrations of up to 320 µg/ml with and without S9 activation, whereas the *in vivo* assay done in NMRI mice at concentrations of up to 2000 mg/kg bw was considered inadequate since Polychromatic Erythrocyte (PCE) value was not reduced in the bone marrow of the treated animals, compared with the vehicle treated animals, indicating that the substance has not reached the target cells.

A bacterial reverse mutation assay conducted according to OECD Test Guidelines 471, 472 and under Good Laboratory Practice (GLP) conditions, used *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 and *Escherichia coli* WP2 uvrA strains, with and without metabolic activation (OECD SIDS, 1994). Negative results were reported in all strains at Litholrubine BK concentrations (reported purity 98%) of 0, 50, 250, 1000 or 5000 µg/plate.

A Chromosomal Aberration Test (CAT) conducted according to OECD Testing Guideline 473 and under GLP conditions, was done on Chinese Hamster Lung (CHL) cells incubated with Litholrubine BK (reported purity 98%) at concentrations of 0, 124, 500, 1000 and 2500 µg/plate. The highest concentration did not show apparent cytotoxic effects. No structural chromosomal aberrations or polyploidy were reported up to the highest concentration tested, with or without metabolic activation (OECD SIDS, 1994).

Litholrubine BK was reported to give negative results in a CAT conducted in CHL/IU cells, tested with or without metabolic activation, at concentrations inducing up to 50% cytotoxicity although no details were provided on the exact concentrations tested (Kusakabe et al., 2002). It is only stated that when cytotoxicity was 50% or less, 5 mg/ml or 10 mM was set as the maximum dose and that the other doses were sequential half dilutions.

The Panel notes 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 notes that a final conclusion cannot be drawn from negative Ames test results obtained under standard conditions.

Azo-reduction of Litholrubine BK may produce sulphonated aromatic amines. 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 naphtylamines, the genotoxicity of sulphonated aromatic amines was compared with their unsulphonated analogues. It was found that in general, sulphonated phenyl- and naphtylamines are non-mutagenic to *Salmonella typhimurium* in Ames tests. For some other sulphonated aromatic amines no genotoxicity was also 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 including the sulphonated phenylamine which would result from azoreduction of Litholrubine BK, 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 present any genotoxic potential.

The Panel noted that the 4-amino-3-hydroxy-2-naphthoic acid which would result from azoreduction of Litholrubine BK was not covered by the review of Jung et al. (1992). However, the Panel considered that 4-amino-3-hydroxy-2-naphthoic acid would be expected to behave similarly as the closely structurally related amino-naphthol-sulphonic acids which have been shown to be negative.

3.2.4. Chronic toxicity and carcinogenicity

The JECFA (1987) evaluation described two long-term studies in mice and rats which, according to the title of the reference, used D&C Red No. 6 (the sodium salt, Litholrubine B) (IRDC, 1981a, 1981b, as referred in JECFA, 1987).

Groups of 60 male and 60 female Charles River CD1 mice were fed diets containing 0, 0.05, 1.0, or 5.0% D&C Red No. 6 (Litholrubine B) for 104 weeks (equivalent to 0 75, 1500 or 7500 mg/kg bw/day Litholrubine B); 2 groups of 60 animals of each sex were used as controls. Individual body weights were recorded weekly, biweekly and monthly. Haematological examinations were carried out on 10 mice/sex/group at 3, 6, 12 and 18 months. At termination of the study, all animals were necropsied and the weights of the brain, kidneys, liver, and spleen were recorded. Complete histopathological examination was carried out on the control and high-dose groups only. No statistically significant differences were observed in food consumption, body weight gain, or haematological parameters mentioned above except for a depressed reticulocyte count in the high-dose groups relative to controls after 18 months, although the counts were within the expected range for mice of that age according to the report.

Beginning at week 64, there was a treatment-related increase in mortality in males, and survival was statistically significantly reduced in the 5% dose group at 91 and at 104 weeks; increased mortality was not observed in females. No toxicologically-significant dose-related differences in organ weights or gross morphology were observed. Histopathological examination of animals in the high-dose group revealed a variety of degenerative, inflammatory, proliferative, or neoplastic lesions which according to the report were commonly associated with aging mice, occurring with similar frequency or sporadic distribution in controls. Exceptions were degenerative renal changes, which occurred with higher incidence among treated males from the high-dose group, and alveolar adenomas, which were the most common tumours occurring in the study. Compared to controls, statistically significant increases in the unadjusted incidence of alveolar adenomas were seen in high-dose group males, but in the report these were considered of dubious toxicological significance because of unequal sampling of the low- and mid-dose groups and because of earlier diagnosis associated with the increased mortality in the high-dose group. No others statistically significant treatment-related increases were reported in tumours or non-neoplastic lesions at other sites, the occurrence of which were considered in the report to be incidental or common for aged mice (IRDC, 1981a).

In a chronic toxicity study with an *in utero* phase, D&C Red No. 6 (Litholrubine B) was administered to Charles River CD rats at dietary concentrations of 0, 0.05, 0.3, or 2% (equivalent to 0, 25, 150, 1000 mg/kg bw/day). In the *in utero* segment of the study, 60 rats of each sex were assigned to each treatment group and then mated after receiving the diet for 60 days. A minimum of 35 litters per dosage level was used to select 70 rats of each sex per group for the long-term segment of the study. In the long-term phase, the pups were weaned onto their respective diets at 21 days and maintained on these diets throughout the remainder of the experiment.

Individual body weights and food consumption measurements were recorded. Ophthalmoscopic examinations, haematology, serum biochemical examinations and urinalysis were performed at regular time-points and after 24 months for females in the long-term phase. An interim sacrifice and necropsy of 10 rats/sex/group was conducted after 12 months of treatment. For animals killed at the interim or terminal sacrifices, brain, kidney, liver, spleen, testes, thyroid, heart, adrenals, uterus, and ovaries were weighed. Complete histopathological examination was carried out on the control and high-dose groups only.

In the long-term phase, mean food consumption values were similar for control and treated rats, but there was a treatment-related depression in body-weight gain, most marked in the high-dose group. Males showed a larger decrement in body-weight gain than females in the same dose group; the deficit compared to controls reaching about 19% for high-dose males by week 91 of the study. There was an accelerated mortality rate in male rats in the high-dose group and, for males, the study was terminated at week 95 when there were only 9 survivors in the high-dose group compared with 17 and 29 in the two control groups.

No changes considered to be related to treatment were reported in the haematological and clinical biochemical examinations and, apart from the colour of the urine, no differences attributable to treatment were observed in urinalysis values. At 20 months, in an additional haematological investigation on 1 low-dose male and 1 high-dose male markedly elevated leucocyte counts were reported, which were attributed to a probable infection with *Mycoplasma pulmonis*. No compound-related macroscopic changes were detected. There were no statistically-significant variations in mean organ weights at the 12-month interim sacrifice, and subsequent statistically-significant variations in the high-dose males were related in the report to the decrement in mean body weight. Histopathological examinations revealed a higher incidence of chronic nephritis, renal tubular epithelial hyperplasia, myocardial fibrosis, reticular hyperplasia, and pigment deposition in the spleen than in the controls. Compared to controls, a higher incidence of atrophy/degeneration of testicular tubules was reported in high-dose male rats that died during the study and from 12 months of treatment to termination. These changes were considered common in aging rats and no specific compound-related effect was identified other than an acceleration of these changes. There was no statistically significant increase in incidence of the above lesions at termination.

The number of malignant tumours in the males rats, was significantly increased (P>0.01) using the Kruskal-Wallis test for adjusted trend, but it was suggested that this single value was not toxicologically significant because of the small number of tumours present (unadjusted incidence was 4% in the controls and 9% in the high-dose group). There was an unusually high incidence of pituitary adenomas in one control group of males; the incidence in the high-dose group was not statistically significant increased over either control group. The unadjusted incidence of Leydig cell adenomas in males was reported to be 2% in controls and 6% in high-dose animals, not statistically significant at the 0.05 level, but tests for unadjusted trend and homogeneity of life table curves were statistically significant at the 0.01 level. However, they were of doubtful toxicological significance because of the small number of tumours involved (IRDC, 1981b).

The JECFA evaluation (1987) concluded that "*in the long-term mouse study, there was a dose-related increase in mortality and renal pathology, but detailed histopathology was not conducted on the low- and intermediate-dose groups. The long-term study in rats was complicated by high mortality rates, which led to premature termination of the study for males. In addition, only limited histopathological examinations were conducted. In view of these limitations, it was not possible to determine an unequivocal no-effect level in either study. Therefore, an ADI could not be established*".

In its evaluation the SCF evaluated toxicological data from long-term studies in the rat and dog (SCF, 1983). However, no further details are provided and the only reference in the opinion that might concern these studies is identified as "IRDC (1981) Unpublished data supplied to EEC Commission". The Panel notes that according to the title of this report the compound tested is Litholrubine B.

The SCCNFP (2004) evaluation summarised one long-term study done with Litholrubine BK in dogs which according to this evaluation was previously described by BIBRA (1993a). Groups of dogs (3 animals/sex/dose) were fed diets containing 0.015, 0.1 and 1.0% Litholrubine BK, (equivalent to 3.75, 25 and 250 mg/kg bw/day) for 2 years. A control group of 6 animals per sex was also included in the study. General parameters for body weight, food consumption, survival rate, blood chemistry, clinical chemistry and urinalysis were investigated. At necropsy, organ weights were determined and macroscopic and microscopic investigations were performed. No specific details were available on which organs were examined. No substance-related adverse effects were noted. It was reported that

thyroid weights were slightly increased with treatment but neither animal sexes nor doses at which this effect was observed are specified. It is stated that the slight increase in thyroid weights were not considered as a pathological effect by the authors of the study. However, it is not clear to the Panel what was the exact basis for this conclusion as the SCCNFP summary only states that this conclusion was based on information provided by BIBRA in which the observed effects were not correlated with macroscopic and microscopic investigations of this tissue.

BIBRA also summarised two chronic toxicity and carcinogenicity studies with Litholrubine B in mice and rats, which according to the references are the same as those evaluated by JECFA in 1987 and described above (BIBRA, 1993; JECFA, 1987). However, additional information was given by BIBRA in relation to the chronic toxicity study in the rats, as it was reported that the US Food and Drug Administration (FDA, 1982) subsequently examined tissue sections from the kidneys of all treated rats in this study, and concluded that Litholrubine B exacerbated a spontaneous kidney disease of aged rats (chronic progressive necrosis) in the mid- and high-dose male groups and in the high-dose female group.

No carcinogenicity study on Lithorubine BK was available for evaluation by the Panel.

3.2.5. Reproductive and developmental toxicity

In the long-term toxicity study which included an *in utero* phase carried out in Charles River CD rats with Litholrubine B (described above), observations were made on the fertility index, gestation anomalies, and effects on parturition and lactation; indices for live births and survival to weaning were calculated (JECFA, 1987). For the *in utero* phase, no compound-related effects were reported on body weight, food consumption, ophthalmoscopic examination, fertility, or gestation and lactation indices. In comparison to controls, a higher incidence of atrophy/degeneration of testicular tubules in high-dose male rats that died during the study and from 12 months of treatment to termination. These changes were considered common in aging rats and no specific compound-related effect was identified other than an acceleration of these changes. There was no statistically significant increase in incidence of the above lesions at termination (JECFA, 1987).

The SCF (1983) opinion only referred to reproduction studies with the calcium salt of Litholrubine (Litholrubine BK), which showed that the reproductive function was not affected (SCF, 1983). No further details were provided in the SCF evaluation and the Panel considers that this study might have been carried out using Litholrubine B, according to the reference included in the SCF opinion.

The BIBRA (1993a) toxicity profile for Litholrubine BK briefly referred to several reproductive toxicity studies described below. The SCCNFP (2004) report referred to the same studies as the BIBRA (1993a) toxicity profile.

Groups of female rats (20 animals/group) were administered Litholrubine BK at dose levels of 5, 16 or 50 mg/kg bw/day by stomach tube on days 6-15 of pregnancy. No adverse effects were observed on maternal weight gain, number of resorptions and fetal deaths, fetal weight and viability, litter size or incidence of fetal malformations or skeletal aberrations (Durloo and Woodard, 1972).

In a 3-generation rat study, groups (10 males and 20 females per group) were administered Litholrubine BK at dose levels of 0.5, 5, 15 or 50 mg/kg bw/day. No treatment-related effects were observed on maternal or fetal body weights, number of resorptions or survival of the offspring. There was a reported reduction in fertility in the second generation at 50 mg/kg bw/day, but this was not reported in the third generation at any dose level (Weil and Carpenter, 1973).

Groups of female rabbits (10 animals/dose) were given Litholrubine BK at dose levels of 5, 16 or 50 mg/kg bw/day by stomach tube on days 6-18 of pregnancy. No adverse treatment-related effects were observed on maternal weight gain, number of resorptions, litter size, fetal weight and viability, or the incidence of foetal malformations (Vettorazzi, 1981).

It is reported by BIBRA that litter size was decreased when mink were fed cheese rind containing Litholrubine BK but no further details were given (Vettorazzi, 1981).

The OECD report summarised a study in rats which had not been considered previously in evaluations by other bodies and was identified by the Panel as the only key study on repeated-dose toxicity of Litholrubine BK (with a reported purity of 98%) (OECD SIDS, 1994). The unpublished study was done according to the OECD combined repeated-dose and reproductive/developmental toxicity Test Guideline 422 and conducted under GLP. Groups of rats (13 animals/sex/dose) were exposed to Litholrubine BK by oral exposure (gavage) at doses of 0, 100, 300 and 1000 mg/kg bw/day. Males were exposed for 42 days including 14 days before mating and females were exposed from 14 days before mating to day 3 of lactation, with a reported frequency of treatment of 7 days/week. All animals survived to the end of the study. The only reported treatment-related effect was red-stained faeces considered not indicative of toxicity. Throughout the study, no differences were reported in mean body weight gains and food consumptions in all dose groups, in both sexes, when compared to the control groups. No statistically significant changes in haematological parameters were noted in any dosed male groups. On serum biochemistry, male rats that received 300 mg/kg bw/day or higher doses showed statistically significant decreased levels for serum calcium and phosphorus compared to controls. Male rats that received 1000 mg/kg bw/day showed significant decreases in serum potassium and total cholesterol levels, and statistically significant increases in chloride and Glutamic Oxaloacetic acid Transaminase (GOT) levels as compared to controls. No other statistically significant differences in clinical parameters were observed in the dosed male groups compared to controls. No clinical chemistry results were reported for the female rats in the study. Male rats that received 1000 mg/kg bw/day showed a statistically significant increase in relative but not in absolute kidney weights as compared to controls.

Female rats that received 100 or 1000 mg/kg bw/day Litholrubine BK showed statistically significant decreases in absolute thymus weights in comparison to the controls. Absolute thymus weights were not statistically significantly decreased in females of the mid-dose group. Relative thymus weight was statistically significantly decreased in females of the low-dose group only. No further statistically significant differences in organ weights were observed in males or females.

Histopathological examinations showed changes in the kidneys of treated animals. The lesions included regenerated renal tubular epithelium in male rats receiving 300 mg/kg bw/day Litholrubine BK or higher. In females, the incidence of foamy tubular epithelial cells was increased compared to controls in all treated groups. Although the incidence was similar, the severity of this lesion was slightly increased in the high-dose group only. In addition, increased incidences of necrotic tubular epithelium were seen in all treated groups compared to controls, however no dose-effect relationship either in incidence or severity was observed.

There were no histopathological changes reported in the other organs of the females.

No adverse effects on reproduction/development toxicity parameters were reported, as evidenced by lack of a statistically significant difference from the control group number of copulated pairs, copulation index, number of pregnant animals, fertility index, pairing days until copulation, gestation index and length in days, implantation index, delivery, live birth and viability index. No difference in the sex ratio and body weight of F1 pups up to the 4th day of lactation and no increase in external and visceral anomalies were reported.

Under the conditions of this study, the OECD SIDS report identified No-Observed-Effect-Levels (NOELs) of 300 mg/kg bw/day Litholrubine BK for males and 100 mg/kg bw/day Litholrubine BK for females.

The Panel, having consulted the tables in the original Japanese study report (FDSC, 1993) (and the English abstract of this study), concluded that there was a NOAEL of 100 mg/kg bw/day for males and that the NOAEL for females was below 100 mg/kg bw/day (the lowest dose tested).

3.2.6. Allergenicity, hypersensitivity and intolerance

Reactions to food colourings, including those triggered by immune (hypersensitivity) and non-immune (intolerance) mechanisms are assumed to be infrequent in the population, and prevalences from 0.14 to around 2% have been reported (Young et al., 1987; Hannuksela and Haahtela, 1987; Fuglsang, 1993, 1994). Recent studies performed under adequately controlled conditions imply that sensitivity to food additives in patients with chronic urticaria/angioedema or asthma is uncommon (Simon, 2003; Supramaniam and Warner, 1986). Adverse reactions after Lithorubine BK intake have not been reported.

The BIBRA (1993a) toxicity profile briefly referred to an unsuccessful attempt to induce sensitisation in 25 healthy subjects given five 48-hour covered patch tests with 50% Litholrubine BK mixed with talc; each exposure was separated by a 24-hour period of treatment with aqueous sodium lauryl sulphate. No skin reactions were seen when the volunteers were challenged (generally after a 10-14 day rest period) with a further 48-hour covered patch test using the same mixture of 50% Litholrubine BK with talc. A continuous 21-day application of a patch test in 10 healthy women with the same 50% Litholrubine BK mixture was reported as producing a minimal irritant effect (BIBRA, 1993a).

One case report of a 26-year-old woman showing erythematous papules and vesiculation, on and around the lower lip, was attributed to D&C Red No. 7 colour (Litholrubine BK) after testing all ingredients of a lipstick (Ha et al., 2003). The patient had a history of frequent inflammation of the lips (cheilitis) from using red-coloured lipsticks for the past 6 years. The authors indicated that cases of allergic contact cheilitis due to D&C Red No. 7 had never been reported before.

The Panel concludes that although after Litholrubine BK exposure from cosmetics cheilitis has been documented in one case report, no conclusion on the induction of hypersensitivity could be drawn from the limited scientific evidence available.

4. Discussion

The Panel was not provided with a newly submitted dossier for Litholrubine BK 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 not all original studies on which previous evaluations were based were available for re-evaluation by the Panel.

Litholrubine BK (E 180) is a red azo dye authorised as a food additive in the EU and previously evaluated by JECFA, with the latest evaluation in 1987 and by the SCF in 1983. JECFA was unable to establish an ADI (JECFA, 1987), whereas the SCF established an ADI of 0-1.5 mg/kg bw/day based apparently, on a NOAEL of 150 mg/kg bw/day from a long-term rat study (SCF, 1983). The JECFA evaluation done on D&C Red No. 6 (Litholrubine B) considered that there were limited histopathological examinations in two long-term studies in mice and rats that did not allow an unequivocal no-effect level to be determined. In order to comprehensively evaluate this dye, JECFA requested results from a complete histopathological examination of all dose groups in the long-term mouse study, results of a new long-term study in rats, and an adequate reproduction/teratology study.

Specifications have been defined in Commission Directive 2008/128/EC and by JECFA, 2006. The purity is specified as not less than 90% of total colouring matters. The remaining 10% may be accounted for by calcium chloride and/or calcium sulphate as the principal uncoloured components but this is never mentioned explicitly, ≤ 0.5% subsidiary colouring matters, ≤ 0.2% 2-amino-5-methylbenzenesulfonic acid, calcium salt, ≤ 0.4% 3-hydroxy-2-naphthalenecarboxylic acid, calcium salt, ≤ 0.01% unsulphonated primary aromatic amines (expressed as aniline) and ≤ 0.2% ether extractable matter.

The Panel notes that the only toxicokinetic data available on Litholrubine BK, suggest that there is very limited absorption in animals; most of the orally administered dose being excreted via the faeces and not via the urine. No additional information was available on Litholrubine B.

A study done in rats for an exposure period of 42 days in males and 17 days in females, in accordance with OECD Test Guideline 422 and GLP conditions, reported decreased general biochemistry parameters in serum and GOT levels as well as increased kidney weights and histopathological lesions in renal tubular epithelium, for males receiving 1000 mg/kg bw/day Litholrubine BK (OECD SIDS, 1994). No effects were reported on reproductive/developmental toxicity parameters measured in this study. Female rats that received 100 or 1000 mg/kg bw/day showed statistically significant decreases in thymus weights in comparison to the controls. Decreased in thymus weight were not statistically significant in females of the mid-dose group. Relative thymus weight was statistically significantly decreased in females of the low-dose group only. No further significant differences in organ weights were observed in both, males and females.

Histopathological examinations showed alterations predominantly occurring in the kidney. The lesions included regenerated renal tubular epithelium in male rats receiving 300 mg/kg bw/day or higher. In all treated females, the incidence of foamy tubular epithelial cells was increased compared to controls. Although the incidence was similar in all dose groups, the severity of this lesion was slightly increased in the high-dose group only. In addition, increased incidences of necrotic tubular epithelium were seen in all treated groups compared to controls, however no dose-effect relationship either in incidence or severity was observed.

The Panel, having consulted the tables in the original Japanese publication (FDSC, 1993) and the English abstract of this study, concluded that there was a NOAEL of 100 mg/kg bw/day for males and that the NOAEL for females was below 100 mg/kg bw/day (the lowest dose tested). Based on the lack of a clear dose-response relationship in this study the Panel was unable to identify a suitable NOAEL, LOAEL or BMD to establish an ADI.

Based on available mutagenicity studies the Panel considers that Litholrubine BK does not show genotoxic activity *in vitro*.

The conversion of Litholrubine BK by azo reduction *in vivo* results in the formation of a sulphonated aromatic amine and 4-amino-3-hydroxy-2-naphthoic acid that may not be formed in the standard *in vitro* genotoxicity tests (Prival and Mitchell, 1982). 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 the sulphonated aromatic amine that could be formed by azo reduction of Litholrubine BK was included in this review, the Panel concludes that the data reviewed by Jung et al. (1992) are sufficiently reassuring to support the conclusion that the sulphonated aromatic amine formed from Lithorubine BK by azo reduction does not give reason for concern with respect to genotoxicity.

The Panel noted that the 4-amino-3-hydroxy-2-naphthoic acid which would result from azoreduction of Litholrubine BK was not covered by the review of Jung et al. (1992). However, the Panel considered that 4-amino-3-hydroxy-2-naphthoic acid would be expected to behave similarly as the closely structurally related amino-naphthol-sulphonic acids which have been shown to be negative.

The Panel also notes that the specifications on the purity of Litholrubine BK permit concentrations of unidentified unsulphonated aromatic amines (expressed as aniline) to be present in concentrations of up to 100 mg/kg Litholrubine BK. Although some aromatic amines may be associated with genotoxicity or even carcinogenicity, the Panel notes that Litholrubine BK was negative in *in vitro* genotoxicity tests.

Allergic reactions after Litholrubine BK intake have not been reported. Recent studies performed under properly controlled conditions imply that sensitivity to food additives in patients with chronic

urticaria/angioedema or asthma is uncommon. Therefore the Panel concludes that while one case report of cheilitis after exposure from cosmetics containing Litholrubine BK has been documented, no conclusion on the induction of hypersensitivity by Litholrubine BK could be drawn from the limited scientific evidence available.

Overall, the Panel concludes that the present database is too limited to continue supporting the ADI for Litholrubine BK previously set by the SCF (1983) or to establish a new ADI. Therefore, the Panel considers that the existing ADI of 0-1.5 mg/kg bw/day established by the SCF should be withdrawn.

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 Tiers (SCOOP Task 4.2). Since Litholrubine BK is only allowed to be present in a single specified foodstuff at QS, estimation of exposure based on the Budget method (Tier 1) was considered inappropriate by the Panel. As no actual levels of use or analytical data were provided to the Panel, only the Tier 2 approach has been assessed. Dietary exposure estimates have been performed for child and adult populations based on the maximum level of use of 100 mg/kg for the food category 'edible cheese rinds' in accordance with the decision rules defined by the Panel to deal with QS authorisations (Appendix A).

The Panel made exposure estimates for children (1-10 years old) for 12 European countries (Belgium, France, UK, the Netherlands, Spain, Czech Republic, Italy, Finland, Germany, Denmark, Cyprus, Greece) and the adult population (>18 years old). In the absence of specific food consumption data for the food category 'edible cheese rinds' as such, in the European database, the Panel decided to apply a general correction factor of 10% to the consumption of the cheese food category in order to express food consumption data for edible cheese rinds for all members states.

The Panel notes that the anticipated exposure to Litholrubine BK from an assumed maximum level of use of 100 mg/kg in edible cheese rinds, based on exposure estimates in high consumers of edible cheese rinds, were from 0.02 to 0.06 mg/kg bw/day in children and from 0.01 to 0.03 mg/kg bw/day in adult population. The highest level of anticipated exposure (95^{th}/97.5^{th}) is 1700-fold lower than the identified effect level of 100 mg/kg bw/day in female rats. The Panel further notes that Litholrubine BK can be used following the QS principle and information on actual use levels in edible cheese rinds is not available. The Panel also notes that from the call of usage data on additives, members of the CIAA did not report using Litholrubine BK in cheese products.

Provided the use level assumed by the Panel (100 mg/kg in edible cheese rinds) is realistic and that otherwise the exposure assessment makes worst case assumptions, and taking into account the lack of a dose-response in the incidence of the renal lesions and the low severity observed in female rats, the Panel considers that it is unlikely there would be a significant safety concern for humans from the current single authorised use of Litholrubine BK in edible cheese rinds.

The Panel further notes that the specifications of Litholrubine BK need to be updated with respect to the percentage of material not accounted for that may represent calcium chloride and/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.

No information was available to suggest that Litholrubine BK could be found as an aluminium lake.

CONCLUSIONS

Litholrubine BK (E180) is a red mono-azo dye that has been previously evaluated by the JECFA and the SCF. JECFA was unable to establish an ADI, considering that there were limited histopathological

examinations in two long-term studies in mice and rats that did not allow an unequivocal no-effect level to be determined. The SCF established an ADI of 0-1.5 mg/kg bw/day.

The Panel concludes that the basis for the establishment of the existing SCF ADI of 0-1.5 mg/kg bw/day based on a reported NOAEL of 150 mg/kg bw/day identified in a long-term rat study is unclear due to the limited information available in this evaluation.

The Panel was unable to identify a suitable NOAEL, LOAEL or BMD to establish an ADI and concludes that the existing SCF ADI of 0-1.5 mg/kg bw/day should be withdrawn.

The Panel notes that the highest anticipated exposure to Litholrubine BK is 1700-fold lower than the identified effect level of 100 mg/kg bw/day in female rats. Therefore, the Panel considers that it is unlikely there would be a significant safety concern for humans from the current single authorised use of Litholrubine BK in edible cheese rinds.

The Panel further notes that the specifications for Litholrubine BK need to be updated with respect to the percentage of material not accounted for that may represent calcium chloride and/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.

DOCUMENTATION PROVIDED TO EFSA

1. Pre-evaluation document prepared by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

2. CIAA (Confederation of the Food and Drink Industries of the EU). Exercise on occurrence data - EFSA re-evaluation of some food colours (December 2009)

REFERENCES

BIBRA (British Industrial Biological Research Association), 1993a. Toxicity Profile Lithol rubine BK. BIBRA Information Services Ltd. Surrey, UK.

BIBRA (British Industrial Biological Research Association), 1993b. Toxicity Profile Lithol rubine B. BIBRA Information Services Ltd. Surrey, UK.

Brown JP, Dietrich PS and Bakner CM, 1979. Mutagenicity testing of some drug and cosmetic dye lakes with the Salmonella/mammalian microsome assay. Mutation Res 66, 181.

Durloo RS and Woodard G, 1972. Safety evaluation of D&C red no. 7 in a teratology study in the rat. Unpublished report from Woodard Research Corporation, submitted to the Inter-Industry Color Committee (cited in Vettorazzi, 1981) (as referred by BIBRA 1993a).

EFSA (European Food Safety Authority), 2008. EFSA, Parma, 17 March 2008 EFSA/DATEX/2008/01.

FDA (US Food and Drug Administration), 1982. D&C red no. 6 and D&C red no. 7. Federal Regulations, 47, 57681 (as referred by BIBRA 1993a).

FDSC (Food and Drug Safety Center), 1993. D&C Red No.7. Food and Drug Safety Center, Hatano Research Institute, 729-5 Ochiai, Hadano-shi, Kanagawa 257, Japan. Available from: http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF5281-04-9d.pdf.

Fuglsang G, Madsen C, Saval P and Osterballe O, 1993. Prevalence of intolerance to food additives among Danish school children. Pediatric Allergy and Immunology 4(3), 123-129.

Fuglsang G, Madsen G, Halken S, Jørgensen S, Ostergaard PA and Osterballe O, 1994. Adverse reactions to food additives in children with atopic symptoms. Allergy 49(1), 31-37.

Green MR and Pastewka JV. 1980. Mutagenicity of some lipsticks and their dyes. Journal of the National Cancer Institute 64(3), 665-669.

Ha JA, Kim HO, Lee JY and Kim CW, 2003. Allergic contact cheilitis from D&C red no. 7 in lipstick. Contact Dermatitis 48, 231.

Hannuksela M and Haahtela T, 1987. Hypersensitivity reactions to food additives. Allergy 42(8), 561-575.

Hansen WH, Fitzhugh OG and Williams MW, 1958. Subacute oral toxicity of nine D and C coal tar colors. Journal of Pharmacology and Experimental Therapeutics 122, 29A (abstract 7789) (as referred by BIBRA 1993a).

IRDC (International Research and Development Corporation) (1981a) D&C Red No. 6. Long-term feeding study in rats exposed in utero. Unpublished report No. 355-014 from International Research and Development Corporation. Submitted to WHO by the Public Health Administration, Leidschendam, The Netherlands. (As referred to by JECFA, 1987 and BIBRA, 1993a).

IRDC (International Research and Development Corporation) (1981b) D&C Red No. 6. Long-term feeding study in mice. Unpublished report No. 355-012 from International Research and Development Corporation. Submitted to WHO by the Public Health Administration, Leidschendam, The Netherlands. (As referred to by JECFA, 1987 and BIBRA, 1993a,b).

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1987. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 21, nos 615 on INCHEM. Available form: http://www.inchem.org/documents/jecfa/jecmono/v21je09.htm.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2006. Compendium of Food Additive and Flavouring Agents Specifications. Monograph 1. Lithol Rubine BK. Available from: http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-254.pdf. Accessed on the 11th October 2009.

Jung R, Steinle D and Anliker R, 1992. A compilation of genotoxicity and carcinogenicity data on aromatic aminosulphonic acids. Food and Chemical Toxicology 30, 635-660.

Kusakabe H, Yamakage K, Wakuri S, Sasaki K, Nakagawa S, Watanabe M, Hayashi M, Sofuni T, Onoa H and Tanaka N. 2002. Relevance of chemical structure and cytotoxicity to the induction of chromosome aberrations based on the testing results of 98 high production volume industrial chemicals. Mutation Research 517, 187–198.

Leist KH, 1982. Sub-acute toxicity studies of selected organic colorants. Ecotoxicological and Environmental Safety 6, 457-463.

Milvy P and Kay K, 1978. Mutagenicity of 19 major graphic arts and printing dyes. Journal of Toxicology and Environmental Health, Part A, Volume 4, Issue 1, 31 - 36

Miyagoshi M, Hayakawa Y and Nagayama T, 1983. Studies on the mutagenicity of cosmetic azodyes. Eisei Kagaku 29, 212 (English abstract and tables) (as referred by BIBRA 1993a).

Muzzall JM and Cook WL, 1979. Mutagenicity test of dyes used in cosmetics with Salmonella/mammalian-mic-rosome test. Mutation Research 67, 1-8.

OECD SIDS (Organisation for Economic Co-operation and Development Screening Information DataSet), 1994. SIDS Initial Assessment Report for SIAM 2. D&C Red No. 7. 2-Naphthalenecarboxylic acid, 3-hydroxy-4-[(4-methyl-2-sulfophenyl)azo]-, calcium salt. CAS N°: 5281-04-9. UNEP Publications. Paris, France. Available from: http://www.inchem.org/documents/sids/sids/5281049.pdf.

Prival MJ and Mitchell VD 1982. Analysis of a method for testing azo dyes for mutagenic activity in Salmonella typhimurium in the presence of flavin mononucleotide and hamster liver S9. Mutation Research 97, 103-116.

SCF (Scientific Committee for Food), 1979. Reports of the Scientific Committee for Food (8th series).

SCF (Scientific Committee for Food), 1983. Reports of the Scientific Committee for Food (14th series). Opinion expressed 1983, 59-60.

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products), 2004. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers. Pigment Red 57. Adopted 25 May 2004.

Scotter MJ and Castle L, 2004. Chemical interactions between additives in foodstuffs: a review. Food Additives and Contaminants, Part A 21(2), 93-124.

Simon RA, 2003. Adverse reactions to food additives. Current Allergy and Asthma Reports 3(1), 62-66.

Supramaniam G and Warner JO, 1986. Artificial food additive intolerance in patients with angio-oedema and urticaria. Lancet 2 (8512), 907-909.

Souci S, Fachmann W and Kraut H, 2000. Food composition and nutrition tables, CRC Press, MedPharm Scientific Publishers, Stuttgart, 6th revised edition.

TemaNord, 2002. Food additives in Europe 2000; Status of safety assessments of food additives presently permitted in the EU. TemaNord 560. Copenhagen.

Tennant D, 2006. Screening of Colour Intakes from Non-Alcoholic Beverages. Report prepared for the Union of European Beverages Associations (UNESDA), December 2006.

Tennant D, 2007. Screening potential intakes of natural food colours. Report provided for the Natural Food Colours Association (NATCOL), July 2007.

Vettorazzi G (editor), 1981. Handbook of International Food Regulatory Toxicology. Volume 2: Profiles. MTP Press Ltd. Lancaster, UK.

Weil CS and Carpenter C, 1973. Results of inclusion in the diet of rats for three generations. Unpublished report from Mellon Institute, submitted to the Inter-Industry Color Committee by the Cosmetic Toiletry and Fragance Association, Inc. (cited in Vettorazzi, 1981) (as referred by BIBRA 1993a).

Young E, Patel S, Stoneham M, Rona R and Wilkinson JD, 1987. The prevalence of reaction to food additives in a survey population. Journal of the Royal College of Physicians of London 21, 241-247 (as referred to by BIBRA, 1990).

APPENDIX

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 foodstuff, 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 than10 analytical data are available, the highest value is used;
    ii. if less than10 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.

2. **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 additives directives and the principles of the Budget method.

b. Tier 2: Estimates are based on MPLs of use, as specified in the additives directives 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
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
BIBRA	British Industrial Biological Research Association
BMD	Benchmark Dose
CAS RN	Chemical Abstracts Service Registry Number
CAT	Chromosomal Aberration Test
CIAA	Confederation of the Food and Drink Industries of the EU
EC	European Commission
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
GLP	Good Laboratory Practice
GOT	Glutamic Oxaloacetic acid Transaminase
HPV	High Production Volume
IRDC	International Research and Development Corporation
JECFA	Joint Expert Committee on Food Additives
LOAEL	Low-Observed-Adverse-Effect-Level
MPL	Maximum permitted use level
NATCOL	Natural Food Colours Association
NDNS	National Diet and Nutrition Survey
NOAEL	No-Observed-Adverse-Effect-Level
NOEL	No-Observed-Effect-Level
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers
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
SIDS	Screening Information DataSet
TWI	Total Weekly Intake
UNESDA	Union of European Beverages Associations

WHO	World Health Organization