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SCIENTIFIC OPINION

Scientific Opinion on the re-evaluation of riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) as food additives[1]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The EFSA ANS Panel provides a scientific opinion re-evaluating the safety of riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) which are authorised as food additives in the EU and have been previously evaluated by JECFA and by the SCF. JECFA allocated a group ADI for riboflavin and riboflavin-5′-phosphate sodium of 0–0.5 mg/kg bw/day. The SCF considered that the use of riboflavin-5′-phosphate sodium as a food colour should not alter significantly the average daily intake of riboflavin for which no ADI was established. 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 considered that riboflavin-5′-phosphate sodium is rapidly dephosphorylated to free riboflavin in the intestinal mucosa and then metabolised using normal metabolic pathways. The Panel noted that no adverse effects were observed in two 90-day studies in rat and that riboflavin and riboflavin-5′-phosphate do not raise concern with respect to genotoxicity. The Panel also noted that there are limited data from clinical studies, in which no significant adverse effects were reported. The Panel considered that the use of riboflavins as food additives will result in an exposure above that from the regular diet and that the available database is insufficient to assess whether potential high intakes from all combined sources cause adverse effects or not. Due to the absence of carcinogenicity/chronic toxicity studies and lack of relevant reproductive and developmental toxicity studies, the Panel considered that it is not appropriate to allocate an ADI. The Panel concluded, despite the uncertainties in the database, that riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) are unlikely to be of safety concern at the currently authorised uses and use levels as food additives.

KEY WORDS

riboflavin, riboflavin-5′-phosphate, E 101, CAS Registry Number 83-88-5, CAS Registry Number 130-40-5, food additive, food colour

[1] On request from the European Commission, Question No EFSA-Q-2011-00361, EFSA-Q-2011-00430, adopted on 12 September 2013.

[2] Panel members: Fernando Aguilar, Riccardo Crebelli, Birgit Dusemund, Pierre Galtier, David Gott, Ursula Gundert-Remy, Jürgen König, Claude Lambré, Jean-Charles Leblanc, Pasquale Mosesso, Alicja Mortensen, Agneta Oskarsson, Dominique Parent-Massin, Martin Rose, Ivan Stankovic, Paul Tobback, Ine Waalkens-Berendsen, Ruud Woutersen and Matthew Wright. Correspondence: ans@efsa.europa.eu

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SUMMARY

Following a request from the European Commission, the Panel on Food Additives and Nutrient Sources added to Food (ANS Panel) of the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion re-evaluating the safety of riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) when used as food additives.

Riboflavins (E 101) are authorised as food additives in the European Union (EU) in accordance with Annex II to Regulation (EC) No 1333/2008 and have been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969, 1981 and 1998, and by the EU Scientific Committee for Food (SCF) in 1977, 1984, 1998 and 2000. Synthetic riboflavin was evaluated by JECFA in 1969, where an acceptable daily intake (ADI) of 0–0.5 mg/kg bw/day was allocated on the basis of limited data. JECFA based this ADI on a "level causing no adverse effects in the rat" (50 mg/kg bw/day, expressed as riboflavin). In 1981, JECFA allocated a group ADI for riboflavin and riboflavin-5′-phosphate of 0–0.5 mg/kg bw/day (expressed as riboflavin). It is not specified in the evaluation on which study this ADI was based. In 1998, JECFA included riboflavin derived by fermentation with a strain of genetically modified *Bacillus subtilis* in the previously established group ADI of 0–0.5 mg/kg bw/day for synthetic riboflavin and riboflavin-5′-phosphate sodium.

In 1977, the SCF classified riboflavin-5′-phosphate sodium as a colour which could be used in food, but no ADI was established. The SCF was of the opinion that the use of this substance as a food colour should not alter significantly the average daily intake of riboflavin. In 1998, the SCF concluded that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* is acceptable for use as a food colour. Furthermore, in 2000, the SCF concluded that it was not possible, based on the available database, to derive a tolerable upper intake level (UL) for riboflavin used as a vitamin because the available data on adverse effects from high oral riboflavin intake were not of sufficient quality and extent as to be used for the determination of a UL. However, the SCF (2000) stated that the limited evidence available from clinical studies indicated that current levels of intake of riboflavin from all sources do not represent a risk to human health.

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 some original studies on which previous evaluations were based were not available for re-evaluation by the Panel.

Riboflavin and riboflavin-5′-phosphate sodium can be obtained by chemical synthesis or from microbiological sources; however, according to industry, chemical synthesis is not currently used.

Riboflavin is relatively stable during thermal and non-thermal food processing and storage in the dark but is very sensitive to light. Riboflavin-5′-phosphate sodium is fairly stable to air but is hygroscopic and sensitive to heat and light. Several compounds are formed from riboflavin and riboflavin-5′-phosphate sodium under the influence of light, including the non-volatile compounds lumichrome and lumiflavin, and the volatile compound 2,3-butanedione.

When administered alone, the absorption of free riboflavin is 30–50 % at the dose range of 5–20 mg and is decreased at higher oral doses. However, the amount of riboflavin absorbed depends on the intake; it is increased when riboflavin is given orally with food. Riboflavin-5′-phosphate sodium and riboflavin are probably absorbed by a specific transport system in the upper gastrointestinal system. In plasma, riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins, and is mainly found as flavin adenine dinucleotide (FAD). Lumichrome and lumiflavin have been identified as metabolites of riboflavin in the rat, while 7-α-hydroxyriboflavin has been identified as a plasma metabolite in humans. Riboflavin-5′-phosphate sodium may be dephosphorylated during absorption but may subsequently be rephosphorylated in the mucosa, transported to the liver, where it is again

dephosphorylated to riboflavin (the form in which it occurs in the circulation), and mainly excreted in urine.

Wistar rats received diets providing 20, 50 or 200 mg riboflavin/kg bw/day for 13 weeks (Buser et al., 1995). The purpose of this study was to assess and compare the toxicity of two preparations of riboflavin produced by a new fermentative method, called "riboflavin 96 % ex fermentation" and "riboflavin 98 % ex fermentation", and a riboflavin produced by chemical synthesis named "riboflavin 98 % ex synthesis". In the absence of any adverse effect, the no observed adverse effect level (NOAEL) identified in this study was 200 mg/kg bw/day for the three grades of riboflavin. The Panel agreed with this NOAEL.

In a study by Bachman et al. (2005), the test item, containing 80.1 % riboflavin, 0.25 % lumichrome, 1.5 % 6,7-dimethyl-8-ribityl-lumazine (DMRL), 0.1 % 8-hydroxymethyl-riboflavin (8-HMR) and 20 % maltodextrin, was tested on SPF-bred Wistar rat of both sexes at target doses of 0, 50, 100 and 200 mg test item/kg bw/day for 13 weeks in accordance with an OECD guideline. In the absence of any adverse effect, the authors of the study considered that the NOAEL for male and female rats was 200 mg test item/kg bw/day, the highest dose level tested, corresponding to 160.2 mg riboflavin/kg bw/day, 0.5 mg lumichrome/kg bw/day, 0.2 mg 8-HMR/kg bw/day and 3 mg DMRL/kg bw/day. The Panel agreed with this NOAEL.

Based on the *in vitro* genotoxicity data available, the Panel concluded that the use of riboflavin and riboflavin-5′-phosphate sodium as food additives does not raise concern with respect to genotoxicity.

No chronic toxicity studies or carcinogenicity studies are available.

In 1981, JECFA concluded, after the evaluation of three studies on reproductive and developmental toxicity, that no adverse effects were observed. The Panel noted that the quality of these studies was not adequate to conclude on the reproductive and developmental toxicity.

The Panel noted that there are several human studies in children, adolescent and adults (including pregnant women) on the possible beneficial effects of riboflavin supplementation in the case of deficiency and clinical trials using riboflavin as migraine prophylaxis. The Panel noted that these studies were not designed as safety studies; however, they provide information on the limited range of observed adverse effects.

Due to the absence of carcinogenicity/chronic toxicity studies and lack of relevant reproductive and developmental toxicity studies, the Panel considered that it is not appropriate to allocate an ADI to riboflavin and riboflavin-5′-phosphate sodium.

In Annex II to Regulation (EC) No 1333/2008, riboflavins are permitted in concentrations up to 100 mg/L in americano, bitter soda and bitter vino, and at *quantum satis* in pasturmas (edible external coating) and vegetables in vinegar, brine or oil (excluding olives). Furthermore, riboflavins may be added to all foodstuffs other than those listed in Annex II to Regulation (EC) No 1333/2008 at *quantum satis*.

The exposure of European children to riboflavins used as food additives calculated by using the data provided by industry ranged from 0.5 to 1.8 mg/kg bw/day at the mean, and from 0.9 to 3.9 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to riboflavins (> 10 % in all countries) were processed fruit and vegetables (up to 51 %), soups and broths (up to 55 %) and sauces (up to 35 %). Estimates calculated for the adult population give a dietary exposure to riboflavins of 0.2–0.7 mg/kg bw/day at the mean and 0.4–1.7 mg/kg bw/day for high-level (95th percentile) consumers. The main contributors (> 10 %) to the total anticipated mean exposure to riboflavins were processed fruit and vegetables (14–65 %) and soups (9–36 %).

Overall, the Panel considered that:

- Riboflavin-5′-phosphate sodium is rapidly dephosphorylated to free riboflavin in the intestinal mucosa then metabolised using normal metabolic pathways.

- Two subchronic toxicity studies in rats, performed in accordance with OECD guidelines, did not report any adverse effects at doses amounting up to 160 and 200 mg riboflavin/kg bw/day, the highest doses tested.

- Riboflavin and riboflavin-5′-phosphate sodium do not raise concern with respect to genotoxicity.

- There are limited data from clinical studies with doses up to 400 mg riboflavin/day, in which no significant adverse effects were reported.

- The use of riboflavin and riboflavin-5′-phosphate sodium as food additives will result in an exposure that is higher than that from the regular diet.

- The available database is insufficient to assess whether or not potential high intakes from all combined sources (food additive, food supplements, diet) cause adverse effects.

Despite the uncertainties in the database, the Panel concluded that riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) are unlikely to be of safety concern at the currently authorised uses and use levels as food additives.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

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

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under the Regulation (EU) No 257/2010[4]. This Regulation also foresees that food additives are re-evaluated whenever necessary in light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[5] of 2001. The report "Food additives in Europe 2000[6]" submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with a highest priority.

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

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

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

ASSESSMENT

1. Introduction

The present opinion deals with the re-evaluation of the safety of riboflavins (E 101), which include riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)), when used as food additives.

The Panel noted that, as only the sodium salt of riboflavin-5′-phosphate (E 101(ii)) is authorised as a food additive, the title of the European Commission (EC) specifications according to the Commission Regulation (EU) No 231/2012[7], "riboflavin-5′-phosphate", does not adequately describe the chemical identity of the substance. The Panel used the term "riboflavin" to refer to the food additive E 101(i) and "riboflavin-5′-phosphate sodium" to refer to the food additive E 101(ii).

Riboflavins are authorised as food additives in the EU in accordance with Annex II to Regulation (EC) No 1333/2008[8] and have been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1969, 1981 and 1998, and by the EU Scientific Committee for Food (SCF) in 1977, 1984, 1998 and 2000.

Riboflavin and riboflavin-5′-phosphate sodium are also authorised sources of vitamin B_2 for food fortification, PARNUTS (foods for particular nutritional uses) and food supplements. In 2000, the SCF concluded that it was not possible, based on the available database, to derive a tolerable upper intake level (UL) for riboflavin used as a vitamin because the available data on adverse effects from high oral riboflavin intake were not of sufficient quality and extent to be used for the determination of a UL. However, the SCF (2000) stated that the limited evidence available from clinical studies indicated that current levels of intake of riboflavin from all sources do not represent a risk to human health.

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.[9] The Panel noted that some original studies, on which previous evaluations were based, were not available for re-evaluation by the Panel.

2. Technical data

2.1. Identity of the substance

The chemical name for riboflavin is 7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)benzo(g)pteridine-2,4(3H,10H)-dione). Its molecular weight is 376.37 g/mol. The molecular formula is $C_{17}H_{20}N_4O_6$. The Chemical Abstracts Service (CAS) Registry Number is 83-88-5 and the European Inventory of Existing Commercial chemical Substances (EINECS) number is 201-507-1. It is a yellow to orange-yellow crystalline powder with a slight odour. It is very slightly soluble in water, practically insoluble in alcohol, chloroform, acetone and ether and very soluble in dilute alkali solutions (JECFA, 2006). Its pK_a is ~ 10 at 20 °C (saqual GmbH, 2010).

The chemical name for riboflavin-5′-phosphate sodium is monosodium (2*R*,3*R*,4*S*)-5-(3′)10′-dihydro-7′,8′-dimethyl-2′,4′-dioxo-10′-benzo[γ]pteridinyl)-2,3,4-trihydroxypentyl phosphate. Its molecular weight is 514.36 g/mol (dehydrated form). The dihydrate form has the molecular formula $C_{17}H_{20}N_4NaO_9P{\cdot}2H_2O$ and the anhydrous form the molecular formula $C_{17}H_{20}N_4NaO_9P$. The CAS Registry Number of the anhydrous form is 130-40-5 and the EINECS number is 204-988-6. The CAS Registry Number of the dihydrate form is 6184-17-4. It is a yellow to orange crystalline hygroscopic

[7] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, pp. 1–295.

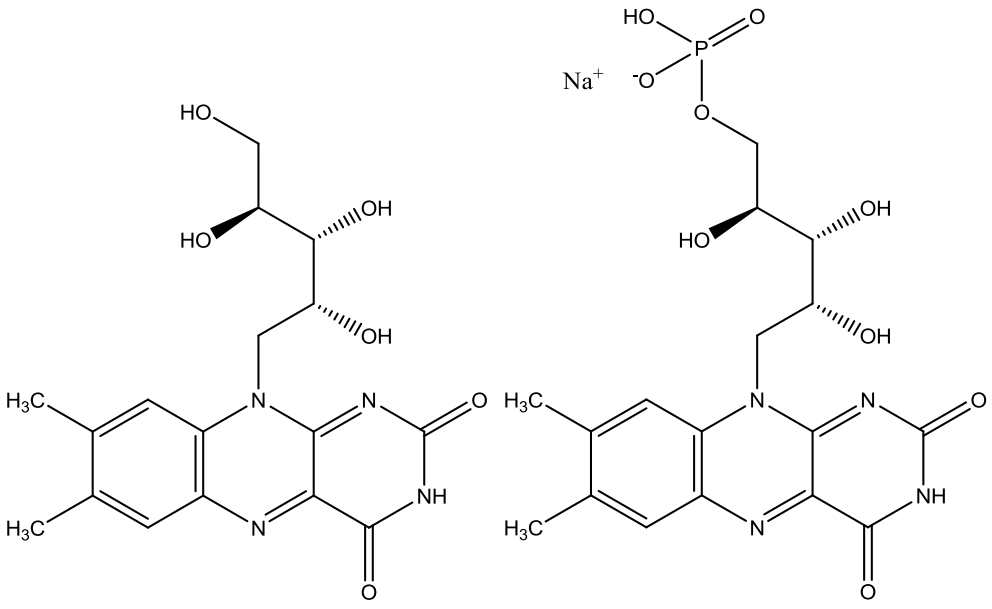
[8] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008.

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

powder with a slight odour, soluble in water and insoluble in ethanol (JECFA, 2006). Its pK_a is ~ 2.5, 6.5 and 10.3 at 20 °C (saqual GmbH, 2010).

More than 40 synonyms are in use for riboflavin and riboflavin-5′-phosphate sodium (ChemIDplus advanced, Internet, 2013). The synonyms most commonly used in the published literature are, for riboflavin, lactoflavin, vitamin B_2, flavaxin, vitamin G and NSC 33298 and, for riboflavin-5′-phosphate sodium, riboflavin-5′-phosphate ester monosodium salt and sodium flavin mononucleotide (FMN).

The structural formulae for riboflavin and riboflavin-5′-phosphate sodium according to ChemIDplus (via Internet, 2013[10]) are given in Figure 1.



Riboflavin Riboflavin-5′-phosphate sodium

Figure 1: Structural formulae of riboflavin and riboflavin-5′-phosphate sodium

2.2. Specifications

Specifications have been defined in Commission Regulation (EU) No 231/2012 and by JECFA (JECFA, 2006) (Tables 1, 2 and 3).

[10] Riboflavin; riboflavin-5′-phosphate sodium.

Table 1: Specifications for riboflavin (E 101(i)) according to Commission Regulation (EU) No 231/2012 and JECFA (2006)

	Commission Regulation (EU) No 231/2012	**JECFA (2006)**
Identification		
Solubility	–	Very slightly soluble in water; practically insoluble in alcohol, chloroform, acetone and ether; very soluble in dilute alkali solutions
Spectrometry	In aqueous solution, the ratio A_{375}/A_{267} is between 0.31 and 0.33 and the ratio A_{444}/A_{267} is between 0.36 and 0.39. Maximum in water at ca. 375 nm	Using the aqueous solution from the assay, the ratio A_{375}/A_{267} is between 0.31 and 0.33 and the ratio A_{444}/A_{267} is between 0.36 and 0.39
Specific rotation	$[\alpha]_D^{20}$: between –115° and –140° in a 0.05 *N* sodium hydroxide solution	$[\alpha]_D^{20}$: between –115° and –140°. Dry the sample at 100 °C for four hours. Dissolve 50.0 mg in 0.05 *N* sodium hydroxide free from carbonate and dilute to 10.0 mL with the same solvent. Measure the optical rotation within 30 minutes of dissolution
Colour reaction	–	Dissolve about 1 mg of sample in 100 mL of water. The solution has a pale greenish-yellow colour by transmitted light, and by reflected light has an intense yellowish-green fluorescence which disappears on the addition of mineral acids and alkalis
Assay		
	Content not less than 98 % (on an anhydrous basis)	Not less than 98 %
Purity		
Loss on drying after four hours at 105 °C	Not more than 1.5 %	Not more than 1.5 %
Sulphated ash	Not more than 0.1 %	Not more than 0.1 % (in 2 g of sample)
Primary aromatic amines (calculated as aniline)	Not more than 100 mg/kg	Not more than 100 mg/kg
Subsidiary colouring matters	–	The absence of lumiflavin should be tested
Arsenic	Not more than 3 mg/kg	
Lead	Not more than 2 mg/kg	Not more than 2 mg/kg
Mercury	Not more than 1 mg/kg	
Cadmium	Not more than 1 mg/kg	

Table 2: Specifications for riboflavin from *Bacillus subtilis* according to JECFA (2006)

	JECFA (2006)
Identification	
Solubility	Practically insoluble in ethanol, acetone and diethyl ether; very soluble in dilute alkali solutions
Spectrophotometry	Using the aqueous solution from the assay, determine the absorbance (*A*) at 267 nm, 375 nm and 444 nm. The ratio A_{375}/A_{267} is between 0.31 and 0.33. The ratio A_{444}/A_{267} is between 0.36 and 0.39
Specific rotation	$[\alpha]_D^{20}$: Between –120° and –135°. Dry the sample at 100 °C for four hours. Dissolve 50.0 mg in 0.05 *N* sodium hydroxide free from carbonate and dilute to 10.0 mL with the same solvent. Measure the optical rotation within 30 minutes of dissolution
Colour reaction	Dissolve about 1 mg of sample in 100 mL of water. The solution has a pale greenish-yellow colour by transmitted light, and by reflected light has an intense yellowish-green fluorescence, which disappears on the addition of mineral acids and alkalis
Assay	
	Not less than 98.0 % and not more than 101.0 %, calculated on the dried basis
Purity	
Loss on drying after four hours at 105 °C	Not more than 2 %
Sulphated ash	Not more than 0.1 %
Lumiflavin	Not more than 0.025 %
Primary aromatic amines	Not more than 100 mg/kg (calculated as aniline)
Lead	Not more than 1 mg/kg

Table 3: Specifications for riboflavin-5′-phosphate sodium (E 101(ii)) according to Commission Regulation (EU) No 231/2012 and riboflavin-5′-phosphate sodium according to JECFA (2006)

	Commission Regulation (EU) No 231/2012	**JECFA (2006)**
Identification		
Solubility	–	Soluble in water; insoluble in ethanol
Spectrometry	In aqueous solution, the ratio A_{375}/A_{267} is between 0.30 and 0.34. The ratio A_{444}/A_{267} is between 0.35 and 0.40. Maximum in water at ca. 375 nm	Using the aqueous solution from the assay, the ratio $A3_{75}/A_{267}$ is between 0.30 and 0.34. The ratio A_{444}/A_{267} is between 0.35 and 0.40
Specific rotation	$[\alpha]_D^{20}$: between +38° and +42° in a 5 molar HCl solution	$[\alpha]_D^{20}$: between +38° and +42° (1.5 % w/v solution of dried sample in 20 % w/v HCl)
Test for sodium	–	Passes test Use the sulphated ash for the test
Assay		
	Content not less than 95 % total colouring matters calculated as $C_{17}H_{20}N_4NaO_9P{\cdot}2H_2O$	Content not less than 95 % total colouring matters calculated as $C_{17}H_{20}N_4NaO_9P{\cdot}2H_2O$
Purity		
Loss on drying after five hours in vacuum over P_2O_5 at 100 °C	Not more than 8 %	Not more than 8 %
Sulphated ash	Not more than 25 %	Not more than 25 %
Inorganic phosphate (calculated as PO_4 on a dried basis)	Not more than 1 %	Not more than 1 %
Subsidiary colouring matters: • Riboflavin (free) • Riboflavin diphosphate • Lumiflavin	 Not more than 6 % Not more than 6 % –	 Not more than 6 % Not more than 6 % Passes test for absence
Primary aromatic amines (calculated as aniline)	Not more than 70 mg/kg	Not more than 70 mg/kg
Arsenic	Not more than 3 mg/kg	–
Lead	Not more than 2 mg/kg	Not more than 2 mg/kg
Mercury	Not more than 1 mg/kg	–
Cadmium	Not more than 1 mg/kg	–

According to industry, primary aromatic amines are produced when riboflavin is chemically synthesised. Biological processes have currently replaced the chemical synthesis of riboflavin, and it is reported that primary aromatic amines as potential by-products are not known from riboflavin fermentation process (saqual GmbH, 2010).

According to industry, the main degradation products of riboflavin and riboflavin-5′-phosphate sodium expected to be present as impurities in the commercial products are: lumiflavin (CAS No 1088-56-8), lumichrome (CAS No 1086-80-2) and 8-hydroxymethyl-riboflavin (8-HMR) (CAS No 52134-62-0) (saqual GmbH, 2010). Although 2,3-butanedione is described as degradation product in the literature (Jung et al., 2007), according to the industry, it is not regarded as impurity likely to occur in riboflavin and riboflavin 5′-phosphate sodium (saqual GmbH, 2010).

The Panel noted that while JECFA defines a limit of < 0.025 % for lumiflavin in the specifications for riboflavin from *Bacillus subtilis*, limits for lumiflavin are absent from the EC specifications for riboflavin and for riboflavin-5′-phosphate sodium. The Panel also noted that EC specifications for riboflavin do not include limits for lumichrome and 8-HMR, while the limits for these impurities in specifications for pharmaceutical grade riboflavin are ≤ 0.2 % (Ph. Eur. 2011).

Regarding the specifications for riboflavin-5′-phosphate sodium, the Panel notes that the subsidiary colouring matters include up to 6 % free riboflavin and up to 6 % riboflavin diphosphate, these substances being part of the total colouring matter, which amounts to ≥ 95 %.

2.3. Manufacturing process

Riboflavin can be synthesised either chemically or produced using microorganisms. According to industry, "*riboflavin is not produced from chemical sources*" (saqual GmbH, 2010). The Panel noted that chemical synthesis is no longer used.

Information on different manufacturing processes was provided by industry.

2.3.1. Riboflavin

The chemical reaction process starts with D-ribose reacting with 3,4-xylidine in methanol. When the ribose is hydrogenated, the secondary amine *N*-(3,4-dimethylphenyl)-D-1′-ribamine is built. In a further step in the synthesis, when riboflavin is produced by cyclocondensation of *N*-(2-phenylazo-4,5-dimethylphenyl)-D-1′-ribamine with barbituric acid, aniline is eliminated and can be found as a trace amount in the chemically synthesised riboflavin (Stahmann, 2000; saqual GmbH, 2011).

Riboflavin can be obtained by controlled fermentation using a genetically modified strain of *Bacillus subtili*[11] growing in a culture broth which produces riboflavin as a secondary metabolite. Fermentation is stopped by inactivating the microorganisms through heating. Riboflavin is isolated by cooling down the solution followed by continuous band filtration and washing. The purification is a continuous operation consisting of filtration, crystallisation and isolation (saqual GmbH, 2010). The Panel noted that *Bacillus subtilis* is recommended for the qualified presumption of safety (QPS) list with the qualifications "absence of toxigenic activity" (EFSA BIOHAZ Panel, 2012). Three genetically modified strains of *Bacillus subtilis* for production of vitamin B_2 are currently under evaluation by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP).[12]

According to industry (saqual GmbH, 2010), riboflavin can also be produced by fermentation using the fungus *Ashbya gossypii*. When grown in a sterile culture broth, the microorganisms use vegetable oil as growing medium to synthesise riboflavin. After fermentation, the microorganism is inactivated by heating. Riboflavin is isolated in the next step, and the crude product is eventually purified to give the final product. It is reported that primary aromatic amines as potential by-products are not known to be produced by the riboflavin fermentation processes (saqual GmbH, 2010). The Panel noted that information on active second metabolites and their toxicological profile would be necessary to assess this method of manufacturing riboflavin as a food additive. The Panel noted that, according to the EFSA Panel on Biological Hazards (BIOHAZ), "*the knowledge concerning the capacity of Ashbya*

[11] The Panel noted that according to the SCF (1998), the strain of *Bacillus subtilis* used in riboflavin production is RB50::$[pRF69]_n$ $[pRF93]_m$ Ade+ (3).
[12] EFSA-Q-2010-00991; EFSA-Q-2010-01391; EFSA-Q-2012-00954.

gossypii to produce biological active secondary metabolites remains limited and this species cannot be proposed for QPS list" (EFSA BIOHAZ Panel, 2012) and that a genetically modified strain of *Ashbya gossypii* for production of vitamin B_2 will be evaluated by the EFSA FEEDAP Panel.[13]

According to industry, (saqual GmbH, 2010), 6,7-dimethyl-8-ribityl-lumazine (DMRL) is a known precursor of riboflavin in the biofermentation process and may occur as an impurity in the final product.

2.3.2. Riboflavin-5′-phosphate sodium

Riboflavin-5′-phosphate sodium is produced by phosphorylation of the starting material riboflavin with phosphorous oxychloride (saqual GmbH, 2010).

2.4. Methods of analysis in food

Arella et al. (1996) reported the results of a collaborative study re-evaluating a proposed official method for the determination of vitamins B_1 and B_2 (riboflavin) in nine different foods: baby food, powdered milk, meal with fruits, yeast, cereals (two), chocolate powder, food complement and tube-feeding solution. Determination was accomplished using reversed-phase high-performance liquid chromatography (RP-HPLC) and fluorimetric detection (FD). The recovery rate in all analysed foodstuffs was higher than 89 %, except for chocolate powder (75 %).

Scotter (2011) reviewed the methods of analysis of permitted natural colours in food. The author concluded that RP-HPLC and FD after acidic and enzymatic hydrolysis of the phosphorylated analogues and/or those bound to food proteins are accepted techniques for the determination of riboflavin when in the free form.

Other analytical techniques for the determination of riboflavin in food have been described by Caelen et al. (2004), Zandomeneghi et al. (2007) (free riboflavin in milk), Aranda and Morlock (2006) (riboflavin in energy drinks) and Zougagh and Rios (2008) (riboflavin in milk powder).

Tang and Lin (2010) described a method for the determination of riboflavin in flour. Wang et al. (2011) described a method for the simultaneous determination of folic acid and riboflavin in nutritional beverages.

The Institute for Reference Materials and Measurements (IRMM, 2013) stated that, despite satisfactory performance characteristics, the applicability of several methods in routine control laboratories is limited because of the required instrumentation. It is further stated that published methods, such as the one described by van den Berg et al. (1996), are adequately sensitive for monitoring of the levels of riboflavin added to food for colouring purposes but that there is room for further improvement in the analytical methods for riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)).

2.5. Stability and reaction and fate in food

2.5.1. Stability under storage conditions

2.5.1.1. Riboflavin

Riboflavin is stable against heat if protected from light and humidity. Riboflavin, in alkaline solution, is rapidly degraded, photochemically, to lumiflavin in the presence of light and oxygen. Exposure to light in neutral or acidic solutions yields lumichrome by cleavage of the D-ribityl side chain of riboflavin (Isler and Brubacher, 1988).

[13] EFSA-Q-2012-00953

According to data submitted by the industry, riboflavin has been shown to be stable for up to 60 months under normal conditions (25 °C and 60 % relative humidity in aluminium laminate foil bags or 30 °C and 70 % relative humidity) and for up to 6 months under accelerated conditions (40 °C and 75 % relative humidity) (saqual GmbH, 2010).

2.5.1.2. Riboflavin 5′-phosphate sodium

Riboflavin 5′-phosphate sodium is fairly stable in air but is hygroscopic and sensitive to heat and light. The product may be stored for 33 months from the date of manufacture in the unopened original container and at a temperature below 15 °C. Neutral and acidic solutions of riboflavin 5′-phosphate sodium are relatively stable if exposure to light is avoided, but in the alkaline range decomposition occurs, which is accelerated by light (saqual GmbH, 2010).

According to data submitted by the industry, riboflavin-5′-phosphate sodium (manufactured with fermentative riboflavin) has been shown to be stable for up to 9 months when stored under normal conditions (25 ± 2 °C and 60 ± 5 % relative humidity in aluminium bags) and for up to 12 months when stored at 15 °C in aluminium bags (saqual GmbH, 2010).

Further, three batches of riboflavin-5′-phosphate sodium (manufactured with synthetic riboflavin) were found to be stable after a storage of 3 years at 5, 23 and 35 °C in closed plasticised aluminium foil bags. According to industry, data are applicable to riboflavin-5′-phosphate sodium produced with biofermented riboflavin (saqual GmbH, 2010).

2.5.2. Stability of riboflavin under conditions of use

Riboflavin stability is affected by oxygen, other components such as metal sulphates or amino acid chelates, and water activity (Dennison et al., 1997). The storage stability of riboflavin in a low-moisture dehydrated model food system was determined by Dennison et al. (1977) as a function of water activity, moisture content and temperature. Fortification of the model system was at a level of 25 % of the recommended daily allowance (RDA) per 100 g. Riboflavin retention was approximately 100 % after 8 months storage at 10–30 °C. However, at 37 °C the loss of riboflavin increased with increasing water activity (Dennison et al., 1977).

Kamman et al. (1981) measured the stability of riboflavin in enriched pasta humidified at 25, 35, 45 and 55 °C for periods of up to one year. Riboflavin was shown to be extremely stable in the absence of light, even at elevated temperatures.

Haddad and Loewenstein (1983) studied the stability of riboflavin during processing of milk, and it appeared that riboflavin is stable during pasteurisation, sterilisation and after two weeks' frozen storage.

Gaylord et al. (1986) quantified riboflavin in milk samples exposed to fluorescent light. The effects of compositional factors were determined by comparing rates of loss of riboflavin in milk with different amounts of milk fat and milk solids. Upon exposure to fluorescent light, rates of riboflavin loss were lower in whole milk than in skimmed milk. Riboflavin was degraded more slowly in skimmed milk with 1 % added non-fat dry milk than in skimmed milk with no added solids. No additional protective effect was found when added solids were increased from 1 to 3 %.

Kristensen et al. (2001) studied the influence of light and temperature on the colour and oxidative stability of processed cheese. Riboflavin was found to degrade on light exposure after 14 days of storage. Further degradation continued until approximately 100 days of storage, at which time a minimum level, corresponding to approximately 25 % of the original content, was reached in the samples exposed to light. No influence of temperature was observed. There was little change in the concentration of riboflavin in the processed cheese samples stored in the dark during the one-year investigation period.

In a review, Choe et al. (2005) assessed a number of studies on the chemical reactions and stability of riboflavin in foods. Riboflavin is relatively stable during thermal and non-thermal food processing and storage but is very sensitive to light. Thermal degradation of riboflavin in soymilk followed first-order kinetics and the degradation rates were 7.05×10^{-4}, 4.26×10^{-3}, and 2.12×10^{-2}/min at 90 °C, 120 °C and 140 °C, respectively. Under light, riboflavin can act as a pro-oxidant for food components. Photosensitisation of riboflavin causes production of reactive oxygen species such as superoxide anion, singlet oxygen, hydroxyl radical and hydrogen peroxide. Radicals and reactive oxygen species accelerate the decomposition of mainly lipids, proteins and vitamins (A, C, D and E), and to a lesser extent carbohydrates, and could cause significant nutrient loss in foods.

2.5.3. Degradation products

Several studies have reported that riboflavin in foods is unstable to light, but very stable in the dark. Several compounds are formed from riboflavin under the influence of light, including the non-volatile compounds lumichrome and lumiflavin, and the volatile compound 2,3-butanedione (Huang et al., 2006; Jung et al., 2007).

According to industry, the following degradation products of riboflavin and riboflavin-5′-phosphate sodium can be expected to be present in food products (saqual GmbH, 2010):

- **8-Hydroxymethyl-riboflavin** (8-HMR) (chemical name: 8-(hydroxymethyl)-7-methyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione)), formed as a product of oxidative degradation of riboflavin in the downstream process.

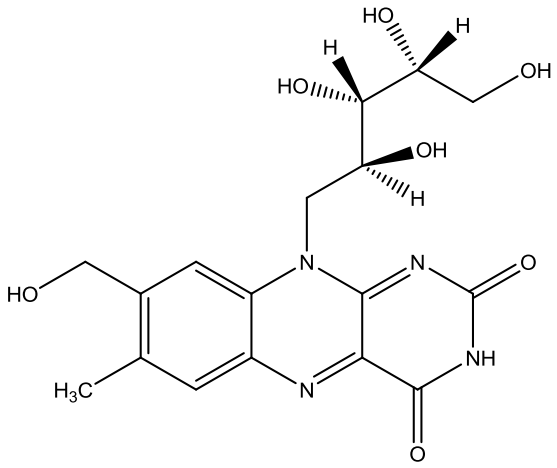


Figure 2: Structural formula of 8-HMR (CAS No 52134-62-0)

- **Lumichrome** (chemical name: 7,8-dimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione), formed either during manufacture or storage by degradation of riboflavin under the influence of light in an acidic environment.

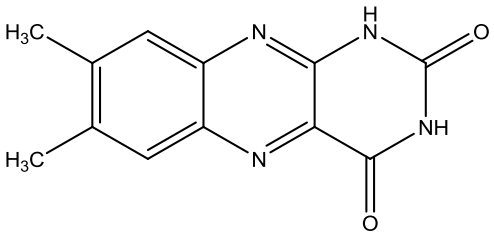


Figure 3: Structural formula of lumichrome (CAS No 1086-80-2)

- **Lumiflavin** (chemical name: 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione), formed as degradation product of riboflavin upon alkaline treatment, preferably under the influence of light (Isler and Brubacher, 1988).

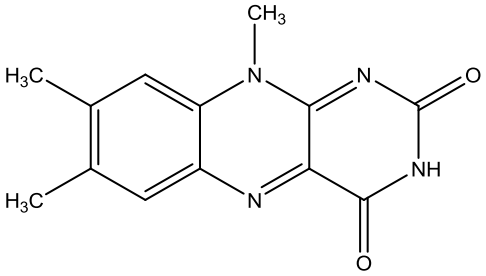


Figure 4: Structural formula of lumiflavin (CAS No.: 1088-56-8)

- **2,3-Butanedione** (chemical name: butane-2,3-dione), formed by reaction between singlet oxygen and riboflavin under light (Jung et al., 2007).

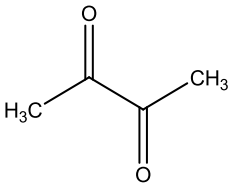


Figure 5: Structural formula of 2,3-butanedione (CAS No 431-03-8)

2.6. Case of need and proposed uses

Maximum permitted levels (MPLs) of riboflavins have been defined in Annex II to Regulation (EC) No 1333/2008.

Currently, riboflavins are authorised food additives in the EU at *quantum satis* in all foodstuffs with the exception of concentrations up to 100 mg/L in aromatised wines and aromatised wine-based drinks (americano, bitter soda).

Table 4 summarises foods that are permitted to contain riboflavins and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

Table 4: MPLs of riboflavins (E 101) in foods according to the Annex II to Regulation (EC) No 1333/2008

Category number	Food categories	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
01.4	Flavoured fermented milk products including heat treated products		*Quantum satis*
01.5	Dehydrated milk as defined by Directive 2001/114/EC	Except unflavoured products	*Quantum satis*
01.6.3	Other creams	Only flavoured creams	*Quantum satis*
01.7.1	Unripened cheese excluding products falling in category 16	Only flavoured unripened cheese	*Quantum satis*
01.7.3	Edible cheese rind		*Quantum satis*
01.7.4	Whey cheese		*Quantum satis*
01.7.5	Processed cheese	Only flavoured processed cheese	*Quantum satis*
01.7.6	Cheese products (excluding products falling in category 16	And only flavoured unripened products	*Quantum satis*
01.8	Dairy analogues, including beverage whiteners		*Quantum satis*
03	Edible ices		*Quantum satis*
0.4.2.1	Dried fruit and vegetables	Only preserves of red fruit	*Quantum satis*
0.4.2.2	Fruit and vegetables in vinegar, oil, or brine	only preserves of red fruit and vegetables (excluding olives)	*Quantum satis*
04.2.3	Canned or bottled fruit and vegetables	Only preserves of red fruit	*Quantum satis*
04.2.4.1	Fruit and vegetable preparations excluding compote	Only mostarda di frutta	*Quantum satis*
04.2.4.1	Fruit and vegetable preparations excluding compote	Only preserves of red fruit	*Quantum satis*
04.2.5.3	Other similar fruit or vegetable spreads	Except crème de pruneaux	*Quantum satis*
05.2	Other confectionery including breath refreshening microsweets		*Quantum satis*
05.3	Chewing gum		*Quantum satis*
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4		*Quantum satis*
06.3	Breakfast cereals	Only breakfast cereals other than extruded, puffed and/or fruit-flavoured breakfast cereals	*Quantum satis*
06.5	Noodles		*Quantum satis*
06.6	Batters		*Quantum satis*
06.7	Pre-cooked or processed cereals		*Quantum satis*
07.2	Fine bakery wares		*Quantum satis*
08.2.1	Non-heat-treated processed meat	Only pasturmas	*Quantum satis*
08.2.3	Casings and coatings and decorations for meat	Except edible external coating of pastrumas	*Quantum satis*
08.2.3	Casings and coatings and decorations for meat	Also edible external coating of pastrumas	*Quantum satis*

Category number	Food categories	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
09.2	Processed fish and fishery products including molluscs and crustaceans	Only surimi and similar products and salmon substitute	*Quantum satis*
09.2	Processed fish and fishery products including molluscs and crustaceans	Only fish paste and crustacean paste, precooked crustacean and smoked fish	*Quantum satis*
09.3	Fish roe	except Sturgeons' eggs (Caviar)	*Quantum satis*
12.2.2	Seasonings and condiments (only seasonings, for example curry powder, tandoori)	Only seasonings, for example curry powder, tandoori	*Quantum satis*
12.4	Mustard		*Quantum satis*
12.5	Soups and broths		*Quantum satis*
12.6	Sauces	Excluding tomato-based sauces	*Quantum satis*
12.7	Salads and savoury-based sandwich spreads		*Quantum satis*
12.9	Protein products, excluding products covered in category 1.8		*Quantum satis*
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC (excluding products from food category 13.1.5)		*Quantum satis*
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)		*Quantum satis*
13.4	Foods suitable for people intolerant to gluten as defined by Regulation (EC) No 41/2009		*Quantum satis*
14.1.4	Flavoured drinks	Excluding chocolate milk and malt products	*Quantum satis*
14.2.3	Cider and perry	Excluding cidre bouché	*Quantum satis*
14.2.4	Fruit wine and made wine		*Quantum satis*
14.2.5	Mead		*Quantum satis*
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Except: spirit drinks as defined in Article 5(1) and sales denominations listed in Annex II, paragraphs 1–14, of Regulation (EC) No 110/2008 and spirits (preceded by the name of the fruit) obtained by maceration and distillation, London gin, sambuca, maraschino, marrasquino or maraskino and mistrà	*Quantum satis*
14.2.7.1	Aromatised wines	Except americano, *bitter* vino	*Quantum satis*
14.2.7.1	Aromatised wines	Only americano, *bitter* vino	100
14.2.7.2	Aromatised wine-based drinks	Except *bitter* soda, sangria, claria, zurra	*Quantum satis*

Category number	Food categories	Restrictions/exception	MPL (mg/L or mg/kg as appropriate)
14.2.7.2	Aromatised wine-based drinks	Only *bitter* soda	100
14.2.7.3	Aromatised wine-product cocktails		*Quantum satis*
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % alcohol		*Quantum satis*
15.1	Potato-, cereal-, flour- or starch-based snacks		*Quantum satis*
15.2	Processed nuts		*Quantum satis*
16	Desserts excluding products covered in categories 1, 3 and 4		*Quantum satis*
17.1	Food supplements supplied in a solid form including capsules and tablets and similar forms, excluding chewable forms		*Quantum satis*
17.2	Food supplements supplied in a liquid form		*Quantum satis*
17.3	Food supplements supplied in a syrup-type or chewable form		*Quantum satis*

2.7. Reported use levels or data on analytical levels of riboflavins

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a lower level than the MPL. For those additives for which no MPL is set and which are authorised as *quantum satis*, information on actual use levels is required. In the framework of Regulation (EC) No 1333/2008 on food additives and of Commission Regulation (EU) No 257/2010[14] regarding the re-evaluation of approved food additives, EFSA issued a public call for scientific data on riboflavins including present use and use patterns (i.e. which food categories and subcategories, proportion of food within categories/subcategories in which it is used, actual use levels (typical and maximum use levels), especially for those uses which are limited only by *quantum satis*).

2.7.1. Summarised data on reported use levels in foods from industries and other sources

Tables 5 provides data on the use levels of riboflavins in foods as reported by industries. Table 5 also shows the levels used for the refined exposure assessment identified by the Panel and based on data for several food categories in finished products reported by industries or from the rules followed to deal with *quantum satis* authorisation, as indicated in Appendix A.

[14] Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 133/2008 of the Europeam Parliament and of the Council on food additives. OJ L 80, 26.3.2010.

1 **Table 5:** Summary of levels used in the refined exposure assessment

Matching FAIM category	Food items	MPL (mg/kg or mg/L)	Industry					Level used for calculation
			Tennant (2007)		FoodDrinkEurope (2013)		Comments from industry	
			Typical (mg/kg)	Highest (mg/kg)	Typical (mg/kg)	Highest (mg/kg)		
1	Dairy products	*Quantum satis*			13–50	100		100
1.4	Milk and dairy-based drinks	*Quantum satis*			4.5–27	30		30
1.4	Desserts including flavoured milk products	*Quantum satis*		20				20
3	Edible ices	*Quantum satis*		20	0–50	50		50
4.2	Processed fruit and vegetables	*Quantum satis*		40	80	80		80
5	Confectionery	*Quantum satis*		10		70–80	Refers to pure riboflavin	80
6.5	Noodles	*Quantum satis*			50	70		70
7.2	Fine bakery wares	*Quantum satis*		30		38		38
16	Desserts, not dairy and cereal based	*Quantum satis*			0–10	10		10
12.6/12.2	Sauces, seasonings, pickles, relishes, chutney, piccalilli	*Quantum satis*		30	0–10	10	A value of 720 mg/kg is reported only for sauce Béarnaise in Denmark	10
12.4	Mustard	*Quantum satis*		50	0–150	150		150
12.5	Soups	*Quantum satis*				195		195
14.1	Non-alcoholic beverages	*Quantum satis*	1–10	10	1–10	10	Maximum use level on the basis of pure pigment/colouring principle equivalents	10
15.1	Processed potato products	*Quantum satis*			90	400		400
9.2	Processed fish and fishery products including molluscs and crustaceans	*Quantum satis*			23	25		25
9.3	Fish roe	*Quantum satis*			<100			100

2.8. Information on existing authorisations and evaluations

Riboflavins are authorised as food additives in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 on food additives. Specific purity criteria on riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) have been defined in the Commission Regulation (EU) No 231/2012.

Synthetic riboflavin was evaluated by JECFA in 1969 (JECFA, 1969), where an acceptable daily intake (ADI) of 0–0.5 mg/kg bw/day was allocated on the basis of limited data. JEFCA based this ADI on a "*level causing no toxicological effect in the rat*" equal to 50 mg/kg bw/day. In 1981, JECFA allocated a group ADI for riboflavin and riboflavin-5′-phosphate of 0–0.5 mg/kg (expressed as riboflavin). In 1998, JECFA included riboflavin derived by fermentation with a strain of genetically modified *Bacillus subtilis*[15] in the previously established group ADI of 0–0.5 mg/kg bw/day for synthetic riboflavin and riboflavin-5′-phosphate sodium.

In 1977, the SCF classified riboflavin-5′-phosphate sodium as a colour which could be used in food, but no ADI was established. The SCF was of the opinion that the use of this substance as a food colour should not alter significantly the average daily intake of riboflavin (SCF, 1977, 1984). In 1998, the SCF concluded that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* is acceptable for use as a food colour.

In 2000, the SCF concluded that it was not possible, based on the available database, to derive a Tolerable Upper Intake Level (UL) for riboflavin used as a vitamin because the available data on adverse effects from high oral riboflavin intake were not of sufficient quality and extent as to be used for the determination of a UL. However, the SCF (2000) stated that the limited evidence available from clinical studies indicated that current levels of intake of riboflavin from all sources do not represent a risk to human health.

The SCF advised on the popular reference intake (PRI), which is 0.8–1.6 mg/day for male children, 0.8–1.3 mg/day for female children, 1.3 mg/day for male adults and 1.1 mg/day for female adults (SCF, 1993). The SCF (2000) referred to the RDA for riboflavin that was established by the Food and Nutrition Board (FNB, 1998) which is 0.5–0.9 mg/day for children, 1.3 mg/day for male adults and 1.0–1.1 mg/day for female adults. According to the D-A-CH (2013), the RDA for riboflavin varies from 0.7–1.6 mg/day for male children, 0.7–1.3 mg/day for female children, 1.2–1.5 mg/day for male adults and 1.2 mg/day for female adults.

According to the Bundesinstitut für Risikobewertung (BfR) report "Use of vitamins in foods: toxicological and nutritional-physiological aspects", there have been no reports of adverse effects in humans as a consequence of high or excessive riboflavin intake from foods or supplements (BfR, 2005).

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) will advise on population reference intakes for riboflavin.[16]

2.9. Exposure

2.9.1. Food consumption data used for exposure assessment

In 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) was built from existing national information on food consumption at a detailed level. Competent Authorities in the European countries provided EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (see EFSA guidance on 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a)).

[15] According to the SCF (1998), the strain of *Bacillus subtilis* used in riboflavin production is RB50::$[pRF69]_n$ $[pRF93]_m$ Ade+ (3).
[16] EFSA-Q-2011-01222

Overall, the food consumption data gathered at EFSA were collected by different methodologies and thus direct country-to-country comparison should be made with caution.

For calculation of chronic exposure, intake statistics have been calculated based on individual average consumption over the total survey period excluding surveys with only one day per subject. High-level consumption was calculated for only those foods and population groups for which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011a). The Panel estimated chronic exposure for the following population groups: toddlers, children, adolescents, adults and the elderly. Calculations were performed using individual body weights.

Thus, for the present assessment, food consumption data were available from 26 different dietary surveys carried out in 17 different European countries as shown in Table 6.

Table 6: Population groups considered for the exposure estimates of riboflavins

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

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from FoodEx classification system has been linked to the Food Classification System as presented in the Commission Regulation (EU) No 1129/2011[18], part D, to perform exposure estimates.

2.9.2. Exposure to riboflavins from their uses as food additives

Since riboflavins are authorised at the *quantum satis* levels for almost all food categories listed, exposure to riboflavins from their uses as food additives has been calculated by using data on reported use levels provided by industry, combined with national consumption data for the five population groups (Table 7). Exposure to riboflavins from their use in aromatised wine and aromatised wine-based drinks at the MPL of 100 mg/kg was considered negligible, as the food additive is authorised for specific alcoholic beverages only (Americano, bitter vino, bitter soda).

High-level exposure (typically 95th percentile of consumers only) was calculated by adding the 95th percentile of exposure from one food group (i.e. the one having the highest value) to the mean exposure resulting from the consumption of all other food groups.

This is based on the assumption that an individual might be a high-level consumer of one food category and would be an average consumer of the others. This approach has been tested several times by the Panel in re-evaluation of food colours and has shown reasonable correlation with high-level

[17] The terms "children" and "the elderly" correspond, respectively, to "other children" and the merged group "elderly and very elderly" in the EFSA guidance on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011b).

[18] Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council establishing a Union list of food additives. OJ L 295, 12.11.2011, pp. 1–177.

total intakes when using the raw food individual consumption data. Therefore, this approach was preferred for the calculations based on the MPLs and maximum reported use levels in order to avoid excessively conservative estimates.

However, the Panel notes that its estimates should be considered as being conservative as it is assumed that all processed foods contain riboflavins added at the maximum reported use levels.

Table 7 summarises the estimated exposure to riboflavins from their use as food additives in the five population groups.

Table 7: Summary of anticipated exposure to riboflavins from their uses as food additive using reported use levels in five population groups (mg/kg bw/day)

Estimated exposure using reported use levels	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (> 65 years)
Mean	0.7–1.3	0.5–1.8	0.2–1.0	0.2–0.7	0.2–0.7
High level[19]	1.2–2.3	0.9–3.9	0.6–2.2	0.4–1.7	0.3–1.6

2.9.3. Main food categories contributing to exposure of riboflavins using maximum reported use levels

Table 8: Main food categories contributing to exposure to riboflavins using reported use levels and number of surveys in which each food categories is contributing

Category number	Foods	Toddlers	Children	Adolescents	Adults	The elderly
		% contribution to total exposure (number of surveys) [(a)]				
1.4	Flavoured fermented milk products including heat treated products	8–17 (3)	7–17 (8)	7–9 (3)	7–10 (2)	5 (1)
1.5	Dehydrated milk as defined by Directive 2001/114/EC		16 (1)			
1.6	Cream		5–7 (2)	5 (1)	7–16 (3)	8–17 (3)
1.7.1	Unripened cheese (excluding category16)	6–8 (3)	5–19 (5)	6–22 (2)	5–25 (6)	5–19 (4)
1.7.2	Ripened cheese	6–18 (2)	5–15 (8)	5–16 (9)	6–16 (13)	6–16 (6)
1.7.5	Processed cheese		5 (2)		6 (1)	5 (1)
3	Edible ices	8 (1)	5–10 (6)	5–6 (3)	5–6 (2)	7–26 (6)
4.2	Processed fruit and vegetables	23–70 (4)	19–51 (15)	11–46 (12)	14–65 (15)	26–66 (7)
5.2.1	Other confectionery with added sugar		5–7 (2)	7 (1)		
7.2	Fine bakery wares	5–18 (1)	6–21 (11)	6–16 (10)	7–18 (8)	5–8 (4)
12.5	Soups and broths	16 (1)	6–55 (8)	9–50 (6)	9–36 (7)	38–66 (2)
12.6	Sauces		10–35 (10)	10–35 (10)		
14.1.4.1	Flavoured drinks with sugar		6–11 (7)	5–14 (7)	5–10 (7)	7 (1)
14.1.4.2	Flavoured drinks with sweeteners			7 (1)	9 (1)	

[19] Typically 95th percentile of consumers only.

Category number	Foods	Toddlers	Children	Adolescents	Adults	The elderly
		% contribution to total exposure (number of surveys) [(a)]				
15.1	Potato-, cereal-, flour- or starch-based snacks	8–26 (3)	6–19 (12)	6–35 (11)	5–20 (10)	6 (1)
16	Desserts excluding products covered in categories 1, 3 and 4	29–47 (2)		6–29 (10)		

[(a)] Total number of surveys may be greater than total number of countries as listed in Table 6, as some countries submitted more than one survey for a specific age range.

2.9.4. Dietary intake via other sources

Riboflavin is present in a wide range of foods, with liver, milk, meat and fish being the most important sources. In Western diets, the contribution of milk to total intake of riboflavin is estimated to be 25–30 % (Powers, 2003). Data on dietary intake of riboflavin from foods excluding riboflavins from their use as food additives was available from the European Nutrition and Health Report 2009 (Elmadfa, 2009).

It should be noted that these intake estimates are based on average food consumption and on average concentrations of riboflavin in foods and are thus less conservative than the approach used for the estimation of exposure to riboflavins from their use as food additives as described above.

Using EFSA default body weight values of 23.1 kg for children aged 3–10 years and 67.2 kg and 82.0 kg for female and male adults, respectively (EFSA Scientific Committee, 2012), the dietary intake of riboflavin via other sources would be in the range of 0.05–0.09 mg/kg bw/day for children, of 0.02–0.04 mg/kg bw/day for female adults and 0.02–0.03 mg/kg bw/day for male adults.

Riboflavin is also widely used in food supplements in form of multi-nutrient supplements sold over the counter. Most supplements provide a daily dose of riboflavin at or below the RDA. However, some supplements, mainly offered for medical or health promotion purposes, may provide intakes of up to 100 mg/day (EVM, 2003). Although only very limited information is available on the pattern of intake of riboflavin from supplemental use, it should be noted that, in view of the amount of riboflavin used as nutrient in some food supplements, it is likely that this may substantially contribute to the overall exposure of some persons.

2.9.5. Dietary exposure to degradation products

Exposure to the degradation products of riboflavin has been estimated using the JECFA specification for lumiflavin of 0.025 % (see Table 2) and the pharmaceutical specifications for 8-HMR (0.2 %) and lumichrome (0.2 %) (Ph. Eur., 2011). Based on the calculations for exposure to riboflavin using the highest reported use levels from industry, the exposure to these degradation products would be as listed in Table 9.

Table 9: Summary of anticipated exposure (µg/kg bw/day) in five population groups to 8-HMR, luminochrome and lumiflavin as degradation products resulting from the usage of riboflavins based on reported use levels

Estimated exposure	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (> 65 years)
8-HMR					
• Mean	1.4–2.6	1.0–3.6	0.4–2.0	0.4–1.4	0.4–1.4
• High level[19]	2.4–4.6	1.8–7.8	1.2–4.4	0.8–3.4	0.6–3.2
Lumichrome					
• Mean	1.4–2.6	1.0–3.6	0.4–2.0	0.4–1.4	0.4–1.4

Estimated exposure	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (> 65 years)
• High level[199]	2.4–4.6	1.8–7.8	1.2–4.4	0.8–3.4	0.6–3.2
Lumiflavin					
• Mean	0.18–0.33	0.13–0.45	0.05–0.25	0.05–0.18	0.05–0.18
• High level[19]	0.30–0.58	0.23–0.98	0.15–0.55	0.10–0.43	0.08–0.40

2.10. Uncertainty analysis

Uncertainties in the exposure assessment of riboflavins have been previously discussed in the present opinion, in the related chapters. According to the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and summarised in Table 10.

Table 10: Qualitative evaluation of influence of uncertainties

Sources of uncertainties	Direction
Consumption data: different methodologies/representativeness/under-reporting/misreporting/no portion size standard	+/–
Extrapolation from food consumption survey of few days to estimate chronic exposure	+
Linkage between reported use levels and food items in the consumption database: uncertainties on the precise types of food to which the use levels refer	+/–
Occurrence data: maximum reported use levels within a food category	+
Exposure model: uncertainty in possible national differences in use levels of food categories, data set not fully representative of foods on the EU market, exposure calculations based on the maximum reported use levels (no use of typical use levels when available)	+

+ = uncertainty with potential to cause overestimation of exposure; – = uncertainty with potential to cause underestimation of exposure.

3. Biological and toxicological data

The biological properties of riboflavin and riboflavin-5′-phosphate sodium have been previously evaluated by JECFA in 1969, 1981 and 1998 (JECFA, 1969, 1981, 1998). The present opinion briefly reports the major studies evaluated in these reports. Additional information has been identified from the literature and the call for data.

3.1. Absorption, distribution, metabolism and excretion

JECFA (1981) evaluated a few studies on toxicokinetics of riboflavin and riboflavin-5′-phosphate sodium and the conclusions are summarised below:

> *"Riboflavin-5'-phosphate is rapidly dephosphorylated to free riboflavin by incubation with intestinal mucosa or intestinal juice from rats (Christensen, 1969). Lumichrome and Lumiflavin were identified as metabolites of riboflavin in the rat (Yang and McCormick, 1967). Hydroxyethylflavin, formylmethylflavin and an unknown metabolite were identified as metabolites in male volunteers (West and Owen, 1969)."*

3.1.1. Absorption

Six male adults were orally exposed to 5, 10, 20, 50, 100 or 500 mg/person of riboflavin-5′-phosphate sodium and the concentration of free riboflavin was measured in plasma and urine (Stripp, 1965). After oral intake of 100, 200 or 500 mg riboflavin-5′-phosphate sodium/person, the peak plasma concentration of free riboflavin (200–300 ng/mL) was observed 1.5 hours after the administration and values were similar, independent of the dose. Regarding urinary excretion, there was approximately linearity between the dose of riboflavin-5′-phosphate sodium (from 5 to 20 mg/person) and the amount of riboflavin excreted in urine (30 to 50% of the dose). However, an increase in the dose above 50 mg

did not cause any further increase in the urinary excretion of riboflavin (17.0, 14.0 and 17.9 mg after administration of 100, 200 and 500 mg riboflavin-5′-phosphate sodium/person, respectively). In addition to the oral administration, riboflavin (84 mg) was given as an intravenous infusion for two hours, which resulted in a peak concentration of 1 800 ng/ml, whereas peak concentration was about 350 ng/ml after an oral dose of 100 mg. The urinary excretion of the intravenous dose was nearly complete (97 % within 24 hours). According to the author, the limited urinary excretion and the similar concentration–time profile would suggest that absorption from the gastrointestinal tract is limited. The Panel agreed with this conclusion and considered that the unchanged values of both plasma profile and urinary excretion of riboflavin for doses above 50 mg/person would indicate a saturable process in the gastrointestinal absorption of riboflavin, occurring for such high oral doses.

Increased absorption of riboflavin-5′-phosphate occurs in humans if the substance is given with a meal (5–30 mg riboflavin-5′-phosphate sodium/person), probably due to a longer intestinal transit time (Jusko and Levy, 1967). From these data, the authors suggested that riboflavin-5′-phosphate and riboflavin are probably absorbed by a specific transport system in the upper gastrointestinal system. Riboflavin-5′-phosphate may be dephosphorylated during absorption but may subsequently be rephosphorylated in the mucosa and transported to the liver, where it is again dephosphorylated to riboflavin (the form in which it occurs in the circulation), before being excreted in the urine.

Further data reviewed by McCormick (1989) indicated that the uptake of riboflavin occurs by an active and saturable transport system at physiological concentrations, as demonstrated *in vivo* or by using sections of gut or isolated enterocytes.

Said and Ma (1994) reported that the intestinal uptake process of riboflavin appeared to be under the regulation of intracellular protein kinase A and calcium/calmodulin-mediated pathways, as demonstrated in Caco-2 cells. Moreover, the amount of riboflavin absorbed depended on the intake; it was increased when riboflavin was given orally with food. The absorption rate of free riboflavin was 50–60 % at a dose range of 2–25 mg/person (Elmadfa and Leitzmann, 1998).

3.1.2. Distribution and metabolism

In human plasma, riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins, and mainly found as flavin adenine dinucleotide (FAD). The significance of this protein binding is not fully understood (Steier et al., 1976; Natraj et al., 1988).

The pharmacokinetics and utilisation (flavocoenzyme synthesis) of orally and intravenously administered riboflavin in healthy humans were assessed in a study by Zempleni et al. (1996a). After determination of the circadian rhythms of riboflavin concentrations in the plasma and urine of four males and five females (control period), each subject received three different oral riboflavin doses (20, 40 and 60 mg) and one intravenous bolus injection of riboflavin (11.6 mg). Pharmacokinetic variables were calculated using a two-compartment open model. The maximal amount of riboflavin absorbed from a single dose was 27 mg per adult. The half-life of absorption was 1.1 hour. First-order rate constants describing distribution and elimination of riboflavin were significantly higher after intravenous than after oral administration ($P < 0.01$). The plasma flavocoenzyme concentration was low compared with the increased riboflavin concentrations. Clearance data indicated that urinary excretion of riboflavin contributes half of the overall removal of riboflavin from plasma. No sex differences were observed for any of the pharmacokinetic variables.

In a complementary study in humans, the same authors (Zempleni et al., 1996b) identified 7-α-hydroxyriboflavin in blood plasma following the administration of different oral (20, 40, 60 mg) or intravenous (11.6 mg) doses of riboflavin to healthy subjects and in females with liver cirrhosis (oral 40-mg dose). Plasma peak concentrations of 40 nmol/L in males and 20 nmol/L in females ($P < 0.01$) were achieved within two hours. Correction of peak concentrations and areas under the plasma curves by the rate constants of disposition led to the finding of approximately equal amounts of 7-α-hydroxyriboflavin released into plasma in both sexes. No significant influence of different oral

riboflavin doses on 7-α-hydroxyriboflavin kinetics was found. Liver cirrhosis had no significant effect on the amount of 7-α-hydroxyriboflavin released into blood plasma. However, the failure to detect this metabolite following intravenous riboflavin administration indicates a substantial influence of gastrointestinal or liver passage.

3.1.3. Urinary excretion

In healthy adults eating varied diets, riboflavin accounts for 60–70 % of the excreted urinary flavins (McCormick, 1989). The urinary excretion of riboflavin varies with intake, metabolism and age.

3.2. Toxicological data

3.2.1. Acute oral toxicity

The oral LD_{50} values of both riboflavin and riboflavin-5′-phosphate sodium are greater than 40 000 mg/kg bw in mice and greater than 20 000 mg/kg bw in rats (Bächtold, 1980). The oral LD_{50} values of lumichrome and lumiflavin in mice are greater than 9 000 mg/kg bw and 6 000 mg/kg bw, respectively (Bächtold, 1980).

An acute oral toxicity study was performed with 8-HMR in rats and an approximate LD_{50} was found to be > 2 000 mg/kg bw (Schöni, 1989).

Acute toxicity studies were performed with riboflavin 96 % (feed grade, spiked with 2.31 % DMRL, corresponding to 46.2 mg/kg bw at the limit dose) and riboflavin 98 % (tablet grade containing 1.2 % DMRL, corresponding to 24 mg/kg bw at the limit dose). No mortality was observed in either study (Wolz et al., 2000a, b). These studies were not available to EFSA.

3.2.2. Short-term and subchronic toxicity

Female Sprague–Dawley rats were fed the monodiethanolamine salt of riboflavin-5′-phosphate five days per week for 29 weeks at daily dose of 1, 4, 10 and 40 mg/animal (Randall, 1950). At the highest dose, a slight decrease in growth rate was observed. A decrease in white blood cells and red blood cells count compared with control animals occurred in week 21 in the group receiving the highest dose. Differential white blood cell counts did not reveal any consistent changes in the relative distribution. At 200 mg/kg bw/day, two rats died and the surviving eight animals showed slight anaemia and decreased weight gain; five animals developed pneumonia. Due to the methodological limitations of this study and the poor health status of the animals no no-observed-adverse-effect level (NOAEL) was identified by the Panel.

Wistar rats (six week olds, 16 animals/sex/group, males weighing 160–170 g and females 130–140 g) received diets providing 20, 50 or 200 mg riboflavin/kg bw/day for 13 weeks (Buser et al., 1995). The purpose of this study was to assess and compare the toxicity of (a) two grades of riboflavin produced by fermentation with rec *Bacillus subtilis*, named "riboflavin 96 % ex fermentation" and "riboflavin 98 % ex fermentation", and (b) a riboflavin produced by chemical synthesis named "riboflavin 98 % ex synthesis". The study was performed in accordance with OECD Guideline 408 and Good Laboratory Practice (GLP) and quality assessment (QA) statements were included. A control group comprised 30 animals of each sex. A satellite group of six animals of each sex was attached to each group for the determination of riboflavin concentrations in blood and urine and for investigating the reversibility of any toxicological parameter. Feed and water were provided *ad libitum*. Samples of the diet were taken at the start of treatment and at weeks 6 and 13 to determine the content, homogeneity and stability of the test compound in the diet. Animals were checked for behaviour and general condition. Alopecic areas were seen frequently in various groups, but these findings were transient and were not dose-related. Ophthalmoscopy showed no abnormalities. Excrement collected from rats at the highest dose groups of the three test substances was yellowish throughout the study. No dose-related differences in food and water intake were seen. During treatment, females receiving 200 mg/kg bw/day "riboflavin 98 % ex fermentation" showed a slightly, but usually statistically significant,

decrease in growth of about 6 %. Males and females receiving 50 mg/kg bw/day "riboflavin 98 % ex synthesis" also showed a slight but statistically significant decrease in growth. These effects were not considered to be toxicologically relevant because they were limited (<10 %), and food consumption was not affected. During the recovery period, body weights were similar in all groups. Haematology conducted on week 6 did not reveal any dose-related changes in red blood cell (RBC) counts or clotting potential. At the end of the treatment period variations were limited to a slightly, though statistically significantly, lower haemoglobin concentration and red blood cell count and higher reticulocyte count in females of the highest dose group receiving "riboflavin 98 % ex fermentation". Individual animal data showed that low values for RBC and haemoglobin were associated with high values for reticulocytes in only two rats. Reticulocyte counts were also relatively high in other groups, but there was no dose–response relationship. Slight increases in thrombocyte count reached the level of statistical significance in females in the highest dose group and in males in the highest dose group. Total white blood cell counts in week 6 and at the end of the treatment period were lower in all treatment groups than in controls, and the differences were statistically significant except in the group receiving the lowest dose of "riboflavin 96 % ex fermentation" and the middle and highest doses of "riboflavin 98 % ex fermentation". However, there was no association with a specific test substance or correlation with dose. Differential white blood cell counts did not reveal any consistent changes in percentage distribution, but, since the absolute numbers were calculated from the total white blood cell count and percentage distribution of each cell type, the changes in total white blood cell count were often accompanied by decreases in the absolute number of lymphocytes and/or neutrophils. As there were no noticeable changes in haematological findings at the end of the recovery period, this effect can be attributed to the test compounds and considered as reversible. At the end of the recovery period thrombocyte count was decreased in females in the group receiving the highest dose of "riboflavin 98 % ex fermentation" but this finding was not observed at the end of the treatment period. Clinical chemical and urinary analyses showed no treatment-related changes. At the end of treatment, a slight but statistically significant increase in relative liver weight was seen in females in the 200 mg/kg bw/day "riboflavin 98 % ex fermentation", group and a slight but statistically significant increase in relative spleen weight was seen in males in the 50 and 200 mg/kg bw/day "riboflavin 96 % ex fermentation" groups, but with no dose–response relationship. Macroscopic and microscopic examination did not reveal any treatment-related abnormality. According to the authors, the changes in organ weights were considered to be of questionable biological relevance because there was no dose–response relationship and they were not accompanied by histopathological changes or changes in clinical chemistry parameters. At the end of the recovery period, all of the haematological parameters were normal, except for a statistically significant decrease in mean thrombocyte counts in females receiving 200 mg/kg bw/day "riboflavin 98 % ex fermentation", and there were no notable changes in organ weights. Riboflavin concentrations in blood and urine were not reported.

The authors concluded that the NOAEL was 200 mg/kg bw/day for all three grades of riboflavin (Buser et al., 1995). The decreases in mean haemoglobin concentration and erythrocyte count in females receiving 200 mg/kg bw/day "riboflavin 98 % ex fermentation" were considered by the authors to be fortuitous findings, because there was no overt association between the low erythrocyte counts and the high reticulocyte counts, except in two females. Moreover, according to the authors, the measured values did not exceed the historical controls, and there was also no clear dose–response relationship in any group. In addition, there were no related changes (e.g. in haemolysis or weight and histopathology of haematopoietic organs) that could be associated with variations in the RBC profile. The Panel agreed with the NOAEL of 200 mg/kg bw/day (the highest dose tested for all three test materials) proposed by the authors.

The Panel noted that the test items contained up to 0.2 % luminochrome, 0.15 % 8-HMR and 0.45 % DMRL, corresponding to 0.4 mg lumichrome/kg bw/day, 0.3 mg 8-HMR/kg bw/day and 0.9 mg DMRL/kg bw/day at the highest dose of 200 mg test item/kg bw/day, which can be considered the NOAELs for these substances from this study.

In the Bachman et al. study (2005), the test item, containing 80.1 % riboflavin, 0.25 % lumichrome, 1.5 % DMRL, 0.1 % 8-HMR and 20 % maltodextrin, was tested in SPF-bred Wistar rats of both sexes

at target doses of 0, 50, 100 and 200 mg test item/kg bw/day for 13 weeks. The formulation consists of 80 % riboflavin (ex fermentation from *Bacillus subtilis*) and 20 % maltodextrin. The riboflavin used was feed grade, which means that it was not subjected to the final purity steps as done for food-grade riboflavin; the batch used had a content of 80.1 % riboflavin; lumiflavin levels in feed-grade riboflavin are lower than 0.025 % (limit test by thin layer chromatography). The effective mean daily intakes of test item were 49.1, 99.3 and 199.1 mg/kg bw/day in males and 50.5, 100.9 and 201.3 mg/kg bw/day in females. The study was performed in accordance with OECD Guideline 408 and in compliance with GLP. The main group comprised 10 animals per sex, which were sacrificed after 13 weeks of treatment. An additional five rats per sex and dose group were treated similarly and then allowed a four-week treatment-free period, after which they were sacrificed. Blood and urine were collected during weeks 4 and 10 for bio-analytical investigations. Clinical signs, feed consumption and body weights were recorded periodically during acclimatisation, at the end of treatment and at the end of the recovery period. At the end of the dosing and treatment-free recovery periods, blood samples were collected for haematology and clinical chemistry analysis. Urine samples were collected for urinalysis. All animals were killed, necropsied and examined post mortem. Histopathological examinations were performed on organs and tissues from all animals in the control and high-dose groups. Kidneys and spleen from males of the lower dose groups and from all recovery male rats were also examined. One male and one female in the control group died during the study. The only clinical sign considered to be related to the treatment, albeit not an adverse effect, was the discolouration (pale/yellow) of the faeces in animals in the high dose group, and, at a minimal incidence only, in males of the middle-dose group. Neither daily observations nor weekly detailed clinical inspections revealed other relevant changes in the appearance or behaviour of the animals. Grip strength during week 13 in both fore- and hind-limbs was greater in males in the two highest dose groups. In the lowest dose group, a slightly higher for forelimb grip strength, in the absence of a similar effect on hindlimbs, was considered incidental. In females, there were also significantly higher values for hind limb grip strength, but with no dose–response relationship. The overall means for locomotory activity measurements performed during week 13 were similar in the control and treated groups. Ocular investigations performed during acclimatisation and during weeks 13 (treatment end) and 16 (recovery end) revealed no treatment-related changes. The overall mean feed and water intakes in control and treated groups were similar. Mean body weight development was similar in animals in the control and treated groups. Haematology, clinical biochemistry and urinary parameters were not considered to be influenced by treatment. The histopathological examinations performed at the end of the treatment period revealed treatment-related microscopic changes in the kidneys. The changes consisted of eosinophilic granules (hyaline droplets) in the renal tubules of males rats in the two highest dose groups (100 and 200 mg/kg bw/day). After a four-week recovery period, the kidney morphology returned to the normal. These observations were not associated with any other morphological change such as degeneration or inflammation, and most probably represent protein complexes associated with alpha-2µ-globulin. The accumulation of hyaline droplets associated to alpha-2µ-globulin is a male rat specific response and therefore was considered by the authors not to be relevant to humans (Bachmann et al., 2005).

Considering the lack of toxicological relevance of the kidney changes for risk assessment and the lack of any other adverse effects, the authors of the study considered that the NOAEL for male and female rats is 200 mg test item/kg bw/day, corresponding to 160 mg riboflavin/kg bw/day, the highest dose level tested. The Panel agreed with this NOAEL.

The Panel noted that the test item contained up to 0.25 % lumichrome, 0.1 % 8-HMR and 1.5 % DMRL, which corresponded to 0.5 lumichrome mg/kg bw/day, 0.2 mg 8-HMR/kg bw/day and 3 mg DMRL/kg bw/day at the highest dose of 200 mg test item/kg bw/day.

The effects of excess riboflavin on body weight gain, food intake, tissue weights and urinary excretion of B-group vitamins were investigated in weanling rats (Fukuwatari, 2009). The weanling rats were freely fed ordinary diet containing 0.0006, 0.1, 0.5 or 1 % riboflavin for 22 days. Excess riboflavin did not affect body weight gain, food intake or tissue weights. The urinary excretion of riboflavin also did not differ among animals receiving the different diets. These results clearly showed that feeding a diet

containing up to 1 % riboflavin did not induce adverse effects in rats, and according to the authors the NOAEL for riboflavin was 1 % in diet, corresponding to 900 mg/kg bw/day.

3.2.3. Genotoxicity

3.2.3.1. *In vitro*

Riboflavin (E 101i), riboflavin-5′-phosphate sodium (E 101ii) and 8-HMR were tested for genotoxicity in a series of *in vitro* assays.

Riboflavin (purity, 96 % ex fermentation[20]) was investigated in a bacterial reverse mutation assay (Ames test) (Albertini, 1995a). The study was performed in compliance with GLP. The Panel noted that the study was carried out in accordance with OECD Guideline 471. A standard plate incorporation assay and a preincubation assay in the absence and in the presence of an exogenous metabolising system (S9) were performed. Five *Salmonella typhimurium* strains (TA1535, TA97, TA98, TA100 and TA102) were employed. Riboflavin was dissolved in dimethylsulphoxide (DMSO) and tested in concentrations ranging from 50 to 5 000 µg/plate. Precipitates were observed at 1 666 and 5 000 µg/plate. No toxicity was noted. Riboflavin was not mutagenic in this assay.

Riboflavin (purity 98 % ex fermentation[20]) was investigated in a separate bacterial reverse mutation assay under the same conditions (Albertini, 1995b). Precipitates were again observed at concentrations of 1 666 µg/plate and above. No bacterial toxicity was noted. Riboflavin was not mutagenic in this assay.

In an unpublished report (BASF, 1994), a substance named "Lutavit B2 SG 80" was tested in the Ames test with the standard plate incorporation and the preincubation methods in the absence and in the presence of an exogenous metabolising system (S9). The purity of the substance was reported to be about 80 %. The identity of the substance was not clear from the report; however, separate technical information indicates that the active ingredient is vitamin B_2 (riboflavin).[21] The Panel noted that the study was carried out in accordance with OECD Guideline 471. The report did not contain a GLP statement. Five concentrations of "Lutavit B2 SG 80", ranging from 20 to 5 000 µg/plate, were tested with four *Salmonella typhimurium* strains (TA1535, TA1537, TA98, and TA100) both with and without S9. No precipitation of the test compound was found. Toxicity was only indicated by occasionally slight decreases in the number of colonies, while a change in the background lawn which would further indicate toxicity was not reported. No mutagenic effects were observed in this study.

These findings were reported to be consistent with the results reported in former studies according to which riboflavin and riboflavin-5′-phosphate sodium were not mutagenic in different *Salmonella typhimurium* strains or in *Saccharomyces cerevisiae* strain D4, either with or without activation (Litton Bionetics, 1977a,b; Fujita and Sasaki, 1986; Kale et al., 1992a). However, only the studies by Kale et al., in which riboflavin was tested with the *Salmonella typhimurium* strains TA100, TA98, TA97a and TA102, were available to the Panel.

Bacterial microsomal reverse mutation tests in *Salmonella typhimurium* were also negative with and without S9 mix for riboflavin 96 % (feed grade, spiked with 2.31 % DMRL) and riboflavin 98 % (tablet grade, spiked with 1.2 % DMRL) (Gocke, 2000a, b). According to industry (saqual GmbH, 2010), DMRL is a known precursor of riboflavin in the biofermentation process and may occur as an impurity in the final product. The studies were performed in compliance with GLP and were in accordance with OECD Guideline 471. In these studies, riboflavin containing DMRL was evaluated in five *Salmonella typhimurium* strains (TA1535, TA97, TA98, TA100 and TA102) using the standard plate incorporation assay and a preincubation method in the presence and absence of S9, respectively. Riboflavin containing DMRL was suspended in DMSO. Upon addition of aliquots to the aqueous

[20] The Panel assumed that the fermentation was with *Bacillus subtilis*.

[21] http://www.basf.com.pe/sac/web/peru/es_ES/function/conversions:/publish/content/peru/nutrition_health/documentos/nutricion_animal/vitaminas/fichas_tecnicas/ficha-tecnica-lutavit-b2-sg-80.pdf

medium, precipitation was observed at 5 mg/plate. Weak toxicity was observed at 1.25 mg/plate in strain TA102. The results obtained with these two riboflavin formulations were similar and no mutagenic effects were observed.

Riboflavin (purity 96 % ex fermentation[20]) was investigated in an *in vitro* chromosomal aberration assay in Chinese hamster ovary cells in the presence and absence of metabolic activation (Chételat and de Vogel, 1996a). The study was conducted in compliance with OECD Guideline 473 and in accordance with GLP. Concentrations ranging from 10 to 75 µg/mL were tested after 18 hours' continuous treatment without metabolic activation. In the presence of metabolic activation, concentrations between 400 and 5 000 µg/ml were tested after three hours' pulse treatment. Two separate experiments were performed at a fixation period of 18 hours. Additionally, the highest concentrations were tested after 32 hours' continuous treatment in the absence of metabolic activation. Riboflavin did not induce structural or numerical chromosome aberrations.

Riboflavin (purity 98 % ex fermentation[20]) was also investigated in an *in vitro* chromosomal aberration assay in Chinese hamster ovary cells in the presence and absence of metabolic activation (Chetelat and de Vogel, 1996b). The study was conducted in compliance with OECD Guideline 473 and in accordance with GLP. Concentrations ranging from 25 to 200 µg/ml were tested after 18 hours' continuous treatment without metabolic activation. In the presence of metabolic activation, concentrations between 400 and 5 000 µg/mL were tested after three hours' pulse treatment. Two separate experiments were performed at a fixation period of 18 hours. Additionally, the highest concentrations were tested after 32 hours' continuous treatment in the absence of metabolic activation. Riboflavin did not induce structural or numerical chromosome aberrations.

The Panel noted that, although these chromosomal aberration assays (Chételat and de Vogel, 1996a, b) had some shortcomings in the choice of solvent and the level of precipitation observed, they were performed in accordance with OECD Guideline 473. The Panel considered that, based on the available data, there is no concern with respect to genotoxicity.

The mutagenic potential of riboflavin and its photodegradation product lumiflavin was also evaluated using two indicator tests (umu test, SOS chromotest) and the Ames/*Salmonella* assay (Kale et al., 1992a). Whereas riboflavin did not induce genotoxicity in the absence and presence of S9, lumiflavin was genotoxic in both indicator tests in the presence of S9 and mutagenic in the Ames/*Salmonella* assay in strains TA100, TA98 and TA97a in the presence of S9. The increases in revertants were dose related (up to 1.9-, 2.9- and 4.9-fold in TA100, TA98 and TA97a, respectively). However, the study has significant shortcomings with respect to the evaluation of toxicity, which was not reported. In this study, mainly in the presence of S9 or caecal cell-free extracts (CCE), untreated controls show a reduced number of revertants possibly caused by cytotoxic effects. In the absence of evaluation of thinning of background lawn in the treated cultures, increases of revertants could be ascribed to toxic effects, that is the utilisation of trace amounts of histidine by auxotrophic surviving bacteria with the formation of micro colonies. Furthermore, preincubation method applied for treatment of cultures did not follow standard recommendations since test compound was removed by centrifugation at the end of treatment. In addition, the number of plates used for each experiment is not clear. On these bases, the Panel considered that the methods implemented were thought not to be sufficiently robust to support the results reported and therefore the study was not considered for risk assessment.

On exposure to visible light, riboflavin and lumiflavin produced reactive oxygen species such as singlet oxygen and superoxide radicals. The reaction was found to be time and concentration dependent. Both riboflavin and lumiflavin, upon illumination, showed genotoxicity in the umu test as well as in the Ames/*Salmonella* assay with *Salmonella typhimurium* TA102 (Kale et al., 1992b). No genotoxicity was observed if the compounds were not illuminated. The study bears the same shortcomings reported for previous study. In addition, although the authors refer to *Salmonella typhimurium* TA102 as the test system, in the Materials and Methods section they report the use of *Salmonella* tester strains TA100, TA98 and TA97a. On these bases, the Panel considered that the

methods implemented were not to be sufficiently robust to support the results reported and therefore the study was not considered for risk assessment.

8-HMR was tested for mutagenic activity in the preincubation version of the Ames test (Albertini, 1989). Five concentrations ranging from 100 to 5 000 µg/plate were tested with seven *Salmonella typhimurium* strains (TA1535, TA1537, TA1538, TA97, TA98, TA100 and TA102) in the absence and in the presence of S9. 8-HMR was dissolved in DMSO. Precipitates were observed at 2 500 and 5 000 µg/plate. No toxic effects were observed. 8-HMR did not induce gene mutations in this assay. The Panel noted that the study was done in accordance with OECD Guideline 471, except that only a single experiment was performed. The study report did not contain a GLP statement.

The Panel noted that there are *in vitro* studies in which riboflavin was illuminated with light of different wavelengths, e.g. Ennever et al. (1983), Sisson (1987) and Kale et al. (1992b). The Panel, however, considered such studies not relevant for the safety evaluation of the use of riboflavin as a food additive.

3.2.3.2. *In vivo*

No *in vivo* studies were available.

Overall, the Panel concluded that, based on the available genotoxicity data, the use of riboflavin and riboflavin-5′-phosphate sodium as food additives does not raise concern with respect to genotoxicity.

3.2.4. Chronic toxicity and carcinogenicity

No data are available.

3.2.5. Reproductive and developmental toxicity

JECFA (1981) evaluated three studies. The first one, by Unna and Greslin (1942), was not considered relevant by the Panel for this assessment. JECFA concluded: *"Weanling male and female rats were fed daily doses of 10 mg (equivalent to 25–100 mg/kg bw/day) of riboflavin for 140 days. The animals were mated and normal litters were obtained from the riboflavin and control groups. At three weeks of age the offspring of the first generation were fed daily with 10 mg riboflavin. Daily feeding over periods of 140 days were continued for three generations. No differences were observed in the development, growth, maturation and reproduction of treated and control animals. Autopsies at the end of the test period did not show any gross change (Unna and Greslin, 1942).*"

The second study evaluated by JECFA (Schumacher et al., 1965) was revisited by the Panel. Female rats of the Long-Evans strain (about 300 g) were fed diet containing 100 mg riboflavin/kg diet, which is 25 times the level in the basal diet, which contained 4 mg riboflavin/kg diet (equivalent to 5 mg/kg bw/day) (Schumacher et al., 1965). The study consisted of 3 experiments in which 5 animals were fed riboflavin and 15 animals the control diet. The females were mated after they had been fed the experimental diets for two weeks, and these diets were continued during gestation and lactation. The number and average weight of the newborn were recorded as soon as possible after the birth of each litter. The young had access to the maternal diet during the nursing period.

The reproduction of the control group was considerably better (68 %, 13 litters for 19 mothers) than that of the high-riboflavin group (38 %, 5 litters for 13 mothers). The value of 68 % was slightly less than that usually found for the stock colony females of this laboratory (70–75 %). The average birth weight, number of young per litter and average weight of the young at weaning were not significantly influenced by increasing the level of riboflavin in the maternal diet. The higher mortality (birth to weaning) in the riboflavin group reflected the complete loss of one litter (10 young). Two control litters were also lost because the mothers failed to nurse. More data are needed to determine whether the high-riboflavin diet increased mortality. The reduced number of litters in the high-riboflavin group may be related to the high maternal intake of these vitamins. With respect to the number of young per litter and the average birth and weaning weights, all of the riboflavin groups and the control group

compared favourably in performance with the laboratory breeding colony. High levels of riboflavin in the maternal diet did not influence the liver storage of this vitamin at weaning. The growth studies found no evidence of any beneficial effect in the young resulting from high maternal intake of riboflavin during pregnancy and lactation.

The authors concluded that high levels riboflavin ingested during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning or vitamin requirement after weaning. However, the authors considered that the reduced number of litters in the high-riboflavin group may be related to the high maternal intake of this vitamin and concluded that more data are needed (Schumacher et al., 1965).

The Panel noted that the number of females used in the experiments is not clear. The authors reported in their publication that 30 female rats were used for each of 3 experiments. In each experiment, 15 females were fed the basal diet. One group was fed a high-pyroxidine diet, a second group a high-thiamine diet and the third group a high-riboflavin diet. However, in one table the number of mothers in the control, thiamine, riboflavin and pyridoxine groups is reported to be 40, 19, 13 and 15, respectively. In addition, the Panel noted that the study design was not in accordance with the current OECD guidelines. The number of females in the high-riboflavin group was very low (5 females) compared with the control group (15 females), and no statistical analyses were performed. Consequently, the Panel considered that this study cannot be used for the risk characterisation.

In the third study, groups of young female Wistar rats were fed diets containing mixtures of vitamins including 4 or 40 ppm riboflavin (equivalent to 0.2 or 2 mg riboflavin/kg bw/day,[22] respectively) during pregnancy and lactation (Le Clerc, 1974). There were no significant differences in the number per litter, mortality or weight gain of offspring between the groups. As, in this study, rats were fed mixtures of vitamins, it is not possible to derive a NOAEL specifically for riboflavin. However, the Panel noted that no adverse effects were induced by a mixture of vitamins resulting in exposure to 2 mg riboflavin/kg bw/day.

3.2.6. Hypersensitivity, allergenicity, intolerance

Occasional cases of anaphylaxis to vitamin B_2 have been reported after consumption of vitamin supplements (Liang-Shiou et al., 2001) or energy drink (Masuda et al., 2009). Such reactions are very rare and the available literature did not indicate that allergenicity/immunotoxicity was a concern for riboflavins used as food additives.

3.2.7. Human data

The SCF (2000) performed a review of the literature of human data available in 2000. There are some case reports describing various adverse effects, such as yellow pigmentation of skin and hair, reversible electroencephalographic abnormalities not associated with clinical symptoms and chronic fatigue (Stripp, 1965; Farhangi and Osserman, 1976; Hirano et al., 1981; Santanelli et al., 1988; Peluchetti et al., 1991).

Schoenen et al. (1994) reported no side effects in 49 patients treated for migraine prophylaxis with 400 mg/day riboflavin taken with meals for at least 3 months.

In a later study by Schoenen et al. (1998), 55 patients with migraine were treated with 400 mg/day riboflavin (or a placebo) in a random trial of 3 months' duration. Only three adverse effects were recorded during the trial. One woman in the riboflavin group experienced diarrhoea two weeks after starting the drug and withdrew from the study. On follow-up, her symptoms disappeared with 72 hours. Another patient receiving riboflavin complained of polyuria but completed the study. In the placebo group, one patient mentioned recurrent abdominal cramps of moderate intensity that did not interrupt the trial.

[22] Calculated by the Panel in accordance with EFSA Scientific Committee (2012).

In a study on 23 adults with migraine, in which riboflavin was given at dose of 400 mg/day, three patients suffered mild effects such as diarrhoea, upper abdominal pain or artificial erythema (Boehnke et al., 2004).

In the studies by MacLennan et al. (2008) and Bruijn et al. (2010), children with migraine were treated with riboflavin at a dose of 200 mg/day (21 patients, 12 weeks, 5–15 years old) and 50 mg/day (20 patients, 16 weeks, 6–13 years old), respectively. No adverse effects were reported.

In another study (Condò et al., 2009), children and adolescents (43; 8–18 years old) with migraine were treated with 400 or 200 mg riboflavin/day for 3, 4 or 6 months. No adverse effects were reported; two patients experienced vomiting and increased appetite, but, according to the authors, this was unrelated to riboflavin use.

No adverse effects were reported in several other clinical studies in which patients from Europe, Thailand and Africa received riboflavin at doses of up to 60 mg/day (Ajayi et al., 1990; Madigan et al., 1998; Fishman et al., 2000; Hustad et al., 2002; McNulty et al., 2006; Powers et al., 2007; Hoey et al., 2009; Tavares et al., 2009).

In two consecutive studies, riboflavin was used in the treatment of anaemia in 366 (Ma et al., 2008) or 164 (Ma et al., 2010) pregnant Chinese women, Riboflavin was administered for two months at a dose of 1 mg/day and no adverse effects were reported

4. Discussion

The Panel was not provided with a newly submitted dossier and based its evaluation on previous evaluations, additional literature that had become available since then and the data available following a public call for data.

Riboflavins (E 101) are authorised as food additives in the EU in accordance with Annex II to Regulation (EC) No 1333/2008 and have been previously evaluated by JECFA in 1969, 1981 and 1998, and by the SCF in 1977, 1984, 1998 and 2000.

Synthetic riboflavin was evaluated by JECFA in 1969, where an ADI of 0–0.5 mg/kg bw/day was allocated on the basis of limited data. JECFA based this ADI on a "level causing no adverse effects in the rat" (50 mg/kg bw/day, expressed as riboflavin). In 1981, JECFA allocated a group ADI for riboflavin and riboflavin-5′-phosphate sodium of 0–0.5 mg/kg bw/day (expressed as riboflavin). It is not specified in the evaluation on which study this ADI was based. In 1998, JECFA included riboflavin derived from a production strain of genetically modified *Bacillus subtilis* in the previously established group ADI of 0–0.5 mg/kg bw/day for synthetic riboflavin and riboflavin-5′-phosphate sodium.

In 1977, the SCF classified riboflavin-5′-phosphate sodium as a food colour which could be used in food, but for which an ADI was not established. The SCF was of the opinion that the use of this substance as a food colour should not significantly alter the average daily intake of riboflavin. In 1998, the SCF concluded that riboflavin produced by fermentation using genetically modified *Bacillus subtilis* is acceptable for use as a food colour. Furthermore, in 2000 the SCF concluded that it was not possible, based on the available database, to derive a UL for riboflavin used as a vitamin because the available data on adverse effects from high oral riboflavin intake were not of sufficient quality and extent to be used for the determination of a UL. However, the SCF (2000) stated that the limited evidence available from clinical studies indicated that current levels of intake of riboflavin from all sources do not represent a risk to human health.

Riboflavin can be obtained by chemical synthesis or from microbiological sources. According to industry, chemical synthesis is not currently used and riboflavin is produced by fermentation using the *Bacillus subtilis* or the fungus *Ashbya gossypii*. The Panel noted that information on active second metabolites of *Ashbya gossypii* and their toxicological profile would be necessary to assess this

method of manufacturing riboflavin as a food additive. The Panel noted that, according to the EFSA BIOHAZ Panel, "*the knowledge concerning the capacity of Ashbya gossypii to produce biological active secondary metabolites remains limited and this species cannot be proposed for QPS list*" and *Bacillus subtilis* is recommended for the QPS list with the qualifications "absence of toxigenic activity". Three genetically modified strains of *Bacillus subtilis* and a genetically modified strain of *Ashbya gossypii* for production of riboflavin are currently under evaluation by the EFSA FEEDAP Panel. The Panel noted that the re-evaluation of riboflavins as food additives applies to riboflavin (E 101(i)) produced in accordance with the manufacturing process that was evaluated by the SCF (1998). This re-evaluation cannot be applied to riboflavin produced by other manufacturing processes. These could be considered as significant changes in the production methods which require an assessment in accordance with relevant legislation. Riboflavin 5′-phosphate sodium is produced by phosphorylation of the starting material riboflavin with phosphorous oxychloride (Saqual GmbH, 2010).

Riboflavin is relatively stable during thermal and non-thermal food processing and storage in the dark but is very sensitive to light. Riboflavin-5′-phosphate sodium is fairly stable to air but is hygroscopic and sensitive to heat and light. Several compounds are formed from riboflavin and riboflavin-5′-phosphate sodium under the influence of light, including the non-volatile compounds lumichrome and lumiflavin, and the volatile compound 2,3-butanedione.

When administered alone, the absorption of free riboflavin is 30–50 % at the dose range of 5–20 mg and is decreased at higher oral doses. However, the amount of riboflavin absorbed depends on the intake; it is increased when riboflavin is given orally with food. Riboflavin-5′-phosphate sodium and riboflavin are probably absorbed by a specific transport system in the upper gastrointestinal system. In plasma, riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins, and is mainly found as FAD. Lumichrome and lumiflavin have been identified as metabolites of riboflavin in the rat, while 7-α–hydroxyriboflavin has been identified as a plasma metabolite in humans. Riboflavin-5′-phosphate sodium may be dephosphorylated during absorption but may subsequently be rephosphorylated in the mucosa, transported to the liver, where it is again dephosphorylated to riboflavin (the form in which it occurs in the circulation), and mainly excreted in urine.

Wistar rats received diets providing 20, 50 or 200 mg/kg bw/day riboflavin for 13 weeks (Buser et al., 1995). The purpose of this study was to assess and compare the toxicity of two preparations of riboflavin produced by a new fermentative method, called "riboflavin 96 % ex fermentation" and "riboflavin 98 % ex fermentation", and a riboflavin produced by chemical synthesis named "riboflavin 98 % ex synthesis". In the absence of any adverse effect, the NOAEL identified in this study was 200 mg/kg bw/day for the three grades of riboflavin. The Panel agreed with this NOAEL.

In a study by Bachman et al. (2005), the test item, containing 80.1 % riboflavin, 0.25 % lumichrome, 1.5 % DMRL, 0.1 % 8-HMR and 20 % maltodextrin, was tested on SPF-bred Wistar rat of both sexes at target doses of 0, 50, 100 and 200 mg test item/kg bw/day for 13 weeks in accordance with an OECD guideline. In the absence of any adverse effect, the authors of the study considered that the NOAEL for male and female rats was 200 mg test item/kg bw/day, the highest dose level tested, corresponding to 160.2 mg riboflavin/kg bw/day, 0.5 mg lumichrome/kg bw/day, 0.2 mg 8-HMR/kg bw/day and 3 mg DMRL/kg bw/day. The Panel agreed with this NOAEL.

Based on the *in vitro* genotoxicity data available, the Panel concluded that the use of riboflavin and riboflavin-5′-phosphate sodium as food additives does not raise concern with respect to genotoxicity.

No chronic toxicity studies or carcinogenicity studies are available.

In 1981, JECFA concluded after the evaluation of three studies on reproductive and developmental toxicity that no adverse effects were observed. The Panel noted that the quality of these studies was not adequate to conclude on the reproductive and developmental toxicity.

The Panel noted that there are several human studies in children, adolescent and adults (including pregnant women) on the possible beneficial effects of riboflavin supplementation in the case of deficiency and clinical trials using riboflavin as migraine prophylaxis. No significant adverse effects were reported at doses up to 400 mg/day, and for up to six months of treatment, equivalent, according to the EFSA Scientific Committee (2012), to 17.3 mg/kg bw/day for children aged 3–10 years with a mean weight of 23.1 kg The Panel noted that these studies were not designed as safety studies; however, they provide information on the limited range of observed adverse effects.

Due to the absence of carcinogenicity/chronic toxicity studies and lack of relevant reproductive and developmental toxicity studies, the Panel considered that it is not appropriate to allocate an ADI to riboflavin and riboflavin-5′-phosphate sodium.

In Annex II to Regulation (EC) No 1333/2008, riboflavins are permitted at concentrations up to 100 mg/L in americano, bitter soda and bitter vino, and at *quantum satis* in pasturmas (edible external coating) and vegetables in vinegar, brine or oil (excluding olives). Furthermore, riboflavins may be added to all foodstuffs other than those listed in Annex II to Regulation (EC) No 1333/2008 at *quantum satis*.

The exposure of European children to riboflavins used as food additives calculated by using the data provided by industry ranged from 0.5 to 1.8 mg/kg bw/day at the mean, and from 0.9 to 3.9 mg/kg bw/day at the 95th percentile. The main contributors to the total anticipated mean exposure to riboflavins (> 10 % in all countries) were processed fruit and vegetables (up to 51 %), soups and broths (up to 55 %) and sauces (up to 35 %). Estimates calculated for the adult population give a dietary exposure to riboflavins 0.2–0.7 mg/kg bw/day at the mean and 0.4–1.7 mg/kg bw/day for high-level (95th percentile) consumers. The main contributors (> 10 %) to the total anticipated mean exposure to riboflavins were processed fruit and vegetables (14–65 %) and soups (9–36 %).

Riboflavin is present in a wide range of foods, with liver, milk, meat and fish being the most important sources. Across Europe the mean dietary intake of riboflavin ranges from 1.1 to 2.0 mg/day for children (equivalent to approximately 0.04–0.08 mg/kg bw/day[23]), and 1.2–2.8 mg/day for adults (equivalent to approximately 0.02–0.05 mg/kg bw/day[23]) (Elmadfa, 2009).

Overall, the Panel considered that:

- Riboflavin-5′-phosphate sodium is rapidly dephosphorylated to free riboflavin in the intestinal mucosa then metabolised using normal metabolic pathways.
- Two subchronic toxicity studies in rats, performed in accordance with OECD guidelines, did not report any adverse effects at doses up to 160 and 200 mg riboflavin/kg bw/day, the highest doses tested.
- Riboflavin and riboflavin-5′-phosphate sodium do not raise concern with respect to genotoxicity.
- There are limited data from clinical studies with doses up to 400 mg riboflavin/day, in which no significant adverse effects were reported.
- The use of riboflavin and riboflavin-5′-phosphate sodium as food additives will result in an exposure that is higher than that from the regular diet.
- The available database is insufficient to assess whether or not potential high intakes from all combined sources (food additive, food supplements, diet) cause adverse effects.

[23] Calculated by the Panel in accordance with EFSA Scientific Committee (2012).

In the opinion of the Panel, taking into account the arguments of safety and the uncertainties in the database, riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) are unlikely to be of safety concern at the currently authorised uses and use levels as food additives.

CONCLUSIONS

The Panel concluded that it is not appropriate to allocate an ADI to riboflavin and riboflavin-5´-phosphate sodium due to the absence of carcinogenicity/chronic toxicity studies and the lack of relevant reproductive and developmental toxicity studies.

Despite the uncertainties in the database, the Panel concluded that riboflavin (E 101(i)) and riboflavin-5′-phosphate sodium (E 101(ii)) are unlikely to be of safety concern at the currently authorised uses and use levels as food additives.

DOCUMENTATION PROVIDED TO EFSA

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APPENDIX

Appendix A. Rules defined by the Panel to deal with *quantum satis* (QS) authorisation, usage data or observed analytical data for all regulated food additives to be re-evaluated

Figure 6: Rules defined by the Panel to deal with usage data or observed analytical data for all regulated food additives to be re-evaluated and procedures for estimating intakes using these rules

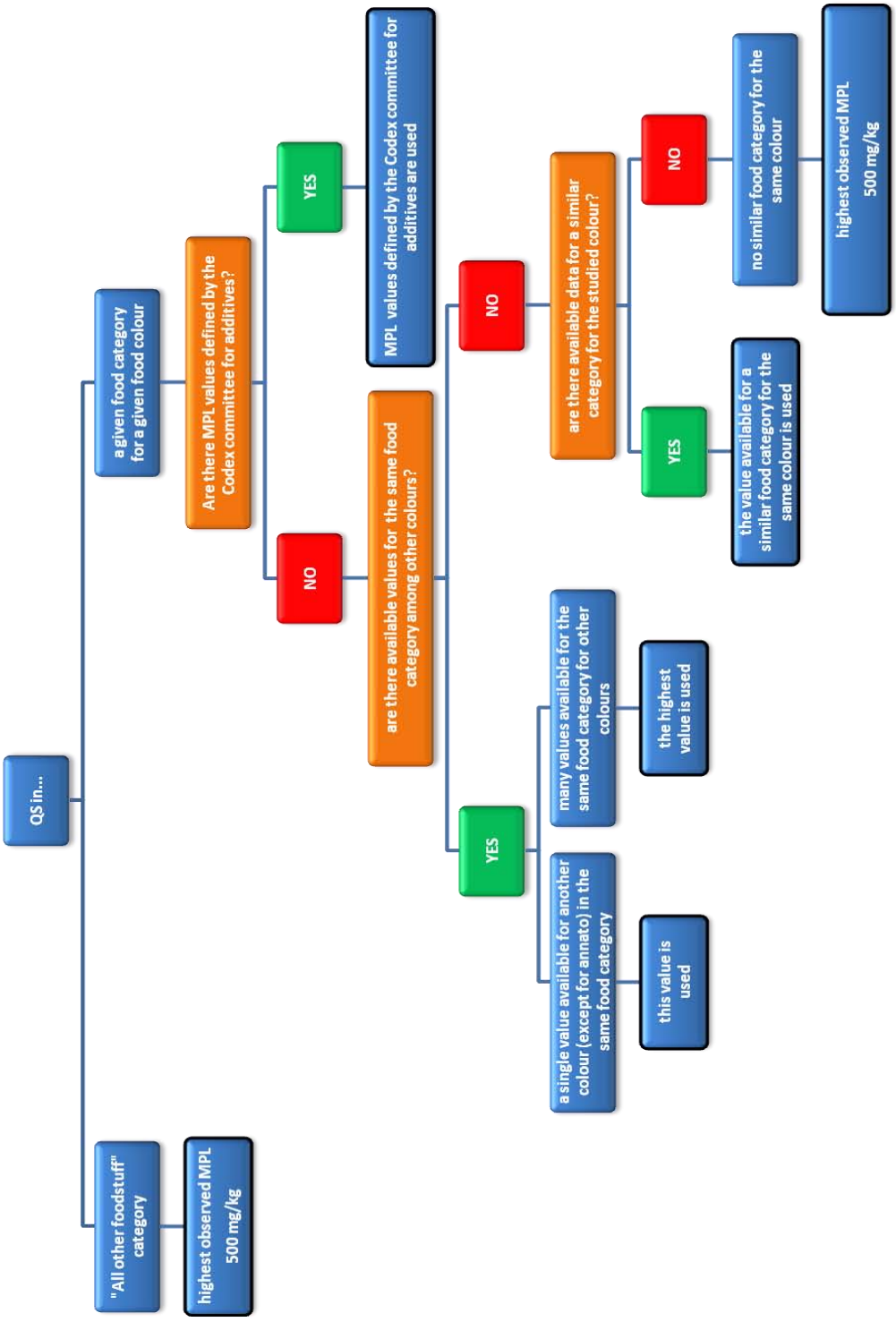

Figure 7: Rules defined by the Panel to deal with *quantum satis* (QS) authorisation

GLOSSARY AND ABBREVIATIONS

ADI	Acceptable daily intake
ANS	EFSA Panel on Food Additives and Nutrient Sources added to Food
BfR	Bundesinstitut für Risikobewertung
BIOHAZ	EFSA Panel on Biological Hazards
bw	Body weight
CAS	Chemical Abstract Service
CCE	Caecal cell-free extracts
CIAA	Confederation of the Food and Drink Industries of the EU
D-A-CH	Deutsche Gesellschaft für Ernährung, Österreichische Gesellschaft für Ernährung Gesellschaft für Ernährungsforschung, Schweizerische Vereinigung für Ernährun
DMSO	Dimethylsulphoxide
DMRL	6,7-dimethyl-8-ribityl-lumazine
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EINECS	European Inventory of Existing Commercial chemical Substances
EVM	Expert Group on Vitamins and Minerals
FAD	Flavin adenine dinucleotide
FAIM	Food additives intake model
FAO/WHO	Food and Agriculture Organisation/World Health Organisation
FD	Fluorimetric detection
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FNB	Food and Nutrition Board
GLP	Good laboratory practice
8-HMR	8-hydroxymethyl-riboflavin
HPLC	High-performance liquid chromatography
IRMM	Institute for Reference Materials and Measurements
JECFA	Joint FAO/WHO/Expert Committee on Food Additives
LC-MS	Liquid chromatography–mass spectrometry
LD_{50}	Lethal dose, 50 %, i.e. dose that causes the death of 50% of treated animals
MPL	Maximum permitted level
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PARNUTS	Foods for particular nutritional uses

PRI	Popular reference intake
QA	Quality assessment
QPS	Qualified presumption of safety
QS	*Quantum satis*
RBC	Red blood cell
RDA	Recommended daily allowance
RP-HPLC	Reversed-phase high-performance liquid chromatography
SCF	Scientific Committee for Food
UL	Tolerable upper intake level