



## RIBOFLAVIN FROM *ASHBYA GOSSYPII*

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### 1. Summary

This Chemical and Technical Assessment summarizes data and information on riboflavin from *Ashbya gossypii* submitted to the Committee of the 89<sup>th</sup> JECFA meeting by BASF<sup>1</sup> in a dossier dated 15 December 2019.

The Committee evaluated riboflavin for the first time in 1959 (4<sup>th</sup> meeting) and published purity criteria for a product containing at least 98% of riboflavin "extracted from natural sources or manufactured synthetically". A comprehensive safety evaluation followed in 1969 (13<sup>th</sup> meeting) as part of the work on "Food Colours Derived from Natural Sources". Routes of production were not mentioned; the ADI was derived from a toxicological study published in 1942. Applying a safety factor of 100 to the No Observed Effect Level of 50 mg/kg bw/d from the study the ADI was determined to be 0.5 mg/kg bw; this ADI was changed to a group ADI of 0-0.5 mg/kg bw for riboflavin and riboflavin-5'-phosphate when the Committee evaluated riboflavin-5'-phosphate in 1981.

The Committee assessed riboflavin produced from a genetically modified *Bacillus subtilis* in 1998, and included it into the group ADI along with synthetic riboflavin, the phosphate salt and riboflavin from genetically modified *B. subtilis*. At its 92<sup>nd</sup> meeting (2021) the Committee established a group ADI "not specified" for riboflavin, riboflavin- 5'-phosphate, riboflavin from *B. subtilis* and riboflavin from *A. gossypii*; the Committee withdrew the previous group ADI of 0 - 0.5 mg/kg bw.

Riboflavin from *A. gossypii* is produced by fermentation using a self-cloned strain of the fungus *A. gossypii*. The fermentation is stopped by autolysis. Purification by several filtration and precipitation/crystallization steps results in a food grade dry powder, free of fermentation media constituents and the production organism. This product meets the JECFA specifications for riboflavin; it contains not less than 98% riboflavin, not more than 1.5% residual moisture and no more than 0.025% lumiflavin.

The complete genomic sequences of the original and self-cloned *A. gossypii* strains were analyzed using antiSMASH 1.2.0, for the possible presence of gene clusters encoding secondary metabolites. It was concluded from the analyses, that the genome of *A. gossypii* has no hidden potential to produce secondary metabolites. Applying good manufacturing practices to the selection of raw materials for the fermentation should additionally guarantee the absence of mycotoxins from the final product.

The specifications for riboflavin from *A. gossypii* are consistent with the monographs for riboflavin

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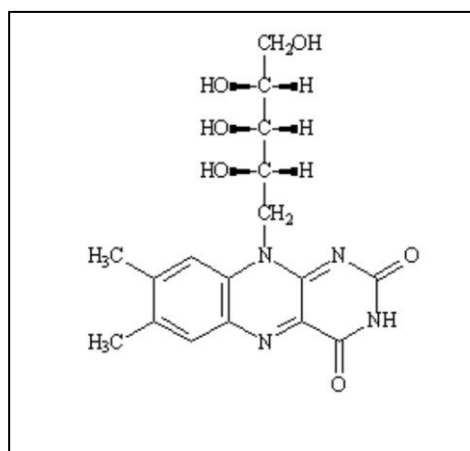
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(INS 101(i)) and riboflavin from *B. subtilis* (INS 101(iii)).

Riboflavin is used as food colour in products subjected to intense processing/storage (which result in partial loss of their natural colour), to standardize colour, or to impart a yellow hue to processed foods. Riboflavin is also a vitamin (B2); therefore, it is also used for the fortification of food and in dietary/food supplements. However, the safety evaluations of these applications were not within the scope of this Committee.

## 2. Description

Riboflavin can be obtained by chemical synthesis or by fermentation. It is a yellow to orange yellow crystalline powder that displays polymorphism. It is soluble in dilute aqueous alkali solutions and practically insoluble in ethanol. It has a molecular weight of 376.37 Da and its structural formula is shown below.



Soon after the discovery of riboflavin in 1933 various manufacturing routes were developed; chemical synthesis was followed by fermentation with microorganisms, and a decade later, during World War II, the first purified riboflavin was available commercially. Due to mandatory food fortification laws enforced in the US in 1943, increased demand for riboflavin led to research by the public and private sectors to develop alternate routes of manufacturing. Two fungal sources, *Eremothecium ashbyi* and *A. gossypii* (classified today as *E. gossypii* in the same genus as *E. ashbyi*) were the most promising. By 1946-47 scale up and successful commercial use of riboflavin was reported.

BASF licensed the original riboflavin-overproducing strain in 1984; marketing of feed-grade riboflavin started in 1990. Five years later food and pharmaceutical grade riboflavin were introduced to commerce using the purification step for riboflavin manufactured by chemical synthesis. Chemical synthesis of riboflavin was phased out by BASF in 1996.

*Ashbya gossypii* is a filamentous fungus that grows well on plant oils like soybean oil or rape seed oil and on sugar substrates like glucose or sucrose. The naturally occurring wild-type fungus produces large amounts of riboflavin, possibly for UV protection (Stahmann *et al.* 2001), leading to yellow colour development after a few days of growth. The *A. gossypii* strain used in this submission is self-cloned for riboflavin overproduction.

Riboflavin from *A. gossypii* complies with the specifications for the synthetic riboflavin and the riboflavin produced by fermentation using *B. subtilis*.

### 3. Manufacturing

#### 3.1. Manufacturing principle

Riboflavin from *A. gossypii* is produced by fermentation which is stopped by autolysis. The final product after several filtration and precipitation/crystallisation steps is a food grade dry powder, containing not less than 98% riboflavin.

#### 3.2 Detailed description

BASF produces a feed grade and a food grade riboflavin by fermentation of soybean oil and other components and with the same self-cloned *A. gossypii* strain. The process of food grade riboflavin production includes the additional purification steps of dissolution, filtration, precipitation and drying.

##### a) Taxonomy of the producing organism *A. gossypii* (LU11439)

The microorganism used for fermentation of Riboflavin is *A. gossypii*. The following taxonomic information applies to the parent and recipient organisms as the strain is self-cloned and thus contains only *A. gossypii* DNA sequences.

Genus:	<i>Eremothecium</i>
Species:	<i>Ashbya gossypii</i>
Strain:	LU11439 (the modified, current production strain)
Deposition number:	DSM 23096
Generic name:	<i>Ashbya gossypii</i>

According to Kurtzman & de Hoog (2011) the appropriate taxonomically valid classification is *E. gossypii*, however, in scientific publications the organism continues to be identified as *A. gossypii*.

##### b) Phenotypic and genetic markers

The detailed phenotypic description was given most recently in The Yeasts, a Taxonomic Study (Vol. 2, Chapter 30.4) by Kurtzman & de Hoog (5<sup>th</sup> Edition, 2011) in their monograph of the genus *Eremothecium*.

The complete sequence and annotation of the *A. gossypii* genome was published in 2004. This shows a 95% homology and gene synteny to the genome of budding yeast, *Saccharomyces cerevisiae* (Dietrich, *et al.* 2004, Gattiker *et al.* 2007). *A. gossypii* has its 4718 protein-coding genes and 9.2 million base pairs (smallest genome of a free-living eukaryote yet characterized) spread over seven chromosomes. *A. gossypii* strains can be identified by comparing their DNA sequences to the whole

genome sequence which is publicly available (Dietrich, *et al.* 2004, Gattiker *et al.* 2007).

BASF's first self-cloned strain of *A. gossypii* (LU9868) was sequenced using the Illumina technique. Based on this sequence it was confirmed that the various strains used by the applicant since 1984 are closely related to *A. gossypii* as defined by the publicly available sequence.

*A. gossypii* is not known to be genetically unstable (Dietrich, *et al.* 2004, Gattiker *et al.* 2007), and one of the reasons for selecting this species as a riboflavin-producing organism was its phenotypic and therefore genetic stability as natural riboflavin overproducer (Demain 1972).

#### **c. Pathogenicity, ecological and physiological traits**

*A. gossypii* and the closely related *E. ashbyii* from the same genus are not listed under higher safety level than 1 in accordance with European Union Directive 2000/54/EC.

According to Kurtzman & de Hoog (2011) this microorganism is “commonly found in tropical and subtropical areas of the world as a pathogen of coffee (*Coffea* spp.), cotton (*Gossypium* spp.), soybean (*Glycine max*) and other crops.” and it is not known to be of clinical importance in animals or humans.

#### **d. Secondary metabolites produced by *A. gossypii***

The complete genomic sequences of BASF's first self-cloned production strain LU9868 and of the published *A. gossypii* wild strain (ATCC 10895) were analysed for presence of gene clusters encoding secondary metabolites using antiSMASH 1.2.0. No gene clusters of polyketide synthases or non-ribosomal peptide synthases (NRPS), the two classes of secondary metabolite gene clusters that are frequently involved in the biosynthesis of toxic secondary metabolites, were identified in the genome.

Three gene clusters were predicted by antiSMASH in both strains. Two of these gene clusters were annotated as type “terpene”, and one gene cluster as type “other”, reflecting that antiSMASH detected a putative gene cluster which does not fit any of the classical secondary metabolite compound classes. Comparison of the central gene of each cluster with *S. cerevisiae* genome displayed high homology with yeast sequences and eventually for each of the three central cluster genes it could be shown that the transcribed enzyme was not involved in secondary but in primary metabolism.

It could be concluded from the analysis, that the genome of *A. gossypii* has no hidden potential to produce secondary metabolites. This result is confirmed by the fact that no production of toxins with *A. gossypii* has been reported during the decades of industrial fermentation, and in the academic community.

#### **e. History of strain development**

BASF started to use the strain LU8907 to produce riboflavin, in 1990. This strain was derived from the original LU3178 strain by UV-induced mutation. LU8907 was replaced in 2001 by a self-cloned strain LU9868 that was replaced by the current production strain LU11439 in which several rib genes encoding the necessary enzymes for the final biosynthetic steps are over-expressed. Absence of foreign DNA in the genome of the LU11439 strain, was confirmed by Southern blot analysis using

genomic DNA from LU11439 and LU8907. The production strain was confirmed to be genetically stable. It contains no transferable resistance marker genes and no other sequences of concern. Furthermore, it carries no heterologous sequences derived from vector DNA.

#### **f. Information relating to the production process**

The production strain is stored in frozen state for maintaining stable and reproducible activity. This master cell bank is used for preparing a “working cell bank” consisting of hundreds of frozen vials which are then used for inoculation of the seed culture.

The growth media used for fermentation are standard media similar to those used in many other laboratory or production scale cultivations. Constituents are of food grade quality and include inorganic salts, amino acids, complex carbon and nitrogen sources like yeast extract and soy bean meal, plant oil (from soy bean or rape seed), carbohydrates and defoamers. Addition of any kind of antimicrobial compounds is not required and is not done.

The culture is grown for several days in the fermentation vessel. During this growth period riboflavin is secreted into the fermentation broth. Fermentation is stopped by autolysis inducing cell disintegration; further temperature increase, simultaneous pH-reduction, and crystallization at acidic conditions ensure complete cell lysis. Riboflavin crystals are separated from the fermentation broth by decantation.

The slurry from the decanters is dissolved in diluted alkaline water, filtered, and precipitated. This process is repeated, and the precipitate is suspended in water, and spray dried. The final product is a fine, yellow to orange-yellow granule (“high flow”) or powder (“fine powder”) with a content of riboflavin of not less than 98 %.

The product is verified to be free of viable production organism. It is also verified by PCR to be free of residual production strain DNA.

### **4. Chemical characterization**

#### **4.1. Composition**

The article in commerce contains not less than 98% and not more than 101% of riboflavin (as dried substance), with typical levels between 99% and 100%. Residual moisture is present at not more than 1.5%, with typical levels of ~ 1%.

#### **4.2 Identification**

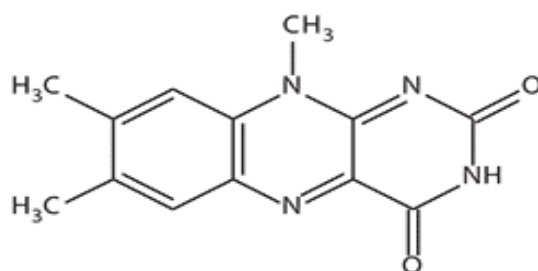
Riboflavin is very soluble in dilute alkaline solutions and practically insoluble in ethanol. It is optically active and identified based on its UV absorbance spectrum and optical rotation.

#### **4.3 Analytical methods**

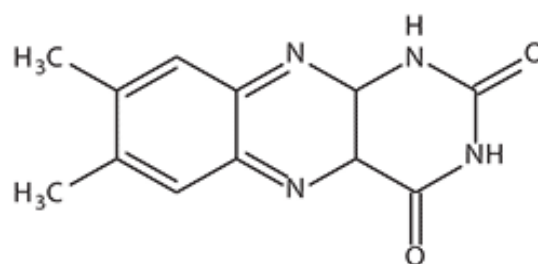
The specifications monograph cites general tests included in the *FAO Combined Compendium of Specifications* (FAO JECFA Monographs 1, vol. 4, 2006), specific tests for the assay (UV absorption) and, for the determination of organic impurities (HPLC-DAD).

#### 4.4 Possible impurities

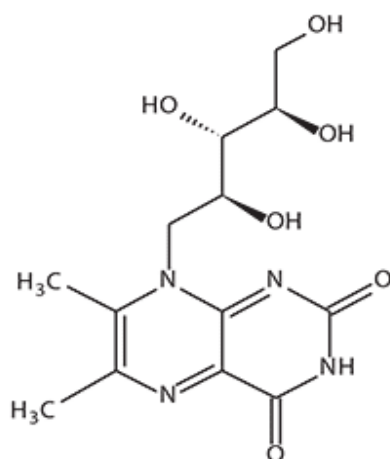
Possible impurities include the precursor 6,7-dimethyl-8-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3*H*,8*H*)-dione (Compound C) (CAS No.: 5118-16-1) and three degradation products. Under exposure to light at alkaline conditions lumiflavin (Compound A, chemical name: 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione), (CAS No.: 1088-56-8) may be formed. At neutral and weak acidic pH the formation of lumichrom (Compound B, chemical name: 7,8-dimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione), (CAS No 1086-80-2) is favoured. Oxidative degradation of riboflavin leads to the formation of 8-Hydroxymethyl-riboflavin (8-HMR) (Compound D, 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)), (CAS No 52134-62-0). The structures of these four by-products are shown below.



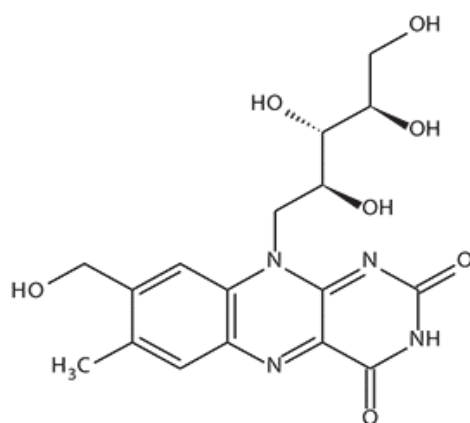
Compound A: 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (lumiflavine)



Compound B: 7,8-dimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione



Compound C. 6,7-dimethyl-8-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3H,8H)-dione



Compound D. 8-(hydroxymethyl)-7-methyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4(3H,10H)-dione.

The current specifications monograph contains limit only for lumiflavin (compound A).

Due to the two-step purification including microfiltration and precipitation, carryover of the yeast and fermentation medium constituents is minimized.

Based on analytical data provided by the sponsor for three non-consecutive batches of riboflavin from *A. gossypii*, lead levels are below the limit of detection (0.5 ppb).

#### 4.6 Rationale for proposed specifications

The proposed specifications for Riboflavin from *A. gossypii* are based on the monographs for riboflavin (INS 101(i)) and riboflavin from *B. subtilis* (INS 101(ii)). The method for the determination of lumiflavin was replaced by the relevant *Ph. Eur.* Method.

Primary Aromatic Amines were not included as they are not anticipated by-products of manufacturing.

## 5. Functional uses

### 5.1. Technological function

Riboflavin is used as food colour in products subjected to intense processing/storage (which result in losses of part of their natural colour), to standardize the colour, or to impart a yellow hue to processed foods. Riboflavin is a vitamin (B2), and it is therefore also used for the fortification of food and in dietary/food supplements, however these uses of riboflavin are not within the scope of this evaluation.

### 5.2. Food categories and use levels

Riboflavin from *A. gossypii* is intended for use as a colour additive in various foods and beverages. The substance does not differ in its purity and properties from evaluations of riboflavins by previous Committees and listed in the GSFA. Riboflavin from *A. gossypii* is intended to be used under the same conditions as 101(i) Riboflavin, synthetic and 101(iii) Riboflavin from *B. subtilis*. The current listed uses, use levels and specific notes for riboflavins are available in the 2019 edition of the GSFA (on-line edition) and are presented in the Appendix. The uses listed therein also apply to riboflavin from *A.gossypii*.

Note of caution: the current listed food categories and levels are for the use of riboflavin and its derivative, the riboflavin phosphate sodium salt (101(ii)). Both compounds differ in their physico-chemical properties and their suitability. Riboflavin is photosensitive and therefore not a food colour of choice when stability to light is required. Riboflavin is also not a strong colourant, so in many food matrices other yellow food colours may be more suitable. Therefore, riboflavin is not a major colourant in many of the food categories listed in Appendix 1.

## 6. Reactions and fate in foods

Riboflavin is relatively stable during food processing and storage; it is very stable under acidic conditions, even when exposed to air and oxidants. It is sensitive to light (visible and UV), by which it is degraded irreversibly to either lumiflavin or lumichrom. Riboflavin is stable if stored in tight, light-resistant containers. 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 (EFSA, 2013).

## 7. References

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## APPENDIX: GSFA Provisions for Riboflavin

Number	Food Category	Max Level	Units	Notes
01.1.4	Flavoured fluid milk drinks	300	mg/kg	52
01.3.2	Beverage whiteners	300	mg/kg	
01.5.2	Milk and cream powder analogues	300	mg/kg	
01.6.1	Unripened cheese	300	mg/kg	
01.6.2.1	Ripened cheese, includes rind	300	mg/kg	XS277 XS276 S274 XS265 XS269 S266 XS267 XS272 XS271 XS268 XS270462
01.6.2.2	Rind of ripened cheese	300	mg/kg	
01.6.4	Processed cheese	300	mg/kg	
01.6.5	Cheese analogues	300	mg/kg	
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	300	mg/kg	
02.2.2	Fat spreads, dairy fat spreads and blended spreads	300	mg/kg	
02.3	Fat emulsions mainly of type oil-in-water, including mixed and/or flavoured products based on fat emulsions	300	mg/kg	
02.4	Fat-based desserts excluding dairy-based dessert products of food category 01.7	300	mg/kg	
03.0	Edible ices, including sherbet and sorbet	500	mg/kg	
04.1.1.2	Surface-treated fresh fruit	300	mg/kg	4 16
04.1.2.4	Canned or bottled (pasteurized) fruit	300	mg/kg	267
04.1.2.5	Jams, jellies, marmelades	200	mg/kg	
04.1.2.6	Fruit-based spreads (e.g. chutney) excluding products of food category 04.1.2.5	500	mg/kg	
04.1.2.7	Candied fruit	300	mg/kg	
04.1.2.8	Fruit preparations, including pulp, purees, fruit toppings and coconut milk	300	mg/kg	182
04.1.2.9	Fruit-based desserts, including fruit-flavoured water-based desserts	300	mg/kg	
04.1.2.10	Fermented fruit products	500	mg/kg	
04.1.2.11	Fruit fillings for pastries	300	mg/kg	

Number	Food Category	Max Level	Units	Notes
04.2.1.2	Surface-treated fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, and nuts and seeds	300	mg/kg	4 16
04.2.2.3	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), and seaweeds in vinegar, oil, brine, or soybean sauce	500	mg/kg	
04.2.2.6	Vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed pulps and preparations (e.g. vegetable desserts and sauces, candied vegetables) other than food category 04.2.2.5	300	mg/kg	92
04.2.2.7	Fermented vegetable (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera) and seaweed products, excluding fermented soybean products of food categories 06.8.6, 06.8.7, 12.9.1, 12.9.2.1 and 12.9.2.3	500	mg/kg	
05.1.5	Imitation chocolate, chocolate substitute products	1,000	mg/kg	
05.2	Confectionery including hard and soft candy, nougats, etc. other than food categories 05.1, 05.3 and 05.4	1,000	mg/kg	XS309R
05.3	Chewing gum	1,000	mg/kg	
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	1,000	mg/kg	
06.3	Breakfast cereals, including rolled oats	300	mg/kg	
06.4.3	Pre-cooked pastas and noodles and like products	300	mg/kg	473153
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	300	mg/kg	
06.6	Batters (e.g. for breading or batters for fish or poultry)	300	mg/kg	
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	300	mg/kg	
09.2.1	Frozen fish, fish fillets, and fish products, including mollusks, crustaceans, and echinoderms	1,000	mg/kg	XS315 XS312 XS292 XS165 XS36 XS190 XS95 XS191 XS9295
09.2.2	Frozen battered fish, fish fillets, and fish products, including mollusks, crustaceans, and echinoderms	300	mg/kg	XS16616
09.2.3	Frozen minced and creamed fish products, including mollusks, crustaceans, and echinoderms	300	mg/kg	16
09.2.4.1	Cooked fish and fish products	300	mg/kg	95

Number	Food Category	Max Level	Units	Notes
09.2.4.2	Cooked mollusks, crustaceans, and echinoderms	300	mg/kg	
09.2.4.3	Fried fish and fish products, including mollusks, crustaceans, and echinoderms	300	mg/kg	16
09.2.5	Smoked, dried, fermented, and/or salted fish and fish products, including mollusks, crustaceans, and echinoderms	300	mg/kg	XS236 XS222 XS189 XS244 XS167 XS31122
09.3.1	Fish and fish products, including mollusks, crustaceans, and echinoderms, marinated and/or in jelly	300	mg/kg	16
09.3.2	Fish and fish products, including mollusks, crustaceans, and echinoderms, pickled and/or in brine	300	mg/kg	16
09.3.3	Salmon substitutes, caviar, and other fish roe products	300	mg/kg	XS291
09.3.4	Semi-preserved fish and fish products, including mollusks, crustaceans, and echinoderms (e.g. fish paste), excluding products of food categories 09.3.1 - 09.3.3	300	mg/kg	
09.4	Fully preserved, including canned or fermented fish and fish products, including mollusks, crustaceans, and echinoderms	500	mg/kg	XS90 XS119 XS3 XS94 XS37 XS70 95
10.1	Fresh eggs	300	mg/kg	4
10.4	Egg-based desserts (e.g. custard)	300	mg/kg	
11.3	Sugar solutions and syrups, also (partially) inverted, including treacle and molasses, excluding products of food category 11.1.3	300	mg/kg	
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	300	mg/kg	
12.2.2	Seasonings and condiments	350	mg/kg	
12.4	Mustards	300	mg/kg	
12.5	Soups and broths	200	mg/kg	344
12.6	Sauces and like products	350	mg/kg	XS302
12.7	Salads (e.g. macaroni salad, potato salad) and sandwich spreads excluding cocoa- and nut-based spreads of food categories 04.2.2.5 and 05.1.3	300	mg/kg	

Number	Food Category	Max Level	Units	Notes
13.3	Dietetic foods intended for special medical purposes (excluding products of food category 13.1)	300	mg/kg	
13.4	Dietetic formulae for slimming purposes and weight reduction	300	mg/kg	
13.5	Dietetic foods (e.g. supplementary foods for dietary use) excluding products of food categories 13.1 - 13.4 and 13.6	300	mg/kg	
13.6	Food supplements	300	mg/kg	
14.1.4	Water-based flavoured drinks, including sport energy or electrolyte drinks and particulated drinks	50	mg/kg	
14.2.2	Cider and perry	300	mg/kg	
14.2.4	Wines (other than grape)	300	mg/kg	
14.2.7	Aromatized alcoholic beverages (e.g. beer, wine and spirituous cooler-type beverages, low alcoholic refreshers)	100	mg/kg	
15.1	Snacks - potato, cereal, flour or starch based (from roots and tubers, pulses and legumes)	1,000	mg/kg	
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	1,000	mg/kg	
08.2	Processed meat, poultry, and game products in whole pieces or cuts	1,000	mg/kg	XS96 XS9716
08.3	Processed comminuted meat, poultry, and game products	1,000	mg/kg	XS89 XS98 XS88 16
08.4	Edible casings (e.g. sausage casings)	1,000	mg/kg	16
06.8.1	Soybean-based beverages	50	mg/kg	
12.9.1	Fermented soybean paste (e.g., miso)	30	mg/kg	