Specifications Monograph prepared by the meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), 92nd Meeting 2021

Riboflavin from *Ashbya gossypii*

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RIBOFLAVIN FROM *ASHBYA GOSSYPII*

New specifications prepared at the 92nd JECFA (2021) and published in FAO JECFA Monographs 27 (2021). A group ADI of not specified for riboflavin from Bacillus subtilis, riboflavin from Ashbya gossypii, synthetic riboflavin and riboflavin-5-phosphate was established at the 92nd JECFA (2021).

SYNONYMS Riboflavin from *Eremothecium gossypii*, vitamin B2; lactoflavin.

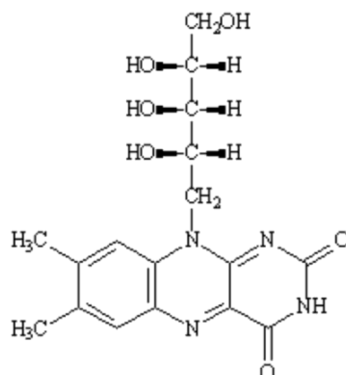
DEFINITION Prepared by fermentation with a non-pathogenic and non-toxicogenic self-cloned strain *of Ashbya gossypii* genetically modified for riboflavin overproduction. The fermentation is stopped by autolysis. Several filtration and precipitation/ crystallisation steps result in a dry product, containing not less than 98% of riboflavin, free of fermentation media components including the production organism.

Chemical names: Riboflavin; 3,10-dihydro-7,8-dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo-[g]pteridine-2,4-dione;
7,8-dimethyl-10-(1'- D-ribityl)isoalloxazine.

C.A.S. number: 83-88-5

Chemical formula: $C_{17}H_{20}N_4O_6$

Structural formula



Formula weight: 376.37

Assay: Not less than 98.0% and not more than 101.0%, calculated on the dried basis.

DESCRIPTION Yellow to orange-yellow crystalline powder.

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

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Solubility (Vol.4) — Very slightly soluble in water; practically insoluble or insoluble in ethanol and acetone.

Spectrophotometry (Vol. 4) — 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] 20, D: Between -115°C and -135°C
Dry the sample at 100°C for 4 h. Dissolve 50.0 mg in 0.05 N sodium hydroxide (free from carbonate) and dilute to 10ml with the same solvent. Measure the optical rotation within 30 min of dissolution.

Colour reaction — Dissolve about 1 mg of sample in 100 ml of water. The pale greenish-yellow colour disappears on the addition of mineral acids and alkalis.

PURITY

Loss on drying (Vol.4) — Not more than 1.5% (105°C, 4 h).

Sulfated ash (Vol.4) — Not more than 0.1%
Test 2 g of the sample.

Impurities — Lumiflavin: not more than 0.025%
See description under TESTS.

Lead (Vol.4) — Not more than 1mg/kg
Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under “General Methods, Metallic Impurities”).

TESTS

PURITY TESTS

Impurities — Impurities are determined by liquid chromatography (Vol. 4)

[Note: The following procedure may be used to determine: lumiflavin (impurity A), lumichrome (impurity B), 6,7-dimethyl-8-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3H,8H)-dione (impurity C), 8-hydroxymethyl riboflavin (impurity D), unspecified impurities and total organic impurities. However, the only requirement for this specification is the determination of lumiflavin.]

Standards and Reagents:

- Riboflavin for peak identification certified reference standard (CRS) (containing impurities C and D), Ph.Eur. (Cat. Code: Y0000757).
- Sodium acetate trihydrate (CAS # 6131-90-4)
- Orthophosphoric acid
- Sodium hydroxide solution (0.5 M and 0.1 M)
- Acetic acid, 30%w/v
- Acetonitrile, HPLC grade with transmittance more than 95% at 210 nm

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- Deionised water, HPLC grade

Preparation of solutions: (Note: Riboflavin is light sensitive. Prepare the solutions immediately before use and protect from light.)

Solution A: Weigh 13.6 g of sodium acetate trihydrate, dissolve in deionized water and make up to volume in a 1 litre volumetric flask.

Test solution: Accurately weigh 0.12 g of sample into a 100 ml volumetric flask, add 10 ml of 0.1 M sodium hydroxide solution, ultrasonicate to dissolve the sample and dilute to 100 ml with Solution A.

Reference solution A: Dilute 1.0 ml of the test solution to 10 ml with solution A. Dilute 1.0 ml of this solution to 100 ml with Solution A.

Reference solution B: Dissolve the contents of a vial of riboflavin peak identification certified reference standard in 1 ml of a mixture of 1 volume of Mobile phase B and 9 volumes of Mobile phase A.

Reference solution C (For impurities A and B): Dissolve 10 mg of sample in 1 ml of 0.5 M sodium hydroxide. Expose to daylight for 1.5 h. Add 0.5 ml of 30 % w/v acetic acid and dilute to 100 ml with deionised water.

Procedure:
Use a HPLC consisting of a high precision binary pump and an autosampler

Column: Octadecylsilyl, end-capped (25-cm x 4.6-mm x 5-µm).

Flow rate: 1.0 ml/min

Detector: UV/Diode array, 267 nm

Injection volume: 10 µl

Gradient conditions:

Mobile phase:

- A: Orthophosphoric acid in deionised water (1:1000 v/v)
- B: Acetonitrile

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0-5	90	10
5-20	90 → 80	10 → 20
20-25	80	20
25-35	80 → 50	20 → 50
35-45	50	50

Identification of impurities: Use the chromatogram supplied with riboflavin for peak identification CRS and the chromatogram obtained with Reference solution B to identify the retention times of impurities C and D.

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Inject the Test solution, Reference solution A, Reference solution B, and Reference solution C and calculate the relative retention times (RRT) with respect to riboflavin (retention time = about 16 min), approximately: Impurity C = 0.2; impurity D = 0.5; impurity A = 1.4; impurity B = 1.9.

System suitability:

Resolution: Minimum 5 between the peaks due to impurities A and B in the chromatogram obtained with Reference solution C.

The chromatogram obtained with Reference solution B is similar to the chromatogram supplied with riboflavin for peak identification CRS.

Correction factors for calculation of impurities:

Multiply the peak areas in the chromatogram of the Test solution corresponding to the following impurities by the appropriate correction factor: lumiflavin, impurity A = 0.7.

[Note: For information only - if calculation of other impurities is desired, the following correction factors may be used: impurity B = 1.4; impurity C = 2.3; impurity D = 1.4.]

Use corrected peak area to determine the content of lumiflavin in the sample (max 0.025%): The corrected peak area for lumiflavin in the chromatogram of the Test solution is less than 0.25 times the area of the principal peak in the chromatogram obtained with Reference solution A.

METHOD OF ASSAY

Carry out the assay in subdued light. Accurately weigh and suspend 65.0 mg of the sample in 5 ml of water, ensuring that it is completely wet, in an amber coloured 500 ml volumetric flask. Add 5 ml of 2 N sodium hydroxide solution and dissolve. As soon as dissolution is complete, add 100 ml of water and 2.5 ml of glacial acetic acid and dilute to 500ml with water. Place 20 ml of this solution in an amber coloured 200 ml volumetric flask, add 3.5 ml of a 1.4% w/v solution of sodium acetate and dilute to 200 ml with water. Measure the absorbance (A) of the solution at 444 nm.

$$\%Riboflavin = \frac{A \times 5000}{328 \times W}$$

A: absorbance of the sample solution at 444 nm
W: weight of sample, g
328: Specific absorbance of riboflavin at 444 nm

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