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Expert Committee on Food Additives (JECFA), 95 Meeting 2022

Spirulina extract

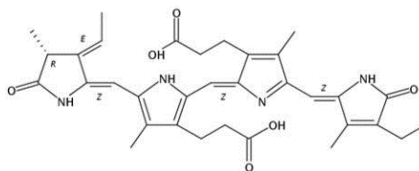
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SPIRULINA EXTRACT

Prepared at the 95th JECFA (2022) and published in FAO JECFA Monographs 30 (2022), superseding tentative specifications prepared at the 86th JECFA (2018) and published in FAO JECFA Monographs 22 (2018). An ADI “not specified” was established at the 95th JECFA (2022).

SYNONYMS	INS 134; Spirulina colour; Phycocyanins from <i>Arthrospira platensis</i> ; Phycocyanins from Spirulina
DEFINITION	<p>Spirulina extract is obtained by aqueous extraction of the biomass of <i>Arthrospira platensis</i>, an edible cyanobacterium. The organism is cultivated and harvested under conditions that prevent the growth of other cyanobacteria and the production of microcystins. The material extracted from the biomass is further treated by steps that may include pH adjustment, centrifugation, filtration, concentration, sterilization, drying, and dilution to the desired degree of pigment concentration. Commercial products are formulated in liquid and powder forms.</p> <p>The main colouring principles in spirulina extract are two phycobiliproteins, C-phycocyanin and allophycocyanin, which are water-soluble pigment-protein complexes in which the chromophore phycocyanobilin is covalently bonded to the protein. Spirulina extract also may contain peptides, other proteins, carbohydrates, and minerals. Extracts from the biomass may contain trace amounts of chlorophyll, beta-carotene, and other carotenoids which are largely removed during production of the colouring material.</p>
CAS numbers	20298-86-6 (Phycocyanobilin) 3-[(2Z,5E)-2-[[3-(2-Carboxyethyl)-5-[(Z)-[(3E,4R)-3-ethylidene-4-methyl-5-oxopyrrolidin-2-ylidene]methyl]-4-methyl-1H-pyrrol-2-yl]methylidene]-5-[(4-ethyl-3-methyl-5-oxopyrrol-2-yl)methylidene]-4-methylpyrrol-3-yl]propanoic acid 21H-Biline-8,12-dipropanoic acid, 18-ethyl-3-ethylidene-1,2,3,19,22,24-hexahydro-2,7,13,17-tetramethyl-1,19-dioxo-, (2R,3E)- 11016-15-2 (C-phycocyanin) 724423-45-4 (Spirulina extract)
Chemical formula	C ₃₃ H ₃₈ N ₄ O ₆ (Phycocyanobilin)

Structural formula



Phycocyanobilin

Formula weight 586.68 (Phycocyanobilin)

Assay Total phycocyanins as the sum of C-phycocyanin and allophycocyanin not less than declared.
See description under TESTS

DESCRIPTION Clear blue liquid or blue powder

FUNCTIONAL USES Colour

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4) Freely soluble in water, insoluble in ethanol.

Colour value Not less than declared.
See description under TESTS

Fluorescence (Vol. 4) A sample dissolved in citrate-phosphate (McIlvaine) buffer, pH 6.0 emits red fluorescence under white light. The fluorescence of the sample disappears when the solution is heated at 90 °C for 30 min.
See description under TESTS

Precipitation A blue precipitate is produced from the addition of ammonium sulfate to a sample dissolved in citrate-phosphate (McIlvaine) buffer, pH 6.0.
See description under TESTS

Spectrophotometry (Vol. 4) Maximum wavelengths approximately 620 nm (c-phycocyanin) and 650 nm (allophycocyanin) for the sample dissolved in citrate-phosphate (McIlvaine) buffer, pH 6.0.
See description under TESTS

PURITY

Loss on drying (Vol. 4) Not more than 7% for the powdered product (105 °C, 4 h)

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

<u>Cadmium</u> (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General Methods, Metallic Impurities").
<u>Lead</u> (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General Methods, Metallic Impurities").
<u>Mercury</u> (Vol. 4)	Not more than 1 mg/kg Determine using a method appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Vol. 4 (under "General Methods, Metallic Impurities").
<u>Microbiological criteria</u> (Vol. 4)	Total (aerobic) plate count: less than 1000 CFU/g Yeast and moulds: less than 100 CFU/g Coliforms: absent in 10 g <i>Salmonella spp.</i> : absent in 25 g <i>S. aureus</i> : absent in 10 g
<u>Microcystins</u>	Less than 0.5 µg/g as microcystin-LR (dried basis) See description under TESTS

TESTS

PURITY TESTS

Colour value The colour value is obtained from the absorbance of a solution of spirulina extract in citrate-phosphate buffer, pH 6.0 at 618 nm.

Reagents

- Citric acid monohydrate (C₆H₈O₇·H₂O), ACS grade
- Disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O), ACS grade
- Water, ultrapure

Preparation of solutions

- M Citric acid: Dissolve 2.28 g of citric acid monohydrate in approximately 25 ml of water, transfer to a 100 ml volumetric flask, and dilute to volume with water.
- 0.2 M Disodium hydrogen phosphate: Dissolve 7.12 g of disodium hydrogen phosphate dihydrate in approximately 50 ml of water, transfer to a 200 ml volumetric flask, and dilute to volume with water.
- Citrate-phosphate buffer, pH 6.0 (per 100 ml): Combine 76.7 ml of 0.1 M citric acid and 126.3 ml of 0.2 M disodium hydrogen phosphate. Adjust the pH to 6.00 using a few drops of 0.1 M citric

acid or 0.2 M disodium hydrogen phosphate.

Equipment

- Filter paper, qualitative, MN 615 grade
- pH meter
- Spectrophotometer with 1 cm cuvettes

Procedure

Dissolve 0.33 g of spirulina extract in 75 ml of water, transfer to a 100 ml volumetric flask, and dilute to volume with water. Filter and dilute 10 ml of the solution to 100 ml with citrate-phosphate buffer, pH 6.0. Determine the absorbance (A_{618}) of the solution at 618 nm by spectrophotometry using citrate-phosphate buffer, pH 6.0 as the reference. Further dilute the solution as needed for an approximate absorbance of 0.6.

Calculations

Calculate the colour value of the spirulina extract as follows:

$$\text{Colour value} = A_{618} \times \text{dilution factor} \times 100 / W_1$$

where

W_1 is the weight of the spirulina extract sample, in g

Fluorescence

Principle

Fluorescence is determined by dissolving a sample of spirulina extract in citrate-phosphate buffer, pH 6.0 and measuring the fluorescence of the sample solution. The solution is then heated at 90 °C for 30 min and its fluorescence is remeasured. Following heating, the fluorescence of the sample disappears and only the fluorescence of the buffer is observed.

Buffer solution

- Citrate-phosphate buffer, pH 6.0: Prepare as described in the colour value test.

Equipment

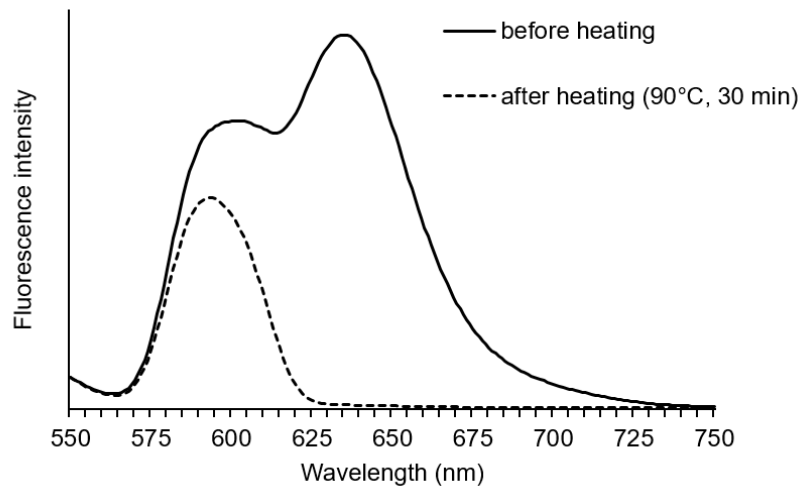
- Fluorescence spectrometer
- Filter paper, qualitative, MN 615 grade
- Heating bath
- Centrifuge or test tubes for heating bath, 12 ml
- pH meter

Procedure

Dissolve a liquid or powder sample equivalent to 0.4 g of spirulina extract with colour value 25 in 100 ml of citrate-phosphate buffer, pH 6.0. Filter and dilute 1 ml to 100 ml with the buffer solution.

Determine the fluorescence of the diluted sample solution using the buffer solution as the reference and an excitation wavelength of 600 nm. Record the fluorescence spectra of the sample solution from 550 nm to 750 nm.

Transfer 10 ml of the undiluted sample solution to a centrifuge or test tube. Heat the solution in the heating bath at 90 °C for 30 min. Filter and dilute 1 ml to 100 ml with the buffer solution. Determine the fluorescence of the diluted solution as described above. Compare the results to the spectra shown below.



Fluorescence spectra of spirulina extract dissolved in citrate-phosphate buffer, pH 6.0 before (—) and after (- -) heating at 90 °C for 30 min

Precipitation

Principle

Spirulina extract dissolved in citrate-phosphate buffer, pH 6.0 is precipitated with ammonium sulfate.

Reagent

– Ammonium sulfate ((NH₄)₂SO₄), ACS grade powder

Buffer solution

– Citrate-phosphate buffer, pH 6.0: Prepare as described in the colour value test.

Equipment

Centrifuge, capable of 10,000 rpm

Centrifuge tubes, 12 ml

Procedure

Dissolve a liquid or powder sample equivalent to 0.4 g of spirulina extract with colour value 25 in 100 ml of citrate-phosphate buffer, pH 6.0. Filter a 10 ml portion of the solution and transfer 5 ml into a 12 ml centrifuge tube. Add 3.3 g of ammonium sulfate in small portions, shaking well between additions. Allow the mixture to stand for 30 min to equilibrate. Centrifuge at 10,000 rpm for 4 min. A blue precipitate is formed.

Microcystins

Principle

Determination of total microcystins is achieved following oxidative cleavage of the unique ADDA side chain ((all-S, all-E)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) found in all microcystins to produce, following clean-up using solid phase extraction (SPE), the analyte 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). MMPB is quantified in the cleaned extract using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the isotopic dilution technique. The variant microcystin-LR (L = leucine, R = arginine) is used as the standard for all variants and matrix-matched calibration curves are constructed using a microcystin-free spirulina substrate.

Reagents:

- Acetic acid, glacial, ACS grade
- Acetonitrile, LC-MS grade
- Methanol, LC-MS grade
- Nitrogen gas, high purity
- Potassium carbonate (K₂CO₃), ACS grade
- Potassium permanganate (KMnO₄), ACS grade
- Sodium bisulfite (NaHSO₃), ACS grade
- Sodium periodate (NaIO₃), ACS grade
- Water, LC-MS grade

Standards:

- (*isotopic standard*) 2-Methyl-3-methoxy-4-phenylbutyric acid sodium salt standard (d₃-MMPB), CAS No. 210890-21-4; m. wt. 208.25, Wako Chemicals, Cat. No. 133-12871 or equivalent
- (*representative standard*) Microcystin-LR (MC-LR), CAS No. 101043-37-2, m. wt. 995.1816, 0.5 ml ampoule containing approximately 10 µmol/kg (9.95 µg/g) of MC-LR dissolved in 50% acetonitrile/water (v/v), certified reference material from National Research Council of Canada, Halifax, cat. no. CRM-MCLR or equivalent

Matrix

Use a sample of spirulina extract powder that has been tested and confirmed to be free of MC as the matrix.

Preparation of solutions

Oxidant solutions:

- 1 M K₂CO₃: Dissolve 13.82 g of K₂CO₃ in 100 ml of water.
- 0.25 M KMnO₄: Dissolve 3.95 g of KMnO₄ in 100 ml of water.
- 0.25 M NaIO₃: Dissolve 53.47 g NaIO₃ in 100 ml of water.
- 40% NaHSO₃ (w/v): Dissolve 40 g of NaHSO₃ in 100 ml of water; make fresh daily.

SPE clean-up solutions:

- 90% Acetonitrile/water (v/v): Dilute 90 ml of acetonitrile to 100 ml with water.
- 5% Methanol/water (v/v): Dilute 5 ml of methanol to 100 ml with water.

Standard solutions for LC-MS/MS:

- d3-MMPB spiking solution: Dissolve 10.0 mg in 0.5 ml of water and dilute to 1.0 ml with water. Serially dilute with water to make a spiking solution of 100 ng/ml.
- MC-LR spiking solutions: Serially dilute the MC-LR certified reference material (approximately 10 µg/ml) with water to make spiking solutions of 1000, 100, and 10 ng/ml.

Eluent solutions for LC-MS/MS:

- 0.05% acetic acid in 5% methanol/water (Mobile phase A): Dilute 0.5 ml of acetic acid and 50 ml of methanol to 1000 ml with water.
- 0.05% acetic acid in 95% methanol/water (Mobile phase B): Dilute 0.5 ml of acetic acid and 50 ml of water to 1000 ml with methanol.

Equipment:

- Autopipettes, 1 µl to 5 ml
- Glass culture tubes, 13 mm x 150 mm, for sample oxidations
- Glass centrifuge vials, 15 ml, for SPE elution capture
- Sonication bath
- SPE cartridges: Strata-X 200 mg (6 cc) (Phenomenex, Torrance, CA, USA) or equivalent
- SPE eluate evaporator: TurboVap-LV (Biotage, Charlotte, NC, USA) or equivalent
- SPE vacuum manifold and pump
- Syringe filters, 0.22 µm polyvinylidene fluoride (PVDF) for HPLC solutions
- Syringes, 3 ml polypropylene for HPLC solutions
- Vials, 2 ml with silicone/PTFE septa for HPLC solutions
- Vortexer

LC-MS/MS system:

- Thermo Surveyor high-performance liquid chromatography (HPLC) system coupled to a TSQ Quantum Access MAX triple quadrupole mass spectrometer (MS) system equipped with a Heated Electrospray Ionization (HESI-II) Probe and Surveyor MS Pump Plus and XCalibur software (Thermo Scientific, Waltham, MA, USA) or equivalent

Autosampler and LC system parameters:

- Full loop injections
- Mobile phase A: 0.05% acetic acid in 5% methanol/water
- Mobile phase B: 0.05% acetic acid in 95% methanol/water
- Column: Waters Symmetry C18 (3.5 µm, 2.1 mm x 150 mm) (Waters Corporation, Milford, MA, USA) or equivalent
- Injection volume: 20 µl (full loop)
- Flow rate: 0.2 ml/min
- Gradient: 25% A over 2 min, 25 – 0% A over 4 min, 0 – 25% A over 2 min, and hold at 25% A for 3 min

MS system parameters

Note: All settings should be optimized for each precursor and product ion.

- Negative ionization mode
- Fragment [M-H]⁻ of MMPB (m/z 207 → 131)
- Fragment [M-H]⁻ of d3-MMPB (m/z 210 → 131)
- Capillary temperature: 275 °C
- Vaporizer temperature: 300 °C
- Spray voltage: 3.5 kV
- Collision energies: 12eV (MMPB) and 15eV (d3-MMPB)
- Isolation width: 1.0 Da
- Sheath and auxiliary gas flow: 45 and 15 gas flow units, respectively

Procedure

1. Spirulina extract samples:

Weigh a 25-mg portion of each sample (dried basis) and transfer to a glass culture tube. Add 10 µl of the 100 ng/ml d3-MMPB spiking solution.

2. Matrix spike and blank samples:

Weigh four 25-mg portions of the matrix and transfer to separate glass culture tubes. Add 10 µL of the 100 ng/ml d3-MMPB spiking solution to each tube. Fortify three of the samples with the MC-LR spiking solutions. For example, add 25 µl of the 1000 ng/ml spiking solution (1.0 µg/g MC-LR), 125 µl of the 100 ng/ml spiking solution (0.5 µg/g MC-LR, the specified level), and 250 µl of the 10 ng/ml spiking solution (0.1 µg/g MC-LR), respectively. Use the fourth sample as the blank.

3. Calibration samples:

Weigh five 25-mg portions of the matrix and transfer to separate glass culture tubes. Add 10 µl of the 100 ng/ml d3-MMPB spiking solution to each tube. Add different aliquots of the 100 ng/ml MC-LR spiking solution ranging from 10 to 500 µL (1 ng to 50 ng) to the five tubes.

4. Oxidations:

Note: Perform oxidations in a fume hood and use safety glasses and gloves.

Add 0.5 ml of 1 M K₂CO₃ to each sample, followed by 1 ml of 0.25 KMnO₄ and 1 ml of 0.25 M NaIO₄. Vortex until the mixture is homogenous, then allow to react at room temperature for 1 h. Stop the reaction with drop-wise addition of 40% (w/v) NaHSO₃ (approx. 500 µl total). This step releases hydrogen sulfide (H₂S) gas and the sample can bubble out of the reaction tube if the reducing agent is added too quickly, so conduct this step slowly. Use disposable wood stirring sticks or slow vortex mixing to ensure the oxidant is fully reduced and the slurry is mixed. The mixture will turn from purple/brown to white/opaque in colour when the reaction is fully stopped. When the solution is finished bubbling, remove from the fume hood for solid phase extraction.

5. Clean-up with solid phase extraction (SPE):

Condition SPE cartridges with 5 ml of methanol followed by equilibration with 5 ml of water. Dilute 3 ml of each sample solution to 6 ml with water and load onto a cartridge. Rinse the cartridge with 3 ml of water followed

by 2 ml of water. Elute with 5 ml of 90% acetonitrile/water and collect the eluate in a 15 ml glass centrifuge vial. Evaporate the eluate to dryness under a stream of N₂ at 60 °C. Redissolve in 1 ml of 5% methanol/water (v/v) using vortex mixing and a sonication bath (2-5 min). Filter the extracted solutions with 0.2 µm PVDF syringe filters and place in HPLC vials. Analyze by LC-MS/MS. Store at 4 °C before analysis.

6. Calculations:

Calculate the concentration of total microcystins (µg/g) as MC-LR using the internal standard method and the matrix-matched calibration curve.

Divide the peak area of MMPB by the corresponding peak area of d3-MMPB measured in each calibration solution and the blank. Construct the calibration curve using linear regression by plotting the normalized MMPB values on the y-axis against the concentrations of the MC-LR standard (µg/g) on the x-axis. Evaluate the results for the matrix spikes using the calibration curve to make sure the results are consistent with the spike values. Determine the results for the samples from the calibration curve.

Microcystins (alternative test)

Principle

Microcystins are determined by enzyme linked immunoassay (ELISA).

Reagents

- Methanol, ACS grade
- Water, ACS grade, negative for microcystins

Preparation of solution

- 75% Methanol/water (v/v): Dilute 75 ml of methanol to 100 ml with water.

Equipment

- Commercially available ELISA kit with cross reactivity for microcystin-LR and other microcystins
- Centrifuge, capable of 4500 rpm
- Centrifuge tubes, 12 ml

Sample preparation

In the absence of other instructions provided by the ELISA kit manufacturer, prepare samples as follows.

Weigh a 3.0 g portion of spirulina extract (dried basis) into a centrifuge tube. Homogenize the material in 20.0 ml of 75% methanol/water for 20 min. Centrifuge the resulting suspension at 4500 rpm for 10 min. Transfer the supernatant to a 50 ml glass Erlenmeyer flask. Add 10.0 ml of 75% methanol/water to the centrifuge tube and homogenize the residue for 30 sec. Centrifuge the resulting suspension at 4500 rpm for 10 min. Add the supernatant to the 50 ml flask and dilute with water to a concentration within the range indicated by the ELISA kit manufacturer.

Procedure

Follow the instructions provided by the ELISA kit manufacturer.

METHOD OF ASSAY

Principle

Total phycocyanins are determined by spectrophotometry as the sum of C-phycocyanin and allophycocyanin using citrate-phosphate buffer, pH 6.0.

Buffer solution

Citrate-phosphate buffer, pH 6.0: Prepare as described in the colour value test.

Equipment

Centrifuge, capable of 3500 rpm

Centrifuge tubes, 12 ml

Heating incubator for 25 ml volumetric flasks

pH meter

Sonicator

Spectrophotometer with 1 cm cuvettes

Procedure

Weigh a 100-mg portion of spirulina extract (dried basis) and transfer into a 25 ml volumetric flask. Dilute to volume with citrate-phosphate buffer, pH 6.0. Sonicate the mixture for 30 min, maintaining the temperature at 8 °C. Incubate at 30 °C for 8 h, shaking manually every hour. Transfer a 10 ml portion to a centrifugation tube and centrifuge at 3500 rpm for 4 min.

Determine the absorbance of the supernatant by spectrophotometry at 620 nm (A_{620}) and 650 nm (A_{650}). Use citrate-phosphate buffer, pH 6.0 as the reference. Dilute the supernatant with additional buffer, if needed, to obtain absorbance values of 0.2 to 0.6 at 620 nm. Record the dilution factor F. A typical absorbance spectrum is shown below.

Calculations

Calculate the C-phycocyanin content (% w/w) as follows:

$$TcPC = [(0.162 \times A_{620}) - (0.098 \times A_{650}) \times V_1 \times 100] / W_1$$

where

W_1 is the weight of spirulina extract, in mg

V is the flask volume, ml

F is the dilution factor

V_1 is the total dilution volume used to prepare the sample solution, equal to $V \times F$, in ml

Calculate the allophycocyanin content (% w/w) as follows:

$$TaPC = [(0.180 \times A_{650}) - (0.042 \times A_{620}) \times V_1 \times 100] / W_1$$

where

W_1 is the weight of spirulina extract taken, in mg

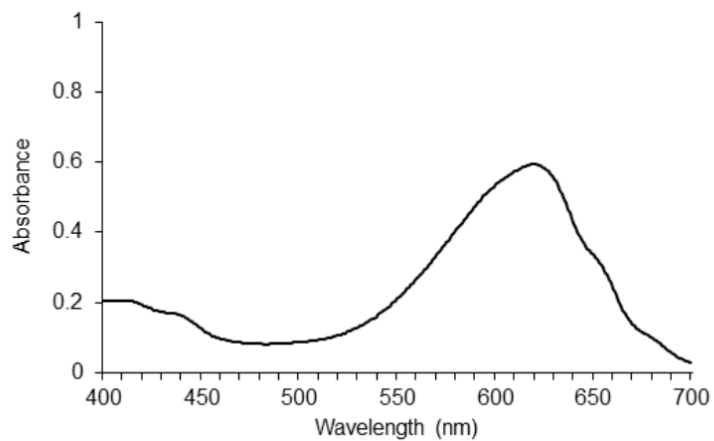
V is the flask volume, ml

F is the dilution factor

V_1 is the total dilution volume used to prepare the sample solution, equal to $V \times F$, in ml

Calculate the total phycocyanin content (% w/w) as follows:

$$TPC = TcPC + TaPC$$



Absorbance spectrum of spirulina extract dissolved in citrate-phosphate buffer, pH 6.0