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# Method of Test for Mycotoxins in Foods-Test of Citrinin

## 1. Scope

This method is applicable to the determination of citrinin in red yeast rice, complex food, food supplements containing red yeast material, and Monascus color.

## 2. Method

After extraction and purification, citrinin is determined by high performance liquid chromatography (HPLC).

**2.1.** Equipment

**2.1.1.** High performance liquid chromatograph.

**2.1.1.1.** Detector: fluorescence detector.

**2.1.1.2.** Column: Atlantis T3, 5 μm, 4.6 mm i.d. x 25 cm, or an equivalent product.

**2.1.2.** Spectrophotometer.

**2.1.3.** Grinder.

**2.1.4.** Blender.

**2.1.5.** Vortex mixer.

**2.1.6.** Ultrasonicator.

**2.1.7.** Centrifuge: centrifugal force > 2000 ×g.

**2.1.8.** pH meter.

**2.2.** Chemicals

Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Formic Acid, reagent grade;
Hydrochloric acid, reagent grade;
Sodium chloride, reagent grade;
Disodium hydrogen phosphate, reagent grade;
Potassium dihydrogen phosphate, reagent grade;
Potassium chloride, reagent grade;
Phosphoric acid (85%), reagent grade;
Ethanol (95%), reagent grade;
Deionized water, resistivity ≥ 18 MΩ • cm (at 25°C);
Citrinin, reference standard.

**2.3.** Apparatus

**2.3.1.** Volumetric flask: 1 mL, 10 mL and 100 mL.

**2.3.2.** Centrifuge tube: 50 mL, PP.

**2.3.3.** Membrane filter: 0.22 μm, Nylon.

**2.3.4.** Filter paper: Whatman No. 1, diameter 11 cm, or an equivalent product.

**2.3.5.** Glass microfiber filter: diameter 9 cm.

**2.3.6.** Immunoaffinity column: a VICAM column containing the monoclonal antibody specific for citrinin, or an equivalent product.

**2.4.** Reagents

**2.4.1.** 2 N hydrochloric acid

Add 16.7 mL of hydrochloric acid slowly into 80 mL of deionized water, and dilute with deionized water to 100 mL.

**2.4.2.** Phosphate buffer

Dissolve 8 g of sodium chloride, 1.2 g of disodium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate and 0.2 g of potassium chloride with 990 mL of deionized water, adjust pH to 7.4 with 2 N hydrochloric acid, and dilute with deionized water to 1000 mL.

**2.4.3.** 0.1% phosphoric acid

Dilute 1.2 mL of phosphoric acid with deionized water to 1000 mL.

**2.4.4.** Methanol: 0.1% phosphoric acid (7:3, v/v)

Mix methanol and 0.1% phosphoric acid at the ratio of 7:3 (v/v).

**2.4.5.** Ethanol: deionized water (1:1, v/v)

Mix ethanol and deionized water at the ratio of 1:1 (v/v).

**2.5.** Mobile phase

Mix 500 mL of acetonitrile, 500 mL of deionized water and 1 mL of formic acid, and filter with a membrane filter.

**2.6.** Standard solution preparation

Transfer about 5 mg of citrinin reference standard accurately weighed to a 10-mL volumetric flask. Dissolve and dilute to volume with methanol as the standard stock solution. Store at -20°C. When to use, dilute appropriate volume of the standard stock solution with methanol to 0.625-6.25 ng/mL as the standard solutions.

**2.7.** Sample solution preparation

Transfer about 1 g of the homogenized sample accurately weighed into a 50-mL centrifuge tube. Add 20 mL of methanol, and ultrasonicate in a water bath

at 70°C for 30 min. After cooling to room temperature, centrifuge at 2000 ×g for 3 min, collect the supernatant, and filter with a filter paper. Accurately take 1 mL of the filtrate, add 39 mL of phosphate buffer, mix thoroughly, and filter with a glass microfiber filter. Accurately transfer 10 mL of the filtrate into the immunoaffinity column (flow rate controlled 1 drop/second). After the filtrate has completely passed through the column, wash the immunoaffinity column twice with 10 mL of deionized water (flow rate controlled 1 drop/second), and discard the eluent. Add 1 mL of methanol: 0.1% phosphoric acid (7:3, v/v) (flow rate controlled 1 drop/second), collect the eluent, and dilute with deionized water to 1 mL. Filter with a Nylon membrane filter, and take the filtrate as the sample solution.

**2.8.** Determination the color value of Monascus color sample

Transfer about 1 g of the homogenized Monascus color sample accurately weighed into a 100-mL volumetric flask, dissolve and dilute to volume with ethanol: deionized water (1:1, v/v), and filtered with a filter paper. Take the filtrate as the test solution, and measure the absorbance at the maximum absorption wavelength in the range of 480-520 nm with a cuvette with an optical path length of 1 cm. The test solution should be adjusted so that its absorbance could fall within the range of 0.2-0.7 or 0.4-1.4 when a single-beam absorption photometer or a double-beam absorption photometer is used, respectively. If the absorbance of the test solution exceeds the range described above, dilute the test solution with ethanol: deionized water (1:1, v/v), and measure the absorbance again. Calculate the color value of the Monascus color sample by the following formula:

$$\text{Color value } (E_{1cm}^{10\%}) = \frac{10 \times A \times F}{M}$$

A: the absorbance of the test solution

F: dilution factor

M: the weight of the sample (g)

**2.9.** Identification and quantification

Accurately inject 20 μL of the sample solution and the standard solutions into HPLC separately, and operate according to the following conditions. Identify citrinin based on the retention time. Calculate the amount of citrinin in the sample by the following formula (μg/kg):

**2.9.1.** Red yeast rice, complex food and food supplements containing red yeast material

$$\text{The amount of citrinin in the sample (μg/kg)} = \frac{C \times V \times F}{M}$$

Where,

C: the concentration of citrinin in the sample solution calculated by the standard curve (ng/mL)

V: the final make-up volume of the sample (mL)

F: dilution factor (80)

M: the weight of the sample (g)

**2.9.2.** Monascus color

$$\text{The amount of citrinin in the sample}^{(note)}\text{ (μg/kg)} = \frac{C \times V \times F \times E}{M \times 50}$$

Where,

C: the concentration of citrinin in the sample solution calculated by the standard curve (ng/mL)

V: the final make-up volume of the sample (mL)

F: dilution factor (80)

M: the weight of the sample (g)

E: the color value (obtained from section 2.8)

Note: the amount of citrinin in the Monascus color sample is calculated as the color value of 50.

HPLC operating conditions[(note)]:

Fluorescence detector: excitation wavelength, 330 nm; emission wavelength, 500 nm.

Column: Atlantis T3, 5 μm, 4.6 mm i.d. x 25 cm.

Injection volume: 20 μL.

Mobile phase: prepared as section 2.5.

Flow rate: 1.0 mL/min.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

**Remark**

1. Limit of quantification (LOQ) for citrinin is 50 μg/kg.

2. When the detected concentration of the sample solution exceeds the range of the standard curve, appropriately dilute the filtrate after centrifugation with phosphate buffer before performing the subsequent purification step of the immunoaffinity column.
3. Further validation should be performed when interfering compounds appear in samples.
4. As confirm by LC-MS/MS, the multiple reaction monitoring (MRM) parameters(note) are shown as follows:

| Analyte | Ionization mode | Ion pair<br>Precursor ion (*m/z*) > product ion (*m/z*) | Declustering potential (V) | Collision energy (eV) |
|---|---|---|---|---|
| Citrinin | $ESI^+$ | 251 > 233* | 24 | 20 |
| | | 251 > 205 | 38 | 30 |

*The quantitative ion.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

**Reference**

1. Ministry of Health, Labour and Welfare, Japan. 2018. General test-color value determination. Specifications and Standards for Food Additives. 9th ed. pp.27-28. Tokyo, Japan.
2. Wu, S. H., Chiu, J. Y., Yu, M. C., Lwo, C. H., Chang, T. P., Chen, J. H. and Shih, W. C. 2019. Development and validation of analytical methods for the determination of mycotoxins and PAHs in foods. Commissioned Research Report of Taiwan Food and Drug Administration.