# GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

# GB 5009.189-2023

# National Food Safety Standard - Determination of Bongkrekic Acid in Foods

食品安全国家标准　食品中米酵菌酸的测定

**Issued on: September 6, 2023** **Implemented on: March 6, 2024**

**Issued by: National Health Commission of the People's Republic of China;**
**State Administration for Market Regulation.**

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# National Food Safety Standard - Determination of Bongkrekic Acid in Foods

## 1 Scope

This Standard specifies the methods for the determination of bongkrekic acid in foods.

In this Standard, Method 1 is applicable to the determination of bongkrekic acid in tremella fungus and its products, fermented rice noodles and its products.

In this Standard, Method 2 is applicable to the determination of bongkrekic acid in tremella fungus and its products, fungus and its products, cereals and its products.

# Method I - Liquid Chromatography

## 2 Principle

The bongkrekic acid in the specimen is extracted by solvent, purified and concentrated by a mixed strong anion exchange column or liquid-liquid extraction method. Adopt a high performance liquid chromatograph for analysis and the external standard method for quantitative determination.

## 3 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are all analytically pure, and the water is Grade-1 water specified in GB/T 6682.

### 3.1 Reagents

**3.1.1** Methanol ($CH_3OH$): chromatographically pure.

**3.1.2** Methanol ($CH_3OH$).

**3.1.3** Ammonia water ($NH_3 \bullet H_2O$): 25% ~ 28%.

**3.1.4** Formic acid ($CH_2O_2$).

**3.1.5** Hydrochloric acid (HCl).

**3.1.6** Phosphoric acid ($H_3PO_4$).

**3.1.7** Sodium bicarbonate ($NaHCO_3$).

**3.1.8** Petroleum ether ($C_5H_{12}O_2$): with a boiling range of 30 °C ~ 60 °C.

**3.1.9** Anhydrous ether ($C_4H_{10}O$).

**3.1.10** Chloroform ($CHCl_3$).

### 3.2 Preparation of Reagents

**3.2.1** Phosphoric acid solution (45.4%): measure-take 45.4 mL of phosphoric acid, place it in a 100 mL volumetric flask, use water to dilute to a constant volume to the scale, and shake it well.

**3.2.2** Sodium bicarbonate solution (40 g/L): weigh-take 40 g of sodium bicarbonate, add water to dissolve it, transfer to a 1,000 mL volumetric flask, reach a constant volume to the scale, and shake it well.

**3.2.3** Hydrochloric acid solution (6 mol/L): measure-take 50 mL of hydrochloric acid, place it in a 100 mL volumetric flask, add water to dilute to a constant volume to the scale, and shake it well.

**3.2.4** Ammonia water-methanol solution: measure-take 80 mL of methanol, add 1.0 mL of ammonia water, add water to reach a constant volume of 100 mL, and shake it well.

**3.2.5** Formic acid-methanol solution (2%): draw-take 2.0 mL of formic acid, add methanol to 100 mL, and shake it well.

**3.2.6** Formic acid aqueous solution (pH 2.5): use formic acid to adjust the pH of water to 2.5 ± 0.1, and shake it well.

### 3.3 Reference Material

Bongkrekic acid ($C_{28}H_{38}O_7$, CAS: 11076-19-0): purity ≥ 95%, or a standard substance certified by the state and awarded a reference material certificate.

**NOTE:** the reference material may use commercial standard solutions that satisfy the traceability requirements.

### 3.4 Preparation of Standard Solutions

**3.4.1** Bongkrekic acid standard stock solution (0.1 mg/mL): accurately weigh-take 10.0 mg (accurate to 0.01 mg) of bongkrekic acid reference material, use methanol to dissolve it, transfer to a 100 mL volumetric flask, and use methanol to reach a constant volume to the scale. Store it in a –20 °C refrigerator away from light. It shall remain valid for 6 months.

**3.4.2** Bongkrekic acid standard series of working solutions: respectively and accurately draw-take bongkrekic acid standard stock solution and use methanol to dilute it to a constant volume. Thus, standard working solutions respectively with a mass concentration of bongkrekic acid of

Use a medium-speed filter paper to filter it, transfer the filtrate to the same 100 mL volumetric flask, and use ammonia water-methanol solution to reach a constant volume to the scale, and evenly mix it. Accurately measure-take 50.0 mL of the extracting solution, place it in 80 °C water bath and concentrate to about 3 mL by rotary evaporation, and reserve it for purification.

#### 5.2.2 Specimen purification

Transfer all the concentrated specimen to the activated solid phase extraction column, successively use 5 mL of water and 5 mL of methanol to rinse it and discard the effluent. Then, use 6 mL of formic acid-methanol solution (2%) to elute it, collect the eluent; in 40 °C water bath, use nitrogen to blow it to dryness. Accurately add 0.5 mL of methanol, vortex to dissolve it, evenly mix it, filter it through a 0.45 μm microporous organic filter membrane, and reserve it for later use.

### 5.3 Liquid-liquid Extraction Method

#### 5.3.1 Specimen extraction

Weigh-take 20 g (accurate to 0.01 g) of specimen and place it in a conical flask. Add about 20 mL of methanol and soak it at room temperature in the dark for 1 hour. Then, add about 70 mL of chloroform and 0.2 mL of phosphoric acid solution (45.4%), oscillate for 30 min, filter it, transfer the filtrate to a 100 mL volumetric flask, and use chloroform to reach a constant volume to the scale, evenly mix it. Accurately measure-take 50.0 mL of the filtrate and reserve it for extraction.

#### 5.3.2 Specimen extraction

Transfer the above-mentioned filtrate into a 150 mL separatory funnel, add sodium bicarbonate solution (40 g/L) that has an equivalent volume with the filtrate, shake it for 2 minutes, and let it stand for stratification. Then, take out the lower layer and place it in another separatory funnel. Use 10 mL of sodium bicarbonate solution (40 g/L) to repeat the extraction twice, and gently shake it. Combine the sodium bicarbonate solutions extracted in three times, add 25 mL of chloroform, shake for 2 minutes and let it stand for stratification. Then, discard the chloroform and slowly add hydrochloric acid solution (6 mol/L) into the separatory funnel, adjust the pH of the solution to 2 ~ 3, add 50 mL of petroleum ether, shake it for 3 minutes, let it stand for stratification, and take out the petroleum ether layer and place it in a rotary evaporator. Then, respectively use 30 mL and 20 mL of petroleum ether to perform the extraction once and combine the petroleum ether layer into the same bottle. In 40 °C water bath, evaporate and concentrate it to dryness; use a small amount of methanol to dissolve the extract in the rotary evaporation bottle in several times and transfer it to a 5.00 mL glass centrifuge tube or concentration bottle. At 40 °C, blow nitrogen to concentrate it to dryness; accurately add 0.5 mL of methanol, vortex to dissolve it, evenly mix it, filter it through a 0.45 μm microporous organic filter membrane and reserve it for later use.

### 5.4 Reference Conditions of Instrument

**5.4.1** Chromatographic column: $C_{18}$ chromatographic column (column length: 250 mm, inner

$V_2$---the volume of the specimen solution taken for purification, expressed in (mL).

The calculation results shall retain 3 significant figures.

## 7 Precision

The absolute difference between the results of two independent determinations obtained under repeatability conditions shall not exceed 10% of the arithmetic mean.

## 8 Others

The detection limit of this Method is 5 μg/kg, and the quantitation limit is 15 μg/kg.

# Method II - Liquid Chromatography - Mass Spectrometry / Mass Spectrometry

## 9 Principle

The bongkrekic acid in the specimen is extracted with ammoniated methanol and purified by a mixed strong anion exchange column. Adopt liquid chromatograph - tandem mass spectrometer for detection, and the external standard method for quantitative determination.

## 10 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are all analytically pure, and the water is Grade-1 water specified in GB/T 6682.

### 10.1 Reagents

**10.1.1** Acetonitrile ($CH_3CN$): chromatographically pure.

**10.1.2** Formic acid ($CH_2O_2$): chromatographically pure.

**10.1.3** Methanol ($CH_3OH$).

**10.1.4** Ammonia water ($NH_3 \bullet H_2O$): 25% ~ 28%.

**10.1.5** Ammonium sulfate ($H_8N_2O_4S$).

### 10.2 Preparation of Reagents

**10.2.1** Ammonia water-methanol solution: measure-take 80 mL of methanol, add 1.0 mL of

ammonia water, add water to reach a constant volume of 100 mL, and evenly mix it.

**10.2.2** Formic acid-methanol solution (2%): draw-take 2.0 mL of formic acid, add methanol to 100 mL and evenly mix it.

**10.2.3** 0.1% formic acid aqueous solution: draw-take 1.0 mL of formic acid, use water to dilute to 1,000 mL and evenly mix it.

**10.2.4** Acetonitrile-water solution (1 : 1): take 50 mL of acetonitrile, use water to dilute to 100 mL and evenly mix it.

### 10.3 Reference Material of Bongkrekic Acid

Bongkrekic acid ($C_{28}H_{38}O_7$, CAS: 11076-19-0): purity ≥ 95%, or a standard substance certified by the state and awarded a reference material certificate.

**NOTE:** the reference material may use commercial standard solutions that satisfy the traceability requirements.

### 10.4 Preparation of Standard Solutions

**10.4.1** Bongkrekic acid standard stock solution (0.1 mg/mL): accurately weigh-take 10.0 mg (accurate to 0.01 mg) of bongkrekic acid reference material, use methanol to dissolve it, transfer to a 100 mL volumetric flask and use methanol to reach a constant volume to the scale. Store it in a –20 °C refrigerator away from light. It shall remain valid for 6 months.

**10.4.2** Bongkrekic acid standard intermediate solution (1 μg/mL): accurately draw-take 100 μL of bongkrekic acid standard stock solution, use methanol to dilute it and reach a constant volume of 10.00 mL. Prepare it right before use.

**10.4.3** Bongkrekic acid standard series of working solutions: respectively and accurately draw-take bongkrekic acid standard intermediate solution, use acetonitrile-water solution (1 : 1) to dilute it and reach a constant volume. Thus, standard working solutions respectively with a mass concentration of bongkrekic acid of 1.0 ng/mL, 2.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 25.0 ng/mL and 50.0 ng/mL are prepared. Prepare them right before use.

### 10.5 Material

Solid phase extraction column: mixed strong anion exchange column (60 mg/3 mL) or equivalent solid phase extraction column. Before use, successively use 5.0 mL of methanol and 5.0 mL of water to activate it and maintain the column moist.

## 11 Instruments and Equipment

**11.1** Liquid chromatograph - tandem mass spectrometer: equipped with electrospray ion source.

**11.2** Balance: with a division value of 0.01 mg and 0.01 g respectively.

**11.3** Solid phase extraction device (equipped with a vacuum pump).

**11.4** Nitrogen blower.

**11.5** Pulverizer (with $\phi$0.425 mm sieve).

**11.6** Ultrasonic oscillator: 30 kHz ~ 50 kHz.

# 12 Analytical Procedures

## 12.1 Specimen Preparation

For cereals and its products, no less than 1 kg; for tremella fungus and its products, fungus and its products, no less than 600 g; dry specimens shall be pulverized and passed through a $\phi$0.425 mm sieve. Fresh (wet) specimens shall be cut or minced, homogenized, and evenly mixed; each sample for testing shall be no less than 200 g; store in a sample bottle or plastic bag, seal and keep it refrigerated at 2 °C ~ 8 °C.

## 12.2 Specimen Extraction

### 12.2.1 Cereals and its products, tremella fungus and its products, and dried fungus

Weigh-take 2.5 g of specimen (accurate to 0.01 g) in a 50 mL centrifuge tube, add 10 mL of methanol-ammonia water solution (for dried fungus, add 20 mL of methanol-ammonia water solution), thoroughly mix it, and soak it at room temperature in the dark for 1 h. Then, perform ultrasonic extraction for 30 minutes, centrifuge for 5 minutes, take the supernatant and place it in a 25.0 mL graduated centrifuge tube (for dried fungus specimens, place in a 50.0 mL graduated centrifuge tube). Add 10 mL of ammonia water-methanol solution (for dried fungus specimens, add 20 mL of ammonia water-methanol solution) to the residue to repeat the extraction once, combine the extracting solutions, and use methanol-ammonia water solution to reach a constant volume of 25.0 mL (for dried fungus specimens, reach a constant volume of 50.0 mL). Accurately transfer-take 5.00 mL of the supernatant (for dried fungus specimens, accurately transfer-take 10.0 mL of the supernatant) and reserve it for purification.

### 12.2.2 Fresh wet fungus

Weigh-take 2.5 g (accurate to 0.01 g) of specimen into a 50 mL centrifuge tube, add 10 mL of methanol-ammonia water solution, thoroughly mix it, and soak it at room temperature in the dark for 1 h. Then, add 3 g of ammonium sulfate, perform ultrasonic extraction for 30 minutes, and centrifuge for 5 minutes. Take the supernatant into a 25.0 mL graduated centrifuge tube. Add 10 mL of methanol-ammonia water solution to the residue to repeat the extraction once, combine the extracting solutions, and use methanol-ammonia water solution to reach a constant volume of 25.0 mL. Accurately transfer-take 5.00 mL of the supernatant and reserve it for purification.

## 12.3 Specimen Purification

standard solution is shown in Figure B.1 in Appendix B.

**12.6.2 Determination of specimen solution**

In accordance with the instrument reference conditions, determine the specimen solution to obtain the corresponding mass chromatographic peak area of the specimen solution. In accordance with the working curve, obtain the mass concentration of bongkrekic acid in the specimen solution.

# 13 Result Calculation and Expression

The content of bongkrekic acid in the specimen is calculated in accordance with Formula (2).

$$X = \frac{\rho \times V_1 \times V_3 \times 1\ 000}{m \times V_2 \times 1\ 000} \qquad \cdots\cdots(2)$$

$X$---the content of bongkrekic acid in the specimen, expressed in (μg/kg);

$\rho$---the mass concentration of bongkrekic acid in the specimen solution obtained through the working curve, expressed in (ng/mL);

$V_1$---the volume of the extracting solution of the specimen solution, expressed in (mL);

$V_3$---the final constant volume of the sample after purification and elution, expressed in (mL);

$m$---the sampling size of the specimen, expressed in (g);

$V_2$---the volume of the specimen solution taken for purification, expressed in (mL);

1,000---the unit conversion factor.

The calculation results shall retain 3 significant figures.

# 14 Precision

The absolute difference between the results of two independent determinations obtained under repeatability conditions shall not exceed 15% of the arithmetic mean.

# 15 Others

The detection limit of this Method is 1 μg/kg, and the quantitation limit is 3 μg/kg.

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