# GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

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# National food safety standard - Shellfishes - Test method of domoic acid in amnesic shellfish poisoning

食品安全国家标准

贝类中失忆性贝类毒素的测定

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## 1 Scope

This Standard specifies enzyme-linked immunosorbent assay, liquid chromatography, and liquid chromatography-tandem mass spectrometry for the determination of amnesic shellfish poisoning in shellfish.

The enzyme-linked immunosorbent assay in this Standard applies to the determination of amnesic shellfish poisoning in shellfish and its products. Liquid chromatography and liquid chromatography-tandem mass spectrometry apply to the determination of domoic acid (DA) in amnesic shellfish poisoning in shellfish and its products (excluding salted products).

## Enzyme-linked immunosorbent assay

## 2 Principle

The basis of this method is a competitive enzyme-linked immunoreaction. The ELISA plate is coated with a capture antibody against the domoic acid antibody. ADD anti-domoic acid antibody, standard solution or sample solution, and domoic acid enzyme marker. The free amnesic shellfish poisoning competes with the domoic acid enzyme marker for the domoic acid antibody, while the domoic acid antibody is linked to the capture antibody. The unbound enzyme marker is removed in the washing step. The enzyme matrix and developer are added to the micropores and incubated. The bound enzyme marker converts the colorless color former into a blue product. After the reaction stop solution is added, the color is changed from blue to yellow. At 450 nm, MEASURE the absorbance value of the microporous solution. The content of amnesic shellfish poisoning in the sample is inversely proportional to the absorbance value and quantitatively calculated according to the drawn standard curve.

**3.2.5** Eluent: PIPETTE 0.5 mL of tween-20; USE PBS solution (pH7.4) to dilute to 1000 mL.

**3.2.6** Sulfuric acid solution (6 mol/L): PIPETTE 319.2 mL of sulfuric acid; ADD slowly it to 600 mL of water; USE water to dilute to 1000 mL.

### 3.3 Standard

Domoic acid (DA, $C_{15}H_{21}NO_6$, CAS No. 14277-97-5) standard solution.

### 3.4 Preparation of standard solutions

DA standard series working solutions: PIPETTE accurately appropriate amount of DA standard solution; USE PBS solution to dissolve and dilute, to prepare the DA standard series working solutions with mass concentrations of 0 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL, 5.0 ng/mL, and 10.0 ng/mL, respectively. They are used right after they are prepared.

### 3.5 Materials

**3.5.1** Microporous plate coated with DA capture antibody.

**Note:** Commercialized kits, if the technical parameters for evaluation meet the requirements of this Standard, are also suitable for this Standard. See Appendix A.

**3.5.2** Aqueous-phase microporous filter membrane: 0.45 μm.

## 4 Instruments and equipment

**4.1** Microplate reader.

**4.2** Balance: The sensitivity is 0.01 g.

**4.3** Homogenizer.

**4.4** Centrifuge: Speed≥6000 r/min.

## 5 Analytical procedures

### 5.1 Sample collection

COLLECT at least 10 shellfish samples and make the shellfish meat more than 200 g. The frozen sample is placed in a heat preservation box for freezing inspection; or it is guaranteed to be in a low temperature state (0 °C~10 °C) for inspection. For a shelled sample, it shall be opened, and after removing the water, frozen for inspection.

microporous rack and labeled, including blank control pore, standard solution pore, and sample solution pore. MAKE parallel pores respectively. ADD 150 μL of PBS solution (pH7.4) to the blank control pore. ADD 50 μL of amnesic shellfish poisoning standard series working solution to the standard solution pore. ADD 50 μL of the sample solution to the sample solution pore. ADD 100 μL of DA enzyme marker working solution to all the above micropores and mix gently; ADD 100 μL of DA antibody working solution and rapidly mix well for 1 min. USE viscose paper to seal the micropores to prevent evaporation of the solutions; incubate at 4 °C for 2 h. After the incubation, pour out the liquid from the pores; inject 300 μL of eluent into each micropore to rinse; overturn the microporous plate; pour out the liquid from the pores; repeat the above plate-washing operation twice and pat dry on the absorbent paper. ADD 150 μL of hydrogen peroxide and TMB to each pore and mix well; incubate at room temperature for 30 min in the dark. ADD 50 μL of sulfuric acid solution (6 mol/L) to each pore and mix rapidly; terminate the reaction; measure in 30 min and record the absorbance value at a wavelength of 450 nm. If the sample diluent is measured beyond the linear range of the standard curve, the dilution factor may be expanded and the determination repeated.

**5.5 Making of standard curve**

USE the base 10 logarithm of the mass concentration of amnesic shellfish poisoning standard series working solution as the abscissa. USE the percentage absorbance value of standard solution calculated according to formula (1) as the ordinate. DRAW the standard curve.

The percentage absorbance value of amnesic shellfish poisoning standard solution (or sample solution) is calculated according to formula (1):

$$A = \frac{S - S_1}{S_0 - S_1} \times 100\% \qquad \cdots\cdots (1)$$

Where:

A - Percent absorbance value;

S - Average absorbance value of amnesic shellfish poisoning standard working solution or sample solution;

$S_1$ - Average absorbance value of blank control pores;

$S_0$ - Average absorbance value of 0 μg/L amnesic shellfish poisoning standard working solution.

# 9 Reagents and materials

Unless otherwise specified, the reagents used in this method are chromatographically pure; the water is Grade 1 water specified in GB/T 6682.

## 9.1 Reagents

**9.1.1** Acetonitrile ($CH_3CN$).

**9.1.2** Methanol ($CH_3OH$).

**9.1.3** Formic acid (HCOOH).

**9.1.4** Glacial acetic acid ($CH_3COOH$).

## 9.2 Reagent preparation

**9.2.1** Acetonitrile solution (10%): MEASURE and take 10 mL of acetonitrile; USE water to dilute to 100 mL.

**9.2.2** Methanol solution (50%): MEASURE and take 50 mL of methanol; USE water to dilute to 100 mL.

**9.2.3** Formic acid solution (0.1 mol/L): PIPETTE 3.81 mL of formic acid; USE water to dilute to 1000 mL.

**9.2.4** Acetic acid solution (0.1%): PIPETTE 1 mL of glacial acetic acid; USE water to dilute to 1000 mL.

## 9.3 Standard

Domoic acid standard (DA, $C_{15}H_{21}NO_6$, CAS No. 14277-97-5): Purity≥90%.

## 9.4 Preparation of standard solutions

**9.4.1** Domoic acid standard stock solution (150 μg/mL): Accurately weigh 8.3 mg (accurate to 0.01 mg) of the domoic acid standard; USE acetonitrile solution (10%) to dissolve and dilute to 50 mL. The concentration of the domoic acid standard stock solution has been converted using the purity of the standard. STORE at 4 °C in the dark, valid for 6 months.

**9.4.2** Domoic acid standard series working solutions: Separately pipette appropriate amount of domoic acid standard stock solution (150 μg/mL). USE acetonitrile solution (10%) to dilute into standard series working solutions with a concentration of 0.3 μg/mL, 0.6 μg/mL, 3 μg/mL, 15 μg/mL, 30 μg/mL, respectively. The standard series working solutions are stored at 4 °C in the

Accurately pipette 5 mL of the extract into the activated strong-anion solid-phase extraction column. After the liquid is discharged at a flow rate of 1 mL/min, successively use 5 mL of acetonitrile solution (10%) and 0.5 mL of formic acid solution (0.1 mol/L) to rinse. KEEP pumping for 2 min and discard the effluent. Finally, USE 3 mL of formic acid solution (0.1 mol/L) to elute; KEEP pumping for 2 min and collect the eluate (3 mL of eluate is equivalent to 1 g of sample). After passing through a 0.22 μm aqueous-phase microporous filter membrane, it is determined by liquid chromatography.

**11.5 Blank test**

With the exception of no sample, USE the same operation procedures as the sample to obtain the blank solution.

**11.6 Instrument reference conditions**

a) Chromatographic column: $C_{18}$ column. Column length is 150 mm. Inner diameter is 4.6 mm. Particle size is 3 μm. Or those with equivalent performance;

b) Mobile phase: Acetonitrile+acetic acid solution (0.1%) (13+87);

c) Flow rate: 1 mL/min;

d) Column temperature: 35 °C;

e) Injection volume: 10 μL;

f) Determination wavelength: 242 nm.

**11.7 Making of standard curve**

The standard series working solutions are separately injected into the liquid chromatograph, to determine the corresponding peak areas. USE the mass concentration of standard working solution as the abscissa. USE the peak area as the ordinate. DRAW a standard curve. The liquid chromatogram of domoic acid standard solution is shown in Figure B.1.

**11.8 Determination of sample solution**

Inject the sample solution into a liquid chromatograph to obtain a peak area. According to the standard curve, obtain the mass concentration of domoic acid in the sample solution.

# 16 Reagents and materials

Unless otherwise specified, the reagents used in this method are chromatographically pure; the water is Grade 1 water specified in GB/T 6682.

## 16.1 Reagents

**16.1.1** Methanol ($CH_3OH$).

**16.1.2** Acetonitrile ($CH_3CN$).

**16.1.3** Formic acid (HCOOH): Analytically pure.

**16.1.4** Ammonium formate ($HCOONH_4$): Analytically pure.

## 16.2 Reagent preparation

**16.2.1** Acetonitrile solution (10%): MEASURE and take 50 mL of acetonitrile; USE water to dilute to 500 mL.

**16.2.2** Methanol solution (50%): MEASURE and take 500 mL of methanol; USE water to dilute to 1000 mL.

**16.2.3** Ammonium formate buffer solution (2 mmol/L): WEIGH 0.126 g of ammonium formate; ADD 200 μL of formic acid; USE water to dissolve and dilute to 1000 mL.

**16.2.4** Formic acid solution (0.3%): PIPETTE 300 μL of formic acid; USE water to dilute to 100 mL.

## 16.3 Standard

Domoic acid standard (DA, $C_{15}H_{21}NO_6$, CAS No. 14277-97-5): Purity≥90%.

## 16.4 Preparation of standard solutions

**16.4.1** Domoic acid standard stock solution (100 μg/mL): Accurately weigh 5.6 mg (accurate to 0.01 mg) of the domoic acid standard; USE acetonitrile solution (10%) to dissolve and dilute to 50 mL. The concentration of the domoic acid standard stock solution has been converted using the purity of the standard. STORE at 4 °C in the dark, valid for 6 months.

**16.4.2** Domoic acid standard intermediate solution (2.0 μg/mL): Accurately pipette 1 mL of domoic acid standard stock solution (100 μg/mL) into a 50 mL volumetric flask; USE acetonitrile solution (10%) to dilute to the mark. STORE at 4 °C in the dark, valid for 3 months.

r/min for 10 min; PIPETTE the supernatant. ADD 5 mL of methanol solution (50%) to the residue to repeatedly extract twice. Combine the supernatant; USE methanol solution (50%) to dilute to 25 mL and mix well. After standing at -18 °C for 2 h, at 5 °C, centrifuge at 10000 r/min for 15 min. The supernatant is to be purified.

**18.4 Sample purification**

Accurately pipette 5 mL of the extract into the activated strong-anion solid-phase extraction column. CONTROL the effluent speed to about 1 drop per second. Then, successively use 5 mL of acetonitrile solution (10%) and 0.3 mL of formic acid solution (0.3%) to rinse; discard the effluent. USE 4 mL of formic acid solution (0.3%) to elute; collect the eluate; use formic acid solution (0.3%) to dilute to 4 mL (equivalent to 1 g of sample). After mixing well, it is filtered through a 0.22 μm aqueous-phase microporous filter membrane. The filtrate is subjected to liquid chromatography-tandem mass spectrometry.

**18.5 Blank test**

**18.5.1** Reagent blank test: With the exception of no sample, USE the same operation procedures as the sample to obtain the blank solution.

**18.5.2** Blank matrix solution: SELECT a blank sample; USE the same operation procedures as the sample to obtain the blank matrix solution.

**18.6 Instrument reference conditions**

**18.6.1 Liquid chromatography reference conditions**

a) Chromatographic column: $C_{18}$ column. Column length is 100 mm. Inner diameter is 2.1 mm. Particle size is 5 μm. Or those with equivalent performance;

b) Mobile phase: Mobile phase A is methanol; mobile phase B is ammonium formate buffer solution (2 mmol/L); gradient elution. Gradient elution conditions are shown in Table 1;

c) Flow rate: 0.35 mL/min;

d) Column temperature: 30 °C;

e) Injection volume: 25 μL.

**Table 1 -- Gradient elution conditions of mobile phase**

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