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National Food Safety Standard - Determination of Diarrheic Shellfish Poison in Shellfish

食品安全国家标准 贝类中腹泻性贝类毒素的测定

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1 Scope

This Standard specifies mouse biology method, enzyme linked immunosorbent assay (ELISA method) and liquid chromatography - tandem mass spectrometry for the determination of diarrheic shellfish poison in shellfish.

In this Standard, mouse biology method and enzyme linked immunosorbent assay (ELISA method) are applicable to the determination of diarrheic shellfish poison in shellfish and shellfish products; liquid chromatography - tandem mass spectrometry is applicable to the determination of diarrheic shellfish poison okadaic acid (OA), dinophysistoxins-1 (DTX-1) and dinophysistoxins-2 (DTX-2) in edible part of shellfish and shellfish products (excluding salted products).

Mouse Biology Method

2 Principle

Use acetone to extract diarrheic shellfish poison (DSP) in shellfish. Through the distribution with anhydrous ether, decompression and evaporation, take saline solution, which contains 1% Tween-60, as the dispersing medium, prepare DSP injectable suspension. Inject the injectable suspension into mouse's peritoneal cavity. Observe the survival of the mouse; calculate its virulence.

3 Reagents and Materials

Unless it is otherwise stipulated, all reagents used in this Method shall be analytically pure. Water shall be Grade-1 water stipulated in GB/T 6682.

3.1 Reagents

3.1.1 Acetone (C_3H_6O).

3.1.2 Anhydrous ether ($C_4H_{10}O$).

3.1.3 Tween-60 ($C_{64}H_{126}O_{26}$).

3.1.4 Sodium chloride (NaCl).

5.2.2 Frozen sample

At room temperature, thaw frozen sample, till it displays semi-frozen state. In terms of shelled frozen sample, in accordance with the methods in 5.2.1, wash, open the shell, rinse and take out the shell meat; remove frozen pieces attached to the external part of the shell meat; wipe off the moisture. Cut the shell meat into pieces.

5.3 Sample Extraction

Weigh-take 200 g of shell meat sample, which is already cut into pieces; place it in a homogeneous cup. In accordance with the volume ratio, add a triple amount of acetone, then, homogenize it for over 2 min. Pour the well-homogenized substance into a Brinell funnel, then, extract and filter it; gather the filtrate. Respectively use acetone, which is 2 times the amount of the residue, to rinse the residue twice. Combine the filtrate and the above-mentioned filtrate. Transfer the filtrate into a 500 mL of round-bottomed flask. At 56 °C ± 1 °C, decompress and concentrate it to remove acetone, till greasy substance is separated from the liquid surface.

Use 100 mL ~ 200 mL of anhydrous ether to dissolve the greasy substance; pour it into a separating funnel. Use a small amount of anhydrous ether to rinse the round-bottomed flask; combine them and pour them into the separating funnel. Use a small amount of water to wash the lower sticky wall part. Slightly oscillate (avoid generating emulsion); place it still for stratification. Then, remove the aqueous layer (the lower layer).

Use water, which is equivalent to half the amount of ether, to wash the ether layer twice. Remove the aqueous layer, then, transfer the ether layer into a 250 mL or 500 mL round-bottomed flask. At 35 °C ± 1 °C, decompress and concentrate it to remove ether. Use a small amount of anhydrous ether to transfer the concentrated substance into a 50 mL or 100 mL round-bottomed flask. Again, decompress and concentrate it to remove ether.

Use 1% Tween-60 saline solution to transfer all the concentrated substance into a scale test tube, then, dilute it into 10 mL. Thoroughly shake it to prepare a homogenous suspension. 1 mL of the suspension is equivalent to 20 g of sample. Take this suspension as test stock solution.

Use the test stock solution to inject mice. When within 24 h, 2 or 3 mice died, oscillate the test stock solution, so that it could become homogenous suspension, then, use 1% Tween-60 saline solution to step-by-step dilute it, in accordance with Table 1, into 4 times or 16 times of diluent. Thoroughly mix it up. Then, in accordance with Table 1, inject mice.

5.4 Mouse Test

Select 6 healthy ICR strain of male mice, weight: 16 g ~ 20 g. Randomly divide them into two groups, namely, test group and solvent control group; 3 mice in each group.

Enzyme Linked Immunosorbent Assay (ELISA method)

7 Principle

In accordance with competitive enzyme-linked immunoreactivity, dissociative diarrheic shellfish poison compete with its enzyme marker for diarrheic shellfish poison antibody. Unbonded enzyme marker is eliminated in the step of rinsing. Add enzyme substrate and color developing reagent to the hole and incubate it. The bonded enzyme marker converts colorless color former into blue product. After adding reaction termination fluid, the color turns from blue to yellow. Under the wavelength of 450 nm, use ELIASA to measure the absorbance value of microporous solution. The content of diarrheic shellfish poison in the sample is inversely proportional to the absorbance value. In accordance with the standard curve being drawn, conduct quantitative calculation.

8 Reagents and Materials

Unless it is otherwise stipulated, all reagents used in this Method shall be analytically pure. Water shall be Grade-1 water stipulated in GB/T 6682.

8.1 Reagents

8.1.1 Methanol (CH_3OH).

8.1.2 Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \bullet 12H_2O$).

8.1.3 Sodium chloride (NaCl).

8.1.4 Potassium chloride (KCl).

8.1.5 Potassium dihydrogen phosphate (KH_2PO_4).

8.1.6 Tween-20 ($C_{58}H_{114}O_{26}$).

8.1.7 Bovine serum albumin (BSA).

8.1.8 Enzyme marker.

8.1.9 Hydrogen peroxide (H_2O_2).

8.1.10 3,3,5,5-tetramethylbenzidine (TMB, $C_{16}H_{20}N_2$).

8.1.11 Sulfuric acid (H_2SO_4).

8.2 Preparation of Reagents

10 Analytical procedures

10.1 Sample Collection

Same as 5.1.

10.2 Sample Preparation

Same as 5.2.

10.3 Sample Extraction

Homogenize the sample that is already cut into pieces. Accurately weigh-take 10 g (accurate to 0.1 g) of the sample; add 50 mL of methanol solution (90%). Conduct homogenization for 1 min ~ 2 min. At 6,000 r/min, conduct centrifugation for 10 min. Transfer the supernatant. In accordance with the volume of the supernatant, add PBS solution, which is twice the volume of the supernatant. Thoroughly mix it up. Absorb 50 μL of the sample diluent for determination.

10.4 Determination

Insert microporous strip, which is coated with diarrheic shellfish poison antibody, into a microporous rack; leave a mark, which includes blank control hole, standard solution hole and sample solution hole. Respectively make parallel holes. Add 50 μL of PBS solution to the blank control hole; add 50 μL of diarrheic shellfish poison standard series working solution to the standard solution hole; add 50 μL of sample solution to the sample solution hole. Add 50 μL of diarrheic shellfish poison enzyme marker to each microporous hole. Promptly and thoroughly mix it up. At 22 °C ~ 25 °C, keep away from light, incubate for 10 min. After the incubation is completed, pour away the liquid in the holes. Inject 250 μL of eluent to rinse each microporous hole. Overturn the microporous board; pour away the liquid in the holes. Then, report the above-mentioned board-rinsing operation for four times. On a water-absorbent paper, pat it dry. Add 50 μL of hydrogen peroxide and TMB to each hole; thoroughly mix them up. At room temperature, keep away from light, incubate for 6 min. Add 50 μL of sulfuric acid solution (1 mol/L) to each hole; promptly and evenly mix them up. Then, terminate the reaction. Within 10 min, under the wavelength of 450 nm, measure and record the absorbance value.

If after the determination, the mass concentration of the extract exceeds the linear rage of the standard curve, properly dilute it, then, re-determine it.

10.5 Draw a Standard Curve

Take the logarithm of diarrheic shellfish poison standard working solution (mass concentration: 10 as the substrate) as the x-coordinate. Take the percent absorbance value of the standard solution calculated through Formula (1) as the y-coordinate.

Liquid Chromatography - Tandem Mass Spectrometry

13 Principle

Use methanol to extract sample. Under alkaline condition, hydrolyze to release esterified state of diarrheic shellfish poison. Use liquid chromatography for separation; use tandem mass spectrometry for determination. Use the matrix standard curve to quantify through external standard method.

14 Reagents and Materials

Unless it is otherwise stipulated, all reagents used in this Method shall be superior grade of purity. Water shall be Grade-1 water stipulated in GB/T 6682.

14.1 Reagents

14.1.1 Methanol (CH_3OH): chromatographic purity.

14.1.2 Acetonitrile (CH_3CN): chromatographic purity.

14.1.3 Ammonia ($NH_3 \bullet H_2O$).

14.1.4 Sodium hydroxide (NaOH).

14.1.5 Hydrochloric acid (HCl).

14.1.6 Ammonium formate (NH_4COOH): chromatographic purity.

14.1.7 Formic acid (HCOOH): chromatographic purity.

14.2 Preparation of Reagents

14.2.1 Methanol solution (30%): measure-take 30 mL of methanol; use water to dilute to 100 mL.

14.2.2 Methanol solution (20%): measure-take 20 mL of methanol; use water to dilute to 100 mL.

14.2.3 Ammonia - methanol solution (0.3%): absorb 0.3 mL of ammonia; use methanol to dilute to 100 mL.

14.2.4 Sodium hydroxide solution (2.5 mol/L): accurately weigh-take 50 g of sodium hydroxide; use water to dissolve and dilute to 500 mL.

14.2.5 Hydrochloric acid solution (2.5 mol/L): accurately measure-take 104.5 mL of hydrochloric acid; use water to dilute to 500 mL.

constant volume of 20 mL.

16.3.2 Hydrolysis release of esterified state of diarrheic shellfish poison

Accurately absorb 1 mL of the extract; place it in a threaded-mouth sample bottle. Add 125 μL of sodium hydroxide solution (2.5 mol/L); mix it up. Then, use sealing film to seal up the bottle. At 76 °C, conduct incubation for 40 min. After cooling it down to room temperature, add 125 μL of hydrochloric acid solution (2.5 mol/L), then, mix it up. The obtained hydrolysate (1.25 mL is equivalent to 0.1 g of sample) may be directly filtered through 0.22 μm organic phase microporous membrane, then, used for liquid chromatography - tandem mass spectrometry determination. Or, if necessary, purification treatment may also be conducted.

16.4 Sample Purification

Use 3 mL of water to dilute the obtained hydrolysate. After vortex mixing, transfer it into previously-activated polymer solid phase extraction column. After the fluid flows out at the flow rate of 1 mL/min, use 1 mL of methanol solution (20%) to rinse it. Discard the effluent. Maintain pumping for 2 min. In the end, use 1 mL of ammonia - methanol solution (0.3%) to elute it. Maintain pumping for 2 min; gather the eluent. Use methanol to reach a constant volume of 1 mL (equivalent to 0.1 g of sample). Filter it through 0.22 μm organic phase microporous membrane; it may be used for liquid chromatography - tandem mass spectrometry determination.

16.5 Blank Test

Except from the step of adding sample, adopt the same steps of operation as the sample. Thus, obtain the blank solution.

16.6 Instrument Reference Conditions

16.6.1 Liquid chromatograph has the following reference conditions:

a) Chromatographic column: C_{18} column; column length: 100 mm; internal diameter: 2.1 mm; particle size: 3.5 μm, or others with equivalent performance;

b) Flow rate: 0.2 mL/min;

c) Column temperature: 35 °C;

d) Injection volume: 10 mL;

e) Mobile phase: mobile phase A: ammonium formate solution (2 mmol/L); mobile phase B: acetonitrile + ammonium formate (2 mmol/L) (95 + 5); gradient elution. Please refer to Table 2 for the conditions of gradient elution.

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