GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

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National Food Safety Standard - Determination of Microcystin in Aquatic Products

食品安全国家标准

水产品中微囊藻毒素的测定

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1 Application Scope

This Standard specifies the method to use liquid chromatography - tandem mass spectrometry and indirect competitive enzyme-linked immunosorbent assay for the test of microcystin (cyclic heptapeptide) in aquatic products.

This Standard applies to the determination of microcyst in aquatic products such as fish, shrimp and river otter.

Method 1 -- Liquid Chromatography – Tandem Mass Spectrometry

2 Principle

Use methanol solution to exact microcystins (MC-LR, MC-RR and MC-YR) in the sample; use solid phase extraction cartridge to purify; apply liquid chromatography - tandem mass spectrometry to determine; use external standard method to fix-column.

3 Reagents and Materials

Unless otherwise specified, all the reagents in this method are analytical reagents; the water is grade-1 water specified by GB/T 6682.

3.1 Reagents

3.1.1 Methanol (CH_4O): chromatographic pure.

3.1.2 Formic acid (CH_2O_2): chromatographic pure.

3.1.3 Ammonium formate (CH_5O_2N): chromatographic pure

3.1.4 Acetonitrile (C_2H_3N): chromatographic purity.

3.1.5 Nitrogen: purity ≥ 99.99%.

source.

4.2 Analytical balance: the sensitivity is 0.001 g and 0.0000 1 g.

4.3 Centrifuge: with the speed larger than 8 000 r/min.

4.4 Homogenizer.

4.5 Rotary evaporator.

4.6 Vortex oscillator.

5 Analysis Steps

5.1 Sample preparation

Take about 500 g of the edible portion of the sample; fully homogenize or pulverize; place in a clean container; seal and mark. Store the sample in the dark below -18°C; complete the test within one month.

5.2 Extraction

Accurately weigh 5 g of sample (accurate to 0.01g); place it in a 50 mL centrifuge tube; add 10 mL of methanol solution (80%); shake well; centrifuge at 8 000 r/min for 10 min; put the supernatant in a new centrifuge tube. Repeatedly extract the residue once; combine both the extracts; fix-volume the extract to 30 mL; use a glass fiber filter paper to filter. Take 6 mL of the filtrate and add 30 mL of water to dilute. To be purified.

5.3 Purification

Use 10 mL of methanol and 10 mL of water successively to activate the C_{18} solid phase extraction cartridge before using; control the flow rate at 1 drop/s ~ 2 drops/s. After all the extract passing through the column, use 10 mL of methanol solution (20%) to rinse; use 10 mL of methanol solution containing 0.1% of formic acid to elute; collect the eluent and use nitrogen to concentrate to dryness. Add 1 mL of methanol solution (20%) to fully dissolve the residue; use organic phase microporous membrane to filter; put it as standby for the machine.

5.4 Blank control test

Respectively weigh 10 blank samples which are the same as the to-be-test sample matrix and does not contain the to-be-test microcystin in a 50 mL centrifuge tube. The following operations are handled in accordance with the operations of 5.2 and 5.3. Combine 10 blank sample solutions; use a microporous membrane to filter. The filtrate can be used as a blank matrix solution to prepare a matrix-matched mixed standard series working solution.

$$X_1 = \frac{A \times V}{m} \times \frac{1\,000}{1\,000} \quad \cdots\cdots (1)$$

Where:

X_1 -- the content of microcystin in the sample, in micrograms per kilogram (μg/kg);

A -- the concentration of microcystin in the sample solution obtained according to the standard curve, in nanograms per milliliter (ng/mL);

V -- the final volume of the sample solution, in milliliters (mL);

m -- sample mass, in grams (g).

1 000 -- unit conversion factor;

The calculation result shall keep three significant figures.

7 Precision

The absolute difference of two independent test results under repeatability cannot exceed 10% of the arithmetic mean value.

8 Others

When the sample amount is 5 g, the detection-limit of MC-LR and MC-RR is 0.3 μg/kg, and the quantitation-limit is 1 μg/kg; the detection-limit of MC-YR is 0.17 μg/kg, and the quantitation-limit is 0.5 μg/ Kg.

Method 2 -- Indirect Competitive Enzyme-linked Immunosorbent Assay

9 Principle

The microcystin in the sample, through extraction and purification, reacts with an excess of specific antibodies against microcystin; the excess free antibody is bound to the pre-coated microcystin artificial antigen in the enzyme-labeled plate. Add enzyme-labeled secondary antibody against microcystin antibody and substrate corresponding to the enzyme for color development; compare it with microcystin standard reaction result; calculate the content of microcystin in the sample.

10.2.3 Phosphate buffer solution (pH 7.4): respectively weigh 0.2 g of potassium dihydrogen phosphate, 2.9 g of disodium hydrogen phosphate dodecahydrate, 8.0 g of sodium chloride and 0.2 g of potassium chloride; mix; then use water to dissolve; fix-volume to 1 000 mL.

10.2.4 Sodium acetate solution (0.1 mol/L): weigh 1.36 g of sodium acetate trihydrate; use water to dissolve; fix-volume to 100 mL.

10.2.5 Acetic acid solution (1 mol/L): measure 5.88 mL of glacial acetic acid; add water to fix-volume to 100 mL.

10.2.6 Sulfuric acid solution (1 mol/L): measure 55.6 mL of sulfuric acid, slowly inject about 200 mL of water along the glass rod; stir; cool to room temperature; add water to fix-volume to 1 000 mL.

10.2.7 Coating solution: weigh 1 mg of artificial antigen; dissolve it in 1 000 mL of phosphate buffer solution (pH 7.4).

10.2.8 Blocking solution: weigh 0.5 g of gelatin; add a small amount of phosphate buffer solution (pH 7.4); heat to dissolve; after cooling, fix-volume to 1 000 mL.

10.2.9 Washing solution (PBS-T): measure 0.5 mL of Tween-20; use phosphate buffer solution (pH7.4) to fix-volume to 1 000 mL.

10.2.10 Antibody dilution solution: weigh 0.5 g gelatin; add a small amount of washing solution; heat to dissolve; after cooling, fix-volume to 1 000 mL.

10.2.11 Secondary antibody solution: mix 1 volume of enzyme-labeled secondary antibody with 5 000 volumes of antibody dilution solution.

10.2.12 Substrate buffer solution (pH 5.0): use acetic acid solution (1 mol/L) to adjust the pH of the sodium acetate solution to 5.0.

10.2.13 Substrate stock solution: weigh 10 mg of tetramethylbenzidine; dissolve it in 1 mL of dimethyl sulfoxide.

10.2.14 Substrate solution: measure 100 μL of substrate stock solution; add 2 μL of 30% hydrogen peroxide and 10 mL of substrate buffer solution. Formulate when needed.

10.3 Standard

Microcystin-LR (MC-LR, $C_{49}H_{74}N_{10}O_{12}$, CAS No. 101043-37-2); pudicity ≥ 95%.

10.4 Preparation of standard solution

Microcystin (MC-LR) standard series solution: weigh an appropriate amount of microcystin (MC-LR); use ethanol solution (20%) to prepare it into solution with MC-

37°C for 2 h, or at 4°C overnight.

12.4.3 Antigen-antibody reaction

Weigh 500 μL of monoclonal antibody against microcystin MC-LR and 500 μL of microcystin standard series solution in a 1.5 mL test tube; mix; then use electric oscillator to shake evenly; place it at room temperature for 30 min. These reaction solutions are used to make the standard competition curve for microcystin.

Weigh 500 μL of monoclonal antibody against microcystin MC-LR and 500 μL of purification solution in a 1.5 mL test tube; mix; then use electric oscillator to shake; place it at room temperature for 30 min. This reaction is used to determine the content of microcystin in the sample.

12.4.4 Competitive reaction

Use washing solution to wash the blocked enzyme-labeled microplate for 3 times (washing for 3 min each time); drop antibody antigen reaction solution (100 μL/well). Do 3 parallel tests at different concentrations. Place at 37°C or room temperature for 90 min. Add antibody dilution solution to the appropriate well of the microplate as a negative control.

12.4.5 Reaction of secondary antibody with monoclonal antibody against microcystin (MC-LR)

Use washing solution to wash the competitive-reacted enzyme-labeled microplate for 3 times (washing for 3 min each time); drop antibody antigen reaction solution (100 μL/well); place at 37°C or room temperature for 30 min.

12.4.6 Color development and determination of absorbance after color development

Use washing solution to wash the enzyme-labeled microplate after the reaction described in 12.4.5 for 5 times (washing for 3 min each time). Add substrate solution (100 μL/well) dropwise; place at 37°C or room temperature for 15 min ~ 20 min for color development. Add sulfuric acid (1 mol/L) (50 μL/well) dropwise to terminate the color reaction.

Use the enzyme-labeled instrument to measure the absorbance after color development at 450 nm within 30 minutes.

12.5 Determination

12.5.1 Apparatus reference conditions

Enzyme-labeled instrument: 450 nm.

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