# National Standard of the People's Republic of China

**GB 5009.24-2016**

# National Food Safety Standard

# Determination of M-Group Aflatoxins in Foods

## 食品安全国家标准

## 食品中黄曲霉毒素 M 族的测定


- Released on 2016-12-23
- Implemented on 2017-06-23
- Issued by NHFPC & CFDA

# Foreword

This standard replaces GB 5413.37-2010 “National food safety standard Determination of aflatoxin M1 in milk and milk products”, GB 5009.24-2010 “National food safety standard Determination of aflatoxin M1 and B1 in foods”, GB/T 23212-2008 “Determination of aflatoxin B1, B2, G1, G2, M1, M2 content in milk and milk products HPLC-fluorescence detection method” and SN/T 1664-2005 “Determination of aflatoxin M1, B1, B2, G1, G2 content in milk and milk powder”.

Compared with 5413.37-2010, major changes of this standard are as follows:

— The standard name has been modified as “National food safety standard Determination of M-group aflatoxins in foods”;

— The scope of application has been added;

— The detection of aflatoxin M2 has been added;

— The enzyme linked immunosorbent assay method has be modified and the Method III has been modified as enzyme linked immunosorbent assay screening method;

— The liquid chromatographic-mass spectrometric method has been modified;

— The pretreatment method of liquid chromatography has been modified;

— The immunochromatography purification fluorescence spectrophotometer method has been deleted.

# National Food Safety Standard

# Determination of M-Group Aflatoxins in Foods

## 1. Scope

This standard specifies the determination method of aflatoxin $M_1$ and aflatoxin $M_2$ (hereinafter referred to as AFT $M_1$ and AFT $M_2$) in foods.

Method I is isotope dilution liquid chromatography-tandem mass spectrometry, applicable to the determination of AFT $M_1$ and AFT $M_2$ in milk, milk products and foods for special dietary purpose containing milk.

Method II is high performance liquid chromatography with the same scope as Method I.

Method III is enzyme linked immunosorbent assay screening method, applicable to screening determination of milk, milk products and foods for special dietary purpose with milk.

### Method I Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

## 2. Principles

Extract aflatoxin $M_1$ and aflatoxin $M_2$ from the test sample with methanol-water solution, dilute the supernate with water or phosphate buffer, purify and enrich by immunoaffinity column, concentrate by purified solution, dilute to scale and filter, then separate by liquid chromatogram, determine by tandem mass spectrum and isotope quantification using an internal standard method.

## 3. Reagents and Materials

Unless otherwise specified, all the reagents in this method are analytically pure and water is Grade I water specified in GB/T 6682.

### 3.1 Reagents

3.1.1 Acetonitriler ($CH_3CN$): chromatographically pure.

3.1.2 Methanol ($CH_3OH$): chromatographically pure.

3.1.3 Ammonium acetate ($CH_3COONH_4$).

3.1.4 Sodium chloride (NaCl).

3.1.5 Disodium hydrogen phosphate ($Na_2HPO_4$).

3.1.6 Monopotassium phosphate ($KH_2PO_4$).

3.1.7 Potassium chloride (KCl).

3.1.8 Hydrochloric acid (HCl).

3.1.9 Petroleum ether ($C_nH_{2n+2}$): boiling range of petroleum ether is 30 ℃~60℃.

### 3.2 Preparation of reagents

3.2.1 Ammonium acetate solution (5mmol/L): weigh 0.39 g of ammonium acetate, dissolve in 1,000 mL of water and mix absolutely.

3.2.2 Acetonitriler-water solution (25+75): add 250 mL of acetonitriler into 750 mL of water and mix absolutely.

3.2.3 Acetonitriler-methanol solution (50+50): add 500 mL of acetonitriler into 500 mL of methanol and mix absolutely.

3.2.4 Phosphate buffered solution (hereinafter referred to as PBS): weigh 8.00 g of sodium chloride, 1.20 g of disodium hydrogen phosphate (or 2.92 g of disodium hydrogen phosphate dodecahydrate), 0.20 g of

monopotassium phosphate, 0.20 g of potassium chloride, dissolve with 900 mL of water, adjust pH to 7.4 with hydrochloric acid and add water to 1,000 mL.

**3.3 Standard substances**

3.3.1 AFT $M_1$ standard substance ($C_{17}H_{12}O_7$, CAS: 6795-23-9): purity is ≥98%, or the standard substance that is identified by China and awarded standard substance certificate.

3.3.2 AFT $M_2$ standard substance ($C_{17}H_{14}O_7$, CAS: 6885-57-0): purity is ≥98%, or the standard substance that is identified by China and awarded standard substance certificate.

3.3.3 $^{13}C_{17}$-AFT $M_1$ isotope solution ($C_{17}H_{14}O_7$): 0.5μg/mL.

**3.4 Preparation of standard solutions**

3.4.1 Standard stock solution (10 μg/mL): weigh 1 mg (accurate to 0.01 mg) of AFT $M_1$ and AFT $M_2$ respectively, and dissolve to 100 mL with acetonitrile solution respectively. Transfer the solutions into brown reagent bottles, seal and store in the dark at -20 ℃. Calibrate the concentration before use (see Annex A for calibration method).

3.4.2 Mixed standard stock solution (1.0 μg/mL): respectively pipette 1.00 mL of 10μg/mL AFT $M_1$ and AFT $M_2$ standard stock solution, accurately put both in a 10mL volumetric flask, add acetonitrile to dilute to 10 mL and obtain the mixed standard solution with concentration of 1.0 μg/mL. Seal the solution and store in the dark at 4 ℃, and period of validity is 3 months.

3.4.3 Mixed standard working solution (100 ng/mL): accurately pipette mixed standard stock solution (1.0 μg/mL) and put into 1.00mL to 10mL volumetric flasks and add acetonitrile to the scale. Seal the solution and store in the dark place at 4 ℃, and period of validity is 3 months.

3.4.4 50 ng/mL isotope internal standard working solution 1 ($^{13}C_{17}$-AFT $M_1$): pipette 1 mL of AFT $M_1$ isotope internal standard (0.5 μg/mL) and dilute to 10 mL with acetonitrile. Store at -20 ℃ and use when measuring liquid sample. Its period of validity is 3 months.

3.4.5 5 ng/mL isotope internal standard working solution 2 ($^{13}C_{17}$-AFT $M_1$): pipette 100 μL of AFT $M_1$ isotope internal standard (0.5 μg/mL) and dilute to 10 mL with acetonitrile. Store at -20 ℃ and use when determining solid sample. Its period of validity is 3 months.

3.4.6 Standard series working solutions: respectively pipette 5 μL, 1 0μL, 50 μL, 100 μL, 200 μL and 500 μL of standard working solution and put in 10mL volumetric flasks accurately, add 100 μL of 50 ng/mL isotope internal working solutions, add the initial mobile phase to 10 mL and the series standard solutions of AFT $M_1$ and AFT $M_1$ with concentrations of 0.05 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL and 5.0 ng/mL are prepared.

## 4. Apparatus and Equipment

**4.1** Balance: sensitive quantities are 0.01 g, 0.001 g and 0.000 01g.

**4.2** Water bath: temperature control is 50 ℃±2℃.

**4.3** Votex mixer.

**4.4** Ultrasonic cleaner.

**4.5** Centrifugal machine: ≥6,000 r/min.

**4.6** Rotary evaporator.

**4.7** Solid phase extraction apparatus (equipped with vacuum pump).

**4.8** Termovap sample concentrator.

**4.9** Liquid chromatogram-tandem mass spectrometer: equipped with electrospray ionization source.

**4.10** Circular screen: 1 mm~2 mm bore diameter.

**4.11** Fiberglass filter paper: rapid speed, high loading capacity, particle retains 1.6 μm in the liquid.
**4.12** Disposable micropore filter head: equipped with 0.22 μm microfiltration membrane (it can be used only after the selected filter membrane is tested with standard solution and confirmed there is no adsorption phenomena).
**4.13** Immunoaffinity column: column capacity is ≥100 ng (refer to Annex B for column capacity, recovery rate and verification method of column recovery rate).
Notes: Quality verification is required for each batch of affinity columns before use.

## 5. Analytical Procedure

Operation of sample injection, drip washing and elution may vary slightly in immunoaffinity columns manufactured by different manufacturers, so their operations shall be carried out according to the requirements in the operating manuals provided by suppliers.

Warning: The whole analysis process shall be conducted within the designated area. This area shall be kept out of light (direct sunlight) and equipped with relatively independent manipulation platform and waste storage device. Operators shall take corresponding protective measures in accordance with requirements of exposure to highly toxic substances.

### 5.1 Sample extraction

5.1.1 Liquid milk and yoghourt

Weigh 4 g (accurate to 0.001 g) of test sample that has been absolutely mixed and put in a 50mL centrifuge tube, add 100 μL of $^{13}C_{17}$-AFT $M_1$ internal standard solution (5 ng/mL), add 10 mL of methanol mix and swirl for 3min. Centrifuge at 4 ℃, 6,000 r/min for 10 min or filter using fiberglass filter paper, transfer appropriate amount of supernate or filtrate into a beaker and add 40 mL of water or PBS to diluter for later use.

5.1.2 Milk powder and foods for special dietary purpose

Weigh 1 g (accurate to 0.001 g) of sample and put in a 50mL centrifuge tube, add 100 μL of $^{13}C_{17}$-AFT $M_1$ internal standard solution (5 ng/mL), vibrate absolutely, then put standing for 30 min, add 4 mL of hot water at 50 ℃, swirl and mix absolutely. Put the centrifuge tube in the water bath at 50 ℃ if milk powder fails to totally dissolve and take out after milk powder dissolves totally. Cool the sample solution to 20 ℃, add 10 mL of methanol and swirl for 3 min. Centrifuge at 4 ℃, 6,000 r/min for 10 min or filter through fiberglass filter paper, transfer appropriate amount of supernate or filtrate into a beaker and add 40 mL of water or PBS to diluter for later use.

5.1.3 Cream

Weigh 1 g (accurate to 0.001 g) of sample and put in a 50mL centrifuge tube, add 100 μL of $^{13}C_{17}$-AFT $M_1$ internal standard solution (5 ng/mL), vibrate absolutely, then put standing for 30 min, add 8 mL of petroleum ether, wait for dissolve cream totally, then add 9 mL of water and 11 mL of methanol, vibrate for 30 min and transfer all the liquid into separating funnel. Add 0.3 g of sodium chloride, shake sufficiently to dissolve, set standing to layer, transfer the lower layer into a round-bottom flask, carry out rotary evaporation to below 10 mL and dilute to 30 mL with PBS.

5.1.4 Cheese

Weigh 1 g (accurate to 0.001 g) of absolutely mixed sample that has been sifted finely and passed through the circular screen with bore diameter of 1 mm~2 mm and put in a 50mL centrifuge tube, add 100 μL of $^{13}C_{17}$-AFT $M_1$ internal standard solution (5 ng/mL), vibrate absolutely, then put standing for 30 min, add 1 mL of water and 18 mL of methanol, vibrate for 30 min, centrifuge at 4 ℃, 6,000 r/min for 10 min or filter using fiberglass filter paper,

transfer appropriate amount of supernate or filtrate into a round-bottom flask, carry out rotary evaporation to below 2 mL and dilute to 30 mL with PBS.

**5.2 Purification**

5.2.1 Preparation of immunoaffinity column

Recover the immunoaffinity column preserved at low temperature to room temperature.

5.2.2 Purification

Discard the liquid in the immunoaffinity column, transfer the above sample solutions into the flow rate of dripping is adjusted to the injection syringe tube and 1 mL/min~3 mL/min. Add 10 mL of water into the injection syringe tube after sample solution drips off, to stabilize flow rate and wash immunoaffinity column. Pump the affinity column to be dry with vacuum pump after water drips off. Take out of the vacuum system, place a 10mL graduated test tube under the affinity column, take out the 50mL injection syringe tube, add 2×2 mL acetonitrile (or methanol) to elute the affinity column, control the dripping rate from 1 mL/minto3 mL/min, pump the affinity column to be dry using vacuum pump, collect all the eluate and put in the graduated test tube for injection, blow the eluate to be nearly dry with nitrogen gas at 50 ℃, add initial mobile phase to 1.0 mL, swirl for 30 s to dissolve the residue, filter with 0.22 μm filter membrane and collect filtrate into the sample injection bottle to inject sample.

Notes: Use the full automatic (online) or semi-automatic (offline) solid phase extraction apparatus after optimizing operating parameters. To prevent the destruction of aflatoxin M, relevant operations shall be conducted in a dark condition (direct sunlight).

**5.3 Reference conditions of liquid chromatogram**

Reference conditions of liquid chromatogram are listed as follows:

a) Liquid chromatographic column: $C_{18}$ column (column length is 100 mm, column inner diameter is 2.1 mm and particle size of padding is 1.7 μm), or other equivalent columns.

b) Chromatographic column temperature: 40 ℃.

c) Mobile phase: A phase, 5 mmol/L ammonium acetate aqueous solution; B phase, acetonitrile-methanol (50+50). Gradient elution: refer to Table 1.

d) Flow rate: 0.3 mL/min.

e) Sample injection volume: 10 μL.

**5.4 Reference conditions of mass spectrum**

Reference conditions of mass spectrum are listed as follows:

a) Detection method: multi-ionic reaction monitoring (MRM);

b) Control conditions of ion source: refer to Table 2;

c) Selection parameters of ion: refer to Table 3;

d) Liquid-chromatogram-mass spectrum and daughter ion scintigram: refer to Annex C.

**Table 1 Gradient elution conditions of liquid chromatogram**

| Time/min | Mobile phase A/% | Mobile phase B/% | Gradient change curve |
|---|---|---|---|
| 0.0 | 68.0 | 32.0 | - |
| 0.5 | 68.0 | 32.0 | 1 |
| 4.2 | 55.0 | 45.0 | 6 |
| 5.0 | 0.0 | 100.0 | 6 |

| | | | |
|---|---|---|---|
| 5.7 | 0.0 | 100.0 | 1 |
| 6.0 | 68.0 | 32.0 | 6 |

**Table 2 Control conditions of ion source**

| | |
|---|---|
| Ionization mode | $ESI^+$ |
| Capillary voltage/kV | 17.5 |
| Cone voltage/V | 45 |
| Voltage of radio-frequency lens 1/V | 12.5 |
| Voltage of radio-frequency lens 2/V | 12.5 |
| Ion source temperature/℃ | 120 |
| Cone blowback air flow/ (L/h) | 50 |
| Desolvation gas temperature/℃ | 350 |
| Desolvation gas flow/ (L/h) | 500 |
| Electron multiplier voltage/V | 650 |

**Table 3 Mass spectrum parameters**

| Compound name | Parent ion (*m/z*) | Quantitative daughter ion (*m/z*) | Collision energy eV | Qualitative daughter ion (*m/z*) | Collision energy eV | Ionization mode |
|---|---|---|---|---|---|---|
| AFT $M_1$ | 329 | 273 | 23 | 259 | 23 | $ESI^+$ |
| $^{13}C_{17}$-AFT$M_1$ | 346 | 317 | 23 | 288 | 24 | $ESI^+$ |
| AFT $M_2$ | 331 | 275 | 23 | 261 | 22 | $ESI^+$ |

**5.5 Qualitative determination**

The retention time of the chromatographic peak of the target compound in the sample is compared with the retention time of the corresponding standard chromatographic peak, and the variation range should be within ±2.5.

Qualitative ion of mass spectrum of each compound must appear, at least including one parent ion and two daughter ions and in the same detection batch, compared relative abundance of two ions of target compound in the sample with the standard solution with equivalent concentration, the allowable deviation shall not exceed the range specified in Table 4.

**Table 4 Maximum allowable deviation of qualitative relative ion abundance**

| Relative ion abundance/% | >50 | 20~50 | 10~20 | ≤10 |
|---|---|---|---|---|
| Allowable relative deviation/% | ±20 | ±25 | ±30 | ±50 |

**5.6 Mapping standard curve**

Detect standard series solutions from low to high concentrations under the liquid chromatogram-mass spectrometer analysis conditions stipulated in 5.3 and 5.4, map by taking peak area ratio of AFT $M_1$ and AFT $M_2$ chromatographic peak with internal standard chromatographic peak $^{13}C_{17}$-AFT $M_1$-concentration and standard

curve regression equation whose linear correlation coefficient shall be greater than0.99.

**5.7 Determination of test sample solution**

Inject the solution to be tested as obtained in 5.2, calculate the mass concentration of the target substance in the solution to be tested by using internal standard method and calculated the content of the substance to be tested in the sample according to Chapter 6.

**5.8 Blank test**

Don't weigh test sample, perform blank test according to the procedure in 5.1 and 5.2 and it shall be confirmed that there is no substance interfering the compound to be tested.

**6. Expression of Analysis Result**

Residue amount of AFT $M_1$ and AFT $M_2$ in the test sample shall be calculated in accordance with Formula (1):

$$X = \frac{\rho \times V \times f \times 1\,000}{m \times 1\,000} \qquad \cdots\cdots(1)$$

Where,

$X$——the content of AFT $M_1$ or AFT $M_2$ in the test sample, μg/kg;

$\rho$——the corresponding concentration of AFT $M_1$ or AFT $M_2$ in the sample injection solution on the standard curve in accordance with internal standard method, ng/mL;

$V$——the final metered volume of sample through immunoaffinity column and column purification elution, mL;

$f$——the dilution factor of test solution;

1 000——the conversion coefficient;

$m$——the mass of test sample, g.

Three decimal places are remained for the calculation result.

**7. Precision**

Absolute difference between two independent determination results obtained under repeatability conditions shall not exceed 20% of arithmetic mean value.

**8. Others**

Detection limit of AFT $M_1$ in this method is 0.005 μg/kg when weighing 4 g of liquid milk and yoghourt, detection limit of AFT $M_2$ is 0.005 μg/kg, limit of quantitation of AFT $M_1$ is 0.015 μg/kg and limit of quantitation of AFT $M_2$ is 0.015 μg/kg.

Detection limit of AFT $M_1$ in this method is 0.02 μg/kg when weighing 1 g of milk powder, foods for special dietary use, cream and cheese, detection limit of AFT $M_2$ is 0.02 μg/kg, limit of quantitation of AFT $M_1$ is 0.05 μg/kg and limit of quantitation of AFT $M_2$ is 0.05 μg/kg.

## Method II High Performance Liquid Chromatography

### 9. Principles

Extract the aflatoxin $M_1$ and aflatoxin $M_2$ in the test sample with methanol-water solution, dilute supernate, purify and enrich with an immunoaffinity column, concentrate, dilute to the scale and filter through liquid chromatogram to separate, and detect using fluorescence detector. Quantify by applying external standard method.

### 10. Reagents and Materials

Unless otherwise specified, all the reagents used in this method are analytically pure and water is Grade I water stipulated in GB/T 6682.

#### 10.1 Reagents

10.1.1 Acetonitrile ($CH_3CN$): chromatographically pure.
10.1.2 Methanol ($CH_3OH$): chromatographically pure.
10.1.3 Sodium chloride (NaCl).
10.1.4 Disodium hydrogen phosphate ($Na_2HPO_4$).
10.1.5 Monopotassium phosphate ($KH_2PO_4$).
10.1.6 Potassium chloride (KCl).
10.1.7 Hydrochloric acid (HCl).
10.1.8 Petroleum ether ($C_nH_{2n+2}$): boiling range is 30 ℃~60 ℃.

#### 10.2 Preparation of reagents

10.2.1 Acetonitrile-water solution (25+75): pipette 250 mL of acetonitrile, add into 750 mL of water and mix absolutely.
10.2.2 Acetonitrile-methanol solution (50+50): pipette 500 mL of acetonitrile, add into 500 mL of methanol and mix absolutely.
10.2.3 Phosphate buffered solution (hereinafter referred to as PBS): weigh 8.00 g of sodium chloride, 1.20 g of disodium hydrogen phosphate (or 2.92 g of disodium hydrogen phosphate dodecahydrate), 0.20 g of monopotassium phosphate, 0.20 g of potassium chloride, dissolve with 900 mL of water, modified pH to 7.4 with hydrochloric acid and add water to 1,000 mL.

#### 10.3 Standard substances

10.3.1 AFT $M_1$ standard substance ($C_{17}H_{12}O_7$, CAS: 6795-23-9): purity is ≥98%, or the standard substance that is identified by China and awarded standard substance certificate.
10.3.2 AFT $M_2$ standard substance ($C_{17}H_{14}O_7$, CAS: 6885-57-0): purity is ≥98%, or the standard substance that is identified by China and awarded standard substance certificate.

#### 10.4 Preparation of standard substances

10.4.1 Standard stock solution (10 μg/mL): respectively weigh 1 mg (accurate to 0.01 mg) of AFT $M_1$ and AFT $M_2$, and dissolve with acetonitrile solution to 100 mL respectively. Transfer the solution into brown reagent bottles, seal and store in the dark at -20 ℃. Concentration calibration before use (see Annex A for calibration method).
10.4.2 Mixed standard stock solution (1.0 μg/mL): respectively pipette 1.00 mL of 10 μg/mL AFT $M_1$ and AFT $M_2$ standard stock solution, put both in a 10mL volumetric flask, add acetonitrile to dilute to 10 mL and obtain the mixed standard solution with concentration of 1.0 μg/mL. Seal the solution and store in the dark at 4 ℃， and

the period of validity is 3 months.

10.4.3 100 ng/mL mixed standard working solution (AFT $M_1$ and AFT $M_2$): accurately pipette mixed standard stock solution (1.0 μg/mL) and put into 1.00mL to 10mL volumetric flasks and add acetonitrile to the scale. Seal the solution and store in the dark at 4 ℃, and the period of validity is 3 months.

10.4.4 Standard series working solutions: respectively pipette 5 μL, 1 0μL, 50 μL, 100 μL, 200 μL and 500 μL of standard working solution and put in 10mL volumetric flasks, add the initial mobile phase to 10 mL and the series standard solutions of AFT $M_1$ and AFT $M_1$ with concentrations of 0.05 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL and 5.0 ng/mL are prepared.

## 11. Apparatus and Equipment

**11.1** Balance: sensitive quantities are 0.01 g, 0.001 g and 0.000 01g.

**11.2** Water bath: temperature control is 50 ℃±2℃.

**11.3** Votex mixer.

**11.4** Ultrasonic cleaner.

**11.5** Centrifugal machine: ≥6,000 r/min.

**11.6** Rotary evaporator.

**11.7** Solid phase extraction apparatus (equipped with vacuum pump).

**11.8** Termovap sample concentrator.

**11.9** Circular screen: 1 mm~2 mm bore diameter.

**11.10** Liquid chromatograph (equipped with fluorescence detector).

**11.11** Fiberglass filter paper: rapid speed, high loading capacity, particle retains 1.6 μm in the liquid.

**11.12** Disposable micropore filter head: equipped with 0.22 μm microfiltration membrane.

**11.13** Immunoaffinity column: column capacity is ≥100 ng. (Refer to Annex B for column capacity, recovery rate and verification method of column recoveryrate).

Notes: Mass of different batches of affinity columns need verified before use.

## 12. Analytical Procedure

Operation of sample injection, drip washing and elution may be slightly different for immunoaffinity columns made by different manufacturers, so their operations shall be carried out in accordance with the requirements in the operating manuals provided by suppliers.

Warning: The whole analysis process shall be conducted in the designated area. This area shall be kept from light (direct sunlight) and equipped with relatively independent manipulation platform and waste storage device. Operators shall take corresponding protective measures according to requirements of contacting with highly toxic substances.

### 12.1 Extraction of test solution

The method is the same as the operations in 5.1 except for the addition of isotope internal standard solution.

### 12.2 Purification

The method is the same as the operations in 5.2.

### 12.3 Reference conditions of liquid chromatogram

Reference conditions of liquid chromatogram are listed as follows:

a) Liquid chromatographic column: $C_{18}$ column (column length is 150 mm, column inner diameter is 4.6 mm and particle size of padding is 5 μm), or other equivalent columns.

b) Chromatographic column temperature: 40℃.

c) Mobile phase: A phase, water; B phase, acetonitrile-methanol (50+50). Gradient elution: A, 70%; B, 30%.

d) Flow rate: 1.0 mL/min.

e) Fluorescence detection wavelength: emission wave length is 360 nm and excitation wave length is 430 nm.

f) Sample injection volume: 50 μL.

**12.4 Determination**

12.4.1 Mapping standard curve

Inject series standard solution from low concentration to high concentration to detect, the peak area-concentration plot was used to obtain the standard curve regression equation.

12.4.2 Determination of test sample solution

Response value of the solution to be tested shall be within the linearity range of standard curve, dilute and re-inject the sample to analyze if exceeding the linearity range.

12.4.3 Blank test

Don't weigh test sample, carry out blank test in accordance with the procedure in 12.1 and 12.2 and it shall confirm that there is no substance interfering the compound to be tested.

**13. Expression of Analysis Result**

Residue quantities of AFT $M_1$ and AFT $M_2$ in the test sample shall be calculated in accordance with Formula (2):

$$X = \frac{\rho \times V \times f \times 1\,000}{m \times 1\,000} \qquad \cdots\cdots (2)$$

Where,

$X$——the content of AFT $M_1$ or AFT $M_2$ in the test sample, μg/kg;

$\rho$——the concentration of AFT $M_1$ and AFT $M_2$ obtained from the standard curve in accordance with chromatographic peak of AFT $M_1$ and AFT $M_2$ in the sample injection solution, ng/mL;

$V$——the final metered volume of sample through immunoaffinity column and column purification elution, mL;

$f$——the dilution factor of test solution;

1 000——the conversion coefficient;

$m$——the mass of test sample, g.

Three decimal places are kept for the calculation result.

**14. Precision**

Absolute difference between two independent determination results obtained under repeatability conditions shall not exceed 20% of arithmetic mean value.

**15. Others**

Detection limit of AFT $M_1$ in this method is 0.005 μg/kg when weighing 4 g of liquid milk and yoghourt, detection limit of AFT $M_2$ is 0.002 5 μg/kg, limit of quantitation of AFT $M_1$ is 0.015 μg/kg and limit of quantitation of AFT $M_2$ is 0.007 5 μg/kg.

Detection limit of AFT $M_1$ in this method is 0.02 μg/kg when weighing 1 g of milk powder, foods for special dietary purpose, cream and cheese, detection limit of AFT $M_2$ is 0.01 μg/kg, limit of quantitation of AFT $M_1$ is 0.05 μg/kg

and limit of quantitation of AFT $M_2$ is 0.025 μg/kg.

**Method III Enzyme Linked Immunosorbent Assay Screening Method**

**16. Principles**

Homogenize, freeze, centrifuge, degrease or extract with organic solvent aflatoxin $M_1$ in the test sample and obtain supernate. The aflatoxin $M_1$ labeled by horseradish peroxidase or fixed in the reaction pore combines with specific antibody competitively with aflatoxin $M_1$ in the sample or standard substance. Add corresponding color developing agent to develop after washing, terminate the reaction by inorganic acid and detect at the 450nm or 630nm wave length. Aflatoxin $M_1$ in the sample is in inverse proportion to absorbance within a certain concentration range.

**17. Reagents and Solvents**

All the reagents required to prepare solutions are analytically pure and water is Grade II water as specified in GB/T 6682.
Prepare required solutions as a guide of the kit.
The commercial kit can be used only after verification is qualified in accordance with the method specified in Annex E.

**18. Apparatus and Equipment**

**18.1** Microplate ELIASA: equipped with 450nm and 630nm (optional) opticalfilter.
**18.2** Balance: the minimum quantity is 0.01 g.
**18.3** Centrifugal machine: revolving speed is ≥6,000 r/min.
**18.4** Vortex mixer.

**19. Analytical Procedure**

**19.1 Pretreatment of test sample**

19.1.1 Liquid sample

Take about 100 g of sample to be tested and shake absolutely, and centrifuge 10 g of sample at 6,000 r/min or above for 10 min by using centrifugal machine. Put about 1 g of liquid in the lower layer in a test tube. This solution can be directly measured or measured according to the method provided on the kit after dilution (solution to betested).

19.1.2 Milk powder and foods for special dietary purpose

Weigh 10 g (accurate to 0.1 g) of sample to be tested and place in a small beaker, add water to dissolve, transfer into a 100mL volumetric flask and add water to 100 mL. The following procedures are the same as those in 19.1.1.

19.1.3 Cheese

Weigh 50 g (accurate to 0.1 g) of sample to be tested, remove the non-edible part on the surface, hard cheese can be crushed directly by using pulverizer; soft cheese need to be frozen at -20 ℃ overnight first, and then crushed by using grinder. Weigh 5 g (accurate to 0.1 g) of absolutely mixed sample to be tested, add the extracting solution provided in the kit, extract according to the instruction book of kit and extracting solution is the solution to be

tested.

**19.2 Quantitative detection**

According to the operation procedure on the enzyme linked immunosorbent assay kit, the test sample (solution) is quantitatively detected.

**20. Expression of Analysis Result**

**20.1 Mapping standard working curve of quantitative determination of enzyme linked immunosorbent assay kit**

Map standard working curve in accordance with the relation of standard substance concentration and absorbance.

**20.2 Concentration calculation of the solution to be tested**

The absorbance of the solution to be measured is substituted into 20.1, and the concentration ρ of the solution to be measured is calculated.

**20.3 Result calculation**

Content of aflatoxin $M_1$ in the food shall be calculated according to Formula (3):

$$X = \frac{\rho \times V \times f}{m} \qquad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (3)$$

Where,

*X*——the content of aflatoxin $M_1$ in the food, μg/kg;

*ρ*——the concentration of aflatoxin $M_1$ in the solution to be tested, μg/mL;

*V*——the metered volume (milk powder, foods for special dietary purpose and liquid samples) or volume of extracting solution (cheese), L;

*f*——the dilution factor;

*m*——the mass of test sample, kg.

Two decimal places are kept for the calculation result.

Notes: Positive sample need further confirmation by Method I or Method II.

**21. Precision**

Absolute difference between two independent determination results obtained under repeatability conditions shall not exceed 20% of arithmetic mean value.

**22. Others**

Detection limit of this method is 0.01 μg/kg and limit of quantitation is 0.03 μg/kg when weighing 10 g of liquid milk.

Detection limit of this method is 0.1 μg/kg and limit of quantitation is 0.3 μg/kg when weighing 10 g of milk powder and foods for special dietary use with milk.

Detection limit of this method is 0.02 μg/kg and limit of quantitation is 0.06 μg/kg when weighing 5 g of cheese.

## Annex A

## Calibration method of standard solutions of AFT $M_1$ and AFT $M_2$

Prepare the AFT $M_1$ and AFT $M_2$ standard solution with concentration of 8 μg/mL~10μg/mL by using acetonitrile solution. Determine absorbance of the solution at the maximum absorption band to determine the actual concentration of AFT $M_1$ and AFT $M_2$. Determine at 340 nm~370 nm wave length by using spectrophotometer, deduct background value of blank reagent of solvent, calibrate system error of cuvette, and then read out absorbance value *A* of standard solution at the maximum absorption wave length ($\lambda_{max}$). The actual concentration of calibration solution ρ is calculated according to formula (A.1):

$$\rho = A \times M \times \frac{1\ 000}{\varepsilon} \qquad \cdots\cdots (A.1)$$

Where,

*ρ*——the determined actual concentration of AFT $M_1$ or AFT $M_2$ after calibration, μg/mL;

*A*——the absorbance value determined at $\lambda_{max}$;

*M*——the molar mass of AFT $M_1$ or AFT $M_2$, g/mol;

*ε*——the absorptivity of AFT $M_1$ or AFT $M_2$, $m^2$/mol.

**Table A.1 Molar mass and molar absorption coefficient of AFT $M_1$**

| Aflatoxin name | Molar mass/ (g/mol) | Solvent | Molar absorption coefficient/ ($m^2$/mol) |
|---|---|---|---|
| AFT $M_1$ | 328 | Acetonitrile | 19 000 |
| AFT $M_2$ | 330 | Acetonitrile | 21 400 |

## Annex B

## Verification method of column capacity of immunoaffinity column

### B.1 Verification of Column Capacity

Add 300 ng of AFT $M_1$ standard stock solution into 30 mL of PBS and thoroughly mix absolutely. Respectively pipette 3 pieces of immunoaffinity columns of the same batch, and sample injection volume of each column is 10 mL. Inject sample, wash, elute and collect eluate, then blow to dry to be 1 mL with nitrogen gas, add the initial mobile phase to 10 mLand separate and determine content of AFT $M_1$ by using liquidchromatograph.
Result judgment: when in the result, AFT $M_1$ is ≥80 ng, it's the commodity that can be used.

### B.2 Verification Method of Column Recovery Rate

Add 300 ng of AFT $M_1$ standard stock solution into 30 mL of PBS and sufficiently thoroughlymix absolutely. Respectively pipette 3 pieces of immunoaffinity columns of the same batch, and sample injection volume of each column is 10 mL. Inject sample, wash, elute and collect eluate, then blow to dry to be 1 mL with nitrogen gas, add the initial mobile phase to 10 mL and separate and determine content of AFT $M_1$ by liquidchromatograph.
Result judgment: when in the result, AFT $M_1$ is ≥80 ng, it's the commodity that can be used.

### B.3 Verification of Cross-Reaction Rate

Add 300 ng of AFT $M_1$ standard stock solution into 30 mL of PBS and thoroughly mix absolutely. Respectively pipette 3 pieces of immunoaffinity columns of the same batch, and sample injection volume of each column is 10 mL. Inject sample, wash, elute and collect eluate, then blow to dry to be 1 mL with nitrogen gas, add the initial mobile phase to 10 mLand separate and determine content of AFT $M_2$ by using liquidchromatograph.
Result judgment: when in the result, AFT $M_2$ is ≥80 ng, it's the commodity that can be used to determine both AFT $M_1$ and AFT $M_2$ simultaneously.

## Annex C
## Liquid chromatogram-mass spectrum and daughter ion scintigram

**C.1** Refer to Figure C.1 for daughter ion scintigram of AFT$M_1$.

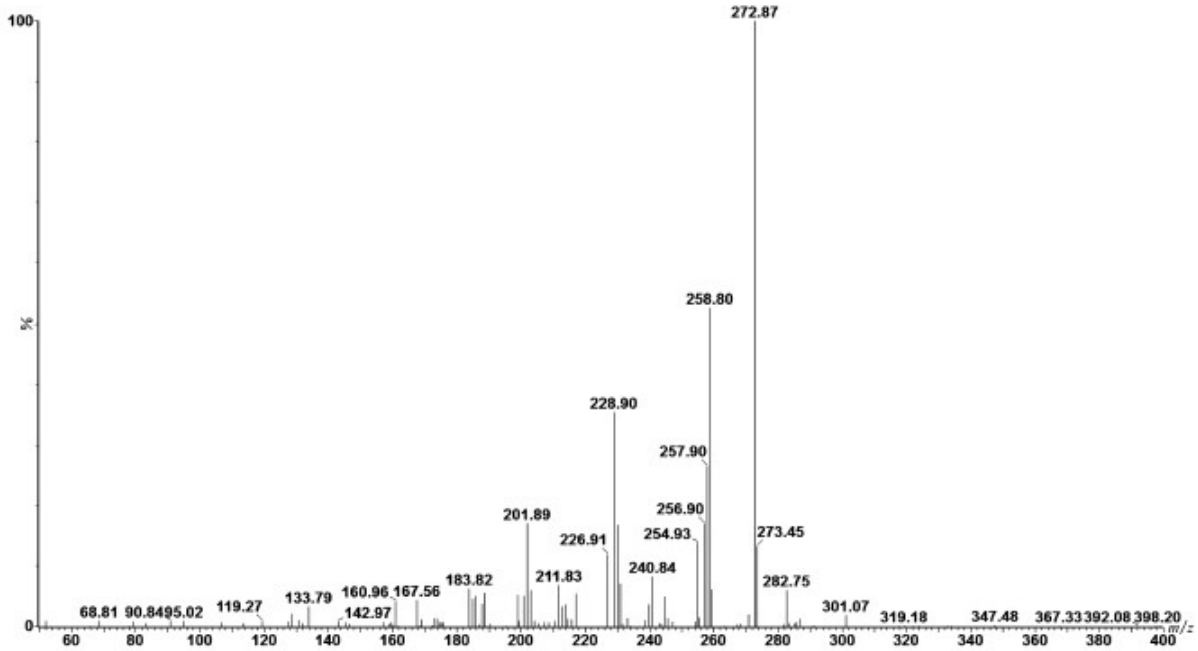


**Figure C.1 Daughter ion scintigram of AFT $M_1$**

**C.2** Refer to Figure C.2 for daughter ion scintigram of AFT$M_2$.

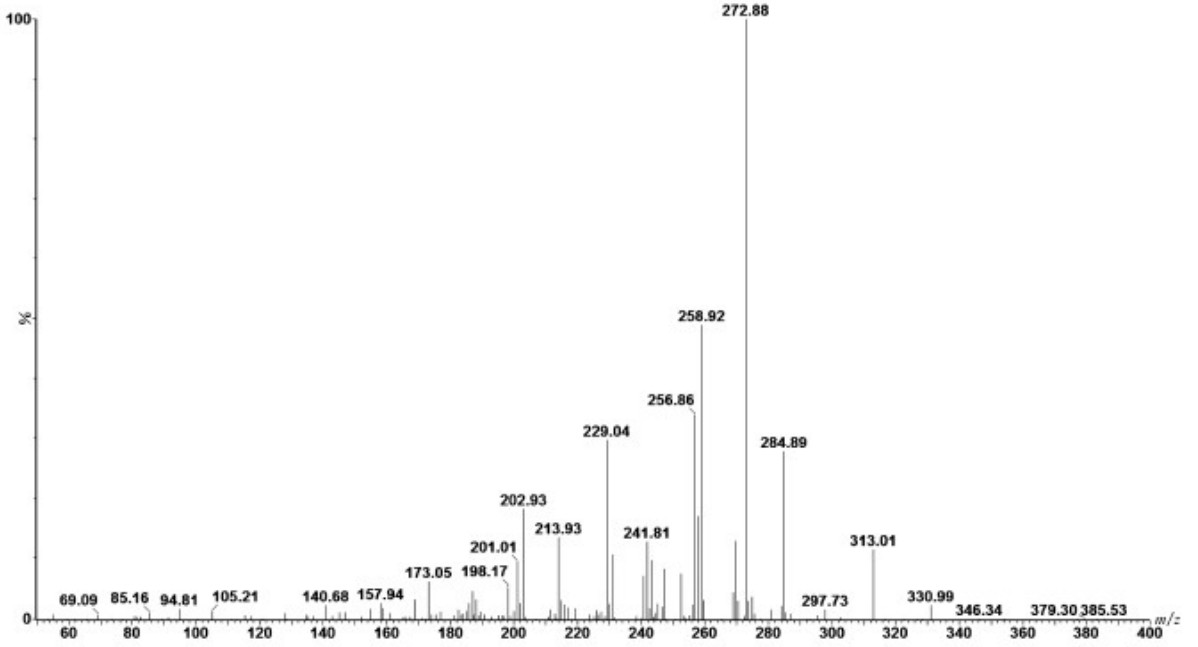


**Figure C.2 Daughter ion scintigram of AFT $M_2$**

**C.3** Refer to Figure C.3 for daughter ion scintigram of $^{13}C_{17}$-AFTM$_1$.

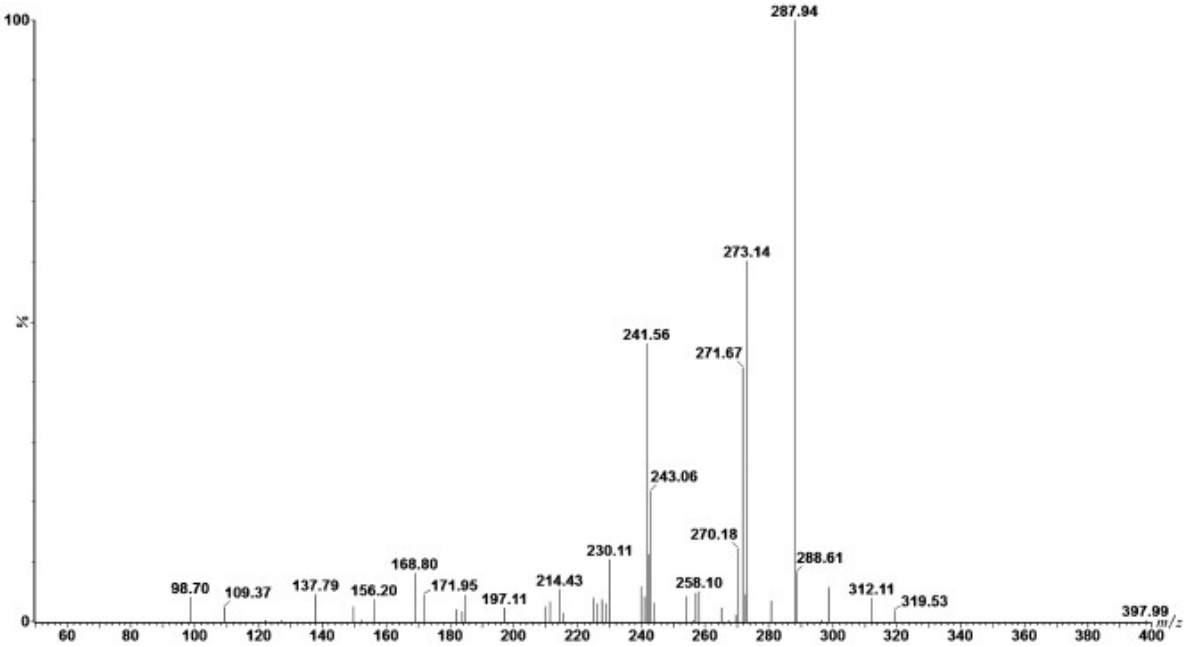


**Figure C.3 Daughter ion scintigram of $^{13}C_{17}$-AFT M$_1$**

**C.4** Refer to Figure C.4 for liquid chromatogram mass spectrum of AFT M$_1$, AFT M$_2$ and $^{13}C_{17}$-AFT M$_1$.

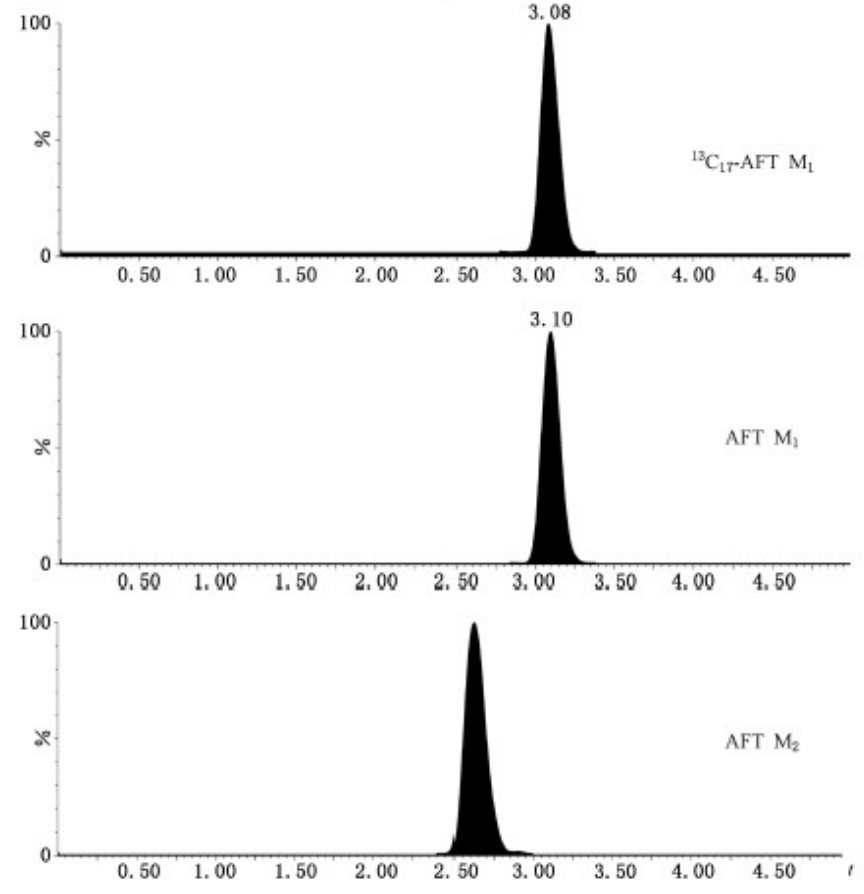


**Figure C.4 Liquid chromatogram mass spectrum of AFT M$_1$, AFT M$_2$ and $^{13}C_{17}$-AFT M$_1$**

## Annex D

## Liquid chromatogram

Refer to Figure D.1 for liquid chromatogram of AFT $M_1$ and AFT $M_2$.

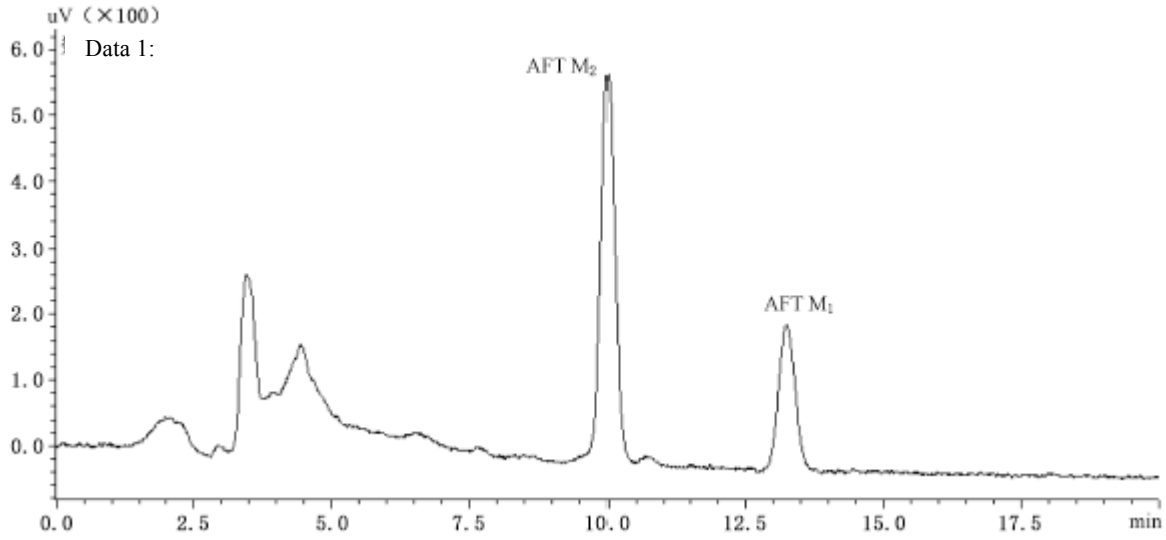


**Figure D.1 Liquid chromatogram of AFT $M_1$ and AFT $M_2$**

## Annex E

## Mass judgment method of enzyme linked immunosorbent assay kit

Select milk or other negative samples and add 3 concentration levels' AFT $M_1$ standard solution (0.1 μg/kg, 0.3 μg/kg and 0.5 μg/kg) into negative matrix in accordance with detection limit of purchased enzyme linked immunosorbent assay kit. Follow the instructions in the instruction manual to read the readings using the readout unit and perform three parallel tests. This batch of products can be used if recovery rate is within the allowable range of 50%~120% according to each standard addition-concentration.