



National Food Safety Standard of the People's Republic of China

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National Food Safety Standard  
Determination of Aflatoxins M<sub>1</sub> and B<sub>1</sub> in Foods

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# Preface

The Standard substitutes the GB/T5009.24-2003 Determination of Aflatoxins M<sub>1</sub> and B<sub>1</sub> in Foods

The replaced former editions are:

-GB/T 5009.24-1985, GB/T 5009.24-1996, GB/T 5009.24-2003.

## National Food Safety Standard

### Determination of Aflatoxins M<sub>1</sub> and B<sub>1</sub> in Foods

#### 1. Scope

The Standard stipulates the determination method of aflatoxins M<sub>1</sub> and B<sub>1</sub> such foods as milk and its products, butter and fresh tissues of pigs (liver, kidney, blood and lean meat).

The Standard applies to the determination of aflatoxins M<sub>1</sub> and B<sub>1</sub> in such foods as milk and its products, butter and fresh tissues of pigs (liver, kidney, blood and lean meat).

#### 2. Principle

After extraction, concentration and thin layer chromatography, the aflatoxins M<sub>1</sub> and B<sub>1</sub> in the sample will produce amethyst fluorescence under UV ( $\lambda=365\text{nm}$ ), the contents are then determined according to the minimum detectable quantity showing on the thin layer.

#### 3. Reagents and materials

3.1 Methanol: analytically pure

3.2 Petroleum ether: analytically pure

3.3 Chloroform: analytical pure

3.4 Anhydrous sodium sulfate: analytically pure

3.5 Isopropanol: analytically pure

3.6 Silica gel G: Used for chromatography.

3.7 Sodium Chloride and its solution (40g/L).

3.8 Sulfuric acid (1+3).

3.9 Glass sand: Clean and dry it after acid treatment. It should be about 20 meshes.

3.10 Standard solution of aflatoxins M<sub>1</sub>: Prepare standard solution of aflatoxin M<sub>1</sub> of 10 $\mu\text{g}/\text{ml}$  with chloroform. Take chloroform as the blank reagent, the wavelength of UV absorption peak of aflatoxin M<sub>1</sub> should be close to 357 nm, and molar extinction coefficient is 19 950. Protect from light, and store in the refrigerator at 4 $^{\circ}\text{C}$ .

3.11 Standard solution of aflatoxins M<sub>1</sub> and B<sub>1</sub> mixture: Prepare solution of aflatoxin M<sub>1</sub> and B<sub>1</sub> mixture of 0.04 $\mu\text{g}/\text{ml}$  respectively with chloroform. Protect from light, and store in the refrigerator at 4 $^{\circ}\text{C}$ .

#### 4. Instruments

4.1 10-mesh round-hole sieve.

4.2 Small pulverizer

4.3 Glass plate: 5cmx20cm

4.4 Expanded groove

4.5 UV lighter: 100W-125W, with 365nm filter glass.

4.6 Micro-injector

#### 5. Analytical procedures

The whole operation should be finished under dark condition.

##### 5.1 Sample extraction

5.1.1 Table 1 shows the details of sample extraction and preparation.

Table 1 Sample preparation

Sample name	Sample weight /g	Water volume /ml	Methanol volume /ml	Extracted solution volume <sup>a</sup> /ml	Volume of 40g/L sodium chloride /ml	Condensed volume /ml	Added volume /μL	Method sensitivity /μg/kg
Milk	30	0	90	62	25	0.4	100	0.1
Condensed milk	30	0	90	52	35	0.4	50	0.2
Milk powder	15	20	90	59	28	0.4	40	0.5
Cheese	15	5	90	56	31	0.4	40	0.5
Butter	10	45	55	80	0	0.4	50	0.5
Pig liver	30	0	90	59	28	0.4	50	0.2
Pig kidney	30	0	90	61	26	0.4	50	0.2
Lean pork	30	0	90	58	29	0.4	50	0.2
Pig blood	30	0	90	61	26	0.4	50	0.2

<sup>a</sup> The volume of extracted solution is calculated per formula (1).

$$X = \frac{8}{15} \times (90 + A + B) \dots\dots\dots(1)$$

Where:

X- volume of extracted solution, with the unit ml;

A- water content in sample, with the unit ml (the sample quantity of milk, condensed milk and pig tissue is 30g, and the sample quantity of milk powder and cheese is 15g);

B- volume of additive water, with the unit ml.

Note: The water content in sample refers to “Food composition table” .

There is 48ml methanol in each extracted solution, and need to add 39ml water to adjust the volume ratio of methanol and water to 55:45, so the volume of additive sodium chloride solution (40g/L) equals 87ml subtracted the volume of the extracted solution (ml).

5.1.2 Milk and condensed milk: weigh thoroughly mixed sample 30.00g, put it into a small beaker, add 90ml methanol and transfer it into a 300ml conical flask with cover, then fix the cover on to prevent leakage. Shake for 30min, filter it into a 100ml mixing cylinder with stopper with folding high speed filter paper. Gather 62ml milk and 52ml condensed milk (each is equivalent to 16g sample) extracted solution respectively according to Table 1.

5.1.3 Milk powder: weigh sample 15.00g, put it into a conical flask with cover, add water 20ml, then add methanol 90ml after the sample is wet, the following operation is as same as “Shake for 30 min...” in article 5.1.3. Gather 59ml extracted solution (equivalent to 8g sample) according to Table 1.

5.1.4 Cheese: weigh 10.00g thoroughly mixed sample which has been fine chopped and passed through 10-mesh circular screen, put it into a conical flask with cover, add 5ml water and 90ml methanol, the following operation is as same as “Shake for 30 min...” in Article 5.1.3. Gather 56ml extracted solution (equivalent to 8g sample) according to Table 1.

5.1.5 Butter: weigh 10.00g sample, put it into a small beaker, solve the butter with 40ml petroleum

ether and transfer it into a conical flask with cover. Add 45ml water and 55ml methanol, shake for 30 min, then transfer all the liquid into a separating funnel. Add 1.5g sodium chloride, shake for solving, and gather 80ml collected solution (equivalent to 8g sample) in a mixing cylinder with stopper according to Table 1 after stratification.

5.1.6 Fresh pig tissues: Take sample of fresh or freeze preservation pig tissues (including liver, kidney, blood and lean pork), fine chopped and homogeneously mixed, weigh 30.00g and put it in a small mortar, add little glass sand and levigate, crush the fresh blood with a beater, or agitate with glass bead for anticoagulation. Weigh thoroughly mixed sample 30.00g, put all the samples into a 300ml conical flask with cover, add 90ml methanol, the following operation is as same as "Shake for 30 min..." in Article 6.1.3. Gather 59ml pig liver, 61ml pig kidney, 58ml lean pork and 61ml pig blood (each is equivalent to 16g sample) extracted solution respectively according to Table 1.

## 5.2 Purification

- 5.2.1 Distribute and purify with petroleum ether: Transfer each of the above extracted solution into a 250ml separating funnel, add specified volume of sodium chloride solution (40g/l) depending on the food (see Table 1). Add 40ml petroleum ether, agitate for 2min, after stratification, transfer the lower layer of methanol-sodium chloride solution into the original measuring cylinder, pour out the upper layer of petroleum ether solution from the back cut of the separating funnel and discard it. Then transfer the solution in the measuring cylinder into the separating funnel. Repeat the above extraction operation with petroleum ether twice more, with 30ml petroleum ether each time, at last, transfer the solution in the measuring cylinder into the separating funnel. For the butter sample solution, it should be extracted with petroleum ether twice in total and with 40ml petroleum ether each time.
- 5.2.2 Distribute and extraction with chloroform: Add 20ml chloroform into the original measuring cylinder, shake the solution well, then pour it back into the original separating funnel, agitate for 2min. After stratification, transfer the lower layer of chloroform into the original measuring cylinder. Repeat the extraction operation twice with chloroform, with 10ml chloroform each time, merge them into the original measuring cylinder. Discard the upper layer of methanol solution.
- 5.2.3 Rinse chloroform layer with water and prepare concentrated product: Pour the merged chloroform into the original separating funnel, add 30ml sodium chloride solution (40g/l), agitate for 30s, and stand for a while. After part of the upper layer of dirty solution has clarified, collect the lower layer of chloroform in the original measuring cylinder. Add 10g anhydrous sodium sulfate, agitate, stand and clarify, filter it with low speed quantitative filter paper with little anhydrous sodium sulfate to a 100ml evaporating dish. Extract the sodium chloride solution with 10ml chloroform one time, filter it to the evaporating dish through filter. Then, pour the anhydrous sodium sulfate onto the filter paper, wash the measuring cylinder and anhydrous sodium sulfate with little chloroform, filter it to the evaporating dish, too. Put the evaporating dish on 65℃ water bath for ventilation and evaporation, use chloroform to transfer the residue in the evaporating dish to an evaporating pipe. If the residue in the evaporating dish is too much, filter it to the evaporating pipe with filter paper. Concentrate the solution to no more than 0.4ml at 65℃ with decompressing air purge, then wash the pipe wall with little chloroform, concentrate the solution to 0.4ml for use.

### 5.3 Determination

#### 5.3.1 Preparation of silica gel G thin layer plate

The thickness of the thin layer plate is 0.3mm. After activation for 2h at 105℃, it can be stored in a desiccator for 1d~2d.

#### 5.3.2 Tap

Take out two thin layer plates of 5cm×20cm, add two drops on the baseline 3cm away from the lower end of each plate, drop 10μL standard solution of aflatoxins M<sub>1</sub> and B<sub>1</sub> mixture on the first and the second plate at the left side where is 0.8~1cm away from the edge, drop the same kind of solution on each plate at the left side where is 2.8~3cm away from the edge, drop 10μL standard solution of aflatoxins M<sub>1</sub> and B<sub>1</sub> mixture at the second point on the second plate for the second time. Generally, the thin layer plate can be placed in a chromatography slot with dry silica gel for dropping, drying with a cold air blower while dropping.

#### 5.3.3 Spread

5.3.3.1 Lateral spread: Add 15ml absolute ether dehydrated with anhydrous sodium sulfate in advance (add 20g anhydrous sodium sulfate into 500ml absolute ether). Put the long side of the thin layer plate which is close to the benchmark into the slot, take out to evaporate after spreading to the end, spread for the second time with the same procedure as above.

5.3.3.2 Longitudinal spread: After lateral spread and evaporation, the thin layer plate is to be longitudinal spread to the point where is 10cm~12cm away from the benchmark with the mixing spreader isopropanol-acetone- benzene- hexane- petroleum ether (boiling range 60℃~90℃)-chloroform (5+10+10+10+10+55), take out and evaporate.

5.3.3.3 Lateral spread: After longitudinal spread and evaporation, the thin layer plate is to be lateral spread again with ethyl ether for once or twice, with the same procedure as 5.3.3.1.

#### 5.3.4 Observation and assessment result

5.3.4.1 Mutually compare and observe the first and the second plate under UV light, if the minimum detectable quantity (the R<sub>f</sub> value of M<sub>1</sub> and B<sub>1</sub> is 0.25 and 0.43 respectively) appears at the corresponding place of aflatoxins M<sub>1</sub> and B<sub>1</sub> benchmark at the second point on the second plate, while there is no phosphor dot at the same place on the first plate, the content of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the sample is below the specified sensitivity of the method (see Table 1).

5.3.4.2 If phosphor dot of aflatoxins M<sub>1</sub> and B<sub>1</sub> appears at the same place on the first plate, check if the sample solution at the second point on the second plate overlaps with the standard dropping point, if they overlap, do the following quantitative and confirmatory test.

#### 5.3.5 Dilute for quantification

If the fluorescence intensity of phosphor dot of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the sample solution corresponds to that of the minimum detectable quantity (0.0004μg) of aflatoxins M<sub>1</sub> and B<sub>1</sub>, the content of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the milk, condensed milk, milk powder, cheese and butter is 0.1, 0.2, 0.5, 0.5 and 0.5μg /kg respectively, and the content of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the fresh pig tissue (liver, kidney, blood and lean pork) sample is 0.2μg /kg each (see Table 1). If the fluorescence intensity of phosphor dot of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the sample solution is stronger than that of the minimum detectable quantity (0.0004μg) of aflatoxins M<sub>1</sub> and B<sub>1</sub>, the contents should be determined one by one according to the fluorescence intensity, decrease the dropping quantity (μL) or drop different quantity (μL) after dilution by estimation, until the fluorescence intensity of phosphor dot of aflatoxins M<sub>1</sub> and B<sub>1</sub> in the sample solution corresponds to that of the minimum detectable quantity.

## 5.3.6 Confirmatory test

Use a pin to circle the point of aflatoxins M<sub>1</sub> and B<sub>1</sub> to be confirmed on the thin layer plate where the qualitative test or quantitative test has been done. Spray sulfate acid solution (1+3), set aside for 5min, observe under a UV light, if the point of aflatoxins M<sub>1</sub> and B<sub>1</sub> of sample solution changes into yellow fluorescence as the standard point does, it can be further confirmed that the substance on the phosphor dot is aflatoxins M<sub>1</sub> and B<sub>1</sub>.

## 6 Formulation of analytical results

The content of aflatoxins M<sub>1</sub> and B<sub>1</sub> is calculated as Formula (2).

$$X = 0.0004 \times \frac{V_1}{V_2} \times D \times \frac{1000}{m} \dots\dots\dots(2)$$

Where:

X- content of aflatoxins M<sub>1</sub> and B<sub>1</sub>, with the unit µg/kg;

V<sub>1</sub>- volume of concentrated sample solution, with the unit ml;

V<sub>2</sub>- dropping volume of sample solution when the minimum fluorescence appears, with the unit ml;

D- total dilution multiple of concentrated sample solution;

m- equivalent sample quantity in the concentrated sample solution, with the unit g;

0.0004- minimum detectable quantity of aflatoxins M<sub>1</sub> and B<sub>1</sub>, with the unit µg.