INTERNATIONAL STANDARD

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# Milk and milk powder — Determination of aflatoxin $M_1$ content — Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography

*Lait et lait en poudre — Détermination de la teneur en aflatoxine $M_1$ — Purification par chromatographie d'immunoaffinité et détermination par chromatographie en phase liquide à haute performance*



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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 14501 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC International), and will also be published by these organizations.

Annex A of this International Standard is for information only.





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# Milk and milk powder — Determination of aflatoxin $M_1$ content — Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography



**WARNINGS**

**1 The method described in this International Standard requires the use of chloroform and aflatoxin $M_1$ solutions. Chloroform is an ozone-depleting substance. Aflatoxins are carcinogenic to human subjects. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) [4, 5].**

**2 Adequately protect from daylight the laboratory where the analyses are performed, and keep aflatoxin standard solutions protected from light, for example by using aluminium foil.**

**3 The use of non-acid-washed glassware (e.g. tubes, vials, flasks, beakers, syringes) for aqueous aflatoxin solutions may cause loss of aflatoxin. Moreover, brand new laboratory glassware coming into contact with aqueous solutions of aflatoxin should be soaked in dilute acid (e.g. sulfuric acid, 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (check to ensure pH is in the range 6 to 8).**

**4 Use a decontamination procedure for laboratory wastes such as solid compounds, solutions in organic solvents, glassware, aqueous solutions and spills. The procedure for decontamination was developed and validated in a programme of the International Agency for Research on Cancer (WHO) [4, 5]**

## 1 Scope

This International Standard specifies a method for the determination of aflatoxin $M_1$ content of milk and milk powder. The lowest level of validation is 0,08 µg/kg for whole milk powder i.e. 0,008 µg/l for reconstituted liquid milk. The method is also applicable to low fat milk, skimmed milk, low fat milk powder and skimmed milk powder.

## 2 Term and definition

For the purposes of this International Standard, the following term and definition apply.

**2.1**
**aflatoxin $M_1$ content**
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The aflatoxin $M_1$ content is expressed as micrograms per litre or micrograms per kilogram.

## 3 Principle

Aflatoxin $M_1$ is extracted by passing the test portion through an immunoaffinity column. The column contains specific antibodies bound onto a solid support material. As the sample passes through the column, the antibodies selectively bind with any aflatoxin $M_1$ (antigen) present and form an antibody-antigen complex. All other components of the sample matrix are washed off the column with water. Then aflatoxin $M_1$ is eluted from the column and the eluate is collected. The amount of aflatoxin $M_1$ present in this eluate is determined by means of high-performance liquid chromatography (HPLC) coupled with fluorimetric detection.

## 4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

### 4.1 Immunoaffinity column

The immunoaffinity column shall contain antibodies against aflatoxin $M_1$. The column shall have a maximum capacity of not less than 100 ng of aflatoxin $M_1$ (which corresponds to 2 µg/l when a volume of 50 ml of test portion is applied), and shall give a recovery of not less than 80 % for aflatoxin $M_1$ when a standard solution containing 4 ng of toxin is applied (which corresponds to 80 ng/l when a volume of 50 ml of sample is applied). Any immunoaffinity column meeting the performance specifications mentioned above can be used. The performance of the columns shall be checked regularly and at least once for every batch of columns (see 4.1.1 and 4.1.2).

#### 4.1.1 Capacity check

By means of a pipette (5.4) transfer 1,0 ml of the stock aflatoxin $M_1$ solution (4.5.2) to a 20 ml conical tube (5.9). Evaporate the solution slowly to dryness using a constant stream of nitrogen (4.3) and dissolve the residue obtained in 10 ml of the 10 % acetonitrile solution (4.2.2). Shake vigorously.

Add this solution to 40 ml of water. Mix well and apply the whole volume to the immunoaffinity column. Be careful to follow the recommendations given by the manufacturer for the use of the columns. Wash the column, elute the toxin and determine the amount bound to the column by HPLC after suitable dilution of the final eluate.

Calculate the recovery for the aflatoxin. Compare the result with the specification under 4.1.

#### 4.1.2 Recovery check

By means of a pipette (5.4) dilute 0,8 ml of the 0,005 µg/ml aflatoxin $M_1$ working solution (4.5.3) to 10 ml with water. Mix well and apply the whole volume to the immunoaffinity column. Be careful to follow the recommendations given by the manufacturer for the use of the columns. Wash the column, elute the toxin and determine the amount bound to the column by HPLC after suitable dilution of the final eluate. Calculate the recovery for the aflatoxin. Compare the result with the specification under 4.1.



### 4.2 Acetonitrile, pure, HPLC grade.

**4.2.1 Acetonitrile,** in water, 25 % solution by volume.

Add 250 ml of acetonitrile (4.2) to 750 ml of water (degas before use).

**4.2.2 Acetonitrile**, in water, 10 % solution by volume.

Add 100 ml of acetonitrile (4.2) to 900 ml of water (degas before use).

### 4.3 Nitrogen gas

**4.4 Chloroform,** stabilized with 0,5 % to 1,0 % ethanol (by mass).

### 4.5 Aflatoxin $M_1$ standard solutions

#### 4.5.1 Calibrant solution

Standard solution of aflatoxin $M_1$ in chloroform with a nominal concentration of 10 µg/ml.

Determine the concentration by measurement of its absorbance at the wavelength for maximum absorption as follows.

By using the spectrometer (5.11), record the absorbance of the calibrant solution against chloroform as blank between 340 nm and 370 nm. Measure the absorbance, $A$, at the wavelength of maximum absorption, $\lambda_{max}$, close to 360 nm. Calculate the concentration, $c_i$ in micrograms per millilitre, using the following equation:

$$c_i = A \times M \times 100/\varepsilon$$

where

$A$ is the numerical value of the absorbance at $\lambda_{max}$;

$M$ is the numerical value of the molar mass of the aflatoxin $M_1$ in grams per mole ($M$ = 328 g/mol);

$\varepsilon$ is the numerical value of the absorption coefficient of the toxin in chloroform, in square metres per mole ($\varepsilon$ = 1 995 $m^2$/mol).

#### 4.5.2 Stock solution

After checking the concentration of the calibrant solution (4.5.1), dilute the calibrant solution in chloroform to give an aflatoxin $M_1$ stock solution of 0,1 µg/ml. The stock solution shall be well-stoppered and wrapped in aluminium foil to exclude light.

Store the stock solution in a refrigerator at a temperature below 5 °C in the dark. Under these conditions the stock solution is stable for about 2 months. After 2 months, the stability should be checked.

#### 4.5.3 Working solutions of aflatoxin $M_1$

Before preparing working dilutions of the aflatoxin $M_1$ standard solution, allow the stock solution (4.5.2) to attain ambient temperature before removing aliquots of the solution for subsequent dilution. Prepare working solutions on the day of use.

Prepare a solution with a concentration of 0,005 µg/ml as follows. By means of a pipette (5.4) transfer 1,0 ml of the stock solution (4.5.2) to a 20 ml conical tube (5.9). Evaporate the solution to dryness using a gentle stream of nitrogen (4.3) and dissolve the residue obtained in 20,0 ml of the diluted acetonitrile (4.2.2). Shake occasionally over a period of 30 min.

Care should be taken when evaporating the solution to dryness to ensure the temperature does not drop so low that condensation occurs.

Use this diluted solution for the preparation of a series of appropriate dilutions of aflatoxin $M_1$ standard solution to provide, depending on the injection loop volume, for injection of 0,05 ng, 0,1 ng, 0,2 ng and 0,4 ng of aflatoxin $M_1$. Dilute by using diluted acetonitrile solution (4.2.2).

## 5 Apparatus

Usual laboratory equipment and, in particular, the following.

**5.1 Disposable syringes**, of capacities 10 ml and 50 ml.

**5.2 Vacuum system** (e.g. Büchner flask, Vac-Elute system[1)] or peristaltic pump).

**5.3 Centrifuge**, capable of producing a radial acceleration of 4 000 × $g$.

**5.4 Pipettes**, of capacities 1,0 ml, 2,0 ml and 50,0 ml.

**5.5 Glass beakers**, of capacity 250 ml.

**5.6 Volumetric flask**, of capacity 100 ml.

**5.7 Water baths**, capable of operating at 30 °C ± 2°C, 50 °C ± 2°C, and between 35 °C and 37 °C.

**5.8 Filter paper** (Whatman No. 4[1)], or equivalent).

**5.9 Graduated conical glass tubes**, with ground glass neck and stopper, of capacities of 5 ml, 10 ml and 20 ml.

**5.10 HPLC-equipment**

**5.10.1 Pulse-free pump**, suitable for constant volume flow rate of about 1 ml/min.

**5.10.2 Injector system**, with fixed or variable injection volume loop, suitable for injection of 50 µl to 500 µl.

**5.10.3 Reversed-phase HPLC analytical column**, with 3 µm or 5 µm packing of octadecyl silicagel plus guard column filled with reverse-phase material.

**5.10.4 Fluorescence detector**, capable of providing about 365 nm excitation and 435 nm emission wavelengths allowing detection of (5 × noise) aflatoxin $M_1$ when 0,02 ng is injected under suitable chromatographic conditions.

**5.10.5 Strip chart recorder,** with a printer or plotter, or electronic integrator or computer-based data processing system.

**5.11 Spectrometer**, capable of measuring at wavelengths from 200 nm to 400 nm, with quartz face cells of optical path length 1 cm.

**5.12 Balance**, capable of weighing to the nearest 0,1 g with readability to 0,01 g.

## 6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

## 7 Procedure

### 7.1 General

Carry out the procedure with daylight excluded, as far as possible.

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1) Vac-Elute system and Whatman are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

Methods for reconstituting the milk powder, loading onto the affinity columns, washing the column and elution vary slightly between column manufacturers. The specific instructions supplied with the columns should be followed precisely. In general, procedures involve reconstitution of milk powder with water or saline buffer, centrifugation, loading under pressure onto (possibly pre-washed) column, washing the column with water and elution of aflatoxin with methanol or acetonitrile. The instructions for flow rates should be followed carefully.

## 7.2 Preparation of test samples

### 7.2.1 Milk

Warm the milk sample to between 35 °C and 37 °C in the water bath (5.7). Either filter the milk through filter paper(s) (5.8) (if necessary use several filters), or centrifuge at a radial acceleration of 4 000 × $g$ for 15 min. Collect at least 50 ml of prepared milk. Continue as specified in 7.4.

### 7.2.2 Milk powder

Weigh, to the nearest 0,1 g, 10 g of sample into a 250 ml beaker (5.5). Take 50 ml of water prewarmed to 50 °C and add this in small amounts to the milk powder. Mix, using a stirring rod, until a homogeneous mixture is obtained.

If the milk powder is not completely dissolved, place the beaker in a water bath (5.7) at 50 °C for at least 30 min. Mix carefully.

Allow the solution of milk powder to cool to 20 °C and then quantitatively transfer it to a 100 ml volumetric flask (5.6) using small amounts of water. Dilute to the 100 ml mark with water. Filter enough reconstituted milk through filter paper(s) (5.8) or centrifuge at a radial acceleration of 4 000 × $g$ for 15 min. Collect at least 50 ml of prepared milk. Continue as specified in 7.4.

## 7.3 Immunoaffinity column preparation

Attach the barrel of a 50 ml disposable syringe (5.1) to the top of the immunoaffinity column (4.1). Connect the immunoaffinity column to the vacuum system (5.2).

## 7.4 Extraction and purification of samples

Pipette 50 ml of the prepared test sample (7.2.1 or 7.2.2) into the 50 ml syringe barrel (5.1) and allow it to pass through the immunoaffinity column at a slow steady volume flow rate of 2 ml/min to 3 ml/min, controlling the volume flow rate by means of the vacuum system (5.2).

Remove the 50 ml syringe barrel and replace it by a clean 10 ml syringe barrel. Wash the column with 10 ml of water. Pass the water through the column at a steady volume flow rate. Blow the column completely dry after washing.

Disconnect the column from the vacuum system. Elute aflatoxin $M_1$ slowly from the column by passing 4 ml of acetonitrile (4.2) through the column using a 10 ml syringe. Allow the acetonitrile to take about 60 s to pass through the column. Control the volume flow rate by means of the syringe plunger. Collect the eluate in a conical tube (5.9). Reduce the volume of the eluate to a volume $V_e$ of 50 µl to 500 µl at 30 °C, using a gentle stream of nitrogen (4.3).

**WARNING: Losses may occur if evaporated to complete dryness.**

Dilute to a final eluate volume $V_f$ of 10 times $V_e$ (i.e. 500 µl to 5 000 µl with water; see Note).

NOTE If the acetonitrile content of the injected extract, containing aflatoxin $M_1$, exceeds the 10 % limit, peak broadening will occur on the HPLC chromatogram. However, a water content of over 90 % has no influence on the peak shape [8].

## 7.5 High-performance liquid chromatography

### 7.5.1 Pump setting

Pump the eluent (4.2.1) at a constant volume flow rate through the HPLC column. If necessary (depending on the type of column used), the acetonitrile-water ratio of the HPLC eluent (4.2.1) may be adjusted to ensure an optimal separation of the aflatoxin $M_1$ from other extract components.

The volume flow rate of the eluent (4.2.1) also depends on the column (5.10.3). As a guideline for conventional columns (with a length of approximately 25 cm and an internal diameter of approximately 4,6 mm), a volume flow rate of approximately 1 ml/min gives optimal results; for HPLC columns with an internal diameter of 3 mm, a volume flow rate of 0,5 ml/min is optimal.

It is advisable to ascertain optimal conditions using a sample extract (preferably free from aflatoxin $M_1$) which is injected separately and in combination with an aflatoxin $M_1$ standard.

### 7.5.2 Chromatographic performance

The linearity of the standard solution injections and the stability of the chromatographic system shall be checked. Repeatedly inject an aflatoxin $M_1$ standard solution (fixed amount) until stable peak areas or heights are achieved. Consecutive injections shall not differ more than 5 % in peak area or peak height.

The responses in retention time of aflatoxin $M_1$ are dependent on the temperature. Therefore compensation shall be made for drift in the detection system. By injecting a fixed amount of aflatoxin $M_1$ standard solution at regular intervals, the results for these standard solutions can be corrected for the drift observed.

### 7.5.3 Calibration curve of aflatoxin $M_1$

Inject in sequence a suitable volume, $V_i$, depending on the injection loop, of aflatoxin $M_1$ standard solutions which contain 0,05 ng, 0,1 ng, 0,2 ng and 0,4 ng of aflatoxin $M_1$ respectively. Prepare a calibration graph by plotting the peak area or peak height against the mass of aflatoxin $M_1$ injected.

### 7.5.4 Analysis of the purified extracts and injection scheme

Inject a suitable volume, $V_i$, of the eluate (7.4) into the HPLC apparatus via the injection loop. Separate all the aflatoxin $M_1$ present, using the same conditions as for the standard solutions. Perform the injection of standards and sample extracts according to a specified injection scheme.

When a series of sample eluates is to be injected one after the other, it is recommended that an aflatoxin $M_1$ standard is injected after every five injections of sample eluates.

Determine the area or height of the aflatoxin $M_1$ peak of the sample eluate. From the calibration graph determine the mass, in nanograms, of aflatoxin $M_1$ in the injected volume of sample extract.

If the peak area or peak height of aflatoxin $M_1$ in the sample eluate is greater than that of the highest standard solution, dilute the eluate quantitatively with water and re-inject the diluted extract into the HPLC apparatus.



# 8 Calculation and expression of results

## 8.1 Milk

### 8.1.1 Calculation

Calculate the aflatoxin $M_1$ content of the test sample, $w_m$, in micrograms per litre, using the following equation:

$$w_m = m_A \times (V_f/V_i) \times (1/V)$$

where

$m_A$ is the numerical value of the mass of aflatoxin $M_1$, in nanograms, corresponding to the area or height of the aflatoxin $M_1$ peak of the sample eluate;

$V_i$ is the numerical value of the volume of the sample eluate injected; in microlitres;

$V_f$ is the numerical value of final volume of the sample eluate; in microlitres;

$V$ is the numerical value of the volume of the prepared test sample passing through the column, in millilitres.

#### 8.1.2 Expression of results

Express the results in micrograms per litre to 3 decimal places.

### 8.2 Milk powder

#### 8.2.1 Calculation

Calculate the aflatoxin $M_1$ content of the sample, $w_p$ , in micrograms per kilogram, using the following equation:

$$w_p = m_A \times (V_f/V_i) \times (1/m)$$

where

$m$ is the numerical value of the mass of milk powder in 50 ml of the prepared test solution (7.4), in grams;

$m_A$, $V_f$ and $V_i$ are as in 8.1.1.

This equation is applicable only when no dilution has been carried out, otherwise the dilution should be taken into account.

#### 8.2.2 Expression of results

Express the results in micrograms per kilogram to 3 decimal places.

## 9 Precision

Details of an interlaboratory test on the precision are summarized in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

## 10 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result(s) obtained; or

— if the repeatability has been checked, the final quoted result(s) obtained.