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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

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 14501 | IDF 171 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

This second edition of ISO 14501 | IDF 171 cancels and replaces the first edition (ISO 14501:1998), which has been technically revised.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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All work was carried out by the Joint IDF-ISO Action Team on *Organic contaminants* of the Standing Committee on *Analytical methods for additives and contaminants* under the aegis of its project leader, Mr. L. Sørensen (DK).

This edition of ISO 14501 | IDF 171 cancels and replaces IDF 171:1995, which has been technically revised.

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

1 Scope

This International Standard specifies a method for the determination of aflatoxin M_1 content in milk and milk powder. The limit of detection 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.

CAUTION

1 **The method described in this protocol requires the use of solutions of aflatoxin M_1. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer** [4], [5].

2 **Protect the laboratory in which the analyses are performed adequately from daylight and keep aflatoxin standard solutions protected from light, e.g. 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, before 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 decontamination procedures for laboratory wastes such as solid compounds, solutions in organic solvents, aqueous solutions and spills, and for glassware coming into contact with carcinogenic materials. Suitable decontamination procedures have been developed and validated by the International Agency for Research on Cancer** [4], [5].

2 Terms and definitions

For the purposes of this document the following terms and definitions apply.

2.1
aflatoxin M_1 content
concentration or mass fraction of substances determined by the procedure specified in this International Standard.

NOTE The aflatoxin M_1 concentration is expressed in micrograms per litre and the mass fraction in micrograms per kilogram.

3 Principle

Aflatoxin M_1 is extracted by passing the test portion through an immunoaffinity column that contains specific antibodies bound onto a solid support material.

As the sample passes through the column, the antibodies are selectively bound 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 only 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 a test portion is applied). It 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 the procedure in 4.1.1 and 4.1.2).

4.1.1 **Capacity check**.

Dilute 1,0 ml of aflatoxin M_1 standard stock solution (4.4.2) to 50 ml with water. Mix well and apply the whole volume to the immunoaffinity column carefully following the recommendations given by the manufacturer for the use of columns. Wash the column and elute the toxin. Determine the amount of aflatoxin M_1 eluted from the column by HPLC after preparing a suitable dilution of the final eluate.

Calculate the capacity for the aflatoxin. Compare the result with the requirements given in 4.1.

4.1.2 **Recovery check**.

Use a pipette (5.4) to dilute 0,8 ml of aflatoxin M_1 standard working solution of 0,005 µg/ml (4.4.3) to 10 ml with water. Mix well and apply the whole volume to the immunoaffinity column carefully following the recommendations given by the manufacturer for the use of columns. Wash the column and elute the toxin. Determine the amount of aflatoxin M_1 eluted from the column by HPLC after preparing a suitable dilution of the final eluate.

Calculate the recovery for the aflatoxin. Compare the result with the requirements given in 4.1.

4.2 **Acetonitrile**, pure, HPLC grade.

4.2.1 **Acetonitrile solution**, 25 %.

Add 250 ml of acetonitrile (4.2) to 750 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution (eluent) before using it.

4.2.2 **Acetonitrile solution**, 10 %.

Add 100 ml of acetonitrile (4.2) to 900 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution before using it.

4.3 **Nitrogen gas**.

4.4 **Aflatoxin M_1 standard solutions**.

4.4.1 **Aflatoxin M_1 standard calibration solution**.

Prepare an aflatoxin M_1 standard calibration solution by dissolving aflatoxin M_1 ($C_{17}H_{12}O_7$) in acetonitrile (4.2) to give a nominal concentration of 10 µg/ml. Determine the actual aflatoxin M_1 concentration by measurement of the absorbance at the maximum absorption wavelength of the solution as follows.

Use the spectrophotometer (5.14) to record the absorbance of the aflatoxin M_1 standard calibration solution against acetonitrile (4.2) as blank at wavelengths between 330 nm and 370 nm. Measure the absorbance, A, at its maximum absorption wavelength, λ_{max}, which is close to 350 nm.

Calculate the concentration, c_1, expressed in micrograms per millilitre, by using Equation (1):

$$c_1 = A \times M \times \frac{100}{d \times \varepsilon} \quad (1)$$

where:

A is the numerical value of the absorbance at λ_{max};

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

d is the optical pathlength, in centimetres (d = 1 cm);

ε is the numerical value of the absorption coefficient, in square metres per mole, of the toxin in acetonitrile (ε = 1 985 $m^2 \cdot mol^{-1}$).

4.4.2 Aflatoxin M_1 standard stock solution.

After checking its concentration, dilute the aflatoxin M_1 standard calibration solution (4.4.1) with acetonitrile (4.2) to an aflatoxin M_1 standard stock solution of 0,1 µg/ml. The standard stock solution shall be well stoppered and wrapped in aluminium foil to protect it from light.

Store the aflatoxin M_1 standard stock solution in a refrigerator at a temperature between 1 °C and 5 °C in the dark. Under these conditions the stock solution is stable for at least two months. If the standard stock solution is more than two months old, determine the aflatoxin M_1 concentration before use. If there is any change, discard the solution and prepare a fresh standard stock solution.

4.4.3 Aflatoxin M_1 standard working solutions.

Before preparing the aflatoxin M_1 standard working solutions, allow the standard stock solution (4.4.2) to attain ambient temperature. Prepare the standard working solutions on the day of use.

Dilute the aflatoxin M_1 standard stock solution (4.4.2) with the 10 % acetonitrile solution (4.2.2) to an aflatoxin M_1 concentration of 0,005 µg/ml.

Remove aliquots of the diluted standard stock solution to prepare a series of standard working solutions containing, for example, 0,05 ng/ml, 0,10 ng/ml, 0,20 ng/ml, and 0,40 ng/ml of aflatoxin M_1 by diluting with the 10 % acetonitrile solution (4.2.2). Other final dilutions may be chosen, depending on the injection loop volume.

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 [i.e. Büchner flask, Vac-Elut system [1)] or peristaltic pump].

5.3 Centrifuge, capable of producing a radial acceleration of at least 2 000*g*.

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

5.5 Glass beakers, of capacity 250 ml.

5.6 One-mark volumetric flask, of capacity 100 ml.

5.7 Water baths, capable of operating at 30 °C ± 2 °C, at between 35 °C and 37 °C and 50 °C ± 5 °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 5 ml, 10 ml and 20 ml.

5.10 HPLC apparatus, equipped with a pulse-free pump, capable of producing a constant volume flow rate of about 1 ml/min, and an injector system, with a fixed or variable injection volume loop, capable of injecting volumes of 20 µl to 500 µl.

5.11 Reversed phase HPLC analytical column, with 3 µm or 5 µm octadecyl silica packing and a guard column filled with reverse phase material.

5.12 Fluorescence detector, capable of providing about 365 nm excitation and 435 nm emission wavelengths and of detecting (signal to noise ratio: 5) aflatoxin M_1 when 0,02 ng is injected under appropriate chromatographic conditions.

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

5.14 Spectrophotometer, capable of measuring at wavelengths from 200 nm to 400 nm, with quartz face cells of optical pathlength 1 cm.

5.15 Analytical balance, capable of weighing to the nearest 0,01 g.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.

7 Procedure

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

Notice that the procedures for reconstituting milk powder, for centrifugation, for loading the sample on to the affinity columns, for washing the column and elution will vary slightly between column manufacturers. Follow precisely, therefore, the specific instructions supplied with the columns.

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1) The Vac-Elut 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 or IDF of these products.

7.1 Preparation of test samples

7.1.1 Milk

Warm the test sample in the water bath (5.7) to between 35 °C and 37 °C. Either filter the sample through filter paper(s) (5.8) using several filters, if necessary, or centrifuge it at a radial acceleration of at least 2 000g for 15 min. Collect at least 50 ml of the thus prepared skimmed milk sample. Continue as specified in 7.3.

7.1.2 Milk powder

Weigh, to the nearest 0,01 g, 10 g of test sample into a 250 ml beaker (5.5). Add 50 ml water, prewarmed in the water bath (5.7) to 50 °C, in small amounts to the test sample. Mix, using a stirring rod, until a homogeneous mixture is obtained.

If the test sample does not become completely suspended, place the beaker in a water bath (5.7) set at 50 °C for at least 30 min. Stir the mixture frequently.

Allow the test solution to cool to between 20 °C and 25 °C. Then, quantitatively transfer the test solution to a 100 ml one-mark volumetric flask (5.6) using small amounts of water. Dilute to the 100 ml mark with water. Filter enough of the reconstituted sample through filter paper(s) (5.8) or centrifuge it at a radial acceleration of at least 2 000g for 15 min. Collect at least 50 ml of the prepared milk powder sample. Continue as specified in 7.3.

7.2 Immunoaffinity column preparation

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

7.3 Extraction and purification of samples

Add 50 ml of the prepared test sample (7.1.1 or 7.1.2) into the 50 ml syringe barrel (5.1). Allow it to pass through the immunoaffinity column at a rate of 2 ml/min to 3 ml/min while controlling the volume flow by using the vacuum system (5.2).

Replace the 50 ml syringe barrel by a clean 10 ml syringe barrel. Wash the column with 10 ml water by allowing it to pass through the column at a steady volume flow rate. Blow the column to completely dry it after washing.

Disconnect the column from the vacuum system. Elute aflatoxin M_1 slowly from the column by passing 4 ml pure acetonitrile (4.2) in about 60 s through the column using a 10 ml syringe. Control the volume flow rate by means of the syringe plunger.

Collect the eluate in a conical tube (5.9). Reduce the eluate to a volume, V_e, of between 20 µl and 500 µl, by placing the tube in the water bath (5.7) set at 30 °C and blowing a gentle stream of nitrogen (4.3) over it.

WARNING — Losses may occur when evaporating the eluate to complete dryness.

Make up to a final eluate volume, $V_f = 10V_e$, 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 % (volume fraction) limit, peak broadening will occur on the HPLC chromatogram. However, a water content of over 90 % (volume fraction) has no influence on the peak-shape [8].

7.4 High performance liquid chromatography

7.4.1 Pump setting

Pump the eluent (4.2.1) at a constant flow rate through the HPLC column. Depending on the type of column used, adjust the acetonitrile/water ratio of the HPLC eluent (4.2.1), if necessary, to ensure an optimal separation of the aflatoxin M_1 from other extract components.

NOTE The flow rate of the eluent (4.2.1) also depends on the column (5.11) used. As guidelines for conventional columns with a length of approximately 25 cm: internal diameter about 4,6 mm, the optimal flow rate is approximately 1 ml/min; with internal diameter about 3 mm, the optimal flow rate is approximately 0,5 ml/min.

It is advisable to ascertain optimal conditions by using a sample extract (preferably free from aflatoxin M_1) which is injected separately and in combination with an aflatoxin M_1 standard working solution (4.4.3).

7.4.2 Chromatographic performance

Check the stability of the chromatographic system by repeatedly injecting a fixed amount of aflatoxin M_1 standard working solution (4.4.3) 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 the aflatoxin M_1 peaks depend on the temperature. Therefore, to compensate for drift in the detection system, inject a fixed amount of aflatoxin M_1 standard working solution (4.4.3) at regular intervals. If needed, the result for the standard working solution used can be corrected for the observed drift.

7.4.3 Calibration curve of aflatoxin M_1

Inject, in sequence, suitable volumes of the standard working solutions (4.4.3) containing 0,05 ng, 0,10 ng, 0,20 ng and 0,40 ng of aflatoxin M_1 into the HPLC apparatus via the injection loop. Prepare a calibration graph by plotting the obtained peak area or peak height for each standard working solution against the mass of aflatoxin M_1 injected.

7.4.4 Analysis of the purified extracts and injection scheme

Inject a similar volume of the eluate (7.3) to that used for the standard working solutions (7.4.3) into the HPLC apparatus via the injection loop. Separate 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 working solution is injected after every five injections of sample eluates.

Determine the area or height of the aflatoxin M_1 peak of the sample eluate. Calculate, from the calibration graph, the mass, in nanograms, of aflatoxin M_1 in the 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. Re-inject the diluted extract into the HPLC apparatus as described above.

8 Calculation and expression of results

8.1 Milk

8.1.1 Calculation

Calculate the aflatoxin M_1 content, as a concentration, of the test sample, c, in micrograms per litre, by using Equation (2):

$$c = m_a \times \left(\frac{V_f}{V_i}\right) \times \left(\frac{1}{V_t}\right) \quad (2)$$

where

m_a is the mass, in nanograms, of aflatoxin M_1 corresponding to the area or height of the aflatoxin M_1 peak of the sample eluate;

V_i is the volume, in microlitres, of the test sample eluate injected;

V_f is the final volume, in microlitres, of the test sample eluate;

V_t is the volume, in millilitres, of the prepared test solution passing through the column.

8.1.2 Expression of results

Express the test results to 3 decimal places.

8.2 Milk powder

8.2.1 Calculation

Calculate the aflatoxin M_1 content, as a mass fraction, of the test sample, w, in micrograms per kilogram, by using Equation (3):

$$w = m_a \times \left(\frac{V_f}{V_i}\right) \times \left(\frac{1}{m_t}\right) \times f \quad (3)$$

where

m_t is the mass, in grams, of the test sample present in 50 ml of the prepared test sample (7.3);

f is the dilution factor of the test sample (for undiluted solutions, $f = 1$).

8.2.2 Expression of results

Express the test results to 3 decimal places.

9 Precision

9.1 Interlaboratory test

The values for repeatability and reproducibility derived from the interlaboratory test were determined in accordance with ISO 5725-1 and ISO 5725-2. Details of the test are summarized in Annex A.

The values obtained may not be applicable to concentration ranges and matrices other than those given.

9.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the values given in Table A.1.

9.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the values given in Table A.1.

10 Test report

The test report shall specify:

a) all information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, together with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident that may have influenced the result(s).

e) the test result(s) obtained, and, if the repeatability has been checked, the final quoted result(s) obtained.