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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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## Forewords

**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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products - Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF.

This third edition cancels and replaces the second edition (ISO 14501 | IDF 171:2007), which has been technically revised. The main changes compared with the previous edition are as follows:

- the lack of detailed explanation in some clauses was leading to variability in the way the method was executed from one laboratory to another. Practical information from skilled end users familiar with routine analysis using this protocol was taken into account in this revision to clarify those ambiguous clauses.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).



**IDF (the International Dairy Federation)** is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by the IDF *Standing Committee on Analytical Methods for Additives and Contaminants* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF-ISO Action Team on A12 of the *Standing Committee on Analytical Methods for Additives and Contaminants* under the aegis of its project leader Mr Paul Jamieson.





# Milk and milk powder — Determination of aflatoxin M<sub>1</sub> content — Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography

**CAUTION 1** — The method described in this document requires the use of solutions of aflatoxin M<sub>1</sub>. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO)<sup>[1][2]</sup>.

**CAUTION 2** — Adequately protect the laboratory in which the analyses are performed from daylight and keep aflatoxin M<sub>1</sub> standard solutions protected from light, e.g. by using aluminium foil.

**CAUTION 3** — The use of non-acid-washed glassware (e.g. tubes, vials, flasks, beakers, syringes) for aqueous aflatoxin solutions can cause loss of aflatoxin M<sub>1</sub>. Moreover, brand new laboratory glassware, before coming into contact with aqueous solutions of aflatoxin M<sub>1</sub>, should be soaked in dilute acid (e.g. sulfuric acid,  $c = 2 \text{ mol/l}$ ) 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).

**CAUTION 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 (WHO)<sup>[1][2]</sup>.

## 1 Scope

This document specifies a method for the determination of aflatoxin M<sub>1</sub> content in 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 limit of detection (LOD) is 0,05 µg/kg for milk powder and 0,005 µg/kg for liquid milk. The limit of quantification (LOQ) is 0,1 µg/kg for milk powder and 0,01 µg/kg for liquid milk.

The method is also applicable to low-fat milk, skimmed milk, low-fat milk powder and skimmed milk powder.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <https://www.iso.org/obp>

### 3.1

#### **aflatoxin M<sub>1</sub> content**

concentration or mass fraction of substances determined by the procedure specified in this document

Note 1 to entry: Concentration is expressed in µg/l and mass fraction is expressed in µg/kg.



- 5) calculate the recovery for the aflatoxin;
- 6) compare the result with the requirements given above. The concentration shall not be less than 0,064 µg/l. Recovery checks can also be conducted with commercially available reference materials.

## 5.2 Acetonitrile, pure, HPLC grade.

### 5.2.1 Acetonitrile solution, volume fraction of 25 % in water.

Add 250 ml of acetonitrile (5.2) to 750 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution (eluent) before using it.

### 5.2.2 Acetonitrile solution, volume fraction of 10 % in water.

Add 100 ml of acetonitrile (5.2) to 900 ml of water and mix. Other volumes in the same proportion may be used. Degas the solution (eluent) before using it.

## 5.3 Nitrogen gas.

## 5.4 Aflatoxin M<sub>1</sub> standard solutions.

### 5.4.1 Aflatoxin M<sub>1</sub> standard calibration solution, (mass concentration $\rho = 10$ µg/ml aflatoxin M<sub>1</sub> in acetonitrile).

Prepare an aflatoxin M<sub>1</sub> standard calibration solution by dissolving aflatoxin M<sub>1</sub> (C<sub>17</sub>H<sub>12</sub>O<sub>7</sub>) in acetonitrile (5.2) to give a nominal concentration of 10 µg/ml. Determine the actual aflatoxin M<sub>1</sub> concentration by measurement of the absorbance at the maximum absorption wavelength of the solution as follows.

Use the spectrophotometer (6.13) to record the absorbance of the aflatoxin M<sub>1</sub> standard calibration solution against acetonitrile (5.2) as blank at wavelengths between 330 nm and 370 nm. Measure the absorbance,  $A$ , at its maximum absorption wavelength,  $\lambda_{\max}$ , which is close to 350 nm.

Calculate the concentration,  $\rho_1$ , expressed in micrograms per millilitre, by using [Formula \(1\)](#):

$$\rho_1 = A \times M \times \frac{100}{d \times \varepsilon} \quad (1)$$

where

- $A$  is the numerical value of the absorbance at  $\lambda_{\max}$ ;
- $M$  is the molar mass of aflatoxin M<sub>1</sub>, in grams per mole ( $M = 328$  g/mol);
- $d$  is the optical path length, in centimetres ( $d = 1$  cm);
- $\varepsilon$  is the numerical value of the absorption coefficient of the toxin in acetonitrile, in square metres per mole ( $\varepsilon = 1\,985$  m<sup>2</sup>·mol<sup>-1</sup>).

Alternatively, certified reference materials are available commercially (for example BCR-423 10 µg/ml aflatoxin M<sub>1</sub> in chloroform).

### 5.4.2 Aflatoxin M<sub>1</sub> standard stock solution, ( $\rho = 0,1$ µg/ml aflatoxin M<sub>1</sub> in acetonitrile).

After checking its concentration, dilute the aflatoxin M<sub>1</sub> standard calibration solution (5.4.1) with 25 % acetonitrile (5.2.1) to an aflatoxin M<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> concentration before use. If there is any change, discard the solution and prepare a fresh standard stock solution.

**5.4.3 Aflatoxin M<sub>1</sub> standard working solutions**, ( $\rho = 0,005 \mu\text{g/ml}$  aflatoxin M<sub>1</sub> in a mixture of nine parts per volume of water and one part per volume of acetonitrile).

Before preparing the aflatoxin M<sub>1</sub> standard working solutions, allow the standard stock solution ([5.4.2](#)) to attain ambient temperature. Prepare the standard working solutions on the day of use.

Dilute the aflatoxin M<sub>1</sub> standard stock solution ([5.4.2](#)) with the 10 % acetonitrile solution ([5.2.2](#)) to an aflatoxin M<sub>1</sub> concentration of 0,005  $\mu\text{g/ml}$ .

Remove aliquots of the diluted standard stock solution to prepare a series of five standard working solutions containing, for example, 0,05 ng/ml, 0,10 ng/ml, 0,20 ng/ml, 0,40 ng/ml, and 0,80 ng/ml of aflatoxin M<sub>1</sub> by diluting with the 10 % acetonitrile solution ([5.2.2](#)). Other final dilutions may be chosen, depending on the injection loop volume.

In some cases, better peak shape may be obtained with diluting the aflatoxin M<sub>1</sub> standard stock solution ([5.4.2](#)) with a mixture of water and acetonitrile in the same acetonitrile/water ratio as the eluent ([5.2.1](#)).

## 6 Apparatus

Usual laboratory equipment and, in particular, the following:

- 6.1 Disposable syringes**, of capacities 10 ml and 50 ml.
- 6.2 Vacuum system**, e.g. Büchner flask, Vac-Elut system<sup>2)</sup> or peristaltic pump.
- 6.3 Centrifuge**, capable of producing a radial acceleration of at least 2 000 *g*.
- 6.4 Pipettes**, of capacities 1,0 ml, 2,0 ml and 50,0 ml, or suitable autopipette.
- 6.5 Glass beakers**, of capacity 250 ml.
- 6.6 One-mark volumetric flask**, of capacity 100 ml.
- 6.7 Water baths**, capable of operating at 30 °C ± 2 °C, at between 35 °C and 37 °C and 50 °C ± 5 °C.
- 6.8 Filter paper**, Whatman No. 4<sup>2</sup> or equivalent.
- 6.9 Graduated conical glass tubes**, with ground glass neck and stopper of capacities 5 ml, 10 ml and 20 ml.
- 6.10 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  $\mu\text{l}$  to 500  $\mu\text{l}$ .

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2) The Vac-Elut system and Whatman are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of these products.



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

**6.12 Fluorescence detector**, capable of providing about 365 nm excitation and 425 nm emission wavelengths and of detecting (signal to noise ratio: 5) aflatoxin M<sub>1</sub> when 0,02 ng is injected under appropriate chromatographic conditions.

**6.13 Spectrophotometer**, capable of measuring at wavelengths from 200 nm to 400 nm, with quartz cuvettes of optical pathlength 1 cm.

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

## 7 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 document. A recommended sampling method is given in ISO 707 | IDF 50<sup>[3]</sup>.

## 8 Procedure

### 8.1 General

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

Notice that the procedures 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.

### 8.2 Preparation of test samples

#### 8.2.1 Milk

Warm the test sample in the water bath (6.7) to between 35 °C and 37 °C. Either filter the sample through filter paper(s) (6.8) using several filters, if necessary, or centrifuge it at a radial acceleration of at least 2 000 *g* for 15 min. Collect at least 50 ml of the thus prepared skimmed milk sample. Continue as specified in 8.4.

Failure to remove fat can cause blockage of the cartridge. To aid in the removal of fat after centrifugation, the sample can be cooled to 4 °C to 10 °C until the fat is solidified.

#### 8.2.2 Milk powder

Weigh, to the nearest 0,01 g, 10 g of test sample into a 250 ml beaker (6.5). Add 50 ml water, prewarmed in the water bath (6.7) 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 (6.7) 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 (6.6) using small amounts of water. Dilute to the 100 ml mark with water. Filter enough of the reconstituted sample through filter paper(s) (6.8) or centrifuge it at a radial acceleration of at least 2 000 *g* for 15 min. Collect at least 50 ml of the prepared milk powder sample. Continue as specified in 8.4.



### 8.3 Immunoaffinity column preparation

Attach the barrel of a 50 ml disposable syringe (6.1) to the top of an immunoaffinity column (5.1). Connect the immunoaffinity column to the vacuum system (6.2).

### 8.4 Test sample purification

Add 50 ml of the prepared test sample (8.2.1 or 8.2.2) into the 50 ml syringe barrel (6.1). 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 (6.2).

Replace the 50 ml syringe barrel by a clean 10 ml syringe barrel. Wash the column with  $2 \times 10$  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 (5.2) in about 60 s through the column using a 10 ml syringe. Control the volume flow rate by means of the syringe plunger. Consider the manufacturer's instructions.

Collect the eluate in a conical tube (6.9). Reduce the eluate to dryness by placing the tube in the water bath (6.7) set at 50 °C and blowing a gentle stream of nitrogen (5.3) over it.

Make up to a final eluate volume,  $V_f = 10V_e$ , i.e. 500 µl to 5 000 µl, with water (see NOTE).

Alternatively, 25 ml of samples can be applied to the immunoaffinity column and the final reconstitution volume decreased to 200 µl.

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<sup>[4]</sup>.

### 8.5 High performance liquid chromatography (HPLC)

#### 8.5.1 Pump setting

Pump the eluent (5.2.1) 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 (5.2.1), if necessary, to ensure an optimal separation of the aflatoxin  $M_1$  from other extract components.

NOTE The flow rate of the eluent (5.2.1) also depends on the column (6.11) 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 and typical elution conditions isocratic eluents (mixture of 80 parts per volume of water, 12 parts per volume of isopropanol and 8 parts per volume of acetonitrile or a mixture of 1 000 parts per volume of water, 275 parts per volume of methanol and 275 parts per volume of acetonitrile) depending on the column.

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 (5.4.3).

#### 8.5.2 Chromatographic performance

Check the stability of the chromatographic system by repeatedly injecting a fixed amount of aflatoxin  $M_1$  standard working solution (5.4.3) until stable peak areas are achieved. Consecutive injections shall not differ more than 5 % in peak area.

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 (5.4.3) at regular intervals. If needed, the result for the standard working solution used can be corrected for the observed drift.



### 8.5.3 Calibration curve of aflatoxin M<sub>1</sub>

Inject, in sequence, suitable volumes of the standard working solutions (5.4.3) containing for example 0,05 ng/ml, 0,10 ng/ml, 0,20 ng/ml, 0,40 ng/ml and 0,80 ng/ml of aflatoxin M<sub>1</sub> into the HPLC apparatus via the injection loop. Prepare a calibration graph by plotting the obtained peak area for each standard working solution against the mass of aflatoxin M<sub>1</sub> injected.

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

Inject a similar volume of the eluate (8.3) to that used for the standard working solutions (5.4.3) into the HPLC apparatus via the injection loop. Separate the aflatoxin M<sub>1</sub> 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<sub>1</sub> standard working solution is injected after every five injections of sample eluates.

Determine the area of the aflatoxin M<sub>1</sub> peak of the sample eluate. Calculate, from the calibration graph, the mass, in nanograms, of aflatoxin M<sub>1</sub> in the sample extract.

If the peak area of aflatoxin M<sub>1</sub> 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.

## 9 Calculation and expression of results

### 9.1 Skimmed milk

#### 9.1.1 Calculation

Calculate the aflatoxin M<sub>1</sub> content, as a concentration, of the test sample,  $\rho$ , in micrograms per litre, by using [Formula \(2\)](#):

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

where

$m_a$  is the mass of aflatoxin M<sub>1</sub> corresponding to the area of the aflatoxin M<sub>1</sub> peak of the sample eluate, in nanograms;

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

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

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

#### 9.1.2 Expression of results

Express the test results to three decimal places for liquid milk.



9.2 Skimmed milk powder

9.2.1 Calculation

Calculate the aflatoxin M<sub>1</sub> content, as a mass fraction, of the test sample, *w*, in micrograms per kilogram, by using [Formula \(3\)](#):

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

where

*m<sub>t</sub>* is the mass of the test sample present in 50 ml of the prepared test sample ([8.4](#)), in grams;

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

9.2.2 Expression of results

Express the test results to two decimal places for milk powders.

10 Precision

10.1 Interlaboratory test

The values for repeatability and reproducibility derived from the interlaboratory test were determined in accordance with ISO 5725-1[\[5\]](#) and ISO 5725-2[\[6\]](#). Details of the test are summarized in [Annex A](#).

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

10.2 Repeatability

In not more than 5 % of cases, 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 be greater than the values given in [Table A.1](#).

10.3 Reproducibility

In not more than 5 % of cases, 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 be greater than the values given in [Table A.1](#).

## **11 Test report**

The test report shall specify at least:

- 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 a reference to this document, i.e. ISO 14501:2021 | IDF 171:2021;
- d) all operating details not specified in this document, or regarded as optional, together with details of any incident that may have influenced the result(s);
- e) the test result(s) obtained, including a reference to the clause which explains how the results were calculated, and, if the repeatability has been checked, the final quoted result(s) obtained;
- f) the date of the test.



Annex A  
(informative)

Results of an interlaboratory trial

An international collaborative test<sup>[7]</sup> involving 16 laboratories was carried out on test samples of milk powder having a fat mass fraction of 1 % (“low fat” test samples 1 and 3) and a fat mass fraction of 28 % (“high fat” test samples 2, 4 and 5). The latter samples were remainders of batches of milk powders used to prepare reference materials<sup>[8]</sup>, hence the aflatoxin M<sub>1</sub> contents were known.

The contamination levels varied from 0,08 µg/kg to 0,6 µg/kg, i.e. 0,008 µg/l to 0,060 µg/l for reconstituted milk.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1<sup>[5]</sup> and ISO 5725-2<sup>[6]</sup> to give the precision data shown in [Table A.1](#).

Table A.1 — Precision data

Sample	1	2	3	4	5
Laboratories participating <sup>a</sup>	12	14	13	11	14
Mean, <i>w</i> , in µg/kg	0,081	0,150	0,080	0,202	0,580
Standard deviation of repeatability, <i>s<sub>r</sub></i> , in µg/kg	0,008	0,022	0,005	0,010	0,073
Repeatability value, <i>r</i> = 2,8 × <i>s<sub>r</sub></i> , in µg/kg	0,023	0,060	0,015	0,027	0,203
Coefficient of variation of repeatability, <i>C<sub>V,r</sub></i> , in %	9,9	14,0	6,8	4,7	12,5
Standard deviation of reproducibility, <i>s<sub>R</sub></i> , in µg/kg	0,019	0,035	0,015	0,022	0,110
Reproducibility value, <i>R</i> = 2,8 × <i>s<sub>R</sub></i> , in µg/kg	0,052	0,098	0,041	0,061	0,310
Coefficient of variation of reproducibility, <i>C<sub>V,R</sub></i> , in %	23	22,7	18,3	10,8	19,1
<sup>a</sup> Laboratories were eliminated using the Cochran and/or Grubbs outliers test.					

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