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**Milk and milk products — Guidelines for a  
standardized description of competitive  
enzyme immunoassays — Determination of  
aflatoxin M<sub>1</sub> content**

*Lait et produits laitiers — Lignes directrices pour une description  
normalisée des tests immuno-enzymatiques — Détermination de la teneur  
en aflatoxine M<sub>1</sub>*

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ISO 14675|IDF 186 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 AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

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**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 and AOAC International 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 National Committees casting a vote.

International Standard ISO 14675|IDF 186 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 AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Organic contaminants*, of the Standing Committee on *Analytical methods for additives and contaminants*, under the aegis of its project leader, Dr E. Märtlbauer (DE).

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## Introduction

Proprietary methods such as ELISA methods cannot be described in separate International Standards. Therefore, this International Standard is intended to provide guidelines on basic parameters required for evaluation/validation of competitive enzyme immunoassays for the quantitative determination of aflatoxin M<sub>1</sub> in milk and milk products.

Currently several quantitative immunochemical test formats are commercially available, which all share the basic principles of the competitive enzyme immunoassay. However, since the test format of the 96-well microtitre plate assay is most commonly used for quantitative measurement purposes, the parameters given in this International Standard are specifically adopted to this test format, and may not necessarily apply in full to a different test format.

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# Milk and milk products — Guidelines for a standardized description of competitive enzyme immunoassays — Determination of aflatoxin M<sub>1</sub> content

## 1 Scope

This International Standard give guidelines on the use of screening methods used for the determination of aflatoxin M<sub>1</sub> content in milk and milk products, based upon competitive enzyme immunoassays.

For legal purposes, positive enzyme immunoassay results require confirmation by an accepted reference method. However, depending on whether the test complies with the specifications given hereafter, enzyme immunoassays can be used for routine quality control, especially when the absence of aflatoxin M<sub>1</sub> above the regulatory limit needs to be documented.

## 2 Principle

Immunochemical methods are based on the ability of antibodies to bind to specific substances. The reversible association between antibodies and their corresponding antigens is called the immunological reaction. The binding forces involved are weak molecular interactions, such as Coulomb and van der Waals forces, as well as hydrogen bonds and hydrophobic binding.

The antigen-antibody reaction is based on the law of mass action and the amount of antigen or antibody present in the reaction mixture can be inferred from the extent of the reaction.

The “quality” of any immunoassay is a function of the immunochemical principle of the method, the properties of the reagents, the assay design and the experimental errors. These basic principles determine the sensitivity, specificity, precision and accuracy of the assay.

Concerning the principle of the method, a distinction exists between competitive methods and non-competitive methods.

For practical reasons, these methods need either labelled antigen or labelled antibody to permit observation of the antigen-antibody reaction.

Competitive methods are based on the competition of free ( $A_g$ ) and labelled ( $A_g^*$ ) antigen for a limited number of antibody-combining sites ( $AB$ ).

Schematically, this immunochemical principle may be presented according to the following formula:



In most cases, the assay response represents the bound-labelled antigen, but any measure of the distribution of the labelled antigen is, in principle, possible.

For the detection of low molecular weight compounds like mycotoxins, which possess only one antibody binding site (epitope), the competitive assay format is mandatory. To provide a distinction between unreacted and complexed reactants, most assays use either antibody (direct competitive assay) or antigen (indirect competitive assay) bound to a solid-phase as immunosorbent. So all the reagents that are not bound by the antibody can be easily removed by “washing” the solid phase.

### 3 Aflatoxin M<sub>1</sub> enzyme immunoassay

Based on the information given in the general principles of immunoassays (see clause 2), it is recommended that an ELISA-method for aflatoxin M<sub>1</sub> should comply with the specifications given in Table 1.

Table 1 — Specification of assay parameters

Parameter	Specification
<b>Antibody</b> Source	Polyclonal or monoclonal
<b>Labelled antigen</b> Marker enzyme Design	Horseradish peroxidase Aflatoxin B <sub>1</sub> or M <sub>1</sub> -oxime-horseradish peroxidase
<b>Assay format</b> Immunochemical principle Design Time	Competitive enzyme immunoassay 96-well microtitre plate assay 3 h to 4 h
<b>Assay sensitivity</b> AFM <sub>1</sub> concentration giving relative absorbance <sup>a</sup> of: 80 % 50 %	< 20 ng/l < 50 ng/l
<b>Assay specificity</b> Cross-reactivity with aflatoxin M <sub>1</sub> Cross-reactivity with other aflatoxins (occurring in milk)	100 % < 20 %
<b>Statistical design</b> Standards — replicates — dilutions — concentration range (ng/ml) Samples — replicates — dilutions	2 or more 6 or more including zero standard between 5 ng/l and 50 ng/l or broader 2 or more if necessary
<b>Calculation</b> Curve fitting	Approximation of a cubic spline, four-parameter-logistic model, linear regression (only linear part of the standard curve) <sup>b</sup>
<b>Precision</b> Standards — coefficient of variation of repeatability of relative absorbance — coefficient of variation of reproducibility of relative absorbance Samples — repeatability limit (ng/kg) — reproducibility limit (ng/kg) — limit of detection (ng/kg) — limit of quantification (ng/kg)	< 10 % < 20 % < 100 ng/kg at the 200 ng/kg level (milk powder) < 150 ng/kg at the 200 ng/kg level (milk powder) < 5 ng/kg (milk) < 10 ng/kg (milk)
<b>Sample preparation</b>	Defat by centrifuging; reconstitute milk powder to a milk (solution)
<b>Recovery</b>	> 80 % for the range from 10 ng/kg to 50 ng/kg (milk) <sup>c</sup>
<sup>a</sup> Absorbance standard or sample/absorbance zero standard × 100. <sup>b</sup> See statistical parameters in 6.3. <sup>c</sup> Correction for recovery usually not necessary.	

## 4 Sensitivity

The potential sensitivity of any immunoassay is directly related to the affinity of the antibody and can be calculated, if the equilibrium constant is known (see reference [2]). Since the antigen-antibody reaction may be described using reaction kinetics as well as thermodynamic equations, time and temperature also have an influence on the assay sensitivity.

## 5 Specificity

Next to sensitivity, the specificity of an immunochemical method is important for the performance of the assay. In principle, a specific reaction in immunology may be defined as follows: *In the presence of different molecules, the specific antibodies must complex only one kind of molecule.*

The probability of forming a “wrong” complex determines the specificity of the reaction. In other words, specificity is determined by the steric (three-dimensional) match of an antigen and antibody as well as by the number of molecular interactions taking place between both molecules. Discussion of specificity requires that both the structure of the antigen and the homogeneity or heterogeneity of the antibodies should be considered.

An antibody preparation is homogenic if all the antibodies bind only the one and same epitope, although with different affinity. This condition is fulfilled by monoclonal antibodies, but also by antisera against compounds of low molecular weight (haptens). On the other hand, an antibody preparation is heterogenic if it contains different antibody populations, specific for different epitopes.

On a molecular basis “true” cross-reactivity describes the case in which at least two different antigens compete for the same antibody-binding site. Almost exclusively, true cross-reactivity is observed in competitive assays using either monoclonal antibodies or antisera against low molecular weight compounds. In practice, this means that a high enough concentration of a truly cross-reacting substance gives the same result as the “right” substances in a competitive assay.

It is important to note that unspecific influences on the assay (e.g. matrix effects) often cannot be distinguished from the specific influence of cross-reacting substances. In both cases, the assay response is the same and a false positive or a false negative result is obtained. Due to the immunoassay principle, however, false negatives are unlikely in competitive assays.

## 6 Statistical parameters

### 6.1 General

For the discussion of statistical parameters influencing the “quality” of immunoassays, it should be noted that general statistical methods to define the limit of detection, the limit of quantification, reproducibility, etc., should also be applied to immunoassays. There are, however, some parameters influencing the within-assay precision (repeatability) and the accuracy of immunoassay results which are typical for enzyme immunoassays set up on 96-well microtitre plates.

For routine analysis, such methods usually specify a number of wells for preparation of the standard curve and a fixed number of replicates for standards and samples, as well as a number of dilutions for each sample.

### 6.2 Precision

In the process described in 6.1, there are two factors that affect the precision of the estimate to a great extent. The first is the location of the observed absorbance values on the calibration curve. The second is the number of replicates used for each sample.

Due to the non-linear shape of the calibration curve, absorbance readings near 50 % relative binding give more precise results than readings near 100 % and 0 % relative binding. For practical purposes the results should be in