

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

The European Standard EN ISO 14675:2003 has the status of a  
British Standard

ICS 67.100.10

## National foreword

This British Standard is the official English language version of EN ISO 14675:2003. It is identical with ISO 14675:2003.

The UK participation in its preparation was entrusted to Technical Committee AW/5, Milk and milk products, which has the responsibility to:

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### Summary of pages

This document comprises a front cover, an inside front cover, the EN ISO title page, the EN ISO foreword page, the ISO title page, pages ii to v, a blank page, pages 1 to 5 and a back cover.

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English version

**Milk and milk products - Guidelines for a standardized  
description of competitive enzyme immunoassays -  
Determination of aflatoxin M1 content (ISO 14675:2003)**

Lait et produits laitiers - Lignes directrices pour une  
description normalisée des tests immuno-enzymatiques -  
Détermination de la teneur en aflatoxine M1 (ISO  
14675:2003)

Milch und Milchprodukte - Leitfaden für eine vereinheitlichte  
Beschreibung kompetitiver Enzym-Immunoassays -  
Bestimmung des Gehalts an Aflatoxin M1 (ISO  
14675:2003)

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## Foreword

This document (EN ISO 14675:2003) has been prepared by Technical Committee ISO/TC 34 "Agricultural food products" in collaboration with Technical Committee CEN/TC 302 "Milk and milk products - Methods of sampling and analysis", the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2003, and conflicting national standards shall be withdrawn at the latest by July 2003.

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## Endorsement notice

The text of ISO 14675:2003 has been approved by CEN as EN ISO 14675:2003 without any modifications.

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standardized description of competitive  
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*Lait et produits laitiers — Lignes directrices pour une description  
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en aflatoxine M<sub>1</sub>*



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## Foreword

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## 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 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.

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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. Märtilbauer (DE).



## 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.



# 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.

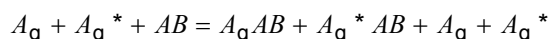
The “quality” of any immunoassay is a function of the immunochemical principle of the method, the properties of the reagents, the assay design and the experimental errors. These basic principles determine the sensitivity, specificity, precision and accuracy of the assay.

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

the range from 20 % to 80 % “relative binding”. Obviously, as with any other methods, the measurement becomes more precise if the number of replicates is increased.

### 6.3 Accuracy

The accuracy of the estimates is mainly affected by the accuracy of the calibration curve. The calibration curve, however, is not known exactly, but is estimated from the absorbance readings of the standard concentrations on the plate. Therefore, the method of curve fitting as well as the number of calibration values used and the true concentrations of the standards determine the accuracy of the calibration curve. Among the mathematical methods used to describe immunoassay standard curves, only the four-parameter-logistic model and the approximation of a cubic spline should be used for enzyme immunoassays (see reference [2]). Both methods give comparable results and at least one of them is implemented in most of the available programs for immunoassay data processing.

## 7 Conclusions

Although the overall quality of an immunoassay is a complex function, which is hard to describe, some factors influencing the major assay quality parameters are summarized in Table 2 in a simplified manner.

**Table 2 — Factors with direct influence on major assay quality parameters of enzyme immunoassays**

Factor	Assay quality parameter			
	Sensitivity	Specificity	Precision	Accuracy
Immunochemical				
— principle of method	yes	yes		
Antibody				
— affinity	yes	no	no	no
— specificity	no	yes		
Labelled reagent				
— activity	yes	no		
— design	yes	yes		
Standards				
— replicates			no	yes
— dilutions			no	yes
Samples	no	no		
— replicates			yes	no
— dilutions			yes	no
Curve fitting			no	yes
Experimental error	yes	yes	yes	yes
Sample preparation	yes	yes	yes	yes

NOTE Concerning the limited specificity as well as the possibility of false positive results due to unspecific inhibition of the assay, confirmation of immunoassay results is still needed for legal and statutory purposes.

## Bibliography

- [1] MÄRTLBAUER E. Basic principles which determine the quality of immunoassays. *IDF Special Issue No. 9302*, 1993, p. 137
- [2] RODBARD D., MUNSON P.J. and DE LEAN A. *Improved curve-fitting, parallelism testing, characterization of sensitivity and specificity, validation and optimization for radioligand assays*. In: *Radioimmunoassay and related procedures in medicine*. Vol. 1, 1977, pp. 469-514. International Atomic Energy Agency, Vienna, 1978

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