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**Traditional Chinese medicine —  
Determination of aflatoxins in natural  
products by LC-FLD**

*Médecine traditionnelle chinoise — Dosage des aflatoxines dans les  
produits naturels par CL-DF*

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CP 401 • Ch. de Blandonnet 8  
CH-1214 Vernier, Geneva  
Phone: +41 22 749 01 11  
Email: [copyright@iso.org](mailto:copyright@iso.org)  
Website: [www.iso.org](http://www.iso.org)

Published in Switzerland

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## Foreword

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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)).

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This document was prepared by Technical Committee ISO/TC 249, *Traditional Chinese medicine*.

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).

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## Introduction

Aflatoxins are naturally occurring mycotoxins produced by certain fungi, which can be found in a variety of agriculture products, contaminated foods and natural medicines, including natural products, decoction pieces and manufactured products. At least 14 different aflatoxins, mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, have been reported to be produced in nature. Among these, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is considered the most toxic. Other important aflatoxins include aflatoxin B<sub>2</sub>, M<sub>1</sub>, M<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, Q<sub>1</sub>, Q<sub>2</sub> and aflatoxicol. AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> are produced by *Aspergillus flavus* and *Aspergillus parasiticus*, while AFM<sub>1</sub> and AFM<sub>2</sub> are formed from AFB<sub>1</sub> and AFB<sub>2</sub> metabolism, respectively. It has been well established that most aflatoxins are highly toxic and carcinogenic. Humans, in particular young children, are less tolerant to aflatoxin toxicity. There are frequent reports of detection of toxic aflatoxins in herbal medicines. Therefore, aflatoxins, in particular AFB<sub>1</sub> and the total amount of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, should be tested and limited as a quality and safety control measure for natural products. There are two main methods to detect aflatoxins in natural products: the liquid chromatography tandem mass spectrometry (LC-MS/MS) method and the liquid chromatography coupled with fluorescence detector (LC-FLD) method. LC-FLD is preferentially chosen due to its high sensitivity, high accuracy and reasonable operating cost (see [Annex A](#), [Table A.1](#)).

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# Traditional Chinese medicine — Determination of aflatoxins in natural products by LC-FLD

## 1 Scope

This document specifies the methods for the determination of aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) in natural products using LC-FLD.

It is applicable to the analysis of aflatoxins in raw materials and manufactured products, including decoction pieces derived from plants and animals.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definition

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:

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

### 3.1

#### aflatoxin

mycotoxin produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*

Note 1 to entry: At least 13 different types of aflatoxin are produced in nature, and most of these are known to be highly toxic and carcinogenic.

Note 2 to entry: Aflatoxin B<sub>1</sub> and the sum of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> shall be tested and limited.

## 4 Symbols and abbreviated terms

AFB <sub>1</sub>	aflatoxin B <sub>1</sub>
AFB <sub>2</sub>	aflatoxin B <sub>2</sub>
AFG <sub>1</sub>	aflatoxin G <sub>1</sub>
AFG <sub>2</sub>	aflatoxin G <sub>2</sub>
HPLC	high-performance liquid chromatography
LC-FLD	liquid chromatography coupled with fluorescence detector
LC-MS/MS	liquid chromatography tandem mass spectrometry

## 5 Reagents

The purity of the reagents used shall be checked by running a blank determination. The chromatogram obtained from the solvents shall have a baseline without noticeable peaks that would interfere with targeted aflatoxins.

**5.1 Water**, of appropriate purity (the resistivity of water shall be at least 18,2 MΩ).

**5.2 Methanol, CH<sub>3</sub>OH**, of HPLC grade.

**5.3 Acetonitrile, CH<sub>3</sub>CN**, of HPLC grade.

**5.4 Sodium chloride, NaCl**, of AR (analytical) grade.

## 6 Apparatus

### 6.1 LC-FLD

The LC-FLD apparatus consists of a solvent pump system, a sample injector, a chromatographic column (a column temperature controller may be used), a detector and a data acquisition system (or an integrator or a chart recorder). The mobile phase is supplied from one or several reservoirs and flows through the column and detector at a constant flow rate. The detector shall be a fluorescence detector.

### 6.2 Chromatographic column

A stainless-steel column sealed with octadecylsilyl silica gel for chromatography shall be used.

### 6.3 Glass sample

All glassware shall be thoroughly cleaned before use. The glassware used for aflatoxin analysis shall be placed in a specific container filled with 0,5 % to 1,0 % sodium hypochlorite solution for more than 2 h and then washed with an adequate amount of fresh running water. Finally, all glassware shall be rinsed with distilled water and dried before use.

### 6.4 Electronic balance

The electronic balance shall be accurate to a minimum of 0,01 mg.

### 6.5 Homogenizer

The homogenizer shall have a rotation speed of up to 15 000 r/min.

### 6.6 Centrifuge

The centrifuge shall have a rotation speed of up to 5 000 r/min.

### 6.7 Volumetric flask

Volumetric flasks with a capacity of 2,0 ml and 50,0 ml shall be used.

## 7 Sample preparation

1) All natural products shall be crushed into powders and screened through a 24-mesh sieve.

- 2) A mixture of 15,0 g powders and 3,0 g sodium chloride shall be added into a 75,0 ml mixed solution of methanol and water at 70:30 volume fraction.
- 3) The mixture shall be homogenized at a speed of higher than 11 000 rpm for 2 min and centrifuged at 2 500 rpm for 5 min.
- 4) 15 ml of supernatant shall be added to a 50,0 ml volumetric flask and diluted with water, then shaken and filtered through a 0,45  $\mu\text{m}$  filter paper.
- 5) About 20,0 ml of the filtrate shall be passed through the immunoaffinity column at a flow rate of 3 ml/min. The column shall be washed with 20,0 ml of water and the eluent shall be abandoned until the air has passed through the column to extrude the water.
- 6) The column shall be eluted with methanol and the eluent shall be collected and concentrated to 0,5 ml by nitrogen. The concentrated eluent shall be diluted with 0,5 ml of the mixed solution of methanol and water at 50:50 volume fraction in the HPLC vial before use.

## 8 Test method

### 8.1 Stock solution and working solution

Stock solution shall be prepared by mixing a solution of aflatoxin standards (1,0  $\mu\text{g/ml}$ , 0,3  $\mu\text{g/ml}$ , 1,0  $\mu\text{g/ml}$  and 0,3  $\mu\text{g/ml}$  of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, respectively). A series of working solutions shall be prepared by diluting the stock solution to 0,10 ng/ml to 100,00 ng/ml (AFB<sub>1</sub> and AFG<sub>1</sub>) and 0,03 ng/ml to 30,00 ng/ml (AFB<sub>2</sub> and AFG<sub>2</sub>), respectively, with mobile phase of methanol and acetonitrile.

### 8.2 LC-FLD conditions

#### 8.2.1 General

The LC-FLD method based on two different methods of derivatization, pre- and post-column derivatization, shall be used for the simultaneous determination of aflatoxins. Commonly, post-column derivatization methods, such as photochemical derivatization, iodine derivatization and electrochemically generated bromine derivatization, have been applied in many countries, regions and organizations including Europe, China, the United States, Japan and South Korea. The LC-FLD method based on iodine derivatization and photochemical derivatization is recommended for the simultaneous determination of aflatoxins (including AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>) in natural products.

#### 8.2.2 LC-FLD conditions and system suitability

- a) A stainless-steel column sealed with octadecylsilyl silica gel for chromatography measurement shall be used.
- b) The mobile phase of methanol-acetonitrile-water shall be used for isocratic elution.
- c) The post-column derivatization system shall be used for detection of aflatoxins using a fluorescence detector.
- d) The excitation and emission wavelengths of the fluorescence detector shall be set at  $\lambda_{\text{ex}} = 360 \text{ nm}$  (or 365 nm) and  $\lambda_{\text{em}} = 450 \text{ nm}$ , respectively.
- e) The resolution of two adjacent chromatographic peaks should be greater than 1,5.

NOTE  $\lambda_{\text{ex}}$  is excitation wavelength (nm) of the fluorescence detector and  $\lambda_{\text{em}}$  is emission wavelength (nm) of the fluorescence detector.

### 8.2.3 Post-column derivatization

#### 8.2.3.1 Iodine derivatization

0,05 % iodine solution prepared by dissolving 0,5 g iodine in 100 ml methanol and diluting to make up to 1 000 ml with water shall be used as the derivatization reagent. The flow rate of the derivatization pump shall be 0,3 ml/min and temperature shall be maintained at 70 °C.

#### 8.2.3.2 Photochemical derivatization

The photochemical derivatization reactor shall be set as 254 nm of UV wavelength. The UV source shall be a mercury lamp ( $\lambda = 254$  nm). The reactor shall consist of a lamp holder with switch, UV lamp, reactor holder and knitted reactor coil.

### 8.2.4 Quantification of aflatoxins in the test sample using calibration curves

25 µl of each working solution of mixed standard solutions shall be injected into the LC-FLD system to record the peak area of each aflatoxin. The chromatogram of AFG<sub>2</sub>, AFG<sub>1</sub>, AFB<sub>2</sub> and AFB<sub>1</sub> is presented in [Annex B](#). The calibration curves of aflatoxins shall be established by plotting peak area versus the serially diluted concentration of aflatoxins. Afterwards, the test sample solution shall also be injected into the LC-FLD system to record the peak area of each aflatoxin. Then the contents of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> in test samples shall be calculated using the calibration curves.

### 8.3 Application of test method

The described method has been shown to be suitable for the following natural products (see [Annex C](#)):

*Zingiber officinale* Rhizome

*Whitmania pigra* Body (*Hirudo nipponica* Body;  
*Whitmania acranulata* Body)

*Pheretima aspergillum* (*Pheretima vulgaris*,  
*Pheretima guillelmi*, *Pheretima pectinifera*)

*Buthus martensii* Body

*Myristica fragrans* Seed

*Cassia obtusifolia* Seed (*Cassia tora* Seed)

*Ziziphus jujuba* Fruit

*Hordeum vulgare* Fruit

*Polygala tenuifolia* Root  
(*Polygala sibirica* Root)

*Citrus reticulata* Peel

*Quisqualis indica* Fruit

*Platycladas orientalis* Seed

*Sterculia lychnophora* Seed

*Nelumbo nucifera* Seed

*Prunus persica* Seed  
(*Prunus davidiana* Seed)

*Scolopendra subspinipes mutilans* Body

*Areca catechu* Seed

*Ziziphus jujuba* Seed

*Bombyx mori* Body

*Coix lacryma-jobi* Seed

This method can also be used in other kinds of natural products, but it shall be demonstrated by method validation. Detailed parameters of method validation are given in [Annex D](#).