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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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Foreword

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

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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_1 (AFB_1) is considered the most toxic. Other important aflatoxins include aflatoxin B_2, M_1, M_2, G_1, G_2, Q_1, Q_2 and aflatoxicol. AFB_1, AFB_2, AFG_1 and AFG_2 are produced by *Aspergillus flavus* and *Aspergillus parasiticus*, while AFM_1 and AFM_2 are formed from AFB_1 and AFB_2 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_1 and the total amount of AFB_1, AFB_2, AFG_1 and AFG_2, 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).

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_1, AFB_2, AFG_1, AFG_2) 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_1 and the sum of aflatoxins B_1, B_2, G_1 and G_2 shall be tested and limited.

4 Symbols and abbreviated terms

AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
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_3OH, of HPLC grade.

5.3 Acetonitrile, CH_3CN, 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 µm 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 µg/ml, 0,3 µg/ml, 1,0 µg/ml and 0,3 µg/ml of AFB_1, AFB_2, AFG_1 and AFG_2, 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_1 and AFG_1) and 0,03 ng/ml to 30,00 ng/ml (AFB_2 and AFG_2), 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_1, AFB_2, AFG_1 and AFG_2) 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 λ_{ex} = 360 nm (or 365 nm) and λ_{em} = 450 nm, respectively.

e) The resolution of two adjacent chromatographic peaks should be greater than 1,5.

NOTE λ_{ex} is excitation wavelength (nm) of the fluorescence detector and λ_{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 (λ = 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_2, AFG_1, AFB_2 and AFB_1 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_1, AFB_2, AFG_1 and AFG_2 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

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

Myristica fragrans Seed

Ziziphus jujuba Fruit

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

Quisqualis indica Fruit

Sterculia lychnophora Seed

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

Areca catechu Seed

Bombyx mori Body

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

Buthus martensii Body

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

Hordeum vulgare Fruit

Citrus reticulata Peel

Platycladas orientalis Seed

Nelumbo nucifera Seed

Scolopendra subspinipes mutilans Body

Ziziphus jujuba Seed

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.

9 Sampling and preservation

9.1 Sampling

For each package the following quantities of samples shall be used: no less than 100 g of general medicinal materials and decoction pieces; no less than 25 g of powdered medicinal materials and decoction pieces; 5 g of precious medicinal materials and decoction pieces.

Natural product samples received by the laboratory shall be labelled with information such as the collected source, date and time, correct species of material and name of the appraiser. The testing samples shall include Chinese materia medica (whole medicinal materials) and decoction pieces derived from plants or animals.

On receipt, a sample shall immediately be assigned a unique identification code, which shall be accompanied through all stages of the analysis to the reporting of the results. Records of samples shall be kept by specified person and place.

9.2 Sample storage

Before testing, the sample shall be dried and powdered. Samples shall be prepared immediately and should be stored in the dark in a refrigerator at 4 °C.

If samples cannot be analysed immediately, they shall be stored below 4 °C away from sunlight. The mass of the flask shall be recorded before and after each measurement of the solution.

Annex A
(informative)

LC-FLD method

Determination of aflatoxins in natural products has various kinds of detection methods, including thin-layer chromatography (TLC), liquid chromatography (LC) and gas chromatography (GC) coupled to a specific detector. Among them, LC-FLD is the most widely used method for aflatoxin detection in natural products, and the liquid chromatography–tandem mass spectrometry (LC-MS/MS) is the technique of choice for the simultaneous determination of various mycotoxins.

Table A.1 — Analytical methods of aflatoxin determination by nation, region and organization

Country or organization	Regulation	Edition	Method
Association of Official Analytical Chemists (AOAC)	AOAC Official Method[9]	2005.08	Liquid chromatography with post-column derivatization using photochemical reactor for enhanced detection (PHRED)
Argentina	Farmacopea Argentina[20]	8	TLC HPLC-FLD
Europe	European Pharmacopoeia[10]	8.0	HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using pyridinium hydrobromide perbromide (PBPB) HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using PHRED HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using electrochemically generated bromine (KOBRA)
South Korea	Korean Pharmacopoeia[17]	11	HPLC-FLD with post-column derivatization using PBPB HPLC-FLD with post-column derivatization using PHRED HPLC-FLD with post-column derivatization using KOBRA
China	Chinese Pharmacopoeia[21]	2015	HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using iodine
			HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using PHRED
			LC-MS/MS detection

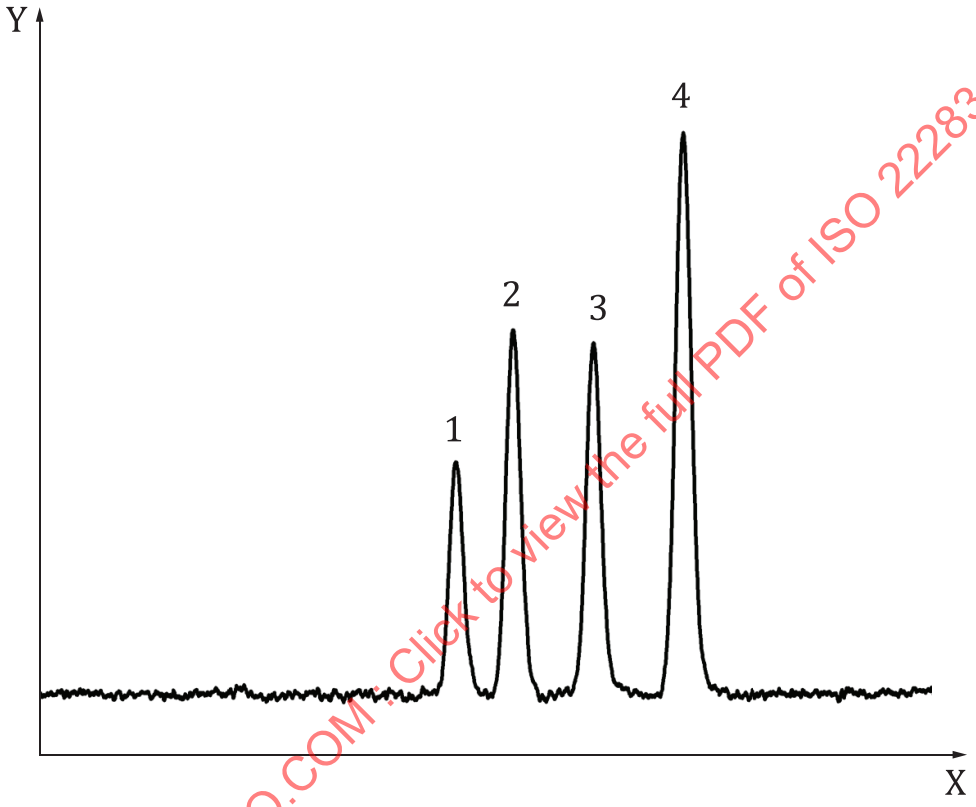
Table A.1 *(continued)*

Country or organization	Regulation	Edition	Method
Japan	Japanese Pharmacopoeia[18]	17	HPLC-FLD with immunoaffinity column clean-up combined with pre-column derivatization using trifluoroacetic acid
			HPLC-FLD with immunoaffinity column clean-up combined with post-column derivatization using PHRED
			Confirmation by LC-MS or LC-MS/MS
United States of America (USA)	United States Pharmacopeia[22]	40	TLC High-performance TLC with immunoaffinity column clean-up HPLC-FLD with immunoaffinity column clean-up

Annex B
(informative)

Chromatogram of AFG_2, AFG_1, AFB_2 and AFB_1

In a referenced chromatogram of AFG_2, AFG_1, AFB_2 and AFB_1 analysed by HPLC-FLD, four main peaks are detected at the four independent retention times, as shown in Figure B.1.



Key

X retention time (min)
Y intensity (uV)
1 peak of AFG_2
2 peak of AFG_1
3 peak of AFB_2
4 peak of AFB_1

Figure B.1 — HPLC chromatogram of aflatoxins in natural product

Annex C
(informative)

Reference of national, regional and organizational limits of aflatoxins in natural products

Regarding the toxicity and presence of aflatoxins in natural products, maximum residue limits (MRLs) are shared globally in pharmacopoeias and national and organizational regulations. In general, the current legal limit for AFB_1 in herbal medicines ranges between 2 $\mu g.kg^{-1}$ and 10 $\mu g.kg^{-1}$, while the limit for combined AFB_1, AFG_1, AFB_2 and G_2 (total aflatoxins) ranges from 4 $\mu g.kg^{-1}$ to 20 $\mu g.kg^{-1}$, as shown in Table C.1.

Table C.1 — Maximum recommended residue limits of aflatoxins and AFB_1 in various types of natural products

Country and organization	Scope of application	Regulation	Edition	Maximum residue limit µg/kg	
				AFB_1	Aflatoxins ($B_1+B_2+G_1+G_2$)
Argentina	Raw materials and preparations	Farmacopea Argentina[20]	8	5	20
Britain	Medicinal plants and herbal products	British Pharmacopoeia[23]	2017	2	4
China	*Prunus persica* seed; *Sterculia lychnophora*; *Citrus reticulata* Peel; *Ziziphus jujuba* seed; *Bombyx mori*; *Platycladus orientalis* seed; *Nelumbo nucifera* seed; *Quisqualis indica* seed; *Areca catechu* seed; *Myristica fragrans* seed; *Cannamomum cassia* peel; *Polygala tenuifolia* Rhizome; *Coix lacryma-jobi* seed; *Ziziphus jujube* fructus; *Pheretima aspergillum*; *Scolopendra subspinipes mutilans*; *Whitmania pigra; Buthus martensii*	Chinese Pharmacopoeia[21]	2015	5	10
China	Medical plants and preparations	Green standards of medicinal plants and preparations for foreign trade and economy (GSMPP)	WM/T 2–2004	5	[a]
EU	Medicinal plant and herbal products	European Pharmacopoeia[10]	8.0	2	4
	Dried *Ficus carica* fructus	European Commission Regulation	EC No 1881/2006 EU No 1058/2012	6	10
	Capsicum annuum fructus; *Piper nigrum* fructus; *Myristica fragrans* seed, *Zingiber officinale* rhizome; *Curcuma longa* rhizome		EC No 1881/2006 EU No 1058/2012 EU No 165/2010	5	10
Japan	*Nelumbo nucifera* seed and its processed products (contains more than five percent)	JETRO	2010	Undetectable	Undetectable
	Herb and herbal preparations	Japanese Pharmacopoeia[18]	17	[a]	10

[a] unrestricted

Table C.1 *(continued)*

Country and organization	Scope of application	Regulation	Edition	Maximum residue limit µg/kg	
				AFB_1	Aflatoxins ($B_1+B_2+G_1+G_2$)
South Korea	*Glycyrrhiza uralensis* rhizome; *Cassia obtusifolia* seed; *Prunus persica* seed; *Pinellia ternata* rhizome; *Platycladus orientalis* seed; *Areca catechu* seed; *Ziziphus jujuba* var. *spinosa* seed; *Polygala tenuifolia* root; *Carthamus tinctorius* flower; *Trichosanthes kirilowii* seed; *Chinemys reevesii* plastrum and carapax; *Chaenomeles speciosa* fruit; *Dolichos lablab* seed; *Curcuma wenyujin* root; *Myristica fragrans* seed; *Croton tiglium* seed; *Prunus armeniaca* seed; *Curcuma longa* root; *Nelumbo nucifera* seed; *Bombyx mori* body	Korean Pharmacopoeia[17]	11	10	15
USA	Botanical origin	United States Pharmacopeia[22]		5	20
	Capsicum annuum fructus paste and powder	Food Code	Issued on Oct, 2009	10	[a]

[a] unrestricted