Determination of Mycotoxins in Food

Government Laboratory

Outline

Determination of Mycotoxins in Food

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Introduction

Contamination with toxins produced by mould

- Natural fungal toxins in food have been affecting our societies for a long time. Human poisoning resulting from mycotoxin contamination has been recorded since the Middle Ages.
- The growth of toxin-producing fungi can happen in large varieties of agricultural products like cereals, oilseeds, fruits etc., that are susceptible to mould infestation under high temperature and humidity.
- The toxins can sometimes find their way into milk, liver and kidney of animals that have consumed contaminated feed.

Mycotoxins can cause a wide range of health effects in humans.

Common Mycotoxins

Mycotoxins（霉菌毒素）

▪ 黃麴黴毒素	Aflatoxin (B_1, B_2, G_1, G_2 and M_1 [#])
▪ 展青黴素	Patulin
▪ 赭麴黴毒素A	Ochratoxin A
▪ 伏馬鐮孢毒素	Fumonisin
▪ 去氧雪腐鐮刀菌烯醇	Deoxynivalenol
▪ 玉米赤黴烯酮	Zearalenone
▪ T-2毒素、HT-2毒素	T-2 and HT-2 toxin
▪ 桔黴素	Citrinin
▪ 麥角生物堿	Ergot alkaloids

Metabolite of Aflatoxin B1

Aflatoxin

Regulatory Level of Aflatoxin

- Different Regulatory control of Aflatoxin

	Hong Kong	Codex
Peanuts or peanut products	20 mg/kg	Unless specified, seed or kernels, after removal of shell or husk; intended for further processing: ▪Peanuts 15 mg/kg
		Whole commodity after removal of shell; *ready-to-eat:* • Almonds, Brazil nuts, Hazelnuts, Pistachios 10mg/kg
Any food other than peanut or its products	15 mg/kg	Whole commodity after removal of shell; *intended for further processing:* ▪Almonds, Brazil nuts, Hazelnuts, Pistachios ▪15 mg/kg
		Whole commodity; ready-to-eat ▪Dried figs: 10 mg/kg Whole commodity: ▪Milks#: 0.5 mg/kg

Aflatoxin M_1

Regulation in Hong Kong

Cap 132AF Harmful Substances in Food Regulations

Regulation	Prohibition of import and sale of food containing certain substances in excessive concentrations
3	A person must not import, consign, deliver, manufacture or sell, for human consumption, any food of a description specified in Column D of the First Schedule which contains any substance specified opposite thereto in Column B, or the description of such substance in Column C, in greater concentration than is specified opposite thereto in Column E.

Regulation in Hong Kong

Cap 132AF Harmful Substances in Food Regulations

Item	Substance	Description of substance	Description of food	Maximum Concentration
1	Aflatoxin	Group of bis-furanocoumarin compounds and includes aflatoxin B 1, B 2, G 1, G 2, M 1, M 2, P 1 and aflatoxicol	Any food other than peanut or its products	15 micrograms per kilogram of the food
			Peanuts or peanut products	20 micrograms per kilogram of the food

Guideline - CODEX

GENERAL STANDARD FOR CONTAMINANTS AND TOXINS IN FOOD AND FEED

CXS 193-1995

A **criteria-based approach**, whereby a set of performance criteria is established with which the analytical method used should comply, is appropriate.

Criterion	Concentration range	Recommended value
Blanks	All	Negligible
Recovery- Aflatoxin Total	1-15 ug/kg	70-110%
	> 15 ug/kg	80-110%
Precision RSD_R	All	As derived from Howitz Equation
Precision RSD_r may be calculated as 0.66 times Precision RSD_R at the conc. of interest		

Guideline - EU regulation

COMMISSION REGULATION (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

Guideline - EU regulation

- General requirements

  Methods of analysis used for food control purposes shall comply with the provisions of items 1 and 2 of Annex III to Regulation (EC) No 882/2004.

- Specific requirements

Criterion	Concentration range	Recommended value
Blanks	All	Negligible
Recovery- Aflatoxin M1	0.01 – 0.05 ug/kg	60-120%
	> 0.05 ug/kg	70-110%
Recovery – Aflatoxin B1, B2, G1, G2	<1.0 ug/ kg	50-120%
	1 -1- ug/kg	70-110%
	> 10 ug/ kg	80-110%

Standard Methods

- Chapter 49: Natural toxins, in Official Methods of Analysis
  - Subchapter 2 – Aflatoxins
  - Subchapter 3 – Aflatoxin M1

- 中華人民共和國國家標准 (食品安全國家標准)
  - GB 5009.22-2016食品中黃麴黴毒素B族和G族的測定
  - GB 5413.37-2010乳和乳製品中黃麴黴毒素M1的測定
  - GB2761-2017食品中真菌毒素限量

Standard Methods

AOAC	Methods
971.24	Aflatoxins in Coco nut, Copra, and Copra Meal (Thin-Layer Chromatographic Method)
993.16	Total Aflatoxins (B1, B2, and G1) in Corn (Enzyme-Linked Immunosorbent Assay Method (Afla-20 Cup Test)
972.26	Aflatoxins in Corn (Thin-Layer Chromatographic Method)
993.17	Aflatoxins in Corn and Peanuts (Thin-Layer Chromatography-Fluorodensitometry Determination)
990.32	Aflatoxin B1 in Corn and Roasted Peanuts (Enzyme-Linked Immunosorbent (Agri-Screen) Screening Assay)
990.33	Aflatoxins in Corn and Peanut Butter (Liquid Chromatographic Method)
991.31	Aflatoxins in Corn, Raw Peanuts, and Peanut Butter (Immunoaffinity Column (Aflatest) Method)
980.20	Aflatoxins in Cottonseed Products (Thin-Layer and Liquid Chromatographic Methods)
994.08	Aflatoxins in Corn, Almonds, Brazil Nuts, Peanuts, and Pistachio Nuts (Multifunctional Column (Mycosep) Method)
989.06	Aflatoxin B1 in Cottonseed Products and Mixed Feed (Enzyme-Linked Immunosorbent Screening Method)
978.15	Aflatoxin B1 in Eggs (Thin-Layer Chromatography Column Chromatography)
970.46	Aflatoxins in Green Coffee (Thin-Layer Chromatographic Method)

Search "**Alfatoxin**"... Total number of methods found: **43**

Standard Methods

BS	Method
15851:2010	Determination of aflatoxin B1 in cereal based foods for infants and young children. HPLC method with immunoaffinity column cleanup and fluorescence detection
14123:2007	Determination of aflatoxin B1 and the sum of aflatoxin B1, B2, G1 and G2 in hazelnuts, peanuts, pistachios, figs, and paprika powder. High performance liquid chromatographic method with post-column derivatisation and immunoaffinity column clean-up.
15851:2010	Determination of aflatoxin B1 in cereal based foods for infants and young children. HPLC method with immunoaffinity column cleanup and fluorescence detection.
ISO 14501: 2007	Milk and milk powder. Determination of aflatoxin M1 content. Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography.
ISO 14675: 2003	Milk and milk products. Guidelines for a standardized description of competitive enzyme immunoassays. Determination of aflatoxin M1 content.
ISO 17375: 2006	Animal feeding stuffs. Determination of aflatoxin B1.
ISO 14674: 2005	Milk and milk powder. Determination of aflatoxin M1 content. Clean-up by immunoaffinity chromatography and determination by thin-layer chromatography.
17424	Determination of aflatoxins in spices other than paprika by IAC clean-up and HPLC-FLD with post-column derivatization.
16050: 2011	Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products. High-performance liquid chromatographic method.

Methods – General Approach

1. Immunoaffinity column clean-up
2. High-performance liquid chromatography
3. Fluorescence detection with Post-column Derivatization
4. Liquid chromatography- Tandem Mass Spectrometry

Sample preparation for HPLC analysis

1. Homogenization
2. Sample weighing
3. Extraction
4. Clean-up by immunoaffinity chromatography
5. Liquid chromatographic method- FLD/MSMS

Immunoaffinity chromatography

- Quick (< 10 minutes)
- Handle variety/complex matrices
- High selectivity, low LOD
- Wide range (up to 300 ppb)
- Higher recovery
- Safe (less toxic solvent consumption)
- Shelf life ~ 6 months
- Usually stored at low temperature

Applications of the method
-different food matrix

- Cereal, grains and products
- Milk and milk products
- Mooncakes
- Bakeries
- Nuts and seeds
- Rice
- Oils and fats

General requirements of method

Spike Level: ≦1 ppb: 50-120%

Spike Level: > 1 to 10 ppb: 70-120%

Precision: ±15%

Experimental Condition of LC-analysis

Column	:	Supelcosil LC-18 DB column, (4.6 mm × 250 mm, 5 µm)
Mobile phase	:	Water: acetonitrile: methanol (3:1:1 v:v:v)
Flow rate	:	1 mL/minute
Excitation wavelength	:	360 nm
Emission wavelength	:	450 nm
Photochemical reactor condition:	:	
Excitation wavelength:		254 nm

Experimental Condition of LC-MS/MS analysis

Injection volume	:	5 µL		
Flow rate	:	0.5 mL/minute		
Column	:	C18, (100 mm × 2.1 mm, 1.8 µm)		
Column temp.	:	20 - 30 °C		
Mobile Phase	:	A: Water with 5 mM ammonium acetate and 2 % acetic acid B: 95 % Methanol with 5 mM ammonium acetate and 2 % acetic acid		
Solvent program	:	Time (minutes)	% A	%B
		0.0	70	30
		6.0	53	47
		6.01	5	95
		7.0	5	95
		7.01	70	30

* Diagnostic ions (MRM)

Experimental Condition

MS/MS system	:	Triple quadrupole mass spectrometer
Ionization mode	:	Electrospray positive ionization
Gas Temp	:	200 °C
Gas Flow	:	16 L/min
Nebulizer Pressure	:	25 psi
Sheath Gas Heater	:	400 °C
Sheath Gas Flow	:	11 mL/ minute
Capillary Voltage	:	2500 V
Nozzle Voltage	:	300 V

Determination by MS/MS

Aflatoxin	MRM	
	Precursor ion (m/z)	Fragment ion (m/z)
B1	313	241
		285*
B2	315	259*
		287
G1	329	243*
		200
G2	331	313*
		245
B1-^{13}C (IS)	330	301
B2-^{13}C (IS)	332	303
G1-^{13}C (IS)	346	212
G2-^{13}C (IS)	348	259

* Diagnostic MRM

Other mycotoxins

Patulin

- There is no specific regulation on patulin in food in Hong Kong.
- Makes reference to the Codex Alimentarius Commission (Codex) standard.
- Standard methods in AOAC
  - 995.10 Patulin in Apple Juice
  - 2000.02 Patulin in Clear and Cloudy Apple Juices and Apple Puree

Sample preparation

1. Homogenization
2. Sample weighing
3. Extraction
4. Clean-up by HLB (optional)
5. UV detection
6. Liquid chromatographic method- tandem Mass Spectrometry (LC-MS/MS)

Experimental Condition

HPLC-UV detection	
Column	: Supelcosil LC-18 DB column, (4.6 mm x 250 mm, 5 µm)
Elution program	: Isocratic program
Mobile Phase	: THF : Water = 0.8 : 100
Flow rate	: 1 mL/min
Wavelength	: 275 nm

Experimental Condition of LC-MS/MS analysis

Flow rate	:	0.25 mL/min		
Column	:	C18 with anionic and cationic ligand, (100 mm × 2.1 mm, 1.8 µm)		
Mobile Phase	:	A: 5 mM ammonium acetate with 3.5 mM acetic acid in water B: Methanol		
Solvent program	:	Time (minutes)	% A	%B
		0.0	80	20
		4.0	80	27
		4.01	0	100
		5.0	0	100
		5.01	100	0
		6.00	100	0

Experimental Condition

MS/MS system	:	Triple quadrupole mass spectrometer
Ionization mode	:	Electrospray negative ionization
Gas Temp	:	290 °C
Gas Flow	:	20 L/min
Nebulizer	:	60 psi
Sheath Gas Heater	:	370 °C
Sheath Gas Flow	:	12 L/min
Capillary Voltage	:	-5000 V
Nozzle Voltage	:	0

Determination of by MS/MS

	MRM	
	Precursor ion (m/z)	Fragment ion (m/z)
Patulin	153	109*
	153	81
Patulin IS	160	115

* Diagnostic MRM

Ochratoxin A

- Ochratoxin A, a potential carcinogenic contaminant, mainly occurs in cereal products. It is also found in a range of other food commodities including coffee.
- Standard methods in AOAC
  - 2000.09 Ochratoxin A in Roasted Coffee
  - 2000.03 Ochratoxin A in Barley
  - 2001.01 Ochratoxin A in Wine and Beer

Sample preparation

1. Homogenization
2. Sample weighing
3. Extraction
4. Clean-up by immunoaffinity chromatography
5. Fluorescence detection
6. Liquid chromatographic method- tandem Mass Spectrometry (LC-MS/MS)

Experimental Condition

HPLC-fluorescence detection		
Column	:	Alltima C18 column, (4.6 mm x 250 mm, 5 µm)
Elution program	:	Isocratic elution
Mobile phase	:	Acetonitrile : 2 % acetic acid in water (1:1)
Flow rate	:	1 mL/min
Excitation wavelength	:	333 nm
Emission wavelength	:	443nm

Experimental Condition

LC-MS/MS				
Flow rate	:	0.5 mL/min		
Column	:	Zorbax Eclipse Plus C18, (100 mm × 2.1 mm, 3.5 µm)		
Mobile Phase	:	A: 20 mM ammonium acetate with 0.5% acetic acid in water B: Methanol		
Solvent program	:	Time (minutes)	% A	%B
		0.0	70	30
		3.0	5	95
		5.0	5	95
		5.01	70	30
		6.00	70	30

Experimental Condition

MS/MS system	:	Triple quadrupole mass spectrometer
Ionization mode	:	Electrospray positive ionization
Source Temp	:	100 °C
Desolvation Temp	:	300 °C
Cone Gas Flow	:	10 L/h
Desolvation Gas Flow	:	600 L/h
Sheath Gas Flow	:	12 L/min
Capillary Voltage	:	2800
Extractor	:	3.0 V

Determination of by MS/MS

	MRM	
Ochratoxin A	Precursor ion (m/z)	Fragment ion (m/z)
	404	239*
	404	102
Ochratoxin A IS	424	250

* Diagnostic MRM

Certified Reference Material

CRM are available from the following reference material producers

- NIST
- IRMM
- European Commission

Quality Control Sample

Quality Control Samples are available from the following organizations:

- LGC
- FAPAS
- NIST

Proficiency Testing Program

Proficiency test program are available from the following PT providers:

- LGC
- FAPAS

Metrology in Chemistry

- Metrology in Chemistry focuses on the study of comparability and traceability of chemical measurements to ensure the reliability of results.
- With support of the results obtained from key comparisons organized by international metrology organizations, GL obtained related Calibration and Measurement Capabilities (CMCs) claim.
- GL participated in key comparisons organized by international metrology organizations such as “Determination of Aflatoxin in figs”.

Way Forward

- To keep up with the latest development in science and technology.
- To make preparation to meet emergency situations to address the needs of the community.
- To develop and equip staff with the skills for service provision under both normal and emergency situations.
- To strengthen international collaborations to enhance service level.

The End

Thank you for your attention !