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Assessment of Aflatoxin levels in food and animal feeds using ELISA and HPLC: Case study at Uganda National Bureau of standards

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About Uganda

- Uganda (East Africa) and is a member of the (EAC), and COMESA.
- Landlocked; Area of Approx. 240,000Sq.km Population of 36 million people
- Predominantly Agricultural country where agriculture employs more than 60 % of the population.
- Shares a big portion of Lake Victoria, the world's largest fresh water lake and the source of River Nile


Uganda
Algiers
Tunis
Rabat
Casablanca
Fès
Oran
Constantine
Valletta
MALTA
Mediterranean Sea
MADEIRA ISLANDS
(PORTUGAL)
Funchal
CANARY ISLANDS
(SPAIN)
Las Palmas
MOROCCO
Marrakech
TUNISIA
Tripoli
Banghāzi
ALGERIA
LIBYA
Laayoune
Western Sahara
Tropic of Cancer
MAURITANIA
Nouakchott
Tombouctou
Agadez
NIGER
MALI
CHAD
Dakar
SENEGAL
Banjul
GAMBIA
Bissau
Bamako
BURKINA FASO
Ouagadougou
Niamey
Zinder
N'Djamena
GUINEA
Conakry
Freetown
SIERRA LEONE
Monrovia
LIBERIA
CÔTE D'IVOIRE
Yamoussoukro
Abidjan
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TOGO
Lomé
Accra
BENIN
Porto-Novo
Lagos
Ogbomoso
Ibadan
Kano
NIGERIA
Abuja
Moundou
CAMEROON
Douala
Yaoundé
Malabo
EQUATORIAL GUINEA
Gulf of Guinea
SAO TOME AND PRINCIPE
São Tomé
Libreville
Annobón
CENTRAL AFRICAN REPUBLIC
Bangui
REP. OF THE
Equator
Brazzaville
Pointe-Noire
Kinshasa
ANGOLA
(Cabinda)
DEM. REP. OF THE CONGO
Mbuji-Mayi
Kisangani
Juba
SUDAN
Omdurman
Khartoum
Port Sudan
Red Sea
Al Jawf
EGYPT
Aswān
Al Jīzah
Cairo
Alexandria
Jerusalem
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Doha
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Jiddah
Mecca
Gulf of Aden
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Asmara
YEMEN
Sanaa
Djibouti
DJIBOUTI
Hargeysa
Addis Ababa
ETHIOPIA
SOMALIA
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Indian Ocean
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Nairobi
Kampala
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RWANDA
Kigali
Bukavu
BURUNDI
Bujumbura
Lake Victoria
Mombasa
Dodoma
Zanzibar
TANZANIA
Dar es Salaam
Lake Tanganyika
Lake Nyasa
Lubumbashi
Kitwe
ZAMBIA
Lusaka
MALAWI
Lilongwe
Blantyre
Cidade de Nacala
Mozambique Channel
MOZAMBIQUE
Beira
Harare
ZIMBABWE
Luanda
Namibe
Lubango
ANGOLA
Windhoek
Walvis Bay
NAMIBIA
BOTSWANA
Gaborone
Pretoria
Johannesburg
Mbabane
SWAZILAND
Maputo
Maseru
LESOTHO
SOUTH AFRICA
Durban
Cape Town
Port Elizabeth
Tropic of Capricorn
South Atlantic Ocean
Ascension
(St. Helena)
St. Helena
(U.K.)
St. Helena
(St. Helena)
COMOROS
Moroni
Mayotte
(admin. by France, claimed by Comoros)
Glorioso Islands
(FRANCE)
Juan de Nova Island
(FRANCE)
Europa Island
(FRANCE)
Bassas da India
(FRANCE)
MADAGASCAR
Mahajanga
Toamasina
Antananarivo
SEYCHELL
St. Denis

- **Uganda grows a variety of fruits, vegetables, cereals & pulses**
  Animals & animal products

Uganda National Bureau of Standards (UNBS)

- UNBS is Uganda's national standards body
- Mandate: **SQMT**
- **Overall objective**:

---To promote local industries

---Ensure fairness in trade through reliable measurement systems

---Protect consumers

UNBS Main Activities

- Laboratory testing
- Standards development
- Imports inspection
- Products and systems certification
- Factory inspection & Market surveillance
- Calibration of measuring and testing equipment
- Verification of weights and measures
- Training and consultancy services
- National enquiry point for WTO TBT/SPS agreements
- Standards information and documentation

Food Safety in Uganda

- Food safety: Handling, Preparation & Storage so as to prevent contamination which can lead to **foodborne illnesses & other health hazards**

- Food safety assurance depends on the nature and risk associated with the food taking care of **good agricultural practices, suitable handling, hygiene, storage, processing, packaging and transportation**

Institutional Framework

Sector/ Regulated area	Regulatory institution	Remarks
Fish	Fisheries Department (MAAIF)	Competent Authority (exports)
Horticulture	Crop Resources Directorate (MAAIF)	MAAIF works with sector Associations, issues SPS certificate
Dairy	Dairy Development Authority (DDA)	Implement Dairy Industry Act
Meat	Animal Resources Directorate (MAAIF), Local Government (DVOs)	District Veterinary officers (DVOs) work with local government, also report to parent Ministry
Coffee	Uganda Coffee Development Authority (UCDA)	Implement coffee production and marketing regulations
Cereals, and Pulses	MAAIF, **UNBS**, Local Government	Monitor Moisture content to avoid aflatoxin
Hygiene and Health aspects	MOH, Local Government, UNBS	Implement Regulations and standards on hygiene
Imported food	**UNBS**	Implement Imports inspection regulations
Imported live animals and plants	MAAIF	Disease control

Common mycotoxins in foodstuffs

Mycotoxin	Main causal agent	Foods commonly contaminated
Aflatoxin	*Aspergillus flavus, A. parasiticus*	All grains, dried fruits
Fumonisin	*Fusarium verticillioides*	Maize
Zearalenone	*Fusarium graminearum*	Maize
Ochratoxin	*Aspergillus ochraceous*	Coffee, cocoa
Trichothecenes (T2 Toxins and deoxynivalenol)	*Fusarium spp*	Cereals (wheat, barley, maize, rice)
Patulin	*Penicillium digitatum*	Apples

Assessment of Mycotoxins

- Toxic Secondary metabolites naturally produced by fungi/molds
- Contaminate agricultural commodities given that environmental conditions are favorable (Field, handling, storage)
  - **Temperature 40-90ºF (4-32ºC)**
  - **Rel Humidity >70%**
  - **Moisture (22-23% esp in grain)**
  - **Oxygen 1-2%**
- Monitoring necessary due to public health concerns; acute, chronic , mutagenic effects observed in humans and animals

Assessment of Mycotoxins

- 2004 - Aflatoxin-contaminated maize in Kenya resulted in 317 cases of hepatic failure and 125 deaths, **(contamination 4,400µg/kg of Aflatoxin B1 220 higher that set limit)**
- 2013, February–March - Contamination with aflatoxins results in a milk recall in Europe and a dog food recall in the United States
- *Analysis is essential to minimize consumption of contaminated food and feed*

Assessment of Mycotoxins

- Method development & evaluation is no easy task

Determining levels for most important mycotoxin in grains at **µg/kg or ppb** is difficult

- **Relatively large primary sample representing a Lot**
- **Reduce sample in bulk & particle size to manageable quantity**
- **Perform analysis on a small representative portion**

- Essential to select a suitable optimum protocol for analysis
- **Selectivity/specificity, Precision, reproducibility, Accuracy recovery etc**

Methods of mycotoxin Detection

- Visual inspection eg in grains, which may locate lots **presumed** to be contaminated with aflatoxin (black light test);
- Rapid screening procedures to determine the presence or absence of aflatoxins (the fluorometric iodine rapid screening and minicolumn tests);
- Laboratory procedures quantifying the **actual** amounts of toxin (thin-layer chromatography, gas-liquid chromatography, high-pressure liquid chromatography, or ELISA tests).

Methods of mycotoxin Detection

- Biological methods
  - Lab animals
  - Larvae
  - Bacteria
- Physicochemical methods
  - Thin layer chromatography
  - High performance liquid chromatography
  - Gas chromatography
  - Mass spectrometry
- Immunological Methods

Enzyme-Linked Immunosorbent Assay (ELISA)

Aflatoxin, Zearalenone, Ochratoxin, DON, T-2

- **Detects and quantifies the presence of an antigen (aflatoxin) in a sample using an enzyme labelled toxin and antibodies specific to aflatoxin**
  - **Polyclonal antibodies**
  - **Monoclonal antibodies**
  - **Recombinant antibodies etc**

Enzyme-Linked Immunosorbent Assay (ELISA)

- ***Accurate*** *– Results are comparable with published* HPLC method
- ***Highly Sensitive***
- ***Reproducible*** *– Consistent results obtained in* intra- and inter-laboratory settings

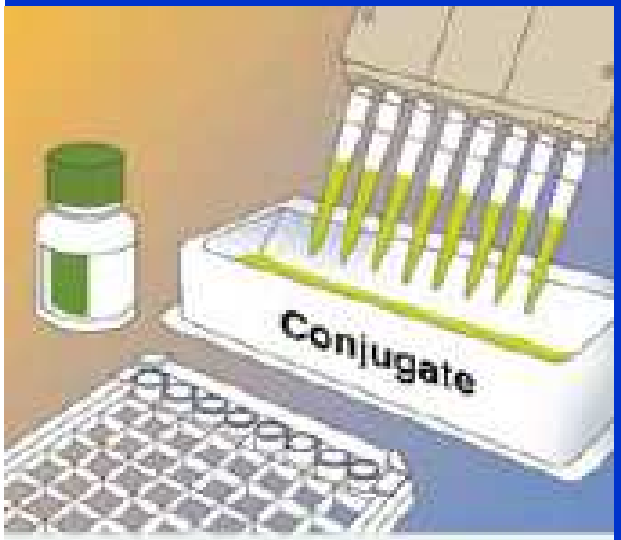
- **Other Benefits**
- ***Rapid*** *– 10-20 minutes total incubation time*
- ***Stable*** *– up to 12 months shelf life*
- ***Easy*** *– Simple sample extraction and no clean up* steps required
- ***Cost-effective*** *– 48 or 96 breakaway microwell* format; minimizes waste and maximizes value
- ***Convenient*** *– Up to 30 minutes reading time after* stopping the reaction

AgraQuant Kit Performance characteristics

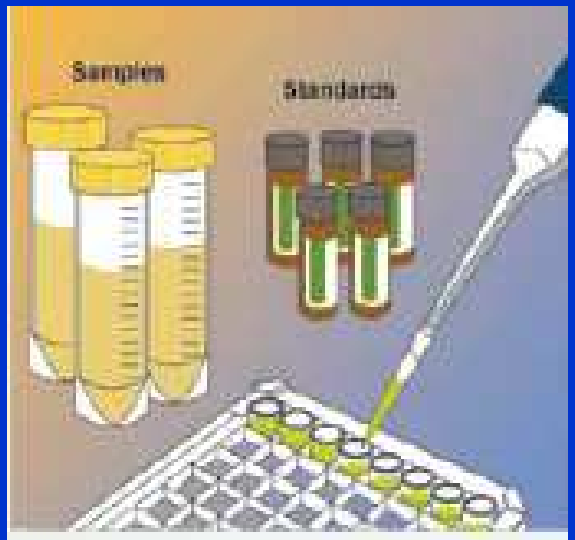
Mycotoxin	Quantitation Range	Limit of detection
Total Aflatoxin	1-20 µg/kg	1 µg/kg
Total Aflatoxin	4-40 µg/kg	3 µg/kg
Rapid Aflatoxin	4-100 µg/kg	3 µg/kg
Ochratoxin	2-40 µg/kg	2µg/kg
Total Fumonisin	0.25-5.0 mg/kg	0.2 mg/kg

ELISA Methodology (Assay perfomed in plastic microwells coated with anti-aflatoxin antibody)

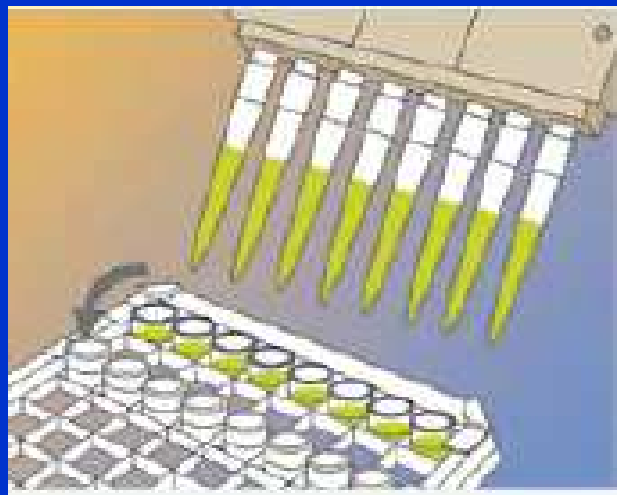
- Extraction of sample: 50g of sample taken + 10g Nacl; extraction done using 250ml (methanol:water; 70:30v/v) in blending jar
- Filter through Whatman 1 and use 50µL aliquot



1) Add 200 µL conjugate into each color-coded dilution well.

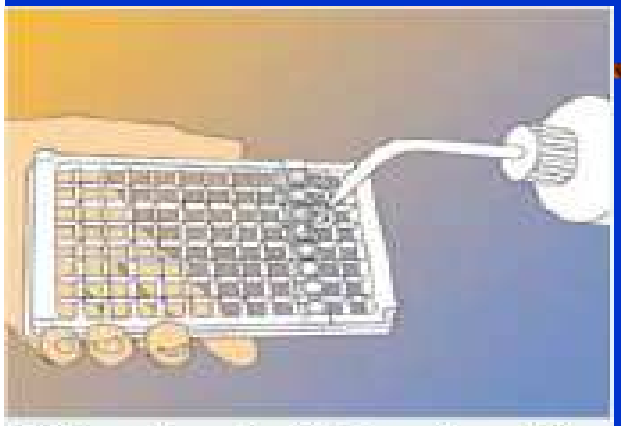


2) Add 100 µL standards or samples to the conjugate.



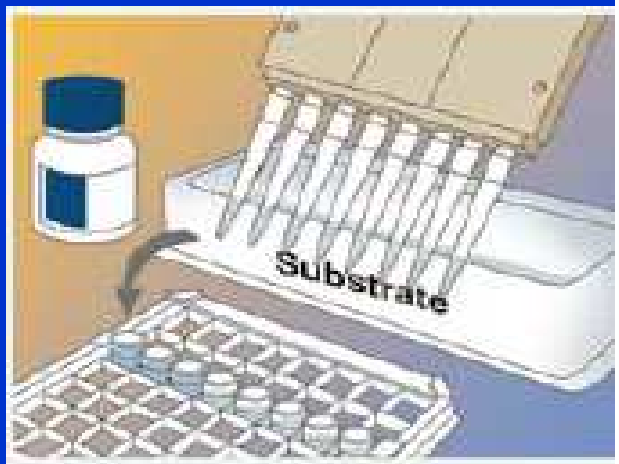
3) Mix well. Transfer 100 µL content to antibody-coated wells. Incubate for 5-15 minutes.

ELISA Methodology



4) Discard contents from the wells and wash wells with deionized water or buffer solution (5x).

5) Tap dry the wells on absorbent paper towel.



6) Add 100 µL substrate into each well. Incubate for 5 minutes.

7) Add 100 µL stop solution into each well.

8) Analyze results using an ELISA reader with 450 nm filter.

Absorbance 450nm;
Colour development inversely proportional to aflatoxin concentration in sample
Y= a. Log (X) + b

High-pressure liquid chromatography (HPLC)

Aflatoxins, Fumonisins

- Grains are extracted and the extract fractionated on either normal or reverse phase columns.
- **1.5 - 3ml HPLC grade methanol was used to elute bound Aflatoxin**
- The aflatoxins are detected using either UV-absorbance or fluorescence detectors.
- Can accurately and quantitatively identify aflatoxin B_1, B_2, G_1, and G_1,
- Expensive equipment /invest

High-pressure liquid chromatography (HPLC)

Operating Conditions

HPLC column	**Column: zorbax Eclipse Plus C18, 4.6x150mm x 5um**
Mobile phase	Mobile Phase A: 1L water containing 238 mg KBr and 700µL 4M Nitric acid Mobile phase B; Methanol=50:50; Isocratic
Flow rate	1.0ml/min
Injection volume	20µL
Column Temp	40ºC
Fluorescence Detection	λ – Excitation: 365 nm; λ – Emission: 460 nm
Runtime	12min
Mathematical model	Y (peak Area, µV.sec) = a.X–b X=(amount of standard solution, µg/kg) Y=(peak Area, µV.sec)

Results comparison of samples analysed from general market surveillance programmes

Coefficients and accuracy indicators . Model equation Y = a.X–b

Mycotoxin	Equation Coefficients		Accuracy indicators	
	a	**b**	**R^2**	**R**
Aflatoxin B1	3.04×10^5	1.45×10^4	0.995	0.999
Aflatoxin B2	4.60×10^5	2.72×10^4	0.999	0.999
Aflatoxin G1	3.56×10^5	6.05×10^3	0.998	0.998
Aflatoxin B2	4.36×10^5	2.06×10^4	0.998	0.999

Results comparison of samples analysed from general market surveillance programmes

Established Limits of quantification

Parameter	HPLC	ELISA
Total Aflatoxin	0.2	3.0
Aflatoxin B1	0.4	1.0

Recoveries for some quality control samples

Reference material	**Total Aflatoxin**		**Aflatoxin B1**		**Recommended value**
	HPLC	ELISA	HPLC	ELISA	
Flour	94%	70%	98%	52%	50-120%

Results ranges as average/number of samples

ND == Not done
NS == Not Specified

Product description	No. of samples	Total aflatoxins (µg/kg)	B1; (µg/kg)	B2; (µg/kg)	M1; (µg/kg)	M2; (µg/kg)	G1; (µg/kg)	G2; (µg/kg)	Maximum tolerable Limit (Codex/National standards)	
									Total aflatoxins (µg/kg)	Aflatoxin B1
Barley malt	1	0-2.3	ND	ND	ND	ND	ND	ND	NS	NS
Flour	30	0-25	<1-2	<0.5	ND	ND	ND	ND	10	5
Peanut butter	40	2-17	<0.5-3	<0.5	ND	ND	ND	ND	10	5
Therapeutic food	40	3-19	0.2-1	<0.5	ND	ND	ND	ND	NS	NS
Rice	20	0.5-3.5	0.8-3	<0.5	ND	ND	ND	ND	10	5
Groundnuts	19	0-12	<1.0	<0.5	ND	ND	ND	ND	10	5
Poultry feed	10	0-32	<1.0	<0.5	ND	ND	ND	ND	NS	NS
Pig feed	5	0-7	<1.0	<0.5	ND	ND	ND	ND	NS	NS
Milk	40	ND	ND	ND	0-0.2	<0.5	ND	ND	NS	NS

Conclusions

- Both methods are sensitive to provide accurate & reproducible results for the set levels
- HPLC is more suitable to quantify low levels and multiple analytes
- Put systems in place for backward traceability for corrective actions and controls against contamination
- Need to increase scope of analysis such as patulin, Zearalenone, Trichothecenes (T2 Toxins and deoxynivalenol)
- Collect more data for standard development and limits
- Increase testing capacity and monitoring (simpler, more sensitive technologies

Acknowledgements