Methods of Estimation of Aflatoxins and Other Contaminants

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Introduction

➢ Aflatoxin is a global food safety concern.

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Farmer → Processer → Distributor → Consumer
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Concerns

Technique
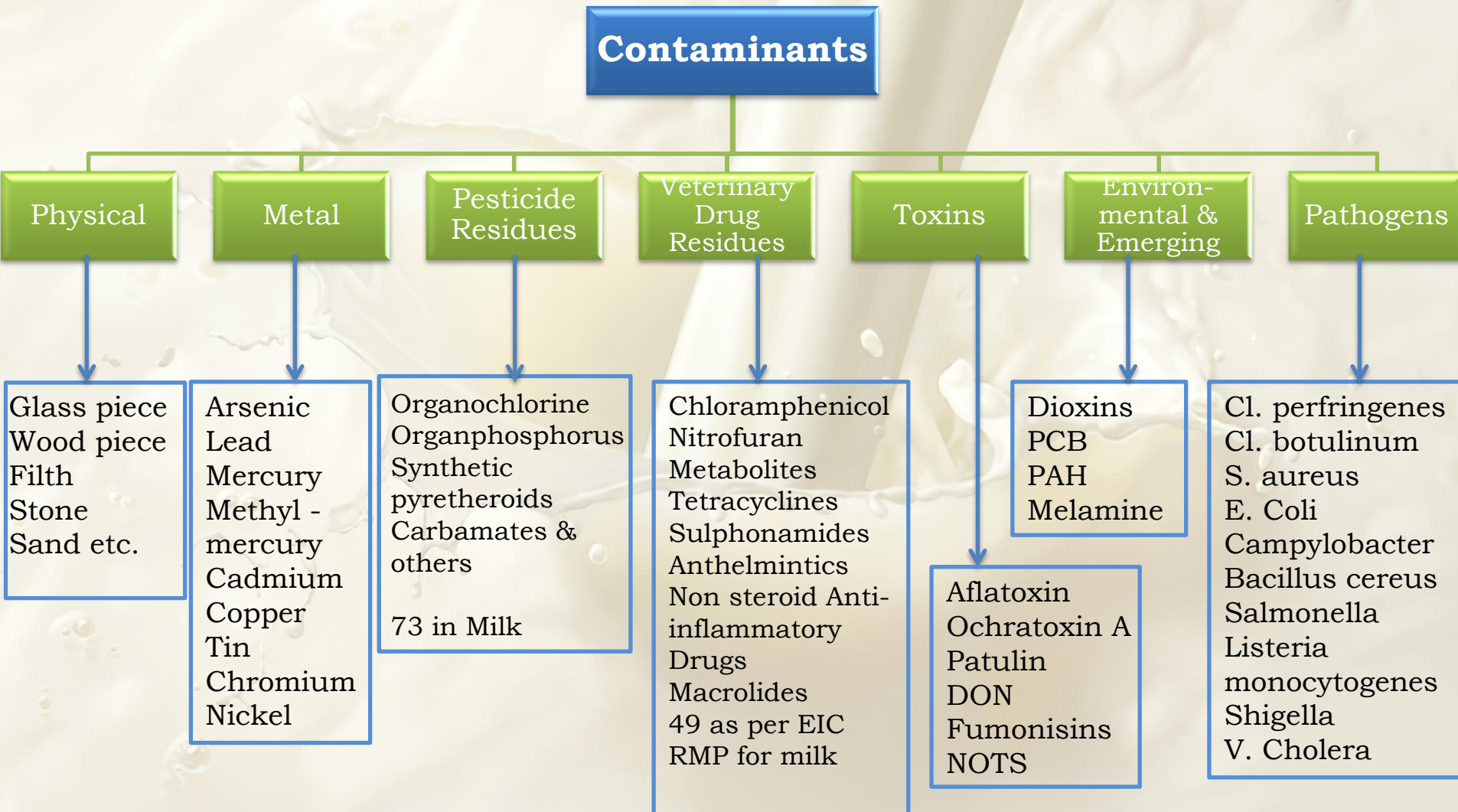Accuracy and precision of Technique
Homogeneity
Competence of the laboratory
Reporting & Compliance

Feed and Food contaminants

Feed and food contaminants can be majorly classified as physical, biological and chemicals.

Contaminants

Physical	Metal	Pesticide Residues	Veterinary Drug Residues	Toxins	Environ-mental & Emerging	Pathogens
Glass piece Wood piece Filth Stone Sand etc.	Arsenic Lead Mercury Methyl - mercury Cadmium Copper Tin Chromium Nickel	Organochlorine Organphosphorus Synthetic pyretheroids Carbamates & others 73 in Milk	Chloramphenicol Nitrofuran Metabolites Tetracyclines Sulphonamides Anthelmintics Non steroid Anti- inflammatory Drugs Macrolides 49 as per EIC RMP for milk	Aflatoxin Ochratoxin A Patulin DON Fumonisins NOTS	Dioxins PCB PAH Melamine	Cl. perfringenes Cl. botulinum S. aureus E. Coli Campylobacter Bacillus cereus Salmonella Listeria monocytogenes Shigella V. Cholera

Mycotoxins

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                        ┌──────────────┐
                        │  Mycotoxins  │
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Aflatoxins	Ochratoxin A	Deoxynivalenol & Zearalenone	Patulin	Fumonisins
Aspergillus flavus and Aspergillus parasiticus	*Aspergillus ostianus*	*Fusarium graminearum Fusarium culmorum*	*Penicillium expansum, Aspergillus clavatus,*	*Fusarium verticillioides Fusarium proliferatum*
• Cereal & Cereal Products • Oil Cakes • Peanut and other nuts	• Wheat • Barley • Rye	• Wheat • Corn • Rye • Barley	• Apple juice • Custard apple	• Corn

Maximum Residue Limits (MRL)

S. No	Name of Contaminants	Name of the Product	Limit (µg/kg)
1	Aflatoxin	**Cattle feed (IS 2052)**	**20**
		Cereal and Cereal Products	15
		Pulses	15
		Nuts Nuts for further processing Ready to eat	 15 10
		Dried figs	10
		Oilseeds or oil Oil seeds for further processing Ready to eat	 15 10
		Spices	30
2	Aflatoxin M1	**Milk**	**0.5**
3	Ochratoxin	Wheat, barley and rye	20
4	Patulin	Apple juice and Apple juice ingredients in other beverages	50
5	Deoxynivalenol	Wheat	1000

Types of Aflatoxin

Feed & Food	Feed & Food	Milk
B_1	G_1	M_1
B_2	G_2	

➢ Aflatoxins B_1, B_2, G_1 and G_2 refer to toxins which give blue (B) or green (G) fluorescence under ultraviolet light.

➢ Aflatoxin M_1 is produced from B_1 in lactating animal/humans when contaminated material is consumed. Since it is found in milk it is designated as 'M'.

Overview of Methods
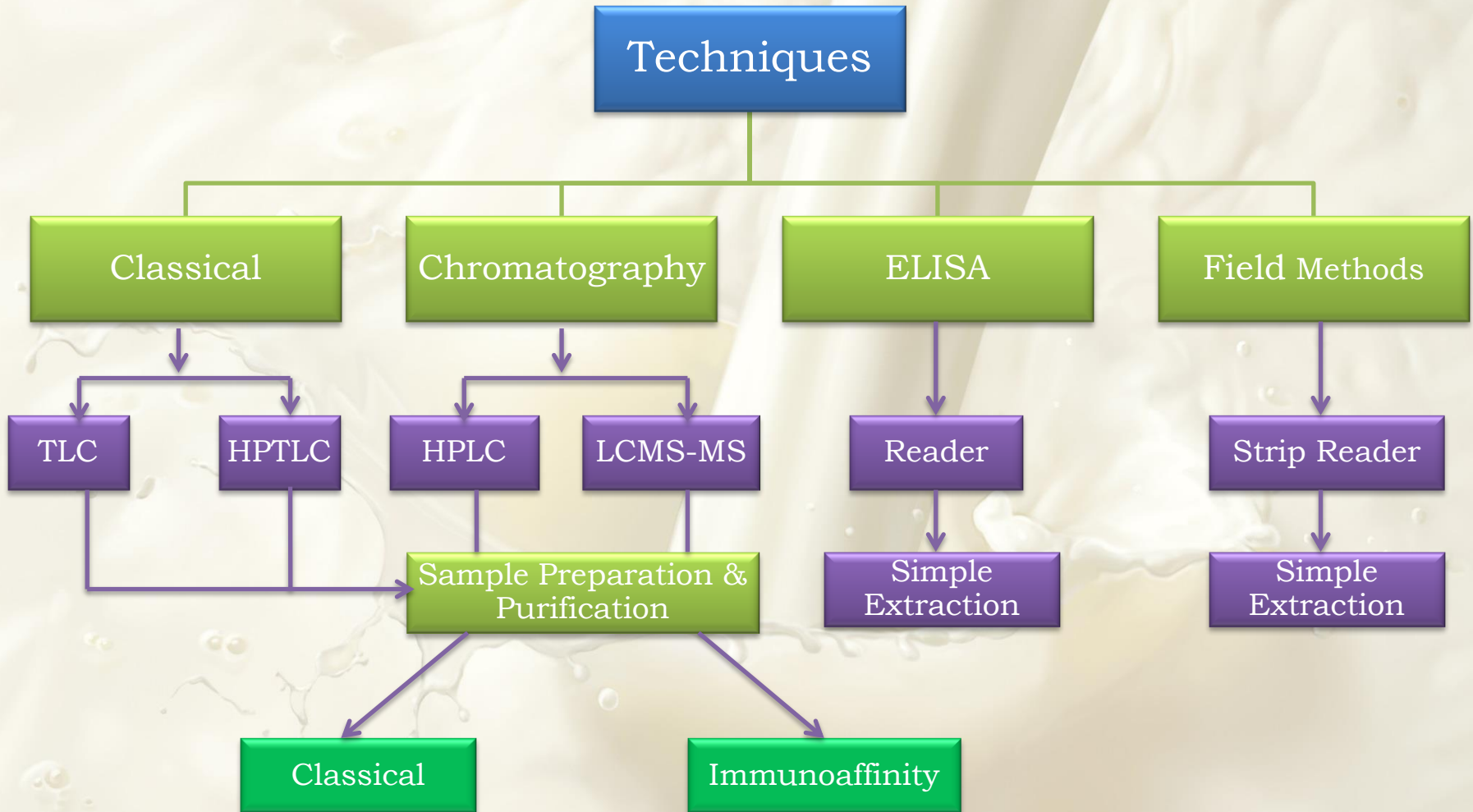
Importance of Sample Preparation (Immunoaffinity Column)

When sample containing (aflatoxin) is passed, aflatoxin binds to the antibodies

Column is washed with PBS or Water to remove remaining matrix particles, if any

Aflatoxin is extracted with Methanol which breaks down the structure of antibodies and releases aflatoxin. It is collected and analysed using technique like HPLC/LCMS-MS

▲ Mycotoxins

◆ ● ■ Other Material

SAMPLE

WASHING

ELUTION

▲ Mycotoxins

WASTE

WASTE
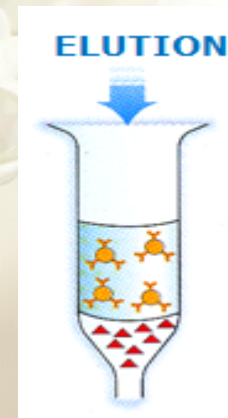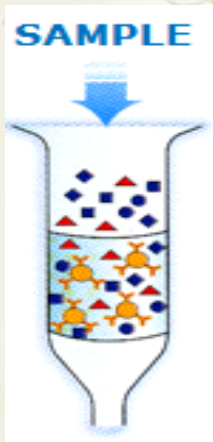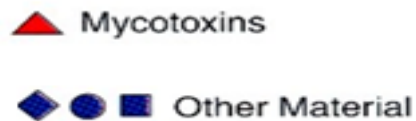
HPLC

TLC & HPLC Technique

The process steps of TLC & HPTLCs are identical. The main difference between them is in the characteristics of the separation plate. HPTLC plates are based on optimized silica gel 60 with a significantly smaller particle size than used for classical TLC, TLC is qualitative and HPTLC is semi quantitative and equipment is involved.

Advantages:

➢ Cheaper compared to advanced methods

Limitation:

➢ TLC is mostly qualitative and HPTLC is semi quantitative, this is not a common technique in food labs due to limitation in quantification.
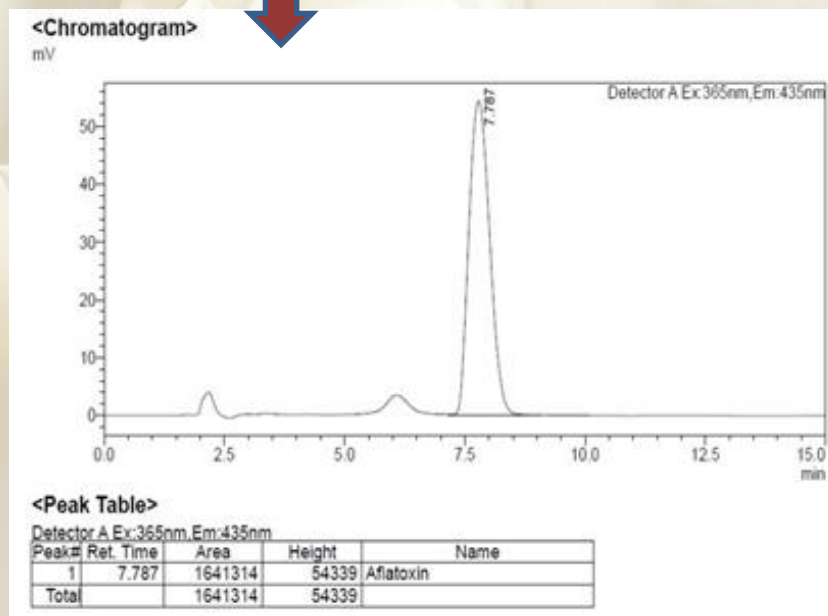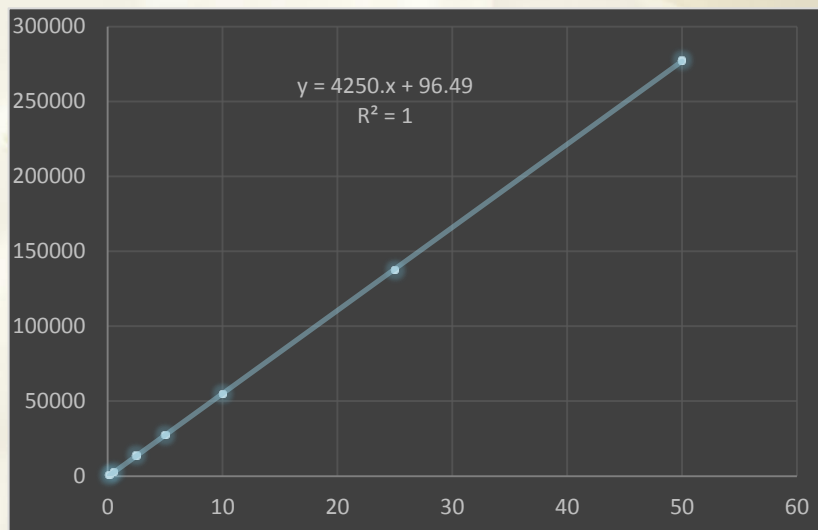
HPLC Technique

HPLC

Kobra Cell

y = 4250.x + 96.49
R² = 1

<Chromatogram>
mV

Detector A Ex:365nm,Em:435nm

7.787

<Peak Table>
Detector A Ex:365nm,Em:435nm

Peak#	Ret. Time	Area	Height	Name
1	7.787	1641314	54339	Aflatoxin
Total		1641314	54339	

HPLC Technique

Advantages:

➢ High performance liquid chromatography provides fast and accurate aflatoxin detection results within a short time.

➢ The detection is as low as **0.025 μg/kg** for Aflatoxin M1 in Milk and **5 μg/kg** for Aflatoxin B1 in Feed using FLD can be achieved.

Limitation:

➢ HPLC for aflatoxin analysis requires rigorous sample purification using immune affinity columns which is expensive.

LCMS/MS Technique

LC MS/MS

LCMS/ MS is one of the most advanced techniques for mycotoxin analysis and many labs are moving towards this technique which is particularly suitable for multi-toxin analysis.

Advantages:

➢No requirement for derivatisation.
➢Multi toxin analysis leads to more efficient use of time.

Limitation:

➢LC-MS/MS is expensive equipment with high running cost which can only be operated by trained and skilled personnel.

Enzyme-Linked Immuno-sorbent Assay (ELISA)

Micro well coated with antigen specific antibodies

Antigen specific antibodies

Antibody immobilized onto a solid support and captures an antigen as analyte (aflatoxin) , which is subsequently detected in the assay.
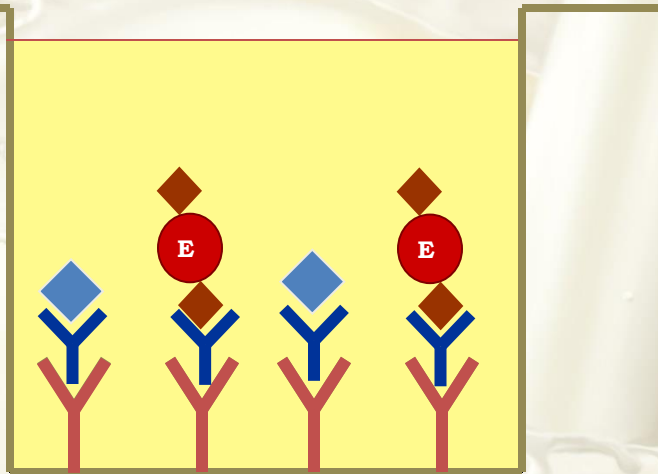
ELISA

Addition of Standard or Sample

ELISA

Color is measured in ELISA Reader

ELISA

Advantages:

➤It is possible to perform the test on a 96-well assay platform, which means that large number of samples can be analysed simultaneously.

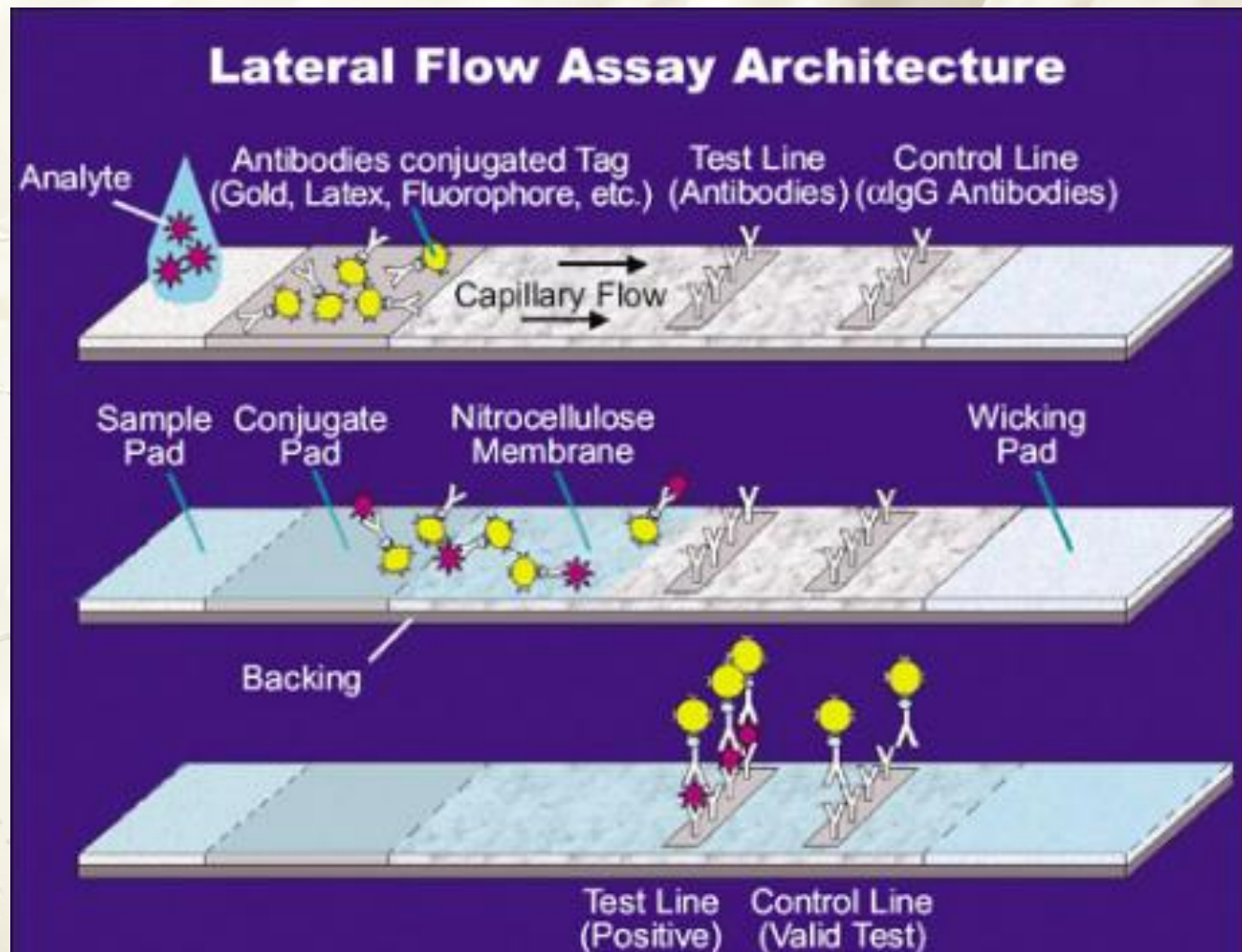➤ELISA kits are cheap and easy to use and do not require extensive sample cleanup.

Limitation:

➤ The ELISA technique is semi quantitative.

Strip Reading / Field Assay

Lateral Flow Technique

Strip Reading

Advantages

➢ It is very fast -Just 3 min

➢ Very Cost Effective

Limitation

➢ Semi-quantitative test

➢ Needs lot of Validation

➢ Difficult to meet regulatory requirements

Techniques for Other Contaminants

Techniques in Microbiology

Conventional Technique

- Requires 9-12 days for confirmation of pathogens.
- More Man-hours required for analysis

VIDAS (Pathogen screening system):

It is an automated, multi-parametric immunoassay testing system internationally used to test food products. It is validated by AOAC/AFNOR.

Advantages:

- Convention technique is cheap
- Result is within 2-3 days in VIDAS
- VIDAS can run many tests simultaneously.
- VIDAS is fully automatic and software controlled.

Limitation:

- VIDAS is expensive and conventional method is time consuming

Techniques in Residues

GC-MS/MS, LC-MS/MS

Pesticides, Antibiotics, Melamine and emerging contaminants

Techniques in Metals

ICP MS and ICP-OES

Metal contaminants and element of nutritional importance

Quality Assurance in Testing

Quality control is a systematic process that controls the validity of analytical results. It checks the accuracy and precision of each method and matrix.

➢External quality control
- ❑ ILC
- ❑ PT

➢Internal quality control
- ❑ Use of Certified reference material (CRM) and Reference standards
- ❑ Use of Control samples
- ❑ Retesting of old sample
- ❑ Spike recovery
- ❑ Positive Samples/ Negative Samples

External Quality Control

Z Score	Result interpretation	Remark
-2 to +2	Satisfactory	No action
2-3	Questionable	Investigate
More than 3	Unsatisfactory	System is out of control

$$z - score = \frac{(\chi_i - \chi_{pt})}{\sigma_{pt}}$$

x_i: Result of participating laboratory

x_{pt}: Assigned value (median)

σ_{pt}: The standard deviation for proficiency testing used for the round (SDPA)

Z score of CALF for aflatoxin B1 and M1 in International Proficiency Testing programs

Year	Name of samples	Name of test	Date of Testing	Organizer	Z Score
2014	Milk Powder	Aflatoxin M1	March-May 2014	FAPAS, England	1.4
2016	Cornmeal	Aflatoxin B1	Jan 2016	APTECA-FAO, USA	0.65
	Cornmeal	Aflatoxin B1	10-Oct-2016	APTECA-FAO, USA	0.72
	Milk Powder	Aflatoxin M1	Oct-Dec 2016	FAPAS, England	-1.2
2017	Cornmeal	Total Aflatoxin	Feb-2017	APTECA-FAO, USA	-1.13
	Cornmeal	Aflatoxin B1	Feb-2017	APTECA-FAO, USA	-0.99
2018	Maize	Aflatoxin B1	Jan-March 2018	APTECA-FAO, USA	1.66
	Cornmeal	Aflatoxin B1	July 2018	FAO-Texas, USA	0.25
	Milk (residues)	Aflatoxin M1	31-Oct-18	DRRR- PT, Germany	0.33

Importance of Sampling

➢ Selective sampling & Representative sampling
➢ Selection of Sample Size
➢ Sample Homogenization
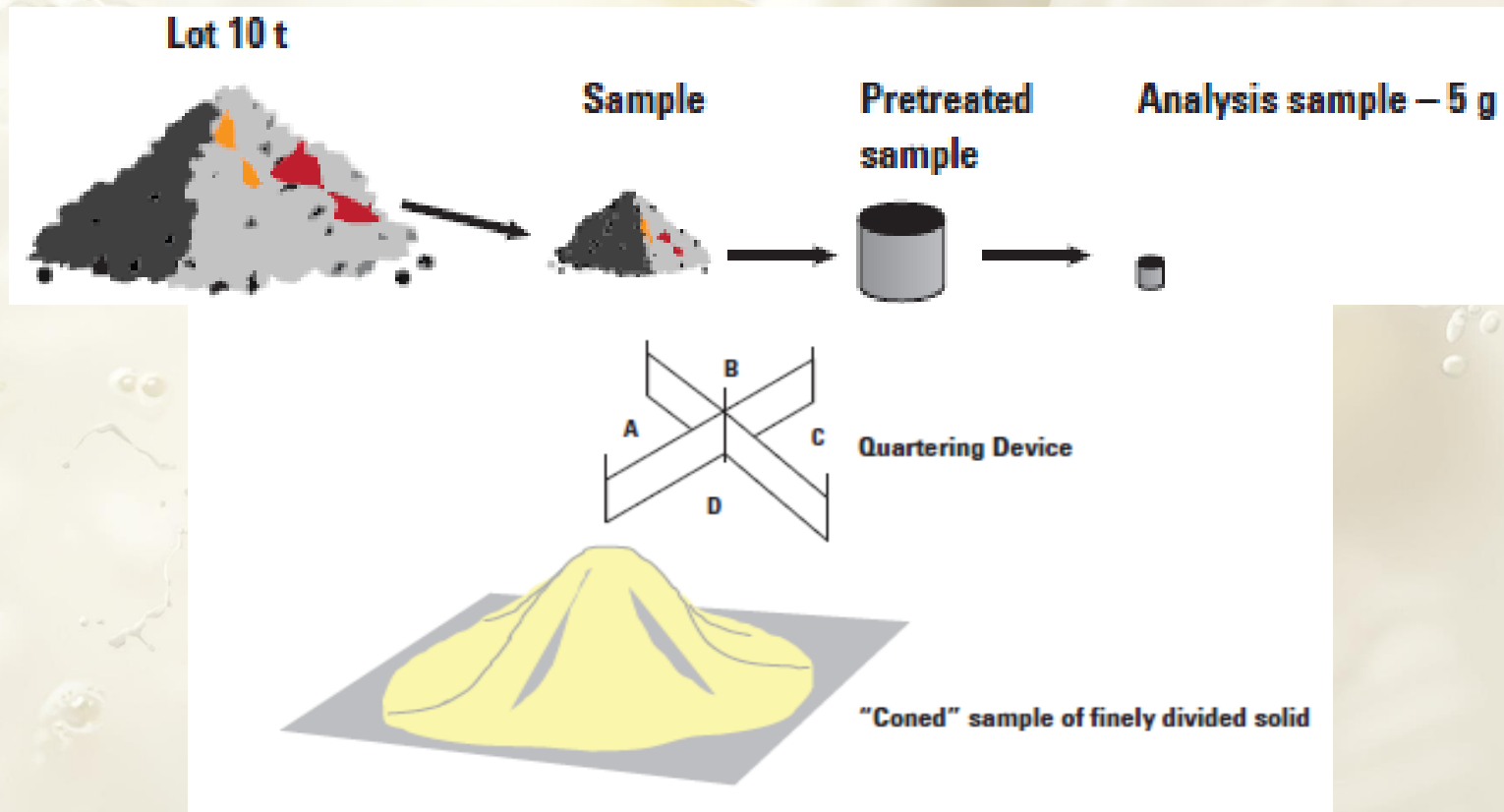
Importance of Reporting for Compliance

Uncertainty:

A parameter associated with the result of a measurement, that characterises the **dispersion of the values** that could reasonably be attributed to the measurand.

Aflatoxin in Feed : 20.0 ± 3.0 ppb

The values can be between 17 to 23 ppb

Decision Rule: Mandatory as per ISO 17025:2017

- For non compliance or rejection with **<u>low probability of false rejection</u>** (Directive 96/23/EC)
- The start of rejection is at the MRL (Limit) + an amount "g" called guard band.

Decision Rule

MRL (Limit) i.e. 20 ppb

G is the guard band or Acceptance zone

Acceptance Zone

Rejection Zone
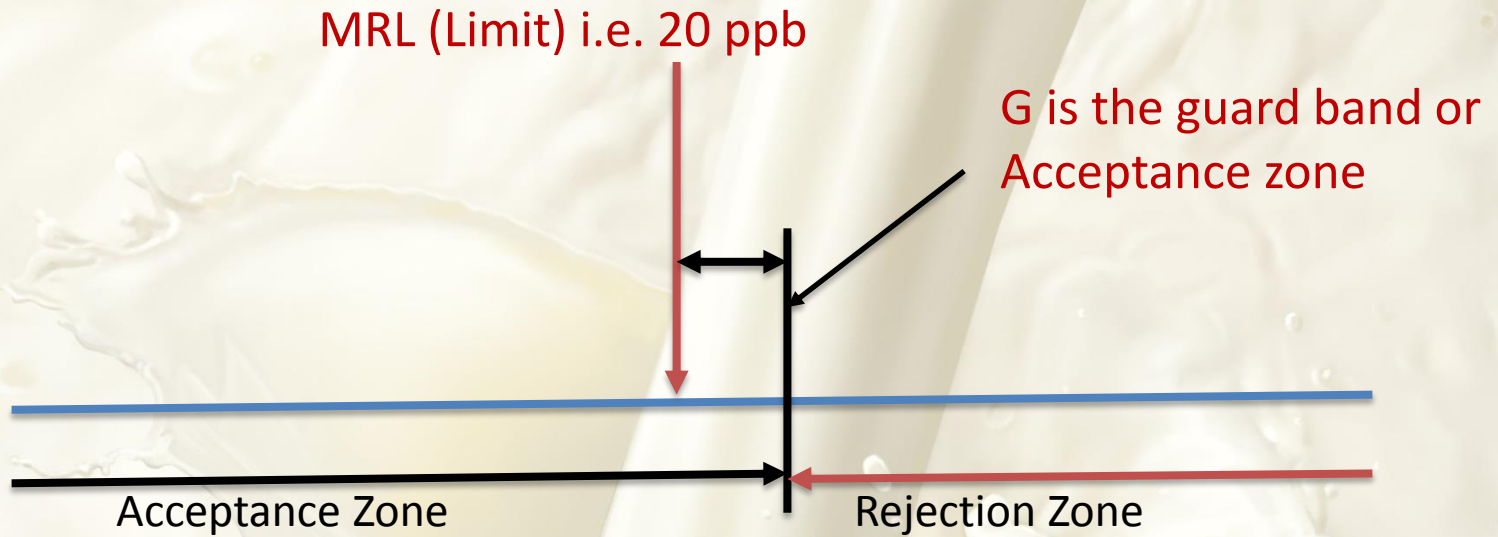
Importance of Accreditation

➢ Implementation of ISO 17025 (NABL Accreditation) results into various improvements w.r.t. analysis

➢ Acceptances of report across the globe by virtue MRA of NABL through APLAC and ILAC.

➢ 8 clauses and 29 requirements.

➢ Risk assessment & Decision rule

➢ Continual improvement

Thank You