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NATIONAL DAIRY DEVELOPMENT BOARD

GUIDELINES

For Production of Quality Distillers Dried Grains with Solubles (DDGS) as Livestock Feed Ingredient

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National Dairy Development Board, Anand-388 001

FOREWORD

The expansion of grain-based ethanol production in the country has significantly enhanced the availability of Distillers Dried Grains with Solubles (DDGS), a valuable co-product with considerable potential for use as a livestock feed ingredient. DDGS provides a cost-effective source of protein, energy and essential nutrients, while contributing to efficient resource utilization and the circular bio-economy.

The nutritive value and consistent quality of DDGS are, however, critically dependent on the quality of raw grains, process control during fermentation and distillation, drying conditions, and post-production storage and handling practices. Variations in these aspects can result in inconsistent nutrient composition, heat damage to proteins, reduced digestibility and concentration of contaminants, particularly aflatoxins. Ensuring standardized production and quality assurance practices is therefore essential for the safe and effective utilization of DDGS in livestock feeding systems.

The document ***"Guidelines for Production of Quality Distillers Dried Grains with Solubles (DDGS) as Livestock Feed Ingredient"*** has been prepared by the Animal Nutrition Group of the National Dairy Development Board (NDDB) to address these issues in a systematic manner. The guidelines cover the complete DDGS production and supply chain, including grain procurement and storage, processing, drying, quality control and feed safety, with emphasis on compliance with proposed Bureau of Indian Standards (BIS) specifications, aflatoxin risk management and adoption of good manufacturing practices.

Adoption of these guidelines by ethanol/DDGS producers, traders and cattle feed plants will promote consistency in quality, enhance confidence of the feed industry, and enable safer and wider inclusion of DDGS in cattle feed formulations. This will support improved animal health and productivity, while safeguarding food safety, particularly in the dairy sector.

All stakeholders are encouraged to adopt and disseminate these guidelines in order to strengthen the livestock feed ecosystem and support national objectives related to ethanol blending, sustainability and feed security.


HEAT
STIR
SPINOT
HEAT
STIR
SPINOT
HEAT
STIR
SPINOT

CONTENTS


GOODS CARRIAGE
NATIONAL PERMIT
TATA
3296

Introduction

Distillers Dried Grains with Solubles (DDGS) is a co-product of the ethanol industry, obtained after fermentation and distillation of cereal grains such as maize, sorghum, wheat, or barley. Over the last two decades, DDGS has emerged as a valuable and cost-effective ingredient in livestock and poultry rations due to its high concentration of protein, energy and essential minerals.

The rising demand for high-quality animal feed, combined with the rapid expansion of ethanol production, has strengthened the role of DDGS as a sustainable feed resource. However, the nutritive value and safety of DDGS depend largely on the quality of raw materials, fermentation efficiency, drying technology, and adherence to standardized production protocols. Inconsistent processing or inadequate quality control can cause wide variations in nutrient composition, digestibility, and presence of contaminants, thereby restricting its effective utilization in livestock feeding.

Although the adoption of DDGS as a feed ingredient is driven by both nutritional and economic considerations, quality consistency remains the most critical determinant of its acceptance in the feed industry. Variations in nutrient profile and digestibility can arise due to:

- Type and quality of grain used.
- Fermentation efficiency (yeast strain, enzymatic treatment, process optimization).
- Distillation and drying parameters, particularly excessive heat that can trigger Maillard reactions and reduce amino acid digestibility (notably lysine).
- Proportion and uniformity of condensed distillers solubles added.
- Storage and handling practices that affect moisture stability, microbial load, and mycotoxin risks.

Ensuring quality production of DDGS is therefore essential to maximize its feeding value, safeguard animal health and performance, and meet feed industry expectations. This requires close monitoring of production processes, precise control of drying conditions to prevent nutrient damage, prevention of aflatoxin contamination, and establishment of reliable nutrient specifications.

Accordingly, the production of high-quality DDGS must be guided by stringent process controls, quality assurance protocols, and industry-wide specifications, including:

- Raw material screening for grain quality and mycotoxin load.

- Controlled fermentation to ensure high ethanol yield and nutrient preservation.
- Optimized drying (typically 85–95 °C with controlled residence time) to prevent protein denaturation while achieving target moisture (<12%).
- Nutrient standardization through accurate blending of distillers grains with solubles.
- Regular analytical testing for proximate composition, amino acid profile, fiber fractions, fat content, sulfur levels, and contaminants.

Adopting such standardized practices ensures that DDGS serves as a reliable, safe, and nutritionally rich feed ingredient, thereby enhancing its market value and supporting its wider adoption across livestock production systems.

This document provides detailed guidelines and best practices for the production of quality DDGS, emphasizing critical control points from raw material selection to final product handling, so that it can be consistently utilized as a high-value livestock feed ingredient.

1.0 Scope

These guidelines prescribe the procedure for production of Quality Distillers Dried Grains with Solubles (DDGS) by grain-based distilleries; describes specifications and test methods to measure the quality of DDGS.

2.0 Objective

Main objective of these guidelines is to produce quality DDGS with label requirements of BIS, so as to assure that an animal receives quality feed item.

3.0 Terminology

Distillers' grain

Residual grain or by-product of a fermentation process in alcohol production from grains (especially corn), which may be fed wet or dry (ISO 20588: 2019).

Distillers dried grains with solubles

Distillers Dried Grain with Solubles (DDGS) is the main by-product of the distillation process from maize grain *(Zea mays L.)*, rice grain *(Oryza sativa)*, barley grain *(Hordeum vulgare)* or wheat grain *(Triticum aestivum)*. DDGS is a dried material produced as a result of extraction of starch from the grains and is considered as valuable feed ingredient for livestock feeding. It is rich in crude protein, moderately rich in crude fat and relatively low in crude fibre.

Foreign matter

Any extraneous matter other than food grains comprising of,

a) inorganic matter includes glass and metallic pieces, dust, sand, gravel, stones, dirt, pebbles, lumps or earth, clay, mud and animal filth etc;

b) organic matter consisting of husk, chaff, straw, weed seeds and other inedible grains etc.

Other edible grains

Any edible grains (including oil seeds) other than the one which is under consideration.

Varietal admixture

The presence of a variety of the same grain other than the variety in consideration.

Damaged grains

Grains that are sprouted or internally damaged as a result of heat, microbes, moisture or weather.

Immature and shrivelled grains

Grains that are not properly developed.

Weevilled grains

Grains that are partially or wholly bored by insects injurious to grains but do not include germ eaten grains and egg spotted grains.

Screening and mixing

The ground grain mass is screened to remove debris and then mixed with water to create a slurry known as "mash."

Starch conversion

Starch, which consists of up to 35% of amylose and the remainder amylopectin, must be converted into glucose before fermentation. This is achieved by adjusting the pH of the mash to 5.5-6.0 and adding the thermo-stable enzyme *alpha-amylase*, which breaks down the starch into soluble dextrins.

Fermentation

The mash is transferred to a fermenter, where yeast (typically *Saccharomyces cerevisiae* due to its efficiency) is added. This fermentation process takes about 60 hours.

Distillation

The fermented mash is pumped into a multi-column distillation system where heat is applied. The columns separate ethanol from water based on their boiling points (ethanol at 78°C and water at 100°C). The distillation process yields a product stream containing approximately 95% ethanol by volume. This ethanol is then passed through a molecular sieve to remove the remaining water, resulting in 100% pure anhydrous ethanol.

Denaturation of ethanol

Before storage, a small amount of denaturant (5% gasoline) is added to the ethanol to make it undrinkable and unfit for human consumption.

Stillage separation

The residue from the distillation process, called "whole stillage," is centrifuged to separate it into thin stillage (a liquid with 5–10% solids) and wet distillers grains (with 33-34% solid).

Drying

The wet distillers grains (WDG) are dried through evaporation to create a feed product. The thick syrup, high in protein and fat, is mixed back to form wet distillers grain.

4.0 Guidelines for quality production of DDGS

Following points need to considered for production of quality DDGS meant for livestock feeding.

4.1 Procurement of grains

- Grains should be

a) the dried mature grains;
b) sweet, hard, clean, wholesome, uniform in size, shape, colour and in sound merchantable condition;
c) free from added colouring matter, moulds, weevils, obnoxious substances, discolouration, poisonous seeds and all other impurities except the limits specified in Table 1; and
d) free from rodent hair and excreta.

- Suggested specifications for grains shall be as per Table 1.

Table 1. Suggested Specifications of Grains

Sr. No.	Characteristic	Requirements
1	Moisture, percent by weight, Max	12.00
2	Foreign matter*	
	Inorganic, percent by weight, Max	0.10
	Organic, percent by weight, Max	Nil
3	Other edible grains, percent by weight, Max	0.50
4	Varietal admixture, percent by weight, Max	5.00
5	Damaged grains, percent by weight, Max	1.00
6	Immature and shrivelled grains, percent by weight, Max	2.00
7	Weevilled grains (percent by count), Max	2.00
8	Aflatoxin, mg/kg, Max	20.00

*In foreign matter, the impurities of animal origin shall not be more than 0.10 percent by weight.

4.2 Storage of grains

- Grains should be stored in a well-ventilated warehouse equipped with drying facilities (if moisture content is higher than the specified limits) and humidity control systems (Figure 1).
- Bags should be stacked at least 2 feet away from walls and other stacks. Plastic or wooden pallets should be used to keep the bags off the floor and prevent moisture ingress.
- Aeration systems and de-humidifiers should be used, if humidity levels rise above acceptable limits (70%).
- If humidity levels rise above acceptable limits, aeration systems and dehumidifiers must be used to restore proper storage conditions.
- Maize grain stock should be tested for moisture and aflatoxin levels at every 15 days intervals.

Figure 1: Well ventilated and proper storage

Figure 2: Grain storage silo

- A hygrometer or hygro-thermometer should be installed in the warehouse to continuously monitor temperature and humidity levels.
- BIS Code of Practices for Food grain Storage Godowns (IS 16144) should be followed.

4.2.1 Storage of grains in bins/ silos:

- Grain storage silos (Figure 2) are essential for preserving harvested grains, preventing spoilage, and ensuring a consistent supply. They play a key role in maintaining grain quality and protecting against losses caused by pests and moisture.
- The selection of grain silos should be based on the capacity and operational needs of the ethanol plant. Silos should be equipped with temperature and humidity monitoring systems, as well as aeration and drying facilities.

4.3 Sampling of grains

The sampling step specifies how the sample will be taken from the bulk lot, the number of samples to be tested, and the size of each sample. It is assumed that the sample is selected in a random manner and is representative (no bias) of the lot. Typically, many small incremental samples are collected from the lot and combined to form an aggregate sample. Representative samples of the material for ascertaining conformity to the requirements of standard should be drawn according to the method given in Annex D of IS 2052.

4.4 Grain milling

The particle size of grain mash plays a critical role in the enzymatic hydrolysis and fermentation stages of bioethanol production. To ensure optimal conversion, particles must be small enough to allow sufficient water penetration and enzyme accessibility. However, excessively fine particles can lead to challenges in downstream processing, particularly in the recovery of co-products.

The final output and particle size are influenced by the quality and moisture content of the raw material, the condition of the hammer mill and screen, and the selected operating parameters. Therefore, maintaining standard raw materials and consistent operational conditions is essential for achieving the desired particle size.

Particle size needs to be checked regularly using sieves or particle size analyzers. The recommended standard particle size for grain mash is given in Table 2.

Table 2. Recommended standard particle size for grain mash

Average particle size (Broken rice)	Standard
Below 0.3 mm	10-15 %
0.3 mm to 1.0 mm	75-80 %
1.0 mm to 1.2 mm	10-12 %
Above 1.2 mm	NIL

In addition to the above, the following areas in the grinding section require attention:

Dust control and explosion prevention

- Install proper dust collection systems (e.g., bag filters, cyclones).
- Ensure adequate ventilation to prevent dust accumulation.
- Use explosion-proof motors and electrical components.
- Install spark detection and suppression systems, especially for large mills.
- Enforce a strict "no smoking" policy around the mill area.

Temperature monitoring and control

- Continuously monitor the mill's operating temperature.
- Ensure cooling systems (air or water jackets) are functional.
- Avoid overfeeding the hammer mill to minimize friction and heat buildup.

Feedstock preparation

- Remove foreign materials (stones, metals, hard objects) from grains before feeding.
- Use magnetic separators to eliminate metal contaminants.
- Install a scalper or sieve to remove oversized or unsuitable material.

Hammer mill maintenance

- Regularly inspect hammers, screens, and liners for wear.
- Maintain proper lubrication of all moving parts.

Operational practices

- Start the mill only when empty and at full RPM before introducing grain.
- Feed material evenly to prevent choking or overloading.
- Follow proper shutdown procedures — allow the mill to clear before switching off.
- Ensure emergency stop buttons are clearly marked and easily accessible.

Quantity of Products

1 kg Corn =
0.40 L Ethanol + 0.32 kg DDGS

4.5 Enzymatic digestion (Liquefaction)

- Maintain optimal temperature and pH for enzyme activity:
  - o Alpha-amylase: typically, 85–90°C (liquefaction).
  - o Glucoamylase: 55–60°C (saccharification).
  - o Maintain pH: ~5.0–5.5, adjusted with acids or buffers.
- Use correct enzyme dosage as per supplier recommendations or process optimization studies.
- Ensure uniform mixing to prevent hot spots and enzyme denaturation.
- Control mash viscosity to allow effective mixing and enzyme distribution.
- Pre-gelatinize starch if necessary to improve enzyme access.
- Prevent microbial contamination by maintaining hygienic conditions.

4.6 Fermentation process

- Select high-performing yeast strains (e.g., *Saccharomyces cerevisiae*) with high ethanol tolerance and productivity.
- Maintain fermentation temperature typically between 30–35°C depending on yeast strain.
- Optimize pH in the range of 4.5–5.5 for yeast activity.
- Ensure adequate nutrient supply (nitrogen, phosphorus, magnesium, etc.) for yeast metabolism.
- Control sugar concentration – avoid osmotic stress from too high an initial sugar level.
- Monitor ethanol and CO_2 levels to track fermentation progress.
- Minimize oxygen exposure after initial yeast activation to maintain anaerobic conditions.
- Avoid contamination by sterilizing equipment and using clean fermentation media.
- Ensure proper agitation and mixing to maintain uniformity in the fermenter.

4.7 DDGS production process

After fermentation, ethanol is separated by distillation while the water and non-fermentable materials (known as whole stillage) are decanted or centrifuged which results in wet cake (e.g., removed suspended solids) and soluble solid-laden water (i.e., thin stillage). Evaporation of the thin stillage produces condensed distillers solubles (known as CDS) which can then mixed into the wet cake, and then dried to produce coproducts (Figure 3). These coproducts are broadly known as "distillers grains", and can be wet or dry, and may or may not have CDS added into the matrix. The following two major products are available from this industry:

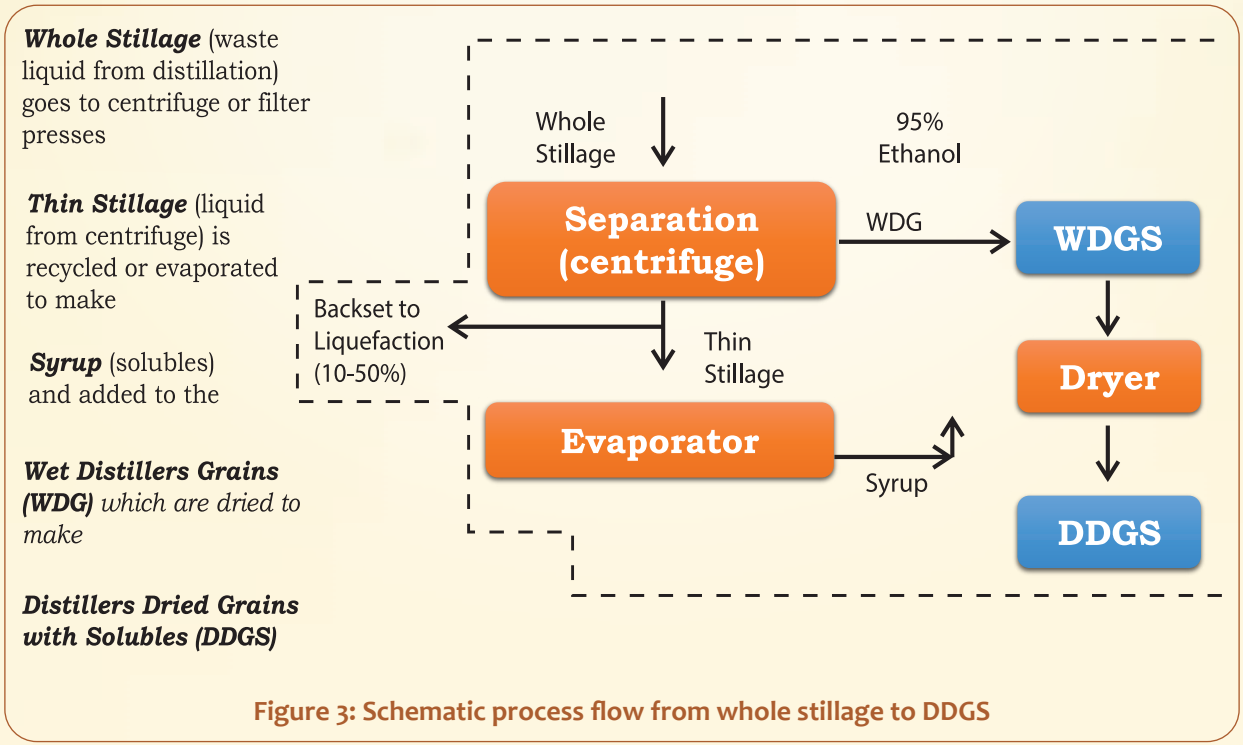


Figure 3: Schematic process flow from whole stillage to DDGS

Wet Distillers Grains (WDG) contain primarily unfermented grain residues (protein, fibre, fat and up to 70% moisture). WDG has a shelf life of four to five days. Dried Distillers Grains with Solubles (DDGS) is WDG that has been dried with the concentrated thin stillage to 10-12 percent moisture.

Distilleries produce alcoholic beverages, industrial ethanol and ethanol biofuel. A schematic flow-diagram indicating the production process of DDGS is illustrated in Figure 4. The process flow for ethanol production from maize through dry milling process is depicted in Figure 5.

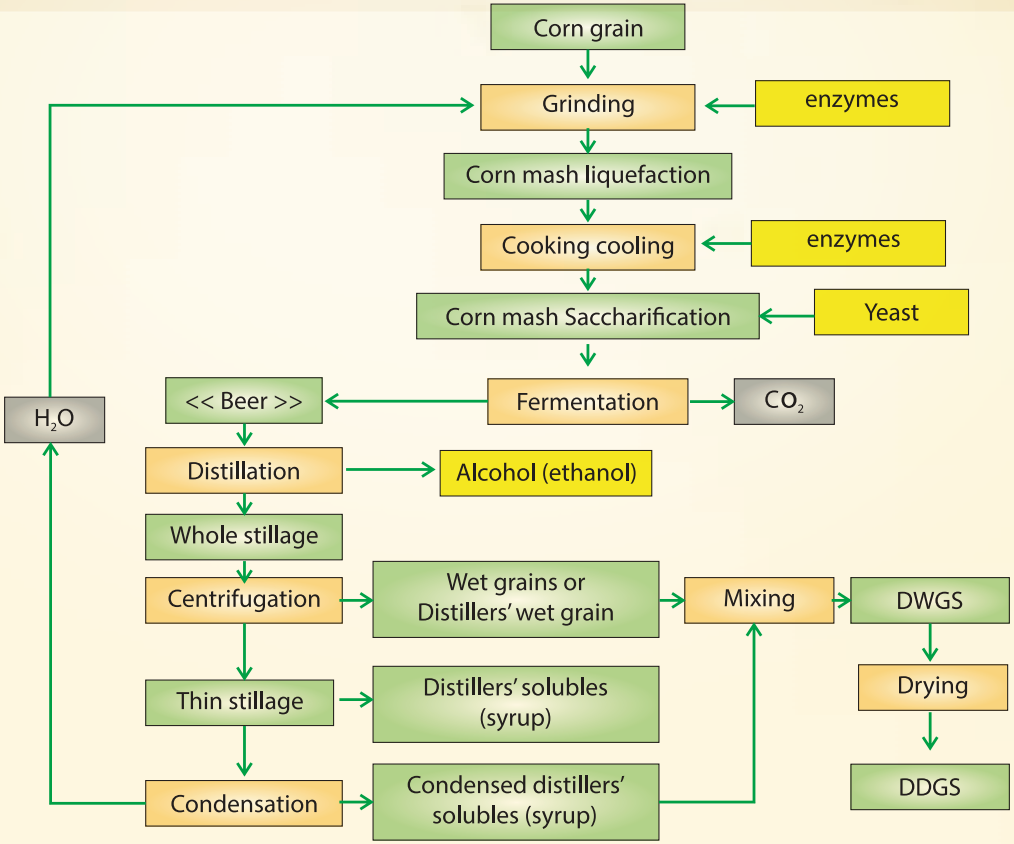
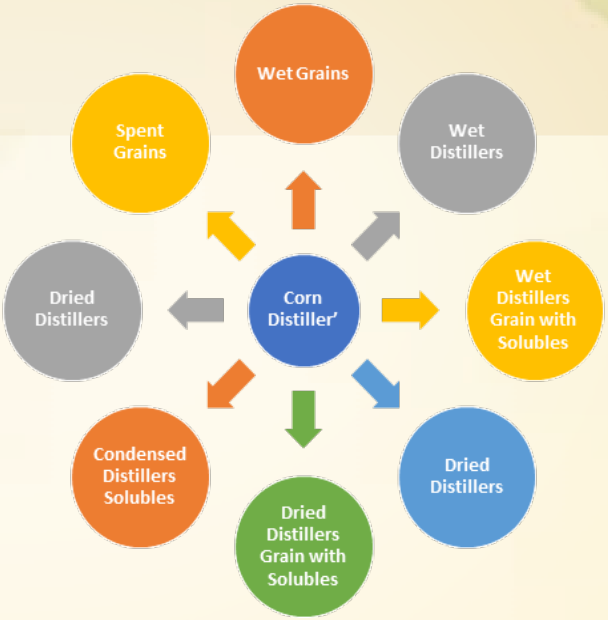


Figure 4: A schematic flow-diagram for production of DDGS (Source : https://www.feedipedia.org/node/71)

4.8 Decanting process

- After fermentation and distillation, the residue known as "whole stillage" - a slurry containing water, ethanol, yeast, dissolved proteins, and grain solids - is collected for separation.
- The whole stillage is pumped into the decanter centrifuge, which spins at high speeds to create strong centrifugal forces.
- Due to density differences, heavier solid particles (fibers, residual grain solids, and yeast) are forced outward, compacting against the bowl wall, while lighter liquid fractions remain closer to the center.
- A rotating scroll within the centrifuge moves the solids (called "Wet Distiller's Grains - WDG") toward discharge openings for collection.
- The clarified liquid known as thin stillage is separated from the solids and typically contains proteins, residual suspended solids, and dissolved nutrients. This 'thin stillage' is often concentrated through evaporation to form a syrup, commonly referred to as 'thick stillage', which is then blended with WDG.
- The resulting mixture is sent to the drying unit for further processing to produce DDGS.
- A typical standard for a decanter in ethanol plants is given in Table 3.



Source: https://www.sciencedirect.com/science/chapter/edited-volume/abs/pii/B9780323991452000136?via%3Dihub

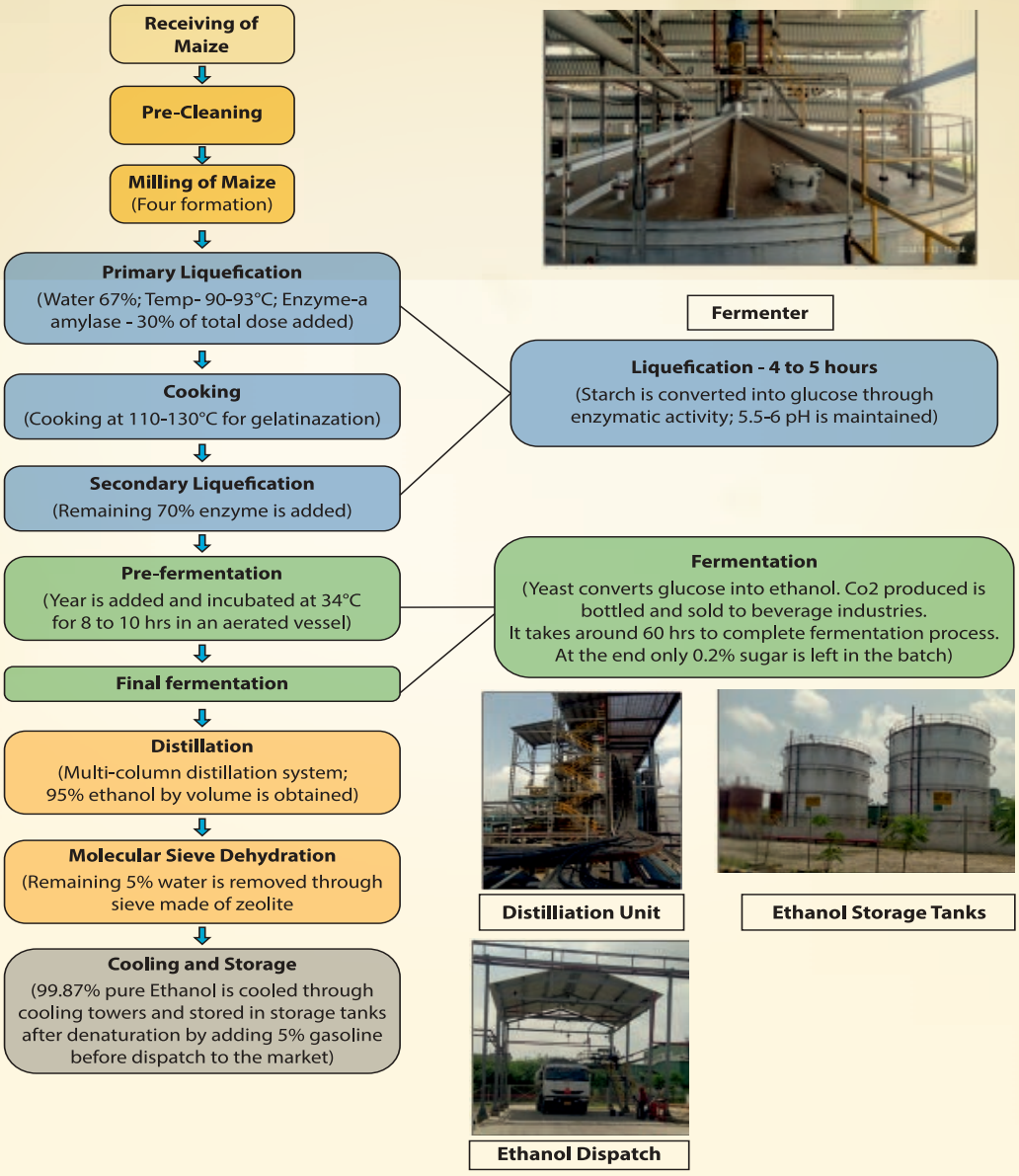


Figure 5: Process flow for ethanol production from maize through dry milling process

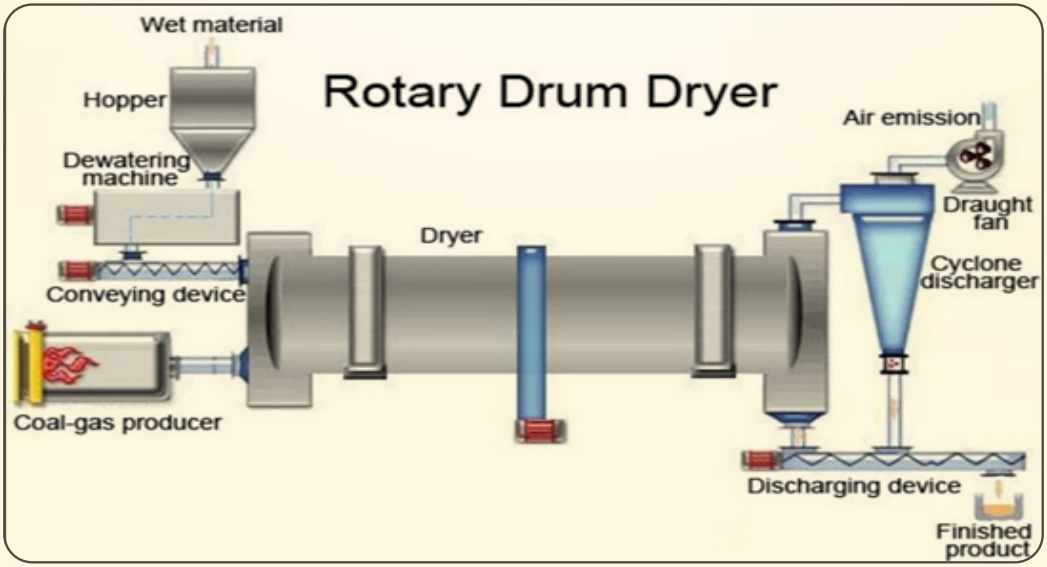
Table 3. Typical standards for a decanter in ethanol plants

Parameter	Typical Standard / Range
Type	Horizontal, solid bowl decanter centrifuge
Capacity	20–100 m^3/h (depends on plant throughput)
G-force (Centrifugal Force)	2,000–4,000 × g
Bowl Diameter	300–700 mm
Bowl Length	900–2500 mm
L/D Ratio	3:1 to 4:1
Operating Speed (RPM)	2,500–4,000 RPM (depending on diameter and feed properties)
Differential Speed	5–30 RPM (scroll vs bowl speed)
Solids Concentration (Output)	30–35% dry solids in wet cake
Motor Power	15–90 kW (depending on size and duty)
Material of Construction	Stainless steel (SS 304/316), wear-resistant alloys
Sealing	Mechanical seals for vapor-tight operation
Control System	PLC/SCADA integrated, with vibration and torque sensors

The decanting process separates whole stillage into solid WDG and liquid thin stillage using high-speed centrifugation based on density differences. Thin stillage is concentrated into syrup and blended back with WDG, followed by drying to produce DDGS as a valuable co-product.

4.9 Drying of wet distiller's grains

- Wet distiller's grains are fed into the dryer housing at a controlled rate using a suitable feeding system. The dryer consists of a rotary tube bundle enclosed within an insulated housing, with external flights attached to facilitate material movement. Dry, saturated steam is supplied to the tube bundle via a rotary joint at one end, while condensate is discharged through a rotary joint mounted at the opposite end.
- As the drum rotates, the attached flights lift and cascade the material over the heated tube bundles, allowing for effective heat transfer primarily through conduction. Water vapor generated during drying is evacuated using an exhaust blower and directed to a cyclone separator, which separates and collects fine particles.
- A portion of the dried product is recycled back to the feed conditioner via a product screw and recycle conveyor to aid in feed conditioning. The entire drying operation is automated and controlled through a centralized control panel.
- A typical standard for DDGS Rotary Dryer in ethanol plants is given in Table 4.



Source: https://vgprocess.com/rotary-dryer-

The picture is indicative and is not intended to promote any particular brand.

Table 4. Typical standard for DDGS Rotary Dryer in ethanol plants

Parameter	Typical Range / Standard
Dryer Type	Rotary drum dryer (direct or indirect fired)
Capacity	8–40 tons/hour (dry basis), depending on plant size
Inlet Temperature	300–500 °C
Outlet Temperature	80–100 °C
Drum Diameter	1.5 – 3.5 meters
Drum Length	10 – 30 meters
Drum Rotation Speed (RPM)	3–12 RPM
Residence Time	30–60 minutes
Heat Source	Natural gas, biogas, steam, or fuel oil
Final Product Moisture Content	10–11%
Material Handling	Screw conveyors, airlocks, and discharge augers
Dust Control	Cyclone separator + bag filter or wet scrubber
Control System	PLC/SCADA-based with temperature and moisture sensors
Construction Material	Carbon steel or stainless steel with refractory insulation
Safety Features	Explosion vents, spark arrestors, and emergency shutoff systems

Drying parameters and operational considerations for DDGS production

- The drying process should be controlled to ensure that the final moisture content of DDGS remains at or below 10–11%, as per product quality standards. Since rotary drum dryers are commonly used in ethanol plants, it is critical to monitor key parameters such as temperature, rotational speed (RPM), and residence time carefully.
- These parameters must be strictly maintained in accordance with the Original Equipment Manufacturer (OEM) guidelines to ensure product consistency, equipment longevity, and operational safety.

- Typical drying conditions:
  - Inlet air temperature: 300–500 °C
  - Outlet air temperature: 80–100 °C
  - Drum rotation speed: 3–12 RPM
  - Residence time: 30–60 minutes
- Rotary drum dryers operate at low RPMs to gently tumble the material, allowing uniform drying. The optimal RPM is influenced by factors such as drum size, design throughput, and material properties.
- The residence time in the DDGS dryer is influenced by factors such as the initial moisture content of the wet cake, inlet and outlet temperatures, feed rate to the dryer, addition of concentrated syrup back into the wet cake etc.
- Careful regulation of these variables ensures efficient drying, prevents scorching or under-drying, and maintains the nutritional and physical quality of the final DDGS product.

If the moisture content of the DDGS exceeds the acceptable range of 10–11%, the material must be reprocessed. Drying parameters such as temperature and residence time should be adjusted accordingly to achieve the desired moisture level. However, excessive drying or excessively high temperatures must be avoided, as they can lead to the production of burnt or discolored DDGS (typically dark brown or black), which negatively affects product quality and market value.

Strict control of dryer temperature, RPM, and residence time is essential to achieve DDGS moisture ≤10–11% while preserving nutritional quality. Adhering to OEM-recommended drying conditions prevents under-drying, reprocessing losses, and quality degradation due to overheating or scorching.

4.10 Colour – an indicator of DDGS quality

Color has been used as a subjective indicator of the nutritional quality of feed ingredients for decades. DDGS color can be an indicator of nutritional value. Although too subjective, color is being used to differentiate real or perceived quality and value among DDGS sources. Research indicates that excessive heating (Maillard reaction) of feed ingredients can result in binding of amino acids and protein to other compounds, such as carbohydrates, thus reducing amino acid digestibility (especially lysine) in monogastric animals. Maillard reactions commonly occur when mid-to-high protein feed ingredients are overheated during the production and drying process, and can be characterized by darkening of color (browning), burned flavor and smell (Figure 7). The nutritional significance of the Maillard reactions in DDGS has been documented in various studies on ruminants, as well as in pigs and chickens, and is responsible for losses in protein quality in DDGS.

In addition, color can give an indication of the maturity of the grain, storage conditions, presence of toxins, contamination due to sand, and possible use of insecticides/fungicides, which give a dull and dusty appearance. Sorghum with an orange to red color may indicate high tannin content. Browning or blackening can indicate excessive heat treatment or spoilage due to improper storage, thus reducing nutritive value. Thus, colorimetric measurements of feed ingredients, especially for DDGS, have become common in the feed industry to assess the extent of heat damage.

Figure 7: DDGS color score card
Source: https://ethanolproducer.com/articles/is-color-the-only-or-best-indicator-of-ddgs-quality-7840

Maize DDGS typically appears as a yellowish, coarse powder with a gritty texture. While, rice DDGS tends to be slightly finer and ranges from light to medium brown in color depending on processing (Figure 8).

Maize DDGS

Rice DDGS

Figure 8: Maize and Rice DDGS

4.11 DDGS storage guidelines

Proper storage of DDGS is a critical step in maintaining product quality and preventing spoilage. The following practices are recommended:

Storage environment

DDGS should be stored in a well-ventilated warehouse equipped with temperature and humidity control systems. The ideal moisture content should be maintained below 10% and must never exceed 12% to prevent microbial growth and spoilage.

Bag stacking and placement

Bags should be stored at least 2 feet away from walls and other stacks to allow adequate air circulation. Plastic pallets must be used to prevent moisture absorption from the floor.

Stack management

Stacks should be turned upside down every 15 days to ensure even exposure and prevent compaction. Routine moisture content monitoring and aflatoxin testing should be conducted during this period.

Cleanliness and pest control

The storage area must be kept clean and dry at all times. Fumigation should be performed as necessary to control pests and mold.

Inventory management

DDGS inventory should be rotated regularly, and storage duration should ideally not exceed 15–20 days to minimize quality degradation.

Moisture sensitivity

As DDGS is hygroscopic in nature, moisture control is a top priority. Installing aeration systems and dehumidifiers in the storage facility can help regulate humidity levels.

Moisture equilibration period

It is recommended to hold DDGS for 3 to 5 days post-drying to allow for complete moisture equilibration. This breaks down any liquid bridges in the cooled mass, thus reducing handling issues like clumping and bridging.

Product conditioning

Grinding or pelleting DDGS is practiced by some ethanol plants to improve bulk density, flowability, and reduce lump formation.

Lipid oxidation risk

Prolonged storage of oil-rich DDGS can lead to lipid oxidation, resulting in rancidity and reduced nutritional value. Therefore, storage time should be minimized for high-fat DDGS.

5.0 DDGS as livestock feed ingredient - Specifications

The material shall be free from harmful constituents, rancidity, adulterants, insect or visible fungus infestation and from fermented, musty or other objectionable odour. It shall be free from dirt and extraneous matter including iron or other metallic pieces. The material shall also conform to the requirements prescribed in Table 5.

Table 5. Requirements for Dried Distillers Grain with Solubles (DDGS) as Livestock Feed Ingredient (Proposed BIS Specifications)

Sl. No.	Characteristic	Requirement		Method of Test, Ref to
		Type-1 (High protein)	Type-2 (Low protein)	
i)	Moisture, percent by mass, Max	11.0	11.0	Clause 4 of IS 7874 (Part 1)
ii)	Crude protein (N×6.25), percent by mass, Min	40.0	25.0	IS 5983 (Part 1)* or IS 5983 (Part 2)
iii)	Crude fat, percent by mass, Min	1.5	5.0	IS 6492
iv)	Crude fibre, percent by mass, Max	5.0	9.0	IS 6865
v)	Acid insoluble ash, percent by mass, Max	1.0	2.0	Annex A of IS 1712 or IS 14826*
vi)	Castor husk or cake	Absent	Absent	Clause 11 of IS 7874 (Part 1)
vii)	Mahua cake	Absent	Absent	Clause 12 of IS 7874 (Part 1)
viii)	Aflatoxin B1 (ppb), Max	40.0	40.0	IS 14718* or AOAC 2003.02 or ISO 17375
ix)	Urea, percent by mass, Max	Absent	Absent	IS 7874 (Part 1) or AOAC 967.07*

NOTES:

1) The values specified for requirements at Sl. No. (ii) to (ix) are on moisture-free-basis.

2) In case of dispute, the test methods given above and wherever indicated by '*' should be the referee method.

3) For crude fibre, the manual method given in IS 6865 should be the referee method.

6.0 Solvent extraction of DDGS (Defatted DDGS)

Solvent extraction of DDGS is primarily performed to remove residual oil (lipids), thereby enhancing its value and enabling better utilization. Below are the key advantages:

- Extracts residual corn oil (typically 3–5%) from DDGS, providing a valuable co-product.
- The extracted oil can be used for biodiesel production, animal feed supplements, or industrial applications.
- De-oiled DDGS has lower fat content, which improves shelf life, pellet durability and handling characteristics.
- It is also reported to have lower aflatoxin levels.
- De-oiled DDGS has a higher relative protein and fiber content, increasing its nutritional density.
- Reduced oil content decreases the risk of oxidative spoilage, thereby extending storage life.
- There is a lower likelihood of mold or microbial growth due to an improved moisture-fat balance.

7.0 Feed safety of DDGS

Feed safety of DDGS has a significant impact on Food safety system because it not only affects animal health and productivity, but also has an impact on safety of animal-derived food products for human consumption. Thus, it is essential for suppliers, buyers and manufacturers of DDGS to not only adhere to regulatory compliances, but also develop and implement programs for continuous improvement in quality and safety aspects of DDGS value chain.

In addition, manufacturers should also implement HAACP (Hazard Analysis and Critical Control Point) systems which are designed to prevent contamination at every step of manufacturing, storage and distribution of DDGS. There are seven principles in developing and implementing a HAACP plan including:

1) Conduct a hazard analysis.
2) Identify critical control points.
3) Establish minimum and maximum limits of the manufacturing process to control potential hazards.
4) Establish critical limits.
5) Establish monitoring procedures and corrective actions.
6) Establish record-keeping procedures.
7) Establish verification procedures.

7.1 Safety related to Aflatoxin B_1 contamination

Aflatoxin is a type of mycotoxin produced by molds, primarily *Aspergillus flavus* and *Aspergillus parasiticus*, which can contaminate grains during production, storage and processing. DDGS, like other grain-based products, is vulnerable to aflatoxin contamination. Contamination can occur at any stage, from grain cultivation to processing and is often worsened by the factors such as poor storage conditions, high moisture and warm temperatures.

Aflatoxins enter DDGS through contaminated grains (such as corn or rice) used in ethanol production. If the grains are already contaminated, the aflatoxins become concentrated in the DDGS because the ethanol production process removes most of the starch, leaving behind protein and fiber, where aflatoxins tend to accumulate.

From a human food safety perspective, aflatoxins are the only class of mycotoxins regulated by the United States Food and Drug Administration (US-FDA) because of its carcinogenic effects. However, if DDGS as livestock feed ingredient contains high concentration of aflatoxin B1, detrimental effects on nutrient utilization, immune function and several other adverse physiological effects can occur that lead to reduced animal health and performance. Thus, production of quality DDGS is of paramount important for feed and food safety point of view.

8.0 Quality control at cattle feed plants for DDGS procurement

- DDGS procurement should be as per the BIS specification.
- Upon arrival of the DDGS consignment or truck, a quality control personnel or designated sampler from the laboratory must collect representative samples from the lot.
- Conduct proper testing at the time of procurement and storage of DDGS at feed plants.
- The consignment should only be accepted if it meets the predefined quality parameters, particularly moisture content and aflatoxin B1 (AFB1) levels.
- Use of rapid detection kits is recommended for on-site screening of contaminants, including aflatoxins.
- The major concern with DDGS is that it could be a potential source of aflatoxins. These

toxins can accumulate during grain storage or processing. Hence proper care needs to be taken for its storage.

- DDGS is hygroscopic in nature and absorbs moisture from the environment. High moisture content can lead to spoilage if not stored properly.
- Screen DDGS for aflatoxin B1 and use appropriate toxin binders for production of quality cattle feed.
- Accordingly, laboratory infrastructure should be upgraded as necessary to support routine AFB1 screening and testing capabilities.

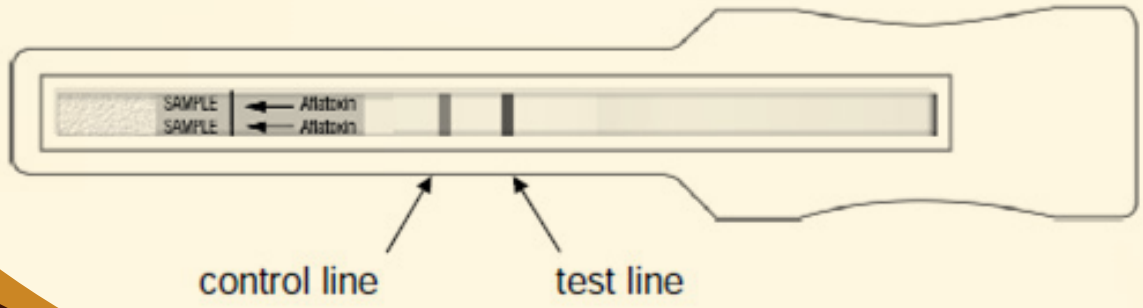
9.0 Test methods for detection of aflatoxins in DDGS

9.1 Rapid Test Strips

Rapid Test Strips, commonly known as Lateral Flow Immunoassays (LFIA), are among the most widely used screening tools for detection of aflatoxin B_1 (AFB_1) in feed and feed raw materials including DDGS. These assays are based on immunochromatographic principles, combining antigen–antibody specificity with capillary flow through a porous membrane. Because of their simplicity, speed, and portability, LFIA kits are particularly suitable for use at feed mills, procurement centers, warehouses, and farms

Sample preparation for LFIA is simple and rapid. Feed or raw material samples are finely ground and extracted using a solvent. After shaking and settling or brief filtration, a small aliquot of the extract is applied directly to the test strip or cassette. The entire procedure from extraction to result interpretation generally takes 5 to 20 minutes, depending on the kit.

The main advantages of LFIA include speed, ease of use, minimal training requirements, and no need for sophisticated laboratory infrastructure. They enable large-scale screening of samples at low cost and



are particularly useful for identifying contaminated batches before feed processing or feeding. However, LFIA methods are generally considered screening tools rather than confirmatory methods

Following are the indicative suppliers for rapid test kits in India.

1) Neogen
2) Charm Sciences Inc.
3) Prognosis Biotech
4) IndiFOSS Analytical Pvt. Ltd.
5) R-Biopharm

9.2 ELISA method for detection of aflatoxin B1

Enzyme-Linked Immunosorbent Assay (ELISA) is one of the most widely used screening methods for detection and quantification of aflatoxin B1 in animal feed and feed raw materials. The method is based on the highly specific antigen–antibody reaction between aflatoxin B1 and anti-AFB1 antibodies and is suitable for rapid analysis of a large number of samples with reasonable sensitivity and accuracy.

In most commercial kits, a competitive ELISA format is employed because aflatoxin B1 is a low molecular weight hapten. In this principle, free aflatoxin B1 present in the feed extract competes with enzyme-labeled aflatoxin (or plate-bound aflatoxin conjugate) for a limited number of antibody binding sites. As the concentration of aflatoxin B1 in the sample increases, less antibody binds to the enzyme-labeled antigen, resulting in a decrease in color intensity after substrate addition. Thus, the color developed is inversely proportional to the concentration of aflatoxin B1 in the sample.

ELISA offers several advantages, including simplicity, rapid turnaround time, high throughput, and relatively low cost compared to HPLC. However, it also has limitations, such as possible cross-reactivity with structurally related aflatoxins (e.g., AFB2, AFG1)

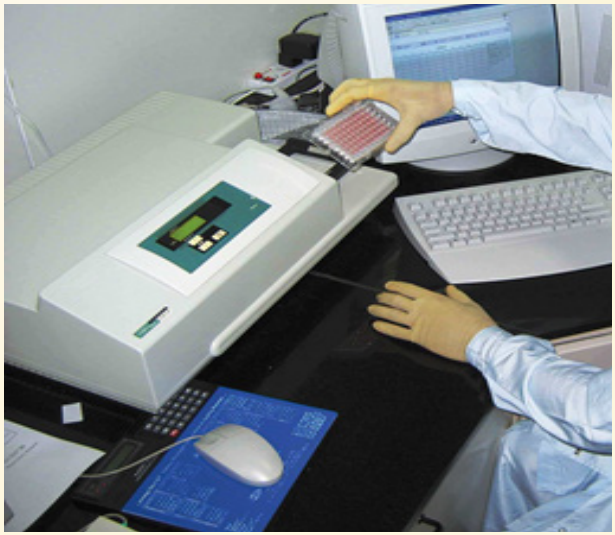


Figure-9 Elisa reader
The picture is indicative and is not intended to promote any particular brand

and matrix effects from complex feed ingredients. Therefore, ELISA is generally recommended as a screening tool, and samples showing levels near or above permissible limits should be confirmed using reference methods such as HPLC or LC–MS/MS.

9.3 HPLC Method for detection of aflatoxin B1

High-Performance Liquid Chromatography (HPLC) is a reference and confirmatory analytical technique widely used for the determination of aflatoxin B_1 in feed, feed raw materials, and food commodities. Because of its high sensitivity, specificity, and reproducibility, the HPLC method is internationally accepted for regulatory monitoring and is prescribed in standards such as ISO 17375, AOAC, and BIS methods. It is commonly used to confirm results obtained from rapid screening methods like LFIA and ELISA.

The HPLC method for AFB_1 detection generally involves four major steps: sample extraction, clean-up, chromatographic separation, and detection/quantification. Feed samples are first finely ground to ensure homogeneity. A representative portion is extracted using organic solvents such as methanol–water or acetonitrile–water, which efficiently solubilize aflatoxins from complex feed matrices. The extract is then filtered or centrifuged to remove particulate matter.

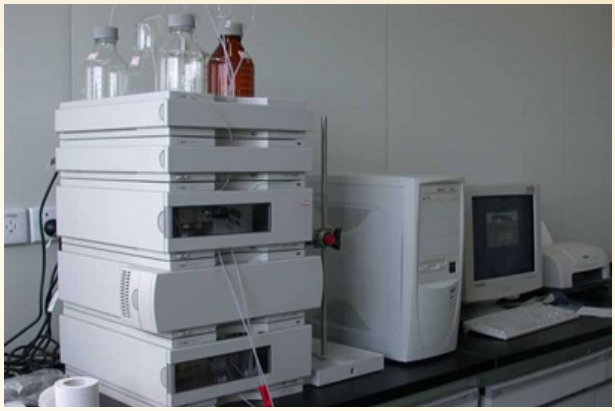


Figure-10 High-performance liquid chromatography

The HPLC method is considered the gold standard for aflatoxin B_1 analysis due to its high analytical reliability. While it requires skilled personnel, laboratory infrastructure, and longer analysis time compared to rapid tests, it provides definitive and legally defensible results. In practical feed safety systems, HPLC is therefore used as a confirmatory tool to support quality assurance programs, regulatory compliance, and risk assessment related to aflatoxin B_1 contamination.

Test Methods for determination of AFB1 in feed and feed ingredients are given as Annexure 1 &2.

10.0 Strategy to mitigate aflatoxin in DDGS and increase its usage in cattle feed manufacturing

The following strategies could be explored to effectively mitigate aflatoxin in DDGS.

- **Screening of maize grain at the time of procurement:** Key parameters such as moisture content, dust levels, presence of foreign material, and damaged grains need to be regularly monitored during procurement. Procurement and storage of grains with a moisture content of below 12% would minimize contamination risks.

Action: NDDB may collaborate with one ethanol plants in screening maize grains by offering investment support for establishing grain sorting machine and aflatoxin testing facilities. This could include the use of rapid test kits for initial screening and comprehensive testing through the NDDB CALF Limited lab to ensure grain quality and safety. The tentative cost of an optical grain sorter with a capacity of 5 MT per hour is about Rs. 35 lakh. Expenses on Aflatoxin testing at NDDB CALF Ltd. would be extra.

- **Proper storage practices of DDGS at production site:** Proper storage of DDGS is essential to prevent aflatoxin contamination. Installing silos with advanced ventilation systems can ensure adequate airflow, inhibiting the growth of mold and fungi. These silos, equipped with moisture control systems, help maintain optimal humidity levels, preventing the grains from becoming too dry or too wet.

Action: NDDB may collaborate with an ethanol plant establishing advanced storage silos to demonstrate the positive impact of effective DDGS storage systems.

- **Linkage for good quality DDGS:** Finally, developing assured forward linkages with cattle feed plants is crucial.

Action: NDDB Dairy Services could be involved to ensure an assured forward linkage.

- Since entire process of monitoring, screening and procurement would be monitored, it may affect the price of maize or price of cattle feed. There may be requirement of gap funding either for procurement of maize or sale of DDGS.
- **Post-harvest management of gains:** There is considerable potential for improvement in this area, particularly in the management of moisture in the grain.

Action: To ensure the procurement of high-quality, low-aflatoxin, or aflatoxin-free maize grain, ethanol plants may engage Farmer Producer Organisations (FPOs) in sourcing good-quality produce. Establishing an assured buy-back arrangement with FPO farmers could incentivize the production of quality maize while ensuring a reliable supply chain for the ethanol plants. NDDB would explore liaison with Fodder Plus FPOs in Gujarat if they can produce and supply maize grain.

NDDB may collaborate with FPOs in developing a **post-harvest cleaning and drying facility** for maize grain to ensure a consistent supply of high-quality maize to ethanol plants. Stationary drying system of various capacity is available in the market.

State-wise grain-based ethanol production and expected DDGS production is given in Table 6.

Table 6. Grain-based ethanol production facilities

Sl. No.	State	Plants (Nos.)	Ethanol production capacity (KLPD)	DDGS output potential (MTPD)
1	Assam	4	720	432
2	Bihar	5	1020	612
3	Chhattisgarh	1	65	39
4	Delhi	13	3245	1947
5	Gujrat	3	1060	636
6	Haryana	7	1430	858
7	Himachal Pradesh	1	200	120
8	Jharkhand	3	650	390
9	Karnataka	3	600	360
10	Madhya Pradesh	8	1485	891
11	Maharashtra	5	820	492
12	Meghalaya	1	100	60
13	Odisha	1	60	36
14	Punjab	9	2215	1329
15	Telangana	3	820	492
16	Uttar Pradesh	7	1500	900
17	Uttarakhand	2	180	108
18	West Bengal	10	2355	1413
	Total	**86**	**18525**	**11115**

Source: Grain Ethanol Manufacturers Association (GEMA) https://gemabharat.com.

11.0 Recommended level of DDGS for inclusion in cattle feed

Most cattle feed plants incorporate DDGS at a level of up to 5% due to the presence of high levels of aflatoxin. However, if aflatoxin B1 can be reduced to a level of about 40 ppb through various mitigation measures during the production, processing and storage of DDGS, it could potentially be used at a higher level of 10-12% in feed formulations as a replacement of oil meals.

12.0 Way forward

Feed is an integral part of the food chain system, particularly milk and thus quality of DDGS must be ensured while ensuring the quality of milk and milk products. To ensure the availability of quality DDGS as livestock feed ingredient, the prescribed guidlines may be followed strictly by the manufacturers, traders and consumers of DDGS in the country.

To effectively mitigate aflatoxin contamination in feed and feed materials, the development of innovative, cost-effective, and residue-free technologies has become a critical necessity. Some of the promising technologies and approaches with potential to prevent or reduce aflatoxin infestation include:

Ozone treatment of maize DDGS

Ozonation of DDGS has emerged as a promising approach to enhance feed safety and quality. Ozone (O_3), owing to its strong oxidation potential, exhibits rapid and broad-spectrum antimicrobial action against spores, pathogenic microorganisms, and viruses. In the ozonation process, commodities are exposed to ozone, which interacts with aflatoxin molecules, leading to their structural breakdown and subsequent loss of toxicity.

Recognized for its safety, ozone has been classified as Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (US-FDA) for applications in food processing. Evidence from global studies indicates that ozonation represents a cost-effective, residue-free method for aflatoxin detoxification in both food and feed raw materials. Thus, ozonation offers significant potential for the safe production and utilization of high-quality DDGS.

Ozonation can be applied to DDGS either during handling or prior to their inclusion in feed formulations, thereby ensuring safer and more stable raw materials. However, when using ozone for aflatoxin control, the dosage and exposure time are critical parameters, as their effectiveness

depends on the initial concentration of aflatoxin B1 present in DDGS. Therefore, careful optimization of both dose and treatment duration is a prerequisite for effective application. Successful adoption of this technology also necessitates investment in ozone generation systems along with the development of standard operating protocols to ensure safe and efficient use.

Use of bio-control measures

Biocontrol is a practical and effective strategy to reduce aflatoxin contamination both in the field and during storage. It relies on the use of beneficial organisms to suppress the growth of toxigenic fungi. International Institute of Tropical Agriculture (IITA), a key partner within the Consultative Group on International Agricultural Research (CGIAR) system, developed '*Aflasafe*' - a biocontrol product (atoxigenic strains of *Aspergillus flavus*) specifically designed to mitigate aflatoxin contamination in crops. Field studies have demonstrated that *Aflasafe* can reduce aflatoxin contamination by 80–99%, ensuring safety from farm to plate.

Developing a regionally tailored biocontrol solution similar to *Aflasafe* for Indian agro-ecological conditions, through collaborative research initiatives, would be highly beneficial for ensuring safer crops and feed.

Use of toxin binder

The incorporation of toxin binders e.g. bentonite into animal feed is a widely adopted strategy to mitigate aflatoxin contamination and subsequently reduce the carryover of aflatoxin M1 into milk. Developing highly effective binders, or utilizing suitable combinations of cost-effective binders, can serve as a practical approach to minimize aflatoxin exposure in cattle and enhance feed safety.

Annexure - 1

AOAC Official Method 2003.02
Aflatoxin B_1 in Cattle Feed

Immunoaffinity Column Liquid Chromatography Method
First Action 2003
Final Action 2006

(Applicable for the determination of aflatoxin B_1 >1 ng/g in cattle feed.)

Caution: This method requires the use of solutions of aflatoxin B_1. Aflatoxins are carcinogenic to humans. Aflatoxins are subject to light degradation. Protect analytical work adequately from the daylight, and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil. Use acid-washed glassware (e.g., vials, tubes, flasks) to prevent loss of aflatoxin. Take special care with new glassware. Soak new glassware in dilute acid (e.g., 2M sulfuric acid) for several hours and rinse extensively with distilled water to remove all traces of acid (check with pH paper).

See Table **2003.02A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

An extract of the test portion is applied to an affinity column containing antibodies specific to aflatoxin B_1. After elution, aflatoxin B_1 is quantified by LC with post-column derivatization and fluorescence detection.

B. Performance Standard and General Procedures for Immunoaffinity Columns

The immunoaffinity columns must contain antibodies raised against aflatoxin B_1. The columns must have a capacity of not less than 40 ng aflatoxin B_1 and must give a recovery of not less than 80% when applied as a standard solution in CH_3OH/H_2O containing 0.25 ng toxin.

Adjust columns to room temperature before conditioning. For conditioning apply 10 mL phosphate-buffered saline (PBS) on top of column and let it pass at a speed of 2–3 mL/min through the column (gravity). Make sure that a small portion (0.5 mL) of the PBS remains on the column until extract is applied. *Note:* Methods for loading onto affinity columns, washing the column, and eluting vary slightly between column manufacturers; follow precisely the specific instructions supplied with the columns.

C. Apparatus

(**a**) *Vertical or horizontal shaker.*

(**b**) *Filter paper.*—24 cm, prefolded.

(**c**) *Erlenmeyer flask.*—500 mL screw-top or glass stopper.

(**d**) *Glass microfiber filter paper.*—Retention 1.6 µm.

(**e**) *Reservoir.*—75 mL with Luer tip connector for affinity column.

(**f**) *Hand pump.*—20 mL syringe with Luer lock or rubber stopper.

(**g**) *Volumetric flask.*—5, 10, and 20 mL (accuracy of at least 0.5%).

(**h**) *LC pump.*—With flow rates between 0.20 and 1.00 mL/min.

(**i**) *Injection system.*—Total loop injection valve with loop between 100 and 1000 µL. Determine RSD (n = 10) for replicate injections of 0.1 ng/mL aflatoxin B_1. RSD must be <5% for option A; if this cannot be met, use option B, **D**(**q**).

(**j**) *Reversed-phase LC column.*—4.6 mm × 25 cm, 5 µm, e.g., LC-18 or ODS-2.

(**k**) *Post-column derivatization system.*—Either with PBPB (second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum 45 cm × 0.5 mm id PTFE) or with electrochemically generated bromine (e.g., Kobra® cell–Rhône Diagnostics Ltd., Lyon, France; www.r-biopharmrhone.com).

(**l**) *Fluorescence detector.*—Wavelength of λ = 360 nm excitation filter and a wavelength of λ > 420 nm cut-off emission filter, or equivalent.

(**m**) *Disposable filter unit.*—Cellulose or cellulose nitrate, 0.45 µm.

(**n**) *Pipet.*—Marked with 1–10 mL capacity.

(**o**) *Analytical balance.*—Readability 0.1 mg.

(**p**) *Laboratory balance.*—Readability 0.01 g.

(**q**) *Calibrated microliter syringe(s) or microliter pipet(s).*—20–500 µL capacity.

(**r**) *Immunoaffinity columns specific for aflatoxin B_1 cleanup.*—*See* **B** for performance standard for column. For example, columns from Vicam (Watertown, MA, USA; www.vicam.com/products/mycotoxins.html) and Rhône Diagnostics (Glasgow, UK; now R-Biopharm Rhône Ltd) have been found to meet these criteria.

D. Reagents

All reagents shall be of recognized analytical grade.

Unless otherwise stated, use water complying with grade 3 of ISO 3696.

Table 2003.02A. Interlaboratory study results for aflatoxin B_1 in cattle feed

Added, ng/g	No. of labs[a]	Average, ng/g	Mean rec., %	s_r, ng/g	s_R, ng/g	RSD_r, %	RSD_R, %	r, ng/g[b]	R, ng/g[b]	HorRat
1.2	20 (1)	1.33	111	0.08	0.26	5.9	19.4	0.22	0.72	1.3
3.6	20 (1)	3.89	106	0.25	0.67	6.4	17.5	0.69	1.87	1.3
NC[c]	21 (0)	<0.02	—	—	—	—	—	—	—	—
NC	18 (3)	0.54	—	0.04	0.10	7.2	17.9	0.11	0.27	1.0
NC	19 (2)	0.87	—	0.08	0.17	8.7	19.4	0.21	0.47	1.2
NC	18 (3)	4.19	—	0.26	0.82	6.2	19.6	0.72	2.30	1.5

[a] Number of laboratories retained after outliers removed; number of outliers are in parentheses.

[b] R and r are calculated according to the IUPAC Harmonized Protocol.

[c] NC = Naturally contaminated.

(**a**) *PBS.*—pH 7.4. Prepare PBS from potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate [1.16 g; disodium hydrogen phosphate 12 H_2O (2.92 g)], and sodium chloride (8.00 g) added to 900 mL purified water. After dissolution, adjust pH to 7.4 (with 0.1M HCl or 0.1M NaOH as appropriate) and dilute to 1.0 L. Alternatively, commercially available PBS tablets with equivalent properties can be used.

(**b**) *Pyridinium hydrobromide perbromide (PBPB).*—CAS No. 39416-48-3.

(**c**) *Potassium bromide.*

(**d**) *Acetonitrile.*—LC grade.

(**e**) *Methanol.*—LC grade.

(**f**) *Acetone.*

(**g**) *Toluene.*

(**h**) *LC grade water.*—Obtained by distillation or deionization. Equivalent to Grade 1 of ISO 3696.

(**i**) *Extraction solvent.*—Acetone–water solution (85 + 15, v/v).

(**j**) *Nitric acid–water.*—1 + 3 (v/v). This will result in a concentration of ca c(HNO_3) = 4 mol/L.

(**k**) *LC mobile phase solvent A.*—Water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v).

(**l**) *LC mobile phase solvent B.*—For use with electrochemically generated bromine: water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v), containing 120 mg potassium bromide and 350 L nitric acid, (**j**), in 1 L final reagent.

(**m**) *Post-column reagent B.*—Dissolve 25 mg PBPB in 500 mL H_2O. Solution can be used for up to 4 days if stored in a dark place at room temperature.

(**n**) *Toluene–acetonitrile.*—98 + 2 (v/v).

(**o**) *Aflatoxin B_1 standard material.*—Crystals or dry film.

ca 10 mL methanol, let aflatoxins dissolve, dilute to volume with methanol, and shake well (*do not* use water as in option A). Transfer exactly 1 mL of this working calibrant into acid-washed glass vial, evaporate to dryness, and redissolve in exactly the same volume that will be used to redissolve the test extract residues before injection. Calculate concentration of aflatoxin B_1 in the evaporated and redissolved standard solutions in ng/mL. Use these concentration values for the calculation (calibration curve). In this case, the calibration range of the method will remain unchanged.

E. Extraction

Weigh, to the nearest 0.1 g, ca 50 g test portion into 500 mL Erlenmeyer flask with screw-top or glass stopper. Add 250 mL acetone–water extraction solvent. Shake intensively by hand for the first 15–30 s and then for 30 min with a shaker. Filter extract using prefolded paper. Pipet 5.0 mL clear filtrate into 100 mL calibrated volumetric flask and dilute to volume with water. The dilution solvent (PBS or water) must be selected according the specifications of the immunoaffinity column (IAC) manufacturer. If not stated differently, make the dilution with PBS. If the solution is not clear, refilter through glass microfiber filter and apply exactly 50 mL clear filtrate on a reservoir placed on a conditioned IAC. If the solution is clear, the diluted solution can be directly applied on the IAC.

F. Immunoaffinity Column Cleanup

Pass filtrate through column at flow rate of ca 1 drop/s (ca 3 mL/min) under gravity. Do not exceed flow rate of 5 mL/min. Wash column with 15 mL water applied in portions of 5 mL, and dry by applying small vacuum for 5–10 s or passing air through by means of syringe for 10 s. This can be done with a 100 mL plastic syringe that is connected to the column (e.g., with a suitable adapter).

(**p**) *Aflatoxin B_1 stock solution*.—Containing 50.0 ng/mL aflatoxin B_1 in toluene–acetonitrile (98 + 2, v/v).

(**q**) *Aflatoxin B_1 working standard solutions (options A and B)*.—Option A is the recommended procedure. Option B includes an additional concentration step and is only applicable if precise signals cannot be obtained with option A. *See also* **C**(**i**).

Option A: Pipet from stock solution, (**o**), the volumes listed in Table **2003.02B** (option A) into a set of 20 mL calibrated volumetric flasks. Evaporate the toluene–acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 7 mL methanol, let aflatoxins dissolve, dilute to volume with water, and shake well.

Option B: Pipet from stock solution, (**o**), the volumes listed in Table **2003.02B** (option B) into a set of 20 mL calibrated volumetric flasks. Evaporate the toluene–acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add

Elute aflatoxins in a 2-step procedure: Collect eluate in volumetric flask (option A), or an LC injection vial (option B). First, apply 0.5 mL methanol on the IAC and let it pass through by gravity. Collect eluate in either a 5 mL volumetric flask (option A below) or an LC injection vial (option B below). Wait 1 min and apply second portion of 1.25 mL methanol. Collect applied elution solvent by pressing air through.

Option A (recommended): This option is recommended, but requires an appropriate fluorescence detector and injection system, **C**(**i**). Option B only applies if the detector signal is insufficient for the analysis according to option A.

Collect eluate in calibrated 5 mL volumetric flask. Dilute to volume with water, and shake well. If solution is clear it can be used directly for LC analysis. If solution is not clear, pass it through a disposable filter unit (0.45 m) before LC injection. The injection by total loop mode produces maximum accuracy. Inject test solution

Table 2003.02B. Preparation of aflatoxin B_1 working standard solutions

	Option A		Option B	
Working standard	Aliquot stock solution, L	Concentration, ng AfB_1/mL	Aliquot stock solution, L	Concentration, ng AfB_1/mL
1	20	0.050	100	0.250
2	70	0.175	350	0.875
3	120	0.300	600	1.500
4	170	0.425	850	2.125
5	220	0.550	1100	2.750

3 times the volume of the injection loop to ensure that injection loop and valve are properly rinsed.

Option B (only if applicable): If the detector signal is not sufficient, an additional evaporation step may be included to meet the requirement.

Collect aflatoxin containing methanol eluate from the affinity column in LC injector vial (acid-washed). In addition, pipet exactly 1.0 mL working standard solutions (option B) into separate injector vials. Evaporate methanol in all vials to dryness under gentle stream of nitrogen at 40 C. Redissolve aflatoxin in aqueous methanol solution (3.5 mL MeOH diluted to 10 mL with water). Use exactly the same volume for the evaporated test residues as for the evaporated working standard solutions, and calculate the new concentration in ng/mL in the redissolved working standard solutions. The volume for redissolving will depend on the size of the injection loop. Use the total loop mode for injection as described in option A.

G. LC Determination with Fluorescence Detection and Post-Column Derivatization

When using PBPB, mount the mixing T-piece and reaction tubing mentioned above, and then operate using the following parameters: flow rates, 1.00 mL/min (mobile phase A); 0.30 mL/min (reagent).

When using electrochemically generated bromine (Kobra cell), follow instructions for installation of the cell as supplied by the manufacturer, and operate using the following parameters: flow rate, 1.00 mL/min (mobile phase B); current,100 A.

Aflatoxins elute in the order G_2, G_1, B_2, and B_1 with retention times ca 6, 8, 9, and 11 min, respectively, and should be base-line resolved, in order to measure aflatoxin B_1 as a discrete peak.

H. Calibration Curve

Prepare a calibration curve using calibration solutions covering the range of 0.5–5.5 ng/g for aflatoxin B_1 and check the curve for linearity according to good laboratory practice guidances. Inject same volume as used for working standards into injector and identify aflatoxin B_1 peak in chromatogram by comparing retention time with corresponding reference standard. Determine quantity of aflatoxin B_1 in injected solution from calibration curve according to **I**.

I. Calculations

Calculate the concentration of aflatoxin in the test sample as follows:

Plot the concentration of aflatoxin (ng/mL) against the peak area (units). Use the resulting function (linear regression) to calculate the concentration of aflatoxin in the measured solution.

Further calculate the contamination level of aflatoxin B_1 in the test material according to:

$$\text{Aflatoxin } B_1, \text{ ng / g} \quad \frac{C_{smp} \quad \text{solvent} \quad \text{elution} \quad \text{dilution}}{W \quad \text{aliquot}_{EX} \quad \text{aliquot}_{IAC}} \quad \frac{\text{ng} \quad \text{mL} \quad \text{mL} \quad \text{mL}}{\text{mL} \quad \text{g} \quad \text{mL} \quad \text{mL}}$$

$$\text{Aflatoxin } B_1, \text{ ng/g} = C_{smp} \quad 10$$

where W = test portion taken for analysis, g; solvent = solvent taken for extraction, mL; aliquot_{Ex} = aliquot taken from extract, mL; dilution = volume achieved after dilution with PBS (water), mL; aliquot_{IAC} = aliquot taken for the immunoaffinity cleanup, mL; elution = final volume achieved after elution from IAC (for option B the volume must be the volume after redissolving), mL; C_{smp} = concentration of aflatoxin in injected solution calculated from linear regression, ng/mL.

In cases where aflatoxin B_1 concentration in the test sample is outside the calibration range, dilute the injection solution appropriately with water–methanol solution (7 + 13, v/v), inject the diluted solution and recalculate.

Annexure - 2

Indian Standard

ANIMAL FEEDING STUFFS — DETERMINATION OF AFLATOXIN B_1 CONTENT OF MIXED FEEDING STUFFS — METHOD USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

1 Scope

This International Standard specifies a high-performance liquid chromatographic (HPLC) method for the determination of aflatoxin B_1 content of animal feeding stuffs including those containing citrus pulp.

The lower limit of determination is 1 µg/kg.

NOTE 1 This International Standard may be applicable for the determination of the aflatoxin B_1 content of a number of raw materials and straight feeding stuffs such as corn gluten, groundnut, palm kernel, copra, citrus pulp, tapioca, soya bean, rice bran, pollard, rape seed, niger seed and cotton seed (see references [1] and [2]). These materials were, however, not included in the collaborative testing of the method.

NOTE 2 This International Standard may also be applicable for the determination of the content of the sum of the aflatoxins B_1, B_2, G_1 and G_2. However, the method has not been validated for this parameter by collaborative testing.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subseqent amendments to, or revisions of, this publication do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of IEC and ISO editions maintain registers of currently valid International Standards.

ISO 6498:1998, *Animal feeding stuffs — Preparation of test sample.*

3 Principle

The sample is extracted with chloroform. The extract is filtered and an aliquot portion is purified on a Florisil®[1)] cartridge and a C_{18} cartridge. The final separation and determination is achieved by high-performance liquid chromatography (HPLC) using a reverse-phase C_{18} column, followed by post-column derivatization with iodine or bromine, and fluorescence detection.

4 Reagents and materials

Use only reagents of recognized analytical grade.

4.1 Water, demineralized or deionized, with resistivity of at least 10 MΩ·cm, or water of at least equivalent purity.

4.2 Concentrated sulfuric acid, $c(H_2SO_4)$ = 18 mol/l, $\rho(H_2SO_4)$ = 1,84 g/ml.

4.3 Sulfuric acid, $c(H_2SO_4)$ = 2 mol/l.

Carefully add 105 ml of concentrated sulfuric acid (4.2) to 895 ml of water and mix well. Avoid excessive heating of the solution.

4.4 Control sample.

Prepare a control sample of about 2 kg of compound feed with an aflatoxin B_1 content of about 5 µg/kg by combining samples of previous determinations with an aflatoxin B_1 content of about 5 µg/kg. Mix thoroughly.

The aflatoxin B_1 content of the control sample should be determined five times by two analysts following the procedure described in clause 8. From the results the mean aflatoxin B_1 content, the standard deviation and the coefficient of variation should be calculated.

4.5 Acid-washed Celite® 545, or product of equivalent quality[2)].

4.6 Florisil® Sep-Pak style cartridge, Waters No. 51960, or product of equivalent qualty[3)].

4.7 C_{18} Sep-Pak style cartridge, Waters No. 51910, or product of equivalent quality[3)].

4.8 Acetone.

4.9 Methanol.

4.10 Acetonitrile.

4.11 Chloroform, stabilized with ethanol (mass fraction 0,5 % to 1,0 %).

WARNING: Chloroform is a toxic substance. Avoid inhalation of and exposure to chloroform. Work in a fumehood when handling the solvent and solutions thereof.

The adsorption characteristics of the Florisil® cartridge (4.6) may change if stabilizers other than ethanol are used. When chloroform as described is not available, the adsorption characteristics should be verified in accordance with clause 8.

4.12 Mixture of acetone and water, 98 + 2 (by volume).

Combine 980 ml of acetone (4.8) and 20 ml of water (4.1). Mix well.

4.13 Mixture of acetone and water, 15 + 85 (by volume).

Combine 150 ml of acetone (4.8) and 850 ml of water (4.1). Mix well.

4.14 Mixture of acetone and water, 5 + 95 (by volume).

4.15 Mixture of methanol and water, 20 + 80 (by volume).

Combine 200 ml of methanol (4.9) and 800 ml of water (4.1). Mix well.

4.16 Concentrated nitric acid, $c(HNO_3)$ = 14 mol/l, $\rho(HNO_3)$ = 1,40 g/ml, for HPLC with bromine derivatization.

4.17 Potassium bromide (KBr), for HPLC with bromine derivatization.

4.18 Mobile phase for HPLC.

4.18.1 Mobile phase for HPLC with iodine derivatization.

Combine 120 ml of acetonitrile (4.10), 210 ml of methanol (4.9) and 390 ml of water (4.1) and mix. Filter the eluent through a 0,45 µm PTFE membrane filter using the solvent filtration system (5.1) and degas for 10 min in the ultrasonic bath (5.2) before use.

NOTE The composition of the mobile phase solvent may need adjustment depending on the characteristics of the HPLC column used.

4.18.2 Mobile phase for HPLC with bromine derivatization.

Combine 400 ml of acetonitrile (4.10), 700 ml of methanol (4.9) and 1 300 ml of water (4.1) and mix. Add to the mixture 286 mg of potassium bromide (4.17) and 152 µl of concentrated nitric acid (4.16). Mix well and degas with a stream of inert gas for 15 min.

4.19 Saturated iodine solution for HPLC with iodine derivatization.

Add 2 g of iodine to 400 ml of water. Mix for at least 90 min and filter through a 0,45 µm PTFE membrane filter (see 5.1). Prepare the solution fresh on the day of use.

Protect the saturated solution from light to prevent photodegradation.

4.20 Sodium hypochlorite solution (household quality), ρ(active chlorine) = 100 g/l.

4.21 Sodium hypochlorite solution, volume fraction 1 %.

Dilute 10 ml of sodium hypochlorite solution (4.20) with 990 ml of water-acetone mixture (4.14).

4.22 Inert gas, e.g. nitrogen.

4.23 Aflatoxin B_1 standard material ($C_{17}H_{12}O_6$), 2,3,6aα,9aα-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]-furo[2,3-*h*][1]benzopyran-1,11-dione; Chemical Abstracts Service Registry (CAS) number 1162-65-8.

4.24 Aflatoxin B_1 standard solution, ρ(aflatoxin B_1) ≈ 10 µg/ml.

Transfer the content of an ampoule containing aflatoxin B_1 (4.23) to a flask and dissolve in chloroform (4.11). Transfer the solution to a convenient size volumetric flask and dilute to the mark with chloroform so as to obtain a solution with an aflatoxin B_1 content of about 10 µg/ml. Mix.

Transfer the solution to amber vials or an airtight screw-cap bottle and store in a cool place (4 °C) in the dark, well sealed and wrapped in aluminium foil.

4.25 Aflatoxin B_1 stock standard solution.

Transfer quantitatively 2,5 ml of the aflatoxin B_1 standard solution (4.24) to a 50 ml volumetric flask and dilute to the mark with chloroform (4.11).

Transfer the solution to amber vials or an airtight screw-cap bottle and store in a cool place (4 °C) in the dark, well sealed and wrapped in aluminium foil.

4.26 Aflatoxin B_1 calibration solutions for HPLC.

4.26.1 Calibration solution I, ρ(aflatoxin B_1) ≈ 4 ng/ml.

Allow the volumetric flask with stock standard solution (4.25) to reach room temperature in the aluminium foil (a few hours).

Transfer 400 µl of the stock standard solution (equivalent to about 200 ng of aflatoxin B_1) to an acid-washed 50 ml volumetric flask, and evaporate the solution to dryness in a stream of inert gas (4.22). Dissolve the residue in 20 ml of the water-acetone mixture (4.13). Dilute to the mark with the water-acetone mixture and mix well.

4.26.2 Calibration solution II, ρ(aflatoxin B_1) ≈ 3 ng/ml.

Transfer quantitatively 7,5 ml of the calibration solution I (4.26.1) to an acid-washed 10 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

4.26.3 Reference calibration solution, ρ(aflatoxin B_1) ≈ 2 ng/ml.

Transfer quantitatively 25 ml of the calibration solution I (4.26.1) to an acid-washed 50 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

This solution is used for repetitive injection during HPLC (8.5).

4.26.4 Calibration solution III, ρ(aflatoxin B_1) ≈ 1 ng/ml.

Transfer quantitatively 2,5 ml of the calibration solution I (4.26.1) to an acid-washed 10 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

4.27 Chromatographic test solution.

Prepare an ampoule containing a mixture of aflatoxins B_1, B_2, G_1 and G_2 in 1 ml of chloroform with concentrations of approximately 1,0 µg/ml, 0,5 µg/ml, 1,0 µg/ml and 0,5 µg/ml respectively.

Transfer the contents of the ampoule to a glass-stoppered test tube or screw-capped vial. Transfer 40 µl of this solution to an acid-washed glass-stoppered test tube (5.4). Evaporate the chloroform in a stream of inert gas (4.22) and dissolve the residue into 10 ml of the water-acetone mixture (4.13).

5 Apparatus

Before use, laboratory glassware coming into contact with aqueous solutions of aflatoxins shall be soaked in sulfuric acid (4.3) for several hours, then rinsed well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

In practice, this treatment is necessary for the round-bottomed flask of the rotary evaporator (5.12), the volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosamplers vials), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

NOTE Laboratory glassware coming into contact with aqueous solutions of aflatoxins has to be soaked in dilute acid because the use of non-acid-washed glassware may cause losses of aflatoxin B_1. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and Pasteur pipettes.

Usual laboratory apparatus and, in particular, the following.

5.1 Solvent filtration system, suitable for PTFE membrane filters with a pore size of 0,45 µm.

5.2 Ultrasonic bath.

5.3 Microsyringe, of capacity 100 µl, for preparation of calibration solutions.

Check by weighing that the inaccuracy does not exceed 2 % of the mass.

5.4 Glass-stoppered calibrated tubes, of capacity 10 ml.

5.5 Spectrometer, suitable for measurements in the UV region of the spectrum, provided with quartz cuvettes of optical path length 10 mm ± 0,1 mm.

5.6 Conical flask, of capacity 500 ml, made of borosilicate glass, with a wide neck and a glass stopper or a screw cap fitted with a PTFE liner.

5.7 Mechanical shaker, horizontal rotation or reciprocating, with frequency 250 min^{-1} to 300 min^{-1}.

5.8 Fluted filter paper, of diameter 24 cm.

5.9 Luer® chloroform-resistant threeway stopcock [4].

5.10 Chemically resistant syringe, 10 ml, with Luer® connector [4].

5.11 Glass column, with internal diameter 10 mm to 15 mm, length about 30 cm to 50 cm, equipped with a Luer® tip[4].

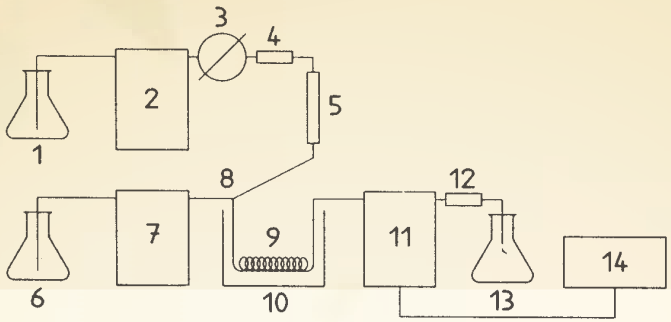
NOTE When a glass column of internal diameter about 10 mm and length about 30 cm is used, it is advisable to use a plastics reservoir (chemically resistant syringe barrel) of at least 70 ml capacity.

5.12 Rotary vacuum evaporator, equipped with a 150 ml to 250 ml round-bottomed flask.

5.13 General HPLC system.

See Figures 1 and 2 for a diagrammatic representation of the HPLC system for derivatization with iodine and bromine respectively.

5.13.1 Pump, pulse free, capable of maintaining a volume flow rate of 0,1 ml/min to 1,0 ml/min.



Key

1	HPLC mobile phase	10	Reaction coil
2	HPLC pump	11	Fluorescence detector
3	Injector	12	Restrictor
4	Guard column	13	Waste
5	Analytical column	14	Recorder/integrator
6	Saturated iodine solution	15	Derivatization cell (KOBRA®)
7	Reagent pump	16	Tension meter
8	Tee joint	17	Power supply, 10 V d.c.
9	Water bath (60 °C)	18	Resistor, 100 kΩ

Figure 1 — Diagrammatic representation of the HPLC system for derivatization with iodine

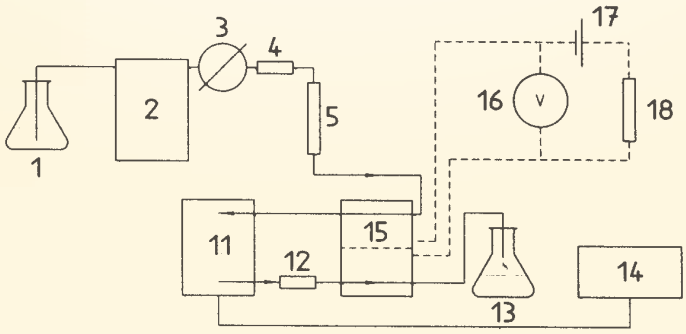


Figure 2 — Diagrammatic representation of the HPLC system for derivatization with bromine

5.13.2 Injection system, with loop suitable for the injection of 250 µl.

5.13.3 Fluorescence detector, with excitation at a wavelength of 365 nm and emission at wavelength of 435 nm (for filter instruments: emission wavelength > 400 nm). Detection of at least 0,05 ng aflatoxin B_1 shall be possible. Some back pressure may be advisable [e.g. by applying a restrictor or a coil of stainless steel or polytetrafluorethylene (PTFE) connected to the outlet of the detector] to suppress air bubbles in the flow cell.

5.13.4 Recorder.

5.13.5 Guard column: C_{18} packing, particle size 37 µm to 50 µm, length 10 mm to 20 mm, internal diameter 3,9 mm; or a guard column of equivalent quality.

5.13.6 Analytical column: C_{18} packing, particle size 3 µm or 5 µm, length 200 mm, internal diameter 3,0 mm; or an analytical column of equivalent quality.

5.13.7 Electronic integrator (optional).

5.14 HPLC system for HPLC with iodine derivatization.

5.14.1 Pump, pulse free, for delivery of the iodine post-column reagent.

5.14.2 Zero dead volume Tee, stainless steel, 1,59 mm × 0,75 mm.

5.14.3 Spiral reaction coil, polytetrafluorethylene (PTFE) or stainless steel.

Dimensions of 3 000 mm × 0,5 mm to 5 000 mm × 0,5 mm have been found to be appropriate in combination with 5 µm or 3 µm HPLC columns.

5.14.4 Thermostatically controlled water bath or **solid-state heating device**, adjusted to 60 °C, capable of temperature regulation to the nearest 0,1 °C.

5.15 HPLC system for HPLC with bromine derivatization.

5.15.1 Electrochemical derivatization cell: Kok's Bromine Apparatus (KOBRA®[5]).

5.15.2 Power supply, 0 V to 20 V d.c.

5.15.3 Tension meter, range 0 V to 10 V d.c., impedance > 50 kΩ.

5.15.4 Resistor, 100 kΩ.

5.16 Syringe, suitable for HPLC injection of 250 µl.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [7].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

7 Preparation of the test sample

Prepare the test sample in accordance with ISO 6498.

Grind the laboratory sample (usually 500 g) so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

8 Procedure

8.1 General

To each series, add a spiked blank sample with an aflatoxin B_1 content of 10 µg/kg and a certified reference material or a control sample (4.4). Addition of a blank sample to each series is strongly recommended to check for contamination from the glassware.

The results shall comply with the criteria in clause 10.

8.2 Determination of the absorption spectrum of the aflatoxin B_1 standard solution

In cuvettes, determine the absorption spectrum of the aflatoxin B_1 standard solution (4.24) between wavelengths of 330 nm and 370 nm by means of the spectrometer (5.5), using chloroform as blank. Measure the absorbance (A) at the maximum near a wavelength of 363 nm.

8.3 Extraction

Weigh, to the nearest 0,1 g, 50,0 g of the prepared test sample (see clause 7) into the conical flask (5.6). Consecutively add 25 g of Celite® (4.5), 250 ml of chloroform (4.11) and 25 ml of water. Stopper the flask, swirl and release the pressure. Restopper the flask and shake it for 30 min on the mechanical shaker (5.7).

NOTE In order to reduce the use of chloroform, one-half of the specified quantities may be used; i.e. 25,0 g of the prepared test sample (see clause 7), 12,5 g of Celite® (4.5), 125 ml of chloroform (4.11) and 12,5 ml of water.

Filter through a fluted filter paper (5.8). If filtration is proceeding slowly, cover the funnel in order to prevent evaporation of chloroform. Collect 50 ml of the filtrate (V_s).

If necessary, take an aliquot portion of the filtrate and dilute to 50 ml (V_f) with chloroform so that the aflatoxin B_1 content does not exceed 4 ng/ml.

Use the filtrate for sample clean-up in accordance with 8.4.

8.4 Clean-up

Carry out the procedure without significant interruptions.

8.4.1 Florisil® purification

8.4.1.1 Preparation of the column-cartridge assembly

Attach a stopcock (5.9) to the shorter stem of a Florisil® cartridge (4.6). Wash the cartridge and remove air by taking 10 ml of chloroform (4.11) and passing 8 ml of it via the stopcock rapidly through the cartridge using a syringe (5.10).

Attach the longer stem of the cartridge to a glass column (5.11) and pass the remaining 2 ml of chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

8.4.1.2 Purification

Add the filtrate (V_s or V_f) collected in step 8.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (4.11), followed by 20 ml of methanol (4.9). Discard the eluates.

During these operations, ensure that the column-cartridge assembly does not run dry.

Elute aflatoxin B_1 with 50 ml of the acetone-water mixture (4.12) and collect the eluate in the round-bottomed flask of the rotary evaporator (5.12).

NOTE 1 The quality of Florisil® varies per batch. Depending on this quality, 50 ml of acetone-water mixture (4.12) may not be sufficient for elution. If so, the use of 60 ml to 70 ml of the acetone-water mixture (4.12) is recommended.

Concentrate the eluate on the rotary evaporator at a temperature of between 40 °C and 50 °C until no more acetone is distilled.

NOTE 2 About 0,5 ml of liquid remains in the flask at this point. Experiments have shown that further evaporation is not harmful and that when 0,5 ml of liquid remains, there is no significant amount of acetone. Residues of acetone may lead to losses of aflatoxin B_1 on the C_{18} cartridge.

Add 1 ml of methanol (4.9), swirl the flask to dissolve aflatoxin B_1 on the sides of the flask, add 4 ml of water and mix. Disconnect and discard the cartridge. Rinse the glass column with water and retain for the C_{18} purification step (8.4.2).

8.4.2 C_{18} purification

8.4.2.1 Preparation of the column-cartridge assembly

Attach a stopcock (5.9) to the shorter stem of a C_{18} cartridge (4.7). Prime the cartridge and remove any air by passing 10 ml of methanol (4.9) via the stopcock rapidly through the cartridge with a syringe (5.10). Air bubbles in the cartridge are visible as light spots on the otherwise greyish background. Take 10 ml of water and pass 8 ml of it through the cartridge. Avoid introduction of air into the cartridge when switching from methanol to water.

Attach the longer stem of the cartridge to a glass column (5.11) and pass the remaining 2 ml of water through the cartridge in the column. Close the stopcock. Remove the syringe.

8.4.2.2 Purification

Transfer the extract obtained in 8.4.1.2 quantitatively to the glass column (5.11), rinsing the flask twice with 5 ml of the water-methanol mixture (4.15) and drain by gravity.

During these operations, ensure that the column-cartridge assembly does not run dry. If air bubbles develop in the constriction near the cartridge, stop the flow and tap the top of the glass column to remove the air bubbles. Then continue.

Elute with 25 ml of the water-methanol mixture (4.15). Discard the eluate. Elute the aflatoxin B_1 with 25 ml of the water-acetone mixture (4.13) and collect the eluate in a 50 ml volumetric flask. Dilute to the mark with water and mix. Use the resulting solution for chromatography (8.5).

NOTE Filtration of the final extract prior to HPLC is normally not necessary. If considered necessary, cellulose filters should not be used because they may lead to losses of aflatoxin B_1. PTFE filters are acceptable.

8.5 High-performance liquid chromatography

8.5.1 General

Allow sufficient time for conditioning and stabilizing the apparatus.

The detector response to aflatoxin B_1 depends on the temperature, therefore compensation should be made for drift.

By injecting a fixed amount of reference calibration solution (4.26.3) at regular intervals (e.g. every third injection), the aflatoxin B_1 peak values between the results for the reference calibration solution can be corrected for using the mean response, provided that the difference between responses of consecutive measurements of reference calibration solution is very small (< 10 %). Therefore injections shall be made without interruptions wherever possible. If interruption is necessary, the last injection before interruption and the first injection after interruption shall be the reference calibration solution (4.26.3).

Because the calibration curve is linear and passes through the origin, the mass of aflatoxin B_1 in the sample extracts is determined directly by reference to the adjacent results for the reference calibration solution.

8.5.2 HPLC pump settings

Set the pump (5.13.1) to give a volume flow rate of the mobile phase (4.18) of 0,5 ml/min or 0,3 ml/min for a 5 µm or a 3 µm HPLC column (5.13.6) respectively.

If iodine derivatization is used, proceed in accordance with 8.5.3.

If bromine derivatization is used, proceed in accordance with 8.5.4.

8.5.3 Post-column pump settings for HPLC with iodine derivatization

Set the pump (5.14.1) to give a volume flow rate of the saturated iodine solution (4.19) of between 0,2 ml/min and 0,4 ml/min. As a rough guide, volume flow rates of about 0,4 ml/min or 0,2 ml/min are advised in combination with volume flow rates of the mobile phase (4.18) of 0,5 ml/min and 0,3 ml/min respectively.

8.5.4 Fluorescence detector

Set the fluorescence detector (5.13.3) to an excitation wavelength of 365 nm and an emission wavelength of 435 nm (filter instrument: > 400 nm). Adjust the detector attenuator to obtain about 80 % full-scale deflection for 1 ng of aflatoxin B_1.

8.5.5 Injector

For all solutions, inject volumes of 250 µl following the instructions of the manufacturer of the injector.

8.5.6 Check of chromatographic separation

Inject the chromatographic test solution (4.27). Valleys should be less than 5 % of the sum of the peak heights of the adjacent peaks.

8.5.7 Check of the stability of the system

Before each series of analyses, repetitively inject the reference calibration solution (4.26.3) until stable peak heights are achieved. Peak responses for aflatoxin B_1 between consecutive injections should not differ by more than 6 %. Proceed without delay with the check of linearity (8.5.8).

8.5.8 Check of linearity

Inject the aflatoxin B_1 calibration solutions (4.26.1 to 4.26.4). Every third injection use the reference calibration solution (4.26.3) for correction of drift in response. Peak responses for this reference calibration solution shall not differ by more than 10 % in 90 min. Correct for drift with the formula given in 9.3.

The calibration graph shall be linear and pass through the origin within twice the standard error of the estimate of the response. Values found shall not differ by more than 3 % from the nominal values.

If these requirements are fulfilled, continue without delay. If not, identify and correct the sources of any problem before continuing.

8.5.9 Injection of sample extracts

Consecutively inject the reference calibration solution (4.26.3), the blank feed extract, the spiked blank feed extract, the reference calibration solution (4.26.3), the extract of a certified reference material or the control sample (4.4) and again the reference calibration solution (4.26.3).

Inject the purified sample extracts (8.4.2.2). After every two sample extracts, repeat the injection of the reference calibration solution (4.26.3). When the series contains more than 10 samples, the last injections should be the aflatoxin B_1 calibration solutions (4.26.1 to 4.26.4).

9 Calculation of results

9.1 Calculation of the aflatoxin B_1 content of the aflatoxin B_1 standard solution

Calculate the aflatoxin B_1 content of the aflatoxin B_1 standard solution (4.24) using the equation:

$$\rho = \frac{M \times A}{d \times \kappa}$$

where

ρ is the aflatoxin B_1 content of the aflatoxin B_1 standard solution (4.24), in mg/ml;

M is the molar mass of aflatoxin B_1, in g/mol (M = 312 g/mol);

A is the absorbance measured in 8.2, corrected for the blank;

d is the optical path length of the cuvette, in cm (d = 1 cm);

κ is the molar absorption coefficient of aflatoxin B_1 in chloroform at 363 nm, in $l \cdot mol^{-1} \cdot cm^{-1}$ (κ = 22 300 $l \cdot mol^{-1} \cdot cm^{-1}$).

9.2 Calculation of the mass of aflatoxin B_1 in the injected reference calibration solution

Calculate the mass of aflatoxin B_1 in the injected reference calibration solution (4.26.3) using the equation:

$$m_c = f \times \rho \times V_{ic}$$

where

m_c is the mass of aflatoxin B_1 in the reference calibration solution (4.26.3) injected, in ng;

f is the dilution and units correction factor, in ng/mg (f = 200 ng/mg);

ρ is the aflatoxin B_1 content of the aflatoxin B_1 standard solution (4.24), calculated in 9.1, in mg/ml;

V_{ic} is the volume of reference calibration solution (4.26.3) injected, in ml (V_{ic} = 0,25 ml).

9.3 Calculation of the mass of aflatoxn B_1 in the test solution

Calculate the mass alfatoxin B_1 in the test solution using the equatio:

13.0 References

IS : P-draft on DDGS (2024). Dried Distillers Grain with Solubles (DDGS) as livestock feed ingredient – Specification.

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