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DRAFT EAST AFRICAN STANDARD

Cereals, Pulses and their products— Test methods

EAST AFRICAN COMMUNITY

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Copyright notice

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Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in the East African Community. It is envisaged that through harmonized standardization, trade barriers that are encountered when goods and services are exchanged within the Community will be removed.

The Community has established an East African Standards Committee (EASC) mandated to develop and issue East African Standards (EAS). The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the public and private sector organizations in the community.

East African Standards are developed through Technical Committees that are representative of key stakeholders including government, academia, consumer groups, private sector and other interested parties. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the Principles and procedures for development of East African Standards.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

The committee responsible for this document is Technical Committee EASC/TC 014, *Cereals, pulses and related products*.

Attention is drawn to the possibility that some of the elements of this document may be subject of patent rights. EAC shall not be held responsible for identifying any or all such patent rights.

Introduction

The lack of harmonized test methods used by laboratories within the EAC Partner States has led to differences in test results. This is due to the availability of several test methods from various International organizations and internally developed and validated methods.

This challenge has prompted the EAC Partner States to develop harmonized test procedures to avoid discrepancies and any trade barriers within the EAC region. The test methods outlined in this standard have taken cognisance of the discrepancies and developed the East African Standard accordingly. Physical test methods for cereals, pulses and their products are similar and have been grouped under the same clause while specific methods have been considered separately.

Cereals, Pulses and their products— Test methods

1 Scope

This draft East African Standard prescribes the test methods for cereals, pulses and their products.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
foreign matter
all organic and inorganic material other than broken kernels and other grains

3.2
inorganic matter
stones, glass, pieces of soil and other mineral matter

3.3
organic matter
any animal or plant matter (seed coats, straws, weeds) other than damaged grain, other grains, inorganic extraneous matter and harmful/toxic seeds.

3.4
pest damaged grain
grain which shows damage or owing attack by rodents, insects, mites or other pests

3.5
rotten and diseased grain
grain affected by mould growth or bacterial decomposition or other causes that may be noticed without having to cut the grains to examine it and renders it unsafe for human consumption

3.6
discoloured grain
grain which is damaged by heat, frost or water

3.7

immature grain
grain which is underdeveloped, thin and papery in appearance. For rice and wheat, grains they have a distinct green colour.

3.8
filth
matter of animal origin (droppings, eggs, larvae, nymphs, or adults of insects and their fragments, rodent hairs and their fragments)

3.9
defective/damaged grain
pest damaged, germinated/sprouted, discoloured, stained, rotten and diseased, immature and shrivelled grains and broken grain

3.10
broken kernels
portions of kernels of which an estimated one-quarter or more of the original entire kernel has been broken off

3.10.1
maize and wheat broken kernels
pieces of grain which pass through a standard sieve

3.10.2
pulses broken kernels
cotyledons which are separated or one or both of the cotyledons have been broken

3.10.3
rice broken kernels
pieces of grain that are less than three-quarters of a whole kernel and includes grains in which part of the endosperm is exposed or grain without a germ. If the piece is more than three-quarters of a kernel, it is considered whole.

3.10.4
sorghum broken kernels
pieces of sorghum grain which pass through a screen having round holes of 1.8mm diameter

3.10.5
barley
pieces of barley that are less than three quarters or whole kernel and with the germ end broke off

3.11
shrivelled grain/shrunken grain
grain which is poorly filled, light and thin whose build-up of reserves has been halted due to physiological or pathological factors

3.12
other edible grains
grains belonging to cereals species other than the grain under consideration

3.13
germinated grain
grain in which the radicle or plumule is clearly visible

3.14
harmful matter/toxic matter
any substances in grain that can have a damaging or dangerous effect on health

3.15
paddy rice
whole or broken un-hulled kernels of rice

3.16
red-streaked kernel
head rice or broken kernel with red bran streaks of length greater than or equal to 50 % of that whole kernel, but where the surface covered by these red streaks is less than 25 % of the total surface.

3.17
chalky kernels
head rice or broken kernel, except wax rice, whose whole surface has an opaque and floury appearance

4 Physical examination

4.1 Principle

Separation of the impurities by sieving and grading

4.2 Apparatus

4.2.1 Analytical balance

4.2.2 Sample divider

4.2.2 Sieves

4.2.3 Magnifying glass

4.2.4 Forceps/spatula

4.2.5 Tweezers, paint brush and scalpels

4.2.6 Dishes

4.2.7 Shallow containers/beakers

4.3 Procedure

4.3.1 Preparation of test sample

Table 1 — Quantity of a working sample

Type of grain	Mass of the working sample (*m*1) g
Maize	200
Wheat	250
sorghum	100
oats	250
barley	250
Rice	100
Millet	100
Bean	200
Soy bean	200
Chick peas	200
Green grams	200
Pigeon peas	200
Cowpeas	200

Carefully mix the laboratory sample to make it as uniform as possible, then proceed to reduce it using a sample divider to obtain a working sample in accordance with Table 1.

Table 1 — Quantity of a working sample

Weigh the test sample so obtained to the nearest 1 g and place it in the shallow containers/beakers (4.2.7).

During the preparation of the test sample, note whether any particular odour or odour foreign to that of grain is detected, and any presence of living or dead, insects or other anomalies.

4.3.2 Determination of impurities

4.3.2.1 Foreign matter

4.3.2.1.1 Procedure

Foreign matter shall be determined by use of appropriate sieve size and/or hand picking.

Select all the foreign matter retained on top of the sieve and add it to that which passed through the sieve. Weigh (*m*2).

4.3.2.1.2 Calculation

Foreign matter, expressed as a percentage of the total mass of the working sample (m1), shall be calculated asfollows:

$$\% \text{ foreign matter} = \frac{m2}{m1} X100$$

4.3.2.2 Inorganic matter

4.3.2.2.1 Procedure

From the foreign matter, select all inorganic matter and weigh (*m*3).

4.3.2.2.2 Calculation

Inorganic matter, expressed as a percentage of the total mass of the working sample(m1), shall be calculated asfollows:

$$\% \text{ inorganic matter} = \frac{m3}{m1} X100$$

4.3.2.3 Filth

4.3.2.3.1 Procedure

Separate filth from both the grain retained on the sieve and that in the holding container and weigh (*m*2).

4.3.2.3.2 Calculation

Filth, expressed as a percentage of the total mass of the working sample(m1), shall be calculated as follows:

$$\% \text{ filth} = \frac{m2}{m1} X100$$

4.3.2.4 Broken grains

4.3.2.4.1 Procedure

Separate the broken grains by use of the appropriate test sieve size, as given in Table 2, or by hand picking and weigh (*m*2).

Table 2 — Size of test sieve

Type of grain	Sieve size
Maize	4.50-mm round hole
Wheat	Perforated sieve with slots measuring 1.6 mmwide and 9.5 mm long
Sorghum	1.8mm

4.3.2.4.2 Calculation

Broken grains, expressed as a percentage of the total mass of the working sample(m1),, shall be calculated asfollows:

$$\% \text{ broken grains} = \frac{m2}{m1} X100$$

4.3.2.5 Shrunken grains

4.3.2.5.1 Procedure

Separate the shrunken grains by use of the appropriate sieve size or by hand picking and weigh (*m*2).

4.3.2.5.2 Calculation

Shrunken grains, expressed as a percentage of the total mass of the working sample (m1), shall be calculated asfollows:

$$\% \text{ shrunken grains} = \frac{m2}{m1} X100$$

4.3.2.6 Immature grains

4.3.2.6.1 Procedure

Select all immature grains and weigh (*m*2).

4.3.2.6.2 Calculation

Immature grains, expressed as a percentage of the total mass of the working sample(m1),, shall be calculated asfollows:

$$\% \text{ immature grains} = \frac{m2}{m1} X100$$

4.3.2.7 Pest damaged grains

4.3.2.7.1 Procedure

Select all grains which are partially eaten (bored) by weevils, grain borers or other crawling pests and weigh (*m*2).

4.3.2.7.2 Calculation

Pest damaged grains, expressed as a percentage of the total mass of the working sample (m1), shall be calculatedas follows:

$$\% \text{ pest damaged grains} = \frac{m2}{m1} X100$$

4.3.2.8 Rotten and diseased grains

4.3.2.8.1 Procedure

Select all rotten and diseased grains and weigh (*m*2).

4.3.2.8.2 Calculation

Rotten and diseased grains, expressed as a percentage of the total mass of the working sample(m1), shall be calculated as follows:

$$\% \text{ rotten and diseased grains} = \frac{m2}{m1} X100$$

4.3.2.9 Discoloured grains

4.3.2.9.1 Procedure

Select all discoloured grains by hand picking and weigh (*m*2).

4.3.2.9.2 Calculation

Discoloured grains, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ discoloured grains} = \frac{m2}{m1} X100$$

4.3.2.10 Other edible grains

4.3.2.10.1 Procedure

Select other edible grains and weigh (*m*2).

4.3.2.10.2 Calculation

Other grains, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ other grains} = \frac{m2}{m1} X100$$

4.3.2.11 Germinated/sprouted grains

4.3.2.11.1 Procedure

Select all germinated/sprouted grains and weigh ($m2$).

4.3.2.11.2 Calculation

Germinated grains, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ germinated grains} = \frac{m2}{m1} X100$$

4.3.2.12 Harmful and noxious grains

4.3.2.12.1 Procedure

Select all harmful and noxious grains and weigh ($m2$).

4.3.2.12.2 Calculation

Harmful and noxious grains, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ harmful and noxious grains} = \frac{m2}{m1} X100$$

4.3.2.13 Contrasting varieties

4.3.2.13 Contrasting varieties

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4.3.2.13.1 Procedure

Select all contrasting variety grains and weigh ($m2$).

4.3.2.13.2 Calculation

Contrasting variety grains, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ contrasting variety grains} = \frac{m2}{m1} X100$$

4.3.2.14 Other defects applicable to milled rice

4.3.2.14.1 Chalky kernels

4.3.2.14.1.1 Procedure

Select all chalky kernels and weigh ($m2$).

4.3.2.14.1.2 Calculation

Chalky kernels, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ chalky kernels} = \frac{m2}{m1} \text{X}100$$

4.3.2.14.2 Red streaked kernels

4.3.2.14.2.1 Procedure

Select all red streaked kernels and weigh (*m*2).

4.3.2.14.2.2 Calculation

Red streaked kernels, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ red streaked kernels} = \frac{m2}{m1} \text{X}100$$

4.3.2.14.3 Paddy rice

4.3.2.14.2.1 Procedure

Select all paddy rice and weigh (*m*2).

4.3.2.14.2.2 Calculation

Paddy rice, expressed as a percentage of the total mass of the working sample, shall be calculated as follows:

$$\% \text{ paddy rice} = \frac{m2}{m1} \text{X}100$$

4.3.2.15 Total defective grains

To obtain the total defective grains, sum up the total of the individual defects.

$$\% \text{ total defective grains} = Y \text{ x } \frac{70}{100}$$

where

Y is the sum total of individual defects, expressed as percentage

5 Determination of moisture content

5.1 Principle

This method determines the moisture content as the loss of mass fraction, expressed as a percentage, of a sample when heated under specified conditions. A pre-conditioning stage is used to minimize moisture loss during the grinding stage.

5.2 Apparatus

5.2.1 Analytical balance

5.2.2 Grinding mill, having the following characteristics:

a) made of materials which do not absorb moisture;

b) easy to clean and having as few dead spaces as possible;

c) adjustable so as to obtain particles of sizes less than or equal to 1.7 mm, less than 10 % (m/m) being over 1 mm and more than 50 % (m/m) being less than 0.5mm; and

d) enable grinding to be carried out rapidly and uniformly without appreciable development of heat and as far as possible without contact with outside air.

NOTE Grinders operating at a speed higher than 3 600 rev/min are unsatisfactory due to excessive moisture lossduring grinding, which results in moisture values lower than actual.

5.2.3 Moisture dish, of suitable dimensions, non-corrodible under the test conditions, or failing this, a glass dish, with sufficiently tight-fitting lid, and having an effective surface area such as to allow distribution of the test portion with no more than 0.3 g/cm^2

5.2.4 Constant-temperature oven, electrically heated, capable of being controlled in such a way that during the normal working, the temperature of the air and of the shelves carrying the test portion is 130 °C ± 3 °C in the neighbourhood of the test portions

5.2.5 Air tight desiccator, containing an efficient desiccant

5.3 Procedure

5.3.1 Preparation of test sample

Carefully mix the laboratory sample to make it as uniform as possible. Grind the sample to obtain the desired particle size as described in 5.2.2 (c). Place the ground sample in an air-tight container.

Products which are likely to undergo changes in moisture content in the course of grinding, in general products with moisture above 15 %, shall undergo pre-conditioning.

5.3.2 Test portion

Rapidly weigh to the nearest 1 mg about 5 g of the test sample in the moisture dish which has been previously dried and weighed, together with its lid to the nearest 1 mg.

5.3.3 Drying

Place the open dishes containing the test portion and the lids in the oven maintained at 130 °C ± 3 °C and leave it for 2 h, taken from the moment when the oven temperature is again at 130 °C ± 3 °C.

Remove the dishes and the lids from the oven, cover rapidly and transfer to the desiccator as quickly as possible. Never place dishes on top of one another in the desiccator.

Weigh dishes and contents after they have reached room temperature (normally 45 min to 60 min) and record the mass of the test portion.

5.4 Calculation and expression of results

5.4.1 Without pre-conditioning

The moisture content, expressed as a percentage, shall be calculated as follows:

$$\% \text{ moisture content} = \frac{(m0 - m1)}{m0} \times 100$$

where

$m0$ is the mass, in grams, of the test portion; and

$m1$ is the mass, in grams, of the test portion after drying.

5.4.2 With pre-conditioning

$$\% \text{ moisture content} = \frac{(1 - m1 - m3)}{m0m2} \times 100$$

where

m_0 is the mass, in grams, of the test portion;

m_1 is the mass, in grams, of the test portion after drying;

m_2 is the mass, in grams, of the test portion before conditioning; and

m_3 is the mass, in grams, after conditioning.

6 Determination of protein

6.1 Principle

The proteinaceous nitrogen is converted into ammonium sulphate by digesting the sample with concentrated sulphuric acid in the presence of a catalyst and boiling point elevator. By adding an excess alkali, the liberated ammonia after neutralization is steam-distilled into boric acid and estimated by titration using standard sulphuric acid solution and converted into percentage protein.

6.2 Apparatus

6.2.1 Digestion unit

6.2.2 Distillation unit

6.2.3 Analytical balance

6.2.4 Digestion tubes, with some glass beads (to prevent superheating)

6.2.5 Conical flasks

6.2.6 Burette

6.2.7 Measuring cylinder

6.2.8 Grinder
6.2.9 Spatula
6.2.10 Dropper

6.3 Reagents

6.3.1 Analytical concentrated sulphuric acid, 93 % - 98 % min. assay

6.3.2 Standard sulphuric acid solution, approximately 0.1 N

6.3.3 Catalyst, copper sulphate

6.3.4 Boiling point elevator, anhydrous potassium sulphate

6.3.5 Indicators, methyl red and phenolphthalein, three to four drops during titration

6.3.6 Sodium hydroxide solution, 40 %

6.3.7 Distilled water

6.3.8 Boric acid, 2 %

6.4 Procedure

6.4.1 Preparation of test sample

Carefully mix and grind the laboratory sample to make it as uniform as possible. In the case of grains, a representative sample is thoroughly cleaned manually or by using a laboratory cleaning machine.

6.4.2 Digestion

Weigh about 1 g of a sample and quantitatively transfer it into clean and dry digestion tube. Add 0.5 g of copper sulphate catalyst, 15 gm of potassium sulphate and 25 ml of concentrated sulphuric acid into the digestion tubes.

Place the digestion tubes into digestion unit and connect it to the manifold. Connect the manifold to the water jet, and slowly adjust the heating knob till it reaches the maximum.

Run a reagent blank. Boil the contents vigorously until the solution becomes clear blue and then allow the digestion tubes to cool.

6.4.3 Distillation

Ensure availability of sufficient distilled water, sodium hydroxide solution and boric acid solution in their respective containers.

Pre-set the distillation apparatus as follows:

- distilled water, 100 ml;
- sodium hydroxide solution, 100 ml;
- 2 % boric acid solution, 100 ml;
- delay time, 30 s; and
- distillation time, 5 min.

Pre-heat and clean the distillation unit for 5 min. This is done by connecting an empty clean digestion tube to the distillation unit and a conical flask under the distillate tube.

Connect the sample digest from the distillation tube and place a conical flask containing boric acid solution and add three to four drops of methyl red under the distillate tube. Run the distillation for 5 min until all ammonia is liberated.

6.4.4 Titration

Add three to four drops of phenolphthalein/methyl red indicator using a dropper to the conical flask containing the sample distillate and titrate it against 0.1 N sulphuric acid till just permanent pink colour and record the volume used.

Add three to four drops of phenolphthalein indicator to the distillate of the blank and titrate it against 0.1 N sulphuric acid till just permanent pink colour and record the volume used.

6.4.5 Calculation

The protein content, expressed as a percentage (as is basis), shall be calculated as follows:

$$\% \text{ protein (as is basis)} = \frac{1.4 \times (T - B) \times N \times F}{W}$$

where

T is the titration volume, in millilitres, for the sample;

B is the titration volume, in millilitres, for blank;

N is the normality of sulphuric acid (0.1N);

F is the correction factor; and

W is the weight, in grams, of the sample taken.

The correction factor, *F*, is

- 5.7 for wheat, and
- 6.25 maize.

The protein content, expressed as a percentage (on dry matter basis), shall be calculated as follows:

$$\% \text{ protein (on dry matter basis)} = \frac{\text{As is result} \times 100}{(100 - \text{Sample moisture})}$$

7 Determination of falling number

7.1 Principle

This method is based on the unique ability of alpha-amylase enzyme to liquefy a starch gel. This starch fraction of the flour is gelatinized in a boiling water bath, then the liquefaction of the starch paste (due to alpha-amylase) is measured. The falling number values bear a complex inverse relationship with the quantity of alpha-amylase in the sample.

7.2 Apparatus

7.2.1 Analytical balance

7.2.2 Falling number machine

7.2.3 Viscometer tube with stoppers

7.2.4 Viscometer stirrer

7.2.5 Automatic dispenser, of 25 ml

7.2.6 Laboratory grinder

7.3 Procedure

7.3.1 Preparation of test sample

Carefully mix and grind the laboratory sample (about 200 g to 300 g) to make it as uniform as possible. In the case of the grains, a representative sample is thoroughly cleaned manually or by using a laboratory cleaning machine.

NOTE To get a uniform particle size, 100 % of the ground sample should pass through 710 microns, between 95 % - 100 % should pass through 500 microns and 80 % or less pass through 210 microns.

Switch on the machine to allow the distilled water in the water bath to boil vigorously. Ensure the water level is adequate and that the cooling system is functional. Mass of the test portion as a function of the moisture content.

Table 3 — Mass of test portion as a function of moisture content

Moisture content of testsample	Mass of test portion	
	for a nominal mass of 7 g [at a moisture content of 15 % (*m/m*)]	**for a nominal mass of 9 g** [at a moisture content of 15 % (*m/m*)]
% (*m/m*)	g	g
9.0	6.40	8.20
9.2	6.45	8.25
9.4	6.45	8.25
9.6	6.45	8.30
9.8	6.50	8.30
10.0	6.50	8.35
10.2	6.55	8.35
10.4	6.55	8.40
10.6	6.55	8.40
10.8	6.60	8.45
11.0	6.60	8.45
11.2	6.60	8.50
11.4	6.65	8.50
11.6	6.65	8.55
11.8	6.70	8.55
12.0	6.70	8.60
12.2	6.70	8.60
12.4	6.75	8.65
12.6	6.75	8.65
12.8	6.80	8.70
13.0	6.80	8.70
13.2	6.80	8.75
13.4	6.85	8.80
13.6	6.85	8.80
13.8	6.90	8.85
14.0	6.90	8.85
14.2	6.90	8.90
14.4	6.95	8.90
14.6	6.95	8.95
14.8	7.00	8.95
15.0	7.00	9.00
15.2	7.00	9.05
15.4	7.05	9.05
15.6	7.05	9.10
15.8	7.10	9.10
16.0	7.10	9.15
16.2	7.15	9.20
16.4	7.15	9.20
16.6	7.15	9.25
16.8	7.20	9.25
17.0	7.20	9.30
17.2	7.25	9.35
17.4	7.25	9.35
17.6	7.30	9.40
17.8	7.30	9.40

18.0	7.30	9.45

Transfer the test portion into the clean, dry viscometer tube and add 25 ml ± 0.2 ml distilled water at 20 °C – 22 °C.

Fit a clean, dry stopper into the top of the viscometer tube and shake vigorously for 20 times or more to obtain homogeneous suspension.

Remove stopper and insert stirrer, scraping down the slurry coating the upper part of tube and scrap all slurry from stopper.

Place the viscometer tube with stirrer in the cassette, then into the boiling water bath within 30 s to 50 s after mixing and lock into position and start the apparatus immediately. The apparatus will carry out the rest of the test automatically.

NOTE First there is the 5-s rest period, then the starch slurry is mixed for 55 s; thereafter the motor stops and the viscometer stirrer is released in its top position to fall free from gelatinized slurry. When the stirrer has sunk the set distance into the starch gel, the timer stops automatically and the red light lit and beeper sounds. The stirring attachment will swing back automatically and the falling number in seconds is read directly from LCD display and printed.

Repeated tests on the same sample within the same laboratory should give results within ± 5 % of the average falling number value.

7.3.2 Interpretation of results

Results for falling number shall be interpreted as follows:

- < 200 s indicates high amylase activity;
- 200 s – 300 s indicates optimal amylase activity; and
- > 300 s indicates low amylase activity

8 Determination of test weight

8.1 Principle

Pouring a sample through a hopper into a container of known volume and weighing

8.2 Apparatus

8.2.1 Digital scale, with a maximum capacity of 5 000 g at a sensitivity of ± 1 g

8.2.2 Hectolitre device with the following parts:

a) blade (slide);

b) pre-filling case;

c) filling case;

d) measuring case; and

e) float (plunger).

8.3 Procedure

8.3.1 Preparation of test sample

Carefully mix the laboratory sample to make it as uniform as possible. In case of the grains a representative sample is thoroughly cleaned manually or by laboratory cleaning machine.

Ensure that the device is placed on a hard, solid and smooth surface.

Fill the pre-filling case to the top with the test sample without compacting the grain, and pour the contents of the measure into the filling hopper with the shutter in place. Open the shutter and let all the grain flow into the measuring case.

Level out using the slide. Weigh the contents of the measuring case.

8.3.2 Expression of results

Express obtained results in kilograms per hectolitre or grams per 0.5 litre

9 Determination of aflatoxin B1, B2, G1 and G2

9.1 Principle

The test sample is extracted with a mixture of methanol and water. The sample extract is filtered and diluted with buffer or water and applied to an affinity column containing antibodies specific for aflatoxins B1, B2, G1 and G2. The aflatoxins are isolated, purified and concentrated on the column then removed from the antibodies with methanol. The aflatoxins are quantified by reverse–phase High Performance Liquid Chromatography (HPLC) with fluorescence detection and post column derivatization.

9.2 Apparatus

9.2.1 Immuno affinity (IA) column, which contains antibodies raised against aflatoxin B1, B2, G1 and G2. The column shall have a minimum binding capacity of not less than 100 ng of aflatoxin B1. It shall give a recovery of not less than 80 % for aflatoxin B1, B2, G1, and not less than 60 % for aflatoxin G2, when a standard solution in 15 ml of a methanol/water mixture [1 part methanol and 3.4 parts water (by volume)] containing 5 ng of each toxin is applied to the IA column. The IA column should be equipped with an appropriate solvent reservoir (for example, a syringe with adapter). It is advisable to carry out recovery experiments for every matrix that the method is used for.

9.2.2 Blender, the use of a high-speed blender is recommended.

9.2.3 Fluted filter paper, for example, 24-cm diameter

9.2.4 Glass microfibre filter paper for example, 11-cm diameter. For example, Whatman 934AH is appropriate for this purpose. Other products may be used if they can be shown to give comparable results.

9.2.5 Volumetric flasks, Class A grade, of 2-ml capacity

9.2.6 Spectrometer, capable measuring wavelengths between 200 nm and 400 nm

9.2.7 Quartz glass cells, of optical path length 1 cm, and with no significant absorption between wavelengths of 300 nm and 370 nm

9.2.8 Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 4 mm and a pore size of 0.45 µm

9.2.9 HPLC apparatus, comprising the components in 9.2.9.1 to 9.2.9.3

9.2.9.1 HPLC pump, capable of producing a flow rate at 1 ml/min

9.2.9.2 Injection system, a syringe-loading injection valve with 50-µl loop or equivalent

9.2.9.3 Analytical reverse-phase separating column, for example, C18, which ensures a baseline resolved resolution of the aflatoxin B1, B2, G1 and G2 peaks from all other peaks, with the following characteristics:

a) length of 250 mm;

b) internal diameter of 4.6 mm; and

c) spherical particle size of 5 µm.

Shorter columns may be used.

9.2.9.4 Post-column derivatization system, consisting of a pulse-free pump and very low dead- volume T- piece, with polytetrafluoroethylene (PTFE) or stainless-steel tubing of length 3 000 mm to 5 000 mm and internal diameter of 0.5 mm, and a heating bath or post-column reactor for the iodine reaction

9.2.10 Fluorescence detector, with excitation at wavelength of 365 nm and emission at wavelength of 435 nm (for filter instruments: emission wavelength at 400 nm), capable of detecting at least 0.05 ng of aflatoxin B1 per injection volume (here 50 µl).

9.3 Reagents

Use only reagents recognized analytical grade, unless otherwise stated.

9.3.1 Water, according to grade 1 of ISO 3696

9.3.2 Sodium chloride.

9.3.3 Iodine, crystalline

9.3.4 Aflatoxin, in crystal form or as a film ampoule

WARNING — Aflatoxins are carcinogenic to human subjects. Adequately protect from daylight the laboratory where the analyses are carried out. This may be achieved effectively by using ultraviolet (UV) absorbing foil on the windows in combination with subdued light (no direct sunlight), or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable).

9.3.5 Acetonitrile, HPLC grade

9.3.6 Methanol, analytical grade

9.3.7 Methanol, HPLC grade

9.3.8 Toluene, analytical grade

WARNING — Toluene is highly flammable and harmful. Standard preparation involving this solvent shall be performed in a fume cupboard. Operations outside the fume cupboard, such as measurement of standards by UV spectrometry, shall be performed with the standards in closed containers.

9.3.9 Toluene/acetonitrile mixture. Mix 98 parts per volume of toluene (9.3.8) with 2 parts per volume of acetonitrile (9.3.5). [See Warning in 9.3.8].

9.3.10 Extraction solvent. Mix 7 parts per volume of methanol (9.3.7) with 3 parts per volume of water (9.3.1).

9.3.11 Mobile phase. Mix 3 parts per volume of water (9.3.1) with 1 part per volume of acetonitrile (9.3.5) and 1 part per volume of methanol (9.3.7). Degas the solution before use.

9.3.12 Post-column derivatization reagent. Dissolve 100 mg of iodine (9.3.3) in 2 ml of methanol (9.3.8). Add 200 ml of water (9.3.1), stir for 1 h, then filter through a 0.45-µm membrane filter (9.2.8). Prepare the solution the week of use and store the solution in the dark or in a brown glass bottle. Before use, stir the solution for 10 min.

9.3.13 Aflatoxin B_1, B_2, G_1 and G_2 stock solutions

WARNING — Protect solutions containing aflatoxin from light as far as possible (keep in the dark, use aluminium foil or amber-coloured glassware).

Dissolve aflatoxin B_1, B_2, G_1 and G_2 separately in the toluene/acetonitrile mixture (9.3.9) to give separate solutions containing 10 µg/ml.

To determine the exact concentration of aflatoxin in each stock solution, record the absorption curve at a wavelength between 330 nm and 370 nm in 1-cm quartz glass cells (9.2.7) using a spectrometer (9.2.6) with a toluene/acetonitrile mixture (9.3.9) as reference.

Calculate the aflatoxin concentration of each aflatoxin, ρ_i, in micrograms per milliliter, using Equation (1) below.

$$\rho i = \frac{A\max \times Mi}{1000\varepsilon i \times d} \quad \ldots (1)$$

where

A_{max} is the absorbance determined at the maximum of the absorption curve;

M_i is the molecular mass, in grams, of each aflatoxin; and

ε_i is the molar absorption coefficient of each aflatoxin in toluene/acetonitrile;

d is the optical path length, in centimeters, of the cell.

NOTE This value is determined in a solution that contains c = 1 mol/l of aflatoxin and in a cell with the optical path length d = 1 cm. The molar absorption coefficient (ε) is usually given without a unit of measurement, but from the equation $A = \varepsilon \times c \times d$, the following unit can be derived for it: 1 mol^{-1} cm^{-1}.

M_i and ε_i are given in Table 4.

Table 4 — Molecular mass and molar absorption coefficient of aflatoxins B_1, B_2, G_1 and G_2

Aflatoxin	M_i	ε_i
B_1	312	19 300
B_2	314	20 400
G_1	328	16 600
G_2	330	17 900
NOTE A mixture of toluene and acetonitrile (98 □ 2) is used as solvent.		

9.3.14 Stock solution of mixed aflatoxins. Prepare a stock solution containing 500 ng/ml of aflatoxin B_1, 125 ng/ml of aflatoxin B_2, 250 ng/ml of aflatoxin G_1 and 125 ng/ml of aflatoxin G_2 in toluene/acetonitrile (9.3.9).
If the solution has to be stored, weigh the flask before storage. Wrap the flask tightly in aluminium foil and store it at approximately 4 °C. Immediately before use, reweigh the flask and record any change in mass after storage.

NOTE Normal exposure to UV light during absorbance measurement results in no observable conversion to photoproducts.

9.3.15 Standard solution of mixed aflatoxins. Transfer each quantity, as specified in Table 5, of mixed aflatoxin stock solution (9.3.14) into a series of four 2-ml volumetric flasks (9.2.5). Evaporate the solutions just to dryness under a stream of nitrogen at room temperature. To each flask, add 1 ml of methanol (9.3.6). Dissolve the dry residue in it, dilute the solution to the mark with water (9.3.1) and mix. Prepare the solution freshly on the day of use.

Table 5 — Preparation of standard solutions

Standard solution	Volume taken from stock solution µl	Concentration of aflatoxin ng/ml			
		B_1	B_2	G_1	G_2
1	60	15.0	3.75	7.50	3.75
2	40	10.0	2.50	5.00	2.50
3	20	5.00	1.25	2.50	1.25
4	10	2.50	0.625	1.25	0.625
NOTE The values given are for guidance only. The standard range includes the concentrations of the samples.					

9.3.15 Sulfuric acid, c (H_2SO_4) = 2 mol/l

9.4 Procedure

9.4.1 Sample preparation and extraction

The sample solution and standard solutions for the HPLC determination shall contain the same solvent or solvent mixture.

Weigh, to the nearest 0.1 g, 25 g of the homogenized test sample into the blender jar (9.2.2). Add 5 g of sodium chloride (9.3.2) and 125 ml of extraction solvent (9.3.10) and homogenize with a mixer for 2 min at high speed.

Check that the blending time and speed do not have a negative influence on the extraction efficiency. Filter the mixture through a fluted filter paper (9.3.2) ($V1$).

Pipette 15 ml ($V2$) of the filtrate into a conical flask of appropriate size with glass stopper. Add 30 ml of water, stopper the flask and mix. Before starting affinity column chromatography, filter the diluted extract through a glass microfibre filter paper (9.2.4). The filtrate ($V3$) should be clear. If not, re-filter it. Proceed immediately in accordance with 9.4.2.

A centrifuge may also be used to obtain a clear solution.

9.4.2 Clean-up

Prepare the Immunoaffinity column (9.2.1) and proceed with the clean-up procedure in accordance with the manufacturer's instructions. Pipette 15 ml (*V*4) of the second filtrate (*V*3) into the solvent reservoir of the Immunoaffinity column. Pass it through the separation column, then wash the column as described in the manufacturer instructions and discard the eluates. Start the elution of the aflatoxins. Collect the methanol or acetonitrile eluate (depending on the product or the manufacturer's instructions) in a 2-ml volumetric flask (9.2.5) (or another volume as specified by the manufacturer). Dilute to the mark with water (*V*5). Mix and proceed in accordance with 9.4.3.

Methods for loading onto Immunoaffinity columns, washing and elution vary slightly between column manufacturers and the specific instructions supplied with the columns should be followed precisely.

NOTE In general, procedures involve sample extraction with a mixture of methanol and water, filtration or centrifugation, possible sample dilution with phosphate buffered solution (PBS) or water, loading under pressure onto a possibly pre-washed column, washing of the column with distilled water and elution of aflatoxins with methanol or acetonitrile (depending on the product and manufacturer's instructions).

Traditional silica gel columns or solid-phase extraction (SPE) columns may also be used. In these cases the manufacturer instructions should also be precisely followed. If the solvent used for elution of aflatoxins is not compatible with the mobile phase, then the eluate should be evaporated into dryness by a Nitrogen stream below 40 °C. The residue should be dissolved in the mobile phase and diluted to 2 ml, or to the volume specified by the manufacturer.

Take care not to exceed the maximum capacity of the column.

9.4.3 HPLC operating conditions

Connect the separation column outlet to one arm of the T-piece of the post-column derivatization system (9.3.12) using a short piece of tubing with an internal diameter of, for example, 0.25 mm. Connect the outlet ofpump which delivers the post-column derivation reagent to the second arm of the T-piece. Connect one end ofa coil of PTFE or stainless steel (see 9.3.12) to the third arm of the T-piece and connect the other end to the detector (9.2.10). Using an oven or water bath, maintain the reaction coil temperature at 70 °C.

When the column specified in 9.2.9.3 was used, the following settings were found to be appropriate:

a) flow rate of mobile phase (column): 1.0 ml/min;

b) flow rate of post-column reagent: 0.3 ml/min; and

c) volume injected: 50 µl.

Allow the entire system to run for 10 min to 20 min to stabilize it. If an integrator is used, adjust the sensitivity controls of the fluorescence detector or integrator to give a ratio of 5:1 for signal response: noise for 0.125 ng of aflatoxin G2 in 50 µl. If a strip chart recorder is used, adjust the fluorescence detector control to give 30 % to 40 % scale deflection with 0.125 ng of aflatoxin G2 in 50 µl.

9.4.4 Identification

Identify each aflatoxin peak in the sample chromatogram by comparing the retention times with those of corresponding reference standards.

Alternatively, the aflatoxins may be identified by simultaneous injection of the sample test solution and standard solutions. Also, the disappearance of the aflatoxin B1 and G1 peaks if no derivatization reagent is added is helpful for identification.

9.4.5 Calibration graph

Prepare the calibration graph for each aflatoxin by injecting 50 µl of standard solutions 1, 2, 3 and 4 (see Table 5). Check the linearity of the curve for details.

9.4.6 Determination

Quantitative determination is performed by the external standard method with integration of the peak area or measurement of the peak height, which is then related to the corresponding value for the standard substance.

Inject volumes of 50 µl of standard solution into the injection loop, following the instructions of the injector manufacturer. Aflatoxins elute in the order G_2, G_1, B_2, B_1 with retention times of approximately 6 min, 8 min, 9 min and 11 min, respectively, and should be baseline-resolved. If necessary, adjust the retention times by changing the methanol concentration of the mobile phase (9.3.11).

Inject 50 µl (V_6) of purified sample extract (9.4.2) into the injection loop.

9.5 Calculation of results

Calculate the mass; m_t, in grams, of the test sample present in the fraction of the second filtrate taken for the Immunoaffinity column (V_4), using Equation (2) below

$$m_t = m_0 \times \frac{V_2}{V_1} \times \frac{V_4}{V_3} \quad \ldots (2)$$

where

m_0 is the mass, in grams, of the test portion (9.4.1), (m_0 = 25 g);

V_1 is the total volume, in millilitres, of the first filtrate (9.4.1), (V_1 = 125 ml);

V_2 is the fraction volume, in millilitres, of the first filtrate (9.4.1) taken for dilution, (V_2 = 15 ml);

V_3 is the total volume, in millilitres, of the second filtrate (9.4.1), (V_3 = 45 ml);

V_4 is the fraction volume, in millilitres, of the second filtrate (9.4.2), (V_4 = 15 ml).

Calculate the mass fraction of each aflatoxin, w_i, in micrograms per kilogram of sample, using Equation (3) below:

$$w_i = \frac{V_5 . m_i}{V_6 . m_t}$$

where

V_5 is the volume, in microlitres, of the eluate (9.4.2), (V_5 = 2 000 µl);

V_6 is the volume, in microlitres, of the purified and injected sample extract (9.4.6), (V_6 = 50 µl);

m_i is the mass, in nanograms, of each aflatoxin *i* present in the injection volume, corresponding to the measured peak area or peak height read off the calibration graph;

m_t is the mass, in grams, of the test sample present in the fraction of the second filtrate taken for the Immuno affinity column (V_4) according to Equation (2).

Add the mass fractions of the four aflatoxins to obtain the mass fraction of total aflatoxins.

NOTE Taking into account the precision data of the method, V1 may be considered equivalent to the volume of the extraction solvent.

10 Determination of total aflatoxin using ELISA

10.1 Principle

ELISA is a solid phase direct competitive enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin is coated to a polystyrene microwell. Toxins are extracted from a ground sample using a suitable solvent based on the manufacturer's instructions. The extracted sample and conjugated aflatoxins are mixed and added to the antibody-coated microwell. Aflatoxin from the extracted sample and conjugated aflatoxins compete to bind with the antibody coated to the microwell. Microwell contents are decanted and non-specific reactants are removed by washing. An enzyme substrate is added and a distinct colour develops. The intensity of the colour is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of aflatoxin in the sample or standard. Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the colour will decrease. An acidic stop solution is added which changes the chromagen colour. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

10.2 Apparatus

10.2.1 Antibody coated microwells

10.2.2 Mixing wells

10.2.3 Aflatoxin controls

10.2.4 Conjugate solution

10.2.5 Substrate solution

10.2.6 Stop solution

10.2.7 Grinder sufficient to render sample to particle size of fine instant coffee

10.2.8 Collection container, minimum 125-ml capacity

10.2.9 Analytical balance

10.2.10 Graduated cylinder

10.2.11 Analytical solvent in accordance with manufacturer's instructions

10.2.12 Distilled or deionized water

10.2.13 Filter paper, Whatman #1 or equivalent

10.2.14 Filter funnel

10.2.15 Pipettor with tips 100 µl and 200 µl

10.2.16 Timer

10.2.17 Wash bottle (if necessary)

10.2.18 Absorbent paper towels

10.2.19 Microplate reader with 450-nm filter

10.3 Precautions

10.3.1 Bring all reagents to room temperature (19 ºC – 27 ºC) before use.

10.3.2 Store reagents at conditions recommended by manufacturer.

10.3.3 Do not return unused reagents back into their original bottles.

10.3.4 Adhere to all time and temperature conditions stated in the procedure.

10.3.5 Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may affect the test results.

10.3.6 Never pipette reagents or samples by mouth.

10.3.7 Solvents are flammable.

10.3.8 The Stop solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.

10.3.9 Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.

10.3.10 Dispose of all materials, containers and devices in the appropriate receptacle after use.

10.4 Method

Sample preparation, extraction, general procedure and interpretation of results will be determined in accordance with the manufacturer's recommendations.

11 Determination of fumonisin using liquid chromatography (LC)

11.1 Principle

Fumonisins are extracted from cereals and cereal products with methanol–acetonitrile–water (25 +25 +50, v/v/v), the filtered extract is cleaned up by an immunoaffinity column, and the fumonisins are eluted with methanol. The eluate is evaporated just to dryness, and the residue is dissolved in acetonitrile–water (50 + 50, v/v). o-Phthaldialdehyde and 2-mercaptoethanol is added to form fluorescent fumonisin derivatives, which are separated by reversed-phase liquid chromatography (LC) with fluorescence detection.

11.2 Apparatus

11.2.1 Centrifuge bottle, plastic, 250 mL, with screw cap

11.2.2 Centrifuge, operating up to 2 500 X g

11.2.3 Filter papers, Whatman No. 4, 12 cm

11.2.4 Glass microfiber filters, Whatman GF/A, 9 cm

11.2.5 Reservoir, 25 mL with Luer-tip connector for immunoaffinity column

11.2.6 Calibrated microliter syringe or microliter pipette, 25 □L –1000 □L

11.2.7 Vacuum manifold to accommodate immunoaffinity columns

11.2.8 Liquid chromatograph, LC pump delivering 1 mL/min constant flow rate and with injection system calibrated to deliver 20 □L; and data system

11.2.9 LC column, stainless steel (150 mm X 4.6 mm id), packed with 5 □m C18 deactivated reversed-phase material, preceded by corresponding reversed phase guard column or guard filter (0.5 □m porosity)

11.2.10 Fluorescence detector, fitted with flow cell and at 335 nm (excitation) and 440 nm (emission)

11.2.11 Shakers
11.2.12 Spatula
11.2.13 Sample divider

11.3 Reagents

11.3.1 Methanol, LC grade.

11.3.2 Acetonitrile, LC grade.

11.3.3 o-Phthaldialdehyde (OPA), CAS 643-79-8.

11.3.4 2-Mercaptoethanol (MCE), CAS 60-24-2.

11.3.5 Sodium dihydrogen phosphate solution, 0.1 M. Dissolve 15.6g $NaH_2PO_4.2H_2O$ in water and dilute to 1 L.

11.3.6 Sodium tetraborate solution, 0.1M. Dissolve 3.8 g $Na_2B_4O_7.10H_2O$ in water and dilute to 100 mL

11.3.7 Hydrochloric acid, 2 M. Dilute HCl (12 M) 1+5 with water

11.3.8 Extraction solvent, Acetonitrile-Methanol-Water (25+25+50, *v/v/v*)

11.3.9 Acetonitrile-water,-50+50, v/v

11.3.10 Phosphate-buffered saline (PBS). Dissolve 8.0 g NaCl, 1.2 g anhydrous Na_2HPO_4, 0.2 g KH_2PO_4, and 0.2 g KCl in approximately 990 mL water. Adjust pH to 7.0 with 2 M HCl, and dilute to 1 L. Phosphate-buffered saline tablets can also be used.

11.3.11 Immunoaffinity columns, specific for fumonisin cleanup with 100 % cross reactivity for both FB_1 and FB_2. The column will have a total capacity of ≥10 µg fumonisins B_1 and B_2 and should give a recovery of ≥ 90 % when a calibrant solution of fumonisins B_1 and B_2 in methanol-PBS containing 5 □g fumonisins is applied. Follow the manufacturer's instructions for the type of column used.

11.3.12 LC mobile phase, Methanol-0.1 M NaH2PO4 (77+23, v/v), adjusted to pH 3.35 with H_3PO_4. Filter mobile phase through 0.45 □m membrane, and pump at 1 mL/min flow rate. Adjust composition to conform with individual LC column characteristics.

11.3.13 OPA reagent. Dissolve 40 mg OPA in 1 mL methanol, and dilute with 5 mL 0.1M $Na_2B_4O_7$ solution. Add 50 □L MCE and mix. Store in the dark for up to one week at room temperature in a capped amber vial.

11.3.14 Fumonisins B_1 and B_2, crystalline form, purity of >95 %

11.3.15 Fumonisin stock solution for LC. Prepare calibrant solution containing fumonisins B_1 and B_2 in acetonitrile-water (50 + 50, v/v) at concentration of 100 □g/mL for FB_1 and 50 □g/mL for FB_2. Fumonisin calibrant solution is stable up to six months when stored at 4 °C. Pipette 500 □L fumonisin calibrant solution intoa 5-mL calibrated volumetric flask. Dilute to volume with acetonitrile–water (50 + 50, v/v), and shake well to obtain stock solution containing FB_1 at 10 ng/□L and FB_2 at 5 ng/□L.

11.3.16 Fumonisin working calibrant solutions for LC. Prepare four LC calibrant solutions in separate 5- mL volumetric flasks according to Table 6. Dilute contents of each flask to volume (5 mL) with acetonitrile- water

(50 + 50, v/v).

Table 6 — Preparation of working calibrant solution

Working calibrant solution	Proportion taken □L	Working solution	Derivatized solution	Working solution	Derivatized solution
1	25	0.05	0.025	0.025	0.0125
2	125	0.25	0.125	0.125	0.0625
3	500	1.00	0.500	0.50	0.250
4	2 000	4.00	2.00	2.00	1.000

11.4 Procedure

11.4.1 Sample preparation and extraction

Permit the materials to reach room temperature before removing test portion. Weigh, to nearest 0.1 g, 20 g of test portion into 250-mL centrifuge bottle, and add 50 mL extraction solvent (11.3.8). Cover the centrifuge bottle, and shake the bottle for 20 min with orbital shaker. Centrifuge for 10 min at 2 500 x g, and filter supernatant through filter paper, (11.2.3), and avoiding transfer of solid material on filter. Again extract remaining solid material by adding 50 mL extraction solvent (11.3.8), to centrifuge bottle and shaking bottle for20 min. Centrifuge for 10 min at 2 500 x g, and filter extract through the same filter paper. Collect and combinethe two filtrates, and pipette 10 mL filtrate into 100 mL flask. Add 40 mL PBS, (11.3.10), and mix well. Filter diluted extract through themicrofibre filter, (11.2.4), and collect 10 mL filtrate (equivalent to 0.4 g test portion) for clean-up through immunoaffinitycolumn.

11.4.2 Immunoaffinity column cleanup

Follow manufacturer's instructions for the type of column used. Remove top cap from column, and connect column with reservoir. Remove end cap from column and attach column to vacuum manifold. Pipette 10 mL filtrate into reservoir. Let filtrate flow through the column at approximately one to two drops per second and discard eluate. Wash column with 10 mL PBS, (11.3.10) at rate of one to two drops per second until air comes through column. Place 4-mL vial under column. Elute fumonisins with 1.5 mL LC grade methanol at one drop per second, and collect fumonisins in vial. Evaporate eluate just to dryness under stream of N at approximately 60 °C. Retain dried residue at approximately 4 °C for derivatization and LC analysis.

11.4.3 Calibration curve

Prepare calibration curves, using working calibrant solutions, (11.3.16). These solutions cover the range of 0.025 □g/g - 2.000 □g/g for FB1 and the range of 0.0125 □g/g -1.000 □g/g for FB2. Prepare calibration curves, before LC analysis, according to Table 6, and check plots for linearity. If curve is not linear, repeat derivatization following instructions carefully and/or reduce the range of the calibrants.

11.4.4 Derivatization and LC Analysis

Re-dissolve purified residue in 200 □L acetonitrile–water (50 + 50, v/v), (11.3.16). Transfer 50 □L aliquots of extract or standards to bottom of 1-mL test tube, and add 50 □L OPA reagent, (11.3.13). Mix solution for 30 s with vortex mixer, and inject 20 □L derivatized solution (equivalent to 20 mg matrix) into LC system exactly 3 min after adding OPA reagent. With the described LC mobile phase, (11.3.12), and column, (11.2.9), satisfactory (baseline) resolution of FB_1–OPA and FB_2–OPA shall be obtained, with expected retention times at approximately 6 min and 15 min, respectively. If fumonisin content of derivatized extract is higher than calibration range, dilute purified extract with acetonitrile-water (50 + 50, v/v), (11.3.16), derivatize with OPA reagent, and repeat LC analysis.

11.4.5 Quantification of fumonisins B_1 and B_2

Quantify FB1 and FB2 by measuring peak area (or peak height) at retention time of each fumonisin and comparing measured value with corresponding calibration curve.

From calibration curves determine amounts of FB1 and FB2 (in nanograms) in aliquot of test solution injected into LC column.

Separately calculate concentrations (CFB) of FB1 and FB2 in micrograms per gram (□g/g), as follows:

$$CF_B = 10^3 M_A \frac{\frac{1}{10^3 M_B}}{\frac{M_A}{20}}$$

where

*M*A is mass, in nanograms, of FB1 or FB2 in aliquot of test solution injected on column, as determined from calibration curve,

*M*B is mass, in milligrams, of matrix injected on column (20 mg), obtained as follows:

$$\frac{20\ g}{mL} \times \text{Extraction step} \times \frac{10}{\text{(dilution)}} \times 10\ mL \times \text{purified aliquot} \times \frac{100\ mL}{1\ \text{reconstitution volume}} \times \frac{50\ mL}{0.2\ mL} \times \frac{0.05\ mL}{0.1\ mL} \times \text{derivatization}$$

□ 0.02 mL □injected aliquot □

and 10^{-3} is factor to convert *M*A and *M*B from nanograms (ng) to micrograms (□g) and from milligrams (mg) to grams (g), respectively.

12 Determination of fumonisin using ELISA

12.1 Principle

ELISA is a solid phase direct competitive enzyme immunoassay. A fumonisin-specific antibody optimized to cross react with the three fumonisin subtypes is coated to a polystyrene microwell. Toxins are extracted from a ground sample with appropriate solvent as per manufacturer's recommendations. The extracted sample and conjugated fumonisin are mixed and added to the antibody-coated microwell. Fumonisin from the extracted sample and conjugated fumonisin compete to bind with the antibody coated to the microwell. Microwell contents are decanted and non-specific reactants are removed by washing. An enzyme substrate is added and a distinct develops. The intensity of the colour is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of fumonisin in the sample or standard. Therefore, as the concentration of fumonisin in the sample or standard increases, the intensity of the colour will decrease. An acidic stop solution is added which changes the chromogen colour. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm (OD 450). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

12.2 Apparatus

12.2.1 Antibody coated microwells

12.2.2 Mixing wells

12.2.3 Fumonisin standards

12.2.4 Fumonisin conjugate solution

12.2.5 Substrate solution

12.2.6 Stop solution

12.2.7 Grinder sufficient to render sample to particle size of fine instant coffee

12.2.8 Collection container, minimum 125-ml capacity

12.2.9 Analytical balance

12.2.10 Graduated cylinder

12.2.11 Analytical solvent in accordance with manufacturer's instructions

12.2.12 Distilled or deionized water

12.2.13 Filter paper, Whatman #1 or equivalent

12.2.14 Filter funnel

12.2.15 Pipette with tips 100 µl and 200 µl

12.2.16 Timer

12.2.17 Wash bottle, if necessary

12.2.18 Absorbent paper towels

12.2.19 Microplate reader with 450-nm filter

12.3 Precautions

12.3.1 Bring all reagents to room temperature (19 ºC – 27 ºC) before use.

12.3.2 Store reagents at conditions recommended by manufacturer.

12.3.3 Do not return unused reagents back into their original bottles.

12.3.4 Adhere to all time and temperature conditions stated in the procedure.

12.3.5 Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may affect the test results.

12.3.6 Never pipette reagents or samples by mouth.

12.3.7 Solvents are flammable.

12.3.8 The Stop solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.

12.3.9 Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.

12.3.10 Dispose of all materials, containers and devices in the appropriate receptacle after use.

12.4 Method

Sample preparation, extraction, general procedure and interpretation of results will be determined in accordance with the manufacturer's recommendations.

12.5 Determination of fats

12. 6 Determination of crude fiber

12.7 Determination of Total Ash content

12.8 Determination of acid insoluble ash for bread

12.9 Determination of urease activity in soybeans

12.10 Determination of tannins in Sorghum

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[15] AOAC 2008.02, *Aflatoxins B1, B2, G1 and G2 and ochratoxin A in ginseng and ginger. Multitoxin Immunoaffinity Column Cleanup and Liquid Cchromatographic Quantitation*

