International Standard 6870

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Animal feeding stuffs — Determination of zearalenone content

Aliments des animaux — Dosage de la zéaralénone

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

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Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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Animal feeding stuffs — Determination of zearalenone content

1 Scope and field of application

This International Standard specifies a method for the determination of the zearalenone content of animal feeding stuffs and, in particular, of maize.

NOTE — Although sorghum gives interfering fluorescent spots identical to those of zearalenone, the method is still applicable to this feed, because the R_f values are different after development of the chromatogram in the second direction. These spots are not developed by the specified confirmation technique.

The limit of determination of zearalenone is approximately 50 µg/kg.

2 Principle

Extraction of a test portion with a mixture of acetonitrile and potassium chloride solution, filtration, taking an aliquot portion and defatting with iso-octane followed by purification in a mixture of acetonitrile, water and lead acetate in the presence of diatomaceous earth. After filtration, taking an aliquot portion and extraction into chloroform which is subsequently evaporated.

Dissolution of the dry extract in a mixture of benzene and acetonitrile and two-dimensional thin layer chromatography of an aliquot portion of this solution. Determination of the zearalenone content by visual measurement or by measurement of the intensity of fluorescence of the spot under UV light by comparison with known quantities of zearalenone applied to the same plate.

Confirmation of the identity of the zearalenone using bis-diazotized benzidine reagent.

3 Reagents

All the reagents shall be of recognized analytical grade and the water used shall be distilled water or water of at least equivalent purity.

3.1 Acetonitrile.

3.2 Iso-octane.

3.3 Chloroform.

3.4 Benzene/acetonitrile, 98 + 2 mixture, by volume.

WARNING — Benzene is toxic by inhalation and contact with skin and is highly flammable.

3.5 Developing solvents.

3.5.1 Toluene/ethyl acetate/formic acid, 6 + 3 + 1 mixture, by volume.

3.5.2 Chloroform/ethanol, 95 + 5 mixture, by volume.

3.6 Potassium chloride, 40 g/l solution.

3.7 Lead acetate solution, prepared as follows.

Weigh 200 g of lead acetate into a 1 000 ml one-mark volumetric flask, add 3 ml of acetic acid, dilute to the mark with water and mix.

3.8 Bis-diazotized benzidine reagent, prepared as follows.

WARNING — Benzidine is a carcinogen, and is toxic by inhalation, contact with the skin and ingestion.

3.8.1 Preparation of 5 g/l benzidine solution

Place 0,5 g of benzidine in a 100 ml flask containing 20 ml of water and 1,5 ml of hydrochloric acid and make up to volume with water.

Keep this solution protected from light in a brown glass bottle.

3.8.2 Preparation of the reagent

Cool equal volumes of the benzidine solution (3.8.1) and of a 100 g/l sodium nitrite solution to between 0 and −5 °C.

Thoroughly mix the two solutions. The solution obtained is dark purple and turbid. Leave to attain room temperature (yellow colour) before use.

Prepare this reagent just before use.

3.9 Diatomaceous earth (Celite 545), hydrochloric acid washed.

3.10 Nitrogen.

3.11 Zearalenone, standard solution of concentration 10 µg/ml, in benzene.

Determine the absorption spectrum of the solution between 300 and 330 nm by means of a spectrometer, using 10 mm silica optical cells and using benzene as reference; record the maximum absorbance, A, which is close to 317 nm.

Calculate the zearalenone concentration, in micrograms per millilitre, of the solution, by means of the formula

$$\frac{318 \times A \times 1\,000}{6\,060}$$

where

318 is the molar mass of zearalenone;

6 060 is the molar extinction coefficient.

4 Apparatus

Usual laboratory equipment and in particular

4.1 Grinder, suitable for preparing a product to pass completely through a sieve of aperture size 1 mm.

4.2 Shaker, capable of producing about 100 oscillations per minute.

4.3 Filter papers, medium grade (a rapid grade filter paper gives a turbid solution; a slow grade filter paper will become clogged).

4.4 Rotary evaporator with round bottom flask.

4.5 Apparatus for thin layer chromatography, i.e. apparatus required for the preparation of the plates (4.6) and application of spots (capillary pipettes or microsyringes), a developing tank, and apparatus for spraying the reagent (3.8) on the plates.

4.6 Glass plates for thin layer chromatography, of dimensions 200 mm × 200 mm, prepared as follows (the quantities indicated are sufficient for the preparation of 5 plates).

Weigh 30 g of silica gel G-HR into a conical flask, add 60 ml of water, stopper and mix thoroughly for 1 min. Spread the slurry over the plates in such a way that a uniform layer of thickness 0,25 mm is obtained. Allow to dry in air and store the plates in a desiccator. Activate the plates before use by placing them in an oven, maintained at 110 °C for 1 h.

Commercially available prepared plates may be used if the results obtained are comparable to the results obtained with plates prepared as specified in the previous paragraph.

4.7 Short wavelength UV lamp (wavelength 253 nm).

The intensity of irradiation shall be such as to clearly distinguish a spot of 25 ng of zearalenone on thin layer plate when the lamp is placed at a distance of 100 mm from the plate.

WARNING — In view of the danger of UV light to the eyes, eye-protection shall be worn.

4.8 Test-tube, of capacity 10 ml, with a polyethylene stopper.

4.9 Fluorodensitometer (optional, but desirable).

4.10 Water-bath, capable of being controlled at 60 °C.

4.11 Conical flask, of capacity 500 ml, with a ground glass stopper.

4.12 Separating funnels, of capacity 250 ml.

4.13 Measuring cylinders, of capacities 100 and 250 ml.

4.14 Pipettes, of capacities 50 and 100 ml.

4.15 Microsyringes.

5 Sampling

Take the laboratory sample of the product to be tested in accordance with the International Standard appropriate to the product concerned, unless sampling for the determination of zearalenone is excluded from its field of application. If an appropriate International Standard does not exist the parties concerned shall reach agreement on this subject, taking into account the characteristics of the product to be sampled.

6 Procedure

6.1 Preparation of the test sample

Grind the sample so that it passes completely through a sieve of aperture size 1 mm. Mix thoroughly.

6.2 Test portion

Weigh, to the nearest 0,01 g, 50 g of the test sample into the conical flask (4.11).

6.3 Extraction

Add 180 ml of the acetonitrile (3.1), and 20 ml of the potassium chloride solution (3.6) carefully measured from a measuring cylinder (4.13). Stopper the flask, mix and shake for 30 min with the shaker (4.2). Filter through a filter paper (4.3).

Transfer, by means of a pipette (4.14), 100 ml of the filtrate to a separating funnel (4.12) and de-fat by carrying out two successive extractions each time with 50 ml of the iso-octane (3.2).

Collect the acetonitrile phase in the round bottom flask of the rotary evaporator (4.4) and evaporate to dryness under reduced pressure.

6.4 Purification

Add to the residue obtained, 20 ml of the acetonitrile (3.1), 60 ml of water and 20 ml of the lead acetate solution (3.7), carefully measured from a measuring cylinder (4.13). Mix and allow to separate for 10 min in the water-bath (4.10) maintained at 60 °C. A precipitate is formed. Add 5 g of the diatomaceous earth (3.9) and filter through a filter paper (4.3).

Transfer by means of a pipette (4.14), 50 ml of the filtrate to a separating funnel (4.12) and carry out three successive extractions, each time with 50 ml of the chloroform (3.3). Dry the chloroform fractions over sodium sulfate. Collect the chloroform fractions in the round bottom flask of the rotary evaporator (4.4) and evaporate almost to dryness under reduced pressure.

Transfer the residue quantitatively to the test tube (4.8) by rinsing with chloroform, then evaporate to dryness under nitrogen (3.10) on the water bath (4.10).

Cautiously add, using a microsyringe, 0,5 ml of the benzene-acetonitrile mixture (3.4) and stopper the tube tightly.

6.5 Two-dimensional thin layer chromatography

6.5.1 Application of solutions (see the figure)

Draw on a plate (4.6) two straight lines parallel to adjacent sides (at 50 mm and 60 mm, respectively, from the edges) to mark the limit of migration of the solvent fronts. Apply the following solutions to the plate by means of microsyringes

- at point A, 25 µl of the purified extract (6.4)
- at point B, 10 µl of the standard solution (3.11)
- at point C, 5 µl of the standard solution (3.11)
- at point D, 10 µl of the standard solution (3.11)
- at point E, 15 µl of the standard solution (3.11).

Dry under a stream of air or of nitrogen (3.10). The spots obtained should have, at most, a diameter of about 5 mm.

6.5.2 Development (see the figure)

Develop the chromatogram in direction I using the developing solvent (3.5.1) (10 mm layer in a saturated tank), protected from light, until the solvent front has reached the marked line. Remove the plate from the tank, and leave to dry for at least 15 min at ambient temperature, protected from light.

NOTE — It may be advantageous, after development in direction I, to view the chromatogram briefly under 253 nm ultraviolet light and outline possible zearalenone spots lightly with a pencil (the spot at point B shows the position of the zearalenone).

Subsequently, develop the chromatogram in direction II using the developing solvent (3.5.2) (10 mm layer in an unsaturated tank), protected from light, until the solvent front has reached the marked line. Remove the plate from the tank, and leave to dry at ambient temperature, protected from light.

6.6 Determination

Two methods of determination can be used: visual or fluorodensitometric measurement. The latter is preferable if apparatus is available.

6.6.1 Visual measurement

Determine the quantity of zearalenone in the sample spot by comparing the intensity of fluorescence under UV light of the spot of the extract with the intensities of spots C, D and E of the standard solution, with the plate placed at a distance of 10 cm from the UV lamp (4.7). Interpolate if necessary.

If the intensity of fluorescence of the 25 µl of extract is greater than that of the 15 µl of the standard solution, apply a smaller volume at point A or dilute the extract with the benzene/acetonitrile mixture (3.4) and repeat the thin layer chromatography (6.5).

6.6.2 Fluorodensitometric measurement

Measure the intensity of fluorescence of the spots using the fluorodensitometer (4.9) at, for example, an excitation wavelength of 313 nm and an emission wavelength of 443 nm (maximum emission at 470 nm).

Determine the zearalenone content of the sample spot by comparing the intensity of fluorescence of the spot of the extract with the intensities of spots C, D and E of the standard solution.

6.7 Confirmation test for zearalenone

Spray the plate obtained in 6.5 with the bis-diazotized benzidine reagent (3.8). Zearalenone gives a bright brick-red spot at room temperature, which fades after being exposed to the air for at least 15 min.

7 Expression of results

7.1 Visual measurement

The zearalenone content, expressed in micrograms per kilogram of product, is equal to

$$\frac{c\ V_1\ V_3}{m\ V_2}$$

where

c is the zearalenone concentration, in micrograms per millilitre, of the standard solution (3.11);

m is the mass, in grams, of test portion corresponding to the volume of the extract subjected to purification (12,5 g);

V_1 is the final volume, in microlitres, of the extract, taking into account possible dilutions;

V_2 and V_3 are, respectively, the volumes, in microlitres, of the extract, and of the zearalenone standard solution (3.11), which show similar intensities of fluorescence.

7.2 Fluorodensitometric measurement

The zearalenone content, expressed in micrograms per kilogram of product, is equal to

$$\frac{m_1 V_1}{m V_2}$$

where

m is the mass, in grams, of test portion corresponding to the volume of the extract subjected to purification (12,5 g);

m_1 is the mass, in nanograms, of zearalenone in the spot of the extract (taking into account volume V_2), deduced from the determinations;

V_1 is the final volume, in microlitres, of the extract taking into account possible dilutions;

V_2 is the volume, in microlitres, of the extract applied to the plate (25 µl).

8 Precision

An international inter-laboratory trial among 20 laboratories (only 16 of which supplied usable results for maize B), each having carried out three determinations, gave the statistical results (evaluated in accordance with ISO 5725[1]) shown in the table.

9 Test report

The test report shall show the method used and the result obtained. It shall also mention all operating details not specified in this International Standard, or regarded as optional, as well as any incidents which may have influenced the result.

The test report shall include all the details required for the complete identification of the sample.

Table — Results expressed in micrograms per kilogram

Sample	Maize A	Maize B (maize A diluted to 1/3)
Number of laboratories remaining after elimination of outliers	20	15
Mean	734	219
Standard deviation of repeatability, S_r	78	34
Coefficient of variation of repeatability	11 %	15 %
Repeatability, 2,83 S_r	221	96
Standard deviation of reproducibility S_R	282	125
Coefficient of variation of reproducibility	38 %	57 %
Reproducibility, 2,83 S_R	798	354

1) ISO 5725, *Precision of test methods — Determination of repeatability and reproducibility by inter-laboratory tests.*

Dimensions in millimetres

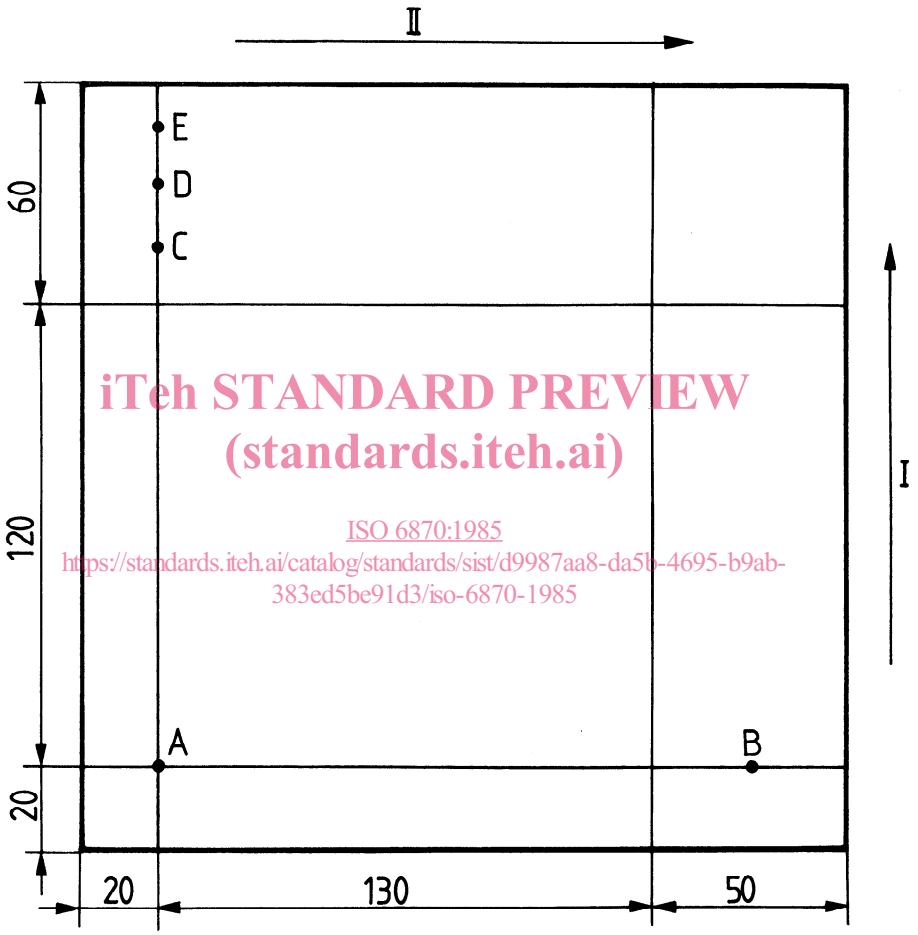

Figure — Application of solutions and development of chromatogram

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