Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

ANNEX V

METHODS OF ANALYSIS TO CONTROL UNDESIRABLE SUBSTANCES IN FEED

A.DETERMINATION OF FREE AND TOTAL GOSSYPOL

1. Purpose and scope

This method makes it possible to determine the levels of free gossypol, total gossypol and chemically related substances in cottonseed, cottonseed meal and cottonseed cake and in compound feed containing these feed materials where more than 20 mg/kg of free gossypol, total gossypol and chemically related substances are present.

2. Principle

The gossypol is extracted in the presence of 3-aminopropan-1-ol, either with a mixture of propan-2-ol and hexane, for the determination of free gossypol, or with dimethylformamide, for the determination of total gossypol. The gossypol is converted by aniline into gossypol-dianiline, the optical density of which is measured at 440 nm.

3. Reagents

3.1. Propan-2-ol-hexane mixture: mix 60 parts by volume of propan-2-ol with 40 parts by volume of *n*-hexane.

3.2. Solvent A: Place in a 1 litre graduated flask approximately 500 ml of propan-2-ol-hexane mixture (3.1), 2 ml of 3-aminopropan-1-ol, 8 ml of glacial acetic acid and 50 ml of water. Make up to volume with the propan-2-ol-hexane mixture (3.1). This reagent is stable for one week.

3.3. Solvent B: Pipette 2 ml of 3-aminopropan-1-ol and 10 ml of glacial acetic acid into a 100 ml graduated flask. Cool to room temperature and make up to volume with N, N-dimethylformamide. This reagent is stable for one week.

3.4. Aniline: *If the optical density in the blank test exceeds 0,022*, distil the aniline over zinc dust, discarding the first and last 10 % fractions of the distillate. Refrigerated and stored in a brown, stoppered glass flask, this reagent will keep for several months.

3.5. Standard gossypol solution A: Place 27,9 mg of gossypol acetate in a 250 ml graduated flask. Dissolve and make up to volume with solvent A (3.2). Pipette 50 ml of this solution into a 250 ml graduated flask and make up to volume with solvent A. The gossypol concentration of this solution is 0,02 mg/ml. Leave to stand for one hour at room temperature before use.

3.6. Standard gossypol solution B: Place 27,9 mg of gossypol acetate in a 50 ml graduated flask, Dissolve and make up to volume with solvent B (3.3). The gossypol concentration of this solution is 0,5 mg/ml.

Standard gossypol solutions A and B will remain stable for 24 hours if protected from the light.

4. Apparatus

4.1. Mixer (tumbler): approximately 35 r.p.m.

4.2. Spectrophotometer.

5. Procedure

5.1. *Test sample*

The amount of test sample used depends on the presumed gossypol content of the sample. It is preferable to work with a small test sample and a relatively large aliquot part of the filtrate, so as to obtain sufficient gossypol for precise photometric measurement to be possible. *For the determination of free gossypol* in cottonseed, cottonseed meal and cottonseed cake, the test sample shall not exceed 1 g; for compound feed, it may be as much as 5 g. A 10 ml aliquot part of filtrate is suitable in most cases; it shall contain 50 to 100 μg of gossypol. *For the determination of total gossypol,* the test sample shall be between 0,5 and 5 g, that a 2 ml aliquot part of filtrate will contain 40 to 200 μg of gossypol.

The analysis shall be carried out at a room temperature of about 20 °C.

5.2. *Determination of free gossypol*

Place the test sample in a ground-necked 250 ml flask, the bottom of the flask having been covered with crushed glass. Using a pipette, add 50 ml of solvent A (3.2), stopper the flask and mix for one hour in the mixer. Filter through a dry filter and collect the filtrate in a small ground-necked flask. During filtration, cover the funnel with a watch glass.

Pipette identical aliquot parts of filtrate containing 50 to 100 μg of gossypol into each of two 25 ml graduated flasks (A and B). If necessary, make up the volume to 10 ml with solvent A (3.2). Then make the contents of flask (A) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the sample solution.

Pipette 10 ml of solvent A (3.2) into each of two other 25 ml graduated flasks (C and D). Make the contents of flask (C) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the blank test solution.

Add 2 ml of aniline (3.4) to each of flasks (D) and (B). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour.

Determine the optical density of the blank test solution (D) by comparison with the reference solution (C), and the optical density of the sample solution (B) by comparison with the reference solution (A), in the spectrophotometer at 440 nm using 1 cm glass cells.

Subtract the optical density of the blank test solution from that of the sample solution (= corrected optical density). From this value calculate the free gossypol content as indicated in 6.

5.3. *Determination of total gossypol*

Place a test sample containing 1 to 5 mg of gossypol in a 50 ml graduated flask and add 10 ml of solvent B (3.3). At the same time, prepare a blank test, placing 10 ml of solvent B (3.3) in another 50 ml graduated flask. Heat the two flasks for 30 minutes over a boiling water bath. Cool to room temperature and make the contents of each flask up to volume with the propan-2-ol-hexane mixture (3.1). Homogenise and leave to settle for 10 to 15 minutes, then filter and collect the filtrates in ground-necked flasks.

Pipette 2 ml of the sample filtrate into each of two 25 ml graduated flasks, and 2 ml of the blank test filtrate into each of two other 25 ml flasks. Make the contents of one flask from each series up to 25 ml with the propan-2-ol-hexane mixture (3.1). These solutions will be used as reference solutions.

Add 2 ml of aniline (3.4) to each of the other two flasks. Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to 25 ml with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour.

Determine the optical density as indicated in 5.2 for free gossypol. From this value calculate the total gossypol content as indicated in 6.

6. Calculation of results

Results may be calculated either from the specific optical density (6.1), or by reference to a calibration curve (6.2).

6.1. *From the specific optical density*

The specific optical densities, under the conditions described, will be the following:

Free gossypol:	$E\frac{1\%}{1\ cm} = 625$
Total gossypol:	$E\frac{1\%}{1\ cm} = 600$

The free or total gossypol content of the sample is calculated by using the following formula:

$$\%\ \text{gossypol:}\frac{E\times 1\ 250}{E^{1\ cm}_{1\ \%}\times p\times a}$$

where:

E = corrected optical density, determined as indicated in 5.2,
p = test sample in g,
a = aliquot part of the filtrate in ml.

6.2. *From a calibration curve*

6.2.1. *Free gossypol*

Prepare 2 series of five 25 ml graduated flasks. Pipette aliquots of 2,0, 4,0, 6,0, 8,0 and 10,0 ml of standard gossypol solution A (3.5) into each series of flasks. Make up the volumes to 10 ml with solvent A (3.2). Complete each series with a 25 ml graduated flask containing only 10 ml of solvent A (3.2) (blank test).

Make the volume of the flasks in the first series (including the flask for the blank test) up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series (including the flask for the blank test). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in μg).

6.2.2. *Total gossypol*

Prepare six 50 ml graduated flasks. In the first flask place 10 ml of solvent B (3.3), and in the others 2,0, 4,0, 6,0, 8,0 and 10,0 ml of standard gossypol solution B (3.6) respectively. Make the contents of each flask up to 10 ml with solvent B (3.3). Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1) and homogenise.

Place 2,0 ml of these solutions in each of two series of six 25 ml graduated flasks. Make the contents of the flasks in the first series up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series. Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenise and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in μg).

6.3. *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed:

— 15 %, in relative value to the higher level, for gossypol contents of less than 500 ppm,

— 75 ppm, in absolute value, for contents of not less than 500 ppm and not more than 750 ppm,

— 10 %, in relative value to the higher value, for contents of more than 750 ppm.

Changes to legislation:
There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, Division A..