METHOD 17.0

Effective 1st January 2018

FREE AND TOTAL GOSSYPOL

1. Scope and Field of Application

This method is for the determination of free gossypol, total gossypol and chemically related substances in cottonseed, cottonseed meal and cotton seed cake, and feeding stuffs containing these substances. The lower limit of determination is 20mg/kg.

2. Principle

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

3. Reagents

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

3.2 Solvent A: place in 1 litre graduated flask about 500ml propan-2-ol/hexane mixture (3.1), 2ml 3-aminopropan-1-ol, 8ml glacial acetic acid and 50ml water. Make up the volume with the propan-2-ol/hexane mixture (3.1). This reagent will remain stable for one week.

3.3 Solvent B: place in a 100ml graduated flask 2ml 3-aminopropan-1-ol and 10ml glacial acetic acid. Cool to room temperature and make up to volume with dimethylformamide. This reagent will remain stable for one week.

3.4 Aniline: if the optical density of the blank test exceeds 0.022, distil the aniline over zinc dust rejecting the first and last 10% fractions of the distillate. This reagent will keep for several months if refrigerated in a stoppered dark glass flask.

3.5 Standard gossypol solution A: place in a 250ml graduated flask 27.9mg gossypol acetate. Dissolve and make up to volume with solvent A (3.2). Pipette 50ml of this solution in a 250ml graduated flask and make up to volume with solvent A. This solution has a gossypol concentration of 0.02mg/ml. Allow to stand for one hour at room temperature before use.

3.6 Standard gossypol solution B: place in a 50ml graduated flask 27.9mg gossypol acetate. Dissolve and make up to volume with solvent B (3.3). This solution has a gossypol concentration of 0.5mg/ml.

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

4. Apparatus

4.1 Rotary shaker; 35-40 revolutions per minute.

4.2 Spectrophotometer.

5. Procedure

5.1 *Sample for analysis*

The weight of sample taken for analysis depends on the supposed level of gossypol in the sample. It is preferable to work on a small sample for analysis together with a relatively large aliquot part of the filtrate, so as to obtain a sufficient quantity of

gossypol in order to be able to carry out a precise photometric measurement.

For the determination of free gossypol, in seeds, flour and cotton seed cake, the sample for analysis must not exceed 1g; for compound feeding stuffs it may be as much as 5g. A 10ml aliquot part of the filtrate is suitable in most cases; it should contain from 50 to 100µg gossypol.

For the determination of total gossypol the sample for analysis may vary from 0.5 to 5g so that a 2ml aliquot part of the filtrate contains 40 to 200µg gossypol. **The analysis must be carried out at a temperature close to 20°C.**

5.2 *Determination of free gossypol*

Place the prepared sample in a 250ml flask with a ground-glass neck, and cover the bottom of the flask with a layer of glass beads of approximately 6mm diameter. Add 50ml solvent A (3.2) stopper the flask and mix for one hour in the mixer (4.1). Filter through a dry filter and collect the filtrate in a small flask with a ground-glass neck. During filtration cover the funnel with a watch glass. Transfer to two 25ml graduated flasks (A and B) identical aliquot parts of the filtrate containing 50 to 100µg gossypol. If necessary make up the volume to 10ml using solvent A (3.2). Then make up to volume the contents of flask (A) with the propan-2-ol/hexane mixture (3.1). This solution is used as a reference solution against which the sample is measured.

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

Add 2ml aniline (3.4) to 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), mix and allow 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 440nm using 1cm 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 amount of free gossypol as indicated in 6.

5.3 *Determination of total gossypol*

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

Transfer 2ml of the sample filtrate to each of two 25ml graduated flasks, and 2ml of the blank test filtrate to two other 25ml flasks. Take one flask from each pair and make up the volumes of each to 25ml with the propan-2-ol/hexane mixture (3.1). These solutions shall be used for reference.

Add 2ml 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 25ml with the propan-2-ol/hexane mixture (3.1), mix and allow to stand for 1 hour.

Determine the optical density as indicated in 5.2. From this value calculate the amount of total gossypol as indicated in 6.

6. Expression of the 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*

In the conditions described, the specific optical densities are as follows:

free gossypol: $E^{1\%}/_{10mm} = 625$

total gossypol: $E^{1\%}/_{10mm} = 600$

The amount of free or total gossypol in the sample is given by the following formula:

$$\text{gossypol \%} = \frac{E \times 1{,}250}{E^{1\%}/_{10mm} \times p \times a}$$

in which:

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 two series of five 25ml graduated flasks. Transfer to each series of flasks respectively 2.0, 4.0, 6.0, 8.0 and 10.0ml aliquots of standard gossypol solution A (3.5). Make up the volumes to 10ml using solvent A (3.2). Complete each series with a blank solution consisting of a 25ml graduated flask containing only 10ml solvent A (3.2).

Make up the volumes of the first series to 25ml (including the blank solution) with the propan-2-ol/hexane mixture (3.1) (reference series).

Add 2ml aniline (3.4) to each flask in the second series (including the blank solution). 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), mix and allow to stand for 1 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. Plot a calibration curve of the optical densities against quantities of gossypol (in µg).

6.2.2 *Total gossypol*

Prepare six 50ml graduated flasks. In the first flask place 10ml solvent B (3.3) and in the

others respectively 2.0, 4.0, 6.0, 8.0 and 10.0ml standard gossypol solution B (3.6). Make up the contents of each flask to 10ml using 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 mix.

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

Add 2ml 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) mix and allow to stand for 1 hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series compared with the corresponding solutions in the reference series. Plot the calibration curve of the optical densities against 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 value, for gossypol contents of less than 500ppm
- 75ppm, in absolute value, for contents of not less than 500ppm and not more than 750ppm
- 10%, in relative value to the higher value, for contents of more than 750ppm.