IS 4876 (1986): Edible Cottonseed Flour (Solvent Extracted)
[FAD 16: Foodgrains, Starches and Ready to Eat Foods]

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Indian Standard

SPECIFICATION FOR EDIBLE COTTONSEED FLOUR (SOLVENT EXTRACTED)

(First Revision)

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Indian Standard

SPECIFICATION FOR EDIBLE COTTONSEED FLOUR (SOLVENT EXTRACTED)

(First Revision)

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AMENDMENT NO. 1 MARCH 2004
TO
IS 4876 : 1986 SPECIFICATION FOR EDIBLE COTTONSEED FLOUR (SOLVENT EXTRACTED)

(First Revision)

[*Page* 6, *Table* 1, *Sl No.* (viii), *col* 2] — Substitute 'Hexane (food grade), ppm, *Max*' *for* 'Residual solvent, mg/kg, *Max*'.

[*Page* 6, *Table* 1, *Sl No.* (viii), *col* 3] — Substitute '10' *for* '170'.

[*Page* 6, *Table* 2, *Sl No.* (xiii), *col* 2] — Substitute 'Salmonella bactena per 25 g' *for* 'Salmonella bactena'.

[*Page* 6, *Table* 1, *Sl No.* (xiii)] — Insert the following at the end of the table:

Sl No.	Characteristic	Requirement	Method of Test, Ref to
(1)	(2)	(3)	(4)
xiv)	Aflatoxin, g/kg, *Max*	30	Annex J of IS 4684:1975*

(FAD 16)

Reprography Unit, BIS, New Delhi, India

Indian Standard

SPECIFICATION FOR EDIBLE COTTONSEED FLOUR (SOLVENT EXTRACTED)

(First Revision)

0. FOREWORD

0.1 This Indian Standard (First Revision) was adopted by the Indian Standards Institution on 21 February 1986, after the draft finalized by the Nutrition Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Protein forms an essential constituent of human diet. Cottonseed oilcakes are now increasingly used for the preparation of edible cottonseed flour and there is considerable scope for commercial production of this product. The flour is rich in protein and lysine and may be used as a protein supplement in human dietaries. It is obtained as a powder which may be used both in blended and processed foods. In view of these possibilities, the Regional Research Laboratory, Hyderabad, carried out pioneering work on the production of edible cottonseed flour and it is now being manufactured in the country on a commercial scale. This standard has been prepared to help in exercising proper quality control of edible cottonseed flour made from solvent extracted cottonseed oilcake.

0.3 This standard was first published in 1968 on the basis of the guidelines recommended by the Protein Advisory Group of WHO/FAO/UNICEF. The research data compiled by the Regional Research Laboratory, Hyderabad, had also been taken into consideration. In addition, assistance had been derived from the work done in USA and in Guatemala.

0.3.1 The question of prescribing free and total gossypol limits had been thoroughly discussed in view of their deleterious effects beyond certain limits, and the committee had adopted these limits recommended by the Protein Advisory Group of FAO WHO/UNICEF.

0.4 In this revision the requirement for acid value of extracted fat has been deleted. The values for free and total gossypol have been revised

to bring them in line with the Protein Advisory Group Guidelines. The sampling clause has also been modified.

0.4.1 A separate Indian Standard (IS : 11581-1986*) has also been brought out on edible cottonseed flour prepared by liquid cyclone process.

0.5 While formulating this standard, due consideration has been given to relevant Rules issued by the Government of India under the Prevention of Food Adulteration Act, 1954 and The Solvent Extracted Oil, Deoiled Meal and Edible Flour (Control) Order, 1967. This standard is, however, subject to the restrictions imposed under these, wherever applicable.

0.6 This standard contains **2.3** which calls for an agreement between the purchaser and the supplier.

0.7 For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS : 2-1960†. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

1. SCOPE

1.1 This standard prescribes the requirements and methods of sampling and test for edible cottonseed flour obtained from cottonseeds by expelling followed by solvent extraction.

2. REQUIREMENTS

2.1 Description — The material shall be obtained by extraction of oil by means of a solvent from oilcake immediately following the single-pressing of the appropriate cottonseeds of good quality, which have been pre-cleaned and are free from infected or otherwise damaged materials and extraneous matter. It shall be in the form of flour of white to pale brownish-yellow colour, uniform in composition and free from insects, rodent hair and excreta, fungal infection, objectionable odour and rancid taste. It shall not contain added flavouring or colouring agents.

NOTE — The appearance, taste and odour shall be determined by organoleptic tests.

*Specification for edible cottonseed flour prepared by liquid cyclone process.

†Rules for rounding off numerical values (*revised*).

2.1.1 Edible cottonseed flour (solvent extracted) shall not contain any other added oilcakes. It shall also be free from harmful foreign oilcakes such as castor and *MAHUA* when tested according to the methods prescribed in **11** and **12** of IS : 7874 (Part 1)-1975*. It shall also be free from *NEEM* cake, *SAL* cake and other foreign materials such as jaggery and molasses.

2.2 The solvent permitted for preparation of edible-grade cottonseed flour shall be food-grade hexane or such other solvents as may be prescribed for food-grade use from time to time

2.3 Particle Size — Unless otherwise specified by the purchaser, the material shall be such that it passes completely through a 150-micron IS Sieve [*see* IS : 460 (Part 1)-1978†].

2.4 The material shall be manufactured, packed, stored and distributed under hygienic conditions (*see* IS : 2491-1972‡).

2.5 The material shall also comply with the requirements given in Table 1.

3. PACKING

3.1 The material shall be packed in sealed metal containers or jute hessian bags with polyethylene lining of 40 to 75 microns

4. MARKING

4.1 The following particulars shall be marked legibly or labelled on each container:

a) Name of the material;

b) Name and address of the manufacturer;

c) Batch or code number;

d) Net mass;

e) Date of manufacture; and

f) Any other requirements under the Standards of Weights and Measures (Packaged Commodities) Rules, 1977

*Method of tests for animal feeds and feeding stuffs: Part 1 General methods

†Specification for test sieves: Part 1 Wirecloth test sieves (*second revision*)

‡Code for hygienic conditions for food processing units (*first revision*)

4.1.1 Each container may also be marked with the **BIS Certification Marking**

4.2 The use of the Standard Mark is governed by the provisions of *Bureau of Indian Standards Act*, 1986 and the Rules and Regulations made thereunder. The details of conditions under which the licence for the use of Standard Mark may be granted to manfucaturers or producers may be obtained from the Bureau of Indian Standards.

TABLE 1 REQUIREMENTS FOR EDIBLE COTTONSEED FLOUR (SOLVENT EXTRACTED)

(*Clause* 2.5)

Sl No.	Characteristic	Requirement	Method of Test, Ref to
(1)	(2)	(3)	(4)
i)	Moisture, percent by mass *Max*	8·0	Appendix B of IS : 4684-1975*
ii)	Crude protein (N × 6·25) on dry basis, percent by mass, *Min*	47	Appendix C of IS : 4684-1975*
iii)	Available lysine, g per 100 g of crude protein, *Min*	3·6	Appendix A of this standard
iv)	Total ash (on dry basis), percent by mass, *Max*	5·0	Appendix D of IS : 4684-1975*
v)	Acid-insoluble ash (on dry basis), percent by mass, *Max*	0·35	Appendix E of IS : 4684-1975*
vi)	Fat (on dry basis), percent by mass, *Max*	1·5	Appendix F of IS : 4684-1975*
vii)	Crude fibre (on dry basis), percent by mass, *Max*	5·0	Appendix H of IS : 4684-1975*
viii)	Residual solvent mg/kg, *Max*	150	IS : 11674-1986†
ix)	Free gossypol, percent by mass, *Max*	0·06	Appendix B of this standard
x)	Total gossypol, percent by mass, *Max*	1·2	Appendix C of this standard
xi)	Total bacterial count per g, *Max*	50 000	IS : 5402-1969‡
xii)	Coliform bacteria per g, *Max*	10	IS : 5401-1969§
xiii)	Salmonella bacteria	Nil	IS : 5887 (Part 3) 1976‖

*Specification for edible groundnut flour (expeller-pressed) (*first revision*).

†Method for determination of residual solvent from flash point determination by modified Pensky-Martens closed tester.

‡Method for standard plate count of bacteria in foodstuffs.

§Method for detection and estimation of coliform bacteria in foodstuffs.

‖Method for detection of bacteria responsible for food poisoning: Part 3 Isolation and identification of salmonella and shigella (*first revision*).

5. SAMPLING

5.1 The method of drawing representative samples of the material and the criteria for conformity shall be as prescribed in IS : 5315-1978*.

5.2 The composite sample of at least 2 kg as given in **7.1** of IS : 5315-1978* shall be divided into three test samples, three samples for moisture determination and three samples for testing microbiological requirements. Each test sample shall comprise about 500 g, each sample for moisture determination about 100 g and each sample for testing microbiological requirements about 50 g.

5.3 The lot shall be declared as conforming to the requirements of the specification if all the test results on the test sample, sample for moisture determination and sample for microbiological requirements are found to be conforming to the relevant specification requirements.

6. TESTS

6.1 Tests shall be carried out in accordance with **2.1, 2.3** and appropriate appendices and standards specified in col 4 of Table 1.

6.2 Quality of Reagents — Unless specified otherwise, pure chemicals shall be employed in tests and distilled water (*see* IS : 1070-1977†) shall be used where the use of water as a reagent is intended.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

APPENDIX A

[*Table* 1, *Item* (iii)]

DETERMINATION OF AVAILABLE LYSINE

A-1. PRINCIPLE OF THE METHOD

A-1.1 The method depends upon the reaction of fluorodinitrobenzene (FDNB) with the—NH_2 groups of the lysine units in intact food proteins and the colorimetric estimation of the DNP-lysine obtained by a subsequent acid hydrolysis.

*Method of sampling for milled cereals and pulses products (*first revision*).

†Specification for water for general laboratory use (*second revision*).

A-2. APPARATUS

A-2.1 Photo-Electric Absorptiometer — set at 435 mμ wavelength or fitted with a blue filter having a maximum transmission close to 435 mμ.

A-2.2 Round-Bottom Flask Fitted with Reflux Condenser — 100-ml capacity.

A-2.3 Water-Bath – maintained at 100°C.

A-2.4 Volumetric Flask – 100-ml and 200-ml capacity.

A-2.5 Burette — 25-ml capacity, graduated to 0·05 ml.

A-2.6 Conical Flask

A-2.7 Pasteur Pipette or Equivalent Pipette

A-3. REAGENTS

A-3.1 Sodium Bicarbonate Solution — 8 percent (*m/v*).

A-3.2 Fluorodinitrobenzene (FDNB) Solution — Prepare fresh daily and for every sample use approximately 0·3 ml of fluorodinitrobenzene (pipetted after warming the bottle) dissolved in approximately 12 ml of ethanol.

A-3.3 Hydrochloric Acid — 8·1 N and 1 N.

A-3.4 Sodium Hydroxide Solution — 2 N.

A-3.5 Phenolphthalein Solution — 1 percent in 70 percent ethyl alcohol.

A-3.6 Buffer Solution — 19 parts of 8 percent sodium bicarbonate + 1 part of 8 percent sodium carbonate, adjusted suitably to 8·5 *p*H with the addition of a little acid or alkali.

A-3.7 Methyl Chloroformate

A-3.8 Di-ethyl Ether — Free from peroxide.

A-3.9 Standard DNP-Lysine Hydrochloride Monohydrate — Dissolve 24 mg of DNP-lysine hydrochloride monohydrate weighed accurately in 500 ml of 1 N hydrochloric acid solution. Two millilitres of this solution will contain 39·85 micrograms of available lysine.

A-4. PROCEDURE

A-4.0 The procedure shall be followed in duplicate and away from direct or strongly reflected sunlight.

NOTE — Pipetting of ether and fluorodinitrobenzene by mouth as routine is not recommended. The automatic sucker, such as pro-pipette has proved a suitable alternative; it may be transferred from one pipette to another.

A-4.1 Weigh accurately about 0·75 g of the material into a 100-ml round-bottom flask and shake gently with 8 ml of sodium bicarbonate solution for 10 minutes. Add fluorodinitrobenzene solution (**A-3.2**). Shake gently but continuously for 2 hours. Evaporate off the ethanol on a boiling water-bath. Add 24 ml of 8 1 N hydrochloric acid and reflux gently for 16 hours. Some yellow crystals may appear on the condenser. These are not DNP-lysine and need not be recovered. Cool suitably for easy filtration. Filter the contents with water washings and make the volume of the filtrate to 200 ml.

A-4.2 Transfer accurately 30 ml of the aliquot of the filtrate to 50-ml flask and make up to volume.

A-4.3 Transfer 2 ml of the diluted filtrate to a glass stoppered tube. Extract with 5 ml portions of di-ethyl ether each time until the final ether fraction is colourless, sucking off the ether layer with the pasteur pipette. Remove dissolved ether by standing the tube in hot water.

A-4.4 Make up the volume to 10 ml with 1 N hydrochloric acid and read the extinction in 1-cm cell with the photo-electric absorptiometer at 435 mμ.

A-4.5 For the blank, take a second aliquot of 2 ml diluted filtrate (*see* **A-4.2**) in a tube and extract with ether as in **A-4.3**, also a third aliquot of 2 ml in a small conical flask for a dummy titration. Dilute and add phenolphthalein as indicator and titrate with 2 N sodium hydroxide taken in a burette. Note the volume of the sodium hydroxide solution needed and discard the flask. Add the same volume to the second tube and then 2 ml of buffer solution of *p*H 8·5 (continue without pause at this stage as DNP-compounds are less stable at this *p*H).

A-4.6 Add 0·05 ml of methyl chloroformate to the second tube. Shake and wait for 5 to 10 minutes. Then add 0·75 ml of concentrated hydrochloric acid cautiously to avoid excessive effervescence. Extract again with two 5 ml portions of di-ethyl ether, evaporate off any residual ether and make up to 10 ml with water. Read the extinction of the tube.

A-4.7 Carry out the experiment from **A-4.3** to **A-4.6** with the standard DNP-lysine solution.

A-5. CALCULATION

A-5.1 Available lysine, grams per 100 g of crude protein

$$= \frac{X_1 - X_2}{S_1 - S_2} \times \frac{39{\cdot}85}{1\,000\,000} \times \frac{50}{2} \times \frac{200}{30} \quad \frac{100}{W\,(100 - M)} \times \frac{100}{P}$$

where

X_1 = absorptiometer reading with unknown;

X_2 = blank absorptiometer reading with unknown;

S_1 = absorptiometer reading with standard;

S_2 = blank absorptiometer reading with standard;

W = mass in g of the material taken;

M = moisture, percent by mass of the material; and

P = crude protein (on dry basis), percent by mass of the material.

APPENDIX B

[*Table* 1, *Item* (ix)]

DETERMINATION OF FREE GOSSYPOL

B-1. PRINCIPLE

B-1.1 Free gossypol (that is gossypol not bound by protein) is extracted by 70 percent aqueous acetone and determined by the colour developed with *p*-anisidine. The difference in absorbance read at 447 mμ before and after reaction with *p*-anisidine, is proportional to the concentration of free gossypol.

B-2. APPARATUS

B-2.1 Mechanical Wrist Action Shaker

B-2.2 Photo-Electric Colorimeter — With a filter having a maximum transmittance between 440 mμ to 460 mμ or a spectrophotometer isolating a band at 447 mμ.

B-2.3 Grinding Mill — a laboratory hammer mill capable of grinding to 1 to 2 mm practicle size.

B-2.4 Solid Glass Beads — approximately 6 mm diameter.

B-2.5 Conical Flask — 250-ml capacity, glass stoppered.

B-2.6 Pipettes, Volumetric — 1-, 2-, 3-, 4-, 5-, 10-, 15-, 20-, 25-, 30-, 35-, 40- and 50-ml.

B-2.7 Flasks, Volumetric — 25-ml and 50-ml capacity.

B-2.8 Insulated Water-Bath — Thermostatically controlled to ±1°C and capable of keeping the water at a gentle boil. For free gossypol determination the water-bath is adjusted to 60 C. Water-bath should be equipped with clamps to securely hold the volumetric flask immersed in water.

B-3. REAGENTS

B-3.1 70 Percent Aqueous Acetone (v v) — 700 ml of redistilled acetone is diluted with 300 ml of distilled water.

B-3.2 95 Percent Ethyl Alcohol, Aldehyde-Free – Reflux 95 percent ethyl alcohol over potassium hydroxide and aluminium (10 g of potassium hydroxide plus 5 g of aluminium granules or dust per litre) for one hour and distil.

B-3.3 Glacial Acetic Acid

B-3.4 *p*-Anisidine — Dissolve about 10 g of technical grade *p*-anisidine in one litre of hot water at approximately 75°C. Add about 2 g of sodium sulphite and 20 g of active carbon or decolourizing carbon. Stir for about 5 minutes. Filter the mixture through a double layer of medium retentivity filter (Whatman No. 1 may be used) on a Buchner funnel under gentle suction. Re-filter through the same paper if the filtrate is turbid. Leave the water-white filtrate in a refrigerator at 0 to 5 C overnight for crystallization. Filter the crystals of *p*-anisidine over a Buchner, and wash them with a minimum amount of cold water. Dry the crystals in a vacuum desiccator over phosphoric pentoxide or concentrated sulphuric acid overnight. Preserve in a brown bottle. Recrystallize the *p*-anisidine if the reagent blank gives an absorbance of more than 0·022.

A-3.5 *p*-Anisidine-Acetic Acid-Ethyl Alcohol Reagent – Dissolve 0·500 g of recrystallized *p*-anisidine in purified ethyl alcohol. Add one millilitre of glacial acetic acid and make up to 50 ml volume with the

ethyl alcohol. Use 3 ml of this reagent for each determination. Since *p*-anisidine is not stable in solution, this reagent shall be made fresh each day.

B-3.6 Ethyl Alcohol-Acetic Acid Reagent — Dilute 1 ml of glacial acetic acid to 50 ml volume with purified ethyl alcohol. Use 3 ml of this reagent for each gossypol blank.

B-3.7 Standard Gossypol Solutions — Weigh accurately 25 mg of pure gossypol, or 27·9 mg of pure gossypol acetate in 70 percent aqueous acetone and make up to 200 ml with 70 percent aqueous acetone. This stock solution contains 0·125 mg of gossypol per millilitre. Dilute 2, 5, 10, 15, 20, 25, 30, 35 and 40 ml of this stock solution to 50 ml with 70 percent aqueous acetone. Two millilitres of each solution are used for determination of standard curve. Pure gossypol acetone is more stable than pure gossypol.

B-4. PROCEDURE

B-4.1 Grind about 50 g of the material to pass through a 1·00 mm IS Sieve [*see* IS : 460 (Part 1)-1978*] in hammer mill. Weigh sufficient quantity of the material to contain about 25 mg of free gossypol into a glass-stoppered 250-ml conical flask. This shall require 1·0 to 1·5 g for screw-pressed cottonseed cakes containing about 0·2 percent or less of free gossypol. Cover the bottom of the flask with the glass beads, add 50 ml of 70 percent aqueous acetone by pipette. Stopper the flask and shake on a mechanical shaker for one hour at room temperature. The rate of shaking should be so adjusted that the material collection around the top of the flask is constantly washed back into solution.

B-4.2 Filter through a dry Whatman No. 1 filter paper into a small glass-stoppered flask. Discard the first portion (about 10 ml) of the filtrate. Cover the funnel with a watch-glass to reduce evaporation. If necessary, gossypol may be determined in the filtrate the next day since gossypol is stable in aqueous acetone.

B-4.3 Pipette duplicate 2-ml aliquots of the filtrate into 25-ml volumetric flasks.

B-4.4 To one of the aliquots add 3 ml of ethyl alcohol acetic acid reagent and make up to volume with ethyl alcohol This is the gossypol blank.

B-4.5 To the second 2-ml aliquot add 3 ml of the *p*-anisidine-acetic acid-ethyl alcohol reagent, and place in a water-bath (with the flask loosely stoppered) at 60°C for 30 minutes.

*Specification for test sieves : Part 1 Wire cloth test sieves (*second revision*).

B-4.6 Simultaneously, to 2 ml of 70 percent aqueous acetone add 3 ml of *p*-anisidine-acetic acid-ethyl alcohol reagent and place in the water bath as in **B-4.5**. This is the reagent blank.

B-4.7 Remove the flasks from the water-bath, cool to room temperature and make up to volume with 95 percent ethyl alcohol.

B-4.8 Prepare a solvent blank consisting of 2 ml of 70 percent aqueous acetone and 3 ml of acetic acid-ethyl alcohol reagent, made up to 25 ml volume with ethyl alcohol.

B-4.9 Determine the absorbance optical density) of the reacted sample solution setting the spectrophotometer to zero absorbance (100 percent transmittance) with the reagent blank.

B-4.9.1 Determine the absorbance of the gossypol blank with the instrument set to zero with the solvent blank.

B-4.9.2 All absorbances shall be read at 417 mμ.

B-5. PREPARATION OF STANDARD CURVE

B-5.1 Carry out the operations from **B-4.3** to **B-4.9.1** using 2-ml aliquots of each of the standard gossypol solutions (*see* **B-3.7**). Plot the values for gossypol concentration expressed as milligrams of gossypol in 25 ml volume against absorbance. For each gossypol concentration determine the factor, milligrams of gossypol in 25 ml volume against corrected absorbance and average the factors thus obtained for all the gossypol standards. When gossypol acetate is used, mg gossypol acetate × 0·896 2 = mg gossypol.

B-6. CALCULATION

B-6.1 Corrected absorbance = $Y_1 - Y_2$

where

Y_1 = absorbance of the reacted sample solution; and

Y_2 = absorbance of the gossypol blank.

B-6.2 Milligrams gossypol in sample aliquot = corrected absorbance × Factor

$$\text{Free gossypol, percent by mass} = \frac{5 \times \text{mg free gossypol in sample aliquot}}{(\text{Mass of sample in g}) \quad (\text{Volume of sample} / \text{aliquot taken})}$$

NOTE 1 — The choice of sample aliquot (*see* B-4.3) depends upon the free gossypol content of the meal and the absorbance values obtained. For cottonseed products in the range of 0·01 to 0·05 percent free gossypol, a 5 ml aliquot is usually optimum. For products containing more free gossypol, a 2-ml aliquot is sufficient.

NOTE 2 — The reading of reagent blank against solvent blank cancels out any interfering colour developed by reaction of acetone with *p*-anisidine. Usually, the reagent blank has about 97 to 98 percent transmittance when measured against solvent alone. If the reagent blank shows less than 95 percent transmittance or an absorbance of more than 0·022, the *p*-anisidine shall be recrystallized again. The gossypol blanks shall read against the solvent and serve to correct for the extraneous colour which is present in cottonseed extracts.

NOTE 3 — For concentrations ranging from 0 to 0·2 mg of gossypol in 25 ml volume, a straight line is obtained on a spectrophotometer. On simpler photoelectric colorimeters with filters, the straight line relationship is usually found for concentrations of gossypol ranging from 0 to 0·10 mg in 25 ml volume.

NOTE 4 — Where 95 percent ethyl alcohol is not available, 80 percent *iso*-propyl alcohol may be substituted for it throughout the procedure.

NOTE 5 — For plant control operations, where rapid analysis is required, the extraction may be done in a Waring Blender using 100 ml of 70 percent aqueous acetone and blending the sample for 5 minutes. The extract is then filtered as usual and procedure thereafter followed as given above.

APPENDIX C

[*Table* 1, *Item* (x)]

DETERMINATION OF TOTAL GOSSYPOL

C-1. PRINCIPLE

C-1.1 The term 'total gossypol' designates 'free gossypol', 'bound gossypol' and closely related pigments which after hydrolysis and reaction with an organic amine (*p*-anisidine or aniline) give a product identical spectrophotometrically with that obtained from pure gossypol and the same reagent. In this method, total gossypol is completely removed from meal in a 30 minute extraction during which gossypol is complexed with neutralized 3-amino-1-propanol in dimethyl formamide. The difference in absorption of aliquot portions of the extract before and after reaction with aniline serves as a measure of the total gossypol content, and allows proper correction for the background absorption of extracts.

C-2. APPARATUS

C-2.1 Photoelectric Colorimeter — With a filter having a maximum transmittance in the vicinity of 440 mμ or a spectrophotometer isolating a band at 440 mμ.

C-2.2 Grinding Mill — a laboratory hammer mill capable of grinding to 1-2 mm particle size.

C-2.3 Pipettes, Volumetric — 1-, 2-, 4-, 6-, 8- and 10-ml.

C-2.4 Flasks, Volumetric — 25-, 50- and 100-ml capacity.

C-2.5 Insulated Water-bath — thermostatically controlled to ± 1°C, and capable of keeping the water at a gentle boil. The water-bath should be equipped with clamps to securely hold the volumetric flasks immersed in water.

C-3. REAGENTS

C-3.1 *iso*-propyl Alcohol-Hexane Mixture — Mix 60 volume of reagent grade *iso*-propyl alcohol and 40 volumes of commercial hexane.

C-3.2 Complexing Reagents — Pipette 2 ml of 3-amino-1-propanol and 10 ml of glacial acetic acid into a 100-ml volumetric flask. Cool to room temperature; and dilute to volume with dimethyl formamide (N-N-dimethyl formamide, redistilled between 152 to 153°C). This reagent is stable for one week after preparation.

C-3.3 Aniline – redistilled over zinc dust. Store in a refrigerator and redistil when the absorbance of the reagent blank exceeds 0 022.

C-3.4 Standard Gossypol Solutions — Weigh accurately 25 mg of pure gossypol or 27·9 mg of pure gossypol-acetate, dissolve in and make up to 50 ml volume with the complexing reagent. If exactly 25 mg of pure gossypol are used, the solution shall contain 0·5 mg/ml.

C-3.4.1 *Preparation of Calibration Curve* — Pipette 2, 4, 6, 8 and 10 ml of standard gossypol solution into 50-ml volumetric flasks. To each standard solution add sufficient complexing reagent to make up the total volume to 10 ml. Use 10 ml of the complexing reagent as a blank. Heat the flask containing the standards and the blank in a boiling water-bath (100°C) for 30 minutes, cool and dilute to volume with the *iso*-propyl alcohol-hexane mixture. Pipette duplicate, 2-ml aliquots of each diluted standard and of the blank into 25-ml volumetric flasks. Dilute one set of aliquots to volume with the *iso*-propanol-hexane mixture and reserve as reference solutions. To the other set of aliquots, add 2 ml of aniline, heat in a boiling water-bath for 30 minutes, cool to room temperature and dilute to volume with *iso*-propyl alcohol-hexane mixture. Allow the flask to stand at room temperature for one hour after dilution and mixing. With a spectrophotometer, determine the absorbance of the reagent blank at 440 mμ using the diluted blank aliquot without aniline as a reference solution. Determine the absorbance of each gossypol standard reacted

with aniline, using the appropriate diluted standard as a reference solution. Substract the absorbance of the reagent blank from each standard to obtain the corrected absorbance. Calculate the calibration factor by dividing the number of milligrams of gossypol in the 2-ml aliquot of each standard by the appropriate corrected absorbance. Average the factor for all the gossypol standards. When a photoelectric colorimeter is used, the factors shall probably vary with each concentration of gossypol in which case a calibration curve shall be plotted and used.

C-4. PROCEDURE

C-4.1 Grind about 50 g of the material to pass through a 1·00-mm IS Sieve [*see* IS : 460 (Part 1)-1978*] in a hammer mill. Take 0·20 g of the material in a 50-ml volumetric flask. Add 10 ml of the complexing reagent.

C-4.2 Use 10 ml of the complexing reagent as the reagent blank.

C-4.3 Heat both the sample and the blank in a boiling water-bath for 30 minutes. Cool to room temperature. Dilute to volume with *iso*-propyl alcohol-hexane mixture and mix. Filter through Whatman No. 1 filter paper and collect the filtrate in a small glass-stoppered flask.

C-4.4 Pipette duplicate 2-ml aliquots of the filtered extract and of the reagent blank into 25-ml volumetric flasks.

C-4.5 Dilute one of the aliquots to volume with the *iso*-propyl alcohol-hexane mixture and reserve as reference solution.

C-4.6 To the other aliquot, add 2 ml of aniline, develop the colour and determine the corrected absorbance at 440 mμ as outlined in **C-3.4.1**.

C-4.7 Determine the mg gossypol in the sample aliquot by means of the calibration curve on the calibration factor.

C-4.8 Total gossypol, percent by mass $= \dfrac{5 \times \text{mg gossypol in sample aliquot}}{(\text{Mass of sample in g}) \times \text{Volume of aliquot (used for analysis)}}$

NOTE — 3-amino-1-propanol may be redistilled, if coloured. Its boiling point is 188°C and it may be conveniently distilled under water pump vacuum.

*Specification for test sieves: Part 1 Wire cloth test sieves (*first revision*).

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