

**DETERMINATION OF
TOTAL GOSSYPOL IN COTTONSEEDS AND
COTTONSEED PRESS CAKES**

A Thesis

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the School of Graduate Studies
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of the Requirements for the Degree
Master of Science in Chemistry**

**by
Atnafseged Admasu
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D E D I C A T I O N

To my father,

Admasu Haile

and to my brother,

Wondwosen Admasu

AawwiTip

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I wish to express my real appreciation to my research advisor Dr. P.B. Oweravanshi for stimulating response and enthusiasm during the development of this research. Special appreciation is also given to Dr. Ibrahim Pesrat who has helped in identifying the research topic and advising in the first part of this project. I wish to express my real appreciation of the indispensable help received from many of my colleagues at the Ethiopian Nutrition Institute, among them W/t Meaza Girina, Ato Keihessa Urga, Ato Melaka Tneta Rto Elias Seyoum W/t Mscut Asfaw. Acknowledgment is made to all ministry staff members and graduate students for their numerous helpful suggestions. The drawings of the figures are the product of the patience and skill of V/o Feseixare Nolcle Giorgio of the Ethiopian Nutrition Institute. A very special thanks is given to my sister W/o Fimebet Asmasu who has done the typing and responded my invitation to give a critique of my work.

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ABSTRACT

DETERMINATION OF TOTAL GOSSYPOL IN COTTON SEEDS

AND COTTON SEED PRESS CAKES

by

Atnafseged Admasu

Advisor: Dr. B. S. Chandravanshi

The reaction of gossypol with 3-amino-1-propanol and iron (III) in acidic medium produces a green colored complex soluble in isopropyl alcohol-hexane mixture (3:2), having a characteristic absorption maximum at 610 nm. The colored reaction product is sufficiently stable and obeys Beer's law in the concentration range 5 - 84 ppm of gossypol. The effective molar absorptivity in terms of gossypol is found to be $64 \times 10^3 \text{ L. mole}^{-1} \text{ cm}^{-1}$ with a photometric sensitivity of 0.08 µg of gossypol per an. On the basis of sensitive color reaction, a new method has been developed for the determination of gossypol. The effect of several experimental variables on the determination of gossypol have been studied. The stoichiometric composition of the green colored gossypol-3-amino-1-propanol-iron (III) complex has been determined and was found to be 1:2 (gossypol to iron).

The precision of the method has been evaluated in terms of standard deviation and the relative standard deviation was found to be $\pm 0.71\%$.

The validity of the method was established by the recovery of standard gossypol added to the cottonseed samples,

The proposed method has been applied for analysis of total gossypol in several varieties of cottonseed grown under different conditions in Ethiopia and the cottonseed press cakes available in the market. The gossypol content of the samples were determined after extraction of total gossypol from the cottonseed and cottonseed press cakes by 30% n-propanol in dimethylformamide. The results obtained by the proposed method have been compared with the results obtained by standard A.O.A.C. method. The two results were found to be almost identical.

1. xtfnmxsra?

Nowadays, the major quantity of edible oil available in Ethiopia is obtained from the cottonseed. In 1980-1981, about 50% of the 10,226,748 kgs. of total oil production comes from the processing of cottonseed. Every year, all the oil mills under the Ethiopian Food Corporation process about 45,000,000 kgs of cottonseed and from those about 5,400,000 kgs. of oil and 22,000,000 kgs. of press cakes are produced.¹

The cottonseed cakes which are produced following the removal of the edible oils are exports to foreign countries at cheap prices. These oil residues are believed to consist of 4 to 12% oil and 18 to 42% protein.² It is estimated that the cottonseed flour (oil residue) potentially available can satisfy the present serious deficiencies in protein in diet-deficient areas of our country, and the protein is of high nutritional quality.

Processed cottonseed protein products in the form of defatted flours with 45 to 50% protein have been produced commercially for food use in several countries for many years. These products have entirely been suitable as protein supplements in cereal food mixtures (maize, wheat, rice, etc.).³ For example, the corn-based product "Incapardma" has been marketed in the Central America since the 1950's.⁵⁻⁸

The utilisation in the human nutrition of the high protein products from cottonseed meal is inhibited by the presence of the phenolic compound, gossypol. This undesirable constituent is toxic to different animal species.⁹⁻¹¹ Therefore, the principal quality deficiencies of cottonseed products (oils and meals) are due to their contents of gossypol pigments. So far at least, no different natural toxicant species has been identified, and gossypol pigments appear to be the significant toxicant of cottonseed meals. Most cottonseed meals are not of uniform quality and there is a wide variability in the gossypol contents of cottonseed meals.¹² For any cottonseed protein product intended for human use, the Protein Advisory Group (PAG) of the United Nations had set limits of 0.06% of free gossypol and 1.2% total gossypol for human consumption in their programs.¹³

Gossypol content in cottonseed is affected by genetic factors as well as by environmental conditions during seed development. Cottonseed grown in Fea Island contained more gossypol than that grown in Egypt¹⁴ for the same species. Frampton et al¹⁵ reported the total gossypol contents of seed from several species of *Gossypium* to range from 0.13 to 6.64%. On the basis of a study of eight varieties of seeds grown at thirteen different locations for three successive years, Pons et al¹⁶ reported that the gossypol contents of the moisture-free kernels ranged from 0.39 to 1.70% and that the variety of seed and environment influenced the content of pigments. Glanded cottonseed grown in Giza was found to contain 2.1% gossypol pigments.¹⁷

The initial content of gossypol pigments of the seed, the conditions selected for the preparation of the seed prior to oil extraction, and the conditions used for extraction, all determine the gossypol pigments in the finished meals. The cottonseed meals produced by any of the commonly used methods of processing are not uniform in composition. The free gossypol contents of typical commercial meal products prepared in the following ways are;⁹

	Free gossypol (%)
Serov; pressing	0.02 - 0.05
Pressing followed by solvent extraction	0.02 - 0.02
Direct solvent extraction	0.10 - 0.50
Hydraulic passing	0.04 - 0.10

The total gossypol content of the meal obtained depends primarily upon the seed used and the conditions used in the preparation of the meals prior to extraction of oil and is in the range 0.5 to 1.2%.⁹ However, processing conditions influence the content of free gossypol in cottonseed meal to a greater extent than they influence the content of total gossypol.

Cottonseed oil is the most valuable economic product of the cottonseed. Highly refined oils with light yellow color are completely free of gossypol.

pigments. However, edible cottonseed oils, mostly available in our country, are deeply colored, ranging from tawny-yellow to reddish-brown in appearance. Gossypol pigments are believed to be the main causes of the dark color. The content of gossypol pigments of crude oils vary from 0.10 to 0.75% depending on the mode of processing;⁹

Present commercial processes for cottonseed meal production all involve the use of heat and other processes to remove, destroy, or bind the gossypol as much as possible (80 - 90%). The major amounts of free gossypol are converted into bound gossypol during the processing of cottonseeds. Some workers tried to reduce the free gossypol by use of different practical methods of extraction. Thus Canella et al.¹⁸ reported a low gossypol cottonseed concentrate when cottonseed meal was extracted with butanol - KOH solution. Using 20-30% of ethyl alcohol in hexane, Liu et al.¹⁹ reduced the free gossypol and total gossypol in cottonseed meal to about 0.013 - 0.04% and 0.32 - 0.55%, respectively.

Commercial development of glandless (without gossypol pigments) cottonseed has been the objective of many plant breeders and such development is the hope of many processors and users of cottonseed meals and oils as a means of overcoming the manifold difficulties resulting from the presence of the gossypol pigments. The first glandless strain derived from a cross between koala

and Hopi Moencopi, a noncultivated primitive cotton from Arizona, had glandless seed but it also had small bolls, low lint yield, inferior fiber properties, late maturity and other undesirable characteristics.²⁰ Although, gossypol free cottonseed appears to be an attainable goal, until such time as the commercial cotton crops in the world market are derived from glandless seeds, the cottonseed processing industry will continue to face the problem of dealing with the gossypol pigments.

2. GENERAL CHARACTERISTIC AND OCCURRENCE

2.1 Occurrence in Cottonseed.

All the deeply coloured gossypol pigments of the seed are concentrated in the pigment glands, sometimes also referred to as gum or resin glands. The pigment glands are spherical or ovoid bodies, measuring from 0.1 to 0.4 mm on the long axis and the average size may differ from seed to seed. The pigment glands comprise from 2.4 to 4.5%^{9,21} of the weight of the kernel. The behaviour of the pigment gland wall during commercial cottonseed processing greatly influences the distribution of gossypol pigments in the final cottonseed meals and oils produced.

2.2 Physiological Effects.

One of the basis for interest in gossypol is the physiological activities of the pigment. Currently, there is much interest in the physiological effects and metabolic fate of gossypol in human, especially because of the substantial contribution cottonseed meal could make in alleviating protein shortages in many of the developing countries.

It is of interest that in 1947, following a presentation
22 of two papers, it was stated that gossypol was an appetite
depressant and could cause intestinal irritations. This prompted
investigation of the effect of daily doses of purified gossypol on
the body weight and food consumption of dogs and, following the sudden
and unexpected death of several dogs which had received relatively
23 low levels of purified gossypol, a warning note was published.
Nevertheless, there are no recorded instances in the technical literature
of gossypol toxicity in humans who have consumed gossypol-containing
products, and on the other hand there are various reports of absence of
deleterious effects when cottonseed products containing relatively
low levels of gossypol are ingested in moderate amounts.

24 Adamova and Ichedeleva reported that daily ingestion for 4.5
months of 60 gm cottonseed cake containing 0.11 to 0.20% free gossypol
produced no harmful effects. The acute oral toxicity of gossypol is
not high for most animals. On a single-dose basis the oral LD
50 (median lethal dose) to rats is about 2.46 - 3.34 g/kg (averaging
2.62 g/kg) when administered in water, it is about 10% more toxic
25 when administered in oil.

The reaction of native gossypol from the seed glands with other
seed components in the oil extraction process is very important to
the practical use of the meal in animal feedings. The free gossypol

reacts with, among other things, available amino acid and in this state is much less physiologically active in the animal gut.²⁶ The gossypol that is thus reacted is called "bound gossypol" and that gossypol that is still not bound to protein by the heat and moisture of processing is designated as "free gossypol". Thus the main concern of the nutritionists is the amount of free, or unreacted, gossypol rather than the total amount of gossypol that is consumed by an animal.

The mechanism by which gossypol causes tissue damage is not yet known. Cardiac irregularity is the most common toxic effect of gossypol, and death is generally ascribed to circulatory failure.⁹ Common symptoms,⁹ with cumulative gossypol or cottonseed meal toxicity are loss of appetite; weight loss; diarrhea; hair discolouration; lowering of hemoglobin, red cell count, and serum protein; degenerative changes in liver and spleen; hemorrhages of liver, small intestine, and stomach; and yolk discoloration and decreased hatchability of eggs.

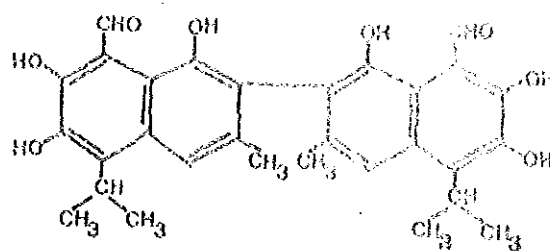
Gossypol forms stable, equimolar chelates of low water solubility with many metallic cations, apparently by linking through the carbonyl and ortho-hydroxyl groups.²⁸

3. CHEMISTRY OF GOSSYPOL

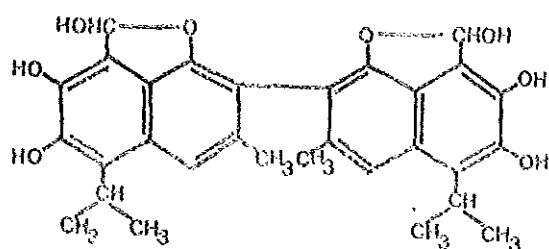
3.1. Structure of Gossypol.

Gossypol, a naturally occurring yellow pigment, has a molecular formula of $C_{30}H_{30}O_8$, and a molecular weight of 518.5. Named by Marchlewski in 1899, gossypol is 1,1', 6,6', 7,7' - hexahydroxy - 5,5' - diisopropyl - 3,3' - dimethyl - [2,2' - binaphthalene] - 8,8' - dicarboxaldehyde³². The structures of tautomeric forms of gossypol derived by Adams et al³³ on the basis of classical studies of the reaction, properties, and degradation products are shown in Fig. 1.

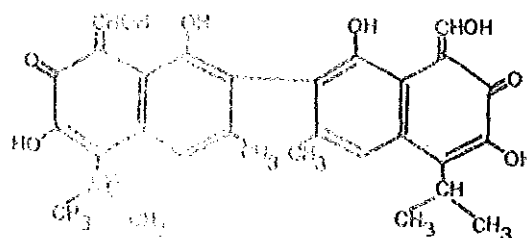
The postulation of three tautomeric structures is necessary to explain many of the reactions of gossypol. Of the three tautomeric modifications of gossypol shown in Fig. 1, structure 1a represents the hydroxy aldehyde tautomer, 1b the lactol tautomer, and 1c the cyclic carbonyl tautomeric form.



(a)



(b)



(c)

FIGURE 1: Structure of Gossypol

- a) hydroxy aldehyde tautomer
- b) lactol tautomer
- c) cyclic carbonyl tautomer

3.2 Reaction of Gossypol

Postulation of the three tautomeric forms of gossypol shown in Fig. 1 was necessary to account for the numerous reaction products encountered in the establishment of the structure of gossypol and their characteristics. Thus, the hydroxy aldehyde tautomer (1a) is responsible for most of the normal aldehyde reactions of gossypol, and this is the predominant form in several organic solvents;³⁴ the hexamethyl ether was formulated from the lactol tautomer (1b) to account for the unusual stability to alkali, as contrasted to the ease of hydrolysis under acidic conditions with the loss of two methoxyl groups. The cyclic carbonyl tautomer (1c) accounts for the readily formation of anhydrogossypol and of identical Diels-Alder type adducts of both gossypol and anhydrogossypol with dienes such as butadiene.

Gossypol reacts with organic acids and the esterification of gossypol has been investigated extensively. The hexaacetate is obtained by reaction of gossypol under rather severe conditions of reaction with acetic anhydride and sodium acetate under reflux. A quite different type of reaction product is readily formed by reaction of gossypol with acetic acid at room temperature. Under these conditions acetic acid combines with gossypol in a one to one molar ratio to produce a gossypol-acetic acid complex. This complex, which is much more stable than

gossypol, has been found very useful in the isolation of gossypol and its preservation for use as a reagent and as a reference standard for analysis.³⁵ Perardi and Frampton³⁶ concluded that gossypol undergoes an ester exchange reaction with triglycerides of crude cottonseed oil and thus accounted for some of the difficulties encountered in refining certain highly coloured crude oils.

The reaction of gossypol with amines has also been studied extensively. Gossypol with liquid ammonia, or in chloroform solution treated with gaseous ammonia, reacts to form dianilinosoyl.^{37, 38} Gossypol reacts readily with two moles of aniline to eliminate two molecules of water and yield a condensation product commonly called dianilinogossypol. Such anils derived from gossypol are generally formulated as Schiff-type bases derived from the hydroxy aldehyde tautomer (1a) in Fig. 1. Part of the toxicity of gossypol can result from rendering certain amino acids unavailable by this reaction, particularly lysine via its epsilon amino group, thus lowering the biological value of the diet.^{39, 40} The reaction with aniline and with p-anisidine has been extensively utilized for the quantitative determination of gossypol related pigments.

Gossypol readily reduces Fehling's and ammoniacal silver nitrate solutions and is extremely sensitive to oxidation. Fundamental to the practical problem associated with the presence of gossypol in cottonseed products is the fate of gossypol under oxidizing condition. Thus, crude cottonseed oils containing gossypol must be refined promptly to prevent formation of highly coloured oxidation products which are not removed by the usual refining and bleaching procedures.

Metal salts of gossypol have aroused considerable interest, particularly because of their potential utility in analysis and the possibility that such salts might counteract toxicity due to gossypol⁴¹. It appears that gossypol produces anaemia by its binding to iron. Gossypol reacts as a strong dibasic acid and forms neutral disodium and dipotassium salts. In order to understand the chemistry of complexes of gossypol, several metal ion-gossypol complexes have been synthesized. The o-hydroxy aldehyde moiety functions a stabilized chelating anion of excellent geometry for metal ion complex formation (Fig.2) and Haas and Shirley⁴² have considered that this was the site of complexation.

The stability constants and stoichiometry of Co(II), Ni(II), and Ca(II) gossypol complexes have been established by several analytical and spectroscopic techniques⁴³. The infrared and the electronic absorption spectra of solid samples indicate the binding of the metal through the hydroxyl and aldehyde groups.

The products from the reaction of Schiff bases such as anilino-gossypol and n-propylanilino-gossypol complexes and Cu(II) acetate indicate 1:2 composition⁴⁴.

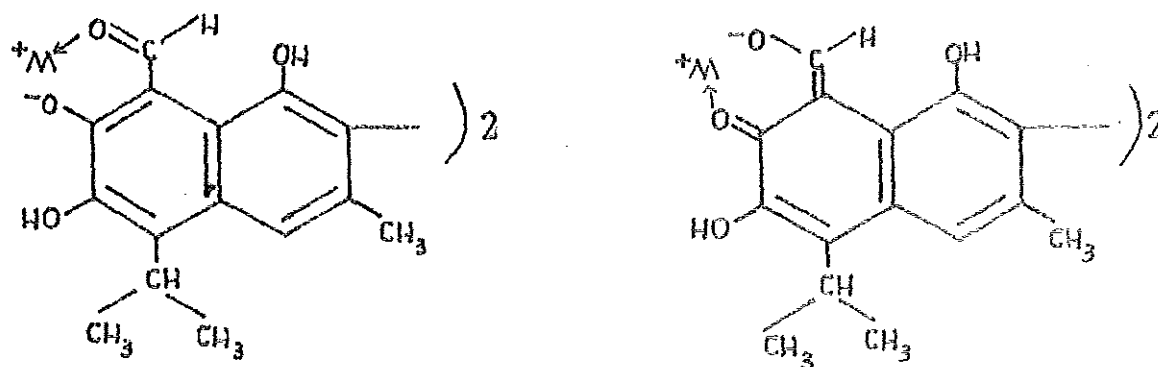


FIGURE 2: Reaction Products Of Metal Ion With Gossypol

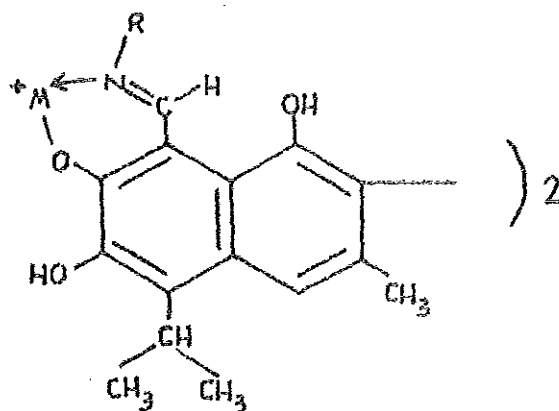


FIGURE 3: Reaction Product Of Metal Ion With Schiff Base.

3.3 Analysis of Gossypol

There has been a recognized need for a reliable routine method for the determination of total gossypol pigments in cottonseed materials. Such method must be fairly rapid and precise. The estimation of total gossypol has proved to be useful for evaluating the influence of processing conditions on the distribution of the gossypol in cottonseed between the meal and oil^{45, 46}.

Numerous procedures have been published for the determination of gossypol pigments⁴⁷. Most of these procedures involved extraction with a suitable solvent, treatment with an aromatic amine (generally aniline or p-anisidine) and determination of the reaction product gravimetrically or spectrophotometrically.

Several gravimetric methods for their determinations have been offered⁴⁸⁻⁵¹, all of which require several days for the complete precipitation of dianilinogossypol. Improved spectrophotometric procedures have generally replaced the earlier gravimetric methods. Aniline is usually employed for the colorimetric determination of gossypol because it reacts with gossypol to form dianiline-gossypol⁵²⁻⁵³. Other reagents which have been used for the development of colored products suitable for spectrophotometric methods include antimony trichloride^{54, 55} phloroglucinol⁵⁶ and borax⁵⁷.

Paper chromatographic methods have been described by several
58,59 workers . Raju and Cater 60 reported use of a triethoxysilyl
ether for the determination of Gossypol by gas-liquid chromatography.

61
The American Oil Chemists' Society has adopted an official
method based on extraction with 3-aminol-propanol in diethylformamide
for the determination of "Total gossypol" in cottonseed and cottonseed
meals. An aliquot of the extracted sample is treated with aniline and
the gossypol content is estimated by spectrophotometry.

Several investigators have used iron salts to inactivate gossypol
29,31 and render the cottonseed meal nontoxic to nonruminants and have
studied the combining ratios of gossypol to ferrous ions 62 .
63
Others use iron salts to oxidize gossypol and convert to other products .
Generally use of iron to counteract gossypol in cottonseed meal goes
64
back more than seventy years . On the other hand, no fundamental inves-
tigation has been made on the use of iron (III) ions for the quantitative
determination of gossypol.

The objective of this project is to propose a new method based on
the complexation of gossypol with iron (III), for the quantitative
determination of total gossypol, and apply the newly proposed method
for the estimation of total gossypol in cottonseed and cottonseed meals.

In the present investigation total gossypol is extracted with 3-amine-1-propanol in dimethyl-formamide.. The complexed gossypol in the extracts readily reacts with iron (III) at room temperature to form an intense green colored gossypol-amino-propanol-iron complex. This reaction is the basis of the colorimetric determination of total gossypol.

With the help of this established method the average contents of total gossypol in most varieties of cottonseeds grown under different conditions in Ethiopia and the cottonseed cakes available in the market are estimated. The data, obtained from these studies will help to select cottonseed varieties and cakes with minimum gossypol pigments, so that they could be used to produce a cottonseed protein concentrate in the form of defatted flours for human food use which will be suitable as protein supplements in cereal food mixtures (maize, wheat, teff, etc.). As a result, this could lead to the development of nutritionally high protein formula at the lowest practical cost that could be afforded by the large target group of the malnourished people of our country.

4. EXPERIMENTAL

4.1 Apparatus and Reagents

4.1.1 Spectrophotometer : A Beckman Model 26 UV-VIS

spectrophotometer equipped with 1 cm matched quartz cells were used for all absorbance measurements.

4.1.2 pH meter : A Beckman Expandometric SS-2

pH meter was used for pH determinations .

4.1.3 Cutting mill: Max Liischer AG. Seon type 1228 with

1 mm and 2 mm screen were used to grind cottonseeds.

4.1.4 Water bath : Water bath operating at 95 - 100 °C was

used for heating the flasks.

4.1.5 Solvent mixture: A solvent mixture was prepared by

mixing redistilled n-hexane and isopropylalcohol in a ratio of 40 to 60 by volume.

4.1.6 Complexing Agent Solution: The solution was prepared

by mixing 2 ml of 3-amino-1-propanol with 10 ml of glacial acetic acid into a 100 ml volumetric flask and diluted to volume with dimethylformamide after cooling to room temperature.

- 4.1.7 Iron (III) Solution: A solution of iron (III) was prepared by transferring exactly 36.2 gm reagent-grade hydrated ferric nitrate into a 1 lt. volumetric flask. The salt was dissolved in a mixture of n-hexane-isopropyl alcohol in a ratio of 40 to 60 and a few drops of concentrated hydrochloric acid was added to it. The solution was diluted to volume with the same mixture of n-hexane and isopropyl alcohol.
- 4.1.8 Stock standard Gossypol Solution: A stock solution of gossypol was prepared by dissolving 0.2790 gm gossypol-acetic acid in complexing agent and diluted to 100 ml with the complexing agent in a volumetric flask. This solution contains 2.5 mg. gossypol per milliliter.
- 4.1.9 Working standard Gossypol Solution: 1 to 10 ml aliquots of the stock solution of gossypol were transferred into 50 ml volumetric flasks. To each standard sufficient volume of complexing agent was added to make total volume of 10 ml. Then the flasks were heated in a water bath for 30 min. Finally, the solutions were cooled and diluted to volume with isopropylalcohol-hexane mixture (60:40).

4.2. Collection of Samples

Samples of cottonseed and cottonseed cake were collected from the following places.

4.2.1 From Institute of Agricultural Research, Melka Werer Research Station.

From this research station seven varieties of cultivated cottonseeds, commonly grown under different conditions in Ethiopia, belonging to G.hirsutum species of the genus Gossypium (subtribe Hibisceae, order Malvales) were collected. These were Acala 1517/70, Albar 637, ABE 1.74, Acala 1517 C, Acala 63-64 (glandless), Fraco Glandless and Fraco Fract Glandless.

4.2.2 From Nazareth and Addis Ababa Oil Mills.

From two oil mills in Nazareth, namely, Nazrawi and Arsina Mertu oil Mills and from three oil mills in Addis Ababa, namely, Teramaj, Akaki and United Oil mills, a total number of 5 samples of cottonseed press cakes were collected.

4.3 Preparation of Samples

4.3.1 Cottonseed: about 50 g of sample was dehulled and the meats were grinded with the help of a laboratory mill to pass a 2 mm screen.

4.3.2 Cottonseed press cake: about 50 g of sample was grinded with the help of a laboratory mill to pass a 1 mm screen.

4.4 Extraction of Gossypol from samples

Analytical sample (approximately 0.5 - 1.5 g) containing 2 - 20 mg of total gossypol was accurately weighed and transferred into a 100 ml Erlenmeyer flask. To the flask 10 ml of complexing agent was added. This was heated in a boiling water bath for 30 min., then cooled and diluted with about 30 ml isopropyl alcohol-hexane mixture. Finally the diluted extract was filtered and made to volume in a 50 ml volumetric flask.

4.5 General Procedure for Determination of Gossypol

Transfer an aliquot of solution containing 0.2-2.0 mg of gossypol to a 25 ml volumetric flask and add 2 to 4 drops of 5N hydrochloric acid. Add 4 ml iron (III) solution and shake the flask.

Allow the mixture to stand for 5 min. to complete the complexation reaction. Then add 1 ml water and immediately dilute to volume with isopropyl alcohol-hexane mixture.

Measure the absorbance at the wavelength of maximum absorbance against isopropyl alcohol-hexane mixture as a reference.

5. RESULTS AND DISCUSSION

5.1 Colorimetric Reaction

Iron (III) reacts with gossypol-aminopropanol in the acidic medium to form a stable, green colored complex, freely soluble in isopropyl alcohol-hexane mixture. This sensitive color reaction forms a basis for the development of a new method for the determination of total gossypol.

5.2 Absorption Spectra

The absorption spectra of the gossypol-aminopropanol, iron (III) solution and the iron-gossypol-aminopropanol complex have been determined in the visible region. The absorption spectra are given in Fig. 4.

The gossypol-aminopropanol and the iron (III) solutions showed negligible absorptions in the region 450-700 nm. However, the gossypol-aminopropanol showed an absorption maximum around 380-400 nm. Iron (III)-gossypol-aminopropanol complex showed an absorption maximum around 620 nm.

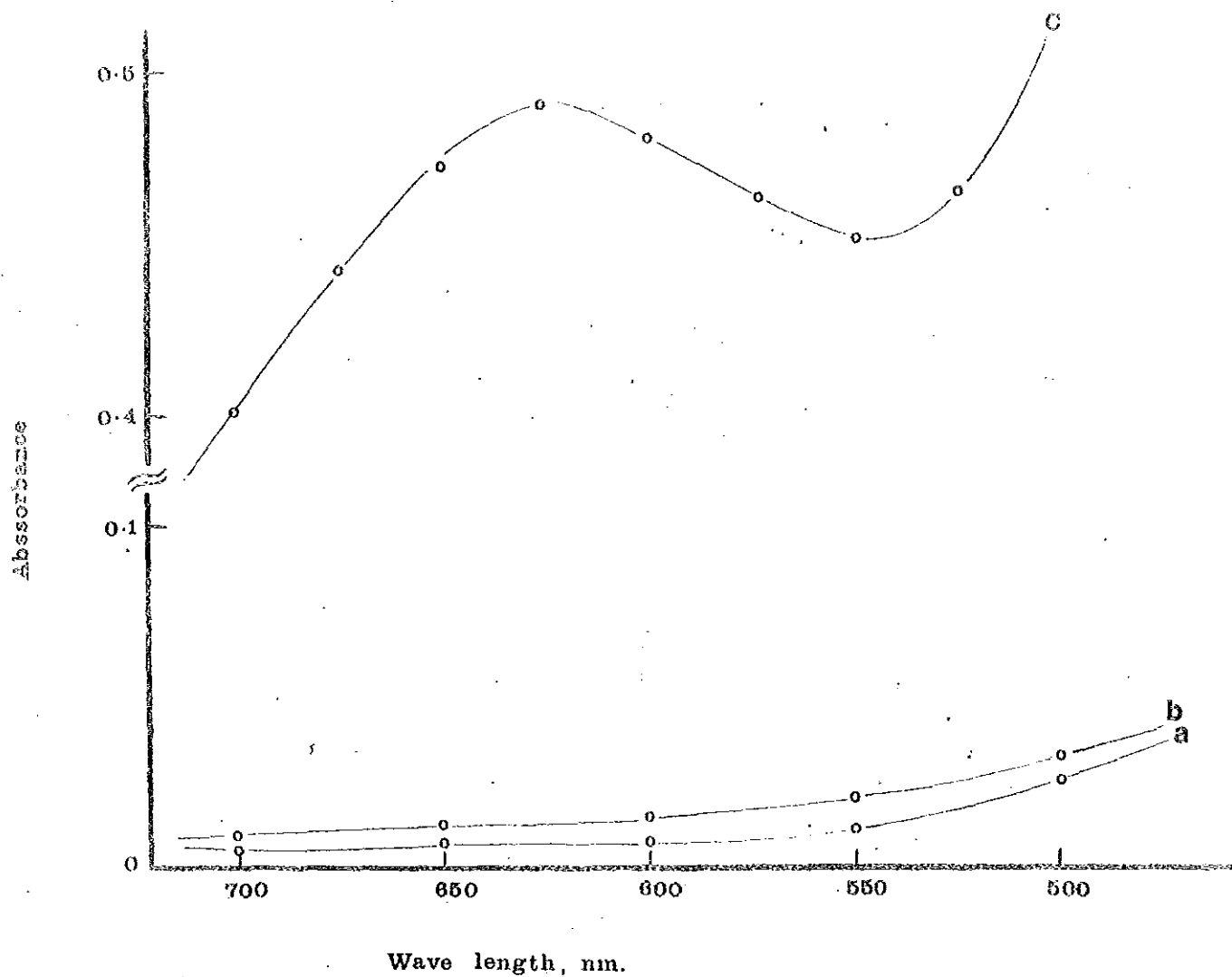


FIGURE 4 : Absorption Spectra of

- (a) gossypol - aminopropanol in isopropylalcohol-hexane (60:40) (10^{-3} M)
- (b) iron(iii) in isopropylalcohol-hexane (60:40) (0.02M)
- (c) iron(iii)-gossypol-aminopropanol complex in isopropylalcohol-hexane (60:40)

After the reaction of iron (III) with gossypol-aminopropanol the resulting solution exhibit no absorption maximum at around 380-400 nm. The disappearance of the peak at this region show that the gossypol-aminopropanol could be completely converted into iron-gossypol-aminopropanol complex on reaction with iron (III).

5.3 Effect of Variables

The effects of several experimental variables on the complexation reaction and color development have been investigated following the general procedure described earlier.

5.3.1 pH

It has been found that the formation of the colored complex between gossypol-aminopropanol and iron (III) is pH dependent. To obtain a complete complexation reaction with constant and maximum absorbance, the pH of the final solution should be in the range of 1.0 - 1.5 (Table I). At higher and lower pH values the solutions exhibit no fixed absorption maxima in the visible region.

Table 1. Effect of PH on the Determination of
Gossypol with Iron (III).

(Concentration of Gossypol. = 25.6 ppm)

PH	Absorbance
	at 620 nm.
0.5	^a 0.112
0.7	0.288
0.9	0.300
1.0	0.302
1.2	0.304
1.4	0.304
1.5	0.304
1.6	0.298
1.7	^a 0.320
2.0	^a 0.325

^a
solutions exhibiting no fixed absorption maxima
in the visible region.

5.3.2. Concentration of Iron (III)

A 1:10 molar ratio of the gossypol to iron (III) is found to be necessary for the maximum color development (Table 2). A large excess of iron upto 500 fold molar excess has no effect on the absorbance measurements of the final solution. In practice usually 4 ml of 0.5% iron (III) solution in isopropyl alcohol-hexane mixture were employed.

5.3.3 Water

With a given concentration of gossypol and iron (III), in an acidic medium, a stable color intensity is developed only in the presence of small amount of water. The amount of water may vary from 0.8 to 1.5 ml (Table 3) in the reaction volume without any change in the color intensity for a final volume of 25ml. The role of water cannot be simply explained. The addition of water is necessary probably to satisfy the coordination number of iron (III) in the complex.

Table 2. Effect of Concentration of iron (III)
in color Development.

(concentration of Gossypol + 36.0 ppm.)

Mole Ratio Gossypol: Iron (III)	Absorbance at 620 nm.
1:1	0.199
1:2	0.273
1:4	0.370
1:8	0.425
1:10	0.440
1:20	0.445
1:50	0.446
1:100	0.447
1:500	0.447

Table 3. Effect of Water in the Color Development for the Reaction of Gossypol-aminopropanol with iron (III).

(Concentration of gossypol = 25.6 ppm)

Volume of Water	Absorbance
	at 620 nm.
0	0.005
0.5	0.202
0.8	0.289
1.0	0.301
1.3	0.294
1.5	0.290
2.0	0.270
2.5	^a

^a
addition of water, >2ml., formed
immiscible solutions.

5.3.4 Order of Addition of Reagents.

It has been found that there is no change in the absorbance or in color intensity of the complex if the order of addition of gossypol and iron (III) is changed prior to the addition of water.

5.3.5 Time for Complexation and Stability of Complex.

The reaction between gossypol-aminopropanol and iron (III) is found to be very fast. When the reaction between the two, in an acidic medium, is allowed to proceed at room temperature, optimum color intensity is reached within 5 minutes time. It was found that the absorbance value of the colored solution remains unchanged for atleast 3 hours and then decreases slowly (Table 1).

5.3.6 Temperature.

The reaction mixture before dilution to the final volume has been subjected to different temperatures.

Table 4. Effect of Reaction Time on Color Development
in the Reaction of Gossypol-aminopropanol with
Iron (III).

(Concentration of gossypol = 36 ppm.)

Time of reaction (min.)	Absorbance at 620 mμ.
0	0.431
60	0.432
120	0.430
180	0.425
240	0.412
300	0.390
360	0.363

Table 5. Effect of Temperature on Color Development in the
Reaction of Gossypol-aminopropanol with Iron (III).

(Concentration of gossypol = 36 ppm)

Temperature ° C	Absorbance at 620 nm.
21	0.429
30	0.427
40	0.415
50	0.395
80	0.374
100	0.332

Variation of the temperature (Table 5)
of the colored solution from 20-30 c did not produce any measurable change in the absorbance of the final solution. At higher temperatures, the color of the solution becomes lighter with decreased absorbance readings.

5.4 Beer's Law, Optimum Concentration Range, Sensitivity and Molar Absorptivity.

The colored system obeys Beer's Law from 0.1 to 2.0 mg of total gossypol in 25 ml solution at 620 nm (Table 6). The standard curve (Fig. 5) has been repeatedly checked and is reproducible throughout the concentration range of 0.1 - 2.0 mg of gossypol in 25 ml volume. The optimum concentration range for the determination as evaluated from the Ringbom's plot⁶⁵ is 0.35 - 1.60 mg of total gossypol per 25 ml (Fig. 6).

The photometric sensitivity⁶⁶ of the colored reaction is 0.08 µg/ml. The molar absorptivity is found to be 6480-50 lt. mole⁻¹ cm⁻¹ at 620 nm.

Table 6. Calibration Curve Data for the Determination of
Total gossypol.

Concentration of gossypol (mg/25 ml)	Absorbance (at 620 nm.)
0.15	0.079
0.30	0.153
0.60	0.305
0.90	0.456
1.20	0.612
1.50	0.769
1.80	0.910
2.10	1.050

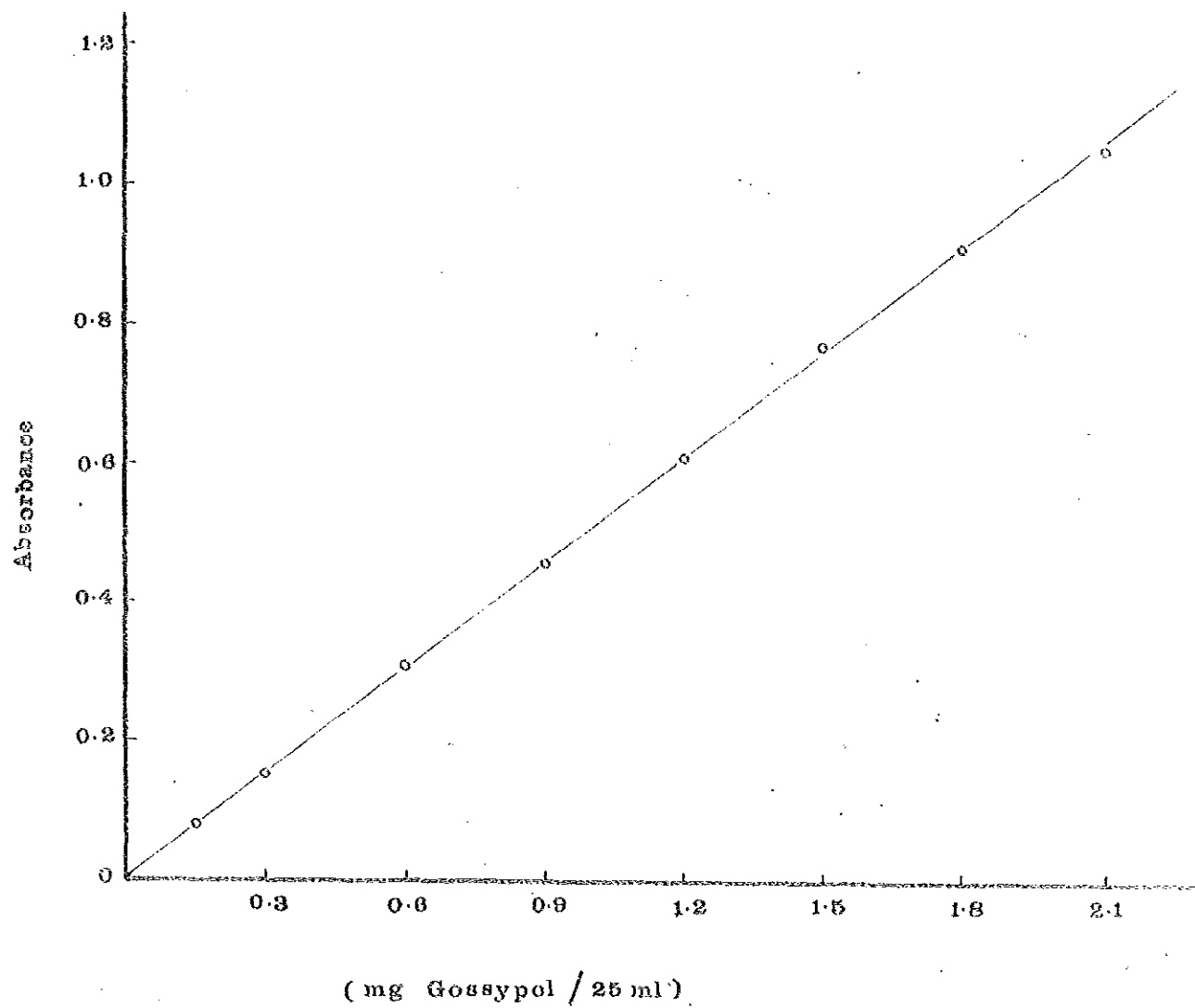


FIGURE 5: Calibration Curve for Determination of Gossypol

5.6.1 Job's Method of Continuous Variations.

The mole fractions of gossypol and iron (III) were continuously varied with a constant total molarity by mixing different volumes of equimolar solutions of gossypol and iron (III). The pH of the solutions were kept constant.

Procedure: Following the general procedure described earlier, X ml of 0.576×10^{-3} M solution of gossypol and $(10-X)$ ml of 0.576×10^{-3} M solution of iron (III) were mixed in 25 ml. volumetric flask. After diluting the reaction mixture with isopropylalcohol- hexane mixture, the absorbance of the solutions were measured at 620 nm (Table 7). This was plotted against the mole fractions of the gossypol.

Maximum absorption occurred at a mole fraction of 0.33 $\frac{[\text{Gossypol}]}{[\text{Gossypol}] + [\text{Iron (III)}]}$ (Fig. 7). This suggests that the combining ratio of gossypol to iron (III) is 1:2.

Table 7. Job's Method of Continuous Variations

(Concentration of gossypol = 0.567×10^{-3} M)

(Concentration of iron (III) = 0.567×10^{-3} M)

Gossypol X ml	Iron (III) (10-X) ml	Absorbance at 620 nm.
9	1	0.081
8	2	0.131
7	3	0.179
6	4	0.225
5	5	0.263
4	6	0.288
3.5	6.5	0.291
3.3	6.7	0.290
3	7	0.285
2.5	7.5	0.265
2	8	0.238
1	9	0.181

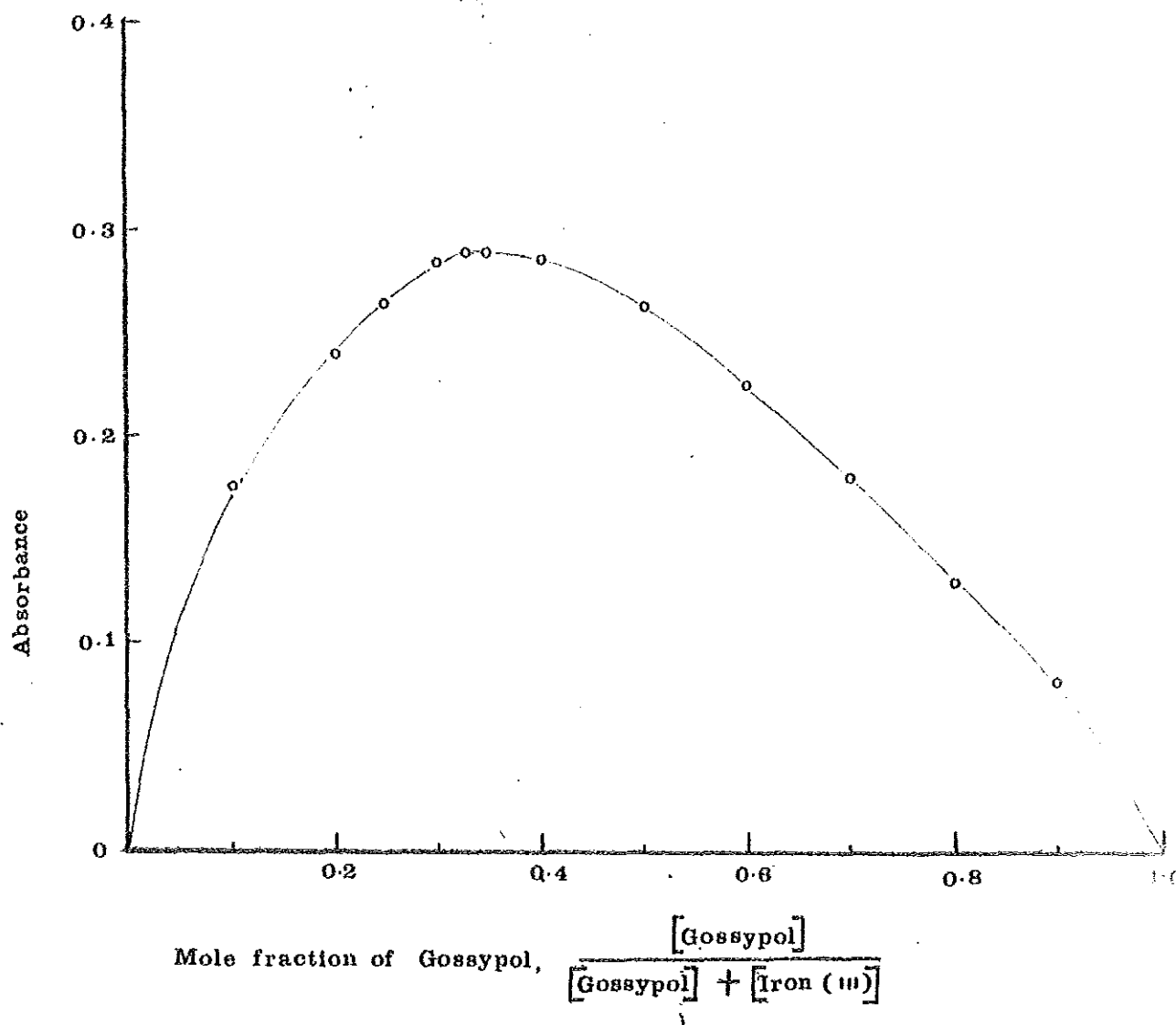


FIGURE 7: Job's Method of Continuous Variations

5.6.2 Mole Ratio Method.

In this method the molar concentration of gossypol was kept constant and the mole ratio of iron (III) was varied. Again the pH of the solution was kept constant.

Procedure: Following the general procedure described earlier, 4 ml of 0.576×10^{-3} M solution of gossypol and 2 ml of 5.76×10^{-3} M solution of iron (III) were mixed in 25 ml volumetric flask. After diluting the reaction mixture with isopropylalcohol-hexane mixture, the absorbance of the solutions were taken at 620 nm (Table 8).

The absorbance were plotted against mole ratio of gossypol to iron (III) (Fig. 8). The intersection of the two tangents corresponds to the mole ratio of 1:2 (gossypol : iron (III)).

The results obtained by both methods indicate that the ratio of gossypol to iron (III) in the complex is 1:2. These results are similar to the result obtained by Haas and Shirley⁴² in which they reported that the ratio of gossypol to iron (III) is 1:2 and indicated that the aldehyde groups and the adjacent, 7-hydroxyl groups are the site of the complexation.

Table 8. Mole Ratio Method

(Concentration of gossypol = 0.567×10^{-3} M)

Mole Ratio		Absorbance
Gossypol: Iron (III)		At 620 mμ.
1:0.5		0.101
1:1		0.190
1:2		0.276
1:4		0.369
1:8		0.425
1:10		0.440
1:14		0.445
1:20		0.446
1:50		0.447

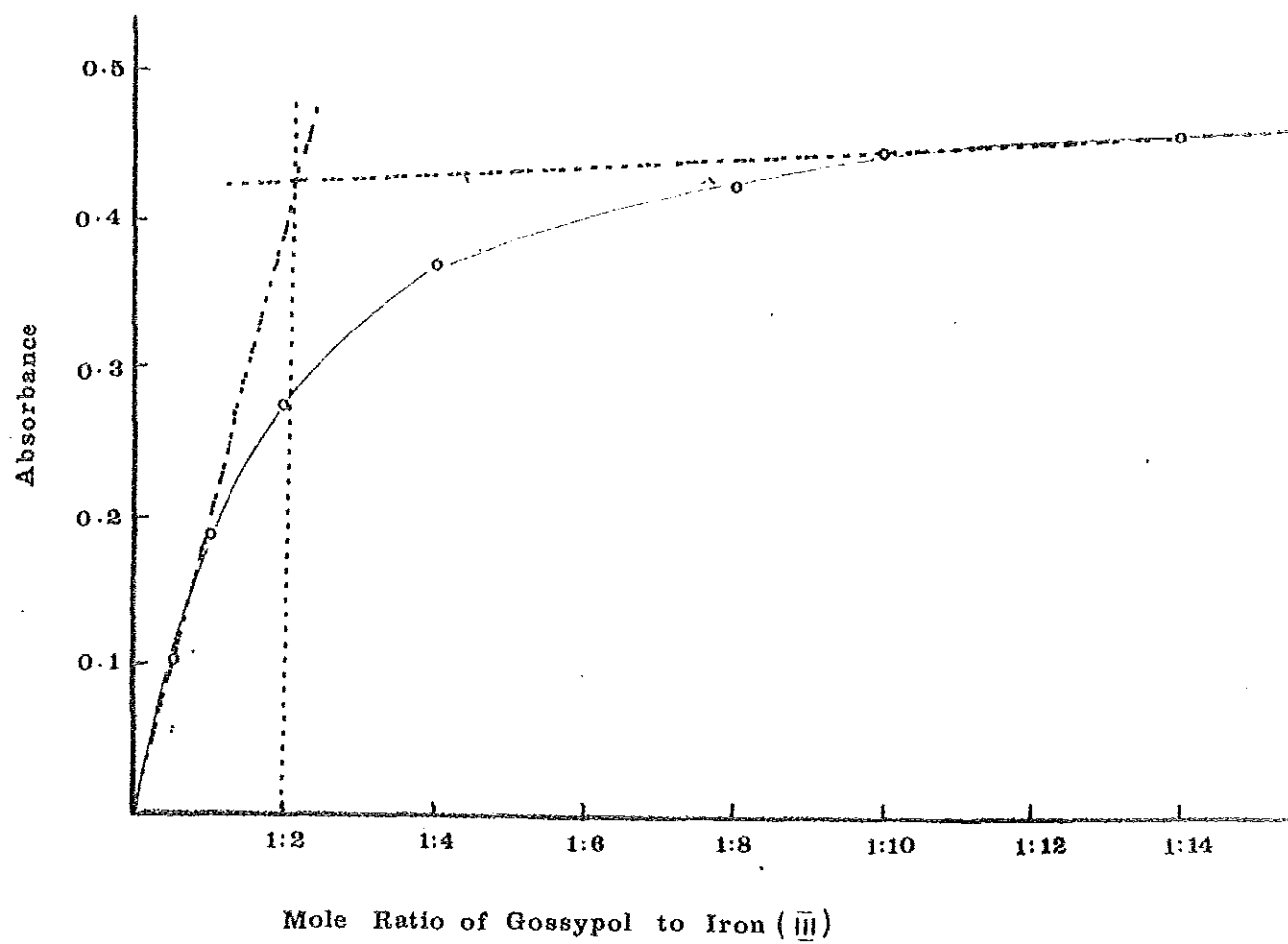


FIGURE 8: Mole Ratio Method

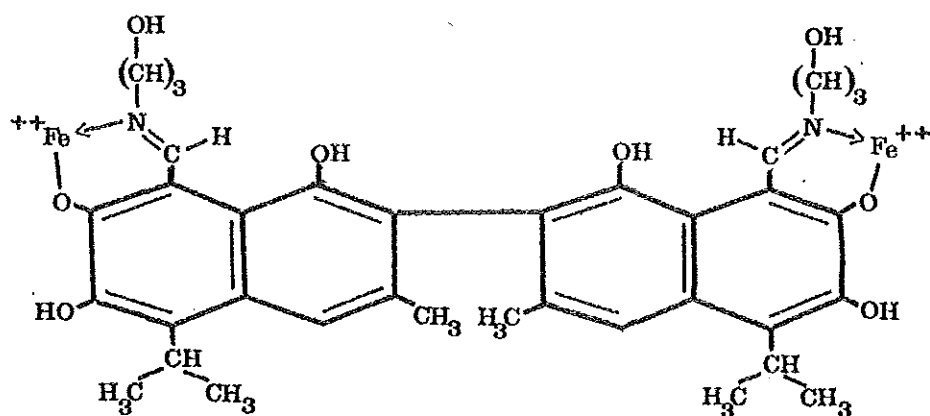


FIGURE 9: Proposed Structure of
Iron-gossypolaminopropanol

Based on the above conclusions, the proposed structure for the iron-gossypol-aminopropanol complex formed upon the reaction of iron (III) with gossypol-aminopropanol is given in Figure 9.

5.7 Application of the Method for the Determination of Total Gossypol in Cottonseed and Cottonseed press Cakes.

The validity of the proposed method has been tested by determining the total gossypol content of cottonseed varieties and cottonseed press cakes.

The absorption curves plotted in Fig 10. demonstrate that the complex formed by treating cottonseed meal extract with iron (III) solution is identical with the complex obtained from pure gossypol similarly treated. The maximum and the minimum occur at the same wavelength and are of the same relative magnitudes. Identity of the spectra after the iron (III) reaction (Fig. 10) is also evidence that meal constituents contribute no interference in the total gossypol determination.

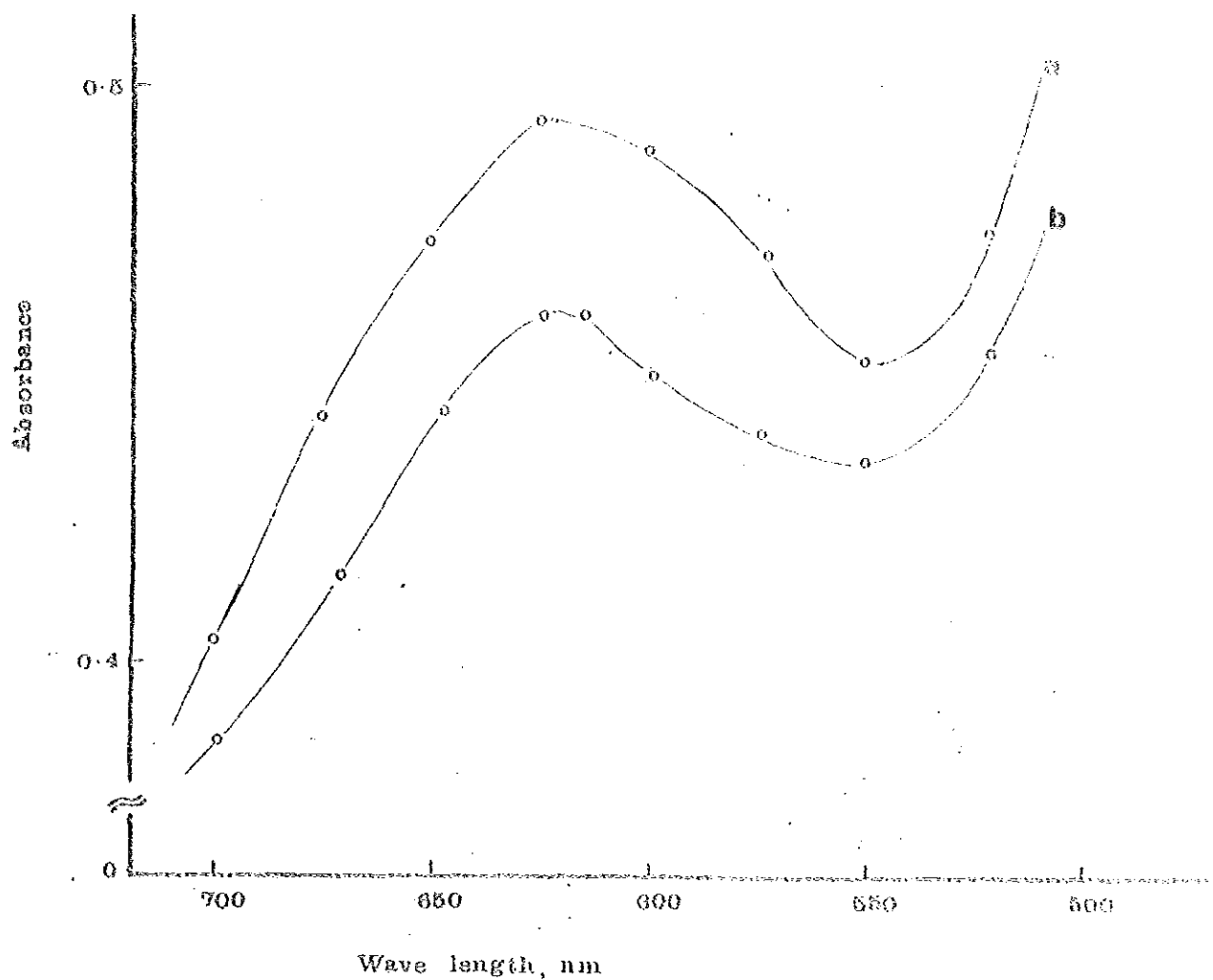


FIGURE 10: Absorption Spectra of Iron(III)-gossypolaminopropionyl complexes

a) from pure gossypol

b) from cottonseed meal extract

The precision of the method was tested on the cottonseed meal extract containing a mean total gossypol content of 0.705%. Ten analyses done on the same sample extract gave a standard deviation of ± 0.005 with a relative standard deviation $\pm 0.71\%$. The mean total gossypol content for the above sample falls in the interval from 0.702 to 0.708%, at 95% confidence levels.

By following the general procedure, the total gossypol contents of cottonseed varieties collected from Institute of Agricultural Research in Melka Woker, cottonseed press cakes collected from different oil mills in Nazareth & Addis Ababa were determined. The results of these determinations are given in Table 10 and 11. Among the national varieties, Acala 1517C (1.063%) and AMS 1.74 (0.33%) contain the highest and the lowest total gossypol respectively. The glandless varieties are found to be not completely free of gossypol pigments.

The total gossypol contents of the press cakes collected from five different oil mills were found to be lower than the recommended
13
value (1.2%) .

5.8 Recovery of Added Gossypol.

To test the effect of interferences in various extracts, known concentrations of pure gossypol treated with aminopropanol were added. Table 9 shows recovery values for two samples. The results show satisfactory recovery of gossypol and these values establish the precision of the method.

5.9 Comparison with other Methods

For the determination of total gossypol, the American Oil Chemists' Society (A.O.C.S) has adopted an official method⁶¹ based on the extraction with 3-amino-1-propanol in dimethylformamide, and then reacting with aniline to form a diaminogossypol chromophore. The total gossypol is estimated from the absorbance of this complex at 440 nm.

The proposed procedure, when applied to a series of cottonseed meal extracts of different types, gave results (Table 10 and 11) in close agreement with those obtained by using the A.O.C.S Tentative method⁶¹ for the determination of total gossypol.

Table 9. Determination of gossypol added to cottonseed extracts.

Type of: sample extracts	Gossypol. in extract (g)	Gossypol added (g)	Total Gossypol Present (g)	Gossypol found (g)	Recovery of gossypol (%)
Cottonseed variety	0.513	0.302	0.815	0.809	99.3 %
Cottonseed press cake	0.497	0.160	0.657	0.654	99.5 %

Table 10. Comparison of proposed Method with A.O.C.S. Method
for Gossypol Content of Cottonseed Varieties.

Type of cottonseed Variety	Total Gossypol. %	
	Proposed method	A.O.C.S. method
Acala 1517/70	0.489	0.483
Albar 637	0.613	0.615
AMC 1.74	0.331	0.329
Acala 1517c	1.069	1.063
Acala 63-64 glandless	0.029	0.034
Prago glandless	0.415	0.411
Prago Bract glandless	0.168	0.166

Table 11. Comparison of proposed Method with A.O.C.S. Method
for Gossypol Content of Cottonseed press Cakes

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Place of collection	Total Gossypol. %	
	Proposed method	A.O.C.S. method
Nazrawi Oil Mills	0.689	0.685
Arsina Mertu Oil Mills	0.518	0.519
Toramaj Oil Mills	0.962	0.956
Akaki Oil Mills	0.850	0.845
United Oil Mills	0.758	0.757

The mean values of the proposed method and the A.O.C.S method have been compared in terms of T test (Table 12). The results obtained indicate that the mean values of the two methods are not significantly different from each other at 80 to 99% probability levels. In addition, the precision of the two methods has been compared in terms of F test (table 13). The result obtained indicates that there is no significant difference in the precision of the two methods at 1 to 10% levels.

The Iron (III) method nearly always indicated a higher gossypol concentration in a given extract than the aniline method of the A.O.C.S. As these samples all contain bound gossypol, the heating of samples in the procedures of A.O.C.S may lead to hydrolysis of some of the bound gossypol.

Although analytical methods currently employed for the estimation of total ⁴⁷gossypol including the A.O.C.S tentative ⁶¹methods are adequate from the view-point of accuracy and precision, they are time consuming. The proposed method is rapid and simple. The accuracy and precision of the proposed method are good and comparable to other methods.

Table 12. Application of t Test to Compare the Mean Values

	Proposed	A.O.C.S.
	Method	Method
Number of determinations	10	10
Mean of Total Gossypol content	0.705	0.703

Pooled Standard Deviation = 0.0045

$t_{\text{expt'l}} = 0.99$

$t_{\text{critical}} = 1.383$ (at 80% probability level)

$t_{\text{critical}} = 1.833$ (at 90% probability level)

$t_{\text{critical}} = 2.262$ (at 95% probability level)

$t_{\text{critical}} = 3.250$ (at 99.9% probability level)

Therefore, $t_{\text{expt'l}} < t_{\text{critical}}$ for all levels.

Table 13. Application of F Test to Compare the Precision

	Proposed Method	A.O.C.S Method
Number of determinations	10	10
Mean of Total Gossypol content	0.705	0.703
Standard deviation, S	0.005	0.004
Square of Standard 2 deviation, S	0.000025	0.000016

$$F_{\text{expt'l}} = \frac{\frac{S_{\text{proposed}}^2}{2}}{\frac{S_{\text{A.O.C.S}}^2}{2}} = 1.56$$

$F_{\text{critical}} = 2.44$ (at upper 10% point)

$F_{\text{critical}} = 3.18$ (at upper 5% point)

$F_{\text{critical}} = 5.35$ (at upper 1% point)

Therefore, $F_{\text{expt'l}} < F_{\text{critical}}$ for all levels.

In addition, a repeated distillation of aniline is avoided and a simple solution of iron (III) in isopropyl-alcohol-hexane was substituted as a color forming agent. Since the reaction between iron (III) and gossypol-aminopropanol is very fast and proceeds at room temperature, in the proposed procedure no heating or cooling of the reaction mixture is required which affect the results.

6. CONCLUSION

A new method is proposed for the spectrophotometric determination of "total gossypol" in cottonseeds based on the extraction of gossypol by 3-amino-1-propanol in dimethylformamide, followed by reaction with iron (III) in an acidic medium to form a stable green colored complex soluble in n-hexane-isopropyl alcohol. The reaction is quite sensitive and specific for gossypol in 3-amino-1-propanol extracts of cottonseeds. The method is simple, rapid, precise and accurate and it can compete with other common methods recommended for the determination of total gossypol in cottonseeds.

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