A STUDY OF GOSSYPOL: MEASUREMENT AND REDUCTION IN COTTONSEED

by

Olive Erinma Akomas

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DEDICATED

to my husband, George, for being a steady source of support and encouragement and to our children, Chiagozie, Adaeze and Chimaihe for being so understanding at the times when Mom was studying.

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Olive Akomas
January 1987.

GENERAL ABSTRACT

The utilization of cottonseed for monogastric and human nutrition is limited by the presence of gossypol, a toxic polyphenolic compound. This compound is toxic when present in the "free" form only, but not when attached to protein in the "bound" form. In the free form, however, gossypol attaches itself primarily to the epsilon amino group of lysine decreasing lysine availability. Since cottonseed is already limiting in lysine any decrease in available lysine will further reduce its protein quality. Cottonseed products intended for human use in the United States must not contain more than 0.045% "free" gossypol as set by the FDA. The Protein Advisory Group of the United Nations set limits of 0.06% and 1.2% for "free" and "bound" gossypol in cottonseed if used for edible food. Many methods have been studied to remove or reduce the level of "free" gossypol in cottonseed including solvent extraction, air classification or blending with other plant proteins. A recent novel approach reported was to heat cottonseed with soybean gums, in which the phospholipids compete with lysine for gossypol.

The measurement of gossypol with titanium tetrachloride was investigated in this study. A stable coloured complex was formed with maximum absorbance at 490 nm which yielded a linear Beer's law plot. Determination of "free" gossypol in cottonseed using the titanium reagent proved unsuccessful due to the interference by other phenolic compounds. A comparison of three other colorimetric methods for gossypol measurement showed p-anisidine and aniline to be the most sensitive with the iron III method the least sensitive. The iron III method was found to be the simplest of the three methods and suitable

for measuring cottonseed products containing relatively large amounts of gossypol.

The development of a cottonseed:gossypol model system was established in which the time, temperature and level of gossypol was adjusted to obtain a 33-38% decrease in available lysine. Available lysine was measured following the dye-binding procedure. Heating a gossypol:cottonseed flour (1:16) model system at $90^{o}C$ for 30 minutes reduced available lysine from 21-22 to 12-14 mMoles/100g protein. This was carried out under alkaline conditions which facilitates the binding of gossypol with the epsilon amino group of lysine typical of the Maillard reaction. Different amounts of choline and ethanolamine (0.4, 0.8 and $1.6x10^{-1}M$ were added to the system) to compete with lysine for gossypol. A significant ($p<0.05$) increase in available lysine was evident in the presence of $0.8x10^{-1}M$ and $1.6x10^{-1}M$ choline or ethanolamine. Ethanolamine was approximately twice as effective as choline in restoring the level of lysine back to 19.1 mMoles/100g protein. This study demonstrates the potential of these bases as a relatively cheap and simple technique for reducing the toxicity of gossypol while at the same time protecting the available lysine.

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CHAPTER 1

GENERAL INTRODUCTION

Approximately 3.5 million tons of cottonseed protein are potentially available for human consumption in many countries where the protein quality of the local diet needs to be improved (Harden, 1975). A major setback to the use of cottonseed protein in edible foods is the presence of free gossypol, a toxic polyphenol present in the pigment glands throughout the kernel. A glandless cottonseed has been developed by plant breeders but this plant is susceptible to disease and poor yields have discouraged its use. Glandless cottonseed accounts for less than 0.5% of the total cotton acreage planted in the United States (Hess, 1977).

Reduction of free gossypol in cottonseed can be achieved by heating which results in gossypol combining chemically with lysine in the protein (Bressani and Elias, 1968). Since cottonseed is already limiting in lysine, further reduction through combination with gossypol adversely affects the quality of the protein (Lyman et al., 1953; Rolz et al., 1972).

Research into methods of improving the quality of cottonseed is being conducted by researchers in the United States, Canada and Latin America. A number of solvent extraction systems have been examined, some of which facilitate a substantial reduction in the level of free gossypol (Cherry and Gray 1981; Damaty and Hudson 1975; Lui et al., 1981; Rahma and Narasinga Rao, 1984). A major drawback associated with organic solvents is the presence of any residual solvent remaining after extraction. Other methods that have been developed include air classification of the glanded cottonseed (Kadan et al., 1979). A cost analysis for the production of cottonseed flour by air classification

indicated a capital investment of 4 to 6 million dollars was required for a 25 ton/day or 50 ton/day plant (Decossas et al., 1982). The development of low gossypol cottonseed products by either solvent extraction or air classification are both costly procedures.

A recent paper by Del Valle et al. (1986) reported the preparation of low free gossypol and high lysine cottonseed/soybean blends using a combination of 50% defatted cottonseed meal/50% extruded soybean flakes. The main advantage of this method was to reduce the need for developing countries to import high priced soybean by incorporating cottonseed meal as a low cost extender.

A study by Yannai and Bensal (1983) showed that heating dehulled and flaked cottonseed with a crude gum mixture obtained from soybeans substantially improved the amount of available lysine as reflected by higher PER values in rats. This effect was attributed to the bases in these phospholipids competing with the cottonseed protein for free gossypol.

The main objectives of this study were:

1. To examine the possibility of using the titanium tetrachloride (20% $TiCl_4$ in conc. HCl) reagent to measure gossypol.
2. To set up a model system composed of cottonseed and gossypol for studying the effect of bases choline and ethanolamine on lysine availability.

CHAPTER 2

REVIEW OF LITERATURE

2.1 COTTONSEED

Malnutrition is a major problem confronting most developing countries. Of particular concern to nutritionists is the short supply of available proteins to meet the rapid expansion of the population in these countries. This problem could be alleviated by increasing the use of plant proteins indigenous to many of the developing countries. Efforts are being made towards more efficient use of such available proteins (Cater et al., 1977).

Cottonseed, a major crop in many developing countries in Africa and Latin America, provides an abundant and cheap source of good quality protein for human use (Martinez et al., 1970; Harden, 1975). Prior to 1942, cottonseed represented the major source of domestic protein concentrate until its subsequent replacement by soybean. Gillham (1969) suggested that approximately one quarter of the flour potentially available from world cottonseed production could alleviate the edible protein shortages of hungry nations. Studies by Bressani (1969) reported excellent growth improvement in children fed Incaparina mixtures composed of 18-30% cottonseed flour and 58% corn flour. As early as 1944, Jones and Divine found that as little as 5 parts of cottonseed flour added to 95 parts of wheat flour produced mixtures containing 16-19% protein compared to wheat flour alone. Berardi and Goldblatt (1980) reported cottonseed meal to be the second largest source of vegetable protein concentrate in the United States. Cottonseed proteins are rarely utilized as a source of edible foods due to the presence of pigment glands that contain a toxic factor, gossypol (Kadan et al, 1979).

2.2 TOXIC COMPONENT: GOSSYPOL

The development of edible protein products from cottonseed has been limited by the presence of a toxic component gossypol present in the pigment glands. This substance has shown to be toxic to monogastic animals including man (Graham, 1961; Harper, 1969; Reber, 1981; Smith, 1970). In order to derive maximum benefit from cottonseed meal, the removal of this toxic component is mandatory. Plant breeders have attempted this by developing glandless cottonseed. (Decossas et al., 1982). Food scientists have examined a variety of solvent-extraction methods to reduce the gossypol content of cottonseed meals (Canella and Sodini, 1977; Cherry and Gray, 1981; Damaty and Hudson, 1975; Liu et al., 1981; Pons and Eaves, 1971; Rahma and Narasinga Rao, 1984). The use of gamma-radiation to reduce gossypol was recently reported by Jaddou et al. (1983).

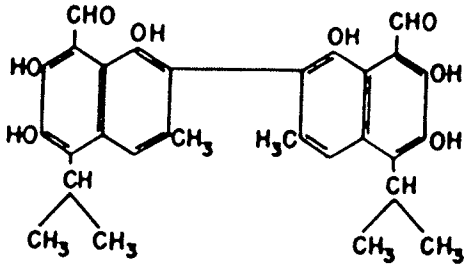
The genetic removal of the pigment glands containing gossypol in the cottonseed plant appeared to be one way of eliminating this toxic component. Difficulties were encountered, however, as the removal of these glands affected the natural defence mechanisms of the cotton plants towards insects, field animals and verticillum wilt disease necessitating the use of insecticides. These glandless cottonseed varieties were low yielding and account for less than 0.5% of the total U.S. cotton crop planted up to 1976 (Hess, 1977). A similar situation was evident in other cotton-producing countries where acreage of glandless cotton remained insignificant (Decossas et al., 1982). Consequently the majority of cottonseed grown is still the traditional gossypol containing cultivars.

2.3 GOSSYPOL: STRUCTURE AND PROPERTIES

Gossypol, a yellow pigmented polyphenolic compound present in various parts of plants belonging to the genera Gossypium (cotton), is found in discrete bodies known as pigment glands (Berardi and Goldblatt, 1980). These are located in the stems, leaves, roots and seeds of the cotton plant (Abou-Donia, 1976). In addition to reacting with the protein, gossypol is also toxic to monogastric animals and man (Liener, 1969; Reber, 1981; Smith, 1970).

The name "gossypol" is derived from "gossyp(ium phen)ol" to indicate its origin and nature (Adams et al., 1960). It has an empirical formula $C_{30}H_{30}O_8$ and a molecular weight of 518:54. Its structure (Figure 1a) shows it has six hydroxyl groups, two carbonyl groups, an aliphatic side chain, a hydroxyl group peri or ortho to the carbonyl group, and a naphthalene nucleus (Berardi and Goldblatt, 1980). The two aldehydic groups and two hydroxyl groups in the ortho position make it particularly reactive. Gossypol is soluble in ordinary organic solvents and can be readily crystallized. It is insoluble in low boiling petroleum ether (b.p. 30-60C) and in water (Jones, 1979). To explain the many reactions that gossypol undergoes at least three tautometric modifications were suggested by Adams et al. (1983). These shown in Figure 1 include (a) the hydroxyaldehyde tautomer, (b) the lactol tautomer and (c) the cyclic tautomeric form.

Gossypol is degraded by simple reactions to remove its aldehyde and isopropyl groups and is esterified by organic acids. Gossypol reacts with acetic acid at room temperature in a 1:1 molar ratio forming a



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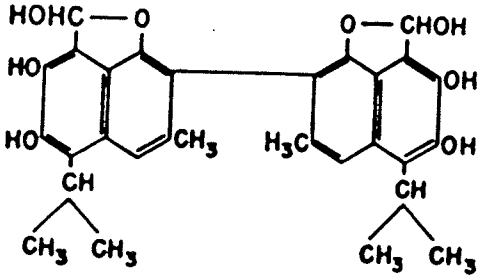

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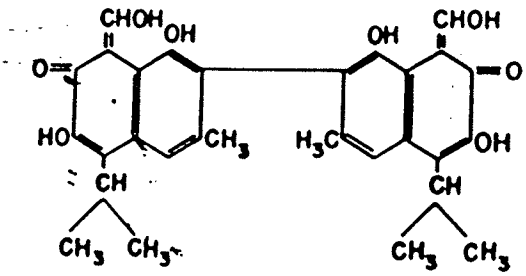

(Ic)

Fig. 1. Structures of the various tautomeric forms of gossypol. (Berardi and Goldblatt, 1980)

gossypol-acetic acid complex that is far more stable than the original gossypol. This complex is particularly useful in isolating gossypol for use as a reagent and reference standard in analysis (Berardi and Goldblatt, 1980). Gossypol also reacts as an acid by forming salts with metals. These metals can be used in the analysis of gossypol (Admasu and Chandravanshi, 1984).

The presence of gossypol is a primary factor when considering cottonseed protein for human use. Gossypol is extremely reactive with both acids and bases as it possesses both phenolic and carbonyl groups. The phenolic groups can react readily to form esters or ethers while the carbonyl groups react with aromatic amines such as aniline or para-anisidine forming Schiff's bases (Adams et al., 1960). The reaction of gossypol with amines has been extensively studied (Alley and Shirley, 1959; Dechary and Brown, 1956; Pominski et al., 1951). It condenses with two molecules of primary amines with the subsequent elimination of two molecules of water. Gossypol's reaction with aniline and para-anisidine forms the basis of gravimetric and colormetric analyses of gossypol (Sherwood, 1926; Clark, 1928; Royce, 1933; Smith, 1966, 1968).

Gossypol is extremely sensitive to oxidation. The crystalline form oxidizes readily in air at room temperature unless protected from light (Pominski et al., 1951). Recent studies by Nomeir and Abou-Donia (1985) demonstrated the decomposition of gossypol and/or its degradation products when continuously exposed to ultraviolet irradiation.

2.4 GOSSYPOL: TOXICITY

The symptoms of gossypol toxicity vary with the particular animal species and generally include depressed appetite and loss of weight.

The most common effect is cardiac irregularity which could lead to circulatory failure and death. The ingestion of gossypol by rats was reported by Ambrose and Robbins (1951) to result in a depression of appetite and loss of body weight. The acute oral toxicity of gossypol in rats produced an LD_{50} value of 2,600 mg/Kg body weight. Couch et al. (1955) observed growth inhibition in chicks fed gossypol while pigs were reported by Lyman and co-workers (1963) to be more sensitive to gossypol with an LD_{50} value of 550 mg/Kg body weight.

The effects of gossypol toxicity has been reported in monogastric animals and young calves until the rumen is fully developed (Adams et al, 1960; Jones, 1979). The absence of toxicity by cottonseed when fed to ruminants is attributed to prolonged mastication, water content, increased time in the rumen, all of which result in the binding of gossypol to protein with less of it absorbed. The presence of unbound gossypol prevents the liberation of oxygen from oxyhemoglobin which has a hemolytic effect on erythrocytes (Menaul, 1923). Gossypol poisoning places an extreme burden on the respiratory and circulatory organs due to the reduced oxygen carrying capacity of the blood. In addition to these effects gossypol also affects the nutritive value of cottonseed protein (Lyman et al., 1953; Rolz, et al., 1972).

The physiological effects of gossypol in humans is of particular interest in view of the potential of cottonseed protein for foods. Toxic symptoms in man are anemia, anorexia, chronic diarrhea and possibly malnutrition. Adamova and Lebedeva (1947) reported that a daily ingestion of 60 g. cottonseed cake containing from 0.11 to 0.20% free gossypol did not produce harmful effects in human subjects fed over

a period of four and half months. Jones (1979) stated that any cottonseed products intended for human use in the United States must not contain more than 0.045% free gossypol as set by the FDA. The Protein Advisory Group of the United Nations (1979) set limits of 0.06 and 1.2% for free and total gossypol in cottonseed products intended for human consumption. Berardi and Goldbatt (1980) noted that a protein concentrate sold as partially defatted cottonseed flour must have a specified free gossypol content not exceeding 450 ppm or .045%. Recommended levels of gossypol for use in cottonseed meat for use in animal and human nutrition is summarized in Table 1.

2.5 GOSSYPOL: COTTONSEED PROTEIN

Conkerton and Frampton (1959) showed the ability of gossypol to react with amino groups in cottonseed protein globulin. The most susceptible amino acid was lysine. The binding of gossypol during the processing of cottonseed is attributed to the formation of Schiff's bases by the reaction of formyl groups in gossypol with the epsilon amino groups of lysine (Conkerton and Frampton, 1959; Martinez et al., 1961). Cottonseed protein is limiting in lysine so that any reduction in its availability adversely affects the quality of the protein (Lyman et al., 1953; Rolz et al., 1972). When free gossypol reacts with amino groups in the cottonseed it is much less toxic. In this form it is referred to as "bound gossypol" to distinguish it from gossypol unbound or "free" gossypol. It is the latter form in which gossypol exerts its toxic effect. Consequently it is the amount of free or unreacted gossypol, rather than the total amount of gossypol consumed by man or animals, that is of major concern. Nevertheless the decrease in free gossypol in cottonseed is usually associated with decreased protein

Table 1

[1] Recommended Levels of Gossypol for Animal and Human Use

Maximum	Free Gossypol	Use
0.04%	In cottonseed meal	broilers, swine, laying hens
0.06%	In cottonseed meal	human consumption (WHO)
0.045%	In cottonseed meal	human consumption (FDA)
	Total Gossypol	
1.2%	In cottonseed meal	human consumption (WHO)

[1] Jones (1979)

quality. In addition the presence of six phenolic groups in the gossypol molecule offers further opportunity for binding with proteins which may become undigestible and unabsorbable (Altschul et al., 1958; Panemangalore et al., 1970). The binding of gossypol to lysine also inhibits the breakdown of protein by enzymes of the digestive system (Damaty and Hudson, 1979). This may be due to etiher gossypol reacting with the substrate and blocking the action of the enzyme or by combining with the enzyme itself.

Smith (1972) showed a decrease in weight gain in weanling rats accompanied a rise in the amount of bound gossypol in the diet. The binding of free gossypol with lysine occurred rapidly at high cooking temperatures (Bressani and Elias, 1968) as well as during prolonged storage at elevated temperatures and humidity (Yannai and Zimmerman, 1970).

2.6 GOSSYPOL: BENEFICIAL PROPERTIES

While gossypol is undesirable economically as far as its presence in food is concerned, it has a number of pharmacological uses. Gossypol has been used as an appetite depressing agent (Berardi and Goldblatt, 1980) although this is illegal in a number of countries due to side effects. It is reportedly used in China as an antifertility agent (Berardi and Goldblatt, 1980). Evaluation of semen produced by more than 4,000 healthy men receiving gossypol suggested it may act by blocking spermatogenesis. An alcohol extract of gossypol from cotton root bark has long been used as an antihemorrhagic agent (Boatner, 1948). The thiosemicarbazone derivative of gossypol (amizon) and isonicotinoylhydrazone derivative of gossypol (ftivazide) are recognized

as important antitubercular drugs (Sadykov, 1965). In addition to these effects Jolad et al. (1975) reported gossypol had antitumor activity. Antiviral activity has also been observed with gossypol (Dorsett et al., 1975). Gossypol has also been found by a number of researchers to serve as an antioxidant and stabilizer for vinyl compounds against polymerization (Lea et al., 1980; Pack et al., 1954).

2.7 EXTRACTION OF FREE GOSSYPOL

A variety of solvent mixtures have been used to extract gossypol. The exact amount of extractable or free gossypol depends on the nature of the solvent used. The following solvents appear to extract similar gossypol derivatives. These include acetone:water (70:30) recommended by Pons and Guthrie (1949) and ethanol:water:ether (57:27:17) by Smith (1946) to extract free gossypol. The acetone:water system was adopted by the American Oil Chemists' Society as it extracted minimal amounts of lipid and caused negligible hydrolysis of bound gossypol. In addition to these two solvent systems, a 2-butanone-aniline-water (90:10:0.5) solvent system was proposed by Storrher and Holly (1954) which appeared to extract similar gossypol derivatives to aqueous acetone (Table 2).

2.8 HYDROLYSIS AND EXTRACTION OF TOTAL GOSSYPOL

Total gossypol cannot be extracted with 70% acetone as "bound" gossypol must be released by hydrolysis from its binding with protein. This is accomplished by acid hydrolysis or hydrolysis with 3-amino-1-propanol and subsequent extraction with a 40:60 mixture of hexane:isopropyl alcohol (Admasu and Chandravanshi, 1984). Bound gossypol is determined as the difference between total and free gossypol values.

Table 2

[1]Comparison of Two Ketone Solvents for Extraction of Free Gossypol

Cottonseed Products	% Free Gossypol	
	Aqueous Acetone	2-Butanane-aniline-water (90:10:0.5)
Hexane-extracted cottonseed kernels	0.44	0.45
Hydraulic-pressed cottonseed meals	0.80	0.82
Screw-pressed cottonseed meals	0.013	0.018

[1]Pons (1977)

2.9 MEASUREMENT OF GOSSYPOL

A number of methods are available for estimating gossypol. Of these, the spectrophotometric methods are the most widely used. These methods include:

1. Reaction of gossypol with aniline to form dianilino-gossypol chromophore with an absorption maximum at 440 nm.
2. Reaction of gossypol with para-anisidine to form a colored complex in the presence of acetic acid with an absorption maximum at 447 nm.
3. Reaction of gossypol with phloroglucinol in strong acid to form a colored complex with an absorption maximum at 550 nm.

The relative sensitivity of these methods are summarized in Table 3. Based on the spectral characteristics of these methods, they all appear to be fairly sensitive with phloroglucinol being the most sensitive followed by p-anisidine and aniline. Nevertheless studies by Storherr and Holly (1954) and Pons (1977) showed all three methods gave comparable results. The American Oil Chemists' Society recommends the aniline procedure for measuring gossypol. A new spectrophotometric method was recently introduced by Admasu and Chandravanshi (1984) for measuring gossypol based on reaction with 3-amino-1-propanol and the subsequent formation of a colored complex with iron III. This method appears to be sensitive compared to the aniline method. Stipanovic et al. (1984) showed that false positive readings were obtained when measuring gossypol by either the aniline or phloroglucinol methods due to interference by oxidation products of hydroxylated unsaturated fatty acids and triglycerides. These researchers recommended extreme caution should be taken when measuring seeds of low gossypol content.

Table 3

Comparison[1] of Different Spectrophotometric Methods for Measuring Gossypol

Reagent and Reference	Maximum Absorption nm	Absorptivity	Relative[2] Sensitivity %
None, only Gossypol	360	39.39	42
Aniline	440	71.20	76
Para-anisidine	447	81.6	87
Antimony trichloride	520	6.6	7
Phloroglucinol	550	94.00	100

[1]Pons, 1977

[2]Relative to phloroglucinol as 100%

Pons (1977) reported that the spectrophotometric methods could detect around 50-100ppm of gossypol. This compares to the gas liquid chromatography method by Raju and Cater (1967) and the HPLC method described by Abou-Donia, et al. (1981) both of which are 50-100 times more sensitive. Nevertheless the limited laboratory facilities in developing countries make the colormetric methods particularly useful.

2.10 REMOVAL OF GOSSYPOL

As reviewed briefly in Section 2.2 gossypol can be removed by a variety of organic solvents. Pons and Eaves (1971) patented a process using aqueous acetone to reduce the free gosyypol content to 0.01-0.02%. A sequential extraction of cottonseed flour using aqueous acetone followed by dry acetone by Damaty and Hudson (1975) reduced gossypol in cottonseed flour to 0.01-0.03% and 0.26-0.36% for free and total gossypol respectively. Major problems associated with the use of acetone were the undesirable flavors and odors arising from the decomposition of sulfur-containing amino acids to hydrogen sulfide. The latter condenses with acetone forming mesityl oxide which gives rise to so-called "catty odor" (Alyevand et al., 1967). A butanol-hydrochloride acid solution was used by Canella and Sodini (1977) to remove gossypol from cottonseed meal, however the level of free gossypol remaining (0.07%) was still higher than that recommended (0.045%) for use of cottonseed products in foods. Cherry and Gray (1981) reduced the free and total gossypol in hexane defatted cottonseed meal by extracting with methylene chloride using the liquid cyclone process. This process normally produces two fractions. A cottonseed flour (overflow fraction) containing 0.2% and 0.6% of total and free gossypol and a large underflow fraction in which free and total gossypol is high and suitable for animal feed only

(Gardner et al., 1976). Cherry and Gray (1981) however, obtained an underflow fraction of glanded cottonseed meal in which the free and total gossypol was reduced from 2.6% and 3.4% to 0.013% and 0.15% respectively. The level of free gossypol in foods was still above the acceptable 0.045% for use in foods. While this method had considerable promise the expense involved using this method made this process uneconomical.

A recent study by Rahma and Narasinga Rao (1984) attempted to remove gossypol from glanded cottonseed using (A) hexane, (B) a 1:1 mixture of isopropanol and hexane and (C) acetone followed by isopropanol:hexane (1:1) mixture. Of the three solvent systems used B produced cottonseed with lowest free gossypol level (0.069%) without altering the functional properties of the protein. This final level was still higher than the 0.045% recommended for use of cottonseed products in food. A mixed solvent extraction consisting of 20-30% (by weight) of ethyl alcohol together with commercial hexane was shown by Liu et al. (1981) to reduce the level of free gossypol to 0.32-0.55%. In addition to reducing the residual oil in the meal to around 0.5% any aflatoxins were also removed by this process. It is obvious from the various studies cited that different degrees of success were obtained in removing gossypol. However a major drawback to the use of organic solvents is the residual remaining in addition to any potential harmful effects.

Kadan et al. (1979) used air-classification of defatted cottonseed flours to produce a low-gossypol, edible cottonseed flour. Several milling methods were examined and of these methods using the fixed hammer disintegrator produced a low gossypol edible product. The yield of this fraction could be increased by heating which lowered the amount of free gossypol by converting to bound form. A cost analysis of the production

of edible cottonseed flour by air classification of glanded cottonseed by Decossas et al. (1982) showed a capital investment of 4 to 6 million dollars was required for a 25 ton/day or 50 ton/day plant. These researchers were confident that the price the air classified cottonseed flour would be competitive with that of soy protein concentrate.

Del Valle et al. (1986) recently prepared low free gossypol and high available lysine cottonseed/soybean blends. By combining 50% defatted cottonseed meal with 50% extruded soybean flakes a low cost edible product was obtained with similar nutritional properties to that of full-fat soy flour. Using this combination the amount of high priced soybeans imported by many developing countries could be reduced by incorporating the cottonseed meal as a low-cost extender.

Many of the methods discussed involve considerable capital investment or the use of organic solvents. A rather novel approach was proposed by Yannai and Bensal (1983) in which cottonseed was treated with soybean gums using heat treatment. These researchers incorporated 3-5% gums into the cottonseed meats, added water to bring moisture content to 20% and adjusting the pH to 7.0 with sodium hydroxide. Cottonseed meats heated at 100^{o}C for 5 minutes in the presence of phospholipids (gums) were superior to the corresponding meats heated at 110^{o}C for 5 minutes in the absence of phospholipids. This was reflected by a lower level of free gossypol and higher available lysine. This was attributed to the ability of the free amino group in the phospholipids to bind with gossypol and thus lower its tendency to bind with lysine.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Cottonseed flour, crystalline choline bitartrate and 2-amino ethanol (ethanolamine) bases were purchased from Sigma Chemical Co., St. Louis, Missouri. Gossypol acetic acid was generously provided by Dr. K.J. Carpenter, Department of Nutritional Sciences, University of California, Berkeley.

The following reagents were used for chemical analyses: Titanium tetrachloride, aniline and para-anisidine (Fisher Scientific Co., Fair Lawn, New Jersey), thiourea (Baker Chemical Co. Phillipsburg, New Jersey) and 3-amino-1-propanol (Fluka AG, Chemischo Fabrik CH-9470, Buchs, West Germany). Measurement of lysine by the dye binding method involved Crocein Orange G (dye) purchased from Sigma Chemical Co., St. Louis, Missouri.

3.2 METHODS

3.2.1. Extraction of Free Gossypol from Cottonseed

Free gossypol was extracted according to the method of Pons and Guthrie (1949):

Extraction Procedure:

1. Weigh approximately 0.3g cottonseed flour into a glass-stoppered 250 ml erlenmeyer flask and record the exact weight.
2. Gently place a stirring bar into the flask and pipet 50 ml of 70% aqueous acetone.
3. Stopper the flask and stir the mixture at room temperature for 1 hour on an Orning PC 353 Stirrer.

4. Filter contents through a funnel containing a Whatman No. 2 filter paper, into a second erlenmeyer flask.
5. Discard the first 10 ml. of filtrate and place a watch glass over the funnel to reduce evaporation. The filtrate contains free gossypol.

3.2.2. Extraction of Total Gossypol from Cottonseed

Total gossypol was extracted according to the method of Pons et al. (1958).

Extraction Procedure:

1. Weigh approximately 0.3g cottonseed flour and transfer to a 50 ml volumetric flask, recording the exact weight.
2. Add 10 ml of a complexing agent to the sample. (Complexing agent: Mix 2ml of 3-amino-1 propanol with 10 ml of glacial acetic acid. Cool the solution to room temperature and dilute to 100 ml with dimethyl formamide in a volumetric flask.)
3. Heat in a boiling water bath for 30 minutes and place marbles on top on the flasks to reduce evaporation.
4. Cool mixture to room temperature, and adjust the volume with hexane-isopropyl alcohol (40:60). Mix well.
5. Filter the final mixture through a funnel containing a Whatman No. 2 filter paper. The filtrate contains total gossypol.

3.2.3. Measurement of Free Gossypol

The following methods were used to measure free gossypol.

3.2.3.1. Titanium Tetrachloride ($TiCl_4$) Method

The titanium method was adapted to measure free and total gossypol.

Procedure:

1. Accurately weigh 2.5 mg of gossypol and transfer to a 250 ml volumetric flask. Dissolve the gossypol in 10 ml acetone, and adjust the final volume with acetone. This provides a stock solution of gossypol (10 ppm) for use as a standard curve.
2. Further dilute the stock solution as follows in 15 ml test tubes to give a concentration range of gossypol from 0-10 ppm:

Stock (ml)	Acetone (ml)	ppm
0	5	0
1	4	2
2	3	4
3	2	6
4	1	8
5	0	10

3. Pipet duplicate 5 ml aliquots of the free gossypol extract into 15 ml test tubes (samples).
4. To each sample and stock solution add 0.2 ml $TiCl_4$ reagent (20% in conc.HCl) and mix on a vortex.
5. Read the absorbance of the samples on a Unicam SP 800 ultraviolet spectrophotometer, to establish the maximum absorbance of the $TiCl_4$-gossypol complex. (Maximum absorbance was at 490 nm).
6. Run standards from (1-10 ppm). Measure the standards using a Pye Unicam SP6-300 spectrophotometer at 490 nm.

7. Since free gossypol is extracted with 70% acetone, prepare a standard curve using gossypol (1-10 ppm) in 70% acetone.

3.2.3.2. Aniline Method

The method was as described in the Official and Tentative Methods of the American Oil Chemists' Society Ba 7-58.

Procedure:

1. Prepare a 20 ppm standard gossypol solution by dissolving 0.001g of gossypol acetic acid in 50 ml of 70% aqueous acetone.
2. Pipet duplicate 2 ml aliquots of free gossypol cottonseed extract and standard into 25 ml volumetric flasks.
3. To one of the aliquots, add 2 drops of 10% thiourea and 1 drop of 1.2N HCl; dilute to volume with 90% isopropyl alcohol as reference samples (10% thiourea solution: Dissolve 10g of reagent grade thiourea in distilled water, and dilute to 100 ml with distilled water.)
4. To the other aliquot, add 2 drops of 10% thiourea, 1 drop of 1.2N HCl and 2 ml aniline.
5. Prepare a reagent blank by mixing 2 ml of 70% acetone, 2 drops of 10% thiourea and 2 ml aniline in a 25 ml volumetric flask.
6. Heat samples (from Step 4) and reagent blank (Step 5) in a boiling water bath for 30 minutes.
7. Remove from bath, and add approximately 10 ml of 80% isopropyl alcohol. Mix. Cool to room temperature and dilute to volume with 80% isopropyl alcohol.

8. Read absorbance of reference samples at 440 mu against 80% isopropyl alcohol, in a Pye Unicam SP6-300 spectrophotometer. Read absorbance of reagent blank against 80% isopropyl alcohol (should be less than 0.022). Read absorbance of samples against reagent blank.

3.2.3.3. Para-Anisidine Method.

The method used was described by Pons and Guthrie (1949).

Procedure:

1. Prepare 20 ppm standard gossypol solution by dissolving 0.0011g of gossypol acetic acid in 50 ml of 70% acetone.
2. Pipet duplicate 2 ml aliquots of cottonseed extract, standards and reagent blank (70% acetone) into 25 ml volumetric flasks.
3. To one of the aliquots add 3 ml of para-anisidine-alcohol-acetic acid, and dilute to volume with ethyl alcohol as reference samples. (Alcohol-acetic acid: In a 50 ml volumetric flask, dissolve 0.5g para-anisidine in 80% isopropanol. Add 1 ml of glacial acetic acid and adjust to volume with 80% isopropanol.
4. To the other aliquot, add 3 ml of para-anisidine-alcohol-acetic acid reagent.
5. Stopper the flasks loosely and heat in a $60^{o}C$ water bath for 30 minutes.
6. Cool and dilute to volume with 95% ethyl alcohol.
7. Read the absorbance of the samples at 447 nm using a Pye Unicam SP6-300 spectrophotometer against the sample blank. Read also the absorbance of the references against the reference blank.

3.2.4. Measurement of Total Gossypol.

Three methods were used to determine total gossypol: Titanium tetrachloride, iron an aniline methods. The absorbance of all samples were read in a Pye Unicam SP 6-300 spectrophotometer. The procedure for the titanium method was the same as for the free gossypol measurement. The only difference is that the sample measured contained total gossypol in which bound gossypol was released from cottonseed with the complexing reagent (section 3.2.2.).

3.2.4.1. Aniline Method

This method was proposed by Pons et al. in 1957.

Procedure:

1. Pipet duplicate 2 ml aliquots of total gossypol cottonseed extract, standards and reagent blank into 25 ml volumetric flasks.
2. Dilute one of the aliquots to volume with isopropyl alcohol hexane (60/40) (references).
3. To the other aliquot, add 2 ml aniline, and heat in boiling waterbath for thirty minutes.
4. Cool and dilute to volume with hexane isopropanol (40:60).
5. Read absorbance of samples at 440 nm against the sample blank. Read absorbance of references against the reference blank.

3.2.4.2. Iron Method.

The method described by Admasu and Chandravanshi (1984) was used.

Procedure:

1. Prepare a 20 ppm gossypol standard solution by dissolving 0.0011g of gossypol acetic acid in 50 ml of 70% aqueous acetone using 70% acetone as reagent blank.
2. Into 20 test tubes, pipet in duplicate 3 ml aliquots of both standard solutions and gossypol extract against the 70% acetone as blank.
3. Add 1 drop of 5M hydrochloric acid, and 1.5 ml of the iron reagent to each test tube. (The iron reagent is a 1.79×10^{-2}M iron III solution: Weigh 0.7232 ferric nitrate. $9H_2O$ onto a watch glass and transfer into a 100 ml volumetric flask. Add 10 ml of hexane-isopropanol (40/60) and 5-10 drops of conc. HCl. Shake until dissolved. Make up to volume with hexane-isopropanol).
4. Mix well on a vortex type mixer and allow to stand for five minutes.
5. Add 0.3 ml of distilled water and 2 ml of hexane-isopropanol (40/60) to each tube, and mix quickly on a vortex mixer.
6. Set a Pye Unican SP6-300 Spectrophotometer at 620nm.
7. Use the blank to set the instrument at an absorbance of zero.
8. Read the absorbance of the standards and samples.

For all the colormetric methods, the amount of gossypol was calculated according to the following formula.

wt of gossypol (mg) in sample =

$$\frac{\text{abs.of sample}}{\text{abs. std.}} \times \text{std.conc.(ppm)} \times \text{vol.of std.} \times \frac{\text{total extract vol}}{\text{extract aliquot vol}} \times \frac{1}{1000}$$

3.2.5. Determination of Available Lysine

The method used to determine available lysine was adapted from Anderson et al. (1984).

Procedure:

1. Weigh cottonseed flour onto weighing paper and transfer to a 15 ml screw-top tube with a teflon lined cap (use 35 mg for A and 46 mg for B).
2. To standard tubes add 600 ul of ethanol. To sample tubes add 600 ul ethanol or 600 ul of gossypol in ethanol in the concentration required.
3. Add 1 ml acetate buffer to all tubes. (Acetate buffer: 16.4 g sodium acetate trihydrate dissolved in 83.6 ml distilled water.)
4. Place tubes in a test tube rack set on top of a Gallenkamp water bath shaker oscillating at 150 rpm for 5 minutes.
5. Cap tubes and heat at required temperature and time in an Eberback water bath shaker.
6. Cool tubes for 5 minutes in cold water.
7. Add 2 ml of ether to tubes, cap tightly and mix on vortex for 15 seconds.
8. Centrifuge tubes for 2 minutes at full speed in a model CS International Centrifuge.
9. With a pasteur pipet, remove ether layer and discard.
10. Repeat steps 7-9.
11. Remove traces of ether by placing tubes in a vacuum oven for 5 minutes using a vacuum but no heat.
12. To standards and A tubes add 0.075 ml water. To B tubes add 0.075 ml propionic anhydride.

13. Cap tubes and place in a test tube rack set on top of a Gallenkamp water bath shaker oscillating at 150 rpm for 15 minutes.
14. Pipet exactly 10 ml of dye solution in all tubes.

    Dye solution: Place a stirring bar in a 1 litre beaker with 500 ml of distilled water. Place the beaker on a Corning Stirrer (PC-353) in a fume hood and let it stir slowly while adding 20 grams of oxalic acid dihydrate, 3:4 grams of potassium dihydrogen phosphate and 1.36 g of crocein orange G (dye). When everything has dissolved, quantitatively transfer the content of the beaker to a 1 litre volumetric flask, and bring the flask to the mark with several rinsings from the beaker.
15. Cap tubes tightly and place tubes horizontally in a metal basket. Place basket in the water bath shaker oscillating at 110 rpm for 60 minutes.
16. Transfer the contents of the tubes to 12 ml polypropylene centrifuge tubes. Mix contents thoroughly to ensure as much of the precipitate is transferred.
17. Centrifuge in the B20 centrifuge for 6 minutes at 10,000 RPM.
18. Using a positive displacement pipet, transfer 100 ul of supernatant to 20 ml test tubes.
19. Using a pipetter bottle add 9.9 ml of dye-binding buffer prepared in an identical manner to that of step 4, omitting the dye.
20. Mix tubes on vortex for 5-10 sec.
21. Read absorbance on Pye Unicam SP6-300 spectrophotometer at 475 nm using water to zero the instrument.

Calculations:

Subtract the absorbance of the samples from that of the standard to give the change in absorbance of the samples. The change of absorbance (4) of the samples is then applied to the following formula:

$$\frac{389 \times \text{abs. sample}}{\%\ \text{Protein} \times \text{abs.std.} \times \text{sample wt(g)}} = \frac{\text{mmoles bound}}{100\text{g Protein}}$$

A values - B values=mmoles lysine bound/100g Protein

22. The standard absorbance corresponds to a value of 3.89 mmoles of dye/litre. To be valid, sample absorbances should correspond to a dye concentration of from 1.3-1.7 mmoles/litre or the analysis must be repeated using an adjusted sample weight.

3.2.6. Studies on Cottonseed Model Systems

The basic model system consisted of cottonseed flour (35 mg for A reading and 46 mg for B reading) in which the pH was adjusted to 8.0 with sodium acetate butter (pH 8.0) as described in 3.2.5. The following parameters were examined:

3.2.6.1. Effect of Temperature on Lysine Availability.

9.25mg and 11.5mg of gossypol were added to the A and B samples (in Step 1) respectively. The samples were heated to 70, 80, 90 and 100 degrees for 30 minutes, to find the optimum temperature at which available lysine is reduced to a minimum in cottonseed flour.

3.2.6.2. Effect of Heating Time on Lysine Availability

The samples described above were heated for 0, 15, 30 and 60 minutes time intervals at the temperature determined in 3.2.6.1. to establish the optimum time needed to reduce lysine availability to a minimum in cottonseed.

3.2.6.3. Effect of Gossypol Level on Lysine Availability

Increasing amounts of gossypol were added to the cottonseed flour corresponding to the following ratios. This was to determine the optimum level of gossypol required to reduce lysine availability in cottonseed flour to a minimum.

Weight of gossypol		
A reading	B reading	Gossypol:Cottonseed ratio
9.25	11.50	1:4
4.625	5.750	1:8
2.313	2.875	1:16
1.156	1.439	1:32
0.578	0.719	1:64

3.2.6.4. Effect of bases (choline and ethanolamine) on Lysine Availability

Increasing amounts (0.4, 0.8 and 1.6 molesx 10^{-1} solutions) of each base were added to the cottonseed flour-gossypol model systems immediately following addition of gossypol. The potential of these bases in reducing the deleterious effects of gossypol on lysine availability was examined.

3.2.6.4.1. Effect of Choline on Lysine Availability.

172.5mg of choline bitartarate was dissolved in 8.5ml of sodium acetate. 1ml of the solution, each containing 20.3mg of sodium acetate was added to eight of the tubes, instead of sodium acetate buffer (Step 3). The weight of the choline bitartrate was reduced to 86.23mg in 8.5ml of sodium acetate, and then doubled to 345.1mg in 8.5ml sodium acetate.

3.2.6.4.2. Effect of Ethanolamine on Lysine Availability

10.3ml of sodium acetate was added to two drops of ethanolamine (2-aminoethanol) weighing 50.1mg. 1ml of the solution (containing 4.86mg ethanolamine) was added to each tube (Step 3) instead of sodium acetate. The weight of the ethanolamine was reduced to 23.2 mg (1 drop) in 9.5ml of sodium acetate, each millilitre containing 2.44mg ethanolamine. The weight of the ethanolamine was finally doubled to 100.4 mg (4 drops) in 10.29ml of sodium acetate (9.76 mg in each millilitre).

3.2.6.4.3. Statistical Analyses

A one way analysis of variance (ANOVA) was used on the studies using the cottonseed flour model system to determine the effect of (i) gossypol level (ii) time and temperature and (iii) bases (choline and ethanolamine) on lysine availability.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 QUANTITATIVE DETERMINATION OF GOSSYPOL

The spectrophotometric data for the gossypol-titanium complex is shown in Table 4. Molar absorptivity is only approximate. The colored complex was stable for several hours and yielded a Linear Beer's law plot as indicated by the coefficient of determination (r^2) value.

Comparison of the methods described for measuring gossypol is shown in Figure 2. All samples were determined in duplicate with less than 1-3% variation between them. Based on the slope of these curves, the para-anisidine method was the most sensitive method followed closely by the aniline method. Both of these procedures were twice as sensitive as the titanium method and twenty times as sensitive as the iron method. The titanium method is limited, however, by the interference of phenolic compounds as discussed later. The iron method, although the least sensitive, nevertheless is the simplest procedure and suitable for measuring levels of gossypol above 10 ppm. This is evident in Figure 3 which shows a linear relationship for gossypol (0-80 ppm) using the iron procedure.

Measurement of the free and total gossypol in extracts of cottonseed flour, used in this study, is summarized in Table 5. The results with the titanium reagent were markedly higher due to the interference by other phenenolic compounds (Eskin et al. 1978). Further refinement would be required to remove these phenols before this method could be used to measure gossypol. Measurement of total gossypol using the iron method was similar to that of the aniline method (Table 5) although the former method is simpler and safer to use. The level of

Table 4

Spectrophotometric Data for Gossypol-Titanium Tetachloride

Compound	Max. nm.	Molar Absorptivity	Measured at nm.	Beer's law plot (r^2)
Gossypol	490	3.8×10^4	490	1.00

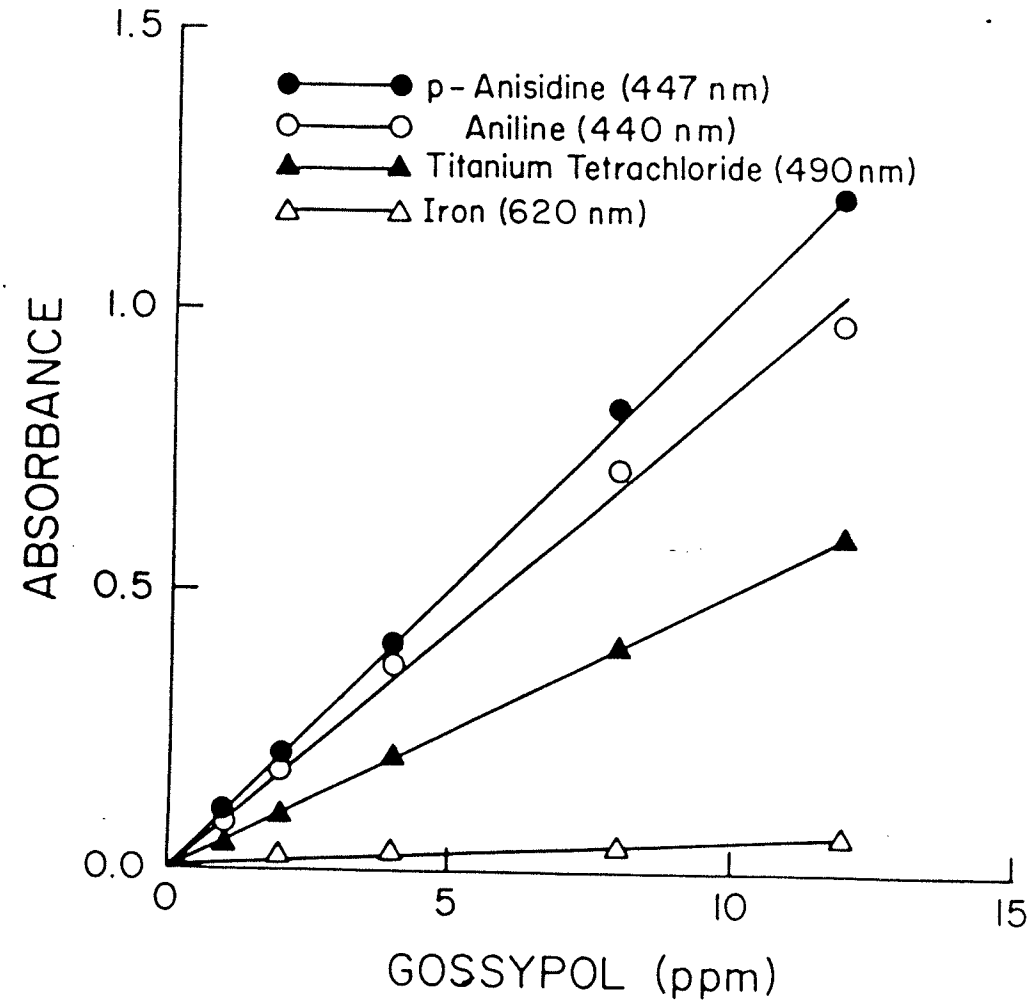

Figure 2
A Comparison of Four Colorimtric Methods
for Measuring "Free" Gossypol

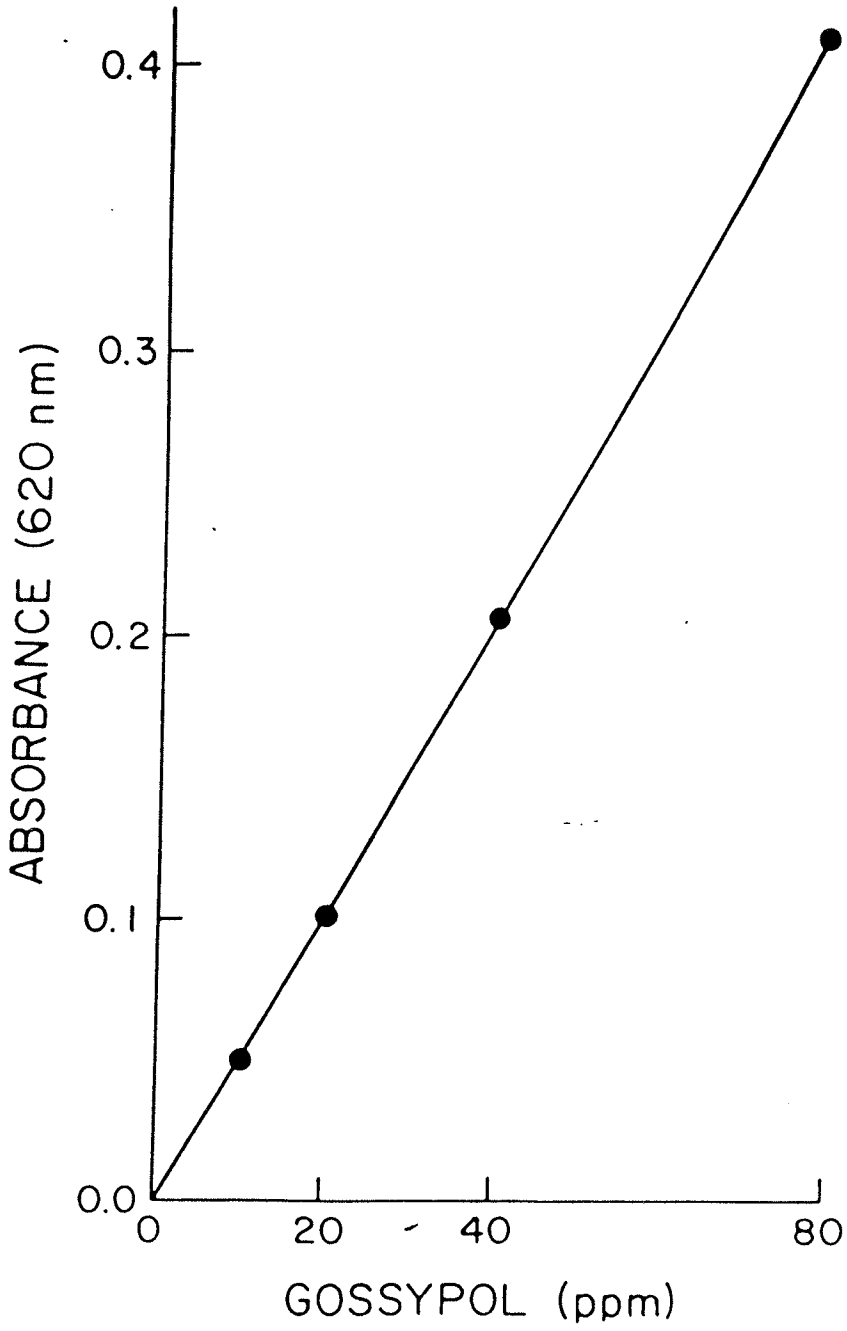

Figure 3
Standard Curve for Gossypol (0-80ppm)
Using the Iron III Method

Table 5

Free and Total Gossypol of Cottonseed Flour using Different Analytical Methods

Methods	Gossypol Content (%)			
	Free	S.D.	Total	S.D.
Titanium Tetrachloride	0.28±0.01		1.02±0.08	
Aniline	0.02±0.00		0.73±0.06	
Para-Anisidine	0.01±0.00		N.D.	
Iron	N.D.		0.83±0.07	

N.D. = Not Determined.

free and total gossypol in the cottonseed flour was well below the recommended limits of 0.06% of free gossypol and 1.2% total gossypol for use in human foods set out by the Protein Advisory Group of the United Nations (Jones, 1979).

4.2 EFFICIENCY OF GOSSYPOL EXTRACTION

Cottonseed flour with and without added gossypol (50 and 100 ppm) was extracted using the method of Pons and Guthrie (1949). The amount of gossypol in the extract was measured by the aniline method. The recovery of gossypol following the extraction process was 100% (Table 6) which confirmed no losses occurred during the extraction procedure.

4.3 STUDIES ON MODEL SYSTEMS:

4.3.1. Effect of time and temperature.

The protein content of conttonseed flour, as measured by the Kjeldahl method was 57.12% using a conversion factor of 6.25. The influence of time and temperature on lysine availability of cottonseed flour incubated with gossypol in a ratio of 16:1 is shown in Figure 4. Irrespective of the temperature used (70,80,90 and 100C) a rapid decrease in lysine availability occurred during the first 15 minutes, which levelled off during the subsequent time period. As the temperature was raised, lysine availability decreased significantly with time ($p<0.05$). No significant difference was observed between 70^{o}C and 80^{o}C although these were significantly ($p<0.05$) higher than model systems heated to 90 and 100C. A significantly ($p<0.05$) lower level of lysine availability was evident for the model system heated at 100C compared to that heated at 90C. These differences were less marked after 15 and 30 minutes. With the exception of the 70C treatment lysine

Table 6

Percentage Recovery of Free Gossypol[1] in Cottonseed Flour

Sample	Free Gossypol (ppm)	Recovery (%)
Cottonseed Flour	241±10	
Cottonseed Flour + 50 ppm Gossypol	292±10	100
Cottonseed Flour + 100 ppm Gossypol	342±12	100

[1]Aniline Method.

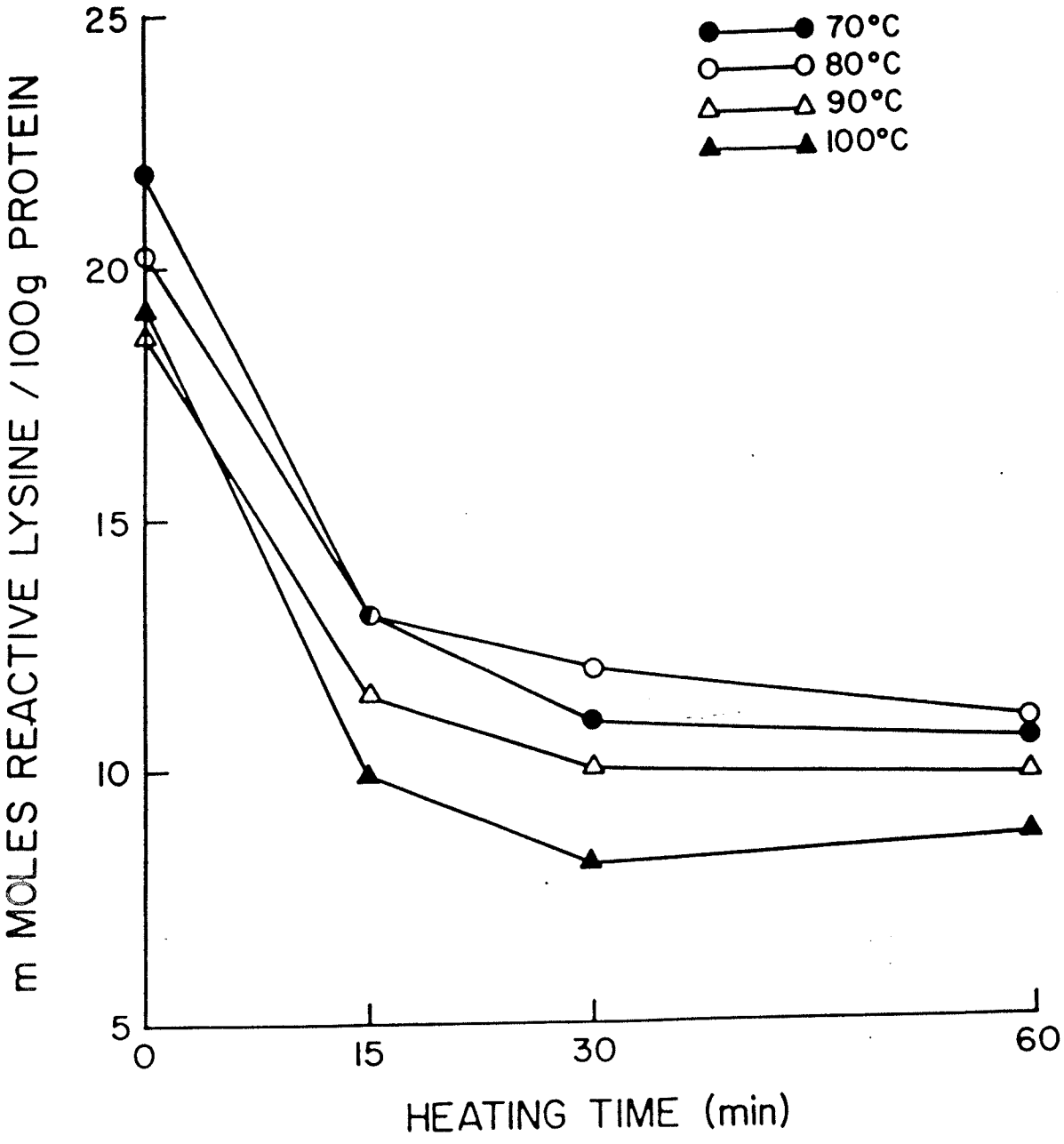

Figure 4
Effect of Time and Temperature on Available Lysine in a Cottonseed Flour:Gossypol (1:16) Model System

availability levelled off at around 30 minutes. A combination of 90C for 30 minutes was adopted as this was considered to provide an adequate reduction in lysine availability.

4.3.2. Effect of Gossypol Level

Effect of the addition of increasing levels of gossypol on lysine availability in cottonseed flour model systems is shown in Figure 5. Increasing gossypol levels to provide ratios of gossypol:cottonseed flour from 1:64 to 1:4 significantly ($p<0.05$) decreased lysine availability. This interaction of gossypol with protein involves the formation of Schiff's bases in which the formyl groups of gossypol condense with the epsilon amino groups of lysine (Martinez and Frampton, 1958; Conkerton and Frampton, 1959). Tilman and Kruse (1962) found protein digestibility was significantly lowered when soybean meal autoclaved with gossypol was fed to sheep. This was attributed to poorer digestibility due to inhibition of proteolytic enzymes by the presence of bound gossypol on the epsilon-amino groups of lysine. Further studies by Tanksley et al. (1970) showed inhibition of pepsinogen by gossypol once the protein had undergone conformational changes. The gossypol-containing portion of pepsinogen was isolated by Wong et al. (1972) who found it to be a decapeptide from the amino-terminal portion of the protein and heptapeptide from the carboxyl terminus. Both peptides were cross-linked by gossypol through the epsilon amino groups of lysine. Damaty and Hudson (1979) found that excess free gossypol followed by heat treatment enhanced the formation of

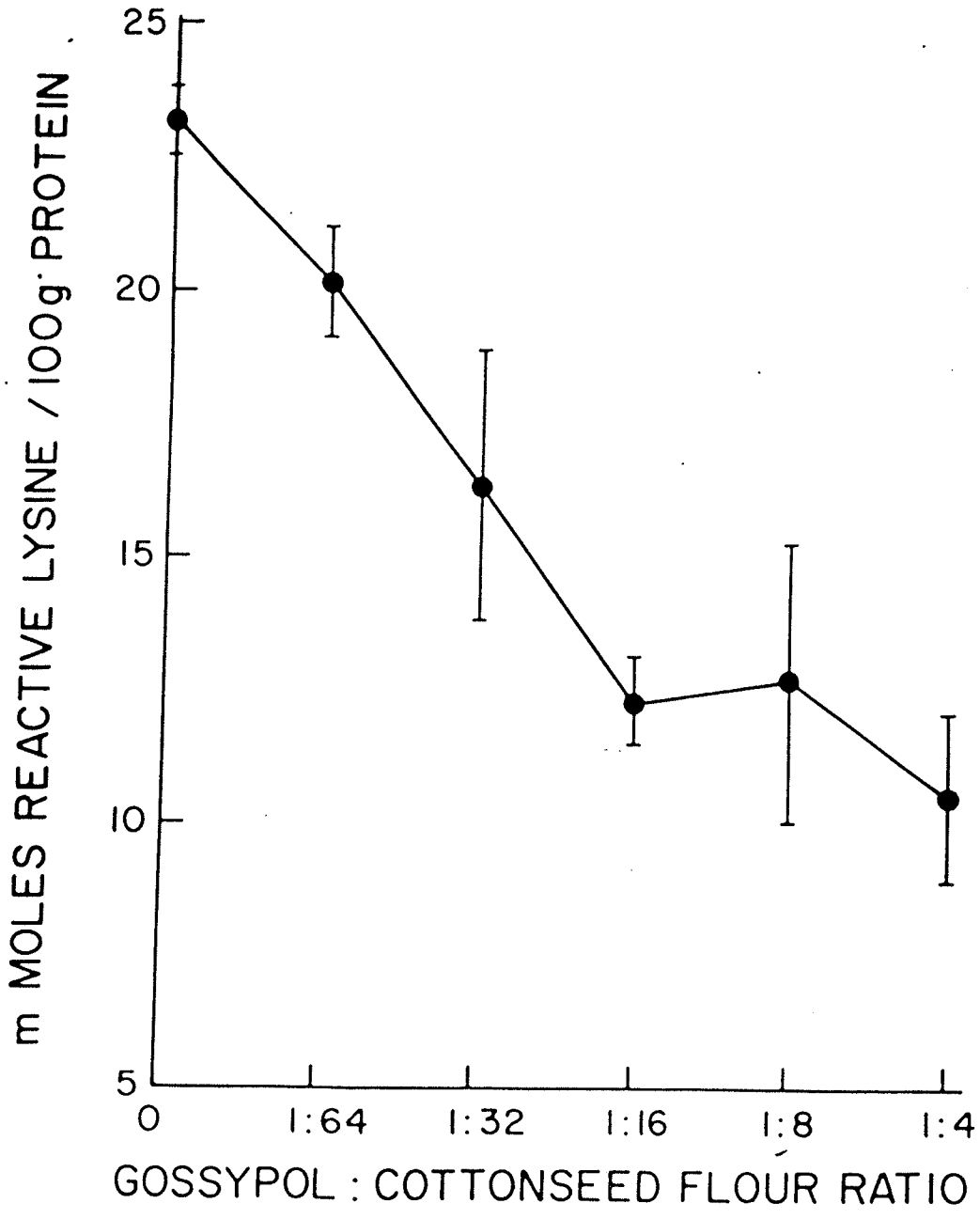

Figure 5
Effect of Increased Cottonseed Flour:Gossypol Ratios on Available Lysine in Model Systems Heated at 90°C for 30 minutes

indigestible insoluble residue. These researchers autoclaved high-gossypol cottonseed flour at 120C for 3 hours in open screw-capped bottles and reported as much as 40% reduction in amino acids liberated by proteolytic enzymes compared to the standard. The data obtained from this study clearly demonstrate a dramatic decline in lysine availability as the ratio of gossypol:cottonseed flour increased which is consistent with the study of Damaty and Hudson (1979). A ratio of 1:16 of gossypol:cottonseed flour was used in subsequent model systems as this produced a decrease of approximately 34.2% in lysine availability.

4.3.3. Effect of Bases (Choline and Ethanolamine)

The interaction of gossypol with protein, as discussed previously involves the formation of a Schiffs base through condensation of the formyl groups of gossypol with the epsilon amino group of lysine. Yannai and Bensal (1983) examined the use of gums from soybean to convert any free gossypol into the bound form. These gums were rich in phospholipids including phosphatidyl choline and phosphatidyl ethanolamine. The amino groups in these phospholipids were thought to compete with the protein for free gossypol. Cottonseed meals pre-treated with these gums were superior to the corresponding untreated cottonseed meals by having a higher lysine and chemical index. When fed to rats the untreated cottonseed meal also resulted in a better growth rate and PER. This improvement was twofold, firstly it reduced toxicity by binding the free gossypol, and secondly it left lysine with more free epsilon groups thus retaining a higher nutritional value. Rather than use phospholipids the potential for the corresponding bases, choline and ethanolamine were examined.

4.3.3.1 Effect of Choline

The addition of three different levels of choline (0.4, 0.8 and $1.6x10^{-1}M$) to cottonseed flour:gossypol model systems is shown in Table 7. One way analysis of variance for groups with unequal replication detected a significant difference ($p<0.05$) in lysine availability for treatments (Table A-1). One way analysis of variance for each level of choline added showed a significant ($p<0.05$) improvement in lysine availability for $0.8x10^{-1}M$ and $1.6x10^{-1}M$ choline (Tables A-3, A-4). This was responsible for the corresponding 21.8 and 17.6% improvement in lysine availability. The addition of $0.4x10^{-1}M$ choline did not significantly improve the available lysine in the cottonseed flour:gossypol model systems (Table A-2).

4.3.3.2 Effect of Ethanolamine

The addition of three levels of ethanolamine on lysine availability is shown in Table 8. A much greater improvement in lysine availability was evident compared to choline treatment. A one way analysis of variance for groups with unequal replication revealed significant ($p<0.05$) treatment effects (Table A-5). Further one way analysis of variance for each of the individual levels of ethanolamine (0.4, 0.8 and $1.6x10^{-1}M$) added showed a significant ($p<0.05$) improvement in lysine availability for model systems to which $0.8x10^{-1}M$ and $1.6x10^{-1}M$ levels of ethanolamine were added (Tables A-6, A-7

Table 7

Effect of Choline on the Reaction Between Gossypol and Cottonseed Protein in Model Systems

Model System	Available Lysine (m moles/100 g protein)	% Decrease in lysine
Control	22.07±1.41	
+ gossypol	13.55±1.57	38.60
+ gossypol + 0.4×10^{-1} M choline	14.37±1.20	34.89
+ gossypol + 0.8×10^{-1} M choline	16.50±1.26	25.23
+ gossypol + 1.6×10^{-1} M choline	15.93±0.99	27.82

Table 8

Effect of Ethanolamine on the Reaction between Gossypol and Cottonseed Protein in Model Systems

Model System	Available Lysine (m moles/100 g protein)	% Decrease in lysine availability
Control	22.01±0.79	
+ Gossypol	14.85±1.16	32.53
+ Gossypol + $0.4x10^{-1}$M Ethanolamine	18.16±1.01	17.76
+ Gossypol + $0.8x10^{-1}$M Ethanolamine	18.53±1.13	15.81
+ Gossypol + $1.6x10^{-1}$M Ethanolamine	19.33±1.13	12.18

and A-8). This corresponded to an increase in lysine availability of 24.8 and 30.2%. Even in the system where $0.4x10^{-1}$M ethanolamine was added there was a 22.3% improvement in available lysine. Addition of $1.6x10^{-1}$M ethanolamine produced a lysine availability value of 19.33 mmoles/100g protein which was 12% lower than the control (22.01 mmoles/100g protein). This contrasted with the model system with gossypol which caused lysine availability to decrease by 32% to 14.85 mmoles/g protein. Ethanolamine appeared to be almost twice as effective as choline in its ability to bind gossypol. The marked improvement of ethanolamine over choline may be due to differences in chemical structure and properties. Choline with its tertiary nitrogen may be less accessible in forming a Schiff's base with the formyl groups of gossypol compared to ethanolamine. Whether the differences between these bases to react with gossypol corresponds to the efficiency of the corresponding phospholipids, phosphatidylcholine and phosphatidylethanolamine, remains to be established.

There have been many attempts to reduce gossypol in cottonseed protein including a variety of solvent extractants, acetone or aqueous acetone, acidic butanol, methylene chloride and isopropanol or aqueous isopropanol (Alyevand et al., 1967; Pons and Eaves, 1971; Harris et al., 1947; Cherry and Gray, 1981). Most of these have a number of drawbacks in particular

the presence of solvent residue. The use of gums by Yannai and Bensal (1983) provides an attractive alternative method for reducing the effect of gossypol. This study demonstrated the potential of using the corresponding bases, choline or ethanolamine, for reducing the deleterious effects of gossypol. Of the bases examined, ethanolamine appeared to be more effective than choline in competing with lysine for gossypol. Once bound to these bases gossypol is no longer toxic in addition to the cottonseed protein being of a higher nutritional value as a consequence of the higher levels of available lysine.

CHAPTER 5

CONCLUSIONS

Measurement of gossypol was conducted using the titanium reagent and compared to three published colorimetric methods. A cottonseed flour:gossypol model system was established and the effects of choline and ethanolamine on lysine availability studied.

5.1 MEASUREMENT OF FREE GOSSYPOL USING THE $TiCL_4$

A stable coloured complex was established between titanium tetrachloride ($TiCl_4$) and gossypol with an absorbance maximum at 490 nm. The method was quite sensitive and yielded a linear Beer's law plot. The inability of $TiCl_4$ to measure free gossypol in cottonseed was due to interference by other phenolic compounds present. Of the colorimetric methods examined the most sensitive was para-anisidine followed by aniline with the least sensitive being the recently published iron III method. Nevertheless the simplicity and safety of the iron method made it quite suitable for measuring cottonseed products containing high levels of free gossypol.

5.2 COTTONSEED MODEL SYSTEMS

A model system composed of cottonseed flour:gossypol was established in which the time, temperature and level of gossypol were optimized to obtain a 30-38% reduction in lysine availability. For example heating for 30 minutes at 90C a model system composed of gossypol:cottonseed flour in the ratio of 1:16 reduced lysine availability from around 21-22 mMoles/100 g protein to 12-14 mMoles/100g protein. Addition of 0.4, 0.8 and $1.6x10^{01}$M solutions of choline or ethanolamine to this system increased the amount of lysine available. Ethanolamine appeared to be almost twice as effective as choline in

competing with cottonseed protein for gossypol. Significant ($p<0.05$) increases in lysine availability were observed when $0.8x10^{-1}M$ and $1.6x10^{-1}M$ levels of both bases were added.

5.3 GENERAL SUMMARY AND CONCLUSIONS

The possible application of $TiCl_4$ for measuring gossypol is limited due to the interference of other phenolic compounds. Nevertheless the iron method was quite suitable when dealing with large levels of free gossypol in spite of its relatively low sensitivity compared to the other methods.

Based on the results from this study. Addition of $1.6x10^{0-1}M$ ethanolamine restored the available lysine in cottonseed flour:gossypol model systems to 19 mMole/100g protein which was 12% less than the original level in the cottonseed flour.

Recommendations for further research include the treatment of cottonseed meal with either of these bases for feeding studies with experimental animals to assess the quality of the protein by PER.

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APPENDICES

Table A-1

One way analysis of variance for groups with unequal replication for the effect of different choline levels on lysine availability in cottonseed model systems

Source of Variation	df	MS	F
Treatment	4	120.69**	60.65**
Error	31	61.59	
Total	35		

**p<0.05

Table A-2

One way analysis of variance for the effect of $0.4x10^{-1}$M choline on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	90.36**	70.59**
Error	8	10.21	1.28
Total	11		

**p<0.05

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	22.83	24.49	14.59	13.87

SE = 0.65

LSE (5%) = 0.65 x 4.53 = 2.94

Treatments A = control
B = control + choline ($0.4x10^{-1}$M)
C = control + gossypol
D = control + gossypol + choline ($0.4x10^{-1}$mM)

Table A-3

One way analysis of variance for the effect of $0.8x10^{-1}M$ choline on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	62.40**	132.77**
Error	8	3.77	0.47
Total	11		

**$p<0.05$

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	22.13	23.55	14.00	16.46

SE = 0.40

LSE (5%) = 0.40 x 4.53 = 1.81

Treatments A = control
B = control + choline ($0.8x10^{-1}M$)
C = control + gossypol
D = control + gossypol + choline ($0.8x10^{-1}M$)

Table A-4

One way analysis of variance for the effect of $1.6x10^{-1}$M choline on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	54.97**	48.22**
Error	8	9.11	1.14
Total	11		

**p<0.05

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	21.67	21.52	13.02	15.93

SE = 0.62

LSE (5%) = 0.62 x 4.53 = 2.81

Treatments A = control
B = control + choline ($1.6x10^{-1}$M)
C = control + gossypol
D = control + gossypol + choline ($1.6x10^{-1}$M)

Table A-5

One way analysis of variance for groups with unequal replication for the effect of different ethanolamine levels on lysine availability in cottonseed model systems

Source of Variation	df	MS	F
Treatment	4	59.99**	34.48**
Error	27	1.74	
Total	31		

**p<0.05

Table A-6

One way analysis of variance for the effect of $0.4x10^{-1}M$ ethanolamine on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	21.22**	7.45**
Error	8	2.85	
Total	11		

**p<0.05

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	20.10	21.15	15.10	18.16

SE = 0.97

LSE (5%) = 0.97 x 4.53 = 4.39

Treatments A = control
B = control + ethanolamine ($0.4x10^{-1}M$)
C = control + gossypol
D = control + gossypol + ethanolamine ($0.4x10^{-1}M$)

Table A-7

One way analysis of variance for the effect of $0.8x10^{-1}M$ ethanolamine on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	56.40**	102.55**
Error	8	0.55	
Total	11		

**p<0.05

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	22.24	24.31	14.49	18.43

SE = 0.43

LSE (5%) = 0.43 x 4.53 = 1.95

Treatments A = control
B = control + ethanolamine ($0.8x10^{-1}M$)
C = control + gossypol
D = control + gossypol + ethanolamine ($0.8x10^{-1}M$)

Table A-8

One way analysis of variance for the effect of $1.6x10^{-1}M$ ethanolamine on lysine availability of cottonseed model systems

Source of Variation	df	MS	F
Treatment	3	39.44**	45.33**
Error	8	0.87	
Total	11		

**$p<0.05$

Tukey's Mean Separation

Treatment	A	B	C	D
Means:	21.90	23.33	15.07	19.33

SE = 0.54

LSE (5%) = 0.54 x 4.53 = 2.45

Treatments A = control
B = control + ethanolamine ($1.6x10^{-1}M$)
C = control + gossypol
D = control + gossypol + ethanolamine ($1.6x10^{-1}M$)