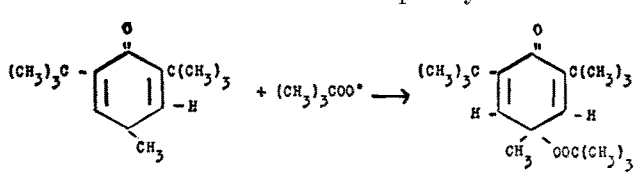
amino groups. Aromatics can supply greater amounts of resonance energy than any other conjugated system. The first free radical found was the triphenyl methyl radical stabilized by the resonance energy of three phenyl groups. The resonance energy of this system has been calculated by L. Pauling and G. W. Wheland (9).

The stability of the triphenyl methyl radical rests on the number of phenyl groups which deactivate the methyl radical by their resonance energy. Many of the effective phenolic antioxidants carry an alkoxy group in the para position to the free phenolic group like methoxy in butylated hydroxyanisole and the heterocyclic part of the coumarans and chromans. These groups have an electron releasing effect on the benzene system, a +M effect, according to C. K. Ingold (10), and thereby decrease the electron deficiency on the site of the odd electron. In addition, the resonance from the 4-site structures of the odd electron diminishes the reactivity of the semiquinone even more. Another corollary of resonance is the shortening of bonds; anything which tends to counteract this will diminish the resonance energy. That is why planarity of the molecule enhances the resonance energy and anything that will strain the bonds of groups attached to the aromatic ring will decrease the resonance energy.

Although 2-tert-butyl-5-methyl-4-methoxyphenol is not as effective as 2-tert-butyl-4-methoxyphenol (BHA), it is nevertheless much better than 2,5-di-tert-butyl-4-methoxyphenol (11). The 5-tert-butyl group acts sterically on the 4-methoxy group, forcing it out of the plane of the aromatic ring, setting up a strain, and diminishing the resonance energy thereby. The effect of the 5-methyl group is much smaller, therefore the inhibitor potency of BHA is much less disturbed. The reactivity of the semiquinone is decreased further by adding a tert-butyl group ortho to the phenolic oxygen carrying the odd electron. Steric hindrance thus created prohibits the approach of any save the smallest atoms, like the hydrogen atom. If the last-mentioned contingency occurs, the inhibitor is reconstituted and could act again. As to the further actions of the semiquinone, T. W. Campbell and G. M. Coppinger (12) have shown for the 2,6-di-tert-butyl-p-cresol that its mesomeric cyclohexadienone free radical can combine with a peroxy free radical:



It is not known whether the semiquinones formed from BHA and the hydroxy-tert-butyl chromans and coumarans will react in the above indicated manner with high reactive radicals and thereby stop another chain reaction. R. H. Rosenwald and J. A. Chenicek (11) suggest dimerization in the ortho position for BHA. As to the fate of the chromans and coumarans, C. Golumbic (13) recognized tocoquinones as the immediate oxidation products of tocopherols in fats. Orthoquinones, namely the chroman-5,6-quinones, were also found but only in autoxidized vegetable fat, never in animal fat.

In view of these experiments in which the oxidation of the antioxidant proceeds by different pathways, even in so closely related substrates as animal and vegetable fats, it is too early to correlate the results C. E. Boozer and G. S. Hammond (14) obtained concerning the fate of the inhibitor when working in simplified systems.

Summary

The inhibitor activity of some 6-hydroxychromans and 5-hydroxycoumarans in gasoline and lard was investigated. The coumarans are the more effective in both substrates. Introduction of a tertiary butyl group ortho to the hydroxyl group increases the activity of 2,2-dimethyl-5-hydroxycoumaran in both substrates, but the activity of the 2,2-dimethyl-6-hydroxychromans is increased only in gasoline.

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Improved Method for Determining Gossypol in Crude Cottonseed Oils

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THE USE OF newer cottonseed processing methods has resulted in the commercial production of crude oils in which the gossypol content can vary from as little as 0.1 to as much as 0.7% (5, 11). With increased use of these methods more consideration may be given to the amount of gossypol in crude oils, particularly since evidence has been presented (2, 5, 11, 12) to indicate that this pigment and its derivatives are primarily responsible for increases in refined and bleached oil color resulting from the storage of some crude oils at elevated temperatures.

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A colorimetric method based on the reaction with p-anisidine (7) has been proposed for the analysis of gossypol pigments in cottonseed oils and has been useful for the analysis of various types of crude oils (2, 5, 6, 7, 11). However, in view of the recent modification of previous methods for the determination of free and total gossypol in cottonseed meats and meals to permit the use of aniline instead of p-anisidine as the reagent for color development (1, 4, 10), attention has been given to a similar modification of the method for the analysis of crude cottonseed oils. Investigations indicated that, when the aniline reaction was applied to certain types of crude oils, significant variations were observed which could be attributed to the type of instrument used for analysis. Since these anomalous results were not encountered in a previous study of the p-anisidine reaction (7), it was deemed advisable to re-examine the suitability of the aniline and p-anisidine reactions for the analysis of crude cottonseed oils for gossypol. As a result of the investigation the use of p-anisidine was found preferable, and certain improvements in the p-anisidine method were indicated, which make it more applicable for the analysis of all types of oils. The modified method is outlined in detail.

Improved p-Anisidine Method

Reagents

1. Isopropyl alcohol-hexane solvent: mix 600 ml. of A.C.S. reagent grade isopropyl alcohol and 400 ml. of commercial hexane (distillation range 146–156°F.).

2. Glacial acetic acid: A.C.S. reagent grade.

3. p-Anisidine: dissolve about 40 g. of technical grade p-anisidine in one liter of distilled water at about 75°C. Add 2 g. of sodium sulfite (Na_2SO_3) and 20 g. of decolorizing carbon. Stir for about 5 min. Filter the hot solution through a double layer of paper on a Buechner funnel. If the filtrate is turbid, refilter through the same paper. Place the solution in a refrigerator or water bath (2–4°C.) for at least 4 hrs., preferably overnight. Separate the crystals by filtration on a Buechner funnel, wash once with cold water (2–4°C.), and dry overnight in a vacuum desiccator over phosphorus pentoxide or concentrated sulfuric acid. Store the dry crystals in a brown bottle in a refrigerator. The reagent purified and stored in this manner is stable for at least one year.

4. p-Anisidine solution: weigh 1 g. of purified p-anisidine into a small brown glass-stoppered bottle, add 48 ml. of the isopropyl alcohol-hexane solvent (measured with a graduate) and 2 ml. of glacial acetic acid by pipet, and swirl to dissolve. Prepare this solution daily as needed.

5. Acetic acid solution: mix 2 ml. of glacial acetic acid and 48 ml. of the isopropyl alcohol-hexane solvent in a small glass-stoppered bottle.

6. Standard gossypol solution: weigh accurately 100 mg. of pure gossypol (3, 9), and dissolve in the isopropyl alcohol-hexane solvent, warming if necessary. Transfer quantitatively to a 250-ml. volumetric flask, dilute to volume with the solvent, and mix well. Pipet 25 ml. of this solution into a 500-ml. volumetric flask, dilute to volume with the solvent, and mix well. If exactly 100 mg. of gossypol are weighed, this solution contains 0.020 mg. of gossypol per ml.

[2] Mention of the names of firms or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

Sample Preparation

The analytical sample must be free from suspended material. Heat the sample to about 50°C., mix well, and filter through creped paper suitable for filtration of oil (Eaton and Dikeman No. 617 or equivalent[2]). Analyze the filtered sample within one day or store in a refrigerator (2–4°C.) prior to analysis. Should the sample require storage for any extended period, it should be stored at about 0°F.(—18°C.).

Sample Weight

For maximum precision it is desirable that the sample aliquot used for analysis contain about 0.1 mg. of gossypol. Suggested sample weights and aliquots are as follows:

Expected gossypol content	Sample weight	Size of flask for oil sample	Aliquot for analysis
%	g.	ml.	ml.
0.00–0.01	5.0	25	5–10
0.01–0.03	3.0	25	5
0.03–0.05	1.0	25	5
0.05–0.10	1.0	25	2
0.10–0.20	0.8	25	2
0.20–0.40	0.5	25	2
0.40–0.60	0.25	25	2
0.60–0.80	0.4	50	2
0.80–1.0	0.25	50	2

Standard Curve

Pipet duplicate 1-, 2-, 3-, 4-, 5-, 7-, 8-, 9-, and 10-ml. aliquots of the standard gossypol solution into 25-ml. volumetric flasks. To one set of the aliquots, add 3 ml. of the acetic acid solution (a rapid delivery pipet may be used), dilute to volume with the isopropyl alcohol-hexane solvent, and reserve as reference solutions (gossypol blanks).

To the other set of aliquots and a reagent blank containing 5 ml. of the solvent, add 3 ml. of the p-anisidine solution, and heat in a water bath at 75°C. for 1 hr. with the stoppers inserted in the flasks. Remove from the bath, cool to room temperature, and dilute to volume with the solvent.

Determine the transmittance of the reagent blank against the isopropyl alcohol-hexane solvent as reference solution. Using the appropriate gossypol blank (standard without p-anisidine) as reference solution, determine the transmittance of each gossypol standard reacted with p-anisidine at the wavelength setting noted below. If a spectrophotometer is used, the transmittance readings should be taken at 460 mμ. For glass filter colorimeters the filter should have a transmission maximum between 450–465 mμ. Corning Glass Company combination filter with 5113 and 3387 glasses, nominal rating of 470 mμ, is satisfactory.

Convert the transmittance readings of the reagent blank and the gossypol standards to optical density (optical density = 2 — logarithm transmittance). Subtract the optical density of the reagent blank from the optical density of each gossypol standard reacted with p-anisidine, and plot the corrected optical density against the mg. of gossypol in the 25-ml. volume to obtain the standard curve. If the calibration curve is non-linear, as with most glass-filter colorimeters, it is necessary to refer to the curve or a table prepared from the curve to determine the concentration of gossypol in sample aliquots. If the calibration curve is linear, as with most spectrophotometers, it is convenient to use a calibration

factor for the calculation of the gossypol concentration of sample aliquots. To obtain the factor for each gossypol standard, divide the mg. of gossypol in the 25-ml. volume by the appropriate corrected optical density:

$$\text{Factor} = \frac{\text{mg. gossypol in 25-ml. volume}}{\text{corrected optical density}}$$

Average the factors for all of the gossypol standards. The concentration of gossypol in the sample aliquots is then found by multiplying the corrected optical density of the sample aliquot by the calibration factor:

mg. gossypol in sample aliquot = corrected optical density X factor

Once obtained, the calibration factor, or calibration curve, need not be redetermined except for periodic check of instrumental response.

Procedure

Weigh an appropriate amount of the filtered crude oil into a suitable volumetric flask, dilute to volume with the solvent, and mix well. Pipet duplicate aliquots of the sample solution, containing about 0.1 mg. of gossypol, into 25-ml. volumetric flasks. To one aliquot add 3 ml. of the acetic acid solution, dilute to volume with the solvent, and reserve as the reference solution. To the other sample aliquot and a reagent blank containing a volume of the solvent equal to that used for the sample aliquot, add 3 ml. of the p-anisidine solution and treat as outlined above for the gossypol standards. Using the diluted sample aliquot without p-anisidine as reference solution, determine the transmittance of the sample aliquot reacted with p-anisidine. For the reagent blank use the solvent as reference solution. Convert the transmittance readings of the sample aliquot and reagent blank to optical density. Subtract the optical density of the reagent blank from that of the sample aliquot reacted with p-anisidine to obtain the corrected optical density. Determine the mg. of gossypol in the sample aliquot by reference to the calibration curve or by use of the calibration factor as outlined above for the gossypol standards. Calculate the percentage of gossypol in the sample as follows:

$$\text{Gossypol, \%} = (V/A \times G)/W \times 10.$$

V = dilution volume of sample before analysis,
A = aliquot of V used for analysis,
G = mg. of gossypol in sample aliquot, and
W = weight of sample in grams.

Experimental and Discussion

The original p-anisidine method (7) was modified to permit the use of larger sample aliquots for analysis, a feature which makes the procedure somewhat more applicable to all oils and lowers the threshold of analysis. These modifications required a change in the composition of the solvent, a higher reaction temperature, and the use of a more concentrated p-anisidine reagent. The use of aniline as a color-developing reagent was explored extensively in comparison with the use of p-anisidine. The aniline method selected for this purpose incorporated the high-temperature aniline reaction suggested by Miller (4) and others (10) for the analysis of free gossypol in cottonseed meals. In addition to the use of these two methods, a pretreatment of crude oils with oxalic acid in methyl ethyl ketone, under conditions shown previously to be adequate for hydrolysis of "bound" gossypol (8), was used to convert modified gossypol pigments to gossypol prior to analysis. This treatment provided a logical basis for comparison of the two methods of analysis as applied to a wide range of untreated oils. The aniline method and the oxalic acid pretreatment are as follows.

ANILINE METHOD

Reagents

1. Hexane-isopropyl alcohol solvent: mix 794 ml. of commercial hexane and 206 ml. of reagent grade isopropyl alcohol.
2. Aniline: distil reagent grade aniline over a small amount of zinc dust, discarding the first and last 10% of the distillate. Stored in a brown bottle in a refrigerator, this reagent is stable for about 2 months.
3. Standard gossypol solution: prepare as described under p-anisidine method except that the hexane-isopropyl alcohol solvent is used for all dilutions.

Standard Curve

Pipet duplicate aliquots of the standard gossypol solutions, covering the range of 0.02–0.20 mg. gossypol, into 25-ml. volumetric flasks. Dilute one set of aliquots to volume with the solvent and reserve as reference solution. To the other set of aliquots and a reagent blank containing 5 ml. of the solvent, add 2 ml. of redistilled aniline and heat in a boiling water bath (100°C.) for 30 min. Cool the flasks and dilute to volume with the solvent. Determine the transmittance of the gossypol standards reacted with aniline and of the reagent blank, using the appropriate reference solutions (standards without aniline and solvent, respectively) at the wavelength settings noted below. If a spectrophotometer is used, measurements are taken at 450 mμ. In the event a glass-filter colorimeter is used, the filter should have a transmission maximum between 450–465 mμ. Convert the transmittance reading to optical density, calculate the corrected optical density of each gossypol standard, and plot the standard curve as outlined in the p-anisidine method. Where appropriate, calibration factors may also be calculated.

Sample Preparation and Sample Weight

As outlined under the p-anisidine procedure.

Procedure

Weigh a suitable sample into a volumetric flask, dilute to volume with the hexane-isopropyl alcohol solvent, and mix well. Pipet duplicate aliquots of the sample solution into 25-ml. volumetric flasks. Dilute one aliquot to volume with the solvent and reserve as reference solution. To the other sample aliquot and an equal volume of solvent, which serves as the reagent blank, add 2 ml. of aniline, develop the color, and determine the corrected optical density as outlined for the standard curve. From the value of the corrected optical density determine the mg. of gossypol present in the sample aliquot by reference to the standard curve or by use of the calibration factor. Calculate the gossypol content of the sample as described in the p-anisidine method.

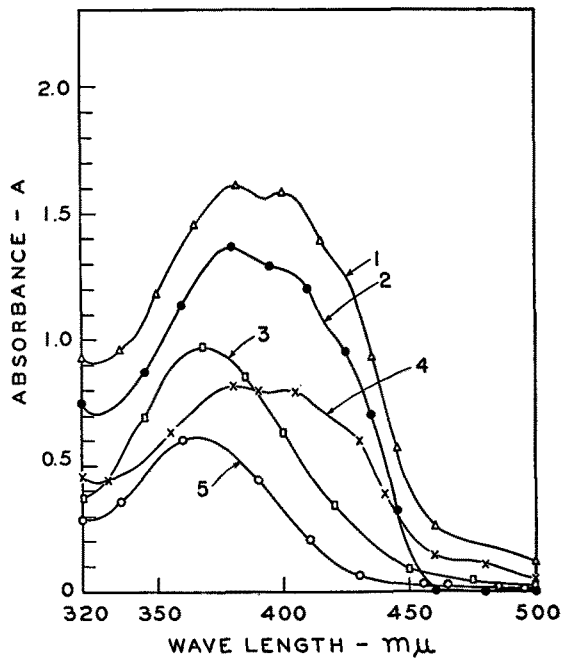

FIG. 1. Absorption spectra of crude cottonseed oils in hexane-isopropyl alcohol.
1. Screw-pressed.
2. Hexane-extracted after prepressing.
3. Prepressed.
4. Hydraulic-pressed.
5. Pure gossypol in refined and bleached oil.

OXALIC ACID PRETREATMENT

Crude oils are weighed into 50-ml. volumetric flasks; 10 ml. of a 0.1 molar solution of oxalic acid in methyl ethyl ketone-water azeotrope (8) are added, and the stoppered flasks heated in a water bath at 75°C. for 6 hrs. About 30 ml. of an isopropyl alcohol-hexane solvent (60:40) are added to each flask, followed by the addition of 2 ml. of 0.5 molar barium acetate, dropped slowly from a burette with frequent swirling. The flasks are diluted to volume with the solvent, mixed, and after 10 min. a portion of the contents of each is filtered through paper. Duplicate aliquots of the filtered solutions, containing about 0.1 mg. of gossypol, are analyzed for gossypol pigments by the p-anisidine and aniline methods.

Erratic results were obtained in the procedure when the barium acetate solution was not completely mixed with the oil-oxalic acid solution. In these cases oxalic acid was not completely removed, and turbidities were encountered on reaction with either aniline or p-anisidine.

PRELIMINARY INVESTIGATIONS

In preliminary investigations of the aniline and p-anisidine reactions, absorption measurements were made at the principal absorption maximum, 440 mμ in the case of aniline and 447 mμ for p-anisidine. The results for certain crude oils were somewhat lower than those obtained on the same oils which had been treated with oxalic acid prior to analysis. The differences between the apparent gossypol content of original and pretreated crude oils were greater in the case of the aniline than for the p-anisidine reaction. Consideration of the principles on which these methods are based offers an explanation for these conflicting results. In order to correct for the background absorption of crude oils, the change in absorption after reaction with the color development reagent is utilized as a measurement of the gossypol content. Since the same technique is used in the standardization with pure gossypol, the system is valid, provided the absorption of gossypol pigments in crude oils does not differ materially from that of pure gossypol. Should the gossypol pigments in crude oils have a higher absorptivity than that of pure gossypol, the net effect would be an over-correction for the background absorption, resulting in low values. Evidence that this effect is responsible for the differences in apparent gossypol content of crude and pretreated crude oils is afforded by a comparison of the absorption spectra of crude oils before and after treatment with oxalic acid (Figures 1 and 2).

Absorption of Crude Oils. The absorption spectra of crude oils dissolved in the isopropyl alcohol-hexane solvent (Figure 1) differ considerably from that of pure gossypol. Hydraulic-, screw-pressed, and solvent-extracted oils exhibit absorption maxima at 380, 400, and 410 mμ, characteristic of gossypol pigments (7), while prepressed oil and pure gossypol show the presence of a single absorption maximum at 367 mμ. Of particular importance, from the standpoint of analysis, is the fact that modified gossypol pigments in hydraulic-, screw-pressed, and solvent-extracted oils have considerable absorption in the region of 420–440 mμ, where pure gossypol has very little absorption. After pretreatment with oxalic acid in methyl ethyl ketone (Figure 2) all of the oils showed the presence of a single absorption maximum at 374 mμ, identical with that of pure gossypol treated in the same manner. This is evidence that the pigments in crude oils exhibiting absorption maxima at 380, 400, and 410 mμ are, in fact, derived gossypol pigments which were converted to behave like gossypol by the

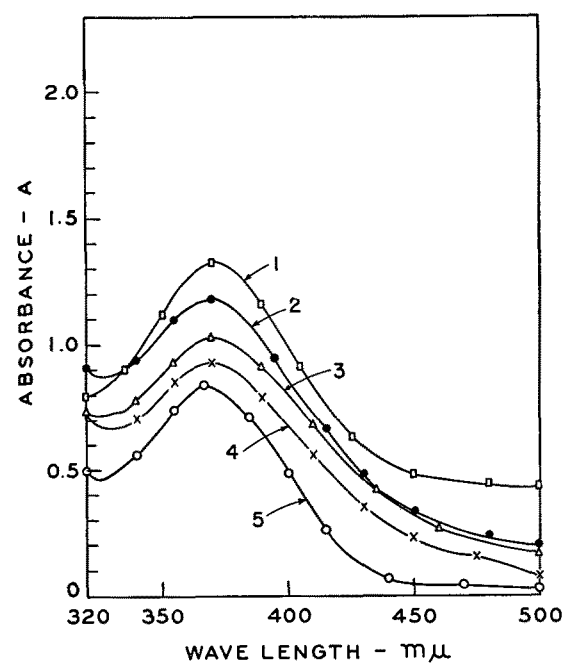

FIG. 2. Absorption spectra of crude cottonseed oils after treatment with oxalic acid in methyl ethyl ketone.
1. Prepressed.
2. Hexane-extracted after prepressing.
3. Screw-pressed.
4. Hydraulic-pressed.
5. Pure gossypol in refined and bleached oil.

oxalic acid treatment. The striking difference in absorption between certain crude oils and pure gossypol (Figure 1) makes it evident that, in the analysis of such oils, an over-correction will be made for the background absorption.

Absorption After Aniline and p-Anisidine Reaction. Under conditions normally employed in analysis the net effect of the over-correction for gossypol pigments is illustrated by the spectra obtained when the sample aliquots of crude oils before aniline or p-anisidine reaction were used as the reference solutions (Figures 3 and 4). In the case of the aniline reaction with hydraulic-, screw-pressed, and solvent-extracted oils (Figure 3) an apparent shift can be noted in the absorption spectra; maximum absorption occurs at 445 mμ rather than at 440 mμ as for prepressed oil and pure gossypol. Since in other data, not reported, the absorption maxima of these oils all occurred at 440 mμ when the isopropyl alcohol-hexane solvent was used as reference solution, the apparent shift in the absorption spectra noted in Figure 3 can be attributed to an erroneous correction for background absorption. These differences in absorption after the aniline reaction (Figure 3) make it evident that absorption measurements at 440 mμ would lead to erroneous gossypol values for certain types of oils.

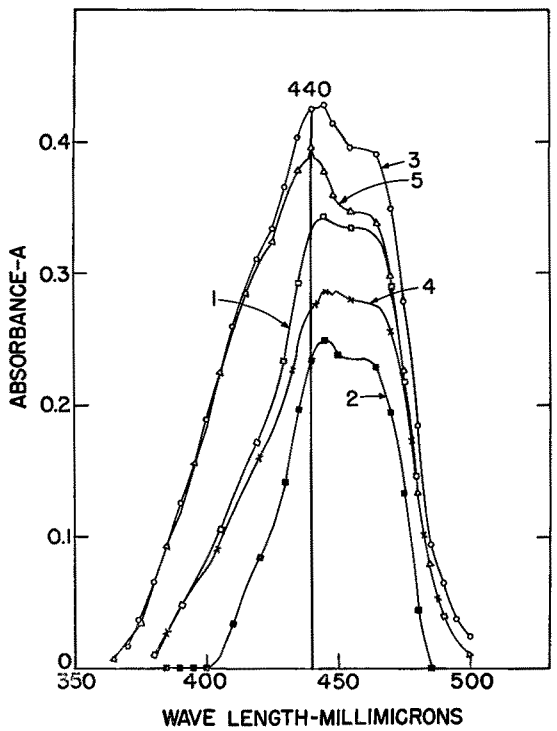

FIG. 3. Changes in absorption spectra of crude cottonseed oils on reaction with aniline. Oil solution before reaction used as reference.

1. Hydraulic-pressed.
2. Screw-pressed.
3. Prepressed.
4. Hexane-extracted after prepressing.
5. Pure gossypol in refined and bleached oil.

The absorption of crude oils after p-anisidine reaction (Figure 4) shows a slight shift in the principal absorption maximum at 447 mμ, while the second maximum at 467 mμ remains unchanged. At the longer wavelength the absorption of modified gossypol pigments is negligible and the net absorption in this region is identical with that of pure gossypol.

It should be emphasized that the over-correction errors noted in Figures 3 and 4 are not constant but will vary with the band-pass of the instrument used for analysis. In the region of 440 mμ these differences will contribute to significant errors in the precision of measurement.

Effect of Wavelength. As suggested by the data presented in Figures 3 and 4, errors introduced by over-correction for gossypol pigments in certain crude oils should be minimized at longer wavelengths. The influence of wavelength on optical density measurements is illustrated by the data tabulated in Table I for a typical hydraulic-pressed oil and for a sample of pure gossypol in refined and bleached oil reacted with aniline and p-anisidine. The colorimetric reactions were applied to the crude and oxalic acid pretreated oils. The optical density values for each sample were calculated to equivalent sample weights to permit direct comparison. For the sample of pure gossypol in the refined and bleached oil, the equivalence of the optical density values throughout the wavelength range for the original and pretreated oil is indicative that no destruction of gossypol occurred as a result of the oxalic acid treatment. In the case of the hydraulic-pressed oil reacted with aniline, optical density values for the crude oil are lower than those obtained on the acid pretreated oil up to 450 mμ, and are equivalent beyond this wavelength. For the p-anisidine reaction the equivalence point for the original and pretreated oil occurs in the region of 460 mμ.

The possible variation in the apparent gossypol

TABLE I

Comparison of Optical Density Measurements on Crude and Oxalic Acid-Treated Cottonseed Oils Reacted with Aniline and p-Anisidine

Wavelength of measurement	Aniline reaction				p-Anisidine reaction			
	Pure gossypol in refined and bleached oil[a]		Hydraulic-pressed oil[a]		Pure gossypol in refined and bleached oil[a]		Hydraulic-pressed oil[a]	
	Crude oil	Treated oil[c]	Crude oil	Treated oil[c]	Crude oil	Treated oil[c]	Crude oil	Treated oil[c]
mμ[b]	O.D.	O.D.	O.D.	O.D.	O.D.	O.D.	O.D.	O.D.
410	0.229	0.255	0.144	0.254	0.240	0.240	0.133	0.241
420	0.281	0.285	0.217	0.311	0.284	0.284	0.202	0.298
430	0.328	0.320	0.295	0.362	0.337	0.337	0.286	0.359
440	0.333	0.339	0.354	0.377	0.377	0.377	0.370	0.408
445	0.329	0.329	0.360	0.370	0.372	0.372	0.393	0.408
450	0.324	0.324	0.364	0.362	0.367	0.367	0.398	0.402
460	0.307	0.306	0.343	0.337	0.352	0.352	0.390	0.391
470	0.237	0.237	0.242	0.254	0.292	0.292	0.333	0.336

[a] Optical density of crude and acid-treated oil adjusted to equivalent sample weights. Sample aliquot without aniline or p-anisidine was reference solution.
[b] Bausch and Lomb Spectronic 20.
[c] Crude oil treated with oxalic acid in methyl ethyl ketone prior to analysis.

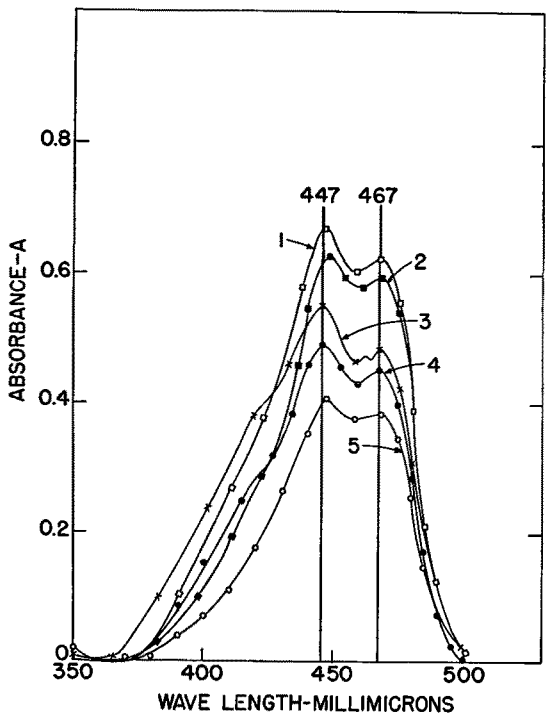

FIG. 4. Changes in absorption spectra of crude cottonseed oils on reaction with p-anisidine. Oil solution before reaction used as reference.

1. Direct hexane-extracted.
2. Hydraulic-pressed.
3. Pure gossypol in refined and bleached oil.
4. Screw-pressed.
5. Hexane-extracted from cooked meats.

content of crude oil as a function of wavelength of measurement and of type of instrument used is illustrated by the data presented in Table II for a sample of screw-pressed oil. Both aniline and p-anisidine reactions were applied to the crude oil before and after pretreatment with oxalic acid. Two spectrophotometers and a photoelectric colorimeter were used; each instrument was calibrated at the appropriate wavelengths with pure gossypol solutions. It is evident in the case of the crude oil reacted with aniline that the values for apparent gossypol content vary throughout the wavelength range. Based on the assumption that the consistent results obtained on the acid pretreated oils by the use of either reagent reflect the actual gossypol content, values obtained by the aniline reaction in the region of 450 mμ are substantially correct when a spectrophotometer is used. The apparently high values noted in the case of the glass-filter colorimeter may be due to the fact that the particular filter used had a transmission maximum at 464 mμ and are an indication of the errors introduced by measurements taken at wavelengths somewhat removed from an absorption maximum.

TABLE II

Effect of Wavelength of Measurement on the Apparent Gossypol Content of Crude Screw-Pressed Cottonseed Oil

Wavelength of measurement	Gossypol content					
	Crude oil			Acid-treated crude oil		
	Aniline		p-Anisidine	Aniline		p-Anisidine
	Beckman [a]	B. & L.[b]	B. & L.[b]	Beckman [a]	B. & L.[b]	B. & L.[b]
mμ	%	%	%	%	%	%
410	0.051	0.050	0.050	0.080	0.084	0.083
420	0.060	0.062	0.060	0.083	0.087	0.085
430	0.067	0.076	0.071	0.087	0.089	0.087
440	0.083	0.084	0.081	0.088	0.088	0.088
445	0.090	0.089	0.086	0.088	0.089	0.088
450	0.092	0.091	0.088	0.088	0.088	0.088
460	0.094	0.095	0.091	0.088	0.089	0.088
470	0.096	0.097	0.091	0.088	0.088	0.089
Colorimeter [c]	0.095		0.091	0.089		0.089

[a] Beckman DU spectrophotometer.
[b] Bausch and Lomb Spectronic 20.
[c] Evelyn photoelectric colorimeter, 470 filter with peak transmission at 464 mμ.

The consistent values obtained on the crude screw-pressed oil between 450–470 mμ for the p-anisidine reaction (Table II) were substantiated by analysis of numerous crude oils. In each case results obtained by use of a spectrophotometer and a colorimeter were in good agreement. Although p-anisidine-gossypol exhibits a second absorption maximum at 467 mμ, with wide band-pass spectrophotometers and colorimeters, it is preferable to measure at 460 mμ, rather than at 467 mμ, in order to combine maximum sensitivity with accuracy of measurement. Calculations indicated that the absorptivity of p-anisidine-gossypol at 460 mμ, obtained by use of a Bausch and Lomb Spectronic 20 (20 mμ band-pass), was 88.0 as compared to a value of 83.3 for dianilinogossypol at its absorption maximum of 440 mμ.

Comparison of Analytical Methods. A comparison of the results obtained by use of aniline and p-anisidine reactions for the analysis of a variety of crude oils is given in Table III. For each sample, analyses by both reactions were obtained from aliquot portions of the same analytical sample. This eliminated

TABLE III

Comparison of Aniline and p-Anisidine Methods for the Analysis of Gossypol in Crude Cottonseed Oils

Type of oil	Gossypol content					Average gossypol content for acid-treated crude oils [c]
	Aniline			p-Anisidine		
	B. & L.[a]		Colorimeter [b]	B. & L.[a]	Colorimeter [b]	
	440 mμ	450 mμ	470 filter	460 mμ	470 filter	
	%	%	%	%	%	%
Hydraulic-pressed	0.039	0.043	0.043	0.044	0.044	0.044
Hydraulic-pressed	0.072	0.075	0.081	0.075	0.076	0.075
Screw-pressed	0.087	0.091	0.095	0.091	0.091	0.090
Screw-pressed	0.186	0.191	0.200	0.187	0.189	0.189
Prepressed	0.166	0.164	0.173	0.169	0.174	0.167
Prepressed	0.245	0.248	0.250	0.249	0.249	0.253
Hexane-extracted after prepress	0.063	0.067	0.072	0.066	0.068	0.067
Hexane-extracted after prepress	0.094	0.098	0.104	0.100	0.101	0.098
Direct hexane-extracted	0.208	0.205	0.221	0.202	0.212	0.198
Refined and bleached, gossypol added	0.100	0.099	0.098	0.100	0.101	0.101

[a] Bausch and Lomb Spectronic 20.
[b] Evelyn colorimeter, 470 filter with peak transmission at 464 mμ.
[c] Crude oils treated with oxalic acid in methyl ethyl ketone prior to analysis. Values listed are averages obtained by aniline and p-anisidine reactions.

sampling errors and permitted direct comparison of the methods. The data for the acid pretreated oils are the averages of fully concordant results obtained by use of both reactions.

The values obtained by use of the aniline reaction emphasize the fact that low results are obtained at 440 mμ for most hydraulic-, screw-pressed, and solvent-extracted oils. Other oils, such as prepressed and direct solvent-extracted, yield comparable results at either 440 or 450 mμ. In practically all cases gossypol estimates with the photoelectric colorimeter (470 filter) tended to be high.

For the p-anisidine method, consistent results were obtained by use of either a spectrophotometer at 460 mμ or a colorimeter (470 filter). In agreement with previous data (Table II) analysis of crude oils by use of the aniline reaction and reading the absorption at 450 mμ or the p-anisidine reaction at 460 mμ gave comparable results which are in good agreement with those for either reaction applied to oxalic acid pretreated crude oils.

From consideration of the data it would appear that use of the p-anisidine reaction at 460 mμ or the aniline reaction at 450 mμ is equally suitable for the analysis of gossypol pigments in crude cottonseed oils. However a serious objection to the use of the aniline reaction is the requirement that measurements be made at 450 mμ, rather than at the dianilinogossypol maximum at 440 mμ, in order to avoid over-correction for background absorption. This qualification will lead to a lowering of the reproducibility of the method, particularly when photoelectric colorimeters are used. Since p-anisidine-gossypol exhibits a second absorption maximum at 467 mμ, where modified gossypol pigments do not interfere with the background correction, the p-anisidine procedure is preferable from the standpoint of accuracy and precision. With a pretreatment of crude oils with oxalic acid in methyl ethyl ketone prior to analysis, measurements can be made at the principal absorption maximum of 440 mμ in the case of aniline-gossypol and at 447 mμ for p-anisidine-gossypol. However the added complexity of analysis does not seem to justify the use of the oxalic acid pretreatment.

Summary

The p-anisidine method for the determination of gossypol in crude cottonseed oils has been reinvestigated and modified to make it applicable to all crude oils obtained by the newer methods of processing cottonseed. The modifications included a change in the composition of the solvent, a higher reaction temperature, and the use of a more concentrated p-anisidine reagent. The modified method was found satisfactory where different colorimeters and spectrophotometers were used for measuring the color developed.

Comparison of aniline and p-anisidine as reagents for the analysis of gossypol pigments showed that the presence of modified gossypol in some crude oils resulted in an over-correction for background absorption and led to significant errors when aniline was used as the color development agent.

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Letter to the Editor

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Lancaster, Bitner, and Beal in a recent paper (1) have described a procedure for determining the induction period in an atmosphere of oxygen as a measure of the stability of an oil. They comment on the small amount of attention required and, by implication, suggest that such a method is superior to the so-called Active Oxygen Method.

The principle of this procedure was suggested by us in 1942 (2), and apparatus of this type has been in constant use in these laboratories for 16 years. Our determinations are made at the temperature of boiling water in an atmosphere of oxygen, and the oxygen absorption is indicated by a continuous record of the mercury level in a manometer.

The apparatus is in current use in several other laboratories in Great Britain.

As a result of our experience we have no hesitation in agreeing with the conclusions of Lancaster and his colleagues. The apparatus is eminently satisfactory for routine work, the services of an analyst are not required, and the results are obtained automatically without attention, whatever the length of the induction period may be.

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