with constant temperature, and data for the effect of extraction temperature are plotted at constant extraction time, the curves cross or touch at the same location. With meat and bone scrap this coincidence occurs at an extraction time of 26 minutes and an extraction temperature of 110°F. This extraction time is the one commonly used in the commercial Iowa State College extractor, and the temperature is that normally used in the laboratory study of many materials. This correlation means that with meat and bone scrap one can estimate the residual extractables obtainable in the pilot plant by making a single laboratory rate extraction.

This empirical approach did not prove to be satisfactory for cottonseed, but this can be explained on the basis of a difference in mechanism. Solvent extraction of meat and bone scrap takes place mainly by a washing process whereas it is believed (5) that with cottonseed it is a diffusional operation.

The toxicity to cattle of certain batches of trichloroethylene-extracted soybean oil meal has raised the question of possible toxicity of other products extracted by trichloroethylene. Since the work presented in this paper was a study in extraction only, the use of trichloroethylene as an experimental solvent should not be construed as a recommendation by the authors that the product resulting from this extraction is or is not suitable as a feed.

Summary

Data are presented to show the effect of the various operating variables on the extraction of meat and bone scrap both in the laboratory and in a pilot plant model of the Iowa State College extractor. From the data presented it has been concluded that the extraction takes place mainly by a washing process with slight diffusion. A possible correlation is suggested for comparing laboratory and pilot plant data.

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[Received April 20, 1954]

A Modification of the p-Anisidine Method for the Determination of Free and Total Gossypol[1]

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A modification of the Pons and Guthrie[1] method for the determination of free gossypol and the Pons[5] method for total gossypol in cottonseed materials is presented. The proposed procedure makes possible the analysis of free and total gossypol in chemically treated products containing dianilinogossypol. The methods presented are spectrophotometric ones in which dianilinogossypol is formed as a measure of the free or total gossypol. The free gossypol method is applicable to the analysis of all types of cottonseed meals available commercially. The advantage of the modified methods is that accurate results can be obtained when the sample contains dianilinogossypol.

The method of Pons and Guthrie for the determination of free gossypol in cottonseed materials is an Official Method of The American Oil Chemists' Society. The total gossypol method has no such official standing. However both have been generally accepted as the most satisfactory methods available.

Neither has previously been evaluated completely with regard to its applicability to materials that have been chemically treated to reduce their free gossypol content. The object of this work was to examine the methods thoroughly with respect to the analysis of cottonseed materials containing dianilinogossypol. Since dianilinogossypol is physiologically inert, it is important that this material not analyze as free gossypol when present in cottonseed meal. It is important, too, that the dianilinogossypol be completely hydrolyzed in the total gossypol method.

Briefly the free gossypol method of Pons and Guthrie requires the ground sample to be extracted with 70% aqueous acetone, using a mechanical shaking device. An aliquot of the acetone solution of gossypol is then allowed to react with p-anisidine, in the presence of acetic acid, for 30 min. at 60° C. A solution of the yellow gossypol-anisidine complex, in alcohol, is measured with a spectrophotometer or a photoelectric colorimeter at 447 mu. Pons' total gossypol method requires the hydrolysis of combined gossypol by oxalic acid in a solution of methyl ethyl ketone-water azeotrope. The liberated gossypol is then measured as in the free gossypol method.

[1] Presented at 45th annual meeting, American Oil Chemists' Society, San Antonio, Tex., Apr. 12-14, 1954.

Discussion

Correlation between feeding tests, using meals containing dianilinogossypol, and free gossypol measurements made by the Official Method have indicated that the method tends to give high results. This was not the case when studying untreated meals. It is likely that dianilinogossypol is extracted along with the free gossypol and that its presence is not entirely corrected for since its extinction coefficient is different from that of the anisidine complex, at 447 mu. It will be shown later that this is only a partial explanation.

Using pure dianilinogossypol, it was shown that it is extracted, to some extent, without hydrolyzing, with 70% aqueous acetone, under the conditions of the test. Reaction with p-anisidine resulted in the measurement of apparent free gossypol.

A study of other solvents was made in the hope that the extraction of dianilinogossypol could be eliminated. This work was unsuccessful. None was as effective as 70% acetone in extracting free gossypol. The stability and solubility of gossypol in aqueous acetone, as pointed out by Pons and Guthrie, make it the most desirable solvent that could be found.

Although all of the aromatic amines will react with gossypol to form highly colored complexes, Pons and Guthrie selected p-anisidine primarily because of its stability and the ease with which it may be purified.

Earlier workers[2,3] used aniline to form dianilinogossypol as a measure of the gossypol content of cottonseed materials. It seemed more correct to use this reagent, rather than p-anisidine, to analyze meal and flakes containing dianilinogossypol. The use of aniline eliminates the introduction of compounds not already in the samples. Possible side reactions, then, are not a danger. The spectrophotometric characteristics of the color produced by the reaction of free gossypol with aniline are, of course, the same as those of the dianilinogossypol in the samples. This means that a blank determination will correct for the traces of dianilinogossypol extracted from the sample.

A calibration curve was prepared from pure gossypol, following the procedure outlined in the Official Method but substituting redistilled aniline for the p-anisidine-acetic acid reagent. A Coleman Jr. spectrophotometer was used to make the absorbance measurements at 445 mu. This wavelength was chosen after preparing the absorption curve for dianilinogossypol in 70% acetone. (See Figure 1.)

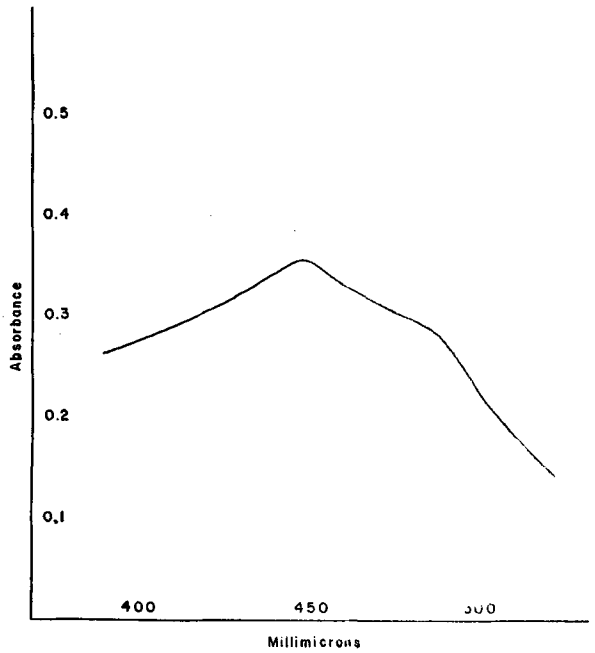

FIG. 1. Absorption curve for dianilinogossypol in 70% acetone. Concentration, 0.1 mgm per ml. Beckman spectrophotometer, model DU. Through 1.0 cm. Peak at 445 mu.

Table I lists a few comparative results by the two procedures. The special chemically processed meals were prepared so as to have varying levels of free gossypol and dianilinogossypol. It should be noted that in all cases the aniline modification gave lower results on meals containing dianilinogossypol but that either method was suitable for untreated samples.

A serious fault in the Official Method is that it tends to be empirical to some degree. This is indicated by the requirements for preparing the standard calibration curve.

Most spectrophotometric methods of analysis permit the preparation of a calibration curve from a single stock solution of the pure material to be measured. A series of different-sized aliquots are taken from the solution, and, after each has been allowed to react with the color-producing reagent, a series of absorbance measurements will show a linear relationship when plotted against concentration, if the solutions conform to Beer's Law.

TABLE I

Meal Sample	% Free Gossypol	
	Official Method	Aniline Modification
Special Chemically Processed Meals		
No. 4	0.035	0.019
5	0.082	0.050
15	0.111	0.100
22	0.141	0.117
23	0.167	0.137
24	0.190	0.165
Hydraulic	0.100	0.097
Screw Press	0.050	0.054
Screw Press	0.050	0.050
Hexane Extr.	0.150	0.146
Hexane Extr.	0.140	0.144

The Official Method requires the preparation of a series of stock solutions, each with a different concentration of pure gossypol. The absorbance measurements are made on equal-sized aliquots from each solution. A calibration curve prepared in this manner is linear. If the customary procedure is followed however, the curve indicates non-conformity to Beer's law. Typical examples are shown in Figure 2.

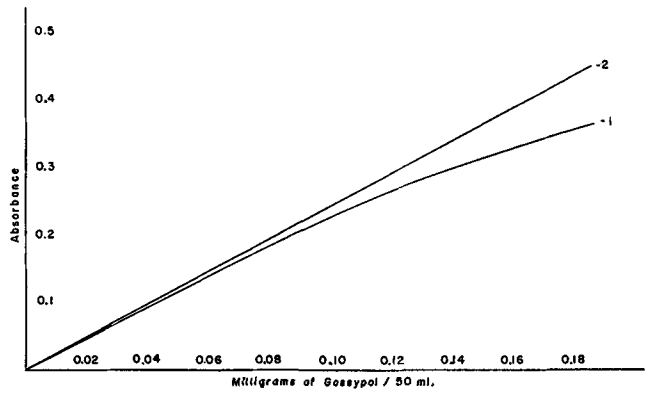

FIG. 2. Calibration curves, Pons and Guthrie method. Coleman Jr. spectrophotometer, model B, with 25 mm cuvettes. 1.—with a series of aliquots from a single stock solution. 2.—with equal sized aliquots from a series of stock solutions.

This weakness in the original method is unfortunate since it requires that the size of the aliquot of the sample extract be kept constant. It must always be the same as that used to prepare the standard curve. Certain types of samples have such a low free gossypol content that the use of large aliquots would be desirable for the required sensitivity and for increased accuracy. The extraction of larger samples, as a partial solution to the problem, was shown to be unsatisfactory.

When a calibration curve was prepared from a series of different-sized aliquots of a single stock solution of gossypol, following the customary procedure, but developing the color with aniline at 60° C., the same non-linear relationship was noted as just described, using p-anisidine. Increasing the aliquot size from the 2 ml. prescribed by Pons and Guthrie met with no success even when reaction time and aniline

excess were increased. Table II shows the results of some of this work.

When the acetone solution of gossypol was allowed to react with aniline at 100° C., the reaction was rapid and apparently complete. It was necessary to have the reaction vessels unstoppered at this temperature however, and most of the acetone evaporated, leaving water and aniline. Fortunately warm aniline is a good solvent for dianilinogossypol, and the whole went into solution readily on the addition of alcohol. The data in Table III show almost perfect recovery of gossypol, even from large volumes, when the high temperature reaction is employed.

The data in Table III were obtained with a reaction time of 15 minutes in a boiling water bath. This choice of time was made from the results shown in Table IV. With this information the data in Table III were supplemented with additional measurements and a

TABLE II

Volume of Aliquot	Total volume less aniline[a]	Volume of aniline added	Reaction time	Mgm. Gossypol		% Recovery
				Present	Found	
ml.	*ml.*	*ml.*	*Min.*			
2	2	3	30	0.0216	0.0216	100.0
5	5	3	30	0.0540	0.0473	87.6
5	5	6	30	0.0540	0.0470	87.1
7	7	3	30	0.0756	0.0607	80.3
2	10	3	30	0.0216	0.0135	62.5
5	10	3	30	0.0540	0.0459	85.0
5	10	6	30	0.0540	0.0460	85.2
7	10	3	30	0.0756	0.0648	85.7
7	10	3	60	0.0756	0.0642	84.9
7	10	3	90	0.0756	0.0650	86.0
7	10	3	120	0.0756	0.0648	85.7
7	10	6	120	0.0756	0.0645	85.3

[a] Diluted with 70% acetone.

TABLE III

Volume of aliquot	Total volume less aniline[a]	Volume of Aniline added	Mgm. Gossypol		% Recovery
			Present	Found	
ml.	*ml.*	*ml.*			
2	2	3	0.0216	0.0214	99.1
2	10	3	0.0216	0.0215	99.5
2	20	3	0.0216	0.0217	100.5
5	5	3	0.0540	0.0542	100.3
5	20	3	0.0540	0.0537	99.4
10	10	3	0.1080	0.1080	100.0
10	10	3	0.1080	0.1083	100.3
20	20	3	0.2160	0.2160	100.0

[a] Diluted with 70% acetone.

TABLE IV

Reaction time	Milligrams of Gossypol	
	Present	Recovered
Min.		
5	0.1880	0.1800
10	0.1880	0.1881
15	0.1880	0.1880
30	0.1880	0.1881
60	0.1880	0.1878

calibration curve was prepared in the conventional manner. (See Figure 3.)

A comparison of results from typical meal samples was necessary as a final test of this high temperature procedure. All of the calibration data recorded thus far had been obtained by using pure gossypol. It is well known that aniline will react with protein-bound gossypol, under the proper conditions, liberating protein and dianilinogossypol.[4] Although bound gossypol is not ether-extractable, it was possible that appreciable amounts were being extracted by 70% acetone. This could cause high results.

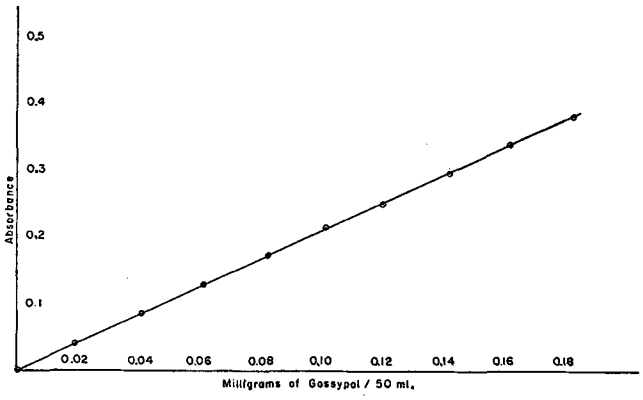

FIG. 3. Calibration curve, proposed method. Coleman Jr. spectrophotometer, model B; 25 mm. cuvettes, 445 mu.

Triplicate aliquots from extracts of solvent, hydraulic, and screw-press meals, as well as samples containing dianilinogossypol, were analyzed by the Official Method, the Official Method modified by using aniline, and the proposed high temperature procedure. The data, shown in Table V, indicate no apparent errors as a result of the high temperature or from excessive reaction times.

TABLE V

Meal Sample	Reaction Time	% Free Gossypol Found		
		Proposed Method	Official Method modified	Official Method
	Min.			
Treated A	15	0.086	0.085	0.100
Treated A	60	0.086		
Treated B	15	0.014	0.016	0.032
Treated B	60	0.014		
Solvent No. 1	15	0.137	0.135	0.137
Solvent No. 1	60	0.135		
Screw Press No. 7	15	0.040	0.040	0.038
Screw Press No. 7	60	0.040		
Hydraulic No. 4	15	0.120	0.121	0.121
Hydraulic No. 4	60	0.121		

Table VI shows several results by the proposed method, compared with data obtained using the Official Method. The special chemically processed meals were prepared so as to have varying levels of free gossypol and dianilinogossypol. As noted before, either method is applicable to untreated meals although the proposed method is a little shorter and cannot be considered empirical. By applying the *t* test to the first group of results in the table, it may be shown that the difference between the method is truly significant when used to analyze meals containing dianilinogossypol. *t*, for these data, is 6.113. The critical value for *t*, 1% probability for 9 degrees of freedom, is 3.250.

Soon after we began to use the modified method for the routine control of meal production, we noticed that in some cases we were unable to reproduce results on replicate samples of chemically treated meals. Checking the procedures carefully, it was found that some analysts were not always duplicating the time taken in handling the samples, especially the time between taking aliquots from the filtered extract and measuring the absorbancies. Further investigation showed that differences of 15-20 min. during this stage of the analysis could change results by as much as 0.03% free gossypol.

TABLE VI

Sample	% Free Gossypol	
	Official Method	Proposed Method
Special Chemically Processed Meal		
1	0.035	0.019
2	0.153	0.138
3	0.082	0.050
10	0.111	0.100
11	0.072	0.062
29	0.094	0.078
34	0.141	0.117
41	0.062	0.040
454	0.167	0.137
455	0.190	0.165
Untreated Meal		
4	0.150	0.146
9	0.140	0.144
10	0.100	0.097
15	0.050	0.055
16	0.050	0.050

It was found that the absorbance of the so-called gossypol blank, *i.e.*, an aliquot of the filtered extract diluted with 80% isopropanol, would fade from, for example, 0.26 to 0.20 in 20 min. However this was noted only when the samples contained dianilinogossypol. Extracts from regular meals were completely stable. Since we measure the free gossypol as a difference in the absorbance of a blank, as described above, and another aliquot treated with an excess of aniline, this fading would give fictitiously high results.

After we found that regular meals did not exhibit this fading, we investigated pure dianilinogossypol, alone and in mixtures with regular meals. The same fading was observed; its extent depended upon the amount of dianilinogossypol present.

It was felt that the fading was probably a result of hydrolysis of dianilinogossypol and that it could be prevented by adding a trace of aniline to shift the equilibrium of the reaction. We investigated this and found that aniline did prevent hydrolysis and that a very slight trace would not react with the free gossypol present, at room temperatures.

Since this fading of the extracted color caused serious differences in results, there was the danger that the hydrolysis probably started as soon as extraction was begun and that some of the free gossypol measured was probably formed as a result of hydrolysis during this time.

To check this we set up a series of replicate samples for extraction. After shaking for one hour, we filtered one and withdrew several aliquots. The other samples were set aside to stand quietly. The absorbance of one aliquot from the first sample was measured immediately and the others at intervals up to two hours. The absorbance of these aliquots fell off seriously during this period, to an amount equivalent to 0.06% free gossypol. The other replicates were filtered at intervals, and the aliquots measured immediately had the same absorbance as that from the first sample. No change was noted even after two hours as long as the extract was in contact with the meal and protected from air.

No attempt is made to explain this although it is felt safe to assume that hydrolysis does not occur during extraction and that our results are correct when the filtered extract is handled with a minimum of delay.

Method—Free Gossypol

Apparatus

1. Mechanical shaker that will provide sufficient agitation of the solution to wash the sample material which collects around the top of the flask back into the solution.
2. Coleman Jr. spectrophotometer, with 19- or 25-mm. cuvettes, or any equivalent photoelectric colorimeter.
3. Grinding mill, Wiley, with 1 mm. screen.
4. Solid glass beads, about 6 mm. diameter.
5. Erlenmeyer flasks, glass-stoppered, 250 ml.
6. Filter paper, medium retentivity, 11 cm.
7. Pipettes and volumetric flasks.
8. Water bath that can be kept at 100° C., with a clamping device to support volumetric flasks in the water. The latter may be replaced by heavy metal washers, slipped over the necks of the flasks, keeping them in a stable, upright position.

Reagents

1. Aqueous acetone. 70% reagent grade in distilled water.
2. Isopropanol. 80% reagent grade in distilled water. Ethyl alcohol, formula 3-A, may be substituted.
3. Aniline. This need not be of high quality, since any grade must be redistilled. Distil through an air condenser, discarding the first and last 5-10%. Store in a tightly stoppered, brown bottle. Redistil when the absorbance of a reagent blank, measured occasionally against alcohol, exceeds 0.02.
4. Standard gossypol solution. Transfer 25 mgm., accurately weighed, of pure gossypol to a 250 ml. volumetric flask. Dissolve and make to volume in 70% acetone. Dilute 25 ml. of this to 250 ml. in 70% acetone for a working stock solution. Each ml. contains 0.01 mgm. of gossypol. If kept cold, dark and tightly stoppered, this solution may be kept for 3-4 days.

Preparation of the Sample

Use a sample prepared as directed in A.O.C.S. Method Ba 1-38. Grind about 50 gm. so that it will pass a 1-mm. screen.

Procedure

Transfer 1. ± 0.001 gm. of sample to a 250-ml., glass-stoppered Erlenmeyer flask. Add enough 6 mm. glass beads to evenly cover the bottom of the flask. Add 50 ml. of 70% acetone. Stopper the flask and shake mechanically for one hour at such a rate that the sample material which collects around the top of the flask will be constantly washed back into solution.

Filter through a dry, 11-cm. paper of medium retentivity, discarding the first portion of the filtrate. Take care to avoid any evaporation of the filtrate. While the sample is filtering, prepare a solvent blank from 70% acetone equal in volume to the sample aliquots to be taken, made to volume with 80% isopropanol. Call this solution A.

Pipet duplicate aliquots of the filtered extract into volumetric flasks (Note 1). Make one of the flasks to volume with 80% isopropanol. This is the gossypol blank. Call it solution B.

With the spectrophotometer set at 445 mu., adjust the instrument to read zero absorbance against solution A. Record the absorbance of solution B. Make this reading as soon as possible after filtering the extract; certainly within 5 minutes (Note 2).

To the other sample aliquot add 3 ml. of aniline and heat in a boiling water bath for 15 min. Do not stopper the flask. This is solution C.

At the same time prepare a reagent blank, solution D, consisting of 3 ml. of aniline and a volume of 70% acetone equal in volume to the sample aliquot. Heat this in the water bath with solution C.

After solutions C and D have been in the water bath for 15 min., remove and make to volume, at room temperature, with 80% isopropanol.

Now adjust the spectrophotometer to read zero absorbance against solution D and record the absorbance of solution C.

Subtract the absorbance of solution B from that of solution C. This is the absorbance of dianilinogossypol formed from the free gossypol in the sample. Calculate % free gossypol from a previously prepared calibration curve.

Calculations

Rather than to make calculations each time by referring to the standard curve or a chart, it is best to calculate the results by the use of a factor. Since the curve must go through the origin, calculate a factor, F, by dividing any concentration in

milligrams per 50 ml. by the corresponding absorbance, taken from the curve.

$$\% \text{ Free Gossypol} = \frac{S \times (C\text{-}B) \times F}{10 \times V}$$

where S = volume of the final colored solution, and
V = volume of the sample aliquot,
assuming the use of a 1.0-gm. sample extracted with 50 ml. of solvent.

Preparation of the Calibration Curve

From a burette add a series of duplicate aliquots of the stock solution of gossypol to two sets of 50 ml. volumetric flasks. Use duplicate 1, 2, 5, 10, 15, and 20 ml. aliquots. Prepare blanks and develop and measure the color as described above. Plot absorbance against milligrams of gossypol per 50 ml. Use rectilinear paper.

Notes

1. The choice of aliquot and volumetric flask size depends upon the free gossypol expected and the sensitivity required. The following table will serve as a guide.
   The figures assume the use of 25-mm. cuvettes with a Coleman Jr. spectrophotometer.

% Gossypol Expected	Ml. of Aliquot	Size of Volumetric (ml. of final solution)
0.10	5	25
0.10-0.15	2	25
0.15-0.20	2	50

2. Occasionally, as a result of processing variables, some samples may exhibit strongly absorbing gossypol blanks from excessive amounts of dianilinogossypol and/or other pigments extracted by the 70% acetone. In the event that the absorbance of the gossypol blank is 0.40 or greater, smaller aliquots must be taken for solutions B and C.
3. Avoid the use of a mouth pipette for handling aniline. Redistillation should be done either in a fume hood or in a well ventilated room.

Method—Total Gossypol

The procedure for this method is described by Pons, Hoffpauir, and O'Connor (5). The changes developed in this work are to use aniline to develop the color and to double the concentrations of the oxalic acid and barium acetate solutions. Dianilinogossypol is more difficult to hydrolyze than protein-bound gossypol.

Summary

A modification of the Pons and Guthrie method for determining free gossypol in cottonseed materials is presented. The use of aniline, rather than p-anisidine, as the color-producing reagent is necessary if meals containing dianilinogossypol are to be analyzed correctly. Increasing the reaction temperature eliminates a serious weakness in the method and results in greater accuracy. The proposed method is applicable to all types of cottonseed meal now marketed.

A slight modification of the Pons method for total gossypol is also presented. By doubling the strength of the oxalic acid used to hydrolyze bound gossypol and by using aniline to develop the color, the method is made applicable to chemically treated meals containing dianilinogossypol.

Acknowledgment

Grateful acknowledgment is made to the Southern Regional Research Laboratory for the pure gossypol used in this work and to C. L. Hoffpauir and W. A. Pons for their help.

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[Received April 21, 1954]

Comparison of Methods for Determining Fatty Acid Oxidation Produced by Ultraviolet Irradiation[1]

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ULTRAVIOLET light catalyzes the oxidation of pure unsaturated fatty acids (2) and the lipids of skin (5, 15), liver slices, and mitochondria (12). The extent of such oxidation can be measured as peroxide, aldehyde, or, in the earlier stages, the degree of conjugation (2), and in the case of linoleic and linolenic acids by a colorimetric reaction with thiobarbituric acid (9). This last reaction is simple and sensitive (3, 13, 15) and has been used with tissues (1, 9, 10, 16) and for dairy products (3, 6, 13). Glavind and Hartman (7) compared the thiobarbituric acid (TBA) reaction with the Kreis test for aldehyde and the dichlorophenolindophenol reaction for peroxide on fatty acids from cod liver oil, methyl oleate hydroperoxide, epihydrin aldehyde, and benzoyl peroxide. They reported that the TBA test paralleled the aldehyde test rather closely but that there was no agreement with peroxide values, as determined by their method. On the other hand, Pool and Prater (14) found a parallel development of peroxides as determined by Wheeler's peroxide method and the substance responsible for the color in the Kreis test. These relationships have been examined further in the present study by comparing peroxide, aldehyde, conjugation, and TBA values for linolenate, linoleate, and oleate oxidized by ultraviolet light.

Methods

Samples of methyl linolenate, methyl linoleate, and methyl oleate (Hormel Foundation, sealed under vacuum) were analyzed after exposure in thin layers to ultraviolet irradiation for various periods (G-E Precision lamp, No. 18A-T10, 6 volts, with glass envelope removed; no filter, distance 10.7 cm., 25.5-27.0°C.). Stock solutions were prepared from samples of unirradiated and irradiated esters diluted with freshly distilled methanol and were kept under oxygen-free nitrogen at 0°C. until analysis. Aliquots (17.9 mg.) of irradiated and unirradiated esters were analyzed by each of the methods given below in rapid succession to minimize differences in degree of autoxidation.

[1] Supported by a grant from the Atomic Energy Commission.