of a binary mixture if it contains not less than 25% of the t,tΔ10,12-isomer and not less than 5% of the t,tΔ9,11-isomer. Comparison of intensities of the diffraction lines given in Table III permits an approximate estimate of the composition.

For mixtures containing less than 25% t,tΔ10,12-linoleic acid or less than 5% t,tΔ9,11-linoleic acid, the solidification point must be measured to determine the composition. This assumes that no components other than these isomers are present in the mixture. As shown in Table II, the solidification point is a sensitive function of composition and in conjunction with the X-ray data defines the composition of any mixture to ± 3%. It is in fact desirable to characterize any mixture by its solidification point since it provides a more precise estimate of the composition than does the diffraction pattern.

The authors are greatly indebted to S. F. Herb, of the Eastern Regional Research Laboratory, for his assistance in preparing the isomers used in the present investigation.

Summary

X-ray diffraction and solidification point data are reported for t,tΔ9,11- and t,tΔ10,12-linoleic acids and their mixtures. The long spacing of the Δ9,11-isomer was 47.95 Å whereas that of the Δ10,12-isomer was 44.09 Å. X-ray diffraction patterns of binary mixtures showed the characteristic pattern of each isomer if the mixture contained not less than 25% of the t,tΔ10,12-isomer or not less than 5% of the t,tΔ9,11-isomer. Outside these composition limits only the pattern of the predominant isomer appeared, and solidification point data were used to define the composition. The solidification point in conjunction with the X-ray data defined the composition of any mixture to ± 3%.

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Effect of Temperature on the Content of Pigments of Stored Cottonseed

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Introduction

CONTROL of color is an exceedingly important problem of the cottonseed processing industry. Only through an understanding of the pigments of the cottonseed and the changes which can occur in them during storage and processing can the processor improve methods for the control of color in his products.

In addition to the effect of the conditions of processing the seed for oil and meal, the original pigment content of the seed and the conditions of storage of the seed and crude oil affect the final color of the oil. Seed of originally high pigment content (1) yields oil of darker color than that produced from less highly pigmented seed. Oil expressed from stored seed is more highly colored than oil expressed from fresh seed and the development of color that cannot be removed by bleaching the refined oil is increased when crude oil is produced from stored seed. Seed stored at high temperatures produces highly colored oils, and storage of the crude oil at high temperatures likewise causes development of pigments which cannot be removed or are removed with difficulty by subsequent refining and bleaching (2).

The investigations of Podolskaya (3) have shown that the cottonseed pigments undergo constant alteration upon storage of prime, low moisture content seed. She found that during storage of variety 114 of *G. hirsutum* the content of gossypol decreased from 1.15 to 0.75% during storage for four months. She has also reported (4, 5) that the initial concentration of yellow and orange-yellow intraglandular pigments, of "red gossypol" which is now known to be a mixture of gossypol and the purple pigment, gossypurpurin, and of the water-dispersable red-purple pigment of mature cottonseed was greater in fresh Egyptian than in fresh Upland seed. The rate of increase in the content of pigments was reported to be more rapid for the Egyptian variety. Pons and coworkers (6) found that the content of "red gossypol" increased during storage of cottonseed at 27°C., but that it did not increase in the case of seed stored at 1°C.

In a recent investigation (7) the effects of environmental and genetic factors upon the pigment contents of several varieties of cottonseed were determined. It was shown that the contents of gossypol and gossypurpurin during storage of seed stored at 80°F. were unrelated to the initial concentration of these pigments. In the 24 varieties investigated gossypol (8) was not found to follow a consistent pattern of change; however, gossypurpurin increased in all samples during storage. The investigation reported here is concerned with the effect of temperature of storage on the changes in the principal intraglandular pigments, gossypol and gossypurpurin, in three varieties of prime, low moisture content cottonseed.

Methods

Samples. Three pure-bred varieties of *G. hirsutum*, namely Stoneville 2B, Delfos 651, and Deltapine 15 were used in this investigation. The three varieties were planted and grown in sandy sarpy soil under similar environmental conditions at the U. S. Cotton Field Station at Stoneville, Mississippi. The seed was harvested early in September 1947 from mature bolls and immediately shipped to New Orleans where it was

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TABLE I

Composition and Initial Content of Gossypol and Gossypurpurin of Different Varieties of *G. hirsutum*

Variety	Composition of the seed					Initial pigment content	
	Kernels			Moisture in delinted seed, %	Free fatty acids in oil, %	Gossypol,[2] %	Gossypurpurin,[2] %
	Lipids,[1] %	Nitrogen,[1] %	Moisture, %				
Stoneville 2B	30.60	5.43	6.37	9.40	0.67	1.53	0.0140
Delfos 651	32.99	5.08	4.53	8.65	0.34	1.61	0.0118
Deltapine 15	26.41	5.56	5.10	9.66	0.93	1.60	0.0112

[1] Calculated on a moisture-free basis. [2] Calculated on basis of weight of kernels, as received.

stored in closed containers at 38°F. until the content of lipids, nitrogen, moisture, gossypol, and gossypurpurin were determined. The length of time between harvesting and the first analysis of the pigments was approximately 60 days. The analyses for lipids, nitrogen, and moisture were made according to the Official Methods of the American Oil Chemists' Society. A sample of each of the three lots of seed was then stored in sealed cans at 38°, 77°, and 85°F., and small samples were withdrawn at periodic intervals for pigment analyses.

Principle of Pigment Analysis Methods. Gossypurpurin in chloroform solution exhibits (9, 10) characteristic absorption maxima at 530 and 568 mμ in the visible wave-length region. Since none of the other pigments of cottonseed exhibit absorption in this region, the specific extinction coefficient at the highest of these maxima (568 mμ) may be used for the determination of gossypurpurin in chloroform extracts of raw seed.

Because of the development during storage of secondary pigments having an absorption similar to gossypol (9) in the region of 364-368 mμ it is necessary to determine the gossypol in such seed by the antimony trichloride-spectrophotometric method (3). Antimony trichloride reacts with pure gossypol to form a stable red-colored reaction product which exhibits a characteristic absorption spectrum in chloroform with maxima at 380 and 520 mμ and a minimum at 430 mμ. The specific extinction coefficients at the maxima are proportional to the amounts of gossypol present. In order to determine whether the results obtained by the antimony trichloride-spectrophotometric method are reliable, it is necessary to determine the ratios of the specific extinction coefficients of the maxima and minimum of a chloroform extract of the seed and compare them with the values for the same ratios obtained with pure gossypol.

When the ratios obtained with chloroform extracts of cottonseed and antimony trichloride are not identical with those obtained with pure gossypol, which is generally the case with extracts of cottonseed after storage for a long period of time, it is necessary to separate the gossypol from interfering substances in the crude seed extracts by extracting it with aqueous alkali (11). An acidified aliquot of the aqueous alkaline extract is transferred to chloroform and the gossypol determined quantitatively after treating the solution with antimony trichloride.

Although gossyfulvin (9) exhibits characteristic absorption in the region of 440 mμ, the presence of very small quantities will be masked by the absorption of gossypol. Gossyfulvin in chloroform solution is converted to gossypol by treatment with a few drops of concentrated hydrochloric acid. Consequently the differences in the specific extinction coefficients of the antimony trichloride reaction product of an extract of cottonseed before and after treatment with hydrochloric acid is used to detect and measure the amount of gossyfulvin in the extract.

Preparation of Chloroform Extracts of Seed. Cottonseed is dehulled in a Bauer laboratory mill, the meats ground in a mortar, and passed through a U. S. No. 50 sieve. One gram of the sieved kernels is added to 25 ml. of chloroform in a low-actinic glass flask, and the mixture equilibrated for 24 hours at 38°F. This time (1, 8) has been found to be sufficient for the complete extraction of gossypol, gossypurpurin, and related pigments. The mixture is then filtered into a pre-cooled low-actinic glass flask. The filtered extract may be maintained at 38°F. when well protected from the action of light, under which conditions the solution of pigments is stable for a period of 48 hours after filtration.

Analysis for Pigments. Using the above-described method of preparation, spectrophotometric analyses of the chloroform extracts were made in the wave-length region from 245 to 650 mμ with a Beckman quartz spectrophotometer. The antimony trichloride-spectrophotometric method was applied to chloroform extracts of the seed and to the alkali-extractable portions of these extracts for the determination of gossypol. The original extracts of cottonseed were treated with concentrated hydrochloric acid prior to applica-

TABLE II

Effect of Temperature and Duration of Storage Upon the Apparent Content of Gossypol in Stoneville 2B Cottonseed

Length of storage, days	Temperature of storage					
	38°F.		77°F.		85°F.	
	$E^{1\%}_{1cm.}$ 364-368 mμ	Apparent gossypol, %[1]	$E^{1\%}_{1cm.}$ 364-368 mμ	Apparent gossypol, %[1]	$E^{1\%}_{1cm.}$ 364-368 mμ	Apparent gossypol, %[1]
0	5.40	1.53	5.40	1.53	5.40	1.53
114	6.05	1.72	5.63	1.60	5.89	1.67
178	5.48	1.56	5.21	1.48	6.10	1.73
277	5.65	1.61	5.93	1.68	6.28	1.78
383	4.48	1.27	4.88	1.49	3.50	0.99

[1] Percentage of apparent gossypol calculated on the basis of the $E^{1\%}_{1cm.}$ = 352 at 364-366 mμ for pure gossypol in chloroform.

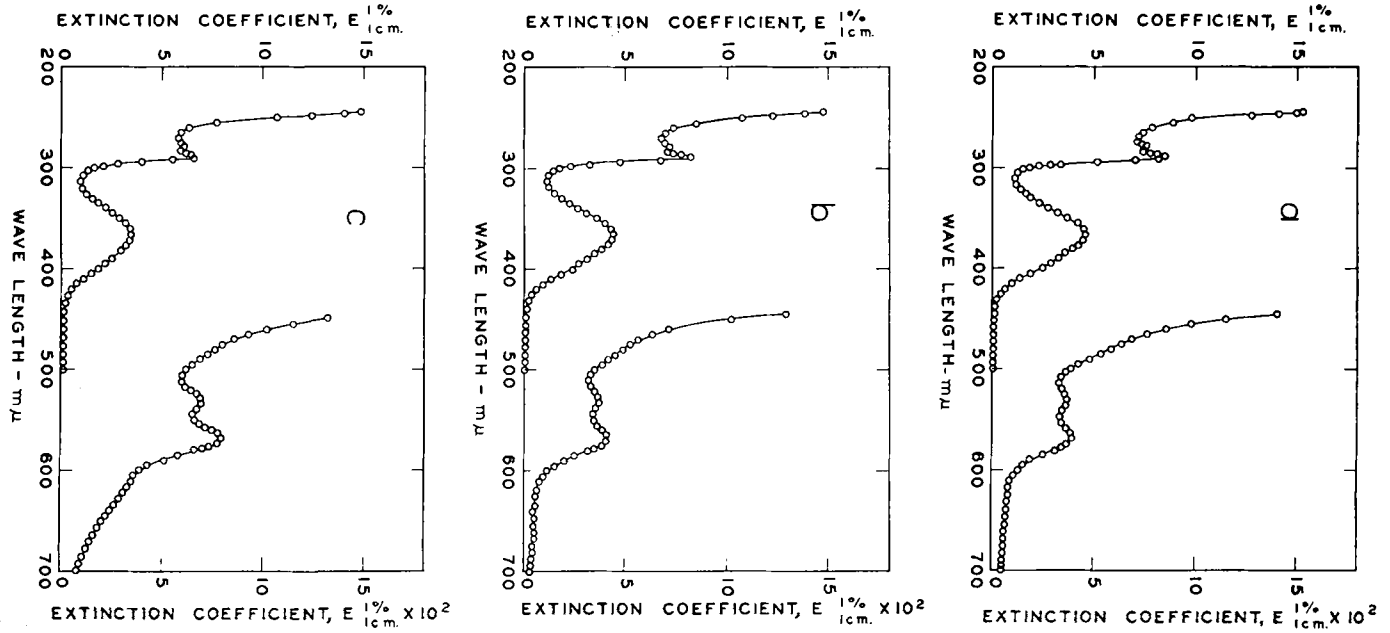

FIG. 1. Absorption spectra of chloroform extracts of Stoneville 2B cottonseed. Curve a, extract of original seed; curve b, extract of seed stored at 38°F. for 380 days; curve c, extract of seed stored at 85°F. for 380 days.

tion of antimony trichloride to detect the presence of gossyfulvin.

Results

Composition and Initial Pigment Content of the Seed. Spectrophotometric analyses of chloroform extracts of the seed did not show the presence of any pigments other than gossypol and gossypurpurin. Gossyfulvin was not found when the extracts were treated with hydrochloric acid. The initial analyses of the three lots of seed are shown in Table I.

Effect of Temperature of Storage on the Content of Gossypurpurin. The absorption spectra of a chloroform extract of the original sample of Stoneville 2B cottonseed and extracts of the same seed after storage for 380 days at 38° and 85°F. are shown in Figure 1. The differences in the heights of the maximum at 568 mμ are indicative of the differences in the gossypurpurin content of each sample. The changes in the contents of gossypurpurin of all three samples of cottonseed as a function of temperature of storage are shown in Figure 2.

Examination of the curves in this figure indicates that the increase in content of gossypurpurin was greatest in cottonseed stored at the higher temperature. There was a pronounced increase in gossypurpurin content in seed of the Deltapine 15 variety even when stored at 38°F. After nearly 13 months' storage at the three different temperatures the content of gossypurpurin of Deltapine 15, which was originally the lowest of the three lots of seed, increased at a faster rate and to a greater extent than in the other two samples.

Effect of Temperature of Storage on the Content of Gossypol. Chloroform extracts of fresh cottonseed gave a red-colored reaction product with antimony

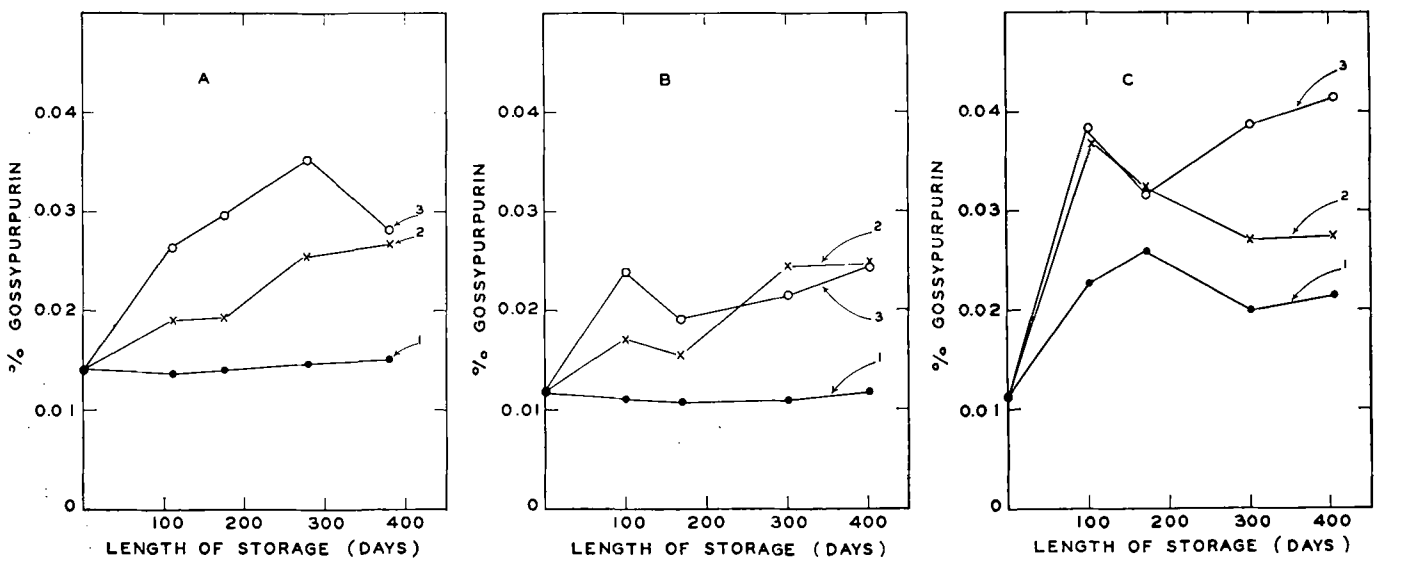

FIG. 2. Variation in gossypurpurin content during storage of (A) Stoneville 2B, (B) Delfos 651, and (C) Deltapine 15 cottonseed. Curves 1, are for seed stored at 38°F., curves 2 for seed stored at 77°F., and curves 3, for seed stored at 85°F.

trichloride which was identical with the antimony trichloride reaction product obtained with pure gossypol. After extraction of the gossypol from these chloroform extracts by alkali, acidification, and treatment with antimony trichloride, the spectral absorption values for gossypol were the same as those obtained by treatment of the original chloroform extracts with antimony trichloride.

When chloroform extracts were prepared from stored cottonseed and treated with antimony trichloride, an orange-colored reaction product was obtained instead of the characteristic bright red product obtained with pure gossypol or with extracts of fresh cottonseed. However, when these chloroform extracts were extracted with dilute aqueous sodium hydroxide and then treated with antimony trichloride, the alkali-extractable portion gave the red-colored reaction product characteristic of gossypol. The product responsible for the formation of the orange-colored reaction product was obviously not soluble in aqueous alkali and remained in the original chloroform extract.

The orange-colored antimony trichloride reaction product of the chloroform extracts prepared from cottonseed after storage for long periods of time was unstable. Its absorption spectrum exhibited maxima at 492-494 mμ and 377-378 mμ, and minima at 434-435 mμ and 362 mμ in contrast to the red gossypol-antimony trichloride reaction product whose absorption spectrum possessed maxima at 380 and 520 mμ and a minimum at 430 mμ. The portion of the extracts prepared from stored seed which was not extractable with aqueous alkali produced a reaction product with antimony trichloride which was dark yellow, very unstable, and having transitory absorption maxima at 380, 460, and 490 mμ. Obviously the presence of this substance(s) will interfere with the antimony trichloride reaction for gossypol by shifting the positions and altering the heights of the maxima.

The non-acidic portions of the chloroform extracts prepared from stored cottonseed were yellow colored and showed in chloroform solution characteristic absorption (373-383 mμ) in the same general wave length region as gossypol (364-366 mμ). It is apparrent that measurement of the specific extinction at 364-366 mμ of chloroform extracts prepared from stored cottonseed will yield unreliable values for gossypol because of the presence of secondary pigments absorbing in the same general region.

In Table II are shown the calculated values for the apparent percentages of gossypol obtained with extracts of Stoneville 2B cottonseed after storage. As seen in Table II, the calculated amount of apparent gossypol only began to decrease after the seed had been stored for more than a year.

In Figure 3 is shown the variation in the content of true gossypol during storage of the three varieties of seed at the three different temperatures of storage, as determined by the antimony trichloride reaction after separation of the non-acidic pigment(s) of the chloroform extracts of the stored seed. The content of gossypol decreased during storage in all three samples at all three temperatures.

Discussion

The absorption spectrum of the non-acidic pigment(s) of stored cottonseed is similar to the absorption spectrum of diaminogossypol (10) which is formed by the treatment of gossypol with ammonia. Diaminogossypol is also non-acidic and therefore non-extractable with alkali, and it produces a yellow-colored reaction product on reaction with antimony trichloride. No evidence has heretofore been presented to indicate that such a product is present in cottonseed, but it is not improbable that *diaminogossypol* constitutes a fraction of the non-alkali extractable portion of the pigmented material observed in stored cottonseed.

It would appear that the transition of gossypol to diaminogossypol through the treatment of gossypol with ammonia (10) and the final conversion of this diaminogossypol to gossypurpurin which occurs *in vitro* may also take place in the living seed. High temperatures would accelerate the transition of gossypol to gossypurpurin during storage. It is possible that during metabolism of the seed changes occur

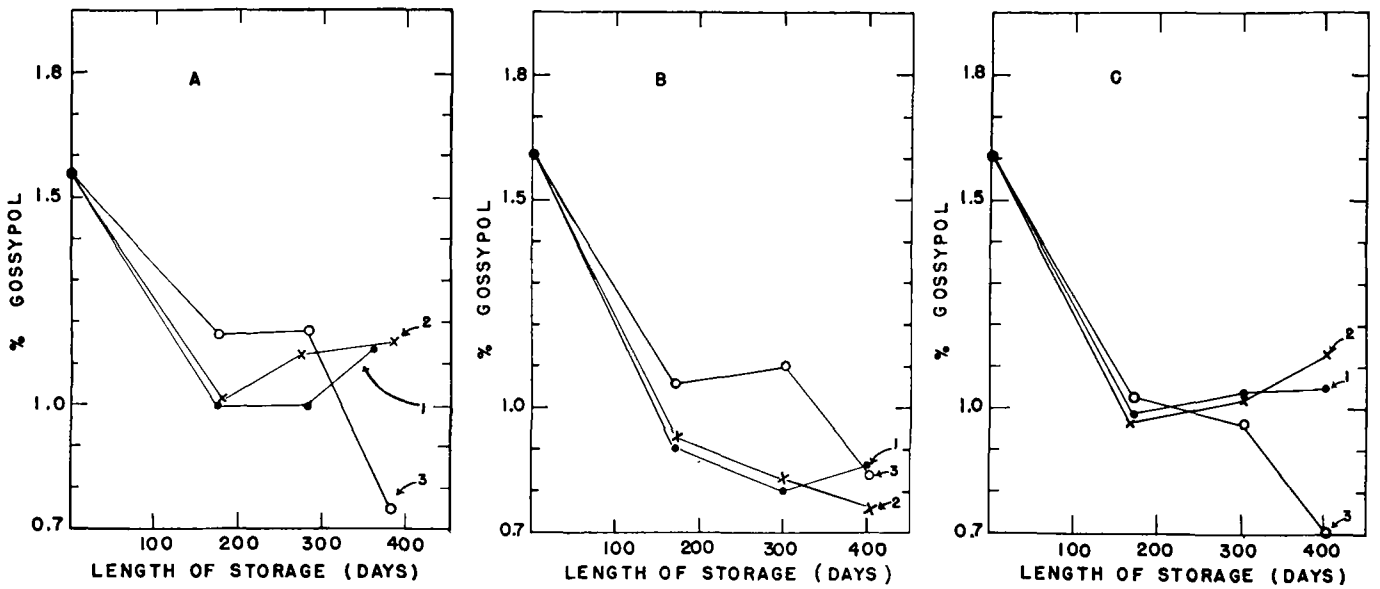

FIG. 3. Variation in content of gossypol of (A) Stoneville 2B, (B) Deltapine 15, and (C) Delfos 651 cottonseed during storage at 38°F. (curve 1), 77°F. (curve 2), and 85°F. (curve 3).

in some of the nitrogenous material of the seed, thereby making available free amino groups or free ammonia which could then react with gossypol to form diaminogossypol.

High temperatures during storage have been shown to increase the color of the oil produced from such seed. It had been previously postulated (7) that gossypurpurin and its decomposition products may be responsible for the dark colors of the expressed crude oils. In this investigation it was confirmed that high temperatures during storage of the seed increased the content of gossypurpurin and this pigment could be one of the major components contributing to the dark colors of the expressed cottonseed oils.

The non-acidic pigment(s), containing diaminogossypol, which are found more abundantly in cottonseed after storage for long periods of time at high temperatures, may account for some of the increased color observed in refined cottonseed oil. During alkali-refining gossypol is removed, but the yellow non-acidic pigment(s) are not removed. Isolation and identification of these non-acidic pigments and other conversion or decomposition products of gossypol and gossypurpurin may make possible a more rational control of the color of finished cottonseed oils.

Summary

Three pure-bred varieties of cottonseed, *G. hirsutum*, which were planted and grown under similar environmental conditions were stored at 38°, 77°, and 85°F. After determining the initial contents of lipids, nitrogen, moisture, gossypol, and gossypurpurin each lot of seed was stored at the different temperatures and analyzed periodically with respect to changes in pigmentation.

The content of gossypurpurin was found to increase during storage in all of the samples. Its increase was proportional to the temperature and length of storage. On the other hand, gossypol decreased during storage of all samples.

The antimony trichloride test for gossypol was found to be applicable only to extracts prepared from fresh cottonseed. During storage of the seed another yellow-colored pigment(s) developed which could be separated from gossypol by alkaline extraction of the original chloroform extract of the stored seed. The alkali extractable portion of the chloroform extract gave a red-colored antimony trichloride reaction product characteristic of gossypol.

It is postulated that at least a fraction of the non-acidic pigment(s) in the crude chloroform extracts obtained from stored cottonseed is diaminogossypol.

Acknowledgment

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A Convenient Heating Apparatus for Isomerization of Oils for Spectrophotometric Analysis*

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THE alkali isomerization of fatty materials during spectrophotometric analysis for polyunsaturated constituents has been carried out in constant temperature baths containing bath wax (1) or mineral oil (2). These baths have disadvantages from the points of view of cleanliness, fumes, and fire hazard. An alternative heating apparatus is described which is rapid in operation and convenient.

The apparatus (Fig. 1) consists of an electrically heated cylindrical brass block 3⅛ in. in diameter and 6 in. long (A), drilled to hold three 6 x 1 in. matched test tubes at a depth of 4½ in. (B), a mercury thermoregulator (C) and a thermometer (D). (A larger cylinder could be used to accommodate more test tubes.) The heating element, 500 watts, (E) is a double winding of No. 20 gauge Chromel wire with the windings insulated from each other by porcelain cement. The element is insulated from the brass block (A) by a layer of asbestos paper (F). To reduce heat loss to the surrounding air the unit is sealed inside a No. 10 can (G) insulated around the circumference with glass wool (H), at the bottom with a half-inch layer of insulating board (I), and at the top with a 3/16 in. sheet of asbestos (J) with openings to match the holes in the block. Two rings of ½ in. insulating board (K and L) center the block and keep it from shifting inside the can.

The thermoregulator is connected to a relay, which may be by-passed by means of a switch. A Variac is

*N. R. C. No. 1963.