Vitamin E Analytical Procedure for Cottonseed and Its Products[1]

BENJAMIN D. DEACON and A. CECIL WAMBLE, Cottonseed Products Research Laboratory, Texas Engineering Experiment Station, Texas A. & M. College System, College Station, Texas

COTTONSEED oil is a relatively economical source of Vitamin E (alpha tocopherol). However there is little information in the literature on the content of Vitamin E in cottonseed and on its fate during processing. Methods of analysis described in the literature have been used with several modifications necessitated by the nature of cottonseed and its products.

There are four known tocopherols (alpha, beta, gamma, and delta). Beta tocopherol has been discovered only in wheat germ oil. Delta tocopherol has been reported to be present to the extent of about 10% of the total tocopherol in cottonseed oil (1). This tocopherol gives a gradual increase in color in the total tocopherol determination (2). It was found that the increases in spectrophotometric readings between 50 seconds and 2 minutes were small. It was therefore concluded that the samples contained very little delta tocopherol. Since delta tocopherol reacts with diazotized *o*-dianisidine in the gamma tocopherol determination (3), the error in determining alpha tocopherol (Vitamin E) would be negligible.

The total tocopherols are determined by the procedure of Emmerie and Engel (4). Interfering substances are removed by molecular distillation as described by Quaife and Harris (3, 5). The hydrogenation step (6) has recently been found to be unnecessary. The gamma tocopherol determination, actually the non-alpha tocopherol determination, is performed by the method of Weisler, Robeson, and Baxter (1). The hydrogenation step might still be necessary for crude oils, etc. No runs have been made without it except on whole seed analyses.

The present procedures are modifications of those described by Morrow (7), a former worker on this project. The most important change is the use of hexane, rather than alcohol, for extracting cottonseed oil. The latter solvent frequently failed to remove the oil properly, probably due to residual moisture. It is not necessary to dry the rolled seed, or other samples prior to extraction with hexane. Hexane also boils at a considerably lower temperature than alcohol so that there is less probability of decomposition of Vitamin E. The extraction is however carried out in an atmosphere of nitrogen.

Analytical Procedure

Preparation of Sample. Samples of whole seed or meats, which contain a relatively large amount of oil, are handled in Sturtevant laboratory rolls which are in fairly tight contact. These rolls are fitted with scrapers. Low oil-content samples, such as meal, are ground in a Bauer laboratory mill at 3,500 r.p.m. to pass a 20-mesh sieve. A 20-g. sample of whole cottonseed, a 10-g. sample of meats, or a 30-g. sample of meal is used.

Liquid samples of crude oil are dissolved in hexane and alkali-washed as described in the section on extraction. Refined or bleached cottonseed oils are weighed into the aluminum cups, and the oil is subjected to molecular distillation as described later.

Extraction. The solid products are extracted in the Soxhlet apparatus for four hours in an atmosphere of nitrogen with 110 ml. of commercial hexane. The extraction is stopped with the minimum volume of hexane in the extraction flask, and the extract is diluted to 50 ml. with hexane. The hexane solution is washed with 40 ml. of a 1% sodium hydroxide solution containing 5% of sodium sulfite in a separatory funnel. The hexane layer is centrifuged for 10 minutes at 1,700 r.p.m. in an International Centrifuge size 2. A 10-ml. aliquot of the hexane layer is evaporated in a small tared aluminum cup (Figure 1) in a vacuum desiccator, and the oil is weighed.

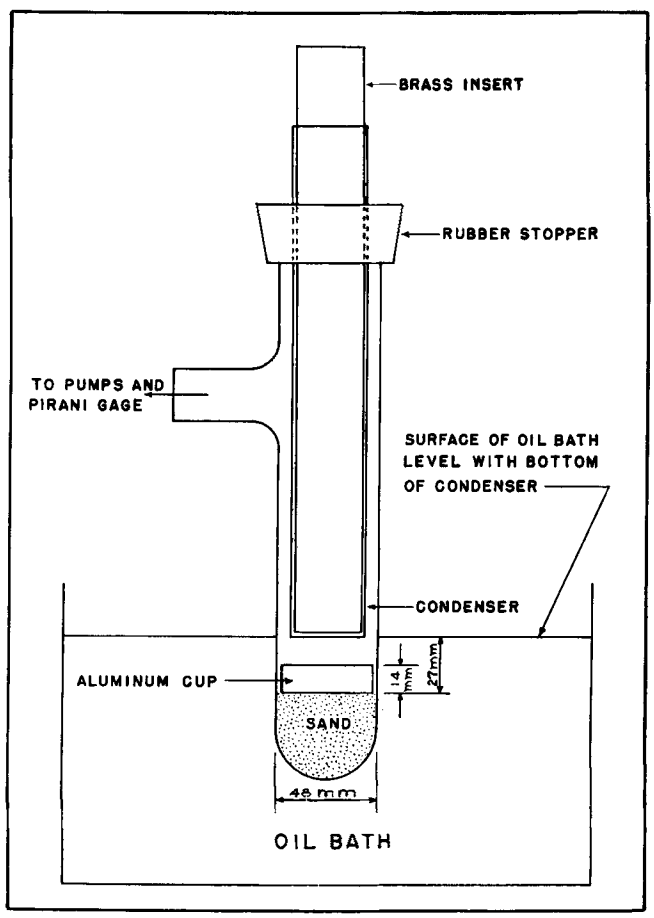

FIG. 1. Semimicromolecular Still.

Molecular Distillation. The aluminum cup, containing the alkali-washed oil, is placed in the semi-micromolecular still (Figure 1), a modification of that described by Quaife and Harris (5). It is connected to a molecular still or high vacuum pump capable of giving a pressure of about 1 micron. About 25 ml. of acetone are placed in each glass condenser tube, and

[1] The Vitamin E research described in this writing was conducted as a cooperative project of the Texas Engineering Experiment Station and the Cotton Research Committee of Texas.

TABLE I

Results of Duplicate Analyses of Whole Cottonseed Grams of Tocopherols Per Ton of Seed

Sample No.	9987		10148		10159		10163		10168		10317	
Total tocopherol	151	158	128	124	105	111	127	132	117	117	130	132
Gamma tocopherol	61	66	39	42	36	33	35	38	36	35	43	41
Alpha tocopherol (Vitamin E)	90	92	89	82	69	78	92	94	81	82	87	91

the brass tubes are inserted. About 30 ml. of acetone are placed in each brass tube, and dry ice is added after the pressure drops below 25 microns. The oil bath is then heated to 230°C. and stirred with a small air stirrer. A 500- and 1,000-watt coil heater are used. When the temperature reaches 230°C., the 500-watt heater is turned off, and the 1,000-watt heater is operated by a variac set at 80-90 to maintain 230°C. for a period of 30 minutes. The brass tubes are removed, and the acetone is sucked out of the glass condenser tubes. These tubes are filled with warm water (50-60°C.), and air is admitted. The glass tubes are then removed and placed in 100-ml. beakers. They are washed down with chloroform and let stand 5 minutes. The tubes are then raised and rinsed with chloroform from a wash bottle. The chloroform is evaporated in vacuum. During this time the 100-ml. beakers are covered with inverted 250-ml. beakers since the solvent sometimes spatters. The vacuum should be regulated so that it will not fall below 30-40 mm. since violent spattering may occur with breakage of the 250-ml. beakers and loss of product.

The residues in the beakers are diluted to 50 ml. with absolute alcohol. The resulting solutions are analyzed for total and gamma tocopherols as described below.

Total Tocopherol Determination

A 5-ml. portion of the alcoholic solution of the molecular distillate, 3 ml. of absolute alcohol, and 1 ml. of dipyridyl solution are pipetted into a 10-ml. volumetric flask and mixed. A Beckman spectrophotometer is adjusted to 100% transmission at 520 mμ, using a 1-cm. cell filled with absolute alcohol. A 1-ml. portion of ferric chloride reagent is added to the 10-ml. volumetric flask and the mixture is shaken for 10 seconds. The optical density of the mixture is read 50 seconds after adding the ferric chloride at 520 mμ in a 1-cm. cell. A blank is run, using 8 ml. of absolute alcohol, 1 ml. of dipyridyl reagent, and 1 ml. of ferric chloride. The optical density of the blank is subtracted from that of the sample.

Calculation

$$\text{Percentage of total tocopherol in oil distilled} = \frac{(\text{Optical density})(0.2326)}{\text{Weight of oil in g.}}$$

NOTE: The method originally used for total tocopherol employed catalytic hydrogenation of the alcoholic solution of the molecular distillate. This would hydrogenate unsaturated substances which might react with ferric chloride, *e.g.*, carotene. Recent results using whole cottonseed have shown that this hydrogenation is not necessary, at least in the case of whole cottonseed.

Gamma Tocopherol Determination [2]

A 15-ml. aliquot of the alcoholic solution of the molecular distillate is pipetted into a 50-ml. glass stoppered cylinder. Seven drops of potassium hydroxide reagent are added from an eye-dropper, and the mixture is shaken once or twice. Seven drops of diazo reagent are then added, and the mixture is shaken about 10 times. The mixture is let stand for 5 minutes, a little anhydrous sodium sulfate, about 15 ml. of water and exactly 10 ml. of purified hexane, are added. The mixture is shaken vigorously 50 times and allowed to settle, and the mixing and settling are repeated twice more. The optical density of the hexane layer is determined at 510 mμ in a 1-cm. cell. The spectrophotometer is balanced against a blank prepared as above, using 15 ml. of absolute alcohol.

Calculation

$$\text{Percentage of gamma tocopherol in the oil distilled} = \frac{(\text{Optical density})(0.1852)}{\text{weight of oil in g.}}$$

$$\text{Percentage of alpha tocopherol} = \text{Percentage of total tocopherol} - \text{Percentage of gamma tocopherol}$$

Reagents

1. *Sodium Hydroxide-Sodium Sulfite Solution.* A stock solution of 1% sodium hydroxide in water is prepared. Ten grams of sodium sulfite are added to 200 ml. of the solution just prior to use.

2. *Purified Hexane.* Commercial hexane is refluxed overnight with 200 ml. of concentrated sulfuric acid per liter. The hexane is distilled, washed with dilute sodium hydroxide, then with water until neutral, and dried with anhydrous sodium sulfate.

3. *2,2'-Dipyridyl Reagent.* 0.125 g. of 2,2'-Dipyridyl are dissolved in 25 ml. of absolute alcohol, then kept in a dark bottle and stored in the refrigerator.

4. *Ferric Chloride Reagent.* 0.2 g. of ferric chloride hexahydrate are dissolved in 100 ml. of absolute alcohol. The solution is kept in the refrigerator until used and returned to the refrigerator immediately afterwards. Blanks in total tocopherol determinations increase rapidly if the ferric chloride solution is allowed to stand at room temperature.

5. *Potassium Hydroxide Reagent.* 20 g. of potassium hydroxide is dissolved in 100 ml. of distilled water.

6. *Diazotized o-Dianisidine.* *o*-Dianisidine dihydrochloride is prpeared from the technical base by the method of Talbot *et al.* (8). 0.5 g. of *o*-dianisidine dihydrochloride is dissolved in 60 ml. of distilled water. Then 6 ml. of concentrated hydrochloric acid and 12 ml. of 5% sodium nitrite solution are added. The solution is mixed well, let stand 5 minutes; and 12 ml. of 5% urea solution are added. The solution is well mixed until effervescence ceases. It is stored in a brown bottle and kept at room temperature for 24 hours prior to use. It is kept in the refrigerator for further use and is stable for about two weeks.

Reproducibility of Results. The results of several recent analyses are collected in Table I. These were performed in duplicate and appear to agree within satisfactory limits.

Tocopherol Analysis of Whole Cottonseed. Total and gamma tocopherol analyses were carried out, and the alpha tocopherol (Vitamin E) content was calcu-

[2] Low results in gamma tocopherol determinations sometimes occur due to reaction of the diazo reagent with alkali on the walls of the tubes. This difficulty is eliminated by shaking, after adding the alkali, and by dropping the diazo reagent directly into the reaction mixture so that it does not flow down the sides of the tube.

lated for various cottonseed samples of several seasons from several localities. Several of these samples were analyzed before and after storage. The results are included in Tables II, III, and IV. Cottonseed varieties represented in the samples were Deltapine, Rowden, HiBred, Texacala, and Stoneville.

TABLE II

Effect of Storage of Cottonseed on Vitamin E
Vitamin E Content in Grams Per Ton of Seed

Crop Season	1949	1949	1950	1950	1951	1951
Date of Analysis	7–51	3–52	8–51	2–52	2–52	6–53
Deltapine						
Beeville	92	89	97	67	66	66
Ysleta	80	79	95	72	69	82
College Station	....	....	....	....	81	69
Lubbock	....	....	....	....	63	69
Rowden						
Beeville	67	60	72	55	58	55
Ysleta	71	71	74	59	59	66
College Station	....	....	....	....	69	65
Lubbock	....	....	....	....	54	54
Mean	77.5	77	84.5	63	65	66

The results of storage of cottonseed on their Vitamin E content are collected in Table II. Reanalysis of the 1951 seed, after storage, showed no significant change in Vitamin E content. The same appears to be true of the second year's storage of the 1949 seed. The 1950 seed appear to have lost a significant proportion of their Vitamin E. This may be due, at least in part, to changes in the method of analysis in the interim.

TABLE III

Effect of Location and Variety on Vitamin E Content of 1951 Cottonseed
Vitamin E Content in Grams Per Ton of Seed

	Deltapine	Rowden	HiBred	Texacala	Stoneville	Mean
Beeville	66	58	69	68	64	65
Ysleta	69	59	68	62	83	68
College Station	81	69	76	78	76	76
Lubbock	63	54	76	63	77	67
Mean	70	60	72	68	75	69

TABLE IV

Effect of Location and Variety on Vitamin E Content of 1952 Cottonseed
Vitamin E Content in Grams Per Ton of Seed

	Deltapine	Rowden	HiBred	Texacala	Stoneville	Mean
Beeville	89	63	84	85	73	79
Ysleta	77	57	92	90	95	82
College Station	98	76	80	81	93	86
Lubbock	78	89	97	98	96	92
Mean	86	71	88	89	89	(84.6)

The cottonseed samples of the 1952 crop were found to contain more Vitamin E than did the 1951 seed (Tables III and IV). The Vitamin E content of cottonseed seems to be very slightly more dependent on variety than on locality.

Tocopherol Analysis of Rolled and Cooked Meats. In order to determine whether Vitamin E is lost during the cooking of rolled meats, samples of these products were analyzed. No significant loss of tocopherols was observed. The results are given in Table V.

Methanol Extraction of Rolled and Cooked Meats. Since the solubility of cottonseed oil in methanol is quite limited, it was anticipated that extraction with this solvent would yield an oil considerably enriched in Vitamin E. Such a concentrate might compete with wheat germ oil as a commercial source of this vitamin.

Rolled and cooked cottonseed meats were extracted with anhydrous methanol for periods of 30 and 90 minutes. The extracts were diluted with water, transferred to hexane, washed with alkali, and evaporated. The resulting oils were analyzed for Vitamin E. The analyses are reported in Table VI.

TABLE V

Analysis of Rolled and Cooked Meats

	Rolled Meats	Cooked Meats
Total tocopherol in oil, percentage	0.065	0.063
Gamma tocopherol in oil, percentage	0.032	0.033
Alpha tocopherol in oil, percentage	0.033	0.030

TABLE VI

Methanol Extraction of Rolled and Cooked Meats

	Rolled Meats		Cooked Meats	
	30 min.	90 min.	30 min.	90 min.
Vitamin E extracted, percentage	55.3	71.2	86.2	90.7
Vitamin E in extract, percentage	0.11	0.08	0.18	0.11

Since the major portion of the Vitamin E is removed during the first 30 minutes, the concentration in the oil decreases on continued extraction. Extraction of cooked meats appears preferable for commercial operation since Vitamin E is more completely removed with about 22% less oil. The Vitamin E concentration obtained after 30 minutes with cooked cottonseed meats (0.18%) is higher than that of wheat germ oil (0.12%) (9), which sells for about 8 to 12 times the price of cottonseed oil. The use of cottonseed oil especially processed for the purpose could reduce the price of Vitamin E, but not in proportion to the cost of these two oils.

The extraction of rolled cottonseed meats by allowing methanol to percolate through them in a column gave oils containing 0.1 to 0.14% of Vitamin E.

Vitamin E Analysis of Commercial Solvent Extracted Meal. Since the extraction of cottonseed oil leaves only small amounts of oil in the meal, it would be expected that the meal would be lower in Vitamin E. A commercial sample was found to contain 0.4% of oil, of which 0.064% is Vitamin E. The meal therefore contained only 2.3 g. of Vitamin E per ton whereas hydraulic or screw press meals have been found to contain 12-30 g. of Vitamin E per ton. However other sources of Vitamin E are normally present in livestock rations containing cottonseed meal.

Effect of Processing on Vitamin E. The cottonseed products from various stages of several runs from the screw press and the hydraulic press in other research have been analyzed for Vitamin E. Within the limits of experimental error there appears to be little or no loss of Vitamin E during rolling, cooking, and expression of crude oil. There is usually, but not always, some loss of Vitamin E during laboratory refining or bleaching.

The results of several runs are collected in Table VII. The vitamin contents of the refined and bleached

TABLE VII

Vitamin E in Products from Screw Press and Hydraulic Operations
Grams Per Ton of Whole Seed

Type of Run	Screw Press	Hydraulic Press	Hydraulic Press
Whole seed	55	....	....
Rolled meats	48	48	55
Cooked meats	51	44	53
Meal	5	14	9
Crude oil	42	40	43
Refined oil	47	41	29
Bleached oil	44	27	29

oils have not been corrected for refining loss. They may be however correct within the limits of experimental error.

Conclusions

A procedure for the analysis of cottonseed and its products for Vitamin E has been developed and improved. The present procedure generally gives good checks on duplicate analyses.

The Vitamin E content of whole cottonseed is somewhat more a function of variety than of locality. The 1952 crop of cottonseed contained more Vitamin E than the 1951 crop. The two crops averaged 84 g. per ton and 68.5 g. per ton, respectively.

Most recent results show that there is no loss of Vitamin E in the storage of cottonseed or during the processing of the seed for its oil and other products.

The hexane-soluble portion of a methanol extract of rolled or cooked cottonseed meats contains Vitamin E in concentrations that have exceeded the values reported for wheat germ oil, the present commercial source.

A sample of commercial solvent extracted cottonseed meal was found to contain considerably less Vitamin E than hydraulic- or screw-pressed meals.

REFERENCES

1. Weisler, L., Robeson, C. D., and Baxter, J. G., Anal. Chem., *19*, 906 (1947).
2. Stern, M. H., and Baxter, J. G., Anal. Chem., *19*, 902 (1947).
3. Quaife, M. L., and Harris, P. L., Anal. Chem., *20*, 1221 (1948).
4. Emmerie, A., and Engel, C., Rec. Trav. Chim., *57*, 1351 (1938).
5. Quaife, M. L., and Harris, P. L., Ind. Eng. Chem. Anal. Ed., *18*, 707 (1946).
6. Quaife, M. L., and Biehler, R., J. Biol. Chem., *159*, 663 (1945).
7. Morrow, H. N., "A Vitamin Study of the Cottonseed and Its Products," Phase Report No. 1, Development of an Analytical Procedure for the Determination of Vitamin E. Unpublished report, Texas Engineering Experiment Station.
8. Talbot, N. B., Wolfe, J. K., MacLechlan, E. A., Karush, F., and Butler, A. M., J. Biol. Chem., *134*, 319 (1940).
9. Harris, P. L., Ann. N. Y. Acad. Sci., *52*, 342-4 (1949).

[Received December 9, 1953]

The Component Acids of Kamala Oil (Mallotus Philippinensis, Muell. Arg.)

S. C. GUPTA, S. S. GUPTA, and J. S. AGGARWAL, National Chemical Laboratory of India, Poona

KAMALA oil from the seeds of *Mallotus Philippinensis*, Muell. Arg. (N. O. Euphorbiaceae), which grows fairly abundantly in this country and to some extent in many other parts of the East, has shown promise as a substitute for tung oil (2, 11). Previously some general studies on the drying properties and characteristics of the oil (1, 7) have been made, but no decisive investigation was carried out. While the present study of kamala oil was in progress, Puntambekar (9) reported that the fatty acids of kamala oil consist of isomeric elaeostearic, ketopolyethenoid C_{18}, oleic, linoleic, stearic, and other saturated acids. These results were deduced from his studies on the physical and/or chemical characteristics of the different fractions obtained by the lead salt alcohol method of the total fatty acids. The inadequacy and limitations of lead salt-alcohol method as an analytical procedure for oils containing conjugated polyethenoid acids are now well known. Moreover his contention regarding the nature of the new acids (mixture of keto polyethenoid acid and isomeric elaeostearic acid) with respect to the positions of keto group and double bonds is not very convincing and lacks substantial evidence. Further, the absence of any keto acid in this oil has been definitely established in our earlier communication (2).

Isolation and characterization (2) of a new acid, kamlolenic acid (ω-hydroxy, 9,11,13-octadeca trienoic acid) prompted further studies on the composition of the oil. When separated by means of its insolubility in petroleum ether (40-60°C.), about 5-10% of this acid was always found in the solvent along with other acids. In the absence of any alternative method for the estimation of this typical acid in the oil, the recently developed photometric method (6) as an analytical procedure has been found of considerable importance in assessing the potentialities of this new drying oil.

Extraction of the oil. The presence of 50-60% kamlolenic acid as a major acid renders the extraction of the entire quantity of the oil from the seeds with petroleum ether impossible. It was therefore found advantageous to extract the oil from the seeds in a soxhlet apparatus first by petroleum ether (40-60°C.) and then by ethyl ether. The oils obtained in both cases were kept separate and analyzed as such. The oils from fresh seeds (Bombay variety) and one-year-old seeds (Uttar Pradesh variety) indicated some differences. The analytical data have been recorded in Table I.

Analytical methods and results. The four specimens of the oil referred to above were separately saponified, and the unsaponifiable matter was removed according to the S.P.A. method (12). The mixed fatty acids obtained in each case were then examined for their components as described below.

Estimation of kamlolenic acid. The spectroscopic values for pure α- and β-kamlolenic acids have already been reported (2). These acids have been isomerized according to the method recommended by Hilditch, Morton, and Riley (6) for elaeostearic acid. The complete data are given in Table II.

The amount of conjugated triene acid in the different samples of the fatty acids from the oil was directly calculated from their respective extinction coefficients at 270.5 mμ. These values however would

TABLE II

$E^{1\%}_{1cm}$ Values for α- and β-kamlolenic acids

	270.5 mμ	268 mμ	234 mμ
α-Kamlolenic acid (unisomerized)	1800[a]	....	176
β-Kamlolenic acid (unisomerized)	....	(1990)[a]	(210)
α-Kamlolenic acid (alkali isomerized, 180°C.–60 mins.)	....	(926)	204
β-Kamlolenic acid (alkali isomerized, 180°C.–60 mins.)	....	(1056)	(188)
α-Kamlolenic acid (alkali isomerized, 170°C.–15 mins.)	....	1510	....
β-Kamlolenic acid (alkali isomerized, 170°C.–15 mins.)	....	(1805)	....

[a] Represent the corresponding values at the highest band head.
(Note: Data not actually required in the analysis of Kamala oil are shown in brackets.)