Determination of Tocopherol in Plant Tissue

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Modifications of the Emmerie-Engel and Furter-Meyer methods for the determination of tocopherol are reported, which consist essentially of the removal of substances that interfere with application to plant extracts. In most cases the results obtained by the two procedures agreed within ±5 to 10%.

DURING the course of investigations on the fat-soluble constituents of vegetable leaf meals, the authors wished to determine the tocopherol content of a number of leaf meals and extracts. A survey of the literature indicated that little work had been done with such materials. The tocopherol content of lettuce leaves has been reported by Karrer and Keller (*8*), and that of alfalfa by Scudi and Buhs (*13*).

The methods used by these workers are lengthy and require specialized apparatus, and attempts to use the latter method with standard tocopherol solutions resulted in poor recoveries. Therefore, the authors attempted to apply the Emmerie-Engel (*3*) and Furter-Meyer (*5*) procedures.

The Furter-Meyer procedure is based on the nitric acid oxidation of tocopherol to the red quinone. The reaction is relatively specific, taking place (*14*) only with compounds that have structures similar to that of tocopherol. The three forms of tocopherol—alpha, beta, and gamma—are not differentiated by this procedure, so that the analytical results show only the total tocopherols present. The method as originally presented by Furter and Meyer was applied only to pure tocopherol solutions and vegetable oils. In leaf extracts carotene, xanthophyll, chlorophyll, tocopherolquinones, and lipoids insoluble in cold ethanol interfered with the determination and had to be removed. In addition Quackenbush *et al.* (*12*) have shown that polyphenols such as pyrogallol interfere with the colorimetric determination. Such compounds, if present, are removed during the purification of the plant extract.

The Emmerie and Engel procedure is indirect. Ferric ion is reduced to the ferrous form by all three tocopherols. The ferrous ion then forms a colored complex with α,α'-dipyridyl. Any compound which can reduce iron will interfere with the method. In leaf extracts carotene, xanthophyll, chlorophyll, and certain lipoids which slowly reduce iron interfere and must be removed.

The modifications of these methods described here consist essentially of the removal of substances which interfere with their application to plant extracts.

EXTRACTION OF TOCOPHEROL

The solutions are kept in the dark except during manipulations and all manipulations are conducted in a room shielded from direct sunlight. All-glass apparatus is used throughout.

DRY SAMPLES. A 25-gram sample of leaf meal, of 30- to 40-mesh, is exhaustively extracted in a Soxhlet with Skellysolve B for 5 to 8 hours, and the extract thus obtained is made to 250 ml. In this manner sufficient sample is obtained for duplicate determinations by both procedures.

FRESH SAMPLES. A 10-gram fresh sample is necessary for the Emmerie-Engel procedure and a 25-gram sample for the Furter-Meyer. The material is extracted in a Waring blender using the foaming mixture of alcohol and Skellysolve B described by Moore and Ely (*9*). This procedure has been successfully used by Devlin and Mattill (*2*) to extract tocopherols from muscle tissue. The alcohol is separated and extracted with Skellysolve B as described by Wall and Kelley (*15*). The Skellysolve solution is then dried with anhydrous sodium sulfate and evaporated under vacuum and nitrogen to approximately 25 ml.

FURTER-MEYER PROCEDURE

The sample taken should contain a minimum of 0.3 mg. of tocopherol and preferably about 1.0 mg. An extract equivalent to 2.5 to 5.0 grams of dry leaf meal usually contains the necessary quantity of tocopherol. The sample dissolved in about 25 ml. of Skellysolve B is passed through a 7 × 2 cm. adsorption column consisting of three parts of Hi-Flow Supercel (Johns-Manville Corporation) and one part of activated magnesia No. 2641 (Westvaco Chlorine Products Company, Newark, Calif.) The preparation of the column and the adsorption technique are identical with those described by Wall and Kelley (*15*) for the determination of carotene. After adsorbing the extract, the adsorption column is washed with about 80 ml. of 5% acetone in Skellysolve B and finally with 20 ml. of 10% acetone in Skellysolve B. The carotene and tocopherol are eluted, chlorophyll and xanthophyll being retained on the column. The eluate is caught in a 250-ml. flat-bottomed boiling flask, ₮24/40.

The solution is evaporated in a water bath at 40° to 50° C. under vacuum and nitrogen to approximately 25 ml. To the concentrated solution are added 2.0 ml. of 85% (by volume) sulfuric acid. The flask is stoppered and vigorously shaken on a shaking machine for 3 minutes. The acid is colored deep blue by the decomposed carotene. Occasionally, if all the pigment is not removed, 2.0 ml. more of acid must be added. The stopper is removed and rinsed with Skellysolve B, and the contents of the flask are rinsed into a 250-ml. separatory funnel with Skellysolve B. The acid layer which separates is withdrawn. The Skellysolve solution is washed once with aqueous 5% sodium sulfate solution, then once with aqueous 1% potassium hydroxide, and finally with more sodium sulfate solution until the aqueous washings are practically neutral.

The solution, which should be colorless or only slightly yellow, is dried with anhydrous sodium sulfate, decanted from the sodium sulfate, and evaporated almost to dryness. The residue is transferred with 3 or 4 washes of Skellysolve B to a 50-ml. Erlenmeyer flask, ₮19/22, and the solution is again evaporated just to dryness. To the flask are added 5.0 ml. of absolute ethanol and then, while the flask is swirled, 1.0 ml. of concentrated nitric acid. The flask, attached to a small Liebig condenser by means of lightly greased interchangeable joints, is then set in a steam bath. The alcoholic solution is refluxed for exactly 3 minutes from the time the alcohol begins to boil. The flask is taken from the steam bath, and cooled with cold water while still connected to the condenser. The flask is then removed, stoppered, and kept in the dark while the color develops. This takes a minimum of 15 minutes; longer periods do not affect the determination.

The nitric acid oxidation of tocopherol results in the formation of a red tocopherolquinone. Prior to making the colorimetric measurements, lipoidal constituents of plant extracts insoluble in cold alcohol must be removed. The alcoholic solution is filtered on a small, dry, sintered, medium porosity Hirsch funnel. A micro colorimeter tube is placed inside a suction flask with the tip of the funnel just inside the tube. The solution is rapidly filtered with minimum suction (pressure filtration may also be used for this operation), the funnel being kept covered with a watch glass to minimize evaporation. The colorimeter tube is then removed and stoppered.

The colorimetric measurement was made with a Fisher Electrophotometer, at a wave length of 480 mμ. To secure this wave length a Corning filter combination consisting of one No. 3389 and two No. 4303 filters is used. A blank composed of 5.0 ml. of absolute ethanol and 1.0 ml. of concentrated nitric acid is the reference solution. Because the blank slowly darkens, it should not stand more than 0.5 hour.

A standard calibration curve, shown in Figure 1, A, was made with solutions of Merck synthetic α-tocopherol in Skellysolve B. The standard solutions were evaporated, oxidized with nitric acid, and filtered as described previously.

EMMERIE-ENGEL PROCEDURE

The best concentration range for this procedure is 0.05 to 0.20 mg. It is more convenient to purify a larger sample containing 0.2 to 1.0 mg. and then take a suitable aliquot for the final determination.

A Supercel–activated magnesia column approximately 4.0 × 2.0 cm. is prepared as described. The sample is adsorbed, eluted, and concentrated as in the Furter-Meyer procedure. After the sample is shaken for 3 minutes with 1 to 2 ml. of 85% sulfuric

Table I. $E^{1\%}_{1\ cm.}$ and 480 and 520 mμ Values for Synthetic α- and Natural α- and γ-Tocopherols

Compound	Furter-Meyer Method			Emmerie-Engel Method		
	Mg.	$E^{1\%}_{1\ cm.}$, 480 mμ		*Mg.*	$E^{1\%}_{1\ cm.}$, 420 mμ	
Synthetic α-tocopherol	1.0	15.5	15.5	0.1	238	238
	2.0	15.5		0.2	238	
Natural α-tocopherol	1.0	16.5	16.4	0.1	249	242
	2.0	16.3		0.2	234	
Natural γ-tocopherol	1.0	13.5	13.9	0.1	174	152
	2.0	14.2		0.2	130	

acid, it is transferred to a separatory funnel and treated as described above. After the last sodium sulfate wash, as much aqueous solution as possible is removed and, without drying, the purified Skellysolve solution is run into a 100-ml. volumetric flask and made to volume.

A 25-ml. aliquot is evaporated almost to dryness on a warm water bath under vacuum and nitrogen. The residue is dissolved in 10 ml. of chloroform and transferred with three chloroform washes totaling 20 to 30 ml. to a 125-ml. separatory funnel. To the chloroform solution are added 1.0 ml. of a 0.5% α,α′-dipyridyl solution in absolute ethanol and 1.0 ml. of a 0.4% ferric chloride solution in absolute ethanol. The reagents are run down the sides of the funnel to minimize mixing. The funnel is then shaken vigorously, approximately 10 ml. distilled water are added at once, and the funnel is again shaken. The stopper and sides of the funnel are then rinsed with a few milliliters of absolute alcohol. Two layers form. The lower chloroform layer is discarded; the upper aqueous alcohol layer, which contains the red ferrous dipyridyl color complex, is run into a 25-ml. volumetric flask and made to volume with absolute ethanol.

The time for color development is 10 minutes, measured from the time the reagents are first mixed with the tocopherol solution. The colorimetric measurement was made with a Fisher Electrophotometer at 520 mμ, obtained by a combination of three Corning filters, one No. 4380, one No. 4303, and one No. 3384. A blank containing 10 ml. of distilled water diluted to 25 ml. with absolute ethanol is used.

A calibration curve was made with Merck synthetic α-tocopherol in Skellysolve B solution. Suitable aliquots were evaporated, and the color was developed as described above. A ferric chloride–α,α′-dipryridyl reagent blank obtained in the same manner as the standards was determined, and the value thus obtained was deducted from the standard readings. Calibration curves obtained before and after deducting the reagent blank are shown in Figure 1, B. Either calibration curve may be used.

DISCUSSION AND RESULTS

Effect of Tocopherol Mixtures. The procedures presented were based on colorimetric standardization with Merck synthetic α-tocopherol. Baxter *et al.* (*1*) and Hove and Hove (*7*) have shown that α-tocopherol is oxidized more rapidly by ferric chloride than are the beta and gamma compounds. On the other hand, when the tocopherols are oxidized to the red *o*-quinone with silver nitrate, this order is reversed (*1*)

In view of the fact that γ-tocopherol might be present in plant tissue and that the synthetic and natural tocopherols might behave differently, the $E^{1\%}_{1\ cm.}$ and 480 and 520 mμ values for synthetic α- and natural α- and γ-tocopherols were determined by modifications of the Furter-Meyer and Emmerie-Engel procedures (Table I).

The $E^{1\%}_{1\ cm}$ values obtained by both methods for synthetic and natural α-tocopherol agree fairly well, the result for natural tocopherol being about 5% high in both cases. As compared with synthetic α-tocopherol, the natural gamma compound gave values that averaged 10% low by the Furter-Meyer and 35% low by the Emmerie-Engel method. Therefore if γ-tocopherol were present in appreciable quantity in leaf material, the error in colorimetry alone would be serious, especially in the Emmerie-Engel determination. As is shown below, in most cases results by the two procedures were in close agreement, indicating that there was little, if any, γ-tocopherol in the products studied.

In this connection, recent papers by Hove and Hove (*7*) and Fisher (*4*) describe the determination of α-tocopherol in the presence of the beta and gamma compounds. It is possible that these procedures in conjunction with the methods described here may be used to determine the individual tocopherol compounds present in plant tissue.

Removal of Pigments. Passage of the extract through activated magnesia not only effectively removes the chlorophyll and xanthophyll but allows quantitative elution of the tocopherol, together with the carotene. Table II shows the results of adsorption experiments with synthetic α- and natural α- and γ-tocopherols. After elution with the acetone–Skellysolve B solutions described previously, the colorimetric determination was made by the Furter-Meyer method, individual calibration curves prepared from each tocopherol compound being used. The results indicate that all the tocopherols can be eluted quantitatively from the magnesia adsorbent.

Table II. Effect of Adsorption and Acid Treatment on Recovery of Synthetic α- and Natural α- and γ-Tocopherols

Compound	Adsorption			Sulfuric Acid Treatment		
	Taken	Found	Recovery	Taken	Found	Recovery
	Mg.	*Mg.*	%	*Mg.*	*Mg.*	%
Synthetic α-tocopherol	2.00	2.00	100	2.0	1.90	95.0
Natural α-tocopherol	2.00	2.03	101.0	2.0	1.95	97.5
Natural γ-tocopherol	2.00	2.05	102.5	2.0	1.90	95.0

Attempts at separating carotene from tocopherol by adsorption on Florisil from benzene solution were unsuccessful. The quantities of carotene and other lipoids are much larger in leaf extracts than in oil and animal tissue extracts, which have been successfully treated with Florisil.

Carotene is removed in both methods by 85% (by volume) sulfuric acid. Parker and McFarlane (*11*) used 85% by weight sulfuric acid to remove the small quantities of carotenoids present in oils. In order to remove the carotene quantitatively, it is necessary to work with a concentrated solution, which must be vigorously shaken with the acid. Under these circumstances the sulfuric acid rapidly decomposes the carotene, and the resultant petroleum ether solution is colorless. Tocopherol losses in this case are low. If the solution is not concentrated, prolonged shaking with the acid is required. Often much larger quantities of acid are necessary. The tocopherol losses in this case amounted to 25 to 50% with the Emmerie-Engel procedure, probably due to oxidation; they were somewhat lower with the Furter-Meyer method. Table II shows the effect of the 85% sulfuric acid treatment on the recovery of snythetic α- and natural α- and γ-tocopherols. The acid treatment was identical with the procedure described above. The colorimetric determination was made by the Furter-Meyer method, individual calibration curves being used. The results show that the acid treatment caused a maximum loss of 5%.

According to Scudi and Buhs (*13*), treatment with 85% sulfuric acid also removes tocopherolquinones, which would otherwise be included with tocopherol values by the Furter-Meyer method. The authors' observations with pure α-tocopherolquinone confirm this. Water-soluble polyphenols, if present, are removed by the water and alkali shakings subsequent to the treatment of the extract with 85% sulfuric acid.

Effect of Unknown Lipoidal Interferences. After the acid treatment, the only subsequent interference observed in the Furter-Meyer procedure came from lipoidal compounds insoluble in cold ethanol. When nitric acid is added to the ethanol containing the tocopherols, and the solution boiled, a clear red product is obtained. On cooling, however, a considerable quantity of material is usually precipitated. The solution thus

becomes turbid and colorimetric readings taken with such a solution would be erroneous. These lipoids are easily removed by filtration, leaving a clear solution. In the Emmerie-Engel cedure, several interfering substances of unknown nature are after adsorption and acid and alkali treatments. One source of interference is a lipoid which slowly reduces iron.

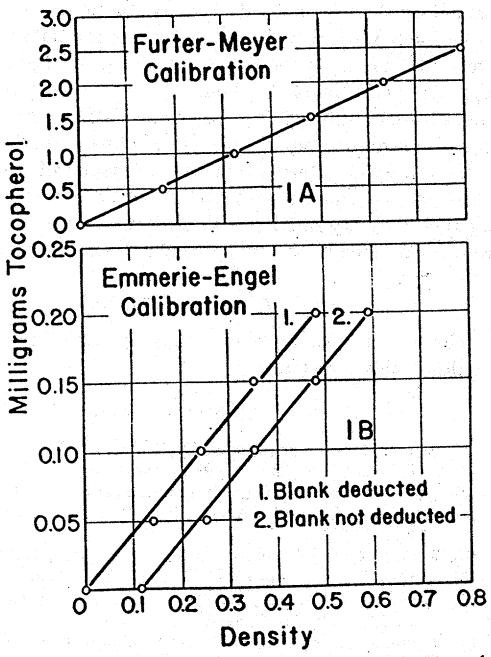

Figure 1. Calibration Curve for Determination of Tocopherol

Purified plant extracts transferred to alcohol and then reacted with the Emmerie-Engel reagents reduced the iron at a slow rate for periods up to 2 or 3 hours. Under the same circumstances pure tocopherol gave a constant reading in 10 minutes. The slow reduction of iron was not linear, for extracts containing twice the quantity of sample gave results 150 to 200% higher than the calculated results. The same effect was noted when pure tocopherol was added to a plant extract, the tocopherol recovery being 150 to 200% higher than the calculated value. The interfering lipoids may be plant sterols, for Devlin and Mattill (*2*) working with muscle extracts noted the same type of interference with the Emmerie-Engel procedure and traced the effect to cholesterol. The authors completely eliminated this source of interference by immediately transferring the colored dipyridyl complex to an aqueous medium, thus taking the lipoidal reducing substances out of contact with the iron. Pure α-tocopherol reduced the iron immediately. Experiments with pure tocopherol solutions treated in this manner gave practically quantitative recovery.

Still another type of interference was noted with certain plant extracts, particularly beet and rhubarb extracts. Recovery of tocopherol added to these extracts and carried through the whole procedure was consistently low. When tocopherol was added directly to the extract after purification of the sample, the recovery was still low. Working on the assumption that some constituent of the purified extract was forming a complex with the ric ion, the concentration of the iron reagent was doubled (from 0.2 to 0.4%), the quantity of sample used for the final tocopherol determination being kept at a minimum. This technique resulted in greatly improved tocopherol recoveries and s adopted for all plant extracts.

Since the high blank shown in Figure 1, B, may have been due to the exposure to suffused light during the manipulation or to the higher concentration of ferric chloride, these two factors were examined. The densities of blank solutions prepared in the conventional manner were compared with those of solutions so handled that there was no exposure to light. The results in both cases were identical.

To determine the effect of ferric chloride concentration, a series of pure synthetic α-tocopherol standards was run by the conventional procedure, using in one case 1 ml. of 0.2% and in the other 1 ml. of 0.4% ferric chloride solution. The results are shown in Table III.

It is apparent that the increased blank is due to the higher ferric chloride concentration. If the blanks are deducted, the results with either concentration are in good agreement. Although a large blank is generally undesirable, it is necessary to use the high ferric chloride concentration for reasons explained above, and apparently there is no loss of accuracy.

Recovery of Tocopherol. Recovery of synthetic α-tocopherol added to plant extracts and carried through the entire procedures is shown in Table IV. In most cases recovery of tocopherol added to a wide variety of vegetable leaf extracts was satisfactory, averaging about 95% with both methods.

Recovery of tocopherol added to a plant extract is probably more a measure of the precision of the method than of its intrinsic accuracy. The fact that tocopherol is determined rather accurately by the two methods is shown by the fairly good agreement of values obtained by them on the same plant materials (Table V). With a few exceptions, the two methods give results checking within ±5 to 10%. In those few cases in which there is a large discrepancy, the values obtained by the Furter-Meyer method are probably more reliable, since this determination is based on a reaction relatively specific for tocopherol.

Only a few analyses reported in the literature compare the results obtained by the Furter-Meyer and the Emmerie-Engel procedures on biological material. Morton (*10*) quotes an analysis of an oil in which the Furter-Meyer result was 400% higher

Table III. Effect of Iron Concentration on Reagent Blanks Found with Emmerie-Engel Method

	0.2% Ferric Chloride		0.4% Ferric Chloride	
Tocopherol	Density	Density (blank deducted)	Density	Density (blank deducted)
0	0.08	0.00	0.15	0.00
50	0.21	0.13	0.27	0.12
100	0.32	0.24	0.38	0.23
200	0.55	0.47	0.61	0.46

Table IV. Recovery of α-Tocopherol Added to Plant Extracts

Dry Leaf Sample	Tocopherol in Sample	Tocopherol in Sample Plus 1.0 Mg. of Added Tocopherol: Calculated value	Observed value	Total Tocopherol Recovered
	Mg.	*Mg.*	*Mg.*	%
Furter-Meyer Method				
Alfalfa	0.47	1.47	1.38	93.8
Beet	1.19	2.19	2.04	93.0
Broccoli	1.25	2.25	2.22	98.6
Kale	0.91	1.91	1.80	94.2
Lima bean	1.79	2.79	2.68	96.0
Rhubarb	2.74	3.74	3.64	97.3
Spinach	1.29	2.29	2.24	97.8
Turnip	0.85	1.85	1.78	96.2
				Av. 95.8
Emmerie-Engel Method				
	γ	Plus 62.5 γ Added Tocopherol: γ	γ	
Alfalfa	65.0	127.5	133.0	104.3
Beet	89.0	151.5	147.0	97.0
Broccoli	100.0	162.5	149.0	91.7
Kale	97.0	159.5	144.0	90.0
Lima bean	175.0	237.5	221.0	93.0
Rhubarb	150.0	212.5	213.0	100.2
Spinach	100.0	162.5	155.0	95.3
Turnip	88.0	150.5	152.0	100.9
				Av. 96.5

Table V. Tocopherol Content of Vegetable Tissues

Sample	Furter-Meyer	Emmerie-Engel
	Mg. per gram	
Alfalfa leaf meal	0.23	0.26
Beet leaf meal	0.44	0.71
Broccoli leaf meal	0.39	0.42
Kale leaf meal	0.36	0.39
Lima bean leaf meal	0.72	0.70
Rhubarb leaf meal	1.24	1.20
Spinach leaf meal	0.42	0.40
Turnip leaf meal	0.34	0.30
Mash, 5% alfalfa	...	0.02
Mash, 2.5% broccoli	...	0.02
Scratch corn	...	0.00
Fresh carrot root	0.14[a]	0.13[a]
Fresh carrot top	0.79[a]	0.56[a]
Frozen spinach	0.47[a]	0.43[a]

[a] Calculated on moisture-free basis.

than the Emmerie-Engel value. Hickman *et al.* (*6*) report an analysis of a mixture of foods in which the Furter-Meyer value was 1400% higher than the Emmerie-Engel result. These extreme discrepancies are undoubtedly due to insufficient purification of the extracts prior to color measurement.

The Emmerie-Engel procedure is much more sensitive than the Furter-Meyer, and hence is more convenient for low potency samples.

SUMMARY

The Furter-Meyer and Emmerie-Engel methods for determination of tocopherol have been applied to plant extracts. In both cases, dry plant materials are extracted with Skellysolve B, and fresh materials with an ethanol–petroleum ether solution, the alcohol then being removed. It is necessary to purify the sample before the final estimation can be made. Chlorophyll and xanthophyll are separated from tocopherols by adsorption on a Supercel–activated magnesia column. Carotene and tocopherolquinones are then destroyed by treatment with 85% sulfuric acid.

In the Furter-Meyer procedure the tocopherols are finally transferred to ethanol solution, oxidized with nitric acid, and, after removal of ethanol-insoluble lipoids, determined with a photoelectric colorimeter at 480 mμ.

In the Emmerie-Engel procedure the tocopherols are finally dissolved in chloroform, and reacted with the ferric chloride–α,α'-dipyridyl reagent. The pink ferrous dipyridyl complex is immediately transferred to aqueous solution to prevent further reduction of the iron by a slowly reducing fat-soluble compound. The colorimetric determination is carried out at 520 mμ in a photoelectric colorimeter. $E^{1\%}_{1\,\text{cm.}}$ and 480 and 520 mμ values obtained by the two methods with synthetic and natural α-tocopherols agreed within 5%. The values for natural γ-tocopherol were 10% lower by the Furter-Meyer method and 35% lower by the Emmerie-Engel method. All these compounds can be quantitatively eluted from a magnesia-Supercel adsorbent and show a maximum loss of 5% when shaken with 85% sulfuric acid.

Tests in which pure synthetic α-tocopherol was added to the extracts and carried through the entire procedure showed an average recovery of 95% with both methods. In most cases the results obtained by the two procedures agreed within $\pm$5 to 10%.

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