MODERN METHODS TO DETERMINE VITAMIN E IN FOODS AND FEEDS

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Summary

Highly specific and sensitive, the proposed fluorometric method for determining α-tocopherol is robust and fairly fast. It has been tested in parallel with a conventional thin layer chromatographic method on foods and feeds. The only necessary cleanup is the usual saponification. The unsaponificable fraction can be extracted with ethyl ether or, preferably, with Extrelut columns. Reagents and their solvents are added to the isooctane solution before each successive reaction and are then eliminated by partition with water. The α-tocopherol (α-T) derivative always remains in isooctane. The first step is nitrosation and elimination of tocopherols and tocotrienols other than α-isomers. α-T is then oxidized to α-tocored (α-TR) with a mixture of sulfuric acid, ferric chloride, and iodine bromide. A-TR is then condensed to a new reagent: 4,5-dimethyl-o-phenylenediamine. The phenazine formed is strongly fluorescent. Iodine and bromine add to the double bonds of α-tocotrienol present and quench the fluorescence of its phenazine.

INTRODUCTION

The determination of vitamin E in food or feedstuffs poses a difficult problem. Eight natural homologs possess a vitamin activity: 4 tocopherols and 4 tocotrienols. Among them, α-T is by far the most active compound. It is generally agreed that α-T accounts for about 80% of vitamin E supply in foods. Moreover, it is the only homolog used in modern feeding. The other homologs have lower activities and it is difficult if not impossible to determine them all. In practice, only α-T is routinley determined. But the other homologs, as well as various substances, interfere in the determination of α-T.

Conventional methods for determination of α-T are lengthy, not specific, and not precise. To separate interfering substances, a tedious cleanup of the sample is necessary. This is usually done by column or thin layer chromatography, and it is still necessary to undertake special treatments before chromatography , such as cleanup with Floridin or sulfuric acid treatment (1,2). Methods using gas chromatography (GC) necessitate a sophisticated cleanup which renders GC almost unnecessary (3). Recently, liquid chromatography (LC) has been proposed. It is more satisfactory than conventional methods, including GC (4). Yet the analysis of complex mixtures containing low levels of α-T and high levels of interfering substances may pose problems. Even with a fluorescence detector, the risk of interference is not negligible. For routine analysis, column pollution may be a severe drawback. Although LC is faster than conventional methods, the separation of α-T in complex mixtures such as foods and feeds require 20 min or more. This is inherent in the technique, to make sure there is no interference due to a compound retained on the column, it is necessary to carry out 2 determinations, which expands further the time required. Finally, the LC technique requires expensive equipment and solvents.

These considerations led us to develop a method based on the succession of several specific reactions, which needs no special cleanup, and which can be automated according to the continuous flow technique.

METHOD

Apparatus

(a) *Glassware*.- Round-bottom flasks, volumetric flasks, and separatory funnels, all low-actinic glass.

(b) *Heating baths*.-With precise temperature regulation (+- 1 ºC).

(c) *Rotary evaporator*.-

(d) *Spectrofluorometer*. Wavelengths for maximum are ca 382 mm (excitation) and 495 mm (emission), but these values depend on instrument used. (We chose 370 and 500 mm to avoid a Raman peak.) Slitwidth: 10 nm (excitation) and 5 nm (emission). Expansion 20. Calibrate fluorometer at 90 % fluorescence with quinine sulfate standard solution, at 348 nm (excitation) and 448 nm (emission). Check these wavelengths when operating another instrument.

(e) *Filter fluorometer*.-Alternatively, use simple filter fluorometer instead of spectrofluorometer, but filters must be adequately chosen. For filter fluorometer used in automated method choose following filters: primary filter, interferential filter with maximum transmission at 366 nm (345 to 390 nm) (ca 25 % transmission); secondary filter: 50 broad-band interferential filter with maximum transmission at 500 nm (460-540 nm) (ca 75 % transmission).

Reagents and Solutions

(a) *Ethanol*.-Absolute

(b) *Potassium hydroxide solution*.-Dissolve 200g KOH (86 %) in 1 L water.

(c) *All-rac-α-tocopherol stock solution*.-80 mg/200 mL ethanol. Store <= 1 month at -18 ºC.

(d) *All-rac-α-tocopherol working solutions*.-Starting from solution (c), prepare daily dilutions (2,4,6 μg/mL) with ethanol.

(e) *Sodium nitrite solution*.-Dissolve 2 g in 50 mL water, then add 50 mL ethanol. Store in refrigerator <= 1 week.

(f) *Iodine bromide solution* 1.2% (w/v) in ethanol (a). Store at -18 ºC <= 1 month.

(g) *Ferric chloride stock solution*.-70% (w/v) $FeCl_3 6H_2O$ in ethanol (a). Store <=1 month at room temperature.

(h) *Oxidizing solution*.-In 500 mL volumetric flask containing ca 300 mL ethanol (a), add successively 10.0 mL $FeCl_3$ solution (g), 10.0 mL Ibr solution (f), and 10.0 mL 98% H_2SO_4. Adjust to volume with ethanol (a). Prepare daily.

(i) *DMPD solution*.-1% (w/v) 4,5-dimethyl-*o*-phenylenediamine (Aldrich) in 100% acetic acid. Prepare daily.

(j) *Buffer solution*.-Prepare aqueous 15% (w/v) $NaH_2PO_4H_2O$ solution and adjust pH to 5.5 with potassium hydroxide (b).

(k) *Quinine sulfate stock solution*.-100 mg/500 mL 0.1N sulfuric acid. Store <= 1 month in refrigerator.

(l) *Quinine sulfate working solution*.- Dilute solution (k) to 0.04 μg/mL with 0.1N sulfuric acid. Store <= 1 week in refrigerator.

(m) *Hydrophobic folded filters*.

Reagents for Sample Preparation

(a) *Pyrogallol solution*.-Dissolve 0.1 g pyrogallol in 4 mL water, add 20 mL ethanol, and adjust volume to 100 mL with methanol.

(b) *Potassium hydroxide solution*.-Dissolve 700 g KOH (86%) in 600 mL water and adjust volume to 1 L.

(c) *Ethyl ether*.-Free of peroxides. Or, instead, Extrelut columns and fillings

(d) *Ascorbic Acid*.-Aqueous solution, 20% (w/v).

Sample Preparation (Saponification and Extraction)

A) *Foods*.-Weigh 10 g sample in 250 mL low-actinic flask, add 50 mL pyrogallol solution (a), add 10 mL KOH solution (b), and heat with reflux under nitrogen for 20 min. Shake flask from time to time, or preferably, use magnetic stirrer. When saponification is ended, rinse cooler with a few mL water and transfer the whole contents to separatory funnel with help of 50 mL water.

Rinse empty flask with 120 mL ethyl ether (c), transfer rinse to separatory funnel, and extract unsaponifiable matter. Extract twice again with 120 mL ethyl ether. Collect ether phases and wash 3 times with 100 mL water. The last water phase must be neutral. Take aliquot from ether solution, add a few mL ethanol (a), and evaporate at 50 ºC under vacuum. Dissolve residue in ethanol (a).

B) *Feeds*.-Saponification A can be employed, but procedure B is preferable in most cases. Weigh 50 g sample in 1 L low-actinic flask. Add 400 mL pyrogallol solution (a) and add 80 mL KOH solution (b). Weigh flask and heat with reflux for 20 min under nitrogen. Shake flask from time to time or, preferably, use magnetic stirrer. Rinse condenser with 20 mL water and weigh flask again. Bring weigh to its initial value plus 20 g with a few mL ethanol (a) if necessary. Take 100 mL aliquot and transfer to separatory funnel. Add 50 mL water and 120 mL ethyl ether (c) and continue extraction as in A. Advantages of procedure B are a higher sample weight, and absence of solid particles during extraction. Disadvantages are that the 100 mL aliquot must be representative of the whole solution (usually it is), and that feeds contain some water not considered. When the water content is <10%, then the error is negligible (<2%). If the water is >10%, then it should be taken into account in the calculation.

C) *Oils and fats*.-Weigh 5 g oil in 250 mL low-actinic flask. Add 100 mL pyrogallol solution (a), add 15 mL KOH solution (b), and heat with reflux for 20 min under nitrogen. Shake flask from time to time or, preferably, use magnetic stirrer. Rinse condenser with a few mL water and transfer contents to separatory funnel with the help of 50 mL water. Add 120 mL ethyl ether (c) and continue as in procedure A.

Note: Heating of saponification solution lasts 20 min, starting from the beginning of reflux.

Substitution of Extrelut columns for ether extractions.-Ethyl ether is dangerous and onerous to use. The 3 extractions of unsaponifiable material followed by 3 washings are time consuming. Use of purification columns requires much less time, is as effective, and is more selective (blanks are reduced) (5).

Instead of transferring saponification solution to separatory funnel, pippet 40.0 mL aliquot into 50 mL volumetric flask and adjust to volume with ascorbic acid solution (d). Shake, and transfer 20.0 mL of this solution to Extrelut column. Wait 15 min after application , set 50 mL volumetric flask under column and elute with 60 mL isooctane. Collect 40 mL during elution =>25 min. Keep flask in freezer (-18 ºC) and adjust to volume with isooctane just before determination. If automated system is used, then isooctane solution is directly passed into automated system. If manual system is used (this article), then an aliquot of the isooctane solution containing 40-120 μg α-T is evaporated, and residue is dissolved in 20.0 mL ethanol (a).

Analytical Procedure

Operate in subdued light. Dilute ethanolic solution of α-T to concentration between 2 and 6 μg/mL.

Nitrosation.-Pour 20.0 mL ethanolic solution inti 250 mL separatory funnel. Add 0.6 mL 100% acetic acid, then 9 mL sodium nitrite (e). Stir, and wait exactly 2 min. Add 6 mL potassium hydroxide (b) and stir. Add 20 mL isooctane, shake 1 min, and let phases separate. Discard bottom phase and wash upper phase with 20mL buffer solution (j). After separation, filter upper phase through hydrophobic filter (m) into 100 mL round-bottom flask. Rinse separator and filter with 10 mL

isooctane.
Oxidation to α-TR.-Add 30 mL oxidizing solution (h), set flask in heating bath at 50 ºC with cooler, and wait 30 min. Quantitatively transfer contents to 250 mL separatory funnel with help of 30 mL water. Shake 1 min, let phases separate, discard bottom phase, and wash upper phase with 30 mL buffer solution (j); separate and filter organic phase through hydrophobic filter (m) into 250 mL round-bottom flask. Rinse separator and filter with 10 mL isooctane.
Reaction of α-TR with DMPD.-Add 40 mL DMPD solution (i). Set flask in heating bath at 90 ºC with cooler (not under nitrogen) and wait 30 min. Cool rapidly and transfer contents to 250 mL separatory funnel. Wash flask with 40 mL 16% Hcl and rinse to separatory funnel, shake 1 min, and let phases separate. Wash upper phase twice with 40 mL buffer solution (j). Filter organic phase through hydrophobic filter (m) into 50 mL volumetric flask. Rinse separator and filter with 10 mL isooctane. Bring volume to 50 mL with isooctane. If fluorometry is performed the next day, keep solution in refrigerator (+ 4 ºC). Before measurement, pipet 10.0 mL isooctane solution, add 10.0 mL ethanol (a), and stir.
Fluorometry
Calibrate fluorometer at 90% fluorescence with quinine sulfate working solution (l) and perform fluorescence measurement.
Blank assay (H_2O).-Only the oxidation (to α-TR) step is changed: Add 30 mL oxidizing solution (h), then add 1.0 mL water and set flask in heating bath at 50 ºC. Let stand 2 min, and stir to obtain one phase. Then wait 28 min, and transfer to 250 mL separatory funnel...(as in normal assay procedure).
Calibration line.-Carry out 2 determinations according to above procedure with each α-T standard working solution (d). Check reagent blank (normal procedure with all reagents but no α-T). Usually it is equal 3-4 %. Subtract water blank assays from values obtained foe each point and plot fluorescence intensity concentration.
Calculation.-For each sample assay, subtract water blank from normal assay value and calculate α-T concentration with help of calibration line.
Operating notes: *(1)* If irregular results occur, then make sure that glassware is clean (in particular, pipets). *(2)* Oxidation of α-T to α-TR is sensitive to water. Absolute ethanol used to prepare solution (h) usually contains 0.1% water. This content varies somewhat, so it is preferable to use the same batch for calibration curve and samples. When determinations are carried out during the whole day, it is better to separate the oxidizing solution (h) in 2 parts (for morning and afternoon). Make sure that glassware used to carry out this reaction is dry. *(3)* Fluorescence measurements must be performed at approximately the same temperature. Use a thermostatically controlled cell holder if temperature varies too much.

RESULTS

Statistical Evaluation

Twelve calibration lines were carried out with standard ethanolic α-T solutions of concentrations 0, 2, 4, 6 μg/mL. The r^2 value of the calibration line was 0.998, and the coefficients of variation (CV) were as follows: 0 μg/mL, 4.8 %; 2 μg/mL, 3.2%; 4 μg/mL, 3.5%; 6μg/mL, 1.5%. The mean CV of the 3 concentrations 2, 4, 6 μg/mL was 2.7%.

The CV obtained with a 6 μg/mL α-T standard solution (highest point of calibration line) over a one month period was 2.8 % (70 determinations).

Selectivity

To check the selectivity of the proposed method, we have run normal determinations and related blank assays with several compounds whose structures are similar to that of α-T, or which are likely to interfere. The results are given in Table 1. As can be seen, no one interferes, even at concentrations one hundred times that of α-T. The β-, γ-, and δ- tocopherols, as well as α-tocotrienol, also do not interfere.

Assays of Foods and Feeds

To test the proposed method, we ran parallel determinations with our former method. We have been using this latter method for about 10 years, although it was published only recently (2).
(a) *Principle of TLC method* (2).-After saponification, the unsaponifiablematter is cleaned on a silica gel chromatography column, the α-T zone is collected and submitted to a sulfuric acid treatment, then α-T is isolated by silica gel thin layer chromatography (TLC). After desorption, it is quantitated with the Emmerie-Engel technique.
(b) *Comparison between methods*.-The samples were sapinified according to the TLC method and aliquots of the unsaponifiable matter were determined by both methods. Two determinations were carried out with each method, blank assays were done with the proposed method. The results are given in Table 2.

It is interesting to note that results agreed well, even for soyabean oil which contains between 5 and 10% α-T relative to the sum of the tocopherols (this vegetable oil contains the lowest ratio of α-T). Moreover, the value found for the blank was very high (43% of the total fluorescence), yet there was no quenching due to blank impurities.

Table 1. Assay of compound which might interferre in fluorometric assay of α-tocopherol

Compound	Concn,[a] μg/mL ethanol	Fluorescence,[b] %	Blank,[b] %
α-T	6	70	3.1
β-T[c]	6	2	0
γ-T[d]	6	6.5	6.5
δ-T	6	0	0
α-T3[d]	6	3	3
BHA	600	2	0
BHT	12000	0	0
Ethoxyquin	600	1.5	0.5
1,2-Dihydroxy naphthalene	600	0	0
Naphthalene-1,2-quinone	12000	0	0
Pyrocatechol	600	0	0
Pyrocatechol monoethyl ether	600	1.5	0.5
Pyrocatechol monomethyl ether	600	0	0
3,4-Dimethylphenol	12000	1	1
Pyrogallol-1-methyl ether	12000	0	0

[a] Concentrations are given for the initial ethanolic solutions.
[b] The reagent blank has been subtracted.
[c] The fluorescence obtained is likely an artifact, since we had difficulties isolating it from a antural extract.
[d] Values found are due to impurities.

Recovery

The recovery factor of α-T measured in several foods and feeds. The samples were first assayed for α-T. Then known amounts of α-T (60 μg) were added to the initial 20 mL ethanolic solutions of the samples. All the determinations were carried out only once. As can be seen in Table 3, the obtained differences were closed to the expected ones (92-110%) with an average of 100.2%.

Extraction of the unsaponifiable matter on Extrelut cartridges gave similar results to the usual ethyl ether extraction.

DISCUSSION

In the first step, saponification, one must make sure that the amount of sample weighed is representative of the product to be analyzed. This depends on the concentration. A general rule is to weigh not less than 10 g supplemented feedstuffs. Usually we weigh 50 g.

It is important to mention that saponification, besides its usual actions, destroys α-tocopherolquinone (α-TQ) and α-tocored (α-TR) if present in the sample.

Table 2. Comparison of proposed method and conventional TLC method for α-tocopherol

Sample	Proposed method			TLC method	
	Fluorescence, %	Blank, %	mg/100g	mg/100g	Diff., %
Croquettes for cats	50.5	11.5	17.1	16.8	+2.2
Sunflower oil	45.5	6.0	74.6	73.1	+2.0
Infant milk	51.6	13.0	11.1	11.1	0
Mineral premix 1	87.8	5.8	2 661	2 786	-4.5
Mineral premix 2	30.0	8.9	7 569	7 679	-1.4
Pet food (for cats)	12.2	5.0	2.15	2.0	+7.5
Corn seed	57.0	10.1	19.9	20.4	-2.3
Spreading paste	47.6	5.0	10.1	10.4	-2.6
Soyabean oil	37.9	16.2	14.3	14.6	-2.4

The nitrosation reaction is done according to Quaife (6), to eliminate the homologs of α-T and the phenolic impurities. A-T does not undergo nitrosation because it has no free aromatic site, but it does not remain unchanged: It undergoes a fast esterification with nitrous acid to form the nitrite ester. We have found no mention of this reaction in the literature. It might be related to the ability of α-T to prevent the formation of nitrosamines. Fortunately, the nitrous ester is oxidized as rapidly as the free form of α-T and also gives α-TR. Furter and Meyer (7) carried out the oxidation with concentrated nitric acid (65%) in ethanolic solution. We had difficulties using 65% HNO_3. The reaction was erratic. Results were even less reproducible with very dilute α-T solutions, as we tried to increase the sensitivity of the method.

We decided to look for another reagent, but the usual oxidizing agents of α-T, such as ferric chloride and ceric sulfate, yield other derivatives, such as the *para*-quinone (α-TQ), instead of the *ortho*-quinone (α-TR) (8). Sulfuric acid is able to produce only limited amounts of α-TR and the reaction is irregular.

Table 3. Recovery factors of proposed manual method

Food/feed[a]	α-T content, mg/100g	Recovery factor, %
Cattle vitamin premix	830	101
Pig vitamin premix	420	102
Cattle mineral premix	16.7	101
Turkey mineral premix	100	94
Chicken feed	1.95	94
Chicken feed[b]	1.95	93
Cattle feed	2.1	95
Cattle feed[b]	2.1	105
Chick feed	4.4	97
Chick feed[b]	4.4	104
Corn seeds soup	2.5	92
Dietetic corn seeds[b]	2.13	106
Dietetic corn seeds	2.13	106
Pig feed	2.44	110
Pig feed[b]	2.44	102
Cocoa breakfast[b]	2.5	101
Cocoa breakfast	2.5	102
Dietetic food	?	99
	Average =	100.22

[a]Unsaponifiable extraction with ethyl ether.
[b]Unsaponifiable extraction with Extrelut cartridges.

CONCLUSIONS

We finally found that a mixture of sulfuric acid, ferric chloride, and iodine bromide, dissolved in ethanol in given proportions, is able to form α-TR. Although the yield is somewhat lower than with HNO_3 (about 50-60%), the results are reproducible because the amount of formed α-TR reaches a plateau. The reaction is carried out in isooctane amd ethanol (isooctane being present at every step, since it is the carrier solvent). Alcohol is necessary for the reaction to proceed.

Simultaneous use of the 3 oxidizing agents is necessary. We tried iodine bromide because we wanted to quench the fluorescence of the phenazines formed by the tocotrienols and especially α-tocotrienol (α-T3). These compounds are homologs of α-T in which the side chain, instead of being saturated, contains 3 double bonds. They have a low biological activity and frequently occur in feedstuffs, where they interfere in the determination of α-T. It is well known that heavy atoms (such as iodine or bromine) when bound to a molecule quench its fluorescence. Iodine bromide is able to add such atoms to the double bonds and it quenches the fluorescence. To save time, the reaction between tocotrienols and iodine bromide was done simultaneously with the formation of α-TR. By chance, the latter reaction was facilitated by iodine bromide.

We found that formation of α-TR is blocked when a small amount of water (3.3%) is added to the ethanolic solvent. But water does not block the oxidation of 1,2-dihydroxy-aromatic impurities. For example, pyrocatechol reacts with ferric chloride in presence or absence of water in the same way. α-T is first oxidized to α-tocopherolquinone (α-TQ) (in presence or absence of water), then α-TQ is oxidized and converted by sulfuric acid to α-TR. This second step is very sensitive to

water. With 3.3% water, the inhibition is 96% effective, so only 4 % of the fluorescence is due to the phenazine. When the water blank gives a higher value than 4% (after subtraction of the reagent blank), then the difference is due to a fluorescent impurity. The possibility of quenching is reduced because the α-T solutions measured are very dilute (vide infra).

We substituted 4,5-dimethyl-*o*-phenylenediamine (DMPD) for OPD. The obtained derivative is likely to be a phenazine too. The reaction rate in the fluorescence intensity are much higher with DMPD. Kofler used acetic acid as solvent, and we used a mixture of acetic acid and isooctane (the latter as carrier). After the phenazine is formed, excess DMPD is extracted with hydrochloric acid. The acetic acid also passes into the aqueous phase.

The isooctane phase contains the phenazine, which fluoresces only after addition of ethanol. The excitation maximum occurs at 382 nm and the emission at 495 nm. These wavelengths are rather remote from the ususal range of fluorescence. To avoid a Raman peak at 437 nm, it is preferable to use 370/500 nm instead of 382/495 nm.

The results obtained with the method are reliable and the precision is satisfactory (CV = 2.8 %). Ethanolic α-T solutions with concentrations as low as 2 μg/mL can be assayed, so the method can be used for most food and feedstuffs.

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