Determination of Total Tocopherols in Grains, Grain Products, and Commercial Oils, with Only Slight Saponification, and by a New Reaction with Cupric Ion

Emilio Contreras-Guzmán* and Frederick C. Strong III

This work describes a sequence of techniques for the extraction, purification, and chemical determination of tocopherols in grains, edible oils, and byproducts of oil refining. It is shown that nontocopherol reducing substances can be removed by gentle treatment with alcoholic KOH, with only slight saponification. Total tocols are determined by a new reaction with cupric ions and complexation with 2,2′-biquinoline (cuproine). It is carried out in a two-phase system, in which the upper phase (heptane) contains the lipids and the lower phase (80% ethanol) the Cu^{2+} ions and cuproine. The reaction occurs when the two phases are mixed by shaking for 2.5 min. The complex, $Cu(cuproine)_2^+$, forms in the 80% ethanol, and in this medium the molar absorptivity, expressed as a function of α-tocopherol, is 14490 L mol^{-1} cm^{-1} at 545 nm. The two-phase system permits control of the time of reaction, eliminating the influence of liposoluble pigments remaining in the heptane phase.

In a preliminary publication, Contreras-Guzmán and Strong (1982) reported the characteristics of a new chemical reaction for the determination of tocopherols and tocotrienols. The reaction is based on the reduction of cupric ions and complexation of the resulting cuprous ions with 2,2′-biquinoline (cuproine) or with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine). The principal advantage of this new method is the exceptional stability of both complexes, permitting working under normal illumination with ordinary glassware. The complex $Cu(cuproine)_2^+$ possesses a molar absorptivity, in terms of tocopherol concentration, of 13 300, a little smaller than that of the complex $Fe(bipyridine)^{2+}$, which, expressed similarly, is 17 185. On the other hand, the complex $Cu(bathocuproine)_2^+$ has a molar absorptivity of 26 650. These data were obtained in absolute ethanol.

For the spectrophotometric determination of total tocopherols (total tocols) in food and feeds, the sensitivity of the complex $Cu(cuproine)_2^+$ is sufficient. However, for the determination of tocopherols separated by TLC or by column chromatography, it is preferable to use the complex $Cu(bathocuproine)_2^+$, which is 2 times more sensitive.

Routine determinations of total tocols are tedious and frequently give erratic results due to not only deficiencies in the chemical reaction but also the introduction of interfering substances in the solvents or loss of tocopherols because of inefficient handling of solutions.

In the work described here, there is given in detail a sequence of techniques that permits the extraction, purification, and evaluation of total tocol content in a simple and reliable way. It should be adequate for laboratories doing routine control in food industries devoted to the formulation of rations and to the extraction and refining of vegetable oils.

EXPERIMENTAL SECTION

Reagents. *all-rac*-α-Tocopherol (Merck) was 0.1% in heptane (I) and in ethanol (II). 2,2′-Biquinoline (cuproine) (Merck) was 0.5% in toluene (III). $Cu(NO_3)_2 \cdot 3H_2O$ was 0.5% in distilled water (IV). Urea was 2.5% in absolute ethanol (V), ascorbic acid was 5% in distilled water (VI), and KOH was 50% in distilled water (VII). 2,2′-Bipiridine and $FeCl_3 \cdot 6H_2O$ solutions for the Emmerie-Engel reaction were prepared according to Müller Mulot (1976). The complexing reagent was 4 mL of III and 20 mL of IV, diluted to 100 mL with V. Ethanolic KOH consisted of 30 mL of VII diluted to 100 mL with absolute ethanol. TLC sheets of silica gel, 0.2 mm thick on aluminum (Merck), were used.

Procedures. *Purification of Solvents.* To each liter of analitical-grade heptane and toluene was added 5.0 mL of saturated cupric acetate monohydrate and the mixture was allowed to stand for 12 h at room temperature. After that, solvents were washed with distilled water, dried with anhydrous sodium sulfate, and distilled. Absolute ethanol, analytical grade, was distilled over KOH (5 g/L). The distillate was then refluxed with 0.1 g of cupric acetate for 30 min and again distilled.

Preparation of Extracts from Grains. Samples should be sufficiently finely ground to pass a sieve of 0.25-mm openings. A total of 2.0–2.5 g ± 0.1 mg of corn or wheat flour and 0.5–0.7 g ± 0.1 mg of soybean or wheat germ were placed in 200 × 25 mm test tubes with screw caps lined with Teflon or PVC (rubber must be avoided). To each tube 20 mL of absolute ethanol was added, and the tubes were closed tightly and immersed for 30 min in a water bath at 85 °C, being shaken ocasionally. After the solution was cooled, 10 mL of heptane was added and the tube shaken 5 min. Then, 20 mL of 1.25% Na_2SO_4 was added, again shaken for 2 min, and allowed to separate into layers. Tocopherols and lipids remained in the heptane layer. A volume of 0.5 mL of α-tocopherol (αT) in ethanol (II) was processed the same as a sample was and used as a standard for calculations.

Preparation of Samples of Oils and Byproducts. Quantities of 0.4–0.5 g ± 0.1 mg of edible oils were made up to 25 mL with heptane. Distillates from the steam deodorization of soybean oil were warmed and homogenized and 0.25–0.3 g ± 0.1 mg made up to 50 mL with heptane; a dilution of 1/10 of this was prepared for chemical analysis. A standard of αT was prepared by diluting 0.5 mL of αT in heptane (I) to 10 mL.

Removal of Interfering Substances by Slight Saponification. Volumes of 7–8 mL of the heptane extracts from samples or standards of αT and 7–8 mL of pure heptane (blank) were mixed with 5 mL of ascorbic acid (VI) and 5 mL of ethanolic KOH (or aqueous 50% KOH in tests for comparison). Tubes were shaken vigorously for 2.5 min, manually or mechanically, and allowed to settle. Then, 5 mL of the heptane layer was measured and shaken with 10 mL of 80% ethanol for 1 min in order to remove de-

Faculdade de Engenharia de Alimentos e Agrícola, Universidade Estadual de Campinas, 13100 Campinas, SP, Brasil.

0021-8561/82/1430-1109$01.25/0

Table I. Determination of Cu(cuproine)$_2^+$ Absorptivity at 545 Nanometers in Units of α-Tocopherol Concentration

concn of αT, μg/mL	A^a	concn of αT, μg/mL	A^a
1.25	0.042, 0.042	10.00	0.337, 0.339
2.50	0.085, 0.085	15.00	0.503, 0.503
5.00	0.169, 0.170	20.00	0.673, 0.676

regression equation	$A = 0.00064 + 0.03364c$
absorptivity	33.64 L g^{-1} cm^{-1}
molar absorptivity	14490 L mol^{-1} cm^{-1}
standard error of estimate	0.0017
coefficient of correlation	0.99998

[a] 1-cm cells.

Table II. Determination of Reducing Substances (Expressed as α-Tocopherol) in Total Lipid Extracts and the Unsaponifiable Fraction of the Same Extracts. Method of Evaluation: Cu(cuproine)$_2^+$

samples	total tocols, mg of αT/100 g dry basis: total lipid extract		unsaponifiable fraction	
soybeans	30.9	31.8	23.0	23.6
wheat flour	13.5	13.6	10.6	10.9
wheat germ	29.3	29.6	23.2	23.7
fresh corn[a]	11.0	11.1	5.1	5.3
mature corn[b]	20.0	20.9	10.0	10.3

[a] Lyophilized Sugary-1. [b] Sugary-1.

graded lipids, ascorbate, and KOH. Layers were allowed to separate.

Analytical Reaction. To 2.5 mL of the purified heptane extracts from samples, standards of αT, and blanks was added 5 mL of the complexing reagent, and the mixture shaken vigorously for 2.5 min (it is essential that the shaking be energetic) and then allowed to settle. The upper layer was discarded and 3 mL of the lower layer (purple) measured. To this was added 0.5 mL of absolute ethanol to clarify it. The absorbance of this mixture was measured at 545 nm in a 1-cm cuvette by means of a Zeiss M 4 Q III spectrophotometer.

Calculations. The total tocol content was obtained with the expression

$$\text{total tocol (mg of } \alpha\text{T/100 g)} = \frac{A_x C_s f}{1000 A_s W_x} \quad (1)$$

where A_x = the absorbance of the sample extract, A_s = the absorbance of the standard, C_s = micrograms of the standard per milliliter, W_x = the weight of the sample in grams, and f = the dilution factor of the sample. It should be emphasized that the expression of total tocopherols in terms of milligrams of αT is not to be taken literally because the principal tocopherol in these samples is γT, but for practical reasons, αT was used. If purified γT becomes available, the standard should be made with this tocopherol.

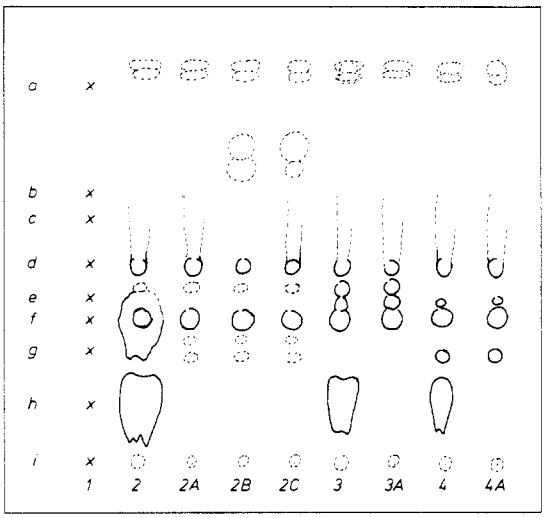

Figure 1. Thin-layer chromatogram of total reducing agents in lipid extracts and of standards. (1) Mixture of standards: (a) β-carotene; (b) tristearin; (c) oleic acid; (d) αT; (e) βT; (f) γT; (g) δT; (h) free sterols and mono- and diglycerides; (i) origin. (2) Corn lipid extract. (3) Wheat flour extract. (4) Soybean extract. (A) Extract treated with ethanolic KOH. (B) Unsaponifiable material extract. (C) Extract purified with alumina.

Recovery of α-Tocopherol. Fifty micrograms of αT was diluted to 10 mL with heptane and 2.5 mL submitted directly to the analytical reaction. Another aliquot of 50 μg of αT was subjected to the complete sequence, like a cereal sample. Another aliquot of 50 μg was only treated by slight saponification, like an oil, and a final 50-μg aliquot was added to 1 g of corn and processed completely. All samples were recovered in 10 mL of heptane, and 2.5 mL of this volume was used for the chemical reaction.

Assays for Comparison. All samples including standards were saponified totally according to the AOAC (1980) method. The nonsaponifiable residue was recovered in heptane, and aliquots of these were used for determining total tocol by reaction with cupric ions and by the conventional method with 2,2′-bipyridine/$FeCl_3$ (Müller Mulot, 1976).

Lipid Content of the Hexane Extracts. The reduction of the lipid content in the hexane extracts submitted to slight saponification was determined by evaporating 2 mL of extract at 60 °C under vacuum.

Thin-Layer Chromatographic Tests for Reducing Substances. Drops of extracts of corn, soybean, and wheat flour in heptane were placed on silica gel plates and developed with hexane/ethyl acetate (92.5/7.5) according to Müller Mulot (1976) and then sprayed with the "complexing reagent". Various standards were used for

Table III. Determination of Reducing Substances (Expressed as α-Tocopherol) in Corn and Soybean Lipid Extracts (1) without Any Treatment, (2) Shaken with Aqueous KOH, (3) Shaken with Ethanolic KOH, and (4) Saponified Conventionally. Method of Evaluation: Cu(cuproine)$_2^+$, Except for the Unsaponifiable Fraction, Which Was Also Evaluated by Fe(bipyridine)$^{2+}$

samples	total tocols, mg of αT/100 g dry basis: without treatment		treated with aqueous KOH		treated with alc	treated with KOH	unsaponifiable fraction: Cu(cuproine)$_2^+$		unsaponifiable fraction: Fe(bipyridine)$^{2+}$	
corn (Opaque-2)	16.2	16.9	6.9	7.8	6.8	6.9	6.9	7.0	7.0	7.2
corn (Maya XII)	13.0	12.2	5.8	6.4	5.3	5.4	5.4	5.5	5.5	5.8
corn (Sugary-1)	18.7	19.0	13.9	14.8	10.2	10.6	10.4	10.8	10.5	10.9
corn (Hybrid)[a]	17.9	18.3	13.1	13.6	8.5	8.8	8.6	8.8	8.9	9.0
soybeans[b]	32.0	32.4	25.2	25.3	21.3	21.7	21.5	20.6	23.9	24.7

[a] Sugary-1/Opaque-2. [b] Santa Rosa.

Table IV. Recovery of α-Tocoferol through the Process of Extraction and Treatment with KOH (Slight Saponification)

samples	absorbance at 545 nm	recovery, %
50 μg of αT not treated	0.120-0.120	
50 μg of αT treated with KOH	0.114-0.117	96.3
50 μg of αT extracted and treated with KOH	0.112-0.110	92.5
1 g of corn[a]	0.140-0.130	
1 g of corn[a] + 50 μg of αT extracted and treated with KOH	0.250-0.246	94.2

[a] Hybrid Sugary-1/Opaque-2.

Table V. Removal of Lipids during Treatment with Ethanolic KOH and Subsequent Washing with 80% EtOH

	concn of lipids, mg/2 mL of heptane			
sample	initial extract	after alc KOH treatment	after washing with 80% EtOH	% removed
corn (Maya)	29.0	27.8	27.1	6.6
corn (Opaque-2)	31.7	28.5	28.5	10.0
corn (Sugary-1)	50.1	48.0	46.2	7.8
corn (hybrid)[a]	50.4	46.0	46.0	8.1
soybean[b]	127.2	123.6	123.0	3.3
	128.5	123.2	123.3	4.1

[a] Sugary-1/Opaque-2. [b] Santa Rosa.

identifying the spots on the chromatogram.

RESULTS AND DISCUSSION

The results of applying the procedures given above to the various types of samples are given in Tables I–V. The chromatogram showing the different reducing compounds in lipid extracts from corn, soybean, and wheat flour is shown in Figure 1.

In considering the results, it is first necessary to explain that the way of determining tocopherols on natural samples is somewhat different from that described in the studies with pure tocopherols (Contreras-Guzmán and Strong, 1982). In that publication, all components reacted in absolute alcohol. In this work, tocopherols and other lipids are in a heptane phase, and cupric ions, cuproine, and urea are in an 80% aqueous ethanol phase. There is reaction only when the two phases are mixed by shaking. Reaction ends when shaking is ended. By means of this artifice, time is controlled, avoiding reaction of nontocopherol-reducing substances. One also avoids absorption due to carotenoid pigments that remain in the heptane phase, together with the oxidation products of the tocopherols. The complexes $Cu(cuproine)_2^+$ and $Cu(bathocuproine)_2^+$ are insoluble in heptane, remaining in the 80% ethanol layer, and their absorbance is measured in this medium.

Table I verifies that the reaction between α-tocopherol and cupric ions in the two-phase system follows Beer's law over a wide range of concentrations, which is sufficient to cover the major part of common products. The molar absorptivity in terms of αT obtained has a value of 14490, a somewhat larger value than that reported in our previous work, 13300. We attribute this increase to the presence of water, the principal difference between the original system and the two-phase system. Water apparently interacts in some way with the cuprous ions and urea, altering the electron distribution of the $Cu(cuproine)_2^+$ complex, which is manifested in a 14% increase in the molar absorptivity. Under these conditions, the determination of α-tocopherol with this complexing agent is only 18.6% less sensitive than the reaction producing $Fe(bipyridine)^{2+}$. The increase in the molar absorptivity does not signify that the molar ratio of 1/2 ($\alpha T/Cu^{2+}$) has changed, since the molar absorptivity in terms of cuprous ion, which was 6580, increased in the two-phase system to 7150, giving a ratio of 14490/7150 = 2.03.

The experiments in Table II were planned to verify whether, with this new method, it will be possible to determine total tocols in the crude lipid extracts and avoid saponification, which is indispensable in all methods in current use. The data of Table II indicate that a large error would be committed by doing the reaction on the crude lipid extracts. They have such large quantities of nontocopherol reducing substances that even the two-phase system did not succeed in reducing their influence. In the corn crude lipids approximately 50% of the absorbance at 545 nm corresponds to interfering substances.

Such substances of nontocopherol nature were observed by developing chromatograms on plates of silica gel. All of them react with cupric ions, with ferric ions, and with DPPH radical. Figure 1 shows a scheme of a chromatogram of reducing substances from crude lipid extracts and vegetable oils. Members of the vitamin E family appear in the central zone (Figure 1 d–g), limited by α-tocopherol and δ-tocopherol. The lower interference (Figure 1h) is the most abundant and reacts on the plate at the same rate as α-tocopherol. It is located in the zone corresponding to free sterols and mono- and diglycerides but did not correspond to campesterol, β-sitosterol, stigmasterol, or cholesterol, which do not reduce any of the reagents for tocopherols. This spot disappears completely after saponification or fractionation of the extract on alumina, silicic acid, or Florisil.

The other group of interfering substances (zone a) were located near the spots given by β-carotene and hydrocarbons in general. They are barely visible components and react more slowly than αT. The large quantity of triglycerides carries some αT, probably because of overloading, in nonsaponified samples. The spots in the zone d–g that do not correspnd to the tocopherol standards must be tocotrienols, which in the hexane–ethyl acetate system appear in these positions (Müller Mulot, 1976). Although saponification removes impurities on the one hand, it also creates a reducing group located between reference substances a and b. According to Whittle and Pennock (1967) and Dicks Bushnell (1967), these may be hydroquinones and other products of the degradation of plastoquinones by KOH. Their influence is controlled in the conventional reaction by chelating the ferric ion remaining after 2.5 min of reaction with phosphoric acid.

Reducing impurities are found in all the extracts, independent of the type of solvent used for extraction. In various tests, acetone, chloroform, heptane, and ethanol were used in a system of continuous extraction and methanol/chloroform/water (Bligh and Dyer, 1959) and ethanol/heptane in a discontinuous system. All of these extracted similar groups of impurities.

The data of Table II indicate that saponification is absolutely necessary to eliminate impurities of a reducing nature. However, in terms of labor and cost, saponification is unsatisfactory for routine control. For this reason, we attempted to determine to what point it is necessary to carry the hydrolysis in order to destroy the interfering substances. Possibly, a more gentle alkaline treatment would be sufficient. It was evident that the large quantity of triglycerides does not disturb the determination of total tocols by chemical means. These compounds do interfere

Table VI. Determination of Reducible Substances (Total Tocols) in Edible Oils and Oil Products (1) without Any Treatment, (2) Shaken with Ethanolic KOH, and (3) Saponified Completely. Method of Evaluation: $Cu(cuproine)_2^+$, Except for the Unsaponifiable Fraction, Which Was Also Evaluated by $Fe(bipyridine)^{2+}$

	total tocols, mg of αT/100 g			
			saponified	
sample	without treatment	shaken with alc KOH	$Cu(cuproine)_2^+$	$Fe(bipyridine)^{2+}$
soybean oil no. 1	92.3	90.9	91.8	92.3
soybean oil no. 2	92.9	91.5	90.9	91.2
soybean oil no. 3	88.3	86.8	86.0	87.0
soybean oil no. 4	83.1	80.7	82.0	82.5
sunflower oil no. 1	71.8	66.0	64.0	68.0
sunflower oil no. 2	91.9	73.0	68.5	72.0
olive oil no. 1	29.5	13.2	13.2	13.9
olive oil no. 2	24.8	12.1	13.0	12.5
corn oil no. 1	142.5	78.4	79.6	80.8
corn oil no. 2	136.9	72.0	71.1	74.2
distillate no. 1[a]	10.00×10^3	5.00×10^3	5.07×10^3	6.10×10^3
distillate no. 2[a]	8.80×10^3	4.10×10^3	4.15×10^3	4.53×10^3
distillate no. 3[a]	11.10×10^3	6.86×10^3	6.63×10^3	7.05×10^3
distillate no. 4[a]	11.34×10^3	7.12×10^3	7.09×10^3	7.38×10^3

[a] From soybean oil steam deodorization.

in the separation of individual tocopherols by TLC or GLC.

Table III shows the results of two types of treatments of lipid extracts with KOH in comparison with those of total saponification. The treatment with aqueous KOH, according to the procedure given under Experimental Section, removed a large quantity of reducing substances, with some results approaching those obtained by saponification. However, it does show poor agreement among duplicates. This was not improved by increasing the time of shaking to 5 min or by changing the concentrations of KOH. Concentrations of less than 25% caused formation of foam, which resulted in loss of tocopherols. On the other hand, when the sample was shaken with ethanolic KOH (approximately 7.5% in the final mixture), the absorbance at 545 nm decreased to reach values similar to those obtained by saponification, foam did not form, and satisfactory duplicates were obtained. The use of ethanolic KOH, in contrast with the method of total saponification, permits doing many determinations at the same time since the complete analysis is carried out in test tubes with screw caps.

Table IV shows recoveries when 50.0 μg of α-tocopherol was treated alone and when added to 1 g of ground corn. The αT was satisfactorily recovered, showing that the ascorbic acid protected it efficiently during the alkaline treatment.

Table V allows one to conclude that the treatment with ethanolic KOH does not cause much hydrolysis since more than 90% of the initial lipids remain in the heptane layer. This means that the reducing material is selectively destroyed without significant hydrolysis of triglycerides. The slight formation of soaps and the high concentration of ethanol permit rapid separation of the phases.

The results in Table VI demonstrate that, for commercial oils, the brief treatment with ethanolic KOH also gives results comparable to those from saponification. Olive, corn, and sunflower oils exhibit significant quantities of nontocopherol, reducing impurities, in contrast to the soybean oils, which are almost "clean". The comparison of the data obtained by the conventional reaction with $Fe(bipyridine)^{2+}$ and those with cupric ions indicates that both reactions basically determine similar substances, a fact that has been verified by TLC. However, the determination with $Fe(bipyridine)^{2+}$ usually gave results slightly higher, probably due to inefficient control of degraded lipids formed during the saponification.

The determination of total tocols in a steam deodorizer distillate from soybean oil is generally more difficult than the determination in vegetable oils, due to the high concentration of reducing substances generated during the process of refining (dimers of tocopherols and other products). The method proposed here is especially adequate for this material and agrees well with data from the literature (Feeter, 1974).

LITERATURE CITED

AOAC "Official Methods of Analysis", 13th ed.; AOAC: Arlington, VA, 1980; Section 43.092.
Bligh, E. C.; Dyer, W. J. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.
Contreras-Guzmán, E.; Strong, F. C., III *J. Assoc. Off. Anal. Chem.* **1982**, *65* (5).
Dicks Bushnell, M. W. *J. Chromatogr.* **1967**, *27*, 96–103.
Feeter, D. K. *J. Am. Oil Chem. Soc.* **1974**, *51*, 184–187.
Müller Mulot, W. *J. Am. Oil Chem. Soc.* **1976**, *53*, 732–736.
Whittle, K. J.; Pennock, J. F. *Analyst (London)* **1967**, *92*, 423–430.

Received for review November 20, 1981. Accepted August 16, 1982. This work was presented at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy in Atlantic City, NJ, March 1981.