(2) Hoppe, J. O., in "Medicinal Chemistry," Vol. 6, Wiley, New York, N. Y., 1963 p. 303.
(3) Chaberek, S., and Martell, A. E., "Organic Sequestering Agents," Wiley, New York, N. Y., 1959, pp. 511–580.
(4) Smith, R. L., in "Progress in Drug Research," Vol. 9, Birkhäuser Verlag, Basel, Switzerland, 1966, pp. 299–360.
(5) Felder, E., Pitre, D., and Fumagalli, L., *Farmaco (Pavia), Ed. Sci.*, **21**, 572(1966).
(6) Grice, R., and Owen, L. N., *J. Chem. Soc.*, **1963**, 1947.
(7) Hebký, J., and Karásek, M., *Coll. Czech. Chem. Commun.*, **29**, 3108(1964).
(8) Hey, D. H., and Kohn, D. H., *J. Chem. Soc.*, **1949**, 3177.
(9) Smith, P. A. S., in "Organic Reactions," Vol. 3, Wiley, New York, N. Y., 1946, pp. 337–449.
(10) Wolff, H., in "Organic Reactions," Vol. 3, Wiley, New York, N. Y., 1946, pp. 307–336.
(11) Dubsky, J. V., and Graenacher, Ch., *Ber.*, **50**, 1962 (1917).
(12) Dwyer, F. P., and Garvan, F. L., *J. Am. Chem. Soc.*, **81**, 2955(1959).

Keyphrases

X-Ray contrast agents—synthesis, X-ray intensity
3-Phenyl-glutaric acid, iodinated derivatives—synthesis
N-Benzyl-iminodiacetic acid, iodinated derivatives—synthesis
NMR spectroscopy—structure
IR spectrophotometry—structure
Toxicity testing—X-ray contrast agents

Assay of Vitamin E in Multivitamin Products Using Thin-Layer Chromatography

By R. R. CRAWFORD, D. C. NARAMORE, and O. K. ESMERIAN

A method for the assay of vitamin E in multivitamin formulas is presented. The sample is saponified and the alpha tocopherol separated from interfering substances by thin-layer chromatography prior to measurement by ferric chloride–α,α'-dipyridyl colorimetry. Assay results of 18 multivitamin formulas comprising three types of dosage forms are in reasonable agreement with label claims. The nature of some interfering substances is discussed.

PRESENT OFFICIAL methods for assaying vitamin E (alpha tocopherol) employ oxidation by either ferric chloride or by ceric sulfate. The amount of alpha tocopherol is determined by the reaction of ferrous ions with α,α'-dipyridyl (red complex) or by titration with ceric sulfate until an excess of ceric ions oxidizes diphenylamine indicator. These methods were critically examined by Lehman before suggesting oxidation by ferric chloride as the method of choice for assaying vitamin E in pharmaceutical products (1). Both of these methods, however, are subject to interference from other reducing materials, among which are antioxidants and vitamin A, included in most formulations. The results from these methods also include responses from the less biologically active nonalpha isomers of tocopherol present in those multivitamin products that use *d*-alpha tocopheryl acetate concentrate or mixed tocopherols concentrate as the vitamin E source.

Early work in this field was directed toward the assay of vitamin E in the presence of only vitamin A, culminating in the hydrogenation procedure reported by Fisher *et al.* (2), and included in the United States Pharmacopeia XVII (3). Paper chromatography has also been applied to vitamin A and E mixtures (4). Recent gas-liquid techniques for identification and estimation of tocopherols have been described (5–7) and applied by Pillsbury *et al.* (8) and by Bowman and West (9) to pharmaceuticals. Several recent references are found pertaining to thin-layer chromatography of tocopherols *per se* (10), in biologic materials (11), in oils and plant tissues (12, 13), and in serum (14). Castrén has reported the determination of vitamin E in pharmaceuticals using TLC separations (silica gel) followed by quantitative estimation of the developed spots by planimetry and visual observation (15, 16).[1]

METHOD

The method developed for multivitamin formulations containing vitamin E in potencies of from 0.15

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[1] For a comprehensive review of vitamin E methodology, see Bunnell, R. H., "The Vitamins," Vol. 6, Academic Press, New York, N. Y., 1967, pp. 261–316.

to 15 I.U. or more per dosage unit consists of three major steps:

(a) Saponification, extraction, and concentration of the alpha tocopherol into a relatively small volume of ether.

(b) Separation of the alpha tocopherol from other ingredients reactive to ferric chloride by TLC.

(c) Determination of the alpha tocopherol by ferric chloride–α,α'-dipyridyl colorimetry.

Reagents

Ethanol, absolute; potassium hydroxide, pellets, 85%; hydrochloric acid, 37%; ethyl ether, anhydrous, ACS reagent grade; sodium sulfate, anhydrous granular; toluene, Eastman White Label 325; α,α'-dipyridyl, 0.25% in ethanol; ferric chloride, 0.1% in ethanol; 2′,7′-dichlorofluorescein, 0.004% in ethanol.

Materials

Thin-layer[2] plates, 20 × 20 cm., silica gel with fluorescent indicator, 250 μ; thin-layer developing apparatus, sandwich type; colorimeter, Evelyn, or other similar instrument having a constant, low-intensity light source and a direct reading, instantaneously responding photocell and galvanometer, equipped with a 515 mμ filter; UV light source, 2,540 Å.

Procedure

(a) Saponification—In a 125-ml. saponification flask obtain a sufficient quantity of the dosage form to result in a total of preferably 8 to 16 I.U.[3] of vitamin E as follows:

Capsules—Place the required number into the flask. Add 10 ml. water, warm, and swirl until digested.

Tablets—Precrush the required number in a mortar and transfer to the flask using 10 ml. water.

Liquids—Using a suitable pipet, transfer the required volume into the flask, and add 5 ml. water.

Add 30 ml. of ethanol, attach an air condenser, and heat with steam to vigorous reflux. Slowly drop 5 g. KOH pellets through the condenser and reflux for 20 min. Remove from heat and immediately add 10 ml. HCl dropwise through the condenser. Cool and transfer to a 500-ml. separator using 50 ml. distilled water followed by three 50-ml. portions of ether. Shake well for 2 min. Separate the ether layer and wash with four 100-ml. portions of distilled water. Dry the extract by passing it through a funnel containing sodium sulfate on filter paper, washing the cake well after passage with additional ether. Reduce the ether volume sufficiently to allow transfer to a 10-ml. or 25-ml. volumetric flask. Make to a final concentration of alpha tocopherol of about 0.5 mg./ml.

(b) Chromatography—Using a suitable pipet, stripe a sufficient quantity of the ether solution (usually 100 to 300 μl.) containing 80 to 120 mcg. alpha tocopherol on a silica gel plate previously activated for 20 min. at 100°. The stripe should be placed parallel to and approximately 2 cm. from one edge of the plate and should extend for a distance of approximately 14 cm. Place a pilot spot of alpha tocopherol next to the unknown. Develop the chromatogram with a migrating solvent of toluene to a distance of about 15 cm. Remove the plate, dry thoroughly with nitrogen, and spray with 2′,7′-dichlorofluorescein solution. Find the alpha tocopherol zone corresponding to the standard at R_f of about 0.5 by observing under UV light (2,540 Å.). Outline the correct band with pencil and scrape the adsorbent into a 125-ml. flask. Add 10 ml. ethanol, swirl for 3 min., and pass through filter paper (Eaton-Dikeman 617 is satisfactory) into a 25-ml. volumetric flask (low actinic) calibrated at 23 ml. or into a 25-ml. graduated cylinder (low actinic, ground glass stopper). Repeat with three 4-ml. portions of alcohol and make to a final volume of 23 ml.

(c) Colorimetry—Add successively to the flask or cylinder 1 ml. dipyridyl solution and 1 ml. ferric chloride solution. Stopper the container and mix well. After 2.5 min., read the percent transmittance of the solution using a 515-mμ filter in the colorimeter. From a calibration chart, determine the corresponding amount of alpha tocopherol, in mcg.[4] Calculate the potency as follows:

a = mcg. alpha tocopherol, from calibration chart
b = final volume of ether extract, ml.
c = volume of ether extract striped, μl.

$$\frac{a \times b}{c} = d = \text{mg. alpha tocopherol in final ether volume}$$

$$\frac{d \times f}{n} = \text{potency, I.U./dosage unit}$$

where f is the weight-unit relationship (17) for *d*-alpha tocopherol (f = 1.49) and *dl*-alpha tocopherol (f = 1.1) and n is the number of dosage units assayed.

DISCUSSION

Analyses in these laboratories of vitamin E in commercial multivitamin capsules and tablets had shown that the USP XVII assay procedure written for decavitamins was in some instances unsatisfactory. The USP procedure removes the interference due to vitamin A but other reducing substances lead to assay results for vitamin E that are sometimes several times too high. Clearly, some means of separation or a more specific assay method was needed. It seemed that TLC would have the capability of separating alpha tocopherol from the other reactants and still allow common methods of determination to be used.

Investigations were done to determine an adsorbent-solvent system to separate alpha tocopherol from interfering substances in capsules and tablets. Information in the literature suggested that an optimum system would be found among two adsorbents (silica gel and alumina) and six solvents (hexane–ethanol 9:1, ether, toluene, toluene–ether 1:1, cyclohexane–ether 4:1, cyclohexane–ethanol 9:1). Test strips of Eastman Chromagram sheets

[2] Suitable are E. Merck plates, Silica Gel F-254.

[3] The following example for tablets labeled to contain 0.15 I.U./tablet illustrates deviations from the procedure when this very low potency product was assayed.
Saponification: 10 tablets crushed and transferred to flask with 30 to 40 ml. water, 110 ml. ethanol, and 7 g. KOH added for reflux. 14 ml. HCl used for acidification. Ether extract transferred to a 10-ml. volumetric flask (concentration of alpha tocopherol of 0.1 mg./ml.).
Chromatography: 1 ml. of ether solution (containing 100 mcg. alpha tocopherol) striped.

[4] For procedures regarding colorimetry, see Lehman, R. W., *J. Pharm. Sci.*, **53**, 201(1964).

(6060 silica gel, 6063 alumina) (Eastman Kodak Co., Rochester, N. Y.) were developed with these solvents using the unsaponifiable fractions from multivitamin-mineral formulations. Reducing substances were visualized by ferric chloride-dipyridyl spray. Table I shows typical R_f values, these obtained after chromatographing the unsaponifiable fraction of a multivitamin-mineral tablet labeled to contain 0.15 I.U. vitamin E/tablet. The best separations were obtained with alumina/toluene, with silica gel/toluene, and with silica gel/cyclohexane–ether. The authors chose silica gel/toluene for further investigation.

Silica gel was quite effective for separating alpha tocopherol from other tocopherols. The unsaponifiable fraction of a mixed tocopheryl acetate concentrate was chromatographed. Of the total tocopherols in the concentrate, 87% was alpha and 13% was a mixture of nonalpha tocopherols. Gas-liquid chromatography (GLC) applied to the eluted alpha tocopherol band showed that only 0.1% nonalpha tocopherols remained.

Some loss of alpha tocopherol, presumably through oxidation, during the separation procedure can be expected. To determine the loss, replicate quantitative chromatograms of *d*-alpha tocopherol were run on 250-μ silica gel plates and the recoveries compared with the unchromatographed input. The results from 10 determinations (Table II) indicated that a 94.7% apparent recovery ±1.6% *SD* of alpha tocopherol could be expected.

The presence of reducing materials in the thin-layer adsorbent that co-elute with alpha tocopherol would result in an inflated recovery value. Several experiments were done in a manner similar to that noted above but in which ether only was striped. Assay of the eluted adsorbent band corresponding in size and position to an alpha tocopherol band from 250-μ silica gel gave values corresponding to about 3 mcg. alpha tocopherol. Since approximately 100 mcg. amounts of alpha tocopherol are striped in the recommended procedure, this value amounts to some 3% of the total and is included in the 94.7% apparent recovery. The corresponding true recovery value was about 92%.

In order to determine whether alpha tocopherol could in fact be recovered from multivitamin preparations, a formulation was obtained typical of one designed for capsules but without any added vitamin E.[5] TLC on Eastman Chromagram sheets (silica gel) of the unsaponifiable fraction indicated that no vitamin E could be detected by visualization with ferric chloride-dipyridyl spray solution. Without addition of alpha tocopherol, USP assay (without hydrogenation) indicated that 6 I.U. of apparent vitamin E/g. was present in the material. Vitamin E, as *d*-alpha tocopheryl acetate concentrate, was added at 2, 3, and 15 I.U./g. before saponification. Recoveries as obtained by the recommended procedure, shown in Table III, were from 98 to 100% of input after correcting for the apparent losses of alpha tocopherol during separation.

The results of the assay procedure applied to multivitamin capsules, tablets, and liquids are given in Table IV. It can be seen that the total apparent amounts of vitamin E in the unsaponifiable fractions (unhydrogenated) ranged from about 1.5 to some 20 times the labeled amount. After chromatography, the assay results (corrected) were found to be generally near label claim: in 14 of the 18 products, they lay between 94 and 110% of this figure.

[5] Kindly supplied by R. P. Scherer Corp., Detroit, Mich.

Table I—R_f Values for Alpha Tocopherol on Test Strips, 100 μ

Test Strip	R_f
Alumina	
Hexane–ethanol 9:1	0.79
Ether	0.82
Toluene	0.37
Toluene–ether 1:1	0.77
Cyclohexane–ether 4:1	0.34
Cyclohexane–ethanol 9:1	0.76
Silica Gel	
Hexane–ethanol 9:1	0.76
Ether	0.95
Toluene	0.61
Toluene–ether 1:1	0.85
Cyclohexane–ether 4:1	0.63
Cyclohexane–ethanol 9:1	0.80

It was of interest to attempt to determine the nature of the interfering substances in these formulas and some work was done in this area. The authors' knowledge of vitamin A would lead them to infer that, since they did not completely remove it by hydrogenation, higher ratios of vitamin A:alpha tocopherol should result in higher apparent amounts of alpha tocopherol in the unsaponifiable fractions. Table V shows that this is generally true. It should be remembered that not only vitamin A but also butylated hydroxyanisole (BHA) and, to some extent, butylated hydroxytoluene (BHT) blended with the vitamin A as antioxidants would be expected to interfere. Figure 1 illustrates a silica gel chromatogram used to aid in identifying these substances. The spot from the unsaponifiable fraction corresponding closely in R_f to that of BHA was confirmed as such by GLC and IR spectral data. The spot closest to the solvent front contained BHT and anhydrovitamin A as shown by GLC and UV ab-

Table II—Replicate Recoveries of *d*-Alpha Tocopherol by Thin-Layer Chromatography

Silica Gel, 250 μ, %
93.8
94.6
96.1
97.0
95.4
93.0
92.6
95.7
96.5
92.7
94.7% mean ± 1.6% *SD*

Table III—Recoveries of Vitamin E Added to Multivitamin Formulation

Input I.U./g.[a]	Recovery, %	
	Found	Corrected[b]
15	95	100
3	94	99
2	93	98

[a] *d*-Alpha tocopheryl acetate concentrate. [b] Correction factor 0.95.

TABLE IV—VITAMIN E CONTENT OF MULTIVITAMIN FORMULAS POTENCY, I.U. PER DOSAGE UNIT

Sample Number	Label Potency	Total Emmerie-Engel	Chromatographed Emmerie-Engel		
			Uncorrected	Corrected[a]	% Label
Capsules					
1	15	25.2	14.8	15.6	104
2	15	26.2	14.6	15.4	103
3	5	10.5	4.1	4.3	86
4	5	12.4	4.8	5.1	102
5	5	7.3	4.5	4.7	94
6	3.7	6.8	3.6	3.8	103
7	3	11.7	2.9	3.1	103
8	2	4.0	2.1	2.2	110
9	2	7.4	2.1	2.2	110
10	1	1.3	0.97	1.0	100
11	0.5	8.1	0.45	0.47	94
Tablets					
12	15	22.7	15.0	15.8	105
13	10	13.7	11.9	12.5	125
14	7.5	11.2	7.3	7.7	103
15	3	4.6	3.0	3.2	107
16	0.15	2.2	0.16	0.17	113
Liquids					
17	50	172.8	44.7	47.1	94
18	5	103.5	6.0	6.3	126

[a] Correction factor 0.95.

sorption, respectively (the anhydrovitamin A is formed upon acidification of the mixture with hydrochloric acid). A summary of the R_f values of known interfering substances is found in Table VI.

The authors have observed the presence of other reducing agents that have not been identified. For example, a thin-layer chromatogram of a multivitamin unsaponifiable fraction showed the presence of at least four reactive ingredients (R_f of 0, 0.25, 0.58, 0.62) in addition to alpha tocopherol and antioxidants. In another example, a thin-layer chromatogram of a hydrogenated multivitamin unsaponifiable fraction revealed four strongly interfering materials (R_f of 0–0.1). This latter example illustrates one instance where grossly high results were obtained with the USP XVII procedure.

TABLE V—COMPARISON OF VITAMIN A: ALPHA TOCOPHEROL RATIO WITH TOTAL APPARENT VITAMIN E

Units Vitamin A/Unit Vitamin E (Label)	Total Emmerie-Engel % of Label
Capsules	
1,000	145
1,350	184
1,670	166
1,670	175
2,500	200
3,000	133
5,000	210
5,000	248
6,250	370
8,300	390
50,000	1,620
Tablets	
400	137
1,330	149
1,670	151
1,670	154
33,400	1,470

Thus, it is possible that some seven or more reducing materials, some of which are not removed by hydrogenation, can be present in a multivitamin formulation.

The use of 250-μ thin-layer plates was necessary in some cases since overloading of 100 μ thicknesses was encountered upon chromatographing sufficient amounts of certain capsule unsaponifiable fractions to provide 80 mcg. or more alpha tocopherol. Overloading appeared to be common with those formulations having large amounts of diluent oils. Three alternate methods that allow smaller amounts to be chromatographed appear as possibilities to eliminate overloading if 100-μ thicknesses are to be employed: (*a*) more sensitive reagents, such as bathophenanthroline (12, 18) or 2,4,6-tri(2′-pyridyl)-*S*-triazine (18, 19), replacing α,α'-dipyridyl; (*b*) alkaline extraction after saponification thereby eliminating most of the fatty acids; or (*c*) reducing the volume of the colorimetric reaction.

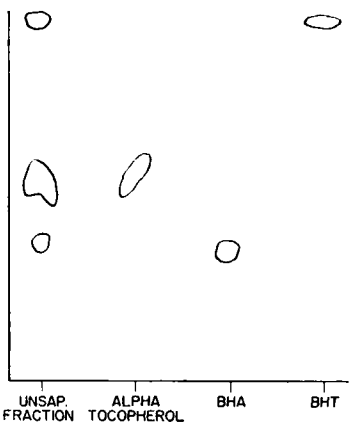

Fig. 1—Identification of some interfering materials

TABLE VI—R_f VALUES OF INTERFERING SUBSTANCES 250 μ SILICA GEL–TOLUENE

Substance	R_f
BHT	0.97
Anhydrovitamin A	0.97
Alpha tocopherol	0.49
BHA	0.38
Beta tocopherol	0.32
Gamma tocopherol	0.30
Delta tocopherol	0.20

Assays Other Than Multivitamins—Thin-layer separation should be applicable to most types of formulations in addition to multivitamins. One such application was to a liquid tonic formula labeled to contain 20 I.U. vitamin E, as *d*-alpha tocopheryl acetate/oz. Assay of the unsaponifiable fraction prior to chromatography indicated 440 I.U. apparent vitamin E/oz. TLC revealed the large amount of reducing substances. Assay of the eluted alpha tocopherol band gave a reasonable result of 20.6 I.U./oz.

REFERENCES

(1) Lehman, R. W., *J. Pharm. Sci.*, **53**, 201(1964).
(2) Fisher, W. T., Edwards, N. M., and Lehman, R. W., *ibid.*, **53**, 294(1964).
(3) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 889–890.
(4) Brown, J. A., *Anal. Chem.*, **25**, 774(1953).
(5) Eisner, J., Iverson, J. L., and Firestone, D., *J. Assoc. Offic. Agr. Chemists*, **49**, 580(1966).
(6) Slover, H. T., Shelley, L. M., and Burks, T. L., *J. Am. Oil Chemists' Soc.*, **44**, 161(1967).
(7) Ishidawa, S., and Katasui, G., *Bitamin*, **31**, 445 (1965).
(8) Pillsbury, H. C., Sheppard, A. J., and Libby, D. A., *J. Assoc. Offic. Anal. Chemists*, **50**, 809(1967).
(9) Bowman, P. B., and West, W. E., *J. Pharm. Sci.*, **57**, 470(1968).
(10) Comer, J. P., and Comer, I., *ibid.*, **56**, 413(1967).
(11) Herting, D. C., and Drury, E. E., *J. Chromatog.*, **30**, 502(1967).
(12) Sturm, P. A., Parkhurst, R. M., and Skinner, W. A., *Anal. Chem.*, **38**, 1244(1966).
(13) Dilley, R. A., and Crane, F. L., *Anal. Biochem.*, **5**, 531(1963).
(14) Herting, D. C., and Drury, E. E., *Am. J. Clin. Nutr.*, **17**, 351(1965).
(15) Castrén, E., *Farm. Aikakauslehti*, **71**, 351(1962).
(16) Castrén, E., *Anales Farm. Hosp.*, **8**, 31(1965).
(17) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 506.
(18) Tsen, C. C., *Anal. Chem.*, **33**, 849(1961).
(19) Martinek, R. G., *Clin. Chem.*, **10**, 1078(1964).

Keyphrases

Vitamin E analysis—multivitamin products
TLC—separation, analysis
UV light—α-tocopherol zone visualization
Colorimetric analysis—spectrophotometer

Alkaloids of *Ochrosia maculata* Jacq. (*Ochrosia borbonica* Gmel.)

Isolation of the Alkaloids and Study of the Antitumor Properties of 9-Methoxyellipticine

By GORDON H. SVOBODA, GERALD A. POORE, and MARILYN L. MONTFORT

Inclusion in the authors' phytochemical screening program of plants of the family *Apocynaceae* related to *Catharanthus roseus* G. Don seemed logical in view of the success realized with the Madagascan periwinkle. Screening of the appropriate extracts of *Ochrosia maculata* elicited both oncolytic and neurosedative activities. The former was found to be associated with 9-methoxyellipticine, the latter with reserpine. While 9-methoxyellipticine possesses experimental antitumor activity of a somewhat lesser order than some of the available clinically active agents, it does exhibit a broader spectrum than most of these compounds. Its moderate degree of potency as an antitumor agent is expressed by its activity against several of the solid mouse neoplasms maintained in these laboratories.

THE GENUS *Ochrosia*, family *Apocynaceae*, consists of approximately 36 species of trees or woody shrubs native to tropical Asia, Oceania, and the Mascarene and Seychelle Islands. *Ochrosia maculata* Jacq., a local name for which is "bois jaune" because of the bark and wood being yellow and bitter (1), is a tree 6–12 m. (20–40 ft.) in height, bearing 3 leaves (rarely 4) in a whorl, being oblong to oblong-lanceolate, 13.5–15.2 cm. (3–6 in.) long, obtuse or somewhat acute, glossy, and often spotted. The flowers are white, being

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