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Note

Comparative analysis of tocopherols by thin-layer chromatography and high-performance liquid chromatography

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The nutritional and biochemical roles of vitamin E as an antioxidant, free-radical scavenger, and membrane lipid stabilizer have generally been recognized[1–4]. In the past, numerous analytical methods have been employed for the separation and quantitation of the various tocopherol isomers and related compounds.

The oldest and most widely employed method is the Emmerie–Engel oxidimetric reaction based on the reduction of Fe^{3+} (from $FeCl_3$) to Fe^{2+} by tocopherols[5], the Fe^{2+} forming a red-colored complex with α,α-dipyridine. This complex is measured colorimetrically at 520 nm. The difficulties and limitations inherent in this method lie in the fact that carotenoids, cholesterol, and vitamin A, along with other non-specific reducing compounds interfere with the colorimetric reaction[6]. The unstable color and variable times for maximum color development are also inherent problems with this method. In 1961, Tsen[7] developed a modified Emmerie–Engel procedure employing bathophenanthroline, which forms a more stable chromatophore with Fe^{2+} increasing the sensitivity of the colorimetric reaction 2- to 3-fold, but still not eliminating the interfering influences.

Alternatively, spectrofluorometry is an extremely sensitive method for assaying free and esterified tocopherols. The original method[8] involved oxidation of tocopherols with nitric acid to form a fluorescent phenazine derivative. Current methods involve measuring fluorescence on tocopherol-containing solvent extracts at 295 nm and 340 nm, the wavelength maxima for excitation and emission, respectively. These methods are preferred over colorimetric procedures due to speed, simplicity, sensitivity and the absence of interference from the non-specific reducing compounds mentioned previously[6].

The most useful chromatographic techniques employed for the separation and quantitation of various tocopherols have been thin-layer chromatography[9,10] (TLC), gas–liquid chromatography (GLC)[11] and column chromatography[12–18].

In the past, TLC and GC methods have required lengthy analysis and allowed the possibility of oxidative loss of the compounds during sample preparation[19]. Until recently, most thin-layer chromatographic procedures involved scraping the resolved tocopherols from silica or alumina plates and eluting the samples with ethanol for subsequent colorimetric or spectrophotometric analysis[6]. With the advent of sensitive

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densitometric methods[20,21], however, the *in situ* separation of a variety of compounds has been made possible, thus reducing analysis time and opportunity for sample loss, and improving the sensitivity and reproducibility of the analyses.

A number of investigators have likewise demonstrated the advantages of high-performance liquid chromatographic (HPLC) methods for tocopherol analysis using normal-phase[12,14,16,18] and reversed-phase[13,15,17,18] systems. The advantages of these systems include the separation of α-, β-, γ-, and δ-tocopherols, high specificity and sensitivity often into the nanogram range, good reproducibility and sample recovery, and speed and ease of sample application.

It should be noted, however, that Hatam and Kayden[13] were unable to resolve the β- and γ-homologues, which chromatographed as a single peak. Vatassery *et al.*[17] encountered similar difficulties in separating the β- and γ-homologues using normal-phase (Corasil II) and reversed-phase (Bondapak C_{18}/Corasil and Bondapak Phenyl/Corasil) columns.

In our laboratory, we sought to develop comparable analytical systems for the study of tocopherols and related compounds using TLC and HPLC.

MATERIALS AND METHODS

The standard compounds used in this investigation (*dl*-α-tocopherol, *dl*-γ-tocopherol, *dl*-δ-tocopherol, *dl*-α-tocotrienol and *dl*-tocol) were obtained from Hoffman-La Roche (Nutley, NJ, U.S.A.). All of the compounds were dissolved in filtered HPLC-grade methanol (Burdick & Jackson, Muskegon, MI, U.S.A.) for subsequent TLC and HPLC analysis.

TLC and HPLC

Silica gel GF plates (Analtech, Newark, DE, U.S.A.) were pre-washed in chloroform–methanol (1:1), and upon drying, activated at 100°C for 10 min. Samples of the various compounds were applied (0.2 to 2.4 μg) under a nitrogen stream in diffuse light to minimize the danger of peroxidation.

Chromatograms were developed in a mobile phase consisting of hexane–isopropyl ether (85:15). Systems employing hexane–ethyl (85:15 and 80:20) ether were attempted, but did not allow for clear separation of α-tocopherol from an unsaponified triglyceride extract spotted on the plates. It was our goal to avoid saponification of our test samples (algal lipids) to reduce any chance of tocopherol damage. Alternatively, a system of acetone–benzene–water (91:30:8) was attempted, yielding excellent separation of the algal lipids but causing the standards to run off the plates. The hexane–isopropyl ether (85:15) system proved the most effective.

The developed plates were air-dried, oven-dried for 15 min at 100°C, and sprayed with 10% copper(II) sulfate–phosphoric acid followed by charring at 190°C for exactly 10 min. The resolved compounds were quantitated using a Shimadzu high-speed TLC scanner (Model-CS920) at 350 nm using a D_2 lamp.

An analytical procedure was likewise developed using HPLC. The tocopherol standards and related compounds were analyzed isocratically on a Varian Model 5000 chromatograph equipped with a Vari-Chrom UV–VIS spectrophotometer. The column (30 cm × 4.0 mm I.D.) was a reversed-phase Varian MCH 10 C_{18} Micropak column (monomeric). The mobile phase consisted of methanol–water (95:5) set at a

flow-rate of 2.0 ml/min. The spectrophotometer was set at a wavelength of 296 nm with a 0.05 absorbance range and band width of 16 nm. Samples ranging from 2 μl to 50 μl were placed on the column.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatogram obtained for TLC analysis of the standard compounds. Although DL-α-tocopherol and DL-tocol chromatographed closely, the scanner was able to quantify distinct peaks, enabling the resolution and quantification *in situ* of all of the compounds of interest. The lower limit of sensitivity approached 0.2 μg sample; at higher concentrations a tailing effect is observed in agree-

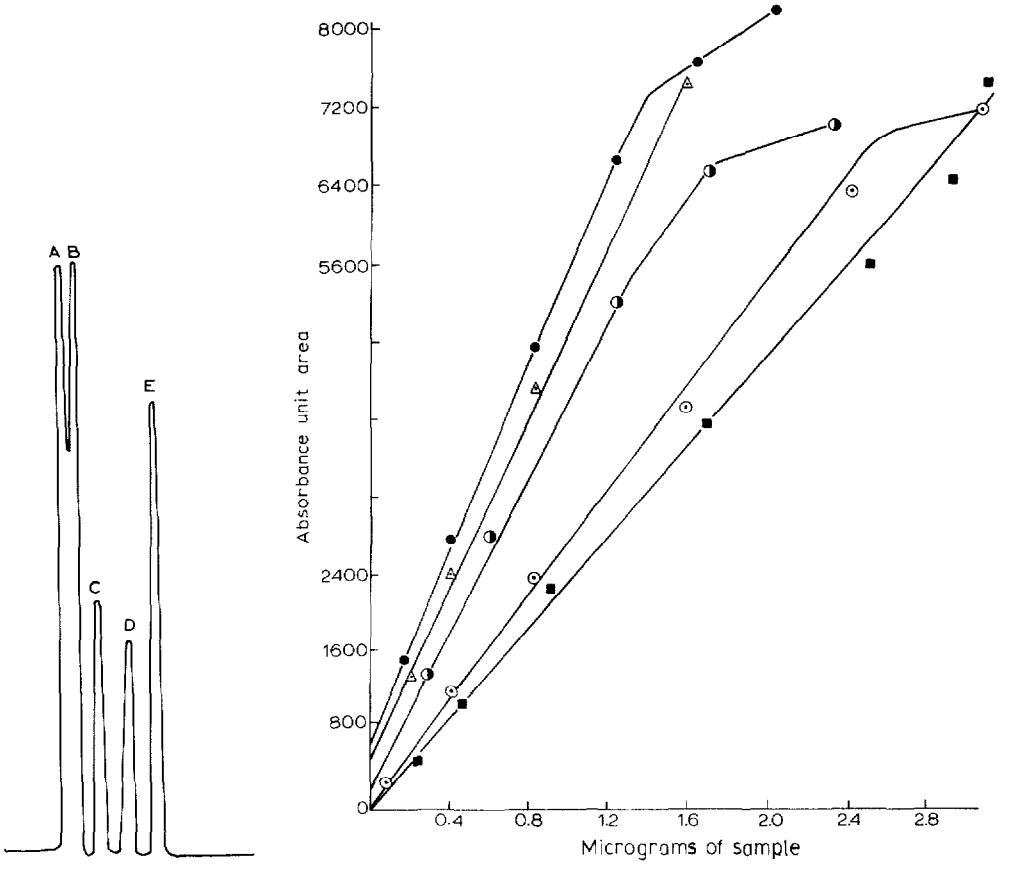

Fig. 1. TLC chromatogram of tocopherol isomers and related compounds. Conditions: plates, silica gel GF; solvent, hexane–isopropyl ether (85:15); detection, λ = 350 nm, D_2 lamp, 10 mV range; visualizing agent, 10% $CuSO_4$–8% H_3PO_4. Peak identities: A = DL-tocol (0.46 μg); B = DL-δ-tocopherol (0.4 μg); C = DL-γ-tocopherol (0.4 μg); D = DL-α-tocotrienol (0.4 μg); E = DL-α-tocopherol (0.4 μg). R_F values: A, 0.27; B, 0.29; C, 0.33; D, 0.37; E, 0.42.

Fig. 2. Calibration curves of tocopherols and related compounds using TLC. Conditions: plates, silica gel GF; solvent, hexane–isopropyl ether (85:15) (one-dimensional); visualizing agent, 10% $CuSO_4$–8% H_3PO_4; detection, λ = 350 nm, D_2 lamp, 10 mV range. ○—○, DL-α-tocopherol; ■--■, DL-γ-tocopherol; ●—●, DL-δ-tocopherol; △—△, DL-α-tocotrienol; ◑—◑, DL-tocol.

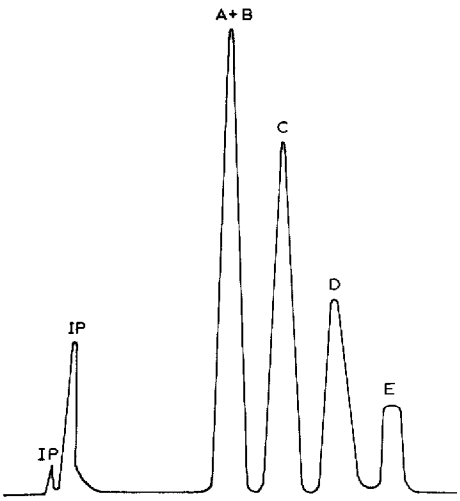

Fig. 3. HPLC chromatogram of tocopherol homologues and related compounds. Conditions: column, MCH 10 C_{18}. Micropak; solvent, methanol–water (95:5); detection, λ = 296 nm, A = 0.05; flow-rate = 2.0 ml/min; p = 158 atm., temperature = 27°C. Peak identities: A + B = DL-tocol (1.4 μg) + DL-α-tocotrienol (2.0 μg); C = DL-δ-tocopherol (2.0 μg); D = DL-γ-tocopherol (2.0 μg); E = DL-α-tocopherol (2.0 μg). Retention times: IP = injection peaks; A + B, 4.9–5.1 min, C = 6.1 min, D = 7.5 min, E = 9.2 min.

ment with the limitation of the Kubelka–Munk theory. Reproducibility of the sample analyses was excellent. Fig. 2 illustrates the standard curves obtained for each compound and the aforementioned tailing effect for some of the compounds.

Recovery studies were conducted on TLC using a lipid extract from *Euglena gracilis* strain Z, to which 500 μg of DL-α-tocopherol was added prior to the extraction of the cells with HPLC-grade acetone (Burdick & Jackson, Muskegon, MO, U.S.A.). Upon exhaustive extraction, rotary evaporation resuspension in HPLC-grade methanol and filtration, the lipid extracts were analyzed. It was determined that recovery of α-tocopherol was 91%.

The results of the HPLC analysis were comparable to those obtained by TLC. Fig. 3 illustrates the chromatogram obtained under the stated operating conditions, along with the retention times for each compound. DL-Tocol and DL-α-tocotrienol coeluted. This could not be alleviated without impairing resolution of the tocopherol homologoes placed on the column. Vatassery *et al.*[17] encountered similar difficulties in separating β- and γ-tocopherol using a variety of normal- and reversed-phase pellicular columns. Variations in operating conditions and solvent composition failed to separate the β- and γ-homologues.

Standard curves were obtained for each compound individually as shown in Fig. 4, and a duplicate recovery experiment was conducted using the same spiked extract. A recovery of 91.6% was demonstrated, comparable to that obtained for TLC.

The TLC and HPLC systems described appear comparable in sensitivity, reproducibility, percent recovery, and ease of application. It should be noted, however, that strict comparisons must be made with caution, as the stationery phase employed for TLC was activated silica, while that employed for the HPLC analyses was bond-

ed-phase system. It has been observed[17] that microparticulate silica gel columns (Partisil PXS10) are far more effective than either normal- or reversed-phase pellicular columns for tocopherol separation. Comparative analyses of tocopherols by TLC and microparticulate silica columns have not been reported.

Of related interest are the findings of Jork and Roth[22] for their comparative analyses of δ-triazine herbicides by GC, HPLC (UV detection), TLC (UV detection), and high-performance thin-layer chromatography (HPTLC) (UV detection). These workers observed comparable sensitivities for HPLC and HPTLC (1 ng and 3–5 ng, respectively). TLC demonstrated a lower sensitivity (8–13 ng), but revealed a linear range (10^2) comparable to that of HPLC and HPTLC.

Fig. 5 is a sample chromatogram from TLC and HPLC analyses of the lipid extracts from a spiked and unspiked sample of cells of *E. gracilis* strain Z for comparison purposes. All of the compounds analyzed were resolvable by TLC as shown in Fig. 1, while DL-tocol and DL-α-tocotrienol were not resolved using HPLC (Fig. 3). This is not the major factor, however, in judging the efficacy of the HPLC system.

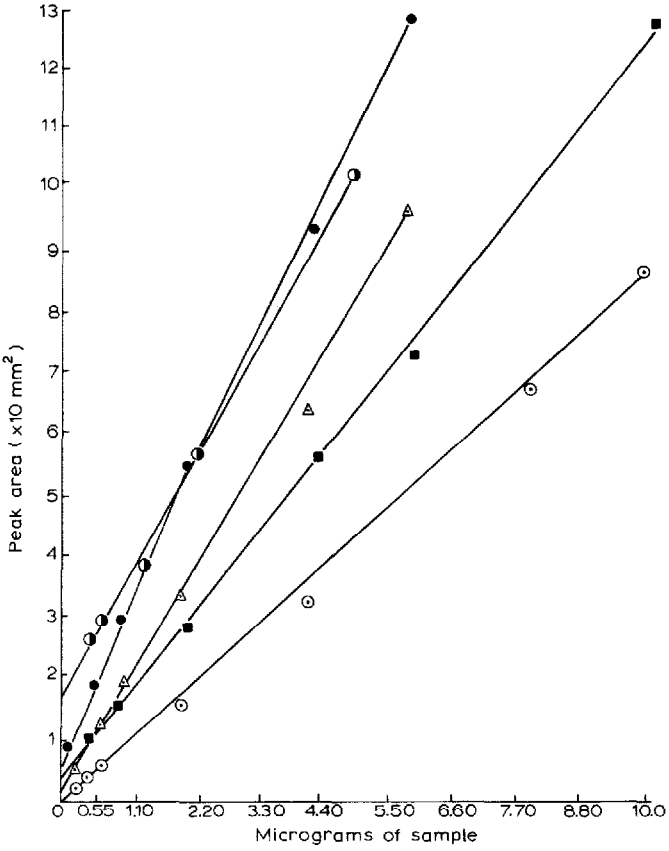

Fig. 4. Calibration curves of tocopherols and related compounds using HPLC. Conditions: column, MCH 10 C_{18} Micropak; solvent: methanol–water (95:5) (isocratic); flow-rate: 2.0 ml/min; detection, λ = 296 nm, range, 0.05; p = 158 atm., temperature, 27°C. ○—○, DL-α-tocopherol; ■—■, DL-γ-tocopherol; ●—●, DL-δ-tocopherol; △—△, DL-α-tocotrienol; ◑—◑, DL-tocol.

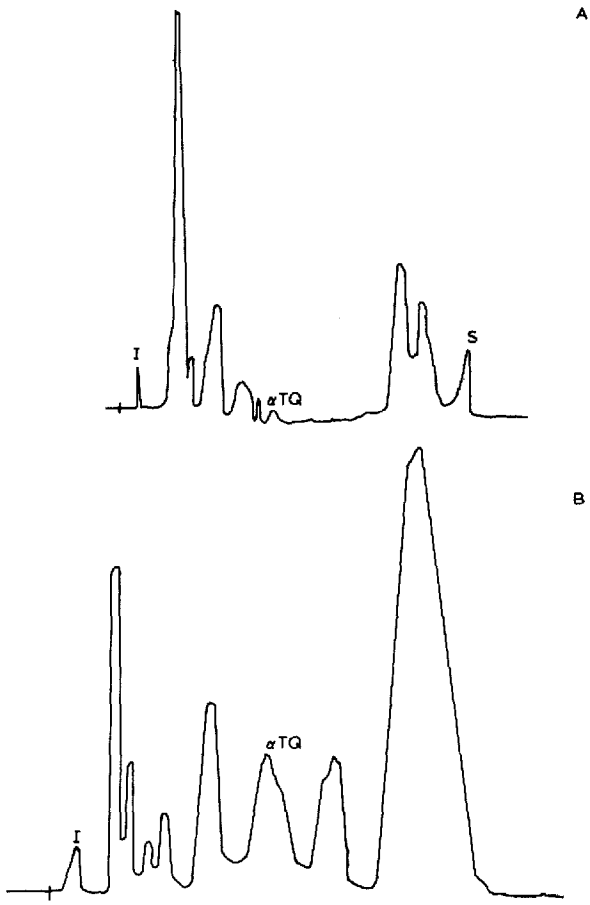

Fig. 5. Sample chromatograms [TLC (A) and HPLC (B)] of lipid extracts of *Euglena gracilis* strain Z. (A) Conditions as in Fig. 1. Sample, lipid extract (chloroform) of *Euglena gracilis* Z cells. Sample volume, 4 μl. I = initial peak; αTQ = α-tocopherol, R_F = 0.42; S = solvent line. (B) Conditions as in Fig. 3. Sample, lipid extract (chloroform) of *Euglena gracilis* cells spiked with 500 μg DL-α-tocopherol. Sample volume, 40 μl. I = injection peak; αTQ = α-tocopherol, t_R = 9.2 min.

The advantage of one system over the other may also be viewed in terms of the analysis time compared to number of samples being analyzed. HPLC appears advantageous for running larger volumes of sample if relatively few in number, along with standards, requiring 10 to 12 min per sample, including a washing between samples. TLC, although necessitating smaller sample volumes, is advantageous when analyzing a larger number of samples; on a single plate one can apply five or six standards and six samples in duplicate and run a complete *in situ* analysis in *ca.* 2 h.

Efforts to optimize the systems described here are currently in progress, including the use of HPTLC and densitometry for *in situ* tocopherol analysis. Nonetheless, the systems described here, while not yet fully optimized, appear to be comparable and complementary tools for tocopherol research.

REFERENCES

1 A. S. M. Giasuddin and A. T. Diplock, *Arch. Biochem. Biophys.*, 210 (1981) 348–362.
2 J. A. Lucy and A. T. Diplock, *FEBS Lett.*, 19 (1973) 205–210.
3 P. B. McCay and M. M. King, in L. J. Machlin (Editor), *Vitamin E —A Comprehensive Treatise*, Marcel Dekker, New York, 1980, pp. 289–317.
4 A. L. Tappel, *Ann. N.Y. Acad. Sci.*, 355 (1980) 18–31.
5 A. Emmerie and C. Engel, *Recl. Trav. Chim. Pays-Bas*, 57 (1938) 1351.
6 I. D. Desai, in L. J. Machlin (Editor), *Vitamin E —A Comprehensive Treatise*, Marcel Dekker, New York, 1980, pp. 67–98.
7 A. Tsen, *Anal. Chem.*, 33 (1961) 849.
8 M. Kofler, *Helv. Chim. Acta*, 25 (1942) 1469.
9 H. R. Bollinger, in E. Stahl (Editor), *Thin-Layer Chromatography*, Academic Press, New York, 1957, pp. 229–232.
10 C. K. Chow, H. H. Draper and A. S. Csallany, *Anal. Biochem.*, 32 (1969) 81–90.
11 P. W. Meijboom and G. A. Jongenotter, *J. Amer. Oil Chem. Soc.*, 56 (1979) 33–35.
12 J. F. Cavins and G. E. Inglett, *Cereal Chem.*, 53 (1974) 605–609.
13 L. J. Hatam and H. J. Kayden, *J. Lipid Res.*, 20 (1979) 639–645.
14 A. Kouchi, Y. Yaguchi and G. Katsu, *J. Nutr. Sci. Vitaminol.*, 21 (1975) 183–188.
15 W. O. Landen, Jr., *J. Chromatogr.*, 211 (1981) 155–159.
16 P. J. Van Niekerk, *Anal. Biochem.*, 52 (1973) 533–537.
17 G. T. Vatassery, V. R. Maynard and D. F. Hagan, *J. Chromatogr.*, 161 (1978) 299–302.
18 R. C. Williams, J. A. Schmitt and R. A. Henry, *J. Chromatogr. Sci.*, 10 (1972) 494–501.
19 A. P. Carpenter, *J. Amer. Oil Chem. Soc.*, 56 (1979) 668–671.
20 V. Novacek, *Amer. Lab. (Fairfield, Conn.)*, 2 (1969) 27–33.
21 J. C. Touchstone, S. S. Levin and T. Murawek, *Anal. Chem.*, 43 (1971) 858–863.
22 H. Jork and B. Both, *J. Chromatogr.*, 144 (1977) 39–56.