J Nutr Sci Vitaminol, 49, 217–220, 2003

Antioxidant Activities of Natural Vitamin E Formulations

Yousry M.A. NAGUIB, Siva P. HARI, Richard PASSWATER, Jr. and Dejian HUANG

J Nutr Sci Vitaminol, 49, 217–220, 2003

Antioxidant Activities of Natural Vitamin E Formulations

Yousry M.A. NAGUIB[1], Siva P. HARI[1], Richard PASSWATER, Jr.[1] and Dejian HUANG[2]

[1]*Soft Gel Technologies, Inc., 6982 Bandini Blvd., Los Angeles, California 90040, USA*
[2]*Brunswick Laboratories, 6 Thacher Lane, Wareham, Massachusetts 02571, USA*

(Received September 12, 2002)

Summary The antioxidant activities of natural *d*-α-tocopherol, mixed tocopherols and tocotrienols, and formulations comprising all forms of vitamin E, providing 400 IU, were determined employing an improved oxygen radical absorbance capacity (ORAC) assay using fluorescein (FL) as the fluorescent probe, randomly methylated β-cyclodextrin (RMCD), 2,2'-azobis(2-amidino-propane)dihydrochloride (AAPH) as the peroxyl radical generator, and Trolox as the standard in 75 mM phosphate buffer. The antioxidant activities, expressed in μmol Trolox equivalent per gram, of *d*-α-tocopherol (87%), mixed tocopherols (70%), and tocotrienols (30%) were found to be 1,293, 1,948, and 1,229, respectively. Some of the vitamin E formulations showed antioxidant activities superior to *d*-α-tocopherol.

Key Words vitamin E, tocopherol, tocotrienol, lipophilic antioxidant, oxygen radical absorbance capacity (ORAC)

Recent research studies have shown that a balanced intake of a full spectrum of vitamin E (γ-, α-, β- and δ-forms of tocopherols and tocotrienols, Fig. 1) is the best way to obtain the benefits of overall health. Research has shown tocotrienols from rice bran to be superior for reducing atherosclerosis lesion size in mice, thereby providing a unique approach to promoting cardiovascular health (*1*).

γ-tocopherol, the principle form of vitamin E in the diet, has been scientifically proven to enhance the health benefits of α-tocopherol (*2*), and is superior in promoting cardiovascular, brain, and immune health (*3*). γ-tocopherol was also found to be superior to α-tocopherol in protecting cells against peroxynitrite, a harmful chemical that alters DNA and causes cancer. The study suggested that a vitamin E supplement should contain at least 20% γ-tocopherol (*4*).

A nested case-control study involving men who developed prostate cancer and matched control subjects showed that men with high levels of γ-tocopherol in the blood had a significantly reduced risk of developing prostate cancer (*2*). The study also found a significant protective association for high levels of selenium and α-tocopherol only in men with a high γ-tocopherol concentration (*2*).

In a recent study, γ-tocopherol and α-carotene were found to be significantly lower in the plasma of coronary heart disease patients as compared to healthy 3 people, suggesting that the plasma level of γ-tocopherol might represent a marker of atherosclerosis in humans (*5*).

In an in vivo study, γ-tocopherol was found to enhance the bio-potency of α-tocopherol. γ-tocopherol induced a marked increase in α-tocopherol concentrations in the serum, nerve tissues, heart, liver, and muscles of rats fed diets containing more of both γ-tocopherol and α-tocopherol than those fed a diet containing α-tocopherol alone (*6*).

In an animal study involving spontaneously hypertensive rats, γ-tocotrienol was also found to prevent the development of increased blood pressure, to reduce lipid peroxidation in the plasma and blood vessels, and to enhance total antioxidant status including superoxide dismutase activity (*7*). A recent study also showed that supplementation with a 100 mg/d tocotrienol-rich fraction of rice bran for a month resulted in a significant reduction in total cholesterol, LDL-cholesterol, and triglycerides (*8*).

The recent considerable interest in developing commercial vitamin E formulations comprising all forms of vitamin E to provide a full spectrum of anticipated health benefits has promoted us to undertake the present study. In developing vitamin E formulations, it is desirable to achieve certain criteria: the required 400 IU, higher antioxidant potency, and a comparable cost. In this paper we report on formulations comprising all forms of tocopherols and tocotrienols with enhanced antioxidant activities.

These formulations were designed to provide 400 IU, based on 1 mg *d*-α-tocopherol equals 1.49 IU. This study provided new information on the antioxidant activity of mixed tocopherols as compared to *d*-α-tocopherol. Furthermore, from the measured antioxidant activities of α-tocopherol, mixed tocopherol, and tocotrienols, it was proven feasible to develop new formulations of vitamin E comprising a full spectrum of all forms of vitamin E, which possess significantly higher antioxidant activity than α-tocopherol.

MATERIALS AND METHODS

Chemicals and apparatus. Randomly methylated β-cyclodextrin (RMCD) was purchased from Cyclolab R&D

E-mail: yousryn@soft-gel.com

Ltd. (Budapest, Hungary). Fluorescein (FL) and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from Aldrich (Milwaukee, WI, USA). 2,2′-Azobis(2-amidino-propane)dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA, USA). Eighty-seven percent *d*-α-tocopherol (containing 13% soy bean oil) and 70% mixed tocopherols (containing 30% soy bean oil) were purchased from Archer Daniels Midland (City, State, Country). Each gram of 70% mixed tocopherols contained 114 mg *d*-α-tocopherol, 11 mg *d*-β-tocopherol, 457 mg *d*-γ-tocopherol, and 131 mg *d*-δ-tocopherol.

Tocotrienol oil was purchased from Oryza Oil and Fat Chemical Co. in Japan. The tocotrienol oil contained 35% total tocopherols and tocotrienols (12.6% γ-tocotrienol, 7.2% α-tocotrienol, and 12.7% α-tocopherol). Palm oil containing 50% total tocopherols and tocotrienols (10% α-tocopherol, 11% α-tocotrienol, 20% γ-tocotrienol, and others) was obtained from Carotech (Edison, NJ, USA).

All other standards were commercially available form Sigma or Aldrich. All ORAC analyses were performed on a COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ, USA) using an excitation wavelength of 493 nm and an emission filter of 515 nm.

Sample preparation. Approximately 0.5 g of sample was dissolved in 20 mL of acetone.

An aliquot of sample solution was appropriately diluted with 7% RMCD solvent (w/v) made in a 50% acetone-water mixture (v/v) and was shaken for 1 h at room temperature on an orbital shaker at 40 rpm. The sample solution was ready for analysis after further dilution with 7% RMCD solvent.

Automatic ORAC assay. The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometer centrifugal analyzer as previously described (*9, 10*). With the exception of samples and Trolox standards, which were made in 7% RMCD solvent, all other reagents were prepared in a 75 mM phosphate buffer (pH 7.4). In the final assay mixture (0.4 mL total volume), FL (6.3×10^{-8} M) was used as the target of free radical attack and AAPH (1.28×10^{-2} M) was used as the peroxyl radical generator. 7% RMCD was used as the blank, and Trolox (12.5, 25, 50, and 100 μM) was used as the control standard. The analyzer was programmed to record the fluorescence of FL every minute after the addition of AAPH. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample. These results were expressed as μmol Trolox equivalent (TE) per gram, as previously described by Ou et al. (*11*).

RESULTS

The antioxidant activity, commonly referred to as oxygen radical absorbance capacity (ORAC), of a lipophilic substance was measured employing a newly developed assay (*9, 10*). This assay is based on the use of (a) fluorescein (FL) as the fluorescent probe, (b) randomly methylated β-cyclodextrin (RMCD) as the molecular host to enhance the solubility of lipophilic antioxidants in aqueous solution, (c) 2,2′-azobis(2-amidino-propane)dihydrochloride (AAPH) as the peroxyl radical generator, and (d) 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) as the standard in 75 mM phosphate buffer (pH 7.4).

In the presence of peroxyl radicals derived from AAPH, the indicator FL gradually loses its fluorescence. The antioxidant activity of a substance is measured by its ability to retain the fluorescence of FL in the presence of peroxyl radicals. The net protection of FL was determined as previously described by Ou et al. (*11*). The ORAC value was calculated as μmol Trolox equivalent per gram sample (μmol TE/g): 1 g of sample has antioxidant activity equal to the number of μmol Trolox. The μmol Trolox equivalent per 400 IU of the sample is calculated as follows: μmol TE/400 IU = μmol TE/g × total weight, in grams, of vitamin E formula which gives 400 IU.

Four formulations of vitamin E were designed to provide 400 IU based on 1 mg of *d*-α-tocopherol equals 1.49 IU One gram of tocotrienols (30%) contains 9% *d*-α-tocopherol, and 1 g mixed tocopherols (70%) contains 8% *d*-α-tocopherol. The following are the compositions of each formula that give 400 IU:
Formula-1: 307 mg α-tocopherol (87%), 15 mg tocotrienols (30%), and 200 mg mixed tocopherols (70%).
Formula-2: 293 mg α-tocopherol (87%), 10 mg tocotrienols (30%), and 150 mg mixed tocopherols (70%).
Formula-3: 303 mg α-tocopherol (87%), 10 mg tocotrienols (30%), and 50 mg mixed tocopherols (70%).
Formula-4: 316 mg α-tocopherol (87%) and 16.5 mg tocotrienols (30%).

Table 1 lists the ORAC data expressed in μmol Trolox equivalent (TE) per gram of tested antioxidant sample. The data in Table 1 revealed that α-tocopherol (87%)

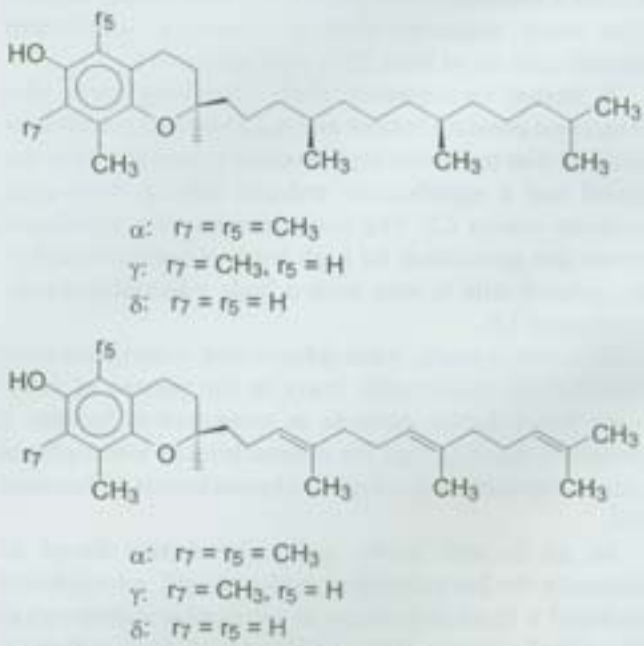

Fig. 1. Structures of studied lipophilic antioxidants tocopherols (top) and tocotrienols (bottom).

Table 1. Antioxidant activities of vitamin E formulations[a].

Sample	μmol TE/g	μmol TE/400 IU[b]
d-α-Tocopherol (87%), 1,300 IU	1,293±64	398
Tocotrienols (30%)[d], 134 IU	1,229±20	
Mixed tocopherols (70%)[e], 120 IU	1,948±76	
α-Tocopherol acetate	14±10	
Formula-1	2,036±116 (1,810)[c]	1,063
Formula-2	1,735±25 (1,738)[c]	786
Formula-3	1,460±156 (1,500)[c]	530
Formula-4	1,303±45 (1,353)[c]	434

[a] The following are the compositions of each formula which gives 400 IU.
Formula-1: 307 mg α-tocopherol (87%), 15 mg tocotrienols (30%), 200 mg mixed tocopherols (70%).
Formula-2: 293 mg α-tocopherol (87%), 10 mg tocotrienols (30%), 150 mg mixed tocopherols (70%).
Formula-3: 303 mg α-tocopherol (87%), 10 mg tocotrienols (30%), 50 mg mixed tocopherols (70%).
Formula-4: 316 mg α-tocopherol (87%), 16.5 mg tocotrienols (50%).
[b] μmol TE/400 IU = μmol TE/g × total weight in grams of vitamin E formula which gives 400 IU.
[c] Calculated μmol TE/g (1 g of sample has antioxidant activity equal to 1 μmol Trolox).
[d] One gram tocotrienols (30%) contains 9% *d*-α-tocopherol.
[e] One gram mixed tocopherols (70%) contains 8% *d*-α-tocopherol.

had a value of 1,293 μmol Trolox equivalent (TE) per gram; from this data one can calculate the μmol TE of 400 IU of α-tocopherol (87%) to be 398 (307.7 mg α-tocopherol is equivalent to 400 IU). Similarly, the μmol TE per 400 IU of each vitamin E formula can be calculated, and the results are given in Table 1.

DISCUSSION

The results of this study show that synthetic vitamin E acetate displayed no antioxidant activity under the current experimental conditions, supporting the essential role of the phenolic-type hydroxyl for the radical trapping antioxidant activity of vitamin E.

As can be seen from the data in Table 1, the antioxidant activities (ORAC value) of natural vitamin E formula-1 and formula-2 are much higher than that of natural vitamin E (*d*-α-tocopherol). Formula-3 also showed an increase (25%) in antioxidant activity as compared to α-tocopherol. Formula-4 showed slightly higher antioxidant activity than that of α-tocopherol. Formulae-1, -2, and -3 contained α-tocopherol, mixed-tocopherols, and tocotrienols, whereas Formula-4 contained only α-tocopherol and tocotrienols. As mentioned above, mixed tocopherols provide additional health benefits, therefore, Formula-1 or Formula-2 would be recommended since they contain all natural mixed tocopherols and tocotrienols and possess significantly higher antioxidant activity than α-tocopherol. Additionally, their cost is comparable to the widely marketed natural *d*-α-tocopherol. Each gram of 70% mixed tocopherols contains 114 mg *d*-α-tocopherol, 11 mg *d*-β-tocopherol, 457 mg *d*-γ-tocopherol, and 131 mg *d*-δ-tocopherol. From these data, the amount of γ-tocopherol in Formula-1 and Formula-2 is calculated to be 24% and 21%, respectively. These values are in accordance with the recommended amounts of γ-tocopherol in vitamin E supplement (*4*). This study was published in Proceedings of the National Academy of Science, USA (1997), and suggested that vitamin E supplement should have at lea[illegible] 20% γ-tocopherol (*4*). Formula-3, on the other hand, contains only 9% γ-tocopherol.

The results of the present study indicate that the various isomers of natural tocopherols and tocotrienols could enhance the antioxidant activity of natural vitamin E (*d*-α-tocopherol). A recent study showed that a combination of α-, γ-, and δ-tocopherols in a concentration found in nature is more potent than α-, γ-, and δ-tocopherol alone in enhancing nitric oxide release, and inhibiting human platelet aggregation and lipid peroxidation (*12*).

The biological activity of vitamin E has generally been associated with its well-defined antioxidant property, specifically against lipid peroxidation in biological membranes. Therefore, it is anticipated that enhancing the antioxidant property of vitamin E as well as the full spectrum of various vitamin E forms might provide better health benefits than α-tocopherol alone.

SUMMARY

The antioxidant activities of α-tocopherol, mixed tocopherol and tocotrienols, and vitamin E formulations comprising all forms of tocopherols and tocotrienols were determined employing an oxygen radical absorbance capacity assay suitable for lipophilic antioxidants. The results of this study clearly indicate that mixed tocopherols possess higher antioxidant activity than *d*-α-tocopherol.

Vitamin E formulations, providing 400 IU, comprising various forms of tocopherols and tocotrienols with enhanced antioxidant activities were developed. Some of these formulations showed antioxidant activities superior to *d*-α-tocopherol.

REFERENCES

1) Qureshi AA, Salser WA, Parmar R, Emeson EE. 2001. Novel tocotrienols of rice bran inhibit atherosclerosis

lesions in C57BL/6 ApoE-deficient mice. *J Nutr* **131**: 2606–2618.

2) Helzlsouer KJ, Huang H-Y, Alberg AJ, Hoffman S, Burke A, Norkus EP, Morris JS, Comstock GW. 2000. Association between alpha-tocopherol, gamma-tocopherol, selenium, and subsequent prostate cancer. *J Natl Cancer Inst* **92**: 2018–2023.

3) Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. 2000. gamma-tocopherol and its metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc Natl Acad Sci USA* **97**: 11494–11499.

4) Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan W, Ames BN. 1997. gamma-Tocopherol traps mutagenic electrophiles such as NO_x and complements alpha-tocopherol: Physiological implications. *Proc Natl Acad Sci USA* **94**: 3217–3222.

5) Kontush A, Spranger T, Reich A, Baum K, Beisiegel U. 1999. Lipophilic antioxidants in blood plasma as markers of atherosclerosis: the role of alpha-carotene and gamma-tocopherol. *Atherosclerosis* **144**: 117–122.

6) Clement M, Bourre J-M. 1997. Graded dietary levels of RRR-gamma-tocopherol induce a marked increase in the concentrations of alpha- and gamma-tocopherol in nervous tissues, heart, liver and muscle of vitamin E deficient rats. *Biochim Biophys Acta* **1334**: 173–181.

7) Newaz MA, Nawal NN. 1999. Effect of gamma-tocotrienol on blood pressure, lipid peroxidation and total antioxidant status in spontaneously hypertensive rats. *Clin Exp Hypertens* **21**: 1297–1313.

8) Qureshi AA, Sami SA, Salser WA, Khan FA. 2002. Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF25) of rice bran in hypercholesterolemic humans. *Atherosclerosis* **161**: 199–207.

9) Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Deemer EK. 2002. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated, β-cyclodextrin as the solubility enhancer. *J Agric Food Chem* **50**: 1815–1821.

10) Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J Agric Food Chem* **50**: 4437–4444.

11) Ou B, Hampsh-Woodill M, Prior R. 2001. Development and validation of an improved oxygen radical absorption capacity assay using fluorescein as a fluorescent probe. *J Agric Food Chem* **49**: 4619–4626.

12) Li D, Saldeen T, Romeo F, Mehta JL. 2001. Different isoforms of tocopherols enhance nitric oxide synthase phosphorylation and inhibit human platelet aggregation and lipid peroxidation: implications in therapy with vitamin E. *J Cardiovasc Pharmacol Ther* **6**: 155–161.