Improved High-Performance Liquid Chromatography of Vitamin E Vitamers on Normal-Phase Columns

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A method is described for improving column stability and reproducibility for analysis of vitamin E vitamers on normal-phase high-performance liquid chromatography columns with isooctane/ethyl acetate/acetic acid/2,2-dimethoxypropane (98.5:0.9:0.85:0.1) mobile phase. Acetic acid in the mobile phase reduced retention times of vitamin E vitamer and increased column stability. A small concentration of 2,2-dimethoxypropane, which reacts with water to form acetone and methanol, reduced the need for column regeneration and stabilized retention times.

KEY WORDS: 2,2-Dimethoxypropane, normal-phase column, rice bran, vitamin E vitamer.

Naturally-occurring vitamin E comprises two vitamer types, tocopherols and tocotrienols. Tocopherols are distributed predominantly in vegetable oils and biological fluids. Tocotrienols have been found in cereal germ and bran, palm oil and rubber latex (1–3). Vitamin E vitamers have attracted the attention of scientists, nutritionists, epidemiologists and clinicians because of their apparent function in cardiovascular disease, as possible anticarcinogenic agents and as compounds that lower cholesterol in blood (4). Increased interest in these compounds created a need for routine analysis in which reproducible separation of all vitamers is obtained.

Normal-phase high-performance liquid chromatography (HPLC) allowed fast and easy separation of positional isomers that reversed-phase HPLC does not. However, the reversed-phase approach is preferred over normal-phase in more than 70% of recently published procedures (5). The reason is that reversed-phase chromatography offers certain practical advantages, *e.g.*, column stability, reproducibility of retention times and quicker equilibration (6). On the other hand, reversed-phase systems, with water and methanol in the mobile phase, have the disadvantages of pronounced deterioration of the peak shape of lipophilic compounds and may cause partial precipitation of lipophilic compounds (6). Normal-phase columns would provide superior separation of vitamin E vitamers if the problems of column deactivation by water or polar compounds could be solved. All organic solvents contain an inherent amount of water in the parts per million range that is sufficient to deactivate normal-phase columns during long-term usage.

2,2-Dimethoxypropane (DMP) has been used to determine water content in solvents (7) and as a drying agent for preparation of samples for infrared analysis (8), or for removing water formed during esterification (9). Bredeweg *et al.* (10) applied DMP for chemical reactivation of silica columns. The procedure is based on the chemical reaction of DMP with water to form 2 moles of methanol and 1 mole acetone in the presence of an acid catalyst (7,8). The purpose of the present study was to develop a mobile phase system that includes DMP to improve column stability and reproducibility on normal-phase columns.

EXPERIMENTAL PROCEDURES

Materials. Isooctane, ethyl acetate and glacial acetic acid were purchased from Mallinckrodt (Paris, KY). DMP (98%) was obtained from Aldrich Chemical Company (Milwaukee, WI). L-ascorbic acid was purchased from Sigma (St. Louis, MO). Tocopherols and tocotrienols were prepared by semi-preparative HPLC from natural sources, as described in a previous study (Shin, T-S., and J.S. Godber, unpublished data).

Sample preparation for HPLC analysis. Five hundred mg stabilized rice bran was placed in a 15-mL test tube with 5 mL ethanol and 0.1 g ascorbic acid. The test tube was placed in an 80°C water bath for 10 min, after which 0.15 mL of 80% KOH was added. The sample was saponified for 10 min at 80°C. After saponification, the flask was placed in an ice bath, and 5 mL water and 5 mL hexane were added. The mixture was transferred to centrifuge bottles and centrifuged at 120 × *g* for 1 min. The upper layer was transferred to a 125-mL separatory funnel. Extraction of the sample with 5 mL hexane was repeated twice. The pooled hexane layer was washed three times with 5 mL water, filtered through Na_2SO_4 and then evaporated under a stream of nitrogen. The oil sample was diluted with 1 mL isooctane.

HPLC. The HPLC system consisted of a Waters (Milford, MA) M-45 pump, a 715 Ultra WISP injector and a 470 scanning fluorescence detector with excitation at 290 and emission at 330. A Supelcosil™ (Supelco, Bellefonte, PA) LC-Si, 5 μm, 25 cm × 4.6 mm i.d. column was used. Eluant 1 was isooctane/ethyl acetate (97.5:2.5), eluant 2 was isooctane/ethyl acetate/acetic acid (97.3:1.8:0.9) and eluant 3 was isooctane/ethyl acetate/acetic acid/DMP (98.15:0.9:0.85:0.1), with a flow rate of 1.6 mL/min. To reactivate the column, it was flushed with methylene chloride, then isopropanol and methanol, after which the solvent sequence was reversed. Alternatively, isooctane/ethyl acetate/acetic acid/DMP (87:10:1:2) was used to reactivate the column (instead of the previous method) with a flow rate of 2.5 mL/min for 20 min.

RESULTS AND DISCUSSION

The effect of modifiers on the retention times for seven vitamers of vitamin E is shown in Table 1. Eluant 1 was used to compare eluants 2 and 3, which were developed in our study to improve column stability. The maximum number of injections with eluant 1 was 24, after which poor separation of early eluting compounds occurred. Seventy-two injections were possible with eluant 2 before resolution was reduced. Resolution remained acceptable after two hundred injections were made with eluant 3.

Ethyl acetate at a concentration of 2.5% (eluant 1) produced good separation of eight vitamers with a maximum

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TABLE 1

High-Performance Liquid Chromatographic Retention Times (mean ± SD) for Vitamin E Vitamers with Various Eluants on a Supelcosil LC-Si Column[a]

Compound[b]	Eluant 1[c]	Eluant 2[c]		Eluant 3[c]	
	24 injections	24 injections	72 injections	24 injections	200 injections
α-T	5.39 ± 0.38	5.07 ± 0.07	4.67 ± 0.36	4.86 ± 0.01	4.84 ± 0.06
α-T3	6.36 ± 0.48	5.85 ± 0.08	5.38 ± 0.43	5.54 ± 0.01	5.53 ± 0.08
β-T	7.72 ± 0.59	7.40 ± 0.09	6.78 ± 0.54	7.07 ± 0.02	7.05 ± 0.09
γ-T	9.10 ± 0.64	8.04 ± 0.10	7.37 ± 0.59	7.77 ± 0.02	7.75 ± 0.12
β-T3	—	—	—	—	—
γ-T3	14.56 ± 0.71	9.61 ± 0.10	8.87 ± 0.66	9.29 ± 0.02	9.28 ± 0.15
δ-T	18.07 ± 0.79	11.35 ± 0.11	10.55 ± 0.67	11.10 ± 0.02	11.07 ± 0.15
δ-T3	22.26 ± 0.89	13.61 ± 0.12	12.45 ± 0.83	12.99 ± 0.03	12.92 ± 0.17

[a]From Supelco (Bellefonte, PA), with 25 cm × 4.6 mm, 5 μm column.
[b]T, tocopherol; T3, tocotrienol.
[c]Eluant 1, isooctane/ethyl acetate (97.5:2.5); eluant 2, isooctane/ethyl acetate/acetic acid (97.3:1.8:0.9); eluant 3, isooctane/ethyl acetate/acetic acid/2,2-dimethoxypropane (8.15:0.9:0.85:0.1).

retention time of 23 min (Fig. 1). Decreased retention times for each compound with increasing injection numbers caused a large standard deviation. The retention time of δ-tocotrienol in the first injection was 23.09; after 24 samples were injected, the retention time decreased to 19.85. Decreasing retention times in eluant 1 are typical of normal-phase columns.

Comparatively shorter retention times were achieved with modification of eluant 1 with acetic acid. Addition of acetic acid especially reduced retention times of late elution compounds. Also, acetic acid increased column stability relative to eluant 1, as indicated by smaller standard deviations with the injection of 24 samples. Acetic acid presumably competed with water and polar material for binding to hydroxyl groups in the column. However, with higher number of injections, retention time gradually decreased, due to column deactivation (Fig. 2). Addition of 0.1% DMP (eluant 3) produced retention times that were similar to eluant 2. As shown in Figure 3, eluant 3 produced relatively constant retention times for each vitamer during 200 injections. Figure 4 depicts the chromatograms of the first (A) and two hundredth (B) injection for eluant 3. Little difference exists between these chromatograms, which illustrated the stabilization obtained by including DMP in the mobile phase.

Changes of retention times with eluant 3 were most likely due to slight differences in extraction conditions and sample matrix, *i.e.*, retention times of each vitamer extracted at the same time were similar. However, it was important to completely remove water with Na_2SO_4 from extracts because samples containing higher water would elute earlier due to an effect of methanol and acetone produced from water through the reaction with DMP.

In our laboratory, vitamin E vitamers in over 600 rice bran samples had been analyzed with eluant 2 prior to development of eluant 3. After every 48 injections, the

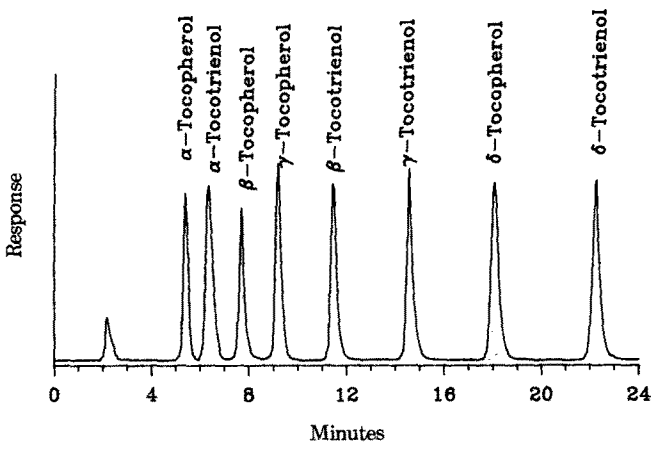

FIG. 1. High-performance liquid chromatography chromatogram of standards of vitamin E vitamers. Chromatographic conditions: Supelcosil LC-Si, 250 × 4.6 mm 5 μm column (Supelco, Bellefonte, PA); mobile phase, isooctane/ethyl acetate (97.5:2.5); flow rate, 1.6 mL/min; detection, fluorescence, excitation 290 nm, emission 330 nm.

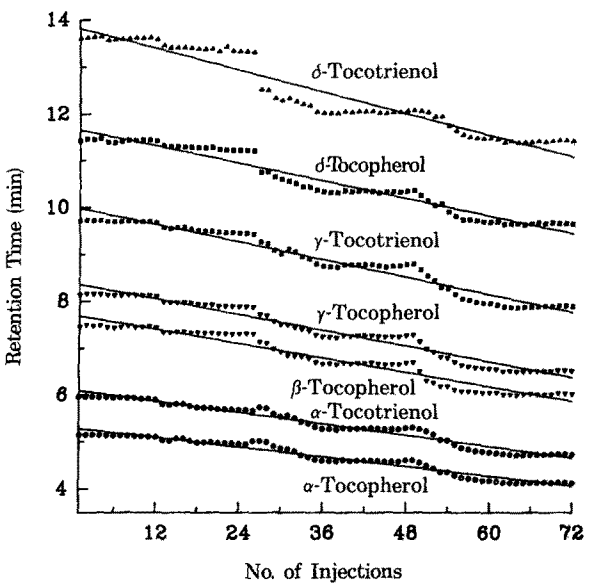

FIG. 2. Changes of retention time of vitamin E vitamers for seventy-two consecutive sample analyses. Twenty-four samples were analyzed each day for three consecutive days without reactivation of column. Solid lines are first-order regression curves. Chromatographic conditions as in Figure 1, except for mobile phase—isooctane/ethyl acetate/acetic acid (97.3:1.8:0.9).

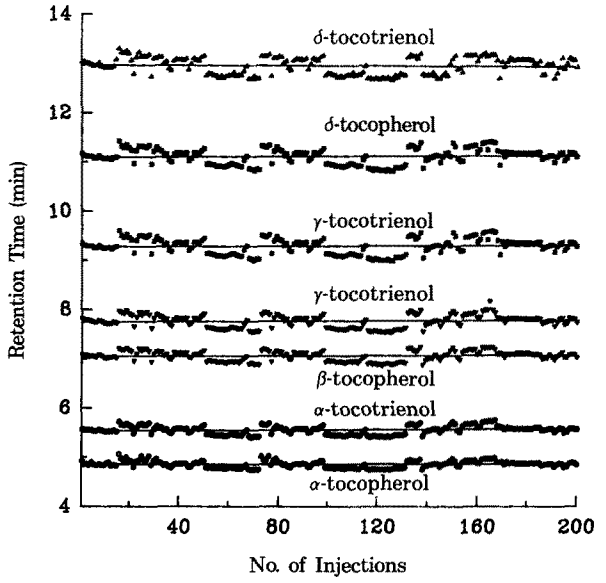

FIG. 3. Changes of retention time of vitamin E vitamers for two hundred consecutive sample analyses. Two hundred samples were analyzed continuously for about 63 h without reactivation. Solid lines are first-order regression curves. Chromatographic conditions as in Figure 1, except for mobile phase—isooctane/ethyl acetate/acetic acid/2,2-dimethyl propane (98.15:0.9:0.85:0.1).

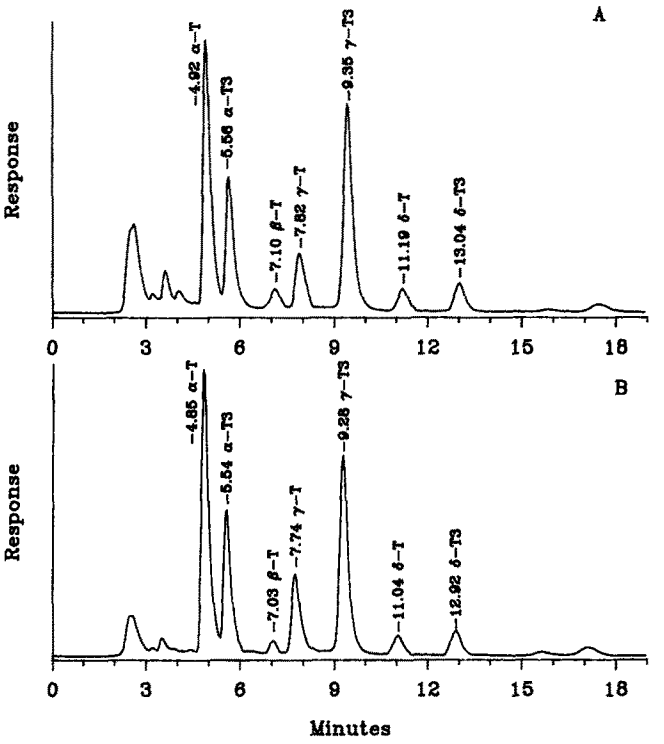

FIG. 4. Chromatograms of rice bran samples. Chromatogram A is first injection and chromatogram B, two hundredth injection. Chromatographic conditions as in Figure 3. T, tocopherol; T3, tocotrienol.

column was reactivated with methylene chloride, isopropanol and methanol. The conventional procedure took three hours. After eluant 3 was developed, column reactivation was obtained within 20 min. The need for long and tedious reactivation of columns was avoided by employing this mixture.

Use of a small amount of acetic acid and DMP in the mobile phase improved column stability and reproducibility of retention times in analysis of vitamin E vitamers on normal-phase columns. A similar modification may also benefit other analyses of lipophilic compounds in normal-phase columns, such as vitamin A.

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