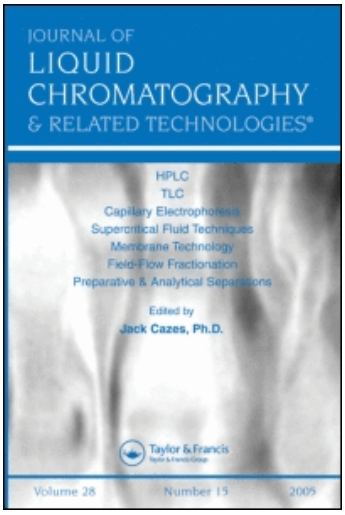


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# Normal Phase High-Performance Liquid Chromatography of Tocopherols on Polar Phases

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# NORMAL PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TOCOPHEROLS ON POLAR PHASES

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## ABSTRACT

Five plant tocopherols were separated by normal phase high performance liquid chromatography (HPLC) on aminopropylsilica or diol-bonded silica with fluorescence detection. HPLC characteristics of these compounds were studied under various mobile phase conditions. Mobile phases employed binary solvent systems comprising a hydrocarbon and an alcohol, an ether or an ester. Separation factors ($\alpha$) of adjacent ring-methylated tocol components were determined.

Employment of aprotic weakly polar modifiers in the mobile phases increased the $\alpha$ values for the pair of 5,8- and 7,8-dimethyltocol, but decreased those for the 5,7-and 5,8-dimethyltocol pair. Both component resolution and detection sensitivity were adversely affected by the use of a diol-bonded silica phase of large particle size. HPLC with cyclohexane-binary eluents invariably led to lower values of analyte capacity factors (k') than with hexane counterparts. Further, regardless of the type of column evaluated, the tocol isomers eluted in an ethereal mobile phase in the order of increasing k' values:

k'(tetrahydrofuran) <k'(dioxane) < k'(t-butyl methyl ether < k'(diisopropyl ether). In HPLC with protic co-solvents, the observed differences in k' values among the three dimetyltocols were interpreted in terms of methyl substitution effects.

## INTRODUCTION

Our continuous interest in distribution patterns of antioxidants in oil seeds has prompted current methodological studies on the separation and quantitation of tocopherol isomers in plant oils. Mobile phase solvent systems employed in the normal phase high-performance liquid chromatographic (HPLC) separation of β- and γ-tocopherols are critical determinants for the accurate analysis of the antioxidant pair at trace levels. To understand factors influencing the separation of closely related tocopherols, comprehensive evaluation of HPLC methods with solvents of variable polarity is of analytical importance.



Complete reverse phase HPLC separations of all soybean tocopherol isomers are difficult. However, normal phase HPLC resolution of the compounds has met with much success. The majority of published methods employed silica columns.[1-13] In contrast, there are few reports in the literature describing the use of polar silica-based columns.[14-16] A recent study[14] showed that normal phase elution characteristics of methylated tocol isomers on cyclodextrin-bonded silica (CDS) were partly dictated by the substitution patterns of the 2-methyl-6-chromanol ring system. As an extension of the previous study, a similar approach was taken to examine the structural effects on the HPLC behavior of the title compounds on different polar phases. The results are reported in this paper.

## EXPERIMENTAL

### Materials

Methyl substituted tocol standards (Figure 1) α-tocopherol (5,7,8-trimethyltocol) (TMT), β-tocopherol (5,8-dimethyltocol) (DBT), γ-tocopherol (7,8-dimethyltocol) (DGT), 5,7-dimethyltocol (DMT), and L-tocopherol (8-methyltocol) (MDT) were obtained from Matreya, Inc. (Pleasant Gap, PA, U.S.A.). All solvents were HPLC- grade and were used as supplied. Hexane (HX), cyclohexane (CHX), dioxane (DIOX), tetrahydrofuran (THF), and ethyl acetate (ETAC) were obtained from Fisher Chemicals (Fair Lawn, NJ, U.S.A.). Other solvents 1-propanol (1-PR), 2-propanol (2-PR), t-butyl methyl ether

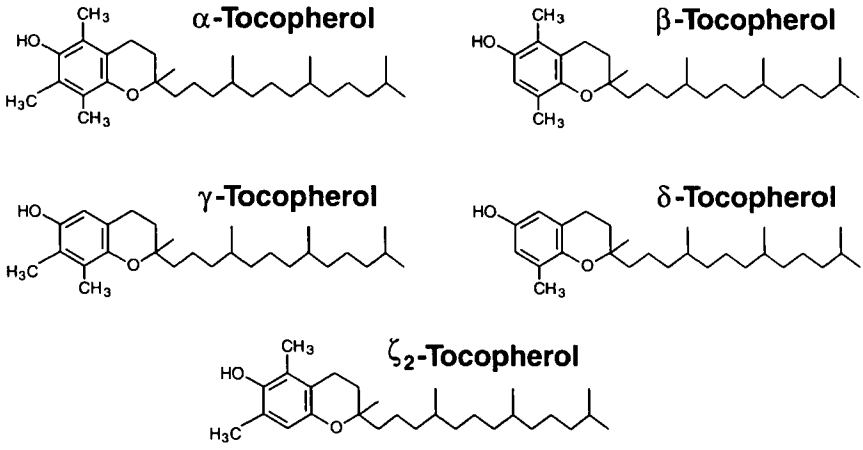


**Figure 1.** Structures of soybean tocopherols and 5,7-dimethyltocol ($\zeta_2$-tocopherol).



(TBM), and diisopropyl ether (IPIP) were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

## Methods

All HPLC experiments were performed on a Thermo Separation Products (formerly Spectra-Physics, San Jose, CA, U.S.A.) liquid chromatograph Model SP8700 solvent delivery unit. An Applied Biosystems (Foster City, CA, U.S.A.) Model 980 programmable fluorescence detector was interfaced with the LC system to monitor column effluents. The detector was set at an excitation wavelength of 298 nm and an emission wavelength of 345 nm. Mobile phases were prepared by mixing hexane or cyclohexane with one of several oxygen-containing solvents (an alcohol, an ether, or an ester) at variable proportions to form binary solvent systems.

Samples containing tocol isomers in hexane (100 μg/mL) were injected onto a column via an Applied Biosystems silica guard column (15 x 3.2 mm I.D.) and a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector fitted with a 10-μL loop. Before HPLC assays, all analytical samples were freshly prepared from individual tocol stock solutions which had been stored in amber vials in a freezer. Capacity factors ($k' = t/t_0 - 1$) were determined for each tocol component, where t and $t_0$ represent average retention times based on three

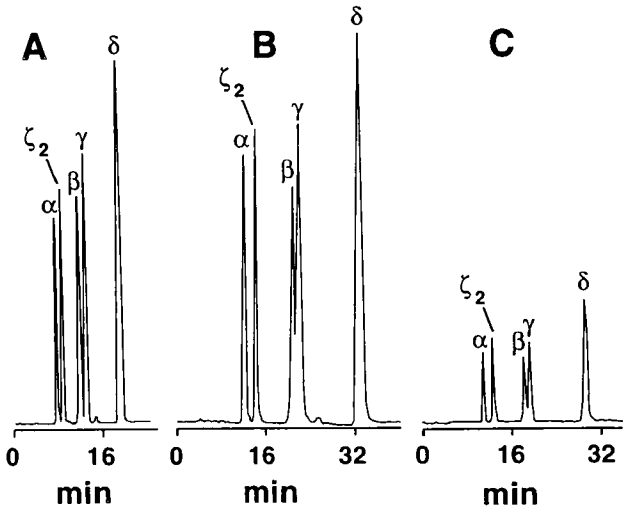


**Figure 2.** Separations of tocopherols with strongly polar modifiers. Columns: (A) amino-Si, (B) diol-Si(10 μm), (C) diol-Si(5 μm). Mobile phases: (A) hexane-dioxane(90:10), (B) hexane-dioxane (95:5), (C) cyclohexane-dioxane (97:3).



replicate determinations of an analyte and an unretained solute, respectively. Separation factors ($\alpha = k'_{i+1} / k'_i$) were obtained for adjacent components, where "i" represents a tocol isomer analyzed.

Stationary phases used in this study include (1) Waters μ-Bondapak $NH_2$ (aminopropylmethylsilyl bonded silica, 10 μm, 300 x 3.9 mm I.D.) (Milford, MA, U.S.A.), (2) Alltech Lichrosorb DIOL (1,2-hydroxy-3-propoxypropylsilyl bonded silica, 10 μm, 250 x 4.6 mm I.D.) (Deerfield, IL, U.S.A.), and (3) ES Industries Chromega Diol (1,2-hydroxypropylsilyl bonded silica, 5 μm, 250 x 4.6 mm I.D.) (Berlin, NJ, U.S.A.). Mobile phases were filtered, degassed, and pumped through the column at a flow rate of 1 mL/min. Prior to sample injections, it normally required 30 min to 1 hr to equilibrate a column.

## RESULTS AND DISCUSSION

Normal phase HPLC results obtained with 10 μm-amino-, 10 μm-diol-, and 5 μm-diolsilica columns are summarized in Tables 1 and 2, Tables 3 and 4, and Tables 5 and 6, respectively. The three stationary phases were evaluated

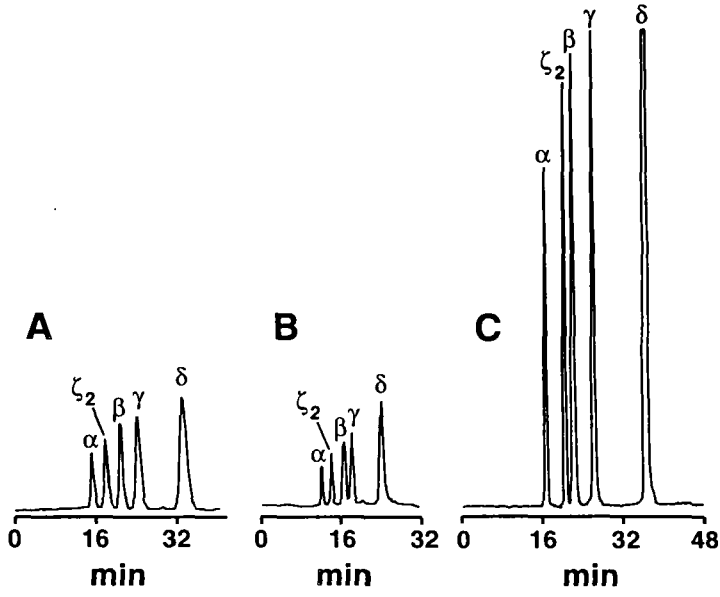


**Figure 3.** Separations of tocopherols with weakly polar modifiers. Columns: same as in Figure 2. Mobile phases: (A) cyclohexane-t-butyl methyl ether(90:10), (B) hexane-t-butyl methyl ether(90:10), (C) hexane-diisopropyl ether(90:10).



individually in conjunction with two hydrocarbon (cyclohexane and hexane)-binary mobile phase systems each of which contained seven different modifiers.

Examples of representative chromatograms obtained under various HPLC conditions are shown in Figures 2 and 3. Examination of the HPLC chromatograms revealed that mobile phase effects on the elution patterns were profound despite little influence of the polar stationary phases on the chromatographic profiles. Retention time differences ($\Delta t$) of $\beta$-$\zeta$ pairs in HPLC with dioxane as co-solvent were generally greater than those of $\gamma$-$\beta$ pairs [i.e., $\Delta t(\beta\text{-}\zeta) > \Delta t(\gamma\text{-}\beta)$] (Figure 2). With mono-oxygenated ether as co-solvent, however, the magnitude of $\Delta t(\beta\text{-}\zeta)$ approached and became smaller than that of $\Delta t(\gamma\text{-}\beta)$ (Figure 3).

For analyses of tocopherols in soybean oils in which the least abundant $\beta$-isomer normally coexists with the most abundant $\gamma$-isomer, HPLC separations showing evenly dispersed tocol components such as those shown in Figure 3

should be the preferred methods of choice to ensure precise quantitation of the trace components in soybean oil samples. Based on our experience with canola oil assays, application of the methods with the weakly polar modifiers in mobile phases (e.g. Figure 3) to the analysis of canola tocopherols should facilitate confirmation of the absence of the β-isomer in the oils. With strongly polar modifiers (e.g. Figure 2), HPLC of the canola oil samples often yields erroneous results because of the appearance a peak at the retention time of the β-isomer in the close proximity of the most abundant γ-isomer on HPLC chromatograms.

As depicted in Figure 1, structures of the five investigated compounds TMT, DMT, DBT, DGT, and MDT differ in the number and position of methyl groups on the aromatic ring of the tocol molecules. Regardless of the type of stationary phases employed, normal phase separations of tocopherols by the number of methyl substitution (monomethyl- *vs* dimethyl- *vs* trimethyltocol) were rather straightforward having α values in the range 1.27-1.75. In HPLC systems where polar modifiers (e.g. 1-, 2-propanol, or dioxane) were used in mobile phases (Figure 2), the two dimethyltocols DBT (β-tocopherol) and DGT (γ-tocopherol) were not as readily resolved (α = 1.00-1.10) as the analogous DMT-DBT pair (α = 1.22-2.10). The differences in the α values among the pairs of the dimethylated tocol compounds can be explained based on molecular polarity and steric factors involving the 5-, 7-, and 8 methyls and the 6-hydroxy group.[14] The two methyl groups in DGT (the γ-isomer) apparently confer a higher degree of disymmetry and polarity in the aromatic moiety than those in DBT (the β-isomer) or DMT (the ζ-isomer) (Figure 1). Thus, the polarity of the three dimethylated compounds in the series appears to be inversely related to their retention times (t) or k' values: t or k' (DMT) < t or k' (DBT) < t or k' (DGT).



Similar to earlier findings from studies[14] with CDS phases, HPLC with mobile phases containing an ester or a mono-functional ether produced well separated β- and γ-tocopherol components (DBT and DGT) (Figure 3) with α values ranging from 1.08 to 1.30 (Tables 1-6). These α values were significantly higher than those obtained with an alcohol- or a polar ether-co-solvent described earlier. In the latter alcohol mobile phase, the adjacent analyte components were separated with α values that decreased in the following order: α (DMT-DBT) > α (DGT-MDT) > α (TMT-DMT) > α (DBT-DGT). Elution with mobile phases containing weakly polar oxygenated modifiers tended to lower the α values for the (DMT-DBT)- and (DGT-MD)-pairs with a concurrent increase in the separation (higher α values) of the β-γ-(DBT-DGT)-pair. Further, as expected, the β-isomer was moderately better separated from the γ-isomer in hexane-binary solvents than in cyclohexane-binary eluents.

## Table 1

### Separations of Methyl Substituted Tocols on Aminosilica (10μm) with Hexane (HX) - Binary Mobile Phases

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile Phase | Component TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.05 | (1.34) | 1.41 | (2.09) | 2.94 | (1.10) | 3.23 | (1.75) | 5.64 |
| HX-1-PR(99:1) | 1.17 | (1.30) | 1.52 | (1.97) | 3.00 | (1.10) | 3.29 | (1.75) | 5.76 |
| HX-THF(90:10) | 1.16 | (1.28) | 1.49 | (1.38) | 2.05 | (1.23) | 2.52 | (1.52) | 3.82 |
| HX-DIOX(90:10) | 1.35 | (1.13) | 1.52 | (1.66) | 2.52 | (1.12) | 2.82 | (1.65) | 4.64 |
| HX-TBM(90:10) | 5.35 | (1.23 | 6.58 | (1.22) | 8.05 | (1.30) | 10.5 | (1.44) | 15.1 |
| HX-IPIP(90:10) | 9.70 | (1.30) | 12.6 | (1.29) | 16.3 | (1.27) | 20.7 | (1.48) | 30.3 |
| HX-ETAC(90:10) | 1.47 | (1.28) | 1.88 | (1.44) | 2.70 | (1.26) | 3.41 | (1.53 | 5.23 |

For solvent and compound abbreviations, see EXPERIMENTAL.



## Table 2

### Separations of Methyl Substituted Tocols on Aminosilica (10μm) with Cyclohexane (CHX) - Binary Mobile Phases

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile Phase | Component TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.00 | (1.35) | 1.35 | (2.00) | 2.70 | (1.07) | 2.88 | (1.74) | 5.00 |
| HX-1-PR(99:1) | 1.11 | (1.33) | 1.48 | (1.96) | 2.91 | (1.06) | 3.08 | (1.73) | 5.24 |
| HX-THF(90:10) | 1.03 | (1.28) | 1.32 | (1.37) | 1.80 | (1.21) | 2.18 | (1.50) | 3.27 |
| HX-DIOL(90:10) | 1.19 | (1.14) | 1.36 | (1.60) | 2.18 | (1.11) | 2.42 | (1.63) | 3.94 |
| HX-TBM(90:10) | 3.47 | (1.24) | 4.29 | (1.22) | 5.23 | (1.19) | 6.23 | (1.40) | 8.70 |
| HX-IPIP(90:10) | 7.61 | (1.33) | 10.1 | (1.29) | 13.1 | (1.18) | 15.4 | (1.47) | 22.6 |
| HX-ETAC(90:10) | 1.21 | (1.27) | 1.54 | (1.50) | 2.31 | (1.23) | 2.84 | (1.49) | 4.23 |

For solvent and compound abbreviations, see EXPERIMENTAL.

The retention data in Tables 1-6 indicate that tocol analytes were generally more strongly adsorbed (higher k' values) on a polar phase in 1-propanol than in 2-propanol. Surprisingly a reversal of this generalization was manifested in normal phase HPLC with a CDS phase.[14] All the tocol components exhibited strongest retention (highest k' values) on the stationary phase in a mobile phase system containing diisopropyl ether. In relation to the

open-chain ethers examined, cyclic ethers (e.g. tetrahydrofuran and dioxane) seemed to contribute to weak analyte adsorption on the column. Thus, the observed general trend of k' values of individual tocols in the presence of ether modifiers was as follows: k' (diisopropyl ether) > k' (t-butyl methyl ether) > k' (Dioxane) > k' (tetrahydrofuran).

Following a parallel approach, attempts at correlating retention characteristics (k' values) of individual tocol components listed in Tables 1-6 with all types of mobile phases of interest failed to delineate systematic orders of variations in k' values with the seven modifier varieties investigated. In all cases studied, the k' values observed in HPLC experiments with hexane-binary solvents were, as anticipated, higher than those obtained with the corresponding cyclohexane-systems. As to the differential mobile phase effects between the cyclic (cyclohexane) and straight-chain hydrocarbon (hexane) structures on the separation of tocopherols were minimal beside the observed polarity implications (Table 1 *vs* Table 2, Table 3 *vs* Table 4, and Table 5 *vs* Table 6).



A comparison of the HPLC data for the amino-column with the corresponding data obtained with diol-phases demonstrated that the selectivity of the amino column (Tables 1 and 2) for the β- and γ-components was somewhat superior (higher α values) to that of both the diol columns (5 μm and 10 μm) used (Tables 3-6). For a given tocol mixture, a highest degree of improvement in the separation of the β-γ pair was observed when a combination of an amino-column and hexane-t-butyl methyl ether (Table 1) was employed in HPLC experiments. To a less extent, enhancement in the resolution of the same pair in the mixture of tocols was found in experiments with the 5-μm-diol-column (Tables 5 and 6) and hexane (or cyclohexane)-t-butyl methyl ether.

Analysis of data obtained with the two diol-columns (Tables 3-6) showed that β- and γ-tocopherols were not separated (α = 1.00) on the 10-μm-diol phase when eluted with a cyclohexane-binary mobile phase containing strongly polar modifiers (alcohol or dioxane) (Table 4). In addition, HPLC with the latter 10-μm low-efficiency diol-column gave detector responses significantly inferior to those obtained with the corresponding 5-μm diol-column probably due to partial adsorption of analytes on the 10-μm column.

On the other hand, under identical mobile phase conditions in the presence of alcohol modifiers, the 10 μm-diol phase seemed to be more retentive than the amino-phase of the same particle size (10 μm) as reflected in the higher k'

**Table 3**

**Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Hexane (HX) - Binary Mobile Phases**

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile | Component | | | | | | | | |
| Phase | TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.44 | (1.39) | 2.00 | (1.89) | 3.77 | (1.07) | 4.05 | (1.59) | 6.44 |
| HX-1-PR(99:1) | 1.55 | (1.36) | 2.11 | (1.90) | 4.00 | (1.07) | 4.27 | (1.64) | 7.00 |
| HX-THF(95:5) | 2.00 | (1.19) | 2.38 | (1.40) | 3.33 | (1.13) | 3.77 | (1.41) | 5.33 |
| HX-DIOX(95:5) | 2.44 | (1.23) | 3.00 | (1.63) | 4.88 | (1.07) | 5.22 | (1.58) | 8.27 |
| HX-TBM(95:5) | 3.61 | (1.26) | 4.55 | (1.46) | 6.66 | (1.12) | 7.44 | (1.49) | 11.1 |
| HX-IPIP(95:5) | 5.27 | (1.30) | 6.83 | (1.57) | 10.7 | (1.10) | 11.8 | (1.53 | 18.1 |
| HX-ETAC(95:5) | 2.33 | (1.17) | 2.72 | (1.55) | 4.22 | (1.10) | 4.66 | (1.53) | 7.11 |

For solvent and compound abbreviations, see EXPERIMENTAL.



**Table 4**

**Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Cyclohexane (CHX) - Binary Mobile Phases**

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile | Component | | | | | | | | |
| Phase | TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.27 | (1.27) | 1.61 | (2.10) | 3.38 | (1.00) | 3.38 | (1.67) | 5.66 |
| HX-1-PR(99:1) | 1.33 | (1.33) | 1.77 | (2.13) | 3.77 | (1.00) | 3.77 | (1.71) | 6.44 |
| HX-THF(95:5) | 1.52 | (1.23) | 1.88 | (1.39) | 2.61 | (1.10) | 2.88 | (1.54) | 4.44 |
| HX-DIOX(95:5) | 1.55 | (1.25) | 1.94 | (1.66) | 3.22 | (1.00) | 3.22 | (1.64) | 5.27 |
| HX-TBM(95:5) | 2.50 | (1.26) | 3.16 | (1.44) | 4.55 | (1.10) | 5.00 | (1.57) | 7.83 |
| HX-IPIP(95:5) | 3.33 | (1.28) | 4.27 | (1.55) | 6.61 | (1.08) | 7.16 | (1.56) | 11.2 |
| HX-ETAC(95:5) | 1.48 | (1.24) | 1.83 | (1.51) | 2.77 | (1.10) | 3.05 | (1.53) | 4.66 |

For solvent and compound abbreviations, see EXPERIMENTAL.

values of the tocol components on the diol phase (Table 1 vs Table 3; Table 2 *vs* Table 4). These particular observations in HPLC with the 10 μm-diol column were comparable to those with a β-CDS column (14) in which k' (β-CDS) > k' (aminosilica) presumably due to interactions between hydroxy groups in the diol- and CDS phases and the hydroxy group in analyte solutes during HPLC separation processes. The use of a relatively high efficiency 5 μm-diol column (such as the one used in this study) and a cyclohexane-binary

**Table 5**

**Separations of Methyl Substituted Tocols on Diol-Bonded Silica (5 μm) with Hexane (HX) - Binary Mobile Phases**

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile Phase | Component TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.44 | (1.31) | 1.88 | (1.80) | 3.38 | (1.06) | 3.58 | (1.53) | 5.48 |
| HX-1-PR(99:1) | 1.47 | (1.35) | 1.98 | (1.89) | 3.75 | (1.07) | 4.01 | (1.62) | 6.50 |
| HX-THF(95:5) | 2.00 | (1.25) | 2.50 | (1.26) | 3.16 | (1.21) | 3.83 | (1.48) | 5.66 |
| HX-DIOX(95:5) | 2.16 | (1.21) | 2.61 | (1.47) | 3.83 | (1.10) | 4.22 | (1.51) | 6.38 |
| HX-TBM(95:5) | 4.05 | (1.27) | 5.16 | (1.21) | 6.22 | (1.21) | 7.50 | (1.48) | 11.1 |
| HX-IPIP(95:5) | 6.33 | (1.35) | 8.55 | (1.27) | 10.9 | (1.21) | 13.2 | (1.55) | 20.4 |
| HX-ETAC(95:5) | 2.05 | (1.27) | 2.61 | (1.36) | 3.55 | (1.20) | 4.27 | (1.51) | 6.44 |

For solvent and compound abbreviations, see EXPERIMENTAL.



**Table 6**

**Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Cyclohexane (CHX) - Binary Mobile Phases**

| | HPLC Characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | k' | (α) | k' | (α) | k' | (α) | k' | (α) | k' |
| Mobile Phase | Component TMT | | DMT | | DBT | | DGT | | MDT |
| HX-2-PR(99:1) | 1.11 | (1.30) | 1.44 | (1.69) | 2.44 | (1.07) | 2.61 | (1.57) | 4.11 |
| HX-1-PR(99:1) | 1.16 | (1.29) | 1.50 | (1.89) | 2.83 | (1.04) | 2.94 | (1.68) | 4.94 |
| HX-THF(95:5) | 1.16 | (1.15) | 1.33 | (1.29) | 1.72 | (1.16) | 2.00 | (1.50) | 3.00 |
| HX-DIOX(95:5) | 1.33 | (1.21) | 1.61 | (1.52) | 2.44 | (1.05) | 2.55 | (1.59) | 4.05 |
| HX-TBM(95:5) | 2.33 | (1.24) | 2.88 | (1.17) | 3.38 | (1.17) | 3.94 | (1.56) | 6.16 |
| HX-IPIP(95:5) | 4.44 | (1.30) | 5.77 | (1.18) | 6.83 | (1.15) | 7.83 | (1.57) | 12.3 |
| HX-ETAC(95:5) | 1.32 | (1.26) | 1.66 | (1.30) | 2.16 | (1.13) | 2.44 | (1.60) | 3.83 |

For solvent and compound abbreviations, see EXPERIMENTAL.

solvent system was advantageous because rapid analyses were achieved without affecting component resolution.

In conclusion, the results of this study provide specific guidelines for the selection of HPLC columns and solvents in the separation of soybean tocopherols and 5,7-dimethyltocol which can be used as an internal standard for reliable quantitation purposes. The ortho-effect of two methyls on the

hydroxy group in tocopherols has significant bearing on separation patterns of components and can be optimized by adjusting mobile phase solvent polarity. In light of the ability of the hydrocarbon mobile phases modified with monofunctional ethers to achieve satisfactory dispersion of analyte components, they are highly recommended for use in the trace HPLC analysis of tocol compounds and related structures including tocotrienols.[17]

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