Comparison of the Column Performance of Narrow-Bore and Standard-Bore Columns for the Chromatographic Determination of α-, β-, γ-, and δ-Tocopherol

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Abstract

A comparison of the performance of narrow-bore (2.1-mm i.d.) and standard-bore (4.6-mm i.d.) analytical silica columns having the same length is completed for the resolution of α-, β-, γ-, and δ-tocopherol. The studies are performed on high-performance liquid chromatographic equipment with minimum extracolumn contribution. Column permeabilities are 1.16×10^{-9} and 2.48×10^{-9} cm^2 for narrow and standard bore, respectively. The narrow-bore column gives up to a 7 times increase in sensitivity compared with a standard-bore column at equivalent running times for the analytes. Approximately one-third solvent savings can be achieved with the narrow-bore column. Theoretical plates of the standard-bore column are higher than that of the narrow-bore column.

Introduction

The core of any high-performance liquid chromatographic (HPLC) system is the column. Without a column having good resolving power when operating at a high linear flow rate, all benefits from high-pressure pumps (especially designed injectors, high-sensitivity detectors, and gradient elution devices) are lost (1).

Narrow-bore columns (2.1-mm i.d.) have diameters between that of microbore (< 1.0-mm i.d.) and standard-bore columns (4 to 5-mm i.d.) (2,3). Therefore, narrow-bore columns have several combined characteristics possessed by microbore and standard-bore columns. Narrow-bore liquid chromatography (LC) can be easily implemented on conventional equipment with little modification and still maintain some advantages of microbore LC such as a reduction in the stationary phase amount, less solvent consumption, and higher mass sensitivity (4–7). Moreover, lower volumetric flow rates cause less damage to pumps, thus extending their life spans and reducing mechanical trouble (3).

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Solid-phase particle sizes that are commonly used as packing materials for LC columns are 3, 5, and 10 µm. Columns with 3-µm particles provide greater resolving power per column length; however, a much higher pressure drop is usually apparent because of fines present in the particle-size distribution. In addition, sharp low-volume peaks are often degraded because of extracolumn effects such as connecting tubing and guard columns (8). A narrow-bore column packed with 10-µm particles often exhibits inadequate efficiency for some routine separations (3). Therefore, choosing 5 µm as the size of the packing material represents a good compromise when considering column efficiency, pressure drop, and extracolumn dispersion effects.

For column testing, the eluent should be comprised of components that are inexpensive, readily available in a pure state, have good UV or fluorescence characteristics or both, low viscosity, and nontoxic. Hexane (containing 1% acetonitrile or 0.5% methanol) has been recommended for silica column testing (9). Solutes should be readily available, stable, moderately soluble, and of low toxicity in solution. Additionally, low relative molecular masses with low volatility are required (9).

Narrow-bore columns have been used in the analysis of fat-soluble vitamins (2,11). However, no reports have been published on the use of narrow-bore silica columns for the analysis of tocopherols to our knowledge. Therefore, the aim of this study was to compare the performance of narrow-bore (2.1-mm i.d.) and standard-bore (4.6-mm i.d.) silica columns for the chromatographic determination of these vitamin E components. A desire to decrease solvent usage in the laboratory influenced the decision to complete this study.

Experimental

Apparatus

The experiments were carried out using an HPLC system equipped with a Waters 2690 separations module (Waters,

Table I. Specific Absorption Coefficients ($E^{1\%}_{1\ cm}$) and Maximum Wavelength (λ_{max}) for Tocopherols in 96% (v/v) Ethanol Solutions*

Analytes	λ_{max} (nm)	$E^{1\%}_{1\ cm}$
α-T	292	71
β-T	297	86.4
γ-T	298	92.8
δ-T	298	91.2

* Obtained from reference 11.

Milford, MA) and a Shimadzu (Columbia, MD) RF-10Axl programmable fluorescence detector attached to a Waters Millennium 2010 version 3.01 chromatography manager on a compatible IBM computer and connected to an HP Deskjet 820Cxi color printer (Hewlett Packard, Hopkins, MN).

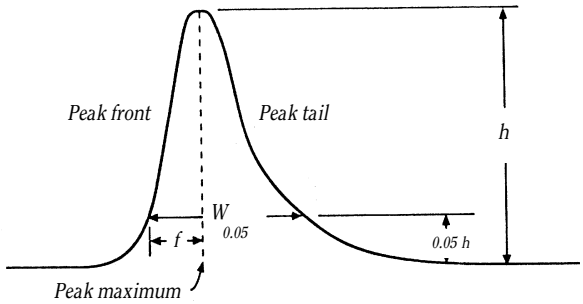

Figure 1. Asymmetrical chromatographic peak (obtained from reference 13).

The columns used were LiChrosorb Si60 (25 cm × 4.6 mm, 5 µm) (Hibar Fertigsaube RT, Darmstadt, Germany) and LiChrosorb Si60 (25 cm × 2.1 mm, 5 µm) (Alltech Associates, Deerfield, IL). Fluorescence parameters were 290 nm for excitation and 330 nm for emission.

The mobile phase (0.8% isopropanol in hexane) was pumped at a flow rate of 1.0 mL/min through the 4.6-mm-i.d. column and 0.32 mL/min through the 2.1-mm-i.d. narrow-bore column. The flow rate of 0.32 mL/min was chosen for the narrow-bore column in order to get retention times comparable with the standard-bore column. The mobile phase was vacuum-filtered through a 0.45-µm nylon membrane filter (MSI, Westboro, MA) and degassed using a FS30 sonicator (Fisher Scientific, Pittsburgh, PA). The chromatographic experiments were carried out at a column temperature of 29°C ± 1°C.

Chemicals and reagents

All reagents were of analytical purity. *n*-Hexane and isopropanol (IPA) were of LC grade and purchased from J.T. Baker (Phillipsburg, NJ). Butylated hydroxytoluene (BHT), all-rac-γ-tocopherol (γ-T), and all-rac-δ-tocopherol (δ-T) were purchased from Sigma (St. Louis, MO). All-rac-α-tocopherol (α-T) was purchased from BASF Corporation (Parsippany, NJ). All-rac-β-tocopherol (β-T) was a gift from Henkel (Fine Chemicals Division, La Grange, IL).

Preparation of stock standard solution

Individual tocopherol standard solutions were created by accurately weighing 20 mg of α-tocopherol and dissolving it in *n*-hexane. The solution was then transferred to a 10-mL volumetric flask and diluted to volume with hexane. A β-T, γ-T, and δ-T solution were similarly prepared. For a purity check, 1.0 mL of each tocopherol solution was pipetted into a 25-mL volumetric flask and diluted to volume with ethanol. The absorbance difference ($A - A^0$) was determined for the solution with a spectrophotometer at a suitable wavelength using λ settings given in Table I. A is the absorbance of the standard solution, and A^0 is the absorbance of the blank (ethanol).

For a stock solution, 8.0 mL of each tocopherol standard solution was pipetted into separate 25-mL volumetric flasks and diluted to volume with a hexane–BHT solution. Concentrations of the stock solution were then calculated from $E^{1\%}_{1\ cm}$ data. Appropriate dilutions were made with the mobile phase to produce the working standard solutions.

Table II. Analytical Figures of Merit for the Chromatographic Determination of α-, β-, γ-, and δ-Tocopherol on Narrow-Bore and Standard-Bore Columns*

Analytes	Linearity	Retention factor	Theoretical plates	Tailing factor	System suitability†	Separation factor	Resolution
α-T	0.9996	0.78	6036	1.0	1.0		
	(0.9999)	(1.0)	(8236)	(0.9)	(0.5)	2.0	6.71
β-T	0.9998	1.57	5182	1.1	0.8	(2.0)	(10.3)
	(0.9999)	(2.0)	(11274)	(1.1)	(0.5)	1.1	1.4
γ-T	0.9998	1.77	5560	1.1	0.9	(1.1)	(2.2)
	(0.9999)	(2.2)	(12777)	(0.9)	(0.5)	1.8	7.72
δ-T	0.9998	3.19	5905	1.1	1.0	(1.8)	(11.5)
	(0.9999)	(4.0)	(10630)	(1.0)	(0.5)		

* 10-µL injection volume and 0.8% IPA in hexane as mobile phase. Values in brackets are from standard bore.
† RSD percentage of five replicate injections at 3.27, 1.54, 3.10, and 3.44 ng/injection in a narrow-bore column for α-T, β-T, γ-T, and δ-T, respectively, and 6.55, 3.09, 6.21, and 6.88 ng/injection in a standard-bore column for α-T, β-T, γ-T, and δ-T, respectively.

Table III. LOD and LOQ for the Chromatographic Determination of α-, β-, γ-, and δ-Tocopherol on Standard-Bore and Narrow-Bore Columns

	Narrow bore				Standard bore	
	LOD (ng)	LOQ (ng)	LOD (ng)	LOQ (ng)	LOD (ng)	LOQ (ng)
Column	LiChrosorb Si60 (25 cm × 2.1 mm, 5 µm)		LiChrosorb Si60 (25 cm × 2.1 mm, 5 µm)		LiChrosorb Si60, (25 cm × 4.6 mm, 5 µm)	
Flow rate	0.32 mL/min		0.32 mL/min		1.0 mL/min	
Mobile phase	0.6% IPA in hexane		0.8% IPA in hexane		0.8% IPA in hexane	
Analytes						
α-T	0.032	0.094	0.021	0.051	0.119	0.307
β-T	0.016	0.046	0.006	0.014	0.029	0.072
γ-T	0.025	0.075	0.012	0.028	0.049	0.119
δ-T	0.039	0.113	0.011	0.024	0.075	0.200

Parameters evaluated

The following parameters were evaluated (1,9,10,12–15):

Column retention factor (k'):

$$k' = (t_R - t_0)/t_0 \quad \text{Eq. 1}$$

where t_R is the retention time and t_0 is the column deadtime (both are in seconds).

Theoretical plates (N):

$$N = 16(t_{Ri}/w_b)^2 \quad \text{Eq. 2}$$

where w_b is the width (s) of the peak measured by extrapolating the relatively straight sides to the baseline and t_{Ri} is the retention time (s) of substance i.

Height equivalent of a theoretical plate (H):

$$H = L/N \quad \text{Eq. 3}$$

where L is the length (mm) of the column.

Reduced plate height (h):

$$h = H/d_p \quad \text{Eq. 4}$$

where d_p is the particle diameter (mm).

Total porosity of the column (ε_T):

$$\varepsilon_T = (Ft_0/Lr_c^2\pi) \quad \text{Eq. 5}$$

where r_c is the radius (mm) of the column and F is the flow rate (mm^3/s).

Column permeability (B_0):

$$B_0 = (\mu\eta L/\Delta p) \quad \text{Eq. 6}$$

where μ is the linear velocity (mm/s), η is the viscosity (Pa•s) of the mobile phase, and Δp is the pressure drop (Pa) across the column.

Tailing factor (T):

$$T = W_{0.05}/2f \quad \text{Eq. 7}$$

where f is the symmetrical chromatographic peak found in Figure 1.

Table IV. Column Characteristics for Standard and Narrow Bore*

Parameters	Narrow bore	Standard bore
H (µm)	41.4	30.4
h	8.28	6.08
ε_T	1.089	0.646
B_o (cm^2)	$1.16 \times 10^{-0.9}$	$2.48 \times 10^{-0.9}$
V_o (mL)	0.943	2.682
V_p (µL)	86	236
E	14788	3715

* Calculated at a component having $k' \approx 1$.

Resolution (Rs):

$$Rs = 2(t_{R2} - t_{R1})/(w_{b2} + w_{b1}) \quad \text{Eq. 8}$$

where t_{R2} and t_{R1} are the retention times (s) of the two components and w_{b2} and w_{b1} are the corresponding widths (s) at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Separation factor (α):

$$\alpha = k_2'/k_1' \quad \text{Eq. 9}$$

where k_2' and k_1' are the column retention factors of the second and first peak, respectively.

Peak volume (V_p):

$$V_p = 4\sigma_V = 4\left(V_0 \frac{1 + k}{\sqrt{N}}\right) \quad \text{Eq. 10}$$

where σ_V is the volume standard deviation and V_0 is the dead volume (µL).

Separation impedance (E):

$$E = H^2/K_F = h^2\phi \quad \text{Eq. 11}$$

where ϕ is the column resistance parameter.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by measuring the magnitude of the analytical background response by injecting a number of blank samples

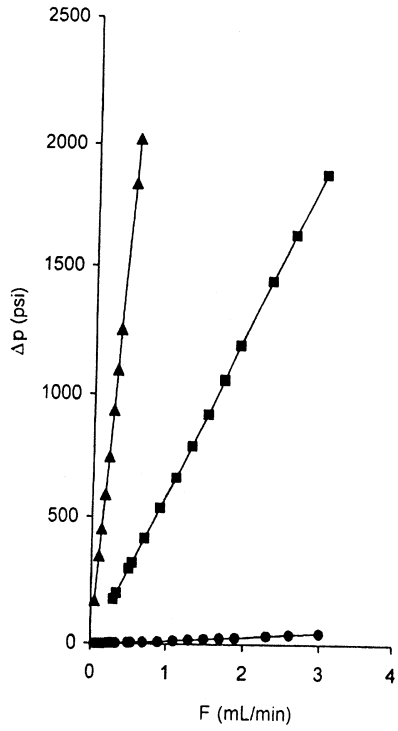

Figure 2. Pressure drop as a function of the flow rate on standard-bore and narrow-bore columns (●) and pressure drop because of equipment (without a column): (▲) narrow bore and (■) standard bore.

and calculating the mean and standard deviation of this response. The mean response of the background plus 3 times the standard deviation provided the LOD, and the mean plus 10 times the standard deviation provided LOQ (16).

Results and Discussion

Linearity

Linearity tests for quantitation were carried out over the range of 0.33 to 16.37, 0.15 to 7.72, 0.31 to 15.52, and 0.34 to 17.19 ng/injection (n = 5) for α-T, β-T, γ-T, and δ-T (narrow bore), respectively, and 0.65 to 32.74, 0.31 to 15.43, 0.62 to 31.03, and 0.69 to 34.38 ng/injection (n = 5) for α-T, β-T, γ-T, and δ-T (standard bore), respectively. Regression analysis showed an excellent linear relationship for both the narrow-bore ($r^2 \geq 0.9996$) and standard-bore ($r^2 \geq 0.9999$) columns (Table II).

Parameters

Standard-bore (4.6-mm i.d.) and narrow-bore (2.1-mm i.d.) columns having the same length (250 mm) and the same particle size (5 µm) were compared in terms of efficiency, tailing factor, separation factor (all from Table II), LOD (from Table III), and dispersion expressed by the peak volume (Table IV) (see tables for the complete listing of all parameters tested). The standard-bore column provided higher efficiency, as indicated by the number of theoretical plates for all components tested (Table II). The higher efficiency was most likely because of the fact that the centrally injected solute failed to reach the column wall of the wide-bore column before it emerged from the foot of the column. Such columns, if uniformly packed very often, show lower plate heights (Table IV) and higher efficiencies than narrow-bore columns in which solute molecules can traverse the bore of the column several times during elution (17).

Determination of the tailing factor showed that the tocopherol peaks of most components tested were slightly asymmetric on both columns (Table II). Tailing can arise from a number of sources, such as (*a*) buildup of "garbage" on the column inlet (this did not apply to our case because the columns tested were new and the analytes for testing were standards); (*b*) sample overload (not a factor because the injected concentrations of vitamin E homologs were in the lower range of the linear curve); (*c*) wrong solvent for the sample (sample was dissolved in the mobile phase); (*d*) extracolumn effects (connecting tubing had been reduced to the minimum); and (*e*) "bad" column (this may indicate that the packing material was not of sufficiently high quality).

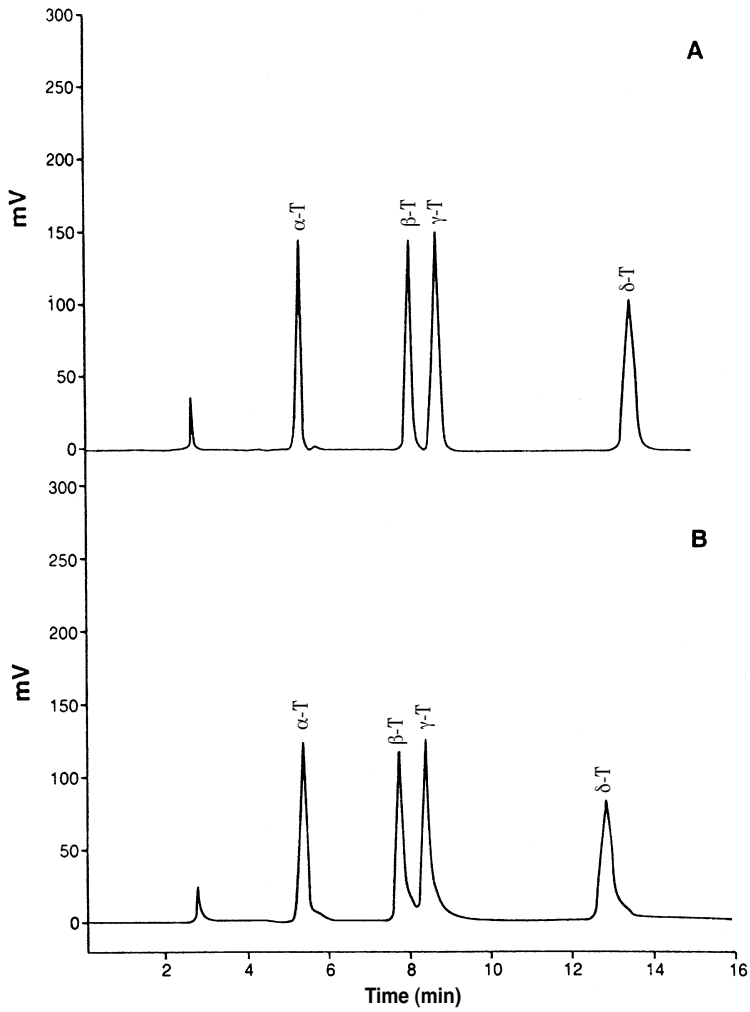

Figure 3. Effect of using different injection volumes on the peak shape eluted from a standard-bore column: (A) 10-µL injection and (B) 20-µL injection.

An inspection of the plots of Figure 2 shows that at a flow rate of 0.3 mL/min, the pressure drop was approximately six times smaller on the standard-bore than on the narrow-bore column. A maximum back-pressure of approximately 2000 psi was seen at 0.54 mL/min on the narrow-bore column.

The standard bore could be operated at 3.0 mL/min with a back-pressure of 1880 psi. The lower pressure drop of the standard-bore column was characteristic of the wider column diameter.

According to Knox (1), the injection volume can be as large as half the peak volume without serious extra band spreading. Using this measurement, the injection volume can be 40 µL and 100 µL for narrow-bore and standard-bore columns (Table IV), respectively. However, more symmetrical peaks were apparent with a 10-µL injection compared with 20-µL injection volumes on the standard-bore column (Figure 3). Therefore, a smaller injection volume is encouraged. The separation impedance (E) was defined in such a way that the lower the E value, the better the

performance. The lowest E value represents the optimum combination of plate height and permeability to flow (9). In our case, the E value of the standard-bore column was approximately 4 times lower than that of the narrow-bore column, indicating that better performance was achieved on a standard-bore than a narrow-bore column based on the separation impedance data (Table IV).

It was verified that peaks elute from the narrow-bore column in much smaller volumes with much less dispersion. The narrow-bore column reduced peak volumes of solutes more than two times for all components. For a concentration sensitive detector such as the fluorescence detector, the signal is proportional to the concentration of the analytes in the flowcell. The narrow-bore column produces signals of higher intensity because of the higher concentration in the flowcell. The increasing factor of the maximum peak concentration was given by the ratio of the squares of the diameters of the columns (10). The enhanced detectability obtained using the 250- × 2.1-mm column with respect to the 250- × 4.6-mm column is illustrated in Figure 4, which compares the LC-fluorescence response of vitamin E forms using the narrow-bore and standard-bore column. For this test, the flow rate was adjusted to give similar analysis times for the two columns by using the same eluent and hardware (pump, autosampler, tubing, and connections). Slightly better separation was achieved on the standard-bore column (Table II). To improve the resolution for the vitamin E homologs on the narrow-bore column, a mobile phase containing 0.6% IPA in hexane was used. The resolution did not improve, and the LOD increased up to 3 times (Table III). The LOD for the vitamin E homologs with the narrow bore were 4 to 7 times lower than with the standard-bore column when using the same mobile phase with a similar running time (Table III). LOQ values on the narrow-bore column were much lower compared with the standard-bore column (up to 8 times lower) (Table III).

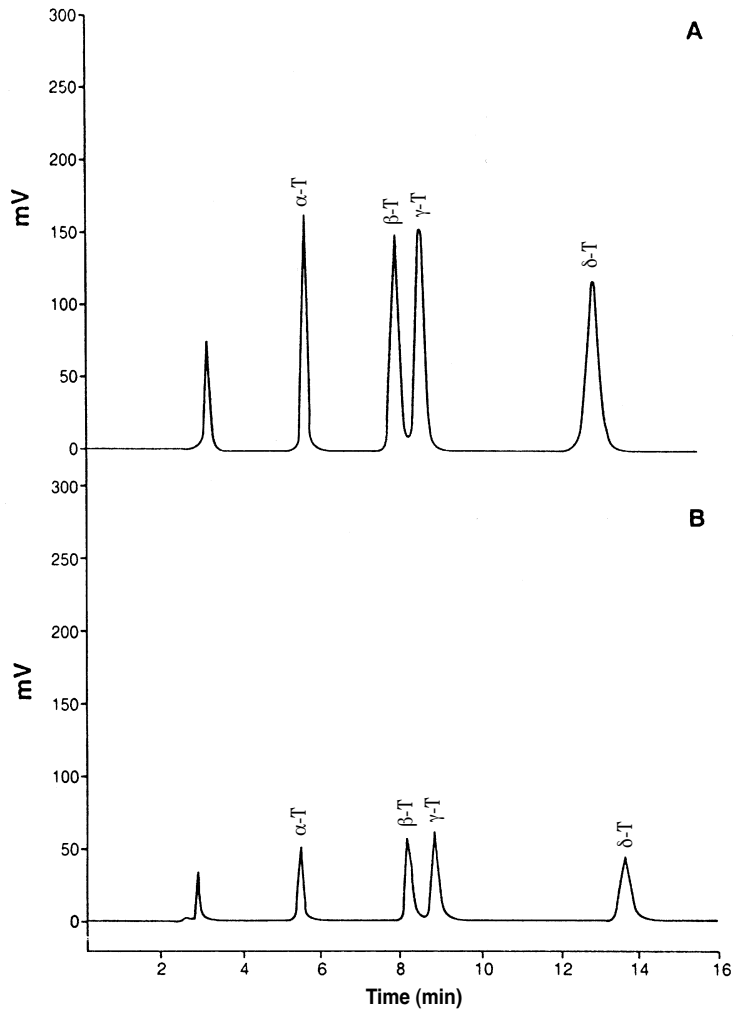

Figure 4. Chromatograms of tocopherols using: (A) a 250- × 2.1-mm-i.d. LiChrosorb Si60 column with a mobile phase of 0.8% IPA in hexane and a flow rate of 0.32 mL/min and (B) a 250- × 4.6-mm-i.d. LiChrosorb Si60 column with a mobile phase of 0.8% IPA in hexane and a flow rate of 1.0 mL/min. The injection volume was 10 µL.

Conclusion

The silica narrow-bore column that was tested on HPLC equipment with minimum dead volume showed differences in column performance when compared with the standard bore for the resolution of tocopherols. Standard bore provided higher theoretical plates, lower pressure drop, and better resolution than narrow bore. Narrow bore gave higher sensitivity and lower solvent consumption. Therefore, narrow-bore chromatography is good for analyzing vitamin E in samples when few interfering compounds are present. Solvent savings of 68% were obtained with the flow rates used in this study. The increased sensitivity obtained with the narrow-bore column could be of significance with some types of low potency biological samples. The main advantage of the standard-bore column for vitamin E analysis is the high theoretical plates that allow greater ability to resolve overlapping peaks from tocopherol peaks. The complexity of food matrices often presents vitamin E chromatograms with such interferences. Therefore, we have maintained routine use of standard-bore columns for vitamin E assay.

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Manuscript accepted August 15, 2000.