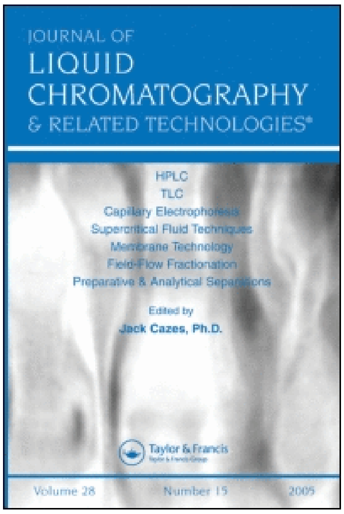

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Characterization of Column Packings in Normal-Phase Liquid Chromatography. II. Retention Behavior of Fat-Soluble Vitamins in Amino- and Cyano-Propyl Bonded Silica Gel and Binary Solvent Systems

Tatsuhiko Ando[ab]; Yoshiyuki Nakayama[a]; Shoji Hara[b]
[a] Department of Biochemistry, Kanagawa Dental College Inaoka-cho, Yokosuka, Japan [b] Division of Organic Chemistry Tokyo College of Pharmacy Horinouchi, Hachioji Tokyo, Japan

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CHARACTERIZATION OF COLUMN PACKINGS IN NORMAL-PHASE LIQUID CHROMATOGRAPHY. II. RETENTION BEHAVIOR OF FAT-SOLUBLE VITAMINS IN AMINO- AND CYANO-PROPYL BONDED SILICA GEL AND BINARY SOLVENT SYSTEMS

TATSUHIKO ANDO, YOSHIYUKI NAKAYAMA[1]
AND SHOJI HARA[2*]
[1]*Department of Biochemistry*
Kanagawa Dental College
Inaoka-cho
Yokosuka 238, Japan
[2]*Division of Organic Chemistry*
Tokyo College of Pharmacy
Horinouchi, Hachioji
Tokyo 192-03, Japan

ABSTRACT

As continuation of our study on the characterization of column packings in normal-phase HPLC analysis, the retention indices of ten fat-soluble vitamins on aminopropyl and cyanopropyl bonded silica columns were systematically estimated using binary solvents containing ethyl acetate (EtOAc), tetrahydrofuran (THF) or 2-propanol (PrOH) in n-hexane. A linear relationship between the logarithm of the capacity ratio and that of the solvent composition was confirmed. The retentivity and selectivity for these chemically bonded packing columns were determined as follows: the amino-type column has stronger and cyano-type column weaker retentivity than the bare silica column for n-hexane-EtOAc or THF binary systems. Specific adsorption of tocopherol derivatives containing phenolic hydroxyl groups on the amino column was observed. To obtain high efficiency in the separation of fat soluble vitamins, peak sharpness and asymmetry factors were measured using three columns and three binary solvents. The bare silica and PrOH binary solvent generally gave superior peak shape for all vitamin samples.

INTRODUCTION

Various chemically bonded silica gel columns have been developed and have gained high repute at many chemical laboratories[1,2]. To clarify the retention characteristics of chemically bonded phases, systematic studies using various solutes were carried out at the laboratory of Hara[3-7].

As continuation of our previous work[8], the present paper describes the retention behavior of fat-soluble vitamins in liquid-solid chromatography using amino- and cyano-bonded columns and binary solvents. The mobile phases used were basic solvents such as ethyl acetate (B1) and tetrahydrofuran (B2) or acidic-basic solvents such as 2-propanol (AB) in n-hexane. Based on the linear relationship between the logarithms of the capacity ratios and those of solvent compositions, as recently established by our experimental data, solute retentivity was systematically determined. To create a phase system that would provide high resolution, peak shape was evaluated by measuring peak sharpness and determining the asymmetry factor.

In a preliminary study of this series[8], the bare silica gel column was characterized in terms of retentivity and column efficiency, using fat-soluble vitamins as solutes and binary solvent systems as mobile phases. The retention characteristics of chemically bonded columns were compared with those obtained using a non-bonded silica gel column as the standard and assessment of column selectivity was made in the present study.

EXPERIMENTAL

LC System and Experimental Procedure

The instruments, samples and reagents were the same as in our previous paper[8], as was also the experimental procedure. Conventional columns packed with amino- and cyano-propylsilyl silica gel (250 x 4.6 mm, Zorbax, Du Pont, Wilmington, DE) were used. Retention indices and peak widths were measured so as to calculate the capacity ratios of the solutes and evaluate peak sharpness and asymmetry, respectively.

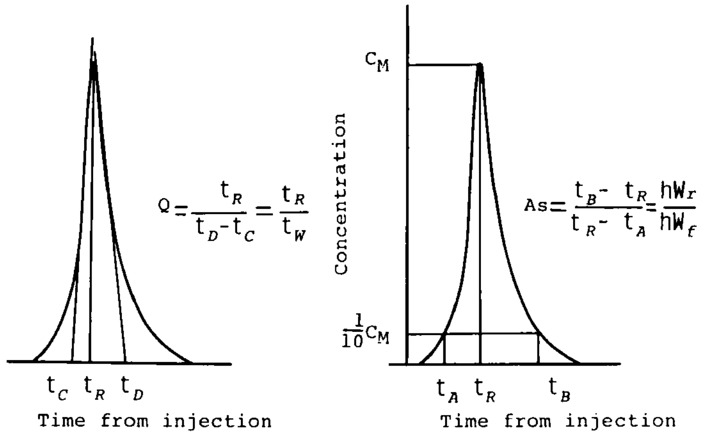

Figure 1. Estimation of the Peak Sharpness and Peak Asymmetry Factor.

The peak sharpness, Q and the asymmetry factor, As, were calculated as follows:

$$Q = t_R/t_W \qquad As = hW_r/hW_f$$

where t_W is peak width measured on a time-scale, hW_r and hW_f are partial peak widths measured from the peak maximum to peak front and then to the peak rear, respectively (Figure 1)[9].

RESULTS AND DISCUSSION

1. Capacity Ratio vs. Solvent Composition

Mechanistic considerations of the retention model for silica gel liquid-solid chromatography indicated the following linear relationship between the logarithm of the capacity ratio and that of the concentration of polar solvents,

$$\log k' = c - n \log Xs \qquad (1)$$

where c and n are constants and Xs is the concentration of the polar solvent[10]. Equation (1) was also found applicable to amino- and cyano-type chemically bonded columns in normal-phase operations using n-hexane binaries[4-7].

The retentivity of the fat-soluble vitamin samples used in our preliminary study was measured systematically using amino- and cyano-propylsilyl silica columns. The mobile phases were binaries containing ethyl acetate (B1), tetrahydrofuran (B2) or 2-propanol (AB) as the polar components in n-hexane as the diluent.

The retention indices were determined at various solvent compositions. The results obtained using amino- and cyano-type columns are presented in Figures 2-4 and Figures 5-7, respectively. A linear relation between the logarithm of capacity ratio and that of polar solvent concentration was confirmed for three binary systems and the correlation coefficient was determined as 0.98.

2. Molecular Structures and Retentivity of the Solutes

The retention behavior of fat-soluble vitamins, using chemically bonded phase systems, was essentially the same as that observed using silica gel systems, though significant differences were noted in some cases. The retention of retinol (1) containing a primary alcohol group was greatest. The retention of ergocalciferol (3) and cholecalciferol (4), each containing a secondary alcohol group, was followed. Separation of ergocalciferol and cholecalciferol was difficult in nearly all phase systems, owing to their structural similarity. This may be explained as due to hydrogen bonding between hydroxyl groups in both the solute molecules and polar groups on the silica gel surface.

The adsorptivity of the formyl group in retinal (2) was weaker than that of the hydroxyl group for cyano-type column systems, whereas in amino-type column systems, interaction between the amino-function located on the packing surface and the aldehyde group in a solute molecule was remarkable; consequently, retinal was not eluted from the amino-type column systems.

The adsorption of vitamin E homologues (5-8) occurred to a lesser extent than that of vitamin A and D homologues for cyano-type column systems, but for amino-type column systems, the retentivity of vitamin E homologues was noted to increase owing to interaction of the amino function introduced into the packing surface, resulting in the forma-

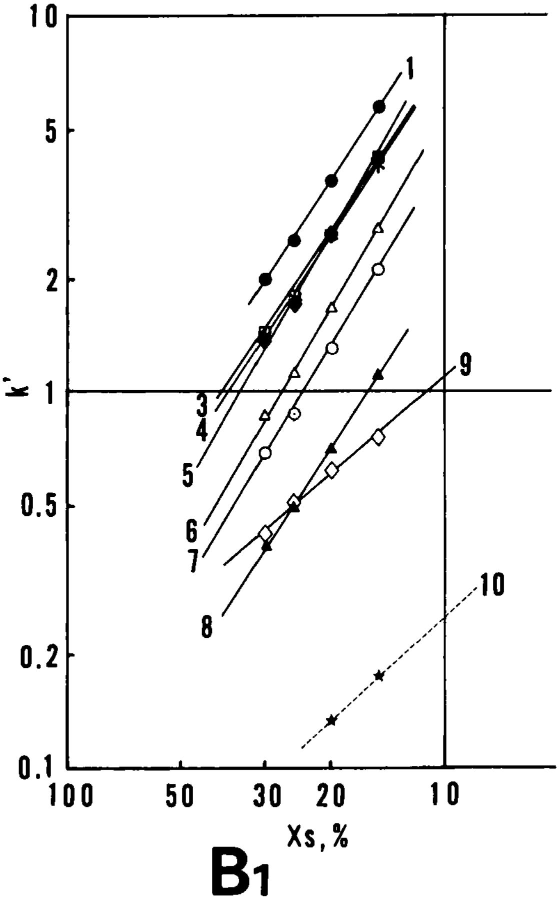

Figure 2. Logarithm of the Capacity Ratio on an Amino-Bonded Silica Column as a Function of That of Ethyl Acetate (B1) Composition in n-Hexane.

Samples: 1=retinol, 2=retinal, 3=ergocalciferol, 4=cholecalciferol, 5=δ-tocopherol, 6=γ-tocopherol, 7=β-tocopherol, 8=α-tocopherol, 9=menadione, 10=phylloquinone.

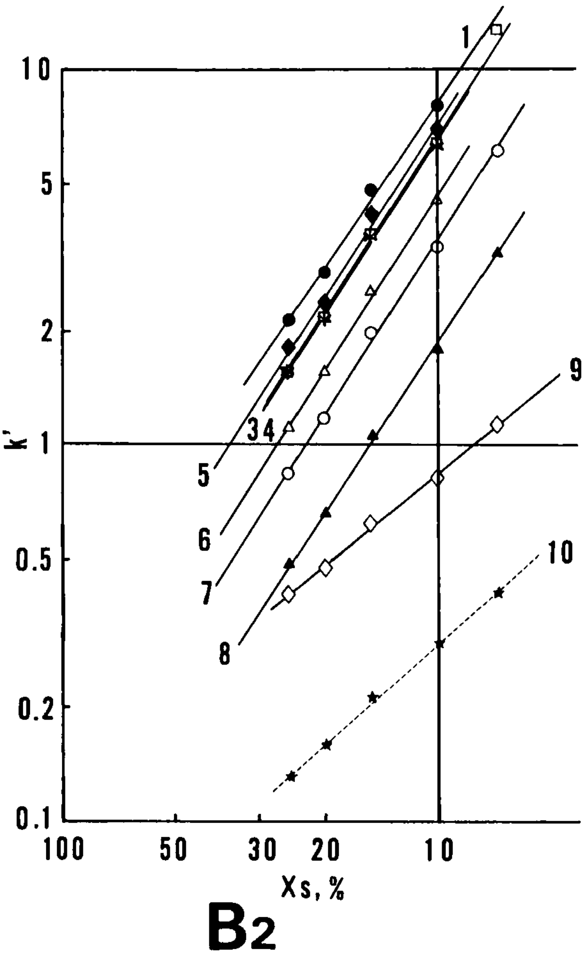

Figure 3. Logarithm of the Capacity Ratio on an Amino-Bonded Silica Column as a Function of That of Tetrahydrofuran (B2) Composition in n-Hexane.

Samples as the same as in Figure 2.

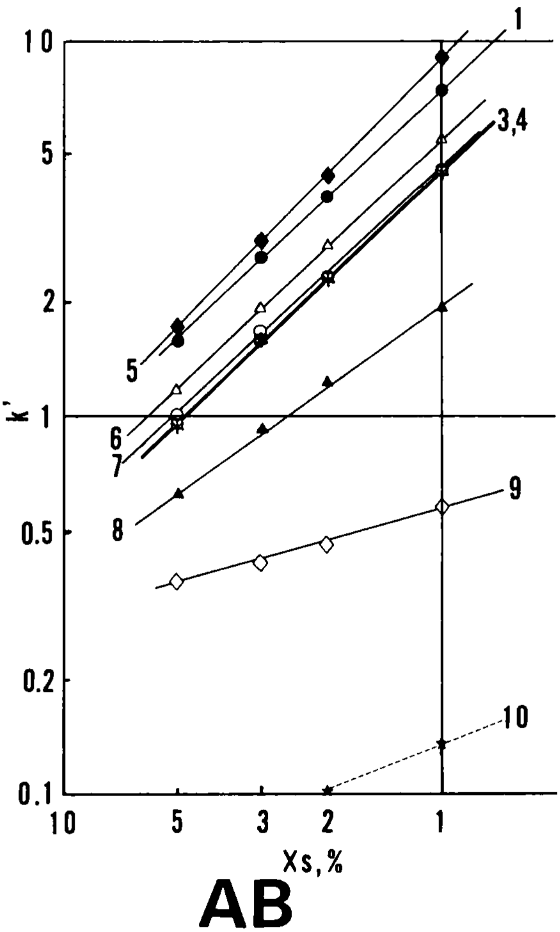

Figure 4. Logarithm of the Capacity Ratio on an Amino-Bonded Silica Column as a Function of That of 2-Propanol (AB) Composition in n-Hexane.

Samples as the same as in Figure 2.

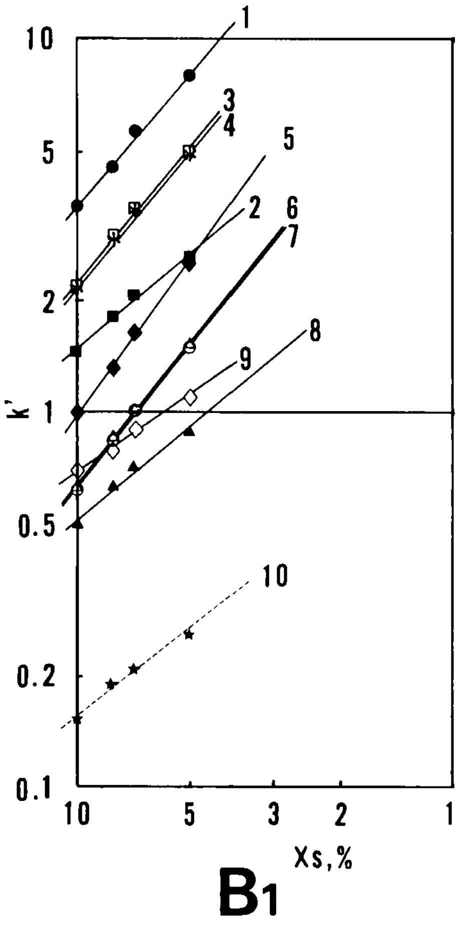

Figure 5. Logarithm of the Capacity Ratio on an Cyano-Bonded Silica Column as a Function of That of Ethyl Acetate (B1) Composition in n-Hexane.

Samples as the same as in Figure 2.

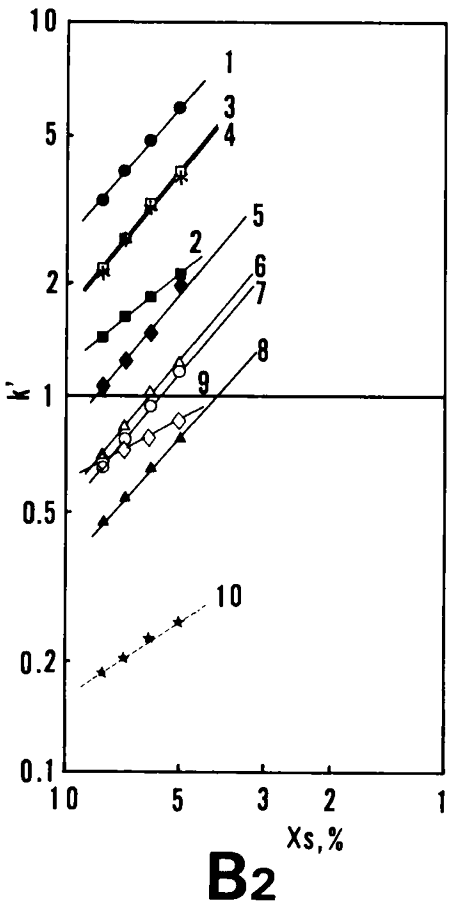

Figure 6. Logarithm of the Capacity Ratio on an Cyano-Bonded Silica Column as a Function of That of Tetrahydrofuran (B2) Composition in n-Hexane.

Samples as the same as in Figure 2.

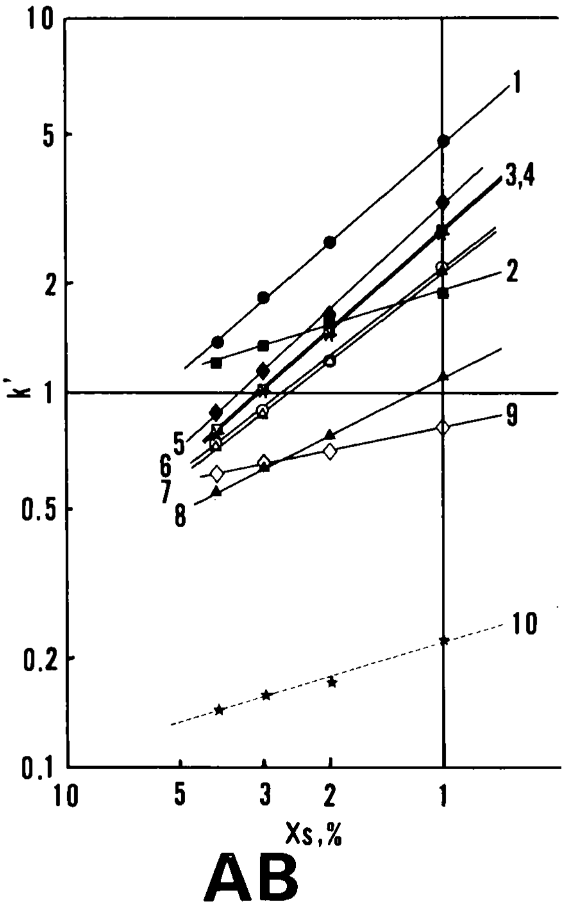

Figure 7. Logarithm of the Capacity Ratio on an Cyano-Bonded Silica Column as a Function of That of 2-Propanol (AB) Composition in n-Hexane.

Samples as the same as in Figure 2.

tion of an ionic bond with the acidic phenol group present in a solute molecule. The adsorption selectivity given by vitamin E homologues, though moderate, was sufficient to bring about a high resolution function of these compounds, as evident from the resolution of vitamin E homologues in Figure 8.

Vitamin K homologues (9,10) gave the weakest adsorption activities in all fat-soluble vitamins examined. The retentivity difference between menadione (9) and phylloquinone (10) was obvious and due to the steric bulkiness of the side chain introduced into phylloquinone.

3. Roles of Polar Solvents in Elution Strength, Selectivity and Column Efficiency

In the previous paper[8], solvent roles in solute retention were examined using an untreated silica gel column. For amino- and cyano-propyl bonded silica columns, these roles were basically the same in most cases for the silica gel column. The retention behavior of fat-soluble vitamins for two binary mobile phase systems in which ethyl acetate (B1) and tetrahydrofuran (B2) served as polar components in n-hexane as the diluent were found reciprocally related, as evident from Figures 2 and 3, and Figures 5 and 6.

The elution strength of 2-propanol (AB) in n-hexane was, in contrast, higher than that of either of the two B type solvent systems, as shown in Figures 4 and 7. The selectivity of the AB solvent as measured by intercept difference (Δlog k') was less than those of the two B solvents except for the vitamin K homologues.

Peak shape, as affected by polar components in binary solvent systems, is a subject to which only scant attention has been directed. Yet, its optimization results in significantly higher resolution[9]. This prompted the authors to determine the peak sharpness (Q) and asymmetry factor (As) for bare silica gel, amino- and cyano-type bonded silica columns using three binary solvent systems. The results are shown in Tables I and II.

From these tables, it is quite evident that Q and As do not remain constant but vary according to the solute and phase system involved in the carrier solvent and packing column used. Among the various phases

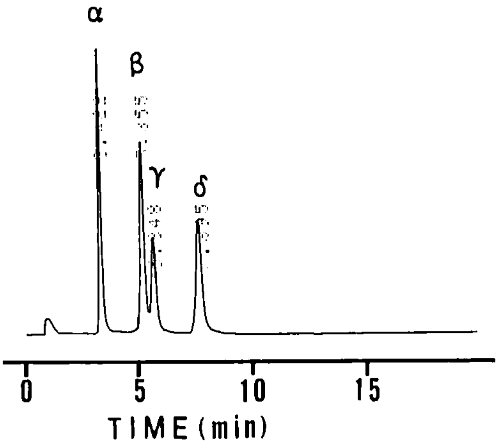

Figure 8. Chromatographic Resolution of Vitamin E Homologues on Amino Column Using 2-Propanol in n-Hexane (2% v/v) as the Mobile Phase.

Table I. Peak Sharpness (Q) of Fat-Soluble Vitamins on Silica Gel, Amino- and Cyano-Bonded Silica Columns Using Three Binary Solvents.

column	sample no. 1	2	3	4	5	6	7	8	9	10	mean	S.D.
solvent system : ethyl acetate(B1)-n-hexane												
silica	28.4	23.4	25.1	26.1	22.0	23.4	21.0	24.8	23.9	13.9	23.2	3.9
amino-type	19.1	----	20.3	20.4	17.9	17.0	16.6	16.2	15.9	9.4	17.0	3.3
cyano-type	22.6	20.1	20.1	19.4	14.8	14.3	12.6	11.5	16.8	11.2	16.3	4.0
solvent system : tetrahydrofuran(B2)-n-hexane												
silica	29.5	21.3	24.6	28.3	20.8	21.1	21.0	20.6	20.8	18.2	22.6	3.7
amino-type	18.3	----	17.7	17.9	15.6	16.0	18.0	18.1	15.2	11.5	16.5	2.2
cyano-type	20.5	21.1	16.9	18.3	7.7	9.4	8.7	14.4	16.2	13.2	14.6	4.8
solvent system : 2-propanol(AB)-n-hexane												
silica	22.5	21.1	21.6	22.3	20.3	19.0	17.0	20.9	19.9	33.4	22.1	4.5
amino-type	14.1	----	14.0	13.9	13.3	13.1	13.7	12.5	11.2	9.9	12.8	1.4
cyano-type	22.9	17.2	16.2	19.1	15.8	16.5	16.1	14.8	18.9	11.6	16.9	3.0

Table II. Asymmetry Factor (As) of Fat-Soluble Vitamins on Silica Gel, Amino- and Cyano-Bonded Silica Columns Using Three Binary Solvents.

	sample no.											
column	1	2	3	4	5	6	7	8	9	10	mean	S.D.
	solvent system : ethyl acetate(B1)-n-hexane											
silica	2.0	1.0	1.2	1.1	1.0	1.5	1.0	1.0	1.0	1.0	1.2	0.3
amino-type	0.7	---	0.7	0.7	0.7	1.0	1.0	1.0	1.0	1.0	1.1	0.2
cyano-type	1.1	1.0	1.2	1.1	1.1	1.4	1.3	1.3	1.0	1.0	1.1	0.1
	solvent system : tetrahydrofuran(B2)-n-hexane											
silica	1.2	1.0	1.3	1.2	1.1	1.0	1.1	1.0	1.0	1.0	1.1	0.1
amino-type	1.1	---	1.1	1.1	1.1	1.4	1.2	1.0	1.0	1.0	1.1	0.1
cyano-type	1.1	1.0	1.1	1.2	2.3	2.7	3.4	2.9	1.0	1.0	1.8	0.9
	solvent system : 2-propanol(AB)-n-hexane											
silica	1.2	1.0	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.1
amino-type	0.9	---	1.0	1.0	1.0	1.2	1.1	1.0	1.0	1.0	1.0	0.1
cyano-type	1.0	1.0	1.0	1.0	1.0	1.2	1.2	1.1	1.0	1.0	1.1	0.1

examined, the silica gel column gave the best peak sharpness, i.e., peak efficiency (Table I), particularly when the 2-propanol (AB)-binary solvent was used on the silica gel column, and the lowest asymmetry factor, thus giving rise to the maximum resolution (Table II).

4. Selectivity of the Column Packings

Different retentivities on bare silica gel and chemically bonded columns in normal-phase operation using fat-soluble vitamins as solutes were measured in the previous paper[8] and first section of this paper. In this section, column selectivity is assessed quantitatively for binary solvent systems. The two constants in equation (1) for a specific solute are considered to depend on the characteristics of the column and thus their quotients for a pair of columns were calculated using bare silica column as the standard. The c-raio and n-ratio for amino/silica and cyano/silica are the quotients of these two constants in equation (1) for the amino column/silica gel column and cyano column/silica gel column, respectively.

Table III. Constants and Constant Ratios of the Linear Relationship between the Retention Index of Fat-Soluble Vitamins and Solvent Composition of Stronger Solvents in n-Hexane for Silica Gel, Amino- and Cyano-Bonded Silica Columns.

Stronger solvent: ethyl acetate.

No. sample	constant c	constant n	constant ratio c-ratio	constant ratio n-ratio	c-ratio	n-ratio
	silica gel		amino/silica		cyano/silica	
1. Retinol	2.23	1.38	1.14	1.10	0.76	0.83
2. Retinal	1.39	1.20	----	----	0.73	0.71
3. Ergocalciferol	2.14	1.45	1.21	1.07	0.72	0.84
4. Cholecalciferol	2.11	1.43	1.15	1.08	0.73	0.85
5. δ-Tocopherol	1.69	1.56	1.54	1.07	0.78	0.85
6. γ-Tocopherol	1.48	1.51	1.59	1.09	0.72	0.83
7. β-Tocopherol	1.36	1.48	1.63	1.08	0.76	0.84
8. α-Tocopherol	1.06	1.31	1.70	1.15	0.68	0.85
9. Menadione	1.01	0.93	0.84	0.88	0.45	0.63
10. Phylloquinone	----	----	----	----	----	----
mean	1.61	1.36	1.35	1.06	0.70	0.80
S.D.	0.44	0.21	0.30	0.07	0.09	0.07

Table III shows constants c and n for the silica gel as the standard and quotients for the two chemically bonded columns connected to a silica column, using ethyl acetate (B1)-n-hexane as the binary solvent. The mean value and standard deviation of the constants and their quotients are also given in the table. The ratios of the constants c and n obtained using the ethyl acetate-n-hexane binary in Table III indicate the relative retentivity of the amino column to be from six to thirty five percent more than those of the silica gel column. In our previous study using steroids as solutes, the retentivity of the amino column was found basically the same as that of a silica gel column[1-7]. The increase in retention observed for an amino-column using vitamins as solutes can be explained as due to selective interactions between the phenolic hydroxyl group in tocopherol derivatives and the amino function introduced onto the packing surface. The mean values of the

Table IV. Constants and Constant Ratios of the Linear Relationship between the Retention Index of Fat-Soluble Vitamins and Solvent Composition of Stronger Solvents in n-Hexane for Silica Gel, Amino- and Cyano-Bonded Silica Columns.

Stronger solvent: tetrahydrofuran.

No. sample	constant		constant ratio			
	c	n	c-ratio	n-ratio	c-ratio	n-ratio
	silica gel		amino/silica		cyano/silica	
1. Retinol	2.27	1.47	1.04	0.99	0.72	0.84
2. Retinal	1.30	1.25	----	----	0.68	0.64
3. Ergocalciferol	2.17	1.47	1.08	1.04	0.69	0.86
4. Cholecalciferol	2.15	1.46	1.07	1.04	0.68	0.86
5. δ-Tocopherol	1.67	1.55	1.40	0.96	0.73	0.85
6. γ-Tocopherol	1.50	1.54	1.44	0.98	0.63	0.80
7. β-Tocopherol	1.32	1.45	1.59	1.07	0.70	0.85
8. α-Tocopherol	1.15	1.39	1.53	1.06	0.57	0.78
9. Menadione	0.79	0.91	0.94	0.88	0.43	0.62
10. Phylloquinone	----	----	----	----	----	----
mean	1.60	1.41	1.26	1.00	0.64	0.78
S.D.	0.50	0.20	0.25	0.06	0.09	0.09

constant ratios for the cyano column in Table III show that the relative retentivity of cyano column may be twenty to thirty percent less than that of a silica gel column.

The ratios of the constants, c and n, obtained using the tetrahydrofuran-binary system in Table IV were essentially the same as those in Table III, obtained using the ethyl acetate-binary system. The retentivity and selectivity of the two basic solvents, ethyl acetate and tetrahydrofuran, on three columns are thus virtually the same.

The retention data obtained using 2-propanol, an acidic-basic solvent, as the polar component and shown in Table V, differ considerablly from those obtained using two binary systems containing basic solvents in n-hexane, as shown in Tables III and IV. The intercepts of the linear relationships between the logarithms of the capacity ratios of vitamins and the logarithms of 2-propanol concentrations in amino and

Table V. Constants and Constant Ratios of the Linear Relationship between the Retention Index of Fat-Soluble Vitamins and Solvent Composition of Stronger Solvents in n-Hexane for Silica Gel, Amino- and Cyano-Bonded Silica Columns.

Stronger solvent: 2-propanol.

No. sample	constant		constant ratio			
	c	n	c-ratio	n-ratio	c-ratio	n-ratio
	silica gel		amino/silica		cyano/silica	
1. Retinol	0.83	1.03	1.05	0.92	0.82	0.87
2. Retinal	0.08	0.61	----	----	3.50	0.52
3. Ergocalciferol	0.63	0.99	1.05	0.97	0.70	0.90
4. Cholecalciferol	0.62	0.99	1.05	0.97	0.69	0.90
5. δ-Tocopherol	0.39	1.12	2.46	0.92	1.31	0.83
6. γ-Tocopherol	0.19	0.99	3.89	0.96	1.79	0.81
7. β-Tocopherol	0.14	0.96	4.86	0.99	2.43	0.83
8. α-Tocopherol	-0.11	0.72	-2.64	0.97	-0.45	1.12
9. Menadione	-0.06	0.48	3.83	0.60	1.33	0.42
10. Phylloquinone	----	----	----	----	----	----
mean	0.29	0.88	1.94	0.91	1.34	0.80
S.D.	0.33	0.22	2.38	0.13	1.13	0.21

cyano-propyl bonded columns generally exceed those on a bare silica column. Conversely, the slopes for the linear relationships determined using 2-propanol binaries on chemically bonded stationary phases are less than those obtained on a silica phase.

CONCLUSIONS

A linear correlation between the logarithm of the capacity ratio versus that of the concentrations of ethyl acetate, tetrahydrofuran and 2-propanol in n-hexane was confirmed experimentally in chemically bonded amino- and cyano-type stationary phases for ten fat-soluble vitamins.

On the basis of the average retention indices of the vitamins in these phase systems, column retentivity was determined quantitatively. The retention selectivity of tocopherol derivatives was observed on the aminopropyl silica column. 2-Propanol-binary solvent generally gave the best peak shape. Column selection and mobile phase optimization should be facilitated by the results presented herein.

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