INVESTIGATION OF ISOPRENOID BENZOATES AND NAPHTHOATES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

ISOCRATIC ELUTION CHARACTERISTICS OF BENZOATES AND NAPHTHOATES OF C_5–C_{20} TERPENOID ALCOHOLS

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SUMMARY

Conditions for derivatization of C_5–C_{20} isoprenoid alcohols and the reversed-phase liquid chromatographic properties of the corresponding benzoate and naphthoate esters are described. A non-linear response of log k' to changes in the composition of the mobile phase was observed on a Radial-Pak C_{18} column. In general, the capacity factors (k') increased with increases in carbon content and degree of saturation in the hydrocarbon chain, and decreased with increases in branching and Z double bond content. Replacement of a single hydrogen in the methyl group at C-3 of geranyl benzoate with fluorine had little effect on k', while the difluoro- and trifluoromethyl derivatives showed regular increases in k'. The large extinction coefficients of the aromatic ester moieties, especially the 2-naphthoates, provide high sensitivity for UV detection.

INTRODUCTION

Metabolites of the terpene biosynthetic pathway fulfill numerous essential roles in plants, animals, and microorganisms. The major building reaction in the pathway is the sequential 1'-4 condensation of isopentenyl pyrophosphate with an allylic pyrophosphate to generate the isoprenylogue of the allylic substrate[1]. Linear chains of one to four isoprene units are very common, and compounds with chain lengths clustering around six, eight, ten and twenty units are also widespread.

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During work on the enzyme catalyzed 1'-4 condensation reaction, it became necessary for us to develop procedures to separate isoprenylogues for spectroscopic studies and radiochemical analyses. Newbery et al.[2] recently studied the separation of isoprenoid alcohols by reversed-phase chromatography with detection at the 0.5-μg level. However, the presence of radioisotopes in our compounds and the necessity of working with small amounts of material required that we use relatively non-volatile derivatives, especially for the smaller molecules in the series. In searching for suitable procedures, we studied the preparation and separation of benzoates and naphthoates of isoprenoid alcohols by reversed-phase chromatography. Since linear isoprenoids often occur naturally as alcohols, or as carboxylate or phosphate derivatives which can easily be converted to alcohols, the methodology can be used for separation and analysis of extracts from in tact plant or animal tissue as well.

EXPERIMENTAL

Materials

3-Methyl-2-buten-1-ol, 3-methyl-3-buten-1-ol, 3-methyl-1-butanol, linalool, geraniol, dicyclohexylcarbodiimide, 4-dimethylaminopyridine and 2-naphthoic acid were obtained from Aldrich and used without further purification. Citronellol was purchased from Matheson, Coleman and Bell. (*E,E*)-Farnesol was purified from a mixture of *E,E*- and *Z,E*-isomers provided by Givaudan. Nerol was also obtained from Givaudan. Fluorinated alcohols were available from previous studies[3], and geranylgeraniol was kindly provided by Professor R. M. Coates. 3,7-Dimethyloctanol was prepared by catalytic hydrogenation (Adam's catalyst) of geraniol in ethanol. Benzoyl chloride and pyridine were freshly distilled and stored over 3 Å molecular sieves prior to use.

Liquid chromatography

A Waters system consisting of a U6K injector, Model 6000A and M45 pumps, a RCM-100 radial compression module, a Model 441 UV detector, a Model 720 system controller and a Model 730 data module were used. All separations were on a Waters Radial-Pak A column (C_{18}, 10 μm, 10 cm × 8 mm). Doubly deionized water and acetonitrile (Merck, chromatographic grade) were passed through type HA (0.45 μm) and type FA (0.5 μm) filters (Millipore), respectively, and degassed just prior to use. Samples were dissolved in acetonitrile, and 5–25-μl portions were injected.

Derivatization

Benzoate esters were prepared by treatment of the terpene alcohols with a 10–50-fold excess of benzoyl chloride using a stock solution of benzoyl chloride–pyridine–diethyl ether (1:4:15, v/v/v). The work-up involved washing the organic layer with 1 *M* hydrochloric acid to remove pyridine, followed by washing with a saturated solution of sodium hydrogen carbonate. The ether layer was then passed through a short column of basic alumina (activity II) followed by two column volumes of ether. Alternatively, the alcohols were treated with benzoyl imidazole and a catalytic amount of imidazolyl sodium in tetrahydrofuran[4]. After 30 min at room temperature, solvent was removed with a gentle stream of dry nitrogen, and the

residue was extracted with hexane. The hexane soluble material was passed through a short column of basic alumina (activity II), followed by five column volumes of hexane–diethyl ether (2:1). Solvent was removed with a gentle stream of nitrogen. Isoprenoid naphthoates were prepared by the method of Neises and Steglich[5]. A solution containing 2 molar equivalents of 2-naphthoic acid and 1 molar equivalent of terpene alcohol in dry methylene chloride at 0°C was treated with a catalytic amount (0.01–0.03 molar equivalents) of 4-dimethylaminopyridine and four equivalents of dicyclohexylcarbodiimide. After 10 min, the mixture was allowed to warm to room temperature. The progress of the reactions was monitored by thin-layer chromatography [silica gel, hexane–ethyl acetate (8:2)] and the reactions were usually complete within 3–5 h. The resulting mixture was filtered through a plug of glass wool and washed in succession with 0.5 M hydrochloric acid and saturated sodium hydrogen carbonate. The organic layer was dried over magnesium sulfate, and solvent was removed at reduced pressure. Pure esters were obtained by flash chromatography on silica gel [hexane–ethyl acetate (95:5)][6]. All esters were characterized by IR, ^{1}H NMR, and mass spectrometry.

RESULTS AND DISCUSSION

Analysis and separation of terpene alcohols by high-performance liquid chromatography is difficult because most of the compounds of interest lack an intense UV chromophore and many are volatile. These problems were circumvented by conversion of the alcohols to their corresponding benzoate or naphthoate esters. The twenty esters reported in this study are listed in Table I. All of the derivatives had low volatilities, and small amounts of samples could be handled without significant losses of material. The high molar extinction coefficients (ε) of the aromatic ester chromophores provided excellent sensitivity for UV detection. We were able to detect the benzoate esters, ε(214 nm) 7500 l mol^{-1} cm^{-1}, at 0.1-μM levels when interference from background contamination was low. A considerable improvement was found for naphthoate esters. Both the shift to longer wavelengths and the higher extinction coefficients, ε(236 nm) 65,200 l mol^{-1} cm^{-1}, permitted detection of 10 nM concentrations of the derivatives. Radioactive compounds can be detected at much lower levels by collecting fractions and determining the radioactivity by liquid scintillation spectrometry.

Capacity factors (k') for isoprenoid derivatives were determined from eqn. 1, where t_R and t_0 are the retention times of

$$k' = \frac{t_R - t_0}{t_0} \tag{1}$$

the aromatic esters and methanol, a t_0 marker, respectively[7]. Methanol was detected at 205 nm, and its retention time did not vary significantly over the range of solvent compositions used in this study. Capacity factors for benzoates are listed in Table II and those for naphthoates, in Table III.

The capacity factors shown in Tables II and III were reproducible to within ±2% for up to a week when the column was in continuous use. Over longer periods, however, the values slowly decreased, with changes as large as 10% for geranyl and neryl naphthoate after 33 days (see Table III). More substantial decreases in k' were

TABLE I

AROMATIC ESTERS OF ISOPRENOID ALCOHOLS STUDIED

Compound	No.
Benzoates	
3-Methyl-2-butenyl benzoate	1
3-Methyl-3-butenyl benzoate	2
3-Methylbutyl benzoate	3
Geranyl benzoate	4
Neryl benzoate	5
Linalyl benzoate	6
Citronellyl benzoate	7
3,7-Dimethyloctyl benzoate	8
Z-3-Fluoromethyl-7-methyl-2,6-octandienyl benzoate	9
Z-3-Difluoromethyl-7-methyl-2,6-octadienyl benzoate	10
Z-3-Trifluoromethyl-7-methyl-2,6-octadienyl benzoate	11
Farnesyl benzoate	12
Geranylgeranyl benzoate	13
Naphthoates	
3-Methyl-3-butenyl naphthoate	14
3-Methyl-2-butenyl naphthoate	15
Geranyl naphthoate	16
Neryl naphthoate	17
Citronellyl naphthoate	18
3,7-Dimethyloctyl	19
Farnesyl naphthoate	20

noted as columns continued to age. Capacity factors for the benzoates listed in Table II were measured shortly after a column was first used, while those for the naphthoates were measured on the same column thirteen months later. A comparison of values in Tables II and III indicates that the capacity factors for the naphthoate esters are only slightly larger than those for benzoates in spite of a difference of four carbon atoms in the ester moieties. Reinjection of geranyl benzoate on the old column gave a k' in 80% acetonitrile which was only 36% of the former value. Installation of a new C_{18} column and injection of geranyl benzoate gave a capacity factor of 5.20 which was close to the initial value of 4.56. These comparisons suggest that the capacity factors decreased as the column aged. Resolution, however, degraded only slightly during the same period, and the elution patterns for the esters did not change. These results emphasize the need for frequent calibration of the chromatograms with standard samples. Measurements for the benzoates (Table II) were taken over a one week period. Those samples run early in the protocol were evaluated again on the last day to insure that the values were reproducible. A similar procedure was followed for the naphthoates.

C₅ isoprene homologues

Plots of log k' *versus* the number of isoprene units (n) for the homologous series of linear allylic terpenoid benzoates (n = 1–4) exhibited excellent linearity in accordance with Martin's rule[8] (eqn. 2, see Fig. 1). The slope (m), determined by a

TABLE II

CAPACITY FACTORS (k') FOR ISOPRENOID BENZOATES ON A RADIAL-PAK C_{18} (10 μm) REVERSED-PHASE COLUMN

Flow-rate, 5 ml/min; temperature, 21°C.

Compound number	Capacity factor							
	Volume fraction acetonitrile							
	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
C_5-Series								
1	117	34.6	12.7	5.53	2.79	1.45	0.69	0.39
2	102	28.6	10.8	4.78	2.74	1.21	0.61	0.31
3	229	58.7	21.1	8.48	3.87	1.95	0.96	0.52
C_{10}-Series								
4			76.3	25.5	10.5	4.56 (1.67)[*] (5.20)[**]	1.77	0.83
5			58.7	19.7	8.32	3.56	1.74	0.76
6			41.5	14.2	6.26	2.65	1.34	0.63
7			102	31.3	12.0	5.13	2.31	1.02
8			209	67.3	20.8	8.48	3.66	1.37
9				10.2	4.32	1.76	0.97	
10				14.2	6.02	2.10	1.05	
11				17.1	7.15	2.84	1.36	
C_{15}-Series								
12				110	30.2	14.0	4.52	1.68
C_{20}-Series								
13					141	43.6	11.4	3.31

[*] Measured on the same column after thirteen months.
[**] Measured on a new column.
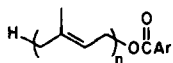
least squares analysis, is a measurement of the sensitivity of capacity factors to isoprene

$$\log k' = mn + b \tag{2}$$

number. The values for m decreased smoothly (Fig. 2) from 0.65 in 0.6 volume fraction (φ) of acetonitrile in water to 0.30 in pure acetonitrile. Similar behaviour was found for the related isoprenoid naphthoates (see Fig. 3) with m decreasing smoothly from 0.55 to 0.34 as φ increased from 0.6 to 0.9 (see Fig. 4). The curvature of the plots in Figs. 2 and 4 is, however, different. With the hemiterpene benzoate derivatives ($n = 1$), it was possible to evaluate capacity factors over the range of solvent compositions $0.3 \leqslant \varphi \leqslant 1.0$. However, the working range shrank rapidly as isoprene

TABLE III

CAPACITY FACTORS (k') FOR ISOPRENOID NAPHTHOATES ON A RADIAL-PAK C_{18} (10 μm) REVERSED-PHASE COLUMN (21°C)

Compound number	Capacity factors				
	Volume fraction acetonitrile				
	0.5	0.6	0.7	0.8	0.9
	Flow-rate (ml/min)				
	7	7	7	4	2
C_5-Series					
14	15.7	6.19	2.77	1.25	0.53
15	14.3	5.74	2.59	1.17	0.49
C_{10}-Series					
16	74.1	22.5	8.35 (7.52)*	3.11 (2.91)*	1.19
17	69.0	21.5	8.06 (7.20)*	2.99	1.16
18	98.9	29.4	10.9	3.85	1.53
19	170	47.8	17.0	5.81	2.22
C_{15}-Series					
20		77.9	24.8	7.65 (6.91)*	2.52

* Measurements taken on the same column after 33 days.

residues were added to the chain, and measurements for geranylgeranyl benzoate were confined to $0.7 \leqslant \varphi \leqslant 1.0$.

Plots of log k' versus φ (Fig. 5) show noticeable curvature for the less highly retained benzoate derivatives that could be examined over a wider range of solvent compositions. The degree of curvature is substantially less for the more highly retained benzoates. Similar, although less pronounced, behaviour was observed for the naphthoates (see Fig. 6). Although many published plots of log k' versus solvent composition are linear, several groups have reported reversed-phase systems that show distinct curvature[9]. In these instances good fits of log k' versus solvent composition were obtained empirically with a quadratic of the type shown in eqn. 3.

$$\log k' = A\varphi^2 + B\varphi + C \tag{3}$$

Using a non-linear least squares analysis, we found that our data also gave excellent fits to eqn. 3 for the parameters listed in Table IV. Wells and Clark[10] recently reported a similar study of a series of benzamides in acetonitrile–water and methanol–water over a wide range of solvent compositions. As φ approached zero (pure water), they found that curvature increased substantially, and their data fit neither a linear nor a quadratic model. One should, therefore, exercise caution in
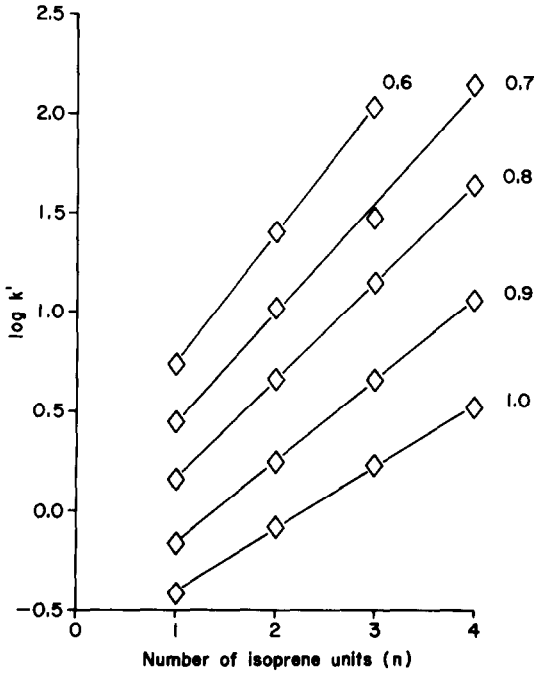
Fig. 1. A plot of log k' *versus* the number of isoprene units (n) in benzoate esters at different volume fractions (φ) of acetonitrile in water.
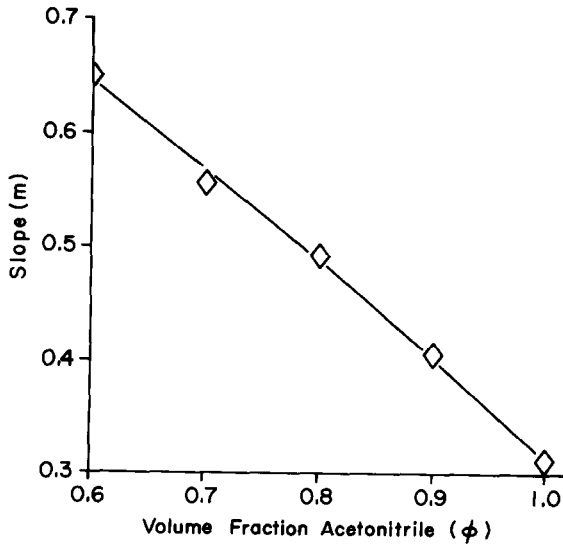

Fig. 2. A plot of slope (m) for isoprenoid benzoates *versus* the volume fraction of acetonitrile (φ).
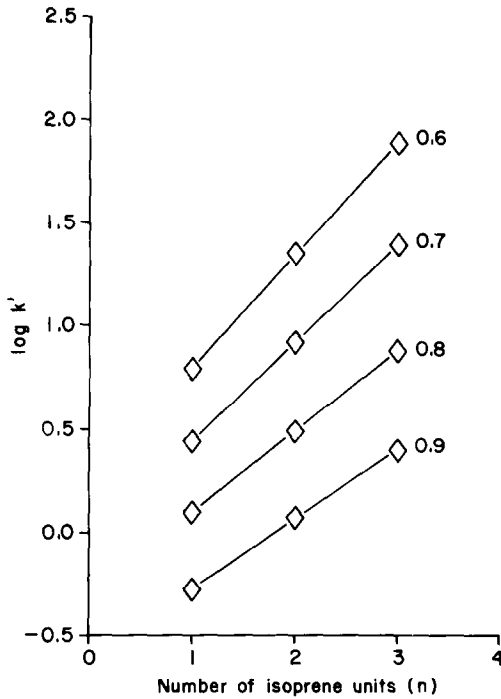
Fig. 3. A plot of log k' *versus* the number of isoprene units (n) in naphthoate esters at different volume fractions (φ) of acetonitrile in water.
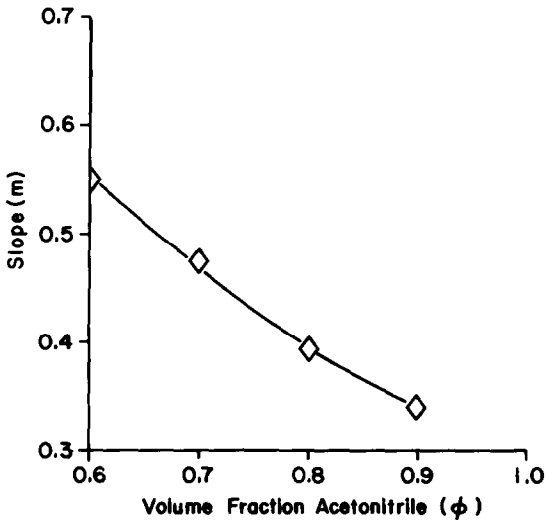

Fig. 4. A plot of slope (m) for isoprenoid naphthoates *versus* the volume fraction of acetonitrile (φ).
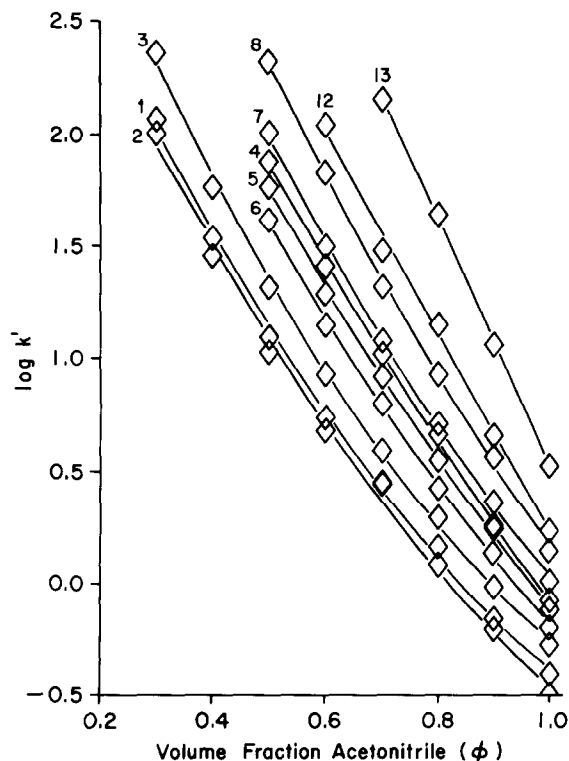
Fig. 5. A plot of log capacity factors (k') *versus* the volume fraction of acetonitrile (φ) for isoprenoid benzoates 1–8, 12 and 13. Lines through the experimental points were computer drawn using eqn. 3 and the parameters listed in Table IV.

extrapolation of our data to values of φ outside of the experimentally determined range even when care has been taken to minimize changes in k' with increasing age of the columns.

Variations in capacity factors within isomeric or closely related structures

Capacity factors for isomeric esters and esters with similar molecular weights varied substantially. Three general trends were noted for the compounds listed in Tables II and III. Capacity factors of compounds with the same general shape (skeletal and stereochemistry of double bonds where appropriate) increased substantially as unsaturation was removed. This trend is illustrated by comparisons of values shown in Fig. 5 for 3-methylbutyl benzoate (compound 3) with the unsaturated C_5 esters 1 and 2 or for 3,7-dimethyloctyl benzoate (compound 8) with C_{10} esters 4 and 7. Similar behaviour was found for C_{10} naphthoates 16, 18 and 19 (Fig. 6). For C_{10} benzoates and for C_{10} naphthoates, k' increased as each double bond was removed from the C_{10}-chain. Within a family of isomers, increased branching or a double bond geometry that produced a more compact structure resulted in a decrease in k'. This trend is noticeable in the series geranyl (compound 4), neryl (compound 5), and linalyl (compound 6) benzoates. Similar relationships for the degree of unsaturation
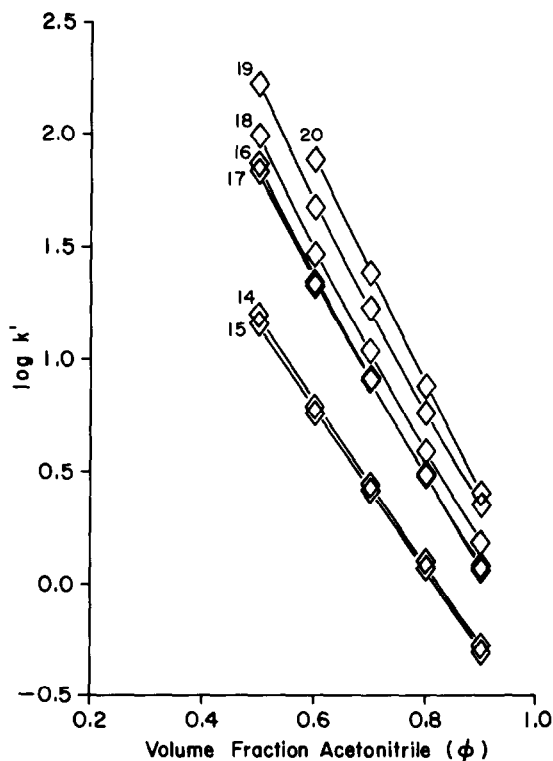
Fig. 6. A plot of log of capacity factors (k') *versus* the volume fraction of acetonitrile (φ) for isoprenoid naphthoates 14-20. Lines through the experimental points were computer drawn using eqn. 3 and the parameters listed in Table IV.

and the stereochemistry of double bonds are seen for the capacity factors of fatty acids[11]. Branching in isomeric compounds is also reported to result in major changes in k' by reducing the hydrophobic effect of individual carbons in a molecule[11].

Introduction of a single fluorine into the methyl group at C-3 of geraniol produced little change in k' despite a substantial increase in molecular weight (see Fig. 7). Successive replacement of the second and third hydrogens at C-3 by fluorine resulted in small, but regular, increases in k'.

Incremental increases in molecular weight per additional isoprene unit are sufficiently large so that the capacity factors for C_5, C_{10}, C_{15}, and C_{20} isoprenoids, with one exception, do not overlap. There is, however, considerable variation within a family as illustrated by the C_{10}-series of benzoates. At one extreme, capacity factors for linalyl benzoate (compound 6) very close to those found for 3-methylbutyl benzoate (compound 3), the most highly retained member of the C_5 family. At the other extreme, values of k' for 3,7-dimethyloctyl benzoate (compound 8) are approximately 70% of those for farnesyl benzoate (compound 12).

When analyzing mixtures containing C_5, C_{10}, C_{15}, and C_{20} isoprenoids, we found that excellent resolution could be obtained by gradient elution chromatography. In most instances the ratio of acetonitrile–water could be altered without

TABLE IV

PARAMETERS FOR ISOPRENOID BENZOATES AND NAPHTHOATES FROM NON-LINEAR LEAST SQUARES FIT TO EQN. 3.

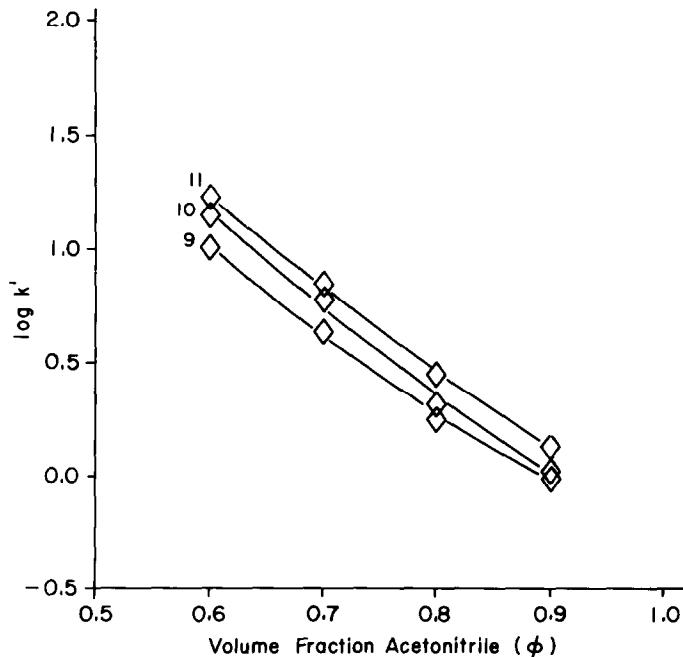| Compound	A	B	C
1	1.95	−5.98	3.65
2	1.71	−5.68	3.50
3	2.30	−6.67	4.11
4	1.12	−5.57	4.38
5	1.17	−5.46	4.18
6	1.68	−6.10	4.24
7	1.51	−6.20	4.70
8	1.25	−6.19	5.08
9	1.58	−5.82	3.93
10	1.39	−5.93	4.22
11	1.40	−5.80	4.20
12	1.31	−6.54	5.48
13	0.83	−6.89	6.59
14	0.50	−4.35	3.24
15	0.43	−4.24	3.17
16	1.59	−6.67	4.79
17	1.63	−6.70	4.78
18	1.60	−6.76	4.97
19	1.52	−6.81	5.24
20	1.55	−7.30	5.72

Fig. 7. A plot of log of capacity factors (k') *versus* the volume fraction of acetonitrile (φ) for isoprenoid benzoates 9–11. Lines through the experimental points were computer drawn using eqn. 3 and the parameters listed in Table IV.

disturbing the relative order in which the aromatic esters eluted. It should be emphasized, however, that capacity factors changed substantially as the C_{18}-columns aged and frequent calibrations with standard samples are essential.

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