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MOVEMENT OF COMPONENTS IN REVERSED-PHASE CHROMATOGRAPHY

II*. EIGENPEAKS IN REVERSED-PHASE CHROMATOGRAPHY WITH SILICA-BOUND HYDROCARBONACEOUS STATIONARY PHASES: EFFECT OF THE ELUITE STRUCTURE

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SUMMARY

Eigenpeaks of the eluent appear in the column effluent upon perturbing the equilibrium composition of the eluent at the column inlet, *e.g.*, by sample introduction. Eigenpeaks move down the column with concentration velocities which are the eigenvectors given by the characteristic solutions of the mass balance equations for the system. The eigenpeak height, as obtained by monitoring the refractive index of the effluent, is related to both the mobile phase composition and chemical structure of the eluite. Eigenpeaks obtained experimentally with eluites of different chemical structure and eluents commonly used in reversed-phase chromatography were examined in order to relate their height to solvation phenomena which occur in the column: (i) solvation of eluite by mobile phase components and (ii) change in solvation of stationary phase and eluite upon eluite binding. The eluite was dissolved in the eluent proper, and the eigenpeak height was found to be proportional to the mass of eluite over the range of sample loads investigated. The relationship observed between the molecular structure of eluite and the height of the attendant eigenpeaks is explained on the basis of the solvation model that takes into account the predominant different solvation effects by using slightly or strongly retained eluites. The results obtained with both binary and ternary eluents could be accounted for by the solvation model. Isomeric eluites which were eluted together in the chromatographic system under study showed significantly different eigenpeak patterns that could be used for identification. The results of this study show that solvation phenomena involving the eluite manifest themselves by the magnitude of the eigenpeak signals that convey a novel type of chromatographic information for possible use in the evaluation of solvation processes as well as for identification of components of elute pairs that are refractory to separation.

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INTRODUCTION

In liquid chromatography, not only the components of the sample to be separated but also constituents of the mobile phase may interact with the stationary phase and are therefore subject to the chromatographic process. However, the concentration of solvent components in the mobile phase is usually so high that they are not bound to the stationary phase according to a simple linear law. Consequently, in linear elution chromatography only the sample components that are conveniently called eluites will be eluted, *i.e.* move down the column with a velocity independent of their concentration whereas the movement of mobile phase components depends on their concentration according to their sorption isotherm[1]. As the liquid volume in the column is conserved, injecting the sample or otherwise perturbing the composition of the eluent containing N components, $N-1$ peaks that are particular to the movement of the mobile phase components are generated, in addition to the eluite peaks. We propose to call such peaks eigenpeaks*. They are characteristic of a given eluent composition, because their area and retention volume are given by the eigenvectors that are the solutions of the differential equations governing the movement of the eluent components through the column[2–4]. Mathematical treatment of the problem goes back to the seminal work by DeVault[2] and has been elaborated in the book by Helfferich and Klein[1]. Recently, Riedo and Kováts[4] gave a detailed analysis of the problem in the context of reversed-phase chromatography. These studies have shed light on the relationship between the sorption isotherm and retention volume of eigenpeaks and confirmed the dependence of the number of eigenpeaks on that of the mobile phase components. On the other hand, McCormick and Karger[5] have investigated eigenpeaks in reversed-phase chromatography with binary hydro-organic eluents. With slightly retained eluites, they attributed the eigenpeaks to displacement of the organic solvent at the stationary phase surface, whereas the "vacancy" peak obtained with strongly retained eluite was assumed to arise from an enhanced binding of the organic solvent component in the presence of the eluite.

Our interest has focused on the relationship between the height of the eigenpeaks, as measured by a refractive index detector, and the molecular structure of the perturbing eluites, because this relationship has received little attention in the literature. We believe that the study of eigenpeaks in high-performance liquid chromatography (HPLC) can shed light on the physico-chemical phenomena underlying the chromatographic process with multi-component eluents, as far as the specific solvation of the eluite and stationary phase is concerned. In this report, we present the results of an investigation concerning the relationship between the molecular structure of the eluite and the concomitant eigenpeaks obtained on varying the composition of the eluent in reversed-phase chromatography. The precision instrumentation available in HPLC facilitates the measurement of the pertinent parameters that yield a novel kind of chromatographic information.

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* When one or more eluites are present, their peaks should also be considered eigenpeaks in a rigorous analysis. Chromatographic practice, however, sufficiently justifies the term "eigenpeaks" being restricted to those peculiar to mobile phase components proper. The term "solvent peak" is preferentially used to denote peaks obtained on injecting labelled eluent components.

Eigenpeaks investigated here are generated by injecting into the eluent stream through the column a small amount of eluite, dissolved in the mobile phase proper. Eigenpeaks arise because the eluite and the attendant perturbation in mobile phase composition, moving down the column, perturbs the equilibrium distribution of the eluent components between the mobile and stationary phases. Consequently, solvent will pass to or from the stationary phase as the equilibrium is readjusted, and the associated change in mobile phase composition will be propagated through the column.

A closer view reveals that eigenpeaks are expected to arise from one or both of two sources under such conditions. The first source of eigenpeaks is the preferential solvation of eluite molecules by those of one mobile phase component in a binary eluent that causes the mobile phase in the surroundings of the eluite to become rich in one component and poor in another with respect to the bulk composition of the eluent. Then, a mobile phase zone depleted in the solvating species will move down the column and its composition will be determined by the appropriate sorption isotherm and the magnitude of the disturbance. The second cause leading to eigenpeaks is the dislodgement of solvent molecules bound to both the stationary phase and the eluite on binding of the eluite at the stationary phase surface. According to this view, therefore, specific solvation of the eluite and release of at least part of the solvation shell from both the stationary phase ligates and the eluite molecules may be responsible for the eigenpeaks that appear when one or more eluites are chromatographed when a multi-component eluent is used in columns packed with chemically bonded stationary phases.

In this study, we examined the relationship between the eigenpeak height and the molecular structure of the eluite in a system comprising silica-bound hydrocarbonaceous stationary phases and hydro-organic eluents, as well as a refractive index detector. After establishing the existence of such a relationship, it is used to assess the relative significance of the two phenomena postulated above.

THEORETICAL

Mobile phase solvation

Suppose 1 mole of an eluite is placed in a mobile phase containing n components, and it is solvated by component A the volume fraction of which is φ_A in the eluent. Under such circumstances, the volume fraction, f_A, of solvent A bound to the eluite is

$$f_A = v_{A,M}/\sum v_{j,M} \tag{1}$$

where $v_{A,M}$ and $v_{j,M}$ are the volumes of species A and j in the mobile phase and the summation is taken over all species. If f_A equals φ_A, the solvation of the eluite causes no eigenpeak.

In general, the volume of unbound component, i, in the bulk mobile phase, V_i, after solvation will be given by

$$V_i = V_{0,c}\varphi_i - v_{i,M} \tag{2}$$

where $V_{0,c}$ is the total mobile phase space in a control volume in the vicinity of the eluite and includes the volume of the solvation shell around the eluite in the mobile phase as it is moving down the column. The local composition, $\varphi_{i,c}$, of i in the bulk mobile phase is then given by

$$\varphi_{i,c} = \varphi_i - V_i/(V_{0,c} - \sum v_j) \tag{3}$$

and the change in composition is given by

$$\varphi_i = V_i/(V_{0,c} - \sum v_j) \tag{4}$$

Effect of retention on eigenpeak

When an eluite is bound to the stationary phase, each species, i, will be released to or taken up from the mobile phase, so that the mobile phase volume is composed as follows:

$$V = V_{0,c} + \sum v_{i,S} \tag{5}$$

where $v_{i,S}$ is the volume of i released from stationary phase. If there is a net movement of solvent species, i, *into* the stationary phase, $v_{j,S}$ will be negative.

If both mobile and stationary phase effects are observed, the change in the volume of i in the mobile phase is given by

$$V_i = V_{0,c}\varphi_i + v_{i,s} - v_{i,M} \tag{6}$$

The total volume, V, of mobile phase in the control element is

$$V = V_{0,c} + \sum v_{i,S} - \sum v_{i,M} \tag{7}$$

Combination of eqns. 6 and 7 yield the volume fraction of i in the control volume, $\varphi_{i,c}$:

$$\varphi_{i,c} = (V_{0,c}\varphi_{i,0} + v_{i,S} - v_{i,M})/(V_{0,c} + \sum v_{j,S} - \sum v_{j,M}) \tag{8}$$

The final change in composition, i, in the volume is

$$\varphi_i = \varphi_i - \varphi_{i,0} \tag{9}$$

If the initial disturbance is so small that the volumes released are small with respect to $V_{0,c}$, the magnitude of the disturbance is approximately given by

$$\varphi_i \approx v_{i,S}/V_{0,c} - v_{i,M}/V_{0,c} \tag{10}$$

Detector response

A refractive index detector was used to monitor the column effluent. The detector response to small changes in eluent composition is

$$\Delta R = \sum (\partial R/\partial \varphi_i)_{\varphi_j} \, \mathrm{d}\varphi_i \tag{11}$$

where $d\varphi_i$ is the difference in the concentration of i between the solution and a reference solution and ΔR is the detector response to the difference in refractive index, n. If the disturbance is large, the response is given by

$$\Delta R = \int (\partial R/\partial \varphi_i)_{\varphi_j} d\varphi_i \tag{12}$$

If v_i is small with respect to V, the response signal will be a linear function of the solvent volume, which will be directly proportional to the sample size. Therefore, the amplitude of the perturbation peak will be directly related to the mass of the sample injected for any given sample.

There are few analytical expressions that relate the refractive index to the composition of a mixture. However, the simplest[6], fairly accurate relationship between the refractive index and the composition of a multi-component system is

$$n = 1 + \rho \sum p_i(n_i - 1)/\rho_i \tag{13}$$

where ρ, p_i, ρ_i, and n_i are the density of the mixture, and the weight fraction, density and refractive index of species i, respectively. Thus,

$$\partial n/\partial \varphi_i = (\partial \rho/\partial \varphi_i) \sum p_i(n_i - 1)/\rho_i + (n_i - 1)/\rho_i \, (\partial p_i/\partial \varphi_i)_{\varphi_j} \tag{14}$$

in this simple model.

Thus, the change in the refractive index signal for a small perturbation in species i in the mobile phase composition will be a function of the initial mobile phase composition, as reflected in the density and the differentials of weight fraction and density with respect to φ_i, as well as in the density and refractive index of the neat solvent i. With small perturbations in the mobile phase, each term on the right-hand side of eqn. 14 should be nearly constant; thus, the refractive index change should be a linear function of the magnitude of the disturbance, $\delta\varphi_i$:

$$\Delta n = \sum (\partial n_i/\partial \varphi_i) \, \delta\varphi_i \tag{15}$$

Larger disturbances, which may be as small as 0.1% of the column volume, cannot be analyzed according to eqn. 15, inasmuch as $\partial n/\partial \varphi_i$ would not be constant. In such cases, the refractive index signal could be obtained by integration of eqn. 14 between the limits given as the initial and final mobile phase composition in the control volume.

In the following, we consider the general relationship between detector response and chromatographic retention factor. If no adsorption of eluite by the stationary phase occurs, the detector response, ΔR, arises solely from solvation effects in the mobile phase and is given by ΔR_M^0, which is evaluated by combination of eqns. 3 and 12 or 15. Likewise, if the eluite is irreversibly bound to the support, the detector response, given by ΔR_M^0, arises from stationary phase solvation effects alone. Solvation effects in both the mobile and stationary phase have to be considered, and the two kinds of contributions to the overall detector response have to be appropriately weighted according to the magnitude of retention, as measured by the retention factor k'. Retention of eluite reduces the amplitude of the detector signal predicted for eluite

solvation in the mobile phase alone. Therefore, the actual detector response due to mobile phase solvation effects, ΔR_M, will be smaller than ΔR_M^0 and proportional to the mass fraction of the eluite present in the mobile phase

$$\Delta R_M = \Delta R_M^0/(1 + k) \tag{16}$$

The mass fraction of eluite found in the stationary phase is $k/(1 + k)$ and, similarly, the actual detector response observed for stationary phase solvation effects can be expressed as

$$\Delta R = \Delta R_S^0 k/(1 + k) \tag{17}$$

where ΔR_S^0 is obtained by use of eqns. 5 and 12.

The overall signal is given as the sum of the two weighted terms by

$$\Delta R = \Delta R_M + \Delta R_S$$

$$= (\Delta R_M^0 + \Delta R_S^0 k)/(1 + k) \tag{18}$$

Relation to eluite structure

It follows from the nature of solvation that solvent molecules will be bound to different structural elements of the eluite molecule with different energies. For example, the hydroxyl group and methylene groups of a long-chain alkyl alcohol, dissolved in a hydro-organic mobile phase, would be expected to attract preferentially water and organic co-solvent, respectively. However, other polar groups in the eluite molecule would probably attract water to a different extent than does the hydroxyl group. Accordingly, the relationship between eluite structure and volume of the mobile phase species adsorbed per mole of eluite can be expressed as

$$\bar{v}_{M,i} = \sum v_{M,ij} N_j \tag{19}$$

where N_j is the number of structural groups, j, in the eluite, and $v_{M,ij}$ is the volume of mobile phase species, i, bound per mole of structural element, j. A similar relationship can be written for solvent release on binding to the stationary phase. If the eluite is equilibrated between the mobile and stationary phases, the volume change of solvent i per mole of eluite is given by

$$\bar{v}_{M,i} + \bar{v}_{S,i} = \sum (v_{M,ij} + k v_{S,ij})/(1 + k) \tag{20}$$

where $v_{S,ij}$ is the volume of solvent removed from the stationary phase per mole of structural element, j, of the eluite binding region. In the particular case, where a series of homologs is examined, the relation between detector response (eigenpeak height) and eluite structure is given by

$$\Delta R = (\Delta R_{M,0}^0 + N\Delta R_{M,i}^0)/(1 + k) + (\Delta R_{S,0}^0 + N\Delta R_{S,i}^0)k/(1 + k) \tag{21}$$

where the subscripts 0 and i refer to the parent molecule and the recurring N structural units, respectively.

EXPERIMENTAL

Equipment

An Altex Model 100A (Altex, Berkeley, CA, U.S.A.) solvent-metering system with a Rheodyne (Berkeley, CA, U.S.A.) Model 7105 sampling valve (syringe-loading sample injector) were used. The column effluent was monitored by a refractive index detector (Perkin-Elmer, Norwalk, CT, U.S.A.) and the chromatograms were obtained with a Model BD-41 strip-chart recorder (Kipp and Zonen, The Netherlands).

Three analytical columns were used: A 250 × 4.6 mm I.D. column, packed with 10-μm Partisil ODS-3, supplied by Whatman (Clifton, NJ, U.S.A.), and 250 × 4.6 mm I.D. Supelcosil LC-1 (5 μm) and 250 × 4.6 mm I.D. Supelcosil LC-8 (5 μm) columns, obtained from Supelco (Bellefonte, PA, U.S.A.).

The temperatures of the column and the flow cell of the detector were controlled by circulating water from a Lauda Model K-2/R constant-temperature bath (Messgerätewerk, Lauda, F.R.G.).

Materials

n-Alkylbenzenes in a homologous series (benzene to *n*-octylbenzene) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Trifluoroethanol was purchased from Merck (Darmstadt, F.R.G.), acetonitrile, tetrahydrofuran, methanol, 1- and 2-propanol, 1-butanol, cyclohexanol, acetic acid and sodium acetate from Fisher (Fair Lawn, NJ, U.S.A.) and absolute ethanol from Publicker Industries (Linfield, PA, U.S.A.). All other compounds used as eluites were purchased from Chem. Service (West Chester, PA, U.S.A.). Distilled water was prepared with a Barnstead unit in our laboratory.

Procedure

Samples were prepared by dissolving a weighed amount of each substance (about 50 mg) in an aliquot of the bulk mobile phase in order to obtain concentrations of solutes ranging from 0.05 to 1 *M*. Sample injection volumes of 5–25 μl were used. The flow-rate was set at 1.0 ml/min, and the column was equilibrated at 25.0°C (in some cases at 50°C) by passing at least 200 ml of mobile phase through it before sample injection. Retention times were evaluated from the positions of peak maxima on the chromatograms. Peak heights were used for quantitative analysis.

RESULTS AND DISCUSSION

Binary mobile phases

When the eluent contains two components, only one eigenpeak is generated. Eqns. 3, 10 and 12 predict a linear relationship between eigenpeak height and the amount of solvent component injected that brings about the underlying perturbation in the eluent composition. Experiments in which samples containing water and acetonitrile were injected into the acetonitrile–water (φ_{ACN} = 0.5) mobile phase in a Partisil ODS-3 column have shown that the eigenpeak height obtained with the differential refractive index detector was almost linear when φ_{ACN} in the sample ranged from 0.4 to 0.6.

A similar linear dependence of the eigenpeak height on the sample size was

observed after injection of various eluites, dissolved in the mobile phase proper. The results obtained with *n*-alkanols in a chromatographic system consisting of a Partisil ODS-3 column and acetonitrile–water (φ_{ACN} = 0.5) as the mobile phase, are given in Table I. In the case of 1-propanol as the eluite, both the concentration of the eluite in the solvent having the same composition as the mobile phase and the sample volume were varied. For other eluites, only the sample volume was varied. The third column in Table I shows the ratio of the eluite peak height to the corresponding eigenpeak height. In the fourth column the number of micromoles of acetonitrile released per micromole of eluite injected are given. These values were calculated from the known amount of eluite injected and the amount of acetonitrile that generated the same eigenpeak height as the eluite proper, when injected in a separate experiment. The latter values were obtained from a quasilinear plot of the eigenpeak height against the amount of acetonitrile injected. It should be noted that another, but likely to be incorrect, interpretation of these numbers is that they are proportional to the number of micromoles of water taken up by the stationary phase after injection of acetonitrile into the column. The proportionality factor would be then 1/2.77.

Inspection of the standard errors of the data in Table I suggests that over a 5–20-fold range of sample size the eigenpeak height is directly proportional to the amount of sample injected. It does not follow, however, that different eluites of equal volume or mass must give peaks of the same height, as their solvation and binding to the stationary phase may be different. Inspection of Table I shows, indeed, that the molar proportionality factors (in the fourth column) have characteristic values for each eluite. As the magnitude of the factor is related to the molecular structure of the eluite, if eqn. 21 holds, these data convey a new type of chromatographic information and may be useful in the identification of eluites.

The eigenpeak heights per mole of an injected eluite are plotted against the logarithmic retention factors in Fig. 1. The eigenpeak height is expressed as the number of moles of acetonitrile that must be injected in order to obtain the same eigenpeak height obtained per mole of eluite. It shows in a general way that the molar

TABLE I

RATIO OF ELUITE PEAK HEIGHT TO EIGENPEAK HEIGHT AND THE NUMBER OF MOLES OF ACETONITRILE RELEASED TO THE MOBILE PHASE PER MOLE OF *n*-ALKANOLS USED AS THE ELUITE

The retention factor, k', and number of experimental determinations, n, are also given. The mobile and stationary phases were acetonitrile–water (φ_{ACN} = 0.50) and Partisil ODS-3, respectively.

Eluite	k'	*Eluite:ACN ratio according to*		n	*Sample*	
		Peak heights	*Moles injected*		*Volume (μl)*	*Eluite concentration (%, v/v)*
1-Propanol	0.086	4.75 ± 0.21	2.19 ± 0.05	6	20	0.5–10
		4.62 ± 0.18	2.31 ± 0.12	7	2.5–25	10
1-Butanol	0.120	8.93 ± 0.15	1.68 ± 0.05	5	5–25	9
1-Pentanol	0.182	15.13 ± 0.08	1.24 ± 0.07	5	5–25	9
1-Hexanol	0.263	21.80 ± 0.84	0.76 ± 0.03	5	5–25	9
1-Heptanol	0.298	33.8 ± 4.27	0.56 ± 0.08	4	5–25	16

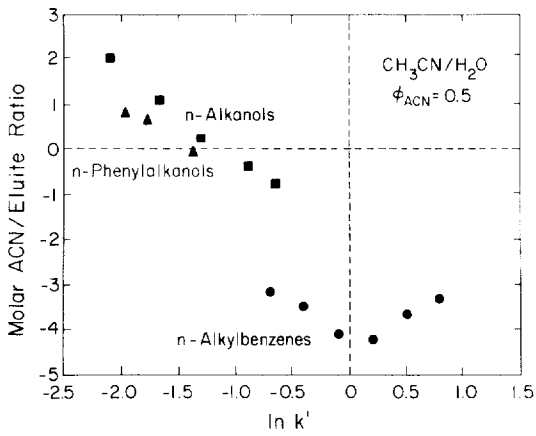


Fig. 1. Relationship between the normalized eigenpeak height and the logarithmic retention factor for members of three homologous series. The normalized eigenpeak height is expressed as the acetonitrile to eluite molar ratio giving the number of moles of acetonitrile that, when injected alone, yields the same detector response as the injection of 1 mole of eluite. The homologous series are *n*-alkanols (■), *n*-phenylalkanols (▲) and *n*-alkylbenzenes (●). The mobile and stationary phases are acetonitrile water (φ_{ACN} = 0.50) and Supelcosil LC-1, respectively.

eigenpeak height changes monotonically with ln k for members of a homologous series, if the retention factors for all members of the series are less than unity. However, series of homologs that have retention factor values both above and below unity, *e.g.*, alkylbenzenes, manifest a parabolic plot, so that two different eluites of such series may have very nearly equal responses. Both patterns can be understood in light of eqn. 20.

In the first case, low retention factors imply that these eluites are found mostly in the mobile phase. Therefore, the eigenpeaks are expected to arise from solvation effects in the mobile phase with only minor contributions from stationary phase interactions. Therefore, the corresponding eigenpeak height can be estimated by eqn. 16. The magnitude of the eluite solvation by the less polar solvent component of the eluent is expected to parallel that of its retention by the non-polar stationary phase in reversed-phase chromatography. Consequently, the greater the retention factor of an eluite, the stronger will be its solvation by acetonitrile in the acetonitrile–water eluent and, concomitantly, the depletion of acetonitrile in the bulk solvent, which is tantamount to an increase in the bulk water concentration. Therefore, if positive values of the term $\Delta R^0_{M,0}$ in eqn. 16 are taken to mean an increase in the acetonitrile concentration in the mobile phase, then for a homologous series $\Delta R^0_{M,0}$ should decrease with increasing retention factor. This effect would manifest itself in an eigenpeak that corresponds to the injection of water.

On the other hand, when an eluite is bound to the stationary phase, solvent bound to the stationary phase or the eluite proper may be released to the mobile phase, or both effects may occur. In either case, the composition of the released solvent is likely to be richer in the organic component of the hydro-organic eluent than the eluent itself. Consequently, we may expect an eigenpeak that corresponds to the injection of the organic solvent component. When the eluite is bound to the hydrocarbonaceous stationary phase via the same regions of the molecule as those

preferentially solvated by the less polar solvent, such solvent molecules are likely to be lost to the mobile phase. Therefore, when all retention factors are greater than unity, the increase in eigenpeak height with the retention factor for members of such a homologous series is believed to reflect the dislodgement of a larger number of the less polar solvent molecules when the eluite is bound to the stationary phase. For the data presented in Fig. 1 this corresponds to the release of more acetonitrile and, as a consequence, this results in an increase in the height of positive eigenpeaks after the injection of alkylbenzenes with increasing carbon number.

Retention factor values equal to unity imply that equal mass fractions of the eluite are to be found in the mobile and stationary phases. Therefore, if the retention factor value is close to unity, eigenpeak heights will be due in significant proportions to both mobile phase and stationary phase effects. Inasmuch as they are opposite in sign, according to the above considerations, and each increases in absolute magnitude with an increase in the retention factor, the peak height would decrease with increases in ln k or carbon number, owing to increases in solvation with carbon number until stationary phase effects begin to predominate, so that the peak height increases with an increase in ln k.

However, an alternative model of binding can also be constructed with opposite predictions. If an amphiphilic solvent component of the eluent is so tightly bound to the apolar stationary phase that it cannot be displaced, then retention of the eluite would have to occur by interactions between the *polar* functions of the solvent molecules, *e.g.*, –OH of alcohols or –CN of nitriles, and those of the eluite molecule or its solvate shell. In this case, no net solvent dislodgement from the stationary phase is expected to give rise to an eigenpeak characteristic of the organic solvent component. Further, the only solvent released from the solvate shell would be water. The height of the eigenpeak would be solely determined by the perturbation of the eluent composition, caused by the solvation of the eluite, and it would, therefore, decrease monotonically in the same fashion as the amount of water released would increase, with an increase in ln k. However, this prediction, is not supported by the data obtained with alkylbenzene eluites and, therefore, this model for the retention mechanism is not defensible.

Although the model predicts that if ln k is less than zero the eigenpeak response of the detector will be due chiefly to mobile phase effects, some influence of the stationary phase is found. The data in Fig. 2 show that eigenpeak heights obtained with *n*-alkanols on two stationary phases, Partisil ODS-3 and Supelcosil LC-1, but the same mobile phase are different. In addition, their rates of change with changing ln k are different. This apparent discrepancy, however, can be understood if the Partisil ODS-3 binds acetonitrile stronger than does Supelcosil LC-1. In that case, on binding more acetonitrile molecules per eluite molecule will be dislodged from Partisil ODS-3 than from Supelcosil LC-1 and the difference in eigenpeak heights obtained with the two columns will become greater as the mass fraction of bound eluite becomes greater. This implies that the divergence in peak heights observed on the two columns should increase as ln k increases and that the eigenpeak height obtained on the two systems should become identical as the retention factor approaches zero. That pattern is observed in Fig. 2. It should be noted that even for the least retained species presented in Fig. 2, the mass fraction bound to the stationary phase exceeds 10%.

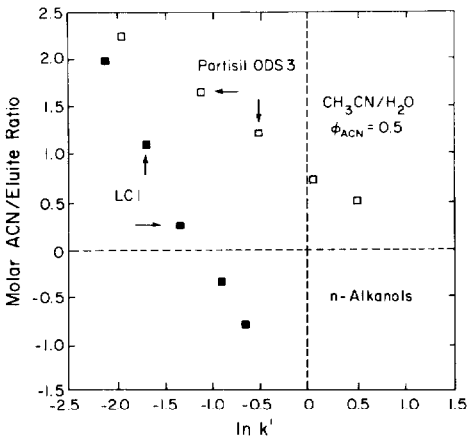


Fig. 2. Relationship between normalized eigenpeak height and logarithmic retention factor for data obtained on two stationary phases. The eluents were alkanols and the mobile phase was acetonitrile–water (φ_{ACN} = 0.5). The stationary phases used were Supelcosil LC-1 (■) and Partisil ODS-3 (□). The normalized eigenpeak height is expressed as the number of moles of organic co-solvent that, when injected alone, yields the same detector response as the injection of 1 mole of eluite.

In Fig. 3, the normalized height of eigenpeaks obtained after injection of alkylbenzene eluites into methanol–water (φ_{MeOH} = 0.7) and acetonitrile–water (φ_{ACN} = 0.5) mobile phases in a methylsilica (Supelcosil LC-1) column are plotted against the logarithmic retention factor. Whereas the results conform to the general pattern already noted, the data points fall into one of two families, according to the mobile phase employed. In light of eqn. 20, the differences observed with the two mobile phases may arise from three sources, as follows.

First, the changes in refractive index on a small change in composition,

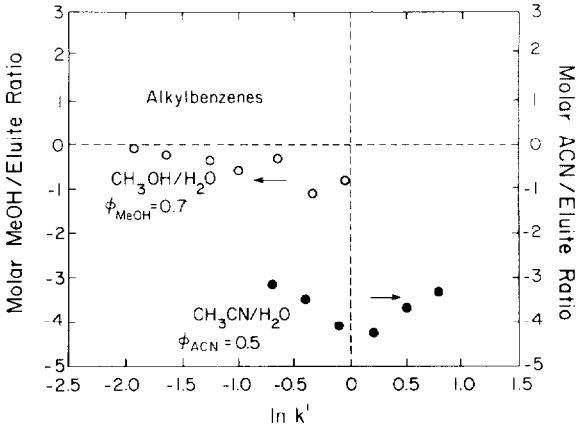


Fig. 3. Relationship between normalized eigenpeak height and logarithmic retention factor for data obtained in two mobile phases. The eluites were alkylbenzenes and the mobile phases were methanol–water (φ_{MeOH} = 0.7) (○) and acetonitrile–water (φ_{ACN} = 0.5) (●). The stationary phase was Supelcosil LC-1. The normalized eigenpeak height is expressed as the number of moles of organic co-solvent that, when injected alone, yields the same detector response as the injection of 1 mole of eluite.

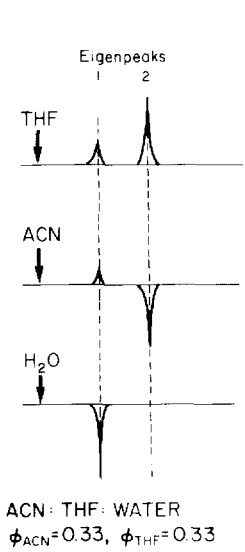
$dn/d\varphi_{ACN}$ and $dn/d\varphi_{MeOH}$, are different for the two mobile phases. Second, the eluent strength of 70% aqueous methanol is greater than that of 50% aqueous acetonitrile so that the retention factors of the eluites under investigation are greater with the latter mobile phase. As a result, the coefficient $(1 + k)$ in the denominator of eqn. 20 is greater for data obtained with aqueous acetonitrile as the mobile phase. Third, the composition change in the eluent on solvation of the stationary phase and/or the eluite proper is expected to be different for two hydro-organic mobile phases having different organic modifiers that do not have the same solvating properties for both the stationary phase and eluites in question. The excess of organic co-solvent bound by hydrocarbonaceous stationary phases, *i.e.*, the difference between its concentrations at the stationary phase and in the bulk eluent, can be determined directly[4]; for methanol and acetonitrile it is found to reach a maximum somewhere between $\varphi = 0.3$ and 0.5. As the maximum volume of bound solvent is significantly greater with acetonitrile than with methanol, the dislodgement of more solvent molecules is expected on binding of the eluite by the stationary phase from an aqueous mobile phase with $\varphi_{ACN} = 0.5$ than with $\varphi_{MeOH} = 0.7$. By a similar argument, the perturbation of eluent composition due to solvation of the eluite should also be greater when $\varphi_{ACN} = 0.5$ than when $\varphi_{MeOH} = 0.7$ in the binary hydro-organic eluents.

Ternary mobile phases

Number of peaks and interpretation. As mentioned before, eigenpeaks cannot represent a perturbation in the concentration of only one mobile component but they are due to perturbations in the concentrations of all components. The amplitude of each peak can be evaluated, in principle, by solving the governing equations with appropriate boundary conditions. The Laplace transform method used by Reilley *et al.*[7], together with eqns. 3 and 15, offers, for example, a method for predicting the magnitude of the eigenpeaks.

Here we examine the two perturbation peaks obtained with the eluent containing acetonitrile–tetrahydrofuran–water ($\varphi_{ACN} = 0.33$, $\varphi_{THF} = 0.33$) after the injection of concentration pulses of each mobile phase component individually. As can be seen in Fig. 4, the first peak engendered by injection of tetrahydrofuran (THF) or acetonitrile (ACN) is relatively small and positive, whereas after the injection of water the first eigenpeak is large and negative. The second eigenpeak is relatively large and positive after injection of tetrahydrofuran, large and negative after acetonitrile, and undetectable after water. The composition change in the mobile phase manifested by the eigenpeak can be inferred from its sign and magnitude. For example, pulses rich in water result in a relatively large negative first eigenpeak in the differential refractometer detector and after tetrahydrofuran in a large positive second eigenpeak. Nonetheless, for convenience it is tempting to regard the first peak as a "water peak" and the second as an "organic solvent" peak, as large first and second eigenpeaks are obtained after the injection of water and organic modifier, respectively.

In light of earlier results, uptake of organic co-solvent is likely to accompany dislodgement of water when an eluite is bound to the stationary phase. If this occurs, a plot of the height (or area) of the first eigenpeak against that of the second eigenpeak should be linear. Such plots of eigenpeak data, obtained with a tetrahydrofuran–acetonitrile–water mixture as the mobile phase and with the homologous series of 1-alkanols, alkylbenzenes and fatty acid methyl esters as the eluites



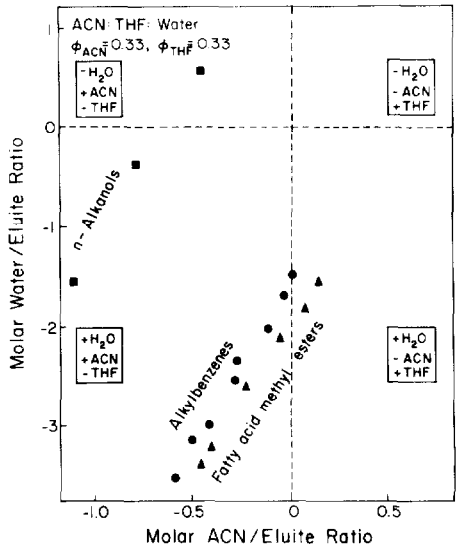


Fig. 4. Eigenpeak pattern arising from injection of each component of the mobile phase individually. The sample injected was slightly enriched in the component indicated, and the concentration ratio of the other two components was fixed. Mobile phase: acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33, φ_{THF} = 0.33).

Fig. 5. Relationships between the normalized first and second eigenpeak heights for members of three homologous series with acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33, φ_{THF} = 0.33) as the mobile phase. The normalized eigenpeak heights are expressed as water to eluite and acetonitrile to eluite molar ratios expressing the number of moles of water and acetonitrile that, when injected alone, yield the same detector response as the injection of 1 mole of eluite.

are depicted in Fig. 5. It can be seen that the data for individual series fall on apparently parallel straight lines. All retention factors exceed unity so that response factors can be described by eqn. 17. The slopes of the plots in Fig. 5 suggest that the amount of water and acetonitrile released to the mobile phase on binding of homologous eluites increase as their carbon number increases. An alternative explanation for the observed behavior is that water dislodgement upon eluite binding by the stationary phase is accompanied by uptake of tetrahydrofuran. This is in agreement with the observation that the peak after excess tetrahydrofuran becomes smaller with increase in excess water.

The eigenpeak pattern obtained with *n*-alkanols, fatty acids esters and alkylbenzenes was investigated by using mobile phases composed of acetonitrile–2-propanol–water (φ_{ACN} = 0.4, φ_{IPA} = 0.2) and acetonitrile–trifluoroethanol–water (φ_{ACN} = 0.5, φ_{TFE} = 0.1). The results are plotted according to the above scheme in Fig. 6. It can be seen that the results obtained with such eluites and ternary mobile phases exhibit a less regular behavior and are not subject to a straightforward interpretation.

The results shown in Fig. 6A for the first eluent suggest that the amount of water released to the mobile phase upon eluite binding by the stationary phase, as indicated by the height of the first eigenpeak, is independent of the carbon number of the alkylbenzene and alkanol homologs or depends only slight on it. On the other hand, the height of the second eigenpeak appears to depend on the eluite carbon

number for alkylbenzenes and alkanols. Thus, the concentration ratio of 2-propanol and acetonitrile appears to be fairly constant in the appropriate eigenpeaks engendered by such eluites. In contradistinction, with fatty acid methyl esters, no net change in water concentration in the mobile phase seems to occur upon eluite binding by the stationary phase, as seen from the invariance of the normalized height of the first eigenpeak in Fig. 6A. Significant changes in solvation by 2-propanol and acetonitrile are suggested by the rather strong dependence of the appropriately normalized height of the second eigenpeak on the carbon number of the eluite.

Similar patterns are observed in the results obtained by using the other mobile phase, composed of acetonitrile–trifluoroethanol–water, as shown in Fig. 6B. With all three homologous series, as the carbon number of the eluite increases, more water is released to the mobile phase on eluite binding by the stationary phase, as suggested by the decreasing height of the first eigenpeak. On the other hand, no regular changes in solvation by trifluoroethanol and acetonitrile are evident from the slight and irregular dependence of the normalized height of the second eigenpeak on the carbon number of the eluite.

Eluite structure and eigenpeak height. With alkylbenzenes and fatty acid methyl esters, the heights of both the first and second molar eigenpeaks of the ternary eluent, as obtained with the differential refractometer detector, show a linear dependence on the number of methylene groups in the eluite molecules, as depicted in Fig. 7. This observation prompted us to analyze the data presented in Figs. 5 and 6 by use of eqn. 20, in which N was taken as the number of methylene groups in the eluite, with the results shown in Table II.

Inspection of Table II reveals that in most cases the contributions to the differential refractometer response associated with the eigenpeaks are opposite in sign

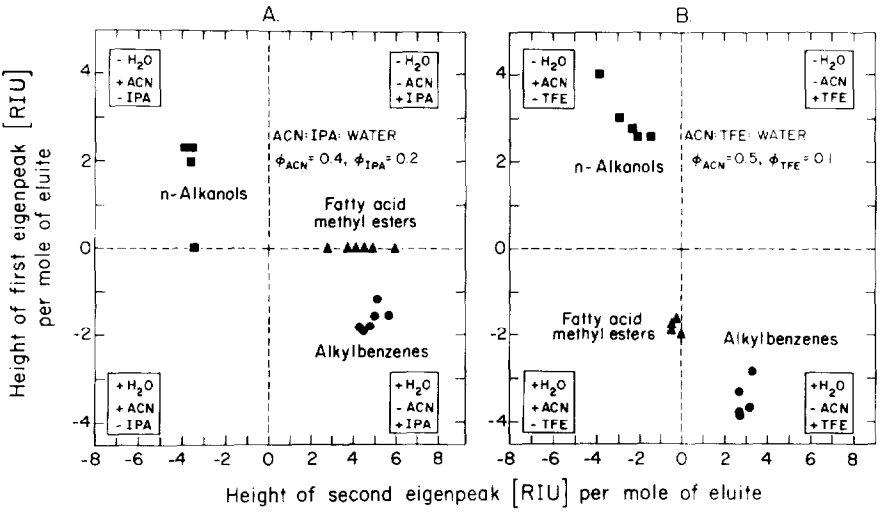


Fig. 6. Relationship between first and second eigenpeak heights in two mobile phases for three homologous series. The eigenpeak heights are expressed as refractive index units (RIU) per mole of eluite injected. The homologous series are *n*-alkanols (■), fatty acid methyl esters (▲) and alkylbenzenes (●). Mobile phases: (A) acetonitrile–2-propanol water (φ_{ACN} = 0.40, φ_{IPA} = 0.20) and (B) acetonitrile–trifluoroethanol water (φ_{ACN} = 0.50, φ_{TFE} = 0.10). Stationary phase: Supelcosil LC-8.

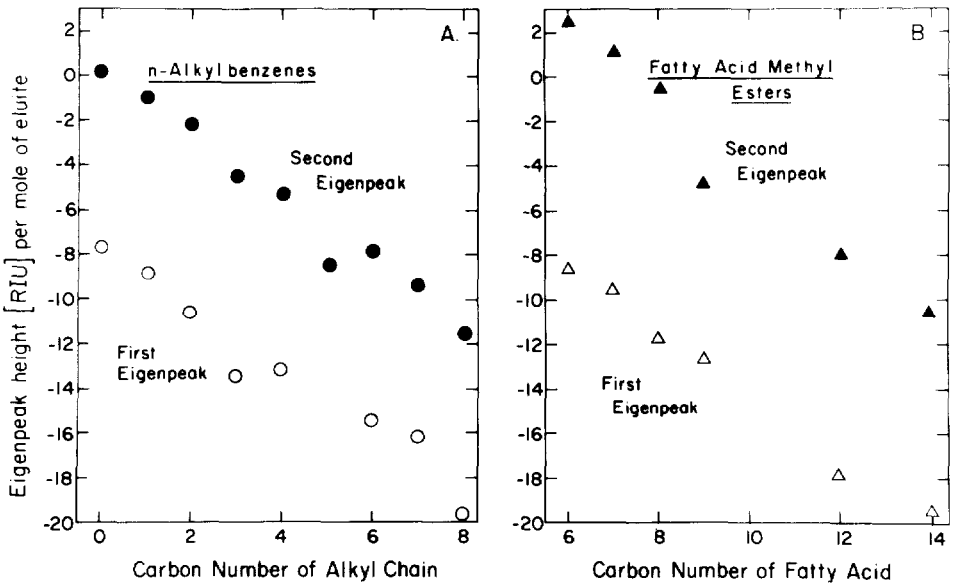


Fig. 7. Relationship between eigenpeak heights and carbon number in the molecule for two homologous eluite series. The eigenpeak heights are expressed as refractive index units (RIU) per mole of eluite, injected for both the first (open symbols) and second eigenpeaks (solid symbols). The homologous series are alkylbenzenes (A) and fatty acid methyl esters (B). Mobile phase: acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33, φ_{THF} = 0.33). Stationary phase: Supelcosil LC-8.

for stationary and mobile phase effects. Where this does not seem to hold true, one of the terms is small and probably experimentally indistinguishable from zero, in agreement with observations made with the binary eluents, noted above. As expected, the values obtained for different core groups of the homologous series are different, and the magnitude of the detector response to the eigenpeaks strongly depends on the mobile phase composition. For the most part, the sign of the eigenpeak for different headgroups is the same in each mobile phase. More interesting results are seen in the acetonitrile–2-propanol–water system with alkylbenzenes or fatty acid methyl esters, because the response of the refractive index detector is opposite in sign to that observed with alkanols. This effect can be ascribed to differences between the solvation shells of an alcoholic hydroxyl group and those of the aromatic or ester moieties.

Eigenpeaks with isomeric eluites. In some chromatographic systems the retention factors of isomers are nearly identical, and the lack of selectivity renders the identification of isomeric eluites difficult. The preceding results suggest that the relationship between the height of the eigenpeaks generated by the injection of eluites dissolved in the mobile phase proper and their molecular structure is different from the dependence of the retention factor on the molecular structure[8]. Therefore, eigenpeak patterns of the isomeric pairs *o*- and *p*-xylene, 2,4,5- and 2,4,6-trichlorophenol, *o*- and *m*-tolyl acetate, 1,2-, 1,3- and 1,4-dichlorobenzene and *o*- and *p*-diethoxybenzene as well as hexanols were investigated in the system acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33, φ_{THF} = 0.33). The detector responses, normalized to micromoles of eluite injected, and the ratio of peak heights are presented in Table III.

TABLE II

DIFFERENTIAL REFRACTOMETER DETECTOR RESPONSES FOR EIGENPEAKS GENERATED BY HOMOLOGOUS ELUITES IN VARIOUS MULTI-COMPONENT ELUENTS AND ASSOCIATED WITH SOLVATION EFFECTS IN THE MOBILE AND STATIONARY PHASES

Respective methylene contribution, $\Delta R_{M,CH_2}$ and $\Delta R_{S,CH_2}$, and core contributions, $\Delta R_{M,core}$ and $\Delta R_{S,core}$, were evaluated by use of eqn. 16 for various homologous series. With ternary eluents, the contributions were calculated for both the first and second eigenpeaks.

*Mobile phase**	*Eluite*	$\Delta R_{M,CH_2}$ *(mobile)*	$\Delta R_{S,CH_2}$ *(binding)*	$\Delta R_{M,core}$ *(mobile)*	$\Delta R_{S,core}$ *(binding)*	*Eigenpeak*
ACN–water (1:1)	Alkanols	−0.276 ± 0.126	1.59 ± 0.126	4.5 ± 0.5	−18 ± 1.6	Only one
	Alkylbenzenes			1.6 ± 0.66	−13 ± 1	
	Phenylalkanols			−13.5 ± 4.4	3 ± 0.8	
MeOH–water (7:3)	Alkylbenzenes	0.13 ± 1.14	0.60 ± 2.65	1.1 ± 3.9	− 7.3 ± 27	Only one
ACN–TFE–water (5:1:4)	Alkylbenzenes	−0.03 ± 0.50	0.42 ± 0.09	13 ± 1.62	− 1.6	First
	Esters			13.7 ± 2.7	− 4.0 ± 1.2	
ACN–THF water (5:1:4)	Alkanols	−0.05 ± 0.31	0.58 ± 0.06	0	7.3 ± 0.7	Second
	Alkylbenzenes			9.8 ± 1.1	0	
	Esters			− 3.5 ± 1.7	5.6 ± 0.7	
ACN–IPA water (4:2:4)	Alkanols	−0.35 ± 2.39	0.26 ± 0.88	9.4 ± 3.7	1.5 ± 3.7	First
	Alkylbenzenes			− 4.2 ± 14	− 0.1 ± 6.8	
	Esters			− 1.6 ± 3.5	3.1 ± 11.2	
ACN–IPA–water (4:2:4)	Alkanols	−0.33 ± 1.97	−0.31 ± 0.72	− 7.1 ± 3.0	0.5 ± 9.4	Second
	Alkylbenzenes			4.2 ± 11.8	6.1 ± 5.6	
	Esters			10.2 ± 2.9	5.2 ± 9.2	
ACN–THF water (1:1:1)	Alkanols	0.06 ± 0.35	0.28 ± 0.05	1.4 ± 9.4	− 0.6 ± 0.8	First
	Alkylbenzenes			1.8 ± 0.6	0.8 ± 0.22	
	Esters			1.4 ± 2.4	0	
ACN–THF water (1:1:1)	Alkanols	0.03 ± 0.07	0.06 ± 0.01	−11.2 ± 0.1	1.3 ± 0.2	Second
	Alkylbenzenes			− 0.1 ± 0.1	0.12 ± 0.04	
	Esters			− 0.5 ± 0.5	0	

* ACN = acetonitrile; MeOH = methanol; TFE = trifluoroethanol; IPA = 2-propanol; THF = tetrahydrofuran.

TABLE III

MOLAR REFRACTIVE INDEX RESPONSES OF THE FIRST AND SECOND EIGENPEAKS OBTAINED WITH ISOMERIC PAIRS OF ELUITES BY USING A TERNARY ELUENT

The retention volume of the eluite and the absolute value of the peak height ratio of the first and second eigenpeaks are also given. The data were obtained with water–acetonitrile–tetrahydrofuran (φ_{ACN} = 0.33, φ_{THF} = 0.33) as the mobile phase and Supelcosil LC-8 as the stationary phase.

Eluite	V_R, *retention volume of eluite*	ΔR		*Eigenpeak height ratio*
		First eigenpeak	*Second eigenpeak*	
o-Xylene	4.76	−11.23	−1.92	0.17
m-Xylene	4.87	−10.34	−2.28	0.22
2,4,5-Trichlorophenol	4.46	−11.59	−20.90	1.80
2,4,6-Trichlorophenol	4.56	−13.20	−17.96	1.36
o-Tolyl acetate	3.53	− 8.91	6.91	0.78
m-Tolyl acetate	3.38	− 7.70	1.98	0.26
Cyclohexanol	3.02	11.27	4.47	0.39
1-Hexanol	3.41	7.16	0	0
2-Ethylbutanol	3.40	5.70	0	0
4-Methylpentanol	3.31	7.09	0	0
1,2-Dichlorobenzene	4.66	−11.03	−6.11	0.55
1.3-Dichlorobenzene	5.02	−13.53	−7.83	0.58
1,4-Dichlorobenzene	4.97	−14.67	−7.62	0.52
o-Diethoxybenzene	3.89	−11.65	−4.89	0.40
p-Diethoxybenzene	4.15	−13.15	0	

The results in Table III show that eigenpeaks by generated by isomeric eluites have distinctly different heights, as measured by the differential refractometer detector. In each case, the normalized height of both the first and second eigenpeaks, particular to a given isomeric eluite pair, are different. Further, the ratio of the first and second eigenpeak heights for a given eluite is usually not the same for isomers.

TABLE IV

SELECTIVITY AND RATIO OF EIGENPEAK HEIGHTS FOR SELECTED PAIRS OF ISOMERIC ELUITES

The mobile phase was acetonitrile tetrahydrofuran water (φ_{ACN} = 0.33, φ_{THF} = 0.33) and the stationary phase was Partisil ODS-3.

Eluite pair	*Relative retention* α	*Ratio of eigenpeak heights for eluite pairs*	
		First	*Second*
o-Xylene–*m*-xylene	1.036	0.925	1.189
2,4,5-Trichlorophenol–2,4,6-trichlorophenol	1.036	1.139	0.859
o-Tolyl acetate–*m*-tolyl acetate	1.089	1.128	3.490
1-Hexanol–2-ethylbutanol	1.001	1.256	0/0

Thus, it is possible, in principle, to distinguish between isomers on the basis of absolute eigenpeak height or the ratio of eigenpeak heights.

The results support the view that eigenpeak height ratios can facilitate the measurement of the difference in the eigenpeak patterns for isomeric eluites. Results are shown in Table IV for four eluite pairs for which the selectivity of the chromatographic system employed was low, as seen from the relative retention values. The ratios of the heights of both the first and second eigenpeaks generated by the two isomeric eluites are distinctly different. The results suggest that the eigenpeaks convey a new type of chromatographic information that may be used to facilitate identification or quantitation of sample components under well controlled experimental conditions.

The eigenpeak pattern generated upon injection of ethylbenzene and 1,4-dichlorobenzene, which have different chemical structures but very similar retention volumes, when acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33; φ_{THF} = 0.33) is used as the mobile phase, is depicted in Fig. 8. It can be seen that the peak height ratio of the second eigenpeak to that of the first eigenpeak is much smaller for ethylbenzene than for 1,4-dichlorobenzene, although the separation factor, α, is practically unity for these eluites in the chromatographic system under investigation. Thus, even if their retention is essentially the same, when each is dissolved in the mobile phase and injected into the column separately, ethylbenzene and 1,4-dichlorobenzene, could be identified on the basis of the height ratio of the two eigenpeaks. If the sample contains both components, dissolved in the eluent proper, the ratio of their concentrations can be determined from the eigenpeak height ratio, provided no other substances are present in the sample. In such a case, the absolute concentration of each component can also be calculated from the height or area of the eluite peak and the ratio of the concentrations, as obtained from the eigenpeak height ratio. Of course,

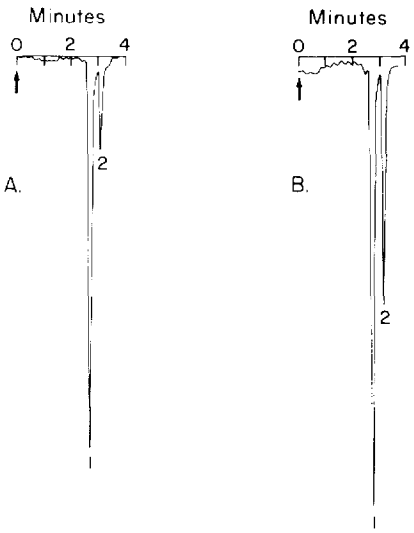


Fig. 8. Eigenpeak pattern obtained with two eluites eluted closely together: (A) ethylbenzene and (B) 1,4-dichlorobenzene. Acetonitrile–tetrahydrofuran–water (φ_{ACN} = 0.33, φ_{THF} = 0.33) and Supelcosil LC-8 were the mobile and stationary phases, respectively.

the calculation is more complicated if the detector response per mole of eluite is not the same for the two substances. It is likely that this approach to eluite identification by the characteristics of the eigenpeaks can also be extended to other groups of species eluted closely together.

The above method may be applied to samples that contain other components in addition to the eluites in question. In such a case, the peak consisting of the two eluites, and only that peak, is directed by use of column switching to a second column, which is operated with the same mobile phase as the first. The eigenpeak pattern generated in the second column is unique to the eluites of interest and can facilitate identification. The usefulness of this approach is, of course, limited when the second chromatographic system can be chosen so that the two components are readily separated.

CONCLUSIONS

Characteristic eigenpeaks are perturbations of the mobile phase composition and can arise from eluite interactions with both the mobile and stationary phases. A simple model was developed to relate the eigenpeak heights, as obtained by the differential refractometer detector to the retention factor and the molecular structure of the eluite. The resulting equations were found to describe the experimentally observed relationship between the eigenpeak height and retention factor. The results suggest that it may be possible to use eigenpeak patterns generated by homologous eluites to gain quantitative information on solvation of eluites in the mobile phase as well on solvation effects associated with reversible binding of the eluite by the stationary phase in the course of the chromatographic process. Thus, this approach may offer an opportunity to throw light on the molecular events involved in the chromatographic retention process.

The simple model for the relationship between the magnitude of the eigenpeak and specific solvation effects was extended through the assumption that at a given mobile phase composition the solvation of a particular structural group in the eluite molecule is independent of the number of such groups in the molecule. This would mean that solvation effects for homologous eluites change in a regular fashion with the number of structural units. Experimental data corroborated this prediction and allowed us to quantify specific solvation effects on the basis of refractive index changes associated with the eigenpeak in the column effluent. The effect is conveniently measured by normalizing eigenpeak height, which expresses the number of moles of eluent component that cause the same eigenpeak height when injected alone as does injection of 1 mole of eluite.

The eigenpeak pattern obtained in the binary or ternary mobile phases investigated here was found to be peculiar to the chemical structure of the eluite molecules. A number of methods were developed for presenting eigenpeak data to facilitate exploitation of this previously ignored chromatographic information, not only for the examination of specific solvation effects attendant on the chromatographic process but also for identification and quantitation of sample components eluted together.

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