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MOVEMENT OF COMPONENTS IN REVERSED-PHASE CHROMATOGRAPHY

I. MOBILE PHASE SPACE WITH MULTI-COMPONENT ELUENTS

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SUMMARY

The retention volume of a peak that is neither retained nor excluded from the mobile phase space in the column is required for determination of retention (capacity) factor and related thermodynamic quantities. In liquid chromatography with multi-component eluents, however, the mobile phase space is theoretically indeterminate owing to interaction between the individual components and the stationary phase and therefore definition and measurement of the mobile phase necessitates that an appropriate convention be found. Here the following four conventions are examined: (i) "all mobile phase components are present in the solvation layer"; (ii) "no solvation layer exists"; (iii) "one given eluent component is not present in the solvation layer"; and (iv) "the most weakly bound solvent component is not present in the solvation layer". Whereas all conventions appear to be acceptable for a thermodynamic description of the interaction between mobile phase components and the stationary phase in most cases, they yield different values of retention factors and associated thermodynamic properties for eluites. Furthermore, the use of some conventions can give rise to practical problems, *e.g.*, experimental determination of void volume by use of the first convention is ill-defined in general and the second and third conventions can lead to negative values for the mobile phase space. The present work suggests that the fourth convention is free of these problems and provides retention factor values which are more appropriate for use in liquid chromatography than those obtained by the other conventions.

INTRODUCTION

Retention factors, k', are probably the most important experimental data in chromatography, because they are used as a dimensionless measure of retention for the calculation of band velocities, relative retention and other parameters. As dimensionless measure of the free-energy change under certain conditions, retention factors are also employed for the calculation of thermodynamic properties, such as

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enthalpy or entropy, associated with the chromatographic process. The evaluation of retention factors requires a knowledge of the chromatographic void volume of the column, *i.e.*, the mobile phase space that is explored by the eluite in the course of the chromatographic process[1]. Ideally, the column void volume is obtained as the elution volume of an inert "homomorph" of the eluite that explores the same mobile phase as the eluite but does not interact with the stationary phase.

In liquid chromatography, numerous difficulties are encountered in accurately mapping the appropriate mobile phase space in the column. Generally, no completely inert tracer substance is available that would qualify as a suitable homomorphic probe for the measurement of the relevant mobile phase hold-up volume. Nonetheless, as long as the pore dimensions of the stationary phase matrix are much greater than the molecular size of the eluite(s), it may be satisfactory to use the actual mobile phase space as the chromatographic void volume.

When the eluent contains only a simple component, the volume of mobile phase space can be measured in a straightforward manner, *e.g.*, by weighing the column filled with a solvent of known density[2–4] or by the elution time of a tagged solvent species[2,5,6]. However, the mobile phase in liquid chromatography is more often composed of more than one component, and in such cases, both the definition and the measurement of the appropriate mobile phase space is encumbered by specific solvation of the stationary phase by components of the eluent.

Recently, the problem received considerable attention in the context of reversed-phase chromatography, which is the most widely used branch of high-performance liquid chromatography (HPLC). In this technique, silica-bound hydrocarbonaceous stationary phases are used with hydro-organic eluents, and the organic solvent is preferentially bound to the stationary phase surface over a wide composition range. A plethora of substances have been suggested for use as inert probes[7–14] but the most convincing case was made for ^2H_2O by McCormick and Karger[2], who investigated the practical aspects of the "void volume problem" in great detail.

A comprehensive theoretical study on the subject was presented by Riedo and Kováts[15]. In applying Gibbs's thermodynamic treatment of interfaces[16], they concluded that no absolute method exists for defining and measuring the volume of the solvation layer at the interface that has a composition different from the bulk mobile phase. In order to evaluate the mobile phase space of uniform composition, therefore, a convention must be applied with respect to the composition of the solvation layer. The consequences of two conventions, "no solvation layer exists" and "one given eluent component is not present in the solvation layer", have been examined in great detail by Riedo and Kováts[15], as well as by Ha *et al.*[17], without unambiguously suggesting the superiority of any of them in chromatographic practice. Martire and Boehm[18] have shown on the basis of statistical thermodynamic arguments that the assumption of no solvation layer gives rise to incorrect results. The use of ^2H_2O as a probe in evaluating the mobile phase space in reversed-phase chromatography, as suggested by McCormick and Karger[2], is tantamount to applying the convention "one given eluent component is not adsorbed". Krstulović *et al.*[19] have found, on investigation of the retention behavior of homologous series, that in many cases this method gave consistent results, although significant failures were noted.

In this study we examined both the theoretical and practical aspects of the "void volume problem" in reversed-phase chromatography. In order to define the

mobile phase space in the column, we suggest the use of the convention "the most weakly bound solvent component is not present in the solvation layer". As the solvent component that is most weakly bound to the stationary phase may vary over a wide range according to this convention, it follows that the mobile phase space is given by the elution volume of the least retained solvent component, as measured by labelled species. This approach can be considered as an additional convention and as a correction to the method concomitant with the "one given solvent component is not adsorbed" convention.

In essence, it yields the smallest mobile phase space in the column that is measured with a tagged eluent component. Analysis of retention data obtained with homologous series showed the most consistent behavior when this approach to the determination of the mobile phase hold-up volume is used for the calculation of retention factors.

THEORETICAL

In liquid–solid chromatography, components of a multi-component mobile phase are known to be adsorbed differentially on the surface of the porous stationary phase[2,17]. The volume occupied by the adsorbed species is generally believed to decrease the actual mobile phase space of the column from its maximum possible value, given by the geometrical void space available for the mobile phase molecules in the column matrix. As the total volume of the column and that of the stationary phase are fixed, determinations of geometrical void space and of the volume of the bulk mobile phase having uniform composition yield the volume of the adsorbed species.

In principle, differences in the bound masses of various species are readily obtained, whereas an unambiguous determination of surface concentrations is impossibile[20]. The essential difficulty in assigning the masses of each component belonging to each phase is the definition of where one phase begins and the other ends. The problem was first treated by Gibbs[16], who recognized that, although heterogeneous masses in contact might be considered as having mathematical surfaces for the sake of simplifing the mathematics of the analysis, this proposition could not be rigorously true, because "if it were so with respect to the densities of the components, it could not be so in general with respect to the density of energy, as the sphere of molecular action is not infinitely small. But we know from observation that it is only with very small distances of such a surface that any mass is sensibly affected by its vicinity —a natural action— and this fact renders possible a simple method of taking account of the variations in the densities of the component substances and of energy and entropy, which occur in the vicinity of surfaces of discontinuity"[16].

Nevertheless, Gibbs has defined a mathematical surface in order to assign all molecules to one of two domains —the mobile phase or the stationary phase— in the context of chromatography. In considerations of liquid–solid interfaces, the dividing surface —if it is a plane— can be arbitrarily placed with respect to its distance from the geometric surface of the solid. Whereas the placement of the dividing plane defines the surface concentrations of each species from the mobile phase, they can take on any value according to the location of the plane. However, the mathematics of the analysis can be reduced by either of two choices of the definition. The approach recommended by Gibbs for practical work was to define the dividing surface so as

to set the concentration of one species in the stationary phase domain equal to zero. A definition that provides equal simplification for binary solvents, given in eqn. 514 in ref. 16, is that the surface concentrations of components of a binary solvent are equal in absolute magnitude and opposite in sign. Each of these definitions reduces the number of variables in the governing thermodynamic expressions by one and, hence, reduces the complexity of the analysis. Before the formulation of these definitions for use in chromatography can be made and insight can be gained from that into the definition of the mobile phase space in the column, it is necessary to examine the general theory of chromatography for its implications regarding sorption to the surface.

Chromatographic velocities

In order to relate these definitions of the locations of stationary phase mobile phase interface to chromatographic practice, the use of the velocity frame is perhaps the most convenient. It is necessary to note that chromatographic movement of one species is characterized by two velocities. Injection of a sample different in composition from the mobile phase brings about perturbations in the the mobile phase concentrations of species; the disturbances move down the column at the concentration velocity that is given by

$$u_{C_{M,i}} = u_0/(1 + \partial C_{S,i}/\partial C_{M,i}) \tag{1}$$

where $C_{S,i}$ and $C_{M,i}$ are the respective concentrations of species i in the stationary and mobile phases, respectively, and u_0 is the mobile phase velocity. Therefore, the rate of movement of a zone of constant composition, $C_{M,i}$, is determined by the slope of the adsorption isotherm of $C_{M,i}$.

However, if no compositional change occurs, as is the case when the composition of the injected sample is identical with that of the mobile phase, except that one or more components is isotopically enriched, the species in the sample move with the species velocity[1], given by

$$u_i^* = u_0 [C_{M,i}/(C_{M,i} + C_{S,i})] \tag{2}$$

$$= u_0/(1 + C_{S,i}/C_{M,i}) \tag{3}$$

Thus, components of the mobile phase also move with their particular species velocity, which can be determined by using their isotopically labelled forms if they have the same retention factor as the unlabelled species[21]. In the case of first-order systems that manifest linear sorption isotherms for the species in consideration, the species and concentration velocities are the same. The definition of both velocities stems from the work of DeVault[22] on the conservation of mass in a chromatographic system. Peaks pertinent to eluent components are conveniently termed "eigenpeaks" and "solvent peaks" when they move with concentration velocity and species velocity, respectively.

The elution volume of i corresponding to the concentration velocity, $V_{R,i}$, is given by

$$V_{R,i} = V_0 (1 + \partial C_{S,i}/\partial C_{M,i}) \tag{4}$$

whereas the retention volume of species i in the column, $V^*_{R,i}$, is

$$V^*_{R,i} = V_0 (1 + C_{S,i}/C_{M,i}) \quad (5)$$

where V_0 is the mobile phase space in the column. Thus, the use of appropriately labelled eluent components as probes yields $V^*_{R,i}$.

Conventions for use in liquid chromatography

Eqns 2–5 describe the movement of eluent constituents and can be used to evaluate the sorption isotherms of mobile phase components, if the mobile phase space is known. Alternatively, they can be used to evaluate the mobile phase space, if the masses of each species bound to the stationary phase are known. In principle, V_0 could be determined by application of eqn. 5 to the species velocity of the various eluites. The obstacle to its use is that it requires an arbitrarily assigned value for the bound volume of one component. However, it is apparent from Gibbs's work that determination of absolute concentration at the surface is impossible; the problem becomes experimentally tractable only after an arbitrary value is assigned to the surface concentration of at least one species. The most general results occur if the concentration of the least-retained species is defined by some non-zero value, and the adsorbed volume of every species, $v_{S,i}$, is related to it by

$$v_{S,i} = (V^*_{R,i} - V^*_{R,0} + v_{S,0}/\varphi_0)\varphi_i \quad (6)$$

where $V^*_{R,i}$, $V^*_{R,0}$ and $v_{S,0}$ are species retention volumes of species i and of the least retained species, and the sorbed volume of the least retained species, respectively. This might be called an "everything is adsorbed" convention. It is unsatisfactory insofar as no insight or guidance into the choice of the $v_{S,i}$ value is given. Consequently, other conventions, which can be regarded special cases of this, have been introduced.

In their analysis of adsorption and chromatography, Riedo and Kováts[15] provided two general conventions, which they called "J is not adsorbed", JNA, and "nothing is adsorbed", NA. The void volume in the JNA convention is given as the species retention volume of a given mobile phase component regarded as unadsorbed, and in the NA convention the void volume is a function of the species retention volumes and the concentration of each species in the mobile phase. The final form of the results depends on the concentration scale used.

Inspection of Fig. 7 in ref. 17 suggests that the JNA convention should be taken to mean that one given mobile phase component is arbitrarily chosen to have zero concentration on the stationary phase over the entire range of mobile phase composition of the multi-component eluent. Indeed, Gibbs in his thermodynamic analysis[16] has suggested that the surface concentration of one species be set equal to zero. For practical applications, the suggestion of McCormick and Karger[2] that ^2H_2O be used as a probe to measure V_0 in reversed-phase chromatography with hydro-organic mixtures as mobile phases anticipated this formulation of more general scope. In the NA convention, the sum of the surface concentrations of sorbed mobile phase species is set equal to zero. This is seen to be similar to the second form of

simplification suggested by the equations presented in Gibbs's analysis. One great advantage of this convention is that, if the volume fraction of each component is used for the mobile phase concentration scale, V_0 obtained by this rubric is identical with the geometric void volume and is therefore related to the maximum possible porosity. For that reason, we denote as $V_{0,max}$ that mobile phase space which is obtained as

$$V_{0,max} = \sum \varphi_i V_i^* \tag{7}$$

where φ_i is the volume fraction of component i in the mobile phase, and the summation is taken over all species.

Complications follow from the use of either of these conventions. In the NA convention, the surface concentration of at least one component is negative. If JNA is taken to mean that the reference species has a surface concentration equal to zero across the entire range of mobile phase compositions, then the surface concentrations of the other species can range in value from greater than zero to less than zero so that the sign of the surface concentration in the JNA convention may also change. This leads to complications in the thermodynamic analysis of the system insofar as chemical potentials, which are functions of the logarithm of the surface concentrations, will become undefined quantities. Ha *et al.*[17] escaped this conundrum by defining a minimum surface layer thickness having the property that the concentration of any mobile phase component in it never equals zero or less. In fact, this is a special method for transforming adsorption data, calculated by use of the NA convention, to values consistent with the "everything is adsorbed" convention. However, this device implies that the volumes of mobile phase components, regarded as sorbed for the purpose of adsorption measurements, are different and larger than those considered for evaluating the mobile phase space in the chromatographic column.

The basis of an alternative convention for the definition of V_0 that avoids these problems comes from the observation that eqn. 5 actually implies that the mobile phase space is given by the species retention volume of that component which is not bound to the stationary phase, *i.e.* $C_{S,i} = 0$. In view of Gibbs's analysis, the surface concentration of any of the species can be equal to zero by definition. If the surface concentrations of the other species are not to be negative and, thus, in order to avoid a physically impossible situation, the species chosen to have a zero surface concentration must have the greatest species velocity and the smallest retention volume. For this reason, we denote this volume by the symbol $V_{0,min}$ and, in accordance with the above requirement,

$$V_{0,min} = V^*_{min} \tag{8}$$

where V^*_{min} is the minimum species retention volume observed at a particular mobile phase composition. This definition of the mobile phase space in the column is similar to one given by the JNA convention of Riedo and Kováts[15] and, thus, corresponds to the use of ^2H_2O as probe in reversed-phase chromatography over a wide range of eluent composition as suggested by McCormick and Karger[2]. However, this convention assumes that the "most weakly bound eluent component is not adsorbed"

and therefore implies that the mobile phase component that probes and defines the void volume may change with the changing proportions of the components that comprise a multi-component mobile phase. In reversed-phase chromatography with binary hydro-organic eluents, for example, the ^2H_2O probe is changed to the isotopically labelled organic solvent component when eluents sufficiently rich in organic solvent are used in order to follow this convention.

EXPERIMENTAL

Equipment

An Altex Model 100A (Altex, Berkeley, CA, U.S.A.) HPLC solvent metering pump with a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve, having a 20-μl loop, were used. In some experiments an automatic injector, Model 725 (Micromeritics, Norcross, GA, U.S.A.), was also used. The column effluent was monitored with a Schoeffel Model 770 (Kratos, Westwood, NJ, U.S.A.) variable-wavelength UV detector and with a refractive-index detector (Perkin-Elmer, Norwalk, CT, U.S.A.). Chromatograms were obtained with a Model BD-41 strip-chart recorder (Kipp and Zonen, The Netherlands).

The 250 × 4.6 mm I.D. column, packed with 10-μm Partisil ODS-3, was supplied by Whatman (Clifton, NJ, U.S.A.). The temperature of the column and of the flow cell of the refractive index detector was controlled by circulating water from a Lauda Model K-2/R constant-temperature bath (Messgerätewerk, Lauda, F.R.G.). Except when methanol or acetonitrile was used alone, the hydro-organic eluent contained 0.1 *M* acetic acid and 0.1 *M* triethylammonium acetate. The flow-rate was 0.5 ml/min.

Materials

Samples that comprised the homologous series of *n*-alkylbenzenes (benzene to *n*-octylbenzene), fatty acid methyl esters (methyl formate to methyl nonanoate), oligoalanines and 2-ketones (propanone to 2-tridecanone) were obtained from Aldrich (Milwaukee, WI, U.S.A.), Chem Service (West Chester, PA, U.S.A.), Sigma (St. Louis, MO, U.S.A.) and Theta Corp. (Media, PA, U.S.A.). Deuterated water was obtained from Baker (Phillipsburg, NJ, U.S.A.) and C^2H_3CN and CH_3O^2H from Sigma. All other chemicals and HPLC-grade solvents were obtained from Fisher (Fair Lawn, NJ, U.S.A.). Distilled water was prepared with a Barnstead unit in our laboratory.

Procedures

Samples were prepared by dissolving approximately 1 mg of each solute in 5 ml of mobile phase. The column was equilibrated by passing at least 200 ml of mobile phase through it before sample injection. Retention time was evaluated from the position of peak maximum in the chromatogram.

The total porosity or maximum void volume was determined gravimetrically[2–4] by use of water, methanol, acetonitrile, carbon tetrachloride and *n*-hexane as solvents. The densities of the solvents were measured with a 25-ml pycnometer at 25°C and were found to agree with the corresponding literature values.

Mean retention volumes and their standard deviations were determined from

replicate determinations of retention volumes. When retention volumes were compared with void volumes according to a particular convention, *t*-statistics[23] were used. In the analysis of homologous series, the void volume was calculated by modification of the method of Berendsen[24], which determines the void volume from the slope and intercept of a plot of retention volume of one homolog against retention volume of the next highest homolog. In our analysis, numerous void volumes were determined for each set of homologs by systematic reduction of the number of retention volumes used in the calculation. The mean void volume value was determined by use of an algorithm for finding and eliminating those values that lie more than three standard deviations from the population mean. Retention factors were calculated from retention volumes and void volume obtained by use of one or more convention.

RESULTS AND DISCUSSION

The four conventions for determining the mobile phase space in solid–liquid chromatography with multi-component eluents have been set out in the theoretical section. According to the most general convention, "everything is adsorbed", and thus the concentration of each mobile phase species is greater than zero at the liquid–solid interface. The other three conventions are "nothing is adsorbed", "J is not adsorbed" and "the most weakly bound eluent component is not adsorbed". According to the last convention, the smallest species retention volume volume, as measured by labelled mobile phase components, is taken as the mobile phase space in the column.

The theoretical basis for NA and JNA have been examined in detail by Kováts and co-workers[15,17]. The model underlying the NA convention is that the dividing plane between the mobile phase and stationary phase, including the species bound to the stationary phase, is chosen so that the net adsorbed volume is zero. The void volume obtained by use of this convention, given by eqn. 7 for use with volume fractions as concentration units, coincides with the void volume that is obtained from the weight difference when the column is filled with neat solvents of different density and then weighed[2–4]. This method gives the maximum possible value of the column void volume, $V_{0,max}$. On the other hand, JNA corresponds to the use of a dividing plane more distant from the stationary phase ligate of bonded stationary phase, chosen in such a way that the surface concentration of J, a given mobile phase component selected as the reference, always remains zero. However, if the species retention volume of this component exceeds that of any of the others, the dividing plane is found to pass through the hydrocarbonaceous ligate or even through the siliceous substrate of bonded supports used in reversed-phase chromatography. In such cases, the ligate, and perhaps the silica, is regarded as part of the mobile phase. An additional complication is that the first three conventions enumerated above can give rise to negative surface concentrations and undefined free energies of retention.

To avoid these problems, we have proposed a fourth convention, which can be considered as a variant of the JNA convention and allows the chemical identity of the non-adsorbed species to change when required by the changing composition of the multi-component mobile phase. According to this convention, one mobile phase is regarded inert at each mobile phase composition as far as binding to the

stationary phase is concerned, but, in contrast to the JNA convention, that species is not specified in advance. Rather, the non-adsorbed component is always taken as the least retained of the species comprising the mobile phase. The applicability of this approach is not confined to binary mobile phase systems, and the definition of the void volume by the elution volume of the least retained, isotopically marked mobile phase species is derived generally from eqn. 5 when $C_{S,i}$ is set equal to zero.

Experimental results illustrating the difference between the three latter conventions are depicted in Fig. 1. The data were obtained with a Partisil ODS-3 column by using methanol–water and acetonitrile–water as the mobile phases. The species retention volumes of eluent components, $V^*_{R,i}$, were evaluated with deuterated species, normalized to $V_{0,max}$ and plotted against the volume fraction of the organic solvent component in the mobile phase. Eqn. 7 was used to calculate $V_{0,max}$ from the species retention volumes of ^2H_2O and deuterated acetonitrile, and $V_{0,max}$ thus obtained was found to be indistinguishable from the column void volume determined pycnometrically. This result is in agreement with that of Ha *et al.*[17]. The species retention volumes used for calculation of $V_{0,max}$ were applied to the evaluation of $V_{0,max}$ by always taking the smallest species retention volume at any mobile phase composition. It can be seen in Fig. 1 that they coincide over most of the mobile phase composition range and diverge only when the volume fraction of acetonitrile exceeds 80%. Similar results are obtained with water–methanol mobile phases for which the maximum difference in the magnitude of $V_{0,max}$ and $V_{0,min}$ is much less than that with the water–acetonitrile system.

The concept according to which the void volume is given by the minimum species retention volume can be considered as a precisely formulated extension of suggestions made by others for the choice of column void volume. Kováts and coworkers[15,17] have shown that both the NA and JNA convention are valid, but have not stated which should be the preferred one. The convention that gives rise to the definition of $V_{0,min}$ can be viewed as an extension of the JNA convention. The use

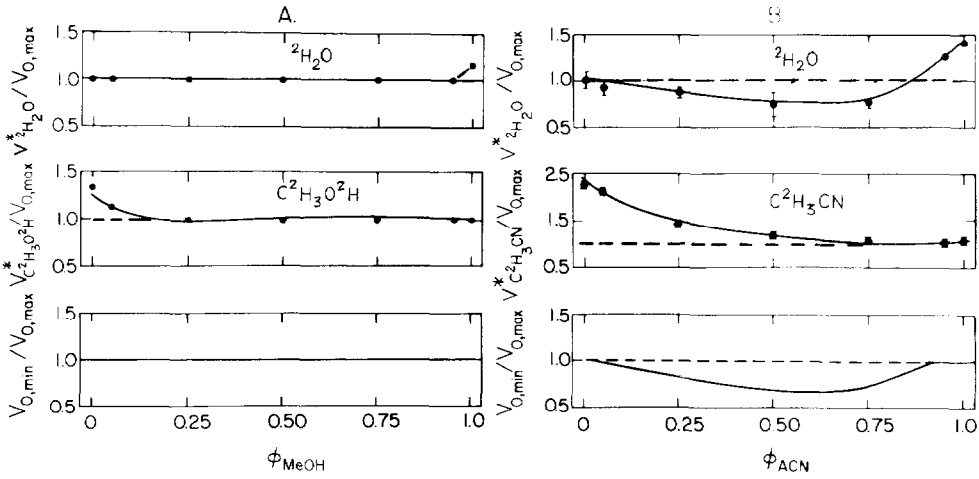

Fig. 1. Dependence of the retention volume of ^2H_2O, deuterated organic co-solvent, and $V_{0,min}$ and $V_{0,max}$ on composition of (A) aqueous methanol and (B) aqueous acetonitrile as mobile phase. $V_{0,min}$ and $V_{0,max}$ are marked by solid and dashed lines, respectively, in the lower frames.

of $V_{0,min}$ may also be in agreement with the view of Martire and Boehm[18], who found the NA convention, *i.e.*, the use of $V_{0,max}$, to be theoretically unsatisfactory on the basis of their statistical mechanics treatment of retention in reversed-phase chromatography. McCormick and Karger[2] suggested the use of ^2H_2O as the most suitable probe in reversed-phase chromatography; indeed, it measures $V_{0,min}$, except at high concentrations of organic solvents in the eluent. This explains why Krstulović *et al.*[19], in a recent critique of the NA convention, found $V^2_{H_2O}$ to be a more appropriate measure of the column dead volume in reversed-phase chromatography than the geometric volume.

The arguments for adopting one convention in preference to another are not primarily thermodynamic in their nature, but are based on the ultimate convenience of use without loss of mathematical vigor. Therefore, it is useful to investigate the implications for chromatographic practice of some of these conventions. Insofar as the evaluation of both $V_{0,min}$ and $V_{0,max}$ require that all species retention volumes be known for use with confidence, no choice can be made between them on the basis of the experimental efforts expended in the use of either.

More interesting are the implications of these conventions for establishing quantitative structure–retention relationships for use in liquid chromatography. Linear dependence of the logarithmic retention factor on the number of recurring molecular units in homologous eluites is as well established in liquid chromatography[25] as it was earlier in gas chromatography[26,27]. Such linear relationships have been exploited to predict retentions from structures or to facilitate the identification of chromatographic peaks by use of retention indices, the best known of which is that of Kováts[26,27] for use in gas chromatography. They are also used to predict physical properties and biological activity from chromatographic retention[28]. In view of the extensive use of such relationships, it is of interest to determine the effect the choice of $V_{0,min}$ convention has on the linearity of such plots of liquid chromatographic data. Linear regression was used to obtain the slopes, intercept and correlation coefficients of plots of logarithmic retention factor *versus* homolog number for which the retention factors had been calculated from the experimental retention volume and V_0 values, determined by the use of the $V_{0,}{}^2{}_{H_2O}$, $V_{0,min}$ and $V_{0,max}$ conventions. The series that were chromatographed on ODS-3 with water–methanol or water–acetonitrile mixtures as mobile phases were alkylbenzenes, 2-ketones, fatty acid methyl esters and oligoalanines. The mobile phase compositions used for each family of homologs are specified in Table I. Linearity was evaluated by use of the correlation coefficients shown for the three conventions; the closer the coefficient to unity, the more linear is the plot. Methods designated as A, B and C in Table I used $V_{0,}{}^2{}_{H_2O}$, $V_{0,max}$ and $V_{0,min}$, respectively, for evaluating the column dead volume. Missing entries for correlation coefficients indicate those plots for which some retention volumes were less than the V_0 value in that convention so that ln k' was undefined. The gaps in Table I confirm the observation of Karger and McCormick[2] that ^2H_2O cannot be used to probe V_0 over the entire range of mobile phase composition. In particular, it fails badly when the eluent is water-lean. For that reason, we shall focus on the comparison of the use of $V_{0,max}$ and $V_{0,min}$.

The results shown in Table I indicate that the choice of convention for the mobile phase space has only a small effect on the linearity, as measured by the correlation coefficient of the dependence of log k' on N_C under the conditions investi-

gated, which are representative of the practice of reversed-phase chromatography, as far as the choice of mobile phases is concerned. Correlation coefficients obtained by use of the two conventions are typically different in only the fourth or fifth decimal place. In view of this closeness, the observation that 60% of the data obtained with aqueous acetonitrile as the mobile phase had larger correlation coefficients when $V_{0,min}$ is used would appear to be an inadequate basis for preferring the convention "the least retained eluent component is not in the solvation layer". Only 50% of the data obtained with aqueous methanol were better fitted by use of $V_{0,min}$. This lower degree of discrimination is expected, inasmuch as the numerical values, obtained for the mobile phase space in the column according to both conventions, are nearly identical when aqueous methanol is used as the mobile phase. These mobile phase systems were selected because of their wide use in reversed-phase chromatography. Methanol and acetonitrile are relatively weakly solvating organic modifiers, but with strongly solvating organic solvents we expect much greater differences between the mobile phase hold-up volumes, as determined according to different conventions.

Three sets of data that showed the worst deviation from linearity in Table I, as measured by correlation coefficients, are plotted in Fig. 2. The points calculated by use of $V_{0,max}$ are marked with crosses and those by use of $V_{0,min}$ with solid circles. Straight lines were drawn through the points, corresponding to the three largest ln k' values in each convention. Fig. 2A shows the results obtained for retention factors of 2-ketones, obtained with aqueous acetonitrile, $\varphi_{ACN} = 0.75$, as the mobile phase. Whereas the data calculated by use of $V_{0,min}$ fall along a straight line, those calculated by use of $V_{0,max}$ form a slightly curved line. Figs. 2B and C show the plots obtained for data on retention of 2-ketones and alkylbenzenes, respectively, with aqueous acetonitrile, $\varphi_{ACN} = 0.50$ as the mobile phase. The points calculated by use of $V_{0,min}$ generally fall along the straight line generated from the largest retention factors. In contrast, the smaller retention factor values obtained by use of $V_{0,max}$ systematically deviate from a similarly generated curve. More pronounced deviations from linearity were observed when data obtained with hydro-organic mobile phases, containing tetrahydrofuran or isopropyl alcohol, were plotted in a similar fashion, because of the greater thickness of the solvation layer of these organic solvent components[29,30].

It is theoretically possible to obtain the void volume from the retention volumes of members of homologous series if a linear relationship between ln k and the number of recurring structural units exists[24,31], Krstulović *et al.*[19] used this method for the evaluation of the mobile phase hold-up volume in order to assess the appropriateness of $V_{0,max}$ or $V_{0,^2H_2O}$.

We also determined the mobile phase space from the retention volumes of alkylbenzenes, 2-ketones, methyl esters of *n*-alkanoic acids and oligoalanines, obtained at selected mobile phase compositions by each of two modifications of the method of Berendsen *et al.*[14,24].

The first modification was the convergence method of Krstulović *et al.*[19] and the second involved the use of a laboratory-designed computer algorithm to remove from a data set all data that lie more than three standard deviations from the mean of the set. In each method void volumes were calculated by use of paired retention volumes of adjacent members of homologous series for all members of the series[24]. Void volumes were then calculated for all subsets of the data obtained by successive elimination of the largest or smallest retention volumes remaining in the set. The

TABLE I

CORRELATION COEFFICIENTS OBTAINED BY LINEAR REGRESSION OF LOGARITHMIC RETENTION FACTOR AGAINST METHYLENE NUMBER, OBTAINED BY USE OF THREE CONVENTIONS FOR THE VOID VOLUME, FOR FOUR HOMOLOGOUS SERIES IN HYDRO-ORGANIC MIXTURES AS ELUENT

The conventions that use methods A, B, C give as void volumes the retention volumes of ^2H_2O, $V_{0,max}$ and $V_{0,min}$, respectively. The values of void volume obtained at each mobile phase by use of the three conventions are shown. The last two columns show the values of void volumes obtained by Berendsen's method[24], by the use of uncorrected retention volumes. The two columns reflect different routes for the minimization of the effects of experimental errors in the final calculation.

Eluent composition	*Homologous series*	*Correlation coefficient*			*Void volume*				
		Method A	*Method B*	*Method C*	*A*	*B*	*C*	*Convergence method*	*Three standard deviations method*
φ_{MeCN}									
1.00	Esters		0.9987515	0.9987515	3.53	2.88	2.88	2.82 ± 0.0055	2.71 ± 0.2506
1.00	Ketones		0.9981855	0.9981855				2.61 ± 0.023	2.54 ± 0.365
1.00	Alkylbenzenes		0.9984275	0.9984725				2.69 ± 0.0344	2.68 ± 0.2187
0.95	Esters	0.9994630	0.9995805	0.9994630	2.59	2.62	2.59	2.68 ± 0.0468	2.42 ± 0.9685
0.95	Ketones	0.9995111	0.9991405	0.9995111				2.46 ± 0.0381	2.45 ± 0.1157
0.95	Ketones	0.9995281	0.9991837	0.9995281				2.48 ± 0.0280	2.48 ± 0.1734
0.95	Alkylbenzenes	0.9998492	0.9998675	0.9998492				2.63 ± 0.0329	2.67 ± 0.1987
0.95	Alkylbenzenes	0.9999296	0.9999138	0.9999296				2.61 ± 0.0354	2.61 ± 0.0873
0.75	Esters	0.9993085	0.9983558	0.9993085	2.49	2.795	2.49	2.43 ± 0.0353	2.44 ± 0.1300
0.75	Ketones	0.9998540	0.9972264	0.9998540				2.49 ± 0.0398	2.50 ± 0.2186
0.75	Alkylbenzenes	0.9999764	0.9999355	0.9999764				2.65	2.62 ± 0.4720
0.50	Esters	0.9998005	0.9968924	0.9998005	2.12	2.78	2.12	1.86 ± 0.0082	1.86 ± 0.1362
0.50	Esters	0.9998808	0.9972452	0.9998808				1.92 ± 0.0229	1.93 ± 0.1192
0.50	Ketones	0.9999710	0.9991980	0.9999710				2.25 ± 0.0159	2.22 ± 0.1474
0.50	Ketones	0.9999888	0.9984578	0.9999888				2.04 ± 0.0227	2.03 ± 0.0767
0.50	Alkylbenzenes	0.9995398	0.9997337	0.9995398				4.04	4.18 ± 2.2315
0.50	Alkylbenzenes	0.9994975	0.9996892	0.9994975				4.49	4.18 ± 2.6676
0.25	Esters	0.9880444	0.9908201	0.9880444	2.46	2.83	2.46	3.45	3.38 ± 0.8372
0.25	Esters	0.9930819	0.9951910	0.9930819				3.15	3.08 ± 0.6076
0.25	Esters	0.9879775	0.9898190	0.9879775				3.42	3.34 ± 0.8288
0.25	Ketones	0.9997429	0.9985064	0.9997429				2.00	1.99 ± 0.3903
0.25	Ketones	0.9999671	0.9994281	0.9999671				2.67 ± 0.0097	2.67 ± 0.2763

0.05	Esters	0.9972595	0.9975947	0.9972595	2.78	2.89	2.78	4.11	4.11
0.05	Esters	0.9970902	0.9974246	0.9970902				4.11	4.11
0.05	Ketones	0.9999999	0.9999904	0.9999999				2.77	2.77
0.05	Ketones	0.9999848	0.9999976	0.9999845				2.95	2.95
0.05	Alanines	0.9565981		0.9566981				1.20	3.09 ± 2.3614
								2.25	3.93 ± 2.6201
								1.53	2.67 ± 2.4888
								7.66	7.66*
Water	Alanines	0.9980985	0.9980985	0.9980985	2.78	2.78	2.78	2.42 ± 0.0388	2.31 ± 0.3630
φ_{MeoH}								2.44	2.42 ± 0.2591*
1.00	Alkylbenzenes		0.9931731	0.9931731	3.52	3.10	3.10	2.79 ± 0.0366	2.77 ± 0.2699
1.00	Esters							2.70 ± 0.0410	2.65 ± 0.1684
1.00	Ketones		0.9845958	0.9845958				2.81 ± 0.0335	2.71 ± 1.3823
0.95	Alkylbenzenes	0.9986830	0.9984551	0.9986830	2.78	2.799	2.78	2.63 ± 0.0489	2.67 ± 0.2644
0.95	Esters	0.9991176	0.9987635	0.9991176				2.72 ± 0.0212	2.68 ± 0.2342
0.95	Ketones	0.9917368	0.9914152	0.9917368				2.62 ± 0.0120	2.62 ± 0.4191
0.75	Alkylbenzenes	0.9990696	0.9990749	0.9990766	2.68	2.665	2.66	1.75	−0.75 ± 7.24
0.75	Alkylbenzenes	0.9996008	0.9996003	0.9995999				2.95	2.62 ± 1.5810
0.75	Alkylbenzenes	0.9992295	0.9991245	0.9991260				1.77	0.52 ± 6.7809
0.75	Esters	0.9999115	0.9998588	0.9998377				2.68 ± 0.4385	2.67 ± 0.1198
0.75	Ketones	0.9996396	0.9996923	0.9997053				2.45	2.52 ± 0.2831
0.50	Alkylbenzenes	0.9996630	0.9996618	0.9996606	2.73	2.715	2.70	5.38	4.86 ± 5.52
0.50	Esters	0.9985308	0.9984298	0.9983266				2.99 ± 0.0549	2.96 ± 0.2136
0.50	Esters	0.9989444	0.9988504	0.9987535				2.92 ± 0.0492	2.91 ± 0.1863
0.50	Ketones	0.9998834	0.9998777	0.9998664				2.51	2.49 ± 0.5256
0.50	Ketones	0.9998118	0.9998177	0.9998188				2.44	2.40 ± 0.5582
0.50	Ketones	0.9998794	0.9998697	0.9998553				2.53	2.50 ± 0.5338
0.25	Esters	0.9977762	0.9978444	0.9977762	2.82	2.833	2.82	3.44 ± 0.0594	3.39 ± 0.0850
0.25	Ketones	0.9998127	0.9998296	0.9998127				3.07	3.07
0.05	Esters	0.9964683	0.9965235	0.9964683	2.84	2.857	2.84	4.35	4.35
0.05	Alanine							2.16	2.34 ± 0.1839
		0.9859244	0.9837641	0.9859244				2.25	2.25 ± 0.0643*

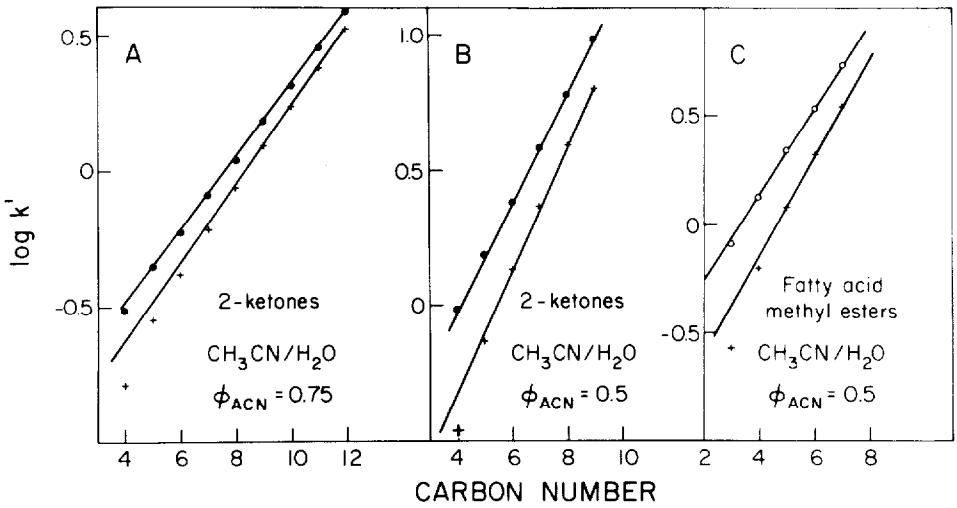

Fig. 2. Plots of logarithmic retention factors against the carbon number of alkylbenzenes and 2-ketones. The retention was measured with the hydro-organic mixture indicated in each frame as the mobile phase on Partisil ODS-3. The retention factor values obtained by the use of $V_{0,min}$ and $V_{0,max}$ are indicated by solid circles and crosses, respectively. The lines were drawn through the uppermost four points on each curve.

values thus obtained were used to calculate the mean and standard deviation of the mobile phase space. In the first approach, all data that fell outside the range specified by the authors[19] were eliminated, and a new mean and standard deviation were calculated. The values of this mean and its three standard deviation limit are given in Table I in the column marked Convergence method. In the second approach, data that were different from the mean by more than three standard deviations were eliminated[23]. A new mean and standard deviation were calculated, and the process was repeated until no more outlying data existed by this test. The mean and three standard deviations, calculated by this method, are given in the column labelled three standard deviation method in Table I.

As seen in Table I, the mean values of the mobile phase hold-up volume, calculated by the two methods, agree fairly well. The major difference between them is the smaller standard deviation in the method of Krstulović *et al.*[19] that forces the standard deviation to be no more than 2.5% of the mean. Inspection of Table I also reveals a generally good agreement between $V_{0,min}$ and the void volume obtained by linearization; when the agreement with $V_{0,max}$ of the value obtained from retention data of homologous eluites is good, the agreement with $V_{0,min}$ is equally good in all cases. Thus, if homologous series can be used to probe the void volume, these results can be taken to argue for the use of $V_{0,min}$ as the mobile phase space in the column. However, determination of dead volume by use this method is known to be unreliable, because the volume obtained is very sensitive to errors in the retention volume[14,31] and because the requirement that a linear relationship exist between logarithm of k and homolog number may not be satisfied. In fact, the data in Fig. 2 suggest that linearity may not be found, even in mobile phases that generally appear to exhibit it[19,24]. Strict linearity is almost certainly not found in isopropyl alcohol-

or tetrahydrofuran-containing mobile phases[24,29,30], inasmuch as the dead volumes obtained by linearization are much smaller than any species retention volume and can have a zero or negative value[29,30].

This caveat notwithstanding, it is interesting to note that the mobile phase spaces obtained by use of the linear ln k–homolog number relationships for the mobile phases investigated here are generally equal to $V_{0,min}$. This is seen in Fig. 3, which shows the void volumes obtained by use of the method of Berendsen[24] with the modifications discussed above for the homologous series of alkylbenzenes, 2-ketones and fatty acid methyl esters. The data are plotted against the volume fractions of methanol or acetonitrile in the mobile phase. $V_{0,max}$ and $V_{0,min}$ are given by the dashed and solid lines, respectively, which are indistinguishable in the methanol systems. The greatest divergence is found when the number of homologs is small and can be regarded as an artifact due to the sensitivity of that method to experimental uncertainties in retention volume. For example, at $\varphi_{ACN} = 0.5$, the value of the dead volume obtained by linearization of alkylbenzene data is much different from $V_{0,min}$, but the values obtained from data on 2-ketones and esters, which are based on more data points in the estimation of the dead volume, agreed well with the value given by $V_{0,min}$.

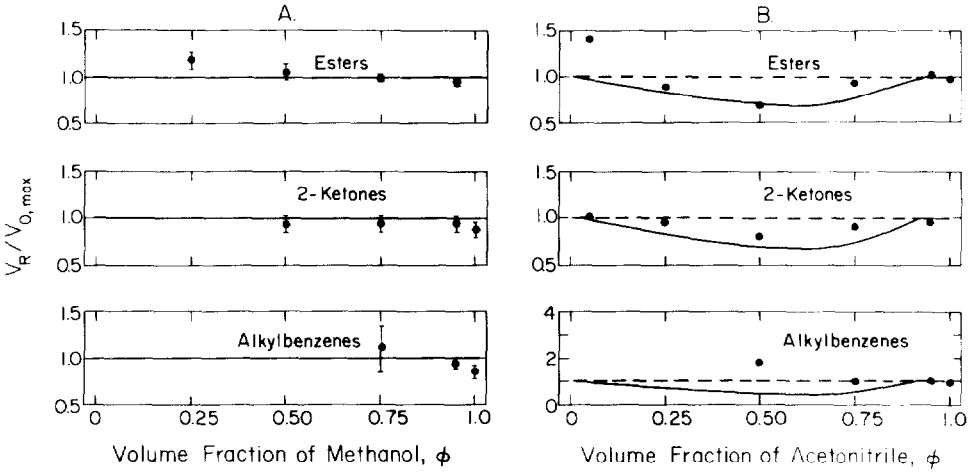

Fig. 3. The ratio of the void volume, V_0, obtained by linearization of the retention volumes of members of homologous series to $V_{0,max}$. The solid line marks $V_{0,min}$. The homologous series used were methyl esters of n-alkanoic acids, 2-ketones and n-alkylbenzenes. The data were obtained with (A) methanol–water and (B) acetonitrile–water mixtures as the mobile phase at 25°C and Partisil ODS-3 as the stationary phase.

Simple, secondary probes of $V_{0,min}$

In the practical determination of mobile phase space, a determination of all species volumes may not be possible. For this reason, it is interesting to know of some simple eluite that would probe $V_{0,min}$. Nitrate[8,13,32], urea and fructose[33] were examined as probes of $V_{0,min}$ for use with aqueous methanol and aqueous acetonitrile mobile phases.

When methanol–water mixtures were used as mobile phases, the probes worked well within the following ranges of methanol composition: urea or fructose,

0–1; and nitrate ion, 0.25–0.75. These results are shown in Fig. 4A. When the mobile phase consisted of aqueous acetonitrile mixtures, $V_{0,min}$ was given by the retention volumes of the following eluites over the indicated range of φ_{ACN}: eigenpeak, 0.5–0.75; urea and fructose, 0–0.75; and nitrate ion, 0.25–0.95. These results, shown in Fig. 4B, indicate that no single eluite can be used as a probe of mobile phase space over the entire composition range, although some do serve well over a wide range of compositions. As noted above, in our experience, nitrate ion ($NaNO_3$ or KNO_3) was an adequate probe, if the mobile phase contained between 25 and 75% of methanol or between 25 and 95% of acetonitrile and the ionic strength of the eluent was sufficiently high to avoid the effect of Donnan exclusion on the retention volume of the salt peak.

CONCLUSIONS

Consideration of the fundamental equation of chromatography, together with the general thermodynamic analysis of adsorption of Gibbs, leads to the conclusion that four conventions for the determination of void volume are possible. They are: "everything in the eluent is adsorbed", "no solvation layer exists", "one particular eluent component is not present in the solvation layer", and "the least-retained mobile phase species is not in the solvation layer". The first convention is not very useful, because it gives no explicit and unambiguous method for the evaluation of the mobile phase volume. The second and third conventions have been thoroughly explored by Riedo and Kováts[15]. The use of the second and third conventions is clear; indeed, the third convention is consistent with the use of ^2H_2O as a probe of mobile phase space in reversed-phase chromatography[2], at least over a wide range of mobile phase compositions. However, calculations according to each of these conventions can lead to negative surface concentrations, complications in the thermodynamic analysis of the system or physically unrealistic, large values of mobile phase

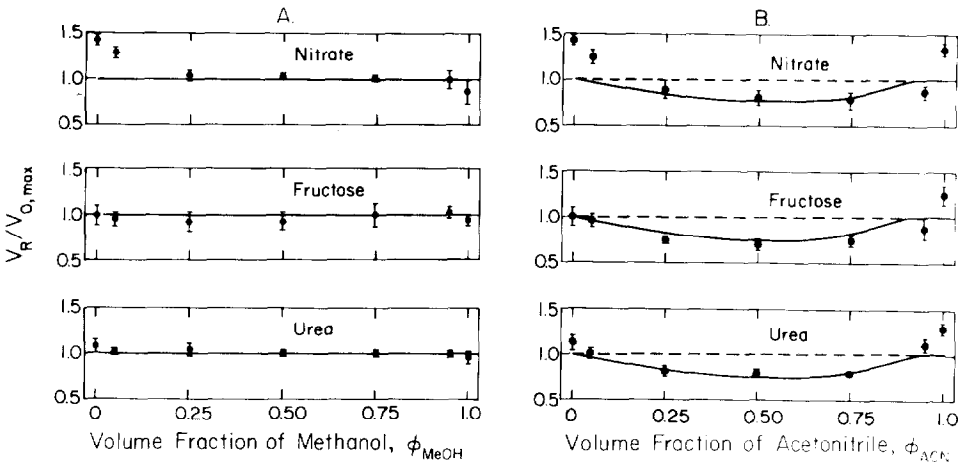

Fig. 4. Elution volume of simple eluite probes, normalized to $V_{0,max}$. The probes are fructose, urea and nitrates, proposed as unretained markers. The dashed and solid lines indicate $V_{0,max}$ and $V_{0,min}$, respectively. The data were obtained at 25°C with (A) methanol–water mixtures and (B) acetonitrile–water mixtures as the mobile phase on a Partisil ODS-3 column (250 × 4.6 mm I.D.).

space. In the fourth convention, the mobile phase space is taken as the species retention volume of the least-retained mobile phase component. This convention can be regarded as an extension of the third. It has the advantage that the problems encountered in the use of the second and third conventions, such as negative concentrations and undefined thermochemical quantities, are avoided.

Insofar as no compelling thermodynamic argument for choosing between these conventions exists, the effects of these conventions on everyday chromatographic practice may be considered. Inasmuch as quantitative structure–retention relationships have a variety of uses, the effects of the conventions on the development of such relationships were examined on the basis of the linearity of plots of logarithmic retention factor *versus* homolog number; in the analysis, different values of the void volume were used according to these conventions. When retention data from homologs were plotted, the use of the fourth convention was found to give more linear plots of ln k *versus* homolog number. Thus, its use will facilitate the development of quantitative structure–retention relationships for use in liquid chromatography. Inasmuch as its use allows escape from problems associated with the other conventions, and it yields —among the four conventions— the simplest structure retention relationships, its adoption for use in liquid chromatography is well merited, particularly if we accept as a guiding principle the rule of intellectual parsimony, attributed to William of Ockham and reformulated by J. Gibbs: "One of the principal objects of theoretical research in any department of knowledge is to find the point of view from which the subject appears in its greatest simplicity"[34].

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