Journal of Chromatography, 158 (1978) 215–225

CHROM. 11,018

ENTHALPY–ENTROPY COMPENSATION IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The enthalpy of hydrophobic interactions, which are believed to account for retention in reversed-phase chromatography, was found to have constant negative values even at temperatures near the freezing point of water. The analysis of data obtained in reversed-phase chromatography with various octadecylsilicas under a wide range of conditions as far as the eluites** and eluents are concerned, showed a linear correlation between the logarithm of the capacity factors, measured at an appropriate reference temperature, and the corresponding enthalpies for the particular chromatographic process. This behavior is due to enthalpy–entropy compensation, which is a manifestation of free-energy relationships. As the compensation temperatures were indistinguishable, it is concluded that the intrinsic mechanism of the interaction of the eluite with the bonded stationary phase was invariant under the chromatographic conditions examined, although the nature and concentration of the organic solvent in the aqueous eluent varied substantially. Whereas compensation behavior was observed with other chromatographic systems consisting of polar stationary phases and a non-polar eluent, the calculated compensation temperatures were significantly lower than those found in reversed-phase chromatography.

INTRODUCTION

Reversed-phase chromatography with hydrocarbonaceous bonded stationary phases and hydro-organic eluents is used extensively for the separation of a wide variety of substances[1]. Recently we investigated the thermodynamic basis of retention in this type of chromatography[2,3]. The theory has satisfactorily accounted for the effect of solvent on the interaction between the eluite and the stationary phase, but it does not give information on the actual mechanism of the process. Much further work is required in order to obtain a sufficiently precise and detailed picture of the mechanism of the solvophobic interactions[4] that are involved in solute retention in reversed-phase chromatography.

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** The substances that are eluted.

In order to further a mechanistic interpretation of reversed-phase chromatography, in the present study we use an extra-thermodynamic approach to analyze retention data. Extra-thermodynamic relationships are frequently encountered in the analysis of analogous chemical processes and used to shed light on certain important features of the underlying physico-chemical phenomena. The term extra-thermodynamic denotes the fact that they fall short of a rigorous thermodynamic foundation. Many such relationships are called linear free-energy relationships[5]. The term originates from the observed linear dependence of the rate or equilibrium constant for a series of chemical transformations on the free-energy change associated with related processes. The most popular representative of such linear free-energy relationships is the Hammett equation[6], which has also been widely used for the interpretation of retention in various chromatographic systems[7,8].

Another extra-thermodynamic approach to the analysis of physico-chemical data is based on enthalpy–entropy compensation[9,10], which manifests itself in a linear dependence of the overall free-energy changes on the corresponding enthalpy change for intrinsically similar physico-chemical phenomena. If such behavior is observed, the fundamentally related equilibrium processes or chemical reactions are said to be isoequilibrium or isokinetic processes, respectively.

Enthalpy–entropy compensation is conveniently expressed by the relationship

$$\Delta H^0 = \beta \Delta S^0 + \Delta G^0_\beta \tag{1}$$

where ΔG^0_β denotes the Gibbs free energy of a physico-chemical interaction at temperature β and ΔH^0 and ΔS^0 are the corresponding standard enthalpy and entropy, respectively. Eqn. 1 implies that in the vicinity of β changes in ΔH^0 are offset by changes in ΔS^0 so that the free-energy change is practically independent of temperature. When enthalpy–entropy compensation is observed with a family of compounds in a particular chemical transformation, then the values of β and ΔG^0_β are invariant and β is called the compensation temperature. The terms isokinetic and isoequilibrium temperature are also used because the respective Arrhenius or Van 't Hoff plots would intersect at $1/\beta$ when compensation occurs. Using the Gibbs relationship for the free energy change, ΔG^0,

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{2}$$

we can rewrite eqn. 1 in order to express the free energy change, ΔG^0_T, measured at a fixed temperature, T, for isoequilibrium processes as

$$\Delta G^0_T = \Delta H^0\left(1 - \frac{T}{\beta}\right) + \frac{T\Delta G^0_\beta}{\beta} \tag{3}$$

According to eqn. 3, for isokinetic or isoequilibrium transformations a plot of ΔG^0_T against the corresponding enthalpy yields a straight line and the compensation temperature can be evaluated from the slope. When this behavior is observed, the species involved in the transformations are believed to share common physico-chemical properties that play an important role in determining the energetics of the process. Conversely, if a particular chemical transformation fails to conform to the common

compensation pattern observed with related processes, it is assumed to be different from them in some significant mechanistic details.

In this paper we consider the existence of enthalpy–entropy compensations in reversed-phase liquid chromatography. As compensation behavior is generally attributed to the effect of the solvent on the interacting species[9], a comparison of the compensation temperatures obtained with appropriate data can serve as a diagnostic tool for variations in the retention mechanism on changing the conditions, *e.g.*, the composition of the eluent.

EXPERIMENTAL

Instrumentation

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 liquid chromatograph equipped with a Model 70-10 sampling valve (Rheodyne, Berkeley, Calif., U.S.A.), a Schoeffel (Westwood, N.J., U.S.A.) Model SF 770 variable-wavelength detector at a setting of 210 or 260 nm and a Perkin-Elmer Model 56 strip-chart recorder was used. A constant-temperature circulating water-bath (Model WB 20D; MGW, Lauda, G.F.R.) was used to maintain the temperature of the jacketed column constant. The eluent flow-rate was 1 or 2 ml/min.

Columns

A Partisil ODS 2 column (250 × 46 × 64 mm) was obtained from Whatman (Clifton, N.J., U.S.A.). This column was packed with irregularly shaped 10-μm octadecylsilica with a carbon load of 17%. The other column (250 × 46 × 64 mm) was packed in our laboratory with 5-μm Spherisorb ODS (Phase Separations, Hauppage, N.Y., U.S.A.), which is a spherical octadecylsilica containing 6.5% of carbon.

Eluents and samples

Phosphate buffers (50 m*M*, pH 2.0 and 7.0) were used neat or mixed with 6 and 30% (v/v) acetonitrile, respectively. Acetonitrile was obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.); H_3PO_4, KH_2PO_4 and Na_2HPO_4 were supplied by Fisher (Pittsburgh, Pa., U.S.A.). The sample substances were purchased from Aldrich (Milwaukee, Wisc., U.S.A.).

Evaluation of the capacity factor

The retention time of $NaNO_3$ was taken as t_0 and the capacity factor, k, was evaluated from the retention time of the eluite, t_R, by the relationship $k = (t_R - t_0)/t_0$. All measurements of k were made in triplicate. Enthalpies were calculated by linear-regression analysis of the capacity factor data. Confidence interval estimates of these enthalpy values and also the slopes of compensation plots were obtained using the *t*-test suggested by Blaedel and Iverson[11]. All calculations were carried out on a PDP 11 mini-computer using BASIC language.

RESULTS AND DISCUSSION

The temperature dependence of the hydrophobic effect for biological sub-

stances has been the subject of controversy[12]. As the solubility of aliphatic hydrocarbons in water is dominated by entropy effects[13], it has been postulated that the strength of hydrophobic interactions increases with temperature[14]. Because the physicochemical phenomena that underlie the hydrophobic effect and solute interaction with the non-polar stationary phase are essentially the same, the widely accepted theory would predict that retention would increase with temperature in reversed-phase chromatography. Such a behavior, however, has not yet been reported in the literature nor found in our laboratory.

Chromatographic retention is conveniently measured by the dimensionless capacity factor, k, which is related to the thermodynamic equilibrium constant, K, for eluite binding by $k = \varphi K$, where φ is the phase ratio of the column. The free energy change for the process is expressed by

$$\Delta G^0 = -RT \ln K = -RT \ln (k/\varphi) \tag{4}$$

and substitution into eqn. 2 yields for the capacity factor

$$\ln k = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \varphi \tag{5}$$

If the mechanism of the process is invariant over the temperature range investigated and the enthalpy is constant, a plot of $\ln k$ against $1/T$, which is commonly referred to as a Van 't Hoff plot, yields a straight line.

In order to examine the dependence of retention on temperature, experiments were carried out over a wide range of temperature from 3 to 60°C and the capacity factors of the eluites were measured. Figs. 1 and 2 show Van 't Hoff plots for the chromatographic retention of aromatic carboxylic acids, substituted hydantoins and allantoin on octadecylsilica. The data presented in Fig. 1 for aromatic acids were obtained by using aqueous phosphate buffer with or without 6% (v/v) of acetonitrile as the eluent. The temperature interval for the data in Fig. 2 was narrower. In this instance, however, in addition to neat aqueous buffer, an eluent containing 30% (v/v) of acetonitrile was also employed. The plots are linear and the slopes are positive; hence, for these substances the enthalpy of association with the hydrocarbonaceous functions of the stationary phase is constant and negative over a wide temperature range in reversed-phase chromatography.

In addition to confirming the general observation concerning the sign of enthalpy, the results in Figs. 1 and 2 demonstrate that the enthalpy for hydrophobic interactions in such systems remains the same even at temperatures near to the freezing point of water where the structure of water changes significantly. Whereas the observed behavior is in accord with our earlier conclusions drawn from a detailed theoretical analysis of such processes[2], it contradicts the predictions often made regarding the sign of the enthalpy for the hydrophobic effect[14]. We believe that further studies on the retention of biochemical substances in reversed-phase chromatography will broaden the theoretical basis for adequate interpretation of experimental observations.

The aim of the present investigation, however, was the analysis of retention data

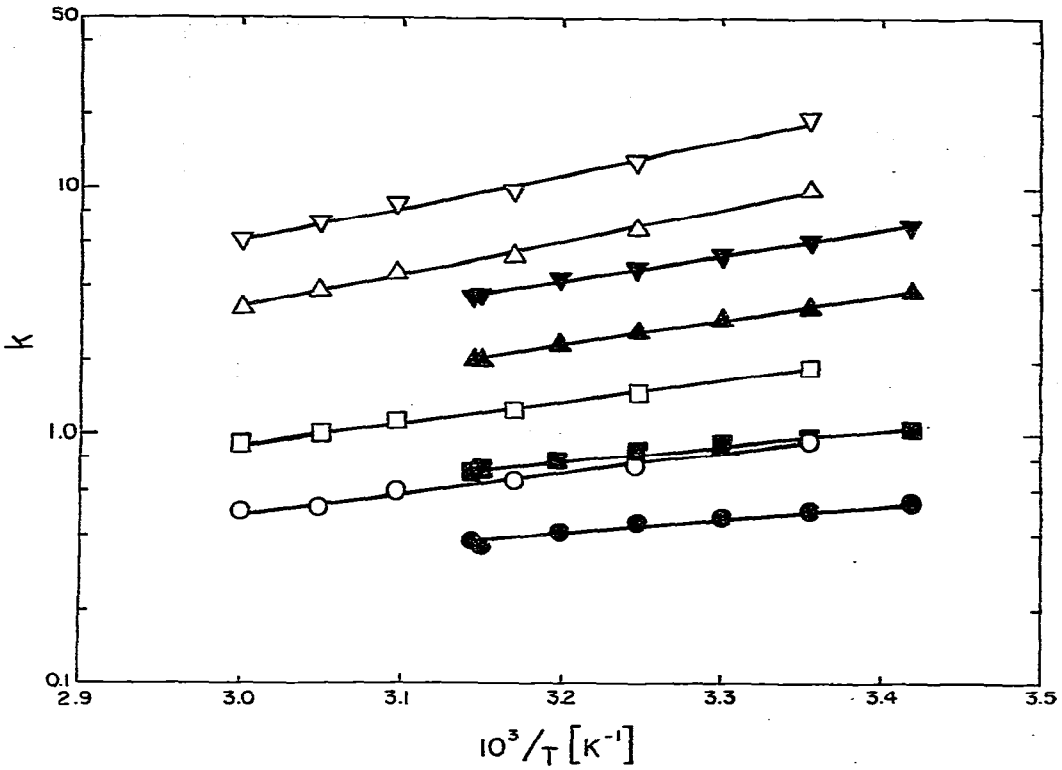

Fig. 1. Van 't Hoff plots for the capacity factors of aromatic acids in reversed-phase chromatography using octadecylsilica as the stationary phase and neat aqueous 50 m*M* NaH_2PO_2 buffer (pH 2.0) (open symbols), or the same buffer containing 6% (v/v) of acetonitrile (closed symbols) as the eluent. Column: 5-μm Spherisorb ODS, 250 × 4.6 mm. Elutes: 3,4-dihydroxymandelic acid (○,●); 4-hydroxymandelic acid (□, ■); 4-hydroxyphenylacetic acid (▽, ▼); 3,4-dihydroxyphenylacetic acid (△, ▲).

by an extra-thermodynamic approach in order to examine whether the reversible binding of various eluites to hydrocarbonaceous stationary phases manifests enthalpy–entropy compensation under a wide range of conditions employed in reversed-phase chromatography. In the case of compensation, a close correspondence of the compensation temperatures calculated for various sets of data can be accepted as proof that the retention mechanisms are essentially identical according to extensive studies on isokinetic processes[10]. In contradistinction, a significant divergence of isoequilibrium temperatures associated with the chromatographic process under different conditions can be taken as evidence of differences in the fundamental mechanism of eluite–stationary phase interactions.

Conventionally, compensation behavior is tested by the linearity of ΔH^0 *versus* ΔS^0 plots as suggested by eqn. 1. Recent work, however, has shown that under such conditions compensation may arise not only from physico-chemical phenomena of interest to us and discussed here, but also from statistical effects due to errors associated with the determination of enthalpy[15,16]. It has been shown that when plots of ΔG^0 *versus* ΔH^0 according to eqn. 3 are used, statistical compensation is minimized and

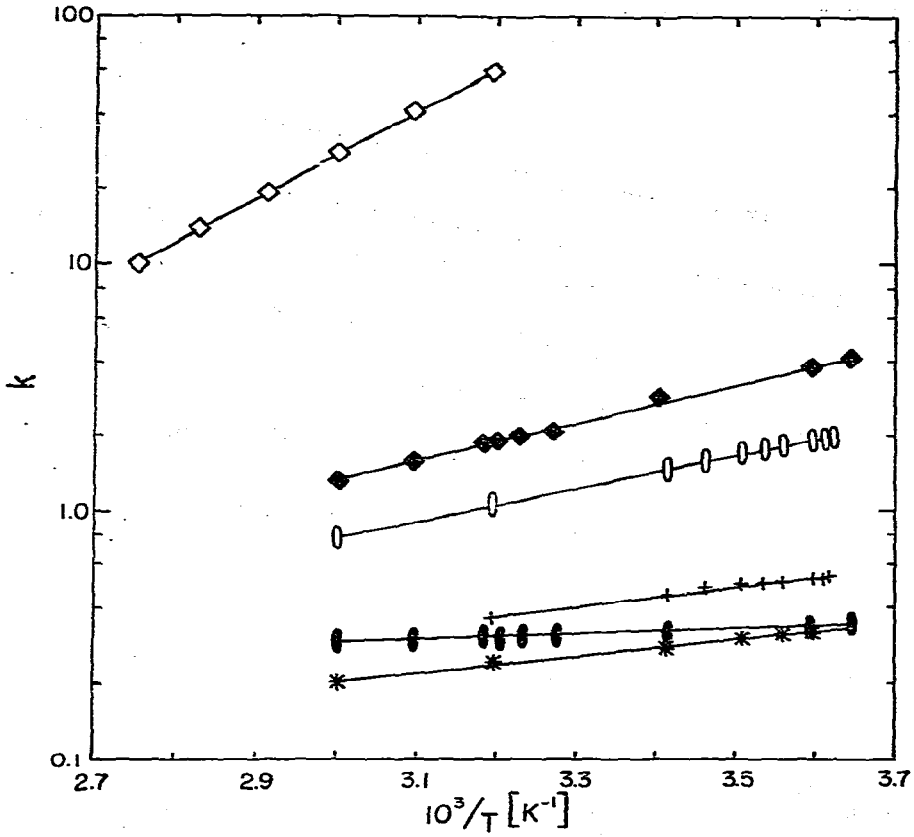

Fig. 2. Van 't Hoff plots for the capacity factors in reversed-phase chromatography using octadecylsilica as the stationary phase and neat aqueous 50 m*M* Na_2HPO_4 buffer (pH 7.0) or a 7:3 (v/v) mixture of this buffer and acetonitrile as the eluent. Column: 10-μm Partisil ODS 2, 250 × 4.6 mm. 5,5'-Dimethylhydantoin (ovals) and 5,5'-diphenylhydantoin (◇, ◆) were eluted with both eluents; open symbols refer to the neat aqueous buffer. Phenylacetic acid (+) and allantoin (*) were eluted only with neat aqueous buffer.

the linearity of such plots is indicative of compensation due to the intrinsic similarity of the chemical transformations under investigation.

Eqns. 3 and 4 can be combined to give

$$\ln k_T = -\frac{\Delta H^0}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G_\beta}{R\beta} + \ln \varphi \qquad (6)$$

where k_T is the capacity factor at temperature T. According to eqn. 6, plots of the capacity factors of various solutes measured at a given temperature under different conditions against the corresponding enthalpy change are linear when compensation occurs, *i.e.*, the reversible binding of the solute by the stationary phase is subject to an essentially identical mechanism. In order to enhance the accuracy of this diagnostic tool, the reference temperature, T, should be near the harmonic mean of the experimental temperatures used for the evaluation of the enthalpies[15].

The data presented in Fig. 1 for the temperature dependence of the retention of aromatic carboxylic acids on octadecylsilica have been re-plotted according to eqn. 6 and the results are shown in Fig. 3. It can be seen that the data obtained with both eluents, neat aqueous phosphate buffer and a mixture containing 6% (v/v) of acetonitrile, show compensation behavior. The correlation coefficient for the total set of data is 0.965. Although the correlation is better when the two data sets are fitted individually, the slopes and intercepts are virtually indistinguishable, *i.e.*, they show no statistically significant difference at the 0.05 confidence level. Consequently, enthalpy–entropy compensation is observed with the system under investigation and the compensation temperature is essentially the same when a neat aqueous or a hydro-organic eluent is used. The mechanism of the reversible binding of the aromatic acids by octadecylsilica, therefore, appears to be indifferent to the presence of the organic modifiers in the aqueous eluent.

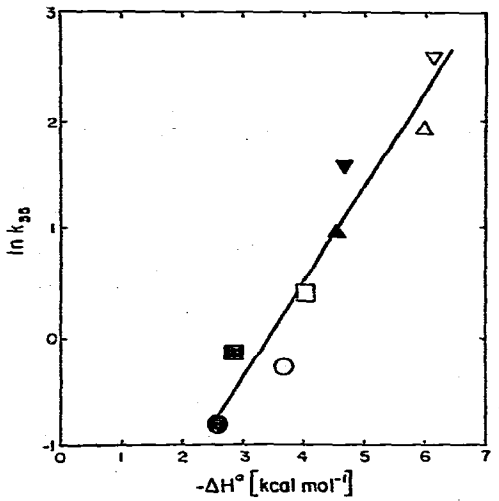

Fig. 3. Compensation plot according to eqn. 6 for the data shown in Fig. 1. The capacity factors on the ordinate were evaluated at 35°C near the harmonic mean of the experimental temperature intervals. Symbols as in Fig. 1; thus, data obtained with neat aqueous phosphate buffer and with buffer containing 6% (v/v) of acetonitrile are shown by open and solid symbols, respectively.

A compensation plot of the data presented in Fig. 2 is shown in Fig. 4. The capacity factors were measured by using aqueous phosphate buffers with and without acetonitrile as the eluent. The data for allantoin, 5,5'-dimethylhydantoin and 5,5'-diphenylhydantoin obtained with the two eluents show compensation behavior. It is especially noteworthy that the data points obtained for phenylacetic acid and dimethylhydantoin with an eluent containing 30% (v/v) of acetonitrile fall on the line generated by the hydantoin data obtained with neat aqueous eluent. Thus, these points share the compensation behavior manifested by the hydantoins even when the solvent composition is drastically changed and it does not seem to make a difference that phenylacetic acid is ionized and the hydantoins are neutral dipoles. In other words, the mechanism of interaction with the hydrocarbonaceous stationary phase appears to be the same, irrespective of the eluent composition and the electrostatic properties of the eluites.

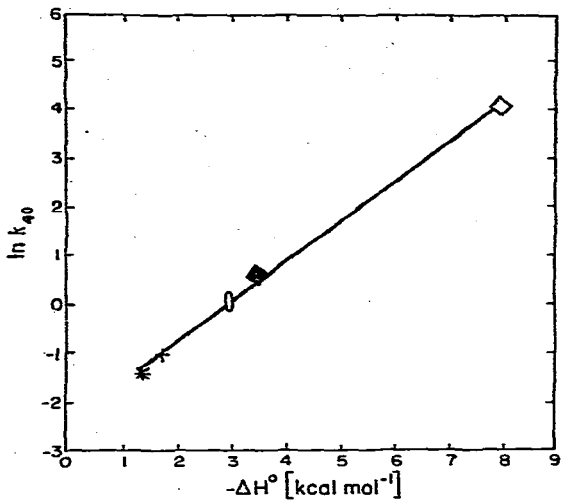

Fig. 4. Compensation plot according to eqn. 6 for the data shown in Fig. 2. The capacity factors on the ordinate were taken at 40°C near the harmonic mean temperature of the experiments. Symbols as in Fig. 2.

The above results were obtained with totally porous octadecylsilicas. On the other hand, Knox and Vasvari[17] studied the temperature dependence of the capacity factor for aromatic compounds on a pellicular reversed-phase material, Permaphase ODS, by using 40% (v/v) methanol in water at 40°C. They also measured the ΔH° values for acetone, benzene, bromobenzene, dibutyl phthalate and 1,3,4-trichlorobenzene and listed the k values at 40°C. It is believed that in this type of stationary phase a relatively thick film of poly(octadecyl)siloxane is deposited in and covalently bound to the thin porous silica layer on the surface of fluid impervious glass beads. It is therefore interesting to see whether compensation would occur by using such a stationary phase in reversed-phase chromatography. Indeed, the data evaluated according to eqn. 6 exhibit compensation behavior with a correlation coefficient of 0.995.

As enthalpy–entropy compensation occurs in all three instances examined, the corresponding compensation temperatures can be calculated according to eqn. 6. Identity or close similarity of the compensation temperatures would strongly suggest that the mechanism of reversed-phase chromatography is invariant under the conditions examined and the solvophobic binding process exhibits isoequilibrium behavior over a wide range of conditions as far as the solutes and the stationary and mobile phases are concerned.

The values of β and the intercept, $\ln \varphi - (\Delta G_{\beta}/R_{\beta})$, as obtained from the analysis of the three data sets are listed together with their 95% confidence intervals in Table I. It can be seen that the values of β vary considerably, if β is defined by the slope of $\ln k$ *versus* ΔH^{0} plots alone. If, however, the uncertainty in the slope is considered and used to establish 95% confidence intervals, the values of β given for the three instances in Table I are indistinguishable. Similar scattering of β has often been observed with isokinetic processes, but nevertheless the identity of such β values is accepted on the basis of statistical analysis[16]. The narrowest temperature

TABLE I

ENTHALPY–ENTROPY COMPENSATION IN REVERSED-PHASE CHROMATOGRAPHY USING OCTADECYL SILICA AS THE STATIONARY PHASE

The intercepts of the compensation plots according to eqn. 6 and the compensation temperatures, β, calculated from the slopes are indicated. P = 95% confidence interval.

Eluite	*Eluent*	*Column*	*T* (°K)	*Intercept*	*β* (°K)	*Range of β at 0.05 P* (°K)
Aromatic carboxylic acids	50 m*M* aqueous phosphate buffer, pH 2.0, with and without 6% (v/v) of acetonitrile	5-μm Spherisorb ODS	308	−2.97±1.08	647	539–897
Substituted hydantoins, allantoin and phenylacetic acid	50 m*M* aqueous phosphate buffer, pH 7.0, with and without 30% (v/v) of acetonitrile	10-μm Partisil ODS 2	313	−2.17±0.49	596	490–745
Substituted benzene derivatives (data from ref. 17)	Water–methanol (60:40, v/v)	Permaphase ODS	313	−3.13±0.56	639	554–755

interval estimated for β is 554–755 °K and the other two data sets yield compensation temperatures that fall in this interval. The results demonstrate that all of the data would yield a general linear plot for enthalpy–entropy compensation. Thus, compensation behavior is observed in reversed-phase chromatography on octadecylsilica with relatively low molecular weight and polar eluites under a wide range of conditions. The close similarity of the isoequilibrium temperatures strongly suggest that the retention mechanism is essentially the same in the instances investigated.

Further support for this conclusion can be gained from the analysis of the data by Kikta and Grushka[18] who measured ΔH^0 and ΔS^0 for the retention of alkylphenones on various nonylsilica stationary phases having a very low surface coverage with the covalently bound hydrocarbonaceous functions. The data obtained from experiments using 50% (v/v) water–methanol as the eluent show compensation behavior when plotted according to eqn. 1. The correlation coefficients for data obtained with three columns containing nonylsilicas of 5.1, 10.3 and 13% surface coverage have been calculated to be 0.991, 0.997 and 0.826, respectively. The respective compensation temperatures were 460, 593 and 512 °K. Although these values may reflect the effect of statistical compensation[15], which is expected in all but the most precise data for ΔH^0 and ΔS^0, they could represent intrinsic compensation. In any case, the β values obtained from such reversed-phase chromatographic experiments are similar to those listed in Table I.

As the present extra-thermodynamic approach has not been applied to the analysis of chromatographic retention, no data are available on compensation effects and isoequilibrium temperatures for other chromatographic systems. For this reason, we analysed the data of Knox and Vasvari[17], who published the capacity factors at 40°C and the enthalpies measured with phenols and acetophenone on a pellicular ether-type stationary phase, Permaphase ETH, with *n*-hexane containing 1% of ethanol as the eluent. The analysis of data showed a compensation behavior less pronounced than that in the previous instances and the correlation coefficient was

0.849. What is most significant, however, is that the compensation temperature for this set of data was 140°K. The relatively low compensation temperature (see Table I) confirms that the retention mechanism on the polar ether-type stationary phase from *n*-hexane as the mobile phase is different from that observed with non-polar hydrocarbonaceous sorbents and hydro-organic eluents.

A similar conclusion can be drawn from the enthalpy and entropy data reported by Kikta and Grushka[18] for the retention of ketones and aldehydes on nonyl- and octylsilicas having 10.3 and 19% surface coverage, respectively, when *n*-hexane was used as the eluent. Plots of their data according to eqn. 1 show compensation behavior with correlation coefficients of 0.828 and 0.912 for the C_9 and C_{18} bonded phases, respectively. The corresponding compensation temperatures were calculated to be 385 and 236 °K. These β values are significantly lower than those shown in Table I for reversed-phase chromatography. This finding is readily explained by the different retention mechanisms in the respective chromatographic processes: interaction of the eluite with the silanol groups at the stationary phase surface in one instance and solvophobic interactions with the bonded hydrocarbonaceous moieties in the other.

CONCLUSIONS

The mechanism of solute retention at the molecular level in solvophobic chromatography is yet to be elucidated. The results of the present extra-thermodynamic analysis, however, show that the reversible binding of eluites to hydrocarbonaceous bonded phases conforms to the same intrinsic mechanism under a wide range of conditions used in reversed-phase chromatography as far as the composition of the eluent, the chemical nature of the eluites and the configuration of the octadecylsilica stationary phase are concerned.

This conclusion is based on the observed free-energy relationships that manifest enthalpy–entropy compensation for retention data obtained with various relatively low-molecular-weight eluites by using different octadecylsilica stationary phases and neat aqueous or hydro-organic eluents. The compensation temperatures determined for different sets of retention data were indistinguishable, suggesting that the essential features of the underlying physico-chemical process are the same in reversed-phase chromatography under the conditions examined in this study. In contradistinction, compensation temperatures with other chromatographic systems employing polar stationary phases and non-polar eluents have been found to be markedly lower than those obtained in reversed-phase chromatography.

According to our analysis, the intrinsic mechanism of the eluite–stationary phase interaction in reversed-phase chromatography does not change as a result of the presence of acetonitrile or methanol in the aqueous mobile phase even at relatively high concentrations.

ACKNOWLEDGEMENTS

This work was supported by grants Nos. CA 21,948 and GM 20,993 from the National Institutes of Health, U.S. Public Health Service.

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