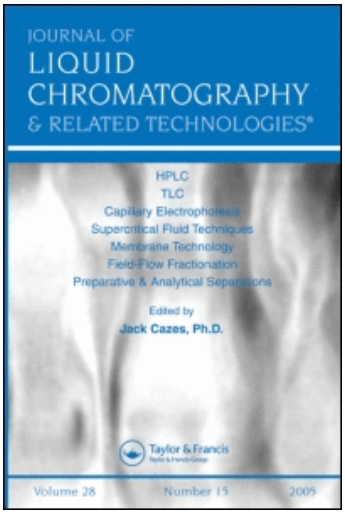

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Estimation of Molecular Parameters By Hplc

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ESTIMATION OF MOLECULAR PARAMETERS BY HPLC

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INTRODUCTION

In addition to quantitative analytical data, high pressure liquid chromatographic (HPLC) methods may be designed to collect correlation data. In particular, chromatographic retention data may be used to characterize the lipophilic nature of the solute and therefore may be used as a parameter in quantitative structure activity relationship (QSAR) which relate drug structure and pharmacological activity. Alternatively, chromatographic retention may be used as a parameter in the prediction of physical chemical properties of solute (solubility, pKa, partition coefficient). Finally, it may be

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used merely for prediction of retention character of a similar compound in the present or related chromatographic system. Some of these studies prior to 1980 have been reviewed (1, 2).

The typical experimental design for such studies proceeds in steps. A basis set of related compounds (homologs and/or analogs) is chosen and chromatographed in one or several systems. A retention parameter, usually the capacity factor, is chosen, measured for each compound, and compared with various activity (IC_{50}, LD_{50}, other drug concentration value), physical chemical property (solubility, pKa, partition coefficient, reaction rate constant), or other chromatographic (retention time, retention volume, R_f) or derived physical chemical parameter (Hansch π, Taft E_s, Hammet σ, molar volume, molar refractivity) to see if a relationship exists. If a relationship exists, the correlation may be used to predict parameters for other compounds which are not contained in the basis set. If the proper basis set has been chosen and a suitable correlation defined, this process may save time in predicting what compound should be chosen for the desired purpose. An assumed linear free energy relationship is used to provide the correlation.

Linear Free Energy Relationships

For any reaction, the change in Gibbs free energy can be expressed as

$$dG = \sum_{i=1}^{k} \mu_i \, d\, n_i = \sum_{i=1}^{k} \mu_i \, \upsilon_i \, d\, E$$

where μ_i is the chemical potential, n_i is the number of moles, and υ_i is the stoichiometric constant for the i^{th} species, and E is the extent of reaction. At equilibrium at constant temperature and pressure, the change in free energy with change in extent of reaction must go to zero since free energy must achieve a minimum value.

$$\frac{dG}{dE} = \sum_{i=1}^{k} \upsilon_i \, \mu_i = 0$$

The chemical potential is a function of activity a_i so

$$\mu_i = \mu_i^o + RT \ln a_i$$

where μ_i^o is the chemical potential when $a_i = 1$

Then $$\sum_{i=1}^{k} \upsilon_i \, \mu_i^o + \sum_{i=1}^{k} \upsilon_i \, RT \ln a_i = 0 \text{ at equilibrium}$$

and $$\sum_{i=1}^{k} \upsilon_i \, \mu_i^o = -RT \sum_{i=1}^{k} \upsilon_i \ln a_i = -RT \ln \prod_i a_i^{\upsilon_i}$$

or $$\Sigma \, \upsilon_i \, \mu_i^o = -RT \ln K \text{ where K is the equilibrium constant.}$$

$$k = \prod i \, a_i^{\upsilon_i}$$

The left hand term is the standard state Gibbs free energy $\Delta G^o = -RT \ln K$ for the case when unmixed reactants in their standard states are converted to unmixed products in their standard states.

For any series of compounds, ΔG^{o} may be measured for the reaction of a parent compound. Then ΔG^{o} in the reaction of the substituted parent compound may be measured. The additional free energy involved is assumed to be directly related to the chemical nature of the substituent and may be used as a characteristic value for that substituent in that reaction on some other "parent compound". This method was applied by Hammett (3) to the ionization process for meta and para substituted benzoic acids and has since been applied to other reaction processes. By rearranging the free energy relationships

$$\frac{\Delta G^{o}_{p} - \Delta G^{o}_{px}}{RT} = \ln K_{px} - \ln K_{p} = \sigma\rho$$

where p represents the parent compound and px the substituted compound, σ is a constant for the substituent and ρ is a constant for the reaction.

This same concept may be applied to the liquid/liquid distribution process. At equilibrium for two immiscible phases, the condition for equilibrium is that the chemical potential be equal in both phases

$$\mu_1^{o} + RT \ln a_1 = \mu_2^{o} + RT \ln a_2$$

where 1 is the aqueous phase and 2 is the organic phase.

$$\ln \left(\frac{a_2}{a_1}\right) = \frac{\mu_1^{o} - \mu_2^{o}}{RT} = \frac{\Delta G^{o}}{RT} \text{ (distribution)}$$

If a_2/a_1 is defined as the partition coefficient P and common logarithms are used

$$\log P = \frac{\Delta G^{o}}{2.3\ RT}\ (\text{distribution}) = \text{a constant}$$

Again, if the distribution experiments are performed in the same two phase liquid/liquid system, at constant temperature and pressure, then the logs of the partition coefficients for the parent compound and the substituted compound should differ according to the free energy contribution of the substituent. Hansch (4) defined this characteristic value (π) as

$$\pi = \log P_{px} - \log P_{p}$$

where, again p represents the parent compound and px the substituted compound.

Partition Coefficients

Reverse phase liquid chromatography may be considered to operate by a partitioning process. (For a better understanding of the other factors involved, see (2) and (33).) The partitioning process occurs between the mobile (usually aqueous) phase and the stationary hydrophobic alkane moiety bonded to the packing.

The relationship between HPLC chromatographic retention and partition coefficient has been derived (5)

$$\log k' = \log P + \log (Vs/Vm)$$

where k' is the chromatographic capacity factor, V_s is the

volume of the stationary phase and V_m the volume of the mobile phase. Also,

$$k' = \left(\frac{t_R - t_o}{t_o} \right)$$

where t_R and t_o are the retention times of the solute and unretained compound respectively. A similar expression can be derived for lipid impregnated paper or TLC plates (6, 7).

McCall (8) used this liquid chromatographic approach to measure partition coefficients for a series of substituted benzene compounds to establish a correlation. This correlation was tested with a series of substituted 2,4-diamino-pyrimidine-3-oxides related to the vasodilator, Minoxidil. The column was packed with a vigorously silylated Corasil C-18. Solvent systems ranged from water to 0.2% triethylamine in water to 1% triethylamine in 40% MeOH/H_2O. All k' values were normalized to 1% TEA in water by the expression

$$\frac{\text{Solvent A eluting Power}}{\text{Solvent B eluting Power}} = \frac{k' \text{ in Solvent B}}{k' \text{ in Solvent A}}$$

By chromatographing the same solute in two systems, the proportionality constants necessary were determined. Retention in H_2O was 8.1 times that in .2% TEA/H_2O, retention in .2% TEA/H_2O was 4.8 times that in 1% TEA/H_2O, and retention in 1% TEA/H_2O was 20 times that in 1% TEA/40% MeOH/H_2O.

Satisfactory correspondence between measured k' and calculated k' using a parent k' and Hansch π constants supported the reported partition coefficient values.

Haky and Young (9) reported a correlation between retention on a commercial octadecyl silane column using a simple phosphate buffer (pH 7)/methanol mixture for 68 compounds in the log P range 0.17 - 4.46. Hydrogen bonding was felt to affect both liquid partitioning data and chromatographic data, to the extent that a separate set of standards was recommended for evaluating partition coefficients with this chromatographic method for hydrogen bonding vs. non-hydrogen bonding compounds. Only phenolic compounds were evaluated, however, and extension of the studies may be necessary to apply the method to other hydrogen bonding species (e.g. those containing nitrogen or sulfur).

Mirrlees et al (10) reviewed some of the previous correlations with TLC methods and were among the first to attempt the direct measure of octanol/water partition coefficients by the use of HPLC retention. They criticized McCall's use of the hydrocarbon column correlations to Hansch π values with the statement that octanol was the only true model of itself. Their approach was to coat a slurry packed Kielselguhr support, which had been thoroughly silanized, with water saturated octanol which was then eluted with octanol saturated water until no more droplets of octanol came off the column. Pyridine N-oxide or tyrosine were used to define t_o,

octanol saturated water was the eluent, and a series of standards were injected to ensure that the published octanol water partition coefficient could be measured by their chromatographic procedure. These standards ranged from caffeine (log P = -.07) to phenol (log P = 1.46) to acridine (log P = 3.40). On each day of operation, two or three standards were chromatographed as well as the unknowns, injected in octanol saturated water solutions, to ensure that determined partition coefficients of the unknowns were valid. Two limitations were recognized in this method. Poorly water soluble compounds could not be determined, although up to 10% of a solubilizing solvent (methanol) could be used without affecting k'. In addition, an apparent partition coefficient for an ionizable compound could not determined and corrected to the true partition coefficient with the aid of the dissociation constant. The data for propranolol were interpreted as suggesting that both the cationic and neutral species partitioned in their system and therefore did not recommend the ion correction procedure in general. This approach of determining octanol water partition coefficients was advocated over the simplified approach of correlating chromatographic retention to some measured biological activity or physical chemical property. The results in the latter case would not be suitably fundamental to allow comparisons to data generated in another laboratory.

Unger (11) also advocated systems which involved octanol saturated chromatographic columns and octanol saturated aqueous mobile phases for the direct determination of octanol/water partition coefficients. Excellent correlations ($r > .99$) between partition coefficients determined this way and by the conventional shake flask approach were observed for some phenothiazines and tricylic antidepressants. Furthermore, a correlation was found ($r = .93$) between the lipophilic parameter determined in this way (essentially the octanol/water partition coefficient) and the activity of the compounds as measured by the histamine releasing activity in rat mast cells. For the same set of compounds for which k' was determined in a 20% CH_3CN in aqueous buffer not saturated with octanol, correlation of partition coefficient and activity was less ($r = .87$).

Two major points must be considered. Mirrlees suggested that often a correlation of $r = .87$ would be perfectly adequate for the purpose, especially since the octanol treatment of the column and mobile phase requires constant attention to maintain the same conditions of saturation. Furthermore, the addition of 20% organic solvent to the mobile phase will drastically change any apparent correlation for partitioning between aqueous (mobile phase) and hydrophobic (column packing) phases. For ionizable species (mobile phase), the greater the amount of added organic solvent the more tenuous the value of apparent pH becomes.

Unger correctly pointed out, however, that for chromatographic/biological correlations to apply, partitioning must be a primary factor in the action of the drug. The lipophilicity of the drug as a determinant of partitioning only operates as a correlation factor when the drug's activity is determined by its ability to partition. If electronic, steric, hydrogen bonding, dispersion forces or any other factors are dominant in drug action then no lipophilicity factor will be well correlated alone.

This was suggested recently for some 7- and 9-substituted 1-methyl isoguanines (12). Correlation between log k' and log D or π were r = 0.86 and r = 0.92 respectively for a commercial octadecyl (IBM) column and 6% acetonitrile/pH 7.4 (0.05M) phosphate buffer solvent system. The D values (distribution ratios which are sometimes referred to as apparent partition coefficients), had been determined by the shake flask method and the π values were calculated for the substituents by Hansch or Rekker fragment method (13). When each of these (log k', log D, or π) was used as the lipophilic parameter for correlation to the benzodiazepine displacement activity of the drugs, however, the log k' was shown to best describe the activity data (r = 0.78) while log P and π were about equal (r values of 0.75 and 0.72 respectively). The addition of a steric descriptor (connectivity) did not increase the correlation in any case, indicating that some mechanism in addition to

partitioning might be partially responsible for activity. If an association factor was important, then the chromatographic system which is known to have both partitioning and adsorptive components (2) in separation would be the better tool. This seemed to be the case. The additional work to determine pKa values for the 28 compounds investigated to allow correction for ionization or the effort necessary to change the chromatographic system to give better correlations were not warranted by the authors.

The lipophilicity of a series of demorphic related oligopeptides as measured by HPLC retention, TLC retention, or by summation of Hansch π values was recently reported (14). Log k' correlated well with R_m and $\sum \pi$ values but no biological data were presented.

Hammers et al (15) determined k' vales in water/methanol mixtures, extrapolated these to a methanol free solvent, and found better correlation with partition coefficient values for some methyl and n-alkyl benzenes, fused arenes, polyphenyls, chlorobenzenes, chloroanilines, and chlorophenols. The activity of the solute in the mobile phase apparently affected the results. An expression was derived to account for this

$$\log k' = \log\left(\frac{n_2\ V\ \text{org}}{n_1\ V\ \text{water}}\right) + \log\left(\frac{\Upsilon_1\ \text{org}}{\Upsilon_2\ \text{interface}}\right) + \log P$$

where n_2 and n_1 are the moles of solute in the stationary or

mobile phase, V_{org} and V_{water} are the molar volumes of the organic and aqueous phases, and γ_1 org and γ_1 interface are the activity coefficients for the solute in the organic portion of the mobile phase and the interfacial boundary with the stationary phase respectively. This reduces to the empirical expression

$$\log k' = A + B \log P$$

suggested by Collander (16) for a displacement adsorption model of retention. For a LiChrosorb RP-18 column, the steric and intramolecular electronic effects could be quantitatively evaluated for the halogenated anilines and phenols studied.

Braumann et al (17) found improved correlation between the k' value extrapolated from binary solvent systems to pure water and partition coefficient for some phenylureas, s-triazines, and phenoxycarbonic acid derivatives. This approach eliminated the selectivity effects and allowed more adequate evaluation of the hydrophobic nature of the solutes.

Valko (18) found a linear expression for log p (octanol/water) as a function of the slope and intercept of the k' vs. volume fraction of acetonitrile in the chromatographic solvent curve, for 26 compounds for the log P range -1.22 to 3.87. The statistical treatment showed that the slope and intercept values were truly independent variables thus allowing multiple linear regression of the expression

$$\log P = a \text{ (slope)} + b \text{ (log } k_o') + c$$

where k_o' is the intercept of the k' vs. volume fraction acetonitrile curve, and a, b, and c are the regression coefficients. The ratio a/b then gave the volume fraction of organic modifier in the chromatographic solvent which allowed the best correlation of retention and partition coefficient. All chromatography was performed at a pH at which all compounds were in the neutral state. The results indicated that a 48% acetonitrile/52% aqueous mobile phase and RP-18 column system was most similar to the water/octanol system for this set of compounds.

A nonlinear relationship between k' and volume fraction of acetonitrile in the chromatographic solvent was reported (19) for several hydrophobic iodo amino acid derivatives. This is the generally observed dependency for peptides and proteins and results in a quadratic expression relating k' to the organic modifier volume fraction (ψ).

$$\log k' = a + b\psi + c\psi^2$$

Such effects must be taken into account before assuming a linear k' vs. solvent fraction relationship to a set of compounds. These results were attributed to adsorptive effects during chromatography which may be related to the accessibility of solvophobic or silanophilic binding sites on the stationary phase to the iodo amino acid derivatives. The availability of these binding sites depends on the amount of water present in the solvent system. These effects should be understood prior to

attempting correlations of retention with biological activity especially for the set of compounds with very different functional groups.

Lins et al (20) used Unger's method of octanol saturated HPLC to determine the partition coefficient for indolizine. This could not be determined by the shake flask approach since the compound was unstable. k' values for several para and meta substituted 2-phenyl indolizines were then determined on an exhaustively silylated column, as suggested by McCall (8), using 40% acetonitrile in water as eluent. These compounds also were so unstable, with detectable degradation in one hour, that shake flask determination of partition coefficients was impossible. Standards with known partition coefficients were chromatographed also to standardize the system. The regression line for known log P vs. determined log k' for these compounds was used to evaluate log P for the substituted indolizines from their determined log k' values. π values were calculated for the substituents and log P for indolizine was estimated by the Hansch method. The log P for indolizine was also calculated by the Rekker fragment method (13). Very similar values for log P for indolizine were found by these three methods: 2.49, 2.41, and 2.45 respectively.

Brent et al (21) traced the history of correlations between partitioning or partition chromatography and biological activity and advocated the use of a heavily silylated reverse phase C18

column, a mobile phase with pH and ionic strength adjusted, and an organic solvent in the ionic phase to adjust k' so that column length could be maintained for compounds with widely varying log P values. The advantages of using HPLC for this purpose, rather than other chromatographic methods were stated. The method is simple, reproducible, accurate, does not require quantitative analysis of solutes, experimental conditions are easily controlled, small quantities of material of low purity can be used (as long as the retention time and true structure can be correlated), it is easily automated, and a broader range of lipophilicity can be evaluated than by TLC or shake flask methods. The objections to the use of octanol coated HPLC columns advocated by Unger and Mirrlees were the problems associated with octanol of viscosity, immiscibility, and objectionable odor. To this must be added the inconvenience of preparing and maintaining a consistent octanol saturated column for the duration of the experiments.

For a set of 10 sulfonamides and 15 barbiturates, excellent correlations were observed between k' and biological activity. For bacteriostatic activity of the sulfonamides, r values of .87 and .99 were seen for regressions either uncorrected or corrected for ionization respectively. Similarly, the hypnotic, inhibition of arbacia egg division, and O_2 uptake activity correlations with k' for the barbiturates were represented by r values of .88, .96, and .99 respectively in their method.

These authors also provided a method for calculation of the pH of aqueous methanol solutions incorporating activity and dielectric constant considerations. This provides the means of adjusting pH and ionic strength of solvent systems to pH 7.4 and the equivalent tonicity of blood for more controlled simulation of this biological fluid in this in vitro test.

Ionization Constants

The lipophilic nature of a compound may be measured by its overall solvent/solvent (usually octanol/water) distribution characteristics or by its chromatographic capacity factor. Another factor which affects lipophilicity is the molecule's ability to ionize. In aqueous systems this ionization is usually the reversible protonation or deprotonation which is described by the equilibrium acid dissociation constant Ka.

Normally, only the neutral species is considered to partition into a hydrophobic phase but for biological correlations the ionized species may also be important. This ionization helps determine aqueous solubility, may be a factor in "active transport" across membranes which are otherwise only poorly permeable to the compound, or may determine ability to act at the biological site of action through its electronic character. For a monoprotic species the distribution ratio which is measured in octanol/water, assuming no distribution of the ionized species into the organic phase nor formation or distribution of an ion pair is given by

$$D = \frac{[HA]\ org}{[HA]aq + [A^-]aq}$$

If ion pair formation occurs and this ion pair can partition, then the following expression would be expected

$$D = \frac{[HA]org + [MA]org}{[HA]aq + [A^-]aq + [MA]aq}$$

where M is the generalized counter ion and HA is the generalized acid. For the generalized basic substance B and the counter ion X

$$D = \frac{[B]org}{[B]aq + [BH^+]aq}$$

$$\text{and} \quad D = \frac{[B]org + [BHX]org}{[B]aq + [BH^+]aq + [BHX]aq}$$

The capacity factor for an ionizable solute has been shown to be (23)

$$k' = \frac{k_o\,[H^+] + k_{-1}\,(Ka)}{Ka + [H^+]} \quad \text{for an acid}$$

$$\text{and} \quad k' = \frac{k_1\,[H^+] + k_o\,(Ka)}{Ka + [H^+]} \quad \text{for a base}$$

in the absence of ion pairing where k_o, k_{-1}, and k_1 are the capacity factors for the neutral, anionic, and cationic species respectively. This approach was used to determine the pKa of several test weak acids and bases (23) for comparison against a spectrophotometric method. Excellent results were

obtained by using an XAD-2 column. This column was more stable than a silica based column to a broader pH range of solvents and also had no adsorptive sites to complicate the chromatography. The advantages of using a chromatographic approach for measurement are that compounds with very low aqueous solubility, or which lack a UV chromophore as part of the molecule which determines its ionization, can be determined even if they are somewhat impure. Recalling Mirrlees observation about stability, the speed of the chromatographic method may allow determination for relatively unstable compounds. The disadvantages lie in determining the capacity factors for the species which only exist at extremes of pH and that some very soluble compounds may not be retained.

Ionizable species may also be chromatographed on alkyl bonded silica columns in mobile phase containing other species which may act as counter ions. Van DeVenne et al (24) derived an expression for the capacity factor which may be expected if ion pairing can occur for a monoprotic acid species

$$k' = \frac{k_o'}{1 + \frac{Ka}{a_{H^+}}} + \frac{k_{-1} + k_{mx}' a_{m^+}}{1 + \frac{a_{H^+}}{Ka}}$$

while Hafkenscheid and Tomlinson (25) have shown the expression for weak bases

$$k' = \frac{k_o'}{1 + \frac{a_{H^+}}{Ka}} + \frac{k_1' + k_{BHX}' a_{x^-}}{1 + \frac{Ka}{a_{H^+}}}$$

where a_H^+ is the activity of hydrogen ions, a_M^+ and a_X^- are the activities of the counter ions, k'_{MX} and k'_{BHX} are the capacity factors for the ion pair, and K_a is the dissociation constant. Melander and Horvath (2) have discussed earlier studies of determining pKa values from chromatograhic data.

These expressions could be used 1) to characterize chromatographic stationary phases with the use of "standard" ionizable compounds, 2) to determine ionization constants from capacity factors, or 3) to determine capacity factors for the ion pair species if the values of K_a, k_o', and k' were already evaluated. By using the method for calculating true pH of the mobile phase reported by Brent et al (21), and by varying the pH of the mobile phase while holding all other parameters constant, the k' as a function of mobile phase pH could be determined. If the method of Schaper (26) for the simultaneous determination of pKa, P ion and P neutral by nonlinear regression analysis of pH dependent partition measurements were applied to the chromatographic retention data, then the HPLC method would allow significant power to the chemist for determination of all these useful molecular parameters. Further studies should address these considerations and the application to the evaluation of microequilibrium constants for compounds with several ionizable groups.

Other Molecular Parameters

In time the HPLC correlation approach may prove very useful for determining other physicochemical constants which may be intractable to measure by another method. For example, Horvath et al (27) used HPLC to measure metal/organic moiety complex association constants. Kaliszan (1) has suggested using chromatographic retention to evaluate steric and electronic effects for QSAR applications. However, correlations between gas chromatographic retention on nematic phases and steric parameters or retention on polar phases and molar refractivity (a measure of solute electronic character) were the only data mentioned. Similar correlations may be possible with the more convenient HPLC which is also intuitively more comparable to biological systems. He also summarized some correlations of pharmacokinetic data with TLC R_m values. However, it has been suggested (12) that HPLC may be the more useful tool for correlations in this regard since it is a flowing system and that this "dynamic interaction, part adsorptive and part partition may better model a biological distribution system". Correlations between retention and absorption, distribution, or elimination rate constants, or other pharmacokinetic parameters may prove to be valuable in drug design or delivery. These ideas should be developed and applied more extensively to the determination of physicochemical parameters which are otherwise difficult or tedious to evaluate.

At the very least these correlations will expand our understanding of HPLC retention. From an applied viewpoint, the information could be used to calculate k' for a given solute based on a knowledge of the chromatographic system (stationary and mobile phase) and chemical intuition about the solute. Baker (28) determined a correlation between the Retention Index for a series of 2-keto alkane standards on a reversed phase column and the sum of the Hansch π values for the substituents. Prediction of the Retention Index for some barbiturates and anthranilic acid derivatives from Hansch π estimates for these structures was possible. Predictions for a series of propranolol analogues were less successful but this was attributed to uncontrollable adsorption of the protonated form of these amines to the column.

Shalaby et al (29, 30) determined a linear correlation between the log P and retention index for series of pyridopyrimidines, either with saturated or unsaturated "A" rings. The 2-ketoalkane series was used to calibrate the system. The saturated compounds were generally more polar than the unsaturated compounds. Retention was attributed to a combination of ion exchange and partition mechanisms.

Iskander et al (31) reported the relationship of retention with molecular structure for a series of substituted strychnines in a normal phase HPLC system. While no quantitative relationship was derived, some quantitative observations were

made. Retention was a complex function of size (molecular weight) and functionality. If pharmacological data were available, it might be possible to correlate bioactivity with chromatographic retention for these compounds.

Assenza and Brown (32) determined quantitative structure retention relationships for 86 purine analogues on four different reverse phase packings. They determined substituent group contribution values for the compounds similar to the Hansch π and Rekker fragment constants. From these relationships prediction of retention of compounds on the various phases and identification of structure from compound capacity factor were possible. Further development of these concepts may allow the absolute calculation of k' for a solute on any well characterized stationary phase. Extension of these calculations to applications of activity in biological systems would be the logical extension of these studies.

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