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# DETERMINATION OF HYDROPHOBIC PARAMETERS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY: THEORY, EXPERIMENTAL TECHNIQUES, AND APPLICATION IN STUDIES ON QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS

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## 1. INTRODUCTION

The hydrophobicity of a compound, defined as its relative tendency to be readily soluble in most non-polar solvents but only sparingly soluble in water[1], plays an important role in phenomena of physico-chemical, biological and environmental interest. The expulsion of a non-polar solute from its aqueous solution due to its energetically unfavourable interaction with the water molecule network, *i.e.*, the "hydrophobic effect"[1], is the "driving force" for liquid–liquid distribution processes, micelle formation, passive membrane transport and soil sorption and bioconcentration of environmental pollutants. In order to relate the hydrophobic nature of a compound to its biological activity or environmental fate by linear free-energy relationships[2,3], many attempts have been made to describe quantitatively this particular molecular property.

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Solute hydrophobicity is usually expressed by the partition coefficient, $P$, derived from distribution studies of the compound between water and an immiscible non-polar solvent. Based on the extensive work of Hansch's group[4,5], it is now generally accepted that $P$ values obtained from the $n$-octanol–water partition system are particularly suitable for characterizing the interactions between chemical substances and biological systems. Although it has been occasionally questioned whether a bulk liquid such as $n$-octanol is really a good model for biological permeation barriers and adsorption sites, large compilations of $n$-octanol–water partition coefficients[2], as well as $\pi$ substituent[2] and $f$ fragmental[2,6] constants derived therefrom, have provided the physico-chemical basis for numerous successful studies on quantitative structure–activity relationships (QSAR). However, both the experimental determination of $P$ by the shake-flask technique and the calculation of $P$ from tabulated hydrophobic constants have a number of disadvantages (as will be discussed later), so there has been an intensive search for alternative procedures.

Since the early studies of Martin and Synge[7] and Consden *et al.*[8], it is well established that chromatography may provide quantitative information on the hydrophobicity of solute molecules. Considering retention in a chromatographic partition system as a dynamic equilibrium process with an equilibrium constant $K_R$, the retention process can be described by

$$\ln K_R = -\Delta G_R/RT \tag{1}$$

where $\Delta G_R$, $R$ and $T$ are the Gibbs free-energy change of retention, gas constant and absolute temperature, respectively. Following a theoretical treatment of the partition behaviour of solutes in thin-layer and paper chromatography[7,8], Martin[9] has shown that the addition of a substituent to the parent molecule changes the chromatographic $R_F$ value by (as a first approximation) a factor depending on the nature of the substituent and the two chromatographic phases, but not on the structure of the parent molecule itself. Thus, the chromatographic parameter $R_M$, given[10] by

$$R_M = \log (1/R_F - 1) \tag{2}$$

is directly related to other free-energy-based hydrophobic parameters[11]. For a complete discussion of the thermodynamic basis for the relationship between $R_M$ and log $P$, the reader is referred to the excellent review by Tomlinson[11], who additionally provided guidelines for the successful application of $R_M$ in studies on QSAR. Tomlinson also noted some limitations of thin-layer and paper chromatographic techniques and suggested that, for example, liquid–liquid partition chromatography "... because of their more quantitative approach, and because they lend themselves to a more precise control of experimental variables, should be seriously considered in the future for providing accurate, reproducible hydrophobic parameters"[11].

Indeed, since the development of high-performance liquid chromatography (HPLC), it soon became apparent that this technique may produce retention data of previously unattainable accuracy which, using appropriate stationary and mobile phases, can be regarded as a measure of a solute's hydrophobicity[12,13]. Haggerty and Murrill[14] were the first to use chemically bonded hydrocarbonaceous phases instead of a physically adsorbed liquid as the non-polar phase[12,13] for the determination of $n$-octanol–water partition coefficients of substituted nitrosoureas. Assum-

ing that the retention times were affected only by liquid–liquid partitioning, they calculated log $P_{OCT}$ (where OCT refers to *n*-octanol as the non-polar solvent) values which were found to agree closely with those measured by the conventional static techniques. Since then, a rapidly increasing number of workers have assimilated the approach of Haggerty and Murrill, although it became apparent that the description of retention in reversed-phase liquid chromatography (RPLC) as being a liquid–liquid partition process may be an oversimplification. The chemically bonded phase is expected not to behave as a true liquid owing to the restricted mobility of the bonded ligands Also, the mobile phase rarely consists of pure water so that the added organic modifier may exert selective effects on retention that are not necessarily related to the hydrophobic nature of the solute.

These considerations, however, seem not to have received sufficient attention. This is indicated by the sometimes mechanical transfer of previous experience from liquid–liquid partition or other chromatographic techniques to RPLC and, consequently, by the existence of numerous different approaches to the measurement of retention data, the use of a particular stationary phase and the proper choice of the mobile phase.

This review attempts to evaluate, on the basis of a short discussion of the retention mechanism in RPLC, the different approaches and their potential for providing reliable hydrophobic parameters that can be successfully employed in studies on QSAR. The discussion will be focused on the use of *n*-alkyl-bonded phases such as octadecyl-silylated silica gel because (i) the overwhelming number of studies published so far are concerned with this particular reversed phase and (ii) *n*-alkyl-bonded phases are known to exert little selective stationary phase effect on retention which may superimpose on the solvophobic effect that usually controls retention in RPLC. The ultimate purpose is to demonstrate that appropriate RPLC retention parameters may not only be used to calculate partition coefficients such as $P_{OCT}$, but may also be considered as a unique hydrophobic parameter superior to those obtained from the conventional liquid–liquid distribution systems.

Although beyond the scope of this review, it is important to note that RPLC has also been applied to the assessment of other physico-chemical solute properties such as acid dissociation constants[15] and complex formation constants[16]. New developments in this field, including also aspects of hydrophobic parameters, can be found in several recent reviews[17–21].

## 2 RETENTION MECHANISM IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

Although there is still some controversy about the appropriate physico-chemical description of retention on chemically bonded phases[22–29], the present theoretical work has provided a substantial basis for the understanding of the retention process in RPLC. It is not the purpose of this section to discuss extensively the different concepts, their merits and shortcomings, but rather to put emphasis on the underlying principles that determine the retention behaviour of polar and non-polar solutes. In this way it should be possible to rationalize similarities and differences between dynamic RPLC and static liquid–liquid partitioning and to judge the capability of RPLC for providing reliable hydrophobic parameters.

TABLE 1

SOLVENT PROPERTIES[2,36,37]

| *Solvent* | $\varepsilon^{*}$ | $\mu^{**}$ | $\gamma^{***}$ | *Proton acceptor*[§] | *Proton donor*[§§] | *Log* $P_{OCT}$[§§§] |
|---|---|---|---|---|---|---|
| Water | 78 5 | 1 84 | 73 | Large | Large | |
| Methanol | 32 7 | 1 66 | 22 | 7 5 | 7 5 | −0 66 |
| Ethanol | 24 5 | 1 68 | 22 | 5 | 5 | −0 32 |
| 1-Propanol | 20 3 | 1 65 | 23 | 4 | 4 | 0 34 |
| Acetonitrile | 38 8 | 3 37 | 29 | 2 5 | 0 | −0 34 |
| Tetrahydrofuran | 7 6 | 1 70 | 28 | 3 | 0 | 0 46 |
| Dioxane | 2 2 | 0 45 | 33 | 3 | 0 | −0 42 |

* Dielectric constant
** Dipole moment (Debye)
*** Surface tension (dyn $cm^{-1}$)
§ Proton acceptor solubility parameter.
§§ Proton donor solubility parameter
§§§ *n*-Octanol–water partition coefficient

### *2.1. Role of mobile phase*

It is now generally accepted that the mobile phase plays the dominant role in the retention process. According to the solvophobic theory developed by Sinanoğlu[30] and adapted to RPLC by Horváth *et al.*[23], the "driving force" for retention is the unfavourable interaction of a solute with the surrounding water molecules present in the mobile phase. This leads to a net free-energy change on exclusion of the solute from the eluent to the non-polar ligands of the support. Implicit in this model is the view that the interaction between solute and the stationary phase is weak and non-selective[24]. The free-energy change is determined by the energy that is needed to create a suitably sized cavity within the water–organic molecules network. This solvophobic (hydrophobic) effect[1,30] increases with increasing surface area of the solute, although polar and/or charged substituents may counteract the expulsion from the eluent by introducing Van der Waals and electrostatic interactions between the solute and solvent that favour solvation. Conversely, the energy of cavity formation decreases with decreasing surface tension of the eluent, which is achieved by the addition of an organic modifier with low dielectric constant. A quantitative treatment of the factors involved in solute–solvent interactions can be found in the work of Horváth *et al.*[23].

Both theoretical predictions have been verified experimentally. The dependence of retention on the hydrophobic surface area is reflected by a linear relationship between the carbon number of a homologous series of compounds[31,32]; the resulting methylene group contribution to retention is constant for many homologous series studied[31–33]. The effect of the surface tension of the eluent on retention is demonstrated by a linear relationship usually observed between the volume fraction of the organic modifier in the eluent and the corresponding capacity factor[34].

However, apart from being simply a reductant of surface tension, the organic modifier is known to exert selective effects on the retention of, in particular, polar solutes[35]. The reason for this becomes apparent on inspection of Table 1, which gives

some solvent properties of organic modifiers commonly used in RPLC. Among these, methanol is the most "water-like" solvent in providing both strong hydrogen-bond donor and strong hydrogen-bond acceptor abilities so that the addition of methanol to an aqueous mobile phase over a wide range of volume fractions will change the ordering of water molecules to a limited extend only[24] and will not affect the interaction potential with polar solutes. In contrast, *e.g.*, acetonitrile and tetrahydrofuran, two commonly employed modifiers, are comparatively weak hydrogen-bond acceptors only and will therefore exert a much more dramatic influence on the structure of the eluent and hence on the energetics of the solvophobic effect[30]. Further, their interaction potential within particular hydrogen-bond acceptor solutes is different and, with acetonitrile, selective dipole–dipole interactions between solute and solvent may occur[38] owing to the high dipole moment of acetonitrile (Table 1).

Indeed, examples of solvent selectivity effects are numerous and it is good chromatographic practice to use ternary mobile phases to exploit these differences for the separation of closely related compounds. As far as the determination of hydrophobic parameters by RPLC is concerned, the nature of the organic modifier is therefore very critical, and does not seem to have received the attention it deserves.

### *2.2. Role of stationary phase*

The stationary phase has been introduced above as a uniform layer of covalently bound alkyl chains that, in terms of the energetics of retention, do not differentiate between solute molecules. Although there is little doubt that the overall features of retention in RPLC can be described by considering the solution behaviour of sample molecules in the mobile phase, the structure and the properties of the hydrocarbonaceous surface do have some important implications on the use of retention data as hydrophobic parameters.

It has been argued[39–44] that the octadecyl-silylated surface is not a good model for either *n*-octanol or a biomembrane because it is alkane-like and possesses no hydrogen-bonding activity. It was therefore attempted to make an RPLC system that would behave exactly like the *n*-octanol–water system by using an *n*-octanol-coated stationary phase and an *n*-octanol-saturated aqueous phase. With this experimental set-up it was possible to obtain retention data that were well correlated with log $P_{OCT}$[39–43]. As will be shown in this section, the basic assumption concerning the structure and polarity of the stationary phase is not correct

For steric reasons, it is impossible to couple alkyl chains with all hydroxyl groups on the silica gel surface so that, depending on the bonding procedure[45,46], a variable number of residual silanol sites remain accessible to "silanophilic"[47] interactions with solute molecules. These may involve dipole interactions with polar or polarizable molecules, hydrogen bonding with hydrogen-bond acceptors and electrostatic interactions with charged molecules due to the acidic nature of the silanol groups[48]. In water-containing mobile phases, however, the silanol groups are strongly hydrated so that a mixed retention mechanism, *i.e.*, retention due to both hydrophobic and silanophilic interactions[47,49–52], is not likely to occur for neutral solutes and weak acids. These solute groups generally show linear plots of the logarithm of the capacity factor (log $k'$) *versus* the organic modifier content of the mobile phase. The dual retention mechanism of protonated bases, for example, is readily apparent from the typically U-shaped plots of log $k'$ *versus* volume fraction of the organic

modifier, which is induced by an increase in retention with increase in modifier concentration at lower water contents (*i.e.*, normal-phase behaviour) and an increase in retention with a decrease in modifier concentration at high water contents (*i.e.*, reversed-phase behaviour)[49,53]. El Tayar *et al.*[53] have recently shown that the position of the minimum in the plots depends on the pH of the mobile phase and hence on the proportion of protonated solute molecules that can participate in silanophilic interactions. It is important to note that a mixed retention mechanism can be converted into a solvophobic mechanism by the addition to the eluent of, *e.g.*, lipophilic amines, which effectively mask the binding sites for silanophilic solutes[47,49].

The structure of the alkyl-bonded phase is not completely resolved. Depending on the functionality of the silane reagents and the bonding procedure, monomeric "brush-type"[45] and polymeric phases are currently available[54]. The alkyl chains of monomeric phases, often idealized as a homogeneous fur, are not evenly distributed on the surface and have a significant proportion of folding in their structure[27,52]. Additionally, their configuration depends on the mobile phase composition. In water, the bristles shrink to form a rigid surface layer of dispersively interacting hydrocarbon chains in which solute penetration is limited[55]. In mixed organic–aqueous eluents, the alkyl ligands are solvated by the organic modifier and the bristles are more or less extended into the mobile phase[56]. Slaats *et al.*[57] have shown that the adsorption of methanol from methanol–water eluents reaches a maximum at about 20% (v/v) methanol and then remains approximately constant. These data have been interpreted as indicating that a monolayer of methanol is formed on the surface of the bonded phase For acetonitrile and tetrahydrofuran, on the other hand, the volume of the extracted layer far exceeds a monolayer[56], indicating much stronger solvent–stationary phase interactions with these more hydrophobic (Table 1) solvents. Co-extraction of variable amounts of water has been noted[50,56,58] and attributed mainly to residual silanol groups[56].

The structure of a polymeric phase is difficult to visualize because little is known about the degree of polymerization and the extent of cross-linking of the alkyl chains. Although the polymeric phase seems to possess a more rigid surface topology[59,60] and hence may interact selectively with rigid polycyclic aromatic hydrocarbons[59], their chromatographic performance resembles in many respects that of monomeric phases.

In summary, the stationary phase in RPLC should be thought of as a heterogeneous interfacial phase of highly anisotropic character[61], composed of (i) a weakly acidic silica gel surface consisting of unreacted silanol groups that are at least partially dissociated, (ii) the solvation shell of the silanol groups consisting of water and probable also the (hydrogen-bonding) organic modifier[58], (iii) patches of interacting alkyl chains more or less extended into the mobile phase and (iv) a layer of adsorbed liquid the composition of which is different from that of the mobile phase. Additionally, the system is in a dynamic state, *i.e.*, re-ordering of the chains and a rapid exchange of adsorbed mobile phase components have been shown to occur[61].

### *2.3. Comparison between static liquid–liquid distribution and dynamic chromatographic retention*

The success of *n*-octanol as a model solvent for the study of the behaviour of bioactive compounds in biosystems has been attributed to its adequate lipophilic–

hydrophilic balance brought about by the *n*-octyl chains, the hydrogen-bonding hydroxyl groups and the relatively high water content at saturation (2.3 *M*)[5,62]. The above treatment has shown that both dispersive interactions and hydrogen-bonding activity are also operating in an RPLC system composed of octadecylsilylated stationary phases and methanol–water eluents, so that it is not necessary to coat the stationary phase with *n*-octanol in order to account for hydrogen-bonding effects, as has been recommended[39–43].

However, *n*-octanol is an isotropic liquid so that the size and shape of solute molecules are not determinants of the partition process. This is in contrast to the strong anisotropic nature of a typical biomembrane for which *n*-octanol is used as a model. The major components of most membranes are phospholipids and cholesterol molecules forming a bilayer in which proteins and other lipids are incorporated. Three structurally different regions constitute the permeation barrier for bioactive solutes, *viz.*, (i) an outer region exposed to the aqueous phase and composed of the charged phospholipid head groups, which are highly polar, (ii) a water–organic interface consisting of tightly packed cholesterol rings, glycerol backbones and the first few methylene units of the hydrocarbon chains, which is a medium-polar and highly inflexible region and (iii) the tail groups of the hydrocarbon chains forming an extremely non-polar, flexible and loosely packed region[62]. Thus, a membrane will not behave as a bulk liquid in its discriminative power with respect to solute partitioning. Rather, the molecular size and shape and the orientation of functional groups relative to the other structural features of the solute will certainly contribute to the "biological" partition coefficient.

According to the picture developed above for RPLC, it is obvious that a number of similarities exist between the mobile phase–stationary phase interface and the membrane–water interface. The chemically bonded phase does not behave as a liquid but resembles much more the ordered array of the membraneous hydrocarbon chains. The residual silanol groups, some of them being charged at neutral pH, and the adsorbed layer of hydrogen-bonding organic modifier and co-extracted water molecules may be expected to figure the polar, outer membrane regions. Finally, both systems are apparently in a dynamic state where true equilibrium is seldom achieved.

These arguments should not be taken to indicate a true identity of the two systems and, further, it should be stressed that specific interactions between solutes and the lipophilic phase are usually outweighed by the hydrophobic effect and do not contribute significantly to the free-energy change of the distribution process in either liquid–liquid partitioning, membrane permeation or RPLC retention. However, a number of instances may exist where minor structural differences within a group of related compounds do have some consequences for transport phenomena in biosystems, and it is expected (and will be discussed below) that in these instances RPLC retention parameters may include additional information about the physicochemical properties of solutes that is not applicable in a liquid–liquid partition system such as *n*-octanol–water. For most systems it is further expected that retention parameters may be equally useful in describing quantitatively the hydrophobic nature of a bioactive compound.

## 3 DETERMINATION OF HYDROPHOBIC PARAMETERS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

### *3.1. Retention parameter*

Retention in liquid chromatography is quantitatively described by the capacity factor, $k'$, given by the normalized retention time (or volume), *viz.*,

$$k' = (t_R - t_0)/t_0 \tag{3}$$

where $t_R$ is the retention time of a retained solute and $t_0$ is the mobile phase hold-up time. The capacity factor is related to the thermodynamic equilibrium constant, $K_R$, via

$$k' = K_R \Phi \tag{4}$$

where $\Phi$ is the phase ratio of the stationary to the mobile phase. Combination of eqns 1 and 4 yields

$$\ln k' = -\frac{\Delta G_R}{RT} \tag{5}$$

or

$$\log k' = -\frac{\Delta G_R}{2.3\ RT} \tag{6}$$

Hence the capacity factor is the fundamental parameter for comparison of retention data and for the quantification of physico-chemical phenomena in terms of linear free-energy relationships. Some workers, however, chose to use retention times or volumes directly as a measure of a solute's hydrophobicity[63–70]. Although reasonable correlations with other hydrophobic parameters have usually been reported, these results are difficult to compare and to interpret[71] owing to the strong dependence of retention times on the specific experimental conditions.

The calculation of the capacity factor requires a knowledge of the mobile phase hold-up time, $t_0$. In theory, $t_0$ is equal to the retention time of a solute "identical" with the mobile phase[72], a value experimentally impossible to determine. In practice, several approaches have been applied to solve this problem. The most frequently used procedures include (i) the use of pure or labelled mobile phase components, (ii) the use of "non-retained" polar solutes, (iii) the use of organic or inorganic salts, (iv) the linearization of the net retention times for homologeous series and (v) differential weighing of the column[72–76]. However, each method has its shortcomings and is regarded as a more or less appropriate approximation for measuring $t_0$. For practical purposes, methods (i)–(iii) appear to be the most suitable and reliable determination techniques[57,71,76,77]. As many laboratories do not possess the equipment required to measure pure, deuteriated or tritiated mobile phase components, salts such as potassium bromide or sodium nitrate are often used as $t_0$ markers. In these instances, it is of prime importance not to use mobile phases of low to zero ionic strength because under these conditions charged solutes are partly excluded from the pores by electrostatic repulsion[78] so that the measured retention time in fact represents the

exclusion time, which is usually much shorter than $t_0$ and further is strongly concentration dependent. Similar problems are known to occur with relatively large $t_0$ markers.

*3.2. Correlation between the capacity factor and the n-octanol–water partition coefficient*

Numerous studies have described the relationship between log $k'$ and other hydrophobic parameters in terms of linear free-energy relationships. Mutual dependences have been reported for log $k'$ and water solubility[79–81], hydrophobic surface area (volume)[79,82–85], molecular connectivity indices[24,82,83,85–89] and quantum mechanically calculated parameters[18,90]. Most reports, however, have described the correlation between log $k'$ and log $P_{OCT}$ (or hydrophobic constants derived therefrom). As the above-mentioned descriptors of molecular properties basically reflect the same solute physico-chemical property and hence are strongly interrelated[2,6,18], the following discussion will be focused on the observed log $k'$–log $P_{OCT}$ relationships for which the relevant literature data are collected in Table 2. In order to organize the data better, the entries in Table 2 are grouped according to the organic modifier used, and within a group are ranked in order of decreasing volume fraction of organic modifier in the mobile phase. Note that additional reports have appeared (given at the bottom of Table 2) that contain related information, but whose style of data presentation precluded their inclusion in Table 2.

Considering first methanol–water eluents, the reported statistical significance of the log $k'$–log $P_{OCT}$ correlations for a wide variety of different solute classes is remarkably high. Haky and Young[98], in a study including 68 different solutes of very diverse chemical character, reported an equation that left only 6.6% of the variance unexplained by the model, and that could be improved only slightly by omitting the strong hydrogen-bonding phenolic compounds from regression analysis. Hammers *et al.*[99], using acidic chlorophenols, basic chloroanilines and apolar solutes, and El Tayar *et al.*[100], studying the retention of 48 *ortho-*, *meta-* and *para-*disubstituted benzene derivatives, obtained regression equations of similar quality. Obviously, the observed mutual relationship between log $k'$, measured in methanol–water eluents, and log $P_{OCT}$ does not depend on the particular structure of the solute group (Table 2) but reflects a general correspondence of the nature of the distribution processes.

The regression equations collected in Table 2 may be regarded as a special case of the Collander equation[121], which relates the partition coefficients measured in two different partitioning systems, *viz.*,

$$\log P_1 = p \log P_2 + q \tag{7}$$

where $p$ and $q$ are constants that are characteristic of the non-polar solvent employed in combination with water. If log $P_{OCT}$ is considered as the reference partition coefficient, reasonable correlations can be expected only when the other non-polar solvent shows similar hydrogen-bonding activity[4,6,121]. In view of the overall degree of correlation between log $P_{OCT}$ and log $k'$ shown in Table 2 for methanol–water eluents, it is clear that the hydrogen-bonding potency of the RPLC system must be very similar to that of the *n*-octanol–water system. This conclusion is further substantiated by the findings of Hammers *et al.*[99] and Hafkenscheid and Tomlinson[101], who ob-

TABLE 2

LITERATURE DATA FOR THE RELATIONSHIP BETWEEN THE *n*-OCTANOL–WATER PARTITION COEFFICIENT (LOG $P_{OCT}$) AND THE CAPACITY FACTOR (LOG $k'$) LOG $P_{OCT} = a$ LOG $k' + b$

The data were either taken directly from the literature or calculated from given retention data using log $P_{OCT}$ values from ref 2* In some instances, Hansch $\pi$ constants or Rekker fragmental constants ($f$) were used instead of log $P_{OCT}$ in the cited references Parameters and abbreviations: $r$, regression correlation coefficient; $n$, number of data points, $\varphi$, volume fraction of organic modifier in the mobile phase; MeOH, methanol, AN, acetonitrile, THF, tetrahydrofuran, EtOH, ethanol, Dio, dioxane, Ace, acetone, n g , not given in the references cited

| *Class of compounds* | *a* | *b* | *r* | *n* | *φ* | *Modifier*** | *Stationary phase* | *Ref* |
|---|---|---|---|---|---|---|---|---|
| Polycyclic aromatic hydrocarbons | 3 20 | 3.24 | 0 977 | 26 | 0.90 | MeOH | Partisil RP-18 | 89 |
| Aromatic hydrocarbons, chlorinated compounds, pesticides | 2.70 | 2 37 | 0.987 | 37 | 0 75 | MeOH | Zorbax RP-18 | 93 |
| Alkylbenzenes | 1.89 | 1 88 | 0.971 | 11 | 0 70 | MeOH | Hypersil RP-18 | 82 |
| Alkylbenzenes | 2 08 | 2.63 | 0 977 | 10 | 0.70 | MeOH | μBondapak RP-18 | 91 |
| Alkylbenzenes | 3.03 | 2 39 | 0.984 | 5 | 0 70 | MeOH | Jasco FineSil RP-18 | 92 |
| Benzophenones | 3 61 | 2.94 | 0.986 | 14 | 0.70 | MeOH | μBondapak RP-18 | 88 |
| Chlorinated benzenes, toluenes, anilines | 2 50 | 2 12 | 0 989 | 20 | 0 70 | MeOH | LiChrosorb RP-18 | 94 |
| Phenoxyacetic acids | 2 31 | 3 40 | 0 941 | 7 | 0 70 | MeOH (1) | LiChrosorb RP-18 | 95 |
| Phenylureas | 2.31 | 2 62 | 0 925 | 11 | 0.70 | MeOH | LiChrosorb RP-18 | 95 |
| Substituted benzenes | 1 72 | 1 34 | 0 932 | 42 | 0 60 | MeOH | Nucleosil RP-18 | 96 |
| Pyridazinones | 1 61 | 1 79 | 0 956 | 8 | 0 60 | MeOH | LiChrosorb RP-18 | 97 |
| Phenols | 1 53 | 1 32 | 0 985 | n g | 0 55 | MeOH (2) | Alltech RP-18 | 98 |
| Miscellaneous | 1 65 | 0.95 | 0 966 | 68 | 0 50 | MeOH (2) | Alltech RP-18 | 98 |
| Halogenated aromatics, anilines, phenols | 1 39 | 1 39 | n g | 36 | 0 50 | MeOH (3) | LiChrosorb RP-18 | 99 |
| Disubstituted benzenes | 1.80 | 0 72 | 0.965 | 48 | 0 50 | MeOH (4) | LiChrosorb RP-18 | 100 |
| Barbiturates | 1 53 | 1.01 | 0 952 | 10 | 0.50 | MeOH | Hypersil RP-18 | 101 |
| Barbiturates | 3.58 | 0 52 | 0.945 | 24 | 0 50 | MeOH | Partisil RP-18 | 87 |
| Hydantoins | 1 63 | 0.83 | 0 963 | 17 | 0.50 | MeOH | Hypersil RP-18 | 101 |
| Aromatic acids | 1.26 | 1 61 | 0.985 | 12 | 0 50 | MeOH (5) | Hypersil RP-18 | 101 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Aryloxoalkanoic acids | 1 66 | 0 95 | 0 992 | 17 | 0 50 | MeOH (6) | μBondapak RP-18 | 102 |
| Basic drugs | 1 91 | 0 97 | 0.974 | 29 | 0.50 | MeOH (7) | Hypersil RP-18 | 103 |
| Phenylureas | 1 32 | 1 29 | 0 950 | 6 | 0 30 | MeOH | Micropak RP-18 | 104 |
| N-Methylcarbamates | 4.53 | −0 77 | 0 946 | 9 | 0 25 | MeOH | Micropak RP-18 | 104 |
| Alkylbenzenes | 1.13 | 2 07 | 0 906 | 19 | 0 75 | AN | Jasco FineSil RP-18 | 105 |
| Aromatic hydrocarbons | 4 66 | 1.92 | 0 990 | 15 | 0 75 | AN | n g | 79 |
| 1-Arylpiperazines | 2 09 | 0 92 | 0 985 | 12 | 0.70 | AN (8) | μBondapak RP-18 | 106 |
| Fused arenes | 3 92 | 1 34 | 0 985 | 10 | 0.70 | AN | Develosil RP-18 | 83 |
| Halogenated benzenes | 3 61 | 1 43 | 0 988 | 13 | 0.70 | AN | Develosil RP-18 | 83 |
| Substituted benzenes | 1 90 | 1 34 | 0.893 | 41 | 0.50 | AN | Nucleosil RP-18 | 96 |
| Alkylbenzenes | 0 58 | 2 57 | 0 906 | 19 | 0 45 | AN | Jasco FineSil RP-18 | 105 |
| Urinary aromatic acids | 1 96 | 1 31 | 0.929 | 37 | 0.40 | AN (9) | Chromosorb RP-18 | 107 |
| Benzamides | 1 90 | 0 79 | 0 987 | 10 | 0 18 | AN | Nucleosil RP-18 | 108 |
| Fused arenes | 11 23 | −3.31 | 0 901 | 10 | 0 50 | THF | Develosil RP-18 | 83 |
| Halogenated benzenes | 4.53 | 0 46 | 0 957 | 13 | 0 50 | THF | Develosil RP-18 | 83 |
| Substituted benzenes | 2 21 | 1 31 | 0 817 | 25 | 0.40 | THF | Nucleosil RP-18 | 96 |
| Pesticides | 2 32 | 1 88 | 0 976 | 21 | 0 65 | EtOH | LiChrosorb RP-8 | 109 |
| N-Methylcarbamates | 1.85 | 0 65 | 0 968 | 9 | 0.25 | Dio | Micropak RP-18 | 104 |
| Phenols | 1 91 | 1.92 | 0 961 | 9 | n g | Ace | μBondapak RP-18 | 110 |
| Anilines | 2 24 | 1 44 | 0 968 | 12 | n g | Ace | μBondapak RP-18 | 110 |

* Related information which by its nature does not fit into the table can be found for 5-nitroimidazoles[111], dermorphin-related oligopeptides[112], benzodiazepines[113], phenols, guaiacols and catechols[114], barbiturates and herbicides[115], barbiturates[87], fatty acids, alcohols and substituted benzenes[116], aliphatic hydrocarbons[117], methylisoguanines[118] and miscellaneous compounds[63,85,119,120]

** In aqueous eluents unless indicated as follows· (1) 0 5 *M* acetate buffer (pH 2 9), (2) 0 05 *M* ammonium phosphate buffer (pH 7), (3) 0.05 *M* acetate buffer (pH 4 5), (4) morpholinopropanesulphonic acid (pH 7.4) + 0.2% *n*-decylamine, (5) 0 08 *M* ammonium phosphate (pH 2 15), (6) 0.0025 *M* phosphate buffer (pH 3), (7) 0 08 *M* ammonium phosphate (acetate) (pH 4 or 7) + 0 8 m*M* N,N-dimethylaminododecane; (8) 0 01 *M* dipotassium hydrogen phosphate (pH 5—7.7), (9) 0 04 *M* phosphoric acid (pH 2)

served a poor correlation between log $k'$, determined in methanol–water eluents, and log $P_{ALKANE}$ for polar solutes capable of forming hydrogen bonds.

For acetonitrile–water eluents, reasonable correlations between log $k'$ and log $P_{OCT}$ have frequently been observed for non-polar solutes possessing at least some structural relationship, *i.e.*, for aromatic hydrocarbons[79], 1-arylpiperazines[106] and halogenated benzenes[83] When heterogeneous solute groups are employed, the capacity factors seem to be only moderately correlated with the corresponding log $P_{OCT}$ values This is readily apparent from the retention data presented by Schoenmakers *et al.*[96] for a large set of substituted polar and non-polar benzene derivatives and those reported by Hanai and Hubert[107] for urinary aromatic acids.

Jinno and Kawasaki[85] have shown that, when using acetonitrile–water eluents and polar solutes, the correlation between log $k'$ and log $P_{OCT}$ (in fact, $\pi$) can be improved by introducing a term that corrects for differential hydrogen bonding:

$$\log k' = 0.14\,\pi - 0.049\,(\mathrm{HA} - \mathrm{HD}) - 0.01 \qquad (8)$$

where the term HA − HD corrects for hydrogen-accepting (HA) and hydrogen-donating (HD) activity. This approach is equivalent to the differentiation of solutes into hydrogen-bond donors and hydrogen-bond acceptors, as has been proposed by Leo *et al.*[4] for the appropriate comparison of partition coefficients measured in solvent systems that contain disparate hydrogen-bonding activities.

Although the limited data set available for tetrahydrofuran–water eluents precludes a detailed discussion, it seems to be clear from the retention data reported by Schoenmakers *et al.*[96] that the range of solute structures for which reasonable correlations between log $k'$ and log $P_{OCT}$ can be established is even more restricted. Other eluent systems cannot be judged owing to the lack of sufficient data.

In summary, a general relationship between log $P_{OCT}$ and log $k'$ for a broad range of structures of biological interest can only be expected to exist for capacity factors determined in methanol–water eluents. This is exactly what one would expect by considering the retention mechanism in RPLC and the organic modifier properties shown in Table 1. As only methanol provides both strong hydrogen-donating and hydrogen-accepting capabilities, (i) the stationary phase–mobile phase interface contains hydrogen-bonding activity owing to adsorbed methanol (and water[122]) molecules and (ii) the water molecule network can incorporate a fairly large amount of methanol, thus in principle maintaining the highly ordered array of water molecules that is the driving force for the hydrophobic effect[1,30]. For acetonitrile and other solvents, the reduced hydrogen-bonding capability of the eluent contributes to a solvophobic effect, the energetics of which are different from those operating in the *n*-octanol–water system and in methanol–water eluents. Finally, the participation of residual silanol groups in the retention process seems to be much more pronounced in solvents other than methanol, as has been demonstrated, for example, for acetonitrile by Hoffman and Liao[123]. Thus, methanol seems to be the organic modifier of choice for the determination of RPLC hydrophobic parameters

Comparing the slope and intercept values of the Collander-type equations collected in Table 2, it is evident that there is no single relationship between solute retention and log $P_{OCT}$. Rather, the individual results depend on the nature of the solute group, the amount of methanol present in the mobile phase and the particular stationary phase used to measure log $k'$.

The slope of the Collander equation is a measure of the solvent system's sensitivity to changes in the hydrophobicity of solutes relative to *n*-octanol, *i.e.* a slope of 1.0 would indicate isodiscriminative behaviour[4]. The reported values vary from 1.26 to as high as 4.53, indicating that a given change in hydrophobicity is detected very differently and less sensitively in terms of log $k'$. Although there is a trend towards lower slopes with decreased organic modifier content in the eluent, the observed exceptions (*e.g.*, barbiturates[87]) also indicate an influence of the structure of particular solute groups.

The intercept of the Collander equation gives a measure of the hydrophobicity of the non-aqueous phase relative to *n*-octanol[4]. Consider, *e.g.*, an equal distribution of a solute molecule between the stationary and mobile phases, *i.e.*, $K_R = 1$, then the capacity factor is equal to the phase ratio (see eqn. 4). Assuming a phase ratio of 0.6–0.7 for reversed-phase material and introducing the resulting capacity factor into the regression equations reported in Table 2, most of the corresponding log $P_{OCT}$ values would be more or less positive. In other words, a solute that is equally distributed between the stationary and mobile phases would prefer *n*-octanol over water as a solvent, *i.e.*, the non-polar phase in RPLC in many instances behaves as if it is more hydrophobic than *n*-octanol. The apparent hydrophobicity of the stationary phase decreases with decreasing methanol concentration in the eluent (Table 2), and additionally depends on the structure of the solute. This has been clearly demonstrated by Haky and Young[98], who showed that the magnitude of the intercept values of the log $k'$–log $P_{OCT}$ plots was related to the extent to which hydrogen bonding was involved in the solute's distribution process.

The results obtained indicate that the expression of hydrophobicity in terms of log $k'$ is relative in nature, and that an established log $k'$–log $P_{OCT}$ correlation for a given class of compounds cannot be extrapolated either to different solutes or to other separation systems, even if the latter consists of an identical mobile phase and a stationary phase of nominally the same composition. The observed variations are induced by the stationary phase, the volume fraction of organic modifier in the eluent and the structure of the solute, which together constitute a major drawback in comparison with the classical *n*-octanol–water system, which provides a single, continuous hydrophobicity scale. Further, the necessity to use reference compounds with known log $P_{OCT}$ values to calibrate the log $k'$–log $P_{OCT}$ plots contains an inherent difficulty. From a theoretical viewpoint, as many standards as possible should be employed to cover all possible interactions between solute, mobile phase and stationary phase, which from a practical viewpoint is impossible to achieve. In practice, this problem is sometimes solved pragmatically by selecting a few standards that simply cover the expected hydrophobicity range of the solute group under study. An inappropriate choice of reference compounds may not only lead to under- or overestimation of the apparent hydrophobicity, but may also reduce the goodness of fit of the data to the log $k'$–log $P_{OCT}$ correlation equation.

It has been claimed[124] that the measurement of hydrophobic parameters for ionizable compounds is not possible using RPLC. For these solutes, the reference log $P^0_{OCT}$ refers to the ratio of the non-ionized compound in each phase. What is actually observed is the ratio of the concentration, assuming that only the uncharged solute can partition into the *n*-octanol phase, of the neutral species to that of all molecular species in the aqueous phase. However, it is known[4] that the non-polar phase may

contain a considerable fraction of charged solutes owing, for example, to dimer formation or partitioning of ion pairs formed with the buffer components, so that the pH of the aqueous phase is usually adjusted to avoid ionization. In RPLC, this approach is applicable to only a limited extent because the silica gel matrix is unstable outside the pH range 1.5–7 5. Therefore, many basic solutes cannot be chromatographed in their non-ionized state

As has been initially shown by Horváth *et al.*[15], the capacity factor can be corrected for ionization effects by the application of the equation

$$k' = [1 + 10^{(pK_a - pH)_{mob}}]^{-1} k^0 + [1 + 10^{(pH - pK_a)_{mob}}]^{-1} k^+ \quad (9)$$

where $k^0$ and $k^+$ are the capacity factors of the non-ionized and fully ionized solute, respectively, and the subscript mob. refers to the actual eluent composition. Hafkenscheid and Tomlinson[103] measured the $pK_a$ values of basic drugs under mobile phase conditions and where able to calculate $k^0$ and to relate it to the corresponding log $P^0_{OCT}$:

$$\log P^0_{OCT} = 1.819\ (\pm\ 0.078) \log k^0 + 0.597\ (\pm\ 0.136) \quad (10)$$

$$n = 28;\ r = 0.977;\ F = 543;\ s = 0.298$$

The observed highly significant correlation shows that RPLC is in principle also suited to provide hydrophobic parameters for charged compounds. As the use of eqn. 10 requires the knowledge of $pK_{a(mob)}$ which is difficult and tedious to determine[103,125], the authors further developed a simple semi-empirical ion correction equation, the solution of which requires only log $k'_{mob}$ and log $k^+$. Log $k^+$ can be easily determined by using a mobile phase with a pH at least 2 units lower than the $pK_a$ values of the bases.

Recently, Fong *et al.*[106] supplied evidence that the problem of correcting for ionization effects on retention may be handled in an even simpler way. Using basic 1-arylpiperazines, they found that the capacity factors at each eluent pH was equally well correlated with log $P^0$ so that the partition coefficient of the neutral species could be adequately described by considering only the pH of the mobile phase and the resulting capacity factor by

$$\log P^0 = 0.029 \log k' - 2.003\ pH_{mob} + 14.923 \quad (11)$$

$$n = 12;\ r = 0.97$$

Additional support comes from the work of Unger and co-workers[40,42,43] and others[53,70,126,127], who also found strong correlations between log $k'$, whether corrected or not for ionization, and other hydrophobic parameters so that ionizable compounds do not seem to represent an insurmountable problem for the determination of hydrophobic parameters by RPLC. It should be stressed, however, that much work is still required for the proper assessment of (i) the effects of organic modifiers on $pK_a$ values of solutes and mobile phase buffer components[125], (ii) the formation of ion pairs in the mobile phase[43] and (iii) changes in the ionization of residual silanol groups and their possible participation in the retention process of charged solutes, before a general procedure can be established for the appropriate handling of such "problem" solutes.

Another possibility for circumventing the above-mentioned problem is to use ion-pair RPLC, where retention of a charged solute is enhanced by complexation with an oppositely charged lipophilic pairing ion (see ref. 128 for a review). For example, Riley *et al.*[129] used ion-pair RPLC to measure the retention behaviour of *s*-triazines, azapurines and benzoic acids on octadecylsilica gel. Functional group contributions, calculated from the capacity factors, were linearly related to the corresponding Hansch $\pi$ substituent constants. Recently, it has been shown that the approach of Riley *et al.* is also valid for benzylbenzoic acid derivatives[130].

In order to summarize the presently available evidence for the capability of RPLC of providing hydrophobic parameters of general significance, the main disadvantage of log $k'$ lies not in its possible limitation to particular solute groups such as neutral compounds, but in its dependence on the stationary phase properties, the organic modifier content of the eluent and the structure of the solute These variables lead to log $k'$–log $P_{OCT}$ relationships of little general significance and may, owing to the necessity to use reference log $P_{OCT}$ values, induce considerable errors in the magnitude of the hydrophobicity of a particular compound and in the relative hydrophobicity within a group of related compounds.

### *3.3. Origin of the variability of log $P_{OCT}$–log $k'$ correlations*

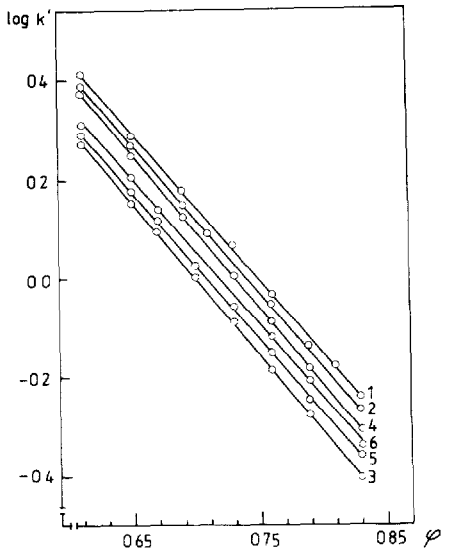
#### *3.3.1. Stationary phase effects*

The lack of uniformity of retention data obtained with identical mobile phases and stationary phases of nominally the same composition for a given solute series is caused by differences in the stationary phase properties[45,131]. Although in water-rich eluents homoenergetic retention, *i.e.*, identical intrinsic thermodynamical behaviour, is observed for most solute classes on many alkyl-bonded phases[132], absolute solute retentions may differ significantly This is shown in Fig. 1 for benzene, the capacity factors of which were determined on six reversed-phase packings at different mobile phase compositions[133]. The variability of absolute retention is the result of differences in the phase ratios per unit volume of the columns[45] so that, depending on the characteristics of the starting material and the bonding procedure used by the manufacturer, a variable number of alkyl chains per unit area are accessible to stationary phase–solute interactions Such differences affect the Collander equation relating the capacity factor to log $P_{OCT}$ by changing the apparent hydrophobicity of the stationary phase and hence the corresponding intercept value. This is readily apparent by comparison of the Collander equations reported by Smith[82] with that reported by Koopmans and Rekker[91] for alkylbenzenes (Table 2).

As long as no standardized procedure for bonded-phase synthesis is employed by the suppliers, column-induced differences in absolute retention will be large. For example, the log $k'$ values for benzene show an absolute variation of 0.16 (on the logarithmic scale), which, considering the relatively narrow range of experimentally available log $k'$ values of $-0.5$ to $+1.0$, will produce a variability in log $k'$ of at least 16%, but usually much more.

#### *3.3.2. Composition of the mobile phase*

The results presented in Table 2 indicate that the slopes and the intercepts of the log $k'$–log $P_{OCT}$ plots were dependent on the organic modifier concentration in



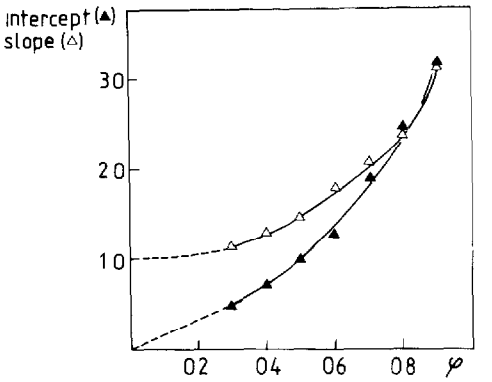




Fig. 1 Plot of log $k'$ *versus* $\varphi$, the volume fraction of methanol in the mobile phase, for benzene Log $k'$ was determined on six different *n*-alkyl-bonded stationary phases[133]

Fig 2 Dependence of the slope (△) and the intercept (▲) of the equation relating log $k'$ to the *n*-octanol–water partition coefficient log $P_{OCT}$ (log $P_{OCT} = a \log k' + b$) on the volume fraction of methanol, $\varphi$, at which log $k'$ was measured The data were taken from ref 96

the eluent. In order to shed light on the supposed dependences, we have related the capacity factors presented by Schoenmakers *et al.*[96] for benzene derivatives to their log $P_{OCT}$ values as given by Hansch and Leo[2]. Using fourteen solutes with log $P_{OCT}$ values from 1.10 (benzyl alcohol) to 4.07 (biphenyl), the capacity factors measured in the methanol volume fraction range 0.3–0.9 were used to calculate the relationship between the slopes and intercepts of the individual Collander-type equations and the methanol content of the eluent (Fig. 2). It is apparent, and indicated by the dotted line in Fig. 2, that the values of the slope converge to 1 0 and that the intercept values converge to zero for a pure aqueous eluent. The same has been shown by Harnisch *et al.*[134] to apply to a series of *n*-alkylbenzenes and seven structurally unrelated OECD (Organization for Economic Cooperation andDevelopment) reference compounds.

In terms of the Collander equation, these findings indicate that the capacity factor obtained in 100% water is identical with log $P_{OCT}$, and that addition of methanol to the eluent leads to a reduced sensitivity with respect to changes in hydrophobicity (slope $>1.0$) and to an enhanced hydrophobicity of the stationary phase (intercept usually $>0.0$).

### *3.3.3 Solute–solvent interactions*

Selective solute–solvent interactions are revealed by Fig. 3, which shows a plot of log $k'$ *versus* the volume fraction of methanol, $\varphi$, for adenine, adenosine and adenosine 3′,5′-monophosphate. This particular example was chosen in order to demonstrate the effect of solute size and polarity differences on the slope of the log $k'$–

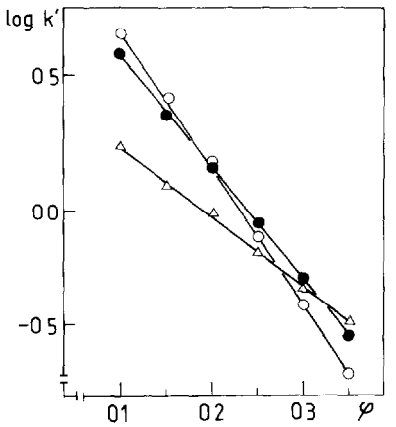



Fig. 3 Relationship between the capacity factors of nucleic acid components, log $k'$, and the volume fraction of methanol, $\varphi$, in the mobile phase The aqueous part of the binary eluent consisted of 100 m$M$ phosphate buffer (pH 6.6). △, Adenine, ●, adenosine, ○, adenosine 3′,5′-monophosphate.

$\varphi$ plots[135]. Regarding the use of the capacity factor as a hydrophobic parameter and considering the data shown in Fig. 3, one would come to completely different conclusions whether log $k'$ is measured at $\varphi$ = 0.35, 0 25 or 0.10 owing to the intersections shown in Fig. 3. Although this example is an extreme case, large slope differences have been also observed for more closely related solutes[99,136]. Thus, selective solute–solvent interactions, if not accounted for, will affect the slope and intercept of the Collander equation and, at worst, will adversely affect the overall correlation owing to the possibility of accidently selecting the "wrong" mobile phase composition (*cf.*, Fig. 3).

We have shown[95,97] that selective solute–solvent interactions can be eliminated by using log $k_w$, the capacity factor obtained by extrapolation of retention data from binary eluents to 100% water, as a hydrophobic parameter. Thus, the discussion of the limitations of log $k'$ indicates log $k_w$ as a possible candidate for overcoming the restricted applicability of RPLC capacity factors for the assessment of the hydrophobic nature of solute molecules.

## 4 THE LOG $k_w$ CONCEPT

The possibility of using log $k_w$ as a hydrophobic parameter was first envisaged and demonstrated by Hulshoff and Perrin[136] for a series of benzodiazepines. More recent studies have added strong evidence that log $k_w$ is more closely related to log $P_{OCT}$ than isocratic capacity factors. However, log $k_w$ values are usually too high to obtain experimentally, and therefore have to be calculated using extrapolation techniques.

According to the solubility parameter concept[25], the relationship between solute retention and the composition of the mobile phase can be described by

$$\log k' = \log k_w + A\varphi^2 - S\varphi \tag{12}$$

where $A$ and $S$ are constants for a given solute–eluent combination and $\varphi$ is the volume fraction of the organic modifier in the aqueous eluent. Schoenmakers *et al.*[28] have recently shown that the validity of eqn. 12 is restricted to mobile phases that contain less than 90% water. When more water was used, the quadratic eqn. 12 turned out to be insufficient and required the inclusion of a correction term. Further, the curvature of the log $k'$–$\varphi$ plot, as described by eqn. 12, was mainly caused by capacity factors measured at the upper or lower end of the volume fraction range. These findings refer to a discontinuity of the retention mechanism at very high or low water concentrations in the eluent. In the first instance, the extended *n*-alkyl chains shrink to form a rigid surface of interacting hydrocarbon chains that are poorly wetted by the eluent[28,55], whereas at very low water concentrations the mobile phase change from a water-like structure to an organic modifier-determined structure that exerts its own solvophobic effect[24]. Hence it is only the intermediate volume fraction range for which the discussed similarities between retention, *n*-octanol–water partitioning and membrane permeations hold, *i.e.*, a hydrogen-bonding non-polar phase and a water-like polar phase. If methanol is regarded as the most suitable organic modifier, its minimum concentration can be derived from the studies of Slaats *et al.*[57] and Scott and Simpson[60], who showed that a sufficient solvation of the bonded phase is attained at $\varphi$ values of 0.1–0.2. For the upper limit, practical experience concerning the curvature of the log $k'$–$\varphi$ plots indicate a value of 0.8–0.9 as being appropriate. Therefore, it is not necessary to describe exactly the retention over the whole volume fraction range[25,28], but to find an adequate expression for the dependence of log $k'$ on $\varphi$ over a volume fraction range of at most 0.1–0.9. Snyder *et al.*[34] showed that in this instance a linear version of eqn. 12 can be used as a good approximation, *viz.*,

$$\log k' = \log k_w - S\varphi \tag{13}$$

Owing to the restriction to the intermediate volume fraction range, log $k_w$ values calculated by means of eqn. 13 are usually lower than those either measured experimentally or calculated using the quadratic eqn. 12[25,28,137]. Further, log $k_w$ is not a solute constant but, owing to the participation of the organic modifier in the retention mechanism, depends on the hydrophobicity of the modifier, being largest in methanol–water eluents and being gradually reduced in more lipophilic solvents such as acetonitrile and tetrahydrofuran[95]. As far as the capability of log $k_w$ in describing the hydrophobic nature of solutes is concerned, the above considerations do not indicate a weakness of the approach, but rather reflect that a *hypothetical* RPLC system composed of an alkyl-bonded phase and an aqueous eluent is different from the *real* aqueous system.

In addition, the use of quadratic or even more complex functions[28] for extrapolation purposes requires the determination of a large number of capacity factors per solute, particularly at low values of $\varphi$, owing to a greatly increased uncertainty in the intercept value log $k_w$ on introduction of a $\varphi^2$ term, so that practical reasons also point to the use of the linear eqn. 13. It should be noted that the linear extrapolation of retention data to 100% water in chromatographic partition-like systems was first suggested and theoretically verified by Soczewiński and Wachtmeister[138] and subsequently applied with much success to thin-layer chromatography by Biagi's group[139,140], so that log $k_w$ is equivalent to $R_M^0$.

The magnitude of the slope, $S$, of the log $k'$–$\varphi$ plots has been interpreted as the number of water molecules removed from the solute on its exclusion from the mobile phase[138,141]. $S$ therefore depends on the size of the solute and the number and structure of polar functional groups. Hafkenscheid and Tomlinson[142] and Braumann *et al.*[95] found that, using methanol–water eluents, $S$ is strongly correlated with log $k_w$ for a broad range of different solute structures. This is not so for eluents containing acetonitrile[95,142], ethanol[143] or tetrahydrofuran (unpublished results), and thus adds support to the previous conclusion that, as far as non-congeneric solutes are concerned, the retention mechanism in methanol–water eluents is unique.

*4.1. Correlation between log $k_w$ and the n-octanol–water partition coefficient*

The judgement of any potential relationship between log $k_w$ and log $P_{OCT}$ suffers from one principal difficulty. In view of the fact that published log $P_{OCT}$ values often differ by up to 50%[2], and that for many highly hydrophobic solutes and more complicated structures no reliable partition coefficients, either determined experimentally or calculated, are at hand, the reason for a possible lack of correlation with log $k_w$ may equally be the result of a failure of the RPLC model or erroneously determined log $P_{OCT}$. To exclude, as far as possible, the latter possibility, we shall discuss first those studies which have dealt with solutes of well established partition behaviour in the *n*-octanol–water system.

El Tayar *et al.*[100] used 49 *ortho*-, *meta*- and *para*-substituted toluenes, anilines, phenols, nitrobenzenes and chlorobenzenes to calculate log $k_w$ by linear extrapolation of retention data from methanol–buffer eluents containing 0.2% (v/v) of the silanol-masking *n*-decylamine. An excellent correlation between log $k_w$ and log $P_{OCT}$ was obtained as

$$\log k_w = 0.899\ (\pm\ 0.051) \log P_{OCT} + 0.226\ (\pm\ 0.111) \qquad (14)$$

$$n = 49;\ r = 0.982;\ F = 1249;\ s = 0.168$$

Eqn. 14 proved to be superior to the corresponding correlation between the isocratic log $k'$ and log $P_{OCT}$ (*cf.*, Table 2), the reason for which was related to an under- or overestimation of the hydrophobicity, in terms of log $k'$, of in polar solutes particular. El Tayar *et al.* additionally observed that adjacent substituents having hydrogen-bond donor and/or acceptor capabilities behaved differently in RPLC than in the octanol–water system. According to the formation of intramolecular hydrogen bonds which compete with intermolecular solute–solvent hydrogen bonds, such *ortho*-substituted compounds should have a higher log $P_{OCT}$ than their *meta*- and *para*-isomers. This was indeed observed for the log $k_w$ values of such compounds, but usually not for the corresponding log $P_{OCT}$ values. El Tayar *et al* therefore concluded that extrapolated log $k_w$ values are more accurate estimates of hydrophobicity and provide a more consistent and precise data set, which they subsequently used to analyse substituent interactions and their possible lack of additivity in terms of hydrophobic constants.

Hammers *et al.*[99] also noted an improved correlation between log $k'$ and log $P_{OCT}$ by using the linearly extrapolated log $k_w$ values from methanol–water eluents for alkylbenzenes, fused arenes, polyphenols and chloro-substituted benzenes, ani-

lines and phenols. They attributed the better data fit to the fact that both log $P_{OCT}$ and log $k_w$ were strongly related to the solute's activity coefficient in water. They formulated the following Collander-type equation:

$$\log P_{OCT} = 0.91\ (\pm\ 0.02) \log k_w + 0.28\ (\pm\ 0.06) \tag{15}$$

$$n = 36;\ s = 0.11$$

Most interestingly, the statistical significance of eqn. 15 dramatically decreased when log $P$ values from $n$-alkane–water, in particular for polar solutes, were used instead of log $P_{OCT}$ values. This finding strongly supports the previous conclusion that the stationary phase–methanol–water interface must not be regarded as alkane-like but certainly has hydrogen-bonding capability. Hammers *et al.*[99] emphasized that, although the solute series incouded acidic chlorophenols, basic chloroanilines and non-polar compounds, the log $k_w$ values could be adequately described with a single regression equation on log $P_{OCT}$ They concluded that the sparingly available silanol groups were not readily accessible to solute molecules and adsorption at these sites could be ignored.

Harnisch *et al.*[134] compared log $k'$ and log $k_w$, measured in methanol–water eluents, as potential hydrophobic parameters for OECD reference substances Again, log $k_w$ proved to be superior to log $k'$ in the correlation with log $P_{OCT}$.

$$\log P_{OCT} = 0.90\ (\pm\ 0.01) \log k_w + 0.08\ (\pm\ 0.09) \tag{16}$$

$$n = 46;\ r = 0.983;\ s = 0.39$$

As the experimental error in log $k_w$ determinations was usually much smaller than that for log $P_{OCT}$, in particular for very hydrophobic compounds, they recommended using log $k_w$ directly as a hydrophobic parameter in studies on the bioaccumulation of potentially hazardous compounds.

Thus and Kraak[144] determined log $k_w$ values for 29 non-congeneric solutes on octadecyl- and phenyl-silylated silica gel using methanol–buffer eluents. While the correlation between log $k_w$ and log $P_{OCT}$ was comparable to those reported in eqns. 14–16, some results and conclusions deserve comment. They measured only three different log $k'$ values in a very narrow volume fraction range ($0.55 \leq \varphi \leq 0.75$) and calculated log $k_w$ by linear extrapolation. Owing to this small data base, the correlation between log $k'$ and $\varphi$ was poor in comparison with the usually reported correlation coefficients and therefore a high uncertainty in the log $k_w$ values resulted because even a small error in $S$ will produce a large error in log $k_w$. Finally, the reported log $k_w$ values of the polar compounds are unreliable because even at the highest water content ($\varphi = 0.55$) used the retention of these compounds is weak and at the limit of accurate measurement. For example, Thus and Kraak[144] reported a log $k_w$ for phenol of 0.09, which is more than one unit lower than those reported in many independent studies (see later in Table 4). Hence the final comment of the authors that phenylsilylated silica gel is a better stationary phase for the determination of hydrophobic parameter may also not be valid (see, *e.g.*, the recent work of Antle *et al.*[131]).

Butte *et al.*[145] correlated experimental log $k_w$ values for 29 mono- to penta-

substituted phenols with their corresponding log $P_{OCT}$ values and found

$$\log k_w = 0.848\ (\pm\ 0.034) \log P_{OCT} - 0.311\ (\pm\ 0.073) \tag{17}$$

$$n = 29;\ r = 0\,979,\ F = 636;\ s = 0.149$$

They noted that the good correlation deteriorated when $\pi$ substituent constants were employed instead of measured log $P_{OCT}$ values and therefore concluded that substituent constants are applicable only to give an approximately orientation and do not replace an exact evaluation. However, the log $k_w$ values reported by Butte *et al.*[145] were consistently lower than those usually observed (see Table 4), which is caused by the relatively large negative intercept of eqn. 17. Butte *et al.* employed 0.01 *M* hydrochloric acid in methanol in order to suppress ionization of the phenols, whereas usually water or buffer at near neutral pH is used as the aqueous component of the eluent At pH 2, however, the structure and polarity of the stationary phase are different from those at pH 7. Owing to the weakly acidic character of the residual silanol groups, an eluent of pH 7 induces a surface with much higher net charge and, concomitantly, with a greater number of water molecules bound to the silica surface[146]. This may result in forcing the partly aggregated *n*-alkyl chains into more of a bristle-type surface whereas at pH 2 the surface consists of more strongly interacting ligands and less water present in the stationary phase–mobile phase interface. Hence the overall polarity of the stationary phase is lower at pH 2 and gives rise to the negative intercept observed for the Collander eqn. 17.

If methanol–water eluents of comparable ionic strength at near neutral pH are used to measure retention, the stationary phase-induced variance of the resulting log $k_w$ values is small. This was demonstrated by Braumann *et al.*[133] for six reversed-phase packings differing in the structure of the bonded phase (monomeric/polymeric), the surface area, the carbon loading and the number of residual silanol groups. From these and additional literature data for solutes of very diverse chemical characters, they obtained an excellent correlation between the mean log $k_w$ values and the *n*-octanol–water partition coefficient, *viz.*,

$$\log k_w = 0.986\ (\pm\ 0.022) \log P_{OCT} + 0.078\ (\pm\ 0.069) \tag{18}$$

$$n = 25,\ r = 0.9938;\ F = 1915;\ s = 0.130$$

In view of the regression coefficients of the Collander-type equation indistinguishable from 1.0 and 0.0, respectively, Braumann *et al.*[133] concluded that log $k_w$ can be used *a priori* as a hydrophobic parameter and needs no additional reference system such as log $P_{OCT}$.

Table 3 summarizes the discussed and additional work on the relationship between log $k_w$ and log $P_{OCT}$. In comparing the reported regression coefficients, it is evident that mean values of 1.0 and 0.0 for the slope and intercept, respectively, can be expected. This proposal is verified by considering the data collected in Table 4. Here, reported and, in some instances, subsequently calculated log $k_w$ values for 60 different solutes are shown for which a minimum of two different values were avail-

TABLE 3

LITERATURE DATA FOR THE RELATIONSHIP BETWEEN THE *n*-OCTANOL–WATER PARTITION COEFFICIENT (LOG $P_{OCT}$) AND THE EXTRAPOLATED CAPACITY FACTOR (LOG $k_w$) LOG $P_{OCT} = A$ LOG $k_w + B$

The retention data were determined in methanol–water or methanol–buffer eluents as indicated In some instances, Hansch $\pi$ constants or Rekker fragmental constants ($f$) were used in the cited references to calculate log $P_{OCT}$ Parameters $r$, regression correlation coefficient; $n$, number of data points.

| *Class of compounds* | *A* | *B* | *r* | *n* | *Eluent* | *Stationary phase* | *Ref* |
|---|---|---|---|---|---|---|---|
| Halogenated aromatics, anilines, phenols | 0.91 | 0 28 | * | 36 | 0 05 *M* acetate (pH 4.5) | LiChrosorb RP-18 | 99 |
| Polar benzenes | 0 93 | −0 07 | * | 12 | 0 05 *M* acetate (pH 4 5) | LiChrosorb RP-18 | 99 |
| Benzene derivatives | 1 12 | −0.36 | 0.974 | 45 | Water | LiChrosorb RP-18 | 95 |
| Benzene derivatives | 1.02 | −0.07 | 0 994 | 25 | Various | Various | 133 |
| Alkylbenzenes | 0 87 | 0.05 | 0.998 | 5 | Water | C-18 Sil-X-5 | 134 |
| OECD reference compounds | 0 90 | 0 08 | 0 983 | 46 | Water | C-18 Sil-X-5 | 134 |
| Disubstituted benzenes | 1.07 | −0.17 | 0.982 | 49 | Morpholinopropane-sulphonic acid (pH 7 4) + 0 2% *n*-decylamine | LiChrosorb RP-18 | 100 |
| Monosubstituted benzenes | 0 97 | 0 0 | 0 993 | * | * | * | 100 |
| Phenols | 1.13 | 0 44 | 0 979 | 29 | 0 01 *M* HCl | LiChrosorb RP-18 | 145 |
| Benzodiazepines | 1.12 | 0 14 | 0 968 | 9 | Ammonia (pH 9) | Corasil RP-18 | 136 |
| Barbiturates | 1.06 | −0 11 | 0 976 | 18 | Water | CO PELL ODS | 87 |

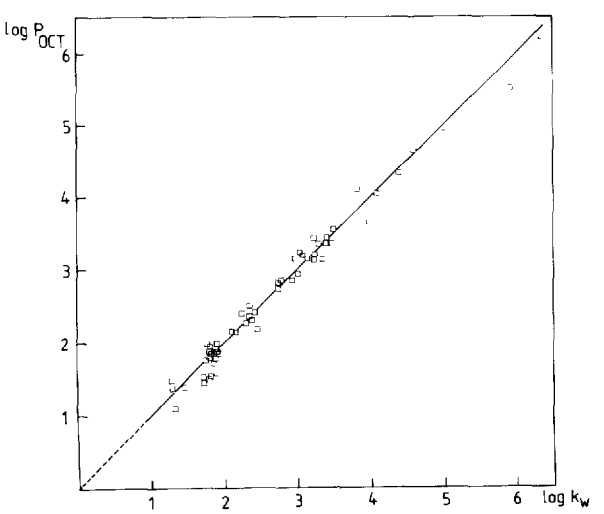
* Not given in the cited references.

able in the literature. From the reported data the mean (log $\overline{k_w}$) and, whenever four or more individual results were available, also the standard deviations of the mean were calculated. The log $\overline{k_w}$ values were subsequently correlated with measured log $P_{OCT}$ values given mainly in ref. 2 to yield

$$\log \overline{k_w} = 0.988\ (\pm\ 0.051) \log P_{OCT} + 0.020\ (\pm\ 0.060) \qquad (19)$$
$$n = 60;\ r = 0.988;\ F = 2456;\ s = 0.176$$

which is shown graphically in Fig. 4. As the solutes employed in the analysis covered a very wide range of possible structures, from very hydrophobic (*p,p'*-DDT) to very polar (aniline), from mono- to multi-substituted compounds, the regression coefficients and the degree of correlation clearly indicate the equivalence of log $k_w$ and log $P_{OCT}$ for chemically very distinct solute classes.

However, there are some exceptions from this general agreement. As noted already, *ortho*-substituted aromatic compounds capable of forming intramolecular hydrogen bonds consistently showed enhanced log $k_w$ values[100], in accordance with the expected decreased ability to form hydrogen bonds with the mobile phase. In contrast, log $P_{OCT}$ values of, *e.g.*, *o*-nitroaniline and *o*-aminophenol were considerably lower than those of their *meta*- and *para*-substituted counterparts[100]. Similar observations have also been made for *ortho*-substituted benzamides[147]. These findings prompted El Tayar *et al.*[100] to postulate the superiority of log $k_w$ over log $P_{OCT}$



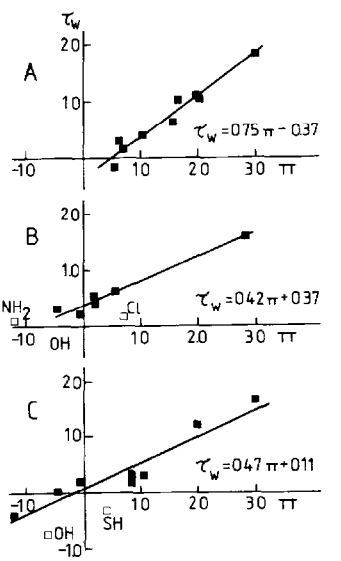




Fig 4. Relationship between the *n*-octanol–water partition coefficient, log $P_{OCT}$, and the extrapolated capacity factor, log $k_w$, for 60 solutes The solid line represents the regression line according to eqn 19

Fig 5 Relationship between the group contribution constant to retention, $\tau_w$, and the Hansch $\pi$ constant (A) C-2-substituted cyclic nucleotides, (B) C-6-substituted cyclic nucleotides, (C) C-8-substituted cyclic nucleotides Outliers are denoted by open squares The solid lines represent the regression lines according to the equations shown Data were taken from ref 147

TABLE 4

LITERATURE LOG $k_w$ DATA AND MEASURED *n*-OCTANOL–WATER PARTITION COEFFICIENTS

Log $k_w$ values were calculated by linear extrapolation of retention data from methanol–aqueous eluents of low to zero ionic strength and near neutral pH, and were mainly from refs 80, 82, 95, 99, 100, 133 and 134. Log $P_{OCT}$ mainly from ref. 2

| *Compound* | *Log $k_w$ reported* | *Mean ± S D ** | *Log $P_{OCT}$* |
|---|---|---|---|
| *Alkylbenzenes* | | | |
| H | 2 11 1 99 2.38 2 16 2 05 2 08 2 18 | 2 14 ± 0.13 | 2.14 |
| $CH_3$ | 2 74 2 71 2.60 3 02 2 72 | 2 76 ± 0.16 | 2.76 |
| 1,2-$CH_3$ | 3 19 3 09 | 3 14 | 3 12 |
| 1,3-$CH_3$ | 3 30 3 19 | 3 25 | 3 20 |
| 1,4-$CH_3$ | 3 29 3.18 3 20 3 29 | 3 24 ± 0 06 | 3 15 |
| $CH_2CH_3$ | 3 27 3.46 3 52 3 18 3 25 | 3.34 ± 0 15 | 3 15 |
| *n*-Propyl | 3 97 4.16 3 82 3 99 | 3.99 ± 0 14 | 3 63 |
| *n*-Butyl | 4.57 4.77 4 75 4.32 | 4.60 ± 0 21 | 4.26 |
| *Halogenated benzenes* | | | |
| F | 2 28 2 31 | 2 30 | 2 27 |
| Cl | 2 80 2 84 2.71 2.80 2 70 2 79 | 2.77 ± 0 06 | 2 84 |
| 1,2-Cl | 3 36 3 32 3 60 3.26 | 3 39 ± 0 15 | 3 38 |
| 1,3-Cl | 3 49 3 41 | 3 45 | 3 38 |
| 1,4-Cl | 3 43 3.32 3 26 3 33 | 3.34 ± 0 07 | 3 39 |
| 1,3,5-Cl | 4 51 4 26 | 4.39 | 4 31 |
| Hexachloro | 5 90 5 96 | 5.93 | 5 50 |
| *Anilines* | | | |
| H | 1 05 0.98 1 39 1 21 1 11 | 1 15 ± 0 16 | 0.91 |
| 2-$NO_2$ | 1 75 1.70 | 1 73 | 1 83 |
| 3-$NO_2$ | 1 42 1.19 | 1 31 | 1 37 |
| 4-$NO_2$ | 1 39 1 52 | 1 46 | 1 39 |
| 2-Cl | 1 89 1.71 | 1 80 | 1 91 |
| 3-Cl | 1 90 1 78 | 1 84 | 1 89 |
| 4-Cl | 1 92 1 75 | 1 84 | 1 83 |
| *Fused arenes and polyphenyls* | | | |
| Naphthalene | 3 48 3 26 3 31 3 22 3.28 | 3 31 ± 0.10 | 3 38 |
| Phenanthrene | 4 30 4 54 | 4 42 | 4.53 |
| Biphenyl | 4 17 4.12 4 26 3 89 4 03 | 4.09 ± 0.14 | 4.06 |
| Anthracene | 4 46 4 73 | 4 60 | 4 63 |
| Pyrene | 4 89 5 10 | 5 00 | 4.88 |
| *Phenols* | | | |
| 2-Cl | 2 02 2 19 | 2 11 | 2 16 |
| 3-Cl | 2 29 2 40 | 2 35 | 2.50 |
| 4-Cl | 2 27 2.33 2 15 2.21 | 2 24 ± 0 08 | 2 40 |
| 2,3-Cl | 2 81 3.12 | 2 97 | 3 15 |
| 2,4-Cl | 2 90 3.23 | 3 07 | 3 21 |
| 2,5-Cl | 2 90 3 19 | 3 05 | 3.24 |
| 2,6-Cl | 2 59 2 92 | 2 76 | 2.84 |
| 3,4-Cl | 3 04 3 41 | 3 23 | 3.44 |
| 3,5-Cl | 3.27 3 68 | 3.49 | 3.56 |
| 2,4,5-Cl | 3 67 3 96 | 3 82 | 4 10 |
| 3,4,5-Cl | 3 81 4 19 | 4 00 | 4 36 |
| H | 1 27 1.26 1 27 1 34 1 37 | 1 30 ± 0 05 | 1 48 |
| 3-$CH_3$ | 1 75 1.81 | 1 78 | 2 00 |
| 4-$CH_3$ | 1 85 1.80 | 1 83 | 1.93 |
| 2-$NO_2$ | 1 90 1 75 | 1 83 | 1 76 |
| 3-$NO_2$ | 1.91 1 80 | 1 86 | 2 00 |
| 4-$NO_2$ | 1 77 1 77 2 00 1 68 1 97 | 1.84 ± 0 14 | 1 91 |

TABLE 4 *(continued)*

| *Compound* | *Log $k_w$ reported* | *Mean ± S D** | *Log $P_{OCT}$* |
|---|---|---|---|
| *Nitrobenzenes* | | | |
| H | 1.91 1 91 1 81 2 03 1.93 1 94 | 1 92 ± 0.07 | 1.84 |
| 2-Cl | 2.32 2 31 | 2 31 | 2.30 |
| 4-Cl | 2 35 2.33 2 42 2 31 | 2 35 ± 0.05 | 2.42 |
| 2-$NO_2$ | 1.96 1 72 2.02 | 1 90 | 1.58 |
| 3-$NO_2$ | 1 66 1 91 | 1 79 | 1.49 |
| 4-$NO_2$ | 1.48 1 86 1 81 1 72 | 1.72 ± 0 17 | 1.46 |
| 4-$CH_3$ | 2 35 2 40 2 46 | 2 40 | 2 42 |
| *Polar benzenes* | | | |
| Acetophenone | 1.78 1 92 | 1 85 | 1 63 |
| Benzyl alcohol | 1 39 1 30 1 47 1.29 1 27 | 1.34 ± 0.08 | 1 10 |
| Benzaldehyde | 1 74 1.65 1 80 | 1 73 | 1 45 |
| $CO_2CH_3$ | 2 44 2.45 | 2 45 | 2 18 |
| Benzonitrile | 1 83 1 77 | 1 80 | 1.56 |
| *Miscellaneous* | | | |
| *p,p'*-DDT | 6 70 6.06 | 6 38 | 6 19 |
| Diuron | 3.02 2 82 | 2.92 | 2 85 |
| Linuron | 2 81 3 07 | 2.94 | 3.00 |
| Chloroxuron | 3 91 3 60 | 3 76 | 4 00 |

* Standard deviation of the mean

for such solute groups. Although the molecular basis for the appropriate discrimination between isomers, which can form intramolecular hydrogen bonds, by RPLC retention parameters is not yet clear, the sensitivity of the RPLC system to such molecular properties could be related to the highly anisotropic character of the eluent–stationary phase interface.

It has been proposed above that retention in RPLC, similarly to transport in biomembranes, is sensitive to the shape of the solute molecule owing to the more or less ordered *n*-alkyl chains of the stationary phase. Indeed, polycyclic aromatic hydrocarbons (PAHs) were successfully separated on octadecylsilica[59], although they contained the same number of rings and hence the same molecular weight. Additionally, Jinno and Kawasaki[85] were able to correlate the retention data of PAHs with their length-to-width ratio and a correlation factor *F*. Ruepert *et al.*[89] showed that the isocratic capacity factors of seven pentacyclic PAHs were significantly different, while the calculation of log $P_{OCT}$ according to Rekker[6] resulted in identical partition coefficients for the isocyclic solutes. These results clearly demonstrate the ability of the stationary phase to discriminate between solute molecules with different shapes, in the case of PAHs between rod-like and disc-like shapes. Additional support comes from the work of Wells *et al.*[84], who showed for *n*-alkylbenzamides that the bulk, branching and site of hydrocarbon branching were controlling factors for retention in RPLC and, further, that retention data could be predicted by using a topology descriptor, *i.e.*, molecular connectivity indices. Thus, log $k_w$ can be expected to include also steric effects on the overall hydrophobicity, which are not applicable in a liquid–liquid distribution system such as *n*-octanol. However, the experimental basis for this conclusion is incomplete, and it is highly desirable not only to collect more log $k_w$ values for non-polar isomeric solutes, but also to analyse the ability of

log $k_w$ to predict transport phenomena in biosystems of differently sized and shaped molecules.

Fig 5 shows the relationship between $\tau_w$, the substituent hydrophobic constant derived from log $k_w$[129], and the Hansch $\pi$ substituent constant for cyclic nucleotides[147]. The purine base of these compounds contains four nitrogen atoms, so that strong electronic interactions between polar substituents and the base are expected to occur. This was indeed the case, as is indicated by the necessity to treat each substituent position (C-2, C-6, C-8) separately in the correlation analysis, and to exclude a significant number of outliers (open squares in Fig. 5). For cAMP (6-$NH_2$) and cIMP (6-OH), for example, it is known that the additional electrons donated to the ring are effectively delocalized, resulting in different tautomeric forms of cAMP and cIMP in solution[148], which, by an increase in the (hydrophobic) surface area, enhance the overall hydrophobicity of the purine base. The $\tau_w$ correctly modelled the expected increase in hydrophobicity while the corresponding $\pi$ values suggest the opposite to be the case. This example clearly demonstrates that the $\pi$ (or $f$) approach is not readily applicable to the description of the hydrophobicity of complex structures where strong perturbing effects are exerted by substituents on the electrons of the heterocyclic ring.

The retention behaviour of cyclic nucleotides is also noteworthy in another respect. These compounds are charged at any pH and therefore cannot be chromatographed in their unionized form. Braumann and Jastorff[149] showed that the negatively charged phosphate moiety of the nucleotides interact with metal cations in the mobile phase to form nucleotide–metal ion complexes with reduced electronic charge and thus enhanced retention. They further demonstrated that two different solute species can be used to determine experimentally the hydrophobicity of the solutes, *viz.*, the charged nucleotide in the absence of cations in the eluent, and second the cyclic nucleotide–metal ion complex at saturating cation concentrations with respect to complex formation. This approach may be regarded as being equivalent to ion-pair RPLC with lipophilic counterions, which has been applied, for example, by Riley *et al.*[129] to the measurement of log $k_w$ of benzoic acids, azapurines and triazines. These results indicate that the hydrophobicity of permanently charged solutes can, at least in principle, be assessed by RPLC, which is very difficult to achieve with the *n*-octanol–water system owing to the high solubility of water in *n*-octanol and the uncontrolled distribution of ion pairs that may be formed between solute ions and buffer components.

In summary, RPLC makes several important solute groups accessible to the experimental determination of their hydrophobicity. These include (i) complex structures of unknown partition behaviour for which the additivity of hydrophobic substituent constants may not hold, (ii) permanently charged solutes and (iii) hydrophobic compounds with log $P_{OCT} > 4$ whose partition coefficients cannot be determined with sufficient accuracy. Hence the comparatively weak correlations sometimes observed between log $P_{OCT}$ and log $k_w$[112,150,151] may be the result of limitations of the *n*-octanol–water partition system for such "problem" solutes.

Pietrogrande *et al.*[113] have related the log $k_w$ values of benzodiazepines to the measured log $P_{OCT}$ values and found, apart from a reasonable data fit, a surprisingly large negative intercept value of the resulting Collander-type equation. In contrast, a small intercept close to 0.0 was reported by Hulshoff and Perrin[136] for a similar

set of benzodiazepines (see Table 3). Whereas Pietrogrande *et al.*[113] determined log $k_w$ for the basic solutes in an eluent of pH 7.4, Hulshoff and Perrin[136], neglecting the instability of the matrix above pH 7.5, used an eluent of pH 9. Thus, different solute states with respect to ionization were related to log $P_{OCT}$, referring by definition to the partition coefficient of the non-ionized species. This example clearly shows the importance of ensuring that the same physico-chemical state of the solute is employed in such correlation studies.

Finally, some limitations of the log $k_w$ approach should be mentioned. Very small polar solutes often yield much higher log $k_w$ values than would be expected from their *n*-octanol–water partitioning[93]. This phenomenon may be explained by the tendency of these solutes to be included in the solvation layer of the stationary phase. Similar problems may also arise with surface-active solutes. For very large compounds, the pore size of the matrix becomes a limiting factor and may lead to exclusion phenomena. Further, solutes that may undergo silanophilic or metallophilic[152] interactions with the bonded phase often show poor peak shapes or may even be irreversibly adsorbed on these sites. Although some of these effects can be eliminated by mobile phase additives such as lipophilic amines, it still has to be established whether this procedure is generally applicable. Finally, the range of hydrophobicity covered by the log $k_w$ approach is not unlimited. Considering (i) the accessible volume fraction range of methanol for which the model holds, *i.e.*, $0.1 \leq \varphi \leq 0.9$, (ii) the limited magnitude of the capacity factor that can be accurately measured (usually $0.5 \leq k' \leq 50$) and (iii) the minimum volume fraction range of 0.25 unit that is required for the appropriate extrapolation of retention data to 100% water, the maximum range of reliable log $k_w$ values is approximately 0.0–7.0 Extension of this range is possible when either isocratic log $k'$ values measured at high values of $\varphi$ are used directly[89] or less polar organic modifiers are employed to calculate log $k_w$. In both instances, selectivity effects on retention, as has been discussed in Section 3, have to be taken into consideration, and a reference system is required in order to calibrate the RPLC hydrophobic parameters. If this can be done successfully, upper limiting partition coefficients of approximately 11–12 are within experimental reach.

### *4.2. Comparison between log $P_{OCT}$ and log $k_w$ as hydrophobic parameters*

The log $k_w$ values collected in Table 3 for 60 different solutes were extracted from studies using different experimental conditions. Temperature, ionic strength and pH of the eluent, mobile phase additives, structure of the bonded phase, mobile phase hold-up time determination and accuracy of the reported data were not the same and contributed to the variability of the log $k_w$ values Nevertheless, the standard deviations from the mean are small. This finding is in contrast to the usual variability of measured log $P_{OCT}$ values, as was demonstrated by several interlaboratory comparison tests (see, *e.g.*, ref. 134). For example, reported log $P_{OCT}$ values for benzene varied from 1.56 to 2.34[133], and even larger discrepancies have been noted for strongly hydrophobic compounds such as *p,p'*-DDT, for which values of 3.98–6.36 have been reported.

The limited accuracy and agreement of log $P_{OCT}$ values measured by the conventional shake-flask technique is the result of the necessity to measure the solute concentration in at least one of the phases. Problems may arise from (i) hydrophobic

compounds with log $P_{OCT} > 4$ owing to the required precision and sensitivity of the analytical technique, (ii) the formation of micelles and microemulsions in the aqueous phase, (iii) the presence of impurities which, if they are *e.g.*, strongly UV-absorbing, may seriously interfere with the quantitative determination of the solute, (iv) instability of the solute in aqueous media, (v) dissociation/association of polar solutes and (vi) volatility of the solute. The latter point has been recently analysed by El Tayar *et al.*[153], who showed that log $P_{OCT}$ for benzene was strongly affected whether or not the volatility of the solute was adequately considered. Using equipment that reduced the escape of benzene to a minimum, they measured a log $P_{OCT}$ of 2.03, which is considerably lower than the "best" value (2.13) usually assumed[2,6]. Thus, the partition coefficient of the cornerstone of many hydrophobic fragmental systems may not be correct.

The calculation of log $P_{OCT}$ from hydrophobic constants[2,6] is a valuable tool for the establishment of hydrophobic parameters, in particular for those solutes whose log $P_{OCT}$ values are at the extremes of, or outside, the normal measurable range. Despite the great success of this approach, a number of imperfections have also been noted. Strictly, the hydrophobic substituent constants apply only to the solute classes from which they were derived, so that numerous corrections are necessary when calculating log $P_{OCT}$ for non-related structures[154] that are not necessarily unambiguous[155]. Further, non-additivity has been found for some multi-substituted solutes, *e.g.*, polychlorinated phenols[156], and also intra- and intermolecular hydrogen bonding and electronic interactions between neighbouring substituents result in large discrepancies between measured and calculated partition coefficients. Hence the calculation of log $P_{OCT}$ should be regarded as an approximation and cannot completely replace experimental determinations.

As for most solute classes log $k_w$ is equivalent to log $P_{OCT}$ (Table 4), the practical advantages of the chromatographic system clearly favour the use of log $k_w$. Retention time measurements are simple, rapid, and reproducible, and require only extremely small amounts of the solute. The average error of log $k_w$ determinations is approximately $\pm 0.05$ unit, which is at least three times better than can be expected for careful log $P_{OCT}$ measurements by the shake-flask technique. There is no need to quantify solute concentrations and to use ultrapure substances because the solute peak in the chromatogram can be easily identified. An important advantage is the possibility of accurately determining the hydrophobicity of very non-polar compounds that were previously inaccessible to experimental techniques. As the interlaboratory reproducibility is reasonable (Table 4), log $k_w$ may enhance data compatibility for, in particular, many "problem" solutes. Finally, unstable, association-forming and dissociable compounds cause fewer problems in RPLC owing to the speed of operation, the possibility of reducing the oxygen concentration in the eluent by degassing, the microgram amounts required and the possibility of accounting for ionization effects on retention.

There is now substantial evidence that, apart from the practical advantages, the special features of the chromatographic interface also indicate the dynamic chromatographic technique as the method of choice for the quantitative determination of hydrophobic parameters. As retention in RPLC is sensitive to size and shape differences in the solute molecules, log $k_w$ includes additional information about topological solute properties which may also control the behaviour of bioactive com-

pounds in biosystems. This proposal, however, has to be verified by careful studies on the QSAR of isomeric compounds.

## 5 QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS

Despite the ease of operation and the well established correspondence with other hydrophobic parameters, comparatively few workers have employed RPLC retention data in studies on QSAR This is in obvious contrast to thin-layer chromatography[11,17,18] and may be related to a general uncertainty concerning the appropriate choice of the RPLC system and the experimental conditions. It is hoped that this review may help to break down existing barriers and to exploit fully the capability of RPLC of providing highly accurate hydrophobic parameters

Table 5 gives some of the pertinent data for reported relationships between biological activity and chromatographic retention. It can be seen that very few studies have employed log $k_w$, which, as has been shown above, is the most appropriate parameter. Nevertheless, the overall impression is that log $k'$ could completely replace log $P_{OCT}$ in describing the dependence of the biological activity on the hydrophobic nature of compounds. There are some important exceptions, however. Baker *et al.*[157] found that a retention index scale based on the relative retention of the drug and a series of $C_3$–$C_{23}$ 2-ketoalkanes gives higher correlations with biological activities of propranolol and barbiturate analogues than was found between log $P_{OCT}$ and biological activity. They argued that the superiority of the retention index was not simply the result of a higher precision in the measurements but was a better model for biological interactions than the liquid–liquid partitioning model. This argument was based on similar considerations put forth in Section 2.3, *i.e.*, that the ordered array of *n*-alkyl chains and the residual silanol groups better represent the structure of biomembranes.

Braumann *et al.*[95] related log $k_w$ values for several groups of herbicides to log $P_{OCT}$ values and their inhibitory action on photosynthetic electron transport. Whereas log $k_w$ was strongly correlated with log $P_{OCT}$ for phenylureas and phenoxycarbonic acid derivatives and thus could be equally well employed in studies on QSAR, a lack of correlation was observed for *s*-triazines. This was caused by the methylthio substituent, which, if present instead of a chloro substituent, should induce a small decrease in the overall hydrophobicity of three different analogues according to the $\pi$ values of +0.71 for Cl and +0.67 for $SCH_3$. What was actually observed was a large increase in retention of the $SCH_3$-substituted derivatives with a corresponding $\Delta$ log $k_w$ of 1.5, indicative of an additional propyl substituent attached to the triazine ring. Thus, a specific non-polar substituent exerted selective effects on retention that were not observed in the classical liquid–liquid distribution system. Using an improved procedure for the calculation of log $P_{OCT}$ as developed by the Hansch group[162], the correlation between log $P_{OCT}$ and log $k_w$ increased over that initially reported[95]. However, if related to the biological activity of the *s*-triazines, log $k_w$ was still much better in describing the dependence of the inhibition of the photosynthetic electron transport on the hydrophobic nature of the *s*-triazines

With the limited data available, it is not possible to generalize these observations to other solute classes. It is expected from the unique retention mechanism in methanol–water eluents, however, that a substantial experimental basis for the pos-

TABLE 5

QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS THAT EMPLOYED CHROMATOGRAPHIC RETENTION PARAMETERS AS DESCRIPTORS FOR THE HYDROPHOBICITY OF BIOACTIVE COMPOUNDS

| *Class of compounds* | *Biological activity* | *Parameter** | *n*** | *r**** | *Ref* |
|---|---|---|---|---|---|
| Propranolol analogues | Antiarrhythmic activity | Index | 12 | 0.866 | 157 |
| Propranolol analogues | Inotropic activity | Index | 12 | 0.818 | 157 |
| Anthranilic acids | Antiinflammatory activity | Index | 5 | 0.853 | 157 |
| Barbiturates | Hypnotic activity | Index | 15 | 0.875 | 157 |
| Barbiturates | Inhibition of cell division | Index | 9 | 0.959 | 157 |
| Sulphonamides | Inhibition of *E. coli* | $V_R$ | 11 | 0.963 | 66 |
| Benzoylacetic acids | Inhibition prostaglandin synthetase | Log $k'$ | 10 | 0.990 | 158 |
| Benzoylacetic acids | Binding to albumin | Log $k'$ | 12 | 0.959 | 158 |
| Benzomorpholine-2-carboxylic acids | Adrenolytic activity | Log $k_w$ | 5 | 0.882 | 159 |
| Polycyclic aromatic hydrocarbons | Bioconcentration in *Daphnia pulex* | Log $k'$ | 6 | 0.939 | 160 |
| Polycyclic aromatic hydrocarbons | Toxicity for *Daphnia pulex* | Log $k'$ | 5 | 0.982 | 160 |
| Barbiturates | Minimum hypnotic dose, rabbit | Log $k'$ | 9 | 0.969 | 87 |
| Barbiturates | Minimum hypnotic dose, rat | Log $k'$ | 14 | 0.916 | 87 |
| Barbiturates | Inhibition of rat brain | Log $k'$ | 8 | 0.996 | 87 |
| Phenols | Antibacterial activity | Log $k'$ | 12 | 0.923 | 161 |
| Phenols | Haemolysis of rat erythrocytes | Log $k'$ | 12 | 0.901 | 161 |
| Phenols | Acute toxicity in mice | Log $k'$ | 12 | 0.905 | 161 |
| Phenols | Cytochrome in rabbit liver | Log $k'$ | 9 | 0.941 | 161 |
| Phenylureas | Inhibition of photosynthetic electron transport | Log $k_w$ | 10 | 0.841 | 97 |
| *s*-Triazines | Inhibition of photosynthetic electron transport | Log $k_w$ | 6 | 0.911 | 97 |

* The *Parameter* column indicates whether the retention volume ($V_R$), the capacity factor (log $k'$) or the capacity factor extrapolated to 100% aqueous eluents (log $k_w$) was used in the correlation analysis "Index" refers to the retention index scale based on 2-ketoalkanes[162]

** $n$, Number of data points

*** $r$, Correlation coefficient.

tulated superiority of log $k_w$ over other hydrophobic parameters may be available in the near future.

## 6 CONCLUSION

Following the application of thin-layer chromatographic retention data in studies on QSAR by Boyce and Milborrow[163], numerous experimental data now suggest the usefulness of a dynamic chromatographic system for the assessment of the hydrophobic nature of bioactive compounds. In particular, HPLC on *n*-alkyl-bonded stationary phases with methanol–water eluents offers a powerful tool for such purposes. Apart from the empirically found excellent correlation between log $P_{OCT}$ and RPLC retention data, substantial theoretical work has also indicated that attempts to relate chromatographic retention to liquid–liquid distribution are thermodynamically valid[23,24,164,165].

However, the acceptance of RPLC for official and standard methods has been hindered by the strong dependence of the capacity factors on the particular experimental conditions employed, making it difficult to compare results from different laboratories. Therefore, the capacity factors have to be calibrated by relating them to log $P_{OCT}$ of standard compounds. As has been discussed, this approach contains inherent limitations related to (i) selective effects resulting mainly from solute–solvent interactions in the eluent, (ii) the appropriate choice of reference compounds and (iii) the uncertainty of many published log $P_{OCT}$ values for, in particular, strongly hydrophobic compounds. Alternatively, retention index scales similar to those used in gas–liquid chromatography[166,167] have been introduced to unify retention data. These scales are based on the relative retentions of homologous series such as 2-ketoalkanes[168] or alkyl aryl ketones[169]. However, as noted by Brent *et al.*[125], it seems doubtful whether a single homologous series can account for all interactions between the solute and stationary and mobile phases that would be experienced by molecules bearing the full range of possible functional groups.

Log $k_w$, the extrapolated capacity factor for an aqueous eluent, may also be regarded as a means of normalizing retention because the magnitude of log $k_w$ is determined by a change in retention induced by a change in the mobile phase composition, rather than on merely absolute retention under fixed chromatographic conditions. At present, log $k_w$ appears to be the most appropriate RPLC hydrophobic parameter because, on extrapolation, selective effects exerted by the stationary and/or mobile phase on solute retention are partly eliminated. For a broad range of solute structures, log $k_w$ is doubtless equivalent to log $P_{OCT}$ in its ability to describe the hydrophobic nature of bioactive compounds so that the practical advantages of RPLC retention time measurements should strengthen the position of RPLC in the field of QSAR, especially if the observation that in some instances RPLC parameters appear to correlate better with biological data than log $P_{OCT}$ could be substantiated by more experimental studies. Here, compounds with comparable hydrophobicities but different molecular sizes and shapes should be employed because, as pointed out by Tulp and Hutzinger[155], there may be an optimal steric configuration and molecular size for biological processes such as membrane transport. As retention in RPLC is sensitive to the topology of the solute molecule, the question raised by Tomlinson[11] more than 10 years ago may turn out to be answered, *viz.*, "... is it possible that the

chromatographic process, being a dynamic one producing a parameter derived from a non-steady-state function, is more analogous to the biological state than those parameters derived from steady-state measurements?"[11].

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## 8 SUMMARY

The use of RPLC retention parameters as descriptors of the hydrophobic nature of bioactive compounds has been evaluated. The relationship between the capacity factor, measured on *n*-alkyl-bonded stationary phases using binary eluents, and the *n*-octanol–water partition coefficient has been illustrated experimentally and theoretically. It is suggested that retention parameters, in particular the capacity factor (log $k_w$) obtained by extrapolation of retention data from binary eluents to 100% water, could successfully replace the *n*-octanol–water partition coefficient in studies on quantitative structure–activity relationship, and that their use may result in a better correlation with biological data.

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