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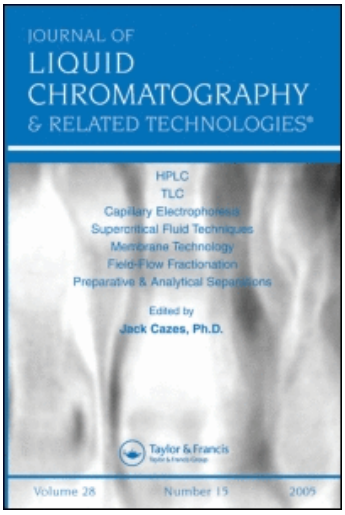

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A REVIEW OF METHODS FOR THE DETERMINATION OF HOLD-UP VOLUME IN MODERN LIQUID CHROMATOGRAPHY

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1.0 Introduction

Arguably, the most important parameter in modern liquid chromatography is the hold-up (or dead) volume, V_M, the volume of mobile phase contained within the chromatographic system between the sample injector and the detector. Without this knowledge many dependent parameters such as capacity factor (k), selectivity (α), and resolution (R_s) cannot be computed (104,15,78). These data are of the utmost importance for the optimization of conditions for the separation of complex mixtures and the identification of solute bands.

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Apart from its significance in the more routine aspects of liquid chromatography such as separation methods development, the determination of the hold-up volume is basic to the reporting of chromatographic data from both intra- and inter-laboratory investigations, the development of schemes for the prediction of retention behaviour (.....) and studies of the retention mechanism itself (.....). Yet there is no universally accepted method for the accurate measurement of this parameter. Some of the methods reported include the measurement of the retention volume of radioactively-labelled eluent, the injection of modified mobile phase (including pure components), static weighing procedures and mathematical determinations based on the retention characteristics of members of various homologous series. Therefore, this paper presents a comprehensive review and critical appraisal of currently used approaches to the determination of hold-up volume in Liquid Chromatography.

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2.0 Definition of Hold-up Volume

In gas chromatography (GC) the dead time can be considered to be the time an infinitesmal amount of non adsorbed gas takes to pass through the chromatographic system under identical conditions as the sample being analysed (17). When dealing with LC however, the situation is not as simple because, although the basic theory of chromatography is common, there are (among others) three basic differences in applying this theory:

(1) The interdiffusion coefficient of liquids is at least 10^4 times smaller than that of gases.

(2) The viscosity of the eluent is some 100 times greater for liquids than for gases.

(3) The interaction between the molecules of the stationary phase and those of the eluent are negligible in gas chromatography, but important in liquid chromatography (18).

An additional consideration is that sample components can be partially or completely excluded from the interior of the porous column material (19).

Mocked and Freyholdt (20) proposed that the dead time of a liquid chromatographic column is that period of time, t, a sample molecule spends in the isotropic mobile phase which flows with the constant rate v $cm^3 min^{-1}$. The hold-up volume, V_M, is therefore equal to the product of these two variables. This definition immediately poses one difficulty which results from the difference between gas and liquid chromatography. For the latter case in general and reversed phase LC (RPLC) in particular, it has been shown by many workers (21-32) that eluent molecules are adsorbed onto the stationary phase, forming a stationary layer of mobile phase components and thus reducing the column void volume. Although the data of some workers (23,26) was limited to adsorbed organic modifier, evidence that water molecules are also part of the adsorbed layer has been produced by others (24,28). It has been proposed by Yonker et al. (24) that the uptake of water by the stationary phase is due to hydrogen bonding with residual silanols.

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The presence of this adsorbed layer led Mockel and Freyholdt (20) to propose the following relationship:

$$V_{mobile} = V_{pore,\ free} + V_{interstitial} \qquad [1]$$

where V_{mobile} = volume of mobile phase (ie. void volume)
$V_{pore,free}$ = pore volume minus volume of sorbed eluent
$V_{interstitial}$ = volume of eluent outside the pores of the column packing.

However this approach ignores the possibility that solute molecules may be partially or completely excluded from the pores of the stationary phase. This view is supported by Scott and Kucera (33)

who pointed out that the void volume for a given solute will not be the sum of the interstitial volume and the total pore volume but the sum of the interstitial volume and that proportion of the pore volume that is accessible to the solute concerned. Knox et al. (34) discussed various exclusion mechanisms and identified two main types of exclusion:

(1) Entropic exclusion due to steric effects
(2) Enthalpic exclusion due to energy effects

This second type of exclusion can be subdivided into two further categories: exclusion of a non-polar solute (partial or total) due to the adsorption onto the pore surfaces of a polar eluent, and exclusion of charged species due to ions adsorbed onto the stationary phase surface.

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The possible exclusion of solute molecules has not always been recognised. For example, Fini et al. (35) assumed that the theoretical hold-up time, t_M, for a given column and eluent flow rate should be the same for all substances and independent of the mobile phase.

The difficulty caused by exclusion of solutes from the pores of the stationary phase has been discussed by several workers. Slaats et al.(28) mention that further complications arise as a result of size or electrostatic exclusion of molecules from the pores. This view is supported by Krstulovic et al. (15) who believe that unlike GC, the void volume in RPLC may be a function of both the mobile phase composition and the molecular size of the solute used for the determination. Laub and Madden (36) state that it is assumed generally in LC that the supposed unretained solute used to measure the column void volume must be of the same hydrodynamic volume as the analyte under investigation.

Experimental evidence on the ability of silica gels to exhibit exlusion properties has been presented by Scott and Kucera (33) who

concluded that the effective pore diameter will influence the measurement of the retention volume or capacity factor of a solute. Similarly Engelhart and Ahr presented data (11) which was explained as being due to the exclusion of the solutes from the relatively small pores of the Zorbax ODS (C_{18}) column.

Several workers take into account this problem in their mathematical treatment of void volume. Larmann et al. (37) and Quarry et al. (38) propose that the capacity factor (k) of a solute is related to its retention time (t_R) by equation [2].

$$k = \frac{t_R - t_{sec}}{t_{sec}} \qquad [2]$$

where t_{sec} is the retention time of a molecule of equivalent size as the solute but which is not retained by the column.

Quarry et al. (38) estimated t_{sec} from a size-exclusion chromatography calibration plot using simplifying assumptions. The results for one set of experimental conditions showed a variation of up to 12% for the dead time measured using deuterium oxide (D_2O) and by 1% - 2% for C_1 to C_5 dialkyl phthalate solutes.

In an essentially similar approach, both Horvath and Lin (19) and Wells and Clark (39) proposed the same equation to account for the partial exclusion of solutes.

$$V_o = V_e + \phi V_i \qquad [3]$$

where V_e = interstitial fluid volume
ϕV_i = intraparticulate fluid volume explored by solute

It should be noted from this equation that for the extreme case in which $\phi = 0$, the unsorbed solute is completely excluded from the pores of the packing material, and when $\phi = 1$, the solute undergoes

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total pore penetration. When there is incomplete pore penetration by the solute, ϕ takes a value between 0 and 1.

Berendsen et al. (40) also recognised this problem and noted that their considerations were not valid if molecular size or other effects play a role as is the case in gel permeation chromatography (GPC).

Exclusion due to ionic effects has also been discussed in the literature. Berendsen et al (40) presented experimental evidence for the effect of increasing electrolyte concentration in the mobile phase upon the retention behaviour of a salt which was explained as follows. When the electrolyte concentration is low, the salt is excluded from the pores of the packing, presumably due to electrical charges on the stationary phase surface. With increasing electrolyte concentration in the mobile phase the ion exclusion effect is suppressed and the pores become accessible to the salt. Obviously at low concentrations an injected salt solution yields the exclusion volume. At high concentration the salt peak maximum may indicate the maximum hold-up time. This view is supported by Wells and Clark (39) who quote GPC data to support their conclusions.

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It can be seen, therefore, that defining the column void volume in LC is not a simple matter. While some investigators (41,42) believe that only the total column porosity has a true physical meaning, Billet et al. (43) doubt that a column possesses a unique void volume. In support of this view, Horvath and Lin (19) discuss various mobile phase hold-up volumes in an attempt to relate experimental data to theoretical considerations.

The foregoing discussion suggests that each solute can be thought of as experiencing its own unique void volume, which is a function not only of the stationary phase, but also of the mobile phase including electrolytes. In fact, when dealing with ionisable salts, even the amount of solute injected will affect the void volume in an

unbuffered system. Thus the calculation of a single void volume is at best , under some conditions, an average value related in some way to the various void volumes experienced by the individual solute molecules. Given these considerations it may be that the most appropriate experimentally determined value, short of determining individual solute void volumes, is the total exclusion volume. This value has the advantage that it ensures that k values are always positive. However, it suffers from the difficulty of measurement under some conditions.

A final complication is the effect of pressure on the void volume of a column. A study by Martin et al. (44) concluded that both retention volumes and retention times are affected by the pressure drop across a column, with the variation in volume being due to the compressibility effect, while the variation in time is mainly due to the viscosity effects. However, it was also found that these are small below a pressure of approximately 200 bars.

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It is therefore clear that the determination of column void volume requires not only an understanding of the retention mechanism involved in the particular system under study and a knowledge of the types of solutes involved in the analysis, but also a knowledge of the end use of the the data. Methods which give acceptable results when used for comparative purposes may lead to unaccepatble errors when used to determine absolute data such as thermodynamic properties.

3. Determination Of Void Volume

Unlike GC, where the discussion in recent years has concentrated upon the merits of various mathematical methods for calculating dead time from the retention data of homologous series (45), the methods for the determination of hold-up volume in LC have been largely experimental. For this reason this section of the review

concentrates on experimental methods. However, since there has been some interest and debate of late concerning the application of the mathematical treatment of retention data for homologous series for the determination of hold-up volume in LC, this topic will be discussed in some detail at the end of this section.

The various techniques for determining the column hold-up volume can be broadly classified into several categories.

3.1 Static Methods

The total column porosity can be determined by successively filling the column with two solvents of different density and then weighing it after each filling. The total volume taken up by the mobile phase (V_M) can be calculated from equation [4].

$$V_M = \frac{W_1 - W_2}{d_1 - d_2} \qquad [4]$$

where W_1 and W_2 = weight of column containing solvents 1 and 2.
d_1 and d_2 = density of solvents 1 and 2.

Various combinations of solvents have been reported in the literature. Slaats et al. (46) suggested weighing a column previously dried with a stream of dry nitrogen and the same column then filled with tetrachloromethane. In a later paper Slaats et al. (28) used psychrometry using pure acetonitrile and methanol. Krstulovic et al. (15) employed acetonitrile and carbon tetrachloride because of the large difference in their densities and also measured the difference in weight between a dry column (purged with pentane and dryed overnight at 60 °C in a stream of helium) and the same column filled with solvent. Methanol and tetrachloromethane have also been used for this purpose (22,40,47).

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In a modification of this technique, Fin et al. (35) weighed the column plus mobile phase. Then having removed the contents of the column, the volume of the column and the weight of the stationary phase were measured, thus allowing the determination of the mobile phase volume. Obviously this method is not applicable to routine analyses.

The value determined by the above procedures represent the maximum volume accessible to the solvent molecules. Therefore, unless the solvent molecules experience entropic or enthalpic exclusion from the column pores, the result places an upper limit on the void volume experienced by a particular solute. As pointed out by McCormick and Karger (22), this value thus serves as a criterion for evaluation of solute retention since elution volumes larger than it are indicative of retention.

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As discussed by Berendsen et al. (40), equation [4] ignores the possibility that the stationary phase is solvated by molecules of the mobile phase. This problem is addressed by Slaats et al. (28,46) who proposed subtracting the volume of the adsorbed mobile phase layer on the silica surface. This volume was determined using breakthrough curves (48) as described by Pannakker et al. (49) or by using the minor disturbance method (28). Without this correction, Krstulovic et al. (15) warn that the use of this measure with liquids which solvate the stationary phase to a significant extent (such as tetrahydrofuran) may lead to negative k values due to the negative slope of the excess isotherm (preferential adsorption of the organic component of the mobile phase rather than the solute). They found that this occurred with alcohols chromatographed with an eluent composed of 60 volume % THF in water.

Riedo and Kovats (42) also supported this correction provided that the density (specific volume) of the liquid in question is the same in the bulk as the absorbed state, that the boundary between liquid and solid is independent of the nature of the liquid and that

exclusion effects can be ignored. Another provision is that if mixtures are used for the determination, the partial molar volumes of the components should remain the same at the composition of the mixture in the surface phase as they are in the bulk.

Scott and Kucera (26) describe a batch method for determining the mass of organic modifier adhering to the stationary phase by measuring the change in mobile phase composition after equilibration with the stationary phase. However, Slaats et al. ( ) point out that this method is not very attractive for use with RPLC columns because the high concentrations of modifier employed lead to a very small difference in the concentrations before and after adsorption.

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A final static method is mentioned by Berendson et al. (40) in their review paper. It is based on a plot of gross retention times versus distribution coefficients determined by static methods. They note, however, that it is difficult to justify the use of this method with chemically bonded stationary phases where bulk partition coefficients are invalid.

3.2 Interstitial Volume

While the previous section considered the measurement of total column porosity by static methods (this property can also be estimated by other experimental methods as discussed in later sections), the other most easily defined void volume is the interstitial volume. Although the concept is clear, its actual value, as discussed by Knox et al. (34) can only be inferred by assuming it equal to V_M for the most excluded solute available, or by estimation from the known pore volume by assuming the density of the matrix and the porosity of the packing (34). This latter method is, however, approximate at best.

One area in which the term is significant is size exclusion chromatography (SEC) where a truly retained substance will only

reside within the mobile phase contained within the interstitial volume. Various substances have been used to measure this interstitial volume. Mori and Suzuki (50) used polystyrene of molecular weight 8.5×10^6 with SEC columns, Scott and Kucera (33) used polystyrene of molecular weight 655,000 with commercially available silica gel columns, while Mocked and Freyholdt (20) used both polystyrene at molecular weight 1.8×10^6 with THF eluents and soluble starch with aqueous alcohol eluents to measure the interstitial volume of chemically bonded octadecyl phases.

It has also been reported (see next section) that injections of weak solutions of ionisable salts into unbuffered aqueous mobile phases are excluded from the pores of RPLC columns, thus allowing their use as a measure of the interstitial volume. However, the dependence of the retention time on sample concentration and the difficulty of ensuring total exclusion limit the usefulness of the technique.

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3.3 Inorganic Salts

Many inorganic salts have been used to measure void volume (51-60) including sodium nitrate (51-57), potassium iodide (58), potassium dichromate (9,30,59), potassium nitrate (27),sodium chloride (60) and sodium nitrite (57). Although an apparently simple technique, the use of inorganic salts presents several difficulties.

Tilly-Melin et al. (29), using phosphate buffered aqueous acetonitrile mobile phases, compared the retention behaviour of potassium nitrate and potassium dichromate each having been prepared in unbuffered mobile phase. They found that potassium nitrate was retained even more than some of the carboxylic acids which they were investigating and therefore was unsuitable for use in determining V_M in that system. On the other hand, the retention volumes obtained using both potassium dichromate and water (modified mobile phase injection) were identical and were taken to be V_M. Unfortunately it

was found that potassium dichromate is retained in systems comprising acid buffered mobile phases as well as all systems containing quaternary ammonium ions and is therefore unsuitable as a measure of V_M in such systems.

A wider study of inorganic salts was carried out by Berendsen et al (40), who compared KI, KBr, NH_4NO_3, $NaNO_3$, $FeCl_3$, K_2CrO_7, $CuSO_4$ and $FeSO_4$ as well as HCl using aqueous methanol mobile phases. It was found that the retention volumes of these salts varied with the salt concentration of the injected solutions. All salts except $CuSO_4$ and $FeSO_4$ (which showed some retention) were reported as exhibiting similar exclusion properties. At low electrolyte concentration the salt is excluded from the pores of the packing, presumably due to electrical charges on the phase surface: with increasing electrolyte concentration in the mobile phase (or with injections of highly concentrated solutions of a salt) the ion exclusion effect is suppressed and the pores become accessible to the salt. Thus Berendson et al. (40) initially concluded that the total exclusion porosity is given by the time elapsed between the injection of a 10^{-4}M KBr solution and the start of the peak, while the time of the peak maximum resulting from an injection of 15 µl of mobile phase saturated with KBr gives an estimate of the true hold-up volume of the column.

Unfortunately the invariance of this latter measurement with mobile phase concentration plus further experiments with KI solutions cast some doubt on the general use of concentrated salt solutions for the determination of hold-up volmes under all conditions. In fact, it was concluded that inorganic salts appear to give good results only for mobile phases composed of nearly equal volumes of water and methanol ($0.4 < \phi < 0.6$).

Commenting on these results, Slaats et al (28) suggested that the difficulty with the use of unretained compounds (including inorganic salts) is that their use cannot be justified in a physical sense. Wells and Clark (39) compared the elution characteristics of both

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sodium nitrate and potassium dichromate with several organic substances and mobile phase components in a study which, in general, supported the observations of Berendson et al. (40). The study confirmed that the elution behaviour of ionic solutes is extremely dependent on the background electrolyte concentration in the mobile phase and that sodium nitrate and potassium dichromate have slightly different retention volumes under some conditions. However, it was shown that the other substances present even greater problems. Therefore, Wells and Clark concluded that when buffered aqueous methanol eluents are used, the injection of any detectable amount of sodium nitrate produces a good estimate of the column void volume. In unbuffered aqueous methanol mobile phases the injection of at least 3×10^{-6} mole of sodium nitrate was found to give a good estimate of the column void volume. Sodium nitrate was recommended over potassium dichromate because of its greater pore penetration, thereby more nearly representing the case where $\phi = 1$ in equation [3].

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These studies illustrate one of the problems with the use of inorganic salts for the determination of void volume, which is the degree of penetration of the salt into the pores of the column packing. Such penetration may vary from zero (total exclusion) to virtually complete penetration depending on a variety of factors including the nature of the mobile phase, type of salt, amount of the salt injected as well as the presence or absence of background electrolyte. This exclusion of co-ions (the Donnan effect) is explained by Knox et al. (34). It arises whenever charged species are confined to a particular region within a thermodynamic system and thus accompanied by a corresponding electrical potential difference between the two regions. Given this problem several authors have recommended against the use of inorganic salts for the determination of void volume. (ref)

Thus, while Krstulovic et al. (15) obtained similar results to previous workers in their investigation of the retention behaviour

of sodium nitrate using mobile phases composed of 0.1 M NaBr in aqueous methanol (except for a larger variation in retention volume with mobile phase composition), they did not recommend its use in determining void volumes. Fini et al. (35) also compared several salts including sodium nitrate and concluded that these should not be used for the determination of V_M. After comparing sodium nitrate and sodium nitrite with several other unretained substances using aqueous methanol mobile phases, Popl and Fahnrich (57) also concluded that inorganic salts are unsuitable for the determination of V_M. This study also revealed a further problem in relation to the use of sodium nitrite, which produced two peaks in acid eluents (pH < 4.0). This was attibuted to the liberation of nitrous acid, which then eluted more slowly than the salt, yielding a second peak.

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A final problem with the use of concentrated salt solutions was noted by Billet et al. (43), who suggested that such methods suffer from the disadvantage that they are not applicable to mobile phases mixtures other than aqueous methanol, since a high salt concentration leads to demixing of aqueous acetonitrile and aqueous tetrahydrofuran eluents. This observation was also made by Berenson et al. (40).

Given the many problems associated with the use of inorganic salts including conflicting evidence on the most appropriate salt to use, extreme caution should be exercised in their use. Where possible, alternative techniques should be considered, especially when using mobile phases other than aqueous methanol.

3.4 Organic Compounds

The use of a large variety of both organic salts and other 'unretained' organic compounds as void volume estimators is widespread. They include sodium benzene sulphonate (6), nitrobenzene (11), β-carotene (49), tetrachloroethane (52), benzoic acid (61), tartrazine (62) fructose (31,63,63), acetone (65,66),

pentane (67), n-hexane (68), iso-octane (67), n-nonane (69), azo-dye ponceau 6R (70), uracil (37,59,71,72), fluorene (66), tetrachlormethane (73,74), chloroform (75), cytosine (76) and N,N-dimethylformamide (77).

A study involving several of these substances (acetone, uracil, N,N-dimethylformamide, sodium benzene sulphonate and tartrazine) was carried out by Wells and Clark (39). When chromatographed with nonbuffered aqueous methanol, the retention behaviour of organic salts (sodium benzene sulphonate and tartrazine) was found to be dependent upon the concentration of the salts but independent of the mobile phase composition: the opposite was observed when phosphate buffered eluents where employed. Thus these compounds are not suitable for use in determining the column void volume. The retention volumes of the other organic substances (acetone, uracil and N,N-dimethylformamide) were found to be dependent upon the mobile phase composition yet independent of the amount of solute injected and so these substances were also rejected for use in determining the column void volume.

Popl and Fahnrich (57), using aqueous methanol mobile phases, compared sodium benzene sulphonate, dimethylformamide and acetone with phlorglucinol which earlier tests had indicated may give a good indication of the column void volume. Sodium benzene sulphonate was rejected for use in determining void volume as it exhibited the same problems as inorganic salts. For non-ionisable substances (acetone and dimethylformamide) the retention volumes were found to be independent of concentration. However, it was found that both of these substances interacted with the stationary phase and the retention volumes were dependent upon the composition of the eluent. On the other hand, the retention behaviour of phloroglucinol was found to be nearly constant for different mobile phase compositions and independent of the amount injected. This substance was therefore recommended as an indicator of the column void volume.

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Krstulovic et al. (15) also investigated the retention behaviour of sodium benzene sulphonate in mobile phases composed of 0.1 m NaBr in aqueous methanol and found that this salt is retained in solutions of low organic modifier concentration. They concluded that this salt appears to be unsuitable for use in the determination of void volume.

In a more extensive study, Fini et al. (35) compared the retention behaviour of picric acid, formamide, urea, thiourea and uracil with water, oxygen and sodium nitrate. They found that while oxygen is retarded by the stationary phase and hence is not suitable for the determination of t_M, formamide, urea and thiourea give good estimates of t_M. Thiourea was recommended because of its strong UV absorption.

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The conclusions of Fini et al. (35) were based on a comparison with the measured amount of liquid in the column and therefore show that these substances were able to penetrate the pores of the column packing, thus giving an estimate of the total column porosity. It is also interesting to note that the retention volumes of formamide and thiourea agreed well with that of sodium nitrate in buffered mobile phases, confirming that the total column porosity was being measured under these conditions. In contrast, the retention volumes of sodium nitrate in unbuffered mobile phases were much lower, as would be expected if the salt was being excluded from the column pores as discussed in section 3.3.

Meanwhile, Engelhardt and Ahr (11) demonstrated some of the problems associated with the use of 'unretained' substances by comparing the retention time of nitromethane (a supposedly unretained substance) with two isomers of aristolochia which they were separating. It was found that in pure methanol the isomers are slightly separated and are eluted shortly after nitromethane.

Although the addition of water might be expected to increase the k values and improve selectivity, it was found that the acidic isomers eluted before nitromethane under these conditions, yielding negative k values. The addition of 1% acetic acid was found to be sufficient to retard the isomers and result in elution after nitromethane. However, it should also be noted that the retention volume of nitromethane was found to be significantly longer under these new conditions.

With regard to the use of neutral species to determine the column hold-up volume, Knox et al. (34) obtained experimental results which apparently invalidate a widely advocated method for determining V_M in adsorption chromatography: this method proposes that for any eluent V_M be taken as the elution volume of the most non-polar solute available (78). Knox et al. (34) suggested that the correct procedure for determining V_M should identify V_M as the elution volume of a solute having the same eluotropic strength as the eluent.

Further, the danger in using 'unretained' compounds can be seen in the use of acetone. While this substance has been used as an unretained compound (65,66), it is also the first member of a homologous series (2-keto alkanes) which has been advocated to determine t_M using the linear relationship between the logarithim of the adjusted retention time and carbon number of substance (16). It is therefore clearly retained to some degree and its use as a void volume marker is questionable.

From the above discussion, it can be seen that organic salts offer no improvement over inorganic salts as they suffer from exactly the same problems. This is particularly true in unbuffered systems where the retention volume of the salt is dependent on the amount of salt injected. While the use of other 'unretained' substances has been recommended by some workers, the wide variety of substances reported in the literature as well as the conflicting evidence

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presented shows that more work need to be done in this area. It is also probable that the substance of choice will depend on the particular experimental conditions being used.

3.5 Isotopically Labelled Compounds

Isotopically labelled compounds have been used to determine column void volume in many studies (18,23,32,42,43,47,79-84). Such compounds, which normally consist of labelled mobile phase components, can generally be classified into one of two categories: deuterated compounds (D_2O is most commonly used) and radioactively labelled compounds.

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The use of labelled mobile phase components has been discussed extensively by Riedo and Kovats (40) who supported the use of compounds containing radioactive carbon. They pointed out that, while deuterated compounds are easier to work with as far as handling and detection are concerned, they are less satisfactory due to the small change in physical properties that commonly occurs with the degree of deuteration. In discussing the relative merit of the two techniques, Riedo and Kovats (42) suggested that the best solution might be the use of a series of compounds with increasing degrees of deuteration. By assuming a linear change in properties, the retention volumes of compounds deuterated to different degrees could be extrapolated to 0% deuteration to give retention volumes of 'labelled but not deuterated compound'.

The two broad classes of isotopic methods of determining V_M are now considered.

(i) Radioactively Labelled Compounds

Knox et al. (34) described a method which involves determining the retention times of radioactively labelled compounds of the eluent. V_M is then precisely obtained from the equation

$$V_M = X_A V_A^* + X_B V_B^* + \ldots\ldots \quad (5)$$

where X_A, X_B are the volume fractions of compounds A,B in the eluent and V_A^*,V_B^* are the retention volumes of radioactively labelled samples of A,B. The great disadvantage of this method is the problem of detection.

A second method using radioactively labelled eluent was also described by Knox et al. (34) and involves collecting single drops of eluate and counting these after dilution with scintillation counting fluid. This method is both time consuming and difficult. Although an apparently obvious solution to the detection problem is the use of a special detector, such a solution is not without problems. Halasz (18) has pointed out that, although such a detector would allow exact V_M values to be achieved by measuring the retention volume of the eluent itself, the volume of the detector would also certainly be different to that of the detector used for routine analyses.

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(ii) Deuterated Compounds

The use of deuterated mobile phase components (D_2O in particular) is far more widespread than the use of radioactively labelled compounds. In particular, Colin and various co-workers (32,47,78-82) have advocated the use of D_2O, even though they warn that the use of this method may not lead to the true single value for t_M, if such a value does exist (79). In a recent paper (47) they compared methods using D_2O, maximum column porosity and linearisation of convergent homologous series and reported that all V_M estimates gave column porosities within the range 0.65 - 0.75.

A more useful study was carried out by Berendson et al. (40) who compared the retention times of water and methanol with their deuterated analogues using various aqueous methanol concentrations and a series of n-alkyl silyl bonded packings. It was found that for a given column and for all mobile phase compositions, except in

the region of $\phi = 0$, all four compounds elute from the column in the same time. At very low concentrations of organic modifier, D_2O elutes faster than methanol or CD_3OD. However, the elution time was found to be dependent on the mobile phase composition with a minimum occurring at $\phi = 0.7$. This phenomenon combined with a slight temperature dependence of retention volume led Berendson et al. (40) to warn that mobile phase components may be subject to chromatographic retention and should be used with care. They suggested that it is only for methanol concentrations around 65% by volume that the true hold-up volume is approached.

A more extensive study was carried out by McCormick and Karger (22). The study involved not only the use of D_2O as a void volume marker in aqueous organic modifier systems where the organic modifier was alternatively methanol, acetonitrile and tetrahydrofuran, but also the use of the deuterated analogues of these three solvents.

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In general, they found that the injection of a sample of mobile phase which had been enriched with deuterated modifier produced two peaks when the eluent was monitored with a refractive index (RI) detector. One of the peaks had the same retention behaviour as non-deuterated modifier concentration pulses, while the other marked the elution of the band containing the deuterated modifier molecules. Furthermore, the first peak could be eliminated by adjusting the total concentration of modifier plus deuterated analogue in the injected sample so that it exactly equalled the concentration of modifier in the bulk mobile phase. The injection of D_2O enriched mobile phase also resulted in the appearance of a vacancy peak.

The origin and properties of vacancy peaks has been discussed by Slais and Krejci (85). The subject has also been treated in more detail by McCormick and Karger in a second paper (23) in which they explain that when equilibrium is established between the mobile and

stationary phases in an RPLC column, the organic modifier is preferentially extracted into the stationary phase by virtue of its hydrophobic expulsion from the mobile phase. If this equilibrium is disturbed by the injection of solute compounds, the composition of the extracted organic modifier system will vary in order to accommodate the changes in the mobile phase-stationary phase equilibrium. In such a case an extra displacement or vacancy band (in addition to the solute band) will appear in the chromatogram.

In their study (22), McCormick and Karger found that the injection of deuterated organic modifier returned retention volumes greater than the maximum column porosity in all cases except at high modifier concentrations. They concluded that deuterated organic modifiers are not
suitable as a measure of void volume. On the other hand, the injection of D_2O gave results consistent with the expected void volume if the layer of modifier extracted into the stationary phase was ignored. The conclusion that D_2O gives a good estimate of the void volume was further supported by comparing isotherms measured by dynamic methods using D_2O as a void volume marker with the isotherms measured by gas chromatography.

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However, as noted by McCormick and Karger (22), the conclusion that D_2O returns a good estimate of the column void volume presumes that these molecules have full access to the pore volumes experienced by more lipophilic molecules and that D_2O does not strongly interact with the residual water-deactivated silanols. This point is also addressed by Slaates et al. (28) who suggest that when D_2O or deuterated methanol is used as a tracer, residual silanol groups present on the adsorbent influence the retention volume. A significant decrease in the void volume measured with 100% water as the mobile phase was attributed to the inability of water molecules to gain access to a significant fraction of the internal pore structure due to poor wetting conditions. In addition, values of void volume greater than the maximum column porosity at 100% organic

modifier were explained as being due to the interaction of the solutes with the residual silanols on the silaceous support. Finally it should be noted that the results of McCormick and Karger (22) show some significant differences compared to those of Berendson et al. (40).

The most common detector used to measure the retention behaviour of D_2O is the refractive index detector. However, Billet et al. (43) have highlighted two problems with the use of this detector. These are the appearance of vacancy peaks as previously discussed and the poor detectability of D_2O in certain mobile phase mixtures. These problems were overcome by using a special detector, the helium microwave induced plasma (MIP) detector. This detector demonstrated greatly increased sensitivity for the detection of D_2O and ensured that the size of the signal was independent of the mobile phase composition, a condition not satisfied by an RI detector.

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This lack of sensitivity of the latter is emphasised by plots which show that in mixtures comprising approximately 80% methanol in water, the D_2O peak cannot be detected by an RI detector, and that only the vacancy peak is observed. This observation casts doubt upon the results of other workers who used high methanol concentrations.

The MIP detector is also effective with other organic solvents. Billet et al. (43) showed that serious problems only occur with high concentrations of THF: a deposition of carbon in the quartz plasma tube occurs thus causing a decrease in the detector's sensitivity to D_2O. The detector also performed well with other deuterated compounds, for which the response of the detector was found to be roughly proportional to the degree of deuteration of the injected compound.

Although an interesting development expecially for fundamental studies of void volume as determined with deuterated compounds, the

MIP detector is unlikely to be used for routine work for the same reasons as those mentioned previously when a specific detector for radioactively labelled compounds was discussed.

In conclusion, the use of deuterated mobile phase components other than D_2O cannot be recommended due to the conflicting data that have been reported (22,40) as well as the probability that such components interact with the layer of mobile phase adsorbed onto the stationary phase. However, the careful use of D_2O does seem to give an acceptable estimate of the column void volume for systems using mobile phases containing up to 70% organic modifier and where reactions with residual silanol groups are not significant. A careful appraisal of such data is necessary, with particular importance being placed on the correct identification of the the D_2O peak, which can present problems due to the occurrance of vacancy peaks. The best results are likely to be obtained by the careful control of D_2O/organic modifier ratio in order to eliminate such vacancy peaks.

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3.6 Injection of Modified Mobile Phase

Another widespread technique for the determination of void volume is the injection of mobile phase components. While some workers (10,71,86,87) have used an injection of mobile phase with a slightly altered organic modifier concentration, others (8,74,75,88-93) have used an injection of pure organic modifier or pure water (26,88, 93-95). A further technique is to measure the retention time of the solvent front (16,96,97).

A refinement of these techniques was described by Scott and Kucera (27), who measured the retention volume of methanol in aqueous methanol mobile phases of compositions between 0% and 70% methanol using a refractive index detector. It was found that as the organic solvent concentration increased, the retention volume of methanol

decreased until it reached a constant minimum value at high modifier concentrations. This constant value was accepted as the void volume. Scott and Kucera (27) also found that the void volume obtained using this method was the same as the value obtained by injecting samples of potassium nitrate.

Colin et al (94) who used the injection of a sample of pure water to define V_M, expressed doubts about the use of an injection of pure organic modifier for the same purpose. In addition they noted problems in identifying the chromatographic response due to water in some organic modifier/water systems because of the oscillating positive and negative absorbance signals produced under such conditions. They recommended the injection of the smallest detectable amount of water in order to minimise this effect. In a further paper, Colin and co-workers (88) overcame this problem for mobile phases within the concentration range 5%-95% water using a Waters Associates model 440 UV absorbance detector. Because of the special optical design of this cell, the perturbations appeared to be eliminated and single, well defined peaks were obtained.

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Berendson et al. (40) conducted a study in which the retention behaviours of pure methanol and water were compared with those of deuterated methanol and D_2O for several n-alkyl silyl bonded packings using aqueous methanol mobile phases. Their results, which were discussed in section 3.5, show that at mobile phase modifier concentrations between 60% and 70% the elution volumes of methanol and water might approach the hold-up volume. This agreement was found on the basis of the temperature independence of the data and its comparision with the maximum column porosity.

In a study of a phosphate-buffered (pH 8.0) aqueous acetonitrile system (29), the retention time of water (introduced as modified mobile phase) was compared with inorganic salts. Tilly-Melin et al. (29) found that the water peak coincided with the least retained salt and thus was accepted as the column void volume. However, a lack of details reduce the usefulness of their study.

A far more extensive study, which was also mentioned section 3.5, was carried out by McCormick and Karger (22) and involved the use of both deuterated and non-deuterated components of three mobile phases (aqueous methanol, aqueous acetonitrile, aqueous tetrahydrofuran). The results of this study showed that the elution volumes of the non-deuterated modifiers exhibit a strong dependence upon the concentration of the modifier in the bulk mobile phase. It was also found that at modifier concentrations less than approximately 30% by volume, the elution volumes of the unlabelled modifiers are generally greater than maximum column void volume. McCormick and Karger (22) therefore concluded that retention of the modifier by the stationary phase was occurring, a result which seems to justify the doubts expressed by Colin et al. (94) with regard to the use of an injection of organic modifier to determine void volume. This study also determined the elution behaviour of water with the results suggesting that the retention behaviour of water-enriched samples is essentially identical to that for the corresponding modifier-rich samples except that the differential refractive index detector response is opposite in direction. Colin et al. (94) concluded that water is retained by the stationary phase and explained this apparent retention by assuming the existence of an extracted modifier layer in the bonded phase and applied the principles of vacancy chromatography. It is interesting to note that for all three mobile phase systems, the minimum retention volume of water occurred for organic modifier concentrations between 60% and 70% and therefore support the findings of Berendsen et al. (40). However, the exact details of the relationship between retention time and modifier concentration differ between the two studies (especially when deuterated mobile phase components are compared to non-deuterated components).

A further problem associated with the use of water-enriched mobile phase as an estimator of the column void colume is the observation of vacancy peaks as discussed in section 3.5.

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Wells and Clark (39) rejected the use of an injection of pure organic modifier to determine the column void volume. In their study of the retention behaviour of methanol in aqueous methanol mobile phases, they found that its elution volume increased with decreasing concentration of organic modifier in the bulk mobile phase. A theoretical understanding of reasons for these difficulties was provided by Slaats et al. (28). While analysing the minor disturbance method for determining the adsorption isotherm for their systems, they derived two relationships (equations 19 and 20 in their paper) which demonstrate that the observed retention volume resulting from the injection of components of a binary system yields a poor estimate of the hold-up volume as it depends strongly on the slope of the adsorption isotherm.

From these studies it is clear that an injection of modifier-enriched mobile phase is not, in general, suitable for the determination of the column hold-up volume. Also, the use of an injection of pure water must be strongly questioned, especially considering the evidence of McCormick and Karger (22) which indicate that the 'true' hold-up volume (if such a value exists) is not being measured under some conditions.

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3.7 Other Methods

This section reviews other methods that have been used to estimate V_M but have experienced limited acceptance.

(a) Halasz (18) developed a relationship for use with a regular packed column where the ratio of the inner diameter (d_c) to particle size (d_p) is greater than 10. For a porous support, Halasz approximated the linear velocity (V) of the eluent by equation [6].

$$V = \frac{1.5\ F}{d_c^{\,2}} \qquad [6]$$

where F is the volumetric flow rate.

For liquid-impenetrable, non-porous supports such as glass beads equation [7] was found to be appropriate

$$V = \frac{3\,F}{d_c^{\,2}} \qquad [7]$$

The retention time of an inert peak can be determined by dividing the column length, L, by the linear velocity, V. Although this relationship has been used by Hemetsberger et al. (103) and Lochmuller and Wilder (54) (who used it to verify the results obtained using $NaNO_3$), such a relationship is at best an approximation.

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(b) Halasz (15) also states that a homologue with a lower carbon number than the eluent is usually unretained. It should be noted, however, that this is not always possible. For example, in aqueous methanol systems an appropriate homologue does not exist.

(c) A third suggestion by Halasz is that if the corrected retention volume of a compound is constant in the temperature range of 20° - 50°C, it may be considered an inert peak.

(d) Neidhart et al. (104) mention a method in which oxygen saturated modified mobile phase is injected and the retention time measured with an RI detector. However results reported by Fini et al. (35) clearly show significant retention of O_2 in aqueous methanol systems at all methanol concentrations.

(e) Neidhart et al (104) also describe a method involving the doping of the mobile phase with a fluorophore (chininesulphate). injection of undoped mobile phase and measuring the decrease in fluorescence. However, this method requires the use of a specific detector and thus is not generally applicable.

(f) In an attempt to overcome the deficiency perceived in most experimental methods (that they do not allow the determination of

the exact value of the hold-up volume because of its dependency upon the porous structure of the stationary phase, thus producing hold-up values which are too high), Neidhart et al. (104) developed a method based on the assumption that the hold-up volume is independent of temperature.

Their method allows the calculation of the zero retention time, t_M, be means of equation [8].

$$t_M = \frac{t^2_R(T_1)-t_R(T_2).\ t_R(T_3)}{2t_R(T_1)-t_R(T_2)-t_R(T_3)} \qquad [8]$$

where $t_R(T)$ values are experimentally determined brutto retention times (sic) at different temperatures, T_i, with the condition that the T_1, T_2 and T_3 are chosen such that T^{-1} values are equidistant. That is

$$T_2 = \frac{2T_1T_3}{T_1 + T_3} \qquad [9]$$

In a two component system, given the condition that the sorption enthalpies of the two components are equal or at least very similar, the zero retention time can be determined by using results at two temperatures as follows:

$$t_M = \frac{t_{R'A}(T_1).t_{R,B}(T_2)-t_{R,B}(T_1).t_{R,A}(T_2)}{t_{R'A}(T_1)+t_{R,B}(T_2)-t_{R,A}(T_2)-t_{R,B}(T_1)} \qquad [10]$$

where $t_{R,A}(T)$ and $t_{R,B}(T)$ are the brutto retention times (sic) of compounds A and B respectively at temperature T.

Also presented was simple graphical technique to solve this last equation. This method was also used by Yi et al. (105) who compared the graphical and mathematical techniques for solving equation [10].

Grushka et al (105) criticised this method on several grounds and while the criticism was answered to some extent (106), as presented,

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the method not only involves many assumptions (thus limiting its usefulness) but also is weakened by the lack of data points used in the determination of the zero retention time. Such a situation can lead to significant errors.

(g) Recently Quarry et al. (38) introduced the concept of t_{sec} (equation [2]), which represents the retention time of a molecule of equivalent size as the solute that is not retained by the column, to take account of steric hinderence effects. The calculation of t_{sec} requires the preparation a size-exclusion chromatography calibration plot for the column of interest using THF as the mobile phase and various polystyrene samples as solutes. The t_{sec} values are then determined using an approach based on a consideration of the fractional pore volume accessible to the solute, and assuming that differences in t_M (measured using D_2O) arise from changes in the volume of mobile phase within the packing pores that is accessible to small solutes. A less sophisticated approach can also be used. As discussed in a previous section, this method produced t_{sec} values which were up to 12% lower than the corresponding t_M value.

However, the difference between individual solutes (C_1 and C_5 dialkyl phthalates) was only 1% to 2% This procedure is certainly a very interesting approach to the question of whether or not a column has a single void volume and seems to support the view that individual solutes experience different void volumes.

In relation to this approach, it is of interest to review earlier work described out by Scott and Kucera (33) regarding the ability of commercially available silica gels to exhibit exclusion properties. Their work indicated that significant exclusion did not occur below a molecular weight of approximately 100 - 150.

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4. Mathematical Determination of Void Volume by Linearisation of Retention Data for Homologous Series

In gas chromatography, the use of Kovats retention indices is widely recognised as the most useful method for presenting comparative retention data (17). The system is based on the observed linear relationship between the logarithm of the adjusted retention time of a substance and its carbon number, where the retention indices of the n-alkanes are defined as 100 times their carbon number.

The retention index of a compound was initially defined (98-100) by equation [11].

$$I_{\text{Substance}}^{\text{Stat. phase}}(T) = 100 \times \frac{\log t'_{Ri} - \log t_{Rz}}{\log t'_{R(z+1)} - \log t'_{Rz}} + 100z \qquad [11]$$

where I = Kovats retention index at a given temperature T.
t'_{Rz} = retention time of a homologue with carbon number z.
t'_{Ri} = adjusted retention time of a substance i.
z = carbon number

However, this equation has been replaced by equation [12] which assumes that a linear relationship exists between the logarithm of the adjusted retention time and the carbon number of a substance.

$$\ln(t_R - t_M) = bz + c \quad (I = 100z) \qquad [12]$$

where b and c are constants.

While adjusted retention times (t'_R) are used in gas chromatography, capacity factors (k) are more common in LC, where $k = t'_R/t_M$. Rewriting equation [12] in terms of capacity factors gives

$$\ln k = b z + c' \qquad [13]$$

where $c' = c - \ln t_M$

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Although the use of equation [12] (or [13]) is not as widespread in LC as it is in GC, a number of workers have discussed its use. (15,16,20, 24,25,35,36,47,72,79,82,90,94,101)

There are two major aspects to be considered in relation to the use of retention data for homologous series. The first is the linearity of the relationship under various experimental conditions and the second is the usefulness of the relationship for determining the column void volume.

One of the first groups to address the question of linearity was Colin et al. (94) who investigated the behaviour of both homologous (n-alkylbenzenes) and pseudo-homologous series (polymethybenzenes, polymethylphenols, chlorobenzenes, nitrobenzenes) using various columns and mobile phases. Rather than simultaneously determine the column hold-up volume, the authors used an injection of pure water for this purpose. In general, their results show that only the n-alkylbenzene homologous series gives consistantly linear relationships of log k vs z. However, a lack of numerical data limits the conclusions which can be drawn from this study.

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A study by Vigh et al (90) improved upon this study by calculating correlation coefficients (R^2) for regression analyses performed upon retention data derived from chromatographing C_6-C_{16} n-alkanols, C_6-C_{12} n-alkanol dinitrophenylhydrazones and C_6 - C_{11} n-alkanone dinitrophenylhydrazones at various temperatures with aqueous methanol eluents. Pure methanol was used to determine t_M. In all cases very high R^2 values were obtained, indicating excellent linearity. This study also showed that the slope of the log k vs z relationship decreased with temperature for all three homologous series while the intercept increased except for the case of the n-alkanols where no trend was apparent.

The question of linearity has also been addressed in two more recent papers by Colin et al. (79, 82). In these papers, the authors rewrite equation [13] as follows

$$\log k = z \log \alpha + \log \beta \qquad [14]$$

where α is the non-specific selectivity of the methylene group
β is the capacity factor of the functional group of a given homologous series.

The authors suggest that log α is a very convenient means for measuring the solvent strength which characterises the hydophilicity of the mobile phase.

While the second paper (82) shows that very high R^2 values are obtained when homologous members only above carbon number 4 are used in the regression, the first (79) introduces a more interesting result. In this earlier study, Colin et al (79) chromatographed several homologous series (alkyl-benzenes, n-methyl esters, n-alkanes, n-alkylbenzenes) over a wide range of binary mobile phases containing water, methanol, THF and acetonitrile. It was found that not only were the results, in general, linear but individual lines for a given homologous series converged to a common point. A procedure was developed for optimizing the intersection point in such a way that the sum of distances between this point and the individual lines was minimised. In all cases except one a single point resulted. It was found that two intersection points existed in the aqueous acetonitrile system, corresponding to those mobile phases containing less than 50% and more than 50% water respectively.

Berendson and Galan (101) had found earlier that the straight lines produced by a given homologous series for different reversed phases also intersect at a common point for those phases with up to 10 carbon atoms. Above 10 carbon atoms, it was found that no further increase in retention occurred and thus the straight lines virtually coincided.

Although most studies have found linear relationships over a wide range of conditions (excluding the lower members of a homologous

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series), a few studies have shown that a distinct break of slope occurs at a critical carbon number. Tchapla et al. (47) found that accurate measurements of the retention volumes of members of homologous series revealed a discontinuity in plots of log k versus carbon number at a point corresponding to the length of the organic ligand of the stationary phase. It was also found that the discontinuity is only observed in monomeric phases and depends slightly on the mobile phase composition but is independent of the functional group of the homologous series. Furthermore, it is not eliminated by adjusting the column hold-up volume. This study also confirmed the observations of Colin et al. (79) that plots of log k versus carbon number at different mobile phase compositions converge to a single point. However, as a break occurred in such plots, two intersection points were found, depending on whether or not homologous members above or below the critical carbon number were used. This 'break of slope' in the log k versus carbon number graph is also confirmed by data presented by Mocked and Freyholdt(20) who used n-alkanes with pure ethanol as the mobile phase. In the same paper, the authors discuss similar results obtained by Engelhard.

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Mocked and Freyholdt (20) suggested that there were three basic requirements for the log k versus carbon number relationship to be linear:

(1) The chain length of the solutes must be kept below that of the bonded phase.
(2) The retention data must be very precise.
(3) The retention data of at least 5 n-alkane homologues are necessary.

The importance of these last two points was also stressed by Smith et al. (45) in their paper which compared various mathematical methods for estimating dead time in GC.

Baker and Ma (16) went further by suggesting the use of a homologous series of C_3-C_{23} 2-keto alkanes to produce a retention index scale equivalent to Kovats retention indices which are regularly used GC.

Using the retention time of the solvent front as a measure of t_M and equation [11] to calculate retention indices, they investigated systems comprising buffered aqueous methanol and acetonitrile mobile phases together with μ-Bondapak C_{18} and μ-Bondapak CN columns. Although the results were linear over a wide range of conditions when using the C_{18} column, the use of the CN column returned non-linear graphs when the modifier concentration was increased beyond 60% methanol: non-linearity was also observed for modifier concentrations above 20% acetonitrile. It was also reported that preliminary studies using μ-Porasil and μ-Bondapak CN (adsorption mode) columns had not been very successful. It appears ,therefore, that more research is required into the linearity of various homologous series under specific experimental conditions.

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The second aspect to this question, the use of the linear relationship to determine column void volume, has also been addressed by several investigators. An early study was conducted by Berendson et al. (40), in which seven homologous series were chromatographed in a mobile phase consisting of 90% by volume methanol in water. The t_M data were obtained using the following equation.

$$t_{Rz+1} = A.t_{Rz} - (A - 1).t_M \qquad [15]$$

where A is a constant

and confirmed graphically by plotting apparent k values using various hold-up times against carbon number. Using this method, even the lowest members of a homologous series were found to lie on the regression line. Therefore Berendson et al. (40) concluded that the most accurate hold-up time using aqueous methanol mobile phases is obtained by the linearization of the logarithm of the net retention times of a homologous series and recommended the use of n-alcohols as the homologous series of choice. A final point to emerge from this study was the fact that t_M decreased with increasing methanol content of the mobile phase. These results were explained in terms of an increasing solution layer as the methanol

concentration in the bulk mobile phase increased. The results were also found to be independent of temperature after a correction for the mobile phase expansion had been applied.

These results were confirmed in a study by Fini et al. (35) who used p-hydroxybenzoic acid esters and aqueous methanol mobile phases of various compositions. It was also found that t_M decreased with increasing methanol concentration.

The equation developed by Berendson et al. (40) (equation [15]) was also used by Yonker et al. in two papers which investigated the behaviour of n-alcohols in various mobile phases (24,25). Their report of a decrease in t_M as the methanol concentration increased thus confirms the results of Berendson et al. (40). In addition, Yonker et al. (25) extended their investigation to both aqueous acetonitrile and THF mobile phases. They found that t_M also decreased with increasing organic modifier concentration in the mobile phase for these systems. The void volume was found to be dependent also on the particular organic modifier used in the mobile phase.

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Equation [15] has also been used by Ambrus (102) in his work on dead time in GC. The method has been reviewed recently (45) and compared with other mathematical approaches to the problem (17).

A further investigation of the use of homologous series to estimate void volume was performed by Krstulovic et al. (15) who chromatographed a number of homologous series using several different columns. Although generally supporting the use of homologous series for the estimation of void volume, some problems were encountered, the main being that when using the equation of Berendson et al. (40) and then maximising the correlation coefficient, there was considerable variation in V_M values.

This was especially a problem when a limited number of homologues with predominantly low carbon numbers were used. This observation contrasts with the findings of Berendson et al. (40) who reported that the individual series gave identical V_M values when chromatographed under identical conditions. Furthermore, Krstulovic et al. (15) observed that the value of V_M depended critically on both the number and choice of homologues used and that R^2 values cannot be used as a test of convergence (it should be noted that R^2 will automatically increase as the number of homologues decreases). These observations are also supported by results presented by Smith et al. (17,45).

To overcome the problem, Krstulovic et al. (15) developed a convergence test in which homologous series which did not give results within a required reproducibility were rejected, thus leading to a greatly reduced scatter of the data.

Two recent studies (36,72) have also broached this subject. Wainwright et al. (72) concluded that the use of n-alkylbenzenes for the mathematical estimation of dead time is probably unwise in either GC or RPLC. In addition, it was concluded that both benzene and toluene show non-linear behaviour and the variation between other members of the series was also significant.

In contrast, Laub and Madden (36) found that the n-alkylbenzene homologues gave very high R^2 values and were suitable (provided that at least four homologues were used) for the estimation of column hold-up volume. However, their data yielded widely varying t_M values (including negative values) depending on the particular homologues used in the regression analysis. Therefore, although high R^2 values were obtained using four homologues, the scatter of the data seems to be excessive. Similar comments can be made with regard to the phenyl-substituted aliphatic alcohols with the additional comment that the authors noted anomalous retention of benzyl alcohol which was explained as being due to hydrogen

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bonding. If this were the case, anomalous retention of the lower members of the n-alcohol homologues would also be expected. In fact, Laub and Madden (36) cite some evidence that this is indeed the case and so cast doubt upon the use of n-alcohols to determine the mathematical hold-up volume.

The above discussion demonstrates the need for caution when using mathematical methods based on homologous series to evaluate HPLC retention data. Unlike GC, where n-alkanes can be used as a standard homologous series for most analyses, other homologous series are required for many HPLC analyses. Combined with the interactions possible between the mobile phase, the stationary phase and the solutes of interest, this new factor creates a situation not experienced in GC. Therefore, the emphasis placed upon the need for a good experimental technique as well as accurate raw retention data for GC investigations (17) is even more relevent to HPLC.

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4.1 Comparison of Mathematical Methods used to Compute V_M from Retention Data for Homologous Series

Several papers (45,107-110) show that there have been a wide range of mathematical approaches to the estimation of dead time in GC. However, the HPLC literature lacks similar comprehensive comparisons of methods used to mathematically determine V_M. The variety of mathematical methods used in HPLC has been far more restricted than those used in GC. The main method is that described by Berendson et al. (40) which uses equation [16]. This equation was later used by Ambrus (12) to interpret G.C. data. Other computational methods include maximisation of the correlation coefficient as suggested by Krystulovic et al (15): the method of Grobler et al (111) as used by Wainwright et al (72): a recent modification of this method by Van Tulder et al (112) and the method of Neidhart et al (104) based on data measures at different temperatures.

Since no extensive comparison of mathematical methods has been made in the HPLC literature and since many liquid chromatographes may not be entirely familiar with the GC literature a brief comparison of the methods used in GC is appropriate here. Of particular use is the recent paper by Smith et al. (45).

The first point that clearly emerges from GC literature is the inaccuracies involved in the use of methods which are retricted to a limited number of data points. The problems with such methods have been pointed out in several publications including those of Guberska (113,114), Sharples and Vernon (115) as well as our own (108). Furthermore, the inaccuracies inherent in the use of limited number of homologues has been mentioned by several workers involved in HPLC (15,20,82). We therefore believe that such methods are of limited use and should be replaced by more sophisticated procedures.

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In a recent paper (45) we made a detailed comparison of the most widely accepted and apparently useful techniques for GC. The methods selected for comparison were:

1. The method of Grobler and Balizs (111) as extended by the technique of van Tulder et al. (112)
2. The method of Guardino et al. (116)
3. The 'exact calculator method' of Dominguez et al (117).
4. The method of Ambrus (102), also extended by the technique of van Tulder et al. (112)
5. The iterative method mentioned by Toth and Zala (118). This method maximises R^2 as suggested by Krstulovic et al. (15)
6. The method of Heog et al (119)
7. The Flexible Simplex method (120)

In addition, a modified approach which allowed the optimisation of t_M while simultaneously fitting a cubic or higher degree polynomial was included in the comparison.

This extensive comparison (45) showed that, of the linear methods, that of Guardino et al. (116) is not only the most accurate, but also the fastest. The study also showed that the accuracy which can be expected from this method depends mainly on the region of the homologue curve that is involved. An accuracy of one unit in Kovats retention indices or better can be expected in most cases when using homologues above carbon number four.

With regard to the polynomial methods it was shown that they offer very little improvement over the linear methods for the higher homologues and only a slight improvement for the lower homologues. Furthermore, these methods can only be used to determine retention indices and are not suitable for the determination of dead volume. It was therefore recommended that these methods only be used where a linear method proves to be unsuitable.

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An examination of the errors introduced into calculations solely due to random fluctation emphasised the need to ensure a high degree of accuracy in the raw data. This observation led to the final recommendation of the study, that not only is it necessary to choose a suitable method for analysis of retention data, but a carefully planned and executed experimental procedure is also required. Such an experimental procedure should involve comparison of the reproducibility of the data with a set of standards at all stages of the procedure. The study showed that such a procedure should allow an inter-laboratory repeatibility of 1 unit in Kovats retention indices for high homologues and 2 units for the lower homologues.

5. Conclusions and Recommendations.

The foregoing review has revealed that there have been many methods adopted for the estimation of the void volume, V_M, in liquid chromatography. Futhermore, many of the methods have been quite specific for the particular application. For example, expensive

detection methods, impractical for conventional quantitative analyses, have been adopted when the aim of the research is to extract fundamental thermodynamic data.

In the case of practical liquid chromatography, where the aim is to enable published retention behaviour to be interpreted for general application, the four main methods used have been injection of inorganic salts, injection of modified mobile phase, injection of suitably unretained organic compounds and mathematical determination of V_M by linearisation of the retention data for homologous series of organic compounds. Of these the inorganic salt method is most open to question. The other three methods may be applied, however, the choice of method depends largely on the system being studied.

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It is therefore recommended that care be taken when choosing a method to estimate V_M. In many cases there will be a choice between the three most applicable methods, However, the detector employed may reduce the number to two. In the case of a UV detector, for example, Uracil (unretained) or an homologous series of aromatic compounds may be used whereas for an RI detector, a series of normal alcohols might be appropriate. In some cases where convenience and the requirement for greater speed pre-empt the need for accuracy, the use of modified mobile phase may be acceptable.

It is obvious that at this time there are no hard and fast rules which can be adopted. However, the mathematical treatment of the retention data for homologous series does appear to offer considerable promise. It is suggested that more research be conducted in this area for a wide range of mobile and stationary phases and packing type. In this way the method may become commonly adopted as it has for the calculation of dead time in gas chromatography.

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