ELSEVIER

Journal of Chromatography A, 965 (2002) 219–232

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

The measurement and meaning of void volumes in reversed-phase liquid chromatography

Catherine A. Rimmer, Carolyn R. Simmons, John G. Dorsey*

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306-4390, USA

Received 14 June 2001; received in revised form 7 May 2002; accepted 17 May 2002

Abstract

The seemingly simple process of measuring the mobile phase volume, V_0, in reversed-phase liquid chromatography has eluded unambiguous agreement for over 25 years. Examples exist in the literature where the reported volume is physically impossible, either equal to or larger than the empty column volume, or being so small that it would represent a total porosity of half the theoretical limit for well-packed columns. Here we review the many proposals for methods of measurement, and compare and critique them. At this time, there is still no consensus for the best method of measurement, and workers are urged to critically examine values they measure, to insure they are at least physically possible.

Keywords: Void volume; Reviews

Contents

1. The definition

''Void volume (V_M): The total volume of mobile

*Corresponding author. Tel.: +1-850-644-4496; fax: +1-850-645-5644.

E-mail address: dorsey@chem.fsu.edu (J.G. Dorsey).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00730-6

phase in the column; the remainder of the column is taken up by packing material. This volume can be determined by injecting an unretained substance'' [1].

2. The need

An accurate determination of the void volume is important for all types of liquid chromatography (LC), but has proven to be especially controversial in reversed-phase LC. It is essential for an accurate determination of the retention factor (k'), which is used for system suitability issues, for theoretical descriptions and prediction of retention of both small and large molecules, for estimation of other partitioning processes, such as octanol–water partition coefficients, for determination of the thermodynamic quantities responsible for chromatographic retention, ΔG, ΔH, ΔS and ΔC_p and for many other issues.

The retention factor is used in fundamental studies for the determination of selectivity (α), which is simply a measure of the ratio of retention factors from a pair of peaks (k'_2/k'_1). It is a thermodynamic value that allows the comparison of a variety of types of columns, base silicas, and bonding densities allowing separations scientists to determine what is happening within the column during the separation. The retention factor is also used for the determination of the distribution coefficient (K), which is used to probe thermodynamic factors in separations. Although these fundamental values are interesting and important to separations scientists, they also have practical applications. In the pharmaceutical industry k' is often used as one of the indicators of system suitability as the retention factor changes as a column ages and loses bonded phase. With a thorough understanding of the chromatographic process it may be possible to model separations and determine the best method for the optimization of separations. It may also lead to a better system for selecting columns, saving a great deal of time and money during the method development process.

LC can also be used as a tool for modeling the biopartioning of solutes. Thus it is proposed as a method of importance for biochemical measurements such as amino acid hydrophobicity determinations and as a method for determining patterns of protein folding.

While it is difficult to deny the importance of accurately determining the void volume, the actual determination in LC has been long debated in the literature. In gas chromatography (GC), the amount of time it takes an unretained solute to elute is usually easily determined by injecting a small amount of air. In some cases detection of the air slug is an issue and a homologous series is used instead. However, in LC the problem is significantly more difficult.

3. The ambiguity

Much of the controversy over determination of the void volume in reversed-phase LC is due to the fact that there is no distinct boundary between the mobile and stationary phases. In liquid chromatography the bonded phase is solvated by the mobile phase and the volume phase ratio depends on the assignment of adsorbed solvent to one phase or another. The stronger components of the mobile phase are preferentially associated with the stationary phase, and it becomes difficult to determine which portion of the organic solvent is considered mobile and which portion is stationary.

Added to the solvation of the bonded phase, the base silica support for the bonded phase is usually porous in nature, which adds to the total surface area of the silica. When the stationary phase is collapsed the pores tend to be more accessible to the solutes. However, as the bonded phase becomes solvated the chains may block access to the pores, thus changing the volume of mobile phase that is actually available to the solutes. Therefore, the pores cause an additional amount of uncertainty in the measurement of the void time. Small solutes and portions of some larger solutes can access the pores, while other solutes may not be able to enter the pores. This indicates that different solutes actually experience different void volumes, making even the definition of the void volume more complicated. Knox and Kaliszan [2] advocated the use of two definitions for dead volume, one for the kinetic dead volume and another for the thermodynamic dead volume. The former included the volume of the interparticle void, and the latter included the additional volume of eluent component contained within the porous stationary phase. Alhedai et al. suggested the kinetic dead

volume was common to all solutes, and the thermodynamic dead volume was unique to each solute [3]. Yun et al. [4] recently explored the theoretical relationships that existed between what they considered to be the components of the dead volume, in particular the column void volume, the mobile phase volume, and the volume of the adsorbed phase. They also suggested a means by which to experimentally measure these quantities.

One important assumption that most of the methods for the determination of the void volume make is that there is only one separation mechanism that occurs. In fact, in reversed-phase LC, partitioning of the solutes into the stationary phase is usually the dominant separation mechanism. However, it is possible to have secondary mechanisms. Solutes may hydrogen bond with unreacted silanols on the silica surface, or adsorb onto the surface of either the silica or the bonded phase. Finally, there may also be size exclusion mechanisms that occur based on the pore diameter.

4. The measurement

Many methods have been proposed for the determination of void volume in LC. While the results of the different methods are significantly different it is not necessarily true that any of them are wrong. It simply means that the void volume is dependent on the definition of the arbitrary boundary between the mobile and stationary phases. It is important to determine the method that is most appropriate for a particular separation or measurement problem.

We discuss several of the different techniques that are used for the determination of the void volume. In particular we examine the use of pyconometry, small neutral ''unretained'' molecules, organic and inorganic salts, minor disturbances, and homologous series for the determination of the void volume in reversed-phase liquid chromatography. These are summarized in Table 1.

4.1. Pyconometry

Techniques employed to find the void volume of a column can be simplified into two categories: static and dynamic methods. The most popular of the static methods used is pyconometry, or the weight difference method. In this technique, the mass of the column is measured when it is sequentially filled with solvents of differing densities. The void volume by weight difference is most often calculated according to the method used by McCormick and Karger [5]

$$V_0 = (W_x - W_y)/(\rho_x - \rho_y)$$

where W is the weight of the column filled with either solvent x or y, and ρ is the density of the solvent. It is generally agreed upon that V_0 determined in this way gives the maximum possible void volume, and is used by many researchers as a reference point. According to McCormick and Karger, this volume also represents the total space within the column accessible to molecules comparable in size to the solvent molecules [5]. They specifically used this V_0 measurement to determine the distribution of organic modifier into the stationary phase.

This method of void volume determination gives the best results when there is a large difference in the densities of the two solvents. The most commonly used solvents for these purposes include methanol ($\rho = 0.7866$), carbon tetrachloride ($\rho = 1.589$), acetonitrile ($\rho = 0.7138$), and chloroform ($\rho = 1.484$). Melander et al. [6] used water, methanol, acetonitrile, carbon tetrachloride, and *n*-hexane to gravimetrically determine the void volume. They found the results from this method to be indistinguishable from the results of other void volume procedures. Djerki and Laub [7] calculated the void volume by the same procedure using methanol and water as the solvents.

In addition to using acetonitrile and carbon tetrachloride to find the column porosity as described above, Krstulovic et al. [8] also found the void volume with the use of only one solvent. By measuring the mass of the column filled with a particular solvent and subtracting the mass of the dry column, they were able to determine the void volume. For this procedure, the column was dried by purging with pentane and drying under helium. A similar procedure was followed by Slaats et al. [9], who used nitrogen gas to dry the column and carbon tetrachloride as the solvent of choice. The value they obtained for the maximum void volume was then corrected for the amount of mobile phase which had

Table 1
Methods for the determination of the void volume in reversed-phase liquid chromatography

Method	Summary	Disadvantages	Advantages
Pyconometry	Volume is calculated from the weight difference of the column which is sequentially filled with solvents of different density.	Difficult to test frequently. Does not account for differences in volume due to bonded phase solvation.	Provides the maximum possible void volume.
Unretained neutral marker	An ''unretained'' solute is injected either individually or as part of a sample mixture. The elution time of the solute is taken as the void time.	Many ''unretained'' markers have slight retention. Therefore, this method should not be used if an accurate measure of retention is necessary.	Fast, simple, and non-destructive. For many applications this method provides an adequate measure of void time.
Organic and inorganic salts	A salt solution is injected. The resulting peak determines the void time.	Buffered mobile phases must be used to prevent Donnan exclusion. The small molecules may see a larger volume than typical solutes.	Fast and non-destructive, may be used with every injection.
Minor disturbance	A mobile phase component, or deuterated mobile phase, is injected. The void time is calculated from the baseline disturbance.	Organic solvent may associate with bonded phase, while water may disrupt the equilibrium.	May be performed with every injection. In most cases it provides an adequate estimate of void time, especially through the use of deuterated mobile phase components.
Homologous series	A plot of retention versus homolog number is extrapolated to the unretained zeroth homolog.	Many variables, including homolog type, the number of homologs, the length of the homologs, and the regression method used for analysis. Labor intensive.	Many variables, may allow for an accurate determination of void volume.

adsorbed onto the silica surface, found by using breakthrough curves. Vespalec and Simek [10] also used the one-solvent method, flushing the column first with water and then drying with a nitrogen stream.

Using the two-solvent method, Alhedai et al. [3] compared the void volumes obtained by three different pairs of solvents and found they were nearly identical. The solvent pairs used were methanol–carbon tetrachloride, acetonitrile–chloroform, and methanol–chloroform. *N*-hexane, methylene chloride, and carbon tetrachloride were solvents used by Möckel [11] to determine the column void volume on a silica column with a pentane eluent. The results agreed with the void volume also determined by the extrapolation of data obtained from *n*-alkane homologs. Möckel also obtained similar values for the void volume of an ODS column using pyconometry, with methanol and methylene chloride as solvents, and the retention volume of deuterated methanol in pure methanol eluent [11].

Rustamov et al. [12] compared the results of three different methods of V_0 calculation: pyconometry using acetonitrile–tetrahydrofuran and acetonitrile–methylene chloride solvents; minor disturbance using acetonitrile, methanol, and tetrahydrofuran; and the retention volume of deuterated acetonitrile. They reported less than 2% RSD for all methods.

The opinions concerning the determination of void volume by the weight difference method are not all positive. Engelhardt et al. [13] concluded that the results are inaccurate due to the small fraction of the column mass actually made up of the solvents. They also found the one-solvent method to be impractical because once the column has been dried, it will only rarely be useful again.

A problem faced by many researchers with regard to the weight difference approach to void volume is the potential solvation of the stationary phase by one or more of the mobile phase components. For this reason, van der Houwen et al. [14] considered the weight difference method useless. According to Melander et al. [6], this method can only successfully determine the void volume when the mobile phase contains a single component. However, as they note, the mobile phase in liquid chromatography is typically a multi-component system, and is rarely composed of a single solvent. Berendsen et al. [15] agreed, claiming the value they calculated using methanol and carbon tetrachloride was merely an upper limit for the total column porosity. Ignoring the possible solvation effects will result in an overestimation of the true void volume. Therefore, they conclude the only accurate method of V_0 determination is by using dynamic chromatographic methods.

4.2. Unretained neutral markers

Many researchers have looked for, and suggested, the ideal ''unretained'' solute for use as a void volume marker. This is probably the most suspect of the methods for the determination of the void volume, in fact Berendsen et al. said that the use of small organic molecules ''is clearly dangerous'' [15]. It has been argued that there will be no truly unretained solutes unless the polarity of the mobile phase is completely different than the polarity of the stationary phase [16]. If the ideal unretained neutral solute exists it must be small enough to access all of the available mobile phase volume, and hydrophilic enough to stay out of the stationary phase. Many small organic molecules have been suggested for this task. However, it has been shown that many of the small molecules yield slightly different void volumes [17]. It has also been shown these differences are accentuated when the molecules are polymeric or ionizable.

In spite of the evidence against the use of small organic molecules, some scientists have suggested that they may provide a reasonable estimate of the void volume. Fini et al. recommended the use of thiourea, as it yielded a void volume comparable to the total column volume [18]. Didaoui et al. [19] also used thiourea as a void marker and compared the results to $NaNO_3$ and a homologous series. The V_0 values from the three methods differed depending on the mobile phase used.

Phloroglucinol has also been cited as a good marker for the void time [20]. A study by Vít et al. [21] compared V_0 from phloroglucinol to the void time calculated using the hydroxybenzene homologous series, and found almost identical results from the two methods. Englehardt et al. used acetone, *N,N*-dimethylformamide, urea, thiourea, and phloroglucinol for the measurement of the void volume in normal-phase chromatography [13].

Acetone is another commonly used solute for the determination of void volume. It is favored as it takes a very small amount of acetone in solution to see a significant peak at a wide range of detection wavelengths. Since the concentration can be so small the acetone should not significantly disrupt the equilibria that is established between the mobile and stationary phases. However, experimental evidence shows that acetone is somewhat retained under all but the strongest mobile phase conditions. A real contradiction occurs when acetone is used as the first member of the 2-ketoalkane homologous series [22]. Thus, by the definition of acetone as the first member of a homologous series it is retained.

The use of uracil as a void marker is also quite common [23]. A study by Bidlingmeyer et al. [24] compared several void markers, including uracil, acetone, and D_2O. The results showed uracil to be less retained than acetone, and thus a better void marker. The behavior of D_2O was quite similar to uracil, but because a refractive index detector is required, D_2O was not recommended for practical use. Nowotnik and Narra [25] also compared several void markers, including uracil, urea, formamide, acetone, and $NaNO_3$ in a buffered mobile phase. Uracil, urea, and formamide showed partial retention, but less than that of acetone, which was

declared unsuitable as a void marker. $NaNO_3$ was concluded to be the best marker in the study.

In tests, most of the small organic molecules were shown to have elution times that increased linearly with decreasing mobile phase strength [14,26,27]. This indicates that retention is occurring, thus these molecules yield a volume that is greater than the ''true'' void volume. Some of the small molecules were also tested using the same mobile phase composition at different temperatures [11,13]. It was found that the measured void volume increased with decreasing temperature. This phenomenon may be due to the increased viscosity of the mobile phase, the decreased diffusion of the test solute, or more likely to changes in retention. These factors all indicate that small organic molecules are not the most appropriate choice for the determination of the void volume, unless only a rough estimate is needed.

4.3. Inorganic and organic salts

Void volume determination by unretained inorganic salts has been a topic of much exploration and dispute. Inorganic salts have been chosen as dead time markers because of their practical simplicity, UV detectability, and their range of size. However, one of the greatest limitations to the use of these salts is the possibility of the exclusion of charged species from the pores of the stationary phase due to the presence of residual silanol groups. This ion exclusion effect is known as Donnan exclusion, and has put restrictions on the use of the salts. Many researchers have suggested using a buffer solution or a large concentration of salt to mask the charges on the silanols. This allows the marker ions to penetrate the pores of the stationary phase, rather than be excluded from them.

The occurrence of the Donnan effect was well illustrated by the work of Engelhardt et al. [13], who worked with $NaNO_3$ and I^-. This work showed that increasing the salt concentration caused a subsequent increase in the elution time of the salt, thereby resulting in a larger observed dead volume. They suggested adding neutral salts to the eluent or using a larger sample of the salt to lessen these exclusion effects. The authors' work also found that the retention of the salts was dependent on not only the sample size, but also the pH of the eluent and the concentration of the organic modifier.

Similar results were obtained by Vít et al. [28], Smith and Burr [29], and Daignault et al. [30]. Daignault suggested using a very small amount of the salt, approximately 10^{-9} moles, to obtain a limiting value for the void volume of the column.

In contrast, work by Djerki and Laub [7] with $NaNO_3$ produced retention times that all agreed within their definition of experimental error. In their opinion, the data showed the size of the salt sample did not greatly influence the retention time.

Alhedai et al. [3] found it was possible to use this ion exclusion effect to determine the interstitial volume of the column. Using sodium nitrate, sodium sulphite, sodium thiosulphate, potassium dichromate, and sodium nitroprusside, the authors attempted to detect any exclusion properties of the column. It was determined that the largest ion, sodium nitroprusside, had the smallest retention time, and that the smallest ion, $NaNO_3$, had the largest. They concluded that the charge on the ion had little or no effect on the retention time, and that $NaNO_3$ was the best choice of salt to use for the determination of the interstitial volume of a reversed-phase column.

Wells and Clark [31] also used $NaNO_3$ to determine the interstitial volume of a reversed-phase column. They then used $NaNO_3$ along with a phosphate buffer in order to prevent ion exclusion from the pores, and found what they determined to be the true void volume. However, due to the less than 3% difference in the capacity factors resulting from each method, their work concluded that it was possible to use either procedure.

In work by Jinno et al. [26], the validity of $NaNO_3$ and $NaNO_2$ as void volume markers was determined on three different reversed-phase columns; these included columns with stationary phases composed of a styrene–divinylbenzene copolymer, a C_{18} silica phase, and a C_8 silica phase. The results showed that both salts had consistent elution times over a range of mobile phase compositions. They did observe a slight increase in elution time as the salt concentration increased. However, they concluded this increase was negligible, and recommended both salts for void time determination. A later paper, also by Jinno [32], reassessed the effectiveness of $NaNO_2$ as a void time marker. The results of the study again

showed a slight increase in elution time as the amount of injected $NaNO_2$ increased with a methanol–water (1:1) mobile phase. A significantly higher increase was observed with a pure methanol eluent. In addition, the study showed the salt did not interact with the C_{18} stationary phase. Jinno again recommended the use of $NaNO_2$ as the void marker, but suggested using a higher concentration of the salt when using methanol as the eluent.

Berendsen et al. [15] found that inorganic salts at low concentrations, $<10^{-3}$ *M*, were subject to the Donnan effect and yield the exclusion volume of the column. Therefore, they recommended using monovalent salt solutions at extremely low ionic strength to find the intraparticle volume. At higher concentrations, $\sim 10^{-1}$ *M*, the salts had full access to the pores of the stationary phase and gave the maximum hold-up time of the column. Therefore, they suggested using highly concentrated salt solutions for the estimation of the hold-up time with methanol–water mobile phases. The salts used in this study included KI, KBr, NH_4NO_3, $NaNO_3$, $FeCl_3$, and $K_2Cr_2O_7$.

Melander et al. [6] continued the work with nitrate ions as the void markers, and found they worked well for methanol–water systems with between 25 and 75% methanol and for acetonitrile–water systems with 25–95% acetonitrile. An additional provision for the use of nitrate was a high ionic strength of the eluent to prevent ion exclusion.

Krstulovic et al. [8] also investigated $NaNO_3$ as the void time marker, and found results similar to the work of others. Without a background electrolyte, the void time was significantly lower than the total porosity of the column found by the weight difference method. However when a NaBr solution was added to the eluent, the salt was no longer excluded and the void time increased. They found positive results when using $NaNO_3$ with salt solutions and a low organic modifier concentration.

Through their work with $NaNO_2$ and $NaNO_3$, Hennion and Rosset [33] found that the elution volume of the salts not only increased for mobile phases that did not contain a buffer, but also for those that did. They concluded the ionic strength of their system was not high enough. The work also showed that concentrated nitrate was a very good void marker for acetonitrile–water mobile phases with 15–90% acetonitrile, and for methanol–water systems with 40–90% methanol. The researchers did suggest that when using concentrated NO_3^- as the void marker, one should compare the results to the void time from the retention of mobile phase components to monitor for exclusion effects.

Wells and Clark [27] found the elution times of $NaNO_3$ and $K_2Cr_2O_7$ to be nearly independent of the solvent composition for both buffered and unbuffered methanol–water systems. However, in unbuffered systems, the salts showed a concentration dependence, which dissipated upon addition of a phosphate buffer. They concluded that any detectable amount of $NaNO_3$ gave a reliable estimate of void volume in buffered eluents, and that approximately 3×10^{-6} mol $NaNO_3$ are required for unbuffered systems.

$LiNO_3$ as a void time marker was the subject of a study by van der Houwen et al. [14]. The elution time of the salt was compared with results from a homologous series test using *n*-alcohols. It was found that the void times for the two methods agreed for all methanol–water compositions above 20% methanol. Charge exclusion effects were studied, and it was determined that the effects were only noticeable with small amounts of injected salt. The addition of other salts or buffer diminished the effects entirely. The authors believed the buffer did not simply mask the charge of the silanol groups as suggested by others, but rather the negligible exclusion effects were due to the reduction of dissociated silanol groups.

A recent article by Oumada et al. [34] studied the behavior of several salts in reversed-phase systems, including $NaNO_3$, $NaNO_2$, KBr, Bu_4NBr, and $K_2Cr_2O_7$. The results of the study showed the elution times of the salts increased with concentration until a maximum was reached at approximately 3×10^{-3} *M*. However, when a buffer was included in the eluent, the retention times were independent of the salt concentration. The authors found that with buffered mobile phases, the void time from the salt is not dependent on the particular salt used, but does vary with the buffer used and the concentration of methanol.

Rather than using the inorganic salts as unretained markers, Shibukawa and Ohta [16] suggested a somewhat different method for using the salts to find

the void volume. Their idea was based on the fact that the ratio of the capacity factors of two analyte ions with the same charge remains constant when the eluent electrolytes are varied. From this relationship, the void volume can then be found using the following equation:

$$V_m = (V_A^{yx} V_B^{wz} - V_A^{wz} V_B^{yx})/(V_A^{yx} + V_B^{wz} - V_A^{wz} - V_b^{yx})$$

with *A* and *B* representing analyte ions, and *yx* and *wz* eluent electrolytes. Because elution times of ionic salts depend on the concentration of the eluent and the amount of injected salt, the authors believed this to be the best of the possible methods to determine the volume of the mobile phase [16].

Over the years, inorganic salts as void time markers have been used in numerous studies [35–46]. Many authors consider salts to be the best choice as a void marker because of the simplicity of use and ease of detection.

Organic salts suffer from the same problems as the inorganic salts. While they are often more easily detected using UV–Vis absorbance detectors than the inorganic salts, they are still charged and can suffer from Donnan exclusion in mobile phases of low ionic strength. This can be addressed by increasing the concentration of the solute or by controlling the ionic strength of the solution, as with inorganic salts.

4.4. Minor disturbance method

The minor disturbance method is one of the most popular methods for the determination of the void volume in a chromatographic system. There are several different ways to create a disturbance that should allow for the calculation of the void volume. Individual mobile phase components can be used, as can isotopically labeled solvents, and the careful observance of system peaks.

A common way to perform the minor disturbance method is to measure the retention of the mobile phase component over the entire range of mobile phase composition. A plot of retention volume versus mobile phase composition will show a dramatic change in V_R. The integral average of the minor disturbance peaks gives the value of V_0. This method was used in several studies by Kazakevich and McNair [47–49]. Their work showed that V_0 calculated in this way was independent of which mobile phase was used. In particular, methanol–water and acetonitrile–water were both used, and gave nearly identical results. Similar results and conclusions were found in a recent study by Rustamov et al. [12], in which acetonitrile, methanol, and tetrahydrofuran minor disturbance data were used.

Kazakevich and McNair compared the void volume from acetonitrile minor disturbance peaks to the void volume calculated from an *n*-alkane homologous series and found the results were nearly the same, with less than 0.2% RSD [49]. In the work of Rustamov et al., V_0 from minor disturbance was compared to that from deuterated acetonitrile and pyconometry, and found to differ by less than 2% RSD [12].

Simply injecting one of the pure eluents of a binary mobile phase mixture will produce a disturbance peak; however, the selection of the eluent component to be used is important. Melander et al. [6] proposed the use of the most weakly bound mobile phase component as it should not be a part of the solvation layer. In RPLC systems, water should provide the best measure of the void volume. However, when a slug of pure water is injected, multiple peaks may be seen [5,13,50]. One peak is due to the elution of the pure water, and a second is a water vacancy peak. The second peak occurs because the pure water disturbs the equilibria between the mobile and stationary phases, so it is due to a slight excess of organic solvent in the mobile phase immediately following the water.

While the appearance of multiple peaks may make the determination of the void volume difficult, the vacancy peak may be removed by carefully matching the solvent composition of the sample with the mobile phase [51]. Unfortunately, the detection of a disturbance peak can be very difficult if the injected solvent has the exact same composition as the mobile phase. For this reason, isotopically labeled mobile phase components have been used for the creation of the minor disturbance. These solvents may be radioactive, or they may be deuterated. When a small amount of radioactive solvent is injected in the same proportions of the mobile phase, it is detected at the column outlet by collecting fractions and using a scintillation counter [22]. While the measurements

may be very sensitive, this technique is impractical as it is time consuming and discontinuous in volume measurement. There are additional problems with the solvent disposal as well as the possible contamination of the column and injector.

Other researchers have advocated the use of deuterated solvents [2,5,8,12,13,22,26,50,52]. The deuterated solvents have a different refractive index than the pure solvents, making it possible to detect the minor disturbance with either a refractive index detector, or a UV–Vis detector set to a low wavelength. As with the regular mobile phase components, it is expected that the deuterated strong solvent will be associated with the stationary phase, while the deuterated water should not be retained. In spite of this, Slaats et al. [51] found good agreement when deuterated water and deuterated acetonitrile were used for the void volume determination. Surprisingly, they found that deuterated methanol did not correlate with the other results. It was hypothesized that the lack of agreement may be due to the exchange of the deuterium with the hydrogens on the unreacted silanols [33,51]. This idea has been supported by other groups using deuterated methanol. If the deuterium is exchanging with hydrogen in the solvent and from the silica the void volume will appear to be larger. Djerki and Laub found that deuterated water provides a good measure of the mobile phase volume when ternary mobile phases are used. In fact, they found that the D_2O gave results which were similar to those they calculated using the linear homologous series method [52]. Using the method proposed by Knox and Kaliszan, in which the retention volumes of isotopically labelled samples of all mobile phase components were used to calculate V_0 [2], Li et al. observed a non-systematically changing value for the dead volume as the percentages of mobile phase components were changed [53].

In spite of these real problems, the most frequently cited disadvantage with this method is that many people feel it is necessary to use a refractive index detector in order to measure the solvent disturbance peak. In many mobile phases this is not the case as UV–Vis detectors will detect changes in refractive index at lower wavelengths. Billet et al. [50] used a microwave-induced plasma (MIP) detector in order to detect D_2O. They found that the MIP provided reproducible measurements of the D_2O in methanol–water mobile phases, as well as for ternary methanol, water, and acetonitrile mobile phases. However, they found that it did not work as well when tetrahydrofuran (THF) was used as the organic solvent or when pure organic solvent was used. They suggested that this was due to the adsorption of the D_2O on the silanols. While the use of the MIP detection scheme was important for showing the validity of using deuterated solvents for the determination of void volume, it is impractical for the average LC laboratory [50].

Gutnikov and Hung suggested the use of oxalodihydroxamic acid as a substitute for D_2O as it has a reasonable UV response that is easier to detect than the solvent disturbance. First, they injected a homologous series of oxalodihydroxamic acids in order to determine the void volume [54]. The solutes were kept in an acidic solution to prevent dissociation from occurring, thus minimizing hydrogen bonding with the unreacted silanols. After evaluating the data from the homologous series they found that oxalodihydroxamic acid provided an accurate estimate of the void volume. In addition it was suggested that hydroxyurea may be a suitable substitute.

Finally, it is possible to determine the void volume by tracing the system peaks which occur when the equilibria is disturbed. Levin and Grushka published two papers in which they discuss the origin of system peaks and propose a method for the determination of the void volume from these peaks [55,56]. Through the measurement of system peaks relative to the concentration of the injected solute and the concentration of the buffer they calculated the amount of solvent in the stationary and mobile phases. The ratio of the injection concentration–buffer concentration that makes the system peaks disappear allowed for the calculation of the k'. From the retention factor they back calculated for V_0. They found the same V_0 using several different system peaks, indicating that they obtained reasonable results [55,56].

4.5. Homologous series

If a homologous series of solutes is injected on a GC column at constant temperature, the plot of the log of the retention time versus homolog number

shows a linear relationship as known from the Martin equation. When the plot is extrapolated back to the zeroth homolog, the *y* intercept gives an accurate measure of the void volume. As this technique has proven to be simple and effective in GC, it has also been attempted in LC.

Several groups have shown that when the homologous series method is used in LC the homologs provide a very straight line as evidenced by an R^2 value close to 1.000. However, the homolog number and number of homologs used for the calculation were shown to be important for the accurate determination of void volume. Some researchers have found that there are deviations from linearity when smaller homologs are used. Most hypothesize that the smaller homologs have greater access to pore volumes compared to their larger counterparts, thus adding a size exclusion mechanism to the retention volume. Others have proposed that the error introduced by hydrogen bonding of the solutes with unreacted silanols is greater when small homologs are used, thus introducing nonlinearity. Berdensen et al. saw that the data points were randomly scattered around the line at the lower homolog numbers. Thus, they concluded that the smaller homologs may provide less precise retention volumes, however, they are not due to additional separation mechanisms [15]. With a series of *n*-alcohols they showed that the lower homologs yield virtually the same void volume as the higher homologs.

Berdensen did see a discontinuity when using homologs greater than 10 units. Others have confirmed this non-linearity at a critical carbon number [13,57–59]. The discontinuity occurs when the length of the homologs exceeds the length of the alkyl chains of a monomeric bonded phase.

The result is a series of suggestions for the selection of homologs. First, the choice of the type of homologous series is based primarily on the solubility in the mobile phase system, detectability of the solutes in the chromatographic system, and availability of the homologs. A minimum of three homologs is necessary in order to extrapolate the line to V_0. However, four are recommended as three may give an unrealistically high correlation coefficient [59]. Additionally, due to possible size exclusion mechanisms, it has been advised that analysis begin with the third homolog, and not exceed the total length of the bonded phase. Finally, the retention data must be very precise in order to avoid miscalculation of the void volume.

Other groups have proposed that it is incorrect to avoid the non-linearity by not using the early homologs. In order to find what they believe to be more accurate void volumes they have proposed more complex calculations for determination of the volume at the *y* intercept. Krstulovic et al., Grobler and Balizs, and van Tulder et al. have shown that it is important to first maximize the correlation coefficient. They have done this by performing iterative linear regressions [8,60,61]. The method of Grobler and Balizs is one of the most commonly used. However, the calculation requires the use of consecutive homologs [60]. Van Tulder et al. extended the Grobler and Balizs method so that it could be used for nonconsecutive homologs [61]. They used non-linear regression analysis to determine the void volume of the column. Didaoui and co-workers evaluated a multiparametric non-linear least-squares regression method [19,62], which they found to yield similar results to those found by Grobler and Balizs with the two successive linear regressions, and by Guardino et al. [63], who used least-squares fitting methods. Wätzig and Ebel [64] developed a non-linear regression algorithm, which they claimed to be substantially faster than the method of Guardino [63]. For this algorithm, they suggested using $n=3$ as the first member of the homologous series.

Bidlingmeyer et al. [24] successfully used the method proposed by Laub and Madden [65] to calculate the void volume for a series of eight alkyl benzenes. This method consists of plotting the log of corrected retention volume against candidate void volumes. The plot with the highest correlation coefficient determines the value of V_0. Bidlingmeyer also used experimental data from the work of Laub and Madden to compare the calculated V_0 obtained in the original work to V_0 obtained using the methods of Berendsen et al. [15] and Al-Thamir et al. [66]. Briefly, the method proposed by Berendsen relies on the fact that the ratio of the capacity factors of two consecutive homologs, or the selectivity, is constant. Plotting the retention time of one homolog versus that of its predecessor allows the calculation of V_0. The method of Al-Thamir is an iterative approach that uses the retention volumes of three adjacent

homologs to plot the difference in the first pair of homolog retention volumes versus the difference in the second pair to obtain a value for V_0. Bidlingmeyer's results showed great discontinuity among the three methods from data using a THF–H_2O mobile phase, and concluded methods such as these should only be used with ''a significant amount of caution'' [24].

Another comparative study using the methods of Berendsen et al. [15], Laub and Madden [65], and Al-Thamir et al. [66] was conducted by Nowotnik and Narra [25] using data from two homologous series, *n*-alkanols and alkylbenzenes. Nowotnik and Narra found all three methods gave reasonably consistent data for the *n*-alkanol series, excluding methanol and ethanol data.

As mentioned above, it has been proposed that pore exclusion results in larger void volumes for smaller solutes. This idea was supported by the works of Möckel and Dreyer [67–69]. Using Berendsen's method [15], which they considered to be the method that yielded the highest data precision, Möckel and Dreyer consistently observed a decrease in void volume when larger members of the *n*-alkane homologous series were used. However, when considering the observed linear dependence of V_R on molecular mass, Kazakevich and McNair [49] disagreed with the idea that the decrease in V_0 was due to size exclusion. They claimed that in true size exclusion cases, there is a linear dependence of V_R on the log of molecular mass.

Using series of symmetrically substituted and nonsymmetrically substituted hydroxybenzenes, Vít et al. [21] calculated quite unreasonable values for the void volume. They concluded using the homologous series method was questionable and described the experimental procedure as ''tedious''.

Theoretically, the homologous series method for the determination of the void volume is ideal. However, the void volumes calculated according to this method should be comparable to one another no matter which homologous series is used; no matter how many homologs are used; and no matter which homolog numbers are used. The fact that this is not the case indicates that the determination of the void volume is much more difficult in LC than it is in GC.

One interesting note is that several researchers found that there was a decrease in mobile phase volume with increasing organic content. This was attributed to the fact that the bonded phase extends as it becomes solvated, thus it blocks the pores. In the case of the lower organic content the bonded phase collapses, allowing the probe molecules to enter the pores. Additionally, Möckel found a slight temperature dependence that was reduced, but not eliminated by the correction for the thermal expansion of the mobile phase [11]. This phenomenon was seen when a series of *n*-alkanes was used for the calculation of the void volume, but not when the minor disturbance method was used.

While this method may be appropriate for large studies and for studying fundamental chromatographic parameters, it is not necessarily appropriate for use with column comparisons. A series of homologs must be injected and eluted using isocratic conditions, and replicate injections must be made in order to insure that the chromatographic system has equilibrated. All of the numbers must then be entered into a computer program in order to perform either the linear or non-linear regression that is used to determine the void volume. While it is assumed that the void volume should not change significantly throughout the lifetime of a column, it is important to test the void volume at regular intervals in order to make sure that the volume phase ratio has not changed due to the loss of bonded phase, the loss of base silica, changes in the solvation of the bonded phase, or changes in temperature. Thus for routine chemical analysis this method is not the most appropriate due to the time that it takes to perform the analysis.

4.6. Other methods

Occasionally a proposed method surfaces that uses non-traditional means to determine the column void volume in reversed-phase LC. Often these methods are not practical or suitable for routine use, but are noteworthy nonetheless. One example is the development of a direct imaging analysis system that uses a charge-coupled device video camera to monitor the progress of solute bands in stepwise elution mode [70]. Using fluorescence detection, the linear flow velocity is determined, and used in the following equation to calculate the void volume

$$V_0 = (L/u) \cdot F$$

In this equation, L is the length of the column, u is the linear flow velocity, and F is the volume flow-rate. The authors in this study proposed this method to avoid using typical nonretained solutes, which they believed had weak interactions with the mobile phase and/or the stationary phase [70].

Another interesting technique employed ^{19}F NMR as a means to calculate void volume [71]. Stationary phase particles were equilibrated with a solution containing fluorinated probe molecules, one of which was NaF. NaF was used for its small size, and the assumption that it would fill the entire void volume of the column. Using a mixture of probe molecules having slightly different resonance frequencies, they were able to study both inter- and intraparticle voids in the column. The ratios of probe molecule signals were used to quantitate the void volume.

5. Capillary electrochromatography

In recent years capillary electrochromatography (CEC) has gained attention with the promise of high efficiency separations and low solvent consumption. While CEC is often viewed as a novel technique, it is a liquid chromatography experiment that uses an electrical potential to drive the mobile phase. This means that void volume measurement in CEC is subject to some of the same limitations as pressure driven LC, while presenting some unique problems.

One of the primary disadvantages of CEC is the lack of flow reproducibility. As silanols on the capillary wall and the stationary phase particles become fouled, the mobile phase velocity slows. This means that it is necessary to measure the void time with every injection in order to accurately compare chromatograms. Therefore, methods such as pyconometry or methods that require special detection schemes are not practical for use in CEC. Additionally, the linearization of a homologous series would be difficult, as it would require the solutes of interest to be well resolved from the homologs. At best this would be difficult and time consuming, at worst it would be impossible.

We are then left with the injection of single component markers, such as inorganic ions, neutral solutes, and solvent deflection. Although solutes in electrochromatography separate primarily based on chromatographic partitioning, it is possible for charged solutes to undergo some differential electrophoretic migration. Thus, in addition to the problems of Donnan exclusion, results may be inaccurate due to electrophoretic migration.

Neutral markers such as thiourea and acetone are commonly used in CEC, as they are easy to see with UV detectors. Unfortunately, since they show evidence of retention in pressure driven separations, it is likely that they are also slightly retained in electroosmotically driven separations. In 1996, Lelievre et al. showed that the elution time of thiourea was highly dependent on the concentration of organic modifier in the mobile phase [72]. Chankevetadze et al. [73] later confirmed this behavior in a paper on chiral separations. They found that in some cases thiourea was retained longer than any of the fully resolved enantiomers. In this case, the retention mechanism was shown to be size exclusion rather than chromatographic partitioning as the phenomena was not seen when larger pore size silica was used.

Solvent deflection methods may provide adequate indication of void volume in CEC. The disadvantages are the same as those seen in pressure driven LC. The injection of solutes in solvent different from the mobile phase composition may temporarily disrupt the equilibrium, or disturb the shape of the peaks. Deuterated solvents may have slightly different mobilities than their non-deuterated analogs. However, the solvent deflection method probably provides the easiest, least expensive, and most accurate method for determining the mobility with every injection in CEC.

CEC is still a developing technique and there has not yet been an exhaustive comparison of the different methods for determining the void time. If CEC becomes a routinely used technique, it will be necessary to experimentally determine the advantages and disadvantages of different methods for measuring void times.

6. The interpretation

It is clear that there is not yet an unambiguous answer to the question of void volume determination.

An answer to the question of assignment of sorbed solvent to either the stationary phase or mobile phase volume may be an easier issue to resolve, which would lead to a less ambiguous choice of methods for mobile phase volume determination. It is also clear in any semi-thorough review of the literature, that many people do not use enough care in measurement or selection of method for measuring this important quantity. Examples exist in the literature where the reported void volume is a physically impossible number!

Is another thorough comparison of methods for void volume determination warranted? Probably. Combined with data from recent molecular dynamic simulations of stationary phase structure, there is hope for agreement of what should be considered *mobile phase volume*.

References

[1] R.E. Majors, P.W. Carr, LC·GC 19 (2001) 124.
[2] J.H. Knox, R. Kaliszan, J. Chromatogr. 349 (1985) 211.
[3] A. Alhedai, D.E. Martire, R.P.W. Scott, Analyst 114 (1989) 869.
[4] K.S. Yun, C. Zhu, J.F. Parcher, Anal. Chem. 67 (1995) 613.
[5] R.M. McCormick, B.L. Karger, Anal. Chem. 52 (1980) 2249.
[6] W.R. Melander, J.F. Erard, Cs. Horváth, J. Chromatogr. 282 (1983) 211.
[7] R.A. Djerki, R.J. Laub, J. Liq. Chromatogr. 10 (1987) 1749.
[8] A.M. Krstulovic, H. Colin, G. Guiochon, Anal. Chem. 54 (1982) 2438.
[9] E.H. Slaats, J.C. Kraak, W.J.T. Brugman, H. Poppe, J. Chromatogr. 149 (1978) 255.
[10] R. Vespalec, Z. Simek, Chromatographia 32 (1991) 130.
[11] H.J. Möckel, J. Chromatogr. A 675 (1994) 13.
[12] I. Rustamov, T. Farcas, F. Ahmed, F. Chan, R. LoBrutto, H.M. McNair, Y.V. Kazakevich, J. Chromatogr. A 913 (2001) 49.
[13] H. Engelhardt, H. Müller, B. Dryer, Chromatographia 19 (1984) 240.
[14] O.A.G.J. van der Houwen, J.A.A. van der Linden, A.W.M. Indemans, J. Liq. Chromatogr. 5 (1982) 2321.
[15] G.E. Berendsen, P.J. Schoenmakers, L. de Galan, J. Liq. Chromatogr. 3 (1980) 1669.
[16] M. Shibukawa, N. Ohta, Chromatographia 25 (1988) 288.
[17] H. Poppe, J. Chromatogr. A 656 (1993) 19.
[18] O. Fini, F. Brusa, L. Chiesa, J. Chromatogr. 210 (1981) 326.
[19] L. Didaoui, A. Touabet, A.Y.B.H. Ahmed, B.Y. Meklati, W. Engewald, J. High Resolut. Chromatogr. 22 (1999) 559.
[20] M. Popl, Z. Voznakova, V. Tatar, J. Strnadova, J. Chromatogr. 21 (1983) 39.
[21] I. Vít, J. Fähnrich, M. Popl, Collect. Czech. Chem. Commun. 54 (1989) 953.
[22] R.J. Smith, C.S. Nieass, M.S. Wainwright, J. Liq. Chromatogr. 9 (1986) 1387.
[23] B.J. Stanley, C.R. Foster, G. Guiochon, J. Chromatogr. A 761 (1997) 41.
[24] B.A. Bidlingmeyer, F.V. Warren, A. Weston, C. Nugent, J. Chromatogr. Sci. 29 (1991) 275.
[25] D.P. Nowotnik, R.K. Narra, J. Liq. Chromatogr. 16 (1993) 3919.
[26] K. Jinno, N. Ozaki, T. Sato, Chromatographia 17 (1983) 341.
[27] M.J.M. Wells, C.R. Clark, Anal. Chem. 53 (1981) 1341.
[28] I. Vít, M. Popl, J. Fähnrich, J. Chromatogr. 281 (1983) 293.
[29] R.M. Smith, C.M. Burr, J. Chromatogr. 475 (1989) 75.
[30] L.G. Daignault, D.C. Jackman, D.P. Rillema, Chromatographia 27 (1989) 156.
[31] M.J.M. Wells, C.R. Clark, J. Chromatogr. 243 (1982) 263.
[32] K. Jinno, Chromatographia 17 (1983) 367.
[33] M.C. Hennion, R. Rosset, Chromatographia 25 (1988) 43.
[34] F.Z. Oumada, M. Rosés, E. Bosch, Talanta 53 (2000) 667.
[35] C.H. Löchmuller, D.R. Wilder, J. Chromatogr. Sci. 17 (1979) 574.
[36] M.T.W. Hearn, B. Grego, J. Chromatogr. 218 (1981) 497.
[37] Cs. Horváth, W.R. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[38] K. Jinno, J. High Res. Chromatogr. Chromatogr. Commun. 5 (1982) 364.
[39] R.M. Smith, J. Chromatogr. 324 (1985) 243.
[40] K. Jinno, K. Kawasaki, Chromatographia 17 (1983) 337.
[41] P. Jandera, J. Kubát, J. Chromatogr. 500 (1990) 281.
[42] M. Rosés, I. Canals, H. Allemann, K. Siigur, E. Bosch, Anal. Chem. 68 (1996) 4094.
[43] R.P.W. Scott, C.F. Simpson, J. Chromatogr. 197 (1980) 11.
[44] J. de Jong, C.F.M. van Valkenburg, U.R. Tjaden, J. Chromatogr. 322 (1985) 43.
[45] J. Hermansson, J. Chromatogr. 152 (1978) 437.
[46] P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen, L. de Galen, J. Chromatogr. 149 (1978) 519.
[47] Y.V. Kazakevich, H.M. McNair, J. Chromatogr. Sci. 31 (1993) 317.
[48] Y.V. Kazakevich, H.M. McNair, J. Chromatogr. Sci. 33 (1995) 321.
[49] Y.V. Kazakevich, H.M. McNair, J. Chromatogr. A 872 (2000) 49.
[50] H.A.H. Billet, J.P.J. van Dalen, P.J. Schoenmakers, L. de Galen, Anal. Chem. 55 (1983) 847.
[51] E.H. Slaats, W. Markovski, J. Fekte, H. Poppe, J. Chromatogr. 207 (1981) 299.
[52] R.A. Djerki, R.L. Laub, J. Liq. Chromatogr. 11 (1988) 327.
[53] L. Li, P.W. Carr, J.F. Evans, J. Chromatogr. A 868 (2000) 153.
[54] G. Gutnikov, L.B. Hung, Chromatographia 19 (1984) 260.
[55] S. Levin, E. Grushka, Anal. Chem. 61 (1989) 2488.
[56] S. Levin, E. Grushka, Anal. Chem. 58 (1986) 1602.
[57] A. Tchapla, H. Colin, G. Guichon, Anal. Chem. 56 (1984) 621.

[58] H. Colin, A.M. Krstulovic, G. Guichon, Chromatographia 17 (1983) 209.
[59] H.J. Möckel, T. Freyholdt, Chromatographia 17 (1983) 215.
[60] A. Grobler, G. Balizs, J. Chromatogr. Sci. 12 (1974) 57.
[61] J.M. van Tulder, J.P. Franke, R.A. de Zeeuw, J. High Resolut. Chromatogr. Chromatogr. Commun. 10 (1987) 191.
[62] L. Didaoui, A. Touabet, B.Y. Meklati, U. Lewin-Kretzschmar, W. Engewald, J. High Resolut. Chromatogr. 22 (1999) 613.
[63] X. Guardino, J. Albaiges, G. Fipor, R. Rodriquez-Vinals, M. Gassiot, J. Chromatogr. 118 (1976) 13.
[64] H. Wätzig, S. Ebel, Chromatographia 31 (1991) 544.
[65] R.J. Laub, S.J. Madden, J. Liq. Chromatogr. 8 (1985) 173.
[66] W.K. Al-Thamir, J.H. Purnell, C.A. Wellington, R.J. Laub, J. Chromatogr. 173 (1979) 388.
[67] U. Dreyer, H. Melzer, H.J. Möckel, J. Chromatogr. 592 (1992) 13.
[68] H.J. Möckel, U. Dreyer, H. Melzer, Fresenius J. Anal. Chem. 342 (1992) 673.
[69] H.J. Möckel, U. Dreyer, Chromatographia 37 (1993) 179.
[70] A. Tamura, K. Tamura, S. Razee, T. Masujima, Anal. Chem. 68 (1996) 4000.
[71] T.W. Perkins, T.W. Root, E.N. Lightfoot, Anal. Chem. 69 (1997) 3293.
[72] F. Lelievre, C. Yan, R.N. Zare, P. Gareil, J. Chromatogr. A 723 (1996) 145.
[73] B. Chankvetadze, I. Kartozia, Y. Okamoto, G. Blaschke, J. Sep. Sci. 24 (2001) 635.