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THE HOLD-UP VOLUME CONCEPT IN COLUMN CHROMATOGRAPHY

(IUPAC Recommendations 2000)

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Contents

ABSTRACT

Revised recommendations are presented for the nomenclature of the *hold-up volume* in chromatography updating those originally presented in IUPAC document *"Nomenclature for Chromatography (IUPAC recommendations 1993)", Pure and Applied Chemistry*, **65** 819-872 (1993). A number of related and derived definitions for retention parameters are described, including a definition of the term "chromatographic system". The paper also compares methods used in the literature to determine the hold-up volume.

1. INTRODUCTION

The IUPAC document *"Nomenclature for Chromatography (IUPAC recommendations 1993)"* (NC) [1] presents a description of the *hold-up volume* (Described in NC 3.7.03) that requires a more precise definition. This paper looks at a number of related and derived definitions for retention parameters and proposes revised recommendations for their nomenclature including a definition of the term "chromatographic system" which was initially mentioned only as a reference to the apparatus used to obtain the *chromatogram* (NC 1.1.02). The particular problems of these definitions in gas chromatography (and to some extent in supercritical fluid chromatography) caused by the compressibility of the mobile phase are described in the following paper by Davankov. The paper also compares methods used in the literature to determine the hold-up volume.

2. DESCRIPTION OF TERMS

2.1 *Chromatographic system and chromatographic process*

A *chromatographic system* is formed by at least two immiscible phases in contact with each other. Occasionally, more than two phases are present, but this will not introduce any changes in the descriptions given here, so only two phases will be considered. One of them (the mobile phase) is continuously moving in a constant direction relative to the other, which normally remains stationary and is that part of the chromatographic system responsible for the retention of the analytes [2]. *Chromatography* (NC 1.1.01) is the name given to the physical method of separation carried out in a chromatographic system. A *chromatographic process* is the process that takes place when an analyte (or a mixture of analytes) is carried along the stationary phase by the movement of the mobile phase. The components of the chromatographic system are often, but not necessarily, contained in a tube, and the whole (the chromatographic system and the tube) is called the column. The separation technique carried out in such a column is termed *column chromatography* (NC 1.3.01), and will be the technique to which this paper will refer.

A *chromatographic process* is initiated when a sample (a substance or a mixture of substances), mixed with the mobile phase, enters the column. This is the real injection of the sample in the chromatographic system. However, the term "sample injection" is currently applied to the introduction of the sample into the mobile phase through the injection device of the *chromatograph* (NC 1.1.04). "*Initiation time*" will be used instead, to refer to the start of the chromatographic process. Throughout this paper, the sample will be considered to have only one component (hereafter, the analyte). The analyte distributes itself between the two phases, and the movement of the mobile phase carries the analyte along the column. The chromatographic process ceases at the point when the analyte leaves the column. This description corresponds to an example of what is termed *"elution chromatography"* (NC 1.2.03). Due to the various dynamic processes that take place inside the column, the analyte is gradually diluted with the mobile phase as it is being transported along the column. The discussion that follows will assume a linear distribution isotherm between the two phases [3]. While travelling along the column, the analyte is assumed to present a Gaussian distribution of concentration, the maximum of this distribution representing the position of the analyte. Accordingly, an analyte "leaves the column" when the point of maximum concentration of the distribution zone reaches the exit of the column.

2.2 *Retention volume, adjusted retention volume, and hold-up volume.*

The volume of mobile phase that leaves the column, from the moment of the entrance of the analyte into the chromatographic system (the *initiation time*) to the moment in which the analyte leaves, is called the "*retention volume*" of the analyte. This description does not correspond to the definition of "*total retention volume*" given in the IUPAC document [1] (NC 3.7.05). The difference will be shown in part 2.3, below. If the analyte is not retained by the stationary phase, then the volume of mobile phase that leaves the column from the *initiation time* to the moment at which the analyte leaves the chromatographic system (that is: the *retention volume* of an unretained substance) is called the "*hold-up volume*" of the chromatographic system. Again, the description in NC 3.7.03 is different from what is given here. Sometimes, this volume is referred to with the term "*dead volume*" (NC 3.2.13.1); this term, however, should only be applied to the volumes that are not swept by the mobile phase, and that are inaccessible to the analyte. Its use is discouraged [1].

During the chromatographic process, there will be molecules of the retained analyte in both the mobile and the stationary phases. Any one of those molecules will spend part of its time in the stationary phase (dissolved, adsorbed, etc.) not moving along the column (diffusion in the stationary phase will be ignored); and the rest of the time in the mobile phase, moving along the column at the same linear velocity as the mobile phase. The volume of mobile phase used to transport a given molecule of the retained analyte while it is in the mobile phase, is the same as

the volume used to transport the molecules of the mobile phase itself along the column: the *hold-up volume*. The retention volume of an analyte is, thus, composed of two parts: the volume of the mobile phase used to transport it along the column while in the mobile phase, and the volume of the mobile phase that left the column while the molecules of the analyte were stationary in the stationary phase. The latter volume is called the *"adjusted retention volume*" (NC 3.7.07) of the analyte, and is the chromatographic parameter that is related to the thermodynamics of the process that takes place. Retention volumes are the parameters that describe the chromatographic process. The symbols accepted to refer to the three volumes just mentioned are V_R for the *retention volume*, V_M for the *hold-up volume*, and V_R' for the *adjusted retention volume* [1]. In *liquid chromatography*, LC (NC 1.4.03), the term "*void volume*" (V_0) is normally applied to the *hold-up volume*. However, the symbol V_0 has been applied to the "*interparticle volume"* or "*interstitial volume"* of the column (NC 3.2.11), which does not always corresponds to the *hold-up volume* of the chromatographic system (see later), so the usual symbol V_M is the recommended symbol to be used for the hold-up volume.

2.3 *Extra-column volume*

The mixing of the analyte with the mobile phase normally occurs before they reach the column (i. e. in the injection device of the chromatograph). Accordingly, a certain amount of mobile phase leaves the column in the time between sample injection and the moment the analyte enters the chromatographic system (the *initiation time*). In addition, when the mobile phase leaves the column, it is usually carried to a detector using some sort of connection (capillary tubes and end pieces) and a further volume of mobile phase is required before the eluted sample is detected. As a result, the volume of mobile phase that leaves the column between sample injection and detection is the sum of three terms: the *adjusted retention volume* (V_R'), the *hold-up volume* (V_M), and the volumes of the injector, detector and connections, known collectively as the "*extra-column volume*" V_{ext} (NC 3.2.13). Extra-column volumes do not have any chromatographic meaning and should be avoided or minimized wherever possible. In the past, the extra-column volume has been considered as part of both the retention volume and the *hold-up volume* [1]. From the description above, it is clear that the extra-column volume is not part of the chromatographic system and should not be included as part of those volumes. Therefore, the experimentally measured retention volume from injection to detection, (the "*gross retention volume"*, V_R^g [4]), will be:

$$V_R^g = V_R' + V_M + V_{ext} = V_R + V_{ext} \qquad (1)$$

The retention volume of an unretained substance, including the extra-column volume, would be the "*gross hold-up volume*", V_M^g. The gross adjusted retention volume is the same as the adjusted retention volume as defined in NC 3.7.07.

The existence of extra-column volumes has often been ignored by practicing chromatographers when calculating system characteristics, such as the phase ratio (NC 3.2.17), the retention factor (NC 3.7.12) or the average linear mobile phase velocity (NC 3.6.05). According to the description above, this is an incorrect practice. The errors involved will depend on the relative value of the various volumes of expression (1). In LC, V_{ext} can be found by an independent experiment (without the column). In *gas chromatography* (GC) (NC 1.2.02), a good experimental approximation to a separation in which there is no extra-column volume would be capillary column gas chromatography (CCGC) with a large split ratio in a low volume injector, the column end entering the detector and a reasonable amount of auxiliary or make up gas that minimises the effect of the detector volume.

2.4 *Mobile phase volume in the chromatographic system*

The *hold-up volume* is defined here as the volume of mobile phase that leaves the column during the transport of an unretained substance along the column. Normally, this volume is equal to the total volume of mobile phase in the chromatographic system, not just the interparticle (exclusion) volume in packed columns. It includes mobile phase inside the pores of the packing material. In some chromatographic techniques, especially in *gas - solid chromatography*, GSC (NC 1.4.01), LC, and *supercritical - fluid chromatography*, SFC (NC 1.4.04), but perhaps also in packed column *gas - liquid chromatography*, GLC (NC 1.4.01), a certain amount of mobile-phase material inside the column could be inaccessible to the analyte, while remaining accessible to other molecules of the mobile phase. The inaccessibility to molecules of the analyte is referred to as exclusion phenomena, caused by sterical or electrostatic reasons. These phenomena are the basis of *size exclusion chromatography* (SEC) and *ion exclusion chromatography* (IEC) (NC 1.5.04). These special cases are not considered here. It must be specially mentioned here that a portion of the mobile phase inside the column may be immobilized (it does not move), but may be accessed by the molecules of the analyte. Calculations based on the diffusion rates of solute molecules in liquids (diffusion rates in gases are orders of magnitude larger) indicate that under chromatographic conditions the analyte molecules will reach the bottom of any normal pore in the time taken by the analyte band to pass over it. Similarly, mobile phase pools formed on the solid surface of particles may be accessible to analytes. These portions of immobilized mobile phase will slow down the overall movement of any analyte, retained or not. They take part in the chromatographic process. If an adequate probe is available (a truly unretained analyte), the measure of the volume of mobile phase that leaves the column during its elution will include the interactions within these immobilized portions.

The volumes of mobile phase mentioned so far, have been described as "volumes measured at the column outlet". The reporting of experimental values of V_M is not always simple. Liquids are considered incompressible, so their volume does not depend on pressure and very little on temperature. However, the volume of a given amount of gas, or fluid under

supercritical or near supercritical conditions, will depend on both temperature and pressure, and these must be recorded when volumes are reported and used. In such cases, in order to specify chromatographic conditions, V_M must be expressed at the column temperature and ambient pressure (NC 3.6.04.2 and NC 3.7.03). The correction factors that must be applied in GC, to express mobile phase volume under different conditions have been intensively discussed [1, 5-14], and the recommended names and definitions are summarized in ref [14] .

Mobile phase volumes have been described in the past as "volume that entered the column...", "volume that passed any cross-section of the column ...", "volume that left the column...", "volume used to transport...". All these expressions are equivalent, as long as the effects of pressure and temperature are not forgotten. Other expressions such as "volume inside the column..." or "volume that moves inside the column...", although normally correct, do not always describe the *hold-up or retention volumes* and should be avoided.

2.5 *Retention parameters based on time*

A chromatogram is the representation of the variation with time, of the amount of the analyte in the mobile phase exiting the chromatographic column (measured at the detector device). Quite often, the chromatographic process is carried out isothermally under conditions of constant flow rate of the mobile phase. Under these circumstances, retention volumes may be related to retention times. Therefore, "*retention time*" (t_R), "*adjusted retention time*" (t'_R), "*hold-up time*" (t_M) and "*extra-column time*" (t_{ext}), may be used instead of the corresponding volumes. Times are measured with reasonable precision with electronic integrators or data systems, as long as the sampling rate of the computer / integrator is adequate. However, when measuring times, an error might be introduced at the moment of initiating the time count. This time error will have the corresponding volume error. For simplicity, this error will not be considered here. The experimental retention time of an analyte, including all possible errors, will be denoted as t_R^g ("*gross retention time*"). The time corresponding to the retention of an unretained substance would be t_M^g ("*gross hold-up time*"), sometimes referred to as t_0. The latter symbol, however, is normally reserved to denote the retention time of an unretained compound in *exclusion chromatography* (EC, NC 1.5.04). Its use in connection with *elution chromatography* is discouraged.

The thermodynamically appropriate retention parameter in chromatography is the retention volume; but as times and not volumes are measured directly, times are normally used in *gas chromatography*. In *liquid chromatography*, conversion from time to volume is straightforward and the normal practice is to use and mention volumes. It must be remembered that for a given chromatographic system at a defined temperature, *retention volumes* and the *hold-up volume* are independent of flow rate, but the corresponding times are not.

In any discussion of the *hold-up time*, two concepts must be clearly distinguished: the *hold-up time* itself, t_M, as defined earlier, and the point of the chromatogram corresponding to the time when a non-retained compound would appear, t_M^g, *gross hold-up time*. According to the previous description, we have:

$$t_M^g = t_M + t_{ext} \quad (2)$$

The values of t_M and t_M^g should be equivalent, and they have been taken as such frequently in the past.

3. THE UTILITY OF THE HOLD-UP TIME (VOLUME)

Hold-up times or volumes are used in chromatography for various purposes. In those expressions in which t_M is used as a term, the exact point of the chromatogram where the unretained analyte should appear (t_M^g) must be exactly known. An example would be the expression used to calculate adjusted retention times: $t_R' = t_R^g - t_M^g$. In cases where the *hold-up time* is used as a factor of the expression, the correct value of t_M must be used. An example will be the retention factor, *k*, which may be obtained as follows:

$$k = (V_R - V_M)/V_M = V_R'/V_M = t_R'/t_M \quad (3)$$

In this case the retention factor may not be obtained with the expression $k = (t_R^g - t_M^g) / t_M^g$, except in the particular case in which $t_M = t_M^g$. However, the expression $k = (t_R^g - t_M^g) / t_M$ is correct.

Some chromatographic and thermodynamic parameters may be accurately calculated using the *gross hold-up time*: adjusted, corrected, net and specific retention volumes (V_R', $V_R°$, V_N, V_g); adjusted retention times (t_R'); distribution constant or partition coefficient (*K*); theoretical and effective plate number of the column (*N*, N_{eff}); separation factor (α); relative retention of peaks (*r*); resolution of two peaks (R_s); peak capacity (*n*), separation number (SN) or Trennzahl (TZ); Kovàts' retention indices (*I*). Other system-dependent parameters may not be calculated with accuracy, unless the correct value of t_M or V_M is known: retention factor (*k*); retardation factor (*R*); phase ratio (β); *hold-up volume* (V_M) if deduced from t_M and the flow rate; average linear mobile phase velocity ($\bar{u}$). Nevertheless, the inaccuracy involved in the use of incorrect *hold-up times or volumes* may be negligible in some cases. Definitions and expressions used to calculate all these parameters may be found in refs. [1, 14].

4. METHODS USED TO ESTIMATE THE HOLD-UP TIME (VOLUME)

The methods used for *hold-up time or volume* determination depend on the physical state of the mobile phase. Accordingly, they will be treated separately. Direct methods (the experiment produces the value of V_M or t_M directly) and indirect methods (results of the experiment are used to calculate the value of V_M or t_M) have been proposed to find the *hold-up time (volume)*. Not all methods produce the same value for V_M or t_M for a given chromatographic system.

4.1 *GAS CHROMATOGRAPHY*

In *gas chromatography*, all methods proposed to estimate hold-up time or volume use experimental retention times and the methods are intended for *hold-up time* estimation. However, none of the methods gives a value of the *hold-up time*; what they really give is the point on the chromatogram where an ideal non-retained substance would appear (t_M^g). All methods assume that sufficient experimental precautions have been taken to assure the absence of time errors due to the existence of extracolumn volumes or to other sources of error. None of them corrects these possible errors. In the following paragraphs, the words "*hold-up time*" will be used, but always "*gross hold-up time*", (t_M^g), will be assumed. It has been mentioned that for many applications, the latter value is perfectly adequate.

4.1.1 *Direct method*

The best estimate of the *hold-up time* should be obtained by the injection of a substance that is not retained by the stationary phase, and that may be monitored by the detector. The retention time of such a substance would show the exact point of the chromatogram to be used for adjusting retention times, but not the true value of t_M, unless the systematic errors mentioned above have been avoided. In GC, such an ideal substance does not exist [15,16], although some permanent gases may be used without much error, if the appropriate detector is available. Methane is not recommended as a *hold-up time* indicator. It may be used, however, to calculate chromatographic or thermodynamic parameters of analytes with a significantly greater retention factors.

4.1.2 *Indirect methods*

The absence of a true non-retained substance for direct estimation of *the hold-up time*, and the widespread use of the flame ionization detector has forced the development of indirect ways of estimation. Reviews may be found in a paper published by Smith *et al.* [17] or the book of Pacáková and Feltl [18]. Only a few will be mentioned in this paper. Sometimes, the *hold-up time* calculated with these methods has been referred to as *"mathematical hold-up time"*, denoted with the symbol t_{MM} .

Linearity methods. The methods that have found widest acceptance are those based on the linearity of the logarithm of the adjusted retention times for the members of a homologous series (preferably n-alkanes) *versus* carbon number, for chain lengths of a minimum of five or six carbon atoms ("the semilog plot"). It is generally accepted that the linearity does not apply to the whole homologous series, but only to a range of chain lengths that seems to depend on the experimental conditions [19]. Early methods relied on the experimental retention times of three homologues. For evenly spaced homologues :

$$t_{MM} = (t_{R,3}\, t_{R,1} - t^2_{R,2})/(t_{R,1} + t_{R,3} - 2\, t_{R,2}) \qquad (4)$$

where $t_{R,x}$ (x = 1, 2 and 3) is the experimental retention time of the homologues with z, z+n and z+2n carbon atoms. For the case of unevenly spaced homologues of z, z+x and z+y carbon atoms, the expression has been generalized [18] to:

$$(t_{R,3} - t_{MM})/(t_{R,2} - t_{MM}) = \{(t_{R,2} - t_{MM})/(t_{R,3} - t_{MM})\}^{x/y} \qquad (5)$$

and from this expression, the *mathematical hold-up time* may be derived. Other methods calculate adjusted retention times directly [17, 18, 20]. The value of t_{MM} is then obtained by subtraction.

A second group of methods based on the linearity principle use four or more homologues (statistical and iterative methods) to compensate for the natural variation of the individual experimental retention times. Perhaps the most popular among this type of methods is that of Guardino *et al.* [21], which starts with an initial estimate of t_{MM} that must be lower than the true *hold-up time*. A similar method producing equivalent results [16, 22] may be used with any initial estimate of t_{MM}.

The value of the *hold-up time* deduced with these linearity methods depends on the homologues used, confirming that the methods may be questionable. This dependence has been explained [19] because the "semilog plot" is not a straight line, but rather a curve, at least up to seventeen carbon atoms. The curvature depends on the actual value of t^g_M used to adjust retention times. It has been shown for the case of the n-alkanes, that the value of t_{MM} obtained by any of the "linearity methods" will approximate the true value of t^g_M if early eluting homologues are used, and will gradually depart from that value as heavier homologues are employed [19]. This assertion is not necessarily true for the case of other homologous series [23]

Non-linearity methods. It has been suggested that the chromatographic behavior of the n-alkanes is best described by an equation that does not imply linearity of the "semilog plot" [15]. A selection from twenty five expressions, including the one that supports the "linearity principle" has brought about the conclusion that under a variety of column types and

chromatographic conditions, the best description of the retention times of the n-alkanes is given by:

$$t_{R,z} = A + \exp (B + C\, z^D) \qquad (6)$$

where z is the carbon number of the n-alkane [15, 16]. From this expression, the *hold-up time* is obtained for a value of z equals zero. A minimum of four n-alkanes plus methane must be injected to deduce the parameters A, B, C and D by a non-linear regression procedure. The value of the calculated *hold-up time* has been checked against the actual retention times of H_2, He, N_2, O_2, Ne and Ar, and against values deduced by linearity methods [16], arriving at the conclusion that this procedure reproduces the retention of an unretained substance better than other methods. Thermodynamic, statistical and structural reasons to support the non-linear chromatographic behavior of n-alkanes have been presented [23, 24],

In the absence of further studies, the non-linear methods are the recommended procedures for gas chromatography.

4.2 *LIQUID CHROMATOGRAPHY*

Numerous papers and reviews [25-29] have discussed the different methods used to measure the *hold-up volume* in liquid chromatography. A short presentation of these methods follows.

Similarly to the situation in gas chromatography, there are no ideal unretained substances that could be conveniently used for a direct measurement of the *hold-up volume* (time) of the chromatographic column. All test compounds suggested in direct methods are either slightly retained by the stationary phase or slightly excluded from certain parts of the mobile phase in the column. The same statement is valid for components of a mixed mobile phase if these components are used in place of an unretained test compound. To evaluate the validity and precision of all techniques described below, one has to take into consideration the balance between the adsorption/exclusion phenomena mentioned above.

4.2.1 *Direct methods*

Methods using inorganic and organic compounds. Inorganic salts, particularly sodium nitrate and sodium nitrite, and some organic compounds, such as uracil, have been used as "markers" for *hold-up volume* determination. It has been shown [29] that *hold-up volumes* measured using inorganic salts depend on experimental condition (mobile phase composition, ionic strength, and sample size) and on the ionic volume of the salt. This has been interpreted

as sample exclusion (due to either electrostatic or steric effect) from the pores of the packing material, which prevents the analyte from fully exploring the intraparticle volume [30]. Consequently, the *hold-up volume* measured using inorganic salts can vary between V_0 and a value close to the interparticle (exclusion) volume, depending on experimental conditions.

The use of organic ions or highly polar analytes for *hold-up volume* determination should be avoided because most of them are retained to some extent, particularly in reversed phase LC (RPLC) with mobile phases containing either moderate or high percentages of water.

4.2.2 *Indirect methods*

Method of column weight. In this technique the column is filled and weighted successively with a light solvent of density δ_1 (e.g., methanol), and a heavy solvent of density δ_2 (e.g., carbon tetrachloride). The column *void volume* can be calculated by:

$$V_0 = (m_2 - m_1)/(\delta_2 - \delta_1) \quad (7)$$

where m_1 and m_2 are the weights of the column filled with the light and the heavy solvent, respectively. It has been suggested that V_0 may be the only measure of the *hold-up volume* with a physical meaning, because it represents the volume within the column accessible to the two solvents and an analyte molecule of a size comparable to that of the solvent molecules. However, with solvents that are strongly adsorbed on the sorbent surface or that solvate the stationary phase to a significant extent (thus increasing the effective volume of the stationary phase at the expense of the mobile phase), this method may give negative k values for unretained compounds [31]. For the same reason, when this method is used for *hold-up volume* determination in RPLC the plots of log k vs. n_C (the number of carbon atoms on the alkyl moiety for each member of the homologous series) and log k vs. $1/T$ (inverse of the column temperature) are non-linear for some solvents (particularly for methanol/water and acetonitrile /water mixtures).

Methods using homologous series. Several methods to calculate the *hold-up volume* rely on the use of homologous series of analytes. These methods are based on a linear relationship between log V_R' *versus* n_C if the free energy of sorption of each analyte on the stationary phase increases by a constant amount from one homologue to the next in the series.

In one approach, the *hold-up volume* is calculated by self-consistent fitting of the equation:

$$\log V_R' = \log (V_R - V_M) = a\, n_C + b \quad (8)$$

With different sets of probes the value corresponding to the highest correlation coefficient is taken as the correct V_M. In another approach, the retention volume of each homologue is plotted against the retention volume of the previous one in the series. The *hold-up volume* can then be calculated from the intercept of this straight line. The size (n_C) of the homologues used in the determination of V_M is of critical importance to obtain self-consistent data. Some authors [31] recommend the use of "convergent" conditions, i.e. chemical series, and chromatographic mobile phases for which the calculated *hold-up volume* does not change significantly (less than 5% being acceptable) with the n_C value and the total number of homologues chromatographed.

This method leads to hold-up values that are systematically smaller than V_0, because the exclusion effect for each homologue increases systematically with the increase of the length of the alkyl chain. Considering what has been said about the validity of equation (8) in GC, further evidence on its application in LC might be desirable.

Methods using isotopically-labeled compounds. Isotopically labeled counterparts of the mobile phase components have been used as *hold-up volume* markers in LC, particularly in RPLC studies. Usually, in these experiments, a deuterated component of the mobile phase (D_2O or a D-labeled-modifier) is employed as the sample and a refractive index monitor or mass spectrometer is used as the detector. Radioactive labeled samples and radioactivity detectors have also been employed in some cases. In these methods the following assumptions are made: 1) The labeled compound used has the same chromatographic behavior as that of the non-labeled counterpart in the mobile phase, and therefore it should explore the same column volume as this component of the mobile phase. 2) There is no difference in retention volume between labeled and non-labeled compounds due to the differences in molecular size. 3) There is no isotopic exchange between the labeled analyte and some components of the stationary phase. Two procedures have been proposed: injection of D_2O, and the injection of all the components of the mobile phase labeled with isotopes (deuterium or radioactive isotopes).

D_2O as a marker. This method uses deuterium oxide to measure the *hold-up volume* of a RPLC chromatographic system. When a sample containing D_2O (either pure or dissolved in the mobile phase) is injected in a LC system fitted with a refractive index detector, two peaks can be expected: one peak corresponds to deuterium oxide and the other to the so-called system peak. The deuterium oxide peak might in fact correspond mostly or even only to the species DHO, because isotopic exchange with water could take place in the mobile phase. Henceforth, for the sake of simplicity, this peak will be referred to as D_2O, the peak of the deuterium oxide or an equivalent expression regardless of its nature. The system peak is because in RPLC a preferential adsorption of the organic modifier on the stationary phase takes place whereas less adsorption of water occurs. Injection of a sample of D_2O, even diluted in the mobile phase, can disturb the adsorption equilibrium and generate a transient enrichment of the mobile phase in one of its components that can be detected (by a refractometer detector) as a system peak. This system peak can be identified because its position is identical to that obtained

by injecting H_2O-rich mobile phase as analyte. The other peak, corresponding to deuterium oxide, has been proposed [32] as a marker of the *hold-up volume* in RPLC.

It has been observed [32] that the retention of D_2O varies significantly with the modifier percentage of the mobile phase. The retention volume of D_2O tends towards V_0 both at high and low concentration of modifier in the mobile phase, whereas values smaller than V_0 are obtained for the retention volume of D_2O at intermediate compositions. It has also been observed [32] that the discrepancy between the retention volume of D_2O and V_0 depends on the hydrophilic character of the modifier used, the highest being for tetrahydrofuran. This variation in the retention volume of D_2O with the mobile phase composition is related to the volume of modifier immobilized on the stationary phase. Since isotopic exchange between D_2O and methanol molecules immobilised in the RP packing takes place, some additional retention of deuterium oxide can be expected in those experiments where RPLC is used with water-methanol mobile phases. Isotopic exchange of injected D_2O with available silanol groups can also contribute to some retention of this substance in RP columns.

Minor disturbance method. In this approach, usually a small volume of the mobile phase with a slightly altered concentration in one of its components is injected in the column. In the simplest case of reversed phase chromatography with a two-component mobile phase and if the effluent of the column is monitored with a suitable detector (generally a refractometer), only one peak due to the disturbance of the mobile phase adsorbed on the stationary phase is observed. The retention volume corresponding to the maximum of this peak is related to V_0 through the slope of the partition isotherm of the organic modifier on the stationary phase at the mobile phase composition at which the experiment is carried out [33, 34]. Therefore, the retention volume of this peak depends on the eluent composition. Only for those mobile phase compositions at which the slope of the partition isotherm approaches a value of unity, the retention volume of the disturbance peak approaches the value of the *void volume* of the column (V_0).

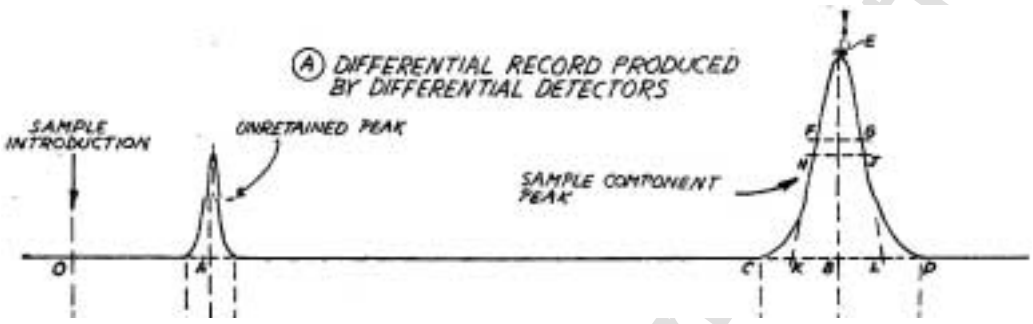
Due to these results, the minor disturbance method has been generally considered to be unreliable in LC. However, it has been shown [29] that using Gibb's model of adsorption a new equation to calculate the *hold-up volume* can be derived. This equation, which uses the retention volume of the disturbance peak at different mobile phase compositions, gives values of the *hold-up volume* which are independent of column temperature and mobile phase composition, and agree, within 5%, with the value of V_0 of the column.

4.3 *SUPERCRITICAL FLUID CHROMATOGRAPHY*

Hold-up times have recently been measured or calculated for capillary SFC with carbon dioxide as mobile phase [35, 36]. *Hold-up times* were measured directly (methane injection), or calculated according to models based on the linearity principle of the semilog plot of the retention factor *vs.* carbon number [35], or based on polynomials that include mobile phase density and temperature terms [36]. The validity of the different evaluation procedures was checked by comparison of calculated *hold-up times*, with those deduced from methane injections. A maximum was found for t_M when plotted versus mobile phase density. Bearing in mind that methane is not recommended as a *hold-up time* indicator in GC [15, 16], and that under SFC conditions, elution order is strongly dependent on both temperature and mobile phase density, it is felt that further evidence should be provided before a conclusion may be drawn about *hold-up times/volumes* in SFC.

5. TERMS AND DEFINITIONS THAT HAVE BEEN INTRODUCED OR MODIFIED

Terms from reference [1] that have been redefined or extended. The Figure mentioned can is in reference [1].



GENERAL TERMS

1.1.01.1 *Chromatographic System*

A system formed by at least two immiscible phases in contact with each other, one of which is continuously moving relative to the other. Normally one of the phases remains stationary, and is that part of the chromatographic system responsible for the retention of the analytes, whereas the moving part is responsible for their transportation.

1.1.01.2 *Chromatographic Process*

The phase distribution that takes place when an analyte (or a mixture of analytes) is carried along the stationary phase by the movement of the mobile phase.

1.1.01.3 *Sample Introduction (Sample Injection)*

The act of introducing the sample to be chromatographed into the apparatus that contains the chromatographic system, to obtain a chromatogram. The term is used to indicate sample introduction into the mobile phase through an injector.

1.1.01.4 *Initiation Time*

The moment at which the chromatographic process starts (when the sample starts to move along the chromatographic system). Normally, in column chromatography the moment when the sample, carried by the mobile phase, enters the column.

TERMS RELATED TO COLUMN CHROMATOGRAPHY

3.7.03.1 *Hold-up Volume (at column temperature and ambient pressure) (V_M)*

The volume of the mobile phase required to elute the unretained compound from the chromatographic column and reported at column temperature and ambient pressure.

$$V_M = t_M \cdot F_c$$

Note: In gas chromatography and supercritical fluid chromatography, this value does not represent the mobile phase volume in the column.

3.7.03.2 *Gross Hold-up Volume (V_M^g)*

The volume of the mobile phase that leaves the column from the moment of sample introduction to the detection of the peak maximum of an unretained compound and reported at column temperature and ambient pressure. It also includes *the extra-column volumes.*

$$V_M^g = t_M^g \,.\, F_c^0$$

Note: This corresponds to the previous definition of *Hold-up Volume* (NC 3.7.03).

3.7.03.3 *Hold-up Time (t_M)*

Time required for the mobile phase to pass through the chromatographic column. (Residence time of an unretained compound in the chromatographic column.)

Note: The *hold-up time* corresponds to the distance OA in Fig. 1, if the extra-column volumes have been eliminated. Sometimes it is also referred to as t_0. This symbol however, should be reserved to denote the retention time of unretained compound in *Exclusion Chromatography*. Its use in *elution chromatography* is discouraged.

Note: *Mathematical Hold-up Time (t_{MM})*

The *hold-up time* deduced by a mathematical treatment of retention data of the chromatogram. The methods available at present produce a value that will normally approximate the "*Gross Hold-up Time*".

3.7.03.4 *Gross Hold-up Time (t_M^g)*

Time elapsed from the sample introduction to the detection of the peak maximum of an unretained compound. It includes the time required for the mobile phase to pass through *the extra-column volumes.*

3.7.05.1 *Retention Volume (at column temperature and ambient pressure) (V_R)*
The volume of the mobile phase required to elute the compound of interest from the chromatographic column and reported at column temperature and ambient pressure.

$$V_R = t_R \cdot F_c$$

3.7.05.2 *Gross Retention Volume (at column temperature and ambient pressure)*, **(V_R^g)**
The volume of the mobile phase that leaves the column from the moment of sample introduction to the detection of the peak maximum of the analyte and reported at column temperature and ambient pressure. It also includes *the Extra-Column Volumes.*

$$V_R^g = t_R^g \cdot F_c^0$$

Note: This corresponds to the previous definition of *Total Retention Volume* (NC 3.7.05).

3.7.05.3 *Retention Time (t_R)*
Time required for the analyte to pass through the chromatographic column. (Residence time of the analyte in the chromatographic column.)

Note: The retention time corresponds to the distance OB in Fig.1A, if the extra column volumes have been eliminated.

3.7.05.4 *Gross Retention Time, (t_R^g)*
Time elapsed from the moment the time count is initiated, to the detection of the maximum of the peak of the analyte in the mobile phase. It includes the time required for the analyte to pass through *the extra-column volumes.*

6. TABLES

Terms and symbols which are new or have been redefined.

LIST OF TERMS

Chromatographic process	1.1.01.2
Chromatographic system	1.1.01.1
Gross hold-up time	3.7.03.4
Gross hold-up volume	3.7.03.2
Gross retention time	3.7.05.4

Gross retention volume	
(at column temperature and ambient pressure)	3.7.05.2
Hold-up time	3.7.03.3
Hold-up volume	
(at column temperature and ambient pressure)	3.7.03.1
Initiation time	1.1.01.4
Mathematical hold-up time	3.7.03.3
Retention time	3.7.05.3
Retention volume	
(at column temperature and ambient pressure)	3.7.05.1
Sample injection	1.1.01.3
Sample introduction	1.1.01.3

LIST OF SYMBOLS

t_M	Hold-up time	3.7.03.3
t_{MM}	Mathematical hold-up time	3.7.03.3
t_M^g	Gross hold-up time	3.7.03.4
t_R	Retention time	3.7.05.3
t_R^g	Gross retention time	3.7.05.4
V_M	Hold-up volume (at column temperature and ambient pressure)	3.7.03.1
V_M^g	Gross hold-up volume	3.7.03.2
V_R	Retention volume (at column temperature and ambient pressure)	3.7.05.1
V_R^g	Gross retention volume (at column temperature and ambient pressure)	3.7.05.2

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