



Towards an accurate method for column void volume determination using liquid chromatography-mass spectrometry

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ARTICLE INFO

Keywords:

Reversed-phase liquid chromatography
Void volume
Void volume markers
Retention mechanism
Modelling

ABSTRACT

Prediction of analyte retention times requires prior knowledge of the column void volume, the measurement of which is still highly contested within the literature and therefore experimental based prediction is often used. In this study, we investigated deuterated acetonitrile as an isotopically labelled mobile phase component to observe its elution behaviour in a binary mixture with water at 25 different mobile phase compositions (from 5 to 95 vol. % of acetonitrile), on two stationary phases (C8 and C18), and at two temperatures (30 and 40 °C) using LC-MS. The same experimental design was additionally used for three commonly used neutral void volume markers: uracil, phloroglucinol and *N,N*-dimethylformamide. Temperature was observed to influence the elution of acetonitrile in an inversely proportional manner with higher temperatures coinciding with lower elution times. By utilizing a three-way ANOVA, the composition of the mobile phase has been shown to have a significant effect on deuterated acetonitrile and other investigated void volume markers, demonstrating the fact that both void volume markers and acetonitrile itself exhibit retention-like behaviour. Excess adsorption isotherms for acetonitrile were calculated using deuterated acetonitrile elution data. The comparison of void volumes, obtained with conventional neutral void volume markers, revealed the former to be 24–36% lower than the void volume obtained using deuterated acetonitrile, as an isotopically labelled mobile phase component. For a water:acetonitrile mobile phase, the minor disturbance method using deuterated acetonitrile to obtain an integral average void volume (2.08 and 2.05 mL for C18 at 30 and 40 °C, respectively and 2.16 and 2.13 mL for C8 at 30 and 40 °C, respectively) was found to be the most appropriate method for determining the elusive column void volume.

1. Introduction

The development of a comprehensive theoretical model for predicting the retention of any given analyte under defined chromatographic conditions is a major goal in current chromatographic research [1–3]. The complexity of this task is largely due to our limited understanding of retention mechanisms on a molecular scale [3–5]. To bridge this knowledge gap, a plethora of different strategies have been implemented over the years [2,3], including statistical QSAR-type approaches [6–8], physico-chemical solvation parameter models [9–13] and molecular simulations [2,14,15] along with various combinations [16].

The void volume, V_0 , is by definition the total volume of mobile phase (MP) moving in the column. The total void volume is a thermodynamic concept of the void volume. In practice, however, it is difficult to unambiguously measure such a freely moving volume of the MP. Ideally, selected compounds classified as void volume markers (VVMs), should not be retained at the stationary phase (SP) and should be small

enough to penetrate all pores. Therefore, the volume determined using VVMs is better represented as the hold-up volume, which is defined as the retention volume of a "non-retained" component [17]. Depending on the extent to which the VVM can access the pores of the column, the hold-up volume can range from the interstitial volume to the total void volume.

The measurement of the column void volume, connected with the column void time via $V_0 = t_0 F$, with F representing the MP flow rate, is not completely unambiguous [18,19]. Rimmer et al. [20] reviewed the different methods used to determine V_0 , which include static (e.g., pycnometry), and dynamic techniques, which include organic and inorganic salts, the minor disturbance method, and unretained neutral markers as probe analytes injected into the system under operating conditions. While there is widespread agreement that the method of using organic and inorganic salts and neutral (unretained) markers is advantageous because of the experimental ease of implementation, opinions differ on the validity of this approach, as different techniques

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<https://doi.org/10.1016/j.chroma.2023.464245>

Received 27 May 2023; Received in revised form 24 July 2023; Accepted 25 July 2023

Available online 26 July 2023

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have been shown to yield different values for void volumes [21]. Inaccurate determination of V_0 directly affects all measured analyte retention factors $k_i = (V_i - V_0) / V_0$ where V_i is the elution volume of analyte i . The method of using putative unretained VVMs is amongst the most popular dynamic methods for void volume determination. The most commonly used neutral VVMs in reversed-phase liquid chromatography (RPLC) include: uracil, urea, thiourea, phloroglucinol (PHL, PIN: benzene-1,3,5-triol), *N,N*-dimethylformamide (DMF), and acetone [18–20,22–26].

The study of adsorption in RPLC is particularly important as a number of studies suggest that adsorption of MP components plays an important and often overlooked role in retention mechanisms [27,28]. In literature, three main retention mechanisms are described: (i) the traditional partitioning mechanism, (ii) an analyte-only adsorption mechanism, and (iii) an adsorption mechanism in which organic MP components form a layer of preferentially adsorbed molecules on the surface of the SP [29,30]. Retention as described by the latter mechanism takes place in the additionally formed adsorbed MP layer, while the SP primarily acts as a medium for the adsorption of the organic MP components. Absorption of organic solvents onto the SP has been experimentally observed and theoretically investigated beforehand [23,29,31]. The minor disturbance method presents a popular and accurate technique for observing thermodynamically driven excess adsorption in RPLC, along with its use in determining the column void volume [32]. The principal idea behind the minor disturbance method is the injection of eluents at compositions that differ from the equilibrated MP composition. The minor disturbance therefore presents a solvent perturbation travelling along the equilibrated column. The result of this procedure are solvent vacancy perturbation peaks, usually detected using photodiode array (PDA) or refractive index detectors. Our primary aim at using deuterated MP components, i.e., deuterated acetonitrile (D_3 ACN) and deuterated water (D_2O), in combination with mass spectrometry (MS) detection, is therefore especially convenient due to the single ion monitoring (SIM) experimental isolation of the isotopically labelled peak. This procedure greatly simplifies the otherwise ambiguous solvent vacancy peaks procedure encountered in the minor disturbance method with classical PDA or refractive index detection. Fóti et al. [33] previously used deuterated water to study excess adsorption of water on three different RP columns, whilst Gritti et al. [34] recently compared different methods for column void volume determination and suggested using D_3 ACN in a pure acetonitrile (ACN) MP, in combination with MS detection, for determining the column void volume. Experimentally demonstrated retention-like profiles for selected neutral (unretained) markers (uracil, acetone and thiourea) for RPLC conditions, and benzene for HILIC conditions were recently reported as well [34].

In this study, we propose a concise and accurate universal thermodynamic method for estimating the void volume as an integral average of the elution volumes of D_3 ACN and D_2O , via the minor disturbance method and MS detection over the entire composition range of the water:ACN mobile phase. We investigated whether the putatively “unretained” VVMs (uracil, PHL and DMF) exhibit retention-like behaviour. By systematically investigating the elution behaviour of selected VVMs and D_3 ACN at each of 100 different experimental conditions via MS detection, we established a detailed and comprehensive publicly available elution dataset that allowed us to investigate the influence of the SP, MP and temperature on the elution times. Using the Kazakevich’s minor disturbance method [27,35–37] the D_3 ACN elution data were used to additionally calculate the excess adsorption isotherms.

2. Material and methods

2.1. Chemicals

Phloroglucinol (99%, reagent grade), *N,N*-dimethylformamide (DMF; 99%), deuterated acetonitrile (D_3 ACN; 99.9% isotopic purity), uracil (99%), acetone ($\geq 99.9\%$), and thiourea (99%, reagent grade)

were purchased from Sigma-Aldrich (Germany). Deuterated water (D_2O ; 99.96% isotopic purity) was purchased from Eurisotop (UK). LCMS grade acetonitrile (ACN; Fisher Scientific, UK, $\geq 99.9\%$) and deionised water, purified with the Mill-Q® system (MQ), were used as chromatographic solvents.

Stock solutions of benzene-1,3,5-triol, DMF, acetone and thiourea at a concentration of 10 mM were prepared in ACN, while a 10 mM stock solution of uracil was prepared in MQ water. Chromatographic experiments were conducted using 1 mM solutions, which were prepared by appropriate dilution of stock solutions with ACN.

2.2. Instrumentation

A Thermo Fisher Scientific Vanquish UPLC system was used for LC experiments, with a binary solvent delivery pump, utilizing an integrated MP mixer and vacuum degasser, autosampler thermostated at 4 °C, column thermostat (set to 30 or 40 °C) with a column preheater element and photodiode array detector. Two standard endcapped RP columns were used; YMC Triart C18 (150 × 4,6 mm, 5 µm, pore size of 12 nm) and YMC Triart C8 (150 × 4,6 mm, 5 µm, pore size of 12 nm). All chromatographic separations were isocratic, with a constant flow of 0.3 mL min⁻¹. Absorption spectra were recorded with a photodiode array detector in the spectral range from 190 to 310 nm at a collection frequency of 20 Hz at 1 nm spectral resolution. 10 µL injection volumes were used for void volume marker solutions and 5 µL for deuterated ACN.

MS analyses were achieved using a Thermo Fisher Scientific TSQ Quantis™ triple quadrupole mass spectrometer with a dual ESI/APCI ion source in positive polarity in the ESI mode. The LCMS hyphenation was achieved by utilizing a six-port valve with a split-less LC infusion into the ESI/APCI ion source. MS experiments were carried out in the single ion monitoring mode (SIM), utilizing the first quadrupole (Q1), with the $m/z \pm 1$ Da, unless stated otherwise. The ESI ion source parameters were kept at the default recommended for the used MP flow-rate, except for the source fragmentation potential, which was set to 10 V.

Thermo Fisher Scientific Xcalibur software was used for LC instrument control, while the Thermo Fisher Scientific TSQ Quantis Tune Application software was used for MS instrument control.

2.3. Experimental design

A full factorial experimental design with variables of temperature (30 °C or 40 °C), MP composition (5–95% ACN with 5% intervals, except for 50–70%, where 2% intervals were used), and SP (C8 or C18 column) was implemented for each investigated VVM and deuterated ACN, with the exception of acetone and thiourea. These VVMs were removed from the study due to their poor ionisation and subsequent MS non-detectability. In total 100 experimental conditions were investigated for uracil, PHL, DMF and D_3 ACN respectively. The limits of 5 to 95% ACN in the MP were chosen in accordance with Fóti et al. [33] who stated that injecting eluents of differing composition outside this range produces solvent perturbations which cannot be taken as negligible in the minor disturbance method, thereby violating the principal assumptions of the method itself. The entire sequence was performed in triplicate for each data point. Each variation of the MP was firstly followed by injection of MQ water as a blank to equilibrate the system. The column oven temperature and the MP preheater element were set to the appropriate temperature with a 0.1 °C tolerance. For VVMs, a combined MS method was created, utilizing three sequentially repeating SIM experiments at the respective nominal m/z values of 74, 113 and 127 Da, which correspond to protonated VVM ions. For D_3 ACN, a single SIM method at (45,0 ± 0.5) Da was used. For details on the MS detection of D_2O , see the Supporting Information file (section S3).

For the detection of VVMs with a classical PDA detector, different wavelengths are recommended: DMF-197 nm, PHL-204 nm, and uracil-

202 nm or 258 nm, based on the maxima of the measured UV–vis absorption spectra given in the Supporting Information (Section S5). If measurements below 205 nm are not possible due to the limitations of the PDA detector, uracil with an absorbance at 258 nm is recommended for use.

2.4. Data analysis

Raw LCMS data (cf. also section S2 in the Supporting Information file) was processed using Thermo Fisher Scientific Xcalibur QualBrowser software. Peak detection was achieved using the integrated Genesis algorithm to obtain retention times. The respective exported PDA and MS chromatograms were processed using Python (ver. 3.10.4) and Visual Studio Code (ver. 1.66.2). Python was also used for figure creation. The three-way ANOVA analysis was implemented in R (ver. 4.05) with RStudio (ver. 2023.03.0).

2.5. Excess adsorption isotherm calculation

McNair et al. [36] defined the minor disturbance column void volume, V_0 , as an integral average of retention volume of the MP component, $V_R(c)$, obtained by the minor disturbance method:

$$V_0 = \frac{1}{c_{\max}} \int_0^{c_{\max}} V_R(c) dc \quad (1)$$

where c is the molar concentration of the MP component, and $c_{\max}$ is the concentration of the pure component (in our case ACN in a binary water:ACN MP). By using the minor disturbance method [27,35–37] with the elution data of D_3ACN , the excess adsorption isotherms were calculated. The mathematical procedure is described in detail in the Supporting Information (section S6 and S7). Briefly, the minor disturbance column void volume is determined as an integral average of the elution of D_3ACN (Eq. (1)). Utilizing this definition of the column void volume enables the calculation of the excess adsorption isotherms (equation S5). Since the specific surface area of the SP is not known for commercial columns used in this work, the adsorption data were normalized per column [32,38]. However, since the properties of the two columns used (C8 and C18) were identical (column dimensions, particles, and pores size), comparison of excess adsorption data, $\tilde{\Gamma}_{ex}$, defined on a per column basis is possible. D_3ACN elution data were first fitted with a quadratic function ($R^2 > 0.992$) and the integrals to calculate V_0 (Eq. (1)) and excess adsorption $\tilde{\Gamma}_{ex}$ (equation S5) were determined analytically (equations S7 and S8). Modelling the elution behaviour of D_3ACN with an analytical function avoids the use of numerical integration. Regression parameters are given in Table S6 in the Supporting Information (section S7).

3. Results and discussion

3.1. General retention behaviour

In a binary MP, the minor disturbance method can in principle be used with both components. We have performed experiments with deuterated acetonitrile, D_3ACN and deuterated water, D_2O (the results for D_2O are detailed in the Supporting Information file, Section S3). However, there are several arguments against the use of D_2O compared to D_3ACN for the determination of the column void volume. Firstly, we observed an extensive isotopic exchange reaction that led to the preferential transformation of the desired $[D_2HO]^+$ ion into the $[DH_2O]^+$ ion. The second reason is that, according to our investigation, the signal intensities are drastically lower than those of D_3ACN , further complicating the use of D_2O . Moreover, the nominal mass of 21 Da is incredibly low from an instrumental point of view (compared to 45 Da in the case of D_3ACN) and may be experimentally unattainable for certain MS systems.

We therefore propose to use D_3ACN instead of D_2O as the desired isotopically labelled water:ACN MP component for the minor disturbance method with detection via MS and therefore the majority of experiments were done with D_3ACN .

Fig. 1 shows the log-transformed MS chromatograms for three VVMs and D_3ACN on a C18 column at 30 °C for various MP compositions (from 5 to 95 vol.% ACN in water:ACN mixtures). The logarithmic transformation serves only to present ion intensity data of different magnitudes on a comparable scale. The raw MS chromatograms from which the elution times were determined, can be found in the Supporting Information file (section S2). The numerical data is presented in Table S1, while the data for C18 separations at 40 °C and for C8 at 30 or 40 °C are given in Tables S2–S4 in the Supporting Information (section S1).

Void volume markers (DMF, uracil, and PHL) all exhibit retention-like behaviour, with a prevalent parabolic profile with respect to the MP composition, which is evident in Fig. 1a–c. Similar profiles for uracil were recently reported by Gritti and Smith using BEH—C18 column at 24 °C and flow rate of 0.2 mL min^{−1} [34]. More interestingly, the elution behaviour of D_3ACN were also observed (Fig. 1d), by utilizing D_3ACN injections and MS detection of the respective $[M + H]^+$ ions. Unlike VVMs, a minimum in the apparent parabolic retention-like profile of D_3ACN is not present in the range of MP compositions under investigation.

To demonstrate more clearly the retention-like behaviour of VVMs and D_3ACN , we show in Fig. 2 the experimentally determined retention times, t_R , for a combination of SP (C18 column) and temperature (30 °C) as a function of MP composition, ϕ , while all investigated combinations are presented in the Supporting Information (section S1, Figure S1). The choice of defining the column void volume as the minima of the respective VVMs retention plot also produces differing values, depending on the VVMs used. As observed in Fig. 2, uracil has a retention minimum at approximately 45 vol.% ACN, DMF at 60 vol.% and PHL at 80 vol.%. Therefore, column void volumes, obtained from retention minima of VVMs, become a function of MP composition.

3.2. Influence of temperature and SP on retention

The influence of temperature on the elution times of investigated VVMs and D_3ACN was explored by conducting HPLC experiments at two different temperatures (30 or 40 °C) on C8 or C18 columns. Fig. 3 presents the difference in elution times, $\Delta t_R = t_{R, 40^\circ C} - t_{R, 30^\circ C}$, for all VVMs and D_3ACN as a function of MP composition. A temperature difference of 10 °C has no drastic effect on elution times. However, at lower ACN content elution times at 40 °C are shorter than at 30 °C, while the opposite trend is observed when the ACN percentage is increased – at high ACN content, Δt_R becomes negative again (Fig. 3a and 3c). The pattern can be compared to the parabolic profile in VVMs elution plots with positive Δt_R values appearing at 40–70 vol.% ACN (Fig. 3a and 3c) where an apparent elution plateau is observed in Fig. 2. The origin of parabolic elution patterns in VVMs can be attributed to the coexistence of MP composition-dependant retention mechanisms, with predominantly hydrophobic interactions at low ACN compositions and HILIC-like interactions in the high ACN composition range. The fact that the retention profile depends on the composition of MP also suggests that VVMs are either slightly retained on the silica-based stationary phase (C8 and C18 columns) or slightly excluded from the organic-rich pore volume, depending on the composition MP [20,23,34]. The data does suggest that increasing the temperature decreases the elution time for D_3ACN (Fig. 3d), which was previously reported in the literature as well [39].

To investigate the influence of the SP, MP and temperature more generally, a three-way ANOVA statistical analysis was performed. The detailed results are given in the Supporting Information file (section S4). All mixed influence variables were found to be statistically insignificant, while the MP composition and type of SP significantly affected the elution times, at a standard 95% confidence interval. Thus, the influence

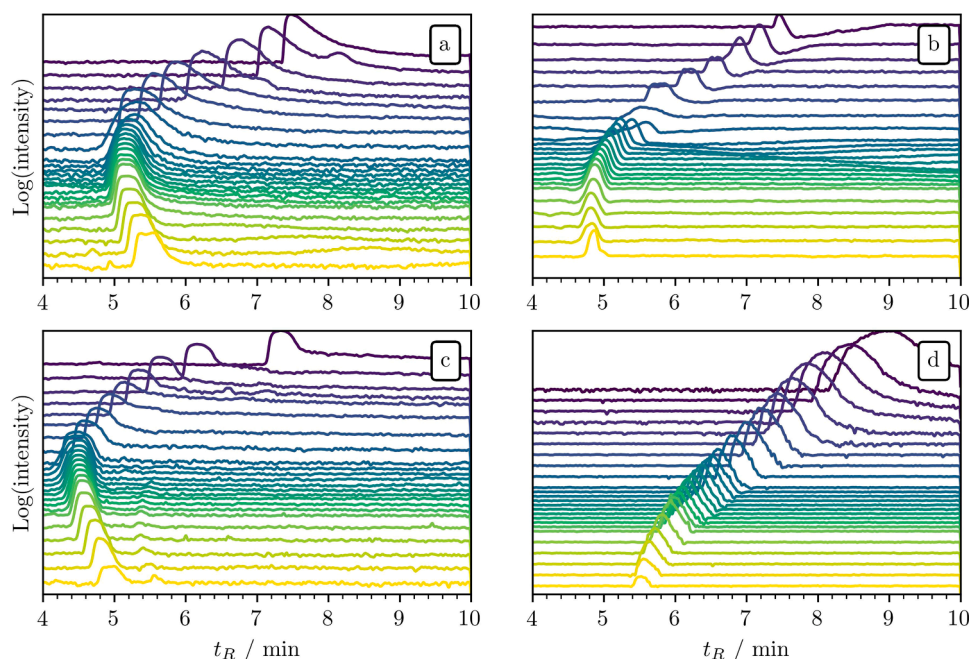


Fig. 1. Log transformed MS chromatograms of a) DMF, b) PHL, c) uracil and d) D₃ACN, recorded in SIM experiments of the respective $[M + H]^+$ ions at 30 °C on a C18 column at a constant flow rate of 0.3 mL min⁻¹. The vol.% of ACN in the MP increases from purple to yellow, from 5 to 95 vol.% (5% intervals, except for 50–70% where 2% intervals were used).

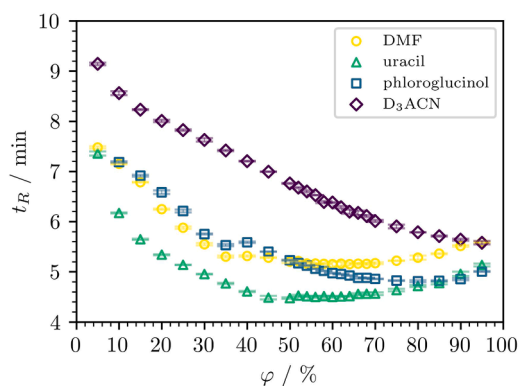


Fig. 2. Elution times for the investigated VVMs and D₃ACN at 30 °C on a C18 column as a function of the MP composition (water:ACN). The error bars were estimated from triplicate measurements.

of MP clearly indicates that all "unretained" neutral void markers exhibit retention-like behaviour. Furthermore, deuterated acetonitrile itself exhibits retention-like behaviour, with elution times differing with a change in MP composition. Temperature was shown to affect only D₃ACN, confirming the conclusions drawn from Fig. 3.

3.3. Excess adsorption isotherms

Excess adsorption isotherms, $\tilde{\Gamma}_{ex}$, are shown on Fig. 4 for both investigated SP (C8 or C18) and at both temperatures (30 or 40 °C) as functions of the vol.% of ACN in the MP, ϕ . In all cases a bell-shaped curve was observed, indicating differential adsorption of D₃ACN as a function of the water:ACN composition. For a given chromatographic column, the $\tilde{\Gamma}_{ex}$ values are lower at higher temperatures (Fig. 4b) For a given temperature and MP composition, the excess adsorption is lower for the C18 column. The composition of the MP at which the maximum of the isotherm occurs also shifts depending on the temperature used – lower temperatures coincide with higher MP composition values of the

isotherm maxima.

3.4. Comparison of D₃ACN and VVMs for void volume determination

The corresponding column void volumes, determined from Eq. (1) (see also equation S7), and measured from the retention minima of the VVMs under investigation are shown in Table 1. Changes in V_0 for the minor disturbance column void volume due to the temperature are very small but are consistent with the trends observed for $\tilde{\Gamma}_{ex}$ (Fig. 4). They are a consequence of the changing elution behaviour of D₃ACN with temperature variations, as already mentioned in Section 3.2. This fact also implies that the minor disturbance void volume, defined as the integral average of the D₃ACN elution data, is a function of temperature and, more importantly, implies the need to thermostat the column. It was observed that the void volume for the C8 column is larger than the void volume for the C18 column at both temperatures. This trend is consistent with the excess adsorption trends seen on Fig. 4 with respect to the SP type used.

Interestingly, for the VVMs used in this study, the elution time minima occur below the minor disturbance void volumes, determined using deuterated ACN, which leads to apparent negative retention factors. Unlike the assumptions of this being the fault of an incorrectly determined void volume, the negative retention factors rather describe less significant affinities towards the SP in comparison to solvent molecules themselves [36]. This is a direct result of the formal definition of column void volumes being related to the MP components rather than any specific VVM molecules. A similar phenomenon was recently observed by Gritti and Smith [34]. The experimentally determined void times of certain neutral unretained void volume markers were shown to be lower than the D₃ACN elution time in a 100% ACN MP by up to 16% for uracil at 75% ACN MP on a selected C18 SP. The degree of positive/negative deviation from the proposed column void time, determined using the D₃ACN elution time in a 100% ACN MP, was observed to be a function of MP composition. Although this may seem counter-intuitive, a lower than expected void volume obtained via VVMs is a consequence of the degree of accessibility of the VVMs to the column pores, with preferential exclusion of the VVMs from the SP-MP interface

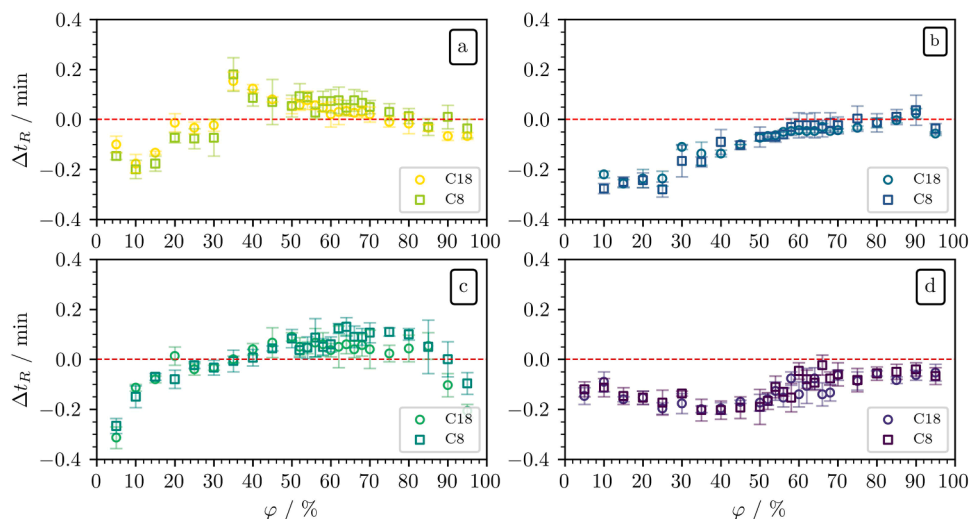


Fig. 3. Differences in elution times, Δt_R , calculated by subtracting elution times obtained at 40 °C from elution times at 30 °C for both SP (C8 or C18) as a function of MP composition: a) DMF, b) PHL, c) uracil and d) D₃ACN. The error bars were estimated from triplicate measurements.

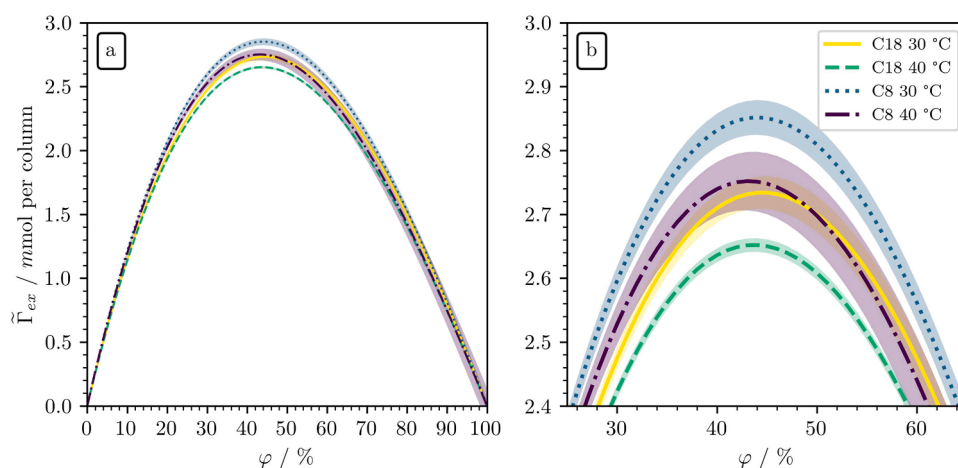


Fig. 4. Excess adsorption isotherms, $\tilde{\Gamma}_{ex}$, calculated using D₃ACN elution data as a function of MP composition, φ , a) excess adsorption isotherms, b) zoomed part of the excess adsorption isotherms. The shaded area represents the uncertainty determined from triplicate measurements. The data applies to C18 or C8 columns at 30 or 40 °C.

Table 1

Minor disturbance void volumes calculated via Eq. (1), and void volumes determined using the minima of the respective VVMs. The uncertainty was estimated from triplicate measurements, as the maximum difference from the mean.

Experimental conditions	V_0 (minor disturbance) [mL]	V_0 (DMF) [mL]	V_0 (PHL) [mL]	V_0 (uracil) [mL]
C18 30 °C	2.082 ± 0.001	1.540 ± 0.003	1.440 ± 0.001	1.339 ± 0.002
C18 40 °C	2.045 ± 0.001	1.548 ± 0.003	1.437 ± 0.001	1.354 ± 0.002
C8 30 °C	2.161 ± 0.002	1.604 ± 0.001	1.512 ± 0.006	1.389 ± 0.003
C8 40 °C	2.126 ± 0.007	1.73 ± 0.01	1.560 ± 0.006	1.548 ± 0.009

region. Additionally, results of a theoretical molecular dynamic simulations study [40] suggest that smaller than expected retention times for uracil indeed are the fault of exclusion from the MP-SP interfacial region. However, the full explanation for this apparent anomaly remains

an open research question. Based on the smallest difference from the minor disturbance void volume, DMF was identified as the most favourable VVM. Since all VVMs exhibit parabolic profile, there is no straightforward way to calculate the void volume. If VVMs are used to determine column void volume, the minimum of the retention profile can serve as an estimate of the void volume.

Gritti et al. [34] recently suggested using deuterated ACN in combination with a MS detector system to determine the column void volume in a 100% ACN MP. This approach, while correct in principle, by following the definition of the void volume being the time needed for solvent molecules to pass through the analytical column, disregards the effect on the solvent molecules incurred by varying MP compositions and temperatures. The limiting case at 100% ACN MP will always result in void volumes lower than the minor disturbance column void volume because the limiting case is the minimum elution volume of ACN, whereas the integral average by definition considers elution volumes at all MP compositions, which are all higher than the limiting case. Additionally, the use of the minor disturbance void volume in retention modelling is more appropriate for gradient experiments, as the varying ACN composition is more consistent with the mathematical treatment of the column void volume as an integral average across different ACN

compositions.

4. Conclusion

In this study D₃ACN and D₂O as the corresponding isotopically labelled water:ACN MP components were used together with MS detection to observe their elution behaviour at different MP compositions on two reversed phase columns at two different temperatures. Extensive isotopic exchange reaction, low ion intensities, and experimental difficulties of measuring low *m/z* values on certain MS systems are arguments against the use of D₂O. Therefore, the majority of experiments were conducted using D₃ACN. The same experimental setup, as for D₃ACN, was used to collect elution data for the three commonly used VVMs, i.e., DMF, uracil and PHL. The results show that all the VVMs studied indeed exhibit retention-like behaviour, which can be observed in the parabolic retention profiles. In addition, it was shown that deuterated acetonitrile also exhibits significant retention-like behaviour, with retention times increasing with decreasing volume fraction of ACN in the MP itself.

A three-way ANOVA analysis revealed that the composition of MP had a statistically significant effect on all investigated compounds. In addition, the SP used was identified as a statistically significant influence for all VVMs and D₃ACN. The trends presented were obtained on two analytical columns and may not apply to all columns in general, especially conclusions with respect to the influence of SP. However, they clearly show that the composition of MP indeed has a major impact on the elution volumes of all compounds studied, refuting the assumption that void volume markers are unretained.

Temperature was shown to have an influence on D₃ACN elution, while no statistical significance was found for the studied VVMs. An inversely proportional effect of temperature on the excess adsorption isotherms, calculated using D₃ACN elution data, was observed, with higher temperatures leading to lower total excess adsorbed acetonitrile.

When VVMs are used for experimental determination of the column void volume, it is recommended that the innate difference of the VVM hold-up volume from the total void volume estimated via minor disturbance method using deuterated MP components is explicitly stated when modelling the retention of analytes, based on the significantly different results obtained in our studies. By using VVMs, a systematic error can be introduced in the model, therefore, their use should be limited to a minimum.

The main advantage of MS detection in the minor disturbance method is the unambiguous identification of the D₃ACN signal, by using SIM experiments of the corresponding isotopically labelled ions. This is in contrast to the conventional implementation of the minor disturbance method, where solvent vacancy peaks detected by PDA/refractive index detectors sometimes appear in a complex and ambiguous manner. If the elution data ideally collected at all MP compositions from 5 to 95% by volume ACN allows curve fitting, the corresponding equations for calculating column void time can be greatly simplified, as demonstrated in our work. The resulting minor disturbance void volume is especially appropriate for gradient experiments, where the change in MP composition, and apparent change in the void volume, is considered due to the integral average of all MP compositions. This approach can significantly improve the robustness of chromatographic methods, which is especially important for accredited methods in various fields such as pharmacy, medicine and industry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All elution times obtained in this study are available in the Supporting Information file (section S1). All excess adsorption model parameters and the associated uncertainties are reported in the Supporting Information file (section S7).

Acknowledgements

The authors acknowledge the financial support from the Slovenian Research Agency, research core funding No. P1-0153 (I.K.C) and P1-0201 (M.L.). The authors would like to thank dr. Uroš Grošelj, Klara Klemenčič, and Luka Ciber for kindly providing PHL, D₂O, and DMF.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2023.464245](https://doi.org/10.1016/j.chroma.2023.464245).

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