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A study of the parameters affecting the accuracy of the total pore blocking method

Anuschka Liekens, Deirdre Cabooter, Joeri Denayer, Gert Desmet*

Vrije Universiteit Brussel, Department of Chemical Engineering, Pleinlaan 2, 1050 Brussels, Belgium

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ABSTRACT

We report on a study wherein we investigate the different factors affecting the accuracy of the total pore blocking method to determine the interstitial volume of reversed-phase packed bed columns. Octane, nonane, decane and dodecane were all found to be suitable blocking agents, whereas heptane already dissolves too well in the applied fully aqueous buffers. The method of moments needs to be used to accurately determine the elution times, and a proper correction for the frit volume is needed. Failing to do so can lead to errors on the observed interstitial volume of the order of 2% or more. It has also been shown that the application of a high flow rate or a high pressure does not force the blocking agent out of the mesopores of the particles. The only potential source of loss of blocking agent is dissolution into the mobile phase (even though this is a buffered fully aqueous solution). This effect however only becomes significant after the elution of 400 geometrical column volumes, i.e., orders more than needed for a regular total pore blocking experiment.

1. Introduction

Recently [1], our group proposed a novel method, the so-called Total Pore Blocking (TPB) method, to determine the external porosity (ε_e) of HPLC columns by measuring the elution time of a non-retained small molecular weight tracer after having filled the micro- and mesopores of the porous support with a hydrophobic solvent that is immiscible with the mobile phase employed during the elution time measurements. This method has since then been used by Gritti and Guiochon [2] to measure the heights equivalent to a theoretical plate of an unretained compound on two packed columns having different mesopore sizes and to investigate the difference in plate height between porous and non-porous particles.

The TPB method provides an alternative to the inverse size exclusion (ISEC) method that is more conventionally used to determine ε_e [3–6] A first advantage of the TPB method over ISEC is that small MW tracers are used instead of the large MW polymer standards used in ISEC. The larger molecules that are used in ISEC to determine the interstitial space are affected by wall effects [7] and are not able to penetrate each corner of the interstitial volume, hence "missing" part of it and leading to an underestimation of ε_e. In the TPB method very small molecules can be used to measure the void volume, so that even the smallest corner of it can be accessed and sampled. Furthermore, in ISEC, the hydrodynamic forces of the flow field can partly unfold the PS strands so that their retention time can be modified by the retention of the disentangled strands in the particle pores. This is not the case in the TPB method, since no polymers are used. Another drawback of ISEC measurements is that ε_e needs to be determined from the extrapolation of two straight regression lines on a graph containing only a limited number of data points. This poor precision of the ISEC method may lead to an error in ε_e of at least a few percent [3]. In the TPB method, the external porosity is immediately calculated from the elution time of the small molecular weight marker. Provided the TPB is used at a sufficiently low flow rate (typically $F < 0.150$ ml/min in a 2.1 mm ID $\times$ 50 mm L column), this value is not affected by the flow conditions [1]. The only error source remaining in the TPB method is the inaccuracy on the measured elution time, caused by small errors on the pumping flow rate and the time registration. These errors are however also present in the ISEC method. A potential uncertainty of the TPB method is the determination of the onset of the asymptotic regime wherein the unretained tracer exactly accesses the interstitial pore space. The onset of this regime can however be simply evaluated from the stabilization of both the detector and the pressure signal [1]. The TPB method is also different from the so-called Donnan-exclusion method, where a mobile phase with a sufficiently low ionic strength is used to prevent ionic t_0-markers to enter the mesopores of the particles. This exclusion occurs when a small amount of the marker is injected at a very low concentration [8]. The fact that such a low concentration has to be used leads to very small eluting peaks and in this way a detection problem occurs [9]. Other potential error sources are the fact that the exclusion of the marker can already become effective in the interstitial space

* Corresponding author. Tel.: +32 0 2 629 32 51; fax: +32 0 2 629 32 48.
E-mail address: gedesmet@vub.ac.be (G. Desmet).

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Table 1
Overview of the different parameters measured for the different columns investigated.

Column	Blocking agent	ε_t (%)	ε_e (%)	p_{max} (bar)	V_i (ml)	$V_{mesopores}$ (ml)	ΔV_{loss} (ml)
Hypersil Gold	C7		37.64	255			0.0019 (±20%)
5 μm particles	C8		36.94	268			0.0023 (±17%)
175 Å pore size	C9	73.24	36.76	289	0.064	0.063	0.0013 (±31%)
	C10		36.67	278			0.0012 (±33%)
	C12		37.13	282			0.0013 (±31%)
Hypersil Gold	C10	74.63	38.87	522	0.067	0.062	Negligible
1.9 μm particles							
175 Å pore size							
Zorbax	C10	72.23	42.08	215	0.073	0.052	Negligible
5 μm particles							
80 Å pore size							
XBridge	C10	76.85	37.98	234	0.066	0.067	0.0029 (±14%)
5 μm particles							
300 Å pore size							

itself, and the fact that the pores of the particles can be so large that complete electrostatic repulsion is difficult to realize [10].

In this work, we present a more in-depth study of the possibilities and limitations of the TPB method. More specifically we focused on the effect of the flow rate, the pressure, the pore blocking agent and the size of the mesopores (Table 1).

2. Experiment

2.1. Chemicals and columns

Uracil (MW = 112.09 g/mol), thiourea (MW = 76.12 g/mol), sodium nitrate ($NaNO_3$, MW = 84.99 g/mol) and potassium iodide (KI, MW = 166.01 g/mol) were provided from Sigma–Aldrich (Steinheim, Germany). Isopropanol was of HPLC grade from Sigma–Aldrich (Steinheim, Germany). Heptane (99+ % pure), octane (99+ % pure), nonane (99+ % pure), decane (99+ % pure) and dodecane (99+ % pure) were purchased from Sigma–Aldrich (Steinheim, Germany). HPLC grade water was prepared in house using a Milli-Q Purification System (Millipore, Billerica, MA, USA). The Hypersil Gold columns C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 5 and 1.9 μm particles respectively were provided by Thermo Fischer Scientific (Runcorn, UK). The column (2.1 mm × 100 mm) filled with non-porous C18 coated 6.55 μm particles was provided by Thermo Fischer Scientific (Runcorn, UK). The Zorbax Bonus RP column C18 (2.1 mm × 50 mm, 80 Å pore size) filled with 5 μm particles was purchased from Agilent Technologies (Diegem, Belgium). The XBridge BEH300 column C18 (2.1 mm × 50 mm, 300 Å pore size) filled with 5 μm particles was purchased from Waters (Zellik, Belgium).

2.2. Buffer

To conduct the TPB experiments a hydrophilic buffer was prepared that consisted of 10 mM ammonium acetate (Sigma–Aldrich, Steinheim, Germany) dissolved in Milli-Q water. The pH was adjusted to pH 3.0 by adding acetic acid (Panreac, Barcelona, Spain).

2.3. Apparatus

Chromatographic data were acquired with an HPLC Agilent 1200 system (Agilent Technologies, Waldbronn, Germany) which can withstand pressures up to 600 bar. This instrument includes an auto-sampler with a 2-μl loop, a diode array detector with a 500-nl flow cell, and a column oven set at 30 °C. Data acquisition, data handling, and instrument control were performed by Chemstation (Agilent Technologies). Samples consisting of 0.1 mg/ml potassium iodide were dissolved in buffer. The injection volume was reduced to 0.5 μl. Absorbances were measured at 254 nm, using a constant rate of 40 Hz. Stainless steel tubing with an internal diameter of 120 μm and a length of 10.5 cm was used to connect the injector with the column. The column was connected to the 500-nl detector by fused silica/PEEK capillary with an internal diameter of 100 μm and a length of 30 cm.

2.4. Pore blocking procedure

The TPB procedure for reversed-phase columns described extensively by Cabooter et al. [1] was closely followed. Briefly, the method starts by rinsing the column with isopropanol that is able to dissolve both hydrophilic and hydrophobic liquids. Subsequently, the column is filled with a so-called blocking agent. This is a hydrophobic liquid that is immiscible with water and can replace the isopropanol in the micro- and mesopores of the particles because of its higher affinity for the hydrophobic layer covering the mesopore walls of reversed-phase columns. In the present study, the following blocking agents were used: heptane (C7), octane (C8), nonane (C9), decane (C10) and dodecane (C12). Finally, the blocking agent is flushed out of the interstitial space of the bed using a hydrophilic buffer which is immiscible with the hydrophobic blocking agent. The hydrophilic buffer used in the present study was obtained with a 10-mM aqueous solution of ammonium acetate adjusted to pH 3.0.

2.4.1. Measurement of the interstitial volumes

When the flushing is finished, the initially porous particles have been transformed into blocked particles that are impermeable to hydrophilic solutes, and hence behave like non-porous particles. Subsequently injecting a non-retained marker into the hydrophilic buffer and recording its mean residence time, the volume of the interstitial space can be readily found (see discussion in Section 3.2). The extra-column volume of the system was measured by replacing the column with a zero dead-volume connection piece and was found to be 0.015 ml.

The mean residence time values were recorded using the method of moments function of the Chemstation software.

To correct the obtained t_0 for the internal frit volume, a pycnometry experiment was run on the frits after having removed them from the column. First the dry mass W_{dry} of the frits was weighted. Then they were submersed in isopropanol (density ρ = 0.786 g/ml) for 5 min. They were then transferred to an Eppendorf tube and centrifuged for 3 s using the short spin option of the Eppendorf 5417 centrifuge. During the centrifugation step the frits were positioned such that their large surface sides were running parallel with the radial direction of the centrifuge. In this position, a short centrifuge step is sufficient to remove the excess of solvent on the outside of the frit, while the solvent on the inside of the frit can be expected to be maximally retained as the centrifugal forces are ori-

ented in the plane of the frits and not in their depths. The frits were subsequently weighted to determine the mass of the frits filled with isopropanol ($W_{isopropanol}$). This experiment was conducted in triplicate. The internal frit volume was then calculated using the following equation:

$$V_{frit} = \frac{W_{isopropanol} - W_{dry}}{\rho_{isopropanol} - \rho_{air}} \quad (1)$$

which leads to the frit porosity using the following equation:

$$\varepsilon_{frit} = \frac{V_{frit}}{V_{geom,frit}} \quad (2)$$

with $V_{geom,frit}$ equal to $\pi r^2 L$ where r is the internal radius of the frit (in this study $r = 1.05$ mm) and L is the length of the frit (in this study $L = 1$ mm).

2.4.2. Application of the TPB method on a non-porous particle column

To investigate whether the hydrophobic blocking agent does not form a thin layer that irreversibly sticks on the outside of the particles, thus reducing the interstitial void space during the actual volume measurements, the TPB procedure was performed on a Thermo column filled with non-porous C18 coated particles. First the elution volume of KI was measured using the ammonium acetate buffer as mobile phase. In a next step the column was blocked with decane using the TPB procedure. The decane was than flushed out of the column using the buffer and the elution volumes of KI were measured during this flushing process.

3. Results

3.1. Effect of pore blocking on the peak shape

Fig. 1 compares the pulse responses obtained in a column with closed and with open mesopores. The excellent overlap of the curves obtained during three subsequent runs demonstrates the robustness of the method. The difference in elution time is characteristic for the volume of the mesopores (difference in time is due to difference in volume that can be accessed by the t_0-marker), and this difference can be used to determine the internal porosity of the particles. Fig. 1 also reveals a clear change in peak shape. The peaks in the blocked pore case are much more tailed and skewed than those obtained in the open pore case. This has two main reasons. The first one is that the peaks in the blocked pore case elute with a smaller volume and are therefore more prone to extra-column band broadening. The second one is that the dispersion in the blocked column is larger than in the non-blocked case. This has been observed both in our lab and in Ref. [2], and was explained there as being the consequence of the fact that, when the pores are blocked, the effect of the packing is more pronounced. When measurements are performed in a column filled with porous particles the effect of the packing on the peak broadening is diminished by the mass transfer occurring in the pores of the particles. In this way velocity gradients existing in the column are less pronounced. On the other hand, when the pores of the particles are blocked, there is no more mass transfer with the stationary phase and so the velocity gradients cannot be diminished as well as when the pores are not blocked. In this way the differences in velocity can only be influenced by diffusion occurring between the particles and so the influence of the packing is more pronounced [2].

The strong tail observed in the blocked pore case also necessitates the use of the method of moments to determine t_0, as the less sophisticated methods that are often used (time at peak maximum, mean time at the peak half height) only correctly represent the total elution volume in the case of a perfectly symmetrical, Gaussian peak. A comparison between the t_0-time determined via the moment method and that via the peak half height was of the order of some 2%. Given that it is our desire that the TPB should be able to spur for differences in interstitial volume of the order of 0.5–1%, this error is intolerable.

3.2. Application of the pore blocking method to a non-porous particle column

As suggested by one reviewer, we also performed the complete TPB procedure on a non-porous particle column. In this case, comparing the measured interstitial void volume before and after having filled the column with blocking agent and having flushed the column with buffer allows to assess whether the flushing buffer is capable of removing all blocking agent from the interstitial void, or leaves a layer of hydrophobic agent sticking to the outer particle surface, thus falsifying the observed insterstitial void volume. Fig. 2 shows a chromatogram of the KI peaks eluting from a column filled with non-porous particles. As can be seen the KI peak eluting before the column was blocked overlaps with the peak eluting after blocking the column and flushing the interstitial volume with ammonium acetate buffer. This shows that the decane can be completely flushed out of the interstitial volume of the column and that the blocking agent does not irreversibly stick to the surface of the particles. Another proof is delivered in Fig. 3. This graph shows the evolution of the external porosity as a function of the flushing time. The external porosity obtained when the curve

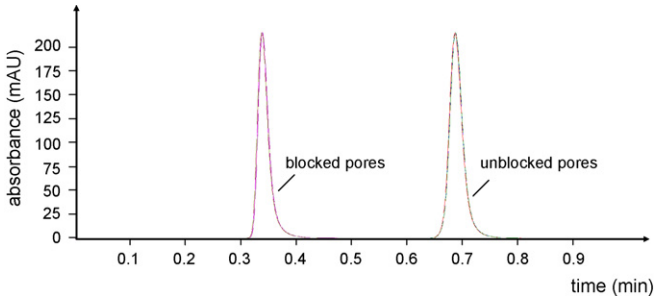

Fig. 1. Chromatogram of KI eluting from a column with blocked and unblocked mesopores. First KI was injected onto the column without having blocked the pores, hence the "unblocked pores" label in the chromatogram. In a next step the pores of the particles were filled with blocking agent and KI was reinjected onto the column after having flushed the interstitial pore space ("blocked pores" label in chromatogram). KI was, for both injection series, dissolved in the mobile phase consisting of an ammonium acetate buffer adjusted to pH 3.0. The employed flow rate was 0.150 ml/min.

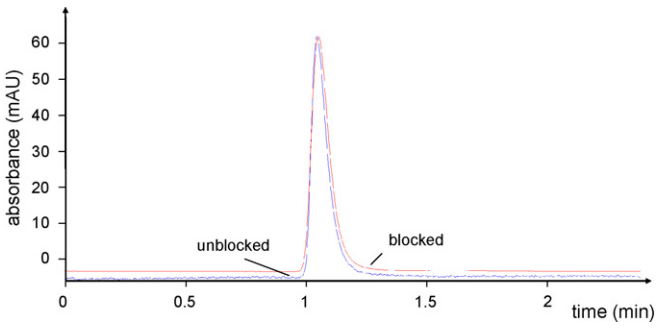

Fig. 2. Chromatogram of KI eluting from a column filled with non-porous particles. KI was first injected onto the column before performing a TPB experiment. The mobile phase employed consisted of an ammonium acetate buffer adjusted to pH 3.0. The employed flow rate was 0.150 ml/min. The KI peak eluting under these conditions ("unblocked") overlaps with the KI peak eluting from the same column after having blocked the column ("blocked") with decane and flushed the decane out of the interstitial volume.

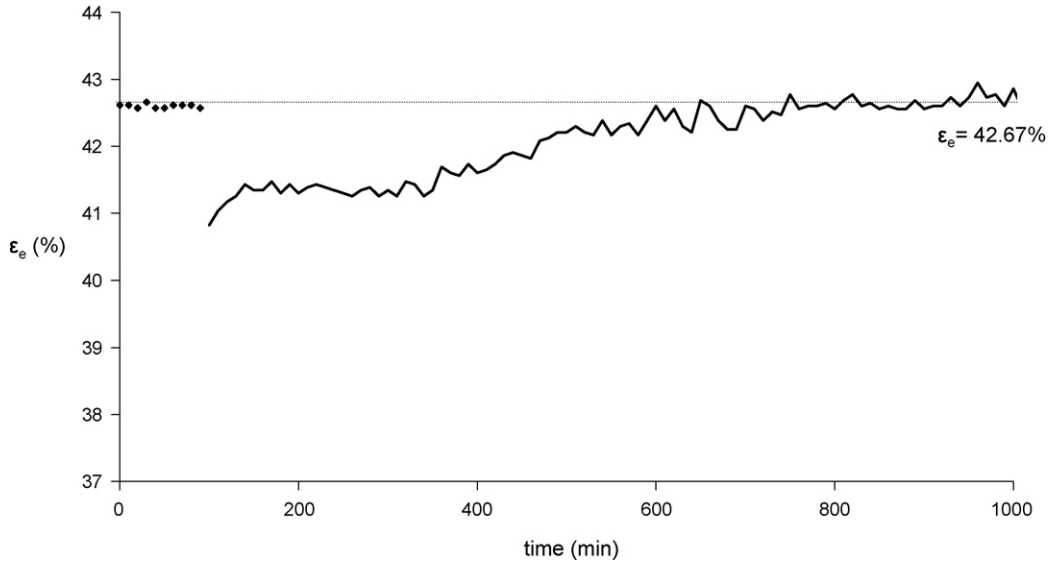

Fig. 3. Evolution of ε_e as a function of time during a TPB experiment performed on a column filled with non-porous particles. The black diamonds represent the measured ε_e values before blocking the column with decane. The full black line represents the ε_e values measured after blocking the column with decane. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase (ammonium acetate buffer adjusted to pH 3.0) in a concentration of 0.1 mg/ml, in consecutive intervals of 10 min at a flow rate of 0.150 ml/min for a total experiment time of 1000 min.

reaches a steady-state is 42.67%. This is in good agreement with the external porosity value obtained before blocking the column (ε_e = 42.59%). The difference between the external porosity measured before and after blocking the column is 0.08% which lies well within the experimental read-out error of 0.2% [1].

3.3. Accuracy of the volume measurement methods

The t_0-time that is determined by injecting the tracer compound in fact relates to the volume V_0, being the sum of V_{frit}, V_{ext} and the interstitial volume V_i:

$$V_0 = V_{frit} + V_{ext} + V_i \quad (3)$$

Hence, to isolate the value of V_i from the measured V_0, the latter value needs to be corrected. The approach (referred to as the "union method") consisted of short circuiting the inlet and outlet tubing using a zero dead-volume union piece. This then yield a value of V_{ext}, assuming the union piece does not introduce any dead-volume.

Subtracting the volume of V_{ext} from V_0 then still leaves the internal frit volume V_{frit} to be in excess of the correct V_i-value. To correct for this volume, a measurement as described in the experimental section was performed. This measurement showed that the internal frit porosity lies around 33%, corresponding to a V_{frit}-volume of 0.0023 ml (value based on the sum of two frits). The latter constitutes a correction of 1.3% to the observed V_i value. Finally ε_e can be calculated as:

$$\varepsilon_{e,union} = \frac{V_0 - V_{ext} - V_{frit}}{V_{geom}} = \frac{V_i}{V_{geom}} \quad (4)$$

with V_{geom} equal to $\pi r^2 L$ where r is the internal radius of the column and L is the length of the column. In this study all the columns had an internal diameter of 2.1 mm and a length of 50 mm, which leads to a geometrical column volume of 0.173 ml. The only exception was the column filled with non-porous particles having an internal diameter of 2.1 mm and a length of 100 mm, which leads to a geometrical column volume of 0.346 ml.

3.4. Effect of the t_0-marker and the blocking agent on the calculated elution volume at low flow rate

To investigate the effect of a possible retention of the t_0-marker on the observed elution volume, several hydrophilic markers were tested (uracil, KI, thiourea, $NaNO_3$). Each of them is usually considered as a non-retained marker, even in the case of fully porous particle columns. In the present application, the mesopores are blocked, leaving even much less possibility for retention. However, since we aim at a very high measurement accuracy (order of 0.5–1% of the elution time), it has to be carefully checked whether or not the employed t_0-marker is retained by the blocking agent itself or even by the hydrophobic coating on the outside of the particles. Doing so, it was found that uracil was most retained, leading to t_0-times that are about 0.9% larger than $NaNO_3$ and thiourea, and about 1.8% larger than sodium iodide (KI). The rest of the study has therefore been conducted with KI.

Fig. 4 shows the evolution of the measured elution volumes (here already translated into the external porosity ε_e via Eq. (4))

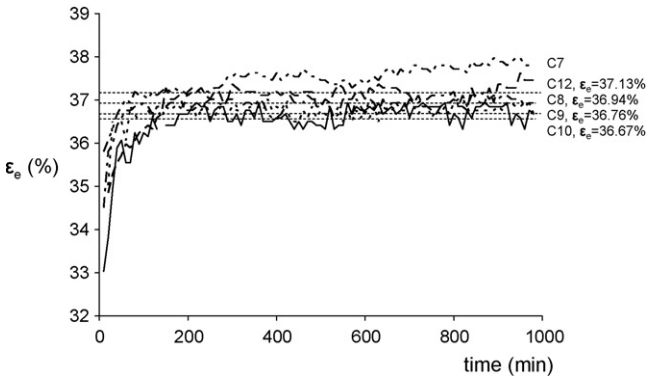

Fig. 4. Evolution of ε_e with time during TPB experiments conducted with different blocking agents. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase (ammonium acetate buffer adjusted to pH 3.0) in a concentration of 0.1 mg/ml, in consecutive intervals of 10 min at a flow rate of 0.150 ml/min for a total experiment time of 1000 min.

with time during a typical TPB experiment. As can be noted, the ε_e-curves first go through a steep rise. This rise corresponds to the period during which the blocking agent that did not enter the mesopores of the particles is gradually flushed out of the interstitial space. After a given time (roughly some 100–200 min), the ε_e-values saturate and reach their final value. The horizontal dashed lines added in Fig. 4 provide a best fit to this final value and provide a direct read-out of the ε_e-value. The obtained ε_e-values are in good agreement with the values traditionally obtained for packed beds (36–40%) [11] and also with the values obtained in Ref. [1]. There it was already shown that the ε_e-values produced by the TPB method lie close to those obtained with ISEC. The latter however showed a larger uncertainty (order 0.01 porosity units) than the former (0.002 porosity units).

As can be noted, there is no significant difference between the ε_e-values obtained with the blocking agents in the range of C8–C12, as all lie within a narrow band of 0.5% and display no particular order. A small yet significant difference can however be noted when C7 is used. In this case, the measured ε_e-values lie slightly above those obtained for C8–C12. They also display a slow yet consistent gradual increase. The latter most probably indicates a gradual loss (leakage) of the blocking agent out of the mesopores.

It should be noted that the ε_e-values presented in Fig. 4 have been obtained using the "union"-method (correction of V_0 using t_0-time of extra-column volume of instrument), and after correction for the experimentally measured frit volume. The ε_e-values we obtained (average value of about 37%) seem very reasonable, and indicate that the amount of blocking agent remaining in the interstitial volume can be neglected (otherwise values well below 37% would have been obtained).

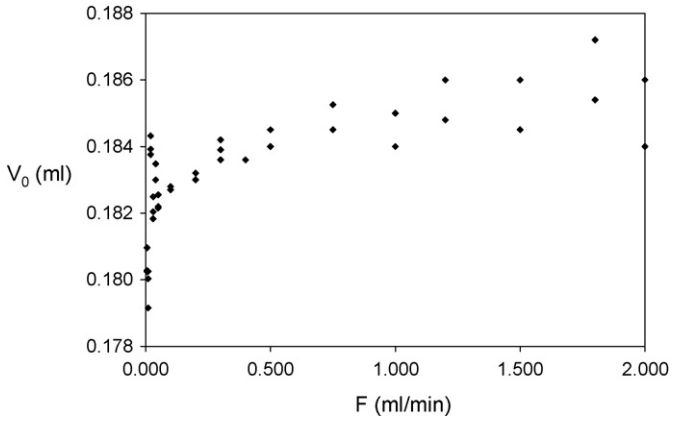

Fig. 5. Evolution of V_0 as a function of the applied flow rate. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase (ammonium acetate buffer adjusted to pH 3.0) in a concentration of 0.1 mg/ml.

Fig. 4 also shows there is a substantial variation on the obtained ε_e-values. These fluctuations (corresponding to a variation of the t_0-time of some 0.002 min) lead to a measurement uncertainty of about 0.3–0.4% of the interstitial volume and are believed to be caused by small pumping rate fluctuations. This uncertainty is not inherent to the TBP-method but is inherent to the pumping accuracy of the employed instrument. A similar variability is for example observed when making a long series of t_0-measurements of a column filled with fully porous (i.e., non-blocked) particles. To demonstrate this, Fig. 5 shows the measured volume as a func-

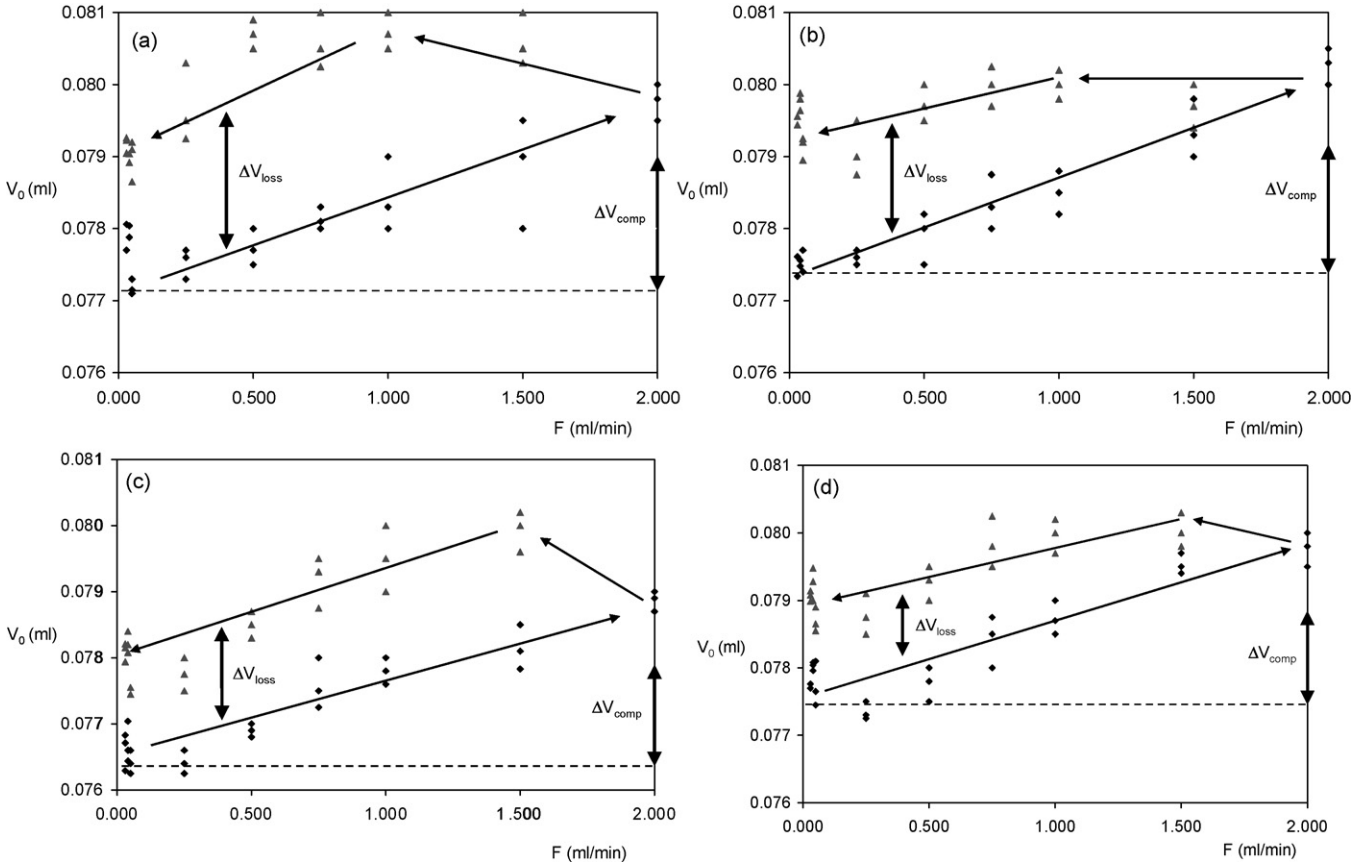

Fig. 6. Observed V_0-values as a function of the applied flow rate for (a) C7, (b) C8, (c) C10, (d) C12. Experiments were run from low to high, and back to low flow rate. The data points belonging to the "upward" series are coloured in black, the data points belonging to the "downward" series are coloured in red. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase in a concentration of 0.1 mg/ml, in triplicate at different flow rates starting from the lower flow rates and gradually going to the higher flow rates and then back to the lower flow rates. Between each change in flow rate the column was allowed to equilibrate for 5 min.

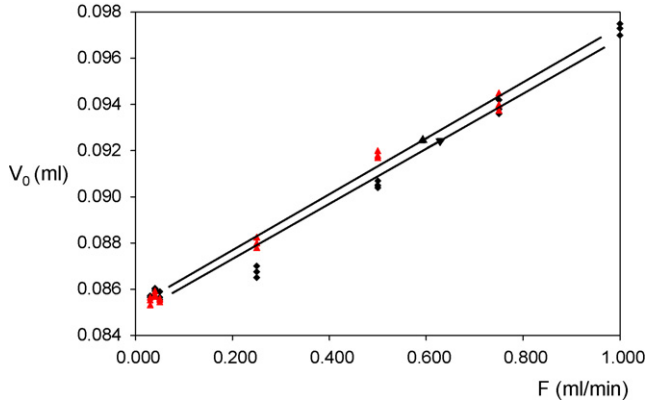

Fig. 7. Repeat of the experiment shown in Fig. 6c, but now in a 1.9-μm particle column. Experiments were run from low to high, and back to low flow rate. The data points belonging to the "upward" series are coloured in black, the data points belonging to the "downward" series are coloured in red. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase in a concentration of 0.1 mg/ml, in triplicate at different flow rates starting from the lower flow rates and gradually going to the higher flow rates and then back to the lower flow rates. Between each change in flow rate the column was allowed to equilibrate for 5 min. Comparison with Fig. 6c allows to assess the effect of pressure.

tion of the flow rate for a column filled with non-blocked particles. The observed fluctuations are of the same order as in a blocked column and can thus be fully attributed to fluctuations in the observed pumping rate. These fluctuations are also observed in the subsequent Figs. 6–9.

3.5. Effect of the flow rate on calculated elution volume

A crucial factor that might influence the ε_e-value obtained using the TPB method is the flow rate at which the experiment is conducted, as one could suspect that at higher flow rates the blocking agent might be expelled from the pores, leading to an incorrect measurement of the interstitial space volume. On the other hand, one could also suspect not all the blocking agent could be removed from the interstitial region when the experiment is run at a too low flow rate. Hence, demonstrating that the observed interstitial volume is independent of the applied flow rate is of the utmost

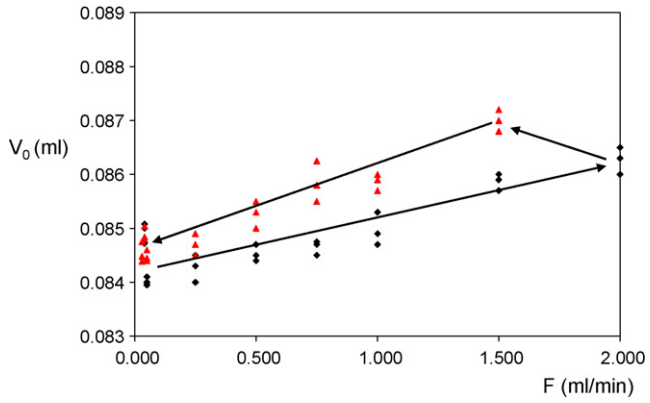

Fig. 8. Repeat of the experiment shown in Fig. 6c, but now in a 5-μm particle column with 80 Å pore size. Experiments were run from low to high, and back to low flow rate. The data points belonging to the "upward" series are coloured in black, the data points belonging to the "downward" series are coloured in red. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase in a concentration of 0.1 mg/ml, in triplicate at different flow rates starting from the lower flow rates and gradually going to the higher flow rates and then back to the lower flow rates. Between each change in flow rate the column was allowed to equilibrate for 5 min. Comparison with Fig. 6c allows to assess the effect of using a column filled with particles having a smaller pore size.

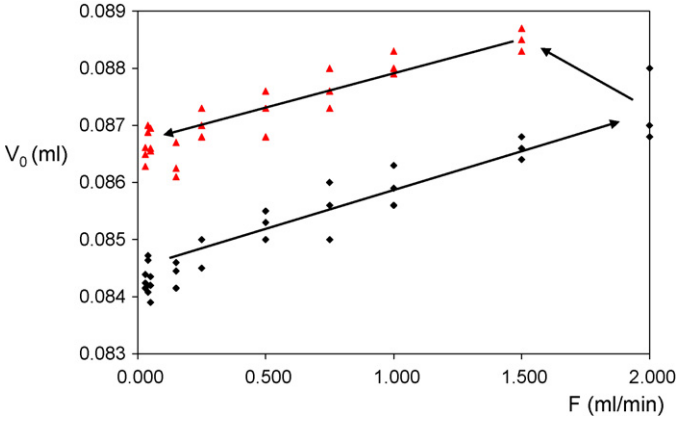

Fig. 9. Repeat of the experiment shown in Fig. 6c, but now in a 5-μm particle column with 300 Å pore size. Experiments were run from low to high, and back to low flow rate. The data points belonging to the "upward" series are coloured in black, the data points belonging to the "downward" series are coloured in red. The plot was obtained by injecting potassium iodide (KI), dissolved in the mobile phase in a concentration of 0.1 mg/ml, in triplicate at different flow rates starting from the lower flow rates and gradually going to the higher flow rates and then back to the lower flow rates. Between each change in flow rate the column was allowed to equilibrate for 5 min. Comparison with Fig. 6c allows to assess the effect of using a column filled with particles having a larger pore size.

importance. An initial exploration of the effect of the flow rate on the observed ε_e-value was already presented in Ref. [1], where it was found that, within the experimental variation, the velocity had no effect on the ε_e-values obtained via the TPB method.

In the present study, a much more elaborate series of experiments was conducted. Several of such series of experiments are plotted in Fig. 6, showing the evolution of the observed column volume with the flow rate for different considered blocking agents (C7–C12). To obtain these data, F was stepwise increased from 0.030 to 2 ml/min and then reduced again to the lowest flow rate. After switching to a new flow rate, the system was allowed 5 min to equilibrate (if ever that would be needed) and three consecutive injections of KI were performed. For a clearer visualization, the data points corresponding to the "upward" series have been coloured differently than those corresponding to the "downward" series. A first remarkable observation is that the observed volume V_0 clearly increases with increasing flow rate. This could be an indication that the blocking agent starts to leak out of the particles at higher flow rates, but as was already suggested by Gritti and Guiochon [2], the larger V_0 could also be due to the compression of the blocking agent caused by the elevated pressure accompanying the larger flow rates.

To verify this, we estimated the volumetric compression that can be expected from the increased pressure using the compressibility of alkanes tabulated in NIST and using the following Eq. (5):

$$V_{\text{blocking agent}} = (\varepsilon_t - \varepsilon_e) \times V_{\text{geom}} \quad (5)$$

which leads to the volumetric compression by:

$$\Delta V_{\text{comp}} = p \times \beta_{\text{blocking agent}} \times V_{\text{blocking agent}} + \beta_{C18} \times V_{C18} \quad (6)$$

wherein V_{C18} was calculated by assuming the same ratio of V_{C18}/V_{pore} as in Ref. [2] and wherein $\beta_{\text{blocking agent}}$ and β_{C18} are the compressibility of the blocking agent and the C18 layer respectively.

For C7, this calculation for example yields a value of $\Delta V_{\text{comp}} = 0.0017$ ml. This value, as well as the values that were calculated for the other considered blocking agents are indicated by the vertical double-headed arrow on the right hand side of each of Fig. 6a–d. As can be noted, the increase in observed V_0 is systematically larger than can be explained from the compression only. This

is in line with another interesting observation, which is that, returning back to the lower flow rate, the observed V_0 does not decrease to its initial value, as would be the case if the increase of V_0 noted during the upward series would have been caused by a pure compression effect, since the latter should be reversible. Instead, a small but significant increase of V_0 is observed for each of the different considered blocking agents. This suggests that some of the blocking agent is lost in the course of the experiment. Obviously, the "loss volume" is larger for C7 and C8 than it is for C10–C12.

3.6. Effect of pressure on calculated elution volume

To decouple the effect of pressure and flow rate, we repeated the experiments conducted on the 5-μm particle column discussed in Section 3.4 on a 1.9-μm particle column. As can be noted in Fig. 7, the upward curve now runs much steeper, but the difference in observed volume before and after the "upward" and "downward" series is much smaller than in the corresponding case in Fig. 6c.

Given the near-perfect reversibility of the variation of V_0 with F, the much steeper relation is perfectly in line with the fact that a 1.9-μm particle column requires a much larger pressure to achieve a given flow rate than a 5-μm particle column.

3.7. Effect of pore size

Comparing Fig. 8 with Fig. 6c allows to investigate the effect of the mesopore size of the particles. The elution volume in function of the applied flow rate was measured for a column filled with 5 μm particles with a 80 Å pore size. In this experiment the maximum flow rate and pressure were the same as in the experiment with the column filled with 5 μm particles with a 175-Å pore size. As can be noted the difference in observed volume before and after the "upward" and "downward" series is less pronounced in the case of the 5-μm particles with a 80-Å pore size. This implies that the blocking agent is less prone to leakage when using a column filled with particles having a low pore size, in agreement with one's physical expectations.

Further increasing the pore size, and considering 5 μm particles with a 300-Å pore size, the observed loss volume was considerably larger (ΔV = 0.0029 ml versus 0.0012 ml) than with the 175 Å material. This can be noted by comparing Fig. 9 with Fig. 6c and is again in full agreement with one's physical expectations. This observation however does not mean that the TPB method cannot be applied to 175 or 300 Å particles, because the reported leakage volumes are only obtained after having conducted the full cycle of flow rates shown in Figs. 6–9, whereas in a normal TPB experiment only one flow rate is needed, so that the leakage losses are much smaller there.

3.8. Dissolution hypothesis as an explanation for the observed variation of V_0 with F

We think the observations made in Figs. 6–9 are consistent with the fact that the blocking agent is not flushed out of the mesopores when the flow rate or the pressure are increased beyond a certain value, but that the observed loss is simply due to the fact that a small fraction of the blocking agent leaves the particles by dissolving into the mobile phase. This dissolution problem is only relevant for the experiments presented in Figs. 6–9, and not for a normal TPB experiment, as this usually lasts less long, so that the amount of blocking agent that can dissolve away is also much smaller.

A first indication for the fact that a high flow or pressure does not "mechanically" push the blocking agent out of the mesopores is that the increase in V_0-volume observed when increasing the flow rate (see Fig. 6a–d) can be largely regained by returning to the initial low flow rate value. A second indication for this fact is that when the series is reversed, the largest difference in observed V_0-value is now not observed in the low F-range but in the large F-range, so that the largest difference again occurs between the start and the end of the experimental series. This indicates that the observed loss is linked to the elapse of a large time, and not to the occurrence of some abrupt flow phenomenon. A third indication is that, the loss does not increase when much high pressures are applied (see Fig. 7), but on the contrary decreases.

To verify the fact that the loss observed in the experimental series shown in Figs. 6–9 is due to a gradual dissolution of the blocking agent, the following calculation has been made. Assuming the absence of any mass transfer limitations, the concentration of dissolved blocking agent in the mobile phase will be equal to the dissolution limit of the alkanes in water $C_{eq,mob}$. The mass of blocking agent (Δm) lost by the particles can then be calculated directly from the amount of blocking agent leaving the column with the mobile phase flow during a given time Δt:

$$\Delta m = F \times C_{eq,mob} \times \Delta t \quad (7)$$

or in terms of total elution volumes:

$$\Delta m = C_{eq,mob} \times n \times V_{geom} = C_{eq,mob} \times n' \times V_i \quad (8)$$

wherein n is the number of eluted column volumes (geometrical volume) and n' is the number of eluted interstitial volumes.

The total elution time for the experiments shown in Fig. 6 was about 4000 min, while the average flow rate was about 0.200 ml/min, corresponding to the elution of some 5000 geometrical column volumes. Considering first the experiment performed with heptane, and considering that the value of $C_{eq,mob}$ equal to the water solubility of heptane given in Ref. [12] as 2.91 mg/l solution, Eqs. (7) and (8) allow to calculate that the total mass of blocking agent that can be lost via dissolution is about 2.33 mg. Translating this in terms of volume using the density of heptane, this loss corresponds to about 0.003 ml which lies relatively closely to the loss volume for heptane observed in Fig. 6a.

Repeating the calculation for octane, and taking the value of $C_{eq,mob}$ equal to 1.39 mg/l solution [12], the total mass of blocking agent that can be lost via dissolution is about 1.11 mg. Translating this in terms of volume using the density of octane, this loss corresponds to about 0.002 ml which again lies relatively closely to the loss volume for octane observed in Fig. 6b.

The water solubilities of C10 and C12 are significantly smaller than for C7 and C8 (respectively, 0.021 mg/l solution and 0.008 mg/l solution), so that the significant "loss" observed in Fig. 6c and d cannot be readily explained from this value.

It should also be noted that the cited values of F and Δt correspond to some 5000 geometrical column volumes (n = 5000) or some 12,000 interstitial volumes (n' = 12,000). These numbers are really huge, and were only reached in the present study because we wanted to make a long-term study of the effect of the flow rate (see Fig. 6a–d). In a normal TPB experiment, the steady-state value for the observed interstitial volume is achieved after the elution of some 200 geometrical column volumes (corresponding to a time of 200–300 min in Fig. 4), or, equivalently, some 500–600 times the interstitial volume.

With such relatively low elution numbers, Eq. (8) only predicts a loss of 0.048 mg, or 0.00007 ml for octane. This constitutes only 0.1% of the interstitial volume, and is an error that can be tolerated, given the uncertainty of about 0.5% on the interstitial volume value that is anyhow unavoidable because of small inaccuracies on the effective flow rate (see variation of ε_e-values observed with time in Fig. 4). If desired, Eq. (8) can be used to correct the observed V_i-value for the loss of blocking agent via dissolution. When decane is used, the loss after the elution of 250 elution volumes (which is what is typically needed for a TPB experiment) can be estimated to

be 0.00005 ml or 0.08% of the interstitial volume (values estimated from the experimentally observed ΔV_{loss}-value shown in Fig. 6c).

The hypothesis that the loss of blocking agent (or increase of observed V_0) observed in Figs. 6–9 is due to its (slow) dissolution into the aqueous mobile phase is further corroborated by the fact that the loss of blocking agent observed in the 1.9 μm particle column (where F is smaller while p is larger compared to the 5 μm particle column) is much smaller than in the 5 μm particle column. This follows readily from Eq. (7), showing that Δm depends on the flow rate and not on the pressure.

4. Conclusions

To accurately determine the interstitial volume V_i of a reversed-phase packed bed column using the total pore blocking method, the following considerations need to be made.

Testing the pore blocking properties of linear alkanes taken from the series heptane, octane, etc. up to dodecane (C7–C12) by measuring V_0 using a buffered pure aqueous solution as the mobile phase, similar V_0-values are obtained for the series C8–C12, while the C7 experiments consistently yielded a larger V_0-value, indicating some loss of the blocking agent during the course of the experiment. This is in line with the smaller hydrophobicity and the better water solubility of the C7. Testing a series of polar, non-retained tracers as t_0-marker (uracil, KI, thiourea, $NaNO_3$), it was found that potassium iodide showed the lowest retention (about 2% in retention volume difference with uracil) and therefore most suited to correctly measure V_0.

In any case, the t_0-times of the eluting peaks need to be determined via the method of moments and not via the time of the peak maximum or the mean time at the peak half height, because the peaks that are obtained during the experiment are typically strongly asymmetric and tailed.

The most correct way to correct the observed t_0-times for the contribution of the system components and the column frits would be to measure the volume of a packed and an empty column, but the latter measurement suffers from a large uncertainty about the integration boundaries. It is therefore preferred to correct via a measurement of the extra-column volume and a separate measurement of the frit volume.

Obviously, a crucial check for the accuracy of the measured V_0-value is that it is independent of the employed flow rate F. Trying to verify this by measuring V_0 as a function of F is however not straightforward as the measured volume V_0 is first of all influenced by the fact that the blocking agent inside the particle pores is compressed by the external pressure of the mobile phase. Obviously, the larger the flow rate is, the larger this compression effect. As a consequence, the blocking agent will occupy a smaller volume and hence a larger (and overestimated) V_0 will be observed. Fortunately, this compression effect is reversible and can be estimated quite accurately via the compressibility value of the blocking agent. Doing long times series (order of several thousands of minutes), the measured V_0-values are however also affected by the slow but gradual dissolution of the blocking agent into the mobile phase. The amount lost is proportional to the number of elution volumes that have been used to sweep the column during the total experimental run. It has been found that typically 200–300 column volumes need to be swept before the interstitial volume has reached a steady-state volume, corresponding to the state wherein the blocking agent has been removed as much as possible from the interstitial space. The loss of blocking agent however only becomes apparent (assuming that a 0.1% difference in observed V_0 would be the desired accuracy limit) after having eluted some 400 geometrical column volumes. This is however much less than needed in a typical TPB experiment, where a steady-state value for V_0 is typically obtained after the elution of some 200 geometrical column volumes.

Nomenclature

L	column length (m)
F	flow rate (ml/min)
t_0	analysis time of an unretained component (min)
W	weight (g)
V_0	void volume of a column (ml)
V_i	interstitial volume of a column (ml)
V_{geom}	geometrical volume of a column (ml)
V_{frit}	volume of a frit (ml)
V_{ext}	external volume (ml)
$C_{eq,mob}$	solubility blocking agent in mobile phase at equilibrium (mg/l)
Δm	mass loss (mg)
n	number of eluted geometrical column volumes
n'	number of eluted interstitial column volumes

Greek symbols

ε_e	external porosity of a column
ε_t	total porosity of a column
ε_{frit}	total porosity of a frit
ρ	density (g/l)
β	compressibility (bar^{-1})

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