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Short communication

Use of pressure drop profiles to assess the accuracy of Total Pore Blocking measurements of the external porosity of chromatographic columns

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ABSTRACT

By comparing the pressure drop in a column where the meso-pores of the particles have been blocked using the Total Pore Blocking (TPB) method to measure the interstitial volume of the column with that in the same column when the particle meso-pores are fully open, it could be demonstrated in a very sensitive way that the interstitial volume is completely devoid of any significant amount of remaining pore blocking agent in the final phase of a TPB experiment. Monitoring the pressure signal until it returns to its original value can hence be used as a reliable indicator that all blocking agent has been removed from the interstitial void at the end of the flushing period. As a consequence, any small molecular weight dead volume marker that is employed in this phase can explore the full interstitial volume, so that the value of the latter can be measured without being underestimated by the fact that some fractions of the interstitial void would still be occupied by the blocking agent.

1. Introduction

In 2007, our group proposed the so-called Total Pore Blocking technique [1] as an alternative to the customarily employed ISEC-method as a means to determine the interstitial volume (volume between the particles) and the external porosity ε_e of reversed phase chromatographic columns [2–5]. Since then, the technique has been used in several studies [6–10]. Correct measurements of ε_e are important as this value influences both the permeability and the band broadening of chromatographic columns (ε_e appears in both the expressions for pressure drop ΔP and for plate height H). Briefly, the TPB method consists of measuring the elution time of a non-retained small molecular weight marker after having reversibly blocked 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 [1].

One of the advantages of the TPB-method over the more conventionally employed ISEC technique is that small MW tracers can be used instead of the large MW polymer standards that need to be used in ISEC. The larger molecules that are used in ISEC can be affected by steric wall effects [11], so that they cannot explore the entire interstitial void volume. In ISEC, the hydrodynamic forces of the flow field can furthermore also lead to a partial unfolding of the PS strands, thus modifying their retention. This is not the case in the TPB-method where no polymers are used. A third 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, which may lead to an error in ε_e of at least a few percent [12]. In the TPB-method, the external porosity is immediately calculated from the elution time of the small molecular weight marker. Provided it could be ascertained that the pore blocking agent exactly fills up the meso- and micro-pore space of the particles, and nothing less or more, the only error source in the TPB-method would be the inaccuracy on the measured elution time, which is only subject to small errors on the pumping flow rate and the time registration errors that anyhow also contribute to the ISEC accuracy [1].

In the Donnan-exclusion method a mobile phase with a sufficiently low ionic strength (such as a 70/30 acetonitrile/water mixture in reversed phase conditions) is used to prevent ionic t_0-markers (such as nitrate ions) to enter the mesopores of the particles. This exclusion only occurs when a small amount of the marker is injected at a very low concentration [13], which may lead to a detection problem [12]. Compared to the ε_e-measurement methods based on the Donnan effect, the TPB has the advantage that it is not based on the electrostatic repulsion of the marker from the mesopores of the particles. Detection problems do not occur in the TPB method since the repulsion of the t_0-marker is based on the fact that it cannot enter the mesopores due to the presence of the blocking agent. Additional potential error sources of the Donnan exclusion are the fact that the exclusion of the marker can already occur in the interstitial space itself and that the pores of the particles can be so large that complete electrostatic repulsion is difficult to realize [14]. The TPB method, in contrary, is valid for the measure-

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ment of the external porosity even for columns filled with particles having large pore sizes, up to 300 Å [15].

However, since there is no standard method with which the external porosity of a chromatographic column can be determined exactly, it is impossible to quantify and compare the accuracy of the TPB-method with respect to these other methods since in a practical experiment the correct ε_e-value is unknown. As a consequence, it is for example not possible to fully remove the doubt over the fact that a possible fraction of the pore blocking agent used in the TPB-method would stick irreversibly to the outer surface of the particles, thus filling up the smallest pockets of the interstitial void and leading to an underestimation of the external porosity.

In the present study, we address this problem by making a detailed study of the pressure drop observed before and after the pore blocking experiment. Representing the established flow via the unretained marker velocity u_0 ($u_0 = L/t_0$, with t_0 the elution time of an unretained marker), the pressure drop in a chromatographic column can be expressed using the following variant of Kozeny Carman's law:

$$\Delta P = \frac{180}{d_p^2}\frac{(1-\varepsilon_e)^2}{\varepsilon_e^2}\frac{\varepsilon_t}{\varepsilon_e}u_0\eta L \quad (1)$$

wherein ΔP is the pressure drop over the column, d_p the diameter of particles making up the packed bed, ε_t the total bed porosity, η the viscosity of the mobile phase and L the length of the column. Taking a typical intra-particle porosity of $\varepsilon_{int} = 0.4$ (needed to calculate the value of the total bed porosity $\varepsilon_t = \varepsilon_e + (1-\varepsilon_e)\varepsilon_{int}$), it can be readily verified from Eq. (1) that if a layer of blocking agent would irreversibly stick to the particles' surface and reduce the effective external porosity experienced by the aqueous buffer flow used during the dead volume marker experiments from for example $\varepsilon_e = 0.38$–0.36 (about 5% change), this would already lead to a difference in pressure drop of about 23%. Similar values are obtained for the other practically relevant values of ε_{int}.

This shows that a measurement of the pressure drop is very sensitive to the value of ε_e. As a consequence, a measurement of ΔP at the beginning and the end of the TPB-experiment (both measurements carried out using the same aqueous buffer liquid) should indicate whether or not there is indeed some blocking agent remaining in the interstitial void of the column.

2. Experimental

2.1. Chemicals and columns

Potassium iodide (KI, MW = 166.01 g/mol) was provided from Sigma–Aldrich (Steinheim, Germany). HPLC grade isopropanol and decane (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). A wide variety of the Hypersil Gold columns C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 3 μm, 5 μm and 1.9 μm particles as well as columns filled with mixtures of 1.9 μm particles with 3 μm and 5 μm particles in ratios of 75:25, 50:50 and 25:75, respectively were provided by Thermo Fischer Scientific (Runcorn, UK).

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). Ammonium acetate buffers are typical buffers that are widely used in HPLC. The pH was deliberately set at 3.0 to protonate the silanol groups present on the silica (pK_a of silanol is ~3.5) and thereby making the silanols less available for interaction with the t_0-marker.

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 2 μl flow cell, and a column oven set at 30 °C. Data acquisition, data handling, and instrument control were performed by Chemstation (Agilent Technologies). Absorbance was measured using a diode array detector with a wavelength set at 254 nm, using a sampling rate of 40 Hz.

2.4. Pore blocking procedure

The TPB-procedure for reversed-phase columns as described in [1] was closely followed. The method starts by rinsing the column with isopropanol that is able to dissolve both hydrophilic and hydrophobic liquids. In the next step, 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 decane was used as blocking agent. In the next step, the blocking agent is flushed out of the interstitial space of the column 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. Samples consisting of potassium iodide (KI) were dissolved in the ammonium acetate buffer to a final concentration of 1 mg/ml and were injected onto the column every 10 min (injection volume = 0.5 μl). At the end of the flushing step, the column is supposed to be in a steady state wherein the pores of the particles are blocked with decane. At this point, the elution time measurement of the unretained marker KI (fully miscible in the aqueous buffer and with negligible affinity for the decane occupying the particle mesopores) can be started to measure the value of the interstitial space V_i given by the following equation:

$$V_i = F \times t_i \quad (2)$$

where F is the flow rate used for flushing the column (ml/min) and t_i is the time upon which the injected marker elutes from the column (min).

The interstitial volume V_i needs to be corrected for the extra-column volume of the system V_{ext}. This volume was measured by replacing the column with a zero dead volume connection piece and was found to be 0.015 ml. The interstitial volume V_i also needs to be corrected for the volume of the frits (V_{frit}) that are present in the column and which correspond to a correction of 0.0023 ml [15]. The external porosity of the column can than be calculated by using the following equation:

$$\varepsilon_e = \frac{V_i - V_{ext} - V_{frit}}{V_{geom}} \quad (3)$$

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 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.

After performing a TPB experiment, the column can be restored to its original state by flushing with isopropanol which will remove the blocking agent from the pores of the particles.

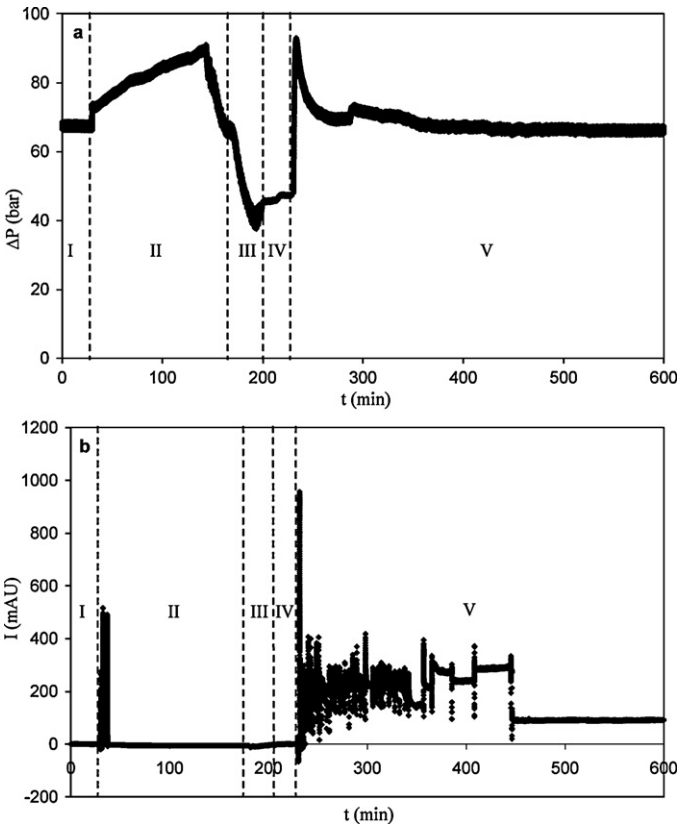

Fig. 1. Overview of (a) the pressure ΔP and (b) the UV-signal intensity I monitored as a function of time of a complete TPB experiment on the Thermo Hypersil Gold column C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 1.9 μm particles. The different phases represent the different steps in the TPB experiment (see text).

3. Results and discussion

3.1. Change in pressure and UV-signal typically measured during a TPB-experiment

Fig. 1a shows the pressure monitoring on the Thermo Hypersil Gold column C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 1.9 μm particles of a complete TPB experiment starting with the flushing of the column with aqueous buffer at 0.150 ml/min during 30 min (phase I). In this phase, the pressure basically remains constant, equalling a value of 68 bar in the presently considered example. In the next phase, phase II, the column is filled with isopropanol at 0.100 ml/min during 120 min. During this phase, the pressure goes through a maximum, caused by the fact that mixtures of water/isopropanol reach a maximal viscosity at some intermediate composition. In phase III, an isopropanol/decane gradient is run from 100/0 to 0/100 (v%/v%) to gradually replace the isopropanol that fills up the column by decane. The gradient runs over 60 min at 0.100 ml/min. In this phase, the pressure gradually decreases as more decane is flushed through the column. This is due to the fact that decane has a lower viscosity than isopropanol. In phase IV, the column is flushed with decane at 0.100 ml/min during 20 min in order to ascertain that the pressure signal is indeed constant, indicating that the column is fully filled with decane. In the last phase (phase V) the interstitial volume of the column is flushed with buffer at 0.150 ml/min during 400 min. It is seen that the pressure displays a sharp rise at the beginning of the flushing. This reflects the moment wherein the major fraction of the decane occupying the interstitial void is pushed out of the column by the immiscible buffer. This requires a certain amount of mechanical force leading to a higher pressure. Once the majority of the decane is flushed out of the interstitial void, the pressure decreases and gradually attains its equilibrium value (66 bar in the example shown in Fig. 1a) and no longer significantly changes beyond this point. This means that the composition of the liquid leaving the column no longer changes. Since the column is continuously flushed with pure aqueous buffer during this period, this implies that the liquid leaving the column also is the pure aqueous buffer.

Fig. 1b shows the UV-signal monitored at 254 nm corresponding to the pressure signal shown in Fig. 1. During phase I, the UV-signal remains constant for obvious reasons. At the beginning of phase II, the UV-signal shows a disturbance, corresponding to the period of increasing pressure due to the replacement of the aqueous buffer by the isopropanol. During phase III, a small gradual disturbance in the UV-signal is noticed corresponding to the gradual replacement of the isopropanol by the decane. During phase IV, the UV-signal remains stable again, corresponding to the constant flow of pure decane passing through the column. In phase V, wherein the decane is supposed to be pushed out of the interstitial void and replaced by the aqueous buffer, the UV-signal undergoes a series of strong disturbances during the 200 first minutes, corresponding to the period wherein also the pressure displayed some strong variations. It is assumed that, in this period, first a large amount of decane is removed from the most accessible spaces of the interstitial void, followed by the removal of some smaller amounts of decane, probably originating from the more stagnant regions of the interstitial void. At the end of phase V, the UV-signal remains stable, in agreement with the constant pressure signal noted in Fig. 1a and indicating that no more decane is leaving the column.

3.2. Change in ε_e-value measured during a TPB-experiment

At the end of stage V, the column is supposed to be in a steady-state wherein the pores of the particles are blocked with decane. Fig. 2 shows the evolution of ε_e as a function of time corresponding to the signals of phase V in Fig. 1a and b on the Thermo Hypersil Gold column C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 1.9 μm particles. It can be seen that the measured ε_e-value increases during the first 200 min of the flushing of the column with buffer. This increase is due to the fact that progressively more and more space of the interstitial void is accessible to the KI marker dissolved in the aqueous buffer as more and more of the decane is flushed out of the interstitial void. After 200 min the ε_e-value stabilizes and after another 200 min it reaches its final stable value of 37.6%.

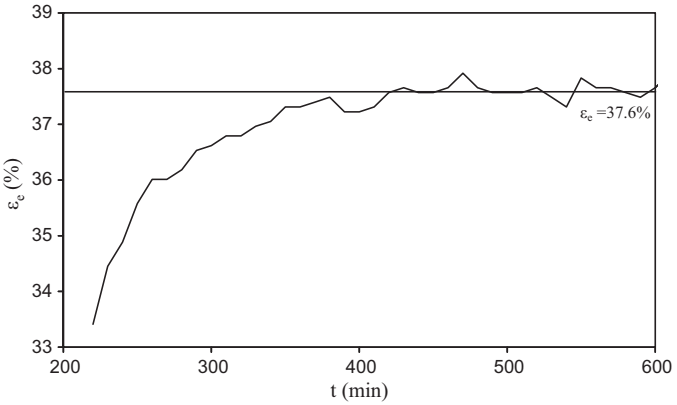

Fig. 2. Evolution of the external porosity ε_e as a function of time during the TPB experiment conducted in phase V of Fig. 1a and b on the Thermo Hypersil Gold column C18 (2.1 mm × 50 mm, 175 Å pore size) filled with 1.9 μm particles.

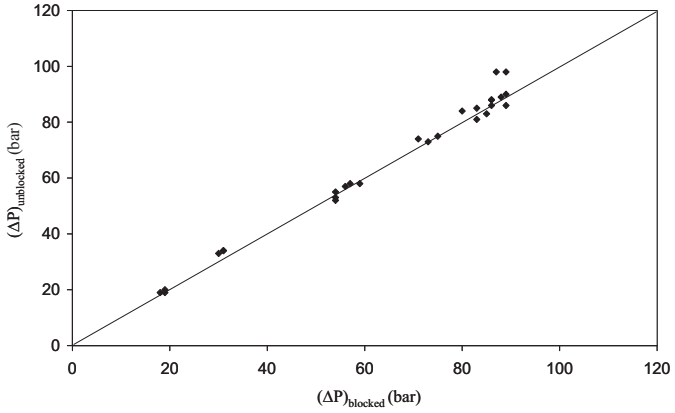

Fig. 3. Overview of all the measured pressure drop data ΔP before and after the blocking of the mesopores. The straight line represents the best fitting straight line passing through the origin.

3.3. Analysis of pressure measurements before and after performing a TPB experiment

Experiments similar to that represented in Figs. 1–2 have been repeated for all considered test columns, all packed with the same particle type but with different size and size mixtures. Fig. 3 compiles all collected pressure drop data, measured with pure aqueous buffer before and after the blocking of the mesopores. As can be noted, all data points nicely cluster around the bisector line, indicating that the pressure drop before and after the filling of the pores with blocking agent is the same. According to Eq. (1) this indicates that the mobile phase flow experiences the same external porosity ε_e before and after the filling of the pores with blocking agent. This in turn indicates that the pore blocking agent that was occupying the entire column during phase IV of the TPB-procedure (see Fig. 1) is again fully removed from the interstitial void space at the end of phase V, at least to such an extent that it does not influence the value of ε_e that is measured via the pressure drop. Given that the pressure measurement is even more sensitive to an error on ε_e than the dead volume measurement carried out during the actual TPB-experiment itself (a deviation of 0.01 in absolute units on the ε_e-value would according to Eq. (1) lead to an error of 10.33% on the pressure drop, whereas the error on the dead volume measurement would still only be 2.58%), it can be concluded that the determination of ε_e via the TPB method is not subject to any significant error originating from a fraction of the blocking agent remaining in the interstitial void volume while the actual ε_e-measurement is being made, i.e., at the end of phase V.

Conducting a regression analysis on the data in Fig. 3 yields a value of 0.993 for the slope of the best fitting straight line passing through the origin, with an R^2-value of 0.998 and a standard deviation of 0.008. With this standard deviation, the slope value of 0.993 falls within the 68%-confidence interval of the theoretically expected value of slope = 1.000 which would be expected if there would be no difference in pressure drop before and after the blocking of the pores. The observed deviation (0.7%) from the bisector line can most probably be fully attributed to the experimental error, as it is of the same order as the typical variation on the pressure signal observed under normal lab conditions.

Moreover, since a slope with a value of 0.993 corresponds to the situation wherein the pressure drop is slightly smaller (some 0.7%) after than before the pore blocking, the hypothesis that any significant amount of pore blocking agent would remain in the interstitial void at the end of the flushing process can be confidently rejected since this would actually lead to an increase of the observed pressure instead of a decrease. These results are in line with those found in a former experiment where a complete TPB experiment was performed on a column filled with non-porous particles. The interstitial volume of the column was measured before and after having filled the column with blocking agent and having flushed this blocking agent from the column with ammonium acetate buffer pH 3.0. It was found that the ε_e-values measured before and after having performed the TPB experiment were in very good agreement (42.6% and 42.7%, respectively) and the difference between these values fell within the experimental error of 0.2% [15].

4. Conclusions

Having collected a large collection of pressure drop data, measured before and after the blocking of the mesopores as is done during the TPB-method, no significant difference in pressure drop could be detected. Since the pressure drop is in itself more sensitive to a deviation in ε_e than the dead time marker experiment that is normally used to determine its value (ΔP depends in a strongly non-linear way on the value of ε_e, whereas the relation between the measured dead time and ε_e is purely linear), this allows to conclude that the mobile phase flow experiences the same external porosity ε_e before and after the blocking of the pores. As a consequence, it can be concluded that the ε_e-value measured at the end of period V is not distorted by any fraction of the blocking agent remaining in the interstitial void volume.

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