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Efficiency of the same neat silica column in hydrophilic interaction chromatography and *per* aqueous liquid chromatography

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ABSTRACT

The dependencies on the mobile phase flow velocity of the efficiency of a column packed with shell particles of neat porous silica (Halo) was measured under two different sets of experimental conditions. These conditions corresponded to the retention mechanisms of *per* aqueous liquid chromatography (PALC) at low acetonitrile concentrations and of hydrophilic interaction chromatography (HILIC) at high acetonitrile concentrations. The results are compared. Small amounts of a diluted solution of caffeine were injected in order to record the chromatograms under strictly linear conditions. These efficiencies were measured in both water-rich (PALC retention mechanism) and acetonitrile-rich (HILIC mechanism) mobile phases for the same retention factors, between 0.25 and 2.5. The mobile phases were mixtures of acetonitrile and water containing neither supporting salt nor buffer component. At low retention factors, the efficiency of caffeine is better in the PALC than in the HILIC mode. For $k' = 0.5$, the minimum reduced height equivalent to a theoretical plate (HETP) is close to 2.5 in PALC while it exceeds 5 in HILIC. The converse is true for high retention factors. For $k' > 2.5$, the HETP is lower in HILIC than in PALC, because the major contribution to band broadening and peak tailing in this latter mode originates from the heterogeneous thermodynamics of retention and eventually restricts column performance in PALC. Most interestingly, the reduced HETP measured in HILIC for caffeine never falls below 4. This suggests that the mass transfer of caffeine between the multilayer adsorbed phase (due to the interactions of the strong solvent and the silanol groups) and the acetonitrile-rich bulk eluent is slow.

1. Introduction

Hydrophilic interactions chromatography (HILIC) is an effective alternative to reversed-phase liquid chromatography (RPLC) for the separation of samples containing very polar compounds [1]. HILIC is based on the distribution of the sample between a polar adsorbent surface (silica, diol, aminopropyl, and zwitterionic phases) and a bulk aqueous phase, usually rich in organic solvent such as acetonitrile [2,3]. While the mechanism of retention in HILIC is still debated [4], the analysis of the elution times of water insoluble samples [5] and the minor plateau perturbation method [6] showed that a water-rich adsorbed multilayer (at least two layers) builds up on the surface of polar stationary phases when acetonitrile-rich (80–90%) bulk mobile phases are used. On the one hand, the more polar the compound, the higher its concentration in the adsorbed layers of water at equilibrium, suggesting that HILIC could have a partition mechanism. On the other hand, the retention of polar compounds increases with increasing water concentration in the mobile phase [7]. These observations demonstrate that partition alone does not govern the retention mechanism of polar samples in HILIC. Analytes compete with water for adsorption onto the polar sites at the stationary phase surface (silanols of silica, amino and zwitterionic groups of silica-bonded phases).

The current shortage of acetonitrile forces scientists in academic and industrial laboratories to consider replacing either acetonitrile as a component of mobile phases or acetonitrile-consuming separation modes with other ones using different solvents. The first option would be to use ethanol or propanol instead of acetonitrile. Ethanol has about the same elution strength as acetonitrile, performs reasonably well, and costs much less, so it is a suitable alternative solvent [8]. A second option is to take advantage of the hydrophobic character of siloxane groups at the surface of silica by using water-rich eluents in *per* aqueous liquid chromatography (PALC) [7]. Although good retention factors can be achieved in PALC, the adsorption mechanism is heterogeneous and involves active adsorption sites, which results in column overloading taking place with rather small samples. In contrast, HILIC shows less peak tailing upon overloading the column. This was illustrated by the measurement of the adsorption isotherm of pyridine by frontal analysis and by the calculation of its adsorption energy

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distribution function onto silica under PALC and HILIC conditions [6].

Regarding the comparative mass transfer of polar samples under PALC and HILIC conditions, few or no data have been reported in the literature [7]. Ikegami et al. recently reported on the separation efficiency of a wide collection of packed and monolithic columns in hydrophilic interaction chromatography [9]. These authors essentially discussed the role of the surface modification of the stationary phase on the performance of packed and monolithic HILIC columns. Bioanalytical liquid chromatography applications were reviewed and the limits observed in their separation efficiencies were discussed [10]. The pH and ionic strength of eluents are also fundamental parameters that still need to be investigated in the retention and adsorption mechanism of polar compounds in both HILIC and PALC modes.

In this work, we investigate the mass transfer of caffeine in the PALC and HILIC modes in order to assess the possible alternative of PALC as a replacement of HILIC processes in liquid chromatography for the analysis and separation of very polar compounds. Neat mixtures of acetonitrile and water were used as the mobile phases. Neither supporting salts nor buffers were added to the eluent so the compound can only compete with the two eluent components for adsorption onto the Halo silica. The analyte was the unprotonated caffeine, which contains four nitrogen atoms, so is suitable for retention under HILIC conditions. At the same time, caffeine is a rather bulky molecule ($M = 194$ g/mol), which is significantly retained in water-rich eluents (PALC mode) by hydrophobic interactions. The minimum sample load was injected in order to reach the linear range of the adsorption isotherm of caffeine and the minimum HETPs measured under PALC and HILIC conditions are compared and discussed. The selection of the chromatographic mode that optimizes the column efficiency at a fixed retention factor is discussed in detail.

2. Theory

The column reduced HETP was measured from the elution time of the peak at its apex and its bandwidth at exactly half its height. This method ignores the consequences of a possible peak asymmetry due to the tailing and/or the fronting of the peak close to its base. It is accurate when the sample concentration is minimum and the adsorption isotherm remains linear (Henry's domain). The three corresponding elution times are t_R (peak apex), $t_{f,1/2}$ (adsorption front) and $t_{r,1/2}$ (desorption rear). The same procedure is respected for the analysis of the extra-column band profiles and the measurement of the elution times $t_{R,ex}$, $t_{f,1/2,ex}$, and $t_{r,1/2,ex}$.

The reduced HETP is given by:

$$h = \frac{L}{d_p} \frac{(t_{r,1/2} - t_{f,1/2})^2 - (t_{r,1/2,ex} - t_{f,1/2,ex})^2}{5.545(t_R - t_{R,ex})^2} \quad (1)$$

where L is the column length and d_p is the average particle diameter.

3. Experimental

3.1. Chemicals

The mobile phases used in this work were *eight* mixtures of acetonitrile and water. These two solvents were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). The mobile phases were filtered before use on a surfactant-free cellulose acetate filter membrane, 0.2 μm pore size (Suwannee, GA, USA). Dichloromethane was also used in the pycnometry experiment. Caffeine (purity > 99%) was purchased from Aldrich (Milwaukee, WI, USA).

3.2. Materials

The experiments were made with a 150 × 4.6 mm HILIC column packed with 2.7 μm Halo particles. It was a gift from the column manufacturer (Advanced Materials Technology, Wilmington, DE, USA). The main characteristics of the bare porous silica and those of the packed columns are summarized in Table 1 of reference [6].

The hold-up volume of this column was derived from pycnometric measurements made at 295 K, under atmospheric pressure, using acetonitrile and dichloromethane as the two solvents. The densities of these two solvents at 295 K are 0.782 and 1.325 ± 0.001 g/cm^3, respectively. The column hold-up volume was 1.537 ± 0.008 mL.

3.3. Apparatus

An Agilent 1090 liquid chromatograph was used to perform the measurements. This instrument includes a ternary solvent delivery system (solvent paths A, B, and C), an auto-sampler with a 25 μL sample loop, a diode-array UV detector (cell volume 1.7 μL, sampling rate 25 Hz), a column oven, and a data station running the data software. From the injector seat to the detector cell, the total extra-column volume of the instrument is 45 μL, measured from the injections of caffeine with a zero-volume union connector in place of the column.

All measurements were carried out at a constant laboratory temperature of 295 K, fixed by the laboratory temperature control system. The daily variation of the ambient temperature never exceeded ±1 K.

3.4. HETP measurements

The sample volume injected was 0.5 μL. The concentration of caffeine was chosen as small as possible so that the minimum signal-to-noise ratio was about 20 and the measurement of the reduced HETP remains precise. An initial concentration of 0.5 g/L was prepared and further diluted until the signal-to-noise ratio was about 20. The final concentration was about 1×10^{-2} g/L and the absorbance of caffeine recorded at 275 nm (maximum of caffeine UV spectrum) was as low as 2.5 mAU. The amplitude of the signal noise is about 0.1 mAU. Fig. 1 shows the corresponding peak profile recorded under PALC conditions (0.4% of acetonitrile in water) for $k' \simeq 2$.

4. Results and discussion

4.1. Retention pattern of caffeine onto Halo HILIC silica

Before measuring the reduced HETPs of caffeine on the Halo silica column, the retention factor of caffeine was measured from water-rich (PALC mode) to acetonitrile-rich (HILIC mode) mobile phases. The volume fractions of acetonitrile in the aqueous mobile phase (0, 1, 2, 3, 4, 5, 10, 20, 30, 50, 75, 90, 95, 99, and 100% acetonitrile in volume) were automatically fixed by the pump mixer (lower pressure compliance) of the HP1090 chromatograph (pump A: pure acetonitrile; pump B: pure water). Fig. 2 shows the plot of the retention factor of caffeine *versus* the volume fraction of acetonitrile in the eluent. An almost perfect quartic U-shaped retention pattern is observed with a minimum of retention for the 50/50 water–acetonitrile mixture ($k' \simeq 0.2$) and a maximum of retention with both pure solvents ($k' \simeq 2.5$).

Similar U-shaped retention pattern have been observed with a wide variety of stationary phases and mobile phase conditions [11–14]. U-shape retention pattern are usually observed with the most hydrophobic neutral HILIC compounds so that, when the water content of the mobile phase is very high, the sample is as

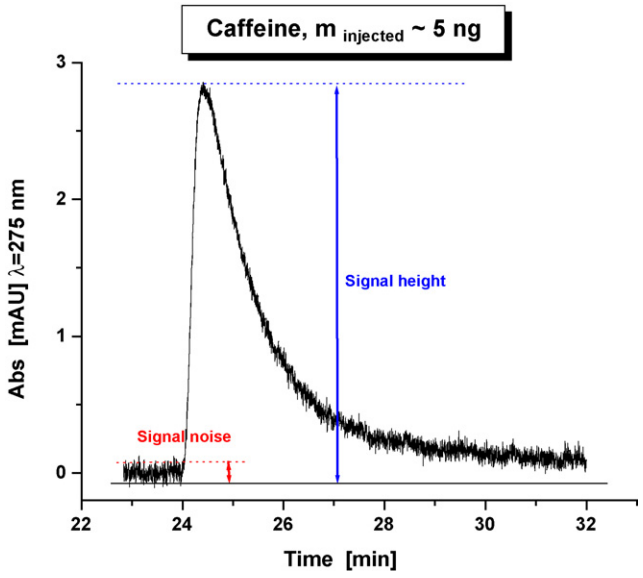

Fig. 1. Example of caffeine peak profile recorded onto the 150 × 4.6 mm Halo silica column under PALC conditions (0.4% of acetonitrile in water). About 5 ng were injected into the column. Flow rate: 0.2 mL/min, $T = 295$ K. Note, despite the low signal-to-noise ratio ($\simeq 20$), the column overloading.

strongly adsorbed as in RPLC. The experimental demonstration was made by Sandra and co-workers [7] who showed that only the most hydrophobic amino-acids (isoleucine, leucine) followed a U-shape pattern on a Zorbax Rx-SIL adsorbent while the less hydrophobic amino acids do not (glutamic acid, lysine). Ionizable HILIC compounds can also be strongly retained in water-rich mobile phases, thanks to electrostatic ion-exchange interactions with the electrically charged stationary phase [15]. In the case of caffeine, strong ion-exchange interactions are not possible because caffeine remains neutral in a water-rich environment. The large increase of retention of caffeine is mainly due to hydrophobic interactions. We note that the variation of k' is very steep when the content of either water or acetonitrile varies between 0 and 5%. Within these concentration ranges, the accumulation of water and acetonitrile onto the Halo silica is maximum as illustrated from their respective excess of adsorption [6]. The change in the composition of the adsorbed eluent multilayer is maximum so the retention factors are strongly sensitive to little change in the bulk concentration.

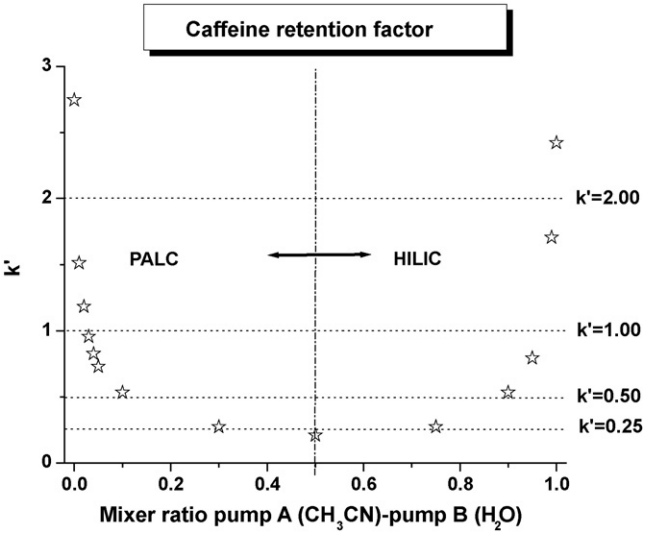

Fig. 2. Plot of the retention factor of caffeine onto the Halo silica as a function of the acetonitrile content in the mobile phase, $T = 295$ K. Note the U-shape of the plot delimiting the PALC mode (at low acetonitrile content) and the HILIC mode (at high acetonitrile content).

In this work, we did not investigate the adsorption mechanism of caffeine by measuring its adsorption isotherm and calculating its adsorption energy distribution under both PALC and HILIC conditions. This work has already been done with pyridine [6]. A similar retention pattern was observed with the minimum of retention shifted towards higher acetonitrile concentrations (80%). Sandra and co-workers [7] found a retention minimum with only 25% of acetonitrile in the mobile phase for the amino acids leucine, proline, and isoleucine. Note that a 20 mM ammonium formate buffer at pH 3 was added to the mobile phase. For even more polar amino acids such as glutamic acid, the increase of retention in water-rich mobile phases even disappears meaning that hydrophobic interactions are eventually negligible. The analysis of the adsorption isotherms and AEDs of pyridine revealed that the retention in PALC originated from the presence of several active (high adsorption energy) sites which represented no more than a few percent of the total saturation capacity of the Halo silica column. In contrast, under HILIC conditions, the retention of pyridine was controlled by the adsorption on weaker but more abundant adsorption sites which gather all types of silanol groups (single, vicinal, geminal).

The question raised in this work is to know whether such a fundamental difference in the adsorption mechanism observed under PALC and HILIC conditions could have repercussions on the mass transfer of caffeine, e.g. on the column efficiency. In other words, do we perform better peak resolution using PALC or HILIC for a fixed retention factor? The answer to that question will be discussed in the next section based on the efficiency data recorded with the sample caffeine.

4.2. Should we prefer PALC or HILIC for better peak resolution?

It is important to recall that neither supporting salt nor buffer additives were used in the acetonitrile-water mixtures in order to maintain the ionic strength and the pH of the mobile phase constant. The sample caffeine remains neutral in either pure water or acetonitrile. Hence, caffeine does not compete for adsorption nor pairs up with any compounds dissolved in the mobile phase. The ionization state of the silanol groups of Halo silica is not controlled. The pK_a distribution of the silanol populations of the Halo silica is unknown. They are likely protonated under HILIC conditions since acetonitrile is a poorly dissociative solvent. In water-rich conditions, some silanol can be at least partially dissociated if their ${}^{W}_{W}pK_a$ is smaller than 8 [16]. This should not have a strong influence on the retention and mass transfer resistance of caffeine which remains neutral for all mobile phase compositions.

Fig. 3 shows the analytical peak profiles recorded under PALC and HILIC conditions at a fixed value of the retention factor k'. The contents of acetonitrile were chosen so that the retention of caffeine was equal to about 0.25, 0.5, 1, and 2. Fig. 2 provides graphically the corresponding volume fractions of acetonitrile as the abscissa taken at the intersections between the horizontal dotted lines representing the different k' values and the U-shaped retention curve. The volume fractions of acetonitrile were then 0.4, 2.5, 13, and 40% along the PALC branch and 99.5, 96, 88, and 60% along the HILIC branch. The retention factors measured in PALC and HILIC match very well except for $k' \simeq 1$. We picked up too small a volume fraction in the PALC branch (2.5%, $k' = 1.25$) as actually required (2.8%, $k' = 1$) because the retention of caffeine is extremely sensitive to any small variation of acetonitrile content in water-rich eluents. Overall, the difference in the retention factor remains small between PALC and HILIC and the comparison between the column efficiencies in PALC and HILIC is made possible.

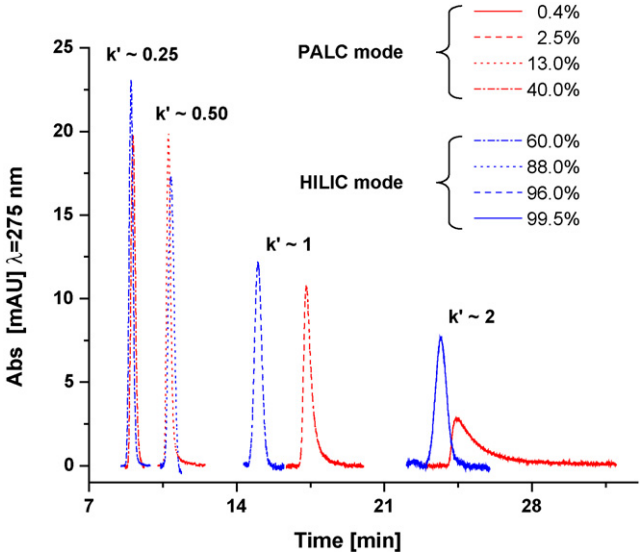

Fig. 3. Peak profiles of caffeine recorded under PALC (red signals) and HILIC (blue signals) conditions for about the same retention factors of 0.25, 0.50, 1, and 2, $T = 295$ K. Injection: 0.5 μL of a 0.01 g/L solution of caffeine. The volume percentage of acetonitrile in the mobile phase is indicated in the legend of the graph (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

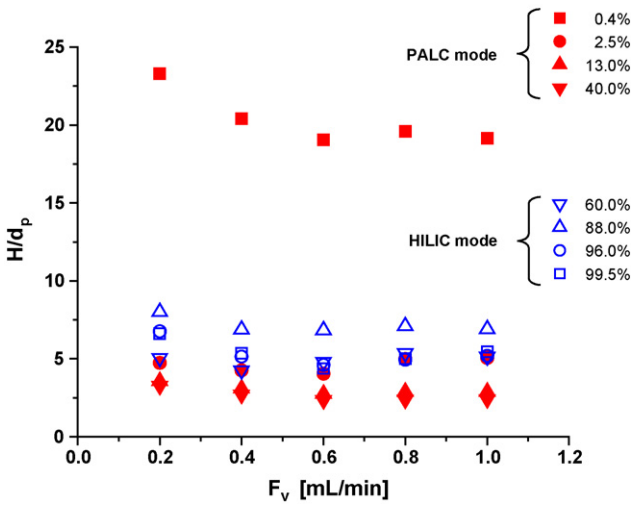

Fig. 5. Reduced HETP plots of caffeine measured in PALC (red full symbols) and HILIC (blue empty symbols). Comparison between PALC and HILIC performance at constant retention factors. Squares: $k' = 2$; circles: $k' = 1$; upwards triangle: $k' = 0.5$; downwards triangle: $k' = 0.25$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

Despite the efforts dedicated to reach the linear range of all the adsorption isotherms, the peak profile of caffeine recorded with 0.4% acetonitrile ($k' = 2$) still tails (see also Fig. 1). This confirms previous experimental studies involving frontal analysis measurements [6] which demonstrated the adsorption heterogeneity and the role of very energetic sites in PALC. Poor apparent column efficiency, which includes thermodynamic effects, are expected since the column is already overloaded when less than 10 ng of caffeine are injected. In contrast, under HILIC conditions and for the same retention factor $k' = 2$, the peak profile is perfectly symmetrical because the active adsorption sites are weaker and more abundant than in PALC. As the retention decreases, the peak tailing considerably decreases in PALC because the active sites are getting saturated with acetonitrile. Fig. 4 plots the asymmetry factors of the peaks measured at half their height and for the same flow

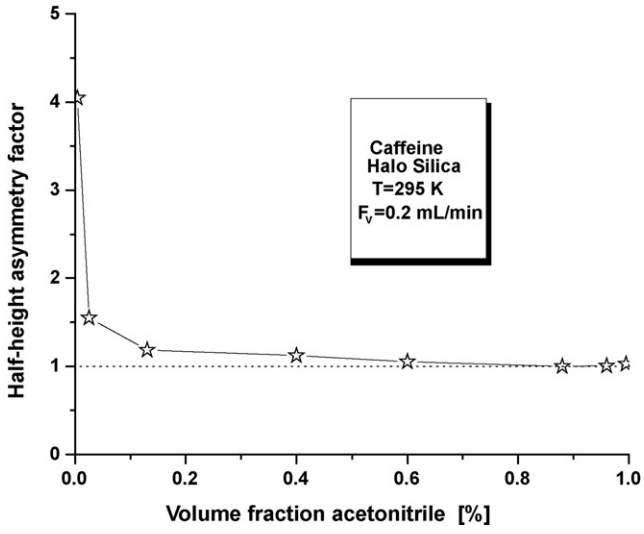

Fig. 4. Plot of the peak asymmetry of caffeine chromatograms measured at half the peak height as a function of the acetonitrile volume fraction in the mobile phase (0.4, 2.5, 13, 40, 60, 88, 96, and 99.5%). Flow rate: 0.2 mL/min, $T = 295$ K. Halo silica column.

rate of 0.2 mL/min. Clearly enough, the thermodynamic peak tailing rapidly decreases as one progressively switches from low to high acetonitrile concentrations. Hence, *a priori*, assuming the column efficiency is independent on the retention mechanism, e.g. PALC or HILIC, the column efficiency should appear larger in HILIC than in PALC.

Fig. 5 shows the corresponding van Deemter plots of caffeine for flow rates increasing from 0.20 to 1.00 mL/min. The minimum of all the HETP curves is observed close to a flow rate of 0.5 mL/min. Not surprisingly, the minimum reduced HETP measured with only 0.4% acetonitrile in water ($k' = 2$) is considerably larger than those measured with the other mobile phases. The experimentally inevitable thermodynamic peak tailing is directly responsible for such a poor column efficiency ($< 20\,000$ plates/m). Accordingly, HILIC is highly preferred to PALC when the analyte is strongly retained. Fig. 6C illustrates this conclusion by comparing the two peak profiles of caffeine for $k' = 2$. HILIC can achieve about 100,000 plates/m, e.g. five times larger column efficiency than in PALC.

Most interestingly, when the retention factors are smaller than 0.5, PALC provides better column efficiency than HILIC, despite a slight peak tailing indebted to column overloading (Fig. 4). The minimum reduced HETPs are about 2.5 in PALC (red full triangles) and around 6.0 in HILIC (blue empty triangles). It is noteworthy that these reduced HETPs are larger than 1.5, the minimum reduced HETPs measured onto Halo-C_{18} columns in RPLC [17] or onto Halo silica in presence of buffer [18]. This plate height excess does not originate from the extra-column contributions since they were systematically subtracted to those measured for the whole system including the extra-column and the column contributions. It can originate in part from a slight column overloading effect under PALC. On the other hand, no obvious explanation stands *a priori* for the higher plate height measured in HILIC. Fig. 6A compares the peak profiles recorded under PALC and HILIC conditions for $k' = 0.5$ and at a flow rate of 0.4 mL/min. It clearly shows that the system peak is much thinner than the band of caffeine in HILIC, which eliminates the hypothesis of a deteriorating column. Possibly, the transfer from a water-rich adsorbed phase onto silanol groups to the acetonitrile-rich mobile phase is a slow kinetic process. As the content of water decreases towards zero, the minimum HETPs become very similar, 4.0 and 4.5 in PALC and HILIC, respectively. Fig. 6B compares the peak profiles recorded in PALC and HILIC

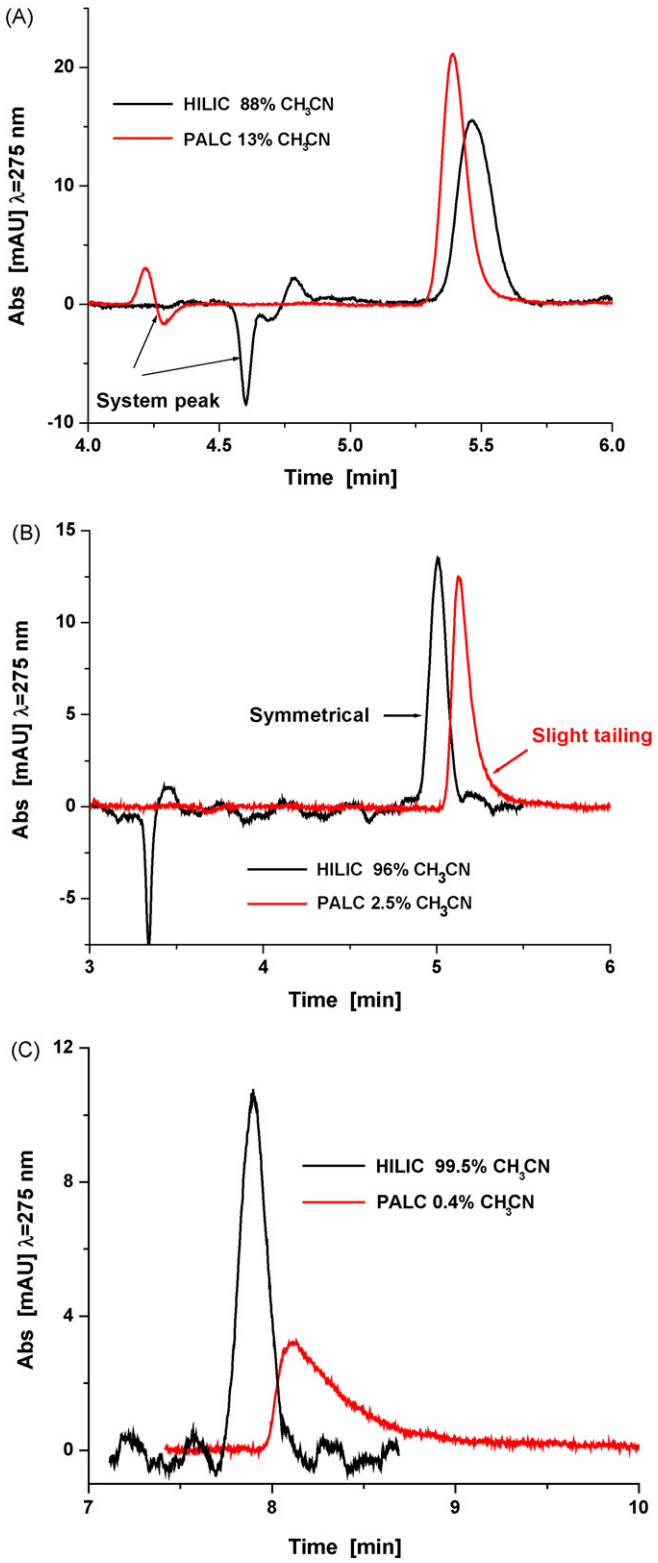

Fig. 6. Comparison between the experimental peak shapes recorded in PALC and HILIC at constant retention factor k'. (A) $k' = 0.5$. Flow rate: 0.6 mL/min. Note the better column efficiency in PALC than in HILIC. (B) $k' = 1$. Flow rate: 0.4 mL/min. Note the comparable column efficiency under PALC and HILIC conditions. (C) $k' = 2$. Flow rate: 0.6 mL/min. Note the better column efficiency in HILIC than in PALC.

for $k' \simeq 1$ and at a flow rate of 0.6 mL/min. Note that the minimum HETP increases from 2.5 to 4.0 in PALC because the peak tailing increases due to a larger column overloading. Most strikingly, the HETP in HILIC decreases from 6.5 to 4.5 after decreasing the content of water in the bulk from 40 to 0.5%. Adsorption–desorption from the stationary phase could possibly be faster when the water content decreases in the water–acetonitrile adsorbed multilayer. Still, the minimum HETP measured in HILIC remains clearly larger than the expected reduced HETP of 1.5 usually observed with the Halo columns.

It is informative to estimate grossly the reduced HETP of the column from the band broadening of the negative system peak recorded in HILIC during injection. The reduced HETP of the system peak is of the order 1.5 and 1.0 in Fig. 6A and B, respectively, which confirms the accepted minimum reduced HETPs measured on the Halo column [17,18].

In conclusion, the poor column efficiency observed in HILIC with caffeine is definitely dependent on the nature of the sample and on its specific kinetic of adsorption–desorption onto neat silica from acetonitrile-rich bulk eluent. Overall, PALC could be preferred to HILIC for weakly retained samples. However, we should keep in mind that too high a water concentration can seriously affect the symmetry of the peaks because of overloading effects [6]. The application of PALC is then limited to the range of mobile phase compositions within which the retention factors of the sample components does not exceed 2. Alternately, the analyst can increase the retention factors of sample components by using a different column but he might face again problems related to column overloading. The trade-off between retention and efficiency has to be carefully evaluated in PALC.

5. Conclusion

In this work, we showed that PALC can provide as much retention as HILIC for polar compounds. Given the current shortage of acetonitrile in academic and industrial research laboratories, PALC could be a suitable mode of chromatography as replacement of HILIC, highly consuming in acetonitrile. This was demonstrated by considering caffeine as the analyte and a column packed with 2.7 μm Halo particles. The analysis of the adsorption and mass transfer behaviors in PALC and HILIC allows to determine the optimum conditions for resolutions of complex mixtures.

1. PALC must be absolutely avoided when analyzing strongly retained samples ($k' > 2$). Their peak shape tails significantly due to the very active adsorption sites so the apparent column efficiency can be no more than 20,000 plates/m when injecting less than 10 ng. HILIC is definitely preferred for the analysis of very retained samples (100,000 plates/m) because the adsorption energies involved are small and the column can be overloaded without a serious risk of efficiency loss.
2. PALC could be preferred to HILIC for the analysis of weakly retained components, e.g. with a larger concentration of acetonitrile in the mobile phase which eventually saturates, blocks the active adsorption sites, and eliminate the peak tailing. For some undetermined reasons, the mass transfer of caffeine was found slower in HILIC (88% CH_3CN) than in PALC (13% CH_3CN) at a constant retention factor of 0.5 with (150,000 plates/m *versus* 60,000 plates/m). A slow adsorption–desorption process from the adsorbed water–acetonitrile multilayer towards the acetonitrile-rich bulk eluent could explain the poor column efficiency in HILIC.
3. HILIC and PALC are equivalent for intermediate retention factors ($k' \simeq 1$, 100, 000 plates/m). Still, the minimum reduced HETP ($\simeq 4$) remains larger than the theoretical minimum expected for

the Halo column (1.5). In PALC, this difference is due to the slight column overloading. In HILIC, this limitation in the column efficiency is most likely due to slow desorption from the silanols adsorption sites.

Finally, we should remember that PALC is limited to the separation of components having moderate retention factors. Still, with the advent of very high efficient columns with minimum plate height around 3 μm, there should be room to obtain rapid separations of complex mixtures with a maximum retention factor of 2 in water-rich mobile phases. If the resolution is eventually insufficient, different columns and stationary phases should be preferred.

In this work, no ionogenic compound was added to the mobile phase so all silanols groups were directly accessible to the analyte. More work is needed in order to establish the role of the pH, of the ionic strength of the eluent, and of the silanol dissociation constants (pK_a) on the performance of HILIC silica columns.

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