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Selection of suitable operating conditions to minimize the gradient equilibration time in the separation of drugs by Ultra-High-Pressure Liquid Chromatography with volatile (mass spectrometry-compatible) buffers

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ABSTRACT

Reversed phase gradient elution is the method of choice for pharmaceuticals analysis since it allows reducing the analysis time while improving both the quality of the separation and the detection limits. The current trends are towards faster separations which can be achieved thanks to equipments withstanding ultra-high pressures and/or high temperatures. Under such conditions, gradient separations can be carried out within a few minutes or even a few tens of seconds. A long equilibration time in addition to the gradient time can be therefore very detrimental. In this work, we investigated the extent to which the gradient equilibration time can be reduced and which parameters mainly affect the retention variability of ionizable compounds when using volatile buffers. We first found out an excellent repeatability between run-to-run experiments whatever the equilibration time and the operating conditions. We then pointed out the key operating parameters which allow achieving reproducible runs when varying the equilibration time between runs. With a view of reducing the equilibration time, the effects of various conditions were examined. The latter include the type of additive for mobile phase pH adjustment, the initial eluent composition, the type of stationary phase, the temperature and the flow-rate. Although much remains to be understood about the equilibration process, our study allows making progress in the knowledge of this phenomenon. Based on the present results, a beneficial effect of both temperature and flow-rate was highlighted and operating conditions leading to faster column equilibration are suggested.

1. Introduction

A gradient elution is most often required for the separation of pharmaceutical compounds because it leads to a reduction of the analysis time along with an improvement in both peak capacity and detection limits. The current trend towards higher throughput involves the need for ultra fast separations. Such separations can be obtained with commercially available equipments which are able to withstand ultra-high pressures and/or high temperatures. Ultra-High-Pressure Liquid Chromatography (UHPLC) [1,2] as well as High-Temperature Liquid Chromatography (HTLC) [3,4] lead to significant gain in speed compared to conventional HPLC while keeping the same peak capacity. The analysis time can even be further decreased if both techniques are combined (HT-UHPLC) [5–9]. In particular, the use of HT-UHPLC can be very attractive for the gradient separation of macromolecules [10]. In such conditions, gradient runs can be carried out within a few minutes or even a few tens of seconds [11]. As a result, any time in addition to the gradient time, i.e. the return to initial composition, the time required for injection and particularly the gradient re-equilibration time represents a limiting step in the pursuit of faster analyses. Once both composition range and gradient slope have been optimized, the only variable left for reducing the total analysis time is the gradient equilibration time. It is then very important to search for conditions that can reduce it. Indeed, any gradient method must include a stage for re-equilibrating the stationary phase with the initial solvent. In reversed phase chromatography, the alkyl chains are solvated by the organic modifier of the mobile phase which forms a layer at the surface of the stationary phase. The amount of solvent adsorbed on the stationary phase depends on the mobile phase composition. The surface of the stationary phase (i.e. the nature and the amount of the adsorbed layer) must always be the same at the beginning of the gradient to get reproducible retention times. In this aim, flushing the column with a few column volumes of the initial solvent is necessary. The key issue is to determine "how much is enough?" as underlined by Dolan [12]. Among chromatographers, a rule of thumb consists in making use of 5–15 column volumes. Yet,

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this number may be overestimated as well as underestimated since the time for column equilibration and hence retention variability strongly depends on both the type of solutes and the operating conditions. Obviously, it also depends on the degree of retention variability which can be accepted. For example, retention variability is more acceptable for routine analysis than for retention modelling. On the other hand, in comprehensive two-dimensional analysis, an excellent repeatability is of prime importance for the second dimension separations to align and reconstruct of 3D plots. Finally, the variability of Δt_r (difference in retention times between two adjacent peaks) is more important than the variability of t_r (absolute retention times) as it directly impacts on the selectivity and the separation quality.

Reaching full stationary phase equilibration is most often demanded and some recent studies deal with the conditions that are required [13–20]. Schellinger et al. [18] were the first to define two different states of equilibration that they have called "repeatable equilibrium" and "full equilibrium". While repeatable equilibrium results in excellent run-to-run repeatability in retention, full equilibrium is only reached when the retention no longer statistically changes as the equilibration time is further increased. It was shown that the addition of a small amount of 1% n-propanol [13] or n-butanol [18] to the mobile phase significantly reduces the time required for full equilibration due to the preferential wetting of the stationary phase by these ancillary solvents. A constant solvation layer is created, thereby reducing the part of the stationary phase to be equilibrated. However, simple mobile phases are always more attractive than complex ones. Adding 1% propanol is yet possible but it is worthwhile wondering if it is absolutely necessary. Moreover the addition of organic solvent reduces the gradient range which can be detrimental for poorly retained compounds which are often present in complex pharmaceutical samples.

Our first objective was to characterize the degree of retention variability which can be expected from the gradient separation of pharmaceutical compounds when working in HT-UHPLC conditions with volatile additives and without addition of ancillary solvent. We then studied to what extent operating conditions (mobile phase pH, stationary phase, solute type, column temperature, flow-rate and gradient conditions) can alter the retention variability and finally how to keep the equilibration time down to a minimum. Gradient methods must often start without organic solvent in the initial eluent and decreasing the equilibration time can be challenging. We therefore paid a particular attention to these gradient conditions.

2. Experimental

2.1. Material and reagents

The gradient runs were performed with different mixtures of acetonitrile and water. Acetonitrile was HPLC grade from SDS (Peypin, France). Water was obtained from an Elga water purification system (Veolia water STI, Le Plessis Robinson, France). The mobile phase pH was controlled thanks to various additives: formic acid, ammonium formate, ammonium acetate, ammonium hydroxide from Sigma–Aldrich (Steinheim, Germany). Buffered eluents prepared from salts were filtered on 0.2 μm nylon filter before use. Solutes used in the test mixture were obtained from Sigma–Aldrich (Steinheim, Germany): acetyl salicylic acid, atenolol, caffeine, nadolol, pindolol, phenol, procaine, methylparaben, propranolol, ethylparaben, diphenhydramine, protriptyline, imipramine, clozapine, NN dimethylaniline, uracil. The latter was used to measure the column dead volume. The chemical structures of the compounds along with their pK_a values are reported in Fig. 1.

2.2. Columns

Four sub-2 μm columns were studied: Acquity BEH-C18 (50 mm × 2.1 mm, 1.7 μm), Acquity BEH-C18 Shield (50 mm × 2.1 mm, 1.7 μm) both from Waters (Milford, MA, USA), Zorbax Stablebond-C18 (50 mm × 2.1 mm, 1.8 μm) from Agilent (Santa Clara, CA, USA) and Nucleodur Gravity C18 (50 mm × 2 mm, 1.8 μm) from Macherey-Nagel (Duren, Germany). A few results were also obtained with a conventional C18 column: Xterra RPC18 (100 mm × 4.6 mm, 3.5 μm) from Waters. The Nucleodur Gravity C18 column was a gift from Macherey-Nagel and the Zorbax Stablebond-C18 was a gift from Agilent.

2.3. Apparatus

The instrument used with sub 2 μm columns was an Acquity UPLC Liquid Chromatograph (Waters, Milford, MA, USA). This instrument includes a high-pressure binary solvent manager with a maximum delivery flow-rate of 2 mL/min, an autosampler with a 5 μL injection loop, a column oven with a maximum temperature of 90 °C and a UV–vis detector with a 500 nL flow-cell. 0.5 μL were injected. The wavelength was set at 220 nm and the time constant at 25 ms. Data acquisition with a 40 Hz sampling rate and instrument control were performed by Empower Software. The maximum backpressure is 1000 bar for flow-rates up to 1 mL/min, 800 bar for flow-rates up to 1.5 mL/min and 630 bar for flow-rates up to 2 mL/min. The mobile phase is preheated prior to entering the column thanks to a coiled stainless steel tube (50 cm × 0.127 mm) located between the Rheodyne injection valve and the column inlet. A polyether ether ketone (PEEK) tube (15 cm × 0.1 mm) is located between the column outlet and the detector. A total extra-column volume of 15 μL was determined using a zero dead volume union connector in place of the column. The measured dwell volume was 120 μL. A time offset of 0.8 s was observed after the zero injection time was recorded. 45 s were required for the injection cycle of the instrument. The needle wash cycle included a strong wash using water–acetonitrile (20/80, v/v) and a weak wash using water–acetonitrile (80/20, v/v).

The instrument used with the conventional Xterra RPC18 column consisted of a Waters 2690 Separation Module and a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The main module included integrated solvent and sample managers as well as an oven to regulate the column temperature up to 90 °C. The quaternary low-pressure pump delivers a maximum flow-rate of 5 mL/min. The injector was equipped with a 100 μL-loop. 5 μL were injected. The detector consisted of an 8 μL UV-cell and acquired channels between 200 and 400 nm at a sampling rate of 10 Hz. The instrument was controlled by Empower Software. Instrumental characteristics are: 65 μL as extra-column volume and 800 μL as dwell volume. The injection time was 45 s and needle wash was performed with a 50/50 (v/v) acetonitrile/water solution.

Data processing (blank signal subtraction, peak integration, chromatographic parameters calculations) was achieved by Azur (Datalys, Saint-Martin d'Hères, France).

2.4. Procedures and gradient conditions

2.4.1. Calculation procedure

All along this work, the equilibration time is expressed through the number of column volumes of initial eluent, N_{cv}, used to flush out the column. This number takes into account the equilibration time, t_{eq}, programmed in the method after the return to initial composition, the time, t_{inj}, required by the instrument for the injection, the dwell volume of the system, t_D and the column dead time, t_0. It

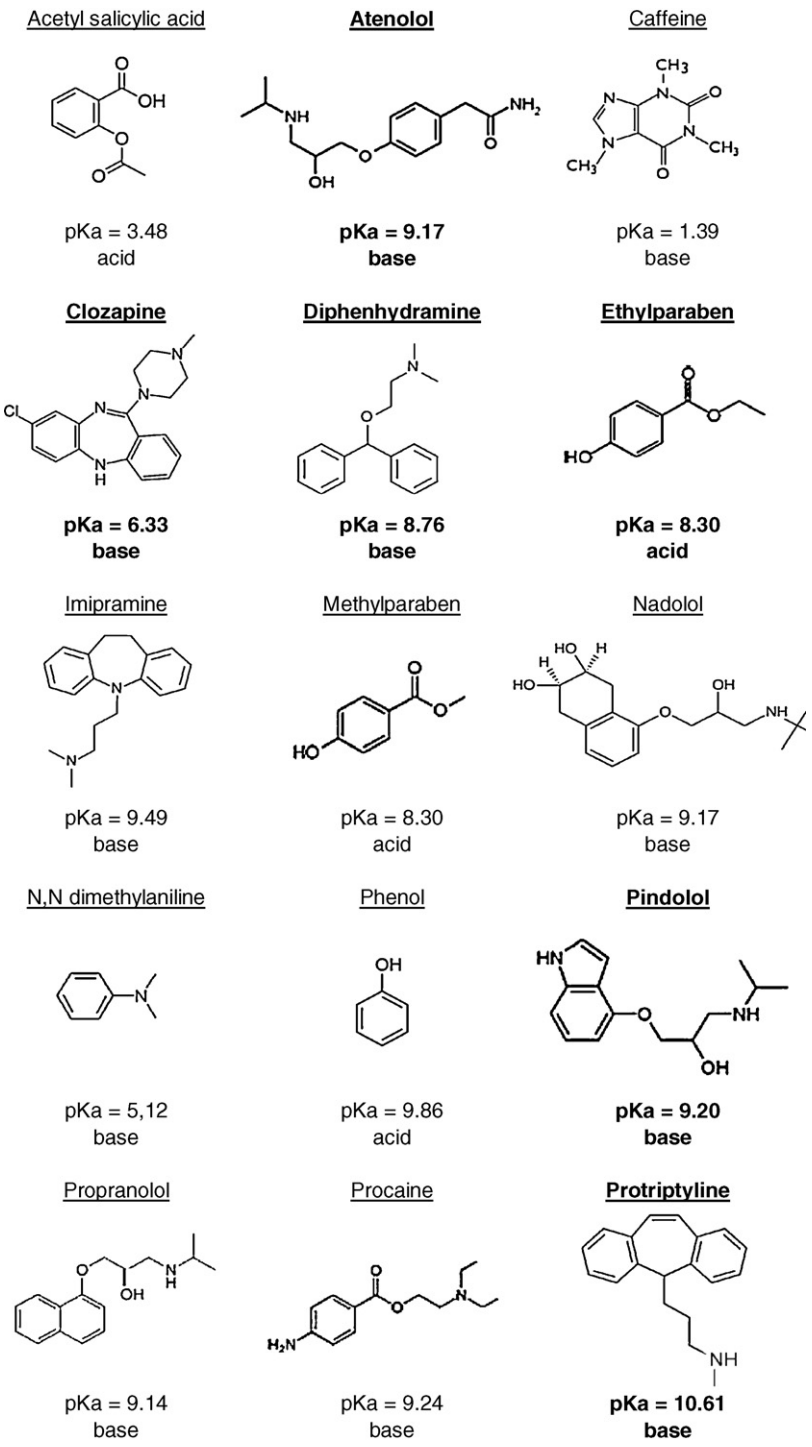

Fig. 1. Molecular structures and dissociation constants of the solutes (pK_a at 25 °C). Compounds involved in the whole equilibration study are in bold.

is given by

$$N_{cv} = \left(\frac{t_{eq} + t_{inj} - t_D}{t_0}\right) \quad (1)$$

For example, when $t_{eq} = 0$, considering 0.66 min for the instrument to inject and 0.22 min for both column dead time and system dwell time, the equilibration time at 0.5 mL/min represents two column dead times ($N_{cv} = 2$), which is the lowest attainable value in these instrument conditions.

The retention variability in gradient elution is assessed by the change in mobile phase composition at elution (ΔC_e) which is given by

$$\Delta(C_e) = \frac{C_f - C_i}{T_G/t_0} \times \frac{\Delta(t_r)}{t_0} \quad (2)$$

where $(C_f - C_i)/(T_G/t_0)$ is the normalized gradient slope with T_G being the gradient time, C_f and C_i being the final and initial mobile phase compositions respectively.

According to Eq. (2), for a given normalized gradient slope, the degree to which the difference in retention times can be acceptable (for repeatability or intermediate precision) is related to the column dead time. Thus, the retention variability can be assessed by the

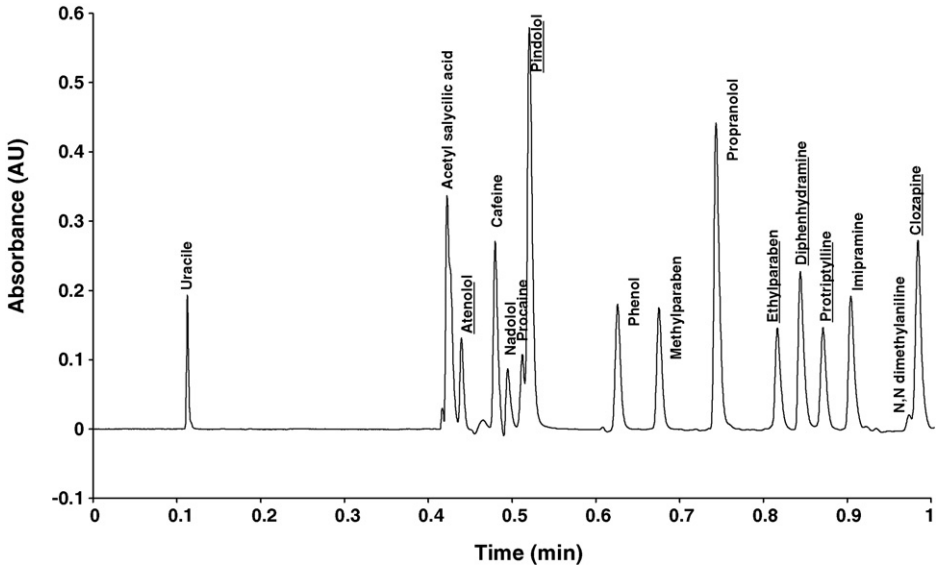

Fig. 2. Typical separation of the drugs mixture. Conditions: 50 mm × 2.1 mm i.d. Acquity BEH-C18 column with 1.7 μm particles; solvent A is water with 10 mM ammonium acetate; solvent B is acetonitrile; 100/0 to 0/90 in 1.2 min at 1 mL/min with 5 column volumes for re-equilibration; 70 °C; 0.5 μL injected; 220 nm detection. The baseline signal has been subtracted. Underlined solutes were selected for the whole study.

ratio $\Delta t_r/t_0$ and not by Δt_r alone which is not correct to compare conditions leading to different column dead times. Hence, with the purpose of obtaining a reliable comparison of our results whatever the flow-rate and the column geometry, the retention variability was calculated as follows:

(1) The retention variability between n runs carried out in the same conditions was assessed by the standard deviation of the retention times, t_r, divided by the column dead time, $\sigma(t_r)/t_0$.
(2) The retention variability between runs carried out in different conditions was controlled by the ratio of the difference between given and reference retention times to the column dead time, $(t_r - t_{r,ref})/t_0$. For the study of retention variability versus N_{cv}, the reference retention time is related to the highest N_{cv} studied.

According to these definitions, the retention variability is then a fraction of the column dead time which should be as small as possible.

2.4.2. Gradient conditions

All our experiments were performed with the same normalized gradient slope, namely with $(C_f - C_i)/(T_G/t_0) = 10\%$ where C_i and C_f are initial and final percentages of acetonitrile in the mobile phase respectively. The mobile phase pH was dependent on the additive: formic acid at 0.1% (v/v) (^w_wpH = 2.7), ammonium formate at 10 mM (^w_wpH = 2.7 adjusted with formic acid), ammonium acetate at 10 mM (^w_wpH = 6.8), ammonium hydroxide at 0.1% (v/v) (^w_wpH = 10.4). In order to keep the ionic strength constant all along the gradient, the pH adjuster was added in both aqueous (eluent A) and organic (eluent B) phases except for ammonium salts which are not soluble in acetonitrile at such concentrations. The gradient was programmed from 100% channel A to 90% channel B followed by a return to 100% channel A in one column dead time. These gradient conditions were always applied unless stated otherwise.

For the repeatability study, series of 10 run-to-run experiments were carried out with different t_{eq} values corresponding to N_{cv} values given by Eq. (1). For the study of retention variability as a function of N_{cv}, retention data were collected with different N_{cv} values starting from the smallest value to the highest one. Each experiment was run in triplicate so that the mean retention time value was considered. Each new N_{cv} series was preceded by a blank gradient.

Sixteen drugs were separated in various operating conditions. A typical separation performed at 70 °C, is given in Fig. 2. After handling the data of all solutes, we selected six representative solutes (those underlined in Figs. 1 and 2) in order to reduce the amount of results presented in this work. The different studied conditions are reported in Table 1.

3. Results and discussion

3.1. Intra-series retention variability (repeatability)

The repeatability can be assessed by measuring the retention variability between n consecutive runs carried out in the same conditions, including the re-equilibration time so that the resulting difference in retention times could be expected to highlight the random experimental error. Schellinger et al. [19] studied the repeatability for basic solutes using a conventional reversed phase column (150 mm × 4.6 mm) in acetonitrile–water with 0.1% TFA and 1% n-butanol as mobile phase and a gradient starting with 10% acetonitrile. In most of the conditions they examined, they found a good repeatability (variation of retention <0.004 min) attainable with only two column volumes of re-equilibration. The repeatability was independent of the equilibration time but they identified many parameters which could affect the repeatability, including the pK_a of the buffer and the type of the stationary phase. They observed that the repeatability of the most retained basic compounds was worse than that of the weakly retained ones. The positive effect of the flow-rate was attributed to a better precision of column temperature at higher flow-rates due to the specific working of the thermostat.

In our different operating conditions listed in Table 1, the repeatability was determined by calculating the ratio of the standard deviation of 10 retention times divided by the column dead time, $\sigma(t_r)/t_0$. Some illustrative results are reported in Figs. 3 and 4. They were obtained with an Acquity BEH-C18 column at 30 °C and a UHPLC system (Fig. 3) and with an XTerra RPC18 column at 30 and 70 °C and a HPLC system (Fig. 4). Both figures show that, in all examined conditions, the equilibration time has no significant effect on repeatability, which is in good agreement with the results

Table 1
Summary of conditions used for this study with corresponding flow-rates (mL/min). Column dead times (min) are given into brackets.

	Mobile phase conditions															
	Formic acid				Ammonium formate				Ammonium acetate				Ammonia			
Temperature	30 °C		70 °C		30 °C		70 °C		30 °C		70 °C		30 °C		70 °C	
Initial acetonitrile percentage	0%	5%	0%	5%	0%	5%	0%	5%	0%	5%	0%	5%	0%	5%	0%	5%
Stationary phases																
Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min))	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min))	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)
Acquity BEH-C18 Shield 50 mm × 2.1 mm, 1.7 μm		0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min)	0.5 mL/min (0.22 min) 1 mL/min (0.11 min)	0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min)
Zorbax SDB-C18 50 mm × 2.1 mm, 1.8 μm	0.5 mL/min (0.22 min)		0.5 mL/min (0.22 min) 1 mL/min (0.11 min)													
Nucleodur C18 Gravity 50 mm × 2 mm, 1.8 μm										0.5 mL/min (0.21 min)		0.5 mL/min (0.21 min)				
Xterra RPC18 100 mm × 4.6 mm, 3.5 μm	1 mL/min (1 min)		2 mL/min (0.5 min)						1 mL/min (1 min)		1 mL/min (1 min)					

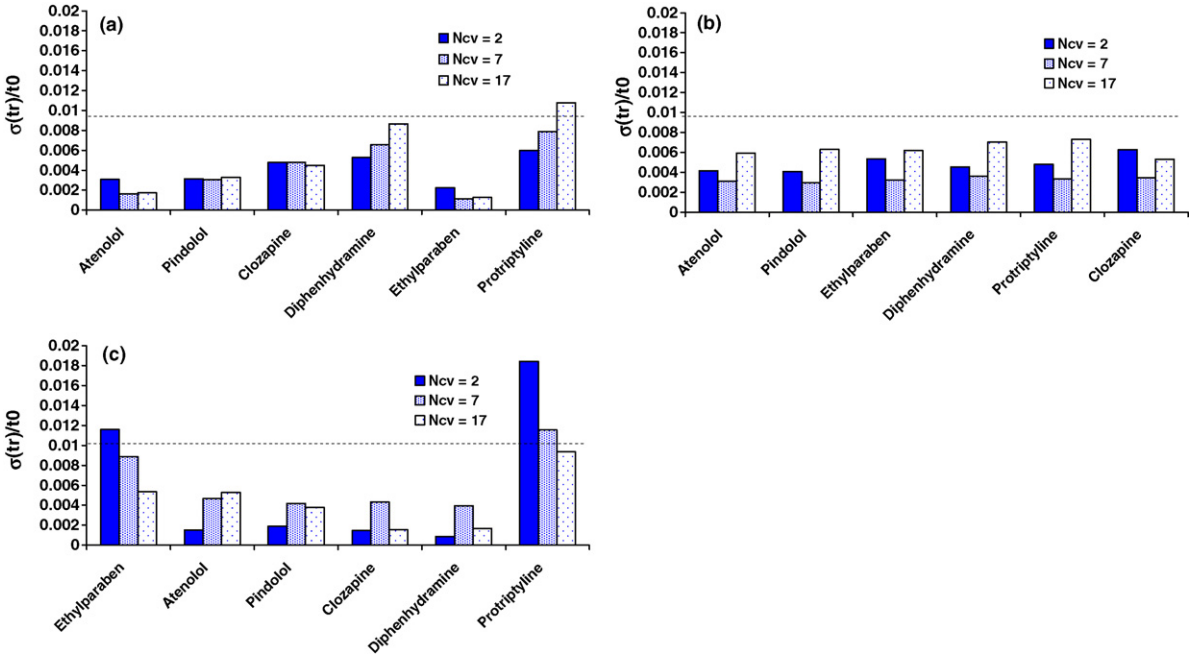

Fig. 3. Repeatability as a function of the solutes and the number of column volumes used for re-equilibration. N_{cv}: 2 ( ); 7 ( ); 17 ( ). Buffers: formic acid (a); ammonium acetate (b); ammonia (c). Column Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm; 30 °C; 0.5 mL/min; initial composition: 0% acetonitrile. Other gradient conditions are given in Section 2.

of Schellinger et al. [19]. Even the lowest number of column dead volumes led indeed to small retention variability with a maximum value ranging from 0.006 to 0.01. In contrast we found no significant trend in retention variability with pH, stationary phase, temperature or flow-rate as shown in both figures, as well as with retention of compounds as shown in Fig. 3 for solutes ranged on the *X*-axis from the least to the most retained. Furthermore, the solute type (ionized or neutral) had no influence and starting with 5% acetontitrile in the initial eluent led to the same level of repeatability as with 0% (results not shown).

With a normalized gradient slope of 10%, the maximum value of 0.01 for $\sigma(t_r)/t_0$ corresponds to a variation of the composition at elution of 0.1% (Eq. (2)) (i.e. a difference in retention times of 0.001 min for a 50 mm × 2.1 mm i.d. column at 1 mL/min in UHPLC conditions). It is important to notice that the same repeatability ($\sigma(t_r)/t_0 < 0.01$) was obtained with a conventional equipment (i.e. a difference in retention times < 0.005 min for a 100 mm × 4.6 mm i.d. column at 2 mL/min as shown in Fig. 4) suggesting that this level of repeatability was only dependent on the studied gradient conditions. For comparison, the $\sigma(t_r)/t_0$ value varied from 0.003 to 0.005 in [19]. However, considering our very unfavourable conditions (no ancillary solvent, initial mobile phase composition of 0% acetontitrile and ultra fast gradient), we were very surprised to obtain such excellent results for repeatability. The repeatability was slightly poorer (<0.02) for protriptyline and ethylparaben with ammonia (Fig. 3c) when only two column volumes were used for re-equilibration. Considering their pK_a, both compounds are partly ionized at pH 10.4 and therefore probably more sensitive to some change in mobile phase pH which may occur from run-to-run if the pumping system has not been completely flushed out before initial eluent enters the column. The flush out volume being about twice the dwell volume [18] (i.e. 240 μL), it is slightly higher, in these conditions, than the re-equilibration volume (220 μl with $N_{cv} = 2$) and this could explain the obtained results with ammonia.

A short equilibration time together with a low retention variability is very attractive for the second dimension of a comprehensive two-dimension liquid chromatography (LC × LC) with mass spectrometry detection. As a matter of fact, any additional time in the second dimension implies an increase in the total analysis time and as a result a decrease in the sampling rate of the first dimension peaks, which is critical to achieve successful 2D-separations.

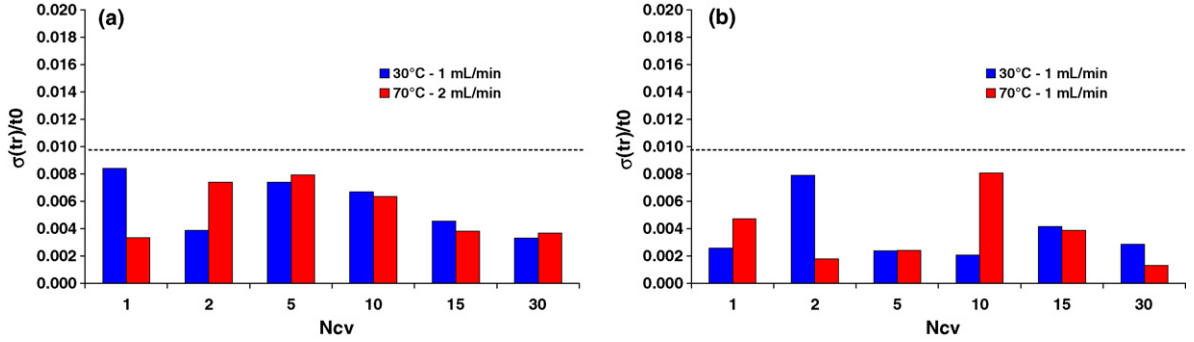

Fig. 4. Average repeatability as a function of the number of column volumes used for re-equilibration and the temperature/flow-rate. Temperature and flow-rate: 30 °C and 1 mL/min ( ); 70 °C and 2 mL/min ( ). Buffers: formic acid (a); ammonium acetate (b). Column XTerra RPC18 100 mm × 4.6 mm, 3.5 μm; initial composition: 0% acetonitrile. Other gradient conditions are given in Section 2.

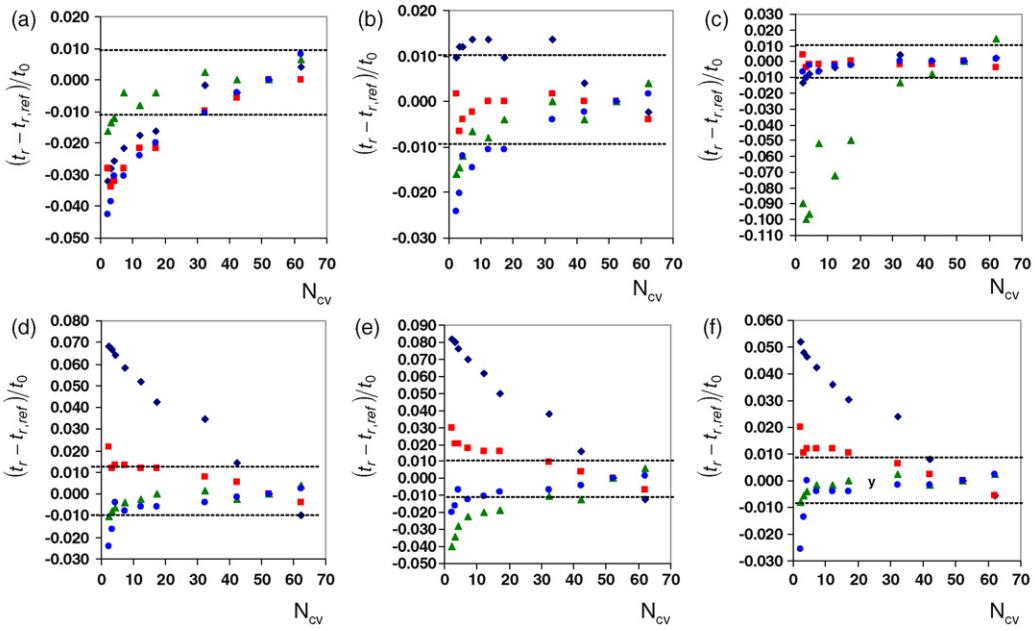

Fig. 5. Effect of the buffer type on retention variability with 0% acetonitrile in the initial eluent. Plot of retention variability as a function of the number of column volumes for six representative solutes: atenolol (a); pindolol (b); ethylparaben (c); diphenhydramine (d); protriptyline (e); clozapine (f). Additives: formic acid (◆); ammonium formate (■); ammonium acetate (●); ammonia (▲). Column Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm; 30 °C; 0.5 mL/min. The dashed lines specify the range where the retention variability is similar to the repeatability (within ±0.01).

3.2. Retention variability between runs when varying the equilibration time

3.2.1. Effect of the type of additive

The effect of the type of additive and the related mobile phase pH on retention variability between runs with different equilibration times is shown in Fig. 5. The buffer influence was studied in the "worst conditions", that are without any organic solvent in the initial eluent. According to various authors [8,13–16,18,20], the full column equilibration is expected to be achieved provided that retention times of all peaks no longer statistically change as the equilibration time is increased. The dashed lines bracket the range where the retention time variability was similar to the repeatability (i.e. within $0.01t_0$). Several conclusions can be drawn from Fig. 5. First, it is clear that ammonium acetate at neutral pH provides the best results. In these conditions, the full column equilibration was established in less than 10 column volumes for all solutes except atenolol as seen in Fig. 5a–f. In contrast, with acidic buffered eluents, 60 column volumes could be insufficient, especially with formic acid as additive and basic compounds which are cationic at this pH. Indeed, the retention variability was very important for the highly retained basic compounds, diphenhydramine (Fig. 5d), protriptyline (Fig. 5e) and clozapine (Fig. 5f). Similarly, full column equilibration took much longer time with ammonia than with ammonium acetate, the worst results being obtained for ethylparaben (Fig. 5c) which is partially anionic in these conditions. Conversely, when this compound is neutral, which was the case with the three other buffers, 5 column volumes or even less were sufficient. It has been reported elsewhere [16,18] that weakly retained compounds such as atenolol in the present study are more affected by low equilibration process because when moving across the column they interact with sites which have been equilibrated by much fewer volumes than more retained compounds. Accordingly full equilibration should be expected when the retention times of early eluted solutes do not vary anymore [17]. Our results clearly show that checking the invariability of retention times of the least retained solute alone is not enough to assess the extent of equilibration as it is clearly both solute and buffer dependent. Obviously, there are much more factors affecting column equilibration in case of ionizable compounds and we were interested in determining the factors leading to faster column equilibration when working with volatile buffers.

3.2.2. Effect of a small amount of acetonitrile in the initial eluent

The same study was repeated with an initial composition of 5% acetonitrile. The data were fitted with a logarithmic function according to Marchand et al. [15] who suggested a first order process to describe column equilibration. The effect of a small amount of acetonitrile in the initial eluent is illustrated in Fig. 6 for the worst pairs of additive-solute. As expected, a small amount of organic solvent significantly reduced the time required to attain the full column equilibration for all solutes and all buffers except for protriptyline with ammonia (Fig. 6c). The removal of the last traces of organic solvent by pure water is time-consuming and therefore maintaining solvation by a small amount of organic modifier leads to faster equilibration [16]. It was observed in Fig. 5 that atenolol was the worst solute with ammonium acetate ($N_{cv} > 30$). Fig. 6a shows that the addition of 5% acetonitrile in the initial eluent considerably decreases the equilibration time needed for full equilibration ($N_{cv} < 5$). The effect of a small amount of acetontitrile is particularly impressive for ethylparaben with ammonia (Fig. 6d). In this latter case, whereas full equilibration was never reached in the absence of organic solvent, it was quickly obtained with 5% acetonitrile ($N_{cv} < 5$). As a matter of fact, the stationary phase must be well solvated by the eluent otherwise the molecules cannot be transferred from the mobile phase to the stationary phase. It is well-known that a small amount of organic modifier is often sufficient to significantly improve the stationary phase wetting which is of prime importance for the equilibration process. However, with formic acid as additive, in spite of a great improvement, the addition of acetonitrile in the initial eluent was not sufficient for the most retained basic compounds to rapidly attain the full equilibration as shown in Fig. 6b for clozapine. More surprisingly, the results for protriptyline with ammonia were even worse with a small amount of acetontitrile in the initial eluent (Fig. 6c).

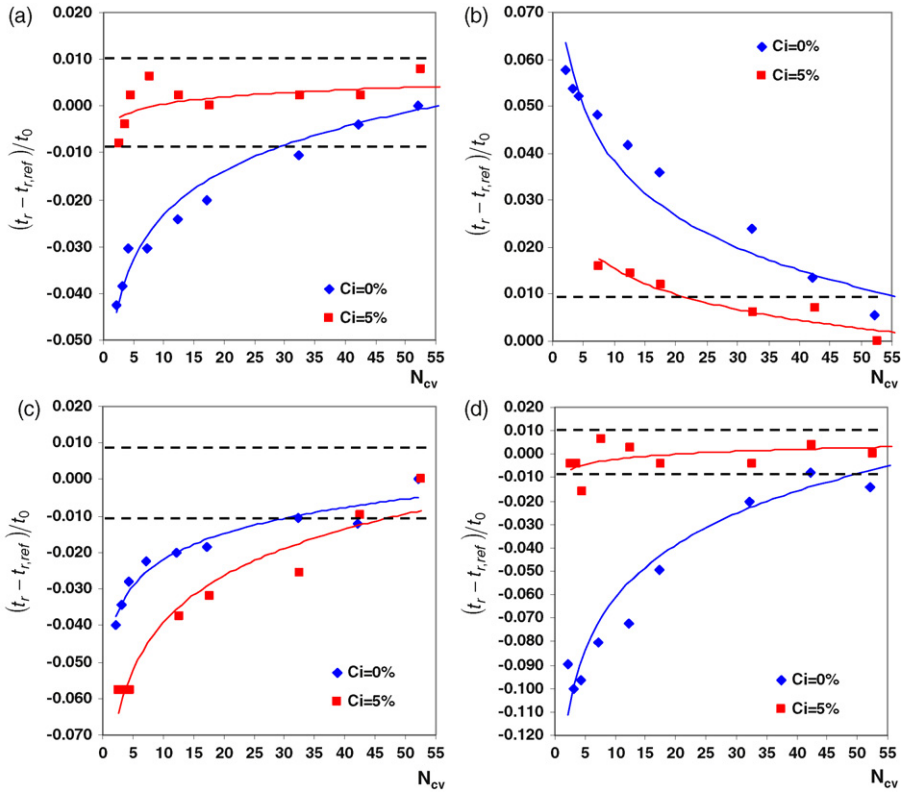

Fig. 6. Effect of a small amount of acetonitrile in the initial eluent. Initial composition: 0% acetonitrile (◆); 5% acetonitrile (■). Buffers: ammonium acetate (a); formic acid (b); ammonia (c and d). Solutes: atenolol (a); clozapine (b); protriptyline (c); ethylparaben (d). Column Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm; 30 °C; 0.5 mL/min.

3.2.3. *Effect of the type of stationary phase*

The previous results were compared to those obtained from the same alkyl phase but with a polar embedded group, other operating conditions being kept identical. Due to poor aqueous wettability, traditional C18-reversed phases are expected to collapse in highly aqueous conditions thereby leading to problems of column equilibration [21,22]. Modified alkyl stationary phases with a polar embedded group were thus developed to prevent collapse due to a better solvation of the stationary phase [23].

Fig. 7 illustrates the comparison between the two stationary phases. Unexpectedly, there was no significant difference between the two stationary phases for atenolol with ammonium acetate (Fig. 7a) when starting the gradient with 0% acetonitrile. For both columns, 30 column volumes were required to attain the full equilibration. It was also worth investigating whether the presence of a polar embedded group could further improve the above results for the clozapine with formic acid. Again, no improvement was observed as shown in Fig. 7b, Moreover a significant deterioration was found for protriptyline with ammonia (Fig. 7c). To summarize these anomalous results: the number of column volumes necessary to attain full column equilibration for protriptyline with ammonia increased from a traditional C18 phase using 0% acetonitrile to the same phase using 5% acetonitrile (Fig. 6c) and finally to a polar embedded phase, also using 5% acetonitrile (Fig. 7c). It is clear that equilibration in the present studied conditions is as slow on a polar embedded phase as on a traditional one whether the gradient starts with a small amount of organic solvent or not.

These results suggest that a poor solvation of the stationary phase is not the major cause of slow equilibration in case of ionizable compounds. While for neutral compounds, the column equilibration is essentially dependent on the initial state of stationary phase wetting, for cationic and anionic solutes, it is also dependent (1) on ionic strength as the cations of the buffer are competing ions for the anionic sites and (2) on mobile phase pH which will fix the number of the anionic sites. The problem of slow equilibration was clearly more important in case of low ionic strength (formic acid or ammonia). As a result, a polar embedded stationary phase does not speed up column equilibration for ionizable compounds. In some cases, it can even worsen the equilibration problem as shown for protriptyline with ammonia as additive.

3.2.4. *Effect of temperature and flow-rate*

The effect of temperature and/or flow-rate on gradient re-equilibration has been little studied and the conclusions were quite different depending on the authors. For instance, Schellinger et al. [20] did not observe any effect of temperature or flow-rate for basic compounds whereas elsewhere a beneficial role of temperature was observed for a neutral compound as probe solute and pure water as initial solvent [16].

In the present work, the role of both temperature and flow-rate in the equilibration process was investigated by studying, on the one hand the effect of temperature and on the other hand the combined effect of temperature and flow-rate. Three series of data are given in Fig. 8 for the four worst solute-additive pairs shown in Fig. 6. The three series correspond to (1) 30 °C and the corresponding Van Deemter optimum flow-rate (0.5 mL/min), (2) 70 °C and the same flow-rate and (3) 70 °C and the corresponding optimum flow-rate (1 mL/min). Similar trends were observed with the polar embedded phase. These results give rise to various comments:

(1) For atenolol which is the least retained compound (Fig. 8a), the rate of achieving column equilibration is neither affected by the temperature nor by the flow-rate. The required N_{cv} is always nearly 30. This is consistent with a previous study involving buffered eluents [20]. It was found that an increase in temperature from 40 to 80 °C had a minimal effect on the number of column volumes required for full equilibration and that full equilibration was independent of the flow-rate. These conclu-

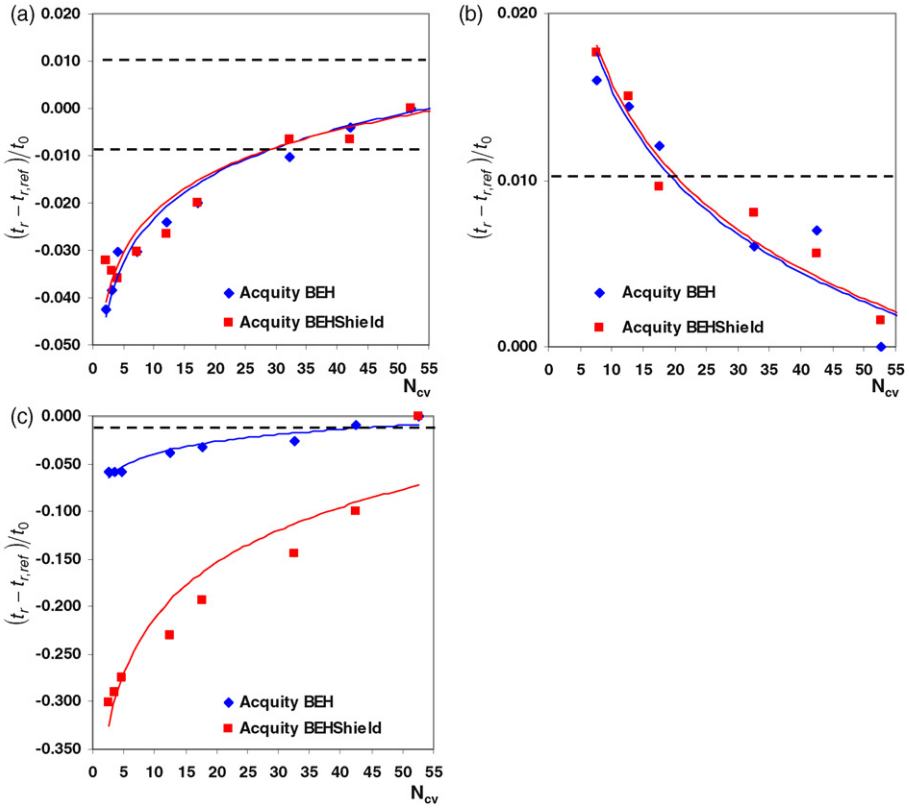

Fig. 7. Effect of a polar embedded group. Buffers: ammonium formate (a); formic acid (b); ammonia (c). Solutes: atenolol (a); clozapine (b); protriptyline (c). Columns: Acquity BEH-C18 (◆); Acquity BEH-C18 Shield (■); 30 °C; 0.5 mL/min; initial composition: 0% acetonitrile.

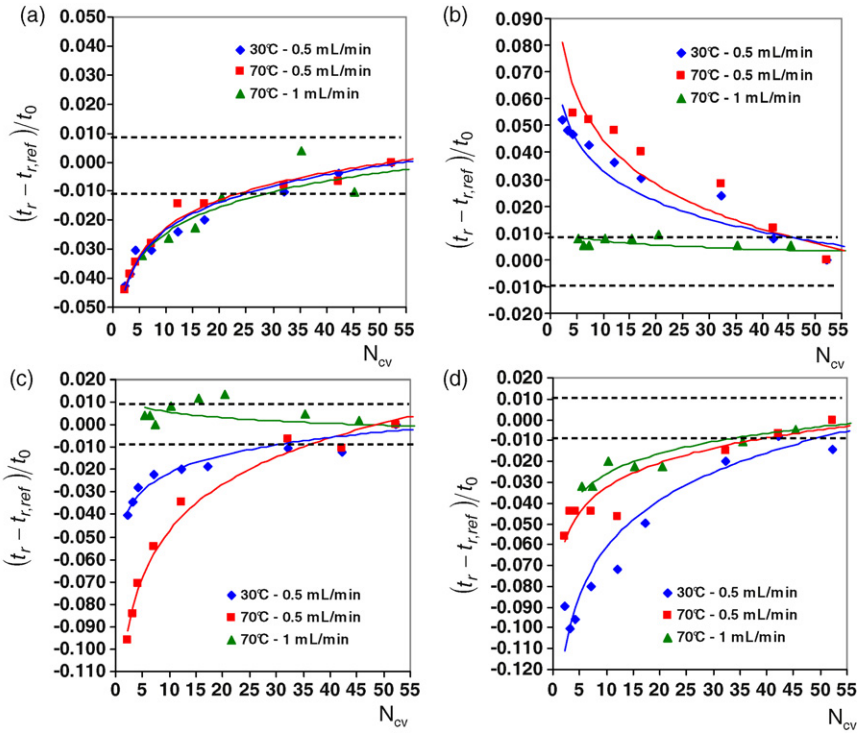

Fig. 8. Effect of temperature and flow-rate for the worst solute-additive pairs of Fig. 5. Additives: ammonium acetate (a); formic acid (b); ammonia (c and d). Temperature and flow-rate: 30 °C and 0.5 mL/min (◆); 70 °C and 0.5 mL/min (■); 70 °C and 1 mL/min (▲). Solutes: atenolol (a); clozapine (b); protriptyline (c); ethylparaben (d). Column Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm; initial composition: 0% acetonitrile.

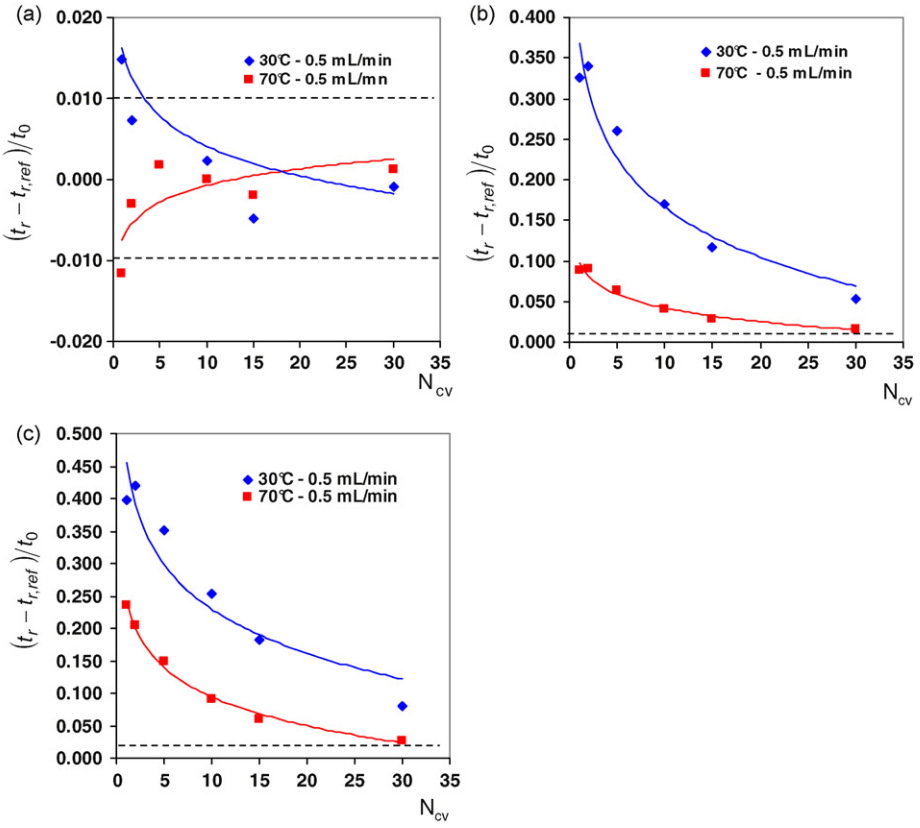

Fig. 9. Effect of temperature alone. Temperature: 30 °C (◆); 70 °C (■). Solutes: ethylparaben (a), clozapine (b), protriptyline (c). Column Nucleodur Gravity C18 50 mm × 2 mm, 1.8 μm; initial composition: 5% acetonitrile; additive: ammonium acetate; 0.5 mL/min.

sions were related to the worst solute which was a weakly retained basic compound as atenolol in the present study. However, it should be pointed out that the gradient runs were started with 10% acetonitrile and carried out with a constant amount of *n*-butanol added to the mobile phase. Such conditions were obviously quite favourable to fast equilibration and therefore a possible effect of temperature and/or flow-rate was probably difficult to highlight. However, it was also noted in the referenced study that this trend differed from a previous study with unbuffered eluents [18] where a significant influence of the flow-rate on the number of column volumes required for full equilibration had been highlighted. According to these previous works and the present study, it can be concluded that early eluted basic compounds are less subjected to thermodynamic and/or kinetic effect.

(2) At acidic pH, the combined effect of temperature and flow-rate was significant for the most retained basic compounds such as clozapine (Fig. 8b), When both temperature and flow-rate are increased, full equilibration is achieved in less than five column volumes with formic acid whereas more than 50 column volumes are necessary at 30 °C. In contrast, increasing the temperature alone is not sufficient to provide a significant improvement.

(3) At basic pH, the number of column volumes is also greatly reduced for basic protriptyline (Fig. 8c) as well as for acidic ethylparaben (Fig. 8d) when increasing both flow-rate and temperature. Again, the influence of temperature alone is not clear.

The evidence of an effect of temperature alone was highlighted on a Nucleodur Gravity C18 column packed with sub-2 μm particles which demanded at 30 °C, even in ammonium acetate conditions, a huge number of column volumes before the full equilibration was achieved. The column temperature was increased to 70 °C while keeping the same flow-rate. The resulting data are shown in Fig. 9 for three different solutes. The number of column volumes for ethylparaben (Fig. 9a) which is neutral at this pH, was very small (<2) and close to that obtained on Acquity columns in similar conditions (see Fig. 5c). In contrast, the number of column volumes needed to equilibrate the Nucleodur Gravity column was considerable for basic compounds at 30 °C (Figs. 9b and c) while less than five column volumes were sufficient for Acquity columns (see Fig. 5e and f). At 70 °C with the same flow-rate, the number of column volumes was greatly reduced even if it remained large (close to 30). These results clearly highlight a beneficial effect of the temperature alone.

A recent study [15] stated that slow equilibration process was probably dependent on the stationary phase and that almost 40% of all commercial columns could be subjected to this effect. The process of slow equilibration was highlighted in the particular case of isocratic separation at low pH with potassium phosphate as buffer, with a high ionic strength (60 mM) and a high content of acetonitrile in the mobile phase (50%). The authors compared the retention times obtained with a column just received from the manufacturer and those obtained after a high pH treatment. They observed an increase in the retention factor followed by a very slow return towards initial values after flushing out the column with a very large volume of mobile phase. Slow equilibration phenomenon was explained by a slow change in column charge, especially at low pH where the number of anionic sites is smaller. These conditions are very far from those of the present study. We nevertheless studied the effect of temperature and flow-rate in formic acid with a Zorbax SB-C18 column which is expected to be less subjected than other columns to slow equilibration according to this previous study. As with all other studied columns, the column equilibration for basic solutes was very slow at 30 °C with formic acid. For illustrative example, Fig. 10b shows the data for diphenhydramine as

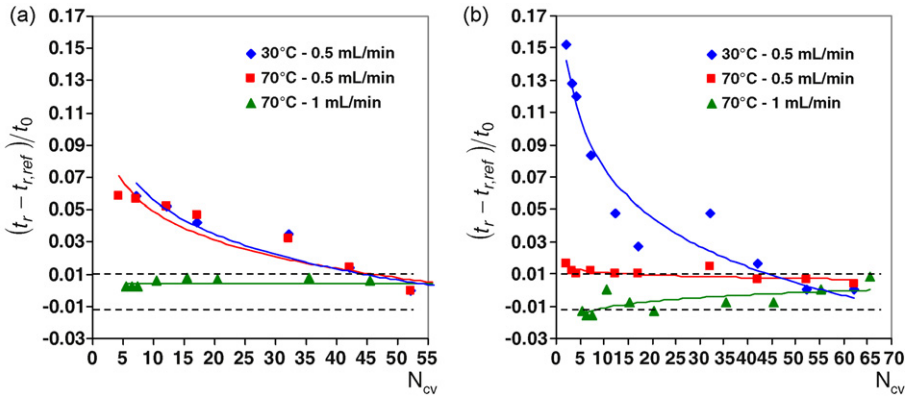

Fig. 10. Comparison of the effect of temperature and flow-rate between two different C18 phases. Columns: Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm (a); Zorbax SB-C18 50 mm × 2.1 mm, 1.8 μm (b). Temperature and flow-rate: 30 °C and 0.5 mL/min (◆); 70 °C and 0.5 mL/min (■); 70 °C and 1 mL/min (▲). Additive: formic acid; solute: diphenhydramine.

basic solute. The trend is quite similar to that with the Acquity BEH column (Fig. 10a). In contrast, a substantial effect of the temperature alone was observed with the Zorbax SB-C18 column (Fig. 10b), similarly as the Nucleodur Gravity phase (Fig. 9). As for all studied columns, the combined effect of temperature and flow-rate is spectacular.

Increasing the temperature may produce the following positive effects on column equilibration: (1) enhancing the sorption/desorption kinetics from mobile phase on to stationary phase; (2) improving the diffusivity of the components within the pores of the stationary phase; (3) modifying the phase conformation; (4) varying the amount of adsorbed solvent; (5) changing the ionization state of the stationary phase.

On the other hand, some authors stressed that the wetting problem was due to the inability of pure water to penetrate the silica pores because of the high surface tension of water [21,24,25] and that pressure could force the water to penetrate the pores and therefore improve the surface wetting. This could account for a positive effect of flow-rate which is related to pressure.

We believe that the temperature alone has a positive effect on the equilibration process but this parameter may be not sufficient due to a concomitant decrease in the column pressure drop which is most probably detrimental for the reason given above. When adjusting the flow-rate at 70 °C, the pressure is increased and this positively affects equilibration. However the pressure was quite similar at 70 °C and 1 mL/min (590 bar for the Acquity BEH) as at 30 °C and 0.5 mL/min (500 bar) since these flow-rates were close to the Van Deemter optimum and therefore nearly inversely proportional to the viscosity. So, interpreting the reason of equilibration improvement with both temperature and flow-rate is not straightforward. Higher temperatures may improve the diffusion of the initial eluent components into the pores while higher flow-rates associated with higher pressures may force the water to penetrate the pores and therefore improve the solvation of the stationary phase [16]. However it is very difficult to ascertain what is determining since the nature of the C18 column (end-capping, bonding density, nature of silica, pore size) is also an important factor [25] as highlighted by the great differences that were obtained between the various C18 columns. Investigations are underway in our laboratory to get definite explanation concerning the effect of temperature, flow-rate and both together.

It is also important to note that when the same volume passes through the column, the time of contact with the initial eluent is divided by a factor two between 0.5 and 1 mL/min. A decrease of the required equilibration volume at higher flow-rate is not in good agreement with other studies [15,26,27] that showed that static equilibration was equivalent to flow-one thereby suggesting that column equilibration should require a certain time of initial eluent exposure rather than a certain volume. According to our results, static equilibration might be much slower, due to the absence of pressure.

It was reported elsewhere that depending on the column oven, high temperatures can be more difficult to control, which may produce a detrimental effect on retention variability [19]. In addition, the extent to which the gradient delivered by a given instrument is reproducible can also affect the retention variability. However, it can be noted that instrumental factors should produce the same effect on retention variability regardless of the column type. We nevertheless studied the effect of temperature with a conventional HPLC instrument and a conventional 3.5 μm particle size column which was also a polar embedded phase. The data obtained at 30 and 70 °C for basic compounds with formic acid are shown in Fig. 11 and can be compared to those obtained from an UHPLC equipment (Fig. 8). As shown, an increase in both flow-rate and temperature also leads to much faster equilibration, suggesting that increasing the temperature and hence the flow-rate is beneficial to column equilibration whatever instrumentation and stationary phase.

3.2.5. *Inter-series retention variability (intermediate precision)*

A major difficulty arises from slow equilibration. It relates to intermediate precision which is given by the retention variability between two gradient runs, both runs being carried out with the same equilibration time but preceded by runs carried out with a different equilibration time. It is important to notice that a given stationary phase may be inevitably exposed to different volumes of initial eluent and is therefore likely to be subjected to this problem. In addition to retention variability, this problem may also lead to variation of selectivity which more strongly affects the robustness of a given method. In this case, a given gradient method related to a given equilibration time turns out to be impossible to duplicate unless full equilibration is achieved.

Indeed, at 30 °C, the retention variability was significantly higher than that expected from repeatability, when formic acid or ammonia were used as additives. This can be observed in Fig. 12 for two series of experiments carried out with $N_{cv} = 7$ and preceded by series carried out with $N_{cv} = 2$ and $N_{cv} = 40$, respectively. This study was performed at 30 °C and 70 °C with flow-rates close to the optimum determined by the Van Deemter curves. At 30 °C, very poor results were obtained with formic acid (Fig. 12a and c) for all basic compounds except for the least retained basic compound (atenolol) and the non-ionized compound (ethylparaben). It is noteworthy that the same trend was observed with both columns, AcquityBEH (Fig. 12a) and ZorbaxSB (Fig. 12c). Furthermore, it was also observed with the polar embedded phase (results not

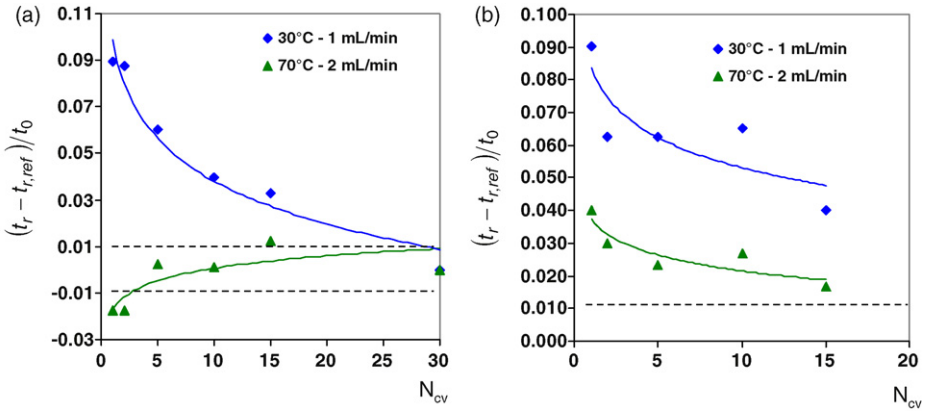

Fig. 11. Effect of temperature and flow-rate using conventional equipment and column. Temperature and flow-rate: 30 °C and 1 mL/min (◆); 70 °C and 2 mL/min (▲). Solutes: pindolol (a), clozapine (b). Column Xterra RPC18 100 mm × 4.6 mm, 3.5 μm; additive: formic acid. Other gradient conditions are given in Section 2.

shown). Similarly, the retention variability was quite significant with ammonia for partially ionized compounds (ethylparaben and protriptyline) (Fig. 12b). At 70 °C, while with 0.5 mL/min, we just observed a small improvement (results not shown), the variability became similar to the repeatability (<0.01) for all solutes with 1 mL/min (Fig. 12), suggesting that the retention times are statistically unchanged at 70 °C with a difference in retention times not greater than 0.001 min. These results point out again that flow-rate together with temperature have a major effect on retention variability. At 30 °C, the quality of the separation (relative retention)

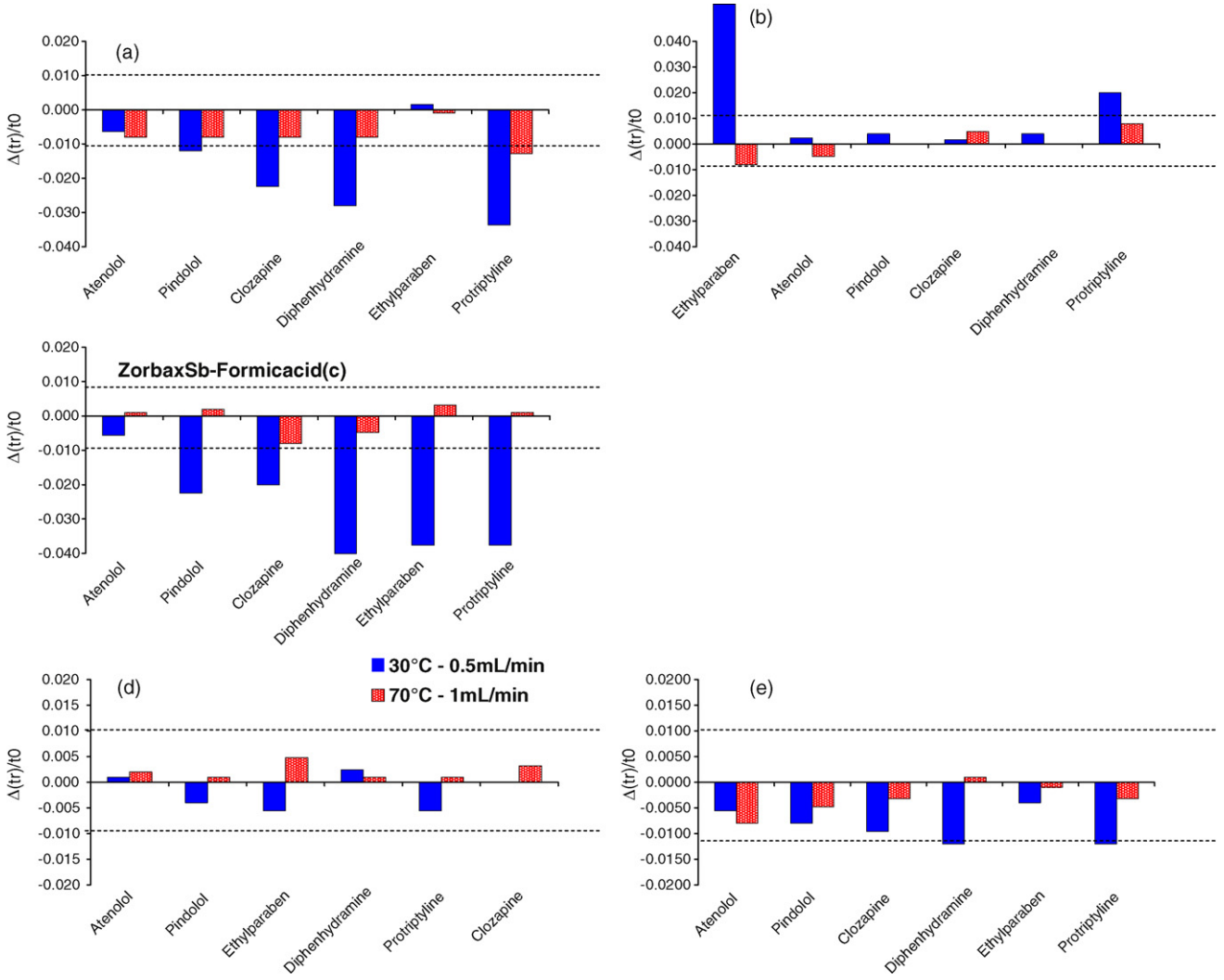

Fig. 12. Retention variability as a function of the solutes and the temperature between two series carried out with the same N_{cv} = 7, preceded by series carried out with N_{cv} = 2 and 40 respectively. Temperature and flow-rate: 30 °C and 0.5 mL/min (■); 70 °C and 1 mL/min (▩). Additives: formic acid (a and c); ammonia (b); ammonium acetate (d); ammonium formate (e). Columns: Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm (a, b, d, and e); Zorbax SB-C18 50 mm × 2.1 mm, 1.8 μm (c). Initial composition: 0% acetonitrile.

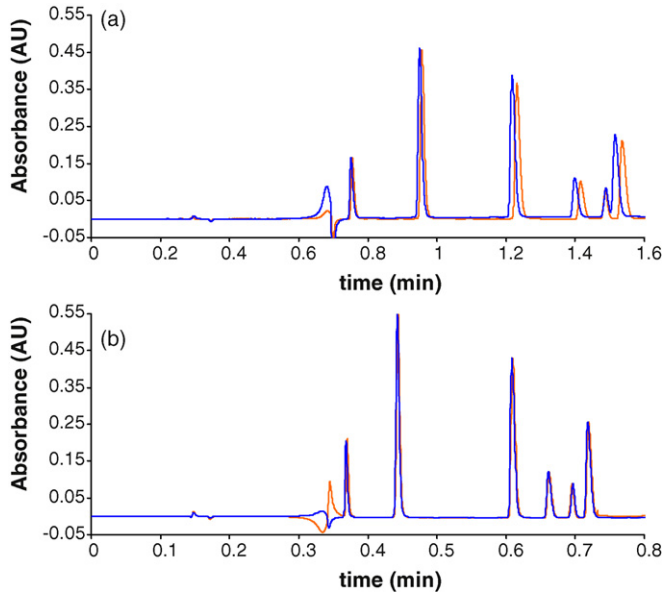

Fig. 13. Two overlaid chromatograms for the separation of six representative solutes (atenolol, pindolol, clozapine, diphenhydramine, ethylparaben and protriptyline) carried out with the same equilibration time (N_{cv} = 7) but preceded by a gradient run performed with a different equilibration time (N_{cv} = 2 and N_{cv} = 40, respectively). Temperature and flow-rate: 30 °C and 0.5 mL/min (a); 70 °C and 1 mL/min (b) Column Acquity BEH-C18 50 mm × 2.1 mm, 1.7 μm; additive: formic acid; initial composition: 0% acetonitrile. Other conditions are given in Section 2. The baseline signal has been subtracted.

may vary between two runs performed with the same equilibration time as shown in Fig. 13 where the last pair of solutes varied considerably depending on the preceding gradient equilibration time while no significant change in retention time and therefore no change in selectivity occurred at 70 °C and 1 mL/min. Neutral species such as ethylparaben at this pH did not exhibit this effect.

The retention variability with both ammonium acetate (Fig. 12d) and ammonium formate (Fig. 12e) was similar to the repeatability even at 30 °C for the atenolol, thereby highlighting again the major role of the ionic strength.

These results are consistent with the preceding ones. For a given equilibration time, there is a given state of the stationary phase, which is modified during the gradient run as the mobile phase composition varies. While the initial state of the stationary phase is dependent on the equilibration time of the run, for ionizable compounds it seems to be also dependent on the column history at the initial mobile phase composition, namely on the duration of the preceding equilibration time. This problem was more important in case of low ionic strengths (formic acid and ammonia) and was reduced at higher temperatures and/or higher flow-rates. Again a slow change in column charge could explain this phenomenon since the maximum column charge is obtained in the initial eluent and most probably dependent on the equilibration time. The total negative charge of the column is likely to decrease during the gradient run because the silanols turn out to be less acidic (i.e. with higher pK_a) when the content of organic modifier in the mobile phase is increased.

Similarly, a day-to-day retention drift has also been observed when the column was stored in an initial butanol/ACN/water/TFA eluent whereas the intra-day repeatability was not affected [19]. According to the authors, these small changes in retention could be due either to instrument performance drifts or to a loss of organic modifier in the initial eluent over time. However, both explanations are not suitable for the present study because (1) the solutes are not affected on the same way, the retention variability depending on both the solute type and the solute retention; (2) our different series were performed within a single day, without organic modifier in the eluent A and (3) we found that, for both ammonia and formic acid, a concomitant increase in column temperature and flow-rate considerably reduced the retention variability, suggesting that a thermodynamic and/or a kinetic effect is involved.

As a conclusion, in order to keep excellent retention variability while using just a few column equilibration volumes, we recommend to circumvent the use of formic acid or ammonia as additives and to prefer the use of ammonium acetate or formate. Otherwise, higher temperatures with appropriate flow-rates prevent excessive variability with either formic acid or ammonia and can therefore offer acceptable results.

4. Conclusions

The aim of this work was to find suitable operating conditions which can provide low re-equilibration times together with low retention variability, working in UHPLC conditions with volatile buffers. The role of both temperature and flow-rate has been emphasized. It was shown that the equilibration process remains strongly dependent on the solute, especially when this latter is ionizable. In this case, the mechanisms which govern re-equilibration in gradient elution remain very complex and require much further work to be elucidated. The major conclusions of the present study are summarized as follows:

- The retention variability between identical runs (i.e. the repeatability) is independent of the operating conditions including re-equilibration time, type of compound (neutral, acidic or basic), type of buffer, composition of initial eluent, temperature, flow-rate and type of stationary phase. The repeatability is quite excellent and corresponds to a difference in composition at elution of 0.1% in spite of the very short gradient times (i.e. 1 min for some of them).
- The retention variability between runs with the same equilibration time but preceded by a run with a different equilibration time (i.e. intermediate precision) can be more critical especially for ionizable compounds with eluents having a low ionic strength (i.e. ammonia or formic acid). It was shown that an increase in temperature together with an increase in flow-rate reduce this retention variability down to the repeatability for all compounds.
- Obtaining the full column equilibration can be sometimes time-consuming. A small amount of 5% acetonitrile in the initial eluent is usually sufficient to reduce the number of required column volumes which is in good agreement with other reported studies. When the required number of column volumes, needed to achieve full equilibration with ionizable compounds, is high (with or without organic solvent in the initial eluent), it appears from the present study that this number can be considerably decreased by selecting appropriate conditions according to the following comments: (1) ammonium acetate or ammonium formate should be preferred to ammonia or formic acid; (2) surprisingly, no improvement can be expected from a polar embedded phase; (3) a beneficial effect of both temperature and flow-rate was highlighted. Although it is difficult to state which parameter between temperature and flow-rate is more advantageous, it should be pointed out that an increase in temperature should always be accompanied by an increase in flow-rate so that the reduced linear velocity is kept constant and as a result it can be stated that high temperatures are always beneficial to column equilibration.

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