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On the extra-column band-broadening contributions of modern, very high pressure liquid chromatographs using 2.1 mm I.D. columns packed with sub-2 μm particles

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ABSTRACT

The efficiencies of two narrow bore columns (100 mm and 50 mm × 2.1 mm) packed with 1.7 μm totally porous BEH-C_{18} particles were measured on two very high pressure liquid chromatographs (Acquity from Waters and 1290 Infinity HPLC System from Agilent) operating at maximum pressures of 1034 and 1200 bar, respectively. The probe compounds were a mixture of uracil, acetophenone, toluene, and naphthalene eluted in a 50/50 (v/v) solution of acetonitrile and water at 303 K with a flow rate of 0.40 mL/min. The apparent efficiencies of columns, which lumps the consequences of band broadening due to the column and the system contributions, may depend much on the extra-column volumes of the instruments used. Actually, it is known for a long time that the apparent column performance is strongly affected by the instrument characteristics, including the diameter of the connecting tubes, the injection technique (with or without needle seat capillary), and the detection cell volume. When the 1290 Infinity HPLC System is equipped with a needle seat, an inlet and an outlet connecting capillary tube with inner diameters around 115 μm, its extra-column variance for a 0.1 μL injection volume is 9.2 μL^2 while that of the Acquity instrument is 6.9 μL^2. Minor modifications suggested by their respective manufacturers allowed significant reductions of these variances, to 6.2 and 3.9 μL^2, respectively. Yet, in their optimized configurations and for weakly retained compounds ($k \simeq 1$), these modern, sophisticated instruments cannot provide more than 75% (1290 Infinity) and 85% (Acquity) of the maximum efficiency of a 2.1 mm × 50 mm BEH column. For more strongly retained compounds ($k > 4$), in contrast, they are both able to provide more than 95% of the maximum expected efficiency.

1. Introduction

A decade ago, the performances of packed columns were practically independent of the HPLC instrument used. The dimensions of the typical columns were 250 mm × 4.6 mm. They were packed with porous 5 μm particles, had a minimum height equivalent to a theoretical plate (HETP) between 10 and 15 μm, and their total porosity, ε_t, was between 0.55 and 0.65. Accordingly, the peak variances of poorly retained compounds, with a retention factor of, e.g. $k = 1$, was as large as 1500 μL^2. The extra-column variance of conventional instruments operating under 400 bar, equipped with a 5 μL needle seat, 60 cm × 170 μm I.D. connecting tubes, and a 8 μL detection cell, with a data sampling frequency of 10-20 Hz is about 75 μL^2 [1]. The extra-column band broadening contribution represents thus 5% of the total variance of a non-retained peak, so the specific instrument used was not a critical factor in the performance delivered by these columns, which provided very similar plate numbers on any HPLC instrument. Things have considerably changed since then.

New packing materials are made of 3, 2.5, or even sub-2 μm porous particles, and now of 2.5 and sub-2 μm shell particles. Columns packed with them are shorter and more efficient. Analysts appreciate the shorter analysis times and high separation power that they provide. To alleviate the consequences of the heat generated in these columns by the percolation of mobile phases at high flow rates, under important pressure gradients, columns must often have narrower inner diameters, 2.1 or 1 mm. Today, columns packed with sub-2 μm totally porous particles [2–5] and sub-3 μm superficially porous particles [6–9] provide column HETPs as low as 3 μm. They are often 5 cm long and 2.1 mm in I.D. Given the total porosity of columns packed with core-shell particles ($\varepsilon_t = 0.53$), the peak variances of compounds with $k = 1$ may be only 2 μL^2. It has become impossible to use these columns with old standard instruments unless some skilled surgical operation reduces their extra-column contributions down to a fraction of the column variance by replacing standard parts with others of smaller volumes

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and the sample band is focused at the column inlet [10]. If it is acceptable for columns packed with 2.5 μm particles, this solution is not so for columns packed with sub-2 μm particles, due to the back pressure limitations of these old instruments.

The low permeability of columns packed with sub-2 μm particles requires that analysts progressively switch toward recent instruments that may deliver the mobile phase at pressures up to 1000–1200 bar. While the problem of delivering the mobile phase under very high pressures is solved, that of achieving full column performance remains, not only due to the heat effects [4,11,12] but also because the extra-column variances of these instruments are still significantly larger than 2 μL^2 [10,13]. It is actually a very challenging task to built up injection systems that deliver extra-column variances as small as a few μL^2.

In this work, we investigate the performances of two recent instruments designed for very high pressure liquid chromatography (VHPLC). Our goal was to determine the apparent efficiency that columns packed with sub-2 μm particles could provide when used with either one of them. These instruments are the Acquity (Waters, Milford, MA, USA) and the 1290 Infinity HPLC system (Agilent, Waldbroen, Germany). Admittedly, these equipments were optimized for different purposes, the first one to allow the successful use of short columns packed with sub-2 μm particles and to provide with them fast analytical results; the other one to allow column operation at the maximum possible inlet pressure, under a high flow rate and to minimize the consequences of the heat generated by the percolation of the mobile phase through the column. Nevertheless, in this study, we focused only on the sources of the extra-column band broadening contributions of these instruments, sources which are related to the behavior of the injection system, the connecting tubes, and the detection cell. Other important instrument properties, such as the solvent composition precision, the minimal delay volume while performing optimal mixing, the sample volume wasted during injection, or the detection sensitivity and dynamic linear range were not checked. For the purposes of many analysts, these instrument characteristics may be as important as the extra-column contributions of the system or even more. All instruments are compromises between requirements that may be incompatible. Before selecting an instrument, analysts must decide what is most important for them and act accordingly.

We used two 2.1 mm I.D. columns, one 50, the other 100 cm long, both packed with BEH-C_{18} particles. We successively measured the true extra-column peak variance contributions of the two instruments, which is necessary to accurately determine the real potential performance of the two columns, and the overall efficiencies of these two columns. We also investigated practical methods to reduce the band spreading contributions of each instrument.

2. Theory

2.1. Peak variance and retention time in a chromatographic system

The different parts of the instrument that are involved in the dispersion of the sample band include the system drawing the sample from its vial into the injection loop, the one ejecting the sample into the needle seat, the needle seat capillary, the injection valve, the tube connecting the valve to the column, the tube connecting the column to the detector, and the detector cell. To these physical sources of dispersion, we must add the acquisition rate of the signal. Note that, in contrast to the 1290 Infinity HPLC system, the Acquity is not equipped with any needle seat capillary. The total observed band variance in time unit is written [10]:

$$\sigma^2_{t,Exp.} = \sigma^2_{t,I} + HL\left[\frac{1+k}{u_0}\right]^2 + \sigma^2_{t,D} \quad (1)$$

where the band variances $\sigma^2_{t,I}$ and $\sigma^2_{t,D}$ account for the sample dispersion before and after the column, respectively, H is the intrinsic column plate height, L is the column length, and u_0 is the chromatographic linear velocity.

The retention time of the sample is given by the classical relationship:

$$t_{R,Exp.} = t_{ex} + \frac{L}{u_0}(1+k) + \frac{t_I}{2} \quad (2)$$

where t_{ex} is the extra-column elution time and t_I is the injection time at the column inlet, in principle equal to the ratio of these respective volumes and the flow rate.

2.2. Apparent column efficiency

The apparent column efficiencies include the combined influence of the band broadening taking place in the column and of the contributions of the extra-column volume. Analysts cannot directly measure the full kinetic performance of a chromatographic column due to the contribution of the extra-column band broadening. The apparent column efficiency is

$$N_{app} = \left[\frac{t_{R,Exp.}}{\sigma_{t,Exp.}}\right]^2 \quad (3)$$

The peak variance was systematically measured according to the peak-width at half-height and assuming a Gaussian concentration distribution:

$$\mu'_{2,ex} = F_v^2 \frac{\left(t^r_{1/2} - t^f_{1/2}\right)^2}{5.545} \quad (4)$$

where F_v is the flow rate and $t^r_{1/2}$ and $t^f_{1/2}$ are the elution times of the rear and front parts of the peak, respectively, at half-height. The comparison between the extra-column second central moment ($\mu'_{2,ex}$) measured from the peak width at half-height on the Acquity and the 1290 Infinity HPLC system very high pressure liquid chromatographs is shown in Fig. 1. Note that the extra-column peak variance contributions are best measured from the second moment of bands eluted through a zero-volume connector, not from the half-height peak-width, because the latter minimizes the consequences of the tailing of injected bands.

3. Experimental

3.1. Chemicals

The mobile phase used in this work was a mixture of acetonitrile and water. Both solvents were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). The mobile phase was filtered before use on a surfactant-free cellulose acetate filter membrane, 0.2 μm pore size (Suwannee, GA, USA). Naphtho[2,3-a]pyrene and 4-*tert*-butylphenol were purchased also from Fisher Scientific. The standard sample mixture containing uracil, acetophenone, toluene, and naphthalene dissolved in 100% acetonitrile was obtained from Phenomenex (Torrance, CA, USA).

3.2. Columns

The two BEH-C_{18} columns (50 mm × 2.1 mm and 100 mm × 2.1 mm) were a generous gift from the column manufacturer (Waters, Milford, MA, USA). The main characteristics of the bare porous silica and those of the derivatized packing material making the column bed are summarized in Table 1. The Zorbax Eclipse C_{18} column (50 mm × 2.1 mm) was generously offered by the manufacturer (Agilent Technologies, Waldbroon,

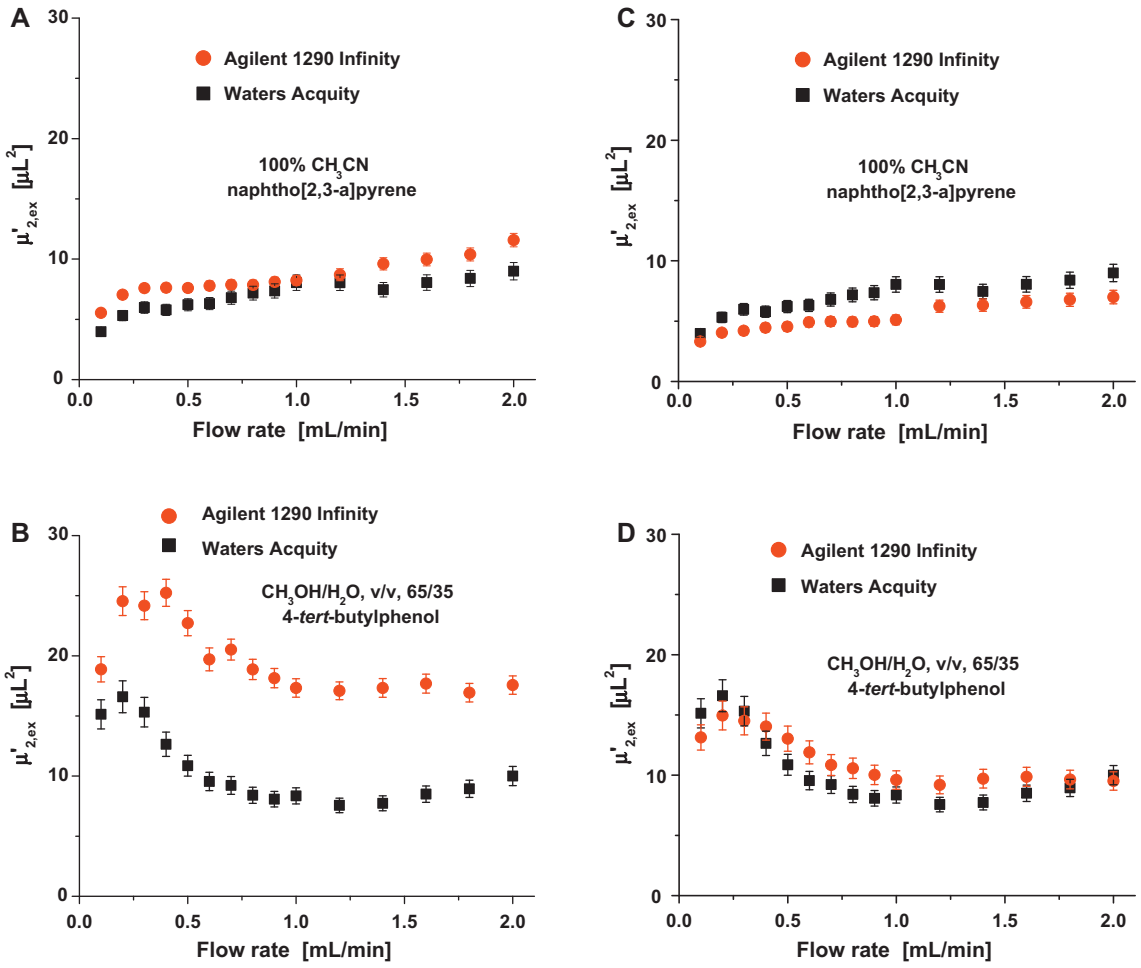

Fig. 1. Comparison between the extra-column second central moment ($\mu'_{2,ex}$) measured from the peak width at half-height on the Acquity and the 1290 Infinity HPLC system very high pressure liquid chromatographs. T = 295 K. Injection volume: 1 μL. The flow rate increases from 0.1 to 2.0 mL/min. (A) Eluent: pure acetonitrile, sample: naphtho[2,3-a]pyrene. 1290 Infinity capillary I.D.: 140 μm. (B) Eluent: methanol–water mixture (65/35, v/v), sample: 4-*tert*-butylphenol. 1290 Infinity capillary I.D.: 140 μm. (C) Same as in (A) except the 1290 Infinity capillary I.D. of 115 μm. (D) Same as in (B) except the 1290 Infinity capillary I.D. of 115 μm. Note that the largest peak variance is observed with the most viscous mobile phase, especially at low flow rates. Note also the comparable extra-column band broadening contributions of both instruments in (C) and (D).

Germany) during the installation of the Infinity 1290 system in our laboratory.

3.3. Apparatus

Two chromatographs capable of supplying an adequate stream of mobile phase at a very high pressure were used in this work: the 1290 Infinity HPLC system and Acquity instruments.

The 1290 Infinity HPLC system (Agilent Technologies, Waldbroon, Germany) liquid chromatograph includes a 1290 Infinity Binary Pump with Solvent Selection Valves and a programmable auto-sampler. The injection volume was set at 0.1 μL. The instrument is equipped with a two-compartment oven and a multi-diode array UV–VIS detection system. The temperature of the detection cell was fixed at 313 K. The system is controlled by the Chemstation software. The sample trajectory in the equipment involves passage through

- A 20 μL injection loop attached to the injection needle. Note that the sample of volume drawn is the same as the volume of sample injected to the column.

Table 1
Physico-chemical properties of the 2.1 mm I.D. BEH-C_{18} columns given by the manufacturer.

Neat silica	**Bridged ethylsiloxane/silica hybrid (BEH)**	
Particle size [μm]	1.7	
Pore diameter [Å]	130	
Surface area [m^2/g]	185	
Bonded phase analysis	**BEH-C_{18}**	
Total carbon [%]	18	
Surface coverage [μmol/m^2]	3.10	
Endcapping	Proprietary	
Packed columns analysis		
Serial number	01672902020A01	01201516010L
Dimension length [mm] × I.D. [mm]	50 × 2.1	100 × 2.1

- A small volume needle seat capillary (red tubing), ≃1.7 μL, between the injection needle and the injection valve. The total volume of the grooves and connection ports in the valve is around 1.2 μL.
- Two connector capillaries (red tubing), with 140 μm I.D., the first being 340 mm long (before the heat exchanger) and the second 220 mm long (after the column and before the detector cell). Their total volume is 8.6 μL.
- One heat exchanger capillary, volume 1.6 μL.
- A small volume detector cell, 2.4 μL, 10 mm path.
- The signal is acquired with a sampling rate of 40 Hz.

The Acquity UPLC (Waters, Milford, MA, USA) liquid chromatograph includes a quaternary solvent delivery system, an auto-sampler with a nominal 5 μL sample loop, actually calibrated at 7.1 μL by the instrument calibration measurement. The injection volume was set at 0.1 μL with partial loop, with needle overfill as the sample loop option. In contrast to the 1290 Infinity HPLC injection system, the sample is placed at one end of the injection loop, which is directly attached to the injection valve, and is directly backflushed to the column stabilizer (filo injection mode). The instrument is also equipped with a column oven, and a data station running the Empower data software from Waters. There is a time delay between the moment when the signal begins to be recorded and the time when the sample physically leaves the injection loop. This offset time is 0.65 s. During an analysis, the sample passes through the following parts which are involved in the extra-column sample band spreading

- There is no needle seat capillary in the Acquity system.
- The 5 μL injection loop is directly connected to the injection switching valve. The dead-volume in the injection valve is approximately 1 μL. Note that 15 μL of sample volume have to be drawn by default for any injected volume.
- One column stabilizer or heat exchanger tube, 127 μm I.D., 550 mm long (before the column) and one outlet capillary, 102 μm I.D., 150 mm long (after the column). The total volume of these tubes is 8.2 μL.
- A monochromatic UV detector, with a 0.5 μL cell and a 10 mm path length.
- The signal is acquired with a sampling rate set at 40 Hz.

The same gold compression screw, collet, and PEEK ferrule (Waters, Milford, MA, USA) were used to connect the columns' inlet to the pre-column capillary of both instruments, which allows a fair comparison between the two instruments. The extra-column contributions were measured by replacing the chromatographic column with a zero dead volume (ZDV) union connector (Thermo Scientific, Suwanee, GA, USA).

The flow rate accuracy was determined by directly pumping the pure mobile phase at 295 K and 1 mL/min during 50 minutes into a volumetric flask of 50 mL in the absence of chromatographic column. The eluent was collected at the exit of the column stabilizer of the Acquity instrument. It was collected at the exit of the heat exchanger of the 1290 Infinity HPLC chromatograph. The relative error was less than 0.2%, so we estimate that the long-term accuracy of the flow-rate at 1 mL/min is around 2 μL/min. Note that the flow rate delivered by the Acquity is true at the column inlet (highest pressure) while this is true at the column outlet (atmospheric pressure) with the 1290 Infinity HPLC pump system. The laboratory temperature was controlled by an air conditioning system set at 295 K. The daily variation of the ambient temperature never exceeded ±1 K.

The temperature of the column inside the oven compartment of both chromatographs was measured with a snap-on strain relief thermocouple of type T (copper-Constantan junction, 1 mm junction size) located at half the length of the column, a handled thermometer with two inputs, and adhesive pads all purchased from Omega Engineering, Inc. (Standford, CT, USA). The precision of the thermocouple is ±0.1 K.

3.4. Samples

The sample mixture contains uracil (<20 μg/mL), acetophenone (200 μg/mL), toluene (9000 μg/mL), naphthalene (1400 μg/mL), and traces of water, all dissolved in pure acetonitrile. Uracil was dissolved in a minimum volume of hot water which was mixed with acetonitrile. All compounds were detected at 254 nm. The mobile phase was a mixture of acetonitrile and water (50/50, v/v). The flow rate was set at 0.40 mL/min on both instruments. The retention factors of acetophenone, toluene, and naphthalene are 1.1, 3.9, and 5.6, respectively, on the 5 cm long column. They are 1.2, 4.3, and 6.3 on the 10 cm long column.

4. Results and discussion

First, we analyze the extra-column contributions of the Acquity and 1290 Infinity HPLC system instruments. Then, we determine the apparent efficiencies of the 2.1 mm × 50 mm and 2.1 mm × 100 mm BEH-C_{18} column on the two instruments and discuss the reasons for the differences observed between the two instruments.

4.1. Extra-column contributions of the standard 1290 Infinity HPLC system and Acquity instruments

The extra-column contributions of both instruments were measured at 295 K in pure acetonitrile with naphtho[2,3-a]pyrene and with 4-*tert*-butylphenol eluted in a methanol/water mixture (65/35, v/v). A 1 μL sample volume was injected. At each applied flow rate (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mL/min), the data were measured in triplicate. Their relative standard deviation (σ_{n-1}) was between 1% and 5% at all flow rates and for both instruments. The accuracy of these measurement is relatively poor because the profiles of bands eluted through the extra-column volumes alone tail systematically. So, by assuming Gaussian profiles for these peaks, we obtained underestimates of the true extra-column peak variance. The lower the flow rate, the more symmetrical the peak eluted, and the closer to the true variance the value given by Eq. (4). Since we are interested in comparing two different instruments, these estimates give fair results because the degree of peak tailing is very similar on the two instruments, at all flow rates.

Fig. 1A and B show plots of the peak variance measured for naphtho[2,3-a]pyrene eluted with pure acetonitrile and for 4-*tert*-butylphenol eluted with a (65/35, v/v) mixture of methanol and water as functions of the applied flow rate. In this section, we emphasize the importance of the role played by the eluent viscosity on the extent of the extra-column band broadening of small molecules. For a given eluent, the widths of the elution profiles of both naphtho[2,3-a]pyrene and 4-*tert*-butylphenol recorded at the exit of a zero-volume connector are very similar. Difference in peak shape and width are only visible when the viscosity of the eluent is significantly changed. The difference between the two plots obtained on the same instrument is particularly striking at low flow rates (<0.2 mL/min). The extra-column band broadening is about three times larger in the aqueous solution of methanol than in pure acetonitrile. This is because band broadening in connecting channels is mostly controlled by radial diffusion of the sample across the diameter of these channels. According to the Aris-Taylor model of band broadening in tubes, which rigorously applies for infinitely long residence times (e.g., at very low flow rates and/or for very long

capillary tubes), the asymptotic value of the increment of variance per time unit is given by [14]:

$$\lim_{t\to\infty}\frac{1}{2}\frac{d\mu'_{2,ex}}{dt} = D_m + \frac{R_c^2 u^2}{48 D_m} \quad (5)$$

where D_m is the molecular diffusivity, R_c the radius of the capillary tube, and u the mean linear velocity along the open connecting capillary tubes. If L is the total length of the capillary tubes, integration of Eq. (5) from $t = 0$ to $t = L/u$ leads to:

$$\mu'_{2,ex} = \frac{2D_m L}{u} + \frac{R_c^2 uL}{24 D_m} \quad (6)$$

The molecular diffusivity of naphtho[2,3-a]pyrene and 4- *tert*-butylphenol under the conditions used are 1.23×10^{-5} cm^2/s and 0.47×10^{-5} cm^2/s, respectively, according to the Wilke and Chang correlation [15]. The connecting tube lengths, L, is typically around 75 cm on both chromatographs and R_c close to 65×10^{-4} cm. For the smallest experimental flow rate applied, 0.1 mL/min, u is 12.6 cm/s. In liquid chromatography, the first term in the right hand side of Eq. (6) (longitudinal diffusion) is completely negligible compared to the second term (axial dispersion due to the parabolic radial flow profile). The peak variance is inversely proportional to the molecular diffusivity of the sample, which is consistent with the observations in Fig. 1A and B. The ratio of the molecular diffusivities of naphtho[2,3-a]pyrene in pure acetonitrile and 4-*tert*-butylphenol in the methanol–water mixture is about 2.6.

At flow rates larger than 0.2 mL/min, the peak variance barely increases or even decreases, as a result of both the coupling between a pure Taylor-Aris diffusion and a pure flow dispersion regimes in the axial direction in short tubes and the inaccuracy of Eq. (4) due to the peak profiles exhibiting increasingly obvious tailing. In a flow controlled regime (for instance at a flow rate of 2 mL/min), the extra-column band broadening is only 15% and 45% larger in methanol–water than in pure acetonitrile with the Acquity and the 1290 Infinity HPLC system, respectively, instead of 200% in a diffusion controlled mechanism, at extremely low flow rates.

Although the data shown in Fig. 1A and B are not very accurate, we observed that the extra-column band broadening contributions measured for the 1290 Infinity HPLC system is always larger than those measured for the Acquity by the same method (Eq. (4)). The difference between the results obtained with the two instruments strongly depends on the viscosity of the eluent. Would the viscosity of the eluent be zero, no radial flow profile would exist and both instruments would provide the same peak variance, due to the sole axial diffusion. The larger the viscosity, the larger the velocity gradient across the connecting tube diameter and the larger the difference in the axial dispersion of the sample between the two instruments as illustrated in Fig. 2.

In this work, we used pure acetonitrile, a 50/50 (v/v) mixture of acetonitrile and water, and a 65/35 (v/v) methanol–water mixture. The viscosities of these fluids are 0.36, 0.86, and 1.64 cP at 295 K, respectively. According to Eq. (4), we measured the extra-column band broadening of the sample test mixture at a flow rate of 0.4 mL/min. The injection volume was set at 0.1 μL, as in the measurements made with the column. The peak variances for the acetonitrile–water mixture were only 3.9 μL^2 on the Acquity and 11.5 μL^2 on the 1290 Infinity HPLC system, values that are about 33% smaller and 100% larger than those measured with pure acetonitrile on the Acquity and the 1290 Infinity HPLC system at the same flow rate, respectively, but for injections of 1 μL. Because the viscosity of the acetonitrile–water eluent is intermediate between those of pure acetonitrile and of the methanol–water mixture, we would rather expect peak variances around 8.4 and 15.0 μL^2 on the Acquity and the 1290 Infinity HPLC system, respectively, for a 1 μL injection. The result for the Acquity is surprisingly smaller than the expected value but is consistent with it for the 1290 Infinity HPLC system. The role played by the injection volume (×1/10) seems to be important with the Acquity injection system while it is marginal with the 1290 Infinity HPLC system. In fact, if we inject 1 instead of 0.1 μL we obtain peak variances of 6.9 instead of 3.9 μL^2 on the Acquity instrument. In theory, would the injection system be ideal, plug volumes of 1 and 0.1 μL should deliver negligible peak variances of 0.08 and 0.0008 μL^2, respectively. This result shows that the behavior of the injection device of the Acquity system is far from ideal and that most of the sample dispersion takes place in the injection loop, before the sample enters into the short tubing when the injection valve operates. This result could be used by the manufacturers of HPLC instruments to improve the design of injection systems and particularly the part where the sample volume is stored before its injection into the column.

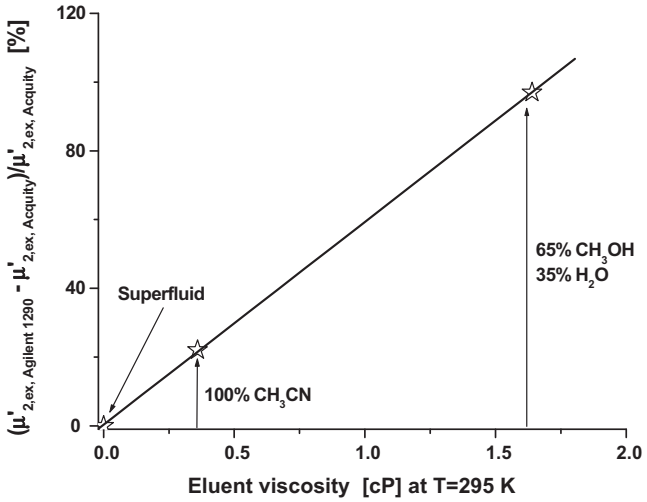

Fig. 2. Average relative increase of the extra-column peak variance contribution measured with the 1290 Infinity HPLC system with respect to that of the Acquity. All measurements were based on the peak width at half-height over a flow rate range of 0.1–2.0 mL/min. Injection volume: 1 μL. Note the quasi-linear increase with the eluent viscosity.

Finally, Fig. 3A compares the extra-column band profiles of a 0.1 μL injection of the probe mixture for a flow rate of 0.4 mL/min. The data were corrected for the offset time of the Acquity (0.65 s) and normalized to the same peak area. The difference of behavior of the Acquity and 1290 Infinity HPLC system is obvious. Most of the difference is accounted by the absence of a needle seat capillary tube in the Acquity. In this instrument, the bolus of sample is placed in the 5 μL injection loop, itself directly connected to the switching valve port. The injection system of the 1290 Infinity HPLC system is designed differently. The sample is first drawn into a 20 μL loop, then flushed backwards towards the switching valve, through a needle and the needle seat capillary tube. Despite the +50% difference between the sums of the volumes of all the connecting parts involved in the injection devices of the Acquity (8.7 μL) and the 1290 Infinity HPLC system (12.0 μL), the extra-column band broadening contribution for a 0.1 μL and 1.0 μL sample injected in a mixture of acetonitrile and water (50/50, v/v) of viscosity $\eta = 0.75$ cP is about 4× and 2.5×, respectively, larger with the 1290 Infinity HPLC system than with the Acquity.

The standard configuration of the 1290 Infinity HPLC system was improved by reducing the inner diameter of the needle seat capillary (volume of about 0.9 μL instead of 1.2 μL initially) and of the connector capillaries (115 μm × 220 mm and 115 μm × 340 mm, total volume, 5.8 μL). The same detector cell volume was used (2.4 μL). The corresponding variances due to extra-column band broadening are shown in Fig. 1C (100% acetonitrile, naphtho[2,3-a]pyrene) and 1D (methanol/water, 65/35, v/v, 4-*tert*-butylphenol)

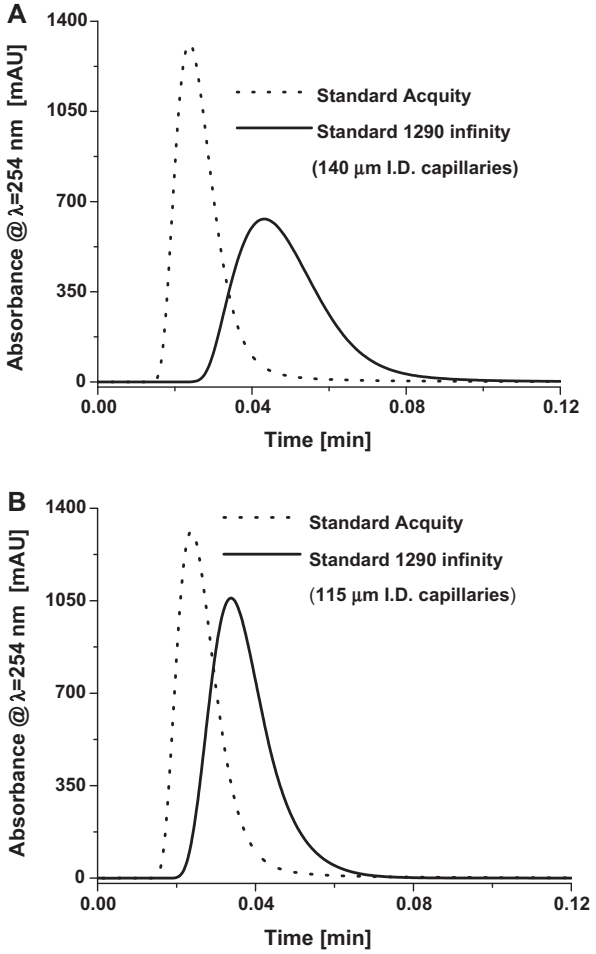

Fig. 3. Comparison between the band profiles of small molecules (uracil, acetophenone, toluene, and naphthalene) eluted through a zero-volume connector measured on the standard Acquity (solid line) and on the standard 1290 Infinity HPLC system (dotted line). Injection volume: 0.1 μL. Flow rate: 0.4 mL/min. The peak profile measured on the Acquity was corrected for the time offset of this instrument (0.65 s) and for the peak area measured with the 1290 Infinity HPLC system chromatograph. (A) The I.D. of the 1290 Infinity capillaries is 140 μm. (B) same as in (A) except the capillaries' I.D. is 115 μm. Note the quasi-equivalence between Acquity and 1290 Infinity system in the standard configuration (B).

and compared to those previously measured with the Acquity system. Under such conditions, the 1290 Infinity HPLC system is equivalent (Fig. 3D) or slightly better (Fig. 3C) than the Acquity system in terms of the extra-column band broadening contributions. For the sake of comparison, the variance of the extra-column band profiles measured from the half-height peak width method in the methanol/water mixture are equal to 13.0 and 10.9 μL^2 for the 1290 Infinity and the Acquity VHPLC systems, respectively. They are, respectively, 4.6 and 6.2 μL^2 in pure acetonitrile. Fig. 3A and B compare the extra-column band profiles of the mixture components (0.1 μL) in a 50/50, v/v, solution of acetonitrile and water on the Acquity (same configuration) and the 1290 Infinity HPLC system instruments. The consequence of the reduction of the extra-column band broadening made is obvious for the 1290 HPLC system. The instrument variance contribution is decreased by half, from 11.5 to 5.2 μL^2 (*versus* 3.9 μL^2 with the standard Acquity instrument). In conclusion, both systems are practically equivalent when the 1290 Infinity HPLC system instrument is equipped with the new needle seat and connecting tubes, having an inner diameter around 115 μm.

4.2. On further possible reductions of the extra-column contributions of the Acquity and 1290 Infinity HPLC System

In this section, we discuss ways to reduce the contribution of the system to overall band broadening and increase detection sensitivity. This can be easily achieved by reducing the detector cell volume and the inner diameters of the connectors and by removing the heat exchangers [10]. The cost of these changes is that the back pressure is increased and the temperature of the eluent entering the system cannot be tuned anymore, which might lead to a change in the column selectivity. From a practical point of view, analysts must adopt the compromise needed to satisfy their requirements concerning the separations performed.

There are possible changes that could be made to further reduce the extra-column band broadening contributions, possibly at the cost of sacrificing the ability of the instrument to make the temperature of the incoming eluent equal to that of the oven. In the case of the Acquity instrument, the column stabilizer is replaced by a shorter capillary tube. In the case of the 1290 instrument, the passage through the heat exchanger is skipped. Thus, these reductions of the extra-column volume of these systems could apply only to separations performed at the ambient temperature of a well

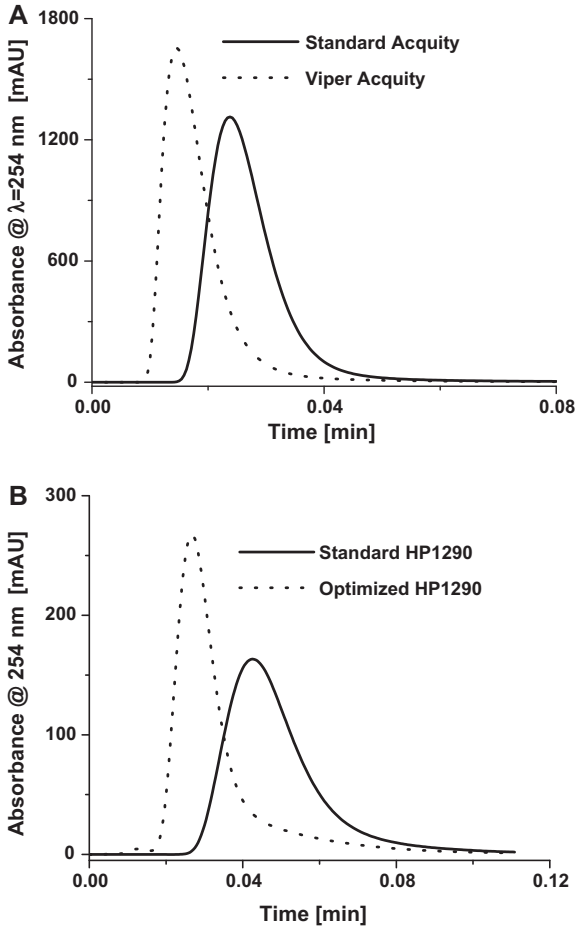

Fig. 4. Comparison between the band profiles of small molecules (uracil, acetophenone, toluene, and naphthalene) eluted through a zero-volume connector and measured on the Acquity (A) equipped with its standard 550 mm × 0.127 mm column stabilizer (solid line) and with a 250 mm × 0.13 mm Viper capillary (dotted line). The peak profiles were corrected for the time offset of the instrument (0.65 s). (B) Same as in (A) except the 1290 Infinity HPLC system under its standard (capillaries' I.D. 140 μm) and optimized configuration. Note the narrower peak-width after reducing the extra-column volume with the appropriate connectors and/or detector cell. Injection volume: 0.1 μL. Flow rate: 0.4 mL/min.

air-conditioned room, when the use of the oven thermostat is not required.

In order to further reduce the extra-column contributions of the Acquity instrument, not only did we inject small (0.1 μL) samples but we replaced the long column stabilizer with a special connecting tube (Viper from Dionex, Sunnyvale, CA, USA) which can fit to any type of instrument and column endfittings, provided they have a standard outer diameter of 1/16th in. This tubing is 25 cm long and 130 μm in diameter. The extra-column peak variance decreased from 3.9 to 2.2 μL^2. Fig. 4A compares the extra-column band broadening of the test mixture (0.1 μL injected) on the Acquity under standard and optimized conditions.

The effect of replacing the standard column stabilizer with the Viper tubing on the retention times of the sample components is illustrated in Figs. 5A and 6A–D. The elution time of uracil decreases by 4%, that of acetophenone is hardly changed while those of toluene and naphthalene are increased by about 2%. In Fig. 6A, because the elution time of uracil is independent of the temperature (unretained compound), it is eluted earlier with the Viper tubing whose hold-up volume is smaller than that of the column stabilizer (3.3 *versus* 7.0 μL). On the other hand, because the Viper tubing does not allow as good a heat exchange between the incoming eluent and the oven compartment as the stabilizer, the eluent enters the column cooler, hence a significant increase of the elution times of compounds with retention factors larger than 4 (see Fig. 6C and D). Both effects compensate each other for acetophenone (Fig. 6B) with a retention factor of 1. Note that Figs. 5 and 6 illustrate the reproducibility of the results given by the Acquity, which is sufficient to observe these small changes in elution times, significantly larger than the reproducibility level of the retention times measured with the Acquity (<0.2%).

Similarly, the extra-column band spreading contributions of the 1290 Infinity HPLC System were reduced by replacing the first and second connector capillaries (140 μm I.D.) by connector capillaries of a smaller diameter (250 mm × 85 μm). The volume of the detector cell was reduced from 2.4 to 0.8 μL. The total extra-column volume decreased from 12.0 to 7.4 μL. Fig. 4B compares the extra-column band broadening of the test mixture (0.1 μL injected) on the 1290 Infinity HPLC System under standard and optimized conditions. The peak variance measured at half the peak height decreased from 11.5 to 3.5 μL^2. Fig. 5B compares the chromatograms obtained with uracil, acetophenone, toluene, and naphthalene for the standard and for the optimized Agilent 1290 systems. In both cases, the heat exchanger was conserved and no shifts in the elution times of the most retained compounds, toluene and naphthalene, were observed. Upon changing the connecting tubes, the average efficiencies of acetophenone, toluene, and naphthalene, measured for six consecutive measurements, increases by 40%, 9%, and 3%, respectively.

The variances of the extra-column contributions to elution peaks measured for the Infinity 1290 and the Acquity systems in their different configurations are summarized in Table 2.

4.2.1. Comparison between the performance of the 2.1 mm × 50 mm and 100 mm BEH-C_{18} columns

In this section, the configurations of the instruments are those indicated in Section 3. These configurations do not necessarily correspond to the optimized configurations recommended to maximize the column efficiency, which are illustrated in the previous sections.

All chromatograms were recorded under the very same conditions of temperature (303.15 K) and flow rate (0.40 mL/min) with the two instruments. The column efficiencies were automatically measured with the new Agilent Chemstation for LC 3D Systems software (revisions B.04.02 [96]) with the following integration parameters: slope sensitivity: 0.001; peak-width: 0.005.

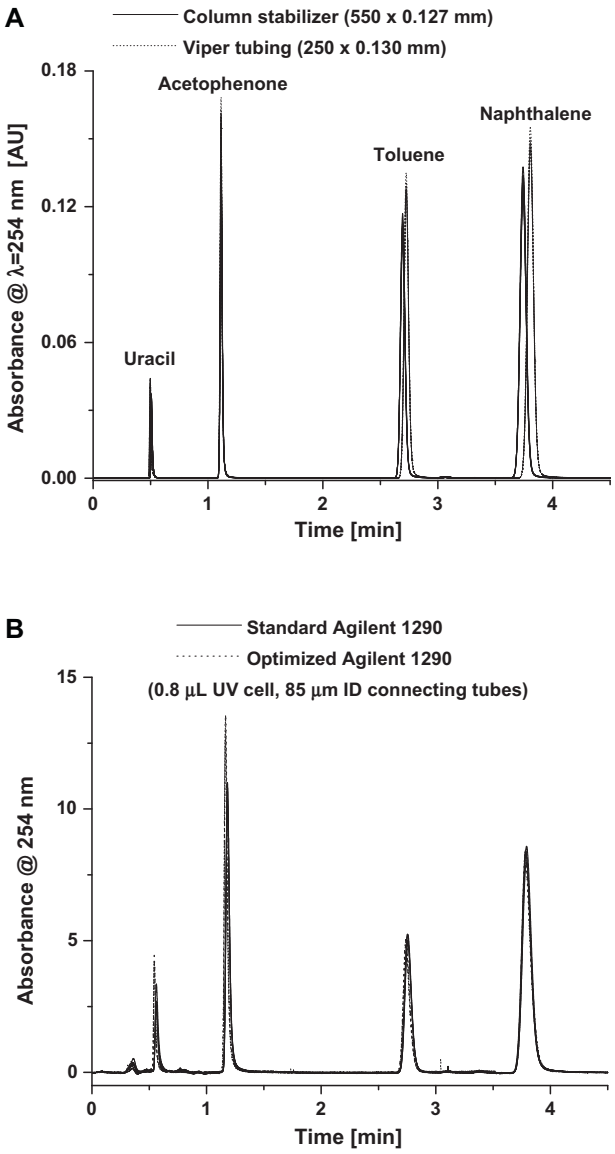

Fig. 5. Comparison between the chromatograms obtained on the Acquity equipped with the column stabilizer (solid lines) or with the Viper capillary tube (dotted lines, 8 replicates, A) and between the Agilent 1290 in his standard configuration (capillaries' I.D. 140 μm) or the optimized 1290 system with a low-volume detection cell and thinner connecting capillaries (dotted lines, 6 replicates, B). Column: 2.1 mm × 100 mm BEH-C_{18}. Sample mixture: uracil, acetophenone, toluene, and naphthalene. Injection volume: 0.1 μL. Oven temperature set at 303.15 K. Flow rate set at 0.4 mL/min. Note that all the chromatograms were superimposed for each configuration of the two instruments demonstrating the high level of reproducibility of the injection systems.

The results are presented in Fig. 7A. Fig. 8A and B compares the chromatograms obtained on the two instruments with the 10 and the 5 cm long columns, respectively. As discussed later, the differences between the elution times of the peaks of each component measured on the two chromatographs is not constant, suggesting that the flow rates and/or the oven temperature are not identical on both instruments. Fig. 8A and B also shows that the peaks eluted from the Acquity are closer to symmetrical. The peak asymmetries calculated with the Chemstation software on the 10 cm long column were 0.78 (acetophenone), 0.87 (toluene), and 0.94 (naphthalene) with the Acquity. They were 0.57, 0.79, and 0.83, respectively, with the 1290 Infinity HPLC system. Similar differences in the peak symmetry were observed with the 5 cm long column: 0.67, 0.81, and 0.87 with the Acquity, and 0.55, 0.74, and

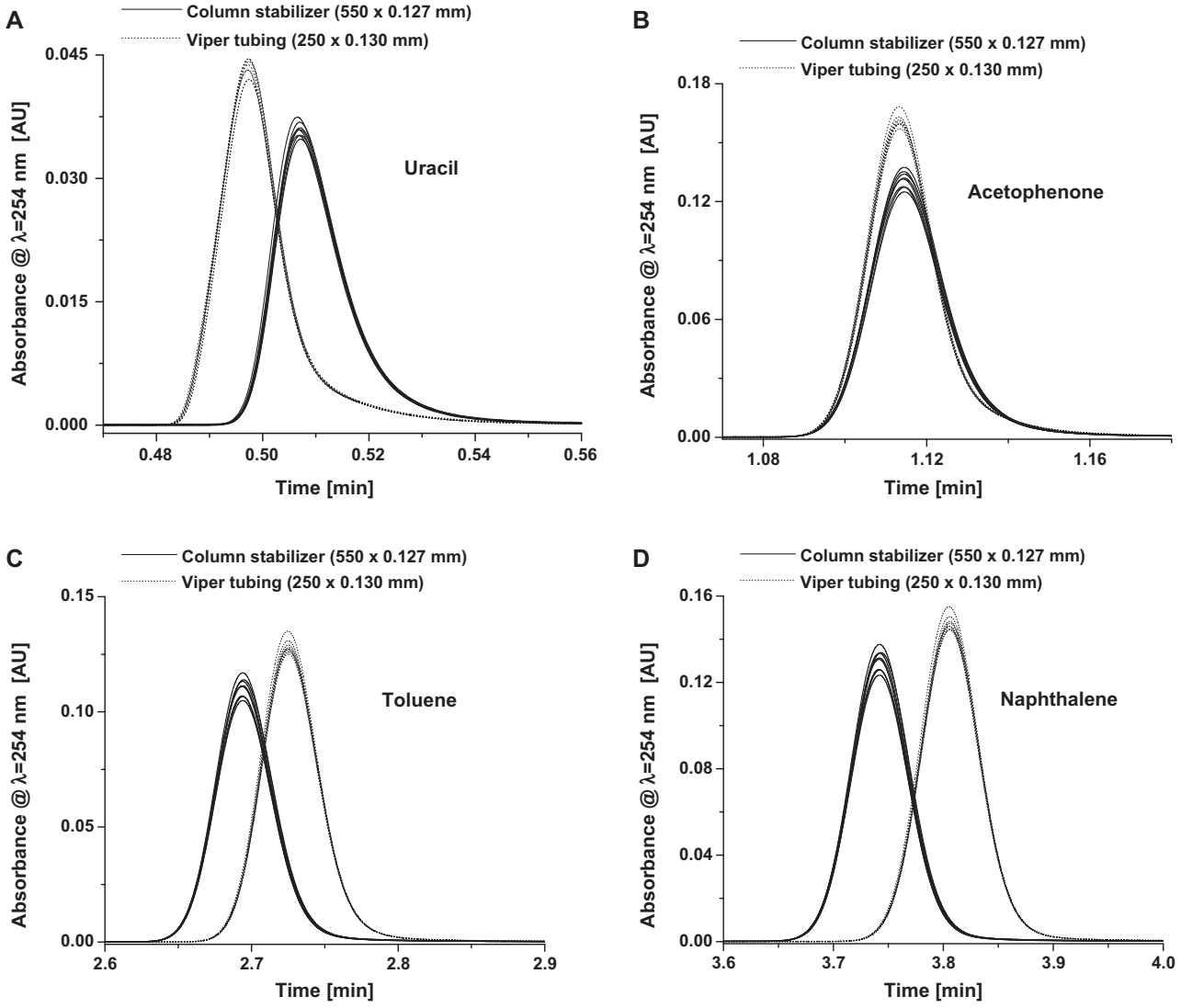

Fig. 6. Same as in Fig. 5, except zooms onto the retention window of each individual compound, uracil (A), acetophenone (B), toluene (C), and naphthalene (D). Note that the difference between the elution times due to the channel modification are much larger than those due to fluctuations of injection-to-injection reproducibility.

0.79 with the 1290 Infinity HPLC system. Such differences in apparent column efficiency and in peak shape reflect the differences between the extra-column band broadening contributions of the two chromatographs. They are qualitatively consistent with the previous results reported in Section 4.1.

Fig. 9 shows the apparent column efficiency of a 2.1 mm × 50 mm column packed with 1.8 μm Zorbax Eclipse C_{18} particles. It illustrates well the improvement in column efficiency achieved by a proper optimization of both instruments. When 115 μm I.D. connecting tubes are used on the Infinity 1290

Table 2
Summary of the extra-column peak variance measured before and after optimization of the system configurations. The eluent was a mixture of acetonitrile and water (50/50, v/v) and the sample was the PTM standard test (0.1 μL injected).

	Carry over seat	Inlet capillary Heat exchanger Outlet capillary	Detection cell volume	Variance (half-height) [μL²]
Acquity Standard	None	550 mm × 127 μm 150 mm × 102 μm	0.5 μL	3.9
Acquity Optimized	None	250 mm × 127 μm 150 mm × 102 μm	0.5 μL	2.2
Infinity 1290 Standard 1	100 mm × 140 μm	340 mm × 140 μm 1.6 μL 220 mm × 140 μm	2.4 μL	11.5
Infinity 1290 Standard 2	100 mm × 115 μm	340 mm × 115 μm 1.6 μL 220 mm × 115 μm	2.4 μL	5.2
Infinity 1290 Optimized	100 mm × 115 μm	250 mm × 85 μm None 250 mm × 85 μm	0.8 μL	3.5

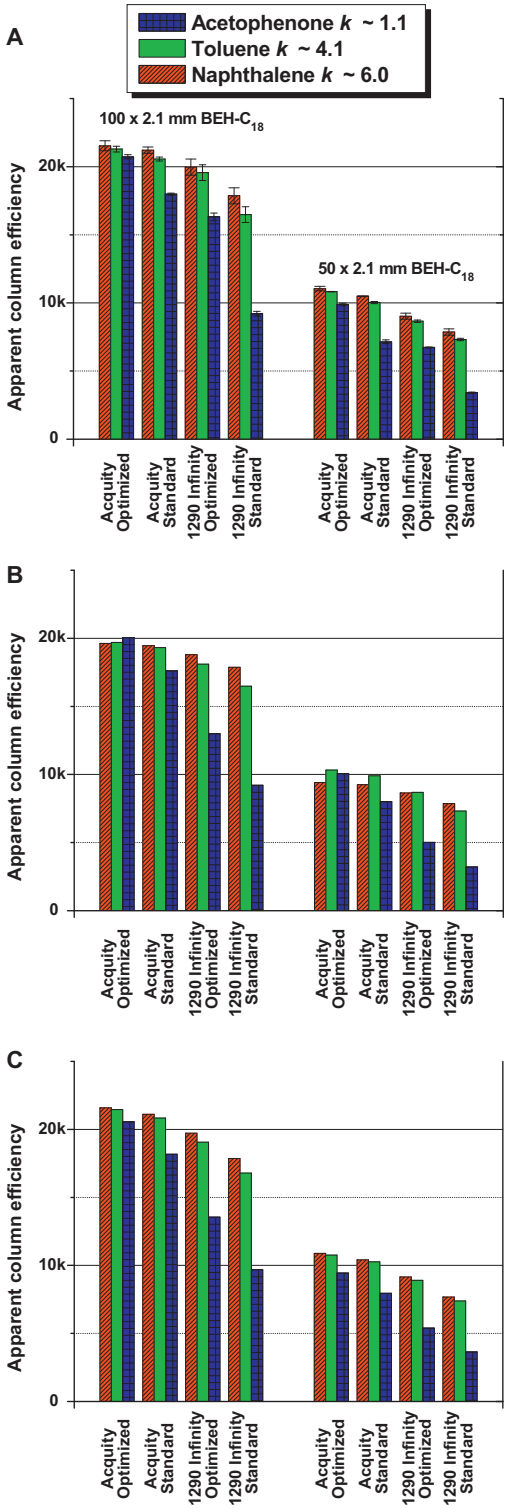

Fig. 7. Efficiencies of the 2.1 mm × 100 mm and 50 mm long BEH-C_{18} columns measured on four different instrument configurations, the 1290 Infinity HPLC system (140 μm capillaries' I.D.), the optimized 1290 Infinity HPLC system, the Acquity equipped with its column stabilizer, and the Acquity equipped with a Viper capillary tube. Temperature set at 295 K. Eluent: premixed acetonitrile–water mixture (50/50, v/v). Flow rate set at 0.4 mL/min. (A) Experimental efficiencies. (B) Calculated efficiencies (Eqs. (1)–(3)) using the best columns' HETPs derived from the extra-column peak variance (half-height peak-width) measured on the 1290 Infinity HPLC instrument (11.5 μL^2). The extra-column variance (half-height peak-width) measured on the standard and Viper-modified Acquity instruments were 3.9 and 2.2 μL^2, respectively. (C) Recalculated efficiencies after adjustment of both the columns' HETPs and the extra-column peak variances (see details in Section 4.2.3).

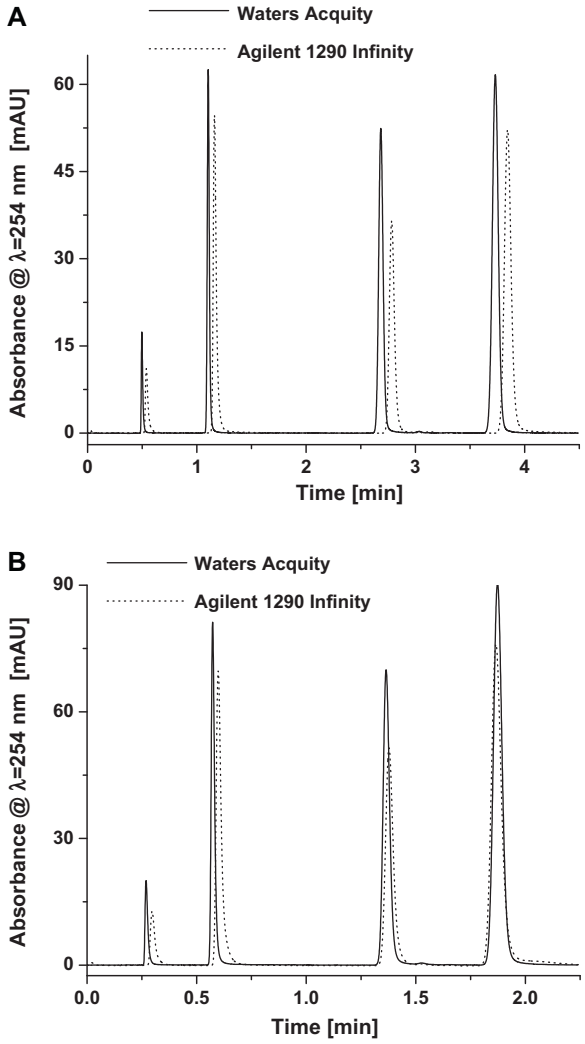

Fig. 8. Comparison between the chromatograms recorded with the standard Acquity (solid line) and the standard 1290 Infinity HPLC system (dotted line, capillaries' I.D. 140 μm). Test mixture by order of elution: uracil, acetophenone, toluene, and naphtho[2,3-a]pyrene. Same experimental conditions as in Fig. 5. (A) 2.1 mm × 50 mm BEH-C_{18} column. (B) 4.6 mm × 100 mm BEH-C_{18} column, flow rate: 1.25 mL/min. Note the better peak efficiency with the Acquity and the relative differences in elution times.

system in its standard configuration, the performance of this system becomes nearly equivalent to that of the Acquity system, in good agreement with the data on the extra-column band variances shown in Fig. 1C and D. Fig. 10A and compares the chromatograms recorded with this same column on both instruments in their respective standard and optimized configurations.

In order to confirm quantitatively these observations, we calculated the apparent column efficiencies under specific conditions. The calculations were made by adding to the column variance the respective extra-column contributions measured in the previous section (and derived from the peak-width at half-height). We estimated the HETPs of each of the two columns for the three retained compounds in our sample according to Eqs. (1)–(3). We considered the 1290 Infinity HPLC system chromatograph as the reference instrument for extra-column contributions because this system delivers no time offset and the flow rate is true (0.40 mL/min) in the detection cell (atmospheric pressure). The extra-column volume and the variance contributions of this instrument were previously measured as 16.7 μL and 11.5 μL^2, respectively. The total column porosities were estimated from the elution time of the

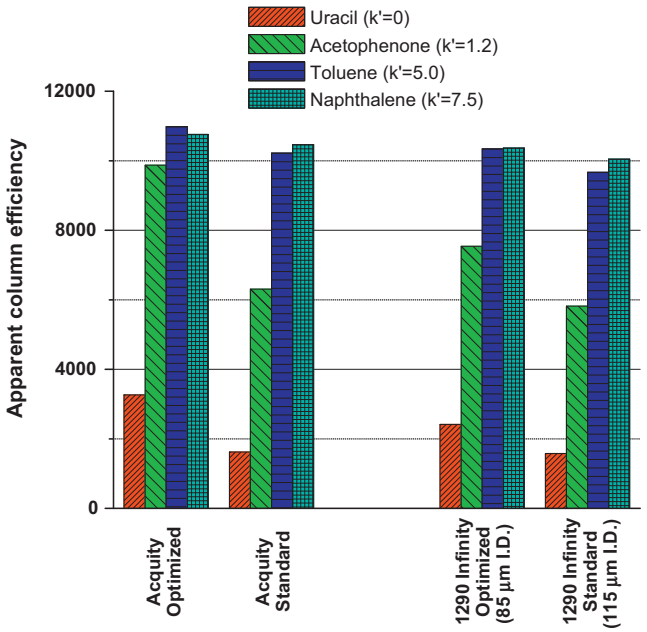

Fig. 9. Efficiencies of the 2.1 mm × 50 mm long column packed with 1.8 μm Zorbax Eclipse C_{18} particles, measured on four different instrument configurations, the 1290 Infinity HPLC system (115 μm capillaries' I.D.), the optimized 1290 Infinity HPLC system (85 μm capillaries' I.D.), the Acquity equipped with its standard column stabilizer, and the Acquity equipped with the Viper capillary tube. Same experimental conditions and probe mixture as in Fig. 7.

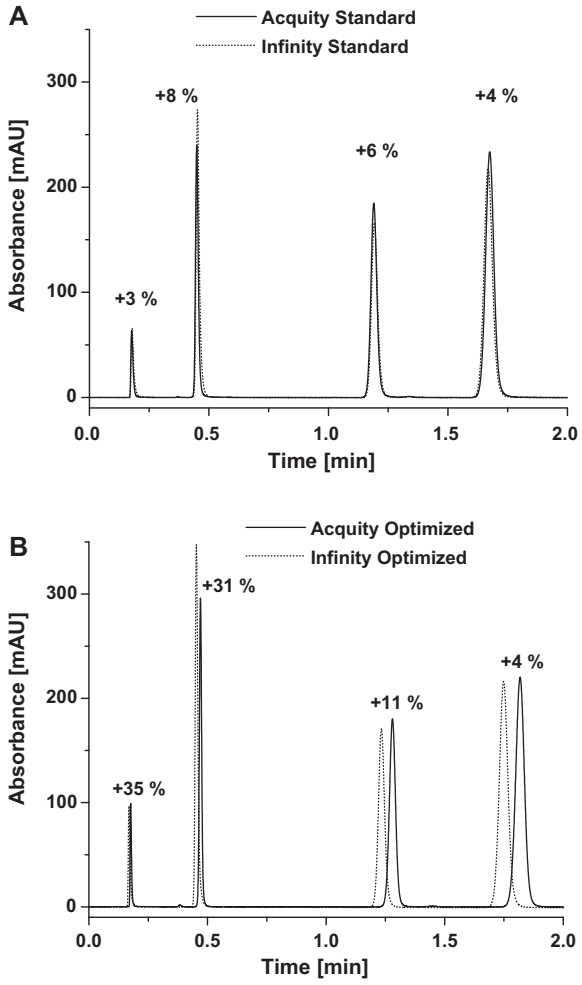

Fig. 10. Comparison between the chromatograms recorded with the Acquity and the 1290 Infinity HPLC systems in their standard (A) and optimized (B) configurations. Same experimental conditions as in Fig. 8. The second standard configuration of the 1290 Infinity system (115 μm I.D. for the carry over seat and connecting capillaries) was considered.

unretained marker, uracil. They are 0.646 and 0.601 for the 5 and the 10 cm long column, respectively. The retention factors of the three analytes were then 1.1, 3.9, and 5.6 on the 5 cm long column and 1.2, 4.3, and 6.3 on the 10 cm long column, obtained as the average of eight consecutive measurements. The best H values were found when the calculated efficiency (Eq. (3)) matched exactly the experimental apparent efficiency given in Fig. 7A. Accordingly, the best plate heights of the 5 cm long column were 3.2, 4.6, and 5.2 μm for acetophenone, toluene, and naphthalene, respectively. Those of the 10 cm long column were 4.1, 5.0, and 5.0 μm.

The values estimated for the most retained compounds (toluene and naphthalene, $k > 4$) are somewhat larger than the minimum HETP previously observed with these same columns (≃3.0 μm) [4,5], especially for the most retained compound. They make sense for poorly retained compounds (acetophenone, $k \simeq 1$). This difference could be due to changes in the experimental conditions, the earlier measurements having been made at the same flow rate but with pure acetonitrile ($\eta = 0.35$ cP) instead of a 50:50 acetonitrile/water mixture ($\eta = 0.93$ cP), so at a 2.6 times higher reduced velocity (according to the Wilke and Chang correlation [15], the diffusion coefficients of low molecular weight, nonpolar compound in pure acetonitrile and a 50/50 water/acetonitrile solution are 0.74×10^{-5} and 1.98×10^{-5} cm^2/s, respectively). Minimum HETPs of 3 μm were observed with pure acetonitrile (viscosity 0.35 cP) at a reduced interstitial velocity of about 7 with the first and second generations of hybrid inorganic/organic BEH-C_{18} columns [4,5]. The flow rate of the acetonitrile/water solution was 0.4 mL/min, corresponding to a reduced interstitial linear velocity of 11–12, nearly twice larger than the optimum velocity. Actually, the plate heights measured in pure acetonitrile at at $\nu = 12$ were 4.1 and 3.7 μm with a 5 and a 10 cm long BEH columns, respectively, for $k \simeq 4$ [5].

Although they are larger than previously measured, we kept these HETP values and calculated the expected apparent efficiencies of the two columns on the Acquity under the two different configurations described above. The results are shown in Fig. 7B. Strikingly, the calculated efficiencies of toluene and naphthalene obtained for the standard Acquity are systematically smaller than those measured. In contrast, the calculated efficiencies of acetophenone match very well with the experimental values.

For instance, the apparent efficiencies of naphthalene and toluene calculated for the 10 cm long BEH column on the standard Acquity should in theory be 9% and 17% larger than those measured on the 1290 Infinity HPLC system, respectively. For the 5 cm long BEH column, these calculated apparent efficiencies should be 18% and 35% larger than those measured on the 1290 Infinity HPLC system. Instead, we observed in Fig. 7A relative increases of the apparent efficiency of 19% and 25% for the 10 cm long column and of 33% and 37% for the 5 cm long column. The difference between the calculated and the measured variations increases with increasing retention factor. The use of the Viper tubing should increase the apparent column efficiency on the Acquity by only 1% and 2% for the 10 cm long column and 2% and 4% for the 5 cm long one, compared to those measured with the column stabilizer. However, experiments show that the actual increases are 2% and 4% (10 cm long column) and 5% and 8% (5 cm long column), about 50% larger.

There is a significant, unexpected difference between the experimental measures of the apparent column efficiency and those

calculated from the independent measurements of the extra-column contributions. There might be several reasons for that. The variance of the instruments were derived from a crude approximation, the peak-width at half-height because integration of the signal is too inaccurate and imprecise. The extra-column contributions assumed for the 1290 Infinity HPLC system could be erroneous (11.5 μL^2) and the estimates of the true column plate heights should be revisited. There could be unsuspected, hence overlooked, differences in the operation of the 1290 Infinity HPLC system and the Acquity, which would cause this significant difference between Fig. 7A and B.

In the next section, we investigate the possible physical causes for such a large difference between the measured and the predicted efficiencies.

4.2.2. Physical differences in the operating conditions of the 1290 Infinity HPLC system and Acquity

We analyze four differences in the operating conditions between the two systems that could affect the final measurement of the apparent column efficiency by the HP Chemstation.

4.2.2.1. Injection time offset. The time offset of the Acquity instrument was measured as 0.65 s [16]. There are none with the 1290 Infinity HPLC system. This means that the zero injection time is set prior to the actual, physical switching time of the injection valve in the chromatograms recorded by the Empower software. This artificially increases the observed elution time of the sample and modifies the performance report of the HP Chemstation which, for this reason, overestimates values of efficiencies. With the shortest column (5 cm long) and the most retained compound (naphthalene, $k \simeq 5$), the correction reduces the apparent column efficiency by less than 1%. Thus, the time offset of the Acquity cannot alone explain differences in column efficiency of 18% (see in Fig. 7A and B the height of the hatched red bars of naphthalene).

4.2.2.2. Differences of the actual flow rates through the column. The flow rate set on the Acquity (0.4 mL/min in this case) is not true at the column outlet but at the column inlet. The isothermal compressibility of a (50/50, v/v) water/acetonitrile mixture at 303.15 K is close to 0.55×10^{-4} bar^{-1} at the atmospheric pressure [17,18]. The inlet column pressure for the 10 cm long column being 551 bar, the actual flow rate at the column outlet and through the UV detector cell is 3.0% larger than the flow rate at the column inlet, a significant difference. The converse situation is true for the 1290 Infinity HPLC system for which the flow rate is true at the column outlet. It is, thus, 3.0% lower at the column inlet. So, there is a maximum velocity difference between the column operated with these two instruments of the order of 6%. Fig. 8A compares the chromatograms recorded on the Acquity and the 1290 Infinity HPLC system, both set at a flow rate of 0.40 mL/min, for the 10 cm long column. They allow to estimate the actual difference in the average flow rates in the two instruments. The retention times measured with the Acquity and corrected for the offset time are smaller than those recorded with the 1290 Infinity HPLC system. But the elution volume difference increases with increasing retention time from 16.6 (uracil, $t_{R,Acquity}$ = 0.496 min), to 23.0 (acetophenone, $t_{R,Acquity}$ = 1.104 min), 40.0 (toluene, $t_{R,Acquity}$ = 2.683 min), and to 45.0 μL (naphthalene, $t_{R,Acquity}$ = 3.731 min). This difference should have been constant and equal to the difference between the extra-column volumes of the two instruments (3.3 μL), would the average flow rates and the temperatures be exactly the same in the column. This is not the case.

Fig. 8B shows a similar chromatogram to the one in Fig. 8A but for the 5 cm long column. Fig. 11 shows a plot of the differences between the elution volumes on the 1290 Infinity HPLC system and Acquity *versus* their elution times. From the data points of the unretained uracil, the retention time of which is independent of the temperature on both columns, we can estimate the difference between the average flow rates at 20 and 16 μL/min in the 10 and 5 cm long columns, respectively (see the initial slope shown in Fig. 11), differences of 5% and 4% with respect to the nominal flow rate of 0.4 mL/min. These values are within the maximum predicted value (6.0%). This actual difference in average flow rates is often neglected by analysts and may render difficult the transfer of a developed method from the Acquity to the 1290 Infinity HPLC system. However, this difference in flow rate should have benefited the 1290 Infinity HPLC system because HETP increases with increasing flow rate along the C-branch of the van Deemter plot. The flow rate difference observed cannot be the source of the difference in column efficiency observed in Fig. 7A and B.

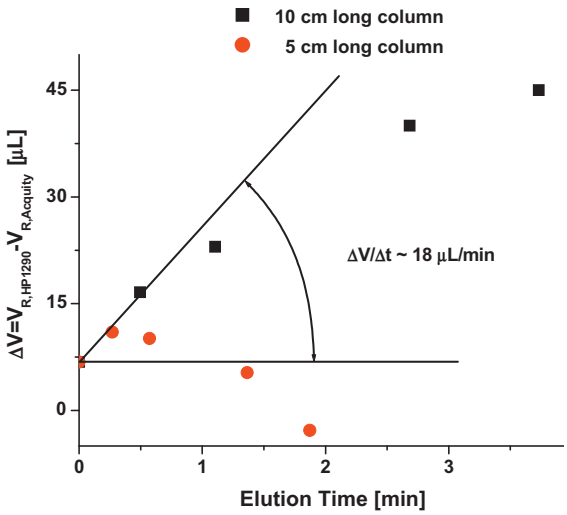

Fig. 11. Plots of the difference in the elution volumes measured on the 2.1 mm × 50 mm and 100 mm BEH-C_{18} columns between the 1290 Infinity HPLC system and Acquity systems as a function of the elution time. The initial increase of the plots (unretained compound uracil) confirms that the average flow rate is slightly larger when using the 1290 Infinity HPLC system than the Acquity instrument (larger by 16 and 20 μL/min with the 5 and 10 cm long columns, respectively). The convex upward shape of the plots confirms that the temperature inside the column is higher in the oven of the 1290 Infinity HPLC system than in that of the Acquity.

4.2.2.3. Differences of the actual temperatures inside the column. We indirectly observed a slight temperature difference between the ovens of the two chromatographs. The convex upward curvatures of the plots in Fig. 9 demonstrate that the temperature of the column is slightly smaller in the Acquity than in the 1290 Infinity HPLC system oven compartment. Would the temperatures be strictly identical, straight lines would have been observed and the difference in elution volumes would linearly increase. Fig. 8B shows that the difference in elution volumes may become negative.

In order to confirm these retention data, the temperature difference was directly measured by sticking a snap-on thermocouple onto the external surface of the stainless steel tube at half the length of the 10 cm long column. After thermal equilibration for nearly an hour while maintaining the flow rate constant at 0.4 mL/min, we measured stable temperatures of 33.3 and 34.3 ± 0.1 °C in the ovens of the Acquity and 1290 Infinity HPLC system, respectively. Overall, the average temperature of the column is about 1 K higher in the 1290 Infinity HPLC system than in the Acquity oven, which explains the observations made in Fig. 8A, B and 9. Note that these two temperatures are larger than the applied temperature of 30 °C, due to the combination of the effects of axial and radial losses of the heat friction released in the column volume [11,12]. The column pressure drops (after correction for extra-column contributions, e.g.

30.5 bar for Acquity, 9.5 bar for 1290 Infinity HPLC system) are 521 and 497 bar with the Acquity and the 1290 Infinity HPLC system, respectively. The difference is essentially explained by the differences in average flow rates of 18 μL/min (0.41 and 0.39 mL/min) in the respective instruments. The respective heat power friction are then 3.56 and 3.23 W/m, at which values the loss of column efficiency is negligible [4]. The HETP of the columns should then be close to their minimum, around 3–4 μm. This contradicts the initial guesses that we made in Section 4.2.1 where plate heights values between 5 and 7 μm were found. In conclusion, the temperature difference of 1 K has a marginal influence and the amount of heat friction released should *a priori* benefit the 1290 Infinity HPLC system. In conclusion, the temperature differences observed do not explain the difference in column efficiency observed in Fig. 7A and B.

4.2.2.4. Presence of weak solvent in the injection loop. We suspected that the nature of the weak needle wash (WNW) eluent of the Acquity could artificially improve the results. Indeed, we used a mixture of 20/80 (v/v) acetonitrile and water as the WNW solvent. The mobile phase contains 50% acetonitrile in water. Because the concentration of water is richer in the WNW eluent than in the mobile phase, provided that the needle is filled with WNW eluent before the sample is drawn, an excess of water-rich eluent could have entered the injection loop and focus the sample at the column entrance such as in gradient elution. This effect was observed and quantified recently [9]. However, the manufacturer stated that the excess volume of the injection loop is filled with the mobile phase when *partial loop filling with needle overfill* mode is used. We checked this information by replacing the WNW solvent with pure acetonitrile. Neither the retention times nor the column efficiencies were changed. So, there was no sample focusing on either the Acquity or the 1290 Infinity HPLC system.

4.2.3. Adjustment of the extra-column peak variances depending on the asymmetry of the peak measured in presence of the column

According to the comparisons made in the previous section, the difference between the apparent column efficiencies of retained compounds should arise from the use of a wrong estimate of the extra-column band variances in the two instruments. When the extra-column variances are derived from the peak-width at half-height, they are underestimated. As a result, the column HETPs calculated from the extra-column variances of the 1290 Infinity HPLC system apparatus (11.5 μL^2) were overestimated. The half-height peak-width method is insufficient to determine the true extra-column peak variances of either the 1290 Infinity HPLC system or the Acquity.

In order to minimize the errors made, we re-evaluated the column HETPs by adjusting the half-height peak variances by a constant factor for both instruments, in order to match experimental and predicted column efficiencies. At the same time, for each column and each compound, a new HETP value was optimized after minimizing the sum of the relative errors made on the efficiencies measured with these instruments. The revised HETPs of the 10 and 5 cm long BEH-C_{18} columns were 4.0 (acetophenone), 4.5 (toluene), and 4.5 μm (naphthalene) and 3.9, 4.4, and 4.3 μm, respectively. The HETP values of retained solutes are more consistent with those previously measured with pure acetonitrile at the same reduced flow velocity of $\nu = 12$ [5]. Fig. 7C shows the revised column efficiencies obtained after this correction. There is now an excellent agreement with the observations in Fig. 7A, in contrast with the results in Fig. 7B. Most importantly, these revised results reveal an obvious correlation between the peak asymmetry and the adjustable factors introduced. For the 5 cm long column, these factors were 0.9, 1.1, and 1.7 for acetophenone (average asymmetry $As = 0.63$), toluene (average asymmetry $As = 0.79$), and naphthalene (average asymmetry $As = 0.85$), respectively. With the 10 cm long column, these factors were 1.0, 1.3, and 1.8 for acetophenone ($As = 0.72$), toluene ($As = 0.83$), and naphthalene ($As = 0.91$), respectively. The more symmetrical the peak, the more important it is to measure the extra-column peak variance from the whole concen-

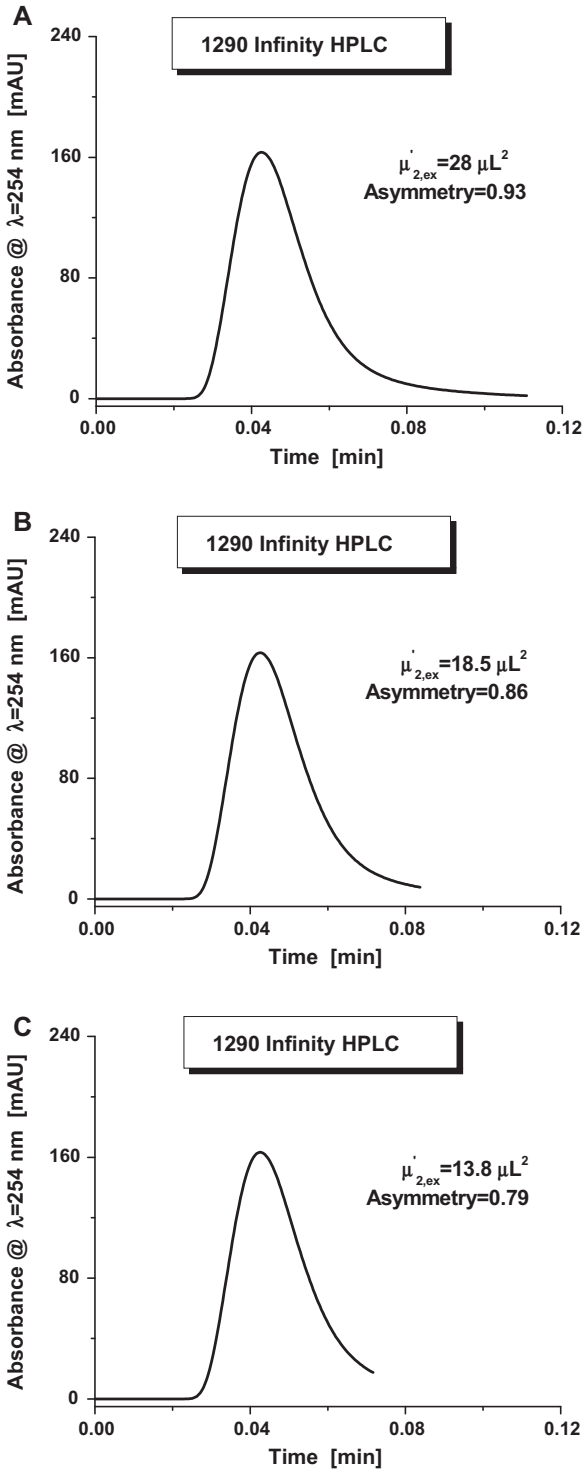

Fig. 12. Representation of the selected cut point in the rear part of the extra-column band profiles (standard 1290 Infinity HPLC sytem, capillaries' I.D. 140 μm) necessary to estimate the correct extra-column band broadening for different peak asymmetry measured in presence of the column. The closer to 1 the peak asymmetry, the closer to the true value the corrected extra-column peak variance. Three degrees of peak asymmetry were considered (A–C).

tration distribution profile of the tailing extra-column band profiles because most of the injected mass contribute to the peak-width of symmetrical peaks.

Indirectly, the true extra-column band broadening due to the instrument is best estimated from the elution of quasi-Gaussian, retained peaks such as the one of naphthalene. In conclusion, the true extra-column band broadening of the 1290 Infinity HPLC system (140 μm I.D. capillaries), optimized 1290 Infinity HPLC system, Acquity, and Acquity-Viper instruments were about 20.2 (initially 11.5), 9.2 (initially 5.2), 6.9 (initially 3.9), and 3.9 (initially 2.2) μL^2, respectively. For instance, Fig. 12 shows where the rear tailing part of the extra-column peak profile measured with the 1290 Infinity HPLC system should be cut in order to obtain a correct estimate of the extra-column peak variance for the eluted compounds having peak symmetries of 0.79, 0.85, and 0.93.

5. Conclusion

The results of this work confirm that the two instruments studied were developed to satisfy different needs and optimized for different sets of conditions. Provided the inner diameter of the connecting tubes of the 1290 Infinity HPLC system are properly chosen (around 115 μm), it delivers the same level of column efficiency as the Acquity instrument. Yet, a slight departure from this tubing inner diameter (e.g., from 115 to 140 μm) may have detrimental effects on the performance of the 1290 Infinity instrument. Then, the standard Acquity provides a higher apparent efficiency than the standard 1290 Infinity HPLC system for 2.1 mm I.D. columns packed with sub-2 μm particles. In contrast, the 1290 Infinity HPLC system can still provide satisfactory results with 4.6 mm I.D. columns packed with the same very fine particles while the Acquity cannot deliver the required high flow rates.

We demonstrated that the difference in column performance is mostly accounted for by the difference in the extra-column band broadening contribution of the HPLC system. The observed differences in flow rate (+4% to 5% with Acquity), column temperature (+1 K with 1290 Infinity HPLC system), and injection time offset (+0.6 s with Acquity) cannot explain the differences in apparent column efficiency measured. The advantage of the Acquity lies in the design of its injection system. Unlike the 1290 Infinity HPLC system, the Acquity has no needle seat capillary tube, which considerably reduces the band width of the injected sample. Yet, the amount of band broadening depends markedly on the injected volume. Eventually, the performance of the 1290 Infinity HPLC system becomes nearly equivalent to that of the Acquity if the inner diameter of its needle seat capillary, inlet and outlet connecting tubes is reduced to about 0.0045 in.

One of the important question raised by this work is how to measure correct estimates of the extra-column peak variance of an instrument when one wants to predict the apparent efficiency of highly efficient columns. No correct estimate can be derived from the peak-width at half-height, due to the important extent of tailing of the band profiles entering the column. The variance derived from the peak-width at half-height can only be considered as a basis of reference for a fair comparison between different instruments. The true peak variance must be obtained by correcting the peak-width at half-height variance with a factor which depends on the peak asymmetry measured on the peaks eluted out of the column, or more precisely, on the difference in peak asymmetry between the peaks recorded with the column and with a zero-volume connector. If the asymmetries of these two profiles are similar, the correction factor is close to unity. However, the asymmetry of the peak eluted from the column is much closer to one than that of the peak eluted from a mere union connector. We found that the correction factor is of the order of 3 for nearly Gaussian peaks with an asymmetry of 0.94. Our work shows that this correction is necessary to predict the actual performances of narrow-bore columns of different length, packed with sub-2 μm particles on two different chromatographs.

The fact that analysts must for a while be more concerned with their instruments than with the column performance in order to achieve high plate counts illustrates the considerable gain in performance achieved on the column front and the limitation of the current instruments. Current research and development in packing technology including in the manufacturing of ultra-fine particles and in that of a new generation of superficially porous particles (Poroshell, Halo, Kinetex) have, for a time, brought columns one step ahead of instruments. It is now unrealistic to operate 1 mm I.D. columns under isocratic conditions with most commercial instruments without extensive modifications. New instruments with smaller hold-up volumes and smaller extra-column band broadening contributions are needed. This seems to require less complicated injection systems.

Finally, the results reported in this work are relevant essentially for work carried out under isocratic conditions, under which the injection system and the connecting tubes prior to the column significantly affect the apparent column efficiency. In gradient elution analyses, the contribution of the HPLC instrument to the recorded peak width is mostly influenced by the post-column volumes, including the outlet tube connector, the detection cell, and the sampling rate of the signal. Given the focusing of band profiles at the column entrance, gradient elution is a far more forgiving mode of elution than the isocratic mode in terms of loss of peak capacity caused by the instrument.

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