Video Article

Curtain Flow Column: Optimization of Efficiency and Sensitivity

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Abstract

Active Flow Technology (AFT) is a form of column technology that increases the separation performance of a HPLC column through the use of a specially purpose built multiport end-fitting(s). Curtain Flow (CF) columns belong to the AFT suite of columns, specifically the CF column is designed so that the sample is injected into the radial central region of the bed and a curtain flow of mobile phase surrounding the injection of solute prevents the radial dispersion of the sample to the wall. The column functions as an 'infinite diameter' column. The purpose of the design is to overcome the radial heterogeneity of the column bed, and at the same time maximize the sample load into the radial central region of the column bed, which serves to increase detection sensitivity. The protocol described herein outlines the system and CF column set up and the tuning process for an optimized infinite diameter 'virtual' column.

Video Link

The video component of this article can be found at https://www.jove.com/video/53471/

Introduction

In recent years column technology for High Performance Liquid Chromatography (HPLC) has advanced greatly; peak capacities have increased considerably thanks largely to the use of smaller particle sizes and the more efficient core shell particles. Since separations are generally more efficient, a flow-on effect has been an increase in sensitivity since peaks are now sharper and hence taller[1-8].

Nevertheless, radial bed heterogeneity is still a limiting factor in the performance of all columns, but this is not a new story since chromatographers have known this for many years. Column beds are heterogeneous in both the radial direction[9-12], and along the column axis[10,12-15]. The wall-effect especially is an important contributor to the loss of separation performance[7,16-18]. Shalliker and Ritchie[7] recently reviewed aspects of column bed heterogeneity and hence this need not be discussed here further. Although suffice to say, that the variation in column bed packing density and the wall effects lead to a distortion of the solute plug, such that bands elute through the column in plugs that resemble partially filled soup bowls rather than thin flat solid discs[7] that are usually depicted in basic teaching texts. When experiments were undertaken such that the solute migration through the bed could be visualized the plug profiles inside the column were partly hollow and the tailing section of the band is largely the wall component of the sample plug. The end result is that it takes many more plates to separate these 'partially hollow' plugs than would be required if the discs were solid and flat[12,14,17]. To overcome the band broadening issues associated with wall effects and the variation in radial packing density, a new form of column technology known as Active Flow Technology (AFT) was designed[7,19]. The purpose of this design was to remove wall effects through the physical separation of solvent eluting along the wall region, from that of mobile phase eluting in the radial central region of the column[19]. There are two main types of AFT columns; Parallel Segmented Flow (PSF) columns and Curtain Flow (CF) columns [7]. Since this protocol is aimed at the use and optimization of CF columns, PSF columns will not be further discussed.

Curtain Flow (CF)

Curtain Flow (CF) column formats utilize AFT end-fittings at both the inlet and the outlet of the column. AFT end-fittings consist of an annular frit located inside a multiport fitting. The frit is made up of three parts: a porous radial central portion that is aligned with the central port of the end fitting, a porous outer portion that is aligned with the peripheral port(s) of the end fitting, and an impermeable ring that separates the two porous portions preventing any cross-flow between the radial central and outer regions of the frit[19]. **Figure 1** illustrates the design of the AFT frit and **Figure 2** illustrates the CF column format. In this mode of operation (CF) the sample is injected into the radial central port of the inlet fitting, whilst additional mobile phase is introduced through the peripheral port of the inlet to 'curtain' the migration of solutes through the radial central region of the column. Hence the sample enters the bed in the radial central region of the column with the outer region of the column having mobile phase only passed through it. Studies have shown that a volumetric flow rate ratio of around 40:60 (central:peripheral port) for the inlet end-fitting of a 4.6 mm internal diameter (i.d.) column is optimal[6,7,16]. The AFT outlet of the CF column allows the adjustment of the central and peripheral flow to their relative portion and can be varied to almost any desired ratio through pressure management. The optimization of a CF column can significantly improve various functional aspects of the column technology, such as separation efficiency or detection sensitivity. In this manner a 'wall-less', 'infinite-diameter' or 'virtual' column is established[6,10,18,20]. The purpose of CF columns is to actively manage the migration of sample through the column to prevent the sample from reaching the wall region. Thus, the solute concentration upon exit to the

detector is maximized, increasing sensitivity of around 2.5 times greater than the conventional column format when using Ultraviolet (UV) detection[16], and even greater when using mass spectral detection[6].

CF columns are ideally suited for low concentration samples, since detection sensitivity is increased. Further, they are ideal when coupled to flow rate limited detectors, such as the mass spectrometer (MS)[6]. An AFT column in a 4.6 mm i.d. format, for example can be tuned to deliver the same volume of solvent to a detector as a standard 2.1 mm i.d. format column when operated at the same linear velocities, by adjusting exiting central flow to 21%. Likewise the AFT column could also be tuned to deliver the same volume load to a detector as a 3.0 mm i.d. column, by adjusting exiting central flow to 43%. In fact any 'virtual' column format could be produced to suit the analytical requirement[6,18,22]. Using these specially designed end-fittings at the inlet and the outlet ensures that a true wall-less column is established.

There are two ways to set up the solvent delivery system to the central and peripheral ports of the inlet: split-flow system[6] and two pump system[6,7]. **Figure 3** illustrates each of these CF system set ups.

Split-flow system

In a split-flow system (**Figure 3A**) the pump flow leading to the injector is split pre-injector using a zero-dead volume T-piece, where one flow stream of mobile phase is connected to the injector, which is then connected to the central port of the inlet end-fitting of the column. The second flow stream of mobile phase by-passes the injector and is connected to the peripheral port on the inlet of the column. During the splitting of flow, the flow stream percentage is adjusted to 40:60 (center:peripheral) before the lines are connected to the column, *i.e.*, from injector to center and pump to peripheral.

Two pump system

The CF column requires two flow streams at the inlet end-fitting of the column. Depending on the type of autosampler/injector of the HPLC instrument, split-flow set up may not be possible, and so CF can then be achieved through 2 pumps (**Figure 3B**[21]). Each pump is allocated and connected to either the central or peripheral port and the flow rate is set to represent 40% of flow for central port and 60% for peripheral port. For example, if the total flow rate is 1.0 ml min^{-1}, the central pump flow rate is set to 0.4 ml min^{-1} and the peripheral pump is set to 0.6 ml min^{-1}.

The choice of which mode of operation is largely dependent on the HPLC instrumentation and chromatographic mode of operation. For example in some autosamplers a change in pressure between sample load position and sample inject position may occur disrupting the split-flow ratio and thus in this case a dual pump set up would be recommended for optimal CF performance. Regardless of the solvent delivery system set up chosen for the inlet of the CF column, the CF outlet optimization remains the same. The outlet central port of the CF column is attached to the Ultraviolet-Visible (UV-Vis) detector with the smallest volume possible of tubing to minimize the effects of post-column dead volume. Since, CF columns emulate narrow-bore columns, dead volume between the column outlet and the detector is detrimental to the separation performance of the CF column. It is critical to ensure the smallest amount of volume of tubing between the central port and the UV-Vis detector to minimize the effects of dead volume such as band broadening, loss in efficiency and sensitivity. Hence, the use of narrow bore tubing (0.1 mm i.d.) is advised to readily allow pressure adjustments without adding inappropriate dead volume. Tubing is also attached to the peripheral port and directed to waste. Upon the outlet of the CF column, the segmentation ratio can be adjusted to any ratio that fits the purpose of the analyst. When a 4.6 mm i.d. CF is used, for example, it is often convenient to set the ratio as either 43:57 or 21:79 (center:peripheral) to emulate a 'virtual' 3.0 mm i.d. column or 2.1 mm i.d. column, respectfully. That way the separation performance is readily bench-marked. The segmentation ratio is measured by weighing the amount of flow exiting from the detector that is connected to the central port and flow exiting the peripheral port over a period time. The percentage flow through each port can then be determined and the ratios can be adjusted by altering the length of tubing attached or using tubing that has a different internal diameter (i.d.).

This video protocol details the operation and optimization procedures of a CF column for enhanced chromatographic performance.

Protocol

Caution: Please refer to material safety data sheets (MSDS) for all materials and reagents before use (*i.e.,* MSDS for methanol). Ensure the use of all appropriate safety practices when handling solvents and High Performance Liquid Chromatography (HPLC) eluent. Ensure appropriate use of engineering controls of HPLC, analytical balance and detector instrumentation, and ensure the use of personal protective equipment (safety glasses, gloves, lab coat, full length pants, and closed-toe shoes).

Note: This protocol contains instructions on how to use a CF column on a HPLC system coupled with a UV-Vis detector. The protocol has been written assuming the reader has basic knowledge and experience in chromatography.

1. Setup of HPLC Instrument

Note: This section can be altered to suit the analysts' needs, *i.e.,* choice of solvents, detector wavelength and flow rate that are appropriate to the sample of interest.

1. Prepare the HPLC instrument with 100% ultrapure water (*e.g.*, Milli-Q water) for line A and 100% methanol for line B as the mobile phase and purge the pumps as per manufacturer requirement.
2. Set the UV-Vis detector to 254 nm.
3. Choose either a pre-injection flow split mode of set-up, or a dual pump flow set-up. For the split-flow mode proceed to Step 2, for the dual pump mode proceed to Step 3.

2. Split-flow System Setup

1. Disconnect the pump line from the injector valve of the auto-sampler.
2. Attach a T-piece to the pump line.
3. Attach a 15 cm piece of 0.13 mm i.d. tubing to each port of the T-piece.
4. Connect one tube from the T-piece to injector valve of auto-sampler.
5. Set the pump to 1.0 ml min^{-1}.
6. Before connecting pump lines to the inlet of the CF column, tune the segmentation ratio of the flow to 40%:60% (center line:peripheral line) as follows in step 2.7.
7. Tuning of CF inlet ratio on split-flow system
    1. Measure the mass of two empty collection vessels using an analytical balance and label one collection vessel central and the other one peripheral (one for the line from the auto-sampler to center port and one for the line from the T-piece to peripheral port).
    2. For 1.0 min, collect the exiting mobile phase from the line coming from the injector (at the point that will be connected to the column) into the collection vessel, whose mass was measured in 2.7.1.
    3. Re-weigh the collection vessel on the analytical scale and determine the mass of mobile phase collected.
    4. Repeat steps 2.7.2 to 2.7.3 for the eluent exiting the line from the T-piece that is to be connected to the peripheral port.
    5. Determine the percentage of flow (ml min^{-1}) from each line of flow according to the following equations:

$$\%\ \text{Central Port} = \frac{\text{Weight of Central Port (g)}}{\text{Weight of Central Port (g)} + \text{Weight Peripheral Port (g)}} \times 100$$

$$\%\ \text{Peripheral Port} = \frac{\text{Weight of Peripheral Port (g)}}{\text{Weight of Central Port (g)} + \text{Weight Peripheral Port (g)}} \times 100$$

    6. Adjust the flow ratio to 40%:60% (± 2%) (line from injector to ***central*** port:line from T-piece to ***peripheral*** port). If the line from the injector to the ***central*** port flow percentage is above 40%, increase the pressure drop by decreasing the internal diameter of the tubing, or increasing its length. If the line from injector to ***central*** port flow percentage is below 40%, increase internal diameter of the tubing or decrease the length of the tube.
    7. Once the flow ratios are tuned turn the pump flow off.
    8. Connect the line from the injector to the central port of the column inlet and the line from the T-piece to the peripheral port of the column inlet.
    9. Slowly ramp the flow rate to 1.0 ml min^{-1} at 100% line B.
    10. Equilibrate the column (4.6 mm i.d. x 100 mm length) by allowing 100% Methanol (line B) mobile phase to flow through the column at 1.0 ml min^{-1} for 10 min. This time is scaled according to the dimensions of other columns the user may employ.
    11. For the tuning of the CF outlet go to Step 4. 'Tuning of CF outlet flow'.

3. Dual Pump System Setup

1. Connect the HPLC system pump to the injector and then connect the line from the injector to the central inlet port of the column.
2. Connect the additional pump directly to the inlet peripheral port of the column. Note that this second pump by-passes the injector.
3. Ramp the flow rate of the system pump attached to the central port to 0.4 ml min^{-1} (representative of 40% of the total flowrate of 1.0 ml min^{-1}) at 100% Methanol (line B).
4. At the same time as Step 3.3, ramp the flow rate of the peripheral pump to 0.6 ml min^{-1} (representative of 60% of the total flowrate of 1.0 ml min^{-1}) at 100% Methanol (line B).
5. Equilibrate the column (4.6 mm i.d. x 100 mm length) by allowing 100 % Methanol (line B) mobile phase to flow through the column at 1.0 ml min^{-1} for 10 min. This time is scaled according to the dimensions of other columns the user may employ.
6. For the tuning of the CF outlet go to Step 4. 'Tuning of CF outlet flow'.

4. Tuning of CF Outlet Flow

1. Connect the central outlet port to the UV-Vis detector using a 15 cm piece of 0.13 mm i.d. tubing.
2. Connect a 15 cm piece of 0.13 mm i.d. tubing to the peripheral outlet port of the CF column.
3. Weigh the mass of two empty collection vessels on the analytical balance and label one vessel central and the other peripheral.
4. For 1.0 min, collect the exiting mobile phase from the UV-Vis detector (central flow) into the collection vessel label central, whose mass was measured in 4.2.
5. Re-weigh the collection vessel containing the collected eluent on the analytical scale and determine the mass of mobile phase collected.
6. Repeat steps 4.4 to 4.5 for the eluent exiting the line from the peripheral outlet port.
7. Determine the percentage of flow from each line of flow according to the following equations:

$$\%\ \text{Central Outlet Port} = \frac{\text{Weight of Central Outlet Port (g)}}{\text{Weight of Central Outlet Port (g)} + \text{Weight Peripheral Outlet Port (g)}} \times 100$$

$$\%\ \text{Peripheral Outlet Port} = \frac{\text{Weight of Peripheral Outlet Port (g)}}{\text{Weight of Central Outlet Port (g)} + \text{Weight Peripheral Outlet Port (g)}} \times 100$$

8. Adjust the flow ratio to 21%:79% (± 2%) (central outlet flow from UV-Vis:peripheral outlet flow from line *)*. If the central flow percentage from the UV-Vis is above 21%, increase the pressure drop by decreasing the internal diameter of the tubing attached to the exit of the UV-Vis detector, or increasing its length. If the central flow percentage from the UV-Vis is below 21%, increase internal diameter of the tubing

attached to the exit of the UV-Vis detector, or decrease the length of the tube. Each time the length of tubing has been changed, repeat steps 4.3 to 4.7.
Note: The CF column in 'virtual' 2.1 mm i.d. mode is ready for analysis.

Representative Results

AFT columns were developed using a specialized frit design (**Figure 1**) in the multiport column end-fittings to overcome the column bed heterogeneity and improve separation performance. An inter-laboratory study on the separation performance of CF chromatography columns (**Figure 2**) was carried out with a dual pump system set up (**Figure 3B**) as described in section 3 of this protocol[23]. A three component test mixture was analyzed under via a 'virtual' 2.1 mm i.d. where 21% of the central outlet flow of the CF column was directed to the detector. The separation of a three component test mixture illustrates the improved performance in terms of efficiency and sensitivity, of a CF column relative to standard columns. The three component test mixture contained phenetole, butylbenzene and pentylbenzene and was analyzed on conventional 4.6 and 2.1 mm i.d. columns and a 4.6 mm i.d. CF column with a segmentation ratio of 22:78 (center:peripheral) to emulate a 2.1 mm i.d. (**Figure 4**). Separation efficiency was evaluated in terms of plate count (N), and sensitivity. The use of CF column for analysis showed a lower limit of detection (**Figure 5**) and an increase in sensitivity (**Figures 4** and **6**) compared to conventional column analyses. It was also found that irrespective of the laboratory or type of HPLC system that is employed, the separation performance outcome for the CF column was relatively the same, all resulting in improved separation performance when employing CF chromatography columns[23].

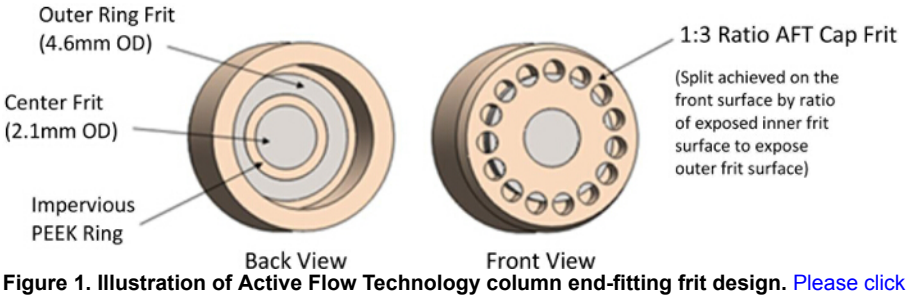

Figure 1. Illustration of Active Flow Technology column end-fitting frit design. Please click here to view a larger version of this figure.

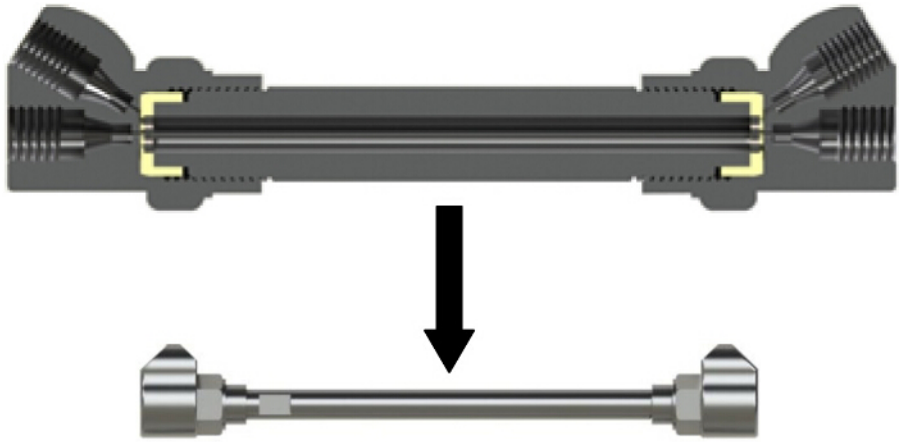

Figure 2. AFT column - CF column format. Please click here to view a larger version of this figure.

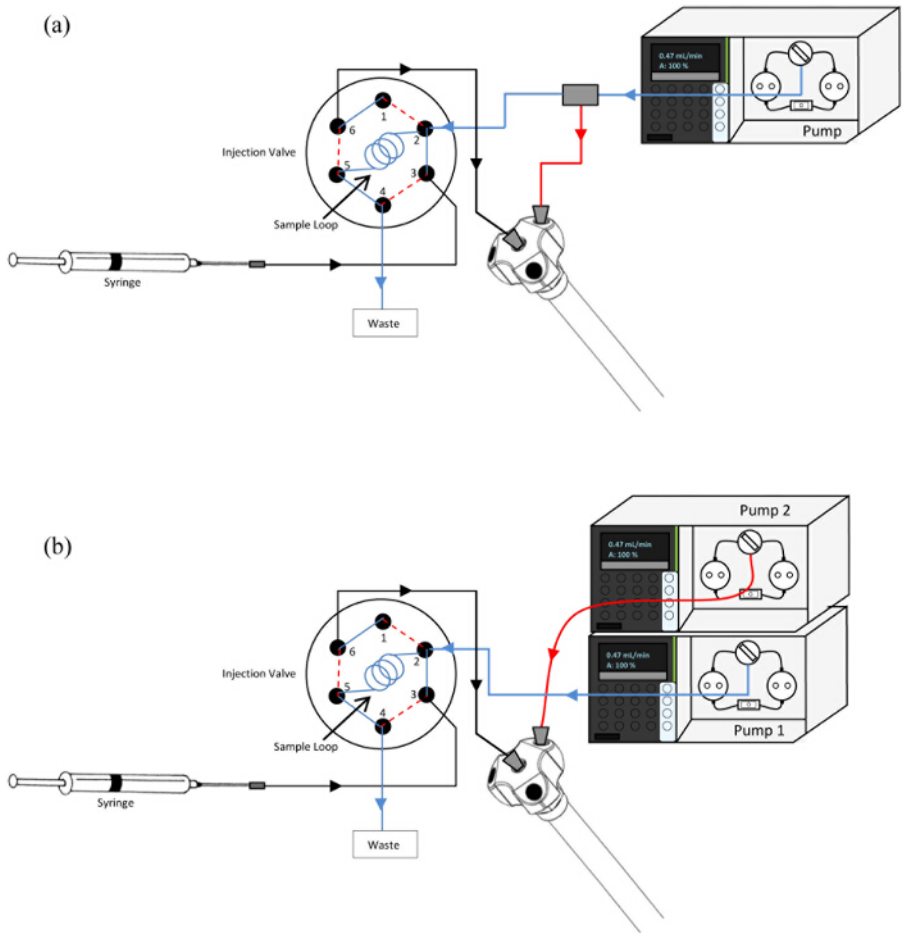

Figure 3. Set up of CF column inlet flow in (A) split-flow system setup and (B) 2 pump system setup. Please click here to view a larger version of this figure.

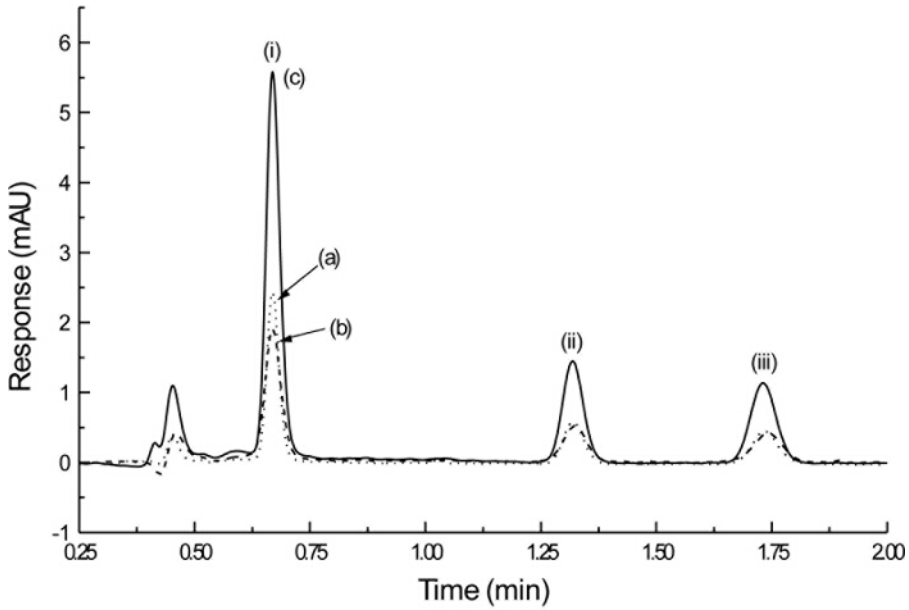

Figure 4. A typical separation of the three component test mixture obtained using the Ultimate 3000 system. (a) Conventional 4.6 mm i.d. column, (b) Conventional 2.1 mm i.d. column, (c) Curtain flow column operating with a 22% outlet segmentation ratio. Solutes: (i) phenetole, (ii) butylbenzene and (iii) pentylbenzene. This figure has been extracted from[23]. Please click here to view a larger version of this figure.

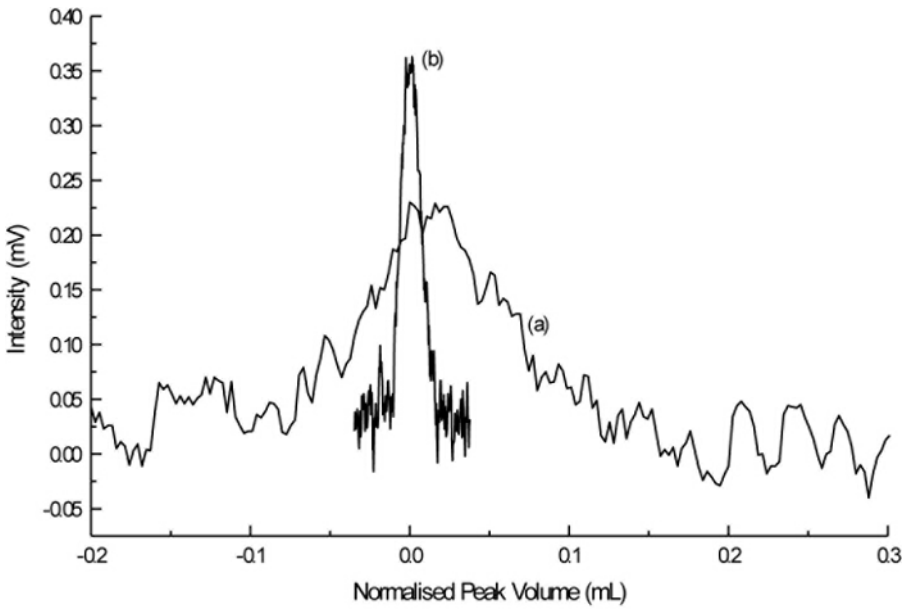

Figure 5. Elution band profiles of butylbenzene at the limit of detection on (a) the conventional column, and (b) the curtain flow column. System: Shimadzu at 2.0 ml/min, 5 µl injection, detection at 254 nm. This figure has been extracted from[23]. Please click here to view a larger version of this figure.

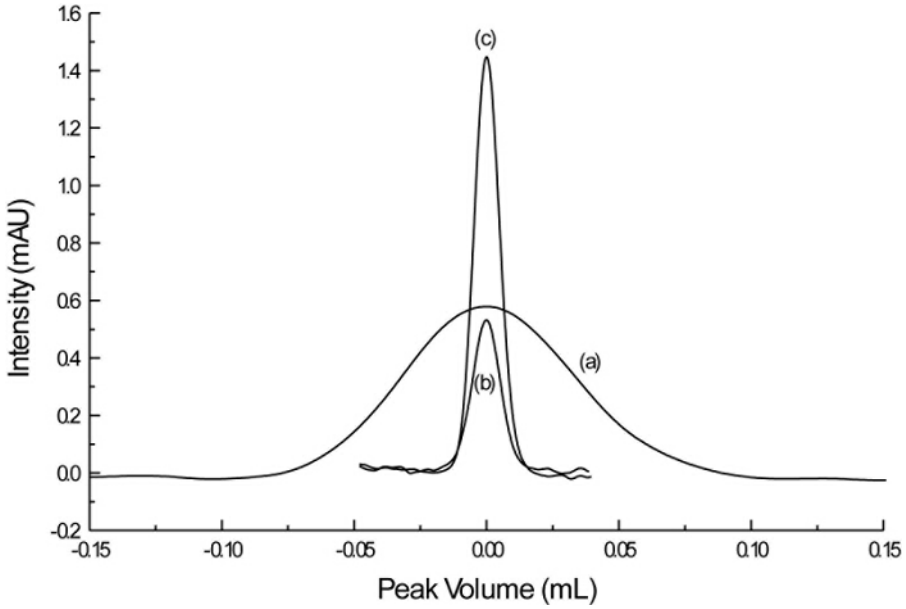

Figure 6. Comparison in the elution profiles of butylbenzene obtained on the Ultimate 3000 system. (a) the conventional 4.6 mm i.d. column, (b) the conventional 2.1 mm i.d. column, (c) the curtain flow column with a 22% outlet segmentation ratio. This figure has been extracted from[23]. Please click here to view a larger version of this figure.

Discussion

This study involved the inter-laboratory analysis of CF chromatography columns to test the analytical performance in terms of efficiency and sensitivity. The CF column was set up with a dual pumping system as described in section '3. Dual pump system set up' to achieve a flow ratio of 40:60 (center:peripheral) on the inlet of the CF column. The 40:60 (center:peripheral) flow ratio was achieved by setting the flow rate of each pump to the value that represents 40% and 60% of the total flow rate, respectively. The CF column outlet was tuned to a 'virtual' column with an i.d. of 2.1 mm by following the procedure described in section '4. Tuning of CF outlet flow'. A sample mixture containing phenetole, butylbenzene and pentylbenzene was used as a test standard for a separation performance comparison between a 4.6 mm i.d. CF column (22:78) and conventional 4.6 and 2.1 mm i.d. columns. **Figure 4** is an overlay of the chromatographic separation of the test mixture undertaken using each of the three columns. The main difference observed in this figure is the significant increase in signal response for the separation obtained using the CF column. The signal response for the 4.6 and 2.1 mm i.d. conventional columns were almost identical as expected as chromatographic conditions were scaled to match the cross-sectional surface of the columns.

Linearity and limits of detection were also assessed between the CF and conventional modes of operation, where a series of standards were prepared and analyzed in replicates on different HPLC systems with different flow rates. Irrespective of which HPLC system was used and at what flow rate the outcome of analysis was essentially the same, where the signal response for CF was always significantly greater than the other conventional columns. Signal response gains were typically between 1.7 and 2.8 times greater than the conventional columns. A 5-fold improvement in the precision of measurements (*i.e.*, relative standard deviation - RSD %) of peak height for the lowest standard series was observed for the CF mode of operation at 22% compared to the conventional 2.1 mm i.d. column. CF improves the precision of peak measurements due to the increase in sensitivity that is obtained by CF. The greater the signal response the lower the RSD value. Thus, as a consequence of improved signal response peak precision is also improved, also efficiency is higher, so bands tail less and hence peak integration is more precise. The limits of detention and quantification using CF columns with an outlet segmentation ratio of 22:78 (center:peripheral) were also improved by up to 2.3 times than conventional 2.1 mm i.d. column[23]. **Figure 5** illustrates the near limit of detection response for butylbenzene peak under the CF conditions and conventional conditions.

An important aspect to the comparison between CF and conventional columns that is not apparent in **Figure 4** is the reduction in peak volume for the analytes in the samples under CF conditions. **Figure 4** presents the peaks with respect to time, however, since in CF mode only a portion of the total flow is being used, peak width could be adjusted with respect to volume. **Figure 6** compares the butylbenzene elution profile with respect to peak volume for CF (22:78) emulating a 'virtual' 2.1 mm i.d., a conventional 4.6 mm i.d. column and a conventional 2.1 mm i.d. The peak volume between the CF and the conventional 2.1 mm columns was almost identical, however, the peak volume of the conventional 4.6 mm column was about 5 times larger than both conventional 2.1 mm and CF (22:78). Importantly, the reduction in peak volume in CF mode did not result in a reduction of signal response, but rather an increase by almost 3 times than that of the conventional columns regardless of internal diameter[23]. Although a reduction in peak volume may not be important for UV-Vis detection, the same cannot be said for detection processes that are flow rate dependent or limited, for example, mass spectrometer or evaporative light scattering detector.

The disadvantage to CF mode of operation like that of narrower-bore conventional columns is its susceptibility to the impact of post-column dead volume, which can significantly deteriorate the separation performance by causing band broadening and decay in signal intensity. However, the dead volume at the inlet is less important. Thus, due care to the post-column tubing is necessary for optimal CF separation performance. CF chromatography is a fairly new form of column technology that has great potential in future applications. For example, the injection of a low concentration sample into the center of the CF column, is 'curtained' by the wall (peripheral) mobile phase concentrating the sample within the center of the CF column, and thus maximizing the signal response. Upon the outlet only the central flow containing the 'concentrated' sample is taken to the detector, providing an increase in sensitivity, ideal for high-speed analysis using high flow rates on flow limited detectors such as MS[6].

Disclosures

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