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# Improving quantification using curtain flow chromatography columns in the analysis of labile compounds: A study on amino acids☆

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ABSTRACT

The performance of curtain flow chromatography column technology with MS detection was evaluated for the analysis of labile compounds. The curtain flow column design allows for separations that are faster and/or more sensitive than conventional columns, depending on how exactly the curtain flow column is configured. For example, when mass spectral detection is employed, the curtain flow column can yield separations that are 5-times faster than conventional columns when the curtain flow and the conventional columns have the same internal diameter. Or when the internal diameter of the conventional column is reduced in order to yield the same analytical through-put as the curtain flow column, the sensitivity on the curtain flow column can be as much as 66-fold higher than the conventional column. As a consequence of the higher analytical through-put less standardization is required in the analysis of labile compounds because less sample degradation is apparent. Consequently the sample integrity is preserved yielding data of a higher quality.



## 1. Introduction

High speed, high resolution separations are an essential component of our modern analytical requirements. Separations coupled with mass spectral detection represent a powerful analytical technique, however, its limitations need to be taken into account. In particular, analysis through-put is limited by the solvent capacity of the MS. The drive towards smaller particles, and the developments in monolithic columns has meant that efficient separations can be undertaken at higher flow velocities. Hence the design strategy of column technology is at variance with the operational limitations of the mass spectrometer, since in MS detection solvent must be removed from the sample, and as the flow rate increases solvent removal becomes more difficult. Strategies that are employed by the analyst to overcome the solvent removal limitation of the MS are either, (1) the use of post-column flow stream splitting, which generally results in a reduction in sensitivity and separation efficiency, or most commonly nowadays, (2) the use of narrow bore columns, rather than analytical scale columns, albeit with perhaps a compromise made in the performance of the chromatographic separation [1–3].

Recently, a new column design has been developed that unites the benefits of both large and small internal diameter columns. This column technology has been referred to as active flow technology (AFT), and in this work we demonstrate the performance advantages of the curtain flow (CF) column, a member of the suite of AFT columns. The concept of the CF column has been discussed in detail in numerous publications [3–8], but it is discussed again here briefly to provide context to the current study. Effectively, AFT columns, including the CF column, provide an environment that establishes a 'virtual' narrow diameter column inside a larger format column [4,5], the flow properties of which emulate that of conventional narrow bore columns that have the same internal diameter as that of the virtual column internal diameter, but with improved performance. The advantage of the virtual column is that it is free from wall effects [4,5]. The key design features of the curtain flow column are the end fittings (both inlet and outlet) and the frits that are housed in these fittings. The frit at both the inlet and the outlet is an annulus, containing a central porous region that is separated from an outer porous region by an impermeable barrier, which prevents cross flow between these two zones. The frit is housed inside a multi-port end fitting, such that the inner porous region of the

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frit aligns with a radial central port, and the outer porous region of the frit aligns with peripheral port(s). Sample and mobile phase enter the column via the radial central inlet port, and pure mobile phase (no sample) enters the column through the peripheral inlet port. This sample introduction process has the purpose of confining the sample to radial central region of the column, and dispersion to the wall is hindered by a curtain flow of mobile phase along the wall region. Mobile phase that flows along the radial central region of the column then exits via the radial central port, and flow along the wall region of the column exits via the peripheral port. The flow proportions at the column outlet can be varied by changing the relative pressure drop across the various exit ports. If for example, 21% of the flow from a 4.6 mm i.d. column exits the column via the radial central exit port, then a virtual 2.1 mm i.d. column is established. If that flow proportion is changed to 43%, then the diameter of the virtual column is 3 mm, and so forth [5]. The advantages of the curtain flow column design are; (1) increased column efficiency, since the most efficient portion of the chromatography column is employed [5], effectively eliminating the wall effect; (2) increased sensitivity, since the entire sample is confined to the radial central region of the column [3] and (3) the solvent flow volume of the virtual column is equal to that of the conventional column having the same internal volume. Hence, a 4.6 mm i.d. CF 'virtual' column can be employed at the same linear velocities and the same exit elution volumes as the narrow bore column that it emulates, making it just as compatible with MS-type detectors. More details of the virtual column, and curtain flow chromatography can be found in Refs. [4,5].

In the present study, we demonstrate another advantage of these columns, greater assay reliability in the analysis of labile compounds. Such reliability arises from the ability of AFT separations with MS detection to be undertaken at higher volumetric flow rates, currently five times faster than conventional columns with the same physical diameter. Consequently, when labile samples are analysed, the overall duration of the assay is decreased and this leads to less variation within the sample during the total analysis time.

## 2. Experimental

### 2.1. Chromatography columns

Columns were supplied by Thermo Fisher Scientific (Runcorn, Cheshire, United Kingdom). Two columns were standard Hypersil GOLD columns with internal diameters of either 2.1 or 4.6 mm. A third column, also supplied by Thermo Fisher Scientific was an AFT column fitted out in curtain flow mode [7]. This column had an internal diameter of 4.6 mm. All columns were 50 mm in length, packed with 5 μm particles.

### 2.2. Chemicals and reagents

All mobile phases were prepared from HPLC-grade solvents purchased from Thermo Fisher Scientific, Australia Pty Ltd. Milli-Q water (18.2 MΩ $cm^{-1}$) was prepared in-house and filtered through a 0.2 μm filter. Formic acid was purchased from Chem-Supply Pty Ltd, Gillman, South Australia. Amino acids were purchased from Sigma–Aldrich (Castle Hill, New South Wales, Australia).

A mixed amino acid stock solution was prepared at the concentration of 100 μg/mL (for each amino acid) in HPLC grade methanol. The amino acids were individually weighed from solid and diluted in methanol. The stock solution was diluted in water to prepare a working range of solutions for calibration from 25 ng/mL to 500 ng/mL.

### 2.3. Instrumentation UPLC–MS/MS

A Thermo Fisher Scientific UHPLC system (Ultimate 3000) equipped with a quaternary pump and auto injector with an in-line degassing unit coupled with a TSQ (triple stage quadropole) Vantage mass spectrometer equipped with HESI II source (Thermo Scientific, San Jose, USA) was used to carry out chromatographic separations with MS detection. The instrument was used as supplied from the manufacturer.

For experiments involving mass spectral analysis the mobile phase flow rate was operated in such a way that the volume presented to the MS detector was always 1 mL/min, irrespective of the column internal diameter, or the whether the column was a conventional column or a curtain flow column. Therefore the 4.6 mm i.d. and the 2.1 mm i.d. conventional columns were operated at 1 mL/min. The 4.6 mm CF column was operated in a manner so as to emulate a 2.1 mm i.d. 'virtual' column. In doing so, the outlet segmentation ratio was set so that 20% of the volumetric flow exited the column from the central outlet port. Hence this column was operated at 5 mL/min so that 1 mL/min entered the MS. The linear flow velocity on the curtain flow column and the 2.1 mm i.d. conventional column was therefore the same. Mobile phase delivery to the curtain flow column was set such that 40% entered the column through the central inlet port and 60% entered through the peripheral ports. This flow proportioning was obtained using a split flow configuration set prior to the injector, hence 40% of flow passed through the injector. Details of the curtain flow operation have been previously published [3].

Gradient elution was used for all separations. The initial mobile phase composition was 95/5 water/methanol (+ 0.1% formic acid) and the final mobile phase composition was 35/65 water/methanol (+ 0.1% formic acid). The duration of the gradient was proportioned to the respective flow velocities on each of the columns. Specifically, on the 4.6 mm i.d. standard column, the gradient duration was 6 min, after which the mobile phase was returned to the original conditions (1 min.). On the 4.6 mm i.d. CF column, where the linear velocity was five-times higher than on the 4.6 mm i.d. conventional column, the gradient duration was 1.2 min, after which the system was returned to the initial conditions in 0.2 min. All columns were equilibrated with five column volumes of mobile phase prior to the next injection. The cycle time of the 4.6 mm i.d. standard column was therefore 5 times that of the 2.1 mm i.d. standard and 4.6 mm i.d. CF columns, In practice, however, the cycle time was not exactly 5× that of the standard 4.6 mm i.d. column since there was a delay between injections that allowed for rinsing and washing of the auto-injector. The effective injection to injection cycle time on the 2.1 mm i.d. and curtain flow columns was thus 3.5× that of the conventional 4.6 mm i.d. column.

Injection volumes were scaled in accordance to the column volume so that sample components at elution, and hence presentation to the MS, would be at constant concentration [3]. Therefore 1.04 μL was injected on the 2.1 mm i.d. column and 5 μL was injected on the 4.6 mm and 4.6 mm CF i.d. columns. Samples and standards were analysed in at least triplicate.

### 2.4. Mass spectrometry parameters

Detection was achieved using the TSQ Vantage mass spectrometer with electrospray ionization in positive ion mode using single reaction monitoring (SRM). The MS parameters were optimized for maximum sensitivity: vaporiser temperature 500 °C, capillary temperature 350 °C, sheath gas rate of 60 units, auxiliary gas flow at 40 and sweep gas flow at 5 units. The spray voltage was maintained at 3.5 kV the tube lens offsets and collision energy were individually optimized for each compound [3]. Data processing was performed using LC Quan software (Thermo Fisher Scientific, San Jose, USA).