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Temperature-assisted on-column solute focusing: A general method to reduce pre-column dispersion in capillary high performance liquid chromatography

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ABSTRACT

Solvent-based on-column focusing is a powerful and well known approach for reducing the impact of pre-column dispersion in liquid chromatography. Here we describe an orthogonal temperature-based approach to focusing called temperature-assisted on-column solute focusing (TASF). TASF is founded on the same principles as the more commonly used solvent-based method wherein transient conditions are created that lead to high solute retention at the column inlet. Combining the low thermal mass of capillary columns and the temperature dependence of solute retention TASF is used effectively to compress injection bands at the head of the column through the transient reduction in column temperature to 5 °C for a defined 7 mm segment of a 6 cm long 150 μm I.D. column. Following the 30 s focusing time, the column temperature is increased rapidly to the separation temperature of 60 °C releasing the focused band of analytes. We developed a model to simulate TASF separations based on solute retention enthalpies, focusing temperature, focusing time, and column parameters. This model guides the systematic study of the influence of sample injection volume on column performance. All samples have solvent compositions matching the mobile phase. Over the 45–1050 nL injection volume range evaluated, TASF reduces the peak width for all solutes with k' greater than or equal to 2.5, relative to controls. Peak widths resulting from injection volumes up to 1.3 times the column fluid volume with TASF are less than 5% larger than peak widths from a 45 nL injection without TASF (0.07 times the column liquid volume). The TASF approach reduced concentration detection limits by a factor of 12.5 relative to a small volume injection for low concentration samples. TASF is orthogonal to the solvent focusing method. Thus, it can be used where on-column focusing is required, but where implementation of solvent-based focusing is difficult.

1. Introduction

Small volume samples are commonly encountered in the fields of metabolomics, proteomics, forensics, neurochemistry and single cell analysis [1–4]. The high complexity and mass limited nature of such small samples necessitates the enhanced detection sensitivity offered by reductions in column diameter that limit sample dilution [5,6]. The recent significant improvements in column technology, i.e. sub-2 μm fully porous and core–shell particles [7–10], while welcome also create a problem—sensitivity to solute dispersion from pre-column processes including volume overload. Volume overload occurs when the relative contribution of the injection plug width to dispersion is large compared to that produced by on-column convective dispersion [11,12]. In this work we describe an approach to mitigate the detrimental effects of pre-column dispersion created by volume overload in capillary columns using temperature-assisted on-column solute focusing (TASF).

One potential solution to the volume overload problem is to reduce the injected volume. For example 5-nL injection volumes are possible in theory with various split- and timed-injection methods [13,14], but achieving them with currently available instrumentation is non-trivial. Such a small injection volume with current valve technology is not possible as a reduction in sample volume does not necessarily produce a proportional reduction in the effective volume loaded onto the column due to dispersion contributions by valve passages and all other pre-column volumes [15]. Fortunately, on-column focusing is a simple and effective process that can minimize the effects of volume overload [5,16]. On-column focusing occurs when injected solute bands are compressed at the head of the column due to high solute retention and their

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subsequent elution at a much higher velocity by the mobile phase. Upon injection the sample solvent system becomes the mobile phase for the period of time required to flush the loaded sample through the injection zone [12,17–19]. This solvent-based focusing works particularly well for aqueous samples injected into a reversed phase column. Recently the effect has seen a resurgence as a means to mitigate pre-column dispersion [20,21] and to counteract the dilution of first dimension analyte bands in online multi-dimensional liquid chromatography [22–28]. Application of solvent-based on-column focusing in capillary scale columns has been more widespread because of their greater susceptibility to volume overload [29–34].

The one fundamental requirement for on-column focusing is the establishment of high retention conditions at the head of the column. The means to attain this retention are not limited to changes in sample solvent composition. The mobile phase has a very strong influence on solute retention; this is why solvent-based focusing works well, but temperature also influences solute retention in LC albeit to a much smaller extent. The effect of temperature on retention is determined by the solute's partial molar enthalpy of retention. Increases in column temperature in RPLC generally decrease solute retention. In fact, high temperature liquid chromatography has exploited elevated temperature's influence on retention, selectivity, solvent viscosity and analyte diffusivity to attain fast, efficient separations in a wide variety of applications discussed in the following reviews and book [35–38].

Increasing column temperature can decrease the effectiveness of solvent-based focusing in reversed phase chromatography in particular. A significant increase in column temperature generally leads to a decrease in retention which may call for a commensurate reduction in solvent strength to maintain constant retention [39]. Thus, in comparison to near-room temperature separations, high temperature separations may use weaker, more aqueous mobile phases. In such cases, the contrast between retention in water from the sample and retention in the more aqueous mobile phase decreases, thus on-column focusing becomes less powerful.

In contrast, TASF benefits from elevated separation temperatures. TASF is based on the premise that the transient reduction in column temperature for a short column segment, ca.<1 cm, for a short period of time, <1 min, will increase solute retention fostering effective on-column focusing. The freezing point of the mobile phase and pressure limitations of the pumping system set the minimum achievable column temperature to near −20 °C for typical aqueous/organic/reversed-phase systems. [40]

Capillary columns offer low thermal mass and small radial temperature gradients allowing rapid changes in column temperature during the chromatographic run. Temperature programming and various temperature 'pulsing' techniques have been successfully used with capillary scale columns. These methods have emphasized the benefits of rapid column heating to generate temperature gradients, increase analysis speed or tune chromatographic selectivity [41–45]. Only the Greibrokk group has explored the potential for sub-ambient column temperatures to focus large injection volumes onto capillary columns. In their work temperature programming initiated at sub-ambient temperatures, ca. 5 °C, was used to focus samples of retinyl esters, polyolefin based Irganox antioxidants and ceramides made in 80–100% acetonitrile prior to separation using a neat acetonitrile mobile phase and C18 column [46–50]. High hydrophobicity and poor analyte solubility in water, necessary for on-column focusing was common to all of this work.

The primary method used to achieve sub-ambient column temperatures involves the use of programmable column ovens with cooling capabilities where the entire column is cooled via convection. There are two noteworthy limitations to this approach: (1) due to air's low heat capacity, rapid changes in column temperature are difficult to obtain, (2) reduction in column temperature significantly increases mobile phase viscosity. Cooling the entire column to sub-ambient temperatures puts serious restrictions on achievable linear velocities due to maximum pump pressure limitations. For example, cooling a 5 cm long column to 5 °C would increase column pressure by a factor of 3, compared to an identical 60 °C isothermal analysis. Reducing the temperature of only short segments of the column is an effective solution to the pressure problem. Cooling 1 cm of the hypothetical 5 cm column to 5 °C, while holding the remaining 4 cm at 60 °C, does significantly increase backpressure, albeit only by 35% relative to the 60 °C isothermal column. Maximum pump pressure is a limitation to TASF and needs to be considered, although the recent improvements in pump technology and increases in maximum operating pressure have lessened its influence.

To solve the problems associated with convection ovens two alternative cooling methods have been suggested. Holm and coworkers [51] developed a device where a single Peltier type thermoelectric cooler was used to cool an aluminum block through which a short segment of the column passes allowing large volume samples of the same Irganox antioxidants described above to be focused. After focusing, the column was moved manually, in space, from the cold zone to a programmable column oven where the separation was performed. Collins et al. presented another application of thermoelectric column cooling where an array of ten independently controlled 1.2 cm square Peltier units were aligned allowing precise temperature control for capillary monolith synthesis and temperature programmed separations of alkylbenzene mixtures [52]. In a second approach to temperature assisted focusing Eghabali et al. cooled a 1 cm long section of the column, near its outlet, cryogenically to approximately −20 °C [53]. Heating was achieved using boiling water. What is unique about this focusing method was placement of the cold trap at the column outlet. This was done to trap and re-focus specific analytes (proteins) within regions of interest improving the observed signal-to-noise ratio (S/N).

In this paper, we describe an efficient approach to on-column focusing we refer to as TASF. We view this method as orthogonal to the conventionally used solvent-based on-column focusing methods so it can be employed independently or in conjunction with solvent-based methods. We report on the efficacy of the TASF method applied injection volumes ranging from about 7% to 150% of the column's fluid volume. Samples of solutes were made in mobile phase to avoid solvent focusing. The TASF approach was found to effectively reduce pre-column dispersion for all injection volumes tested. This method is ideal for applications where on-column focusing is required to mitigate volume overload and focus analyte bands, but where sample solvent compositions are fixed and not significantly different from the mobile phase, i.e. where implementation of solvent-based focusing is difficult.

2. Theory

The signal observed in a chromatographic separation is influenced by all the components in the system and their individual contributions to the observed variance of the chromatographic band. The variance of the signal observed by the detector, in time units ($\sigma^2_{t,obs}$) is given by:

$$\sigma^2_{t,obs} = \sigma^2_{t,inj} + \sigma^2_{t,col} + \sigma^2_{t,det} + \sigma^2_{t,o} \quad (1)$$

where $\sigma^2_{t,inj}$, $\sigma^2_{t,col}$ and $\sigma^2_{t,\mathrm{det}}$ are the variances induced by the injector, column and detector; $\sigma^2_{t,o}$ accounts for the sum of all other sources of dispersion in the chromatographic system, i.e. tubing, connections, etc. We note that the formulation shown is based on the assumption of independence of the processes contributing each term and that there is no mass overload (i.e., the system operates where the solute distribution isotherms are linear). In the ideal case