



Volume based vs. time based chromatograms: Reproducibility of data for gradient separations under high and low pressure conditions



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ABSTRACT

A critical aspect in fast gradient separations carried out under constant pressure, in the very high pressure liquid chromatography (VHPLC) mode is that time-based chromatograms may not yield highly reproducible separations. A proposed solution to improve the reproducibility of these separations involves plotting the chromatograms as functions of the volume eluted vs. UV absorbance instead of time vs. UV. To study the consequences of using the volume-based rather than the time-based chromatograms, separations were first performed under low pressures that do not generate significant amounts of heat and for which the variations of the eluent density along the columns are negligible. Secondly, they were performed under very high pressures that do generate heat and measurable variations of the local retention factor and eluent density along the column. Comparison of the results provides estimates of the improvements obtained when volume based chromatograms are used in gradient analyses. Using a column packed with fully porous particles, four different types of methods and several sets for each method were used to perform the gradient elution runs: two sets of constant flow rate operations, four sets of constant pressure operations, two sets of constant pressure operations with programmed flow rate, and one set using the constant heat loss approach. The differences between time-based and volume-based chromatograms are demonstrated by using eight replicates of early, middle, and last eluting peaks. The results show that volume-based chromatograms improve the retention time reproducibility of the four constant pressure methods by a factor of 3.7 on average. If the column is not thermally conditioned prior to performing a long series of separations, flow controlled methods (constant flow rate, programmed constant pressure, and constant wall heat approaches) are more precise. If one gradient run is used to bring the column to a relatively stable temperature, constant pressure separations have a factor of 3 times better reproducibility of retention times with respect to constant flow rate gradient separations.

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1. Introduction

The desire to accelerate chromatographic separations caused scientists to conduct constant pressure operations in which separation speed is increased by operating the instrument and the column under the highest pressure that they can withstand. The revival of the use of constant pressure gradients was recently suggested [1–4]. Previous work confirmed the advantages of constant pressure techniques [5–10]. However, if the column permeability changes during a series of analyses due to deterioration of the packed beds or to a poor control of the eluent temperature, the

instrument will perform operations at flow rates that will fluctuate. Experiments are needed to better understand the constant pressure process. The performance of different column packing materials under different separation conditions, the different means of delivering the gradient affects on the reproducibility of chromatograms, and what errors may be encountered in quantitation using ultraviolet detection should be investigated thoroughly.

In theory if there is neither appreciable frictional heating during the separation nor change of the eluent density along the column, the time-based chromatogram should be as reproducible as volume-based ones under constant flow rate separations. When the temperature distribution across and along the column is neither uniform nor stationary, time-based and volume-based chromatograms cannot be as reproducible due to the uncontrollable fluctuations in the retention of the analytes [10]. Constant pressure operation in very high pressure liquid chromatography (VHPLC)

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exhibit lower reproducibility of retention times when time-based chromatograms are used to compare methods in which the flow rates are input into the instrument by the analyst [6–10]. Lack of reproducibility occurs under very high pressures due to the heating caused by friction affecting the mobile phase viscosity and density, and may take place at low pressures when the column permeability changes between runs. The goal of this work was to determine how much volume-based chromatograms correct for undesirable but unavoidable fluctuations in flow rate and thermal environment.

Frictional heating in chromatographic columns is a well-known physical phenomenon, which affects retention and band broadening in liquid chromatography [10–21]. The heat generated by friction when the mobile phase is percolating through the column increases with decreasing average size of the particles in the column. Increasing the flow rate also generates more frictional heat. This is not of great concern in isocratic separations since the mobile phase viscosity and its thermal conductivity do not change over time. In gradient separations, these parameters vary during a run and during the isocratic periods preceding or following each analysis [19,22]. There are two potential remedies for this situation. First, an adequate post-run time after each separation allows the column to return to the same starting temperature after each analysis [8]. Second, a very brief post-run time is used, and after one or two separations a reproducible starting temperature is achieved for subsequent separations [6,7]. The first separation(s) are used strictly for column equilibration, and the following runs are excellent reproducibility. For many practitioners this is a reasonable solution to quickly achieve highly reproducible separations.

To evaluate the volume based chromatograms generated by Agilent ChemStation Rev. C.01.03, a column packed with fully porous particles was selected because such columns do not dissipate heat as quickly as those packed with core-shell particles [23,24] and thus their use permits a more rigorous assessment of the software generating the volume-based chromatograms in the ChemStation. Both time-based and volume-based chromatograms were integrated and the response factors for each method compared.

Nine gradient elution methods were used to test various experimental conditions including two sets of constant flow rate separations, four sets of constant pressure separations, two sets of programmed flow constant pressure experiments, and one set of experiments involving experimental conditions which should produce a constant amount of heat at the column wall. Details of these methods are discussed in the next sections.

2. Material and methods

2.1. Instruments, columns, and reagents

Experiments were conducted in a 24 °C room, using a prototype Agilent 1290 Infinity System capable of constant pressure operations (Agilent Technologies, Waldbroen, Germany). A Thermo

Viper (130 μm I.D.) column connection unit (Dionex-Fisher, Sunnyvale, CA, USA) was used to connect a Waters XBridge BEH XP C₁₈ (4.6 × 100 mm; 2.5 μm particle size) column and a Waters XBridge BEH XP Vanguard Pre-Column (2.1 × 5 mm; 2.5 μm particle size) to the instrument. Fisher Scientific (Fair Lawn, NJ, USA) HPLC grade water and acetonitrile were used. The test mixture for all the experiments was a 2 μL sample of the Agilent RPLC checkout sample consisting of (in order of elution) acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone, benzophenone, and acetanilide dissolved in water/acetonitrile (65:35 v/v) in concentrations of 100 μg/mL each (+/– 0.5%). Chemstation Rev. C.01.03(37) was used for online and offline analyses.

2.2. Experimental conditions

The instrument was set to provide the briefest possible equilibration periods (post-run times) allowable by the software. Eight consecutive 2 μL injections of the sample were performed for all experiments. The column was insulated in a one inch thick layer of Styrofoam insulation. In previous work [6,8], the column was left in open air for constant heat loss experiments. It was determined that a marginal difference between constant heat loss experiments took place whether the column was insulated or exposed to open air, as long as an adequate time allowed temperature to equilibrate across the column and between column and Styrofoam insulation before a new separation was performed. For separations made with an inlet column pressure less than 300 bar, the frictional heat generated by the mobile phase is small and separations should be highly reproducible. To ensure that the column temperature was stable for each set of separations, the mobile phase was flowed through the column for 10 min before each series of runs. A 50 to 95% gradient of acetonitrile in water was used for all experiments. Table 1 gives a quick reference to the experimental conditions. It lists the method parameters for the low pressure experiments. Table 2 lists the method parameters for the high flow experiments.

2.2.1. Constant flow rate experiments

The parameters of constant flow rate experiments were input into the instrument software in the conventional way. The first set of constant flow rate experiments was conducted at a flow rate of 0.5 mL/min, with a starting inlet pressure of approximately 141 bar and a total run time of 11.4 min; this generated an average of 0.84 W/m of heat inside the column. The second set of constant flow rate separations was conducted at 2.25 mL/min and a total runtime of 2.55 min with a starting inlet pressure of approximately 600 bar generating an average of 16.99 W/m of heat inside the column.

2.2.2. Constant pressure experiments

Both constant pressure experiments were based on the volumetric flow profile from the constant flow rate experiments using the macro provided by the manufacturer. The ChemStation takes

Table 1
Summary of the experimental conditions and results at low pressures.

Experiment set	Solvent gradient 50% to 95% acetonitrile in water for all experiments			
	Constant flow	Constant pressure 1	Constant pressure 2	Programmed flow constant pressure
Gradient time	10.0 min	10.3 min	7.6 min	7.6 min
Total time	11.4 min	11.2 min	8.3 min	8.3 min
Flow rate	0.5 mL/min	Variable	Variable	Variable
Pressure	Variable	105.5 bar	141 bar	≈141 bar
Retention time RSD (time chromatogram)	0.029%	0.15%	0.15%	0.021%
Retention time RSD (volume chromatogram)	0.026%	0.034%	0.078%	0.026%
Peak width RSD (time chromatogram)	0.56%	0.56%	0.95%	0.62%
Peak width (volume chromatogram)	0.57%	0.41%	0.84%	0.68%

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