Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1192 (2008) 41–53
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

High-speed gradient elution reversed-phase liquid chromatography of bases in buffered eluents
Part I. Retention repeatability and column re-equilibration

Adam P. Schellinger, Dwight R. Stoll, Peter W. Carr *

Department of Chemistry, Smith and Kolthoff Halls, University of Minnesota, 207 Pleasant Street S.E., Minneapolis, MN 55455, USA

Received 19 May 2006; received in revised form 16 January 2008; accepted 22 January 2008
Available online 31 January 2008

Abstract

We studied the run-to-run repeatability of the retention times of both non-ionizable and basic compounds chromatographed using buffered eluents. The effect of flow rate, organic modifier and other additives, buffer type/concentration, stationary phase type, batch-to-batch preparation of the initial eluent, gradient time, sample type and intra-day changes on retention repeatability were examined. We also assessed the effect of column storage solvent conditions on the inter-day repeatability. Although retention repeatability is strongly influenced by many parameters (flow rate, solvent compressibility compensation, precision of temperature control, and buffer/stationary phase type), our primary finding is that with a reasonable size column (15 cm × 4.6 mm (i.d.)) two column volumes of re-equilibration with initial eluent suffices to provide acceptable repeatability (no worse than 0.004 min) for both non-ionizable and basic analytes under a wide variety of conditions. Under ideal conditions (e.g. the right buffer, flow rate, etc.) it is possible to obtain truly extraordinary repeatability often as good as 0.0004 min. These absolute fluctuations in retention translate to worst case changes in resolution of 0.2 units and average changes of only 0.02 units.

Keywords: Gradient elution; Speed; Equilibration; Repeatability; Basic compounds

1. Introduction

There have been many investigations of speed in chromatography [1–11] to reach the ultimate goal of a separation: obtaining an acceptable separation in a reasonable time. The cycle time (injection-to-injection), which is the sum of the gradient development time (t_G) and the re-equilibration time (t_{re}), sets the throughput of gradient elution methods [12]. Although one can decrease t_G and maintain resolution by using smaller particles [13], the pressure limitation of conventional instrumentation (∼40 MPa) sets the upper limit of speed. To solve this problem, Jorgenson and coworkers [14–16], and Lee and coworkers [17–20] have studied ultra-high pressure liquid chromatography with instrumentation that can withstand significantly higher backpressures.

Although there are many ways to minimize t_G without sacrificing resolution, once the gradient time is set the only way to further increase the speed of gradient elution is to minimize re-equilibration time. The current rule of thumb for re-equilibrating a column is to flush it with at least 10 column volumes of initial eluent before beginning the next run [21]. Dorsey and coworkers suggested that the addition of a small amount of *n*-propanol to the eluent can substantially decrease the re-equilibration time [22,23]; however, they did not quantify the state of column equilibration as a function of the re-equilibration time [23]. Furthermore, Dorsey assumed that the retention of acetone, a relatively unretained species, accurately represents the state of column equilibration under various conditions [22]. Our recent work shows that this assumption is often wrong [12].

To clarify the term "re-equilibration", we define two distinct states of re-equilibration: repeatable equilibrium and full equilibrium. Repeatable equilibrium occurs at a value of t_{re} which suffices to provide excellent run-to-run reproducibility in retention (typically a standard deviation of <0.002 min for four replicates) based on the experimental conditions used (a 150 mm × 4.6 mm column with 5 μm particles at 1 mL/min). This degree of reproducibility is probably acceptable for all but the most exacting work (*vide infra*). On the other hand, full

* Corresponding author. Fax: +1 612 626 7541.
E-mail address: carr@chem.umn.edu (P.W. Carr).

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.01.062

equilibrium under gradient occurs at a value of t_{re} where the retentions of all peaks no longer statistically change as t_{re} is increased. This can take quite a bit longer than the state of reproducible equilibrium. Although others have investigated column equilibration phenomena [21–31], we believe our previous study was the first to distinguish between these two different states of re-equilibration [12].

It should be evident that high reproducibility under isocratic elution requires that the column has reached a state of full, i.e. thermodynamic, equilibrium. It is more than possible that reproducible retention, even very reproducible retention, can be obtained in gradient elution chromatography under certain conditions even if a state of full equilibrium with the initial eluent has not been achieved. One can easily imagine that if the final eluent, say one which very readily wets the pores and establishes thermodynamic equilibrium of the mobile and stationary phases, effectively re-initializes the column then all that is needed to achieve reproducible retention is that the instrument deliver a very reproducible gradient.

Previously, based on a test mixture of non-ionizable solutes in unbuffered eluents, we found that repeatable equilibrium was achieved in at most two column volumes of re-equilibration under all conditions investigated [12]. However, when full equilibrium was required with flushing limited to one to two column volumes it was absolutely essential that both 1% (v/v) *n*-butanol be added to the eluent and that the system flush-out volume be minimized; when this was not done it was under some conditions necessary to flush the column with more than 20 column volumes of initial eluent.

Although gradient analysis of non-ionizable solutes in unbuffered eluents requires very little time to re-equilibrate the system, other work indicated that column equilibration, under isocratic conditions, measured with ionized compounds in buffered eluents could require several hours [24,32]. Thus, we here investigated the re-equilibration time needed to achieve repeatable retention in gradient chromatography for cationic solutes in buffered eluents. We also studied the effect of flow rate, organic modifier, buffer type/concentration, stationary phase, *n*-butanol concentration, batch of the initial eluent and sample mixture on the intra-day repeatability and the effect of the storage solvent on the inter-day repeatability. In a second companion study we will report on the re-equilibration time required for full equilibrium. The results in that study are rather different. Specifically we find that one must add *n*-butanol to the initial eluent to rapidly achieve a state of *full equilibrium*. Such is not necessary to achieve *repeatable equilibrium*. However, to minimize confusion in these two studies we decided at the outset to keep conditions as similar as possible.

The degree to which retention variability is acceptable is strongly dependent on the application. Some may be willing to accept a variability of 0.010 min for routine analytical work. However, when the data is subjected to chemometric analysis and the powerful trilinearity condition is imposed, high repeatability becomes very valuable if not absolutely vital. In this case, the potential advantage of obtaining excellent repeatability in retention (<0.002 min) is that data pre-processing steps such as parametric time warping, dynamic time warping (DTW) or correlation optimized warping (COW) procedures [33–35] can be simplified or sometimes avoided before performing chemometric curve resolution [36]. Although such time warping algorithms show great potential for aligning chromatograms with poor repeatability (i.e. >0.01 min), the results of time warping are highly dependent on the sample and experimental conditions. 2DLC is another case where extremely high repeatability presents an advantage because one needs to align the chromatograms with high precision to construct the 2D picture from a long series of 1D runs.

Another advantage of obtaining excellent repeatability is that the potential to identify components using their retention will be improved considerably [37]. Furthermore, the ability to obtain excellent repeatability with a short re-equilibration time is of great importance to increase sample throughput in fields such as forensic toxicology and drug discovery [38–44]. Obviously, the ability to obtain excellent repeatability with minimal re-equilibration time for basic compounds separated in buffered eluents has practical implications in many fields and applications.

2. Experimental

2.1. Instrumentation

All chromatographic experiments were conducted using an HP 1100 chromatographic instrument controlled by version A.10.01 Chemstation software (Agilent Technologies; Palo Alto, CA). The HP 1100 was equipped with a low pressure mixing chamber, autosampler, block heater, quaternary pump, and variable wavelength UV detector. The dwell volume of the HP 1100 instrument, including all tubing required to connect the column, was determined to be 0.90 mL at 1 mL/min using the technique found in chapter 8 of Ref. [45]. The flush-out volume of the HP 1100 was determined to be 2.5 mL (99%) using the technique described previously [12]. The flow rate was checked using a 10 mL volumetric flask and a stopwatch, and was determined to be consistently accurate to within 1% of the set point.

Temperature was monitored at the column outlet using a thermistor (part # 44008) from Omega Engineering Inc. (Stamford, CT). The thermistor was wrapped in a piece of copper metal soldered to 5 cm of pre-cut stainless steel tubing (0.007 in.) obtained from Upchurch Scientific Inc. (Oak Harbor, WA). An OA-2 OP-AMP Designer (E & L Instruments Inc.; Derby, CT) was used to supply a 5 V input to a Wheatstone bridge circuit containing the appropriate resistances to output a 0–1 V signal around temperatures of 40 °C. The analog signal was recorded in Chemstation using an HP 35900E A/D Interface.

2.2. Reagents

All solutes were of reagent grade or better and were used as obtained from the manufacturer without further purification. Uracil, acetone, pheniramine, methapyrilene, chlorpheniramine, *N*-benzyl formamide, 3-pentanone, alprenolol, protriptyline,