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"Measure Your Gradient": A new way to measure gradients in high performance liquid chromatography by mass spectrometric or absorbance detection

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ABSTRACT

The gradient produced by an HPLC is never the same as the one it is programmed to produce, but non-idealities in the gradient can be taken into account if they are measured. Such measurements are routine, yet only one general approach has been described to make them: both HPLC solvents are replaced with water, solvent B is spiked with 0.1% acetone, and the gradient is measured by UV absorbance. Despite the widespread use of this procedure, we found a number of problems and complications with it, mostly stemming from the fact that it measures the gradient under abnormal conditions (e.g. both solvents are water). It is also generally not amenable to MS detection, leaving those with only an MS detector no way to accurately measure their gradients. We describe a new approach called "Measure Your Gradient" that potentially solves these problems. One runs a test mixture containing 20 standards on a standard stationary phase and enters their gradient retention times into open-source software available at www.measureyourgradient.org. *The software uses the retention times to back-calculate the gradient that was truly produced by the HPLC.* Here we present a preliminary investigation of the new approach. We found that gradients measured this way are comparable to those measured by a more accurate, albeit impractical, version of the conventional approach. The new procedure worked with different gradients, flow rates, column lengths, inner diameters, on two different HPLCs, and with six different batches of the standard stationary phase.

1. Introduction

Gradient elution solves some important problems while adding complications of its own [1]. One major complication is that HPLC systems, without exception, are incapable of producing the precise gradient they are programmed to produce [2–5]. For example, Fig. 1a shows a gradient produced by one of the HPLC systems in our lab that is less than five years old and in good repair. There are some major differences (non-idealities) between it and the programmed (ideal) gradient.

Gradient non-idealities are usually categorized into three types: gradient delay, gradient dispersion, and solvent misproportioning [1,2]. Gradient delay time (also called the "dwell" time) is the delay from the time the gradient is programmed to be produced to when it actually reaches the point where the sample is injected. Gradient dispersion is the rounding out of the gradient, resulting in more gradual changes in slope as if a low-pass filter were applied to the gradient profile. Any other gradient non-ideality that is not described by the former two categories, we call solvent misproportioning.

Gradient delay and gradient dispersion originate from volume in the pump, tubing, fittings, and valves, starting at the point where the solvents are proportioned to where the mixed solvent reaches the point of injection (see Supporting Information for more details). One can think of the gradient delay volume as the sum of two parts: mixing volume, V_{mix}, and non-mixing volume, $V_{non\text{-}mix}$ (Fig. 1b). Non-mixing volume can be represented by a long piece of narrow tubing; it takes a significant amount of time for solvent to travel

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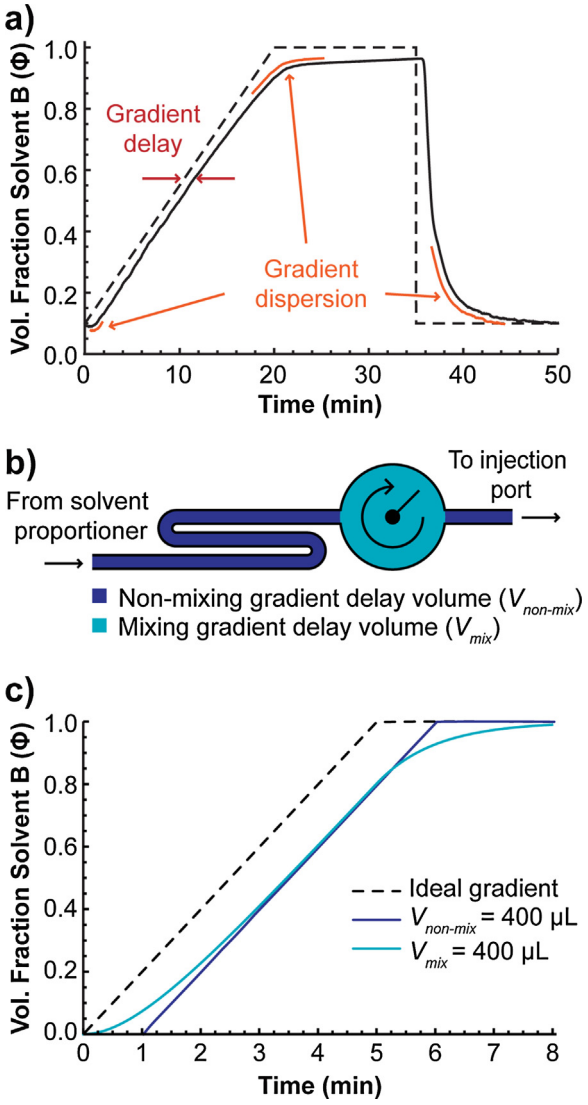

Fig. 1. (a) Comparison of the ideal (programmed) gradient and the actual gradient produced by an HPLC system as measured from the point of sample injection, (b) depiction of the sources of gradient delay volume, (c) two simulated gradients, one with 400 µL non-mixing volume and no mixing volume (the gradient is just delayed), and the other with 400 µL non-mixing volume and no non-mixing volume (the gradient is delayed and dispersed).

its length, but the solvent does not mix with the solvent on either side of it as it travels through (of course, in reality it would mix to some extent by Aris–Taylor dispersion [6]). On the other hand, mixing volume may be approximately represented by a thoroughly mixed reservoir. Newly proportioned solvent entering the reservoir is mixed with the solvent that is already there before it leaves the reservoir at the other end, slowing the rate at which the solvent composition can change. Therefore, while non-mixing volume contributes only gradient delay, mixing volume contributes both gradient delay and gradient dispersion.

If one is unaware of the gradient non-idealities produced by their instrument, they can be a major source of trouble. One important problem arises when attempting to transfer a method developed on one HPLC to a different HPLC. Differences between the gradients produced by each instrument can cause shifts in retention times and even relative retention times (i.e. the selectivity is different) [1,3,4,7–9]. Another common problem arises when running a series of consecutive gradients. If insufficient time is provided between the gradients, the solvent composition will not return all the way back to the initial composition, thereby altering the separation [1]. One may avoid this irreproducibility by simply ignoring the first gradient of each series, but it is difficult to optimize the subsequent separations in the series without understanding the behavior of the HPLC in them. All of these problems are magnified in LC–MS where gradient non-idealities are exaggerated by the relatively low flow rates typically used (100–800 µL/min).

Therefore, it is important to measure the actual gradient produced by an HPLC. There is no "rule of thumb" that can be used to avoid measuring gradient non-idealities; gradient delay volume alone can span well over an order of magnitude (e.g., the Agilent 1290 binary pump specifies a gradient delay volume of <45 µL while the Agilent 1200 quaternary pump specifies a gradient delay volume of up to 1100 µL). But by measuring gradient non-idealities, one can take them into account, optimize methods by running their instruments close to their limits, and troubleshoot instrument problems.

1.1. The conventional approach to measure HPLC gradients

Despite the importance of measuring gradients, we are aware of only one basic approach to measure them [2,3,10]:

1) Replace the column with a piece of tubing narrow and/or long enough to generate the minimum required back-pressure for the HPLC instrument.
2) Replace solvent A with water and solvent B with water containing 0.1% acetone.
3) Measure the "instrument dead time" (the time it takes for an injected solute to reach the detector with the column bypassed) by injecting a detectable compound at a relatively low flow rate of solvent A and recording its retention time.
4) Run a relatively fast gradient (e.g. 5 min) from 0% B to 100% B and record the absorbance at 265 nm as a function of time.
5) Shift the timescale of the absorbance data back by an amount equal to the instrument dead time.

Then, to measure the total gradient delay volume, one line is fit to the baseline (before the gradient) and another line is fit to the gradient. The two lines are extrapolated and the gradient delay time is determined from their intersection. To measure the mixing volume, the error in the volume fraction of solvent B, $\delta\phi$, at the gradient delay time (see Fig. S-1) is used in the following equation [2,3]:

$$V_{\text{mix}} = \frac{\delta\phi V_g}{0.37(\phi_f - \phi_i)} \quad (1)$$

where V_g is the gradient volume ($V_g = t_g F$) and ϕ_i and ϕ_f are the initial and final volume fractions of solvent B.

Despite widespread use of this methodology (a small sampling of articles that describe/use it are as follows: [1,2,8,10–17,4,18]), we are not aware of any report in which its accuracy has been validated. In fact, we find two major problems with it. First, the gradient is not measured under the same conditions as a typical HPLC run: both solvents are water and the back-pressure differs from when the column is in place. These differences can cause bias in the measured gradient. On one of our HPLC systems, the gradient measured this way was drastically different than when acetonitrile was used in solvent B (see Section 3). Of course, the most obvious way to fix the problem is to change the second solvent to acetonitrile, but as we discuss below, this causes a number of complications and the effort required to accommodate them makes the approach impractical for most users.

The second problem is that the approach requires an absorbance detector (or a conductivity detector if a salt is used instead of acetone). We could not find any reports describing a gradient successfully measured with MS detection and in our experience, the