Back to Basics

Gradient Anatomy

Protocols for LC Instruments
2020

Why is Gradient Chromatography Needed?

Analytical samples are quite frequently made of complex analytes with varying physico-chemical properties, such as polarity. The analysis of these compounds using isocratic conditions could be impractical for a few reasons:

- There could be poor separation between the compounds, or
- Some analytes could be retained significantly with unrealistic run times and broad, inefficient peaks, or
- Some analytes could lack retentivity thus elute close to the void with limited interaction with the stationary phase.

The best option for compounds with a broad range of physico-chemical properties, such as hydrophobicities, is to use gradient chromatography.
Reversed phase gradient chromatography typically alters the composition of organic modifier throughout the analysis. The organic composition starts low to sufficiently retain polar compounds, and increases through the course of the gradient to elute the more hydrophobic compounds within a reasonable time frame. An example of a typical gradient timetable and the %B versus time plot can be observed in *Figure 1.*

What Does a Gradient Profile Look Like?

The most frequently used gradient profile is the linear gradient. However, there are a variety of profiles which could be applied, including step, segmented and curved (*Figure 2*).

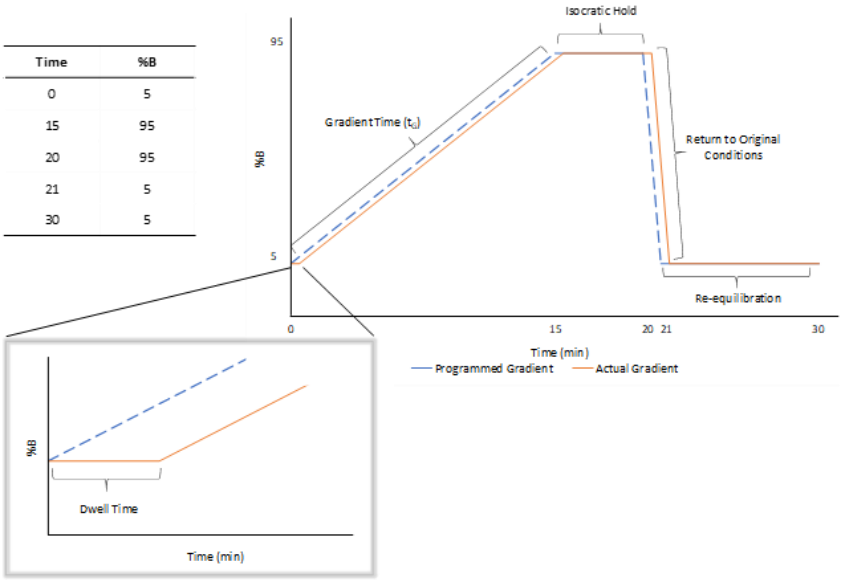

Figure 1 Schematic of the programmed gradient, with the corresponding gradient timetable and the actual gradient

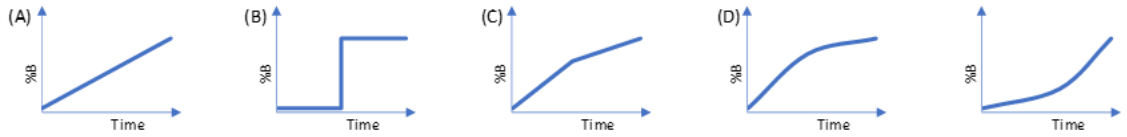

Figure 2 Schematics of different gradient profiles; (A) linear, (B) step, (C) segmented and (D) curved profiles

Gradients are controlled in the method via a gradient timetable program. It is regularly written in terms of % of the strong B solvent, as shown in *Figure 1.* The gradient begins at a lower %B composition, then increases over a period of time, before it either is held isocratically or dropped back to the original starting composition. It is then held isocratically at the original composition in order to re-equilibrate the column for the next injection.

The blue dashed profile follows the timetable described in *Figure 1*. However, in reality there is a slight deviation in the programmed gradient due to the dwell volume (orange trace). The dwell volume is the volume between the point the two solvents first mix to the head of the column. This value is different for each instrument based on the volume of tubing, mixers, injectors and valves. This dwell causes a delay in when the column experiences the change in mobile phase composition, as illustrated.

When is Gradient Chromatography Used?

The use of isocratic or gradient conditions are completely application driven, thus care should be given to select the most appropriate conditions. Some examples where gradient analysis is applied include:

- To elute a range of polar to non-polar compounds within a reasonable timeframe.
- To clean the column in preparation for the next run. This is particularly important for samples with matrices to ensure all contaminants are removed from the stationary phase.
- To find the optimum method conditions using gradient scouting runs to build a retention model. The retention model can enable the most robust and suitable resolution to be achieved for a given separation.
- For peptide, protein and oligo analysis, which are highly susceptible to small changes in organic.

Useful Things to Know and Possible Pitfalls

The column needs to be sufficiently re-equilibrated in order to obtain reproducible results. Ten column volumes are typically sufficient, however, it is application dependent and should be verified. A rough estimate of column volume can be calculated using *Eq. 2,* where *L* and d_c are the column length and internal diameter respectively in cm.

$$V_M = 0.5 \times L \times {d_c}^2 \qquad \textit{Eq. 1}$$

$$1\ Column\ Volume = V_M \times F \qquad \textit{Eq. 2}$$

Each LC has a characteristic dwell volume which needs to be experimentally measured in order to transfer methods between different instrumentation to obtain identical results. Dwell volume can impact significantly

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on selectivity and resolution, and can be a source of error if ignored. The experimental conditions for the dwell volume test are in “Back to Basics: Pump and Dwell Volume Determination”.
Similarly to isocratic analyses which requires a retention factor (k) between 2-10, gradient chromatography requires an average retention factor (k^*) between 2-10. This is calculated using *Eq. 3*, where t_G is the gradient time, F is the flow rate, $\Delta\phi$ is the difference in %B gradient range as a decimal, V_M is the column volume and S is a constant dependent on the molecular weight of the analytes (usually 5 for small molecules).

$$k^* = \frac{t_G F}{\Delta\phi V_m S} \qquad \textit{Eq. 3}$$

Within isocratic separations, the chromatographic performance can be measured using efficiency (N). However, within gradient chromatography, the performance is measured using peak capacity. Peak capacity is defined as the maximum number of peaks which can be separated with $R_s = 1$ within a given t_G.

$$PC = \frac{t_G}{W} + 1 \qquad \textit{Eq. 4}$$

The purity of the solvents can be critical, where gradient grade should be utilised if possible. This is because impurities are often retained on the column at low %B compositions, but with the increase in elution strength, impurity peaks elute off the column. These appear in the chromatogram trace and could potentially interfere with critical peaks.

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