

Impact of Solvent Mixing on Liquid Chromatographic Performance

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INTRODUCTION

Gradient elution, e.g., increasing the organic content over analysis time, is currently the most common technique in reversed-phase (RP)-HPLC. There are two different technical solutions for online (dynamic) gradient formation: high-pressure gradient proportioning (HPG) and low-pressure gradient proportioning (LPG). In both solutions, compositional fluctuation occurs during gradient formation, causing a deterioration of chromatographic performance. This can be reduced with the use of a highly efficient mixer to homogenize the mobile phase composition. Here, the experimental determination of mixer performance is described and the impact on liquid chromatographic performance is discussed for two selected applications using ion-pairing agent.

EXPERIMENTAL DETERMINATION OF MIXER PERFORMANCE

Compositional fluctuations were simulated by a sinus-like gradient oscillating between 10–70% B. The gradient was programmed using the Thermo Scientific Dionex Chromeleon® Chromatography Data System and performed using an Thermo Scientific Dionex UltiMate® 3000 Binary Rapid Separation LC (RSLC) system. Figure 1 illustrates the observed UV-detector signals with and without a mixer. The mixer performance is determined as the percentage of signal attenuation compared to the same instrument without any mixer as the reference. The frequency of the sinus-like gradient multiplied by the flow rate gives the simulated volume period. For the characterization of UHPLC mixers with a typical dwell volume of 30–150 μL , a volume period of 20 μL was chosen. For the characterization of mixers with dwell volumes larger than 400 μL (still the standard for conventional HPLC systems), the experiments were performed with a volume of 200 μL . For an objective and precise comparison of mixer performances, the mixer dwell volume was experimentally determined according to Dolan and Snyder.¹

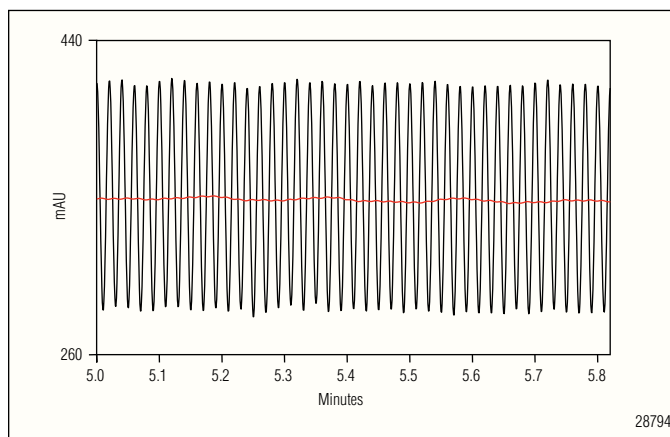


Figure 1. UV-detector absorbance signal of the sinus-like gradient with (red) and without (black) a mixer. The signal attenuation quantifies the mixer performance.

The plot of the mixer dwell volume against the remaining baseline ripple matches an exponential decay curve quite well, e.g., the one found in discharge of a capacitor. Figure 2 illustrates the decay curve for the two volume periods of 20 μL and 200 μL . The volume period for this set of experiments shows the typical behavior of a decay constant, λ .

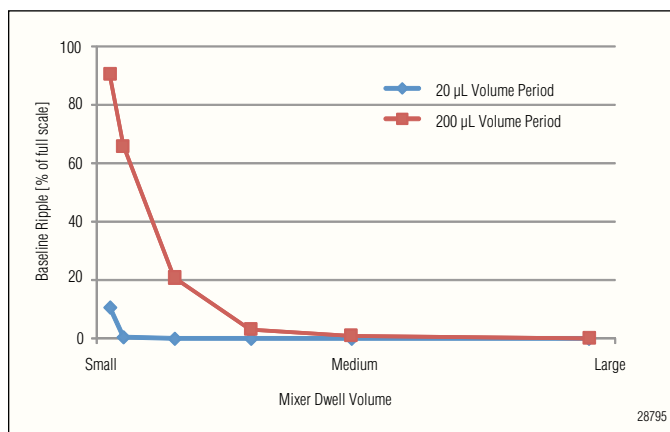


Figure 2. Exponential decay-curve behavior of remaining baseline ripples as a function of the mixer volume.

ON-COLUMN AMPLIFICATION OF MIXING RIPPLE

Ion-pairing agents are widely used for RP-HPLC to manipulate the pH and the interaction of the analytes with the stationary phase in order to enhance separation. Trifluoroacetic acid (TFA) is the most common ion-pairing agent used for peptide and protein separations. Unfortunately, TFA also causes some undesirable effects. The absorbance of TFA below 250 nm dramatically changes depending on the water/acetonitrile ratio. This causes a strong baseline shift during gradient elution. A compensation of this effect is possible if the TFA concentration in acetonitrile is approximately 15% lower than in the aqueous solvent.

The other side effect results from the retentive interaction of TFA with RP columns. Therefore, the TFA concentration of the mobile phase stream behind the column fluctuates with varying organic solvent concentration. In case of incompletely mixed or fluctuating mobile phase content, the dynamic TFA equilibrium on the column is disturbed. This causes a strong amplification of mixing ripples by the column. Since TFA absorbs UV light considerably stronger than water or acetonitrile, significant baseline ripples are observed.² This results in a decreasing signal-to-noise ratio, which significantly reduces the limit of detection (LOD) and limit of quantification (LOQ).

To quantify the amplification effect of the column on the baseline ripple, two sets of experiments were performed. In the first set, the baseline ripples were determined without a column. A 50 μm i.d. fused-silica capillary was used as a restrictor to generate a back-pressure of 40 MPa. In the second set of experiments, the baseline ripples were determined using a column (Thermo Scientific Dionex Acclaim[®] 120 C18, 3 μm , 120 \AA , 250 \times 3.0 mm). For a reproducible and evaluable determination of baseline ripples, each run started with an isocratic elution of 5% B (dial-a-mix) for five minutes. The total absorption difference between 100% A and 100% B was 160 mAU. Figure 3 depicts the baseline with and without column.

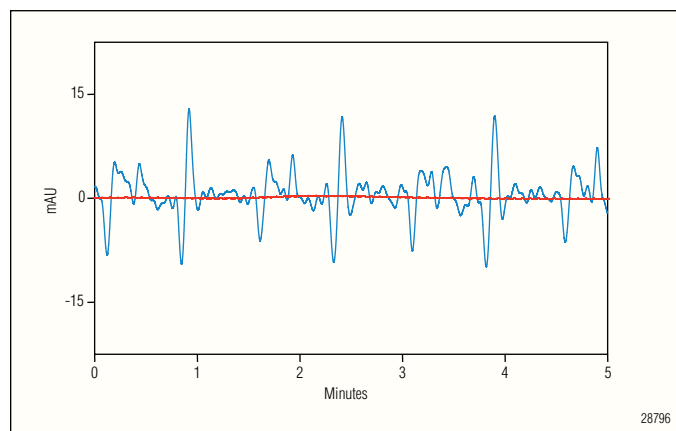


Figure 3. Observed baseline with (blue) and without (red) column.

Plotting the mixer performance against the remaining baseline ripple roughly shows an exponential decay curve as already found in the previous experiments. It also clearly illustrates the strong amplifying behavior of the column. Figure 4 shows the comparison between the baseline ripple with column and the baseline ripple with a restrictor capillary. The amplification of the baseline ripple varies between 37 and 58 depending on the mixer performance, with an average of 44, as shown in Figure 5.

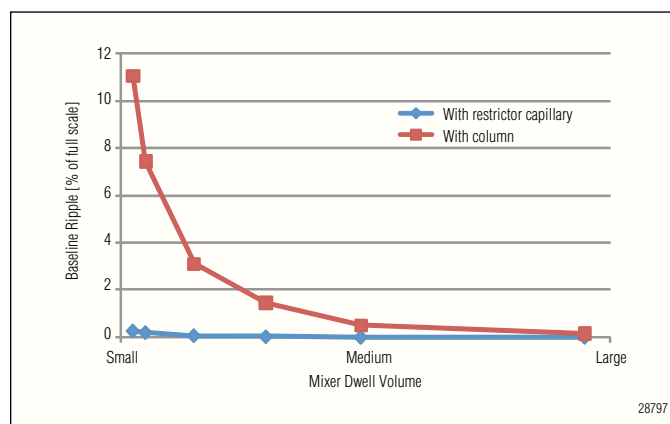


Figure 4. Influence of the mixer volume on the remaining baseline ripple with (red) and without (blue) a column.

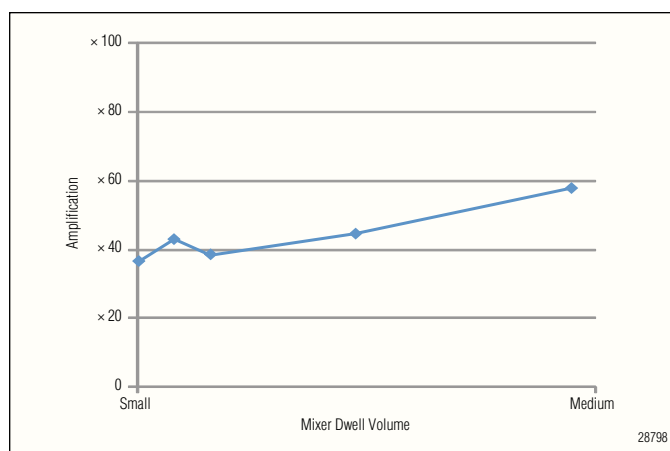


Figure 5. Amplification in baseline ripple by the column as a function of the mixer volume.

MIXING RIPPLE INDUCED PEAK SHAPE DISTORTION

The retention mechanism of RP-HPLC differs between small and large molecules. Because of the higher adsorption enthalpy, proteins are adsorbed onto the hydrophobic surface of the column and remain there until the concentration of the organic modifier is high enough to elute the molecules from the hydrophobic surface. The elution order is related to the hydrophobic nature; the more hydrophilic the proteins, the earlier it will be eluted from the hydrophobic surface under gradient conditions. This concentration-dependant adsorption/desorption process makes the application sensitive to slight deviations in the solvent composition. On the critical concentration range of the organic modifier, fluctuation can cause partial elution of proteins followed by re-adsorption on a later point in the column, thus resulting in distorted peak shapes.³ With shallow gradients and short columns, this effect is more pronounced.

Figure 6 shows the expected peak distortion for lysozyme if a small mixing volume is applied, although no baseline ripple is visible. Regular peak shapes are observed after changing to a larger mixer. Furthermore, the distorted peak shape has a negative impact on the repeatability. Figure 7 shows the influence of the mixer volume on peak height precision and peak width (50%) precision.

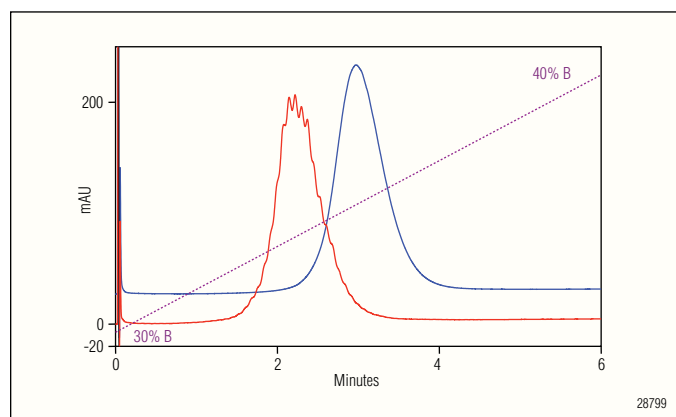


Figure 6. Observed peak shapes of lysozyme with small (red) and large (blue) mixer.

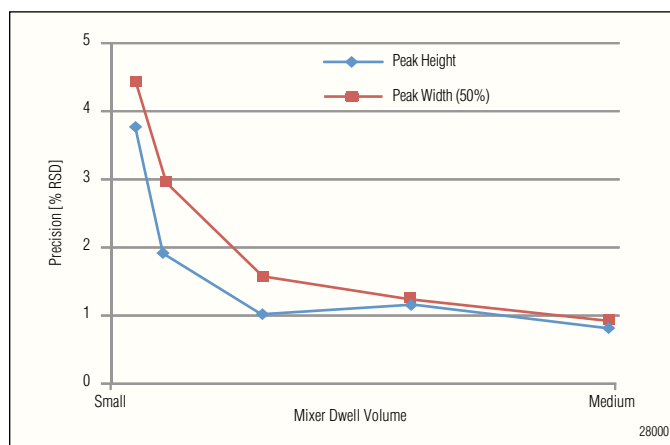


Figure 7. Influence of the mixer volume on peak height (blue) and peak width (50%, red) precision for six replicates.

CHROMATOGRAPHIC CONDITIONS AND EQUIPMENT

	Experimental Determination of Mixer Performance	On-Column Amplification of Mixing Ripple	Mixing Ripple-Induced Peak Shape Distortion
System	UltiMate 3000 Binary RSLC	UltiMate 3000 Binary RSLC	UltiMate 3000 Quaternary RSLC
Solvent A	Water	Water/Acetonitrile 99:1 + 0.1 % TFA	Water + 0.12%TFA
Solvent B	Water + 0.07% Acetone	Acetonitrile 100% + 0.1% TFA	ACN + 0.12% TFA
Gradient	Sinus-like gradient between 10 –70% B	5% B (dial-a-mix)	30–40% for 6 min, 100% for 1 min
Column		Acclaim 120 C18, 3 μm, 120 Å, 250 × 3.0 mm	Phenomenex SecurityGuard™, C18, 4 × 3.0 mm
Temperature		35 °C	30 °C
Flow Rate	1.00 mL/min	1.00 mL/min	1.00 mL/min

The UltiMate 3000 Binary RSLC System consisted of the following modules:

- SRD-3400 Integrated Solvent and Degasser Rack, Four Channels
- HPG-3200RS Binary Rapid Separation Pump
- WPS-3000TRS Rapid Separation Well Plate Sampler, Thermostatted
- TCC-3000RS Rapid Separation Thermostatted Column Compartment
- VWD-3400RS Rapid Separation Four-Channel Variable Wavelength Detector
- Semi-Micro Flow Cell, 2.5 μ L, Stainless Steel
- Thermo Scientific Dionex Viper™ System Capillaries

The UltiMate 3000 Quaternary RSLC System consisting of the following modules:

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- WPS-3000TRS Rapid Separation Well Plate Sampler, Thermostatted
- TCC-3000RS Rapid Separation Thermostatted Column Compartment
- VWD-3400RS Rapid Separation Four-Channel Variable Wavelength Detector
- Semi-Micro Flow Cell, 2.5 μ L, Stainless Steel
- Viper System Capillaries

SUMMARY

An accurate way to experimentally determine the mixing performance using a sinus-like gradient was described. The correlation between signal attenuation and mixer dwell volume follows an exponential decay curve.

It was shown that the amplifying effect of the column on mixing ripples in case of TFA as an ion-pairing agent is significant. With an experimentally-determined amplification factor of up to 58, special attention to the mixer is needed for best possible LOD.

Mixing ripples can have diverse impacts on the liquid chromatographic performance, which does not necessarily provide conclusions based on its origin. It was shown that mixing ripples can significantly influence the peak shape, although they aren't seen in the baseline. The partial elution of lysozyme can be seen as additional ripples on top of the peak.

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2. Winkler, G. Increasing the Sensitivity of UV Detection in Protein and Peptide Separations When Using TFA [trifluoroacetic acid]-Acetonitrile Gradients. *LC-GC* **1987** *5* (12) 1044–1045.
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