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Reliable Solvent Mixing in UHPLC

INTRODUCTION

Solvent mixing and gradient delay volume (GDV) were rarely discussed in the first decades of high-performance liquid chromatography (HPLC). With packing materials of 5 µm to 10 µm and run times of >30 min, the GDV was of minor importance and typically not mentioned in the data sheets for HPLC instruments. This started to change with the introduction of columns using sub-3 µm packing material. The smaller particle size provided higher efficiency; hence it was possible to use shorter columns and significantly reduce run times by a factor of 5 or more. Also, smaller column i.d.s of 3.0 mm and 2.1 mm became standard, resulting in up to 5× smaller flow rates at equal linear velocity. This provided the advantage of lower solvent consumption and higher mass sensitivity. This innovation arrived when HPLC analysts needed higher sample throughput and lower costs per sample to increase productivity in a competitive marketplace, and to cope with the growing need for testing due to steadily increasing regulations (e.g., in drug development).

However, with typical pump dwell volumes of 2 mL to 6 mL,[1] and the increased system backpressure required for running small particle size columns, a standard HPLC system is not well suited to run fast LC methods. For example, in gradient elution at a flow rate of 1 mL/min, the gradient will need several minutes after the injection to arrive on the column. With run times of <10 min, this is far too slow for fast LC applications. The same is also true for peptide mapping, as there is a strong interest in accelerating existing methods with common run times of <40 min. However, with the commonly used UV-active ion-pairing agent trifluoroacetic acid (TFA), a considerable amplification of baseline ripples is observed. These ripples are caused by slight fluctuations in organic solvent concentration. For this kind of application, highly efficient solvent mixing is essential to obtain a smooth baseline even at small GDVs.

To deal with these challenges, the main focus of attention is on reduction of the mixer dwell volume while at the same time maintaining (or even improving) the mixing performance. In addition, the flow paths of recent ultra HPLC (UHPLC) instruments are highly optimized using shorter capillaries with smaller internal diameters.

In this study, the performance of the unique Thermo Scientific Dionex UltiMate® 3000 SpinFlow™ mixing technology and the chromatographic impact of insufficient mixing on a TFA application example is discussed.

Equipment

Dionex UltiMate 3000 Binary Rapid Separation LC (RSLC) System including:

- SRD-3400 – Dionex UltiMate 3000 Integrated Solvent and Degasser Rack, 4 Channels
- HPG-3200RS – Dionex UltiMate 3000 Binary RSLC Pump
- VWD-3400RS – Dionex UltiMate 3000 RSLC Four Channel Variable Wavelength Detector
- Semi-Micro Flow Cell
- Thermo Scientific Dionex Viper™ Capillaries and Dionex Viper Unions

Chromatographic Conditions

Solvent A: Water

Solvent B: Water + 0.07% acetone

Flow Rate: 1 mL/min

Gradient: Sinus-like gradient (as shown in Figure 6) between 10% B and 70% B

WHY IS SOLVENT MIXING NEEDED?

Gradient elution—increasing the organic content over analysis time—is now the most common technique in reversed-phase (RP) HPLC. There are two different technical solutions for on-line (dynamic) gradient formation: high-pressure gradient (HPG) proportioning and low-pressure gradient (LPG) proportioning.

In the HPG configuration, two independent pumps deliver the two solvents and combine them using a tee-manifold immediately after the pump head outlets (at high pressure). The distinctive flow rates of both pumps result in a well-defined solvent composition. Once the streams are combined, they move directly into the mixer. Figure 1 illustrates the typical flow path for HPG pumps.

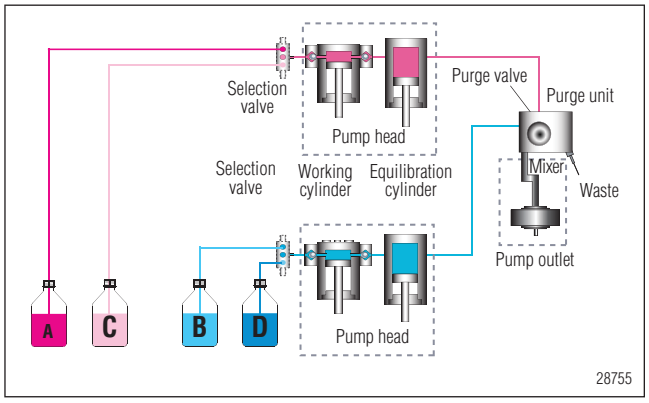

Figure 1. Typical flow path for an HPG pump.

In the LPG configuration, the proportioning of typically up to four solvents is performed using solenoid valves ahead of the pump head inlet. The percentage of each solvent is selected using the timed opening and closing of the valves for the individual solvent channels to achieve the correct mobile phase composition. The mobile phase passes through the pump head before arriving at the mixer. The volume of the pump head therefore adds to the GDV of the pump. Figure 2 illustrates the typical flow path for LPG pumps.

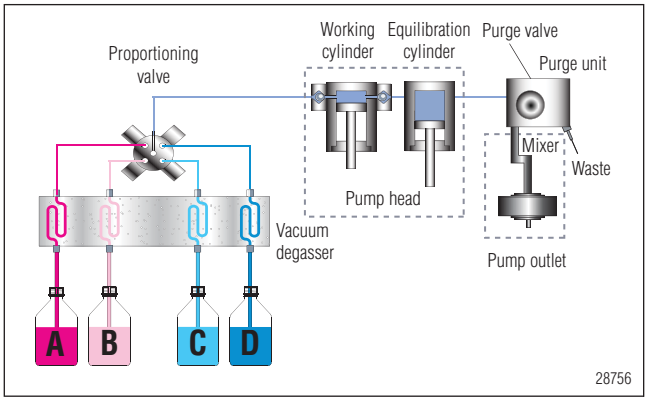

Figure 2. Typical flow path for an LPG pump.

In both pump variants, mixing ripples occur—although in different ways—during gradient formation. In the LPG configuration, the proportioning is performed by opening and closing of valves for each solvent during the aspiration phase of the pump. This forms discrete solvent plugs as illustrated in Figure 3A. For a perfect homogenization of the resulting solvent mixture, highly efficient longitudinal mixing is essential. In the HPG configuration, the composition is formed by two solvent streams. In this case, the solvent composition can fluctuate due to pressure pulsation in the two solvent delivery lines, as illustrated in Figure 3B. The lower the pressure pulsation—which occurs with all piston pumps—the more constant the solvent composition. For a perfect homogenization of the solvent mixture, radial and longitudinal mixing is needed.

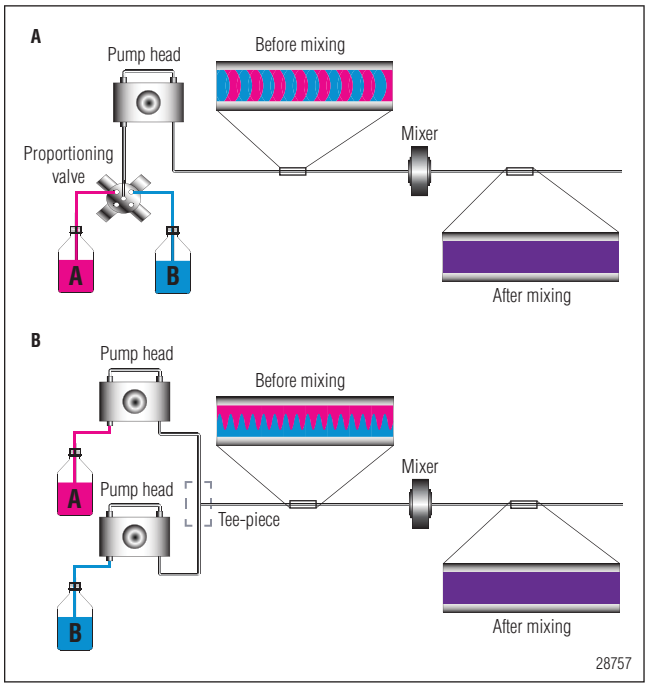

Figure 3. Schematic illustrations of the compositional oscillations in low-pressure mixing systems A) and high-pressure mixing systems B).

In cases in which the solvents mixed for the gradient elution generate different detector response (e.g., different absorption in case of UV detection), incomplete mixing is observed as baseline ripples in chromatograms as shown in Figure 4. This ripple can significantly reduce the limit of detection (LOD) for analytes. The LOD is defined as the lowest analyte concentration that can be detected over baseline noise and is usually expressed as the concentration at a signal-to-noise ratio of at least 3:1.

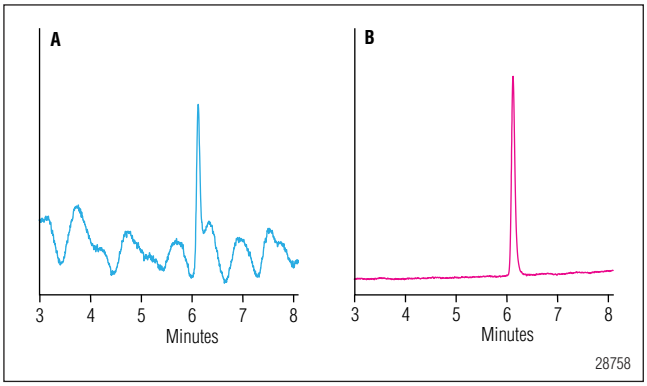

Figure 4. Sections of two chromatograms with baseline ripples A) and without baseline ripples B).

Besides a reduced LOD, incomplete solvent mixing can have also a negative impact on chromatographic performance, such as poor retention time or peak area precision. In protein separations on short columns under RP conditions, insufficiently mixed solvents can even result in chopped peaks, as observed, for example, for the analysis of lysozyme.[2]

THE FUNCTIONAL PRINCIPLE OF THE DIONEX ULTIMATE 3000 SPINFLOW MIXERS

The HPLC mixer requirements are challenging because the mixer must reliably provide the highest possible mixing performance over a wide application range with varying solvent ratios, solvent miscibility, and flow rates. Furthermore, the mixer should be able to homogenize solvent mixtures having both radial and longitudinal fluctuations in varying magnitude and frequency, as illustrated in Figure 3.

To meet these requirements, it is essential that the first step solvent composition is fully homogeneous in the radial direction; otherwise, longitudinal mixing cannot be achieved with the best possible efficiency. With the Dionex SpinFlow mixing design, a patented capillary mixer[3] with microfabricated helical structures ensures high-performance continuous radial mixing. In the second step, the solvent enters the frit-based longitudinal mixing device to homogenize the remaining compositional fluctuation, as illustrated in Figure 5.

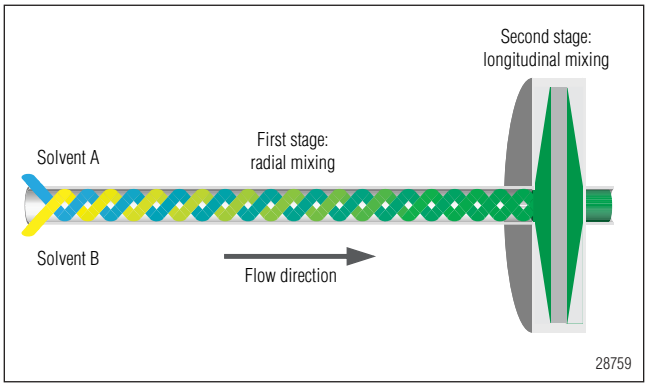

Figure 5. Dionex SpinFlow mixing design with two-step mixing principle for reliable mixing performance.

The remarkably high number of flow paths provided by a frit reliably ensures the highest possible mixing performance under virtually any working conditions. The complete Dionex SpinFlow mixer portfolio is compatible with pressures up to 103 MPa and is therefore ideal for UHPLC.

COMPARISON OF MIXER PERFORMANCE

To demonstrate the advantage of the Dionex SpinFlow mixer design and to determine its mixing efficiency, a series of experiments was performed. Compositional fluctuations were simulated by a gradient oscillating between 10% B and 70% B. The gradient was programmed using Thermo Scientific Dionex Chromeleon® Chromatography Data System software and performed using a Dionex UltiMate 3000 Binary RSLC system. Figure 6 illustrates the observed UV detector signals without a column for a 100 µL Dionex SpinFlow mixer and in the absence of 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 µL to 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 common HPLC systems—the experiments were performed with a volume period of 200 µL.

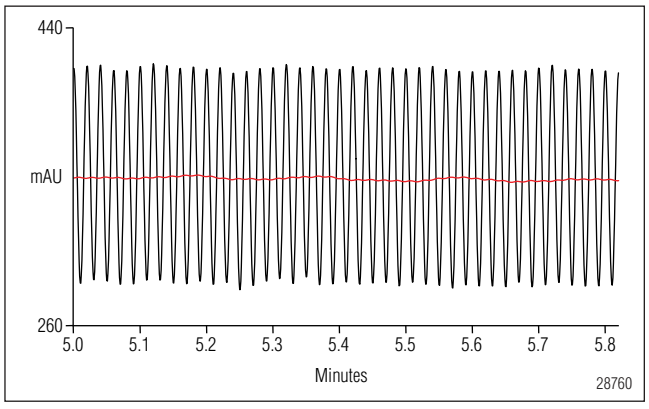

Figure 6. UV detector absorbance signal of the sinus-like gradient without (black) and with 100 µL Dionex SpinFlow mixer (red). The signal attenuation quantifies the mixer performance.

For an objective and precise comparison of mixer performances, the mixer dwell volume was experimentally determined according to J. W. Dolan and L. R. Snyder.[4] A linear gradient from 0% to 100% B over 10 min was run and the time where 50% of the maximum absorbance occurred was determined as illustrated in Figure 7. This time minus half the gradient time equals the dwell time. The system dwell volume is the dwell time multiplied by the flow rate (1 mL/min).

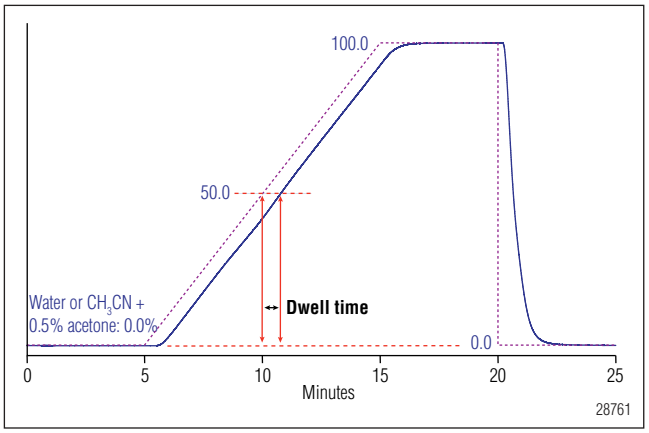

Figure 7. Experimental determination of the system dwell volume.

The mixer dwell volume is the measured system dwell volume with the installed mixer minus the measured system dwell volume when the respective mixer was replaced by a dead-volume-free Dionex Viper union.

RESULTS

The first set of experiments characterized the mixing performance of the Dionex SpinFlow mixers. The plot of the determined mixer dwell volume against the remaining baseline ripple closely matched an exponential decay curve like the one found in the discharge of a capacitor. Figure 8 illustrates the decay curve for the two volume periods, 20 µL and 200 µL. The volume periods for this set of experiments showed the typical behavior of a decay constant, λ.

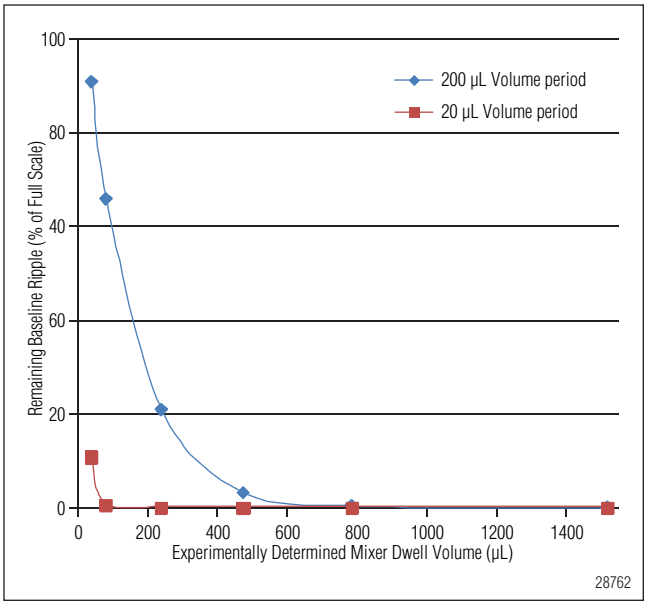

Figure 8. Exponential decay curve behavior of remaining baseline ripples as a function of the mixing performance shown for the Dionex SpinFlow mixers for two different volume periods (20 µL and 200 µL).

The result indicates that the Dionex SpinFlow mixers provide uniform mixing performance per mixing volume for the entire Dionex SpinFlow mixer portfolio.

Figure 9 shows a comparison of mixer performance between the 35 µL and 100 µL Dionex SpinFlow mixers and five different UHPLC mixers from three different vendors.

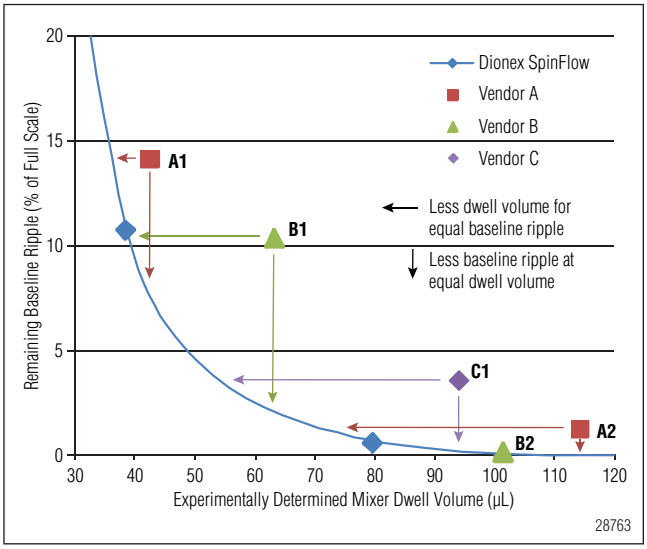

Figure 9. Comparison of the mixing performance for the 35 µL and 100 µL Dionex SpinFLow mixers and UHPLC mixers of other vendors achieved for the 20 µL volume period.

The distinctive number of flow paths that vendor A uses in its UHPLC mixers is not favorable. The comparison shows that the 35 µL Dionex SpinFlow mixer outperforms mixer A1 with 10% less dwell volume for 25% less remaining baseline ripples. The 100 µL Dionex SpinFlow mixer provides better results, with 30% less dwell volume for 55% less remaining baseline ripples compared to mixer A2. The comparison with the frit-based UHPLC mixer B1 shows that the 35 µL Dionex SpinFlow mixer requires 40% less mixer dwell volume to achieve equal mixing performance. The mixer C1 which uses cross-flow shearing achieves a significant lower mixing performance compared to the 100 µL Dionex SpinFlow mixer (85% higher baseline ripples and 15% more dwell volume). Only the mixer B2 of vendor B shows comparable mixing performance to the Dionex SpinFlow mixer.

A similar result is shown for the comparison of the 400 µL Dionex SpinFlow mixer with three HPLC mixers from other vendors, as illustrated in Figure 10.

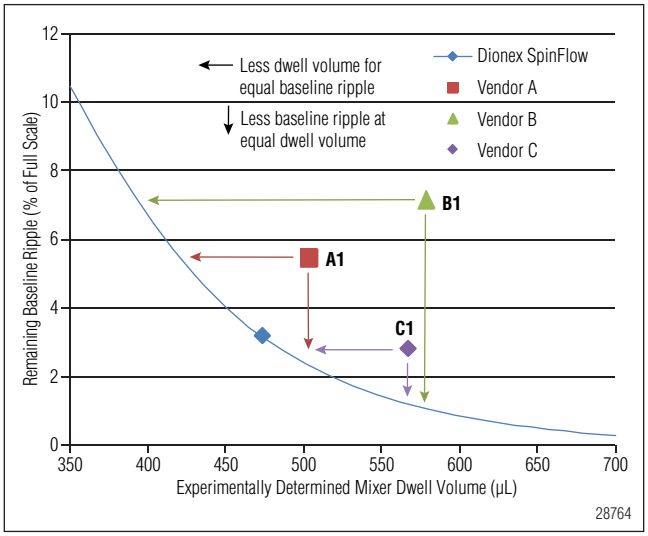

Figure 10. Comparison of the mixing performance for the 400 µL Dionex SpinFlow mixer and HPLC mixers from other vendors for the 200 µL volume period.

Bead-filled columns, such as those from vendors A and B, no longer represent state-of-the-art mixing technology and are easily outperformed by the Dionex SpinFlow mixing design. The Dionex SpinFlow mixer demonstrates 40% less remaining baseline ripples compared to mixer A1 and 55% less remaining baseline ripples compared to mixer B1 with significantly less mixer dwell volume. Also, the distinctive number of flow divider paths that vendor C uses does not provide any advantage. The comparison shows that mixer C1 requires ~20% more dwell volume to achieve mixing performance comparable to the Dionex SpinFlow mixer.

TFA APPLICATIONS: THE CHALLENGE REGARDING BASELINE RIPPLES

Equipment

Dionex UltiMate 3000 Binary Rapid Separation System including:

- SRD-3400 – Dionex UltiMate 3000 Integrated Solvent and Degasser Rack, 4 Channels
- HPG-3200RS – Dionex UltiMate 3000 Binary Rapid Separation Pump
- WPS-3000TRS – Dionex UltiMate 3000 Rapid Separation Wellplate Sampler, Thermostatted
- TCC-3000RS – Dionex UltiMate 3000 Rapid Separation Thermostatted Column Compartment
- VWD-3400RS – Dionex UltiMate 3000 Rapid Separation Four Channel Variable Wavelength Detector
- Semi-Micro Flow Cell
- Dionex Viper System Capillaries

Chromatographic Conditions

Solvent A:	Water/acetonitrile 99:1 + 0.1 % TFA
Solvent B:	Acetonitrile 100 % + 0.1 % TFA
Column:	Dionex Acclaim RP C18, 3 µm, 120 Å, 250 × 3.0 mm
Flow Rate:	1.00 mL/min
Temp.:	35 °C

Ion-pairing agents are widely used for RP HPLC to manipulate the pH and 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 changes dramatically, depending on the water/acetonitrile ratio. For example, at the common detection wavelength of 220 nm, the absorption difference between water/acetonitrile 99:1 + 0.1% TFA and acetonitrile + 0.1% TFA is approximately 160 mAU. This causes a strong shift in baseline during gradient elution. It is possible to compensate for this effect if the TFA concentration in acetonitrile is approximately 15% lower than in the aqueous solvent.

Another negative side effect of using TFA is that it is retained on RP columns. Therefore, the TFA concentration of the mobile phase in the column fluctuates with varying organic solvent concentration. In the 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 noise by the column. Because TFA absorbs 50–100 times stronger than water or acetonitrile in the UV range, significant baseline ripples are observed.[5] Therefore, the requirements on the mixing performance are exceptionally high. However, as of yet, no experimental data is available to quantify the amplification effect of the column on the baseline ripple.

In the first set of experiments, the baseline ripples for the complete Dionex SpinFlow mixer portfolio were determined without column. A 50 µm i.d. fused silica capillary was used as restrictor to generate backpressure of 40 MPa. For a reproducible and evaluable determination of baseline ripples, in each experiment isocratic elution with 5% B (dial-a-mix) was run for 5 min. The full-scale absorption difference between 100% A and 100% B was 160 mAU.

The 35 µL Dionex SpinFlow mixer demonstrated an excellent low residual baseline ripple of 0.25% of full scale. With the 400 µL Dionex SpinFlow mixer, almost no baseline ripple was observed. The same set of experiments was then performed with a column installed in the system, resulting in drastically increased baseline ripples as illustrated in Figure 11.

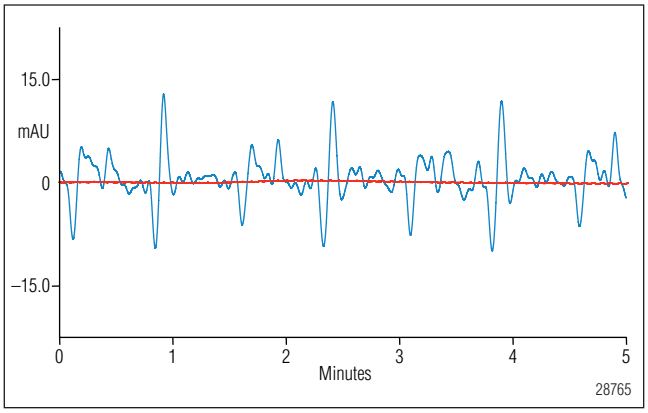

Figure 11. Comparison of the baseline ripples at the 5% B step with column (blue) and without column (red), 35 µL mixer installed.

The baseline ripple for the system configuration with the 35 µL Dionex SpinFlow mixer was calculated as 16.88% of full scale, which represents a 43-fold amplification by the column. Also, with the 400 µL Dionex SpinFlow mixer, a significant increase in baseline ripples of 1.45% of full scale was observed. In that case, the column amplified the baseline ripple by a factor as high as 58.

Plotting the mixer volume—which is proportional to the mixing performance—against the remaining baseline ripple showed a roughly exponential decay curve as shown in the previous experiments. It also clearly showed the strong amplifying behavior of the column. Figure 12 shows the comparison between baseline ripple with column compared to the baseline ripple with a restrictor capillary.

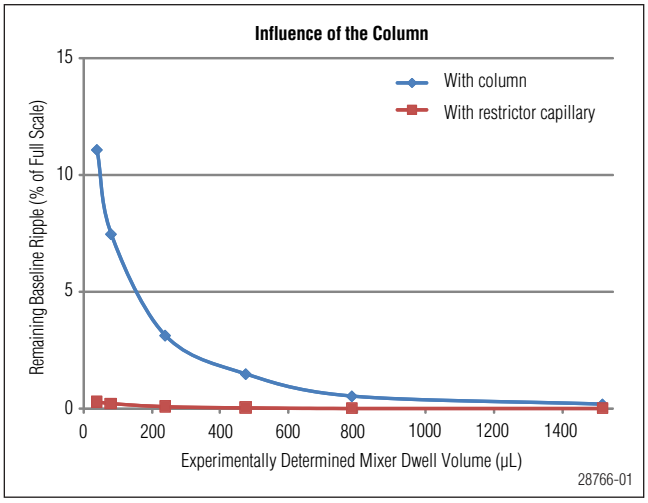

Figure 12. Influence of the column on the remaining baseline ripple.

The amplification of the baseline ripple varies between 37 and 58, depending on the Dionex SpinFlow mixer, with an average of 44, as shown in Table 1.

Table 1. Amplification in Baseline Ripple by the Column

Mixer Volume (µL)	With Restrictor Capillary Base-Line Ripple (% Full Scale)	With Column Baseline Ripple (% Full Scale)	Amplification in Baseline Ripple by the Column
2 (in-line filter)	0.37	13.37	36.6
35	0.26	11.05	43.0
100	0.19	7.44	38.6
200	0.07	3.10	44.6
400	0.03	1.45	57.8

CONCLUSION

The Dionex SpinFlow mixing design—with its unique two-step mixing process of radial mixing followed by longitudinal mixing—provides the best results in attenuating baseline ripples. The exceptionally high mixing performance of the Dionex SpinFlow mixers outperforms mixers available from other vendors. This performance ensures optimum chromatographic resolution at maximum detection sensitivity without pump-related baseline noise. Furthermore, the outstanding flexibility of reliable solvent mixing under virtually any working conditions—from UHPLC (at up to 103 MPa) to conventional HPLC—is proven. The comprehensive Dionex SpinFlow mixer portfolio (Figure 13) enables analysts to perfectly balance the gradient delay volume against mobile phase mixing efficiency within seconds for the widest possible application ranges.

Figure 13. The range of static mixers within the Dionex UltiMate 3000 SpinFlow product line.

General guidelines for selecting the right mixer for an application:

- For fast separations where the mixing ripple does not interfere with the detection (e.g., Thermo Scientific ESA Corona® CAD® Charged Aerosol Detectors or MS detectors), use low mixer volumes (35 µL, P/N 6040.5000, and 100 µL, P/N 6040.5100).
- Use the medium sized mixers (200 µL, P/N 6040.5110, and 400 µL, P/N 6040.5310) for the best balance between fast separation and low mixing ripple in UV detection.
- For highest sensitivity and when mixing ripples interfere with the detection (e.g., due to use of UV-absorbing solvents), use a larger mixer volume (400 µL, P/N 6040.5310, and 800 µL, P/N 6040.5750).
- For UV-absorbing solvent additives that amplify the mixing ripples by interaction with the stationary phase (e.g., TFA applications), use the largest mixer volumes (800 µL, P/N 6040.5750, and 1550 µL, P/N 6040.5450) in order to achieve the highest sensitivity.

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