TECHNICAL NOTE 73582

Intelligent UHPLC pump stroke control

Ensures sensitive peptide analysis by ripple-free baseline in TFA applications

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Goal

Increase detection sensitivity of UV-based detection when using TFA as eluent additive without increasing gradient delay volume

Introduction

The separation of peptides and proteins with reversed-phase high performance liquid chromatography (HPLC) is commonly performed with trifluoroacetic acid (TFA) as additive to water and acetonitrile (ACN) based mobile phases. TFA offers several benefits as modifier. It is an ion-pairing agent, and as such, it reduces secondary interactions of the molecules with the stationary phase, which are the main causes of peak tailing. At the same time, it modulates the selectivity of charged molecules, which otherwise may not be properly retained. These characteristics make TFA a modifier that is hard to replace. All this comes at a price and chromatographers have learned to recognize the two main side effects of using TFA, namely the UV baseline drift and baseline ripples.

TFA presents a strong UV absorption below 250 nm. This is in the same UV range used for the detection of peptides and proteins. These molecules are generally analyzed via a shallow water/acetonitrile gradient and detected at 210–220 nm. However, the TFA absorbance is dependent on the water/acetonitrile ratio. Therefore, during gradient elution, a strong UV baseline drift is observed.[1,2] As rule of thumb, a slightly lower concentration of TFA used in solvent B of the mobile phase helps to level out the baseline shift without compromising retention behavior too much. Generally, for 0.10% (v/v) of TFA in the aqueous solvent A, 0.085% (v/v) of TFA is used in the organic solvent B.

TFA, as ion-pair reagent, is adsorbed on the stationary phase. The mechanism of TFA adsorption is therefore also responsible for the unusually high amplitude of TFA baseline ripples: what the UV baseline reflects is not merely caused by variations in the refractive index of the eluent components, but the retaining stationary RP phase acts as an amplifier due to the TFA enrichment. Consequently, the baseline ripples caused by TFA fluctuations increase the baseline noise. This impacts the detection and quantitation of low abundant molecules which can be indistinguishable from this "noise".

This technical note will present the performance that can be achieved with Thermo Scientific™ Vanquish™ Horizon UHPLC system. Here, we introduce SmartStroke™: a new special stroke behavior to virtually eliminate the TFA baseline ripples even with small mixer volumes to guarantee lower gradient delay volume (GDV) and higher sensitivity.

Experimental

Chemicals	Part number
Deionized water, 18.2 MΩ·cm resistivity or higher	N/A
Fisher Scientific™ Acetonitrile Optima™ LC/MS grade	A955-212
Thermo Scientific™ Pierce™ Trifluoroacetic acid LC/MS grade	85183
Thermo Scientific™ Dionex™ Cytochrome C digest (lyophilized)	161089
Sample handling	**Part number**
Thermo Scientific™ SUN-SRi™ 12 × 32 mm Standard Opening Crimp Vials (amber, 2.0 mL)	22-313375
Thermo Scientific™ SUN-SRi 11 mm Orange Snap Caps	14-823-381

Instrumentation	Part number
Thermo Scientific Vanquish Horizon UHPLC system consisting of:	
System Base Vanquish Horizon/Flex	VF-S01-A-02
Binary Pump H	VH-P10-A-02
Split Sampler HT	VH-A10-A-02
Column Compartment H	VH-C10-A-02
Diode Array Detector HL	VH-D10-A
Lightpipe™ Standard flow cell, 10 mm	6083.0100B
Mixer kit, 200 µL, 150 MPa, VH-P1	6268.5120
Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:	
System Base Vanquish Horizon/Flex	VF-S01-A-02
Binary Pump F	VF-P10-A-01
Static mixer, volume: 350 µL (for total volume of mixing system: 400 µL)	6044.5310
Split Sampler FT	VF-A10-A-02
Column Compartment H	VH-C10-A-02
Diode Array Detector HL	VH-D10-A
Lightpipe Standard flow cell, 10 mm	6083.0100B

Sample preparation	Part number
Water containing 0.1% TFA (v/v) (solvent A)	
Fisher Scientific™ Fisherbrand™ Mini Centrifuge	P/N 10243043
Fisher Scientific™ Fisherbrand™ S-Series Heated Ultrasonic Cleaning Bath	P/N 10551783
Add 1.0 mL solvent A (water containing 0.1% TFA) to the vial containing the cytochrome C digest (lyophilized). Place in an ultrasonication bath for 10 min. Centrifuge for 10 min. Carefully transfer the supernatant to the 1.5 mL amber vial via pipette.	

LC conditions	
Column	Thermo Scientific™ Accucore™ C18 2.6 µm, 2.1 × 100 mm (P/N 17326-102130)
Mobile phase	A: Water containing 0.1% TFA (v/v) B: Acetonitrile containing 0.085% TFA (v/v)
Gradient	Time (min) / A / B 0 / 95 / 5 0.1 / 95 / 5 30.0 / 45 / 55 30.02 / 0 / 100 32.0 / 0 / 100 32.02 / 95 / 5 37.0 / 95 / 5
Flow rate	0.6 mL/min
Pressure	Up to 450 bar
Column temperature	25 °C
Autosampler temperature	5 °C
Detector settings	214 nm; 10 Hz 0.5 s response time

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

TFA baseline ripples – how are they generated?

TFA is adsorbed on the reversed-phase stationary phase and is in equilibrium with the TFA content of the mobile phase. Like the analytes, an increase of ACN in the mobile phase causes TFA to desorb from the stationary phase into the mobile phase. The TFA "elution" can also be triggered by small and local fluctuations of the mobile phase. As reported,[3] a local increase of ACN in the eluent initially causes a desorption of TFA into the mobile phase, resulting in a positive peak. After this ACN fluctuation has passed and the concentration of ACN returns to the original amount, TFA is extracted from the mobile phase and adsorbs again onto the stationary phase. This results in a negative peak (Figure 1a). The opposite effect is observed for mobile phase fluctuations with a lesser amount of ACN causing more TFA to be adsorbed onto the stationary phase, therefore, generating a negative peak. When the ACN content increases again, it extracts TFA from the stationary phase into the mobile phase, producing a positive peak (Figure 1b).

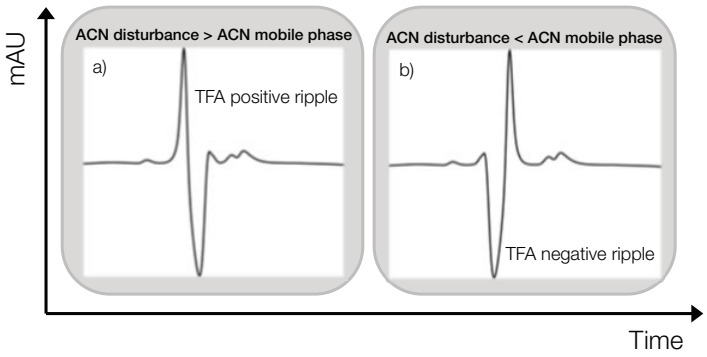

Figure 1. Simulation of a TFA baseline ripple caused by local fluctuations of ACN content in the mobile phase: a) the local variation of ACN content is more than the ACN content in the mobile phase, generating a positive TFA ripple; b) the local variation of ACN content is less than the ACN content in the mobile phase, generating a negative TFA ripple.

High performing binary pumps are indispensable for TFA applications

Local fluctuations of the mobile phase composition and the subsequent baseline ripples depend on the performance and the settings of the (U)HPLC pump unit. In the following discussion, we will focus on binary pumps (high pressure mixing system), since local fluctuation of the mobile phase are more of a challenge for this type of pumps.

Binary pumps consist of two independent pump blocks generally called A and B. Each provide flow for a specific solvent. A and B solvents are then combined in a mixer unit to form the final flow and the final eluent composition. The mixer is located after the pump units. Therefore, the solvents are already under pressure when they reach the mix-point where the mixing occurs. The two pump blocks work with different solvents. In the most common case for TFA applications, the block "A" pumps an aqueous solvent and the block "B" pumps ACN. A very simplified description of the pump operation principle can be summarized in three main phases: draw solvent, compress the solvent, and deliver the solvent.[4] Warming up during the compression phase of ACN are one of the main causes of the local fluctuation of the mobile phase.

Organic solvents (i.e. ACN) warm up during the compression phase. After the precompression phase, the fluid cools down and decompresses slightly overtime. This is then reflected in a flow disturbance, which can cause TFA baseline ripples. For completeness, it is worth mentioning that this effect, although also present with aqueous solvents, is negligible due to the different thermal properties of water. In comparison to organic solvents, water has a lower compressibility, a higher heat capacity, and a lower thermal expansion coefficient. Consequently, TFA applications require the use of high performing pumps able to deliver high flow consistency.

The Vanquish Horizon UHPLC system is based on a binary pump with a two-channel, parallel dual-piston design. With its independent piston drives, it is designed to provide superior flow consistency.[5] Two intelligent algorithms are implemented:

1. *Advanced Thermal Effect Compensation (ATEC™)* – calculating the time for the solvent to cool down after the compression phase
2. *SmartFlow™* – compensating for the flow variation due to the solvent compressibility at high pressures

These two factors work together to offer a very precise and ripple-free flow independent of the gradient slope and mobile phase composition.

Mixer volume and stroke volume – effect on TFA applications

High performing UHPLC pumps are not the only requirement to achieve a ripple-free baseline. The mixer and pump stroke volumes need to also be considered.

TFA ripples are not visible when the volume period of A and B is smaller than the mixer.[6] The reason for that may be not intuitive. First, let us clarify what the *volume period* is. The volume period (V_A) of solvent A is defined as:

$$V_A = \frac{100\%}{A\%} \cdot \textit{pump stroke volume (µL)}$$

If we consider a binary pump with a 120 µL stroke volume (as the Vanquish Horizon pump) delivering a solvent composition of 80% water (solvent A) and 20% acetonitrile (solvent B), the volume period of each solvent is calculated like so:

$$V_A = \frac{100\%}{80\%} \cdot 120\ \mu L = 150\ \mu L$$

$$V_B = \frac{100\%}{20\%} \cdot 120\ \mu L = 600\ \mu L$$

In other terms, it means that a single stroke of pump head B extends over the delivery of 600 µL of final mobile phase. If the mixer has a volume of at least 600 µL, it will therefore "contain" the entire B stroke (Figure 2a). The transition from one stroke to the next one is a very delicate phase. Even though there are check valves to prevent flow of mobile phase backwards from the column into the pump, short-term deviations in the flow from each high-pressure pump are very difficult to avoid. A mixer large enough to contain all the stroke volume will smooth any temporary solvent composition variation. However, in several cases the use of larger mixers is not a feasible solution due to the impact on the GDV.

In case the mixer volume is smaller than the B volume period, there will be moments at the end of the B stroke in which the composition of the combined fluid will be enriched by fluid A (Figure 2b). In this situation, there will be a local and temporary mobile phase variation that will cause a baseline ripple as described in Figure 1. A good solution is to reduce the volume period of B and still use a small mixer. In this case, the volume period of B can be still contained in the mixer and temporary mobile phase variations at the end of the stroke will be smoothed by the mixer (Figure 2c).[7]

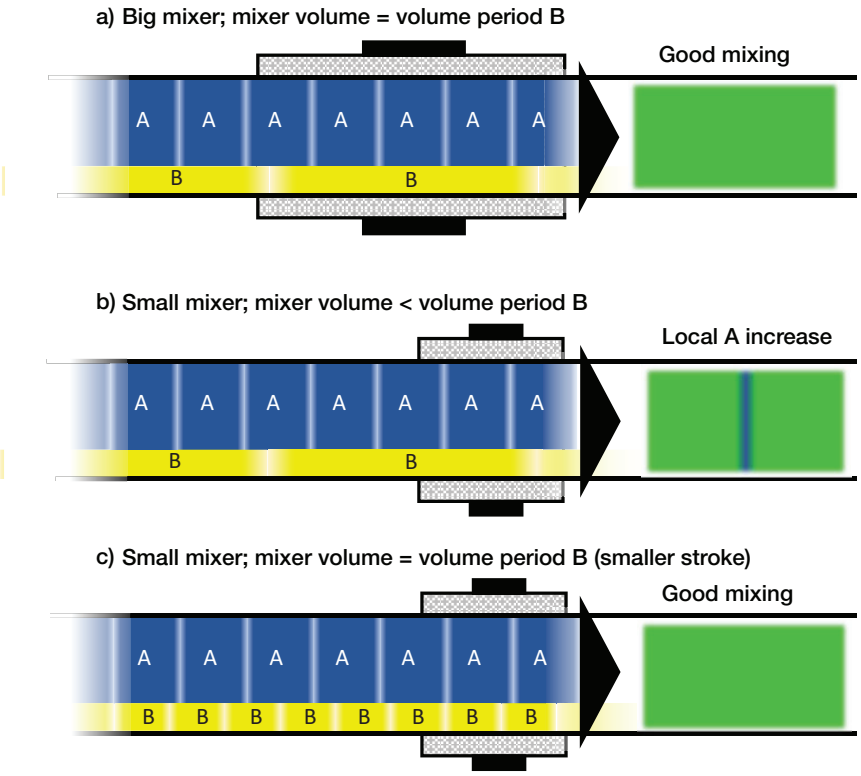

Figure 2. Representations of the flow from individual high-pressure pump blocks in a binary high-pressure mixing system delivering a solvent composition of 80% water (solvent A) and 20% acetonitrile (solvent B). The individual strokes coming from the pump block A are shown in blue, and the strokes from the pump block B are shown in yellow. Flow variations at the end of each stroke are represented in lighter colors. a) When the mixer volume is comparable to the B stroke volume, there is perfect mixing in the final mobile phase; b) When the mixer volume is smaller than the B stroke volume, there will be a moment when the composition of the combined fluid will be enriched in fluid A; c) When the mixer volume is small, but also comparable to the B stroke volume, there is perfect mixing in the final mobile phase.

The volume period of B is dependent on the pump stroke. However, many pumps have a fixed pump stroke volume not permitting any adjustment. The Binary Pump H of the Vanquish Horizon system is based on a parallel dual-piston design with independent piston drives that allows changing the stroke volume of each pump block up to 120 µL.[8] SmartStroke is an intelligent algorithm that activates an optimized piston stroke behavior to reduce the volume period based on the flow rate to guarantee a smooth baseline even with small mixer volumes.

Enhanced peptide detection with SmartStroke

The SmartStroke algorithm can be activated for all applications where a smooth baseline is crucial to achieve the lowest limits of detection and quantitation. This function automatically improves the stroke behavior of both A and B pump blocks. Figure 3 shows baseline traces recorded at 214 nm with the Vanquish Horizon system with SmartStroke activated (black trace) or deactivated (blue trace) for a generic water/acetonitrile gradient containing TFA in both solvents. A significant reduction of the TFA baseline ripples can be observed when the SmartStroke function is activated.

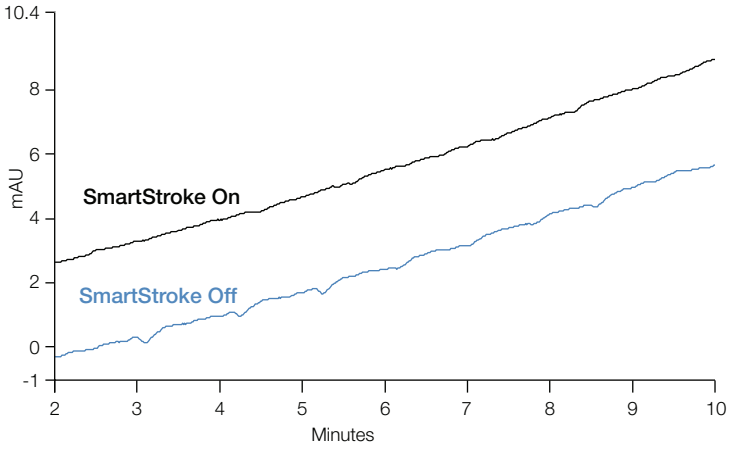

Figure 3. Comparison of UV trace profiles recorded at 214 nm. Black trace: Vanquish Horizon UHPLC system with 200 µL mixer with SmartStroke On; Blue trace: Vanquish Horizon UHPLC system with 200 µL mixer with SmartStroke Off. Details about the chromatographic method are described in the LC condition section. Chromatograms are displayed with a signal offset of 15%.

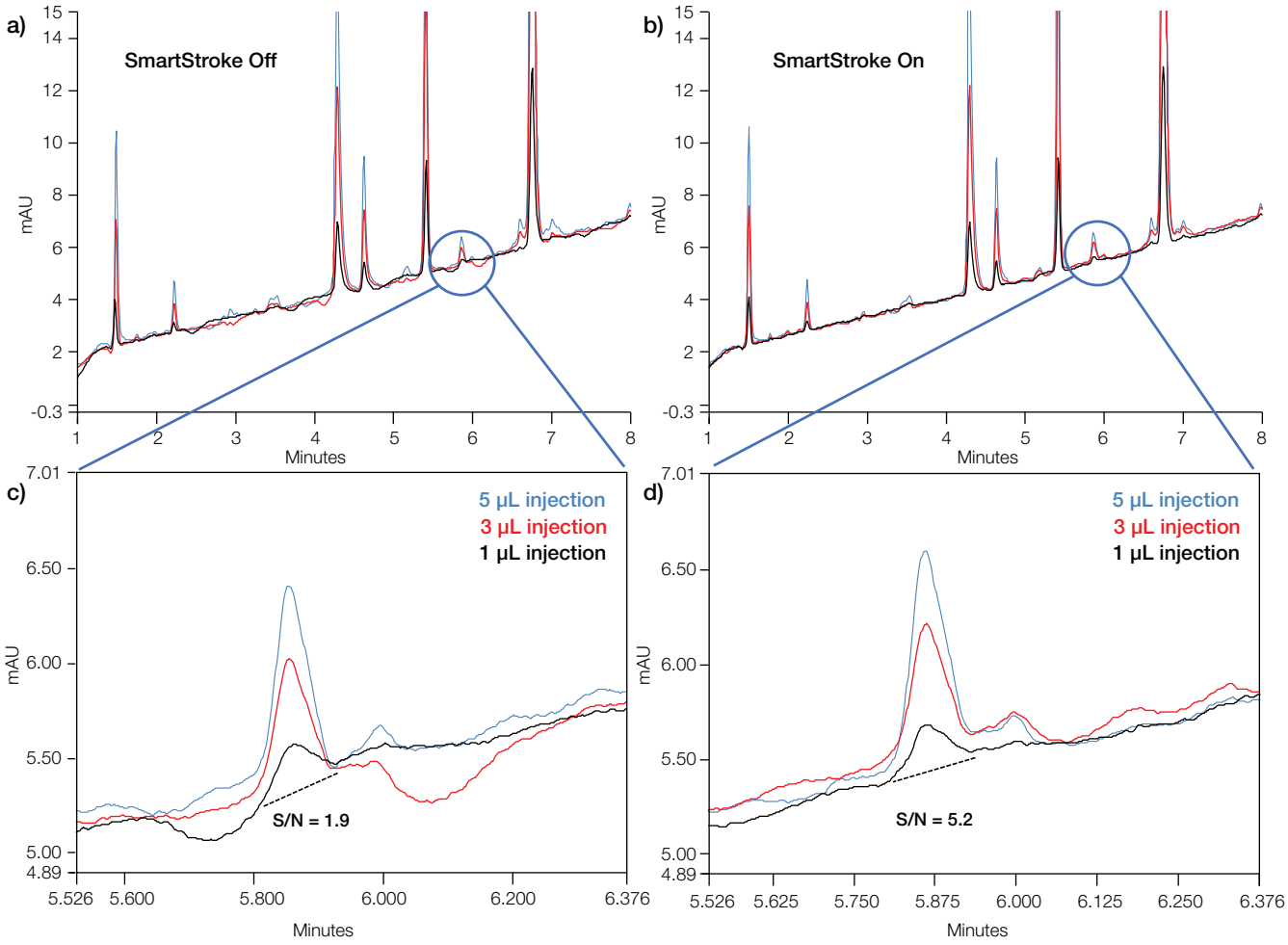

Figure 4. Overlaid chromatograms of cytochrome C digest analyzed with the Vanquish Horizon system with a) and c) SmartStroke Off and b) and d) SmartStroke On at different injection volumes (blue trace 5 µL, red trace 3 µL, black trace 1 µL). c) and d) plots show the signal-to-noise ratio (S/N) of the peptide eluting at 5.87 min at the smallest injection volume of 1 µL.

A smoother baseline is a fundamental requirement for achieving lower limits of detection (LOD) and quantitation (LOQ). The main analytical benefit performed with the Vanquish Horizon system is the improvement of the detection and quantitation for a UHPLC peptide analysis using TFA as mobile phase additive. Figure 4 shows the peptide analysis of a cytochrome C digest at different injection volumes with SmartStroke Off (a-c) and with SmartStroke On (b-d). Although there is no appreciable difference for the most intense peptides, SmartStroke helps to improve the LOD of the less intense compounds. A signal-to-noise ratio (S/N) of 3 is generally acceptable for estimating LOD. A small peptide can be observed eluting at 5.87 min. When the SmartStroke function is activated it can be detected with confidence (S/N = 5.2) even at the lowest injection volume of 1 µL (Figure 4c). Under the same conditions but with the SmartStroke function deactivated, this peptide cannot be detected because it is confounded in the noise (S/N = 1.9).

Comparison study

The UV baseline recorded when running the same TFA gradient was compared between the Vanquish Horizon system with the SmartStroke function and the Vanquish Flex Binary system. For the Vanquish Flex Binary system a 400 µL mixer system is recommend to achieve a very low-noise UV trace.[2] Both systems showed a very smooth baseline (Figure 5). The Vanquish Horizon system is unquestionably the system of choice for demanding applications when small GDV is required. Under the same chromatographic conditions, superior performance and smoother baseline of the Vanquish Horizon system are observed in comparison to the Vanquish Flex Binary system.

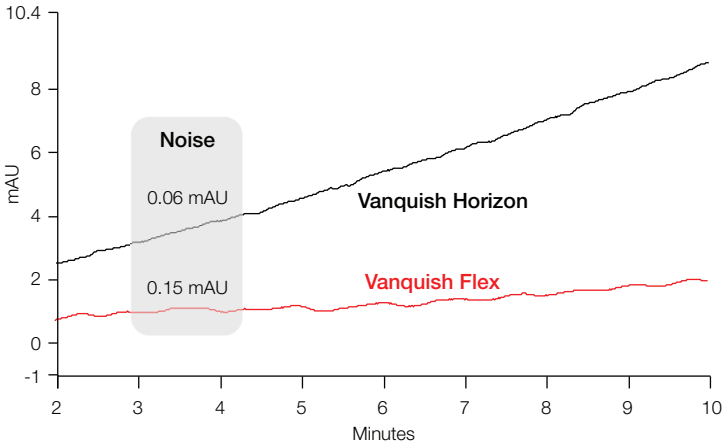

Figure 5. Comparison of UV trace profiles recorded at 214 nm. Black trace: Vanquish Horizon UHPLC system with 200 µL mixer with SmartStroke On; red trace: Vanquish Flex Binary UHPLC system with 400 µL mixer. All chromatograms were recorded with a Vanquish Diode Array Detector HL. The noise level displayed in the figure is measured in the range 3–4 min (although it is an overlay of noise and ripple, an apparent noise was calculated according to ASTM). Details about the chromatographic method are described in the LC condition section. Chromatograms are displayed with a signal offset of 15%.

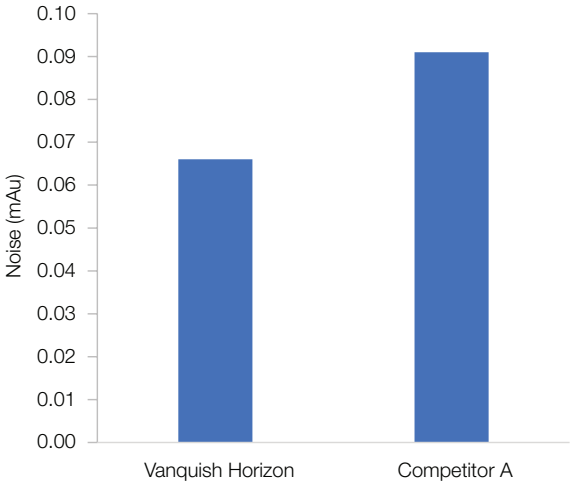

Figure 6. Comparison of noise levels for chromatograms recorded at 214 nm. Left bar: Vanquish Horizon UHPLC system with SmartStroke On; Right bar: Competitor A UHPLC system. The noise level displayed in the graph is measured in the range 2–3 min. Details about the chromatographic method are described in the LC condition section.

To further evaluate the performance of the SmartStroke function, the UV baseline was recorded when running the same TFA gradient and was compared between two high-end binary UHPLC systems able to operate above 1000 bar: Vanquish Horizon and Competitor A. The Competitor A system comprises a high-pressure mixing binary pump, a sampler, a column compartment, and a DAD detector. The system was operated with a 100 µL mixer, as recommended by Competitor A for TFA gradient applications. The Vanquish Horizon system showed a smoother baseline (Figure 6).

SmartStroke function in Chromeleon CDS

The SmartStroke function can be activated for demanding applications when an ultra-smooth baseline is required. SmartStroke activates an optimized piston stroke behavior for the Vanquish Horizon binary pump to reduce baseline ripples observed in special applications containing (but not exclusively) mobile phases with TFA. This function can be easily selected in Chromeleon CDS, version SR5 MUk or higher. The option SmartStroke can be enabled/disabled for an instrument method (Figure 7) as well as for the intervals between queue runs (Figure 8). By default, SmartStroke is disabled. The function activates an intelligent more frequent and variable piston stroke. It is however recommended to enable the SmartStroke option just for the aforementioned applications for the best balance between piston wear and baseline smoothness.

The Vanquish Binary Pump ePanel offers an option to set the behavior of SmartStroke outside of a queue run (SmartStroke Outside Queue Run), e.g., if SmartStroke should be applied during Monitor Baseline (Figure 8).

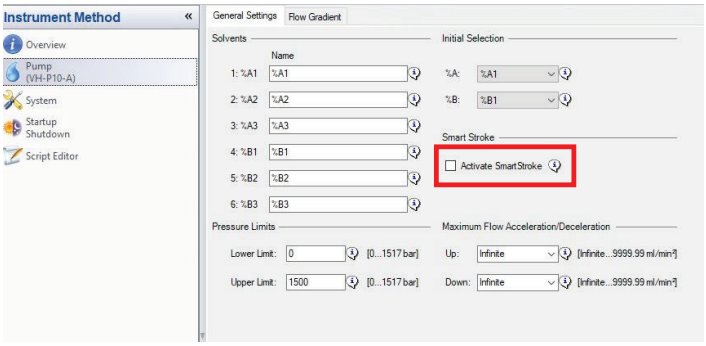

Figure 7. Overview of the pump settings in the Instrument Method in Chromeleon CDS. SmartStroke function can be activated ticking the box "Activate SmartStroke".

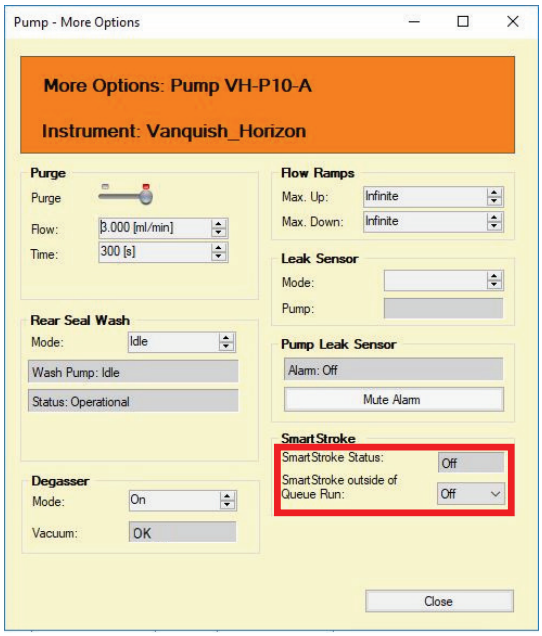

Figure 8. Overview of the "More Options" window accessed by the pump ePanel in Chromeleon CDS. SmartStroke outside of Queue Run can be set to "On" to activate the intelligent stroke.

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For older instrument methods not supporting the SmartStroke function, the settings for SmartStroke and SmartStroke Outside Queue Run are undefined and behave the same as SmartStroke “Off” and SmartStroke Outside Queue Run “Off”. In Chromeleon software versions prior to SR5 MUk, it can be activated via a command script.

Conclusion

- The Vanquish Horizon UHPLC system is the system of choice for demanding TFA applications that require a “ripple-free” baseline.
- SmartStroke is an intelligent algorithm that regulates a new special stroke behavior to virtually eliminate TFA baseline ripples.
- The Vanquish Horizon system with the SmartStroke function achieves the highest sensitivity for the detection and the quantitation of peptides, even with small mixer volumes, to guarantee low gradient delay volume (GDV).

References

1. Thermo Scientific Technical Note 108, Reliable Solvent Mixing in UHPLC https://tools.thermofisher.com/content/sfs/brochures/TN-108-Reliable-Solvent-Mixing-LPN2851-EN.pdf
2. Strobl, C.; Krajewski, M.; Swart, R. Trifluoroacetic acid performance of the Vanquish Flex Binary UHPLC system. Thermo Scientific Technical Note TN72505-EN 0917S https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-72505-lc-trifluoroacetic-acid-reducing-mixer-ripples-tn72505-en.pdf
3. Choikhet, K.; Glatz, B.; Rozing, G. The Physicochemical Causes of Baseline Disturbances in HPLC, Part I – TFA-Containing Eluents, LC GC Europe, February 2003.
4. Steiner, F. The Gradient; Requirements, Optimal Use, Hints, and Pitfalls. Chapter 4 in The HPLC Expert II: Find and Optimize the Benefits of your HPLC / UHPLC Ed. S. Kromidas, Wiley, 2017.
5. Thermo Scientific Product Spotlight SP71188-EN 0714S, How Solvent Delivery Technology Can Improve Confidence in Peak Identification and Quantification https://assets.thermofisher.com/TFS-Assets/CMD/brochures/SP-71188-Solvent-Delivery-Highest-Confidence-Peak-Identification-Quantification-SP71188-EN.pdf
6. Steiner, F. Instrumental Influences on the Quality and Performance of Gradient Methods and Their Transfer Between Different HPLC Devices. Chapter 2 in Gradient HPLC for Practitioners: RP, LC-MS, Ion Analytics, Biochromatography, SFC, HILIC Ed. S. Kromidas, Wiley, 2019.
7. Stoll, D.R. Mixing and Mixers in Liquid Chromatography—Why, When, and How Much? Part I, The Pump, LC GC North Am, October 2018.
8. Thermo Scientific Production Specification PS73056-EN 0220S, Vanquish Pumps https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FCMD%2FSpecification-Sheets%2Fps-73056-lc-vanquish-pumps-ps73056-en.pdf&title=UHJvZHVjdCBzcGVjaWZpY2F0aW9uczogVmFucXVpc2ggUHVtcHM=

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