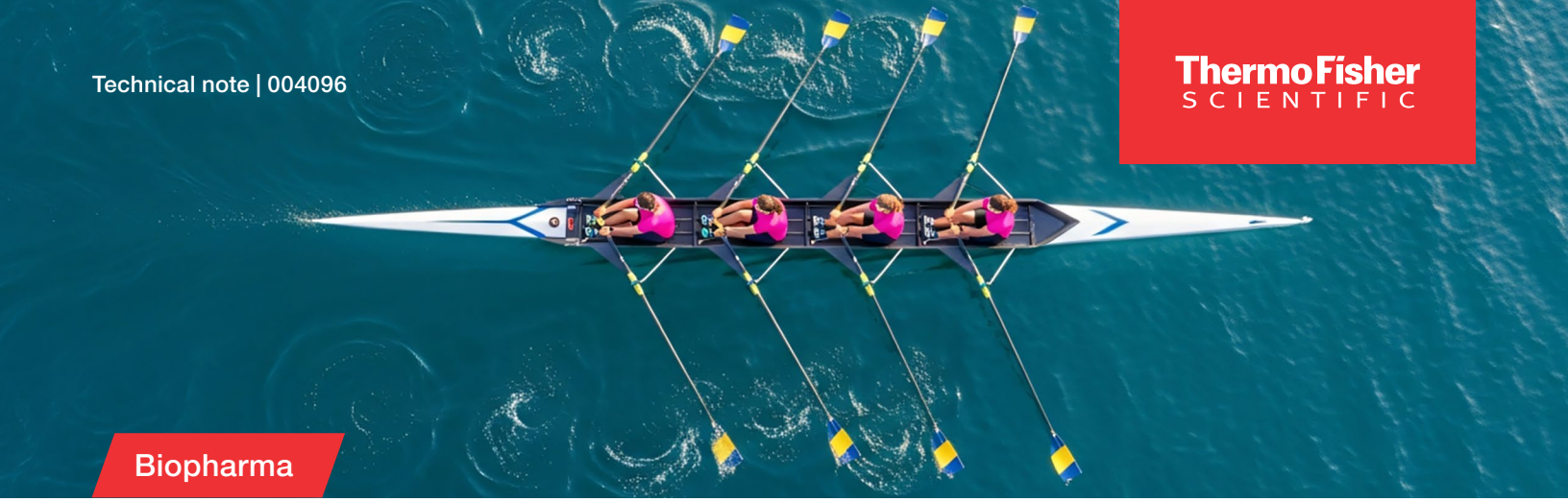
Technical note | 004096



Biopharma

The benefit of binary pump stroke synchronization for more reliable peak identification based on improved retention time precision

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Keywords

Vanquish Flex Binary UHPLC, UHPLC-UV, protein digest, peptide mapping, biopharma, HPG Sync feature, binary pump stroke synchronization, Chromeleon 7.4 CDS, retention time stability

Benefits

- **Repeatability:** Stable retention times in peptide mapping ensure that the same peptide elutes at exactly the same time in repeated runs. This precision is essential for comparing results across different experiments and for validating the consistency of the method.
- **Accurate peak identification:** Consistent retention times help in accurately identifying peaks corresponding to specific peptides. When retention times are stable, it is easier to assign peaks to known peptides based on their expected elution times.
- **Cost savings:** Traditionally, LC-MS is the standard method utilizing top-tier UHPLC-MS instrumentation. The Thermo Scientific™ Vanquish™ Flex Binary UHPLC System configured with a variable wavelength detector (VWD) complemented with the binary pump stroke synchronization (HPG Sync) feature provides a biocompatible, cost-effective, robust, UHPLC instrument to standardize the peptide mapping application.

Goal

In this study, the goal was to show the application robustness benefits of the binary pump stroke synchronization feature on LC-UV peptide mapping, as an example for a challenging gradient UHPLC application.

Introduction

Retention time stability in liquid chromatography (LC) is a critical factor that significantly impacts the robustness of an analytical application. Consistent retention times ensure reproducibility, which is essential for the reliable identification and quantification of analytes. When the same compound elutes at the same time in repeated analyses, it will reduce the risk of misidentification and incorrect quantification. This reproducibility is vital for method validation, as it demonstrates that the method can produce consistent results over time, thereby ensuring the accuracy and reliability of the analytical data.

Moreover, stable retention times are crucial for quality control processes, as they help ensure that products meet required specifications. Any fluctuation in retention time can indicate potential issues with the method or the system, necessitating re-analysis, troubleshooting, or, at the very least, manual peak assignment changes.

Retention time stability in peptide mapping is crucial for ensuring the accuracy and reproducibility of the analysis. Peptide mapping is a key technique used in the characterization of proteins where peptides generated from enzymatic digestion are separated and identified. Stable retention times allow for consistent identification of peptides across different runs, which is essential for comparing results and detecting any modifications or variations in the protein structure. Variability in retention times can lead to difficulties in matching peptides to their corresponding proteins, potentially compromising the integrity of the analysis. As highlighted by Gilar et al. in their study on peptide mapping, maintaining stable retention times is fundamental for reliable peptide identification and quantification, which is critical for applications in proteomics and biopharmaceutical development.[1]

The binary pump stroke synchronization, or HPG Sync, is a feature for the Thermo Scientific™ Vanquish™ Binary Pump F that was developed initially for the Thermo Scientific™ Vanquish™ Binary Pump H. The activated HPG Sync feature times the injection to a specific piston position of the pumping cycle, ensuring that the delivery of solvent composition along the gradient ramp is identical for each run. The injection is held until the piston position, which is controlled by the cam shaft position, is at the same point for every injection. This feature, now available on the Vanquish Flex Binary UHPLC system, makes for a more cost-effective UHPLC-UV configuration versus the advanced UHPLC-MS method. The HPG Sync feature has shown improved retention time stability for all components of this application for over 24 hours for Vanquish Flex Binary UHPLC systems. This improvement increases the data quality and confidence and reduces erroneous and costly measurements (Figure 1).

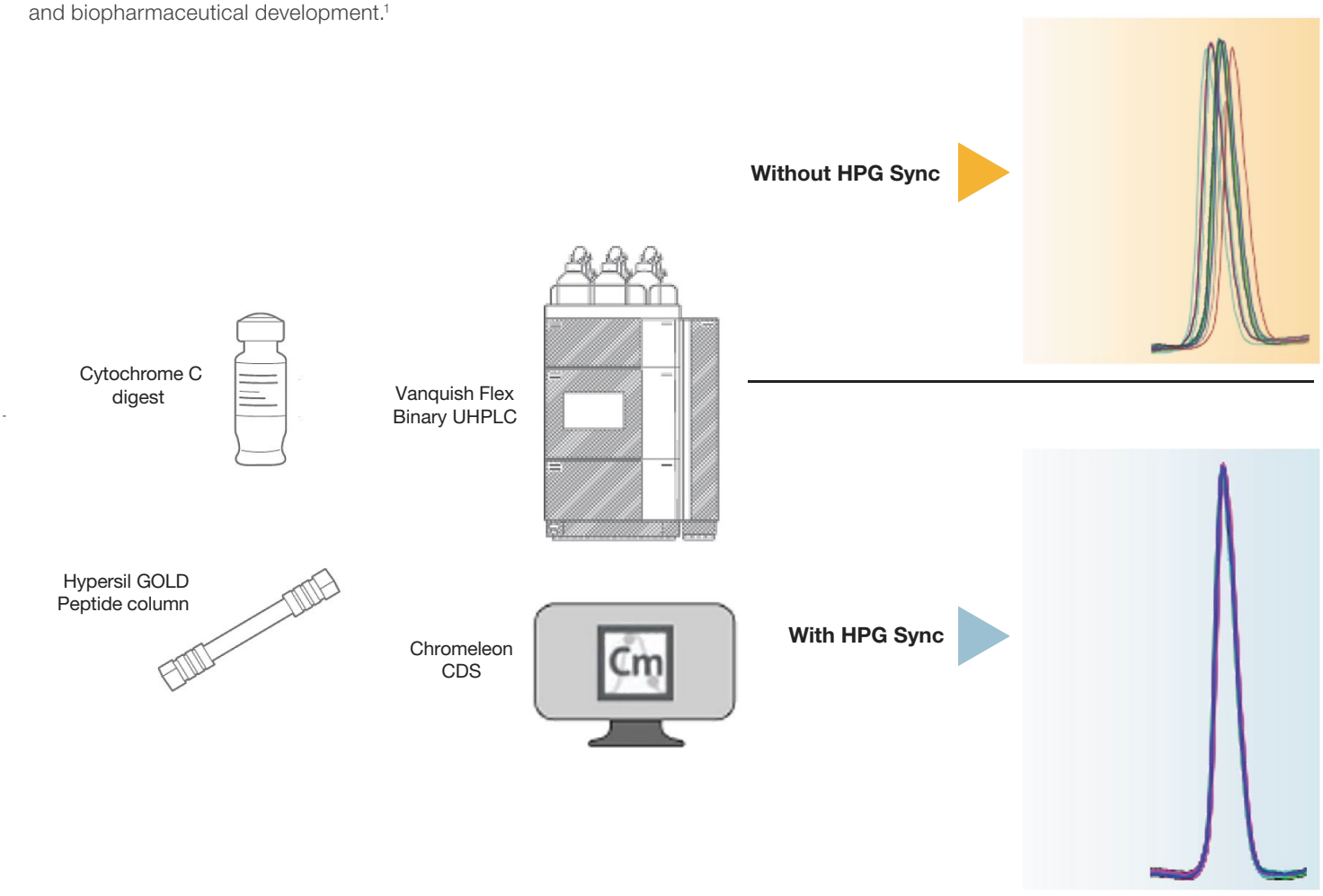


Figure 1. The reconstituted Cytochrome C digest is analyzed via UHPLC-UV using a Thermo Scientific™ Hypersil™ GOLD™ Peptide Column. The new HPG Sync feature improves the retention time precision making for increased data quality, simpler data processing, and application robustness.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (Cat. No. 50136149)
- Fisher Scientific™ Acetonitrile (ACN), Optima™ LC/MS grade (Cat. No. A955)
- Fisher Scientific™ Isopropanol, Optima™ LC/MS grade (Cat. No. A461)
- Fisher Scientific™ Formic acid (FA), Optima™ LC/MS grade (Cat. No. A117-50)
- Thermo Scientific™ Dionex™ Cytochrome C Digest (Cat. No. 161089)

Sample handling

- Thermo Scientific™ Finnpipette™ F1 Variable Volume Single-Channel Pipette: 100–1,000 µL (Cat. No. 4641100N)
- Thermo Scientific™ Finnpipette™ F1 Variable Volume Single-Channel Pipette: 10–100 µL (Cat. No. 4641070N)
- Thermo Scientific™ Finnpipette™ F1 Variable Volume Single-Channel Pipette: 1–10 µL (Cat. No. 4641030N)
- Fisherbrand™ Mini Vortexer (Cat. No. 4-955-152)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Screw Top Microvials for <2 mL Samples, Level 1 Everyday Analysis (Cat. No. 6ESV9-04PP)
- Thermo Scientific™ SureSTART™ Blue Polypropylene 9 mm AVCS™ Screw Caps with Soft Blue Silicone/Clear PTFE Septa, 100/pack, Level 3 (Cat. No. 6PSC9ST101)

Instrumentation

Thermo Scientific™ Vanquish™ Flex UHPLC System consisting of:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex (Cat. No. VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump F (Cat. No. VF-P10-A-01)
- Thermo Scientific™ Vanquish™ Split Sampler FT (Cat. No. VF-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (Cat. No. VH-C10-A-03)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F (Cat. No. VF-D40-A)
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector F Semi-micro Bio Flow Cell, 2.5 µL, 7 mm, PEEK (Cat. No. 6077.0300)
- Thermo Scientific™ Active Pre-heater TQ 0.10 × 380 mm MP35N (Cat. No. 6732.0700)
- Thermo Scientific™ Post-column Cooler TQ 1 µL, 0.10 × 240 mm MP35N (Cat. No. 6732.0540)

Sample preparation

The lyophilized sample (Cytochrome C digest) was reconstituted in 200 µL 95% water, 5% ACN with 0.1% FA (5% solvent B in solvent A). The sample was vortexed and allowed to sit for at least 10 minutes to allow reconstitution of all peptides.

Chromatographic conditions

Table 1. Chromatographic conditions.

Parameter	Value
Column	Hypersil GOLD Peptide, 150 × 2.1 mm, 1.9 µm (Cat. No. 26002-152130)
Solvent A	Water, 0.1% formic acid
Solvent B	Acetonitrile, 0.1% formic acid
Gradient	Time [min] B [%] 0.0 1 5.0 1 6.0 10 70.0 35 72.0 90 77.0 90 79.0 1 81.0 1 83.5 10 91.5 45 93.0 90 99.0 90 101.0 1 115.0 1
Flow rate	0.25 mL/min
Column temperature	50°C, still-air mode 50°C, active pre-heater
Autosampler temperature	8°C
Needle wash solution	25% solvent B in solvent A
Needle wash mode	After draw
Injection volume	10 µL
UV detector settings	220 nm, data collection rate = 20 Hz, response time = 0.2 s

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.4 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

The peptide mapping application using tryptic digested Cytochrome C was based on the LC-MS method[2] with a variable wavelength detector in place of the MS. A binary pump consists of two pump blocks/drives: A and B. At 250 µL/min and an initial solvent B composition of 1%, pump B responsible for solvent B is delivering 2.5 µL/min. At 2.5 µL/min, pump block B is moving relatively slowly and requires time to come into position for the synchronization. Conversely, the waiting time between injections is reduced when the B block delivers more than 2.5 µL/min. The purpose of the HPG Sync is to time the injection with the exact same piston position for pump block B, which in turn delivers the exact same conditions for pump block B for every injection. This, as a result, increases the retention time stability for all components, especially over a long shallow gradient.

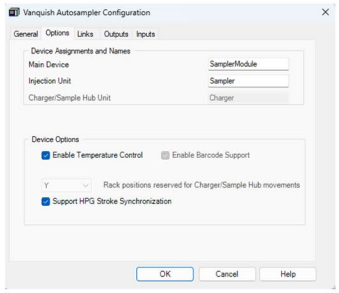
The implementation of the HPG synchronization (Figure 2) begins in Chromeleon CDS with the instrument configuration where the "Support HPG Stroke Synchronization" is activated to enable the synchronization feature (Figure 2A). Next, the instrument method is created with the "Enable Stroke Synchronization" so that the specific instrument method utilizes the HPG Sync feature (Figure 2B). Finally, the mode in which the HPG stroke synchronization takes place is specified (Figure 2C). The option is scheduling the "prepare inject" command to prepare the injection (needle wash, sample draw, etc). The waiting for the correct piston position is then an extra.

- **None:** The sample preparation is not scheduled (not recommended).
- **Prepare in equilibration phase:** This sets a negative time segment to the instrument method where the sampler can prepare the injection. However, this is not reducing run time.
- **Prepare in run stage:** Utilize the equilibration phase of the column to prepare the injection for the next run. This requires the knowledge of approximate preparation time and sufficient time in the equilibration phase of the gradient (recommended).

Note: The first injection of a sequence is not synchronized. Therefore, we recommend a blank run for the first injection.

Observing the resulting chromatograms with HPG Sync OFF versus HPG Sync ON, one can immediately visualize the improvement in the trace overlays. The retention time performance already allowed for accurate peak identification, though the precision improvement can be better visualized in the zoomed regions at the beginning of the gradient (Components 1–5) for HPG Sync OFF (Figure 3A) and HPG Sync ON (Figure 3C). Additionally, the retetion time precision improvement can also be seen at the end of the gradient for HPG Sync ON (Figure 3D) versus HPG Sync OFF (Figure 3B).

A



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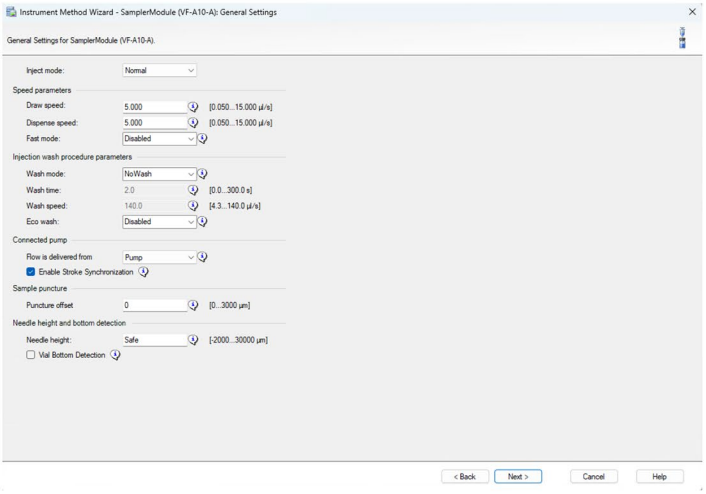

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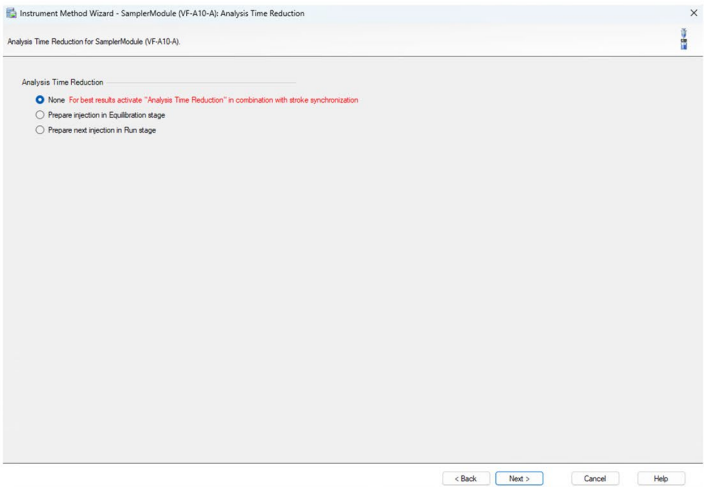

Figure 2. The implementation of the HPG stroke synchronization in Chromeleon CDS where (A) it is first configured, (B) it is activated for the autosampler, and (C) the mode in which it is to be implemented.

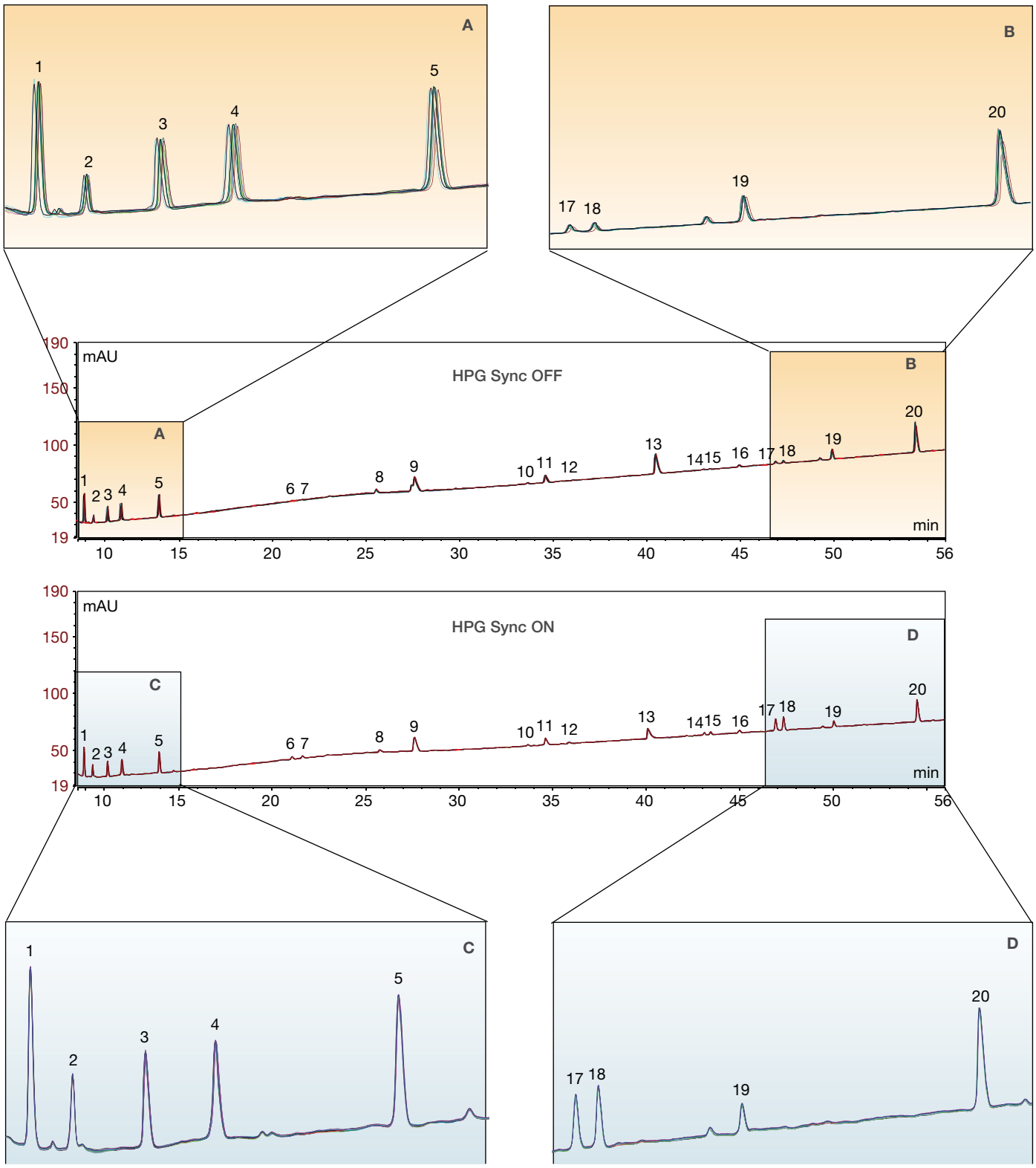

Figure 3. Cytochrome C digest was run on the Vanquish Flex Binary UHPLC system with a Hypersil GOLD Peptide column over the course of 24 hours with n=10. The top chromatograms represent the 20 selected components throughout the gradient. Early and late sections of the chromatograms were analyzed for a zoomed visualization. (A) Early eluting components using HPG Sync OFF show slight retention time shifts as well as (B) late eluting peaks with HPG Sync OFF. In contrast, the improvement using HPG Sync ON can clearly be seen as the 10 chromatograms perfectly overlay and look like one chromatogram, not only in the above reference chromatogram but also in the zoomed (C) early eluting peaks and (D) late eluting peaks.

Transitioning from visual to statistical characterization, Table 2 illustrates each component's response to the HPG Sync feature (ON versus OFF) through the standard deviation over the 24-hour sequence. Component 3 profited the most, with a factor of 5 reduction in the retention time standard deviation, where component 15 decreased only by a factor of 2. When assessing the quality of any peptide mapping LC-UV application, the average reduction of retention time error significantly increases the method quality and robustness through confident peak assignments. For the components in Cytochrome C, the retention time repeatability was improved by an average factor of 3 for all components over the gradient (Figure 4).

Table 2. Component response to HPG Sync feature.

	HPG Sync ON				HPG Sync OFF			
Component	Avg. RT time (min)	% RSD	SD (min)	Max RT shift (min)	Avg. RT time (min)	% RSD	SD (min)	Max RT shift (min)
1	9.949	0.04%	0.004	0.017	9.926	0.26%	0.026	0.065
2	10.410	0.03%	0.003	0.015	10.418	0.17%	0.018	0.049
3	11.205	0.05%	0.006	0.029	11.160	0.26%	0.029	0.083
4	11.972	0.07%	0.009	0.041	11.889	0.29%	0.034	0.103
5	13.968	0.06%	0.008	0.025	13.912	0.18%	0.025	0.09
6	21.075	0.03%	0.006	0.015	21.031	0.10%	0.022	0.088
7	21.633	0.03%	0.006	0.012	21.578	0.11%	0.025	0.095
8	25.733	0.02%	0.006	0.035	25.497	0.08%	0.02	0.074
9	27.607	0.02%	0.005	0.009	27.557	0.08%	0.022	0.075
10	33.680	0.02%	0.006	0.019	33.620	0.07%	0.022	0.085
11	34.621	0.02%	0.006	0.009	34.548	0.06%	0.022	0.085
12	35.865	0.02%	0.006	0.063	36.050	0.06%	0.023	0.093
13	40.107	0.02%	0.008	0.041	40.448	0.05%	0.021	0.078
14	43.124	0.02%	0.009	0.024	43.027	0.05%	0.021	0.078
15	43.460	0.02%	0.009	0.021	43.368	0.04%	0.018	0.068
16	45.005	0.02%	0.009	0.014	44.947	0.04%	0.02	0.075
17	46.937	0.02%	0.009	0.012	46.894	0.05%	0.022	0.084
18	47.362	0.02%	0.009	0.012	47.322	0.04%	0.02	0.078
19	50.056	0.02%	0.009	0.017	49.910	0.04%	0.02	0.074
20	54.510	0.02%	0.009	0.018	54.376	0.05%	0.026	0.091
Average (n=20)		**0.03%**	**0.0071**	**0.0224**		**0.10%**	**0.0228**	**0.08055**

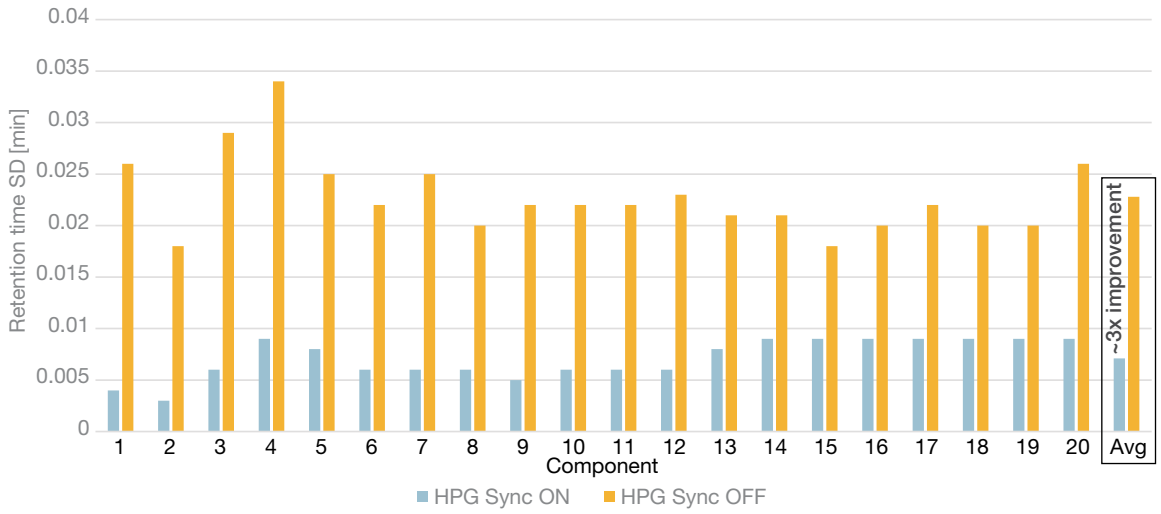

Figure 4. Twenty components of the Cytochrome C digest were selected throughout the gradient, and represented is the standard deviation (n=10) with HPG Sync OFF versus HPG Sync ON. The resulting standard deviation averaged over a 3× improvement on the retention time reproducibility for all components.

Conclusion

The positive effects of the HPG Sync implementation on the retention time precision is expressed in a significant decrease in the standard deviation of the retention time of every component during the eluting gradient. The impact of the HPG Sync ON for this example of peptide mapping was shown to improve the application robustness in terms of:

- **Retention time precision:** The average retention time standard deviation with HPG Sync OFF is ±0.023 minutes, and the average retention time standard deviation with HPG Sync ON is ±0.007 minutes for all components over the gradient, highlighting an average improvement of retention time repeatability by a factor of more than three times. With a selected customer's criterion of 0.03 minutes, both modes provide passing results; the HPG Sync ON increases confidence on peak identification.
- **Accurate peak identification:** Cytochrome C digest was used as a proxy sample representing a monoclonal antibody digest. The peptide mapping application presents, in general, a challenging gradient method for most HPLC systems, resulting in fluctuating peak retention times, potentially leading to inaccurate peak identification. The impact of the HPG Sync feature on the retention time reproducibility makes for simpler and more accurate component identification, increasing the compatibility for automated workflow integration.
- **Cost savings:** Elimination of the MS dependency after the component characterization has taken place can substantially reduce the investment for the routine quality control workflow for peptide mapping. Retention time stability due to exactly controlled cam shaft position at the time of injection increases the method robustness and component mapping and could, therefore, not require the additional MS peak characterization.

References

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