Application note | 004312


Biopharma

# Stability-indicating HPLC-UV method development for GLP-1 receptor analogue semaglutide

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**Keywords**

GLP-1, semaglutide, related impurities, stability-indicating, method development, Vanquish Flex UHPLC, Hypersil GOLD Peptide column

## Application benefits

- A stability-indicating HPLC-UV method is provided for analysis of the GLP-1 receptor analogue semaglutide and its related impurities.
- The data presented provide valuable insights into the influence of column temperature, gradient, and mobile phase additives on reversed-phase separation.
- Column lot-to-lot reproducibility confirms the long-term robustness and stability of the method for routine laboratory applications.

## Goal

Development of a stability-indicating HPLC-UV method for semaglutide analysis using the Thermo Scientific™ Hypersil GOLD™ Peptide Column.

## Introduction

Semaglutide is a 32-amino acid glucagon-like peptide-1 (GLP-1) receptor agonist used to treat type 2 diabetes and support long-term body weight management. Following approval by the U.S. Food and Drug Administration (FDA) in 2017, formulations containing semaglutide became the third best-selling drug worldwide in 2024.[1] Moreover, the expiration of patents for some GLP-1 receptor agonists in several countries is encouraging numerous pharmaceutical companies to develop generic equivalents. According to guidelines published by the FDA for the Abbreviated New Drug Application of highly purified synthetic peptides, peptide-related impurities must be rigorously

identified and controlled.[2] Any variations in the amino acid sequence can potentially interfere with the peptide's biological activity, affecting its efficacy and safety.

Peptide-related impurities commonly arise from amino acid deletions, insertions, substitutions, isomerization, or modifications of amino acid side chains. Many peptide-related impurities share similar characteristics with the target peptide, making them challenging to separate. Reversed-phase HPLC with gradient elution is the most widely used method for peptide analysis. Ion pairing reagents, such as trifluoroacetic acid (TFA), are often added to the mobile phase to form an ion pair with positively charged peptides, thereby increasing their hydrophobicity and retention.[3] However, TFA causes ion suppression in mass spectrometry (MS), significantly reducing the sensitivity. Thus, formic acid (FA) is generally preferred for use with MS detection as it facilitates efficient ionization. However, FA typically results in less effective chromatographic resolution compared to TFA.

This application note describes the development of an HPLC-UV method for the analysis of semaglutide and its related impurities. Figure 1 illustrates the complete layout of the experimental design. To ensure comprehensive coverage of potential impurities that may arise during synthesis, production, transportation, and storage, the semaglutide sample was subjected to thermal and oxidative stress conditions to generate potential impurities. Generated stressed samples were used for optimization of column temperature and gradient conditions to achieve the best separation performance using a Hypersil GOLD Peptide column. The method was optimized by using FA or TFA as mobile phase additives, respectively, and the obtained separation was compared and evaluated. Finally, to ensure the robustness of the method across different column batches, column lot-to-lot reproducibility was evaluated based on injection series on columns from three different lots.

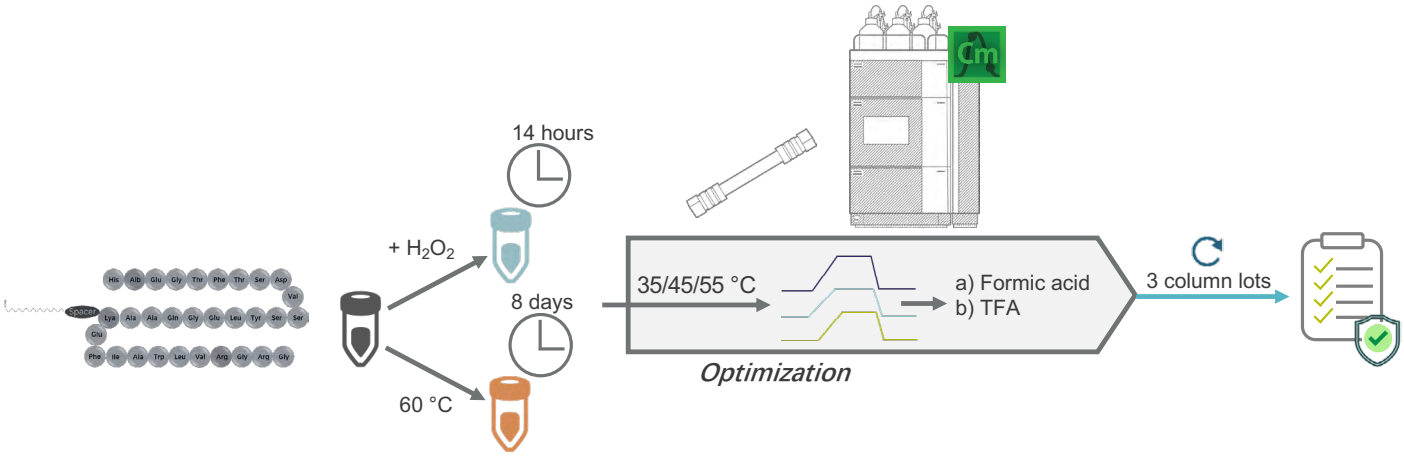


**Figure 1. Summary of stability-indicating HPLC-UV method optimization for semaglutide and its impurities using thermally and oxidatively stressed samples with separation performed on a Hypersil GOLD Peptide column.**

## Experimental

### Chemicals

| Chemical name | Cat. No. |
|---|---|
| Deionized water with 18.2 MΩ·cm resistivity or higher, purified by a Thermo Scientific™ Barnstead™ GenPure™ Pro Water Purification System | 50131948 |
| Fisher Chemical™, Acetonitrile (HPLC) | A998-4 |
| Fisher Chemical™, Formic acid, Optima™ LC/MS grade | A117-50 |
| Fisher Chemical™, Trifluoroacetic acid, suitable for HPLC, ≥99.5% | 044630 |
| Fisher Chemical™, Hydrogen peroxide 30% | H325 |

### Sample handling

| Item name | Cat. No. |
|---|---|
| Thermo Scientific™ SureSTART™ 2 mL Glass Vials (amber) | 6ASV9-2P |
| Thermo Scientific™ SureSTART™ 9 mm Vial Caps with Septum | 6ASC9ST1 |
| Thermo Scientific™ Finnpipette™ F3 Variable Volume Single Channel Pipettes | 4640030, 4640040, 4640050, 4640060 |
| Thermo Scientific™ Thermal Mixer | 13687711 |

## Instrumentation

| Module | Cat. No. |
|---|---|
| **Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:** | |
| Thermo Scientific™ System Base Vanquish™ Horizon/Flex | VF-S01-A-03 |
| Thermo Scientific™ Vanquish™ Binary Pump F | VF-P10-A-01 |
| Thermo Scientific™ Vanquish™ Split Sampler FT | VF-A10-A-03 |
| Thermo Scientific™ Vanquish™ Column Compartment H | VH-C10-A-03 |
| Thermo Scientific™ Vanquish™ Variable Wavelength Detector F | VF-D40-A |
| Thermo Scientific™ Vanquish™ Standard Bio Flow cell, 11 µL, 10 mm | 6077.0200 |

## Sample preparation

Semaglutide active pharmaceutical ingredient with a purity higher than 99.0% was purchased from Shandong Mopai Biotechnology Co., Ltd.

Five milligrams of the semaglutide sample were transferred into an amber glass vial and dissolved upon addition of 5 mL solvent (20% acetonitrile in water, v/v), followed by rigorous vortexing for 5 minutes to achieve a 1.0 mg/mL sample solution.

The semaglutide solution was then split into six aliquots. Three aliquots were used for the triplicate preparation of the thermally stressed sample by heating the vials at 60 °C for 8 days. The other three aliquots were used for the triplicate preparation of the oxidatively stressed sample by adding a 30% hydrogen peroxide solution to a final concentration of 0.01% (v/v) and incubating at room temperature in the dark for 14 hours, followed by immediate analysis.

## Chromatographic conditions

**Table 1. Chromatographic conditions.**

| | |
|---|---|
| Column | Thermo Scientific™ Hypersil GOLD™ Peptide, 150 × 2.1 mm, 1.9 µm (Cat. No. 26002-152130) |
| Eluent A | 0.1% FA or TFA, respectively, in water (v/v) |
| Eluent B | 0.1% FA or TFA, respectively, in acetonitrile (v/v) |
| Final optimized gradient | Time (min) / A% / B%<br>-5 / 95 / 5<br>5 / 95 / 5<br>6 / 70 / 30<br>46 / 50 / 50<br>50 / 5 / 95<br>55 / 5 / 95<br>56 / 95 / 5<br>60 / 95 / 5 |
| Flow rate | 0.3 mL/min |
| Column temperature | 55 °C with active pre-heater at 55 °C, forced air mode |
| Autosampler temperature | 4 °C |
| Needle wash solution | 10% MeOH / 90% water (v/v) |
| Needle wash mode | After draw |
| Injection volume | 5 µL |
| VWD settings | 280 nm, 2.0 Hz, 2.0 s |

## Chromatography Data System (CDS)

Thermo Scientific™ Chromeleon™ CDS 7.3.2 was used for data acquisition and processing.

## Results and discussion

### Thermally and oxidatively stressed sample study

To identify potential impurities that may arise during production, storage, and transportation, semaglutide sample solutions were periodically analyzed under thermal and oxidative stress conditions to achieve a degradation level of approximately 5% to 10%. The untreated semaglutide sample served as the control, and the average semaglutide peak area of the three prepared degradation samples was used to calculate the percentage of degradation. Ultimately, samples exposed to 60 °C for 8 days (thermally stressed) and to 0.01% $H_2O_2$ for 14 hours (oxidatively stressed), yielding degradation ratios of 10.1% and 4.9%, respectively, were used for further analysis and method optimization.

### Column temperature and gradient optimization

Column temperature and gradient conditions were investigated to improve the separation of semaglutide and related impurities by using FA as an eluent additive at first. Figure 2 presents the UV chromatograms of the thermally stressed sample, illustrating the impact of column temperature on separation, which was increased from 35 °C to 45 °C and then to 55 °C. A gradient from 25% to 60% eluent B over the course of 40 minutes with 0.1% FA as eluent additive was used for all analyses. The results indicate that increasing the column temperature significantly enhances the separation, particularly for the semaglutide peak and the closely eluting peak preceding semaglutide (peak 1). As the column temperature was increased, the resolution between these two peaks improved from 0.45 to 0.72. Table 2 summarizes the changes in resolution resulting from the increased column temperature. These results confirm that column temperature plays a critical role in the separation of semaglutide and its related impurities. Accurate control of the column temperature during analysis is essential to ensure optimal separation efficiency and reproducibility. The active column pre-heater was used to minimize temperature differences between the mobile phase and column, resulting in more stable retention time and better peak shape.[4] A column temperature of 55 °C was selected for subsequent optimization as this temperature provided the best separation and meets the temperature specifications of the column.

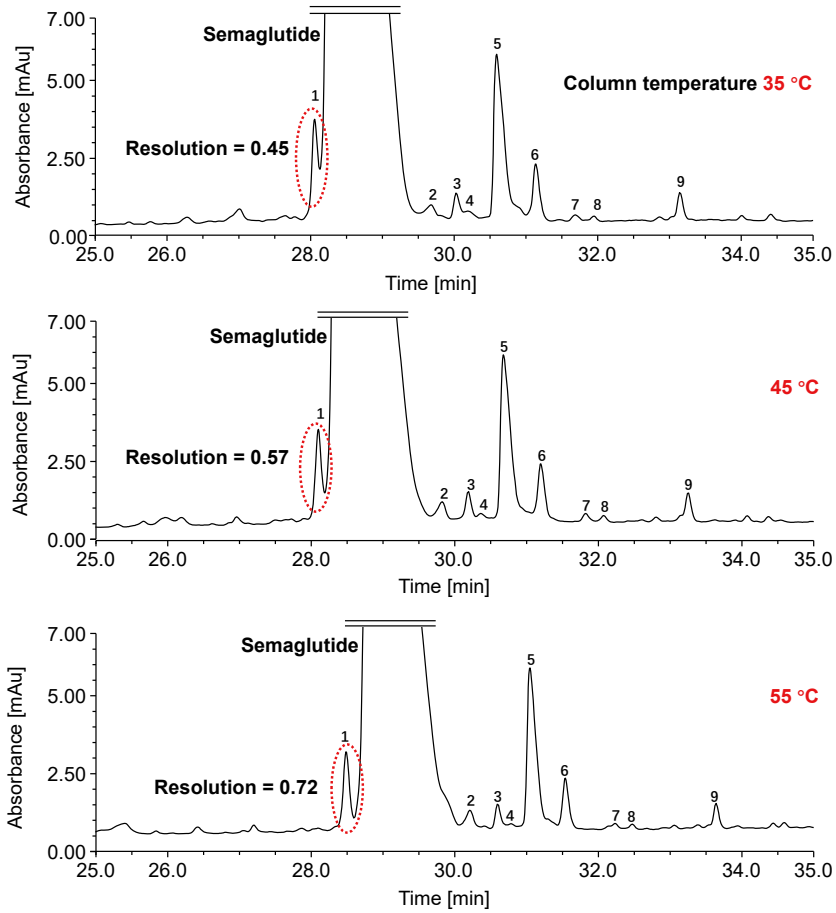


**Figure 2. UV chromatograms of the thermally stressed semaglutide sample illustrating the impact of column temperature on separation.** The labeled peaks 1–9 indicate the peak selection used to calculate the peak resolution provided in Table 2. Gradient: 25% to 60% eluent B over 40 minutes, using FA as an additive.

**Table 2. Resolution calculated based on the selected nine peaks as indicated in Figure 2 from the chromatograms obtained using column temperatures of 35 °C, 45 °C, and 55 °C, respectively.**

| Peak number | Resolution | | | Δ Resolution (45 °C–35 °C) | Δ Resolution (55 °C–45 °C) |
|---|---|---|---|---|---|
| | 35 °C | 45 °C | 55 °C | | |
| Peak 1 | 0.45 | 0.57 | 0.72 | 0.12 | 0.15 |
| Peak 2 | 1.66 | 1.97 | 2.50 | 0.31 | 0.53 |
| Peak 3 | 0.55 | 1.13 | 1.64 | 0.58 | 0.51 |
| Peak 4 | 1.11 | 1.50 | 1.62 | 0.39 | 0.12 |
| Peak 5 | 2.40 | 2.39 | 2.52 | -0.01 | 0.13 |
| Peak 6 | 3.06 | 3.68 | 4.36 | 0.62 | 0.68 |
| Peak 7 | 1.56 | 1.60 | 1.70 | 0.04 | 0.10 |
| Peak 8 | 8.10 | 7.75 | 8.96 | -0.35 | 1.21 |

Subsequently, the gradient was optimized to enhance the separation of impurities. While in reversed-phase HPLC, a shallow gradient can improve the separation of semaglutide and related impurities, it may also broaden the peaks and therefore reduce sensitivity. Figure 3 illustrates chromatograms obtained from gradients with different starting conditions (25%, 30%, 35%) and different end points (50%, 60%), while maintaining a consistent gradient run time of 40 minutes. Most efficient separation of the degradation species while maintaining good sensitivity was achieved with the gradient from 30% to 50% B (Figure 3 A2/B2).

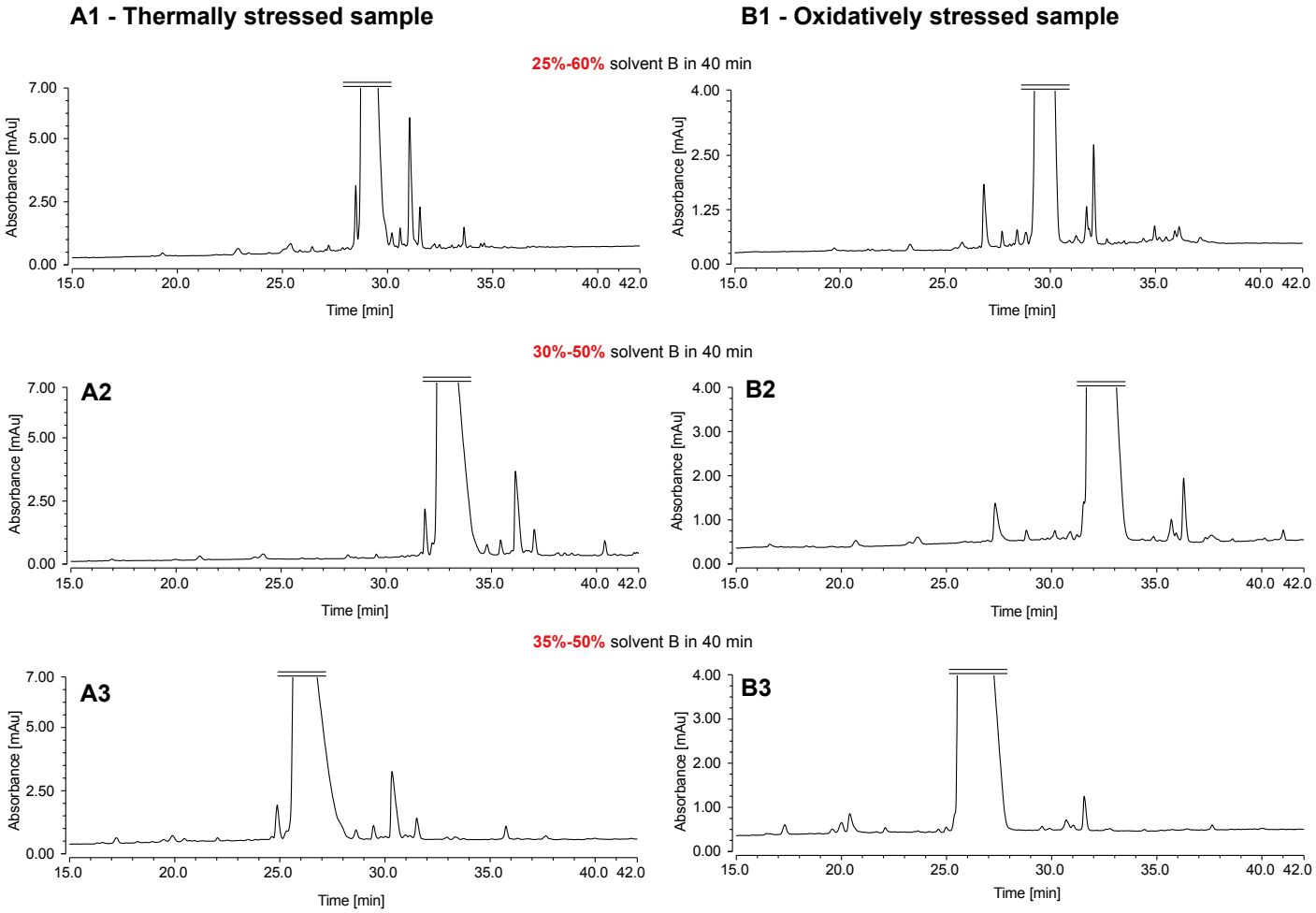


**Figure 3. UV chromatograms of the thermally stressed (A1–A3) and oxidatively stressed (B1–B3) samples, run on the Hypersil GOLD Peptide column with three different gradients.** Column temperature: 55 °C; eluent additive FA.

While TFA is not the preferred eluent additive for LC-MS analysis due to its ion suppression effects, it is well known for providing superior chromatographic resolution and peak shape due to its ability to form strong ion pairs with peptides. In this study, we also optimized the HPLC–UV method using TFA as the eluent additive, following the same optimization strategy based on different column temperatures and gradients. Also here, optimal chromatographic conditions were achieved with a column temperature of 55 °C and a gradient from 30% to 50% eluent B over 40 minutes, as illustrated in Figure 4. According to our expectations, the use of TFA as an additive resulted in superior chromatographic resolution and narrower peak widths compared to FA. Specifically, the peak width at 50% height of semaglutide decreased from 0.83 min to 0.32 min, thereby improving the sensitivity for impurity detection. Notably, one coeluting peak in the thermally stressed sample and two coeluting peaks in the oxidatively stressed sample when FA was used as an additive were successfully resolved under the TFA-based conditions, demonstrating enhanced separation. In conclusion, these findings underline that TFA serves as a more effective additive for the development of HPLC–UV methods aimed at quantitative analysis.

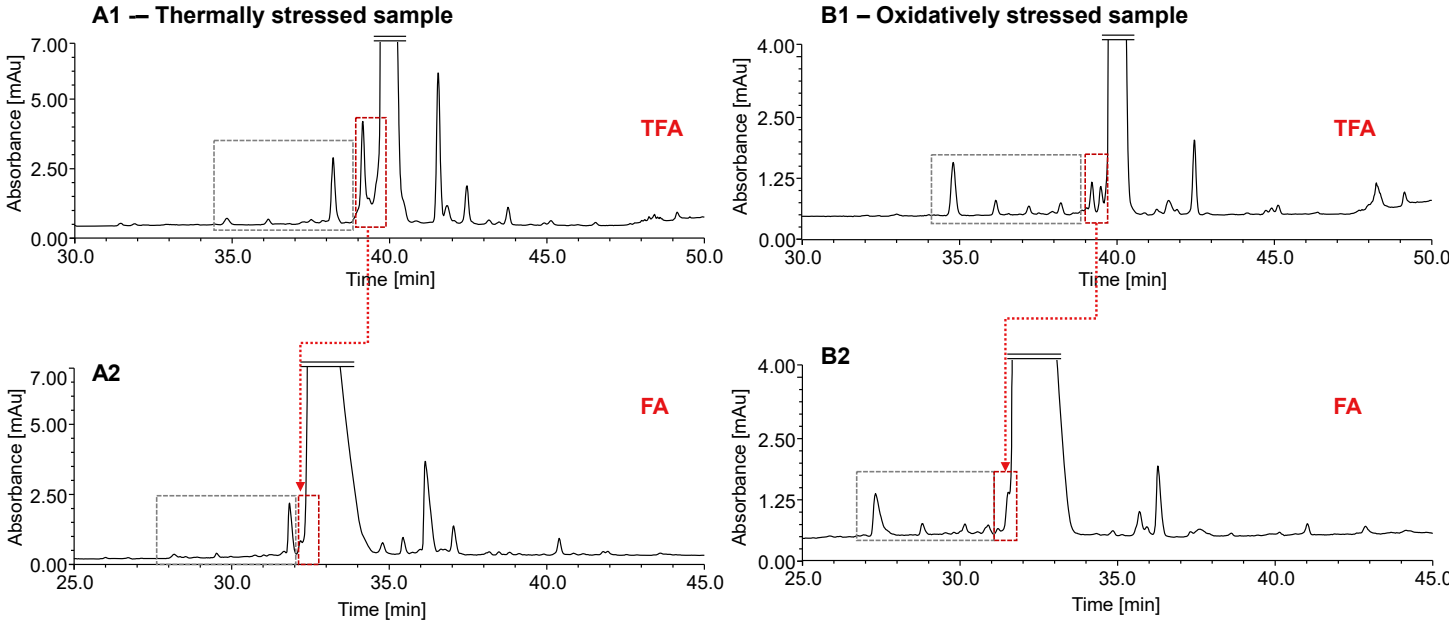


**Figure 4. UV chromatograms obtained from thermally stressed (A1, A2) and oxidatively stressed (B1, B2) samples using TFA (A1 and B1) versus FA (A2 and B2) as eluent additive.** The optimized method was applied with column temperature set to 55 °C and a gradient of 30%–50% B in 40 min. Regions boxed in red in the chromatograms indicate significant differences in separation, while the grey boxes frame preceding peaks that have shifted in retention, while separation is not impacted.

## Assessment of column lot-to-lot reproducibility

Method development is typically conducted using a limited number of column lots. To ensure long-term reproducibility across different laboratories, it is essential to utilize columns that demonstrate high lot-to-lot consistency. Therefore, column lot-to-lot reproducibility was assessed using the final optimized TFA-based method with the Hypersil GOLD Peptide column. The thermally stressed sample was analyzed through six consecutive injections on columns from three different manufacturing lots.

Based on the injection series across different column lots, relative standard deviation (%RSD) values for retention time, relative retention time (RRT) of the impurity eluting before semaglutide, peak area, peak width (50%), and peak height of the semaglutide peak were calculated. All values were ≤1.3%, as summarized in Table 3. These results confirm excellent reproducibility among the columns from different lots, demonstrating the robustness and reliability of the developed HPLC–UV method.

**Table 3. Results and %RSD of the semaglutide peak calculated from six consecutive injections of the thermally stressed sample using Hypersil GOLD Peptide columns from three different lots.**

| Column | Retention time (min) | Relative retention time (RRT) | Peak area (mAu*min) | Peak width (50%) (min) | Peak height (mAu) |
|---|---|---|---|---|---|
| Lot 1 | 40.409 | 0.984 | 17.917 | 0.325 | 51.43 |
| | 40.408 | 0.984 | 17.941 | 0.325 | 51.44 |
| | 40.417 | 0.984 | 17.951 | 0.327 | 51.24 |
| | 40.417 | 0.984 | 17.952 | 0.329 | 51.01 |
| | 40.400 | 0.984 | 17.947 | 0.328 | 51.17 |
| | 40.400 | 0.984 | 17.950 | 0.326 | 51.41 |
| Lot 2 | 40.334 | 0.984 | 17.703 | 0.329 | 50.74 |
| | 40.333 | 0.984 | 17.740 | 0.326 | 51.26 |
| | 40.333 | 0.984 | 17.740 | 0.323 | 51.64 |
| | 40.333 | 0.984 | 17.732 | 0.326 | 51.24 |
| | 40.333 | 0.984 | 17.730 | 0.326 | 51.24 |
| | 40.334 | 0.984 | 17.732 | 0.324 | 51.50 |
| Lot 3 | 40.842 | 0.984 | 17.560 | 0.316 | 52.06 |
| | 40.842 | 0.984 | 17.625 | 0.320 | 51.72 |
| | 40.850 | 0.984 | 17.619 | 0.319 | 51.79 |
| | 40.850 | 0.984 | 17.619 | 0.316 | 52.07 |
| | 40.850 | 0.984 | 17.611 | 0.319 | 51.89 |
| | 40.858 | 0.984 | 17.757 | 0.318 | 51.69 |
| %RSD | 0.58 | 0.00 | 0.83 | 1.30 | 0.70 |

## Conclusion

In this study, a stability-indicating HPLC–UV method for the analysis of semaglutide was developed. Column temperature and gradient were optimized to achieve the best separation of closely eluting peaks. Additionally, the effects of the two mobile phase additives, FA and TFA, were compared.

- The final optimized method used the Hypersil GOLD Peptide column set to 55 °C, including the use of an active column pre-heater. Separation was achieved over 40 minutes using a gradient from 30% to 50% organic solvent, with 0.1% TFA as an additive in both the aqueous and organic mobile phases.
- Column lot-to-lot reproducibility assessment using columns from three different lots demonstrated excellent consistency, with %RSD values of ≤1.3% across all critical parameters.

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