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Optimization of RPLC-UV methods for impurity analysis of the GLP-1 receptor agonists semaglutide and tirzepatide

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Keywords

GLP-1, semaglutide, tirzepatide, method development, Vanquish Flex UHPLC system, Hypersil GOLD Peptide column, Hypersil GOLD C4 column

Application benefits

- Optimized, ready-to-use reversed-phase liquid chromatography (RPLC) methods for impurity analysis of semaglutide and tirzepatide
- Systematic RPLC method development strategy for glucagon-like peptide-1 (GLP-1) receptor agonists (RAs)
- Insight into the impact of key chromatographic parameters on impurity separation to enable more efficient and robust method development

Goal

Assessment of critical chromatographic parameters on GLP-1 peptide impurity separation to derive optimized RPLC-UV methods for impurity analysis of semaglutide and tirzepatide.

Introduction

GLP-1 RAs are a class of medications used to treat type 2 diabetes and for chronic weight management. They are synthetic peptide analogues of the endogenous hormone GLP-1, structurally engineered through amino acid substitutions and fatty acid conjugation to confer resistance to dipeptidyl peptidase 4 degradation and to prolong half-life via enhanced albumin binding. In recent years, several GLP-1 RAs have been approved by the U.S. Food and Drug Administration (FDA). Due to their well-established efficacy in weight management, these agents are rapidly gaining widespread adoption. Notably, semaglutide and tirzepatide ranked among the top ten best-selling pharmaceutical products worldwide in 2025, highlighting the growing demand for these agents.¹

According to FDA guidelines for abbreviated new drug applications of highly purified synthetic peptides, peptide-related impurities must be comprehensively characterized and appropriately controlled.² Any variations in the amino acid sequence can alter the peptide's biological activity, potentially affecting its efficacy and safety. The prevalence of these drugs prompts a need for robust liquid chromatography (LC) methods to effectively analyze and control impurities that may arise during manufacturing, storage, and transportation. However, the development of LC methods for peptide impurity analysis remains challenging. Peptide-related impurities predominantly arise from amino acid deletions, insertions, substitutions, isomerization, or side-chain modifications, producing species that are structurally very similar to the target peptide. This high degree of similarity significantly challenges chromatographic separation, and consequently, no single LC method can be universally applied to impurity analysis across different GLP-1 based therapeutics.

This application note presents the development of optimized RPLC-UV methods for impurity separation of semaglutide and tirzepatide. Commonly used organic solvents and mobile phase additives for peptide separations—including methanol (MeOH), acetonitrile (ACN), formic acid (FA), and trifluoroacetic acid (TFA)—were evaluated in combination with two columns featuring distinct ligand chemistry: Thermo Scientific™ Hypersil™ GOLD C4 Column and Thermo Scientific™ Hypersil™ GOLD Peptide Column. In addition, the effects of column temperature and gradient conditions on separation were investigated and optimized. Thermally stressed samples were used to generate representative impurity profiles. The overall experimental design is summarized in Figure 1. The described workflow provides practical guidance and ready-to-use RPLC-UV methods for impurity analysis of semaglutide and tirzepatide.

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™ Methanol (HPLC)	A452-4
Fisher Chemical™ Formic acid, Optima™ LC/MS grade	A117-50
Fisher Chemical™ Trifluoroacetic acid, HPLC grade, ≥99.5%	AA44630AP

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™ Finnpiptette™ F1 Variable Volume Pipettes	4641100N, 4641070N
Thermo Scientific™ Thermal Mixer	13687711
Thermo Scientific™ Basic Vortex Mixers	88882012

Instrumentation

Module	Cat. No.
Thermo Scientific™ Vanquish™ Flex UHPLC System consisting of:	
Thermo Scientific™ Vanquish™ System Base	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™ Diode Array Detector FG	VF-D11-A-01
Thermo Scientific™ Vanquish™ Standard Bio Flow cell, 11 µL, 10 mm	6077.0200

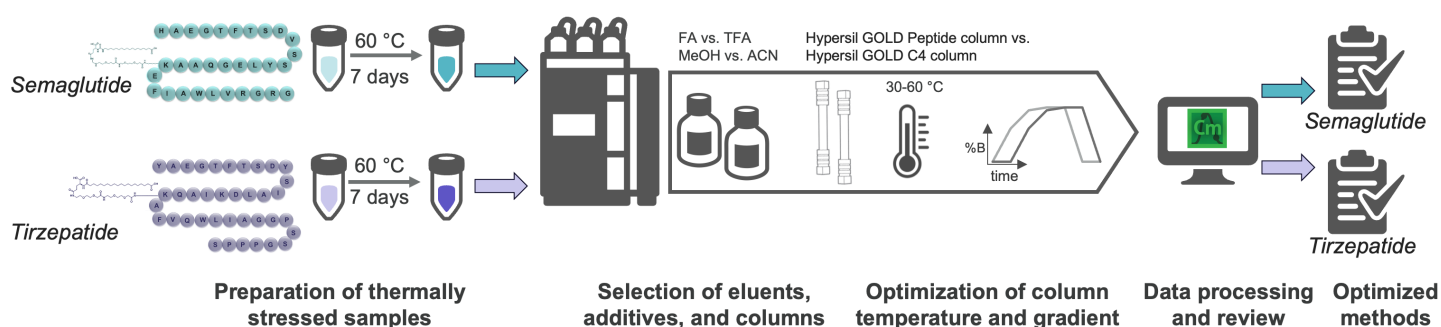


Figure 1. Overview of RPLC-UV method development for impurity analysis of semaglutide and tirzepatide.

Sample preparation

Semaglutide and tirzepatide active pharmaceutical ingredients (APIs, purity >99.0%) were purchased from Shandong Mopai Biotechnology Co., Ltd.

For each API, 5.0 mg was weighed and transferred into an individual amber glass vial and dissolved in 5 mL of diluent (20% acetonitrile in water, v/v). The vials were vortexed for 5 min to obtain homogenous API solutions with a concentration of 1.0 mg/mL.

The sample vials were incubated at 60 °C with shaking at 50 rpm for 7 days to generate thermally stressed samples.

For all experiments in this study, 5 µL of thermally stressed samples were used per injection, corresponding to 5 µg of API.

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3.2 was used for data acquisition and processing.

Chromatographic conditions

Table 1. Summary of LC conditions evaluated for column temperature optimization and mobile phase, additive, and column selection.

Column	Hypersil GOLD Peptide, 150 X 2.1 mm, 1.9 µm (Cat. No. 26002-152130) Hypersil GOLD C4, 150 X 2.1 mm, 1.9 µm (Cat. No. 25502-152130)			
Mobile phases	A: 0.1% FA in water; B: 0.1% FA in ACN A: 0.1% FA in water; B: 0.1% FA in MeOH A: 0.1% TFA in water; B: 0.1% TFA in ACN A: 0.1% TFA in water; B: 0.1% TFA in MeOH			
Gradient	Time (min)	%B (ACN-based methods)	Time (min)	%B (MeOH-based methods)
	-5	5	-5	5
	0	5	0	5
	2	30	2	60
	37	65	37	90
	39	95	39	95
	44	95	44	95
	45	5	45	5
	50	5	50	5
Flow rate	0.3 mL/min			
Column temperature	30 °C, 40 °C, 50 °C, 60 °C, with active pre-heater, still air mode			
Autosampler temperature	5 °C			
Needle wash solution	10/90 MeOH/water (v/v)			
Needle wash mode	Before draw			
Injection volume	5 µL			
DAD settings	Wavelength: 280 nm, data collection rate: 2.0 Hz, response time: 2.0 s			

Results and discussion

Column and eluent selection

As an initial step in method development, a systematic evaluation of stationary phase chemistry and mobile phase composition was performed to identify suitable conditions for chromatographic optimization. Two columns with distinct ligand chain length and hydrophobicity, the Hypersil GOLD C4 column and the Hypersil GOLD Peptide column, were selected for comparison. In parallel, two commonly used organic solvents for RPLC, MeOH and ACN, together with two mobile phase additives, FA and TFA, were evaluated for the separation of semaglutide and tirzepatide. All screening experiments were conducted using fixed gradient conditions specific to the organic solvent employed, as listed in Table 1, with a column temperature of 60 °C, to enable direct comparison of chromatographic performance.

Table 2. Final optimized method parameters for impurity analysis of semaglutide and tirzepatide.

TFA-based methods (same gradient but different column temps.)			FA-based methods (same column temp. but different gradients)			
Semaglutide		Tirzepatide	Semaglutide		Tirzepatide	
Column	Hypersil GOLD C4, 150 X 2.1 mm, 1.9 µm (Cat. No. 25502-152130)		Hypersil GOLD Peptide, 150 X 2.1 mm, 1.9 µm (Cat. No. 26002-152130)		Hypersil GOLD C4, 150 X 2.1 mm, 1.9 µm (Cat. No. 25502-152130)	
Column temperature	30 °C	60 °C	60 °C			
With active pre-heater, still air mode						
Mobile phase	A: 0.1% TFA in water B: 0.1% TFA in MeOH		A: 0.1% FA in water B: 0.1% FA in ACN			
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	-5	5	-5	5	-5	5
	0	5	0	5	0	5
	2	60	2	35	2	40
	42	75	42	50	52	50
	44	95	44	95	54	95
	45	95	45	95	55	95
	46	5	46	5	56	5
	50	5	50	5	60	5
Flow rate	0.3 mL/min					
Autosampler temperature	5 °C					
Needle wash solution	10/90 MeOH/water (v/v)					
Needle wash mode	Before draw					
Injection volume	5 µL					
DAD settings	Wavelength: 280 nm, data collection rate: 2.0 Hz, response time: 2.0 s					

The performance of the column and eluent combinations evaluated for each analyte is summarized in Figures 2 and 3 for semaglutide and tirzepatide, respectively. Performance was assessed using a ranking system based on the number of detected impurity peaks with relative peak areas greater than 0.1%. For equivalent peak counts, the peak shape (peak asymmetry) of the API peak and the resolution between the API peak and the adjacent impurity peaks were considered.

Based on these rankings, the choice of combinations could be effectively reduced for the subsequent gradient optimization while maintaining a high likelihood of achieving adequate separation. For semaglutide and tirzepatide, both ranking 1 conditions applied the Hypersil GOLD C4 column with MeOH–TFA, yielding total relative impurity peak areas of 14.2% and 20.2%, respectively (sum of all impurity peak areas calculated as percentage of total peak area from all integrated peaks in the chromatogram). These top-ranked conditions yielded higher

total relative impurity areas than the other evaluated conditions, confirming the suitability of the selected conditions for further method optimization.

A comparison of the separation performance obtained on the two columns indicates that the mobile phase composition, including the additives, was the primary factor governing the selectivity of GLP-1 peptides, whereas the column ligand functionality played a less significant role. Under identical mobile phase conditions, the C4 column exhibited weaker retention, particularly when MeOH was used as the organic solvent, which is likely attributed to its shorter ligand length. No clear advantage of one organic solvent over the other could be seen, as the performance of separation was highly dependent on the specific combination of column, mobile phase, and analyte. In contrast, for the acidic modifier, the use of TFA resulted in a marked improvement in peak shape and resolution compared to FA.

Semaglutide

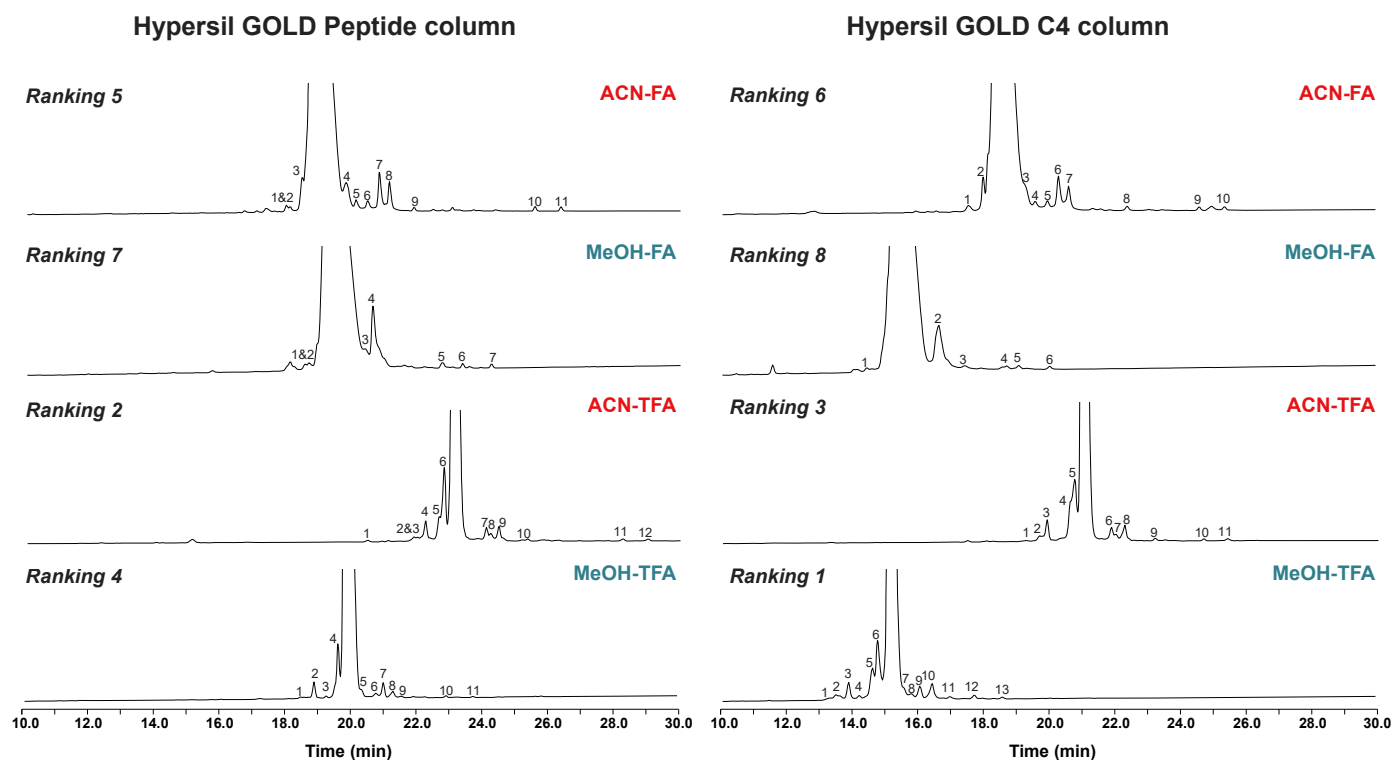


Figure 2. Chromatograms obtained from the degraded semaglutide sample using different combinations of columns and mobile phases. The applied LC conditions are listed in Table 1.

Tirzepatide

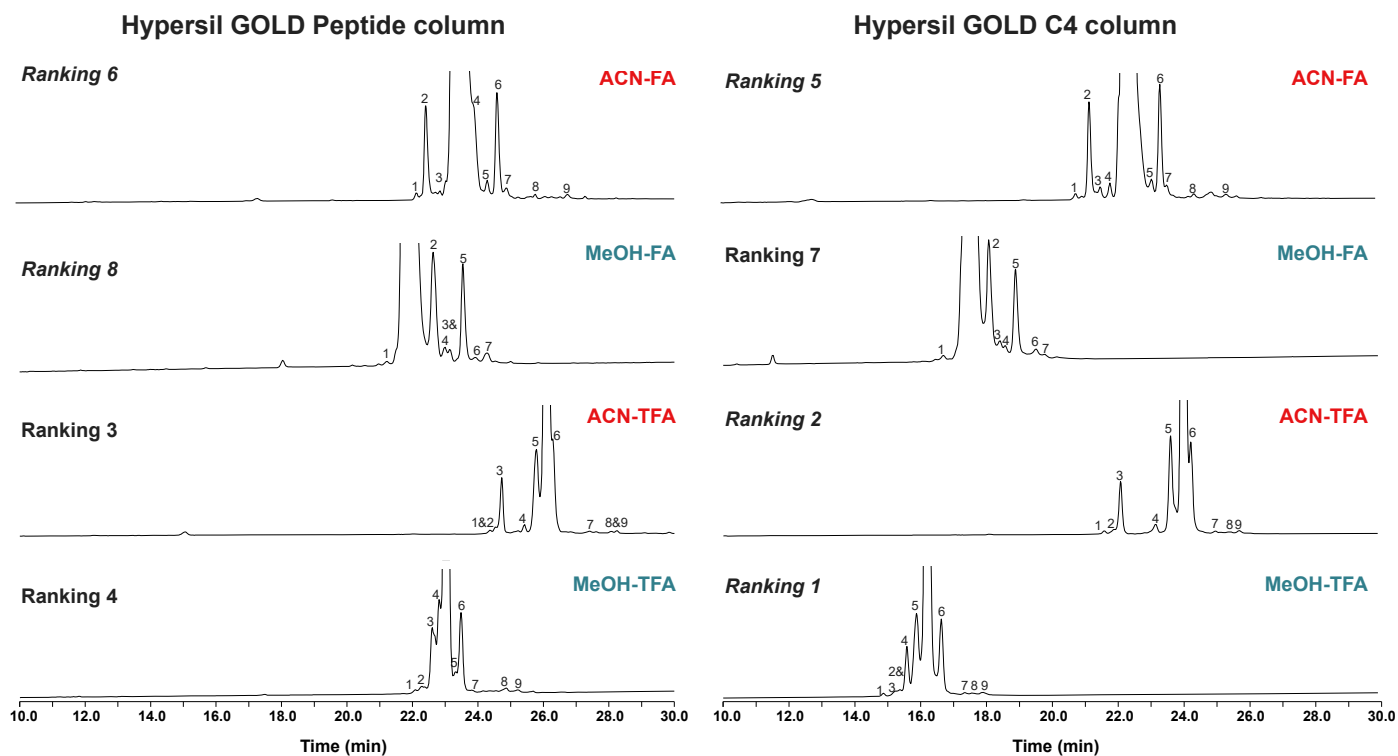


Figure 3. Chromatograms obtained from the degraded tirzepatide sample using different combinations of columns and mobile phases. The applied LC conditions are listed in Table 1.

The results demonstrate that minor structural differences between semaglutide and tirzepatide result in different chromatographic behavior. Under identical conditions, tirzepatide exhibited longer retention on both columns. Furthermore, the ranking of column–mobile phase combinations varied depending on the samples. These findings indicate that method optimization must be compound-specific, and a universal platform method cannot be directly applied to different GLP-1 RAs. Therefore, chromatographic conditions should be tailored to each analyte, and systematic comparison of columns and mobile phases represents a critical first step in LC method development.

Effect of column temperature

For RPLC, the separation of different compounds often depends on column temperature, as temperature changes can substantially affect the selectivity, even if other parameters are unchanged. Column temperature was evaluated at 30 °C, 40 °C, 50 °C, and 60 °C for all screened method combinations, as temperature-dependent effects on retention and resolution varied across the tested mobile phase–column combinations. The results showed that, in most cases, increasing the column temperature significantly improved the resolution of impurity peaks for both semaglutide and tirzepatide, as illustrated in Figure 4. Two representative combinations of mobile phases and columns were selected to illustrate the separation changes.

As the column temperature increased from 30 °C to 60 °C, a clear improvement in the resolution of the major impurity peaks was observed, and two impurities that co-eluted at lower temperatures became resolved at 60 °C for tirzepatide.

However, this trend was not universal, and under a few conditions (e.g., semaglutide on the Hypersil GOLD C4 column with TFA-containing mobile phases), increased temperature resulted in reduced resolution, as shown in Figure 5, where two impurities co-eluted when the column temperature rose from 30 °C to 60 °C. These observations highlight the importance of column temperature optimization during method development; an appropriate column temperature should be selected for each combination of column and mobile phase before subsequent gradient optimization.

A pronounced difference in temperature-dependent retention behavior was observed between ACN and MeOH-based mobile phases. With MeOH-based mobile phases, retention times decreased noticeably as column temperature increased, whereas with ACN-based mobile phases, retention times increased slightly with increasing temperature, as shown in Figures 4 and 5. This behavior was consistently observed across the other column and mobile phase combinations evaluated in this study for both semaglutide and tirzepatide.

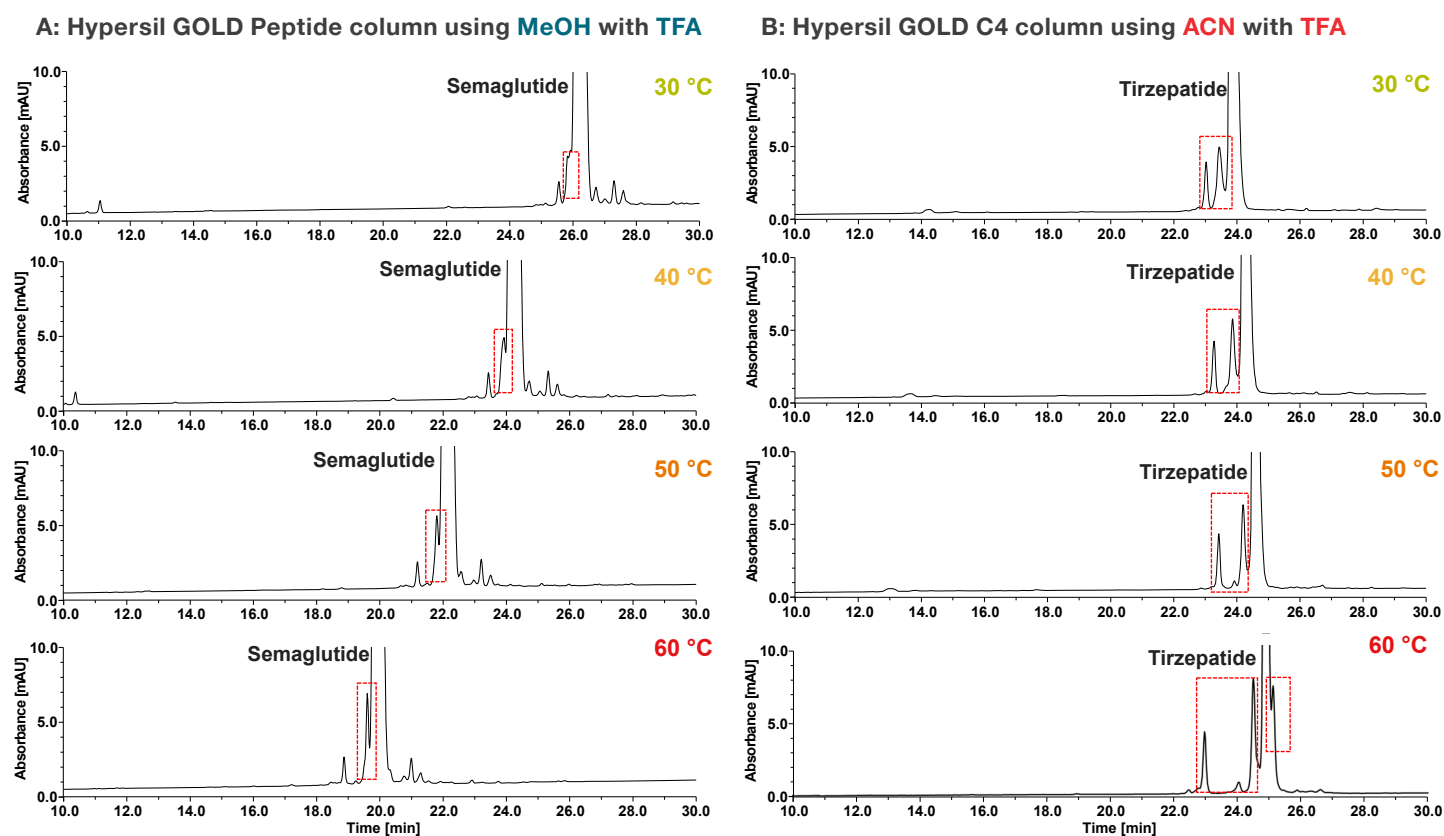


Figure 4. Chromatograms illustrating the effect of column temperature on the chromatographic separation of semaglutide (A) and tirzepatide (B) samples. Peaks boxed in red highlight temperature-dependent changes in resolution.

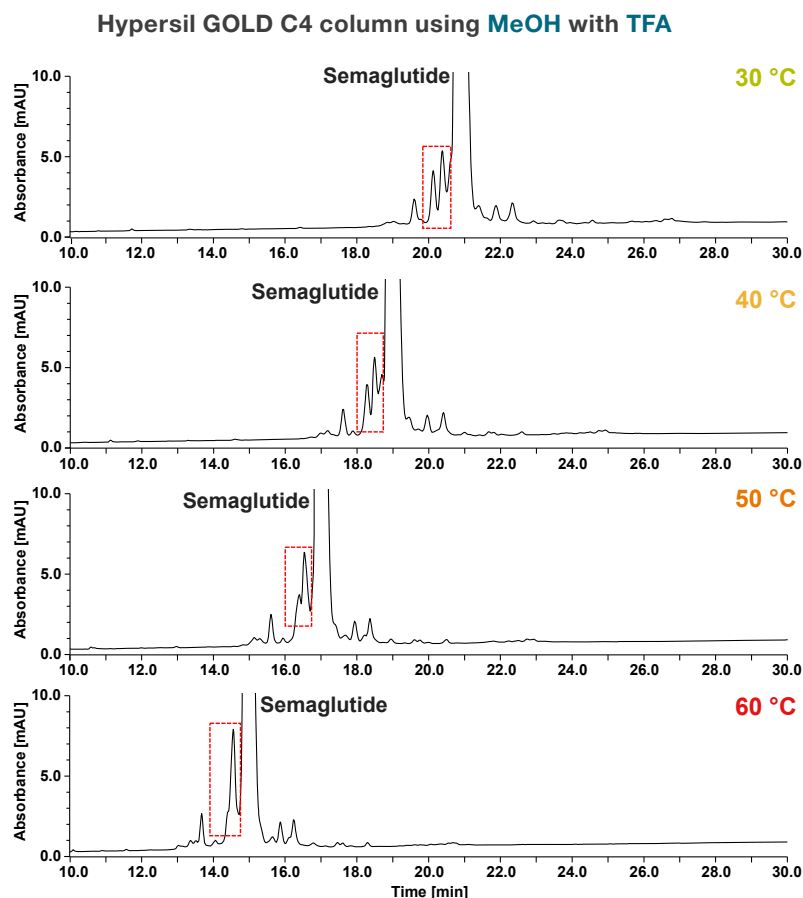


Figure 5. Chromatograms illustrating the effect of column temperature on the separation of the semaglutide sample on a Hypersil GOLD C4 column. Peaks boxed in red highlight temperature-dependent changes in resolution.

The opposite trends observed with MeOH and ACN are likely related to differences in solvent–peptide interactions and temperature-induced changes in peptide conformation, particularly for fatty acid–modified GLP-1 RAs.^{3,4}

Gradient optimization

For the column and mobile phase screening experiments described above, fixed generic gradients specific to the organic solvent were initially applied to different columns and GLP-1 RAs to enable direct comparison of performance. After suitable combinations of column, mobile phase, and column temperature were identified, gradient optimization was performed as the final step to further enhance impurity resolution. Different organic solvent compositions, shallower gradients, and extended gradient times were investigated to identify the most suitable gradient conditions for impurity separation in this step.

For semaglutide and tirzepatide, one top-ranked condition employing TFA-containing mobile phases and one top-ranked condition employing FA-containing mobile phases, listed in

Table 3, were selected for gradient optimization. Despite the generally lower rankings, FA-containing mobile phases were retained in the experimental optimization steps due to their compatibility with mass spectrometry (MS), which can complement the TFA-based methods by enabling subsequent MS-based impurity analysis.

Figures 6 and 7 show the final optimized results using FA and TFA for semaglutide and tirzepatide, respectively. Compared to the initial screening conditions, the optimized gradients significantly improved impurity resolution and enabled the detection of additional species that were not apparent previously. Notably, several additional co-eluted peaks were observed after gradient optimization, highlighting its importance during late-stage method development. The total relative impurity area increased by 1.5% for semaglutide and 2.1% for tirzepatide for TFA-based methods after gradient optimization, indicating that additional impurities were resolved from the API. The final optimized method conditions are listed in Table 2.

Table 3. Mobile phase and column combinations used for gradient optimization.

Sample	Column	Column temperature	Mobile phase additive	Organic phase
Semaglutide	Hypersil GOLD C4	30 °C	TFA	MeOH
	Hypersil GOLD Peptide	60 °C	FA	ACN
Tirzepatide	Hypersil GOLD C4	60 °C	TFA	MeOH
	Hypersil GOLD C4	60 °C	FA	ACN

Hypersil GOLD Peptide column using ACN with FA

Hypersil GOLD C4 column using MeOH with TFA

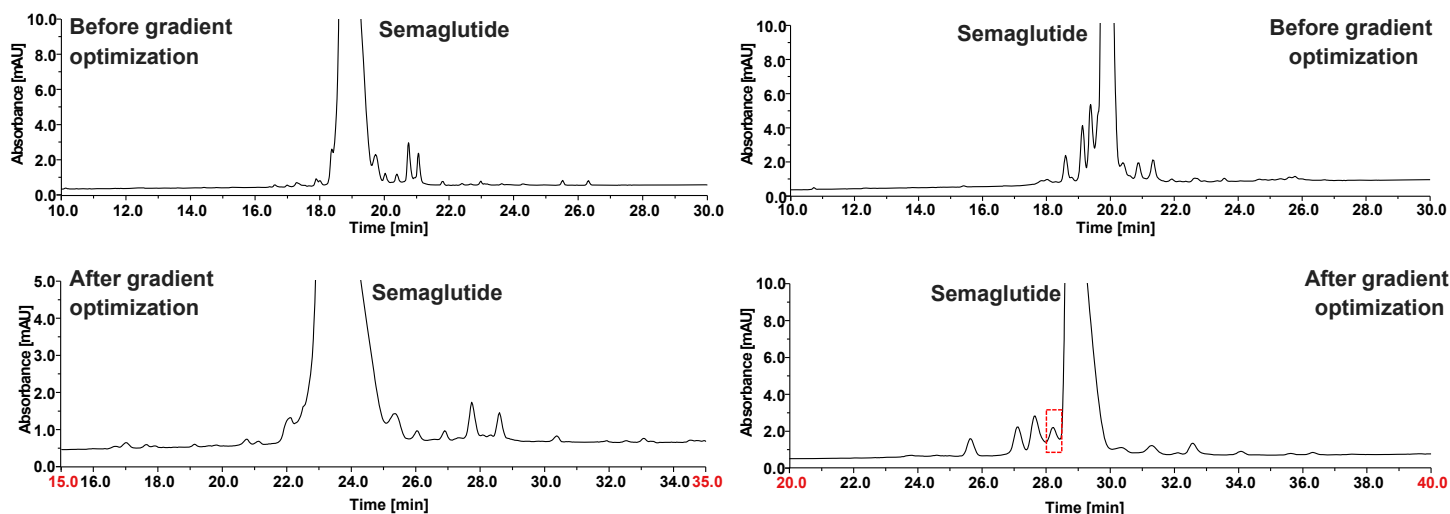


Figure 6. Chromatograms before and after gradient optimization for semaglutide. The area boxed in red highlights improved peak separation after gradient optimization. The final optimized method parameters are listed in Table 2. Red time-axis labels indicate that these panes represent different time ranges, so peaks remain centered.

Hypersil GOLD C4 column using ACN with FA

Hypersil GOLD C4 column using MeOH with TFA

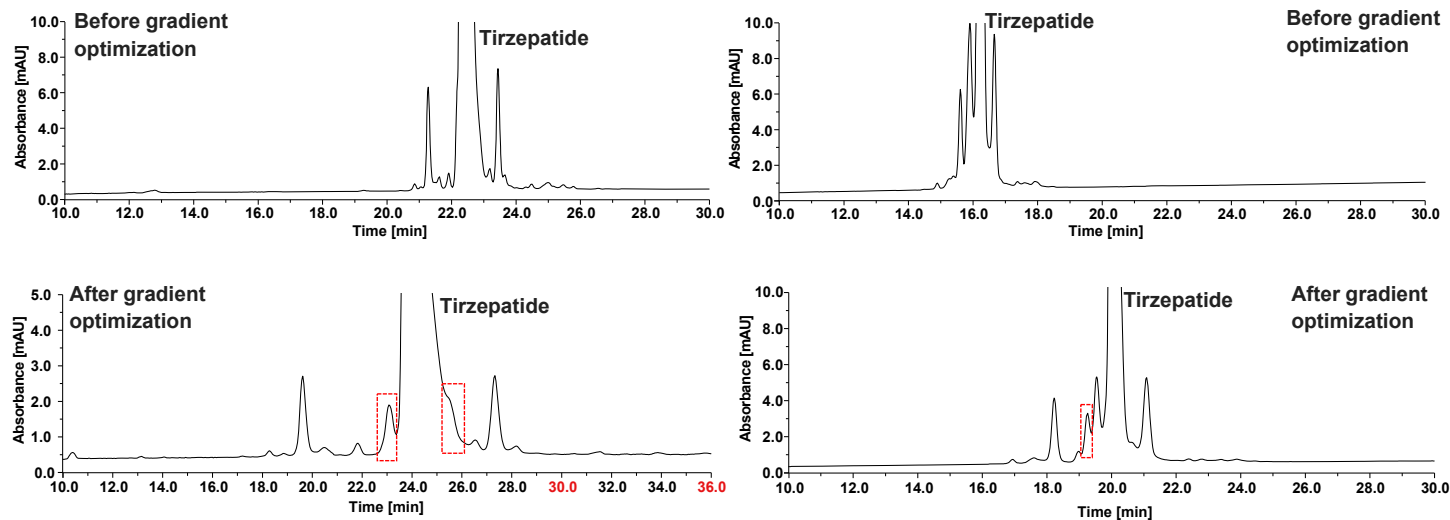


Figure 7. Chromatograms before and after gradient optimization for tirzepatide. Peaks boxed in red highlight improved peak separation after gradient optimization. The final optimized method conditions are listed in Table 2. Red time-axis labels indicate that this pane represents a different time range, so peaks remain centered.

Conclusion

In this study, RPLC-UV methods for the impurity analysis of semaglutide and tirzepatide were successfully developed using Hypersil GOLD C4 and Hypersil GOLD Peptide columns. The effects of stationary phase chemistry, organic modifier (MeOH vs. ACN), and acidic additives (FA vs. TFA) were systematically evaluated. Further optimization of column temperature and gradient conditions significantly improved separation performance. In addition, this study provides practical guidance to support method development for GLP-1 RA analysis.

- Optimal separation conditions—including column selection and mobile phase composition—differed between GLP-1 RAs, underscoring the need for compound-specific method optimization.
- Mobile phase composition was the primary driver of GLP-1 peptide selectivity, whereas differences in column ligand functionality had a comparatively smaller impact.
- Increasing column temperature generally improved resolution; however, for a few combinations, elevated temperatures resulted in reduced resolution.
- TFA-based mobile phases provided improved peak shape and chromatographic performance, while FA-based conditions offer compatibility with complementary LC-MS impurity analysis workflows.

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