Application Note

Biopharma

Evaluation of Chromatographic Parameters Influencing GLP-1 Peptide Impurity Profiling

Using the Agilent Altura Peptide Plus column and the Agilent 1260 Infinity III Prime Bio LC system

Authors

Shawn Ehlers-Cheang,
Randall Robinson,
Oscar Potter,
Ace Gita Galermo,
Mohammad Aref Khalily,
Charu Kumar, Chenchen He,
Ta-Chen Wei, and Jordy Hsiao
Agilent Technologies, Inc.

Abstract

The rapid growth of glucagon-like peptide-1 receptor agonists (GLP-1 RAs) has increased the demand for robust analytical methods for accurately separating and quantifying closely related impurities produced during synthesis and degradation pathways, including amino acid sequence variants, oxidation products, and stereoisomers. As these impurities often differ only slightly from the active pharmaceutical ingredient (API), high-resolution chromatographic separation is essential by both liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography with ultraviolet detection (LC-UV) analysis. This application note presents the rapid development of reversed-phase liquid chromatography (RPLC) methods for GLP-1 RAs such as semaglutide, liraglutide, tirzepatide, and their related impurities. The workflow combines the Blend Assist function of the Agilent 1260 Infinity III Prime Bio LC system with a biocompatible flow path, coupled with the Agilent InfinityLab Pro iQ Plus, and the Agilent Altura Peptide Plus column. Key parameters, including trifluoroacetic acid (TFA) and formic acid (FA) concentration as mobile-phase additives, sample loading amount, and column compartment temperatures, were systematically evaluated to improve separation selectivity and chromatographic performance.

Introduction

GLP-1 RAs are a rapidly growing class of peptide therapeutics used for the treatment of type 2 diabetes and obesity. The traditional peptide-based molecules in this class typically consist of 30 to 40 amino acids and often incorporate structural modifications, such as fatty acid conjugation for extending half-life and improving dosing convenience.[1,2] As a result of synthesis and degradation pathways, GLP-1 RAs can contain a variety of impurities.

Accurate characterization and quantification of these impurities present significant analytical challenges. Many GLP-1-related impurities differ from the API by only a single amino acid substitution, truncation, or stereochemical change, resulting in highly similar physicochemical properties and in some cases, identical molecular masses.[3] Therefore, high-resolution chromatographic separation is critical for their reliable detection, identification, and quantification by LC-UV or LC/MS analysis.

While RPLC is widely used for peptide analysis, GLP-1 peptides are susceptible to nonspecific interactions with metal surfaces in conventional LC systems and columns. These interactions can negatively impact analyte recovery, peak shape, and method reproducibility.[4] Therefore, the use of biocompatible instrumentation and inert column hardware can help minimize these effects and improve overall chromatographic performance.

In this study, an optimized RPLC method was developed using an Agilent 1260 Infinity III Prime Bio LC system, an Agilent InfinityLab Pro iQ Series single quadrupole mass detector, and an Agilent Altura Peptide Plus column featuring Ultra Inert technology. The objective of this study is to systematically investigate how mobile-phase additive concentration, column compartment temperatures, and sample loading influence the chromatographic resolution and overall separation performance of GLP-1 RAs (semaglutide, liraglutide, and tirzepatide) and their related impurities.

Experimental

Chemicals and standards

- Trifluoracetic acid, (LC/MS grade), Fisher Chemical (A116-50)
- Formic acid, 98% (LC/MS grade), Fisher Chemical (A117-50)
- Agilent InfinityLab Acetonitrile for LC/MS, 1x1 liters (part number 5191-5101-001)
- Milli-Q Ultrapure Water
- Semaglutide, Cayman Chemical (40231)
- Liraglutide, Cayman Chemical (24727)
- Tirzepatide, Cayman Chemical (39748)

Sample preparation

Purified semaglutide, liraglutide, and tirzepatide stock solutions were prepared at a concentration of 0.6 g/L in a diluent consisting of 40% dimethyl sulfoxide (DMSO) and 60% of 5% acetonitrile in 0.1% TFA. Each GLP-1 stock solution was subsequently spiked with its corresponding impurity standards to yield a final impurity level of 20% (relative to the API).

Analytical column

Agilent Altura Peptide Plus column, 2.1 × 150 mm, 2.7 µm (part number 227215-903)

Instrumentation

The Agilent 1260 Infinity III Prime Bio LC system comprised the following modules:

- Agilent 1260 Infinity III Flexible Pump (part number G7131A)
- Agilent 1290 Infinity III Bio Multisampler (part number G7137A) with Sample Thermostat (option #101)
- Agilent 1260 Infinity III Multicolumn Thermostat (part number G7116B) equipped with Agilent InfinityLab Quick Connect Heat Exchanger 1290 Bio Standard Flow (option #065)
- Agilent 1260 Infinity III Diode Array Detector HS (part number G4212A) with Agilent InfinityLab Bio-Inert Max-Light Cartridge Cell, 10 mm (part number G5615-60018)
- Agilent InfinityLab Pro iQ Plus (part number G6170A)

Software and data processing

- Agilent OpenLab CDS (version 2.8)

Table 1. Agilent 1260 Infinity III Bio Flexible Pump LC parameters.

Parameter	Value
Instrument	Agilent 1260 Infinity III Bio Flexible Pump LC with DAD (Agilent InfinityLab Bio-inert MaxLight cartridge cell) and InfinityLab Pro iQ Plus
Columns	Agilent Altura Peptide Plus column, 120 Å, 2.1 × 150 mm, 2.7 µm (p/n 227215-903)
Mobile Phase	A) Water B) Acetonitrile C) 1% TFA in water D) 1% TFA in acetonitrile
Flow Rates	0.50 mL/min
Gradient Program	Time %B 0 30 2.0 30 39.0 52 43.0 80 44.0 80 44.5 30 50.0 30
Stop Time	50.00 min
Post Run	0.0 mins
Column Temperature	40, 50, 60, 70, 80 °C
Injection Volume	1.0 µL
Autosampler Temperature	10 °C
DAD Flow Cell	Agilent InfinityLab Bio-inert Max-Light cartridge cell, 10 mm
DAD Settings	Signal measured 220 nm (liraglutide) or 280 nm (semaglutide and tirzepatide) Bandwidth/slit: 4 nm Reference wavelength 360 nm with bandwidth 100 nm Acquisition rate: 2.5 Hz

Table 3. SIM transitions.

Peptide	$[M+3H]^{3+}$ (*m/z*)	Dwell (ms)	Frag (V)	Gain
Liraglutide D-allo-Thr(13)-Liraglutide	1,251.4	50	125	1
Kyn(31)-Liraglutide	1,252.8	50	125	1
Liraglutide-Formaldehyde Adduct	1,255.4	50	125	1
Des-His(1)-Semaglutide	1,326.3	50	125	1
Semaglutide D-Ser(8)-Semaglutide D-Asp(9)-Semaglutide D-His(1)-Semaglutide	1,372.1	50	125	1
Tirzepatide D-Asp(15)-Tirzepatide	1,605.2	50	125	1
Glu(24) Deamidation-Tirzepatide	1,605.5	50	125	1
Endo-Gly(4)-Tirzepatide	1,624.5	50	125	1
Des-Tyr(1)-Tirzepatide	1,551.1	50	125	1
Des-γ-Glu Tirzepatide	1,562.3	50	125	1
Des-AEEA4'-Tirzepatide	1,556.7	50	125	1

Table 2. Agilent InfinityLab Pro iQ Plus parameters.

Parameter	Value
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Gas Flow	13 L/min
Nebulizer	24 psi
Sheath Gas Flow	12 L/min
Capillary Voltage	5,500 V
Nozzle Voltage	400 V
Gas Temperature	270 °C
Sheath Gas Temperature	200 °C

Results and discussion

Optimizing the mobile phase flow rate and gradient is normally a critical step when developing methods for GLP-1 RA impurity analysis. However, the flow rate and gradient were held constant for the purpose of this study, enabling easier comparison across different GLP-1 RAs, temperatures, and mobile-phase additives.

Impact of mobile-phase additives on the separation of GLP-1 RA impurities

Compared to FA, TFA produced differences in retention, selectivity, and peak shape for all GLP-1 RAs evaluated. In general, TFA increased analyte retention and improved peak shape, particularly for semaglutide and liraglutide (Figure 1), resulting in improved impurity resolution in several critical separations.

For semaglutide, the separation between the API and D-His(1)-semaglutide was substantially improved when TFA was used as the mobile-phase additive (Figure 1A). Similarly, liraglutide analyses performed with FA exhibited lower selectivity toward several impurities, resulting in reduced chromatographic resolution compared to TFA-containing mobile phases (Figure 1B).

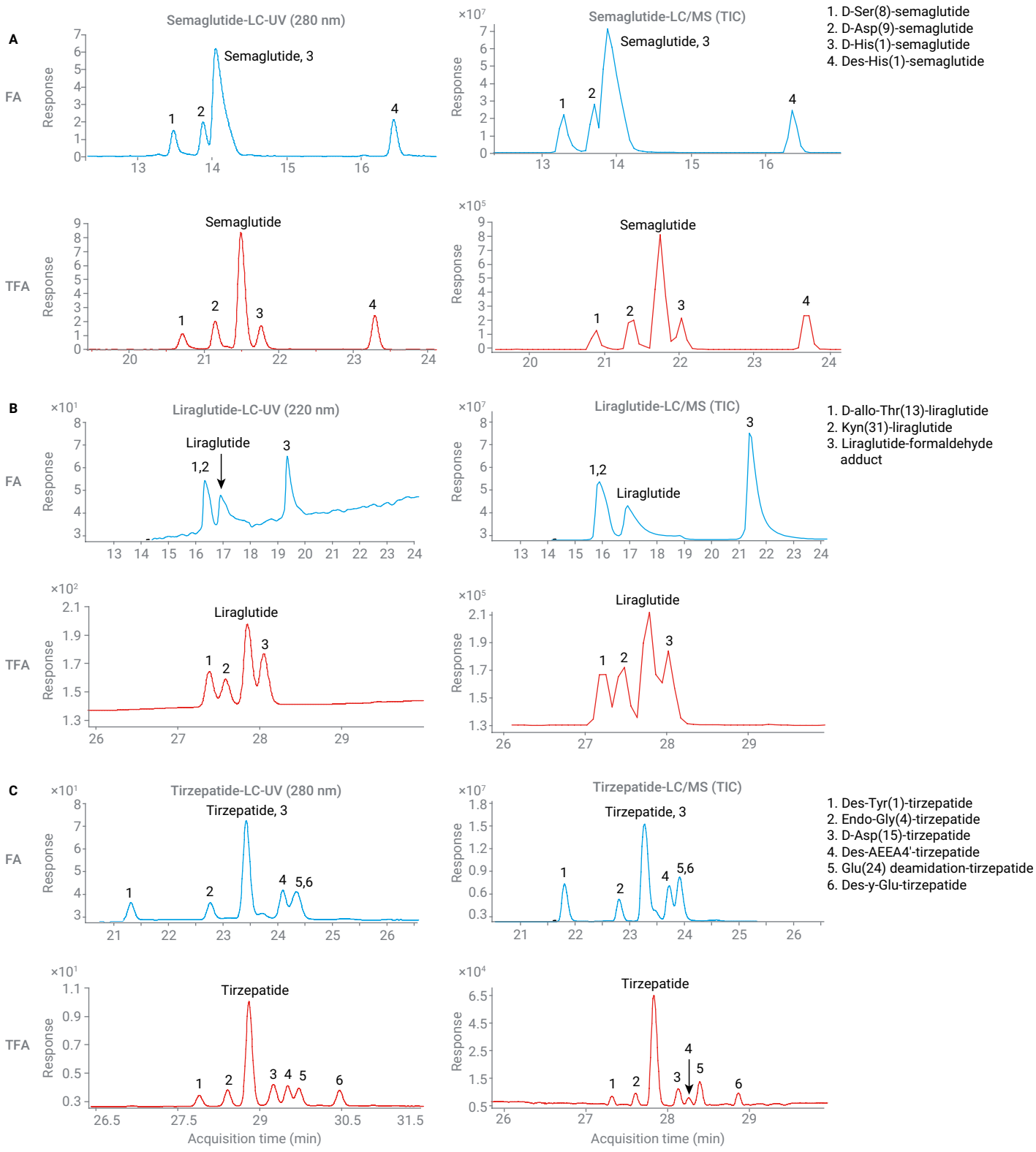


Figure 1. Chromatographic comparison of (A) semaglutide, (B) liraglutide, (C) tirzepatide, and related impurities using 0.1 % formic acid (FA) and 0.1% trifluoroacetic acid (TFA) as mobile-phase additives with column temperature at 60 °C.

In the case of tirzepatide, FA provided increased separation between the Des-Tyr(1) and Endo-Gly(4) impurities. However, TFA provided improved resolution for several other impurity classes, including stereoisomers, deamidated, and degraded impurities (Figure 1C).

Effect of TFA concentration on GLP-1 RA impurity separation

To evaluate the influence of TFA concentration on GLP-1 RA separations, the Blend Assist functionality of the 1260 Infinity III PrimeBio LC was used to generate mobile phases containing TFA concentrations ranging from 0.05% to 0.5% (v/v) (Figure 2). This approach enabled rapid and reproducible screening of additive concentrations without the need to prepare multiple mobile-phase bottles manually.

The results showed that TFA concentration significantly influenced retention, selectivity, and impurity resolution for all GLP-1 RAs evaluated (Figure 3). As TFA concentration increased, retention generally increased for both the APIs and their related impurities. These changes in retention altered impurity selectivity and resolution, demonstrating that TFA concentration is a critical variable during GLP-1 method development.

For semaglutide, increased TFA concentration improved the separation of several impurities. The most notable improvement was observed for D-His(1)-semaglutide, an isobaric impurity that is particularly challenging to chromatographically resolve from the API (Figure 3A).

In contrast, liraglutide exhibited reduced impurity resolution and lower signal intensity at elevated TFA concentrations. The best overall chromatographic performance was observed at 0.05% TFA, which provided the most favorable balance of resolution, peak shape, and detector response (Figure 3B).

Similarly, tirzepatide achieved the best overall impurity separation at 0.1% TFA (Figure 3C). Although increasing TFA concentration had limited impact on peak shape, resolution of critical impurities, including D-Asp(15), Des-AEEA4', and Glu(24), decreased as TFA concentration increased.

These results demonstrate that optimal TFA concentration is dependent upon both the GLP-1 analog and the impurity profile under investigation. Therefore, additive concentration should be systematically evaluated during method development.

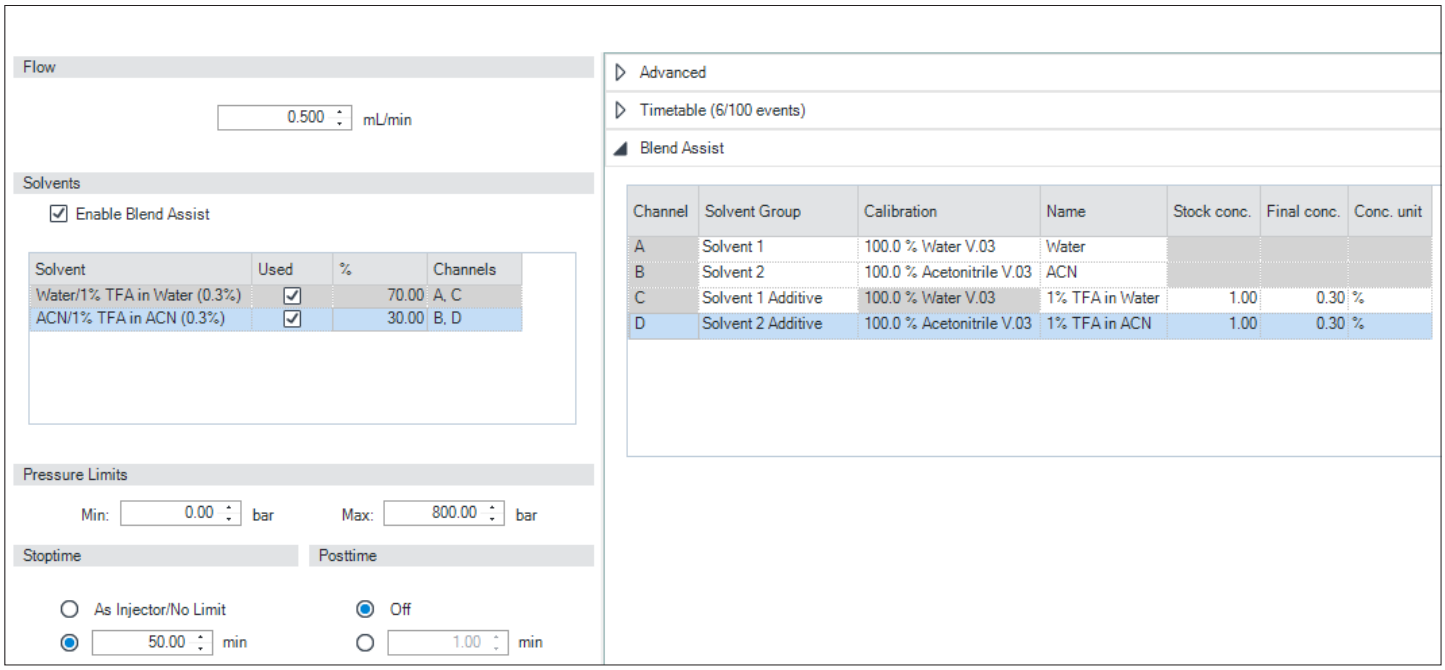
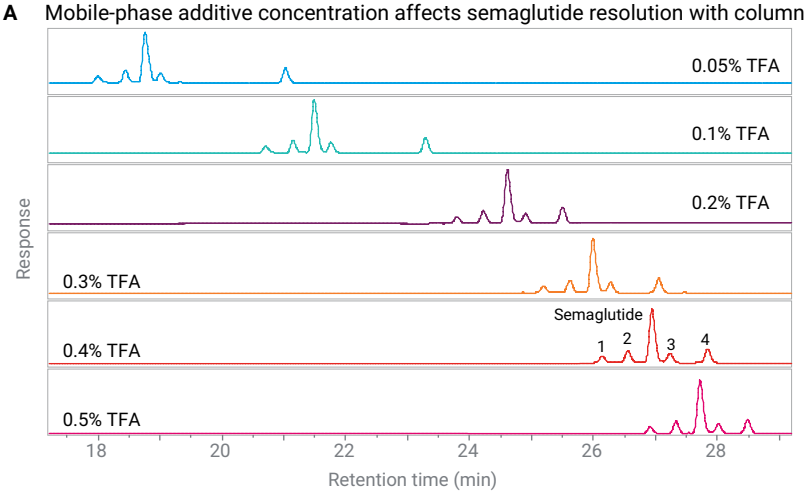


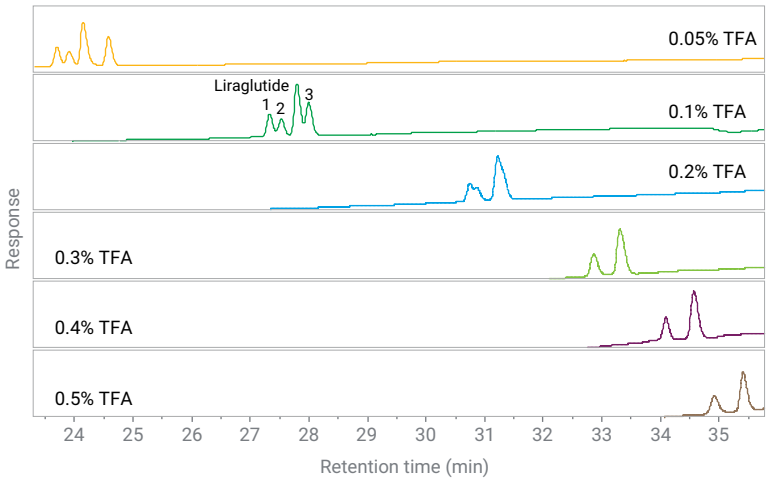
Figure 2. Example of Blend Assist settings for configuring the 0.3% TFA condition using 1% TFA.

A Mobile-phase additive concentration affects semaglutide resolution with column compartment temperature at 60 °C.



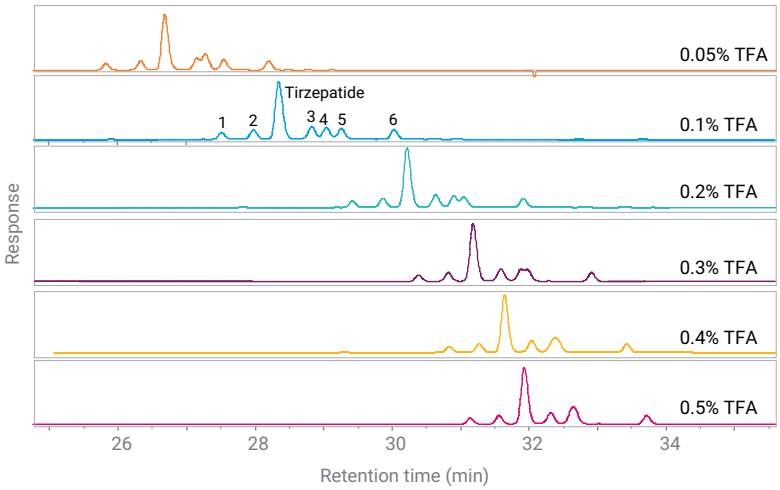
1. D-Ser(8)-semaglutide
2. D-Asp(9)-semaglutide
3. D-His(1)-semaglutide
4. Des-His(1)-semaglutide

B Mobile-phase additive concentration affects liraglutide resolution with column compartment temperature at 60 °C.



1. D-allo-Thr(13)-liraglutide
2. Kyn(31)-liraglutide
3. Liraglutide-formaldehyde adduct

C Mobile-phase additive concentration affects tirzepatide resolution with column compartment temperature at 70 °C.



1. Des-Tyr(1)-tirzepatide
2. Endo-Gly(4)-tirzepatide
3. D-Asp(15)-tirzepatide
4. Des-AEEA4'-tirzepatide
5. Glu(24) deamidation-tirzepatide
6. Des-y-Glu-tirzepatide

Figure 3. Evaluation of the effect of TFA concentration on (A) semaglutide, (B) liraglutide, and (C) tirzepatide impurity separation.

Effect of column compartment temperature on GLP-1 RA impurity resolution

Column temperature plays a critical role in chromatographic separation by influencing analyte mass transfer and interactions with the stationary phase. Using the optimized TFA concentrations identified in the previous study, the effect of column compartment temperature on the separation of semaglutide, liraglutide, and tirzepatide impurities was subsequently evaluated (Figure 4).

Semaglutide impurities generally showed decreasing resolution from the main peak as column temperature increased, although peak widths became noticeably narrower (Figure 4A). For liraglutide, temperature substantially affected the retention of Kyn(31) impurity, causing shifts in impurity selectivity and coelution across the temperature range studied (Figure 4B).

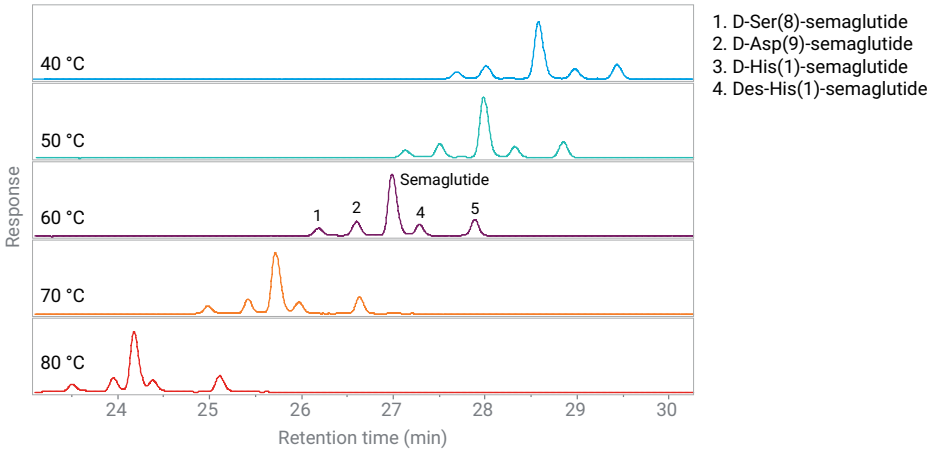
Overall, a column temperature of 60 °C provided the best balance of selectivity, resolution, and peak shape for semaglutide and liraglutide. Conversely, tirzepatide benefited from elevated temperatures, with the highest overall impurity resolution observed at 70 °C (Figure 4C). These findings further demonstrate that temperature is an important method parameter which should be optimized for the GLP-1 RA of interest.

Effect of sample load for semaglutide, liraglutide, and tirzepatide under their respective optimized conditions

Using the mobile-phase compositions and column temperatures identified during additive and temperature studies, the effects of increasing sample load were evaluated for semaglutide, liraglutide, and tirzepatide on both the Altura Peptide Plus column and a charged-C18 competitor column. Increasing sample load affected chromatographic performance to varying extents across GLP-1 RAs and their impurities, with liraglutide showing the greatest reduction in resolution and overall separation performance (Figure 5).

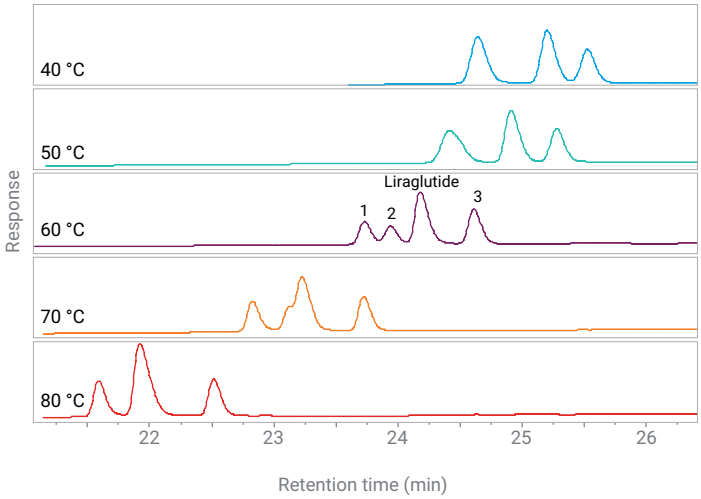
The Altura Peptide Plus column maintained chromatographic performance across the sample loading range examined and provided effective impurity separations under the evaluated conditions. Additional work is needed to determine whether the load sensitivity observed for liraglutide is primarily related to the molecular characteristics of liraglutide itself or to the chromatographic conditions selected for that analysis, including the lower TFA concentration employed.

A Column compartment temperature affects semaglutide resolution with 0.4% TFA concentration.



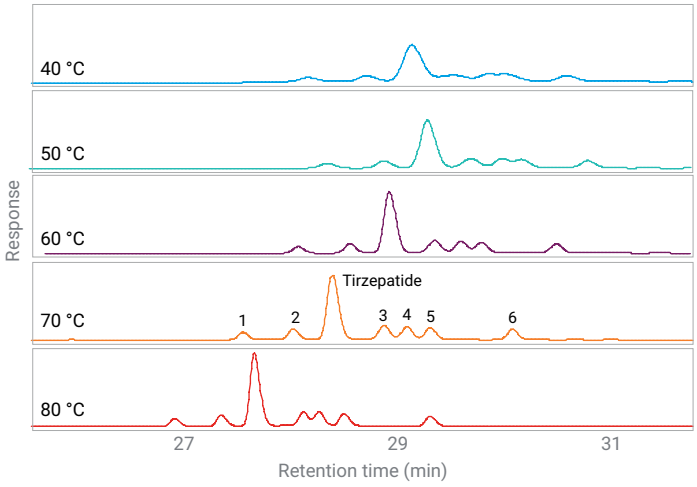
1. D-Ser(8)-semaglutide
2. D-Asp(9)-semaglutide
3. D-His(1)-semaglutide
4. Des-His(1)-semaglutide

B Column compartment temperature affects liraglutide resolution with 0.05% TFA concentration.



1. D-allo-Thr(13)-liraglutide
2. Kyn(31)-liraglutide
3. Liraglutide-formaldehyde adduct

C Column compartment temperature affects tirzepatide resolution with 0.1% TFA concentration.



1. Des-Tyr(1)-tirzepatide
2. Endo-Gly(4)-tirzepatide
3. D-Asp(15)-tirzepatide
4. Des-AEEA4'-tirzepatide
5. Glu(24) deamidation-tirzepatide
6. Des-γ-Glu-tirzepatide

Figure 4. Evaluation of the effect of column compartment temperature on (A) semaglutide, (B) liraglutide, and (C) tirzepatide impurity separation.

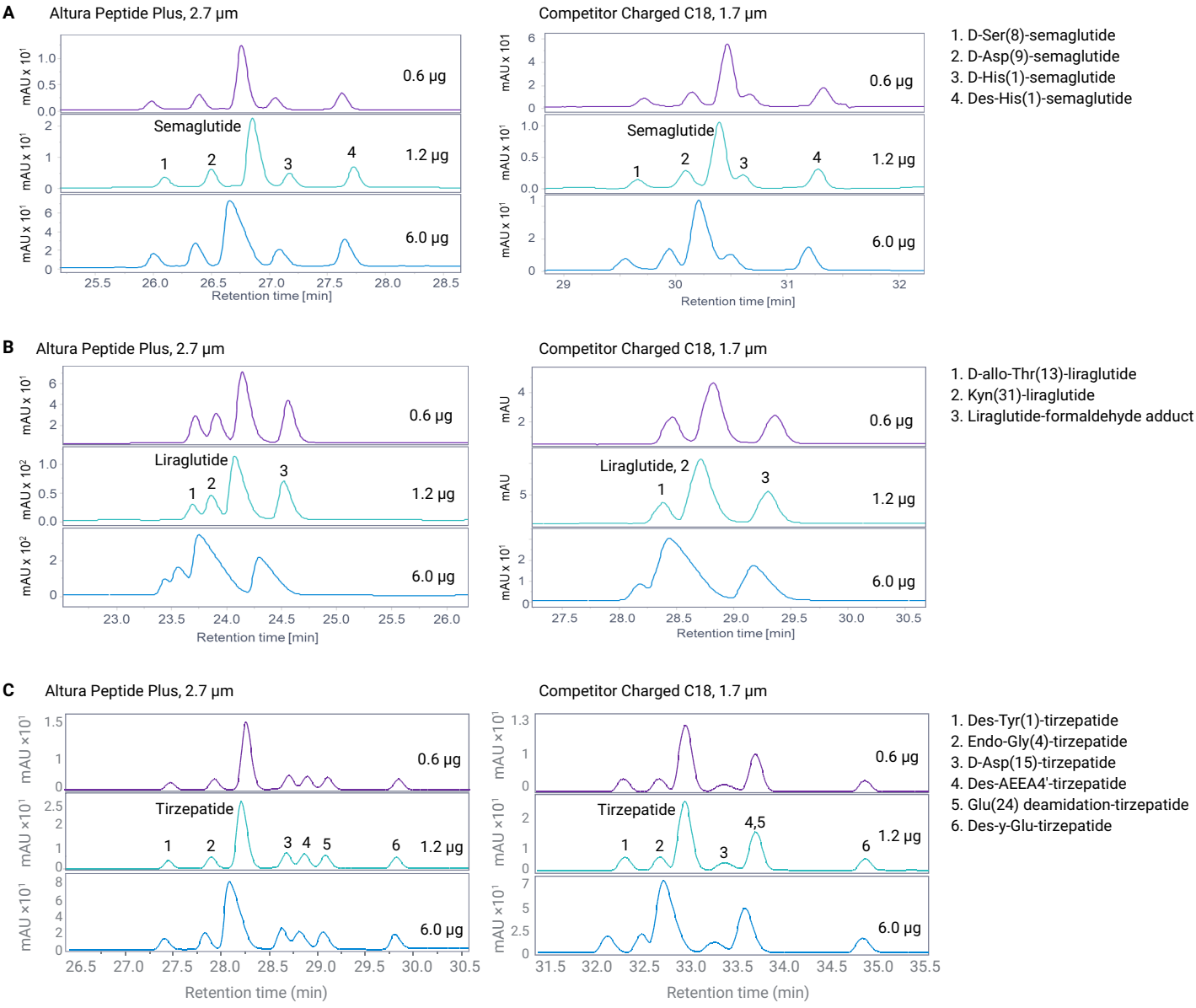


Figure 5. Effect of loading capacity on selectivity and peak shape for (A) semaglutide, (B) liraglutide, and (C) tirzepatide with different RP column chemistries.

Conclusion

This application note shows how the use of an Agilent 1260 Infinity III Prime Bio LC system coupled with the Agilent Altura Peptide Plus column enabled high-resolution separations of semaglutide, liraglutide, tirzepatide, and their related impurities. Evaluation of mobile-phase additives demonstrated that TFA generally provides improved peak shape, retention, and impurity resolution compared to FA, although FA remains an attractive alternative for LC/MS workflows because of its MS compatibility. The influence of TFA concentration was found to be analyte-dependent, with semaglutide showing improved impurity resolution at higher TFA concentrations, while liraglutide and tirzepatide achieved their best overall impurity separations at lower TFA levels.

Column temperature also had a significant impact on separation selectivity. The most effective impurity separations were achieved at 60 °C for semaglutide and liraglutide and at 70 °C for tirzepatide, highlighting the importance of temperature screening during GLP-1 method development. These findings demonstrate that no single set of chromatographic conditions is universally optimal across all GLP-1 RAs and impurity profiles.

The Blend Assist workflow enabled rapid evaluation of TFA concentrations without manual mobile-phase preparation, accelerating method development while supporting transfer of optimized conditions to simplified solvent configurations for improved baseline stability. Under the conditions evaluated, the Altura Peptide Plus column effectively resolved closely related impurities across three GLP-1 RAs with further improvements in separation observed following the optimization of temperature and TFA concentration.

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