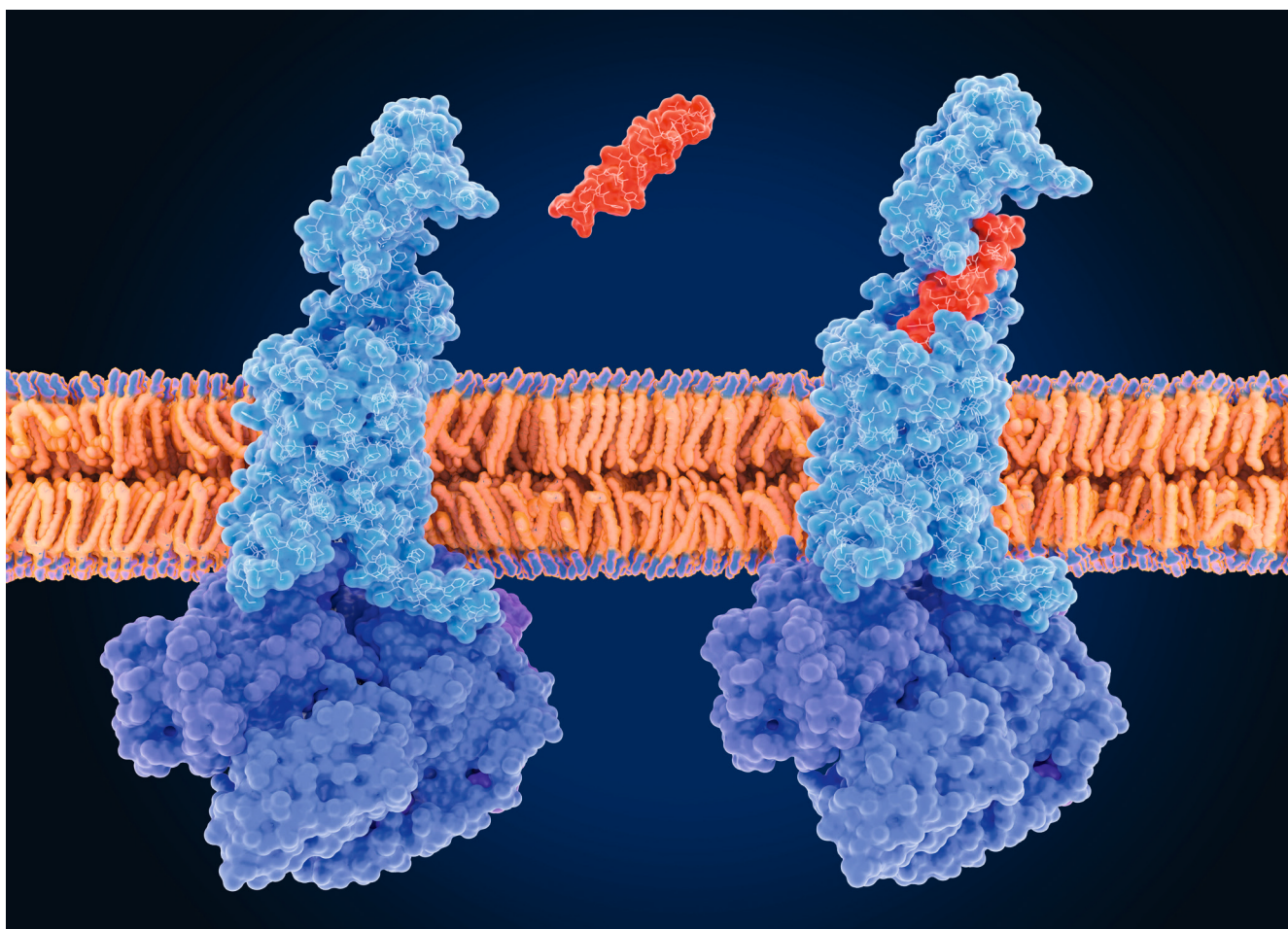


How to achieve a robust method for GLP-1 agonist tirzepatide – a step-by-step method development study

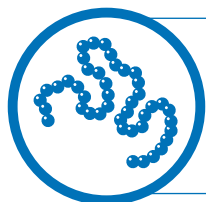
Tirzepatide acts as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) receptor agonist, making it an effective treatment of type 2 diabetes with hypoglycemic and weight-loss effects. Due to their clinical value and pharmacological reliability, GLP-1

receptor agonists occupy a firm position in the global pharmaceutical landscape. Therefore, accurate quality control is essential, especially as the amino acid sequence and higher order structure have a significant impact on the therapeutic performance.



This Application Note outlines a structured method development for the analysis of tirzepatide. The screening protocol evaluates pH and organic modifier in the mobile

phase, column temperature, stationary phase chemistry and the impact of bioinert column hardware.



Tirzepatide

- Molecular weight: 4813.45 Da
- Molecular formula: $C_{225}H_{348}N_{48}O_{68}$
- Structure:

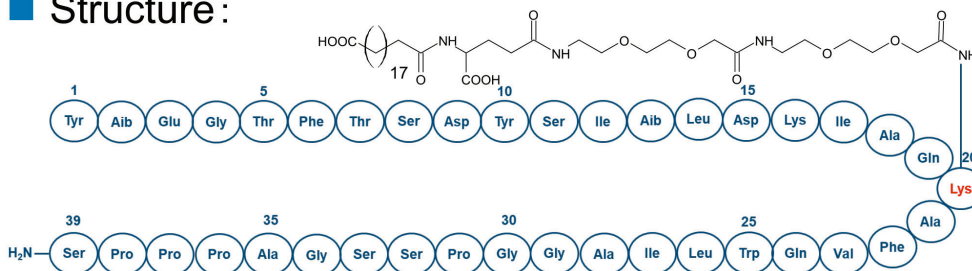
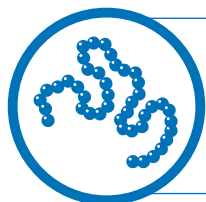


Figure 1: Structure of tirzepatide.

Table 1: Chromatographic screening conditions.

Columns:	YMC-Triart C18 (12 nm, 1.9 μ m) 100 x 2.1 mm ID YMC-Triart C8 (12 nm, 1.9 μ m) 100 x 2.1 mm ID YMC-Triart Bio C18 (30 nm, 1.9 μ m) 100 x 2.1 mm ID
Eluents:	A) aqueous solution B) acetonitrile or methanol
Gradient:	slope 1 %B/min
Flow rate:	0.2 mL/min
Temperature:	40 °C, 60 °C
Injection:	2 μ L
Sample:	crude tirzepatide (20.5 % purity) and standard (2.0 mg/mL in 20 mM ammonium bicarbonat)
Detection:	UV at 220 nm



Influence of mobile phase composition

As a first step a YMC-Triart C18 column is used to investigate aqueous eluents of different pHs of 2.0 – 2.5 – 6.7 – 8.0 (0.1% trifluoroacetic acid, 0.1% formic acid, 20 mM ammonium acetate, and 50 mM ammonium bicarbonate) in combination with acetonitrile or methanol. The column temperature is

set to 40°C for the pH screening. The peak of tirzepatide is marked with a blue box (Figure 2).

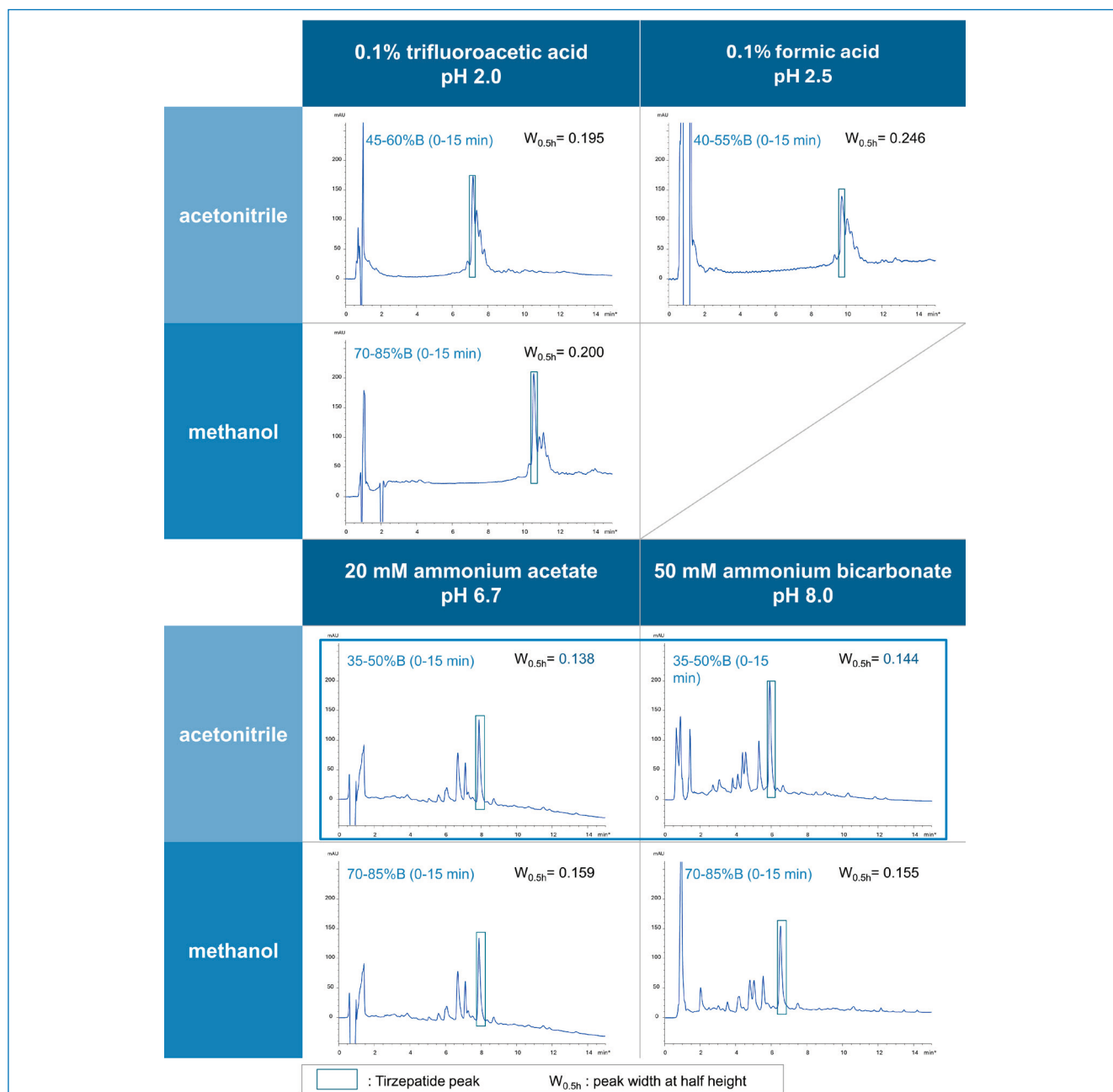
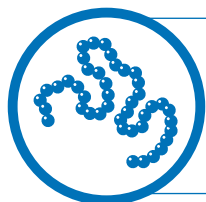


Figure 2. Results of the eluent screening of a crude tirzepatide sample.

Acetonitrile achieves sharper peak profiles across all tested conditions. Using a neutral to weakly alkaline mobile phase results in an improved resolution and sharper peaks compared to the lower pH mobile phases. The good solubility

of tirzepatide in alkaline solutions can be one possible reason for this. Both, ammonium acetate and ammonium bicarbonate provide high resolution and are considered for further testing.



Elevating the column temperature

Raising the column temperature from 40°C to 60°C increases resolution. This observation is based on ammonium acetate eluent only, as ammonium bicarbonate's volatility restricts its application at higher temperatures.

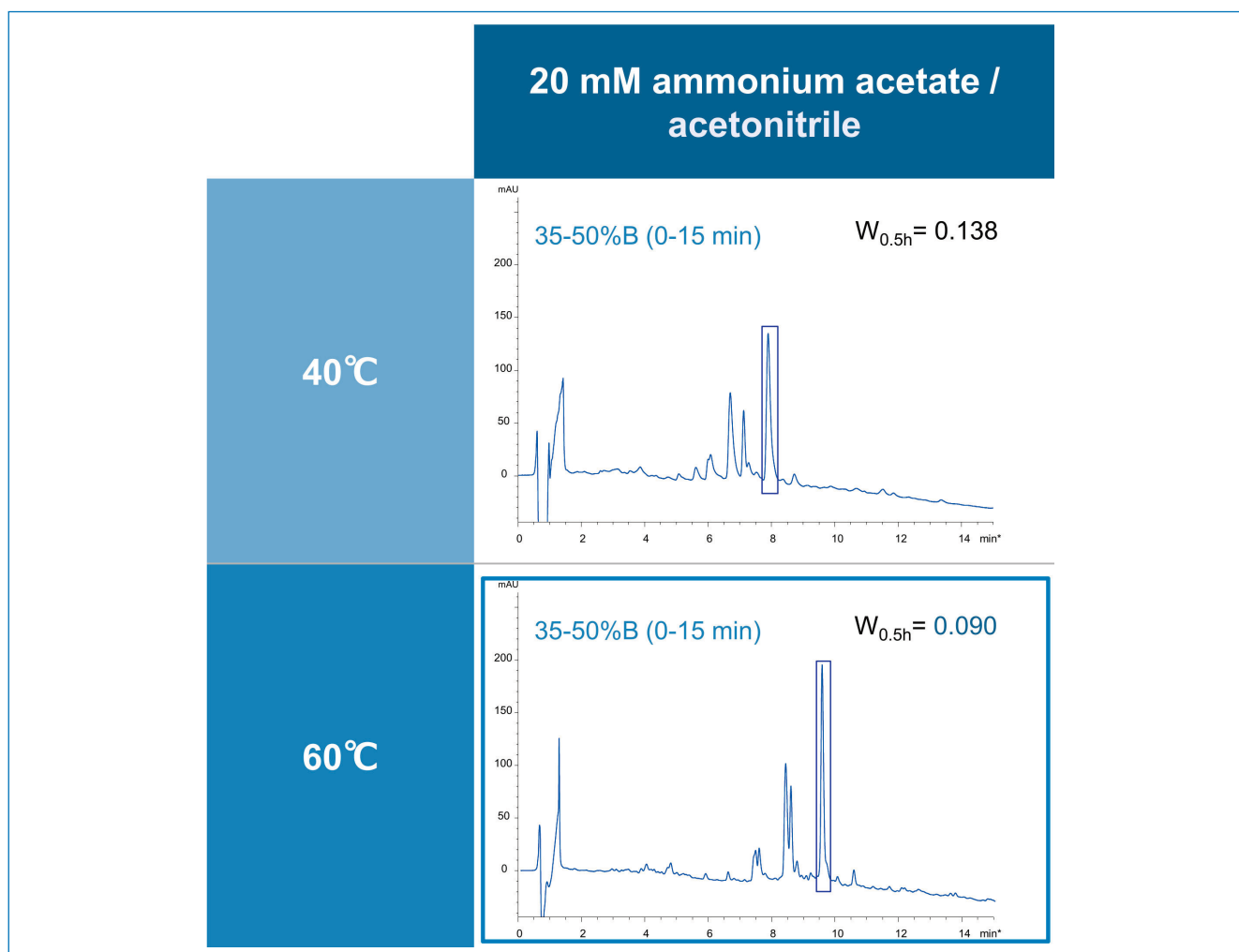
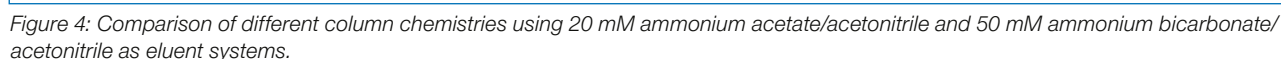


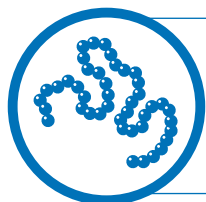
Figure 3: Influence of the column temperature on crude tirzepatide analysis.



Three columns matching the general requirements of the peptide are compared: YMC-Triart C18 with 12 nm pores, YMC-Triart C8 with reduced chain length, and YMC-Triart Bio C18 featuring a 30 nm pore size.



impurity peak. In combination with ammonium acetate, it provides significantly better resolution und a narrow peak width.



The benefit of bioinert column hardware

A comparison of bioinert coated column hardware with stainless-steel hardware (SUS column) at different sample concentrations shows that with the bioinert coated YMC Accura column higher peak areas and improved peak

symmetry can be obtained. It is assumed that non-specific adsorption occurs on the surface of the stainless-steel column.

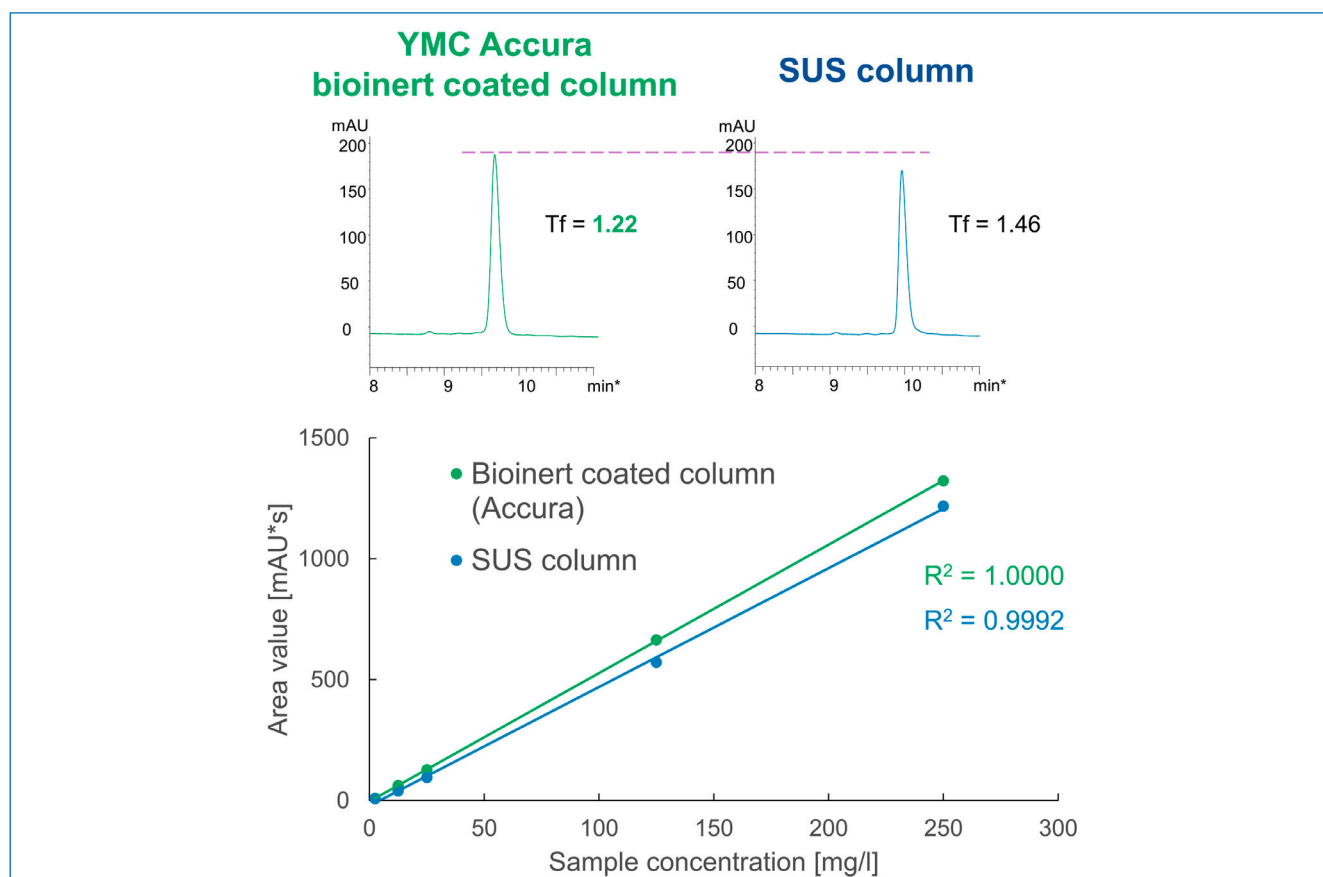
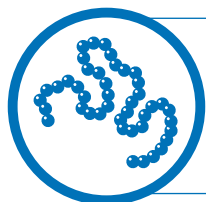


Figure 5: Comparison of the peak symmetry and peak area using a bioinert coated YMC Accura Triart Bio C18 column (green) and the corresponding stainless-steel column (blue).



Optimised analysis conditions

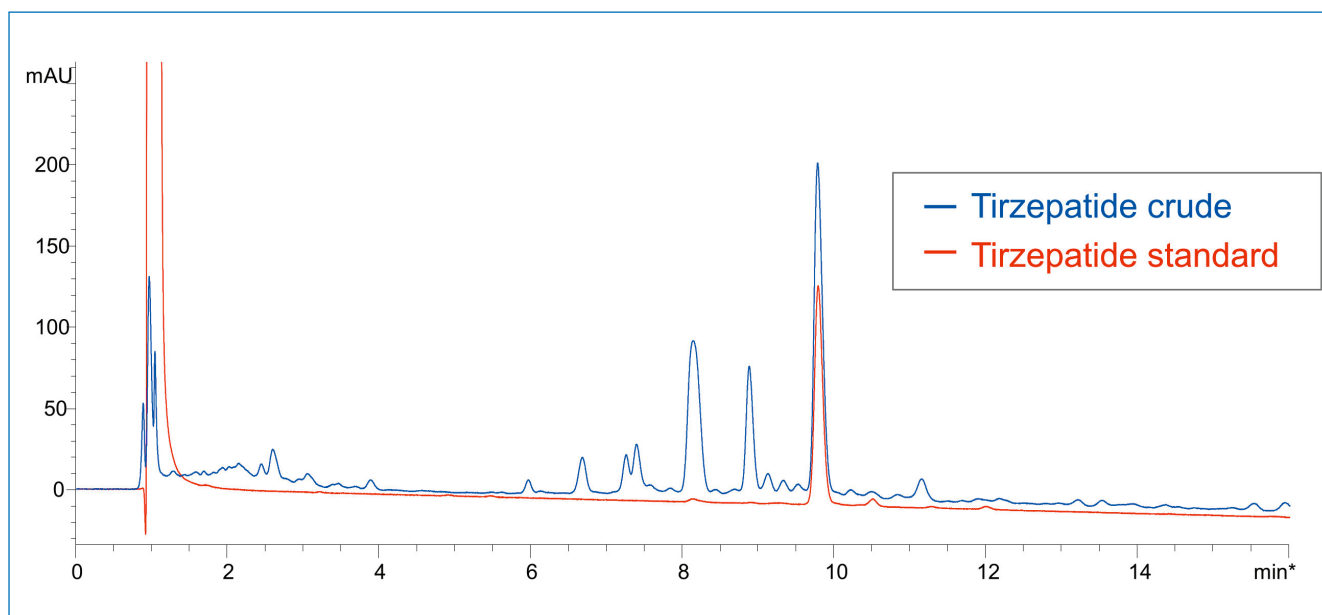


Figure 6: Analysis of a crude (blue) and standard (red) tirzepatide simple under optimised conditions.

Additional adjustments of the gradient slope and organic solvent ratios resulted in a gradient of 36–44 %B in 16 min.

Table 2: Optimised chromatographic conditions.

Column:	YMC Accura Triart Bio C18 (30 nm, 1.9 µm) 100 x 2.1 mm ID
Eluents:	A) 20 mM ammonium acetate (pH 6.7) B) acetonitrile
Gradient:	36–44 %B (0–16 min), 100 %B (16–20 min)
Flow rate:	0.2 mL/min
Temperature:	60 °C
Injection:	2 µL
Sample:	crude tirzepatide (20.5 % purity, 2.0 mg/mL) standard tirzepatide (0.5 mg/mL in DMSO)
Detection:	UV at 220 nm

Conclusion

This method establishes a robust and precise analytical workflow for purity testing of tirzepatide. Neutral to weakly alkaline conditions at elevated temperatures provide high resolution and sharp peaks. Furthermore, using the bioinert coated YMC Accura Triart Bio C18 column improves the

peak shape and recovery, even for small sample volumes. This screening protocol supports method development for other GLP-1 receptor agonists and meets the demands of regulated pharmaceutical analysis.