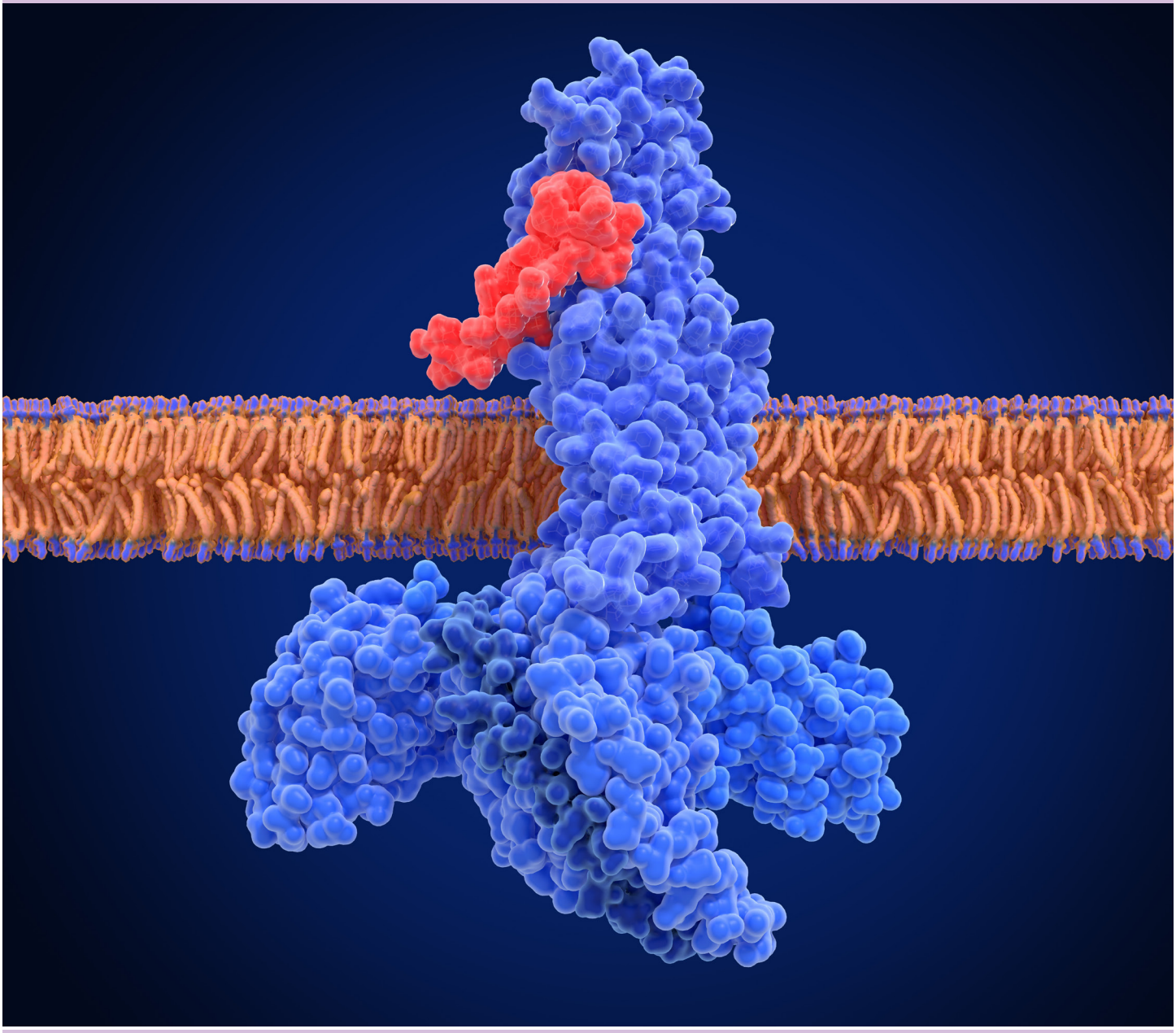
Therapeutic Peptides:

Challenges and solutions for drug developers



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Analyze, purify, validate:
End-to-end analytics for GLP-1 agonists and other therapeutic peptides

Therapeutic peptides are at the center of attention in the biopharmaceutical industry, making waves following recent methodological advancements in synthesis techniques, lipidation, and peptide mimetics. These innovations dramatically expand the range of potential applications for this promising class of drugs.[1,2]

"With half-lives that can now be measured in days rather than minutes and the ability for developers to target multiple receptors and tailor potent biological effects, peptide drugs have launched into an era of explosive growth," says Mike Knierman, biopharma workflow manager at Agilent Technologies.

Demand has especially surged in response to the recent clinical popularity and public notoriety of glucagon-like peptide-1 (GLP-1) receptor agonists such as semaglutide, tirzepatide, and liraglutide. These drugs are currently approved by the US Food and Drug Administration for cardiovascular risk reduction and treatment of conditions including type 2 diabetes, obesity, metabolic-associated steatohepatitis, and obstructive sleep apnea. Additional possible indications are still emerging.[3,4]

Scaling processes and facilities to meet this demand is challenging, especially for pharmaceutical developers who are venturing into the therapeutic peptide modality for the first time after previously focusing on small-molecule drugs. Coeluting impurities, larger and more complex product molecules, and difficult-to-detect sequence errors all require advanced analytical tools and techniques. These analyses ensure that the correct drug reaches patients in the correct doses without contamination.

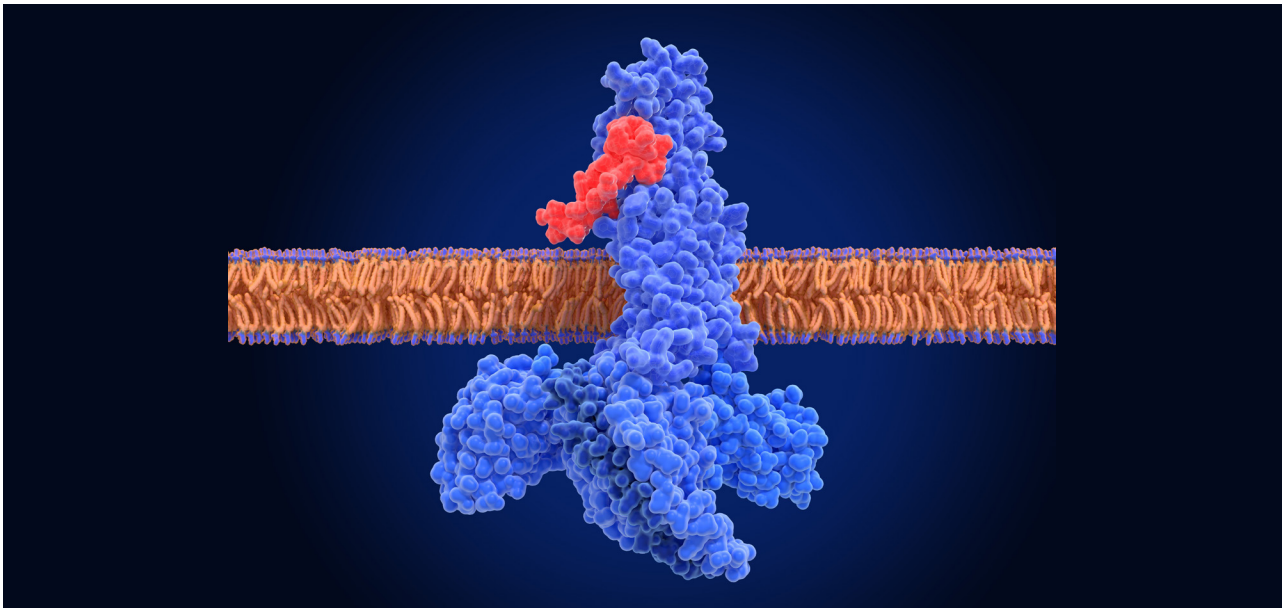

Figure 1. Model of the glucagon like peptide-1 receptor (GLPr) that mediates the action of GLP-1, a peptide hormone. The activated receptor has a strong effect on the management of type 2 diabetes mellitus and obesity. **Credit: Shutterstock**

"This modality has the potential to address some major unmet needs, especially when it comes to treating obesity and related conditions," Knierman says. "It's worth tackling the inherent analytical challenges so we as an industry can scale up to meet the demand."

To bring the full potential of GLP-1 drugs and other therapeutic peptides to market, pharmaceutical developers and manufacturers must ensure quality and safety while maximizing efficiency to deliver treatments to the patients who need them most. Regulators increasingly expect data integrity and orthogonal (multilayered) confirmation of product and impurity identities and quantification.[5] Industry leaders must prove to stakeholders that their manufacturing and quality control methods are reliable and reproducible under Good Manufacturing Practice (GMP), across instruments, operators, and global sites.

This resource focuses on three critical analytical checkpoints in the peptide-manufacturing workflow:

1. **Raw material analysis:** Rapid, noninvasive verification of starting materials to reduce errors and streamline warehouse operations.
2. **Purity and impurity analysis:** Confident detection and characterization of both product related and process related impurities, with sufficient resolution to resolve coeluting species and sensitivity to detect contaminants at low concentrations.
3. **Sequence confirmation:** Detection of insertions, deletions, and substitutions, and identification of isomeric residues that can alter potency, stability, and immunogenicity.

Developing rigorous plans and tools to address each of these challenges will enable biopharmaceutical industry leaders to achieve reproducible results.

Challenge 1:

Raw material analysis

Warehouse mistakes, supplier variability, and look-alike packaging create real risk — especially when different amino acids or grades of solvent are stored side by side. The biopharmaceutical industry remembers the heparin recall of 2008. Counterfeit starting materials with convincingly similar chemical properties infiltrated the heparin manufacturing pipeline. The contamination ultimately led to hundreds of deaths and thousands of adverse events in unsuspecting patients.[6] While heparin is not a therapeutic peptide, the episode offers a stark lesson about the importance of verifying the quality of raw materials.

A therapeutic peptide's quality is established well before its synthesis. "Even the most advanced process design and highly skilled experts cannot compensate for starting materials that are incorrect or out of specification when producing high-quality peptide drugs," says Parul Angrish, director of growth strategy at Agilent. "Quality assurance has to start at Phase zero."

Traditional identity verification often involves opening drums or bottles to collect samples, which are then analyzed in a laboratory. This analysis can be conducted using chromatography or conventional backscatter Raman spectroscopy, which measures changes in monochromatic (laser) light as it interacts with an analyte. When a Raman event is observed, energy from incident laser photons is transferred to the analyte, resulting in emitted photons that are shifted to different frequencies.

Regardless of laboratory technique, the sampling-based verification approach is slow, exposes potentially volatile compounds to light and air, and wastes consumables. More importantly, it introduces variability because different warehouses and operators may have different methods for sampling. The result is a reproducibility problem that begins at the dock and can shows up later as uneven yields, recurrent deviations, or tech transfer issues arising from inconsistent practices across different sites.[7]

A fast, unambiguous identity test that can be performed at receipt with minimal disruption to a substance is one of the most straightforward solutions to slow, irregular sampling-based approaches. Reducing the amount of time raw materials spend in quarantine and verification before entering the production pipeline means materials flow to production without delay or contamination risk. In a market characterized by high incoming volumes and line use, fast, on-site identity tests increase capacity without adding reactors.

By overcoming verification challenges, biopharmaceutical developers can confidently identify and ensure the quality of the starting materials with which they will synthesize each therapeutic peptide.

Solution: Spatially offset Raman spectroscopy (SORS) technology

Spatially offset Raman spectroscopy (SORS) addresses the core limitation of conventional backscatter Raman spectroscopy by allowing substances to be identified accurately while still fully sealed in their packaging.

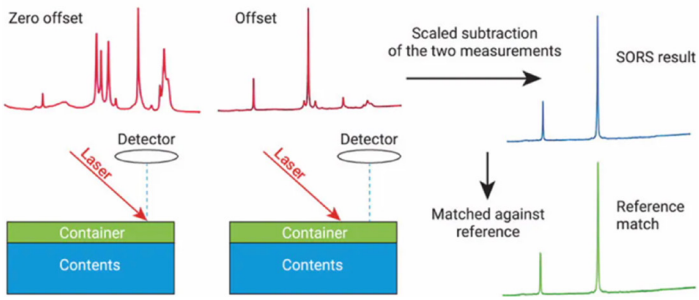

Figure 2: Workflow of spatially offset Raman spectroscopy (SORS). Raman spectra are first collected with a detector aligned directly with a laser (zero offset, left) and then at a lateral offset position (middle). Finding the difference between these two measurements isolates the spectral features of a sample's contents (blue), which can then be compared with known reference spectra (green) to confirm a material's identity.

Instead of collecting a spectrum at the same spot where the laser illuminates the surface, SORS acquires at a physical offset between the illumination and detection points (Figure 2). This offset geometry collects Raman photons in the detection area that originate mostly from beneath the sample surface, allowing the instrument to model and subtract the container's spectral contribution and isolate the chemical fingerprint of the material inside. In contrast, a conventional Raman spectroscopy system with no physical offset yields a spectrum dominated by information about the top layer of a sample — in this case, the packaging (Figure 3).[8]

In practice, this distinction means a trained operator can forego sampling and simply point a handheld SORS unit at an amber bottle, a white high-density polyethylene (HDPE) drum, or even paper packaging to initiate verification. They can confidently verify the identity of the material without opening the container and risking contamination.

The Agilent Vaya Handheld Raman Spectrometer is a handheld SORS device that operationalizes SORS for warehouse environments, making it easy for any worker within an organization — including nonscientists — to get reliable answers for raw material identification. Methods can be built directly on the device: the on-instrument software guides the user through model creation and ensures that spectral differences are sufficient to differentiate among chemically similar materials.

Once a method is validated, a scan can take as little as 10 seconds (depending on the packaging material) and returns a simple pass-fail answer tied to the appropriate spectral library. The system is designed for GMP-compliant processes, so it provides audit trails, role-based access, electronic signatures, barcode and laboratory information management system integration, and validated processing methods. As a result, identity verification becomes a repeatable, documented, and transferable step.

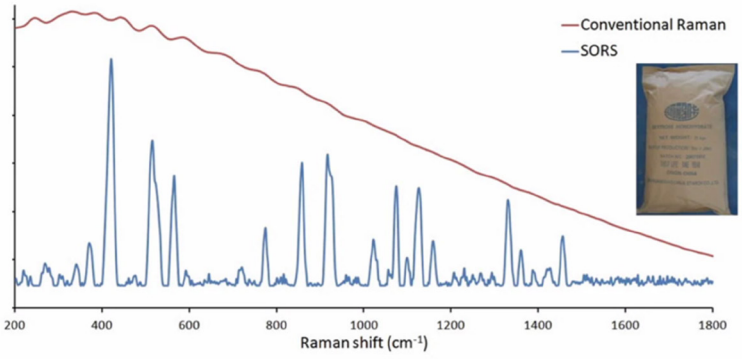

Figure 3: Comparison of Raman techniques for packaged material. Conventional Raman spectroscopy (red) of dextrose packaged in paper shows broad background signals dominated by the multilayer paper sack, while spatially offset Raman spectroscopy, or SORS (blue), reveals sharp spectral peaks corresponding to the dextrose inside the package.

Vaya in action

Agilent scientists demonstrated Vaya's SORS-enabled identification of four different amino acids protected by fluorenylmethoxycarbonyl (Fmoc) — the building blocks of GLP-1 receptor agonists and other therapeutic peptides. The team tested commercial lots supplied in white HDPE and amber bottles, developed methods on Vaya without opening the containers, and validated models using Vaya's guided software.

Despite chemical similarity across Fmoc-protected residues, Vaya SORS quickly distinguished and verified the identities of Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-His(Trt)-OH, and Fmoc-Phe-OH through the containers. The spectral evidence was both intuitive and specific (Figure 4).

Operators did not open containers throughout this entire process, so light- and air-sensitive Fmoc materials maintained integrity and material quarantine time could be lowered from days to hours.

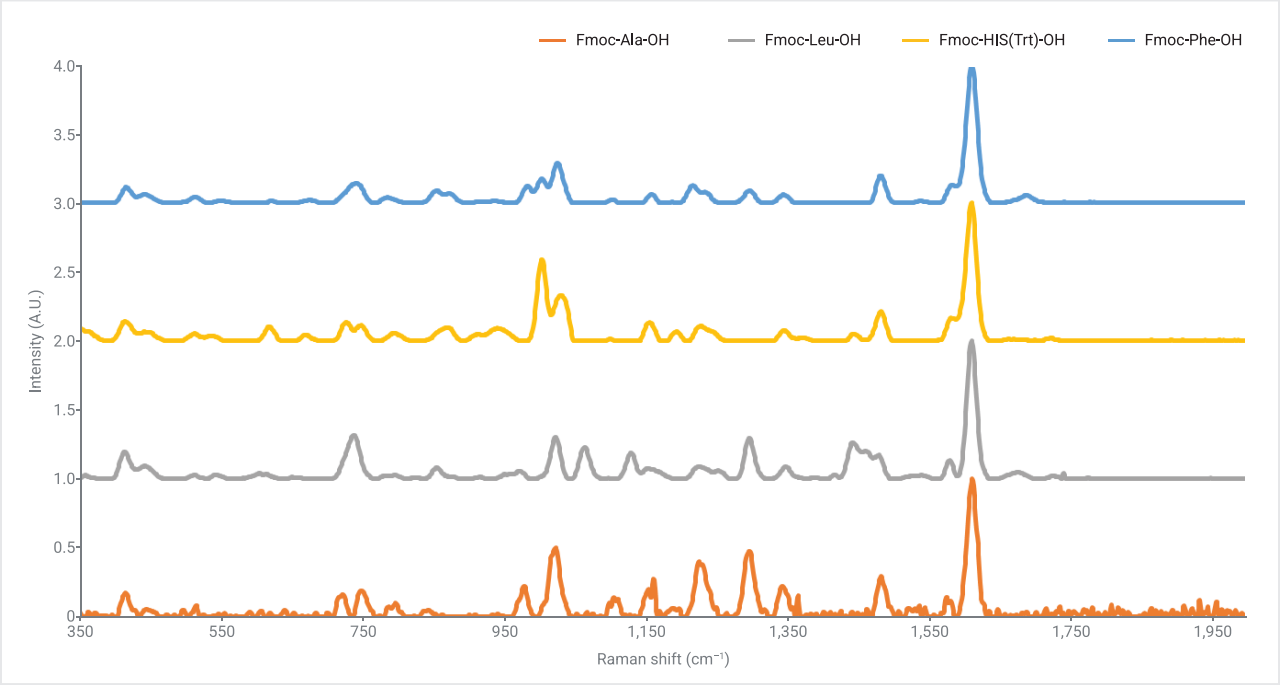

Figure 4: Spectra of amino acids protected by fluorenylmethoxycarbonyl (Fmoc). A band at approximately 1,481 cm⁻¹ attributable to the Fmoc protecting group appeared across the class and served as a marker that distinguished protected residues from their unprotected counterparts. Side chain characteristics, such as aromatic ring modes and carbonyl features provided further resolution among closely related materials.

Challenge 2:

Purity and impurity analysis

The synthesis of peptides includes product-related and process-related species. Product-related species can include amino acid deletions and insertions arising from coupling errors, truncations or incompletely deprotected sequences, or oxidation or deamidation of susceptible residues. Process-related species can include residual solvents or elemental impurities introduced from catalysts, water, or glassware.

Impurities are vectors for clinical and commercial risk.[5] "With GLP-1 drugs poised to reach massive patient populations, developers must make drug quality, safety, and efficacy their foremost priority," Angrish says.

Impurities can have a range of negative effects and implications:

- **Low-level oxidized variants** in a peptide product can alter receptor binding.
- **Deamidation** erodes potency and shelf life.
- **Aggregates** elevate immunogenicity risk.
- **Residual solvents and metals** have direct toxicity concerns and compendial limits (for example, USP <467> and ICH Q3D guidelines).

"The regulations suggest demonstrating the sameness of peptide with a Reference Listed Drug. Any impurity above the threshold of 0.1% needs to be identified. These guidelines bring sensitive analytical requirements to detect, identify and quantify the product-related impurities," says Ashish Pargaonkar, biopharma market development specialist at Agilent for the Asia-Pacific region. "Most [contract development and manufacturing organizations] and generic manufacturers of these peptide drugs are trying to assess the impurities level and quality to satisfy these regulatory guidelines. We often find ourselves helping customers optimize their methods to fit their end product within strict parameters." Every impurity must be reduced below specified thresholds to ensure patient safety and therapeutic effectiveness.

In the GLP-1 agonist market, where multiple drug developers may target similar receptors, quality is a differentiator among competitors. Clean chromatograms, stable impurity profiles, and consistent release data demonstrate process maturity to regulators, partners, and payers alike. On the other hand, coeluting unknowns and drifting retention times can cause out-of-specification (OOS) events and slow tech transfers. Accurate and comprehensive impurity profiles help control quality and mitigate risks associated with peptide drug production and storage.

Purity assessment solutions

For straightforward purity assessments, manufacturers can achieve reliable analyses using liquid chromatography (LC) coupled with mass spectrometry (MS) and ultraviolet (UV) detectors. A single-quadrupole LC/MS system, such as the Agilent InfinityLab LC/MS Detector Pro iQ, paired with a Variable Wavelength Detector, provides simultaneous UV purity measurement and intact mass analysis with spectral deconvolution in software. Spectral deconvolution converts analysis of multiply charged envelopes into intact masses that aid straightforward pass/fail checks. The simple single quadrupole LC/MS system works well with in process monitoring and routine release assays.

However, many assessments are not compatible with single-quadrupole LC/MS systems. "Certain impurities can actually co-elute right alongside your product, in which case you really need advanced separation tools," Angrish warned. "At that point, two-dimensional LC solutions

coupled with MS become a real workhorse for determining what impurities are coeluting under the main peak. This advanced method can help to decide how to analyze and separate impurities and understand the overall effectiveness of your purification process."

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– Parul Angrish
Director of growth strategy, Agilent

One-dimensional LC (1D-LC) uses a single column with just one separation chemistry, while 2D-LC chains two different separation chemistries, multiplying peak capacity and revealing differences a single column can miss. In practice, 2D-LC enables users to capture the most chemically dense time slice in the first round of analysis and pass it to a second, methodologically distinct column. The payoff is cleaner baselines, more trustworthy quantitation, and unambiguous identification of specific variants.[9] It is a fast, reproducible way to turn an ambiguous signal into discrete, actionable components.

Ultra-high-performance LC (UHPLC) tools like the Agilent 1290 Infinity III 2D-LC system are ideal for this kind of multilayered approach. Peptide-appropriate column chemistries are available to help scientists obtain different interaction profiles that can tease apart lipidated analytes and their variants more effectively. Two-dimensional configurations can also perform online desalting so salt -heavy methods become compatible with MS without sacrificing separation quality.

Agilent also recently launched its new Altura HPLC Columns with Ultra Inert technology, which feature biocompatible hardware with low adsorption to prevent nonspecific interaction of metal-sensitive analytes with stainless steel column housing. This allows the stationary phase to be used to its maximum potential for reliable HPLC analyses. Agilent scientists have demonstrated excellent separation of GLP-1 peptides and impurities using an Agilent Altura Poroshell HILIC-Z column and Agilent 1290 Infinity III Bio LC system coupled to the Agilent InfinityLab LC/MS Detector iQ (Figure 5).

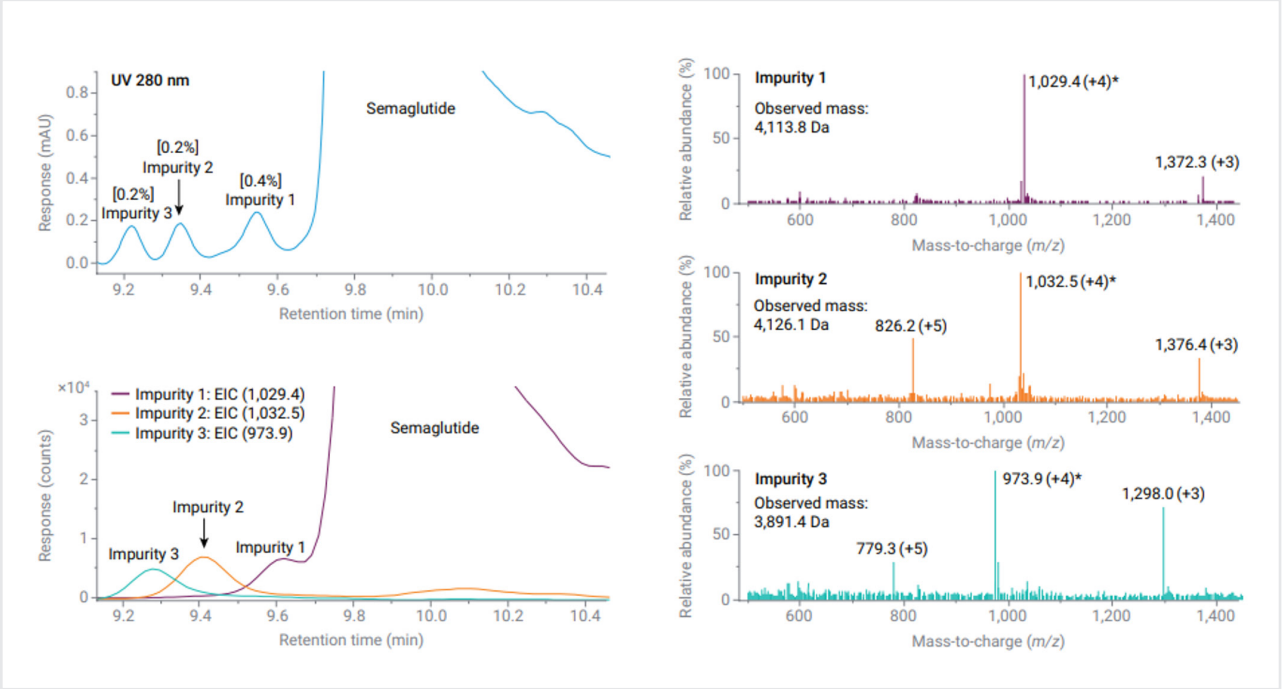

Figure 5. Zoomed HILIC-UV 280 nm chromatogram of semaglutide (top left) and overlaid extracted ion chromatograms (EICs) of semaglutide impurities (bottom left). Mass spectra of the impurities are depicted on the right.

Pargaonkar also highlights that while developers need proper analytical methodologies to assess the quality of these therapeutic peptide drugs, it is important to assess various formulation constituents in the drug products. These include inorganic ions, sugars and amino acids added as buffering or tonicity agents, stabilizers, and antioxidants.

Agilent scientists showcased a unique solution to address this requirement. Combining diode array detection (DAD) and evaporative light-scattering detection (ELSD) technology with the abovementioned chromatography solutions enables the simultaneous measurement of the therapeutic peptide, various formulation constituents, and product-related impurities in GLP-1 receptor agonist drug products (Figure 6).

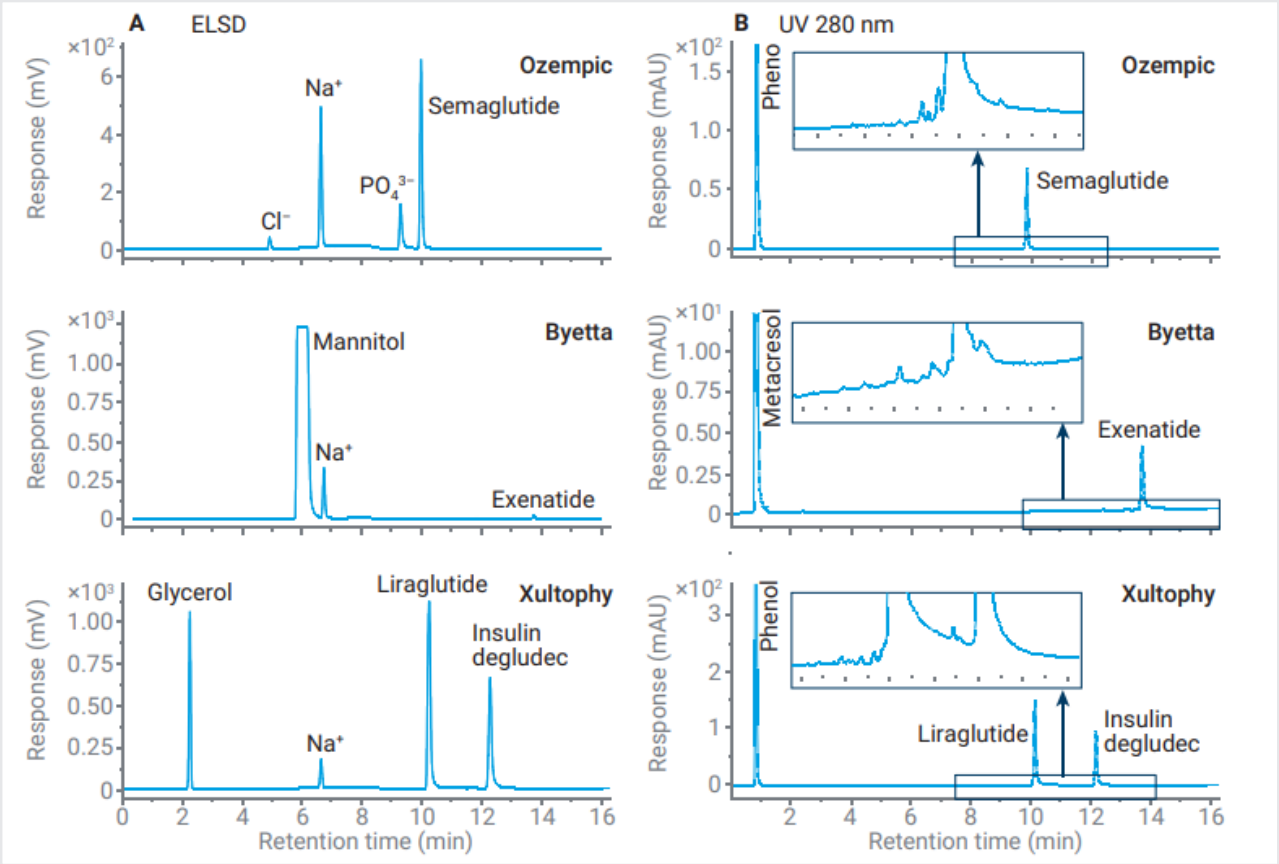

Figure 6: (A) HILIC-ELSD and (B) ultraviolet (UV) 280 nm chromatograms of three glucagon-like peptide-1 (GLP-1) receptor agonists. Experiments performed using an Agilent Altura Poroshell HILIC-Z column with Ultra Inert technology installed on an Agilent 1290 Infinity III Bio liquid chromatography system. The ELSD data provide a readout of cosolvents, tonicity agents, and buffers or pH adjustment substances, while the UV data allow detection of the synthetic peptides and their impurities, as well as the unretained preservative.

"Biopharmaceutical experts find themselves on the knife edge of chromatography, Separating out impurities that are so close to the properties of the parent requires really exceptional column stability and clear mass spectrometry results."

– Mike Knierman
Biopharma workflow manager, Agilent

After achieving thorough separation, manufacturers can rely on LC–quadrupole time-of-flight (LC/Q-TOF) technology for identification. In LC/Q-TOF, the quadrupole first isolates a narrow window corresponding to the precursor of interest and directs it into a collision cell for gas-phase fragmentation. The resulting product ions then enter the TOF analyzer, where their flight times are converted into high-resolution mass measurements across the full MS/MS spectrum, accurate to the ppm level.

The Agilent 6545XT AdvanceBio LC/Q-TOF system helps experts achieve deep peptide mapping and sift through hard-to-detect and low-charge peptides. The system is specifically configured to distinguish biopharma analytes, covering the charge states and masses common to peptides. This instrument reliably captures information including intact mass, isotope distribution, and fragment ion evidence. Analysts can link an unknown peak to a common well-defined issue (such as N-terminal truncation or deamidation) rather than a generic related substance, smoothing both process corrections and communications with regulators.

Challenge 3:

Sequence confirmation

Patients using GLP-1 agonists to treat conditions like type 2 diabetes or obesity typically take weekly injections of the drug over months or years. This long-term dosing makes it especially important that the composition of the GLP-1 agonist drug is correct and consistent. Otherwise, the toxic effect of contaminated substances may accumulate over time. It is crucial to confirm that therapeutic peptide products have the expected sequence with no unintended insertions, deletions, or substitutions.

Many sequence issues can be identified with a core trio of biopharma tools, each answering a different question:[10]

- **Intact-mass LC/MS asks "Did we build the right molecule?"** by measuring the whole peptide's mass after electrospray ionization; software deconvolutes multiple charge states to a neutral mass and compares it with the theoretical value. Any gross composition error — deletions or insertions, truncations, missed lipidation or capping, or unexpected adducts — shows up as a clear mass shift or extra envelope, making this a fast identity check.
- **Bottom-up peptide mapping asks "Is every residue where it should be?"** by enzymatically digesting the peptide, separating the fragments by LC, and acquiring MS/MS to confirm sequences against the expected map. From these spectra, it is possible to localize posttranslational or process-induced changes (such as oxidation, deamidation, or truncations), estimate site-specific levels from extracted-ion chromatograms, and track key areas under stress.
- **Chromatographic retention-time fingerprints ask "Does this lot behave like the reference?"** The main peak's time and shape and the pattern of known minor peaks are remarkably reproducible, so the appearance of shifts, shoulders, or new peaks flag potential substitutions or modifications even before MS interpretation.

Unfortunately, some of the most consequential problems are also among the most challenging to detect. Manufacturers need specialized approaches and instruments to account for chemistries that are not detected by conventional fragmentation, including:

- **Isobaric substitutions** (such as leucine ↔ isoleucine) that leave mass unchanged but can affect binding affinity.
- **Isomerization** (such as aspartate → isoaspartate), which can occur spontaneously and is linked to peptide degradation and potential loss of potency.
- **Noncanonical residues and lipidations** that challenge standard bioanalytical algorithms and can skew other analytics, rendering quality assurance efforts less effective overall and potentially endangering patients.

"Teasing apart closely related substances and achieving reproducible chromatography can be difficult in these molecule classes, especially when you start adding mimetics and lipidation, which drastically change the physical properties," says Knierman.

Solutions for sequence confirmation

Pragmatically, one can start a sequence-confirmation workflow with the fundamentals and escalate only as needed. HPLC can identify peptide impurities by differences in retention times, while paired LC/MS workflows confirm composition by determining exact molecular weights and fragmentation patterns. As previously

mentioned, intact mass measurements with validated deconvolution provide a rapid screen. Bottom-up mapping after enzymatic digestion offers residue level confirmation. For lipidated or otherwise modified GLP-1 analogs, the choice of enzyme, the digestion conditions, and the search parameters must be configured so coverage remains high despite the presence of noncanonical residues.

But when questions remain — especially about isobaric substitutions or isoaspartate formation — electron capture dissociation (ECD) on a high resolution LC/Q TOF platform emerges as the optimal tool. An ECD-enabled Q-TOF platform resolves those ambiguities in both peptides and small proteins. The Agilent 6545XT AdvanceBio LC/Q-TOF can be equipped with an ExD cell that delivers electron-based fragmentation. Spectra are acquired in MassHunter and analyzed in ExDViewer, a tool designed to annotate the full range of backbone and side-chain fragments in Q-TOF datasets.

The primary advantage of ECD for isobaric and isomeric questions is that its unique fragmentation pattern yields localized and informative c- and z-ion series that collision-induced dissociation (CID) lacks. When present, the resulting spectral features are unique enough that they allow scientists to make unambiguous judgments. This additional layer of information is what's necessary to distinguish isobaric and isomeric amino acid substitutions.

ExD precision in action

Characterization of synthetic peptides using Agilent high-resolution LC/Q-TOF systems provides critical quality information about GLP-1 analogs. Implementing electron-based fragmentation in LC/MS workflows is an effective technique for sequence and modification analysis of proteins and peptides. Agilent scientists characterized three GLP-1 analogs — liraglutide, semaglutide, and tirzepatide — using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell for ECD.

Unlike CID, ECD is a gentle technique that fragments the peptide backbone while preserving important labile chemical modifications. Comparing ion scores of GLP-1 analogs analyzed with ECD or CID reveals that ECD fragmentation generates more high-quality, modification-containing fragments than CID (Figure 7).

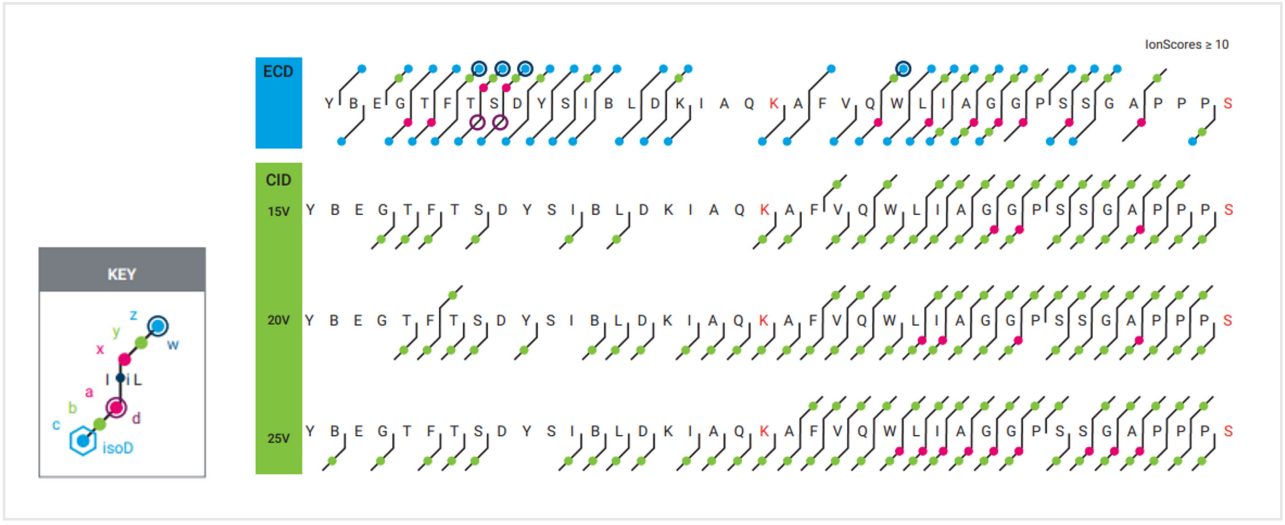

Figure 7: Tirzepatide fragments identified in electron capture dissociation (ECD) or collision-induced dissociation (CID) experiments with ion scores of 10 or greater. ECD provided more high-quality modification-containing fragments, particularly on the N-terminal side of the modification. ECD yielded a more complete ion series with several complementary ion types.

Abundant fragments of amino acid side chains are commonly produced using the ExD cell, which provides additional information about isomeric amino acids such as leucine and isoleucine. Secondary fragmentation of amino acid side chains from ECD radical ions are referred to as w-ions.

Agilent:

Your therapeutic peptide manufacturing partner

Peptide therapeutics will scale only as fast as their analytics. If you are launching a GLP-1 therapeutic, especially if your organization is new to the therapeutic peptide modality, you should take time to build reproducibility into your entire process. This includes aligning critical quality attributes to primary and orthogonal assays, standardizing consumables and mobile phases, and locking in validated electronic processing methods. The result will be faster tech transfer, fewer deviations, and confident lot release — exactly what patients, investors, and regulators expect from modern peptide manufacturing.

Agilent provides reliable advanced analytical tools and support for the biopharmaceutical industry. With deep expertise in therapeutic peptides, Agilent's experts can provide invaluable education to teams who are new to this class of therapeutics. Experienced developers can also benefit from assistance with method refinement and relocation services.

"Agilent Technologies provides a comprehensive portfolio of solutions to support every stage of peptide drug development and assessment. We work closely with our partners, leveraging a deep understanding of their unique needs, processes and controls to deliver impactful results. We are proud to be a trusted partner in advancing these critical drug development efforts, contributing our comprehensive portfolio of solutions and deep scientific expertise to help accelerate innovation and shape the future of GLP-1 therapies."

– Parul Angrish
Director of growth strategy, Agilent

Start your journey to therapeutic peptide success with Agilent Technologies today.

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RA260105.578

Published in the USA, April 01, 2026
5994-9132EN