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Fast Track to Certainty: Confident Biopharma Decisions with LC-Single Quadrupole Mass Detection

How single quadrupole LC/MS simplifies identification, purification, and stability assessment in biopharma

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Introduction

Biopharmaceuticals have transformed the therapeutic landscape—offering highly targeted treatments for a range of conditions, from autoimmune disorders and infectious diseases to cancer and genetic conditions. To ensure product quality, safety, and efficacy, laboratories closely monitor these biomolecules—analyzing critical factors such as molecular identity, purity, stability, and post-translational modifications like glycosylation. In these fast-paced environments, access to a unified approach that provides timely and accurate analytical data is essential.[1]

The Agilent InfinityLab Pro iQ Series mass detectors are built to maximize results with less effort, delivering fast scan speed, a wide mass range, and unmatched sensitivity, with demonstrated performance in biomolecular analyses. Advanced separation and detection capabilities enable confident identification of challenging, coeluting small molecule, peptide, and oligonucleotide active pharmaceutical ingredients (APIs) and impurities.

The seamless integration of the InfinityLab Pro iQ Series with best-in-class Agilent Infinity III LC separation and Agilent OpenLab CDS helps labs manage diverse analytical needs with confidence. User-friendly LC/MS instrument intelligence features simplify operation and maintenance. OpenLab CDS provides complete control in a single software package, offering advanced analysis, flexible reporting, and robust data integrity controls.

This application compendium presents compelling examples of how the powerful Pro iQ Plus mass detector can significantly elevate the analysis and quality control of biomolecules in your lab. Discover how you can accelerate biopharma workflows with a single quadrupole mass spectrometer.

The 1290 Infinity III bio LC system combined with the Pro iQ Plus, boasting a wide mass range (*m/z* 2–3,000), and OpenLab CDS, is ideal for biomolecule molecular weight confirmation.

1. **Pack, B. W.; Manheim, J.; Chahrour, O.; Wood, B.; Zhang, L.; Vogel, U.; Sheng, G.** *Therapeutic Peptides Control Strategy: Perspective on Current Industry Practices.* **Org. Process Res. Dev.** *2025,* **29** (1), DOI: 10.1021/acs.oprd.4c00386

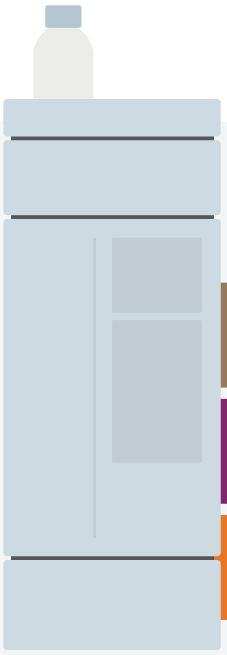
Consolidating Biopharma QC Workflows:

LC/UV Versus Single Quadrupole LC/UV/MS

Ever-evolving demands and regulations in biopharma QC environments require next-level readiness and performance. Traditional LC/UV workflows often rely on three separate methods for identity confirmation, purity assessment, and impurity profiling. By switching to single quadrupole LC/UV/MS, these methods can be consolidated into a single workflow, delivering all three outputs in one run.

Experience the benefits:

- Optimize instrument utilization, analyst time, and lab space
- Improve specificity and confidence
- Streamline data analysis and compliance



Traditional LC/UV

Method 1
Identity confirmation

Method 2
Purity assessment

Method 3
Impurity profiling

Next-generation LC/UV/MS

Single workflow for three methods:
- Identity confirmation
- Purity assessment
- Impurity profiling

Expert Perspective

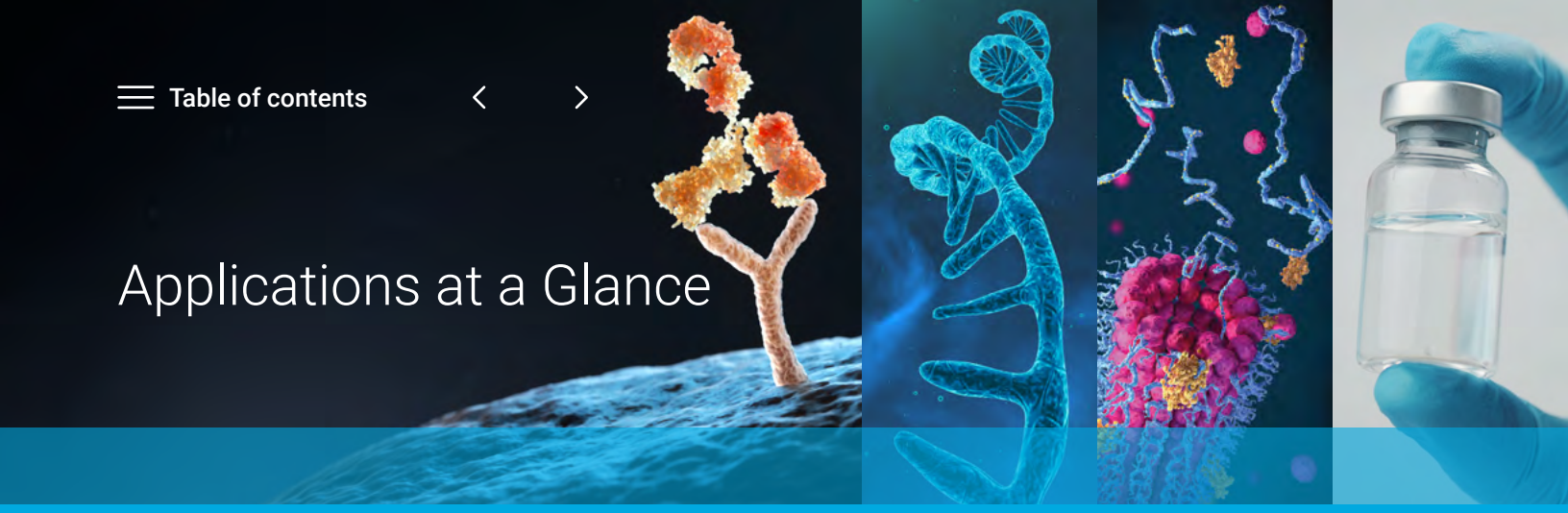
Drug discovery chemistry

"The addition of mass detection to our purification LC/UV workflow has been a game changer. With mass-based fraction collection (MBFC), we can pinpoint a compound to 1–2 vials relative to collecting 10–20 with UV only—saving time. For a single separation, the time for fraction analysis postseparation is reduced from approximately 30 minutes with UV to only 5 minutes with MBFC, accelerating our process for purifying final products and helping us to be more productive as we use the saved time to prepare other products."

— **Bertrand Arnaud,**
Scientific Associate, WELMEDIS

Agilent 1290 Infinity II Autoscale Preparative LC/MSD System

The ultimate system for seamless scaleup—from method scouting to gram-level compound purification with unmatched precision and efficiency using mass-directed fraction collection.



Applications at a Glance

Description	Pro iQ Plus and OpenLab CDS Performance Highlights
1. Glycosylation profiling for monoclonal antibodies	– Excellent MS spectral peak shapes and effective deconvolution – Reliable calculations of relative glycoform abundances – Results comparable to Agilent high-resolution accurate mass spectrometry systems
2. Ion-pairing alternative for synthetic oligonucleotide confirmation	– Superior sensitivity and resolution, even at high *m/z* ranges – Robust performance without toxic, costly ion-pairing reagents – Automated, simplified deconvolution and highly reproducible results across triplicates
3. Impurity profiling of therapeutic peptides	– Robust detection of oxidative modifications – High-quality and accurate mass spectra, even for trace impurities – Unified and streamlined platform for peptide characterization
4. Routine purity assessment of antisense oligonucleotides	– Sensitive and selective detection of low-level oligonucleotide impurities – Excellent ion transmission for accurate analysis of large oligonucleotides – Enhanced peak integration and spectral interpretation with Agilent Oligo Analysis Accelerator (OAA)
5. Improving the productivity of purification workflows with preparative HPLC	– Simplified single ion monitoring (SIM) setup using target compound editor, reducing manual effort and minimizing errors in adduct selection – Ability to tailor triggers for each target compound, enabling clean collection across intensity ranges – Exclusion of coeluting impurities using NOT-based trigger logic – Multiple override columns for fine-tuning of methods for each sample in a sequence

Note:
For applications 1–4, LC/MS analysis was performed on an Agilent 1290 Infinity II bio LC system coupled to an Agilent InfinityLab Pro iQ Plus single quadrupole LC/MS system. For application 5, LC/MS analysis was performed on an Agilent 1290 Infinity II preparative LC system coupled to a Pro iQ Plus.

Application Note

Biopharma/Pharma

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Monitoring Antibody Glycosylation at Intact and Subunit Levels Using a Single Quadrupole LC/MS

Author

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Abstract

This application note describes the use of the Agilent InfinityLab Pro iQ Plus single quadrupole mass spectrometer (MS) to monitor antibody glycosylation relative abundances at the intact and subunit levels of trastuzumab. At the intact level, five glycosylated components were identified from the MS spectral deconvolution software with relative abundances that were consistent with that measured previously on Agilent high-resolution accurate mass systems. At the subunit level, the light chain of trastuzumab showed a single component, which was expected based on its amino acid sequence, while the trastuzumab heavy chain identified four peaks consistent with the G0, G0F, G1F, and G2F glycans. The combination of reliable relative abundances for glycosylation monitoring, as well as excellent MS spectral peak shapes highlights the performance of the Agilent Pro iQ Plus mass spectrometer.

Introduction

Glycosylation is an important parameter to monitor in the biopharmaceutical industry because glycans play a role in the structural stability, safety, and efficacy of a therapeutic drug.[1] A common quality metric that is monitored repeatedly is to ensure that the relative abundances of protein glycosylation fall within accepted tolerances. If relative abundances do not pass predefined standards established by the quality control (QC) team, additional investigation into the manufacturing process for the therapeutic drug is required. Since mass spectrometry provides sensitive and robust methods for the characterization of monoclonal antibodies (mAbs), it is ideal to use mass spectrometry for glycan characterization.

In this study, the Agilent InfinityLab Pro iQ Plus liquid chromatography/mass spectrometer (LC/MS) with improved mass range and superior mass spectral peak shape was used to detect glycosylation at the intact and subunit levels (HC, LC). The relative abundances of glycosylation after deconvolution illustrate that key information about protein glycosylation[2] can be measured on this instrument platform.

Experimental

Chemicals and preparation of solutions

The following solutions from Sigma-Aldrich (St. Louis, MO) were used for the reduction reaction:

A. 8 M guanidine hydrochloride, pH 8.5 (part number G7294, 100 mL)
B. 0.2 M DTT (DL-Dithiothreitol, part number D9779, 1 g)
C. 1 M Tris buffer, pH 8.0 (part number 648314, 100 mL)

Denaturing: 6.4 M guanidine-HCl, 200 mM tris-HCl buffer, pH 8.1

Add 80 mL of 8.0 M guanidine HCl solution to 20 mL of 1 M Tris-HCl buffer in a 100 mL volumetric flask. Mix thoroughly by inversion and measure the pH using a pipette and pH indicator strip and record. If the pH falls between 7.2 and 8.5, this will be sufficient to proceed with the reducing step.

Reduction: 200 mM DTT in 50 mM Tris-HCl solution

Measure 31 mg of DTT using an analytical balance and add 1 mL of 50 mM Tris-HCl solution to dissolve. Vortex solution prior to the reducing step.

Standards and sample preparation

The monoclonal antibody (mAb) trastuzumab at a concentration of 22 mg/mL was acquired from Genentech (South San Francisco, CA). This solution was diluted to a concentration of 1 µg/µL by adding 10 µL of the stock solution to 210 µL of 0.1% formic acid in water. The final solution used for LC/MS analysis was diluted to 250 ng/µL by adding 55 µL of the 1 µg/µL solution and adding it to 165 µL of 0.1% formic acid in water.

The sample preparation procedure used for reduction of trastuzumab was:

1. Take 2.3 µL of trastuzumab stock solution (22 mg/mL) and add to 27.4 µL of 6.4 M guanidine chloride, 200 mM Tris-HCl.
2. Add 6.3 µL of 200 mM DTT in 50 mM Tris-HCl to the trastuzumab solution. Mix gently and spin down, then incubate at 37 °C for 30 minutes (alternate: 60 °C for 30 minutes).
3. To quench the reduction reaction, the solution should be acidified to a final v/v% of 1% formic acid. Add 4 µL of 10% FA to the reduced solution from step 2. Vortex gently and spin down.
4. Add 160 µL of 0.1% formic acid to bring the final volume to 200 µL. The reduced trastuzumab solution has a concentration of 250 ng/µL and is ready for LC/MS analysis.

LC/MS analysis

LC/MS analysis was performed on an Agilent 1290 Infinity II Bio LC system coupled to an Agilent InfinityLab Pro iQ Plus single quadrupole LC/MS system (Figure 1). An Agilent PLRP-S column (2.1 × 50 mm, 5 µm) was used for chromatographic separation. LC and MS parameters are listed in Tables 1 and 2.

Figure 1. Agilent 1290 Infinity II Bio LC system and Agilent Pro iQ Plus single quadrupole mass spectrometer.

Liquid chromatography

Table 1. Agilent 1290 Infinity II LC method.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent PLRP-S, 2.1 × 50 mm, 5 µm (part number PL1912-1502)
Sampler Temperature	5 °C
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Flow Rate	0.5 mL/min
Injection Volume	2 µL
Column Temperature	80 °C
Gradient Program (Intact)	Time (min) %B 0 10 5 60 6 10 8 10
Gradient Program (Reduced)	0 5 0.1 20 8 40 8.1 70 9.1 70 9.2 5 11 5

Mass spectrometry

Table 2. MS parameters.

Agilent Pro iQ Plus Single Quadrupole Mass Spectrometer	
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Time Filter Window	0.1 min
Stop Time	As pump/No limit
MS1 Scan Range	*m/z* 1,000 to 3,000 (intact) *m/z* 600 to 2,400 (reduced)
Scan Time	1,500 ms
Detector Gain Factor	1
Fragmentor	275 V (intact) 175 V (reduced)
Fragmentor Ramp?	Not checked
Data Storage	Profile
Gas Flow	12 L/min
Nebulizer	50 psi
Sheath Gas Flow	11 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Gas Temperature	350 °C
Sheath Gas Temperature	360 °C
Divert Valve	Enabled; LC flow to waste from 0 to 1 min, LC flow to MS from 1 to 8 min (intact) or 1 to 11 min for reduced
Postrun Diverter Position	To waste

Data processing

The LC/MS data were processed using Agilent OpenLab CDS software, version 2.8. Intact deconvolution parameters are shown in Figure 2. For reduced mAb deconvolution, the external background time range was set from 3.0 to 4.3 minutes, automatic deconvolution RT window from 4.3 to 5.6 minutes, deconvoluted mass range from 10,000 to 60,000 Da, Absolute noise threshold to 2,000, relative abundance threshold to 25%, and MW algorithm to centroid.

Results and discussion

Total ion chromatogram for intact trastuzumab

The total ion chromatogram (TIC) for 500 ng of intact trastuzumab is illustrated in Figure 3A. As expected, the TIC shows a single peak that elutes at ~2.6 minutes. To prevent high aqueous content and salts from entering the mass spectrometer, the LC flow is diverted to waste during the first minute of the LC/MS method. From 1 to 8 minutes, the diverter valve was switched to direct flow to the Pro iQ Plus, where the mAb signal was measured.

Raw MS and deconvoluted spectrum for intact trastuzumab

The raw MS and deconvoluted spectra for intact trastuzumab are illustrated in Figures 3B and 3C, respectively. The Agilent Pro iQ Plus single quadrupole mass spectrometer has a scan range of *m/z* 3,000, which allowed for the detection of many charge states for intact trastuzumab and providing excellent deconvoluted spectra. In fact, the inset of Figure 3B shows the raw peak shapes for one of the charge states of trastuzumab. The relative abundances shown here match what was observed in the deconvoluted spectrum in Figure 3C,

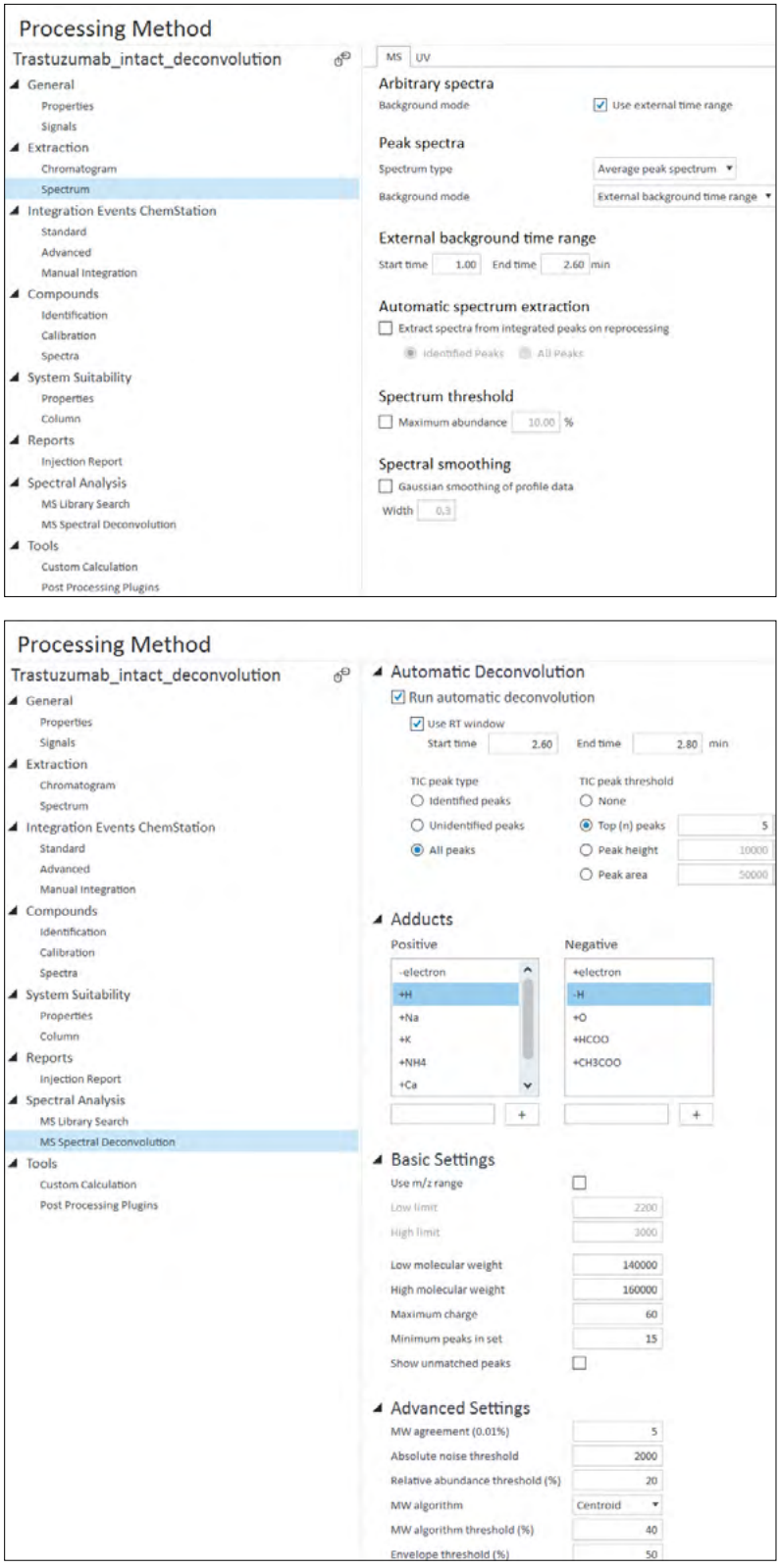


Figure 2. Agilent OpenLab CDS software, version 2.8 processing parameters that were used for intact mAb deconvolution.

giving the user additional confidence in the deconvoluted spectrum and the five main glycosylated peaks identified. A noteworthy processing parameter that reduced ghost peaks in the deconvoluted spectrum was the Minimum Peaks in Set parameter shown in the Basic Settings of the OpenLab CDS MS Spectral Deconvolution tab (Figure 2). For intact and reduced mAb deconvolution this setting was set to 15, which tells the software to deconvolute peaks that have at least 15 charge states. This is an important parameter because it improves the quality of peaks detected in the deconvoluted spectrum. This parameter can be adjusted based on the number of charge states observed in the raw mass spectrum for a molecule of interest.

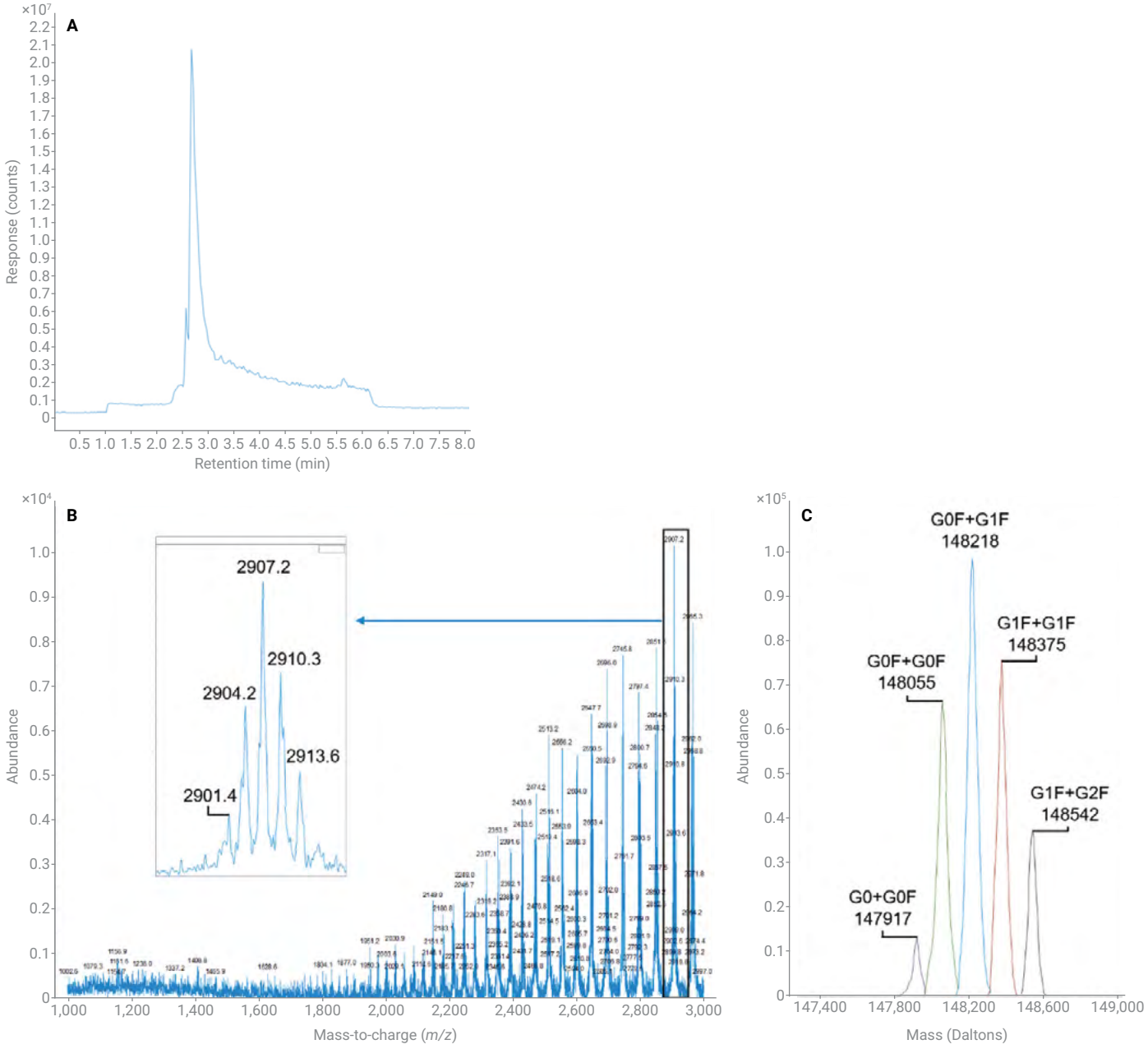


Figure 3. (A) TIC for intact trastuzumab measured on the Agilent Pro iQ Plus single quadrupole mass spectrometer. (B) Raw MS for trastuzumab. The inset shows the zoomed-in region for one of the charge states. (C) Deconvoluted mass spectrum for trastuzumab with measured masses and proposed glycoforms. LC/MS settings are listed in Tables 1 and 2, processing parameters are shown in Figure 2, and calculated mass errors in Table 3.

Reduced mAb chromatogram, raw MS, and deconvoluted spectra

To add an additional dimension to glycosylation profiling, an intact mAb can be reduced into subunits to reduce complexity. The TIC chromatogram for reduced trastuzumab is shown in Figure 4A, where two peaks for the light chain and heavy chain are observed. The measured MS spectral intensity for the light chain was 4-fold higher than the heavy chain. However, considerable glycosylation information for the HC and excellent MS spectral quality was obtained on the Agilent Pro iQ Plus. The insets of Figures 4B and 4D show a zoomed in region for the trastuzumab LC and HC, respectively. Since the site of glycosylation follows a consensus sequence for trastuzumab (i.e., glycosylation occurs at asparagine residues that follow an NXS/T motif), it is expected that the LC would show a single peak since its amino acid sequence should not allow glycan modification. On the other hand, the trastuzumab

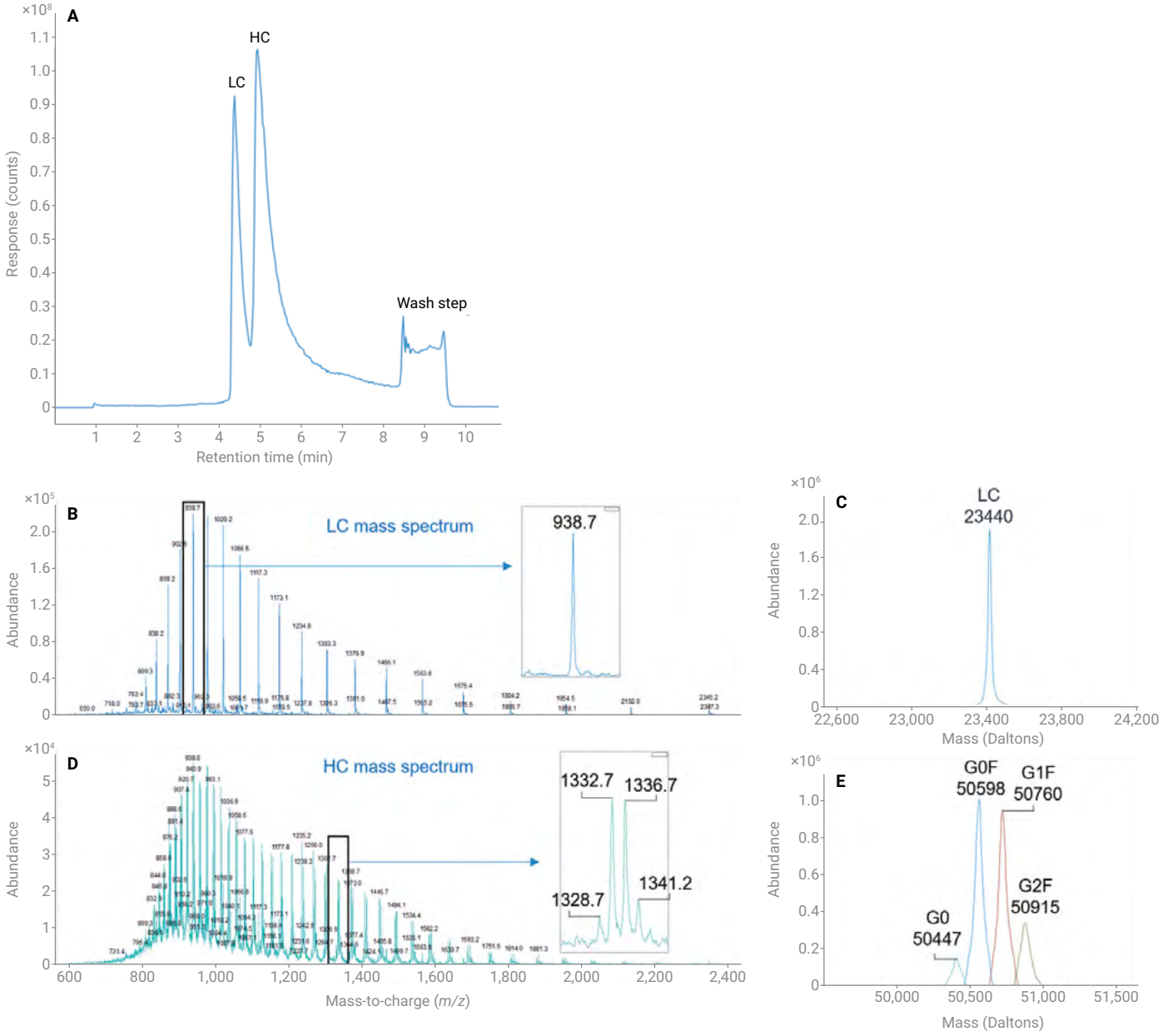


Figure 4. (A) TIC for reduced trastuzumab measured on the Agilent Pro iQ Plus single quadrupole mass spectrometer. Raw MS (B+D) for the trastuzumab light chain and heavy chain, respectively. The insets show the raw spectral quality on the instrument. Deconvoluted mass spectrum (C+E) for trastuzumab light chain and heavy chain, respectively. Calculated mass errors are displayed in Table 3.

HC has an NXS/T motif, which would permit glycosylation at the asparagine residue in the Fc region. The inset of Figure 4D shows four peaks in the raw MS spectrum that are consistent with the G0, G0F, G1F, and G2F glycans. The deconvoluted spectra for the LC and HC are shown in Figures 4C and 4E, respectively. The processing parameters used for reduced trastuzumab were similar to that shown in Figure 2 for the intact mAb. The only difference was that the deconvoluted mass range was changed to 10,000 to 60,000 Da and the relative abundance threshold was set to 25%. Noteworthy in the deconvoluted spectrum for the trastuzumab HC is that the relative intensities of the glycosylated peaks are similar to that observed in the raw MS spectrum inset. While each charge state for the HC will show different relative intensities for the glycosylated peaks, being able to correlate the relative abundances from the input MS spectrum to the deconvoluted spectrum provides confidence in the deconvoluted spectra. The ability to report on the relative abundances of glycosylation at the reduced and intact levels is an important measurement because deviation of these abundances from established tolerances will prompt the QA/QC team in a biopharmaceutical lab to perform additional investigation.

Relative abundances at intact/subunit levels and MS Spectral Deconvolution report

Table 4 shows the measured relative abundances for trastuzumab at the intact and subunit levels, respectively. The relative abundance (%) is calculated by normalizing abundances relative to the most intense component, whereas the relative quantitation (%) will measure the absolute abundance of a component relative to the total abundance of all components. Both the relative abundance (%) and relative quantitation (%) can be used to monitor product reproducibility and quality.

Figure 5 shows an example MS spectral deconvolution software report for intact trastuzumab. The report is easily customizable in the report editor tab of OpenLab CDS software. Here, the report displays details about the acquisition and processing methods, injection volume, vial position, and injection date for traceability. In addition, raw MS and deconvoluted spectra with detected components are shown. The report will also list relative abundance (%) and relative quantitation (%) of the components in the deconvoluted spectrum.

Table 3. Comparison of theoretical average molecular mass and experimentally observed molecular masses after deconvolution in Agilent OpenLab CDS software.

Measured Experimental Errors on the Agilent Pro iQ Plus Mass Spectrometer					
Molecule	**Modification**	**Theoretical Mass (Da)**	**Experimental Mass (Da)**	**Δ Mass (Da)**	**Mass Error (ppm)**
Intact Trastuzumab	G0+G0F	147,912.7	147,916.7	4.0	27
	G0F+G0F	148,058.8	148,055.1	−3.7	−25
	G0F+G1F	148,221.0	148,217.7	−3.3	−22
	G1F+G1F	148,383.1	148,374.7	−8.4	−57
	G1F+G2F	148,545.3	148,542.0	−3.3	−22
Trastuzumab HC	G0	50,456.1	50,447.3	−8.8	−174
	G0F	50,602.2	50,597.9	−4.3	−85
	G1F	50,764.4	50,759.9	−4.5	−89
	G2F	50,926.5	50,915.4	−11.1	−218
Trastuzumab LC	None	23,443.3	23,440.5	−2.8	−119

Table 4. Relative abundances after deconvolution at the (A) intact and (B) reduced levels.

A

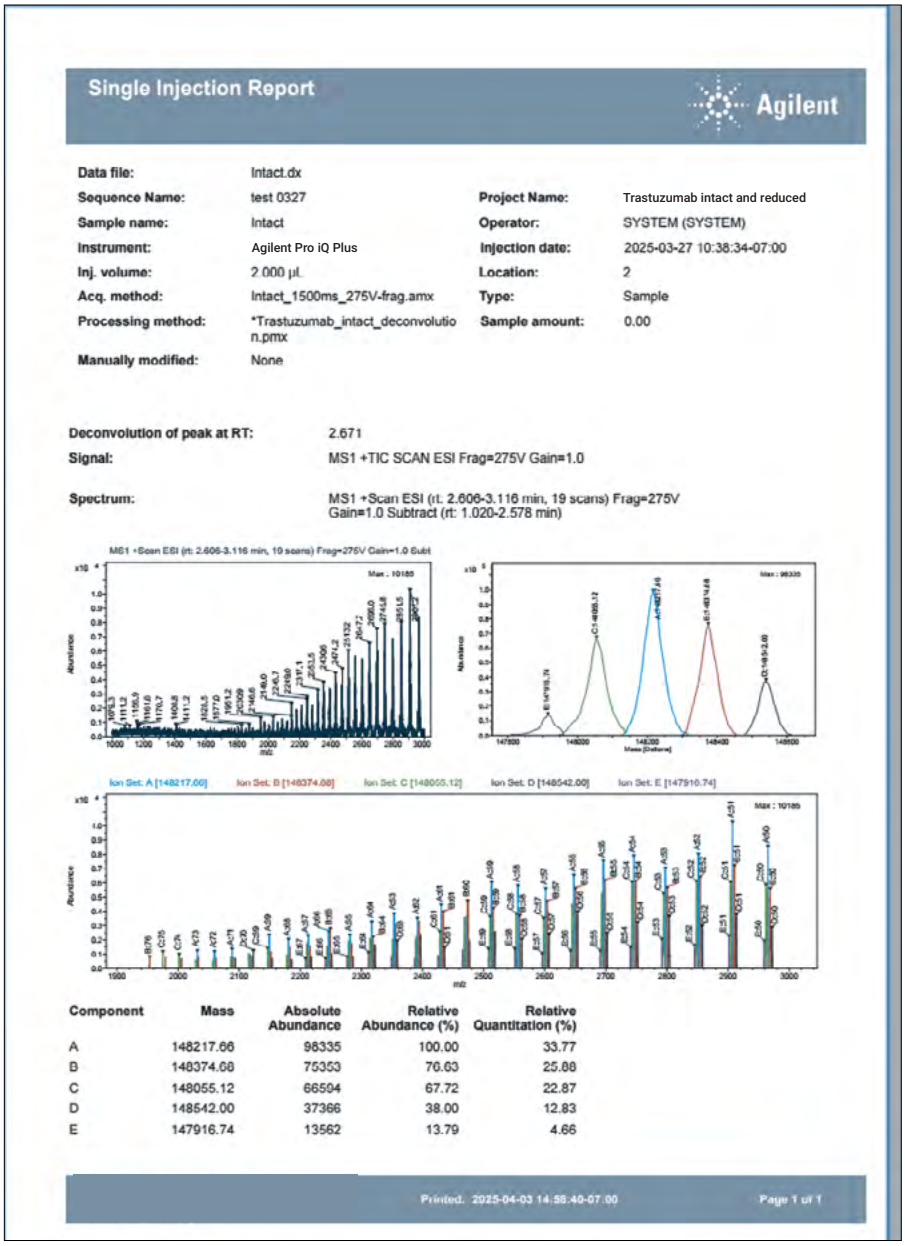
Trastuzumab Intact Relative Abundances				
Component	**Measured Mass**	**Proposed Identity**	**Relative Abundance (%)**	**Relative Quantitation (%)**
A	148,218 Da	G0F+G1F	100	33.77
B	148,375 Da	G1F+G1F	76.63	25.88
C	148,055 Da	G0F+G0F	67.72	22.87
D	148,542 Da	G1F+G2F	38.00	12.83
E	147,917 Da	G0+G0F	13.79	4.66

B

Trastuzumab Heavy Chain Relative Abundances				
Component	**Measured Mass**	**Proposed Identity**	**Relative Abundance (%)**	**Relative Quantitation (%)**
A	50,598 Da	G0F	100	41.22
B	50,760 Da	G1F	94.13	38.80
C	50,915 Da	G2F	33.71	13.90
D	50,447 Da	G0	14.73	6.07

Conclusion

Intact mass analysis by LC/MS is a technique that is commonly employed in the biopharmaceutical lab to monitor the relative abundances of glycosylation. These analyses are important for product efficacy and safety. The Agilent 1290 Infinity II Bio LC system coupled to an Agilent InfinityLab Pro iQ Plus provides a small and cost-effective LC/MS instrument for labs that require robust systems for routine analysis. In this study, the Pro iQ Plus LC/MS system was used to report on the relative abundances of glycosylated peaks at the intact and reduced levels in a therapeutic drug. Five glycosylated peaks were identified at the intact level with relative abundances that were consistent with results measured on Agilent high-resolution accurate mass spectrometry systems. Relative abundances were also monitored for the trastuzumab heavy chain, where four peaks were identified that are consistent with the G0, G0F, G1F, and G2F glycans. The combination of reliable relative abundances for monitoring glycosylation as well as excellent MS spectral peak shapes highlights the performance of the Agilent Pro iQ Plus single quadrupole mass spectrometer.



Single Injection Report

Agilent

Data file:	Intact.dx		
Sequence Name:	test 0327	**Project Name:**	Trastuzumab intact and reduced
Sample name:	Intact	**Operator:**	SYSTEM (SYSTEM)
Instrument:	Agilent Pro iQ Plus	**Injection date:**	2025-03-27 10:38:34-07:00
Inj. volume:	2.000 µL	**Location:**	2
Acq. method:	Intact_1500ms_275V-frag.amx	**Type:**	Sample
Processing method:	*Trastuzumab_intact_deconvolution.pmx	**Sample amount:**	0.00
Manually modified:	None		

Deconvolution of peak at RT: 2.671

Signal: MS1 +TIC SCAN ESI Frag=275V Gain=1.0

Spectrum: MS1 +Scan ESI (rt: 2.606-3.116 min, 19 scans) Frag=275V Gain=1.0 Subtract (rt: 1.020-2.578 min)

Component	Mass	Absolute Abundance	Relative Abundance (%)	Relative Quantitation (%)
A	148217.66	98335	100.00	33.77
B	148374.68	75353	76.63	25.88
C	148055.12	66504	67.72	22.87
D	148542.00	37366	38.00	12.83
E	147916.74	13562	13.79	4.66

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Figure 5. MS spectral deconvolution report for intact trastuzumab.

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2. Wang, D.; Baudys, J.; Bundy, J.; Solano, M.; Keppel, T.; Barr, J. Comprehensive Analysis of the Glycan Component of SARS-CoV-2 Spike Proteins Using Signature Ions-Triggered Electron-Transfer/Higher-Energy Collisional Dissociation (EThcD) Mass spectrometry. *Anal. Chem.* **2020**, *92*, *21*, 14730–14739. https://doi.org/10.1021/acs.analchem.0c03301

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Analysis of Oligonucleotides Using Ion-Pairing Alternatives on the Agilent Pro iQ Plus

Authors

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Agilent Technologies, Inc.

Abstract

This application note presents a practical approach for medium- to high-throughput molecular confirmation of synthetic oligonucleotides using the Agilent InfinityLab Pro iQ Plus LC/MS system. Traditional ion-pair reversed-phase LC/MS methods often require dedicated instrumentation and toxic, costly reagents. In contrast, the method described here uses an ammonium bicarbonate-based method, eliminating the need for ion-pairing agents while maintaining sufficient chromatographic retention and MS sensitivity. Agilent OpenLab CDS MS spectral deconvolution simplifies data analysis workflows, enabling automated processing with minimal optimization. This approach provides robust LC/MS performance for various oligonucleotide types, including antisense oligonucleotides and siRNAs.

Introduction

Oligonucleotides are an emerging therapeutic modality that target and modulate gene expression through the silencing or degradation of mRNA. Once an mRNA target is identified, the antisense sequence must be optimized with strategically incorporated chemical modifications to improve pharmacokinetics[1], affinity, and minimize off-targeting or mismatching.[2]

Ion-pair reversed-phase LC/MS is commonly used to confirm the molecular weights of target oligo sequences to ensure proper synthesis. However, using alkylamines as ion-pairing agents often requires dedicated instrumentation. Furthermore, toxic and cost-prohibitive perfluorinated alcohols such as HFIP (hexafluoroisopropanol) are required for optimal chromatographic separation and MS sensitivity.

In this application, an alternative reversed-phase approach was used for molecular confirmation of oligonucleotides. The method used ammonium bicarbonate instead of ion pairing, while still providing sufficient chromatographic retention and MS sensitivity. Twenty replicate injections of three different antisense oligonucleotides and five replicates of a single siRNA were performed to ensure the applicability and reproducibility of the method.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

- Agilent InfinityLab Pro iQ Plus LC/MS system (G6170A)
- Agilent Infinity II 1290 bio binary pump (G7120A)
- Agilent Infinity II 1290 bio multisampler (G7167B)
- Agilent Infinity II 1290 bio column compartment (G7116B)
- Agilent Infinity II 1260 diode array detector HS (G7117C)

Although this analysis used an Infinity II LC configuration, comparable results can be achieved on the Infinity III LC system with no changes to method parameters.

Sample preparation

All samples were resuspended in deionized (DI) water to a concentration of 50 µM and stored at –80 °C. Samples were transferred to polypropylene vials and stored in the temperature-controlled autosampler for up to two days prior to analysis. Oligonucleotide sample sequences are shown in Table 1.

Table 1. Oligonucleotide sample sequences.

Oligonucleotide Name	Length	Sequence
ASO-1	18	dU/MOErC//MOErA//MOErC/dUdUdU/MOErC//MOErA/dU/MOErA//MOErA/dU/MOErG/CdU/MOErG/G
ASO-2	20	dU/MOErC/dUdU/MOErG/TT/MOErA//MOErC//MOErA//MOErT//MOErG//MOErA//MOErA//MOErA/dU/MOErC//MOErC//MOErC/C
Fomivirsen	21	G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G
Givosiran	22 S	mC*mA*mGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA/L96/
	23 AS	mU*mG*mGfUmCfUmUfUfCmUfCfAmCfAmGfAmGfUmAmGfA*fA*mU

Code	Description
/MOErA/	Methoxyethoxy A
/MOErC/	Methoxyethoxy C
/MOErT/	Methoxyethoxy T
/MOErG/	Methoxyethoxy G
dU	Deoxyuridine
fA	2-fluoroadenosine
fC	2-fluorocytidine
fG	2-fluoroguanadine
fU	2-fluorouridine
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mC	2'-O-methyl C
mG	2'-O-methyl G
mU	2'-O-methyl U
rA	Ribose adenine
rC	Ribose cytosine
rG	Ribose guanine
rU	Ribose uracil

LC/MS analysis

Source parameters for the Pro iQ Plus system are provided in Table 2, while high-performance liquid chromatography (HPLC) parameters are provided in Table 3.

Table 2. Source parameters for the Agilent Pro iQ Plus system.

Mass Spectrometry Parameters	
Parameter	**Value**
MS	Agilent Pro iQ Plus
Source	Agilent Jet Stream Electrospray Ionization (AJS-ESI) Source
Drying Gas Flow	13.0 L/min
Gas Temp	300 °C
Nebulizer Pressure	35 psi
Capillary Voltage	3,000 V
Sheath Gas Temp	250 °C
Sheath Gas Flow	11 mL/min
Nozzle Voltage	1,500 V
Mode	Positive
Scan	*m/z* 700–2,800
Scan Time	1,250 ms
Fragmentor	180 V
Gain Factor	5

Table 3. HPLC parameters used.

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide column, 2.1 × 50 mm, 2.7 µm
Sampler Temperature	8 °C
UV Detection	260/4 nm (Ref 360/20 nm) Peak width > 0.1 min (2.5 Hz)
Mobile Phase A	20 mM ammonium bicarbonate in DI water
Mobile Phase B	Methanol
Flow Rate	0.7 mL/min
Injection Volume	2 µL
Multiwash	20:80 water:methanol; flush port; 5 seconds 90:10 water:methanol; flush port; 3 seconds
Column Temperature	75 °C
Post Time	1.0 min
Gradient Program	Time (min) %B 0 5 0.1 5 3.0 40 3.1 80 3.5 80 3.6 5

Results and discussion

Established in 1997, an alkylamine ion pair with perfluorinated alcohol as the acidic modifier is the preferred mobile phase for LC/MS analysis of oligonucleotides.[3] This is due to its chromatographic performance and electrospray efficiency, especially when compared to mobile phases using acetate as the counter ion. Extensive work has further demonstrated a wide experimental design space when using this powerful ion-pairing system.[4]

However, there are consequences when using alkylamine and HFIP for LC/MS methods. First, optimization of buffer components is necessary since oligo modifications and sequence affect electrospray desorption and chromatography.[5] Second, alkylamine may contaminate ionization sources and LC systems, leading to background peaks if polarity is switched back to positive mode. Consequently, this requires extensive cleaning/passivation of LC components and ion source surfaces. Even then, some labs may dedicate systems to negative mode due to the adsorption of alkylamines onto the LC/MS system. Finally, alkylamine-containing mobile phases have a short shelf life if not kept sealed under argon gas, and thus, mobile phases must be made fresh, sometimes daily, to ensure consistent method performance.

For labs performing molecular weight confirmation workflows where many oligos are analyzed, optimizing mobile phase conditions may not be feasible. Additionally, LC/MS downtime due to instrument maintenance and daily preparation of buffers may lead to sample backlogs, which for many labs can be a considerable challenge. A more practical approach would be to use a non-ion-pairing, reversed-phase methodology.

Recent work provides a more practical and cost-effective alternative to ion pairs, using an ammonium bicarbonate (NH_4HCO_3) buffer and methanol as the strong solvent.[6] This is an advantageous method for molecular weight confirmation, as ESI sensitivity and chromatographic performance are sufficient, even with minimal optimization of the mobile phase and gradient. Further, this referenced work postulates that carbon dioxide outgassing facilitates droplet formation, while ammonia evaporation contributes to proton adduction, thus allowing for positive mode analysis. This, therefore, eliminates the need to dedicate a system for negative mode analysis.

Because oligonucleotides are polar and negatively charged, there may be concerns that these analytes would not be retained or separate chromatographically without ion pairing. However, ammonium bicarbonate-based methods retain a wide variety of oligonucleotides and perform particularly well with modified oligos, such as antisense oligos (ASOs; Figure 1). The method demonstrated here provides a proof-of-concept for medium- to high-throughput analysis. Therefore, the gradient is 5% to 40% Mobile Phase B over three minutes. This relatively "ballistic" gradient provides excellent retention and peak shape, and the 0.7 mL/min flow rate does not negatively impact the spectral quality nor sensitivity.

Like most large molecules with multiple ionizable functional groups, oligos appear as multiply charged ions when analyzed by ESI-MS. Importantly, depending on the alkylamine ion pair and source parameters, charge state distribution of the oligo may vary.[7] This can be problematic with heated ionization sources, which tend to yield higher charge states. Consequently, deconvolution of the spectrum may lead to artifacts and potential misidentifications. On the other hand, ammonium bicarbonate (ABC) tends to yield oligos with lower charge states (Figure 2). The higher *m/z* values will have less spectral overlap with matrix interferences, impurities, or other components, thus improving confidence in the deconvoluted data.

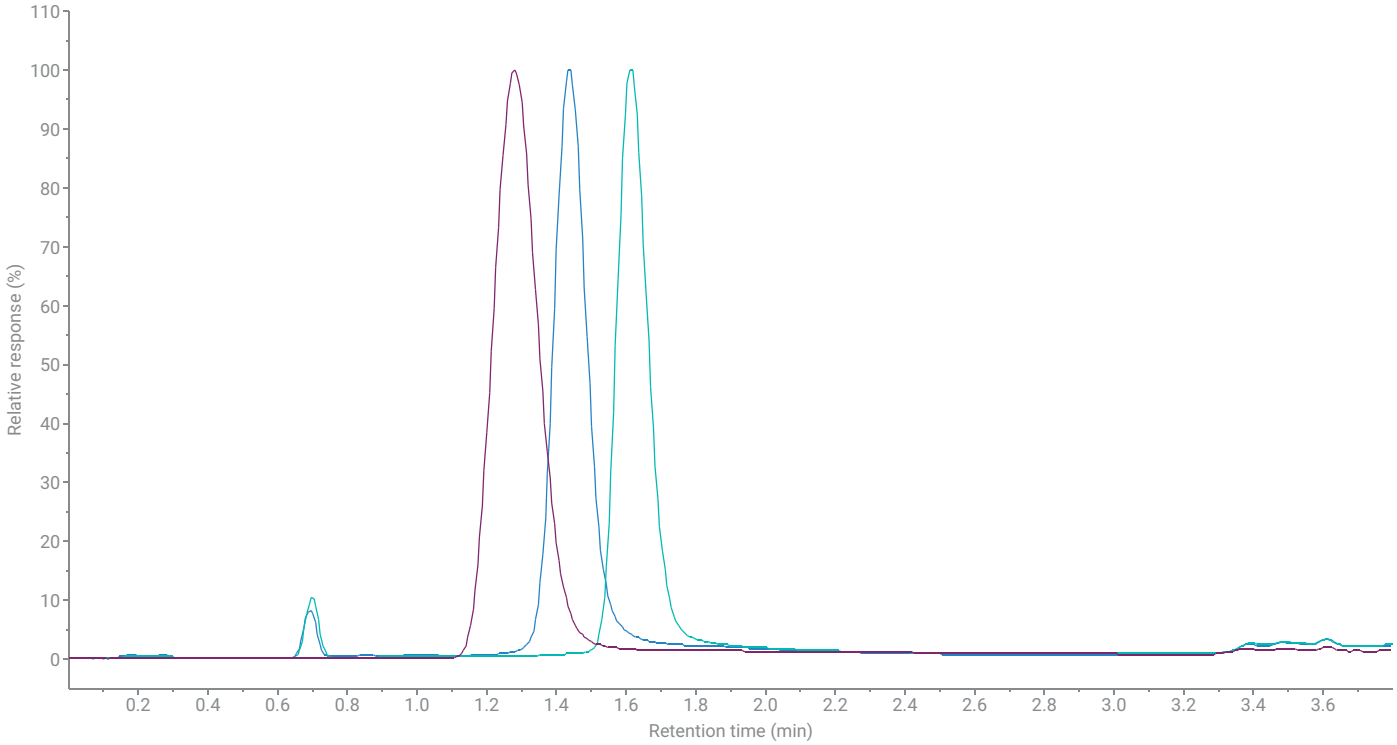

Figure 1. UV chromatograms for three antisense oligos (ASOs). Fomivirsen is a 21mer ASO with no 2'-modifications. Thus, it elutes earlier than the two shorter, fully thioated, 2'-modified ASOs.

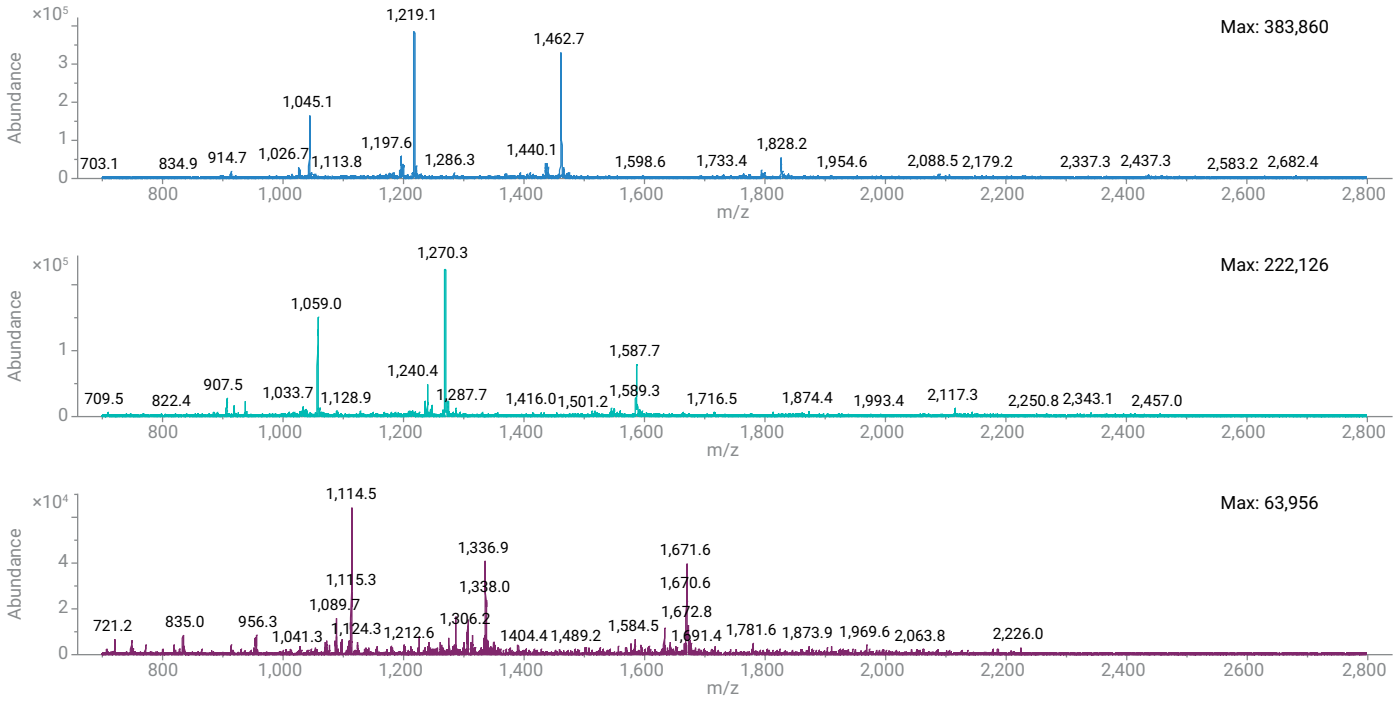

Figure 2. Full-scan spectra for samples. Charge states of 4 to 6 are predominant for each ASO.

Agilent OpenLab CDS uses a unique spectral deconvolution that is optimized for unit mass detectors. Maximum entropy calculates the most probable mass from a spectrum by increasing dominant features while minimizing noise. In contrast, OpenLab CDS spectral deconvolution simply identifies ions in the spectrum and matches them to target masses based on user inputs. These values are then fit to a linear regression or centroided to calculate a molecular weight average. This simple approach allows for easy molecular weight confirmation using generic processing methods, which can be fully automated with minimal optimization. Triplicate injections of each sample yield nearly identical results, with relative standard deviations (%RSDs) < 0.1%. The automated deconvolution settings used in the sample sequence for acquisition are shown in Table 4, and a summary of deconvolution results is shown in Table 5.

Table 4. MS spectral deconvolution settings.

Parameter	Value
Automatic Deconvolution Settings	
Run Automatic Deconvolution	Enabled
RT Window	1–2.7 min
TIC Peak Type	All peaks
TIC Top (n) Peaks	3
Basic Settings	
Use *m/z* Range	Disabled
Low/High Molecular Weight	3,000–10,000
Maximum Charge	10
Minimum Peaks in Set	3
Advanced Settings	
MW Agreement (0.01%)	10
Absolute Noise Threshold	100
Relative Abundance Threshold	10
MW Algorithm	Curve fit
MW Algorithm Threshold (%)	40
Envelope Threshold (%)	50

Figure 3 shows the deconvoluted spectrum of ASO-1, an 18mer phosphorothioate with 2'-MOE modifications. Component A is a full-length product, showing good agreement with the calculated mass. Component B is likely depurination (loss of guanine). Component C is likely a sodium adduct. As shown, the InfinityLab Pro iQ Plus, which combines unit mass accuracy with simple deconvolution, can monitor oligonucleotides and identify unexpected impurities or components. Further characterization can be performed by high-resolution MS.

Table 5. Deconvoluted results of twenty replicates of each ASO.

Oligonucleotide Name	Calculated Mass (Da)	Average Measured Mass (Da)	Delta Mass (Da)
ASO-1	6,348.3	6,347.9	−0.4
ASO-2	7,309.2	7,308.8	−0.4
Fomivirsen	6,682.4	6,681.8	−0.6

A duplex, tri-antennary GalNAc-conjugated siRNA (Givosiran) was also analyzed to demonstrate broad applicability of the method. Slight modifications to the LC/MS parameters were required with the duplex conjugate. Specifically, the gradient program was adjusted slightly (5% to 45% in 3 minutes) to ensure baseline separation of the sense and antisense strands. Additionally, the fragmentor voltage was decreased to 120 V to minimize in-source fragmentation of the fragile oligosaccharide conjugate. Figure 4 shows an overlay of UV chromatograms for five injections of Givosiran.

Although no ion pair is used, the ABC mobile phase still provides baseline separation of sense and antisense strands (Figure 5). This separation is required because any spectral overlap may result in misidentifications in deconvolution. Spectra are shown in Figure 5, with similar charge state distributions to the previously mentioned ASOs. Analysis of the deconvoluted spectrum for each peak show the antisense strand eluting earlier than the sense strand. Interestingly, the sense strand may indicate GalNAc losses (Figure 6). A summary of Givosiran results is shown in Table 6.

Table 6. Deconvoluted results for each injection of Givosiran.

Oligonucleotide Name	Calculated Mass (Da)	Average Measured Mass (Da)	Delta Mass (Da)
Givosiran, Antisense	7,563.8	7,563.1	0.7
Givosiran, Sense	8,736.5	8,735.6	0.9

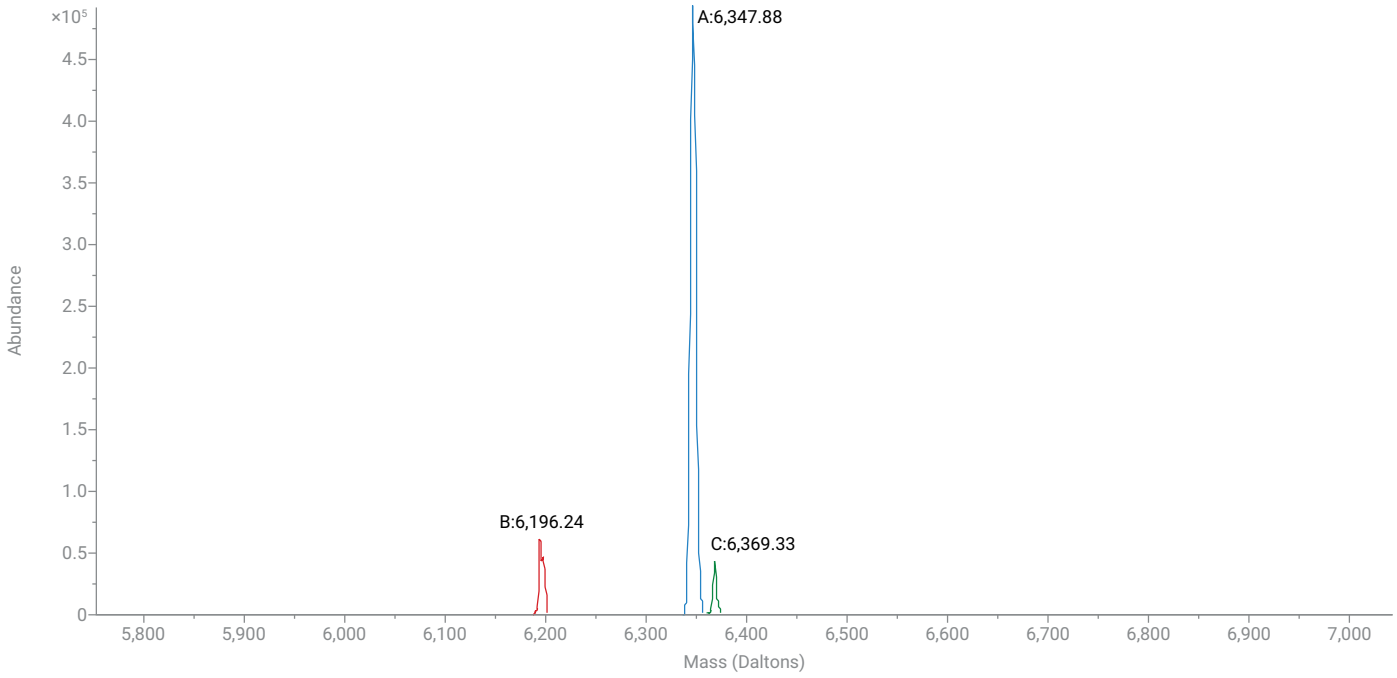

Figure 3. Deconvoluted spectrum for ASO-1.

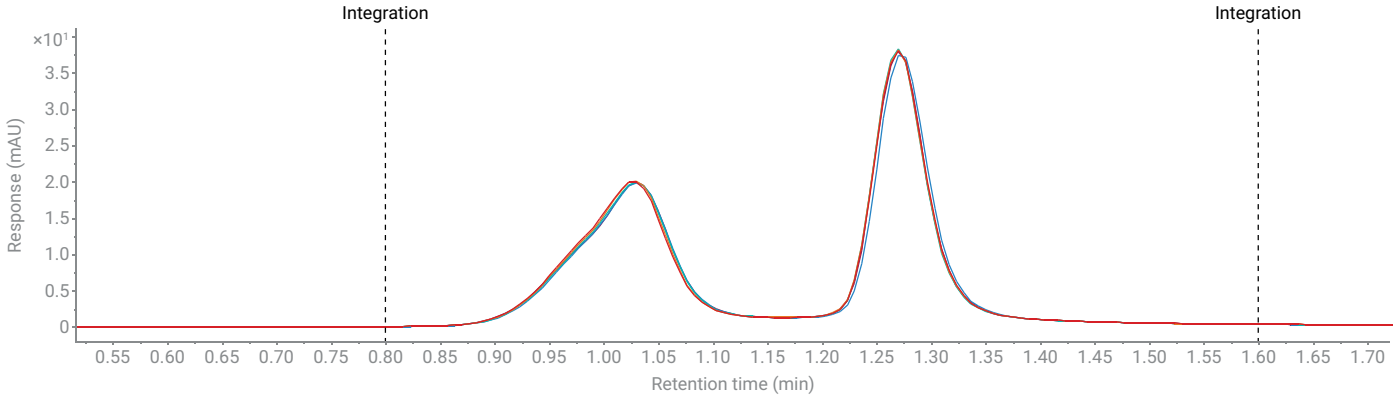

Figure 4. Overlay of UV chromatograms for five injections of Givosiran.

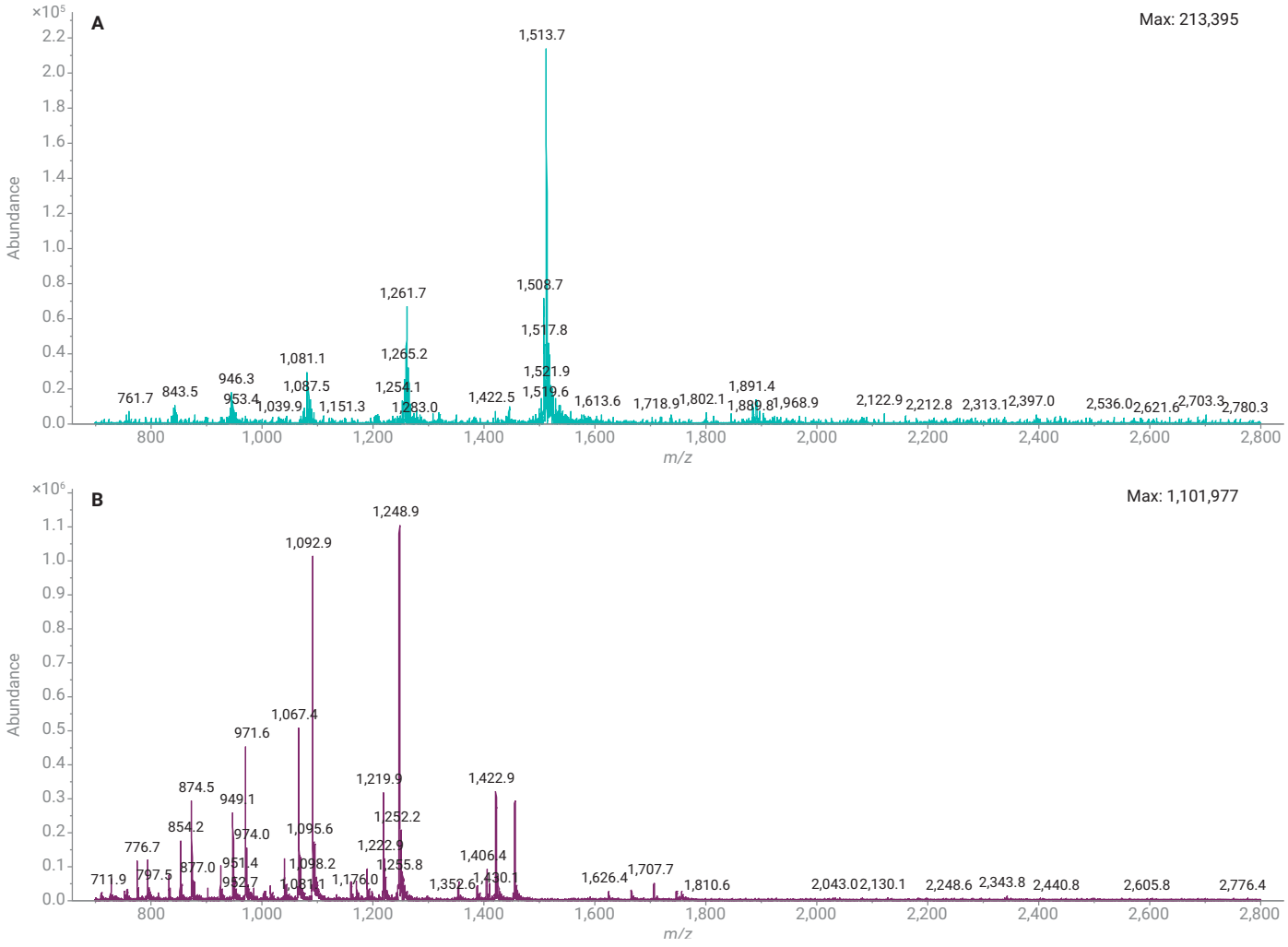

Figure 5. Spectra for antisense (A) and sense (B) RNA strands. Charge state distribution is similar to that of antisense oligos analyzed using a similar method. The 5– charge state is the most abundant for antisense, and the 6– charge state is the most abundant for the GalNAc-conjugated sense strand.

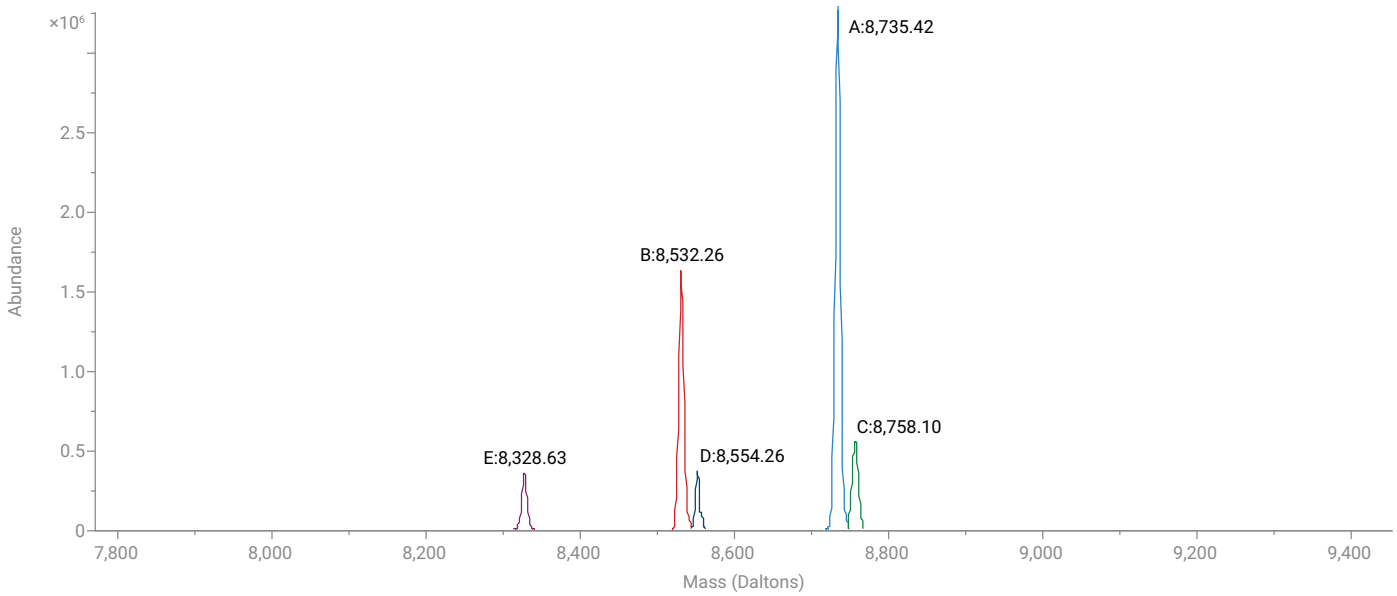

Figure 6. Deconvoluted spectrum for the Givosiran sense strand. The approximately 200 Da shift observed could be loss of GalNAc.

Conclusion

The method described in this application note provides a practical approach for medium- to high-throughput molecular confirmation of synthetic oligonucleotides. The Agilent Pro iQ Plus demonstrates excellent resolution and sensitivity for a unit mass detector, even at high *m/z* ranges that exceed the capabilities of many single quadrupole detectors. The method uses a novel ammonium bicarbonate mobile phase, providing sufficient LC/MS performance for labs analyzing many types of oligos, including antisense oligonucleotides and siRNAs. LC and MS results described in this application note are reproducible. Data analysis workflows are simplified using Agilent OpenLab CDS MS spectral deconvolution, which is automated by unattended processing methods that require minimal optimization.

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Impurity Profiling of Tirzepatide Under Stress Conditions

Using Agilent Pro iQ Plus

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Abstract

The increased demand and use of glucagon-like peptide-1 (GLP-1) agonists prompts a need for robust analytical methods to screen for impurities that may impact their safety and efficacy. Synthetic GLP-1 peptides are susceptible to degradation through pathways such as oxidation under stress conditions.[1] In this study, a high-performance liquid chromatography (HPLC) system coupled with an Agilent InfinityLab Pro iQ Plus mass detector was used to monitor tirzepatide impurities under various pH and storage conditions. This study demonstrates that a high-sensitivity single quadrupole LC/MS system can be used to streamline the detection and monitoring of low-level peptide impurities, making it suitable for implementation in routine quality control (QC) and quality assurance (QA) environments.

Introduction

Glucagon-like peptide-1 (GLP-1) agonists are a class of compounds utilized to treat type-2 diabetes mellitus (T2DM) and obesity. The function of GLP-1 agonists is to lower serum glucose levels and thereby manage metabolism in affected patients. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are both incretin hormones inactivated by dipeptidyl peptidase-4. These hormones stimulate insulin secretion after an oral glucose load through the incretin effect.[2,3] In T2DM, this process can become blunted or absent; however, pharmacological levels of GLP-1 can restore insulin secretion.

The US Food and Drug Administration (FDA) considers the types and amounts of impurities present in a proposed generic drug compared to its reference listed drug (RLD). According to the FDA, a proposed generic synthetic peptide should not contain (i) impurities at levels greater than those found in the RLD nor (ii) any new specified peptide-related impurity that is greater than 0.5% of the drug substance.[4]

Tirzepatide, a dual GIP and GLP-1 receptor agonist, offers promising therapeutic potential but presents analytical challenges due to its susceptibility to chemical degradation, including deamidation, oxidation, and peptide backbone cleavage. Monitoring such degradation pathways is essential for ensuring product integrity, safety, and efficacy.

The Agilent InfinityLab Pro iQ Plus single quadrupole mass spectrometer (MS) offers enhanced sensitivity and dynamic range for low-abundance (sub-ppm) impurity detection. The Agilent 1290 Infinity II LC system coupled with the Pro iQ Plus LC/MS instrument brings a cost-effective mass detection workflow for routine analysis to QC laboratories. This application note details the results from analyzing tirzepatide and its related impurities under varying pH and storage conditions.

Experimental

Chemicals and standards

- Formic acid, 98% (LC-MS grade LiChropur), Millipore Sigma (part number 5.33002)
- InfinityLab acetonitrile (LC/MS grade), 1 × 1 liters, Agilent (part number 5191-5101-001)
- Tirzepatide, AstaTech (part number AT40456)
- Ammonium formate (LC-MS grade LiChropur), Millipore Sigma (part number 70221)
- Ammonium hydroxide, for HPLC, 35% solution in water, Thermo Fisher Scientific (catalog number 460801000)

Sample preparation

Tirzepatide stock solution was prepared at 2 mg/mL in 15% acetonitrile/de-ionized water. Working solutions were diluted to 50 ng/µL in buffer solutions adjusted to pH 5, 7, and 9. Samples were injected directly without additional purification. Aliquots were stored in the autosampler at 5 °C and analyzed on different days after storage for up to seven days.

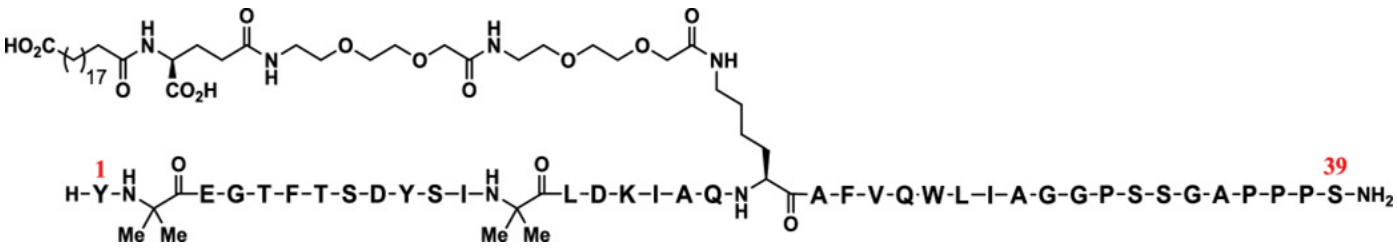

Figure 1. Tirzepatide amino acid composition.[5]

Instrumentation

LC/MS analysis was performed using the Agilent 1290 Infinity II bio LC system coupled with the Pro iQ Plus mass detector. Chromatographic separation was achieved on an Agilent ZORBAX RRHD 300 Å StableBond C18 column. The detailed LC and MS operating parameters are summarized in Tables 1 and 2.

Table 1. LC method parameters.

1290 Infinity II Bio LC System	
Column	ZORBAX RRHD 300 Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)
Mobile Phase A	LC/MS-grade water + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate	0.400 mL/min
Injection	Standard
Injection Volume	1 µL
Column Temperature	60 °C
Gradient Program	Time (min) %B 0 20 5 48 10 58 11 60 12 80 14 80 14.1 20 15 20

Table 2. MS parameters.

Pro iQ Plus Single Quadrupole LC/MS	
Source	
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Gas Temperature	300 °C
Drying Gas Flow	11 L/min
Nebulizer	30 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Pro iQ Plus	
Fragmentor	95 V
Scan Type	Scan
Scan Time	500 ms
Data Storage	Profile
MS Spectrum Range	*m/z* 500–2,500

Software and data analysis

The Pro iQ Plus LC/MS system was operated using Agilent OpenLab CDS 2.8 software, which includes built-in spectral deconvolution capabilities for processing GLP-1 peptide data. The deconvolution algorithm in OpenLab CDS is specifically optimized to simplify complex spectra generated from multiply charged ions, particularly those acquired using unit mass resolution instruments. Table 3 outlines the specific data processing parameters used in this study.

Table 3. Data processing parameters used in OpenLab CDS 2.8 for GLP-1 peptide analysis.

Parameter	Value
Spectrum Extraction Type	Peak apex spectrum
Background Mode	Spectrum at peak start and end
Use *m/z* Range	Disabled
Run Automatic Deconvolution	Enabled
Low Molecular Weight	500
High Molecular Weight	10,000
Maximum Charge	6
Minimum Peaks in Set	3
MW Agreement (0.01%)	5
Absolute Noise Threshold	1,000
Relative Abundance Threshold (%)	10
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	50

Results and discussion

Tirzepatide degradation products were highly dependent on pH and storage duration. The Pro iQ Plus single quadrupole LC/MS system successfully detected trace levels of impurity-related products.

Peptide analysis using OpenLab CDS

OpenLab CDS software provides a unified platform for peptide characterization and impurity assessment. Figure 2 shows the data review for tirzepatide after seven days of storage at 5 °C and pH 7. As shown in Figure 2, impurity peaks were detected at trace levels, eluting close to the main tirzepatide peak. It should be noted that accurate deconvolution relies on acquiring high-quality mass spectra. The Pro iQ Plus mass detector provides such high-quality mass spectra, even for trace impurities.

The software layout includes helpful tools such as chromatogram view, MS spectrum window, and spectral deconvolution results, allowing for efficient review of both the main peptide and any low-level impurities.

The total ion chromatogram (TIC) shows peaks corresponding to tirzepatide and its related impurity products. The mass spectrum at 7.89 minutes confirms the elution of tirzepatide, with the presence of +3, +4, and +5 charge states (Figure 2). The deconvoluted mass spectrum shows a molecular weight of 4,813.1 Da, consistent with the theoretical average mass of tirzepatide (4,813.5 Da) and within the expected mass accuracy (±0.3 Da) for the instrument.

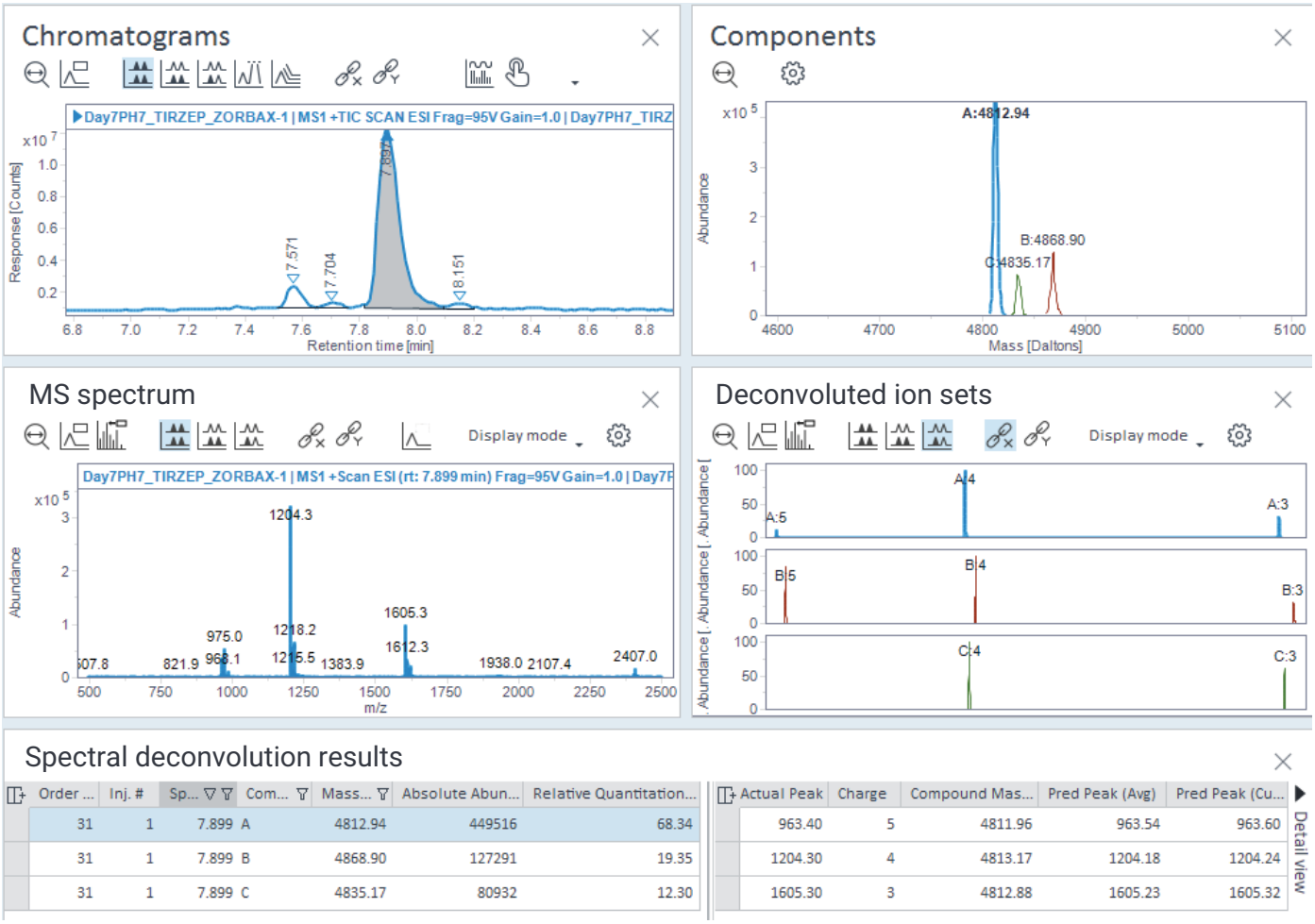

Figure 2. Data review for tirzepatide after seven days of storage at 5 °C and pH 7.

The Pro iQ Plus LC/MS system enables sensitive detection and characterization of low-level impurities in tirzepatide samples. Figure 3 shows the mass spectra of two impurities eluting at 7.57 and 7.71 minutes (as seen in Figure 2), with deconvoluted masses of 4,845.2 and 4,817.9 Da, respectively. These species correspond to mass shifts of +32 and +4.4 Da relative to the theoretical average mass of native tirzepatide (4,813.5 Da). The mass shifts are consistent with oxidative modifications, which are common degradation pathways for peptides under stress conditions. Amino acids such as methionine, tryptophan, and histidine are particularly susceptible to oxidation. Notably, tryptophan oxidation can lead to a +4 Da shift due to specific structural modifications ($+O_2-CO$).[6]

Table 4 summarizes the deconvoluted molecular weights of tirzepatide and its related impurities, including oxidized forms and an unknown impurity.

Table 4. Deconvoluted molecular weights of tirzepatide and its related impurities.

GLP-1 Peptide	Native Peptide Molecular Weight (Da)	Impurity Product		
		Oxidation ($+O_2$)	Oxidation (O_2-CO)	Unknown Impurity
Tirzepatide	4,813.1	4,845.2	4,817.9	4,689.6

In addition to the oxidation-related impurities, an unknown species was detected at a retention time of 8.15 minutes. The deconvoluted mass spectrum of this species indicates a molecular weight of ~ 4,689.6 Da, corresponding to a mass difference of +124 Da relative to native tirzepatide. Further MS characterization (that is, MS/MS) is required to identify this impurity.

The high sensitivity and dynamic range of the Pro iQ Plus LC/MS instrument allows for confident detection of both major and trace-level species in a single run. Importantly, the MS successfully detects impurities present at a relative peak area < 2%, highlighting the suitability of the Pro iQ Plus LC/MS for monitoring minor degradants in peptide samples. Furthermore, the integrated spectral deconvolution within OpenLab CDS streamlines data analysis by simplifying interpretation of multiply charged spectra, eliminating the need for external software.

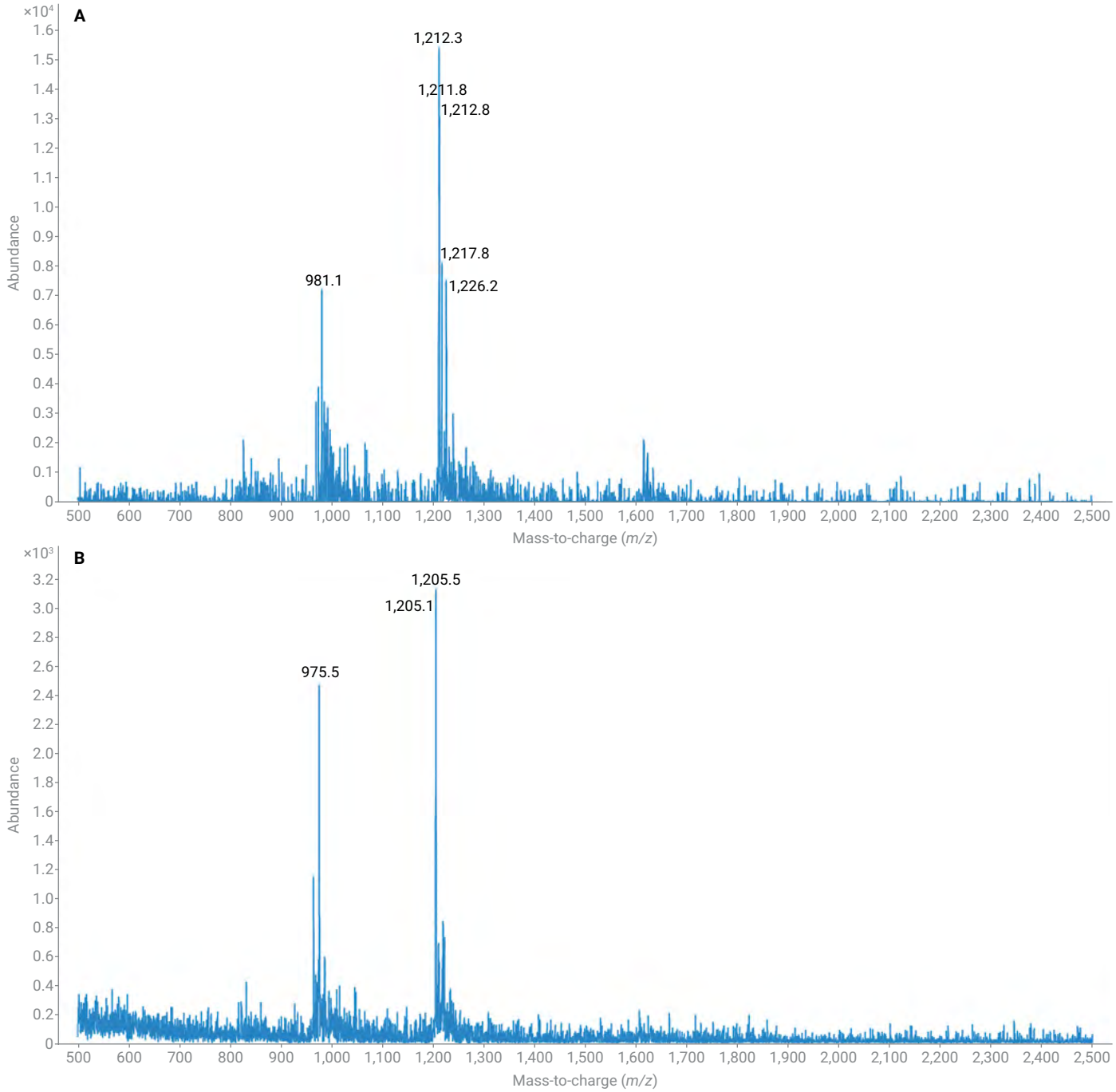

Figure 3. Mass spectra of tirzepatide impurities eluting at retention times 7.57 minutes (Panel A) and 7.71 minutes (Panel B). Mass spectral deconvolution reveals molecular weights of 4,845.2 Da for the impurity in Panel A and 4,817.9 Da for the impurity in Panel B.

Temporal monitoring of impurity generation

Proper storage and handling of GLP-1 medications are essential to preserve their stability, potency, and overall effectiveness. Inappropriate storage conditions can lead to degradation, reduced therapeutic efficacy, and potential safety risks. Therefore, evaluating how different storage durations and conditions influence the formation of degradation products is critical for ensuring product quality and patient safety.

Figure 4 shows temporal plots for the generation of the oxidation products of tirzepatide at various solution pH values. The data in Figure 4 suggest that tirzepatide is least stable at pH 5 and can undergo significant oxidation, even at 5 °C.

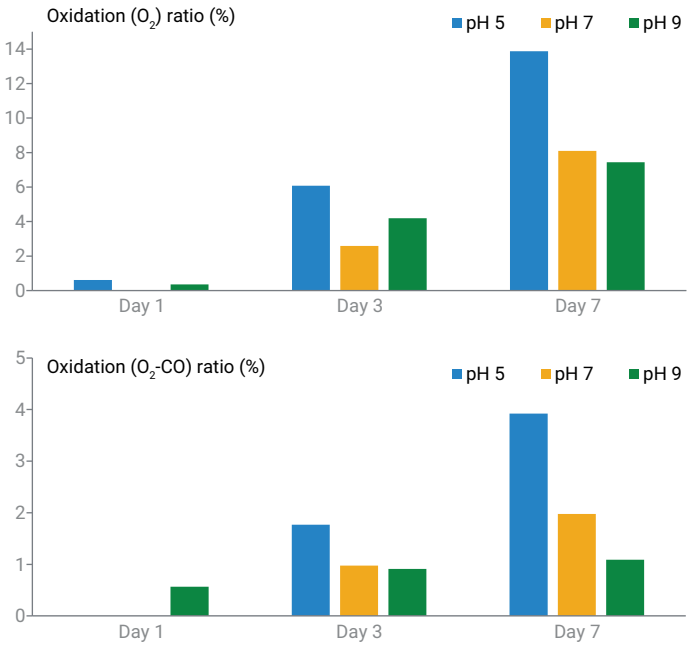

Figure 4. Oxidation ratio of tirzepatide related impurities over time under different pH conditions.

Conclusion

In conclusion, impurity profiling of tirzepatide under stress conditions using the Agilent Pro iQ Plus mass detector demonstrates the effectiveness of this analytical method for detecting and monitoring low-level peptide impurities. Notably, the method was able to detect impurities with relative peak areas less than 2%, showcasing its sensitivity for low-level impurity identification. The study highlights the susceptibility of tirzepatide to degradation through oxidation, particularly under varying pH and storage conditions. These findings underscore the importance of proper storage and handling to maintain the stability and efficacy of GLP-1 medications. The high sensitivity, cost-effectiveness, and simplicity of this single quadrupole LC/MS make it a practical and accessible tool for routine pharmaceutical QC and QA workflows.

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Assessing the Purity of an Antisense Oligonucleotide Sample by LC/MS

Using a high-sensitivity unit mass detector

Authors

Lee Bertram and Brian Rivera
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Abstract

This application note outlines a method for assessing the purity of antisense oligonucleotides (ASOs) using the Agilent InfinityLab Pro iQ Plus LC/MS system. While high-resolution mass spectrometry is typically employed for analyzing large biomolecules, such as oligonucleotides, the LC/MS approach described here enables the use of a unit mass detector in a more robust and routine fashion. The Agilent Oligo Analysis Accelerator (OAA) enhances peak integration and spectral interpretation, streamlining the quality control workflow. This method supports rapid analysis of oligonucleotide purity, assay performance, and basic impurity profiling.

Introduction

Antisense oligonucleotides (ASOs) are an established therapeutic nucleic acid modality, manufactured by solid-phase synthesis. Because of their complexity, a common way to assess the purity of ASOs is liquid chromatography/mass spectrometry (LC/MS). Although high-resolution MS might be appropriate for workflows such as characterization and sequence confirmation, unit mass detection provides a more robust and practical method for routine testing labs.

Single quadrupole LC/MS has previously been shown to effectively support purity, assay, and impurity profiling for routine testing of single-stranded therapeutic oligos, such as ASOs.[1] This method relies on MS full scan to quantitate closely related impurities that elute under the main peak. The LC/MS conditions ensure that analytes are predominantly in the 4– charge state when entering the gas phase. For example, an oligo that is ~ 8 kDa would have a predominant *m/z* value of ~ 2,000. This would either be at the upper limit or possibly exceed the scan range for some unit mass detectors. Furthermore, this method has a limit of quantitation (LOQ) of ≥ 0.2%, requiring a sensitive mass detector with efficient high mass transmission.

In this application note, the Agilent InfinityLab Pro iQ Plus LC/MS system is used to determine the purity of an ASO. The full scan spectrum from this unit mass detector enables the identification and quantification of low-level impurities at method specifications. This study establishes proof of concept for the use of this system in the routine testing of oligonucleotides for QC lot release.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

- Agilent InfinityLab Pro iQ Plus LC/MS system (G6170A)
- Agilent 1290 Infinity II bio binary pump (G7120A)
- Agilent 1290 Infinity II bio multisampler (G7167B)
- Agilent 1290 Infinity II bio column compartment (G7116B)
- Agilent 1260 Infinity II diode array detector HS (G7117C)

Although this analysis used an Agilent Infinity II LC configuration, comparable results can be achieved on the Agilent Infinity III LC system with no changes to method parameters.

Sample preparation

A 20-mer, non-HPLC purified antisense oligo (ASO-1) was obtained from Integrated DNA Technologies (Coralville, IA, USA). Samples were reconstituted in de-ionized water to 1 mg/mL, then further diluted to 0.1 mg/mL in de-ionized water.

Software

Data acquisition was performed in Agilent OpenLab CDS, version 2.8, using the LC/MS parameters shown in Tables 1 and 2. Data analysis was performed in Oligo Analysis Accelerator (OAA) for OpenLab CDS, version 1.0.

LC/MS settings

Table 1. Source parameters for the Agilent InfinityLab Pro iQ Plus (6170A).

Mass Spectrometry Parameters	
MS	6170A
Source	Electrospray ionization (ESI)
Scan Mode	Negative polarity
Drying Gas Flow	12.0 L/min (standard) 13.0 L/min (harsh)
Gas Temperature	260 °C (standard) 350 °C (harsh)
Nebulizer Pressure	25 psi
Capillary Voltage	4,000 V
Scan	*m/z* 1,450 to 2,175
Scan Time	1,000 ms
Fragmentor	100 V
Gain Factor	1

Table 2. HPLC parameters.

Parameter	Value
Column	Agilent AdvanceBio Oligonucleotide, 2.1 × 150 mm, 2.7 µm (p/n 659750-702)
Sampler Temperature	8 °C
UV Detection	260/4 nm (Ref 400/80 nm) Peak width > 0.05 min (5 Hz)
Mobile Phase A	10% ACN, 5 mM tributylammonium acetate, 1 µM EDTA
Mobile Phase B	80% ACN, 5 mM tributylammonium acetate, 1 µM EDTA
Flow Rate	0.25 mL/min
Multi Wash	20:80 Water:methanol, flush port, 5 seconds 90:10 Water:methanol, flush port, 3 seconds
Column Temperature	50 °C
Post Time	1.0 min
Gradient Program	Time (min) %B 0 45 22 80 25 80 26 45

Results and discussion

This method demands exceptional sensitivity, precision, and a broad mass range, making the Agilent InfinityLab Pro iQ Plus an ideal solution for the task. To determine the purity of an ASO, a full scan spectrum is extracted from the main peak, which tends to be broad (2 to 3 minutes) due to the shallow gradient program. Any *m/z* values in the average spectrum that exceed an established threshold can then be used to generate an extracted ion chromatogram (EIC). The limit of quantification (LOQ) is established at 0.2%, and because many impurities are near isobaric, any spectral overlap may interfere with the data. Thus, a sensitive and selective method is required for this analytical workflow.

The Pro iQ Plus has a mass range of *m/z* 2 to 3,000. This method typically requires a full scan of *m/z* ± 150 around the 4– charge state of the full-length product (FLP). For example, an oligo with a molecular weight of 8,000 Da would require a scan range from *m/z* 1,750 to 2,150. Additionally, sufficient ion transmission at higher *m/z* values is important, as EICs are used for the relative quantitation of each impurity. Figure 1 shows the ion transmission stability for mass axis assignment and peak width at *m/z* 2,234, monitored over 24 hours. These data demonstrate the robustness and stability of large molecule transmission on the Pro iQ Plus.

Prior to selecting the ions that exceed the threshold for EIC integration, the method requires a comparison of the sample under different MS conditions. Figure 2 shows the overlay of so-called "standard" and "harsh" spectra from the ASO-1 sample. This overlay of spectra on a relative scale is used to determine if any ions exceeding the threshold are adducts. Ions that exceed the threshold under standard conditions, but not under harsh conditions, are regarded as adducts. This is because the higher temperature conditions used for harsh conditions minimize adduct formation during electrospray desorption of the ASO. The spectra obtained under both conditions demonstrate excellent selectivity and sensitivity for the ions in the 4– charge state.

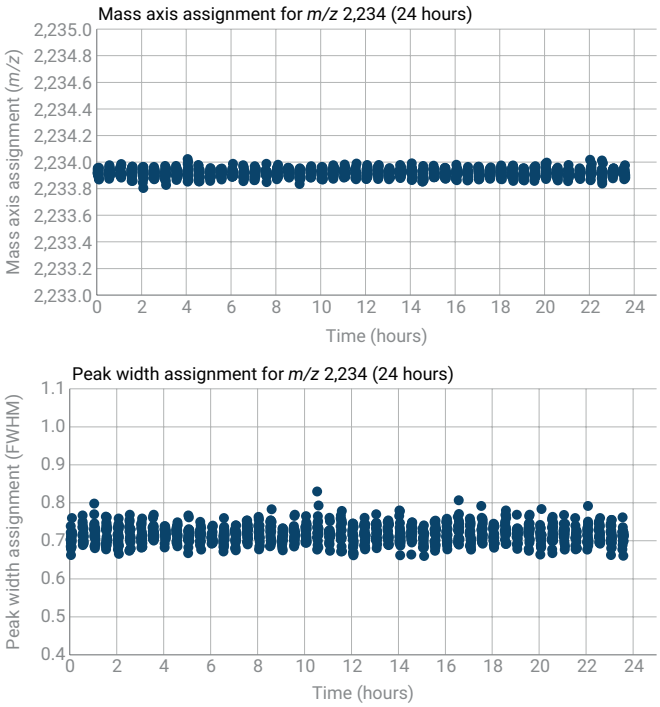

Figure 1. Mass axis assignment (*m/z*) and peak width (FWHM) of the calibrant ion (*m/z* 2,234) monitored over a continuous, 24-hour time period to demonstrate the stability of large molecule transmission on the Agilent Pro iQ Plus.

Table 3 shows the ions identified using the OAA algorithm. These ions exceed thresholds under both standard and harsh conditions, with any "known" ions being within *m/z* ± 0.5 of values in the imported ion list. Interestingly, several unknown ions with *m/z* values greater than the FLP are observed. These may be longmers (for example, n + 1) or other process-related impurities. These again are not chromatographically resolved, and thus it is critically important that they are measured by the MS detector.

The ions in Table 3 were used to generate EICs, which were then integrated into the next step. This information is required for two purposes. First, the relative peak areas are used to calculate the MS purity of the peak. Second, the earliest- and latest-eluting impurities are used to determine the UV integration of the main peak (Figure 3). Thus, both MS and UV purities can be used to determine the overall purity of the ASO sample.

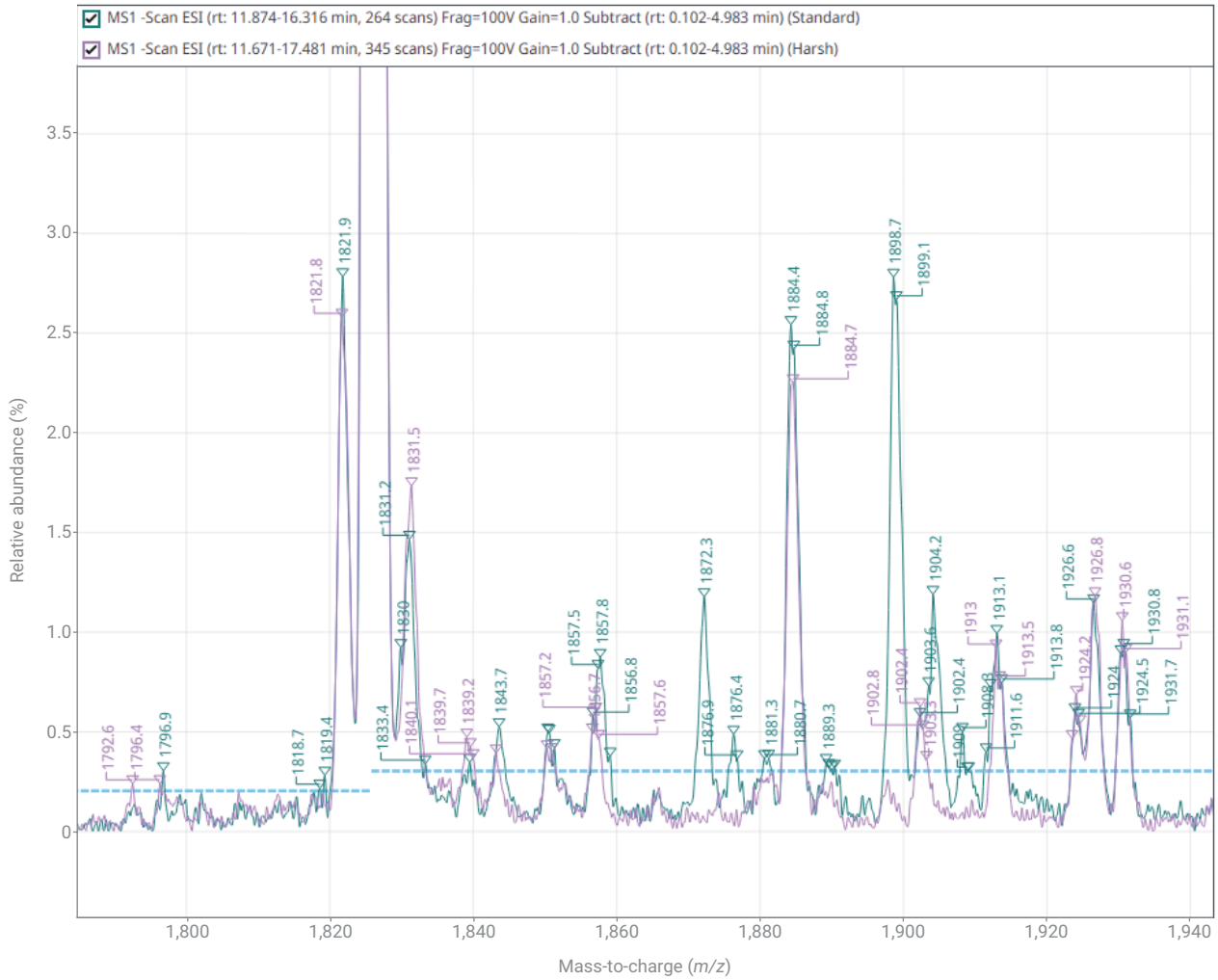

Figure 2. Overlay of ASO-1 full scan spectra under standard (teal) and harsh (violet) conditions. The Oligo Analysis Accelerator software user interface enables direct inspection of the overlaid spectra. Dashed lines indicate the thresholds: 0.2% "prepeak" (*m/z* values less than the 4– charge state of the full-length product) and 0.3% "postpeak" (*m/z* values greater than the 4– charge state of the full-length product). The software then automatically classifies ions accordingly based on whether the harsh-condition ions are still above the threshold.

Table 3. Classified ions from ASO-1 that exceed method thresholds.

Name	*m/z*
Full-Length n	1,826.3
Full-Length n (P=O)1*/Loss of Methylene	1,822.3
N – p(MOE A)	1,725.5
N – p(MOE MeU)/n – p(MOE MeC)	1,727.9
N – p(dA)	1,744
Abasic Depurination Species (Loss of Ade + H_20)	1,797
n + p(dA)	1,908.5
n + p(dG)	1,912.5
Dithioate/Thioate	1,830.2

Name	*m/z*
N3-(2-cyanoethyl)thymine (CNET)	1,839.5
Unknown	1,884.4
Unknown	2,087.1
Unknown	1,857.8
Unknown	1,651.1
Unknown	1,902.4
Unknown	1,774.1
Unknown	1,843.7
Unknown	1,850.7

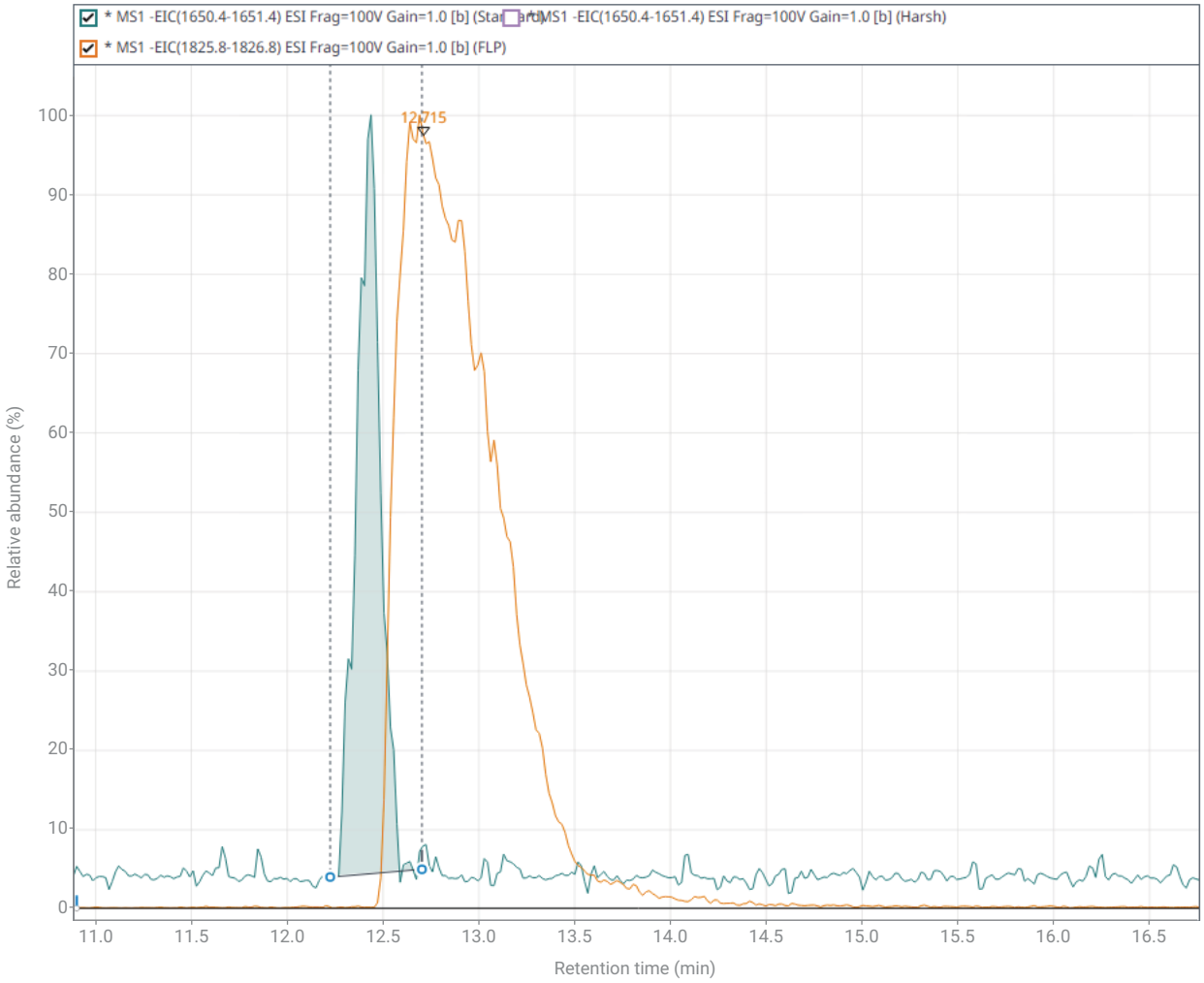

Figure 3. Extracted ion chromatogram integration within the Oligo Analysis Accelerator software, which allows for direct integration within the application. The *m/z* 1,650 ion (unknown) is used to determine the integration of the UV peak. The start of integration for the earliest-eluting impurity is applied as the start of integration for the UV peak, with UV and MS retention time offsets also considered in this automated calculation.

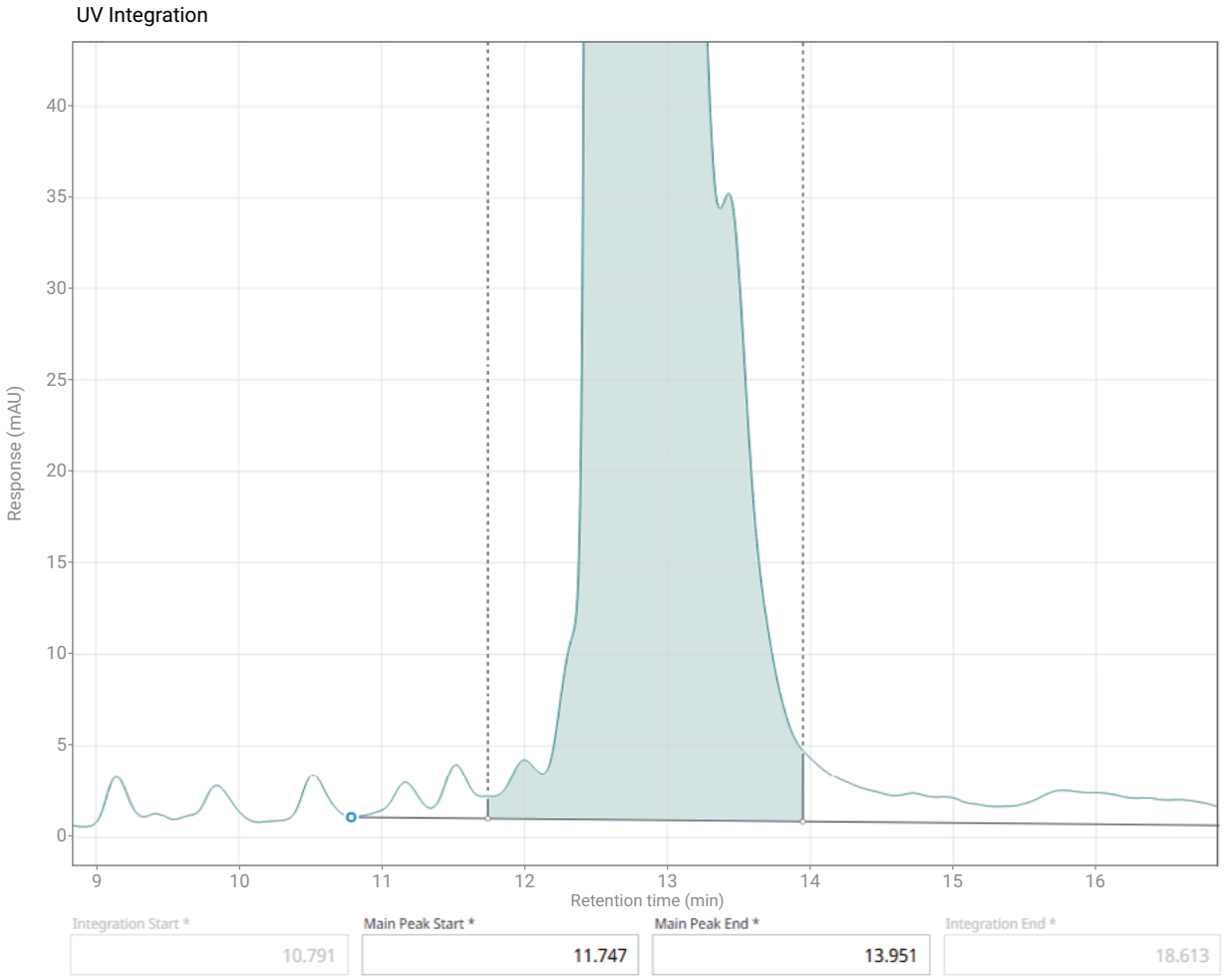

Figure 4. UV integration of the main peak, based on EIC integration for early- and late-eluting impurities.

The relative peak areas for each EIC are shown in Table 4. The resulting MS purity of 86.9% contrasts with the calculated UV purity of 98.0% (Table 5). Although this method was developed to allow for the coelution of closely related impurities, chromatographic method development may help ensure more accurate quantitation. This is especially important considering that many of the unknown impurities are likely n – 1 or alkylated impurities, which elute later than the FLP. In either case, a sensitive and selective MS detector is crucial for determining the purity of an oligonucleotide.

Table 4. Relative peak area results for all ions.

Name	*m/z*	Category	EIC Peak Area (%)
Full-Length n	1,826.3	Full-length n	85.126
Full-Length n (P=O)1*/Loss of Methylene	1,822.3	Full-length (P=O)	2.184
n – p(MOE A)	1,725.5	n – 1	0.197
n – p(MOE MeU)/n – p(MOE MeC)	1,727.9	n – 1	0.489
Abasic Depurination Species (Loss of Ade + H_2O)	1,797	Abasic	0.271
n + p(dA)	1,908.5	n + 1	0.271
n + p(dG)	1,912.5	n + 1	0.578
n + p(MOE MeU)/n + p(MOE MeC)	1,924.6	n + 1	0.338
n + p(MOE A)	1,927	n + 1	0.663
n + p(MOE + G)	1,931	n + 1	0.624
Dithioate/Thioate	1,830.2	Others	0.688
N3-(2-cyanoethyl)thymine (CNET)	1,839.5	Others	0.21
Unknown	1,884.4	–	1.831
Unknown	2,087.1	–	0.59
Unknown	1,857.8	–	0.545
Unknown	1,913.8	–	0.614
Unknown	1,651.1	–	0.334
Unknown	1,902.4	–	0.47
Unknown	1,774.1	–	0.453
Unknown	1,843.7	–	0.356
Unknown	1,850.7	–	0.25
Unknown	1,749.7	–	0.256
Unknown	1,833.4	–	0.216
Unknown	1,478.4	–	0.242
Unknown	1,457	–	0.162
Unknown	1,507.4	–	0.113

Table 5. UV and MS purity results.

Criteria	Result	Expected Result
UV Purity	98.0%	98.0%
MS Purity	86.9%	–
Most Abundant Mass (Da)	7,307.6	7,307.4

Conclusion

This method, as cited in previous literature[1], has become the gold standard for LC/MS-based purity, assay, and impurity profiling of therapeutic, single-stranded oligonucleotides. The method requires a selective and sensitive detector capable of acquiring full scan spectra from the main peak to ensure accurate quantitation of impurities. Additionally, depending on sample, the method may require scans above *m/z* 2,000, which is a limiting factor for many unit mass detectors. Due to its excellent ion transmission at high mass ranges, the Agilent InfinityLab Pro iQ Plus can meet these method requirements for both sensitivity and selectivity, thereby allowing QC analysis of oligonucleotide impurities.

Reference

1. Rentel, C.; Gaus, H.; Bradley, K.; Luu, N.; Kolkey, K.; Mai, B.; Madsen, M.; Pearce, M.; Bock, B.; Capaldi, D. Assay, Purity, and Impurity Profile of Phosphorothioate Oligonucleotide Therapeutics by Ion Pair–HPLC–MS. *Nucleic Acid Ther.* **2022**, *32(3)*, 206–220. DOI: 10.1089/nat.2021.0056

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Technical Overview

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Improve the Productivity of Purification Workflows

Mass-based fraction collection with the Agilent InfinityLab Pro iQ Series Mass Detector in Agilent OpenLab CDS

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Abstract

Preparative high-performance liquid chromatography (HPLC) is a common technique to purify single compounds from a crude sample. In complex mixtures, the selectivity of a UV detector might not suffice to isolate a target peak from the matrix. In these cases, a mass detector is a useful addition to increase selectivity and ensure the highest purity of the collected fractions. This technical overview shows the benefits of the Agilent InfinityLab Pro iQ Series Mass Detector in conjunction with Agilent OpenLab CDS for mass-based purification workflows. Software and hardware features have been improved to increase productivity and ease of use.

Introduction

Preparative HPLC is the method of choice to separate and isolate single compounds from complex mixtures. Whether it is an extract from a natural product, the result of an organic or peptide synthesis to create a new pharmaceutical, or a reaction mixture of a chemical process, the more complex the sample, the more byproducts are to be expected. This makes the isolation of single target compounds more challenging. To increase the selectivity of a fraction collection method, mass detectors are frequently used.

Agilent InfinityLab Pro iQ Series Mass Detectors[1] integrate seamlessly with Agilent preparative HPLC systems. With mass-based fraction collection, compounds of interest can be collected with the highest selectivity and sensitivity. The definition of target compounds by chemical formula enables specific selection of adducts and charge states for each target. Different trigger thresholds may be set, and single or multiple masses can be excluded from collection using a novel trigger combination. To increase flexibility in purification campaigns with a variety of targets, each line of a sample sequence may contain override columns for the target compound/formula and trigger thresholds.

This technical overview presents the benefits of the InfinityLab Pro iQ Series Mass Detector in different preparative HPLC applications.

Experimental

Instrumentation

All experiments were conducted on an Agilent 1290 Infinity II Preparative LC System comprising the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B)
- Agilent 1260 Infinity III Diode Array Detector WR (G7115A) with 0.3 mm Preparative Flow Cell (option #084)
- Agilent 1260 Infinity III Isocratic Pump (G7110B)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A) with delay coils for 4–8 mL/min (option #211)
- Agilent InfinityLab Pro iQ Plus LC/MS (G6170A)

Column

Agilent Prep 100Å C18, 10 × 50 mm, 5 µm, preparative LC column (part number 446905-802).

Software

Agilent OpenLab CDS, version 2.8 FR2 or later

Solvents/chemicals

InfinityLab Acetonitrile (ACN) Gradient Grade for LC (part number 5191-5100*) was used as mobile phase B. InfinityLab ACN for LC/MS (part number 5191-5101*) and LC/MS grade Formic Acid (part number 5191-4549) was used for the preparation of the MS makeup solvent. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

*** Only available in select countries.**

Results and discussion

Defining target compounds and adducts

Unlike universal detectors such as refractive index detectors, mass detectors will produce varying signal intensities for different compounds. Depending on their size and chemical structure, two molecules of the same concentration might not ionize to the same extent and thus produce different signal intensities. Moreover, some molecular substructures will favor the generation of adducts, whereas others are prone to cleave an entire functional group. The formation of proton versus sodium or potassium adducts also depends on the sample matrix and solvent.

This varying behavior for different molecules poses a challenge to the chromatographer who needs to monitor a target compound and trigger its collection into a fraction with the highest sensitivity possible. It is not always easy to predict the ion species a molecule will form in a mass detector based solely on its chemical structure. Take for example, the dye Patent Blue VF, which contains an iminium and two sulphonate groups that may be charged (Figure 1). In solid form, one of the sulphonate groups is coordinated with a sodium ion to yield an overall neutral charge. In solution, however, depending on the pH and ion strength, both sulphonates can be either negatively charged or carry a proton or sodium adduct.

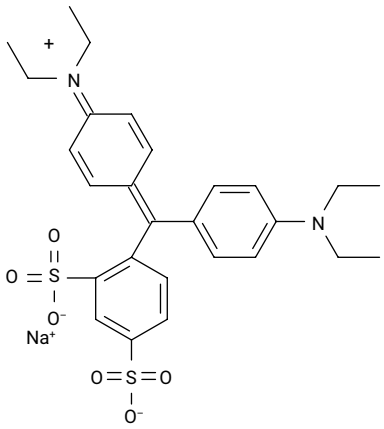

Figure 1. Patent Blue VF.

With the target compound editor in OpenLab CDS (Figure 2), users can simply enter the chemical formula or molecular mass of a compound of interest and pick all positive and/or negative ions that might be expected. The software will take care of calculating the resulting mass-to-charge ratios in each polarity and create single ion monitoring (SIM) signals. All signals of a single target compound are then combined into one trigger signal. In the example of Patent Blue VF, the formula as depicted in Figure 1 was entered as a target compound, resulting in a monoisotopic mass of 566.2 Da. Both the proton and sodium adduct were selected as positive ions; the loss of a sodium ion was selected as the negative ion. The table in the lower half of Figure 2 shows the resulting *m/z* signals that will be monitored in positive and negative ionization mode and serve as peak trigger.

When the fraction collector method is then defined, it will consider the sum of all single *m/z* traces of a compound as trigger. Therefore, it does not matter if, for example, the proton or the sodium adduct is more abundant – fraction collection will be triggered as soon as the sum or any single signal is above the threshold. This takes away the stress and error source of selecting the most abundant adduct.

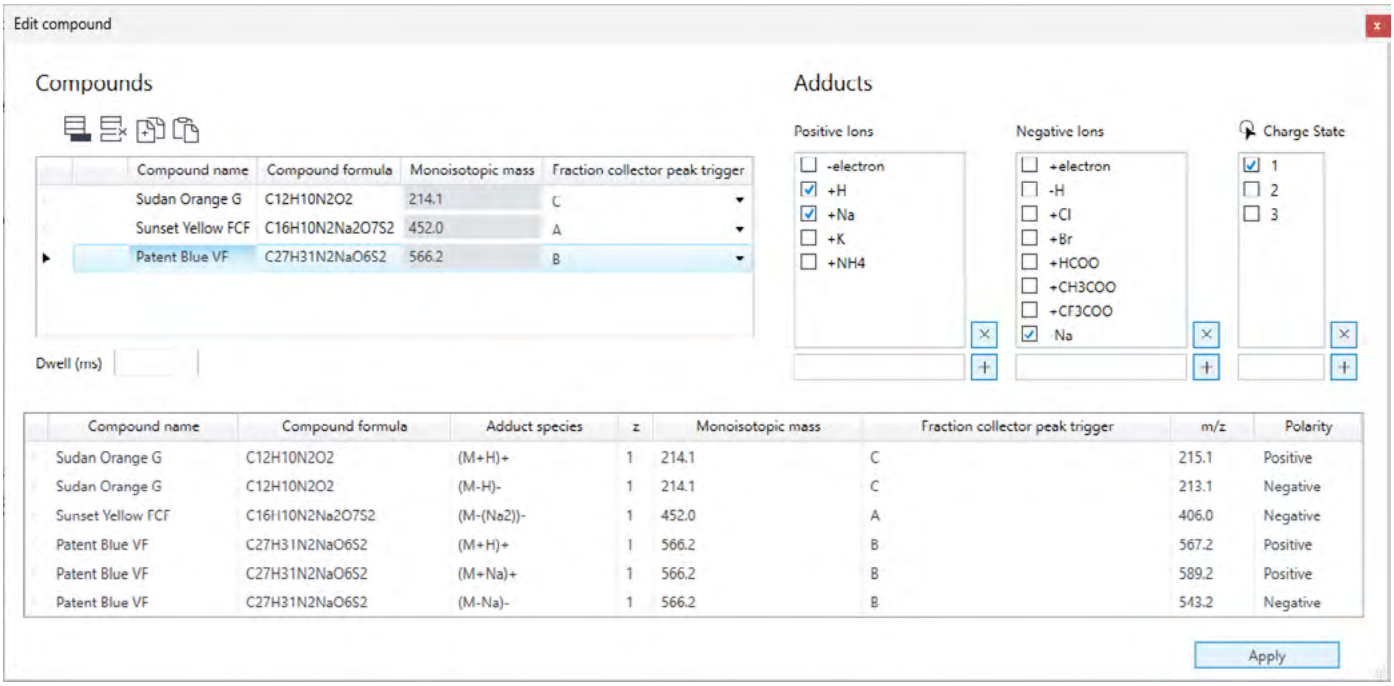

Figure 2. Target compounds window of the advanced acquisition method in Agilent OpenLab CDS using an Agilent InfinityLab Pro iQ Mass Detector.

To account for the different ionization intensities of different target compounds, users can link up to four triggers (A–D) to the target compounds. In the fraction collector method (Figure 3) these triggers can have different threshold and slope settings, allowing, for example, a more conservative setting for lower-abundant ions.

Figure 3. Fraction collector method setup showing threshold settings for up to four mass-based triggers.

Figure 3 shows three different thresholds for triggers A, B, and C, which are linked to three dyes of the delay and checkout calibrant (set up as target compounds in Figure 2). These settings were used to trigger the collection of these dyes after separation on a preparative column. The results are shown in Figure 4. Each target compound is represented in a combined chromatogram showing the sum of the SIM traces that were monitored as trigger signals by the Pro iQ method. These summed chromatograms are created automatically by OpenLab CDS, reducing the time spent on data analysis.

The signal intensities in Figure 4 differ by a factor of 1,000 between the first and third compound. The fact that all compounds were collected into distinct fractions shows the benefit of multiple mass-based triggers: a clean collection would not have been possible by using just a single trigger setting for all targets.

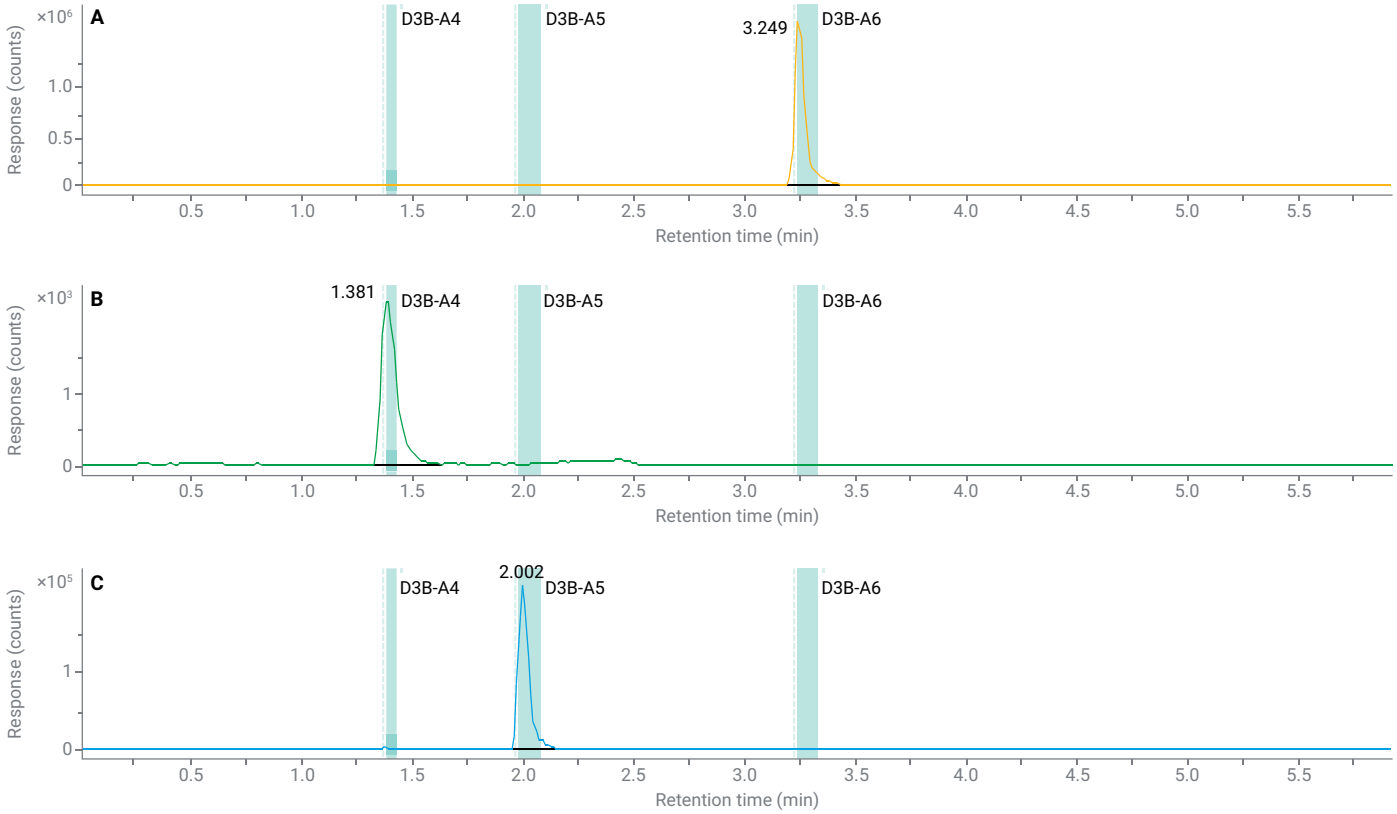

Figure 4. Summed SIM traces of three dyes collected with three different trigger settings. Collected fractions are shown as blue bars. Note the different Y-axis scale.

Targets with multiple charges

Another example where one compound creates multiple ions in mass detection is the analysis of large molecules such as peptides or oligonucleotides. The sequence of amino acids or a phosphate backbone presents multiple opportunities for protonation or deprotonation, causing a range of multiple charged ions in the mass spectrum.

For the chromatographer who wants to trigger fraction collection of a multiply-charged molecule, it is hard to predict which will be the most abundant ion. For this reason, the compound editor in the Pro iQ method enables the user to add multiple customized charge states and connect them to a single target molecule (Figure 5). Only the formula or neutral monoisotopic mass needs to be entered. The software calculates the correct mass-to-charge ratio even for multiple charges and records SIM signals that serve as a peak trigger.

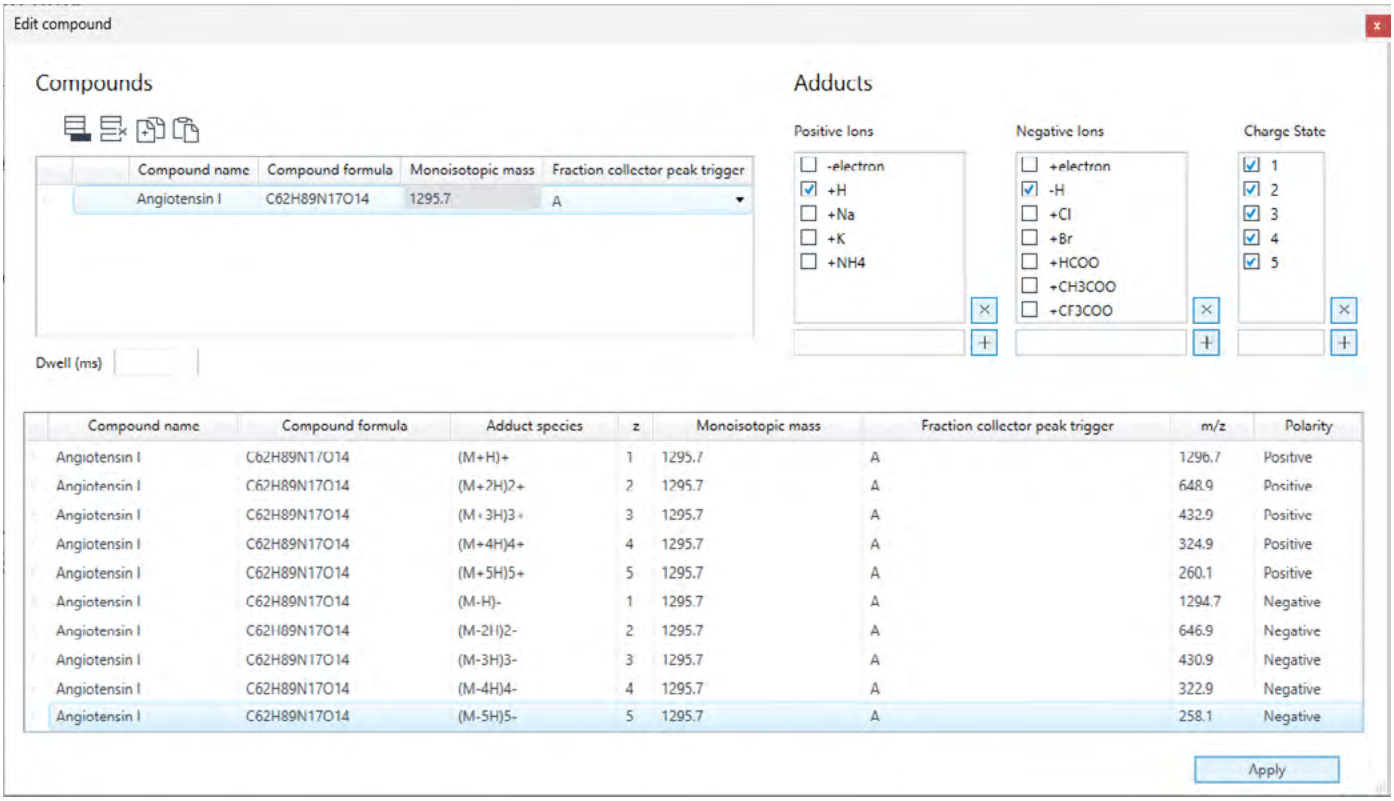

Figure 5. Target compounds window of the Agilent Pro iQ method in Agilent OpenLab CDS showing a peptide as target with +H/−H adducts and charge states of one through five. Note the automatic calculation of the *m/z* traces based on the monoisotopic mass and charges.

To illustrate this feature, a crude sample of angiotensin I was separated and purified using the mass detector settings depicted in Figure 5, combined with a threshold of 10,000 cps and chromatographic conditions as described in a previous application note.[2] Figure 6 depicts the summed chromatogram and single traces of the monitored ions with charges from one through five. The different scales of the y axes visualize the abundance of each charge and show that the double and triple charged ions contributed mainly to the overall signal that triggered the fraction collection. These results underline the gain in confidence of successful fraction collection when the new software features are used with the Pro iQ Mass Detector.

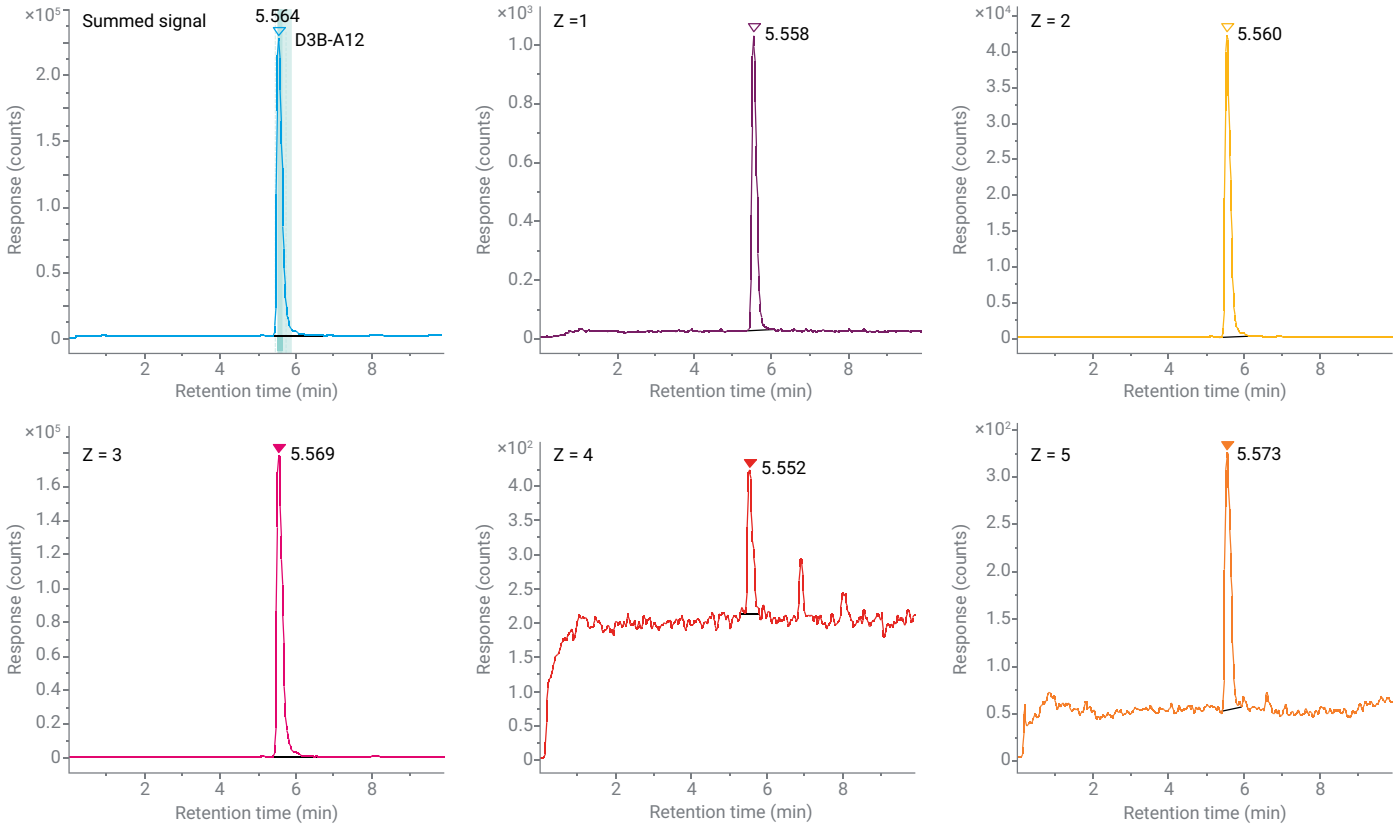

Figure 6. Summed and single SIM traces of angiotensin I monitored as protonated species with different charges (Z = 1 to 5). Note the different Y-axis scales.
Z = 2 and 3 were the main contributors to the summed signal that triggered fraction collection by a threshold of 10,000 cps (blue bar in top left chromatogram).

Trigger combination to exclude impurities

A major benefit of mass-based fraction collection is the ability to detect analytes with high sensitivity and selectivity, independent of their signal in a UV detector. Compounds that coelute in the UV signal can (at least from a signal point of view) be separated based on their specific mass. The new trigger combination logic of the Pro iQ mass detector makes use of this attribute by combining signals intended as fraction trigger with another signal that contains unwanted impurities. The latter signal is linked with the others by the Boolean logical operator not (capitalized NOT for clarity). With a dedicated threshold setting in the fraction collector, the NOT trigger will pause fraction collection of any other trigger until the compound(s) linked with the NOT trigger are below the threshold again.

Figure 7 illustrates the challenge of purifying coeluting compounds. A mixture of several analytes is to be separated, and caffeine is to be purified from this mixture. There is, however, an orange dye that elutes close to caffeine. In the UV trace, it is visible that the two compounds are not perfectly separated. The SIM trace of the target caffeine, however, does not show the impurity and will trigger based on the threshold settings of 50,000 cps.

If the orange dye is added to the Pro iQ method as an exclusion mass (NOT trigger), a dedicated threshold may be set, above which no other fraction will be collected (Figure 8). The same sample was separated and purified again, but with the orange dye excluded. Figure 9 shows the UV and SIM traces for the target and exclusion masses. The threshold for caffeine is exceeded after 0.85 minutes, but no collection happens because the exclusion mass is also above its threshold. Only after the exclusion mass signal has fallen below the threshold will the collection start at 0.94 minutes.

The practical relevance of this exclusion trigger becomes evident when the collected caffeine fractions are re-analyzed for purity. Figure 10 displays the re-analysis of a fraction collected with and without excluding the coeluting impurity. The purity of caffeine by UV peak area increased from 93% to more than 99%, which saves time in the purification process by making a second polishing step obsolete.

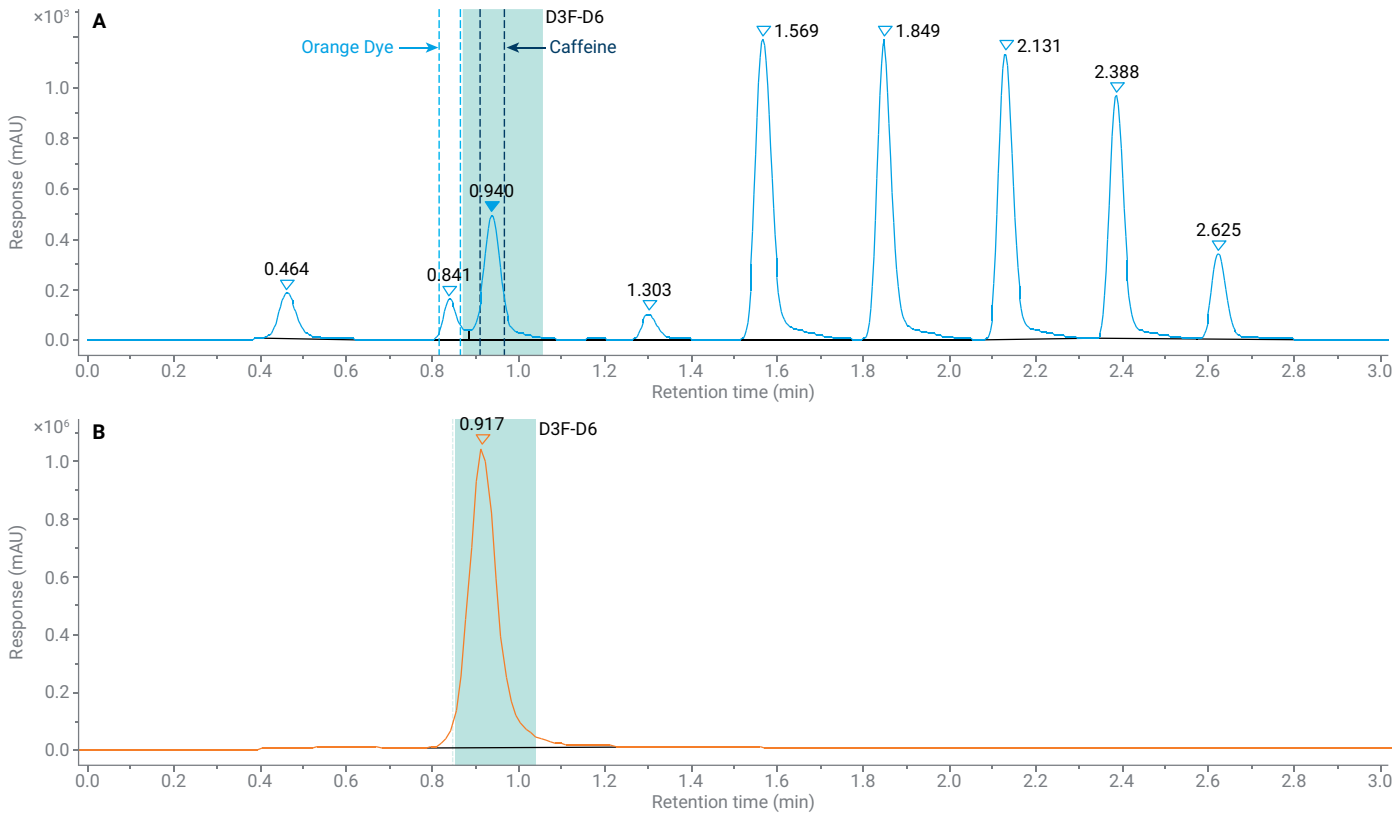

Figure 7. Purification of caffeine from a mixture of compounds. The possible contamination by the orange dye is not clearly visible in the UV (blue) and caffeine (orange) traces.

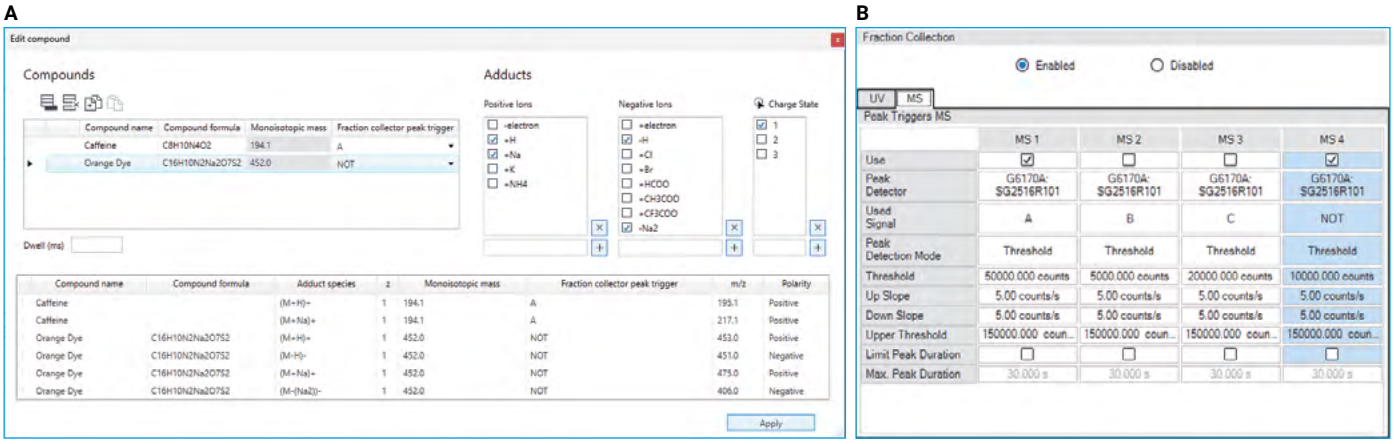

Figure 8. Adding a compound to be excluded from the fraction collection with dedicated threshold settings. (A) Agilent InfinityLab Pro iQ method UI. (B) Fraction collector UI.

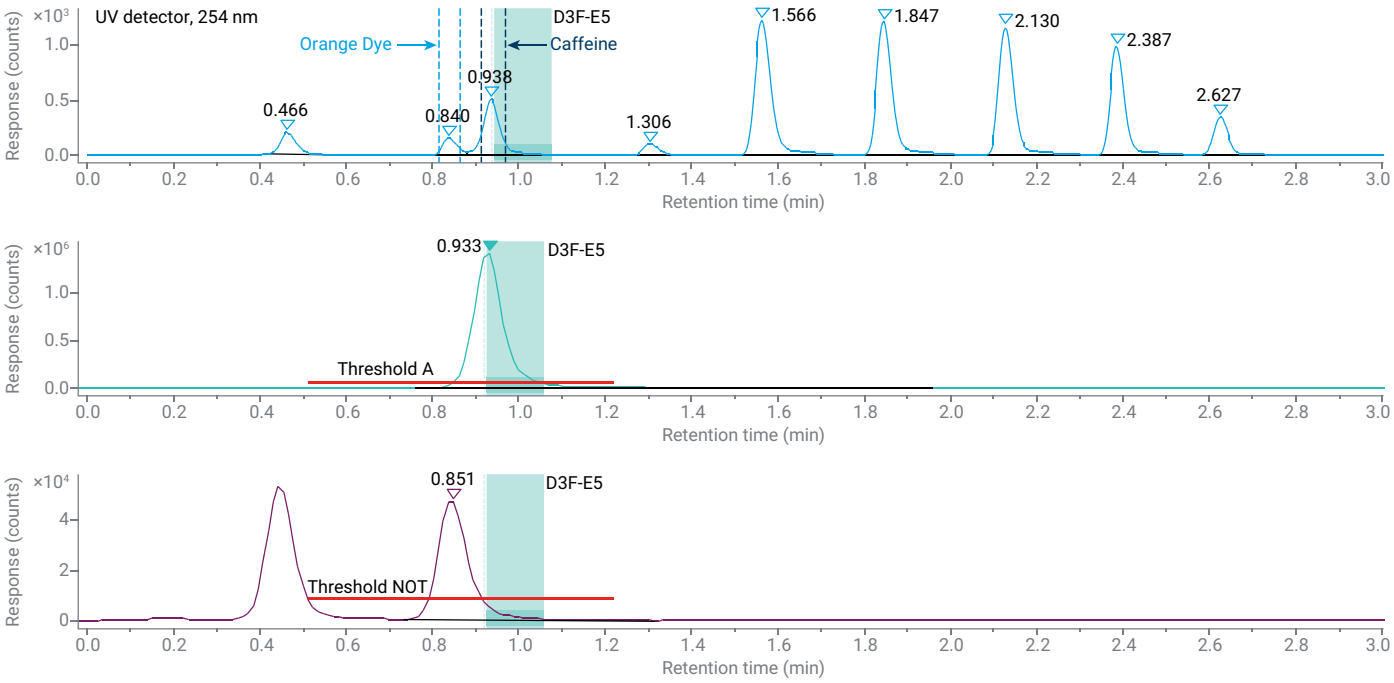

Figure 9. Using the exclusion trigger (NOT logic) to avoid contamination of the caffeine fraction (turquoise trace) by the orange dye (purple trace). The collection is delayed until the NOT signal is below the threshold. In the UV signal (blue trace), the contamination is not visible.

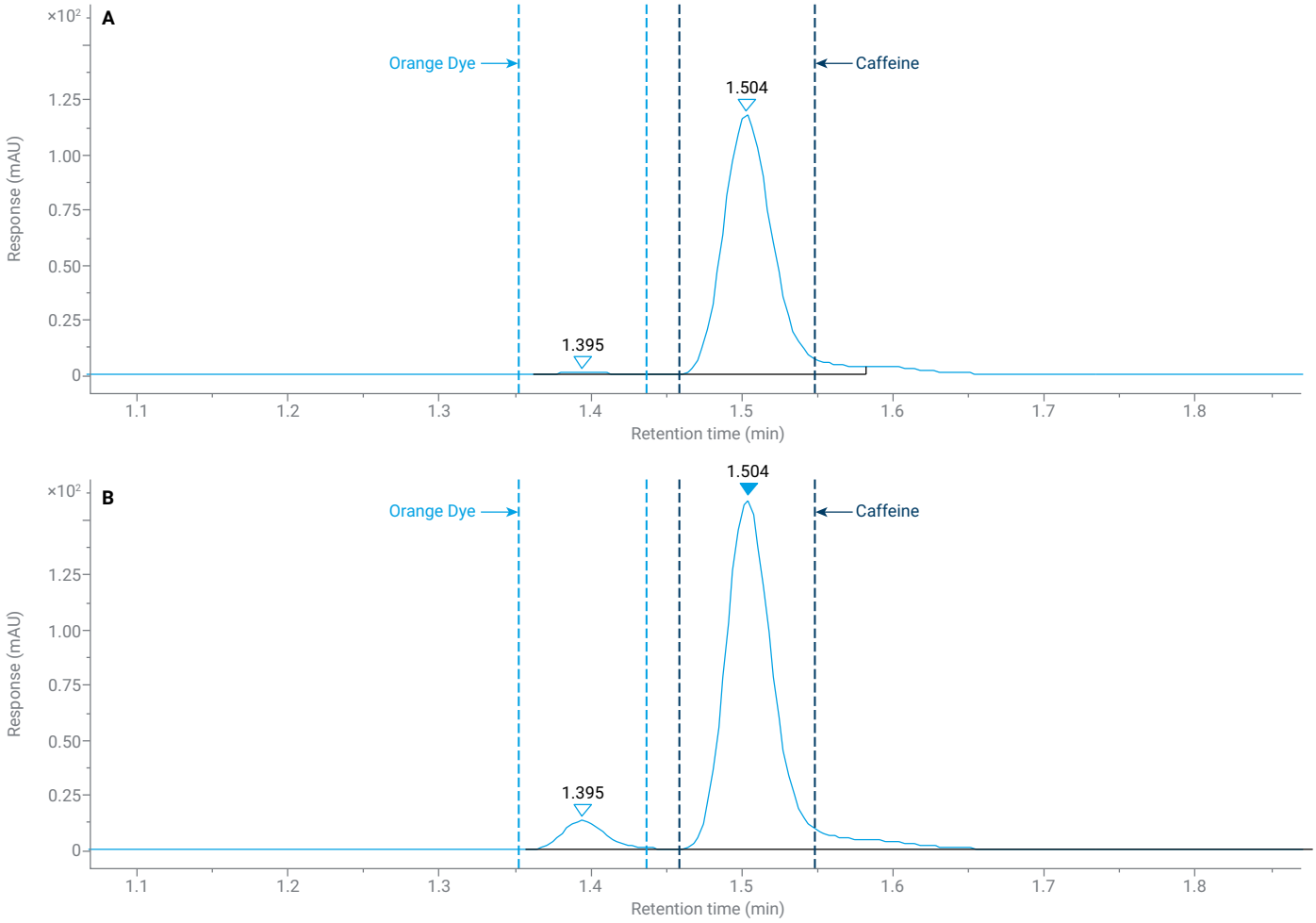

Figure 10. Re-analysis of the caffeine fraction collected with (A) or without (B) the orange dye being excluded by a NOT logic trigger. The purity based on the UV peak area is 93% versus > 99%.

Sequence override columns

To facilitate the use of multiple triggers in daily practice, OpenLab CDS enables the use of override columns during sample submission. The threshold of all four available UV and MS triggers can be adjusted for each sample in a sequence. Moreover, there are three columns to override target masses or groups of targets. These three target columns, for example, enable using a template method that has the following fraction collection settings:

- **Target 1:** main compound(s), trigger A: high abundance, high threshold
- **Target 2:** lower abundant compounds, trigger B: low threshold
- **Target 3:** impurities to be excluded – NOT trigger

By filling one or all of these override columns, users can fine-tune methods specifically to each sample in a sequence without changing the original method or creating multiple versions with only minor changes in target mass or threshold. All adjustments are logged in the data file and can be printed in a report for documentation. A list of all method override columns available in OpenLab CDS, version 2.8 FR2 is depicted in Figure 11.

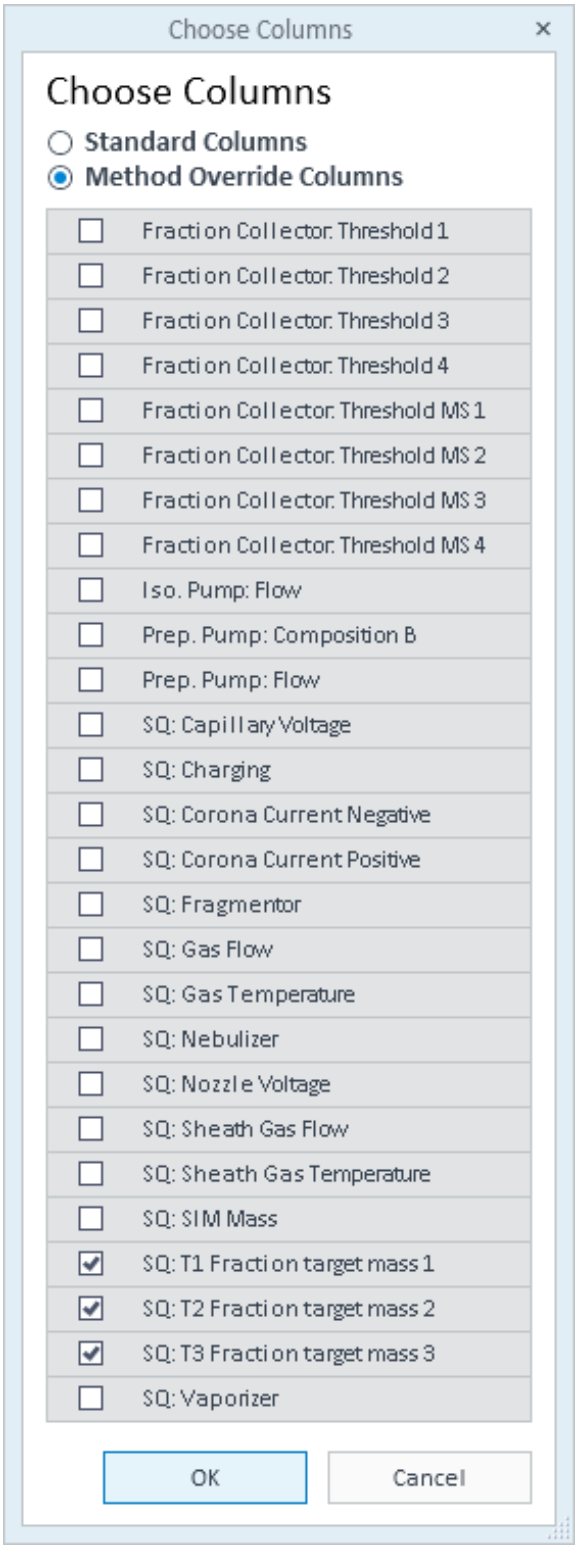

Figure 11. Method override columns available in Agilent OpenLab CDS, version 2.8 FR2.

Conclusion

Mass-based fraction collection in Agilent OpenLab CDS has been designed with the challenges of purification workflows in mind. Defining target compounds by formulas linked with specific adducts and charge states facilitates the purification method setup and reduces errors. The extension to four different mass-based triggers, including a NOT logic to exclude impurities from contaminating a coeluting target, enables a more precise fraction collection definition with higher purity outcomes. Fine-tuning methods to single samples of a sequence has never been easier with multiple override columns for target mass, trigger threshold, and more. These features, combined with an Agilent InfinityLab Pro iQ Series Mass Detector, greatly increase the productivity of purification workflows.

References

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