Technical Overview

Improve the Productivity of Purification Workflows

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

Author

Florian Rieck
Agilent Technologies, Inc.

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.

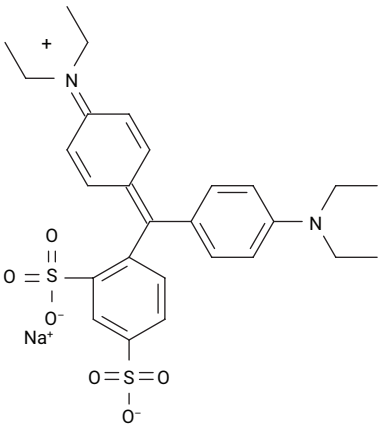

Figure 1. Patent Blue VF.

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.

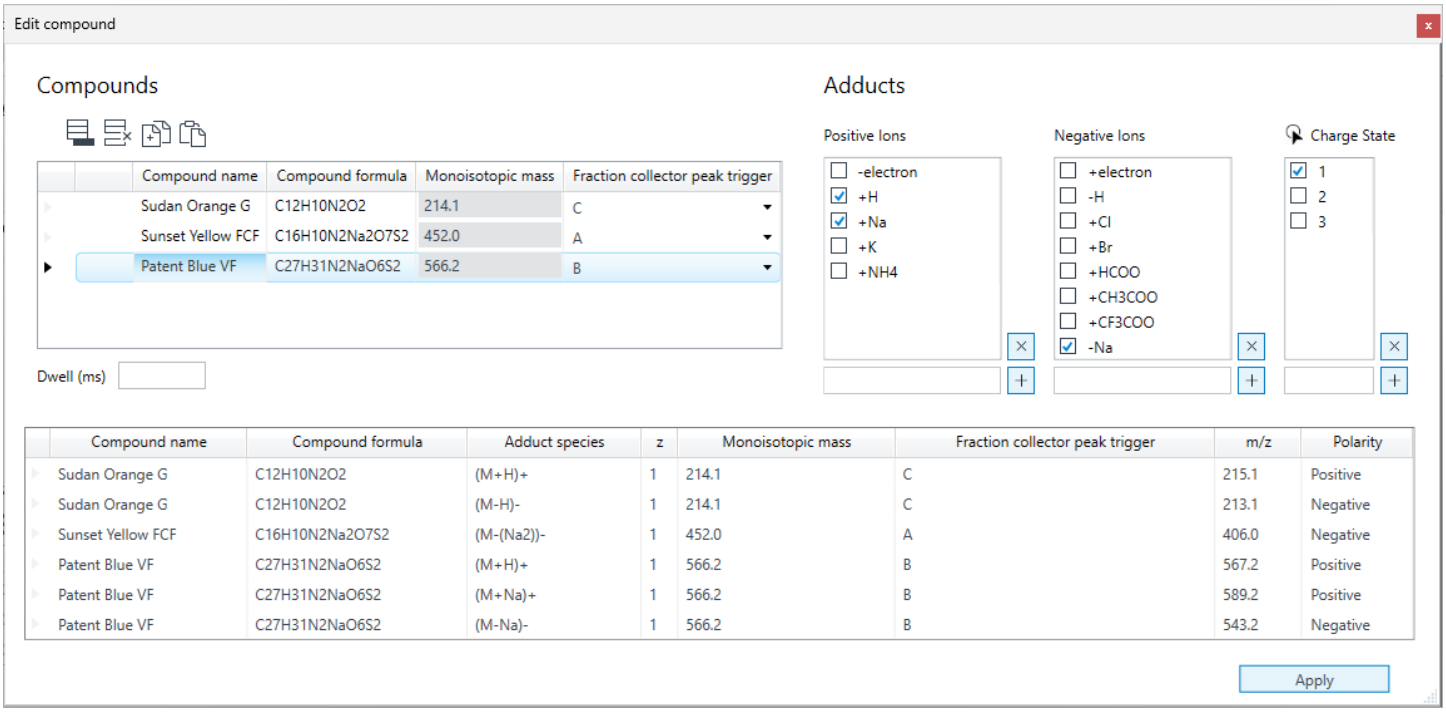

Figure 2. Target compounds window of the advanced acquisition method in Agilent OpenLab CDS using an Agilent InfinityLab Pro iQ Mass Detector.

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.

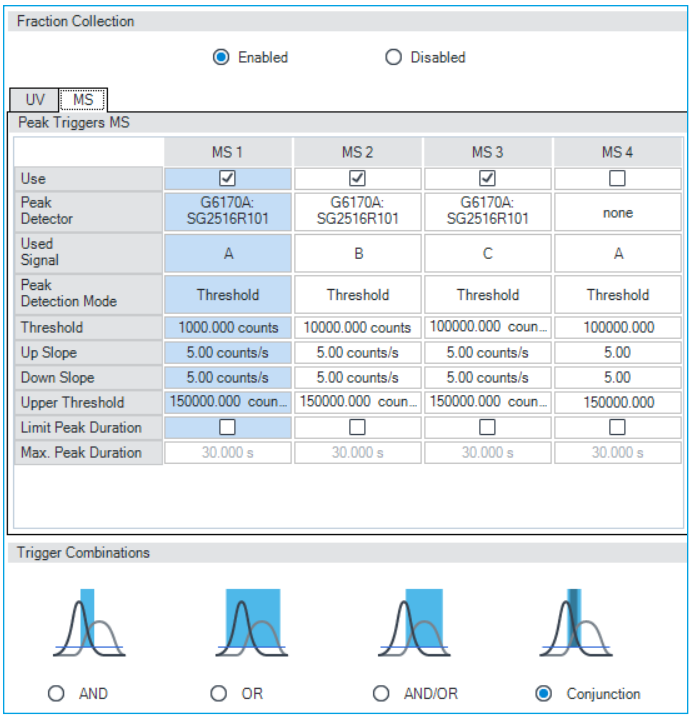

Figure 3. Fraction collector method setup showing threshold settings for up to four mass-based triggers.

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.

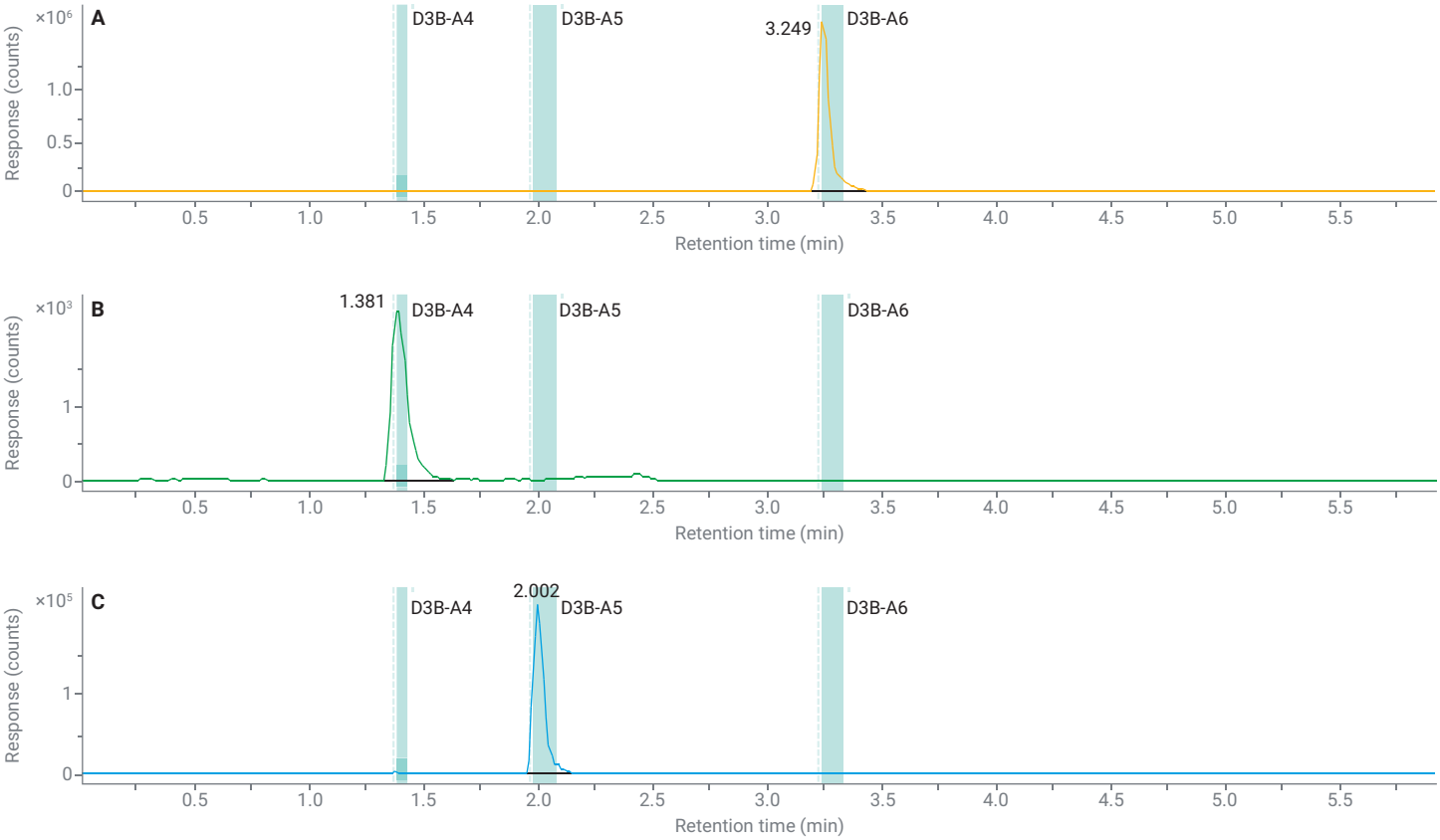

Figure 4. Summed SIM traces of three dyes collected with three different trigger settings. Collected fractions are shown as blue bars. Note the different Y-axis scale.

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.

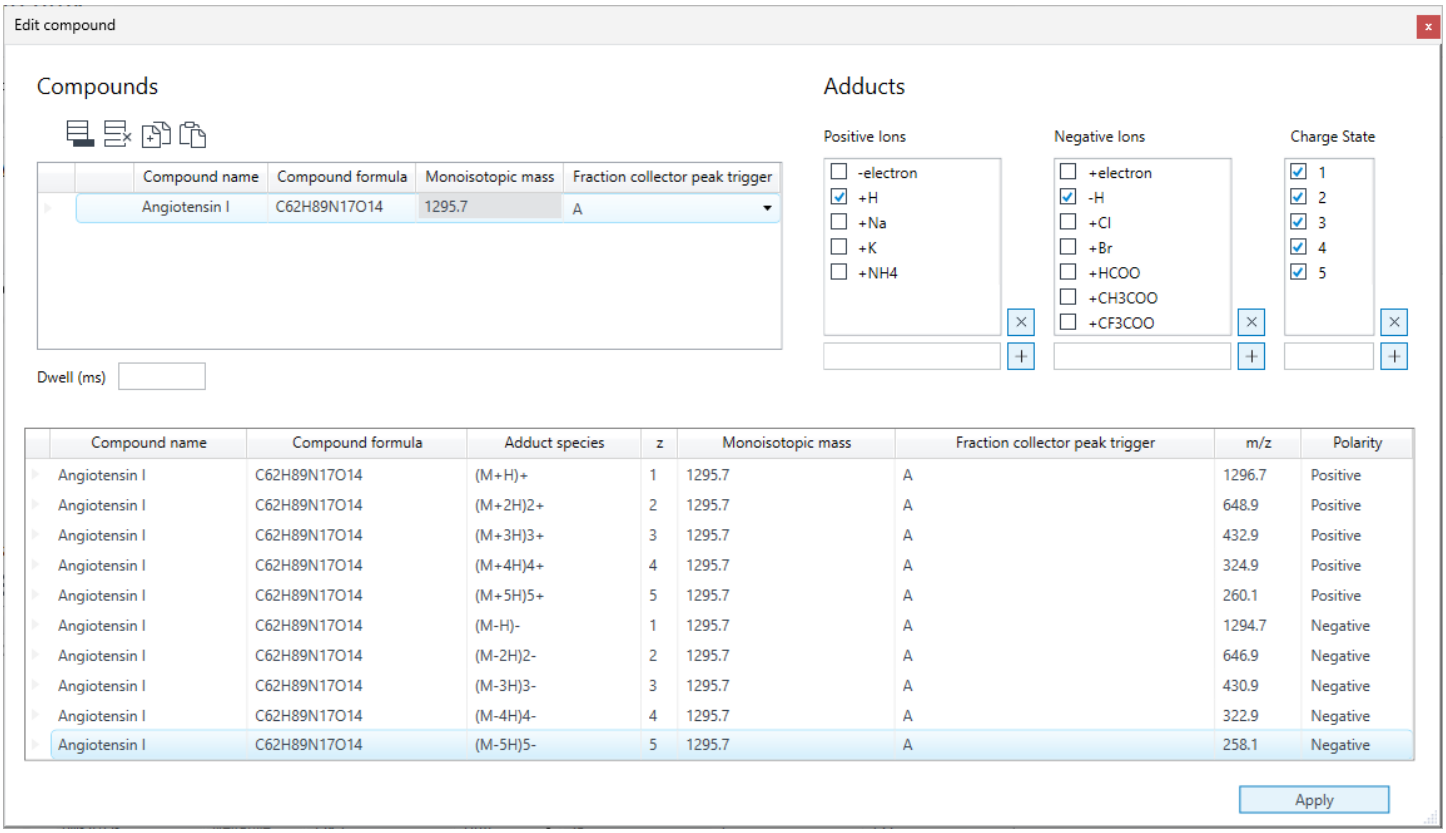

Figure 5. Target compounds window of the Agilent Pro iQ method in Agilent OpenLab CDS showing a peptide as target with +H/−H adducts and charge states of one through five. Note the automatic calculation of the *m/z* traces based on the monoisotopic mass and charges.

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.

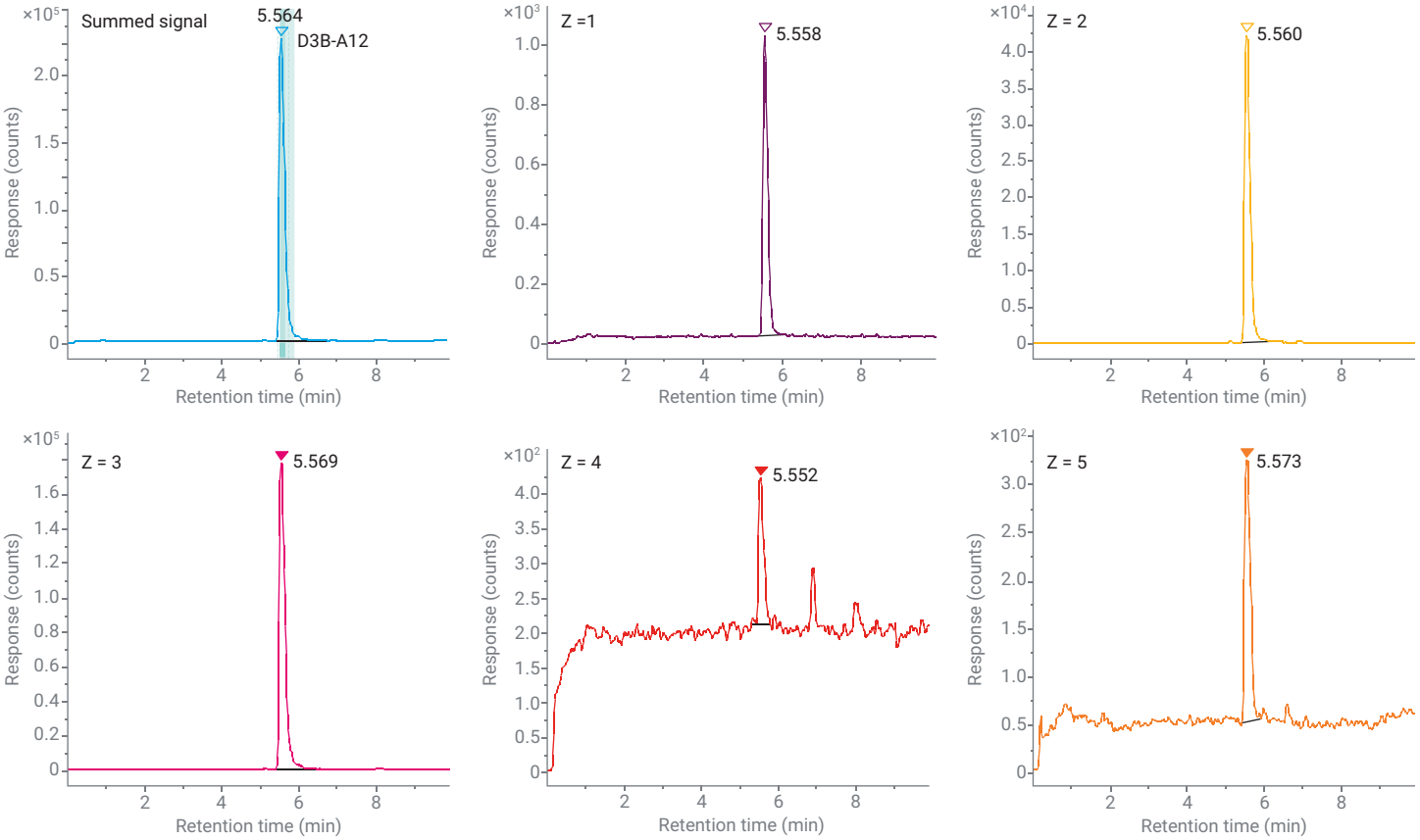

Figure 6. Summed and single SIM traces of angiotensin I monitored as protonated species with different charges (Z = 1 to 5). Note the different Y-axis scales. Z = 2 and 3 were the main contributors to the summed signal that triggered fraction collection by a threshold of 10,000 cps (blue bar in top left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

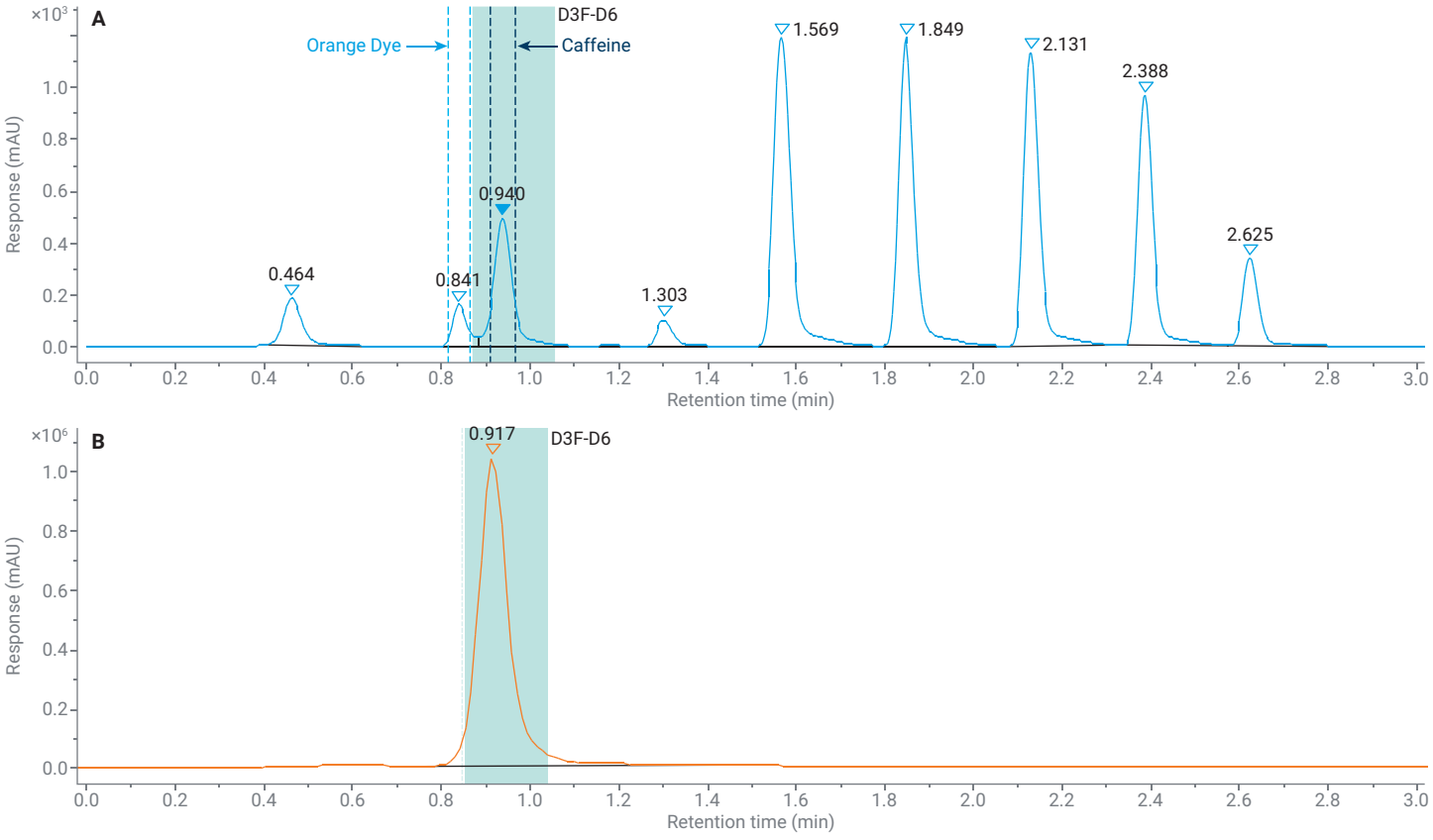

Figure 7. Purification of caffeine from a mixture of compounds. The possible contamination by the orange dye is not clearly visible in the UV (blue) and caffeine (orange) traces.

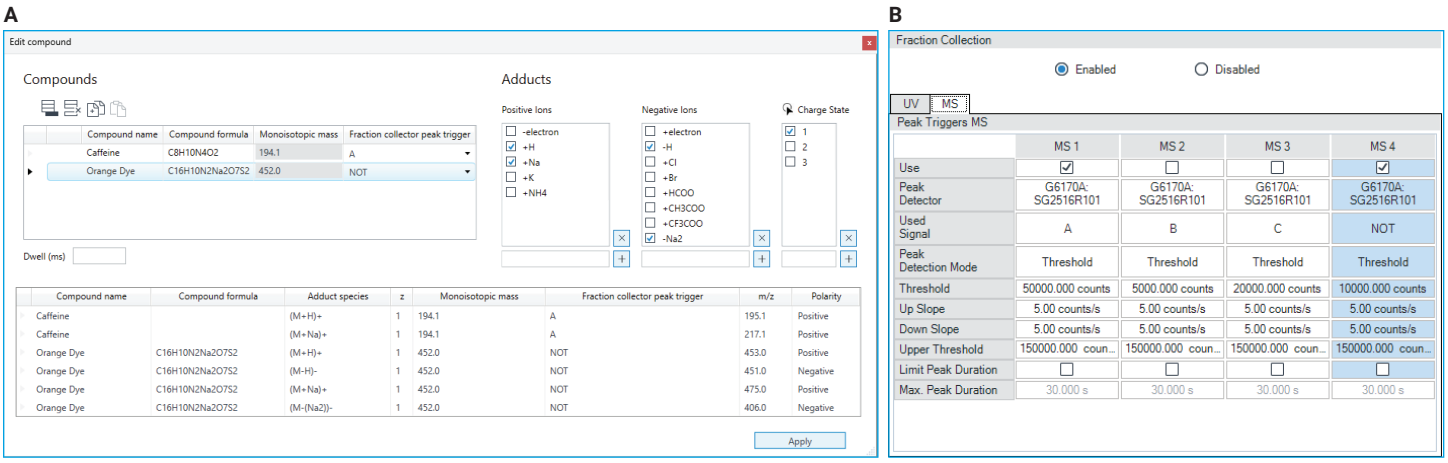

Figure 8. Adding a compound to be excluded from the fraction collection with dedicated threshold settings. (A) Agilent InfinityLab Pro iQ method UI. (B) Fraction collector UI.

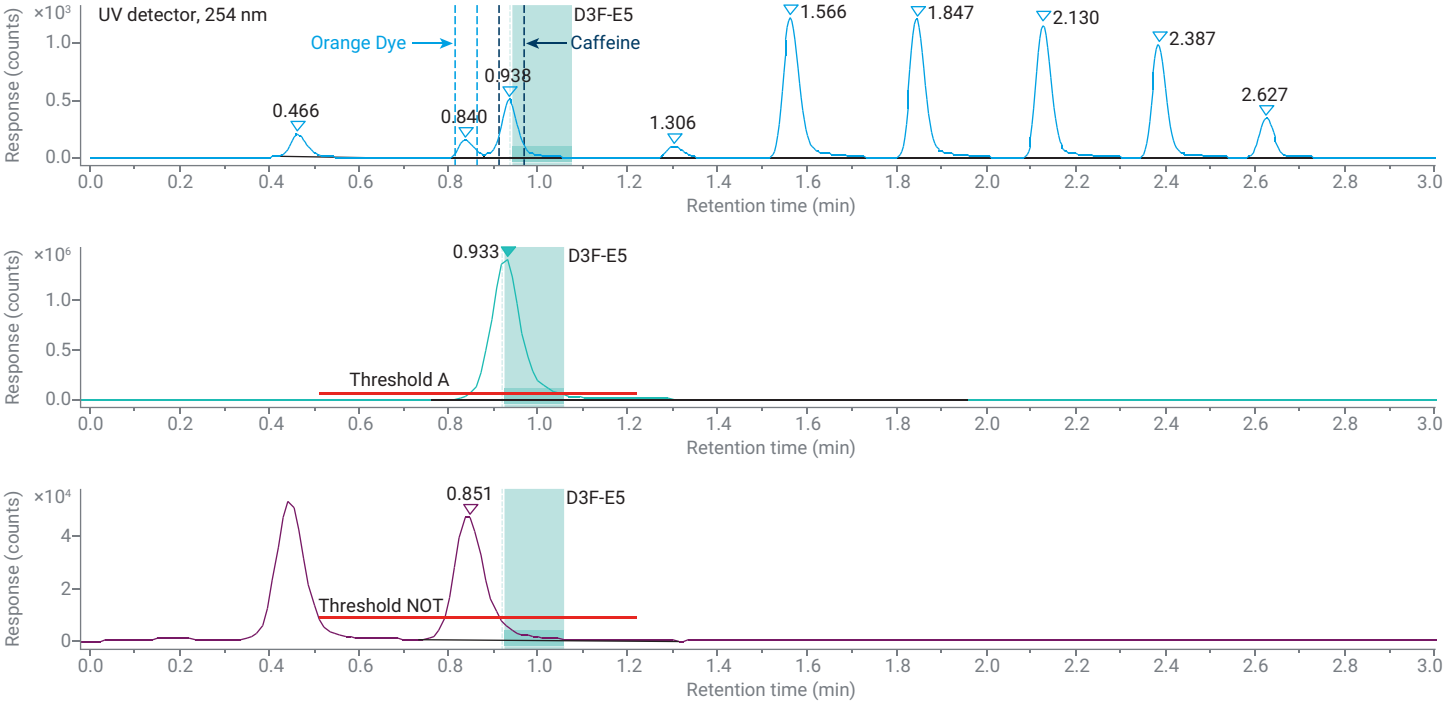

Figure 9. Using the exclusion trigger (NOT logic) to avoid contamination of the caffeine fraction (turquoise trace) by the orange dye (purple trace). The collection is delayed until the NOT signal is below the threshold. In the UV signal (blue trace), the contamination is not visible.

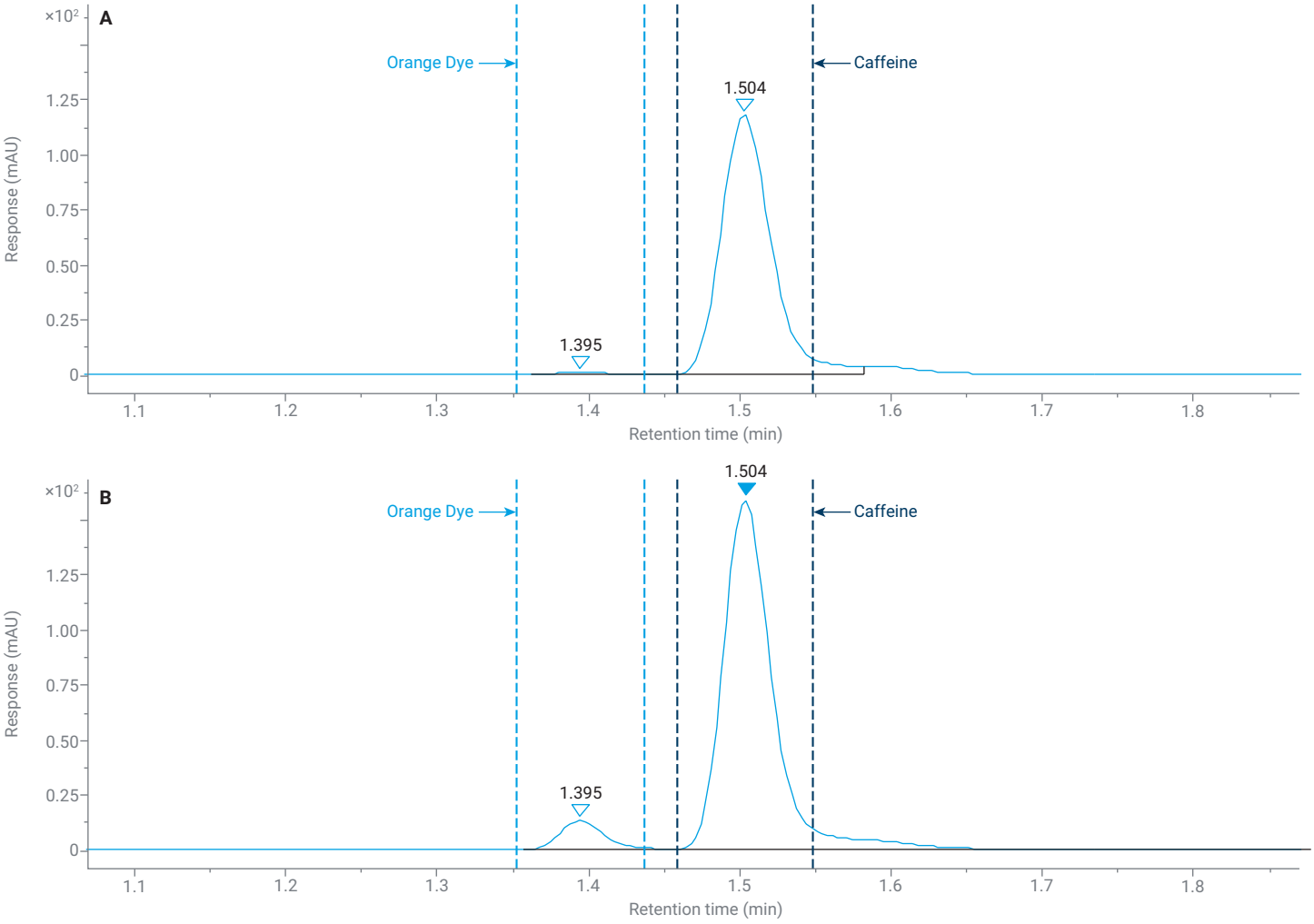

Figure 10. Re-analysis of the caffeine fraction collected with (A) or without (B) the orange dye being excluded by a NOT logic trigger. The purity based on the UV peak area is 93% versus > 99%.

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.

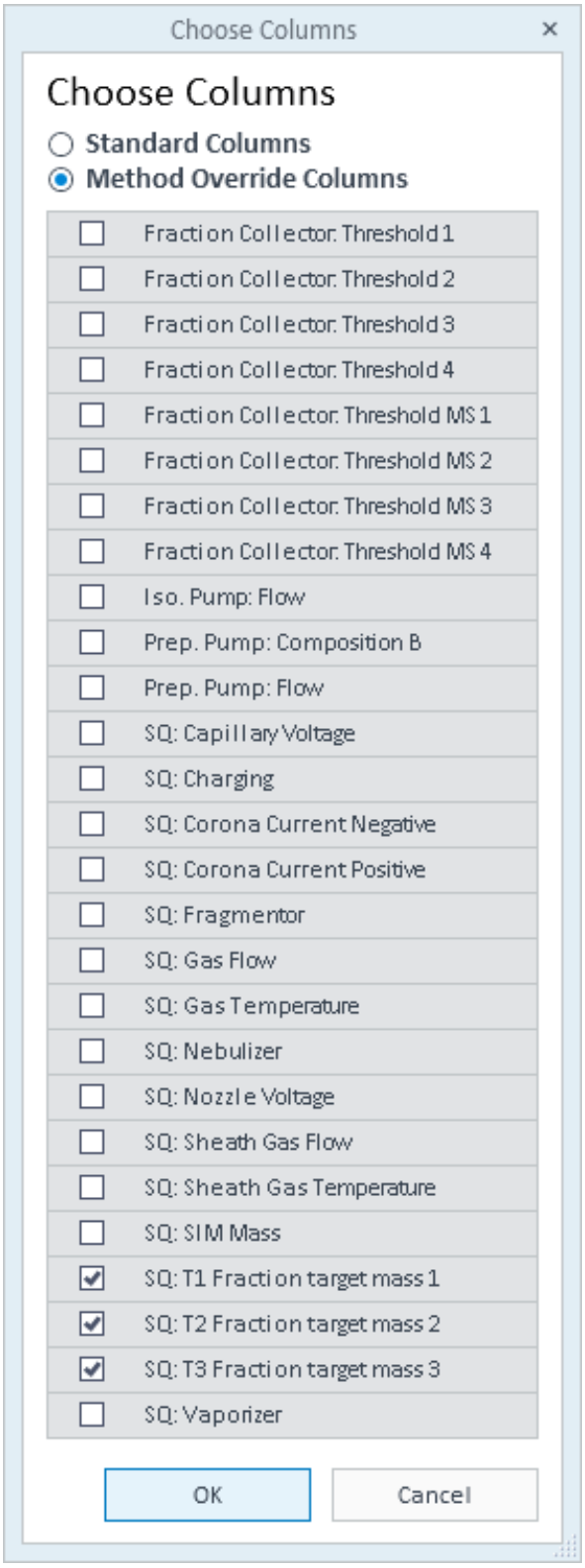

Figure 11. Method override columns available in Agilent OpenLab CDS, version 2.8 FR2.

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

1. Harness the Power of Pro. *Agilent Technologies brochure*, publication number 5994-8330EN, **2025**.
2. Efficient Purification of Synthetic Peptides at High and Low pH. *Agilent Technologies application note*, publication number 5994-5311EN, **2022**.

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