

# Developing Purification Strategies for the Agilent 1260 Infinity II Preparative LC/MSD System



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## Abstract

Peak-based fraction collection triggered by a UV signal threshold leads to satisfactory results in most cases. Some samples and applications, however, require special attention when fraction collection parameters are set. This Technical Overview describes different strategies on how to achieve best collection results using different settings on an Agilent 1260 Infinity II Preparative LC/MSD System. UV and mass-based fraction collection, fraction pooling, as well as recovery collection are discussed in detail to provide knowledge that can be turned into successful and confident fraction collection.

## Introduction

Agilent InfinityLab LC Purification Solutions<sup>1</sup> mark a giant leap in efficient purification. The Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector offers the highest flexibility regarding fraction collection modes and configuration of the fraction bed.<sup>2</sup> The Agilent 1260 Infinity II Preparative LC/MSD System provides the base for successful fraction collection with a plus in usability.

In a compact housing, the Agilent 1260 Infinity II Preparative Binary Pump delivers high performance for preparative separations using columns from 9.4 up to 30 mm inside diameter. Samples are injected much faster with the Agilent 1260 Infinity II Preparative Autosampler, while carryover has been reduced significantly by a redesigned needle seat and integrated needle wash. With the Agilent extended portfolio of preparative fraction collectors, the system can be tailored to every need. For extra reliability, add an Agilent 1260 Infinity II Preparative Valve-Based Fraction Collector as a recovery collector, diverting the fraction collector waste outlet to distinct recovery positions.

To get the most out of the 1260 Infinity II Preparative LC/MSD System, fraction collection parameters need to be set properly. Although, in most cases, simple peak-based fraction collection triggered by the signal of an ultraviolet (UV) detector will produce satisfactory results, some scenarios might require special attention during method setup. This Technical Overview demonstrates different strategies on how to exploit the vast possibilities of the Agilent OpenLAB CDS ChemStation software to succeed with fraction collection even with challenging samples.

## Experimental

### Instrumentation

The system used for the experiments comprised the following modules:

- Agilent 1260 Infinity II Preparative Binary Pump (G7161A)
- Agilent 1260 Infinity II Preparative Autosampler (G7157A)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with a 0.3-mm preparative flow cell (Option #024)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Isocratic Pump (G7110B)
- Agilent LC/MSD (G6125B)
- Agilent 1260 Infinity II Column Organizer (G9328A)
- Agilent Delay Coil Organizer (G7163 60010) with knitted delay coil 5 m × 1.0 mm (5067-6184)
- Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector (G7159B)
- Agilent 1260 Infinity II Preparative Valve-Based Fraction Collector (G7166A), configured as recovery collector

Fraction reanalyses were conducted on an Agilent 1260 Infinity II Binary LC in a configuration as follows:

- Agilent 1260 Infinity II Binary Pump (G7112B)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Diode Array Detector (G7115A), equipped with a 10-mm standard cell (G1315-60022)

### Columns

**Preparative column:** Agilent Prep C18, 21.2 × 50 mm, 5 µm PrepHT cartridge (p/n 446905-102) with PrepHT end fittings (p/n 820400-901)

**Analytical column:** Agilent ZORBAX SB-C18, 4.6 × 50 mm, 5 µm (p/n 846975-902)

### Software

Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, version C.01.07 SR2 [263]

### Solvents and samples

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). Acetaminophen, acetanilide, acetylsalicylic acid, benzocaine, benzyl-4-hydroxybenzoate, caffeine, ethyl-4-hydroxybenzoate, methyl-4-hydroxybenzoate, propyl-4-hydroxybenzoate, salicylic acid, sulfamerazine sodium salt, and dimethylsulfoxide were bought from Sigma-Aldrich, Taufkirchen, Germany.

**Table 1.** Preparative gradients, used with an Agilent Prep C18, 21.2 × 50 mm, 5 µm column.

Parameter	Description	
Mobile Phase	A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile	
Flow Rate	25 mL/min	
Gradient	Figures 1 and 2 0.00 minutes – 2% B 0.30 minutes – 2% B 3.50 minutes – 98% B 4.00 minutes – 98% B 4.10 minutes – 2% B	Figures 3 to 6 0.00 minutes – 2% B 0.30 minutes – 2% B 4.20 minutes – 60% B 4.30 minutes – 98% B 4.80 minutes – 98% B 4.90 minutes – 2% B
Stop Time	5.10 minutes	5.90 minutes
Detection	UV 254 nm Peak width >0.1 minutes (5 Hz)	UV 230 nm Peak width >0.1 minutes (5 Hz)
Injection Volume	50 µL	
Flow modulator	Mode M4, split ratio 2,500:1, dilution factor 1:150	
Fraction collection	Peak-based; threshold and slope settings described per example	

**Table 2.** Analytical gradient for fraction re-analysis, used with an Agilent ZORBAX SB-C18, 4.6 × 50 mm, 5 µm column.

Parameter	Description
Mobile Phase	A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile
Flow Rate	1.5 mL/min
Gradient	0.00 minutes – 10% B 0.10 minutes – 10% B 3.70 minutes – 64% B 3.80 minutes – 98% B 4.30 minutes – 98% B 4.40 minutes – 10% B
Stop Time	5.50 minutes
Injection Volume	5 µL
Detection	UV 254 nm, no reference Peak width >0.013 minutes (20 Hz)

**Table 3.** MSD Spray chamber and signal settings.

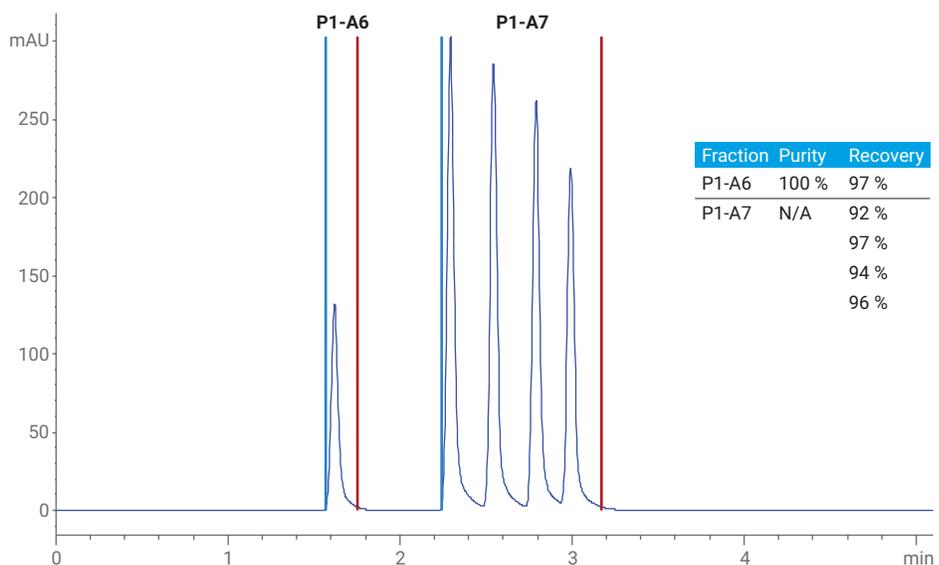
Parameter	Description
Make-Up Flow	1.5 mL/min
Make-Up Solvent	Methanol/water 70:30 + 0.1% formic acid
Spray Chamber	Agilent API Electrospray
Signal 1	Positive scan 125 to 750 Fragmentor 100 V
Signal 2	Negative scan 125 to 750 Fragmentor 100 V
Drying Gas Flow	9.0 L/min
Nebulizer Pressure	40 psig
Drying Gas Temperature	300 °C
Capillary Voltage	±3,000 V

## Results and discussion

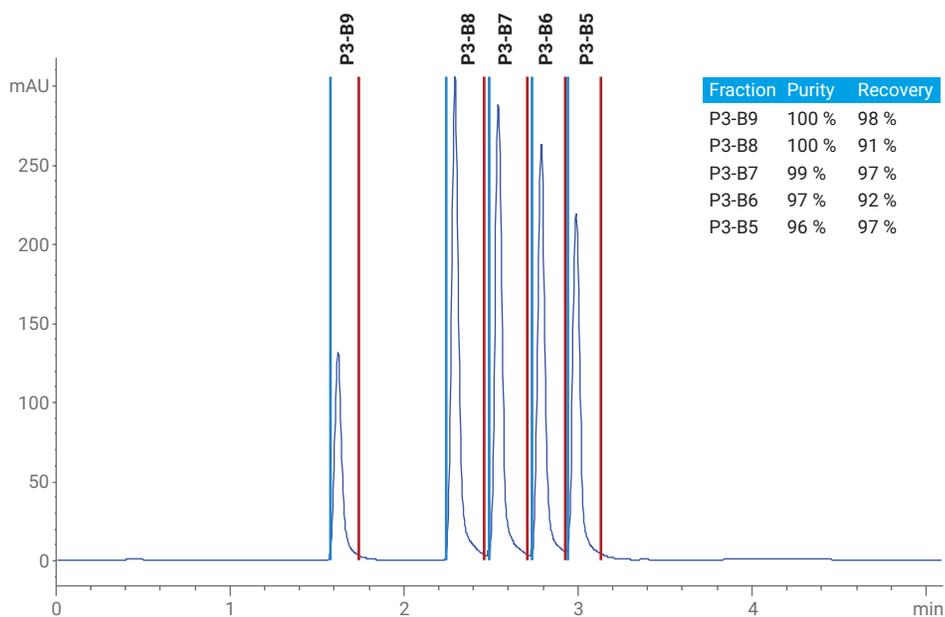
### Correct use of threshold and slope settings

Peak-based fraction collection is the most straightforward way to collect all components of a sample without specific focus on a main compound. Fraction starts are usually triggered by exceeding a signal threshold that is defined in the method of the fraction collector. To achieve the highest recovery of the compounds, this threshold should be set very low. In some cases, however, fraction collection triggered by signal threshold can fail. Depending on the wavelength of the UV detector, the absorbance of the solvent can become visible towards the end of a gradient with increasing organic solvent percentage. This can cause the baseline to rise above the threshold and trigger continuous fraction collection. Peak tailing, which occurs frequently with highly concentrated preparative samples, can prevent the baseline from falling below the threshold between insufficiently resolved peaks. Fraction collection does not stop after one peak, in this case, and decreases sample purity (Figure 1).

To work around these potential problems, use the signal slope or a combination of signal threshold and slope as the fraction trigger. The sample separation and collection depicted in Figure 1 was repeated with the same gradient but an upslope and downslope fraction trigger of 10 mAU/s and 1 mAU/s, respectively. Figure 2 illustrates that, with these settings, all peaks were collected into distinct fractions with high recovery and purity.

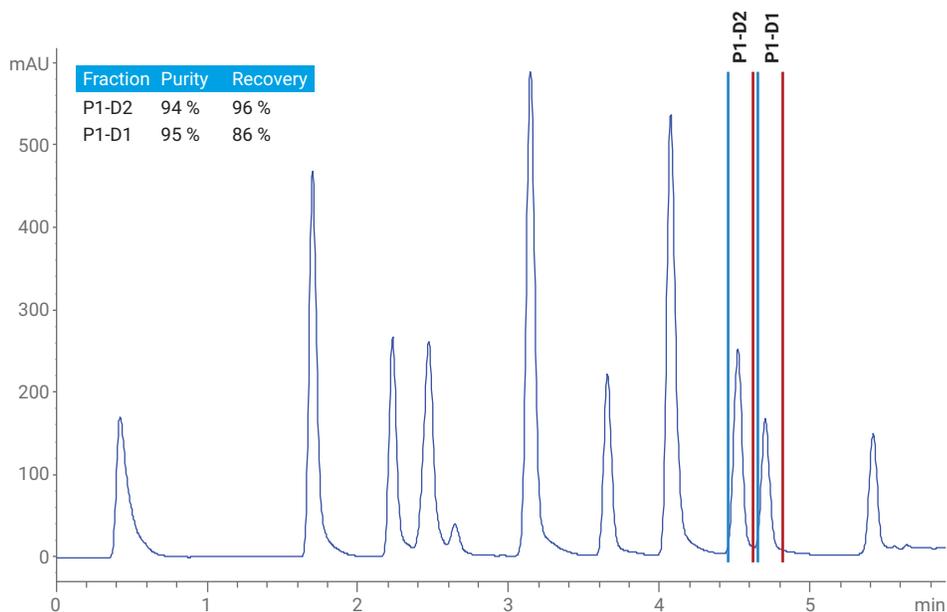


**Figure 1.** Fraction collection triggered by UV signal threshold (254 nm, 2 mAU). Blue and red vertical lines illustrate fraction start and end, respectively. Recovery of all compounds was high, but peaks 2 to 5 were collected into a single fraction because the baseline was too high between the peaks.



**Figure 2.** Fraction collection triggered by UV signal slope (254 nm, 10 mAU/s upslope, 1 mAU/s downslope). Despite slight peak tailing, all compounds were collected in distinct fractions with high recovery and purity.

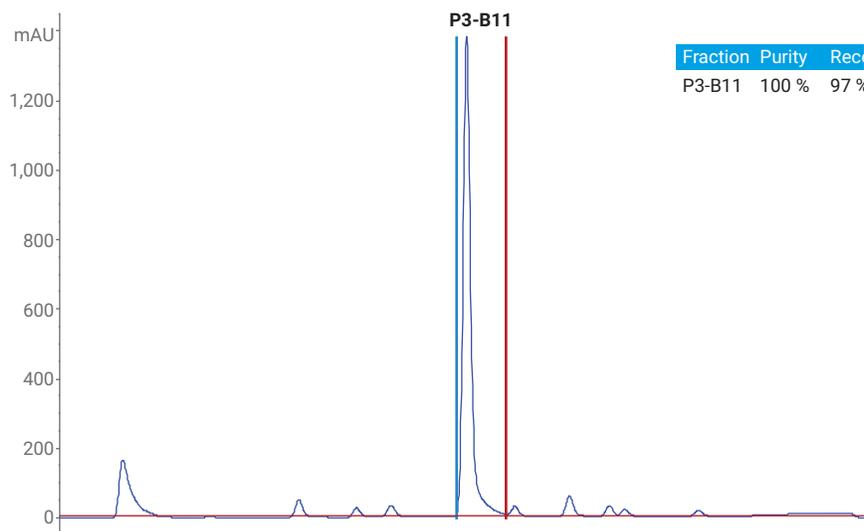
Even with the selectivity of a mass selective detector (MSD), the correct use of UV signal threshold and slope parameters can be crucial for correct fraction collection. Figure 3 depicts the chromatogram (230 nm) of a separation with mass-based fraction collection. Benzocaine (target mass 165.1) was to be isolated from the sample without contamination by ethylparaben (target mass 166.1), which elutes closely after benzocaine. Due to the isotopic pattern of both analytes, fraction collection, triggered only by the MSD, would cause both peaks to be collected into a single fraction. However, combining the MSD with a UV signal and setting an up- and downslope trigger of 10 mAU/s and 1 mAU/s enables the collection of benzocaine separately from ethylparaben.



**Figure 3.** Mass-based fraction collection of benzocaine (P1-D2,  $m/z$  166.1 in +ESI). Only the combination of MSD with the UV signal slope allowed fractionation separately from ethylparaben (P1-D1,  $m/z$  165.1 in -ESI).

### Use slope for high selectivity

When fraction collection with high selectivity is desired, the combination of UV detection with an MSD is the method of choice. With the right trigger settings for the UV signal slope, samples containing impurities at low concentrations can selectively be purified even without MSD. Figure 4 shows the UV chromatogram of a purification triggered by UV detection only. The crude sample contained approximately 84% of the target compound (estimated by peak areas at 230 nm). With fraction collection driven by a signal threshold at 5 mAU, all compounds, including impurities, would have been collected. Setting the trigger to threshold and slope with settings of 30 mAU/s upslope and 1 mAU/s downslope, allowed specific collection of the pure target compound with high recovery.



**Figure 4.** Purification of a crude sample (84% purity) with fraction collection triggered by UV signal (230 nm) with threshold 5 mAU, upslope 30 mAU/s, and downslope 1 mAU/s. Minor impurities did not exceed the upslope parameter, and were not collected. The red horizontal line illustrates the threshold of 5 mAU – all peaks would have been collected with a setting of threshold only.

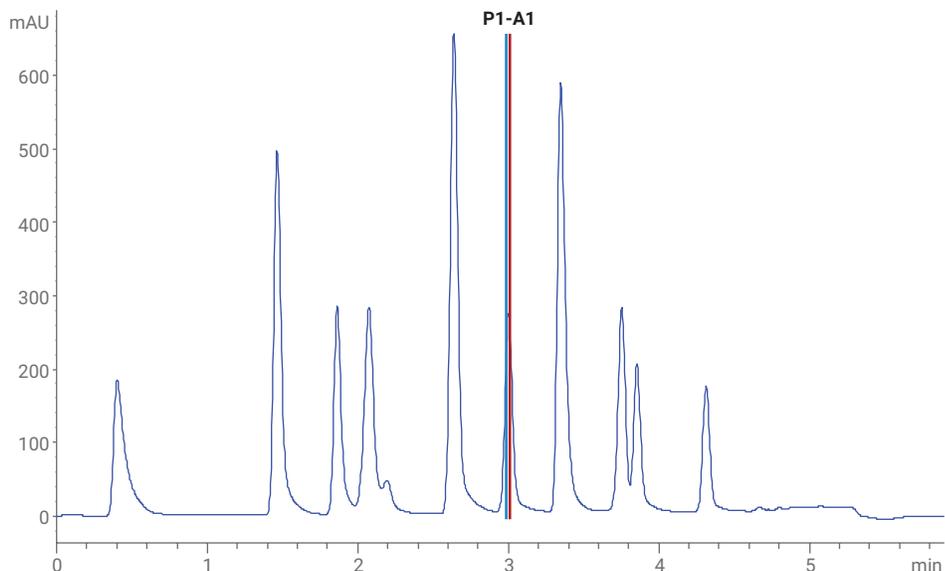
### Mass-based fraction collection

Purifying single components of samples with multiple, higher concentrated impurities, requires mass-based fraction collection (or tedious method development to drastically increase resolution around the target peak and restrict fraction collection to a small time window – which is a waste of time and solvent and might not be possible for every sample). The general MSD method used for fraction collection will most likely monitor both positive and negative signals in electrospray ionization mode (ESI). Similar to the UV signal, fraction collection can be triggered by signal threshold, slope, or a combination of both. Each sample can then be assigned a maximum of eight target masses (up to 16 if only one MSD signal is monitored) that are monitored and used as a trigger. Different ion species can be selected in the MSD method, in most cases  $[M+H]^+$  and  $[M-H]^-$ , although other adducts are selectable from a list, or can be defined by the user.

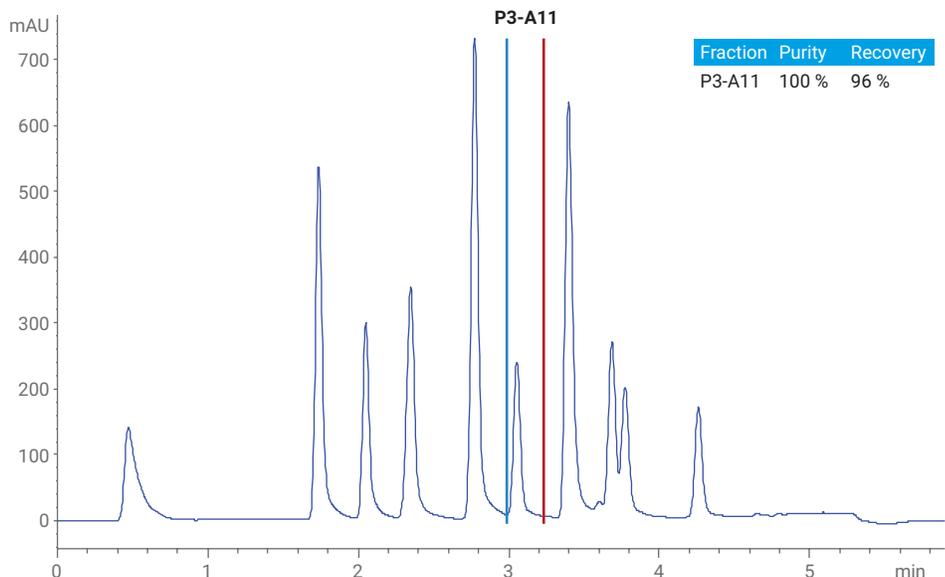
Monitoring the  $[M+H]^+$  and  $[M-H]^-$  ion species might seem the best approach to cover all ions likely to be created in the ESI source. Some compounds are much more likely to form adducts with sodium or potassium, which can be omnipresent in crude samples. Figure 5 illustrates the separation and mass-based purification of acetyl salicylic acid. MSD method parameters were set to monitor both the  $[M+H]^+$  and  $[M-H]^-$  ion species, and trigger above a signal height of 20,000 cps with a target mass of 180.0. The resulting fraction was 100% pure, but collected only the apex of the peak, yielding low recovery.

In another experiment, the same sample and gradient were applied, but MSD parameters were set to monitor only  $[M+Na]^+$  adducts (again with a target mass of 180.0 and fraction trigger threshold of 20,000 cps). The trigger on the sodium adduct still collected the pure acetyl salicylic acid peak selectively,

but with much higher peak width, and recovery of 96% (Figure 6). These two experiments clearly illustrate that even a specific detection method such as mass spectrometry requires careful setting of parameters to yield optimum collection results.



**Figure 5.** Mass-based fraction collection of acetyl salicylic acid, triggered by a UV threshold of 5 mAU (230 nm) combined with an MSD threshold of 20,000 cps. Target mass: 180.0, ion species  $[M+H]^+$ ,  $[M-H]^-$ , trigger:  $m/z$  179.0 (-ESI).



**Figure 6.** Mass-based fraction collection of acetyl salicylic acid, triggered by a UV threshold of 5 mAU (230 nm) combined with an MSD threshold of 20,000 cps. Target mass: 180.0, ion species  $[M+Na]^+$ , trigger:  $m/z$  203.0 (+ESI).

## Fraction pooling

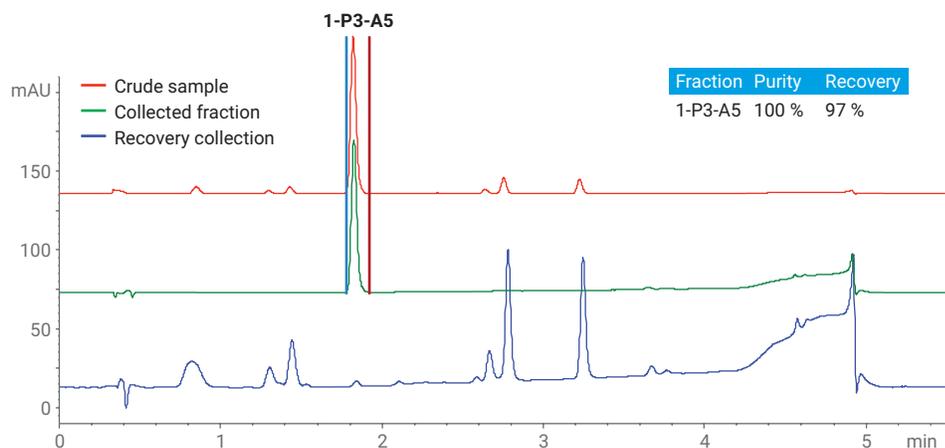
Purifying large-volume preparative samples can require multiple injections. To facilitate processing of the collected fractions, all Agilent fraction collectors support fraction pooling. This functionality allows the user to set a fraction start location for the first sample of a sequence only, and set Pooling as the fraction start location of all following injections of that sample (Figure 7). Fraction collection will then always start at the same position whether single or multiple fractions have been collected during the first run. The fraction collector keeps track of the fill state of each location. Once the selected pooling location is  $\geq 90\%$  filled, no further run will start until a free location has been selected.

## Recovery collection

The 1290 Infinity II Preparative Open-Bed Fraction Collector can be configured with a 1260 Infinity II Preparative Valve-Based Fraction Collector as a cluster for fraction and recovery collection. A simple fraction collector diverts everything not collected as a fraction to the waste. If fraction trigger parameters are set incorrectly, a precious compound might be lost this way. A recovery collector can prevent this scenario. Connected to the waste outlet of the fraction collector, the 1260 Infinity II Valve-Based Fraction Collector can be configured to save each run into a separate recovery location. A maximum of 11 recovery fractions are supported, giving the user the opportunity to review the collected fractions every 11 runs. If all target compounds have been collected, the recovery collection of that particular run can be discarded. If fraction collection failed, the target compound can be recovered by evaporating the solvent and re-injecting the recovery fraction. Figure 8 illustrates the principle of recovery collection.

Line	Sample Container	Sample Location	Sample Name	Method Name	Inj/Loc	Target Mass	Frac Start Pos
1	Use Current C...	P2-B3	Caffeine + Impurities	D:\Florian...	1	194.1	P6-A1
2	Use Current C...	P2-B3	Caffeine + Impurities	D:\Florian...	1	194.1	Pooling
3	Use Current C...	P2-B3	Caffeine + Impurities	D:\Florian...	1	194.1	Pooling
4	Use Current C...	P2-B3	Caffeine + Impurities	D:\Florian...	1	194.1	Pooling

**Figure 7.** Sequence table entries for fraction pooling. The fraction collection start position needs to be defined only for the first injection. Set Pooling for all following injections of the same sample.



**Figure 8.** Fraction collection with recovery collection (re-analysis at 254 nm). The sample (red) was separated, and the target compound was collected into a single fraction (green). The recovery collection (blue) demonstrates that all other compounds can be recovered in case fraction collection had not been triggered correctly.

## Conclusion

The Agilent 1260 Infinity II Preparative LC/MSD System excels in usability and focus on applications. The broad flexibility of the hardware is complemented by a large number of software features that provide the basis for successful fraction collection under variable conditions. If peak-based fraction collection triggered by a UV signal threshold is not enough to attack a challenging purification scenario, slope triggers, combined with MSD signals, fraction pooling, and recovery collection are only few of the possibilities a 1260 Infinity II Preparative LC/MS System has to offer.

## References

1. Agilent InfinityLab LC Purification Solutions, *Agilent Technologies Brochure*, publication number 5991-8009EN, **2017**.
2. Rieck, F. Time-, Peak-, and Mass-Based Fraction Collection with the Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector, *Agilent Technologies Technical Overview*, publication number 5991-7654EN, **2016**.

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