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Reduce Your Purification Footprint: Scouting, Collection, and Reanalysis in Well Plates on a Single System

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Abstract

Typical purification workflows using high-performance liquid chromatography (HPLC) involve two systems: the analytical system is used for scouting, method development, and fraction reanalysis; the preparative system is used for fraction collection. Moving samples and fractions between these systems and calculating the correct scale-up is time-consuming and prone to errors. This technical overview describes how analytical and preparative hardware can be combined and smartly operated by a single software package using Agilent InfinityLab LC Purification Solutions, reducing bench space, error sources, and the time needed for manually handling samples and fractions.

Introduction

Purification of small molecules by preparative HPLC has been established as a standard technique in many laboratories. Nevertheless, the process still involves different steps that require manual interaction and some thorough considerations and calculations. In most cases, the workflow includes a scouting step under analytical conditions to identify the target (preferably by mass selective detection (MSD)) and determine whether it is separated from other compounds in the sample. Analytical columns often do not tolerate strong injection solvents, which means the sample needs to be diluted in a weaker solvent. Once a suitable gradient has been found, it needs to be scaled up to enable higher sample loads under preparative conditions. Fraction collection must be triggered either by detector signal or in a time-based fashion. Finally, the purity of the collected fractions needs to be determined. All these steps can be prone to errors whenever the operator needs to dilute the samples, calculate gradients and scale-ups, and move different vials from the analytical to the preparative instrument and back for reanalysis.

This technical overview demonstrates how the interaction of hardware and smart software facilitates, or even eliminates, these steps of the purification workflow. The Agilent 1290 Infinity II Autoscale Preparative LC/MSD System combines both analytical and preparative flow paths in a single instrument. Autosampler and MSD are connected to both flow paths by a valve, which keeps the required bench space and cost of investment low. The autosampler has two sample loops for analytical and preparative injections, respectively, and doubles as a fraction collector. This combination enables easy reinjection of collected fractions, as well as basic liquid handling such as dilution and homogenization of samples and/or fractions. The entire system is controlled by the Automated Purification software for OpenLab ChemStation, which adds smart algorithms to the hardware: target peaks can be automatically identified, scale-ups and focused gradient calculations are done behind the scenes, and fraction reanalysis sequences are set up by a single click. Mass-based fraction collection and focused gradients enable highly specific fraction collection, which enables both samples and fractions to be held on a single 48-well plate. Keeping samples and fractions together largely reduces the risk of mixing up or losing track of a specific user's fractions, and thus adds confidence to the workflow.

Experimental

Instrumentation

The Agilent 1290 Infinity II Autoscale Preparative LC/MSD system used in this experiment comprised the following modules:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with 200 mL pump heads (option 206)

- Agilent 1260 Infinity II Quaternary Pump (G7111B) with active seal wash (option 30) and active inlet valves (option 032)

- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B) with 5 mL sample loop (option 241)

- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with 0.3 mm preparative flow cell (option 024)

- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm flow cell (option 018)

- Agilent 1290 Infinity II MS Flow Modulator (G7170B)

- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/14-port valve head (G4738A)

- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)

- Agilent 1260 Infinity II Delay Coil Organizer (G9324A)

- Agilent LC/MSD XT (G6135B)

Columns
- **Analytical column:** Agilent Prep-C18 Scalar 4.6 × 100 mm, 5 µm (p/n 449905-902)
- **Preparative column:** Agilent Prep-C18 21.2 × 100 mm, 5 µm (p/n 449905-702)

Software
- Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, version C.01.10 [239]
- Agilent Automated Purification software for OpenLab ChemStation, version A.01.08 [043]

Solvents

LC-grade acetonitrile and methanol, as well as analytical-grade dimethylformamide and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Samples

Six samples of different polarity, each containing at least three compounds, were created to represent a wide range of applications. Samples were dissolved in acetonitrile, dimethylformamide, dimethyl sulfoxide, 25% methanol in water, and methanol. Dilution of the concentrated samples for analytical injections was carried out by the autosampler using 25% methanol.

Sandwich plug solvents for preparative injections were 25% methanol in water or pure methanol, depending on the polarity of the sample.

Results and discussion

A sequence of six samples was submitted on a 1290 Infinity II Autoscale Preparative LC/MSD system controlled by Automated Purification software. As a sample and fraction container, a 48-well plate with 7.5 mL volume per cavity was chosen (see Figure 1). The six-by-eight layout allows for eight cavities per sample, which were used for:

- Concentrated sample
- Sample solvent needed for sandwich injection
- Dilution solvent
- Diluted sample for analytical scouting
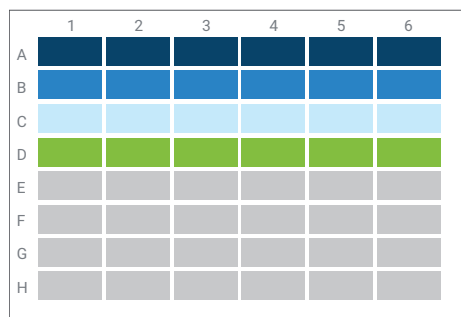- Fraction collection (four cavities)

Figure 1. Sample plate layout. The reaction microplate (part number 201236-100) offers enough space for six preparative samples (dark blue), sample-specific sandwich solvents (mid blue), dilution solvents (light blue), diluted samples (green), as well as 30 mL of fractions (grey).

Method settings

Table 1. Chromatographic conditions of analytical and preparative runs.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in methanol	
Flow Rate	1.5 mL/min	31.86 mL/min
Gradient	Scouting and focused gradients calculated by the software	
Dilution	100 ×	N/A
Injection Volume	10 μL	1000 μL
Sampler Method Preset	Preset 2: Nonpolar sample matrix Plug solvent: 25% methanol	Preset 3: Saturated nonpolar sample matrix Plug solvent: methanol
Temperature	Ambient	Ambient
UV Detection	Signal A: 210 nm Signal B: 254 nm Signal C: 280 nm 10 Hz data rate	Signal picked by the software based on target peak 10 Hz data rate
MS Detection	Signal 1: positive scan m/z 150 to 600 Signal 2: negative scan m/z 150 to 600	Signal picked by the software based on target peak
Split Ratio to MSD	Full flow	5,000:1 (mode M6) Turn ON after 2.00 min
Fraction Collection	Not applicable	Peak-based, UV and MSD UV threshold: 10 mAU UV upslope: 5 mAU/s UV downslope: 10 mAU/s MSD threshold: 15,000 cps

Table 2. MSD spray chamber and fraction collection settings.

Parameter	Value
Make Up Solvent	0.1% formic acid in methanol:water (70:30)
Make Up Flow	1.5 mL/min
Ionization Source	Agilent Jet Stream Electrospray
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Drying Gas Flow	12.0 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11.0 L/min
Capillary Voltage	±4,000 V
Nozzle Voltage	±600 V
Target Mass (m/z)	Entered per sample
Ion Species	$[M+H]^+$, $[M-H]^-$

One advantage of this well plate approach is to have both sample and fractions on a single plate, minimizing the risk of mixing up fractions of different instrument users. Moreover, the dedicated locations for sample-specific injection and dilution solvents reduce the risk of cross-contamination among samples and enable optimum chromatographic performance in both analytical and preparative scale. Strong sample solvents needed for sandwich injections in preparative scale would lead to sample breakthrough on analytical scale. Therefore, a weaker solvent (25% methanol) was used to prepare the diluted samples. The dilution solvent was also used as post-sample plug solvent for the analytical injections.

To enable drawing of sample-specific dilution or injection solvents, the locations of these solvents were saved in the method as positions relative to the sample. Sandwich injection solvent, for example, was set to *Location + 1 Row*, dilution solvent to *Location + 2 Rows*. This way, a single method could be used for all samples.

By setting the dilution position to *Location + 3 Rows*, the autosampler was able to dilute each sample automatically before the analytical injections. Without pausing, the autosampler draws from a preparative sample but injects from the dilution position after dilution and mixing is completed. Automatic sample dilution is a feature available for analytical injections on the 1290 Infinity II

Preparative Open-Bed Sampler/Collector. Saved as part of the method, it reduces manual interaction with the samples and exposure of the operator to dangerous solvents.

Using Automated Purification software further facilitated the workflow of analytical scouting, scale-up, and purification. One analytical method is enough to analyze the complete sequence, as the software calculates the scouting gradient based on the column dimensions and fills it in the method. Aligning the UV and extracted ion chromatogram (EIC) of the scouting run, the software detected the peak of interest in each sample based on a target mass entered in the sequence table (Figure 2).
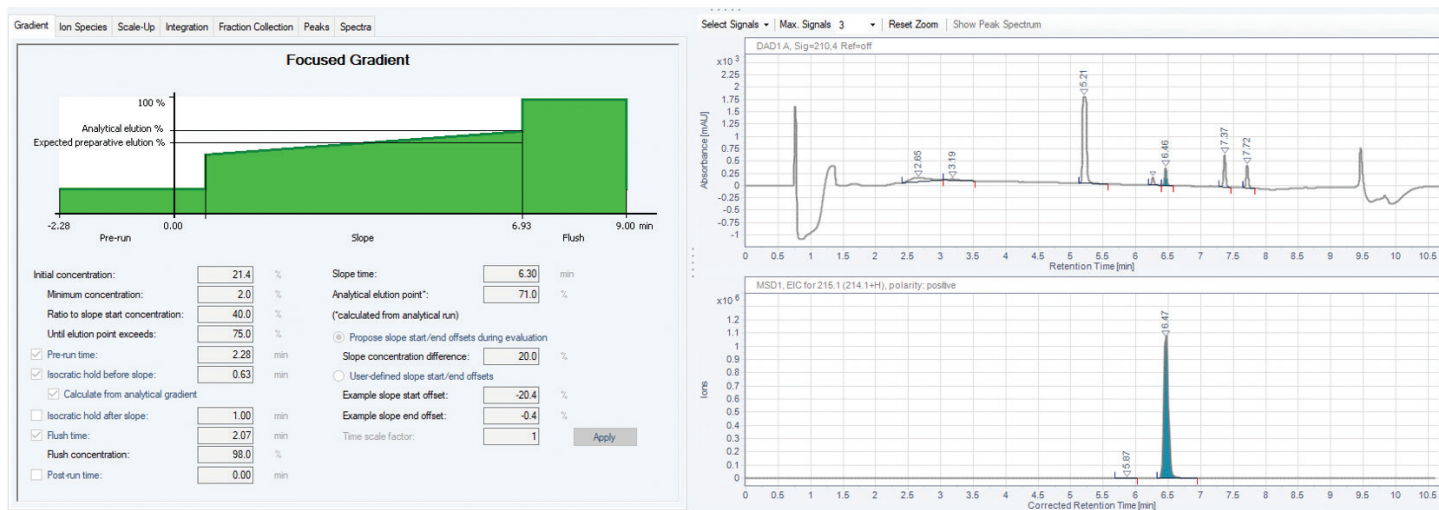

Figure 2. Analytical results view in Automated Purification software. Once the target is identified by overlaying the UV and MSD signals (blue peak on the right), the software calculates a focused gradient (left) for optimum separation under preparative conditions.

A second method was used as a preparative base method, where the software calculated a focused gradient for each target peak based on its retention time. Using a focused gradient resulted in a shorter run time (9.0 minutes in preparative scale versus 10.67 minutes in analytical scale) and consequently a minimized solvent consumption. The main advantage of a tailored focused gradient, however, is that resolution around the target peak is maintained despite heavy sample loading. Between the analytical scouting and preparative purification runs, the sample amount was scaled up by a factor of 10,000. Still, the target peak was separated well enough to allow specific fraction collection (see Figure 3). Only in the challenging sample 6 was the resolution not high enough to stop fraction collection at the tail of the target peak. The rising baseline of the following peak, however, triggered a new fraction, which prevented contamination of the third fraction. For cases like this, fraction reanalysis (Figure 6) proves especially helpful.
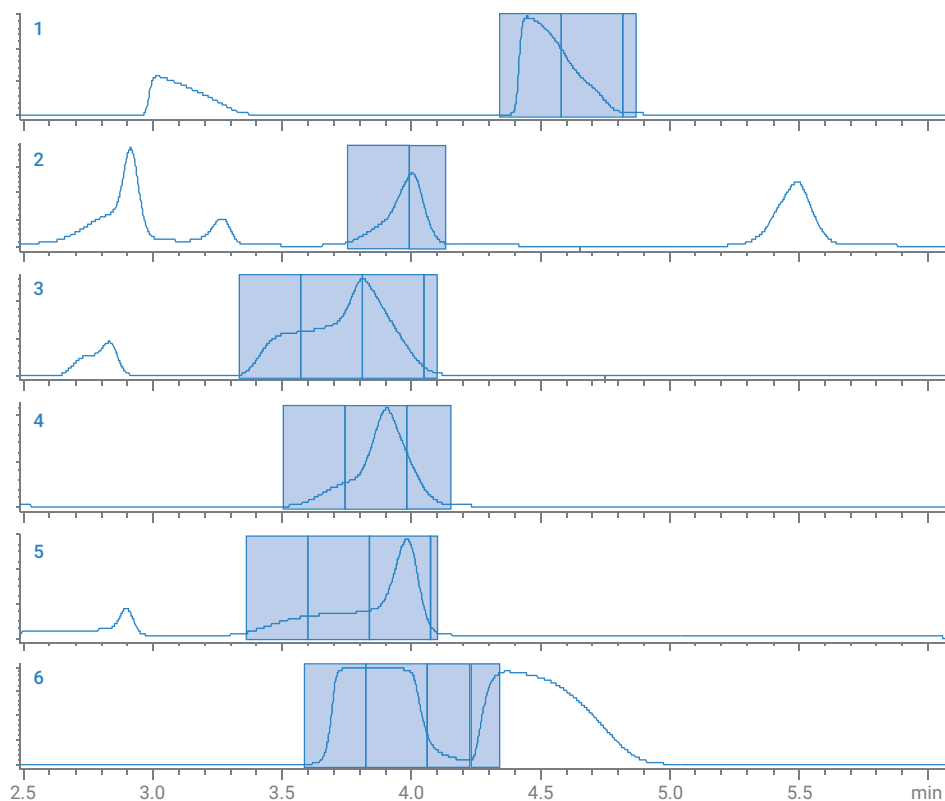

Figure 3. Chromatogram overlay (cutout) of purification runs for the six samples. Blue boxes indicate episodes of fraction collection. Calculation of specific focused gradients resulted in optimized resolution of the target peak approximately halfway through the gradient.

Figure 4 displays the preparative results view in Automated Purification software. In an interactive view, the operator sees sample positions, fraction locations, as well as chromatograms and spectra at one glance. The graphical fraction view on the upper right side can also be changed to a tabulated view, which is best for selecting single fractions to generate and export a liquid handler .csv file or reanalysis sequence. Depending on the time and scope of the workflow, either all fractions or only critical ones (e.g., collected at the target peak front or tail) can be selected for reanalysis.

Using this function, a reinjection sequence for the fractions of sample 6 was created. The first two fractions collected from this sample seemed to contain only the target peak, but fractions 3 and 4 might have already been contaminated with the peak eluting afterwards (see Figure 3). The two fractions were selected, and a ready-to-submit ChemStation sequence including method and injection volume was created using the fraction export dialogue in Purification software (Figure 5). As the experiment was conducted on an Autoscale system with a combined autosampler/fraction collector, all reinjections could be carried out without moving the fractions from the collector bed. Homogenization of the fractions before reinjection was selected in the reanalysis method and carried out automatically by the autosampler. This feature is described in detail in another publication.[1]
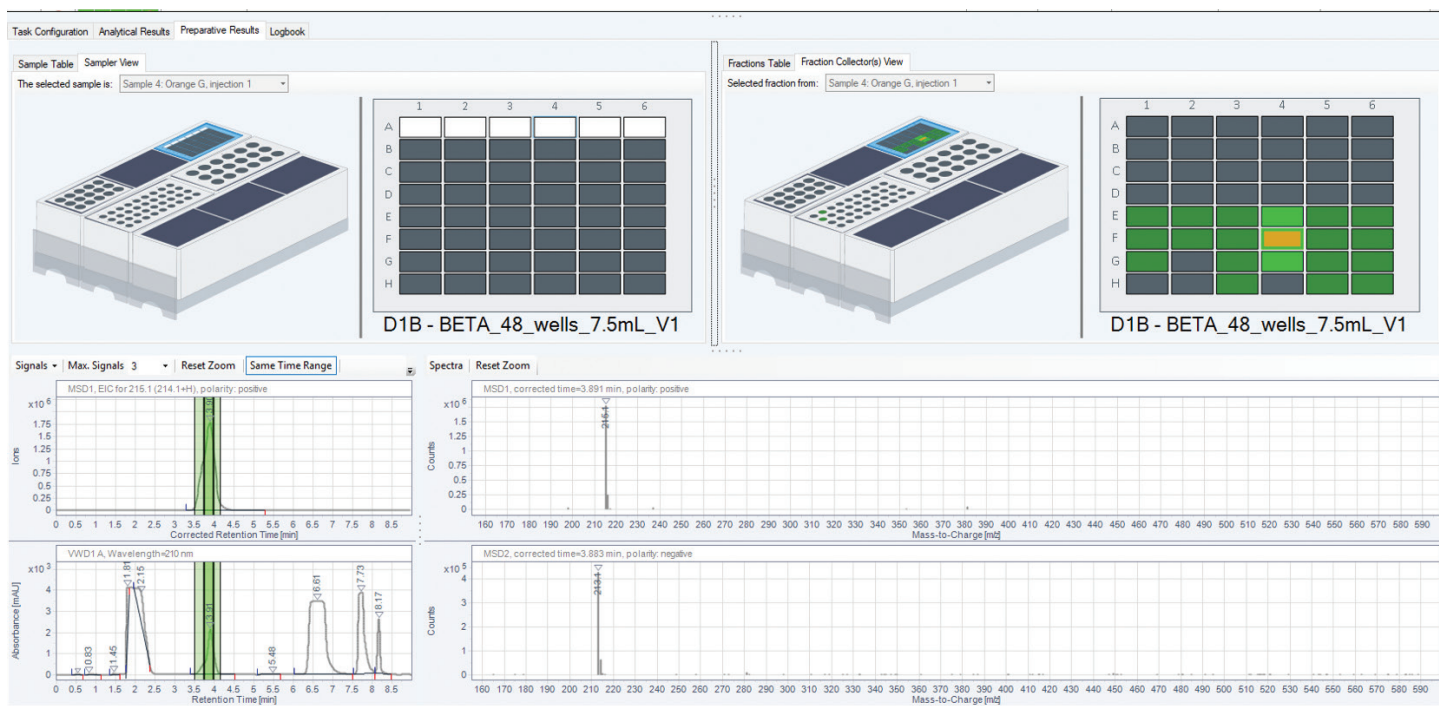

Figure 4. Preparative results view in Automated Purification software. Using focused gradients and mass-based fraction collection the fraction volume of a 48-well plate was sufficient for all samples (see upper right).

Figure 6 shows the reanalysis results for fractions 3 and 4 of sample 6. The target peak eluted at 6.6 minutes, the main impurity at 7.0 minutes. Overlaying the UV traces at 254 nm, it became apparent that fraction 3 was almost void of the main impurity, whereas fraction 4 still contained small amounts of the target peak. The purity of fraction 3, determined as UV peak area percentage at 254 nm, was 98%. Both fraction 3 and 4 contained small amounts of another impurity eluting at 6.8 minutes, which had not been detected during the analytical scouting runs. To further purify fraction 3 and investigate this new impurity, a larger amount of fraction 3 could now be injected into the preparative flow path of the system, with fraction collection aimed at both target peak and impurity.

Conclusion

A set of six samples was successfully analyzed and purified on a 1290 Infinity II Autoscale Preparative LC/MSD system. Using elaborate hardware features in conjunction with the smart algorithms of Automated Purification software facilitated the purification workflow: dilution of the concentrated sample for analytical scouting, identification of the target peak by mass, creation of a focused gradient for each sample, mass-based fraction collection, and reinjection of collected fractions were all carried out automatically. Eliminating the need to manually handle samples or fractions saved time and largely reduced the risk of errors. The specificity of mass-based fraction collection allowed samples and fractions to be contained in a single 48-well plate, which added more confidence due to easier traceability and lower risk of mixing up fractions.

Figure 5. The export function in Automated Purification software can be used to create fraction reanalysis sequences.
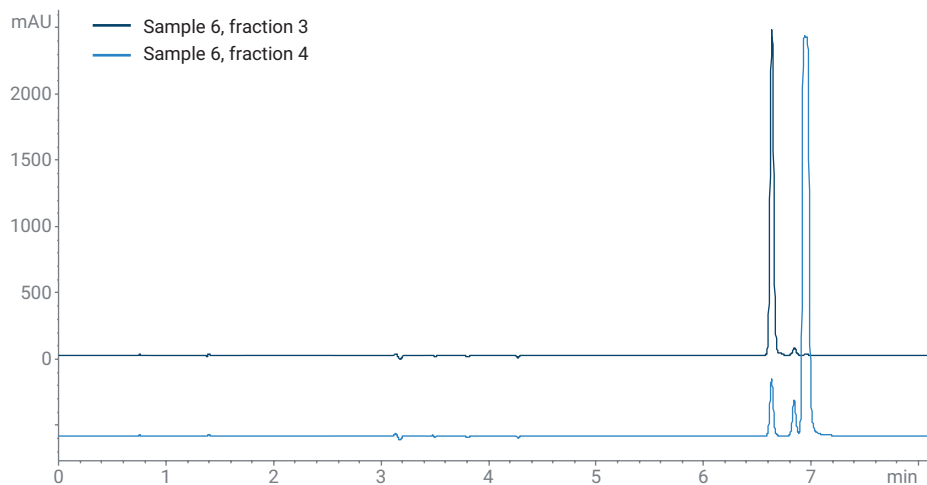

Figure 6. Reanalysis of fractions 3 and 4 of sample 6. The dark blue trace shows that fraction 3 has a purity of 98%. Fraction 4, on the other hand (light blue trace), contains the main impurity eluting after the target, along with small amounts of the target peak. Both fractions contain a small impurity that was previously undetected.

Reference

1. Spuling, I.; Rieck, F. Spotting Fraction Impurities with More Confidence Using the Agilent 1290 Infinity II Preparative Open-Bed Sampler/ Collector. *Agilent Technologies technical overview*, publication number 5994-1643, **2019**.

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