# EVALUATING THE TOOLS FOR IMPROVING PURIFICATION THROUGHPUT

Waters

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## INTRODUCTION

Chemists are constantly looking for ways to improve the overall throughput of their purification system. Time is the limiting factor for throughput, and there are 2 areas where time savings can be achieved. These are in the amount of time required to perform a separation and the amount of time between injections. Making the purification system as efficient as possible requires optimizing and minimizing both of these times. The challenge, however, is to minimize these times without impacting the purity and recovery of the fractions.

In order to correctly compare time saving techniques, we first established a baseline separation to define a standard analysis and collection time. We purified ten drug like compounds with a generic 10 minute preparative gradient. This baseline analysis time was then used as the comparison time for the analysis performed when the different time saving chromatographic functionalities were applied.

An approach for decreasing the analysis time includes the to use of shallow or narrow gradients. Approaches for decreasing the time between injections include column regeneration techniques, and automatically ending the purification run after the desired target has been collected.

## SYSTEM

Waters® 2525 Binary Gradient Modul (BGM), 2767 Sample Manager, Column Fluidics Organizer (CFO), 2996 Photodiode Array Detector, ZQ Mass Spectrometer, 515 Makeup Pump, and a 1:1000 Passive Flow Splitter. Another 2525 BGM for regeneration as needed. All components are controlled by MassLynx™ and FractionLynx™ .

*Figure 1. Waters® Mass–Directed ZQ™–Based AutoPurification™ System*

The 10 sample library consisted of various drug-like compounds at a sample concentration of about 20 mg/mL dissolved in DMSO.

## METHODS

The chromatographic methods used water with 0.1% formic acid as mobile phase A, and acetonitrile with 0.1% formic acid as mobile phase B. Methanol was used as the makeup solvent for the preparative analysis.

**Generic Analytical and Preparative Gradient**
SunFire™ C18 4.6 or 19 x 50 mm 5 µm, 1.5 or 25 mL/min total flow gradient and a 10 minute total run time.

| | Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|---|
| 1 | Initial | 1.50 | 95.0 | 5.0 | Initial |
| 2 | 7.00 | 1.50 | 5.0 | 95.0 | 6 |
| 3 | 8.00 | 1.50 | 5.0 | 95.0 | 6 |
| 4 | 8.25 | 1.50 | 95.0 | 5.0 | 6 |
| 5 | | | | | |
| 6 | | | | | |

*Figure 2. Analytical gradient table*

**Narrow or Shallow Preparative Gradient**
SunFire™ C18 19 x 50 mm 5 µm, 25 mL/min total gradient. The start and end %B composition is variable and dependant on the sample retention time during its analytical analysis. The time window in which the analytical sample eluted defines the conditions for the prep run. For example, if the compound eluted at 4.04 min, then the prep method would ramp up the organic to 50% at 0.5 min.

| Time (Minutes) | Composition (%B) |
|---|---|
| 0.00—0.5 | 5—%B Start |
| 0.50—1.67 | %B Start—%B End |
| 1.67—2 | %B End—95 |
| 2—3 | 95 |
| 3 - 5 | End |

*Table 1. Narrow gradient table. See Table 2 for %B Start and End*

| Gradient Name | Analytical Retention | % B Start | % B End |
|---|---|---|---|
| A | 0.00 - 1.67 | 5 | 20 |
| B | 1.67 - 2.84 | 20 | 35 |
| C | 2.84 - 4.0 | 35 | 50 |
| D | 4.00 - 5.17 | 50 | 65 |
| E | 5.17 - 6.34 | 65 | 80 |
| F | 6.34 - 7.5 | 80 | 95 |

*Table 2. The various narrow gradients used relative to the analytical Rt.*

## BASELINE THROUGHPUT

The generic gradient was used to perform the purification of 10 samples and the overall run time was measured. These times is used to compare throughput increases against.

| Sample | Retention Time (min) | Run Time (min) | Time Between Injections (min) |
|---|---|---|---|
| 1 | 1.18 | 10 | 2 |
| 2 | 5.2 | 10 | 2 |
| 3 | 1.35 | 10 | 2 |
| 4 | 4.67 | 10 | 2 |
| 5 | 3.18 | 10 | 2 |
| 6 | 2.55 | 10 | 2 |
| 7 | 2.41 | 10 | 2 |
| 8 | 5.06 | 10 | 2 |
| 9 | 2.02 | 10 | 2 |
| 10 | 2.63 | 10 | 2 |
| Total Run Time | | **120 minutes** | |

*Table 3. The overall throughput with the generic gradient. The total run time was 120 minutes.*

## NARROW GRADIENTS

Shallow gradients can be used to improve preparative chromatographic resolution [1]. However, if the resolution is adequate in the analytical separation, a shorter shallow gradient can be used to increase throughput. The short method will focus its gradient on the same organic concentration but in a shorter time frame.

Figures 4 A and B show an example of one of the ten samples being purified by both a generic and narrow gradients. The target was successfully isolated using Narrow gradient D. The results show that the resolution is maintain over the focused section of the gradient (the blue bracket). Note that there is a loss in resolution in the non-focused areas of the gradient. This could have to be considered when the compound elutes at the very beginning or end of the focused gradient.

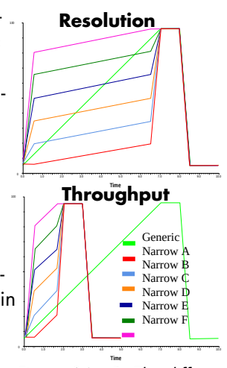


*Figure 3A—B. The different narrow gradients possible to focus on either improved resolution or throughput.*

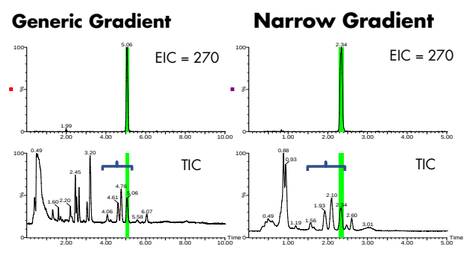


*Figure 4A—B. A comparison of the 10 minute generic and the 5 minute narrow purification. The blue bracket corresponds to the focused area of the gradient, where the resolution is maintained.*

| Sample | Generic Retention Time (min) | Narrow Gradient | Narrow Retention Time (min) | Run Time (min) | Time Between Injections (min) |
|---|---|---|---|---|---|
| 1 | 1.18 | A | 1.38 | 5 | 2 |
| 2 | 5.2 | E | 1.65 | 5 | 2 |
| 3 | 1.35 | A | 1.74 | 5 | 2 |
| 4 | 4.67 | D | 1.94 | 5 | 2 |
| 5 | 3.18 | C | 1.75 | 5 | 2 |
| 6 | 2.55 | B | 1.90 | 5 | 2 |
| 7 | 2.41 | B | 1.95 | 5 | 2 |
| 8 | 5.06 | D | 2.34 | 5 | 2 |
| 9 | 2.02 | B | 1.30 | 5 | 2 |
| 10 | 2.63 | B | 2.08 | 5 | 2 |
| Total Run Time | | | **70 minutes = 1.7 Fold Increase** | | |

*Table 4. The overall throughput increases by1.7 fold when incorporating narrow gradients, compared to using a generic gradient.*

## RINSING AND EQUILIBRATION

It is important for high quality chromatography, that the column is rinsed and re-equilibrated with the appropriate volume of solvent, typically defined in column volumes. Insufficient rinsing can cause carryover, and equilibration time also has a significant impact on the overall throughput, with inadequate equilibration leading to retention time variability, poor chromatographic peak shape, or even sample breakthrough. The quantity of rinsing solvent is dependant upon the sample matrix, the retentiveness of the column, and the elutropic strength of the rinsing solvent. Typically, 2-3 column volumes is required to rinse. For equilibration, various articles report anywhere from 3-20 column volumes can be used [2-3].

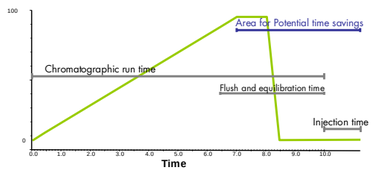


*Figure 5. Illustration of an injection cycle, with chromatographic analysis time, equilibration and flush time, and injection cycle for next injections time displayed. The area where time could potentially be saved is noted.*

For example, a 19 x 50 mm column has a volume of about 12 mL. Two column volumes or 24 mL of 95% B were used to flush the column, and 60 mL of 5% B were used to re-equilibrate the column. With the gradient flow of 25 mL/min, the flush takes about 1 minute, and the equilibration takes about 2.5 minutes. However, the flow rate can be elevated above optimal chromatographic conditions (30 mL/min for 5 um packing), so long as the system can withstand the overall pressure increase. We found that the flow could be increased to 40 ml/min, only generating an additional 1300 psi of backpressure, reducing the flush time to 0.6 minutes and the re-equilibration time to 1.5 minutes, a 1.5 min savings.

## OFF-LINE REGENERATION

To increase throughput, a regeneration pump can be used to flush and re-equilibrate the first column off-line, while the next sample is running on a second column.

**Method:**
The run is terminated at 2.5 minutes for the narrow gradients, or 7 minutes for the generic and the next injection started. The first column is switched offline and its flush started, while the second column is put in line to receive the next sample. As mentioned earlier, the time required for the injection to be performed is 2 minutes.

**Run Time Savings:**
Generic: Reduction of 3 minutes / sample, for a reduction in the total run time from 120 minutes to 90 minutes = **1.2 Fold Increase**

Narrow: Injection to injection time was reduced from 12 minutes with the generic method to 4.5 minutes using narrow gradients and off-line column regeneration. This reduced the total run time from 120 minutes to 45 minutes, a **2.7 Fold Increase**

## EARLY RUN TERMINATION

To further reduce the time required for analysis, a software tool may be used which can automatically end the run after the target has been collected. The throughput improvements of this feature will be illustrated for both generic and narrow gradients. For either gradient approach used, once the target has finished collecting, the gradient will stop and a flush with 95% B will wash the remaining material off the column. After a defined time of rinsing, the column will then be re-equilibrated with the initial gradient solvent. Note: 2 minutes of equilibration time is performed between injections. Table 5 shows the throughput improvements.

| Sample | Generic Run Time | Generic with Regeneration | Narrow Run Time | Narrow with Regeneration |
|---|---|---|---|---|
| 1 | 4.03 | 3.43 | 4.23 | 3.63 |
| 2 | 8.05 | 7.45 | 4.50 | 3.90 |
| 3 | 4.20 | 3.50 | 4.59 | 3.99 |
| 4 | 7.52 | 6.92 | 4.79 | 3.19 |
| 5 | 6.03 | 5.43 | 4.60 | 4.00 |
| 6 | 5.40 | 4.80 | 4.75 | 4.15 |
| 7 | 5.26 | 4.66 | 4.80 | 4.20 |
| 8 | 7.91 | 7.31 | 5.19 | 4.59 |
| 9 | 4.97 | 4.27 | 4.15 | 3.55 |
| 10 | 5.48 | 4.88 | 4.93 | 4.33 |
| Total Run Time | **58.75 min = 2.0 Fold Increase** | **52.75 min = 2.3 Fold Increase** | **46.53 min = 2.6 Fold Increase** | **40.53 min = 3.0 Fold Increase** |

*Table 5. The overall throughput improvement using the run termination function can range from a 2 to 3 fold increase, depending on what additional tools are used. Using the regeneration pumps saves 0.6 minutes per injection when compared to a single column method. This corresponds to the time required to rinse the column. The re-equilibration time is incorporated into the 2 minutes to make an injection.*

## DIRECT INJECTION

Throughput can be further improved by reducing the time required to make an injection. One approach is to use a feature newly incorporated into the 2767, called direct aspiration injection. With this injection technique, the sample is aspirated directly onto the sample loop (see Figure 6). This saves time by reducing the number of injection steps and the number of surfaces requiring rinsing (no injection port is required). Furthermore, because the rinsing time is reduced to under 30 seconds, the needle rinsing can be performed at the start of the injection. Previously, this was done after fraction collection because of the potential for losing the early eluting peaks.

**Direct Injection Time Savings**

The time required to inject and rinse was reduced from 2 minutes with the standard partial loop injection to 0.4 minutes for the direct injection. Table 6 show the throughput possible by combining direct injection with the various other tools.

| Tool | Original Total Run Time (min) | Direct Inject Total Run Time | Without Direct Inject | Overall Increase using Direct Inject |
|---|---|---|---|---|
| Generic | 120 | 104 | — | **1.2** |
| Generic + End Run | 58.75 | 53.75 | 2.0 | **2.2** |
| Generic + End Run + Regeneration | 52.75 | 36.75 | 2.3 | **3.3 Fold Increase** |
| Narrow | 70 | 54 | 1.7 | **2.2** |
| Narrow + End Run | 46.53 | 41.63 | 2.6 | **2.9** |
| Narrow + End Run + Regeneration | 40.53 | 24.53 | 3.0 | **4.9 Fold Increase** |

*Table 6: Using direct injection can improves the overall throughput. Direct injection has a greater impact when using regeneration because the 2 minutes for the normal injection is used to re-equilibrate with a single column. But with regeneration, the re-equilibration is down off-line and the injection time is dead time.*

## CONCLUSIONS

- **Throughput can be increased by about 5 fold using a combination of narrow gradients, early run termination, off-line column regeneration and direct injection. This correlates to an 80% decrease in run time.**
- **Narrow gradients can be used to improve throughput, but requires additional information about the target.**
- **Off-line column regeneration has a greater impact on throughput as the run time is reduced.**
- **Early run termination improves throughput and reduces the amount of consumed solvent saving both time and money.**
- **Direct injection reduces the injection-to-injection cycle time from 2 to 0.4 minutes and has the greatest impact on throughput when combined with regeneration.**
- **Various combinations of throughput enhancing tools can be used based on the specific requirements**

## REFERENCES

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