Optimizing Purified Sample Recovery on Reverse-Phase HPLC Systems

Application Note 209

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Introduction

Today's researchers process hundreds of thousands of compounds per month. They face many challenges when trying to accomplish this daunting task. Two factors that directly affect the outcome of this task are purity and recovery. Acceptable limits for purity and recovery need to be established before the compounds can be processed. If time is taken to develop, optimize, and validate an analytical method, then a robust analytical method can be scaled to preparative when processing the samples.

Many columns allow the direct transfer from analytical to preparative chromatography, since the same packing media is used in both types of columns. Although transferring to a preparative mode may seem linear, there are many factors that need to be addressed in order to achieve optimum purity and recovery. The purpose of this application is to present the factors that can directly affect purity and recovery when processing compounds on a preparative level. The following parameters will be addressed:

- Characteristics of short, fast preparative columns
- General gradients for classes of compounds
- Determination of collection valve switch time
- Determination of detector time constant
- Injection techniques that optimize sample loading
- Accurate determination of delay volume from detector to fraction collection valve
- Determination of software response speed

Materials & Methods

Instruments and Accessories

Gilson 215 Liquid Handler, equipped with: 175-mm Z-arm, 819 Injection Module with 5.0-mL stainless steel loop, and beveled-tip probe (269 x 1.3 x 0.8 mm ID)

Gilson VALVEMATE® Valve Actuator, equipped with: 7610 Rheodyne valve (10-port, 2-position)

Gilson 322 HPLC Pump, equipped with H2 pump heads (flow rate up to 30 mL/min.)

Gilson 307 HPLC Pump, equipped with 10 mL/min. pump head (regeneration pump)

Gilson 155 UV/VIS Dual-wavelength Detector (0.2-mm pathlength, 1.0 AUFS)

MetaChem Inertsil® ODS-2 Column (50 x 21.2 mm, 5 µ)

YMC™ ODS Column (50 x 50 mm, 10 µ)

Purity vs. Recovery

Purity and recovery are used to define the capabilities of a system to collect fractions. Although each describes a unique feature of fraction collection, the two concepts are related.

Purity is usually determined by means other than UV detection (e.g., MS, DAD). This allows for the evaluation of the component that is responsible for the absorbance peak in the chromatogram. Determination of whether one compound or more than one compound is responsible for the peak is what defines the level of purity.

Recovery is based on the amount of product collected; the purer the compound, the greater the recovery—which leads to enhanced synthesis and assay development.

Key Factors of Fast Preparative Reverse-Phase HPLC

- Cycle time per sample is important
- What purity is required? (80, 85, 90, 95, >95%?)
- How to define purity? (UV, ELSD, MS,?)
- What recovery is acceptable? (80, 85, 90, 95, >95%?)
- Acceptable limits of purity and recovery may limit cycle time, and, therefore, productivity

Key Factors of Fast Preparative Methods

- Develop general gradients for classes of compounds

For more polar molecules:	3–50% AcCN/water
For average polarity molecules:	10–95% AcCN/water
For more lipophilic molecules:	40–100% AcCN/water

- Use short columns (50–100 mm), small diameter packing material (5 or 10 µ), and high flow rate
- Cycle time per injection of 3 minutes to 10 minutes
- Approximately 100-mg injections. Based on 21.2-mm ID columns. Sample purity requirements may limit injected sample mass

Key Factors of Fast Preparative Collection Applications

- Conventional prep peak widths are from 1 to 20 minutes
- Fast preparative chromatography may have total run times of 3 to 10 minutes per sample
- Fast preparative peak widths are from 10 to 60 seconds
- Time resolution of all hardware components becomes significant

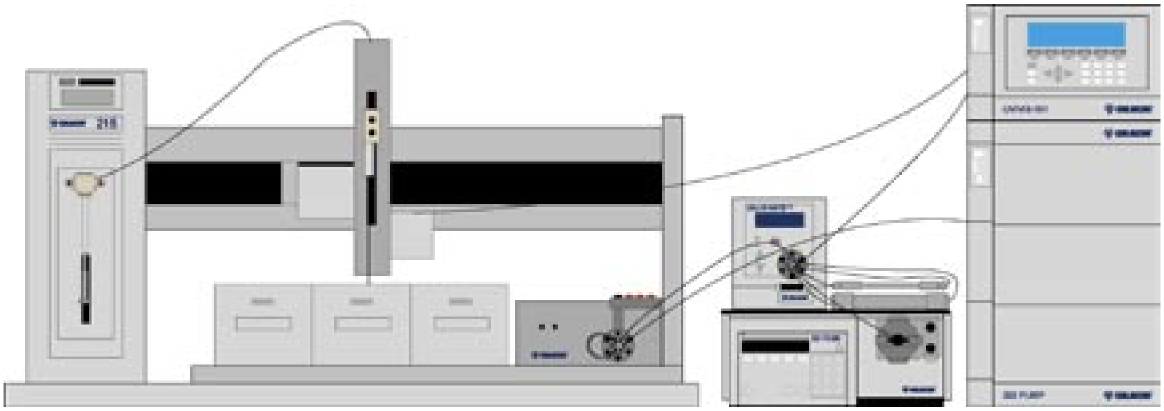


Figure 1: Preparative System Hardware

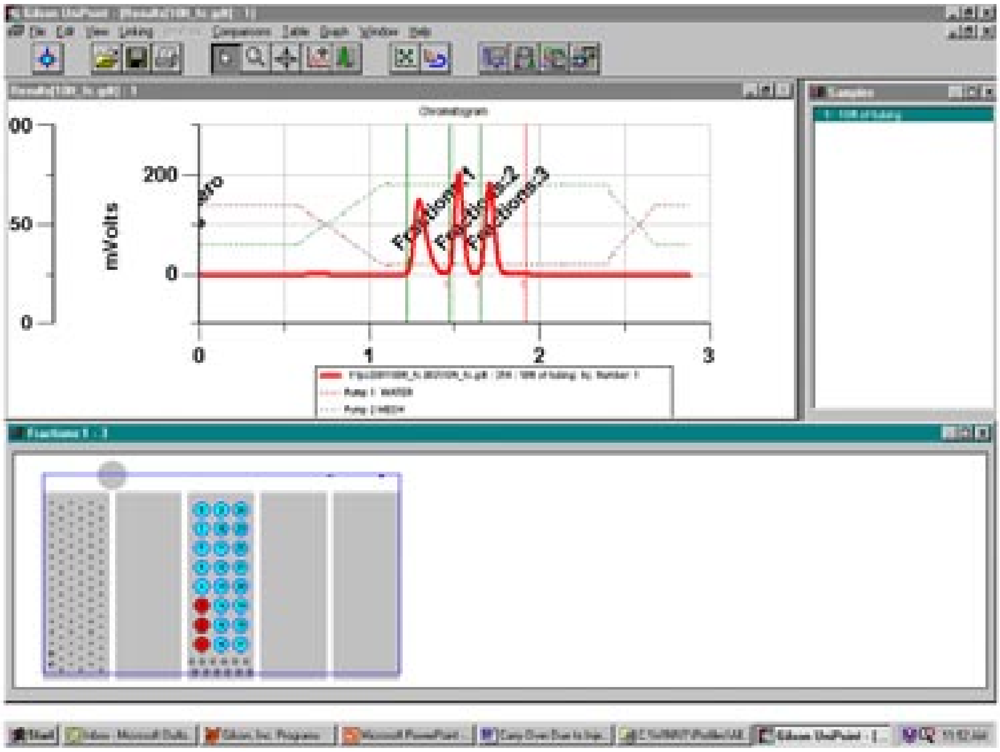


Figure 2: Fast Preparative Chromatogram

Collection Valve Switch Time

- Switch time should only be a few milliseconds
- A switch time of 1 or more seconds is too long
- At 40 mL/min. and less than 5-minute run times, the peak widths can be between 10 and 20 seconds

Detector Time Constant

- Most detectors have a detector noise filter which smooths the data output signal. This may be labeled:

  Peak Width

  Time Constant

  Rise Time
- Detector signal smoothing takes time; set the time constant to the fastest setting
- With fast flow rates and narrow peaks, the sample may reach the fraction collector before the software receives the signal from the detector
- A delay of 2 or 3 seconds can result in the loss of 50% of the sample, relative to a peak width of 5 seconds for a given peak
- With fast chromatography, as the value for the detector time constant increases, mass recovery decreases

Detector Time Constant (sec.)	Yield (%)
0	>95
25	93
50	77
75	47

Table 1: Detector Time Constant

Using a DMSO Solvent Sandwich

Using a small amount of a strong solvent to buffer the sample from the highly aqueous mobile phase (in the plumbing before the column, at initial conditions) can be beneficial in preventing precipitation. However, a sandwich solvent can cause degradation of resolution. Therefore, care should be taken with how much additional strong solvent is injected with the sample. The use of a sandwich solvent should not affect mass recovery.

Sandwich Solvent (µL)	Yield (%)
0	84
50	89
100	>90
150	85
200	84
250	84

Table 2: Using a DMSO Solvent Sandwich

Delay Volume (I)

For example: 5 feet of 0.030 inch internal diameter tubing contains approximately 695 microliters. At a flow rate of 20 mL/min., the delay time is 2.1 seconds.

10 feet of 0.040 inch internal diameter tubing between the detector and fraction collector contains 2,470 microliters. At a flow rate of 40 mL/min., the delay time is 3.7 seconds.

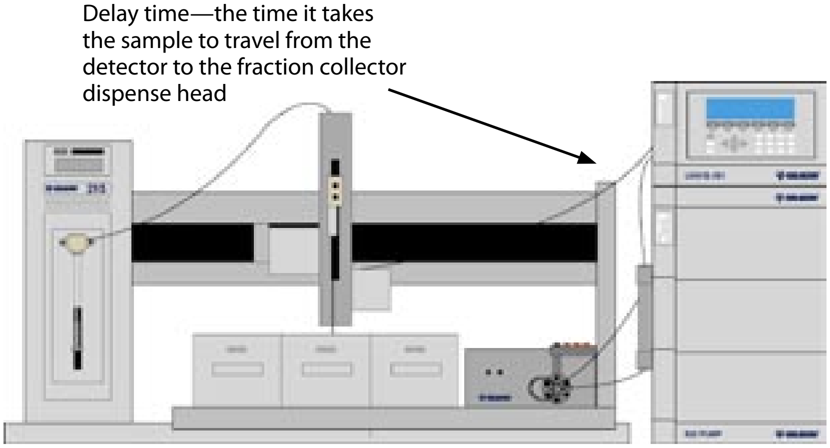
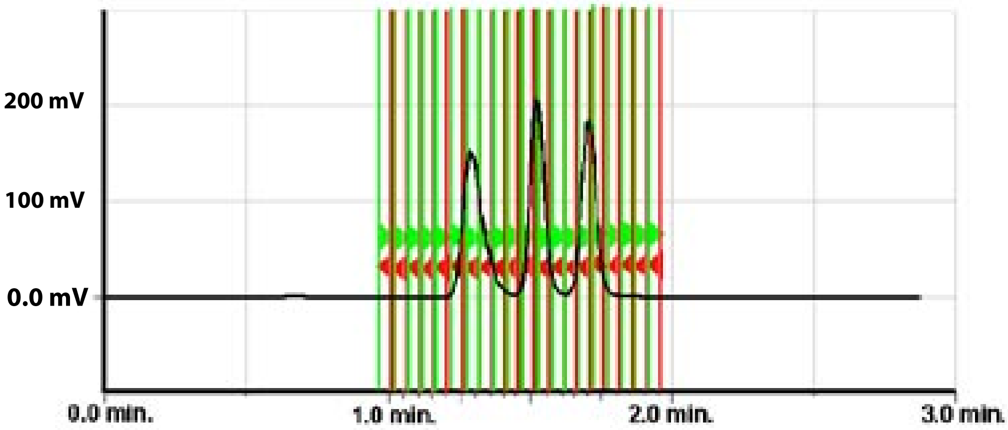

Delay time—the time it takes the sample to travel from the detector to the fraction collector dispense head

Figure 3: Delay Volume or Time

It is important to accurately determine the delay volume or time to optimize the collection of desired fractions.

Method Used to Determine Delay Time

1) 1 mg of Coumarin 314 was injected. A gradient from 50–100% AcCN/water was used to elute the sample. Detector wavelength was set at 436 nm.
2) The signal readout on the front of the UV detector was carefully monitored.
3) A stopwatch was started as soon as the signal appeared on the UV detector.
4) The stopwatch was stopped as soon as the dye reached the fraction collector valve.
5) Using a minimal amount of tubing (1.1 mL) between the detector and fraction collector, at 20 mL/min., the delay time is 3.3 seconds.



Graph 1: Delay Time Setting Confirmed by Reanalyzing Collected Fractions

Individual fractions collected by time. Approximately 20 fractions per minute .

Software Processing Speed

At a flow rate of 20 mL/min., it takes a sample 2.1 seconds to travel through 5 feet of 0.030 inch internal diameter tubing.

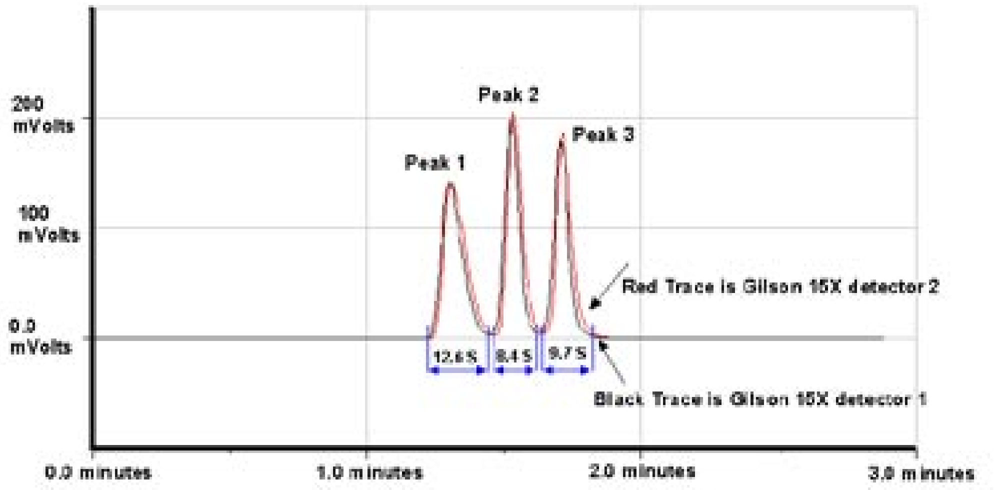
It may take the software data controller longer than 2 seconds to process the following data:

- Is a peak being eluted from the column?
- Does this peak meet set peak identification parameters?
- What is the delay from the detector to the fraction collector?
- Start collection

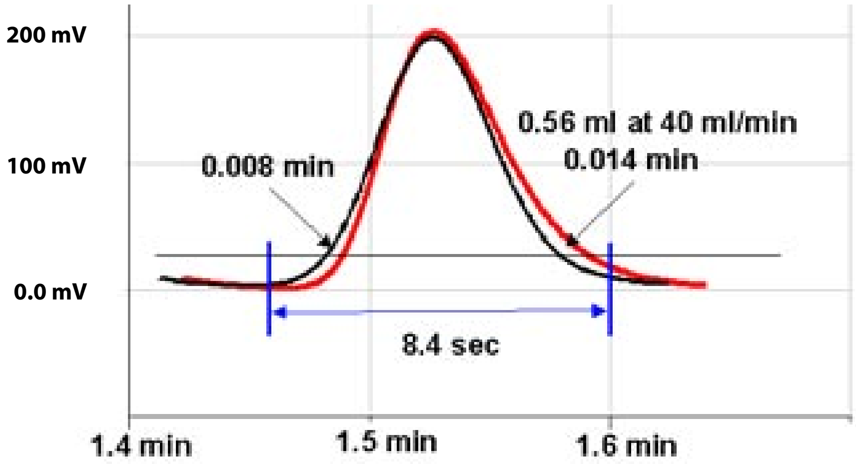
Delay Volume (II)

It may be necessary to add tubing between the detector and fraction collector to increase the delay volume. This will overcome the inherent delay times associated with the detector time constant, fraction collector valve switch time, and data processing.

One of the problems associated with additional tubing lengths is peak spreading. However, at these increased flow rates, peak spreading through 10 feet of tubing is negligible.



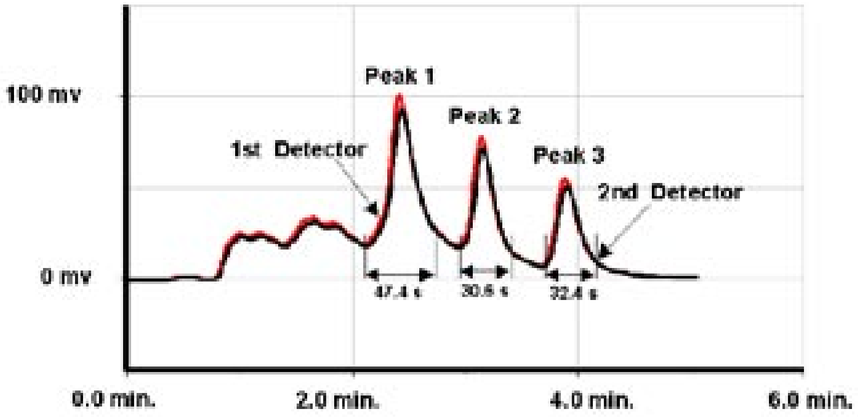
Graph 2: Example of the Peak Spreading in 10 ft. of 0.040 in. ID Tubing at a Flow Rate of 40 mL/min.
Peak spreading was determined by placing two detectors in series within an HPLC system with 10 ft. of 0.040 in. ID tubing between them. The sample was injected and the response from each detector recorded. Column used: Metachem Inertsil® ODS-2 (50 x 21.2 mm, 5µ).



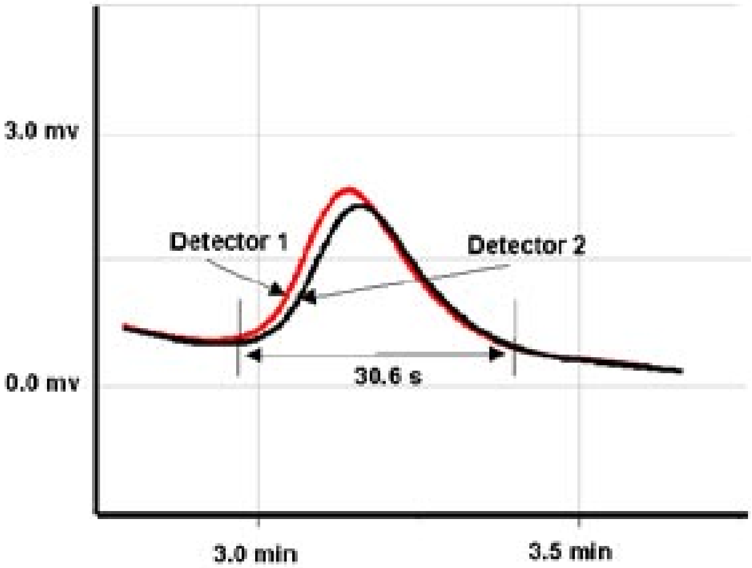
Graph 3: Closeup view of the Peak Spreading in 10 ft. of 0.040 in. ID Tubing at a Flow Rate of 40 mL/min.

Delay Volume (III)

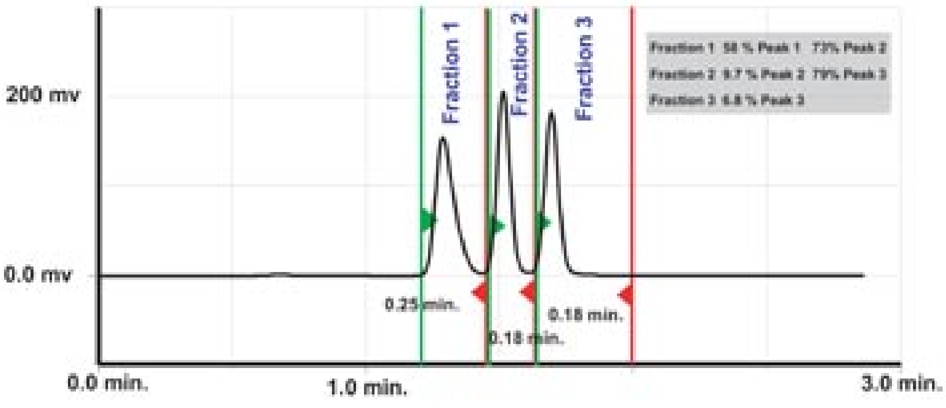
10 feet of 1/8 inch outer diameter and 0.062 inch inner diameter tubing contains 5,937 microliters. At a flow rate of 150 mL/min., the delay time is 2.4 seconds.



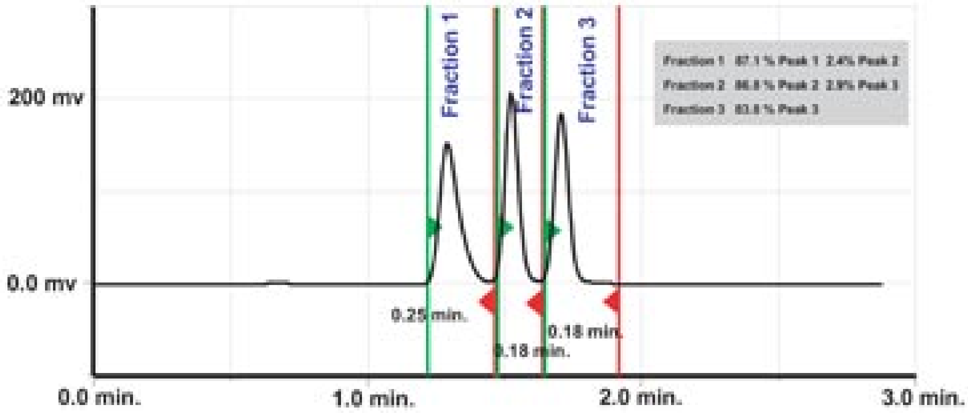
Graph 4: Peak Spreading from 10 ft. of 0.062 in. ID Tubing at a Flow Rate of 150 mL/min. The procedure is the same as desribed for Graph 2. Column used: YMC™ ODS (50 x 50 mm, 10 µ).



Graph 5: Peak Spreading from 10 ft. of 0.062 in. ID Tubing at a Flow Rate of 150 mL/min.



Graph 6: Recoveries <u>without</u> 10 ft. of Tubing to Increase Delay Volume Tubing ID: 0.040 inches; flow rate: 40 mL/min.



Graph 7: Recoveries with 10 ft. of Tubing to Increase Delay Volume
Tubing ID: 0.040 inches; flow rate: 40 mL/min.

Conclusion

Paying attention to system parameters directly affects purity level and percent recovery of compounds processed via fast preparative HPLC. The following system parameters were evaluated:

- Collection valve switch time
- Detector time constant delay
- DMSO sandwich solvent
- Delay volume from detector to collection valve
- Software response

Time restraints and sample throughput may not allow optimization of all of the system parameters evaluated in this application. This application represents the guidelines and avenues for testing the parameters, whether one or all are required.

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August 2003 Page 8 of 8 319309-01