A NOVEL APPROACH FOR REDUCING FRACTION DRYDOWN TIME

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INTRODUCTION

Recent advances in purification technology have shifted the throughput bottleneck from purifying the samples to fraction drying. Some of the technologies employed for sample drying include vacuum centrifugation, heated nitrogen blow-down and lyophilization. However, each one has the same rate limiting factor, the quantity of water present. This quantity is dependant on the separation technique used to generate the fractions. The most commonly used technique is reverse phase- (RP-) HPLC which can generate fraction with the water content as great as 95%.

One approach experimented with, is to collect fractions directly onto SPE cartridges. In theory this method is perfect, but making it automated and rugged has continued to be the challenge. A drawback to this approach is that a very high flow dilution pump is required to trap the compound on the cartridge. This high flow rate requires a large quantity of sorbent with large volume cartridges, and generates large volume fractions. Another problem with collection onto SPE cartridges is the possible change in selectivity that could result in poor trapping or breakthrough of the analyte.

OVERVIEW

This poster shows the development and optimization of a method that removes the water and reduces the overall volume of the collected fraction. This method works by injecting and trapping the previously collected fraction onto a preparative column. The fraction is trapped by diluting the loading flow with 100 % aqueous mobile phase. After the trapping has been completed, 100% organic mobile phase is passed through the column to elute the sample. Collection of the target is triggered by the MS detector and the collected fraction is now in 100% organic mobile.

SYSTEM DIAGRAM

The standard components of the Waters AutoPurification System were used to perform the fraction concentration. In the plumbing diagram shown in Figure 1, the aqueous flow out of the gradient pump is directed into the first tee (T1). This tee acts as a mixer, diluting the organic concentration of the injected fraction, so that it will not breakthrough the trapping column. The organic flow out of the gradient pump is directed to a second tee (T2) and is used to elute sample from the column.

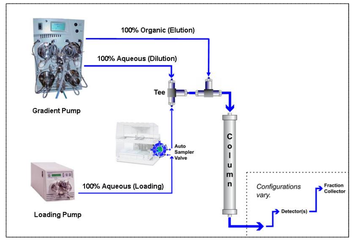

Figure 1. Plumbing diagram for the concentration system. Both fraction collection and concentration was performed on the same mass– directed AutoPurification system. Fraction collection was triggered by MS.

PROOF OF PRINCIPLE

To establish a baseline performance of the method parameters,10 drug-like compounds were initially purified. These purified fractions collected in various different concentrations of organic solvent, were then used as the samples to evaluate the concentration method. The samples were loaded onto a trapping column and eluted in 100% organic solvent. Once it was determined that the initial method was successful, the process was optimized for minimum fraction volume and maximum throughput. The examples shown have initial fraction volumes as great as 30mL of aqueous/organic and are reduced to as little as 1.5mL of organic solvent.

Purification Method

- 10 mg sample load
- Generic 5-95 gradient w/ Water: ACN: formic acid
- Fraction volume of 5 – 8 mL with recoveries of > 95%

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	0.00	20.00	95.0	5.0	0.00
2	1.00	20.00	95.0	5.0	6
3	8.00	20.00	5.0	95.0	6
4	8.50	20.00	95.0	5.0	11
5	10.00	20.00	95.0	5.0	6

Figure 2: Generic 5-95% gradient

Concentration Method

The collected fractions were injected onto the same column as was used for the purification. The samples were loaded onto the column with a loading pump at 6mL/min 100%A, and 29mL/min aqueous from a dilution pump. After 6.5 minutes, the loading and dilution flow is stopped. Now that the sample is retained on the column, the elution is started at 29mL/min of 100% B.

Results

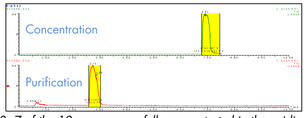

Figure 3. 7 of the 10 were successfully concentrated in the acidic mobile phase in which they were collected. All recoveries were > 85%.

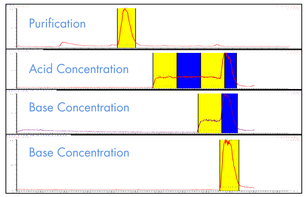

Figure 4. 2 of the remaining 3 were successful after adding base to fraction. This indicates that these sample should have been purified at a basic pH to keep the target neutral.

Although the remaining sample was purified using the SunFire™ column, it was not retained on the column during the concentration process. However, because fraction collection was triggered by MS, no sample was lost. Additional work is required to determine why it was not retained.

METHOD OPTIMIZATION

Once the trapping method was determined to be successful, we looked into optimizing the conditions. The parameters evaluated included the column dimension and packing, the dilution ratio and the elution flow rate. An initial fraction of 10 mg of diphenhydramine collected in 8 ml of 60% water was the concentration test sample.

Column Dimensions

The column must be able to trap the target fraction and yet give a minimum elution volume for the concentrated fraction.

The maximum flow rate and the minimum loading time were determined to establish a minimum run time. These factors are dependant upon the column ID, particle size and injection loop.

19 x 50 mm Trap Column

- 5 and 10 µm packing gave same fraction volume. The only difference was the system back pressure.

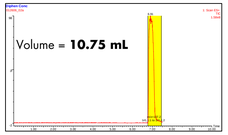

Figure 5: Concentration of the test fraction on a 19 x 50mm column.

10 x 50 5µm Trap Column

The overall flow rate was reduced when the method was transferred to the 10 mm column. By reducing the elution flow rate, from 24 to 12 mL/min, the concentrated fraction volume was reduced from 8ml to 2.9 mL.

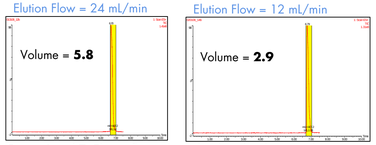

Figure 6 and 7: Elution flow adjusted reduced with minimal adjustment to the peak width.

By reducing the flow rate even further to 8 ml/min, the original 8 ml of 60% water was reduced to 1.6 mL of 100% organic solvent.

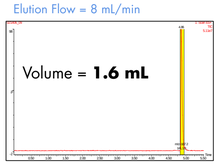

Figure 8: Concentration of the test fraction on a 10 x 50mm 5µm column at an elution flow rate of 8ml/min.

There is minimal loss of the overall speed of the analysis with the reduced elution flow rate. The loading and dilution pump operate at 24 and 4 ml/min respectively until 6.5 minute. The flow rate was then reduced to the lower elution flow, accounting for the smaller volume, concentrated fractions.

IMPROVING THROUGHPUT

Sample Loading Rules

1. The injection volume must be less than ½ the volume of the sample loop .
   - Because the injection volume was 8 mL, the minimum loop volume was found to be 15 mL.
2. 3 to 5 times the loop volume is required to clear the sample from the sample loop.
   - The minimum volume found to clear all the sample was 45 mL.

Dilution Ratio

The dilution ratio (dilution flow: loading flow) is a critical factor in this method. The dilution ratio is a measure of the amount of aqueous solvent used to dilute the fraction's organic content, to allow it to be trapped onto the column. If the dilution ratio is too small, it will cause breakthrough. If it is too large, it will decease the throughput because of the additional time required to load the sample. Figure 9 shows the effect of the concentration with varying dilution ratios. The results show that at a ratio of 4.5 there is a jagged breakthrough of the target compound that is not present at a ratio of 5 or higher.

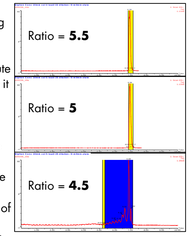

Figure 9 A-C: Concentration of test fraction with varying dilution ratios.

Scaling the Method

Based on the minimum loading time and dilution ratio, it is possible to establish the relationship between the loading time and the total flow rate (Table 1).

To reduce the loading time to under 5 minutes, the table shows that a loading and dilution flow of 10 and 50 mL/min, respectively, are required. This gives a total flow of 60 mL/min across the column.

Loading Flow (mL/min)	Loading Time (minutes)	Dilution Flow (mL/min)	Total Flow (mL/min)
5	9.0	25	30
6	7.5	30	36
7	6.43	35	42
8	5.63	40	48
9	5.0	45	54
10	4.5	50	**60**
15	3.0	75	90
20	2.25	100	120

Table1. The relationship between the loading time and the total flow.

How to handle the increased back pressure?

- Increase the particle size. A 2x increase = 1/4 the back pressure
- Waters SunFire 10 x 50 mm 10 µm, P/N 186003840
- 60 mL/min only generated 2300 psi.

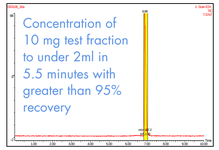

Figure 10. The results from the optimized method

MASS LOAD

One concern with these optimized parameters is the mass load on the smaller trapping column. To evaluated this, the compounds were purified with increasing mass load on the preparative column, until overload conditions were achieved. The collected fractions were concentrated using the optimized method. Two examples are shown below.

***Example 1**: 60 mg of Ketoprofen*

The initial purification generated a 10 mL fraction containing about 50% water. The concentration method successfully reduced the volume to 3.6 mL of organic solvent with a recovery greater than 95%.

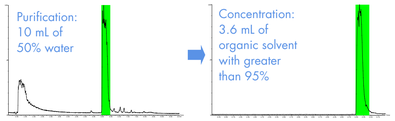

Figure 11A-B: The purification and concentration of 60 mg of ketoprofen.

Example 2: 20 mg Phenyl-tetrazole

The purification generated 2 fractions with a total volume of 18 mL containing about 60% water. The concentration successfully reduced the volume to 3.2 mL of organic solvent with the recovery greater than 95%.

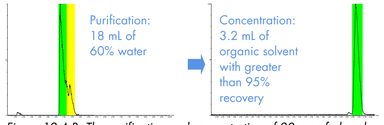

Figure 12 A-B: The purification and concentration of 20 mg of phenyl-tetrazole.

Rule: When the chromatography begins to overload for the purification on a 19 x 50 mm, the fraction will not be completely trapped on the 10 x 50 mm column.

AUTOMATIC POOLING

Fraction pooling on the trapping column can also increase throughput. A 3 mL fraction was collected for each of the 10 injections. The ten 3mL fractions were then individually loaded onto the trap column and then concentrated. A single 1.5 mL 100% organic fraction was collected.

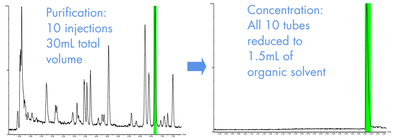

Figure 13: An example of automatic pooling of 10 fraction tubes into a single concentrated fraction

CONSIDERATIONS

- The pKa of the target compound should be considered when performing the purification. The target compound should be neutral during the purification. This means that a basic compound should be run in a basic mobile phase and conversely an acidic target should be run in an acidic mobile phase. This will result in better loading and chromatography [1], and also ensure that the collected fraction is not ionized in solution. By being neutral it is more likely to be successfully trapped during the concentration process.
- The amount of collected material, in both mass and volume, will dictate the required system configuration. The volume of the fraction will determine the size loop required. The mass of collected material will determine the column size. Both the loop and column size will determine the overall throughput of the system.
- In the examples shown, all of the concentrated fractions were triggered by MS. However, this was done only for method development purposes. It is possible to collect these fractions by UV or even just by time. When collecting by time, each tube has the same volume and organic concentration, so the time required for drying is constant. With typical fractionation, each tube can have a different volume and organic concentration, so the time required for drying is variable. This variability can lead to inefficiency, by either needlessly drying for too long, or by stopping too early then checking multiple tubes, to find that you need to restart for only a few of the tubes.

BENEFITS

Dry down Time

Composition	Volume	Dry Down
Aqueous/ Organic	5-30 mL	5—15 hours
100% organic	1– 3 mL	**< 30 min**

- Concentrating the fraction to about 3 mL of organic solvent can be accomplished in 6 minutes.

Process Enhancements

- Shorter drying times equals more efficient use of the driers.
- Automatic pooling of multiple fraction tubes reduces the post-purification sample handling

ACKNOWLEDGEMENTS

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