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Analytical and Measuring Instruments

Key Parameter Concepts

Part 2 - How Does System Flow Rate Affect the Chromatography of a Fermentation Sample?

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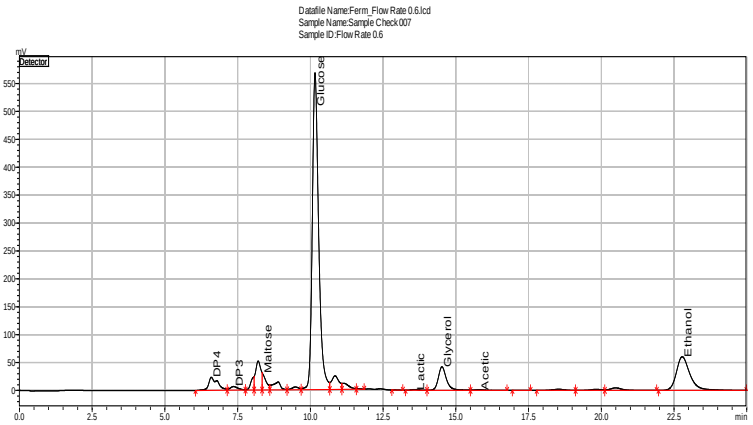
■ Introduction

During the performance of fermentation monitoring by high performance liquid chromatography (HPLC), bioethanol plant laboratories use methods that may have been set up by instrument manufacturers or installers, based on a set of operational parameters that "seem appropriate". How do these labs know if the method parameters they are using are truly the best parameters for securing the most reliable data from their analyses? Certainly, many of the parameters are appropriate based on the recommendations from the column manufacturer, but what does that mean in the actual evaluation of the results?



Part 1 of this series ("Effect of Column Temperature on Bioethanol High Performance Liquid Chromatography") showed that the best overall compromise for the column temperature was approximately 70°C. Part 2 looks at the effect, if any, on the chromatography when different pump flow rates are used. Is there any benefit to a reduced mobile phase flow rate? How about an increased flow rate?

For this analysis, the new Shimadzu i-Series Bioethanol Analyzer HPLC was used. This is a compact HPLC system with a high-performance autosampler and built-in column heater. Peak detection was performed with the new RID-20A Refractive Index Detector and data processing was accomplished by Lab Solutions LC software (Ver. 5.87). The column was a Phenomenex Rezex ROA (7.8 x 300 mm) with Security Guard cartridge to protect the analytical column from unwanted cationic intrusion. The manufacturer's recommended conditions for this column are a 0.600 mL/min flow rate, a maximum operating pressure of 1000 psi, and a maximum column temperature of 80°C. For this study, the column temperature was set to 70°C with an injected sample size of 5 µL. The detector flow cell was maintained at 40°C and data was collected at 5 Hz. The mobile phase used was 0.005 N sulfuric acid from Chata Biosystems.

The pump flow rate was tested from 0.400 mL/min to 1.000 mL/min, in 0.100 mL/min increments. Injections of a standard were made at each flow rate, followed by a single injection of a fermentation sample before changing to the next flow rate.

■ System Pressure

The backpressure measured at the pump is a reflection of the amount of resistance to flow produced by the entire flow path. Pressure is always the highest at the most upstream point: the pump. As the mobile phase passes through more and more of the flow path, the effective pressure is reduced. In most HPLC systems, the column represents the largest contributor to the pump backpressure reading.

With all other components remaining constant (tubing, connections, column, down-stream components), the expectation should be that the system pressure will increase proportionally to the mobile phase flow rate being delivered. As a result of the different flow rates used in this study, the following pressures were measured at the pump's pressure transducer.

Flow Rate (mL/min)	**0.400**	**0.500**	**0.600**	**0.700**	**0.800**	**0.900**	**1.000**
System Pressure (psi)	241	294	350	405	461	516	572

Table 1: System Backpressure vs. Pump Flow Rate.

There is always a fear that having too much pressure could damage the column, so many bioethanol labs have adopted the practice of setting a maximum allowable system pressure of 600 psi. While this is an admirable goal

for column longevity, it is understating the capabilities of the column somewhat. Several years ago, the column manufacturer (Phenomenex) changed their manufacturing procedure for the column packing. This was to give a slightly better resolution for the peaks early in the chromatogram, but it also had the side benefit of better pressure resistance. The Phenomenex website now states that the maximum operating pressure is 1000 psi.

Always follow your column manufacturer's recommendations for operational parameters to avoid column damage.

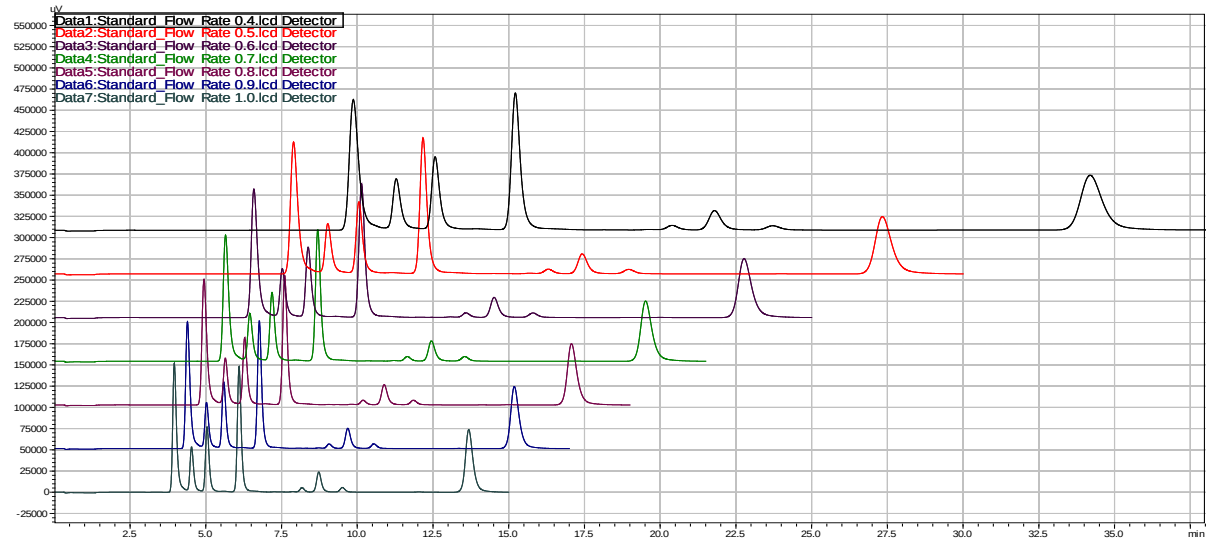
■ Key Parameters

To assess the chromatographic effect of the different flow rates on the separation of the standard components being monitored during this analysis, there are certain observable parameters that can be used for comparison purposes. The parameters that have been selected are: **Retention Time, USP Width, Area/Height Ratio, Tailing, Theoretical Plate Number, Resolution, and Signal/Noise Ratio**. Please see the description in Part 1 of this series for more details on these performance parameters. In addition to these parameters, **Peak Area and Peak Height** will be observed.

■ The Effect of Pump Flow Rate on a Standard Sample

Retention Time

The overlay below is of the same standard sample injected at seven different pump flow rates, ranging from 0.400 – 1.000 mL/min.



Fermentation Calibration Standard Mix at 7 Different Pump Flow Rates.

In nearly every case where the same separation mechanisms are being used, the elution of a substance from the HPLC column is based on the volume of mobile phase delivered. In a "standard" chromatogram, where the run time is 25 minutes and the flow rate is 0.600 mL/min, the entire elution of peaks must be accomplished in 15 mL of mobile phase delivered (25 min x 0.6 mL/min). As the flow rate changes, the retention time for a specific peak may change, but not its elution volume.

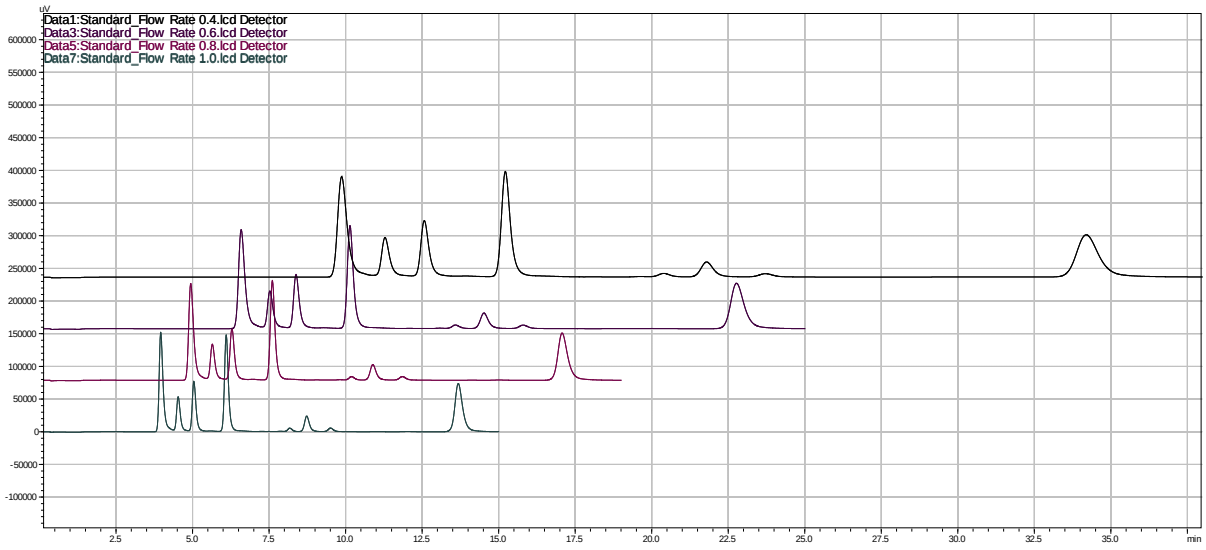
Using the peak apex retention time for the ethanol peak in the standard chromatograms shown above, it is clear that the absolute elution volume (flow rate x retention time) is essentially unchanged for all seven sets of data. It is clear that increasing the flow rate "compresses" the chromatography into a shorter time frame, but the same amount of mobile phase will be used for each analysis.

Flow Rate (mL/min)	Ethanol RT (min.)	Ethanol Elution Volume (mL)
0.4	34.21	13.684
0.5	27.345	13.673
0.6	22.782	13.669
0.7	19.527	13.669
0.8	17.079	13.663
0.9	15.195	13.676
1	13.686	13.686
		13.674
		0.008
		0.061%

Average
Std. Dev.
%RSD

Table 2: The Relationship between Ethanol Retention Time, Elution Volume, and the Delivered Flow Rate.

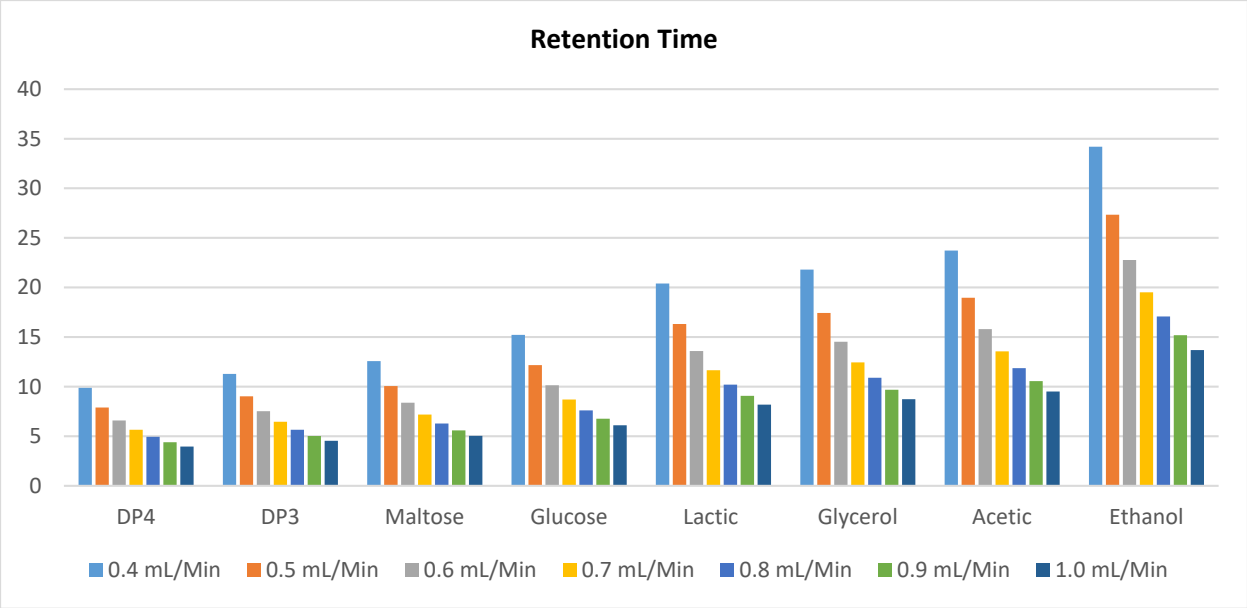
By removing some of the data files so that the differences in peak dimensions and placement can be seen more easily, it is obvious that the peak sizes are becoming smaller, but not necessarily shorter. The biggest difference, other than the dramatic change in retention time, is that the peaks become steadily narrower with an increasing flow rate.



Fermentation Calibration Standard Mix at 0.4, 0.6, 0.8, and 1.0 mL/min Pump Flow Rates.

	Peak Retention Time						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	9.871	7.90	6.589	5.653	4.941	4.395	3.961
DP3	11.288	9.03	7.527	6.455	5.646	5.025	4.531
Maltose	12.572	10.06	8.381	7.187	6.286	5.594	5.043
Glucose	15.219	12.17	10.143	8.697	7.607	6.768	6.1
Lactic	20.396	16.31	13.59	11.654	10.196	9.072	8.177
Glycerol	21.796	17.43	14.52	12.449	10.89	9.688	8.73
Acetic	23.718	18.97	15.802	13.55	11.856	10.55	9.51
Ethanol	34.196	27.34	22.771	19.519	17.073	15.186	13.681

Table 3: The Relationship Between Peak Retention Time and the Delivered Flow Rate for an 8 Component Standard.



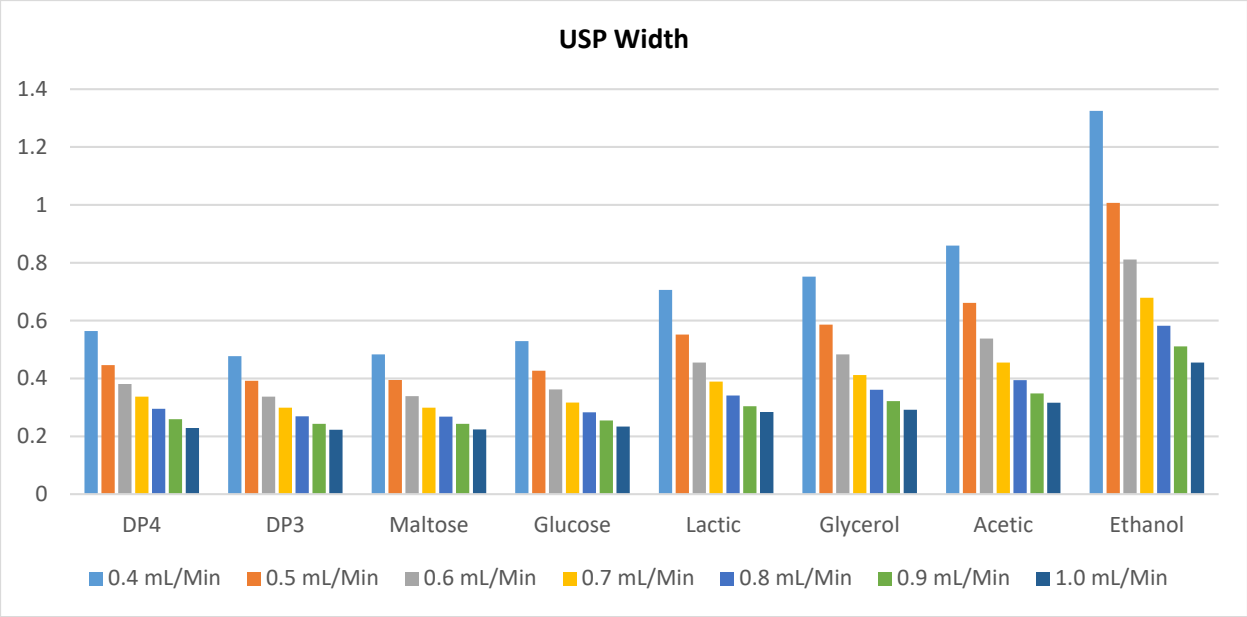
Plot of the Retention Time vs. Delivered Flow Rate

USP Width

It is easily observed from the chromatograms that increasing the flow rate reduces the width for all peaks. This is due to the higher flow rate decreasing the residency time of the sample through the detector flow cell. Simply put, the higher flow rate clears the peak through the detector flow cell in less time, so the data system has less time to detect the peak. This does, however, place a certain amount of importance on the rate of data acquisition, as narrower peaks can require a higher rate (data points per second, Hz) to adequately measure the peak size. Because all other analysis parameters are being held constant, the effect on the USP Width is consistent for all peaks; they all have narrower widths as the flow rate increases. Therefore, the relationship between retention time and USP Width are similar.

	USP Width						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	0.564	0.45	0.381	0.337	0.295	0.259	0.229
DP3	0.477	0.39	0.337	0.299	0.269	0.243	0.223
Maltose	0.483	0.40	0.339	0.299	0.268	0.243	0.224
Glucose	0.529	0.43	0.362	0.317	0.283	0.255	0.234
Lactic	0.706	0.55	0.455	0.389	0.341	0.304	0.284
Glycerol	0.752	0.59	0.483	0.412	0.361	0.322	0.292
Acetic	0.859	0.66	0.538	0.455	0.394	0.348	0.316
Ethanol	1.325	1.01	0.811	0.679	0.582	0.511	0.455

Table 4: The Relationship between Peak USP Width and Delivered Pump Flow Rate.



Plot of USP Peak Width for Fermentation Calibration Standard Peaks at 7 Different Flow Rates.

Area/Height Ratio

It makes sense that a narrower peak can have less peak area that a wide peak. This is a concept of residency time in the detector flow cell. All the time that a peak, or portions of it, spend in the flow cell, the data system is adding up area for that peak. Peak area is typically stated as a number, but it is an obvious expression of peak intensity times time. In the most general of terms, this would be µV x Seconds.

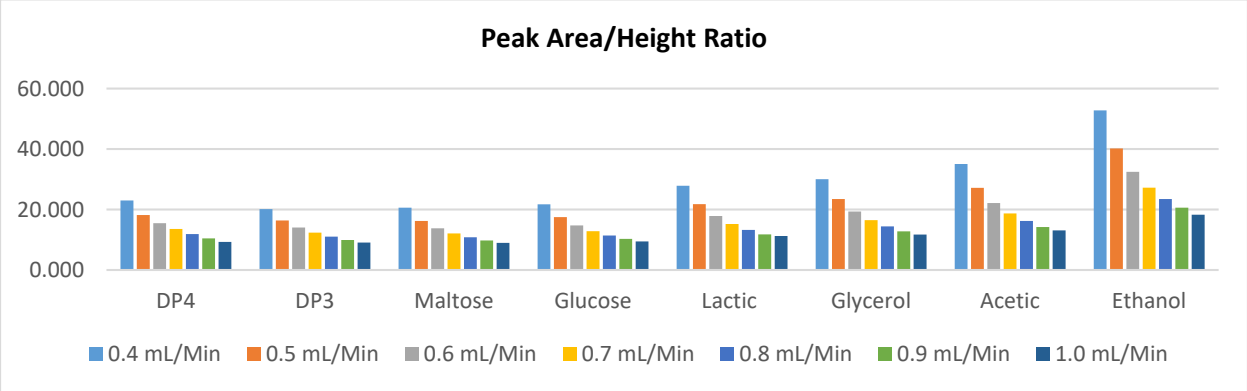
The concern will be if the reducing peak area will make it more difficult to detect very small quantities of each analyte. This will remain to be seen.

The observed chromatograms make it easy to see that the peak areas will be reduced in a similar manner to the peak width. Yet, while the peak areas are becoming smaller, the effect on the peak height is not as obvious. The peak height does change, but much more subtly than the peak area. Some peaks have a relatively consistent peak height, while some have slightly decreasing heights and others slightly increasing heights.

	Peak Area						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	3539728	2824343	2348788	2025170	1763599	1569674	1411832
DP3	1219652	970931	810227	697585	608139	539341	485682
Maltose	1782074	1375188	1145845	983589	859565	765363	692077
Glucose	3509880	2799484	2327237	1992795	1740294	1547013	1406189
Lactic	150288	118770	96859	81624	70735	62331	60939
Glycerol	688532	551133	458442	392764	343364	305137	280449
Acetic	178680	143616	119327	102085	89237	78331	73184
Ethanol	3406370	2713034	2256549	1936097	1695094	1508637	1351624
	Peak Height						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	153697	155333	151593	148685	147950	149563	152082
DP3	60437	59188	57655	56344	55080	54309	53507
Maltose	86341	84764	82856	81029	79317	78258	77102
Glucose	161453	160097	157552	154787	152154	150317	148266
Lactic	5395	5445	5411	5365	5321	5281	5415
Glycerol	22931	23474	23695	23777	23791	23794	23836
Acetic	5095	5283	5388	5456	5495	5513	5586
Ethanol	64522	67498	69520	71010	72187	73162	73830

	Area/Height Ratio						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	23.031	18.182	15.494	13.620	11.920	10.495	9.283
DP3	20.180	16.404	14.053	12.381	11.041	9.931	9.077
Maltose	20.640	16.224	13.829	12.139	10.837	9.780	8.976
Glucose	21.739	17.486	14.771	12.874	11.438	10.292	9.484
Lactic	27.858	21.813	17.900	15.214	13.293	11.803	11.254
Glycerol	30.026	23.478	19.347	16.519	14.433	12.824	11.766
Acetic	35.068	27.183	22.145	18.710	16.239	14.207	13.101
Ethanol	52.794	40.195	32.459	27.265	23.482	20.621	18.307

Table 5: The Relationships of Peak Area, Peak Height, and A/H Ratio with Delivered Flow Rate.



Plot of Area/Height Ratio for Fermentation Calibration Standard Peaks at 7 Different Pump Flow Rates.

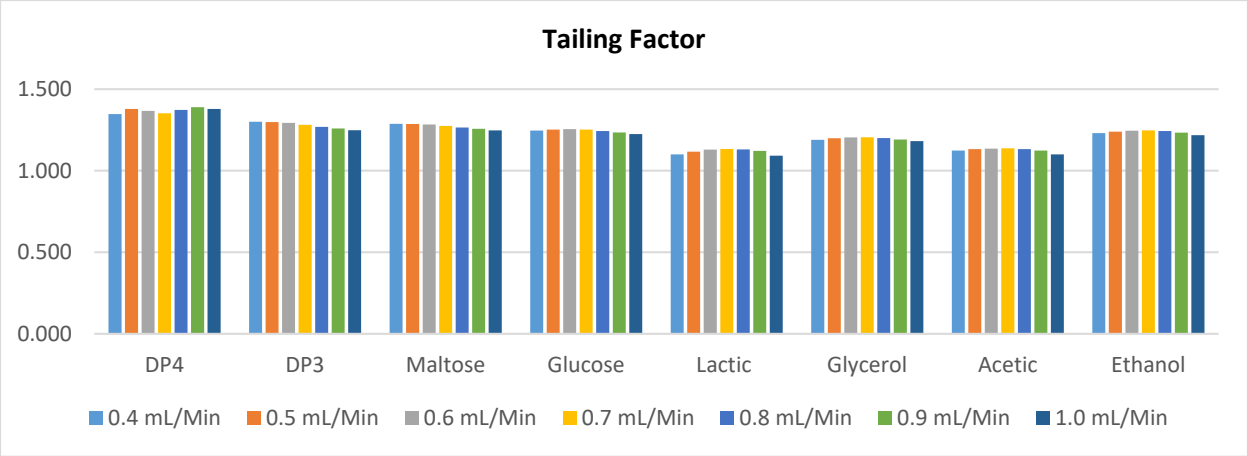
Tailing Factor

The amount of tailing of a peak is presented in the Tailing Factor. This, in simple terms, compares the amount of peak time after the apex time to the amount of peak time ahead of the apex time. A perfectly symmetrical peak would have a tailing factor of 1.000. To try to eliminate the effect of baseline drift and noise, this measurement is made at 10% of the peak height.

The results from the seven different flow rates show slight variations, but nothing highly significant. This would lead to the conclusion that the changing flow rate is having very little influence on the tailing found in these peaks.

	Tailing Factor						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	1.347	1.379	1.367	1.352	1.373	1.389	1.379
DP3	1.300	1.298	1.293	1.281	1.269	1.259	1.248
Maltose	1.287	1.286	1.283	1.275	1.265	1.257	1.247
Glucose	1.246	1.252	1.255	1.252	1.243	1.234	1.225
Lactic	1.100	1.117	1.129	1.133	1.130	1.122	1.092
Glycerol	1.189	1.199	1.204	1.205	1.200	1.191	1.181
Acetic	1.123	1.132	1.135	1.137	1.132	1.123	1.100
Ethanol	1.230	1.239	1.245	1.247	1.243	1.233	1.218

Table 6: The Relationship between Tailing Factor and Delivered Flow Rate.



Plot of Tailing Factor for Fermentation Calibration Standard Peaks at 7 Different Pump Flow Rates.

Theoretical Plate Number

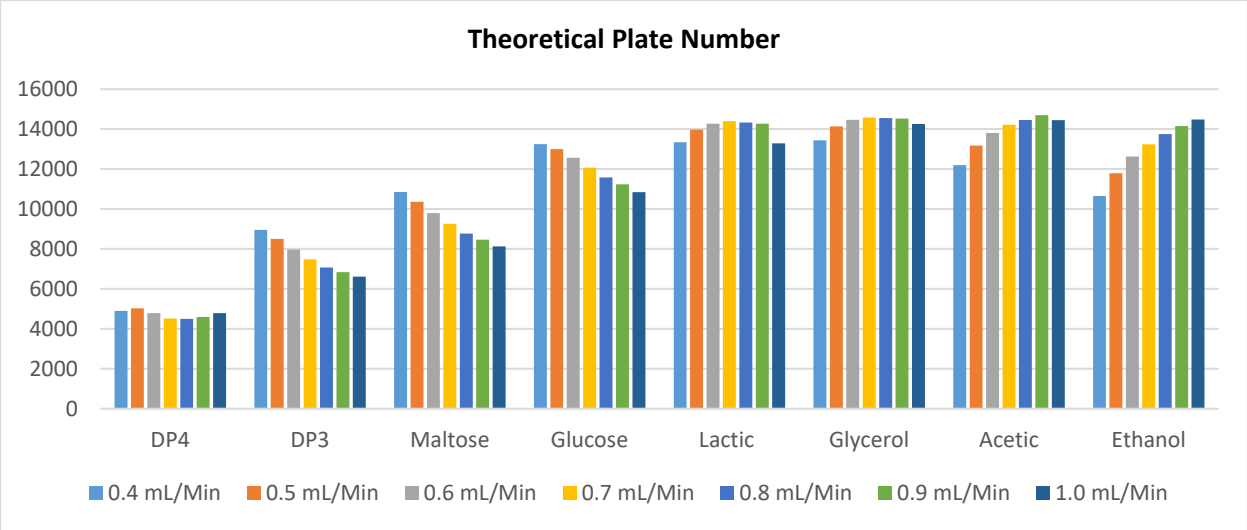
The Theoretical Plate Number (NTP, see Part 1 of this series) is a combination of peak retention time and peak width. This parameter is often considered to be an indication of how actively each analyte interacts with the column. To put it in very basic terms, the theoretical plate number is a measurement of the quality of interaction of an analyte with the column, while also factoring in the tendency of peaks to experience diffusion as they pass through the column. In general, the higher the number, the better the analyte interacts with the column. With all other factors being equal, peaks eluting late in the chromatogram will have a higher NTP than peaks eluting early in the chromatogram.

While the entire sample flow path affects this value, it is the column that produces the vast majority of any interaction. The ion exclusion chromatography being used for this separation is a mixed bag of separation mechanisms, using components of size exclusion, ionic interaction, and hydrophobic interaction. Each of these separation mechanism components has a different kinetic rate of interaction, so the change in flow rate may show how each kinetic interaction is being affected.

The table below shows the maximum NTP values (using the USP calculation method) for each analyte over the range of tested flow rates. The pure carbohydrates (DP4+, but especially DP3, Maltose, and Glucose) seem to prefer the slower flow rates, while the smaller molecules, including the organic acids, tend to prefer the slightly higher flow rates. Ethanol shows a significant increase in NTP as the flow rate increases.

	Theoretical Plate Number (USP Method)						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4+	4901	5027	4784	4514	4497	4592	4787
DP3	8952	8505	7964	7480	7073	6839	6611
Maltose	10851	10359	9791	9256	8772	8465	8131
Glucose	13249	12999	12568	12071	11577	11232	10840
Lactic	13345	13973	14271	14396	14329	14269	13286
Glycerol	13440	14134	14467	14577	14549	14526	14258
Acetic	12195	13172	13807	14214	14459	14693	14447
Ethanol	10652	11791	12627	13239	13747	14147	14478

Table 7: The Relationship between Theoretical Plate Number and Delivered Flow Rate.



Plot of Theoretical Plate Number for Fermentation Calibration Standard Peaks at 7 Different Pump Flow Rates.

Size exclusion is a separation mechanism that sorts molecules solely on their relative size. The column packing particles have small pores that some molecules cannot fit into. These "size excluded" molecules have, therefore, a shorter flow path than molecules that can meander into and out of the pores. In this way, a totally excluded molecule will be larger in size (like a starch molecule) and will elute early in the chromatogram. This helps to explain why the DP4+ peak is the first peak out of the column.

Smaller molecules have a longer flow path due to the migration into the column bed pores, so they will be further separated by size. The smallest molecules can reach further into the pores than molecules that barely fit into the pores. Add to this the ion exclusion mechanism, where carbohydrates actually interact with the hydrogen ion clusters resident in the column due the sulfonic acid groups of the column packing and the sulfuric acid mobile phase, and further separation is attained by more selective interaction with the carbohydrates and alcohols. The organic acids, lactic acid and acetic acid, should exist primarily in their protonated form at the pH of the mobile phase. But, this still allows interaction of these ionizable compounds with the hydrogen ion clusters. This provides an additional ion exchange quality to the retention of these molecules.

Ethanol is subject to the ion exclusion separation mechanism, but it can also experience a hydrophobic interaction separation mechanism with the polymer backbone of the column packing. This causes alcohols that have increasing hydrocarbon content (more methylene groups), relative to the hydroxyl content, to be retained longer. This helps to explain why the fusel alcohols are so well retained in this separation system, as they have more C-H content than ethanol.

Because ethanol can stay on the column for a very long time, it is more susceptible to simple diffusion while it migrates through the column, leading to "band spreading" or a wider peak. Using an increased flow rate reduces the residency time of all peaks, thereby reducing the time for diffusion to take place. Ethanol seems to very prominently show this effect.

In looking at the plot above, some general conclusions may be considered. First of all, the NTP for DP4+ is relatively constant. This may suggest that the separation of DP4+, mostly due to the size exclusion mechanism, may not be particularly affected by changes in flow rate, at least over the range tested.

The DP3, Maltose, and Glucose results all show the same general effect. The NTP decreases as flow rate increases. This would tend to indicate that the partial size exclusion effect and the ion exclusion effect are very slow, kinetically, and increasing the flow rate is detrimental to achieving high theoretical plate numbers.

The next three peaks, lactic acid, glycerol, and acetic acid, seem to hit a plateau or peak in the 0.6 – 0.9 mL/min range. This can be useful information for trying to optimize this area of the chromatogram.

Over the flow rate range tested, the ethanol peak steadily increases the NTP value. This indicates that the optimum flow rate for theoretical plate generation has not yet been attained, even at 1.000 mL/min.

Resolution

For many chromatographers, improved peak resolution is one of the primary goals when developing separation methodology. The resolution calculation combines factors of retentivity, selectivity, and theoretical plates. Retentivity is a measure of how well an analyte is retained by the column. Selectivity is the ratio of the retentivity of adjacent analytes in a chromatographic system.

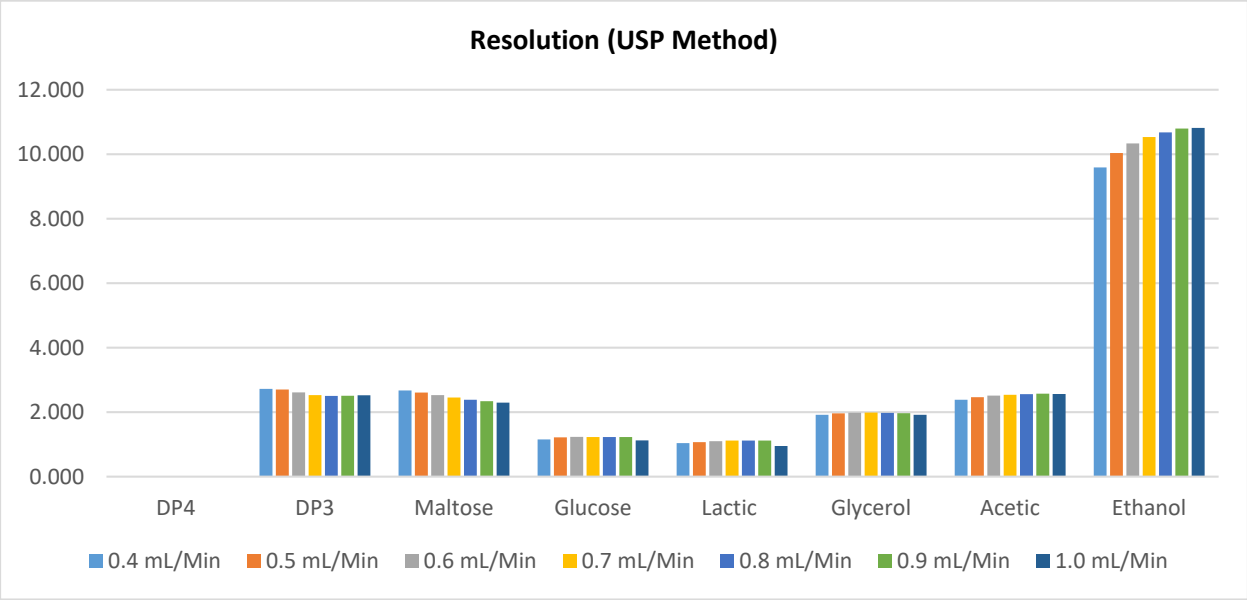
In a chromatographic separation, an analyte either exists adsorbed to the stationary phase (column) or is in the mobile phase. While in the stationary phase, the analyte is not moving toward the detector. While in the mobile phase, the analyte is moving toward the detector at the same rate as the mobile phase. Separation only occurs when there is a partitioning of the analyte between the mobile phase and the stationary phase. It spends a certain amount of time in the mobile phase and a certain amount of time adsorbed to the stationary phase. The more time the analyte spends in the mobile phase than adsorbed to the stationary phase, the earlier it will elute from the column.

When only the mobile phase flow rate is changed, all other factors remaining constant, the retentivity of the analytes does not change. Therefore, the selectivity does not change either, unless the flow rate can elicit a new interaction that favors one analyte's retention over another. In the case of the standards used in this study, there is no effect.

By definition, resolution is measured as the separation of a peak from the peak ahead of it, and the first peak cannot have a value. In terms of a general goal, **a resolution of 1.5 or higher is considered "full separation".**

	Resolution (USP Method)						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4	--	--	--	--	--	--	--
DP3	2.722	2.701	2.610	2.526	2.505	2.508	2.523
Maltose	2.674	2.609	2.527	2.451	2.384	2.341	2.295
Glucose	1.153	1.219	1.234	1.227	1.228	1.225	1.123
Lactic	1.038	1.070	1.099	1.119	1.119	1.118	0.947
Glycerol	1.919	1.964	1.982	1.986	1.979	1.969	1.919
Acetic	2.386	2.464	2.512	2.540	2.558	2.575	2.561
Ethanol	9.594	10.039	10.337	10.535	10.681	10.796	10.816

Table 8: The Relationship between Chromatographic Resolution and Delivered Flow Rate.



Plot of Resolution Value for Fermentation Calibration Standard Peaks at 7 Different Pump Flow Rates.

An examination of the plot of resolutions values versus flow rate shows interesting results. Clearly, some of the analytes, most noticeably maltose, see a modest loss of resolution as the flow rate increases. Most do not have a significant change in resolution. Ethanol, on the other hand, shows a steady increase in resolution.
Based on the performance of the standard sample analytes, there does not seem to be much of an argument to prohibit using a higher flow rate to attain nearly the same separation quality.

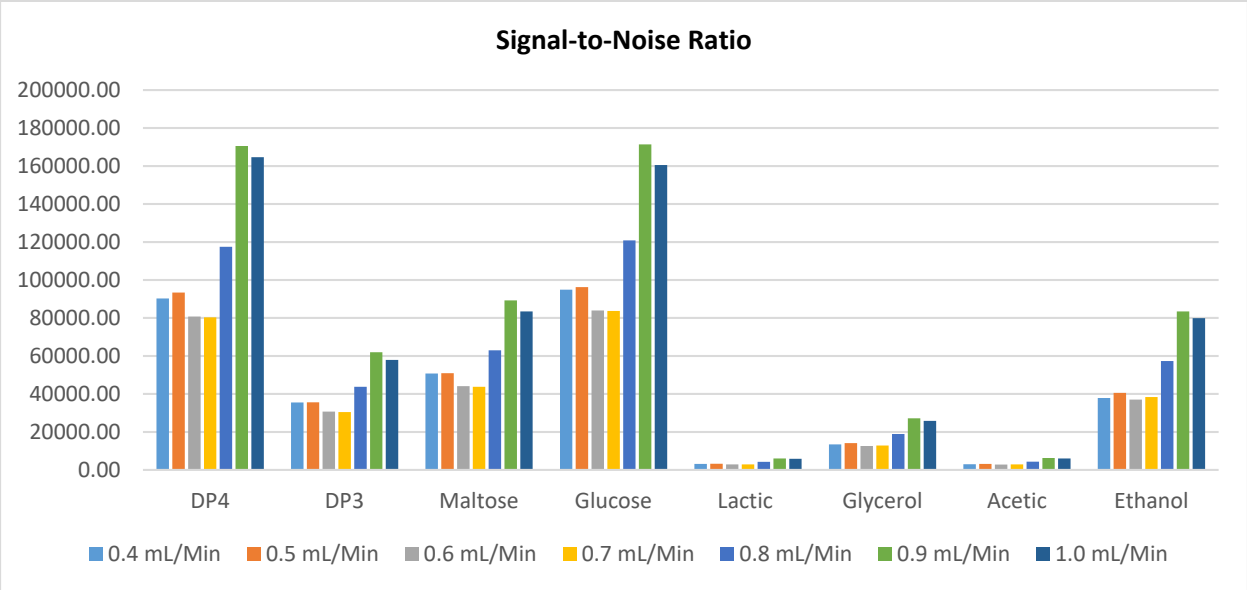
Signal-to-Noise Ratio

Where peak resolution will help to describe the quality of chromatographic separation, the signal-to-noise (S/N) ratio will describe the quantitative capability of the separation. The higher the S/N ratio, the lower the concentration of an analyte that can be detected and/or quantitated. Effectively, S/N ratio is a good estimate for sensitivity.

For this measurement, the signal intensity (net peak height) is compared as a ratio to the average noise taken in the time segment of the chromatogram immediately prior to the elution of the DP4+ peak. This noise segment is before any peaks should elute from the standard sample. However, with a changing flow rate that changes the retention time of the DP4+ peak, the noise segment is a different time range in each chromatogram. The ASTM noise algorithm was used to calculate the average noise from 10 subsections of the assigned time range.

	Signal - to - Noise Ratio						
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**
DP4	90311.90	93377.45	80740.55	80371.62	117493.19	170570.36	164682.44
DP3	35512.69	35580.25	30707.80	30456.54	43741.45	61937.25	57940.38
Maltose	50733.53	50955.53	44130.40	43800.00	62988.85	89250.59	83490.14
Glucose	94869.18	96240.84	83914.12	83669.91	120831.34	171430.59	160550.26
Lactic	3169.93	3273.16	2882.12	2900.02	4225.79	6022.64	5863.54
Glycerol	13474.38	14111.41	12620.54	12852.69	18893.09	27135.96	25810.88
Acetic	2993.95	3176.07	2869.91	2949.30	4363.94	6287.76	6049.13
Ethanol	37913.12	40575.67	37027.29	38384.39	57326.76	83438.10	79947.37
ASTM Noise	1.70	1.66	1.88	1.85	1.26	0.88	0.92

Table 9: The Relationship between Signal-to-Noise Ratio and Delivered Flow Rate.



Plot of Signal-to-Noise Ratio for Fermentation Calibration Standard Peaks at 7 Pump Flow Rates.

The S/N ratio is an effective measure of quantitative capability, but it is highly dependent on getting a reliable average noise value. As can be seen from Table 8, the noise, in general, decreases with an increasing pump flow rate. This can be a case where the higher pump speed smooths out any contribution from pump pulsation. If so, it seems that the general trend is that the S/N ratio improves with an increasing flow rate.

■ Conclusions from Standard Chromatograms

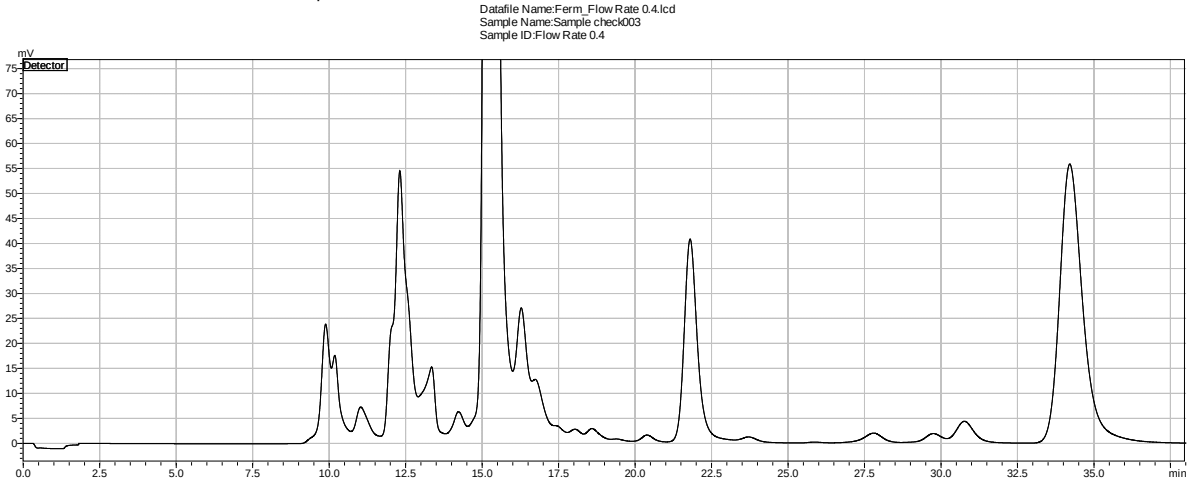
Several of the key parameter comparisons have predictable results. Increasing the flow rate reduces the retention time and peak width. But, these are largely inconclusive when it comes to deciding if the chromatography is being positively or negatively affected by the change in flow rate. Perhaps the most interesting parameter is the Theoretical Plate Number, as it seems that there is more of a positive effect of higher flow rates for analyte peaks that come out later. This is very likely due to a reduction in the peak diffusion that takes place when peaks are slow moving.

When the wider scope parameters, Resolution and Signal-to-Noise Ratio, are considered, some interesting concepts begin to form. First of all, there seems to be very little, if any, negative consequence from running the standards at higher flow rates. In fact, the resolution for ethanol seems to continue to improve with an increasing flow rate. Secondly, though the peak areas reduce with higher flow rates due to peak width reduction, the signal-to-noise ratio trends higher at higher flow rates. This is primarily due to a slight reduction in pump pulsation noise in the baseline.

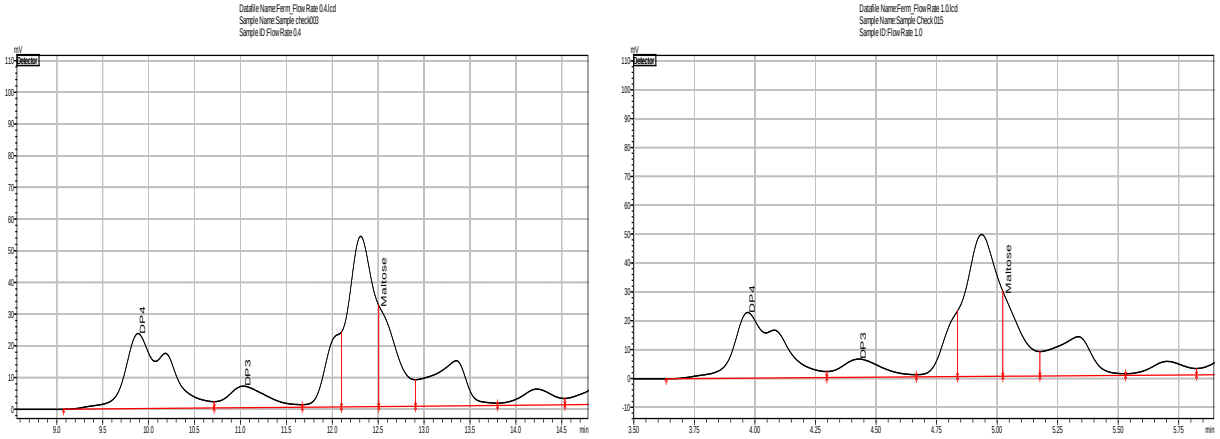
Finally, based on the chromatograms of standards only, there seems to be very little difference in resolution or sensitivity when the analysis is done at any flow rate from 0.4 – 1.0 mL/min. The biggest advantage, operationally, is that higher flow rates lead to shortened analysis times.

■ The Effect of Temperature on a Fermentation Sample

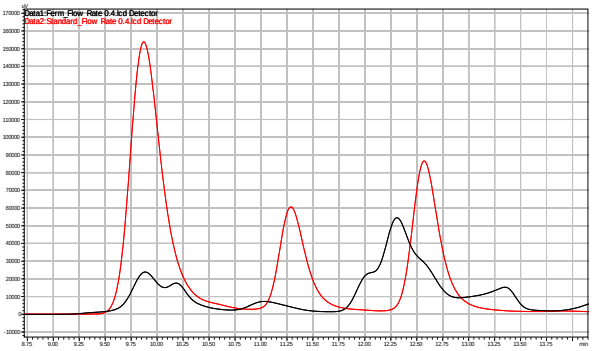
All the previous data was performed by running the same standard sample at different temperatures. Turning to a much more complex chromatogram, a well-aged fermentation sample, it should be possible if the same concepts hold true. In this sample, a fermentation sample was aged by maintaining at room temperature to allow residual enzymatic activity to very nearly completely convert the starches and polysaccharides into glucose. Therefore, the glucose peak is very large and all other peaks are quite small. This places the secondary metabolites in the same approximate size as the maltose peak.



Aged Fermentation Sample Chromatogram, Analyzed at 0.4 mL/min Pump Flow Rate



Fermentation Sample Analyzed at 0.4 mL/min (Left) and 1.0 mL/min (Right)



Comparison: DP4+ to Maltose, Standard versus Unknown

It is worth noting that the analytical method is very repeatable. Excluding the effect of small, late-eluting peaks, the retention times for all peaks is very repeatable (see Part 1). Therefore, it can be critical to the analysis of results to recognize that the identity of peaks is based on correlation of the retention time of the standard material to the corresponding peak in any fermentation samples. If the retention time seems to have shifted, it is likely that the wrong peak is being identified.

The comparison of the maltose area at 0.4 and 1.0 mL/min shows that the chromatography is nearly identical. If anything can be said as a detraction from running at the higher flow rate, it would be that the perceived "shoulder" for maltose is harder to discern at 1.0 mL/min. The shoulders are easier to find at a slower flow rate, but manual processing may still be needed to split the correct peak away from its neighbor.

This begs the question: is it worth the additional analysis time to get a slightly improved indication of the maltose shoulder?

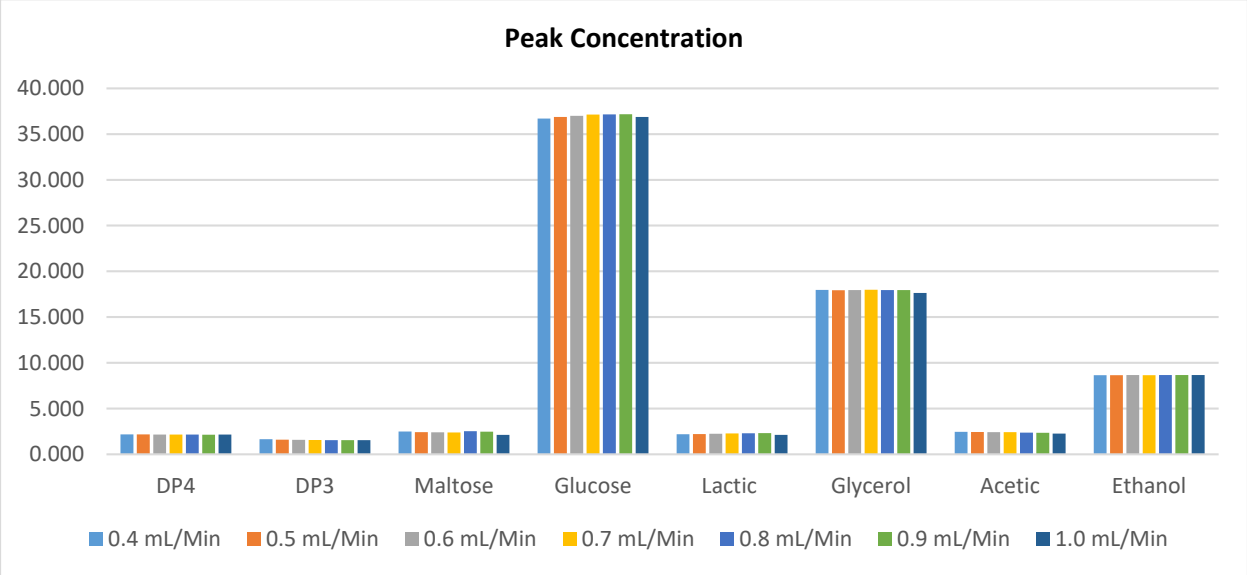
Quantitative Testing

To determine if flow rate has much effect on quantitative results, a standard was used to generate a single-point calibration of each method. Strictly for comparison purposes, the concentration of each of the eight analytes in the standard was set to 10 for the calibration. This would allow for a relative concentration value to be determined for those analytes in the aged fermentation sample. The intent was to see if changing the flow rate resulted in a significant change in the calculated concentration value for each of the analytes of interest.

The raw data was processed to have a nearly identical integration pattern for all chromatograms. In the case of the maltose peak, manual processing was employed to get the best estimate of concentration.

	Aged Fermentation Sample Peak Concentration							
	0.4 mL/Min	**0.5 mL/Min**	**0.6 mL/Min**	**0.7 mL/Min**	**0.8 mL/Min**	**0.9 mL/Min**	**1.0 mL/Min**	**Rel. Calib. Conc.**
DP4	2.177	2.176 (-0.046%)	2.169 (-0.368%)	2.154 (-1.060%)	2.158 (-0.882%)	2.146 (-1.437%)	2.159 (-0.839%)	10
DP3	1.659	1.596 (-3.797%)	1.577 (-5.138%)	1.563 (-6.088%)	1.557 (-6.526%)	1.543 (-7.450%)	1.556 (-6.675%)	10
Maltose	2.494	2.428 (-2.646%)	2.401 (-3.830%)	2.391 (-4.290%)	2.532 (+1.589%)	2.484 (-0.395%)	2.125 (-14.855%)	10
Glucose	36.695	36.873 (+0.485%)	37.004 (+0.838%)	37.130 (+1.176%)	37.161 (+1.255%)	37.171 (+1.281%)	36.874 (+0.482%)	10
Lactic	2.199	2.217 (+0.819%)	2.253 (+2.436%)	2.284 (+3.773%)	2.301 (+4.446%)	2.313 (+4.954%)	2.133 (-2.853%)	10
Glycerol	17.963	17.939 (-0.134%)	17.956 (-0.039%)	17.983 (+0.111%)	17.954 (-0.050%)	17.950 (-0.072%)	17.634 (-1.833%)	10
Acetic	2.454	2.439 (-0.611%)	2.430 (-0.984%)	2.426 (-1.152%)	2.379 (-3.092%)	2.347 (-4.498%)	2.260 (-8.266%)	10
Ethanol	8.658	8.653 (-0.058%)	8.659 (+0.012%)	8.654 (-0.046%)	8.666 (+0.092%)	8.671 (+0.150%)	8.670 (+0.138%)	10

Table 10: Calculated Peak Amounts for an Aged Fermentation Sample at Different Delivered Flow Rates
(% Difference from the Value at 0.4 mL/min)



Plot of Concentration Values for an Aged Fermentation Sample at 7 Pump Flow Rates.

The results of this experiment show that there are some differences in the calculated concentration of analyte peaks over the range of flow rates tested. Very close inspection shows that the more significant differences occur in the maltose-to-acetic acid elution range. There seems to be some trending of concentrations, but the differences are small, relative to their actual concentration. The exception may be at 1.0 mL/min, where it seems that there is a larger drop in some of the concentrations for some of the analytes.

If the results for 1.0 mL/min are ignored, the actual averages for the analytes would be as seen in the table below. This shows that the quantitative result variation is quite narrow over flow rates of 0.4 – 0.9 mL/min.

	Concentration Average	Standard Deviation	%RSD
DP4	2.163	0.013	0.58%
DP3	1.583	0.042	2.63%
Maltose	2.455	0.057	2.31%
Glucose	37.006	0.190	0.51%
Lactic	2.261	0.046	2.04%
Glycerol	17.958	0.015	0.08%
Acetic	2.413	0.041	1.69%
Ethanol	8.660	0.007	0.08%

Table 11: Average Concentration, Standard Deviation, and %RSD of Peak Values for 0.4 – 0.9 mL/min Flow Rate

■ Conclusions from Fermentation Sample Chromatograms

From examination of the experimental results, it appears that the delivered pump flow rate has very little effect on the elution of the many peaks that can be present in a fermentation sample. With a standard method flow rate of 0.6 mL/min, it is very doubtful that anyone would desire to perform this analysis methodology at a lower flow rate. It seems entirely feasible that use of a faster flow rate does not have much effect on the quantitative accuracy of the analysis.

In most forms of chromatography, there is a flow rate range that represents a "sweet spot" for analysis. Operating below that range will typically not improve the chromatography, but will unnecessarily extend the time needed to complete an analysis. Performing analysis above the desired range can start to introduce effects that have some negative quality. In some cases, this is caused by flowing too fast for the kinetic interactions between analytes and the stationary phase (column) to be as effective. This can result in loss of theoretical plates. In some cases, the faster flow may be introducing a new type of interaction that changes the separation profile.

A summary of the individual test parameters showing the effect of increasing the flow rate from 0.4 – 1.0 mL/min is as follows:

System Pressure	Proportionally More
Retention Time	Proportionally Less
Peak Width	Proportionally Less
Peak Height	Minor Affect
Tailing Factor	Mostly Unaffected
NTP	Affected Differently by Analyte, based on the predominating separation mechanism
Resolution	Minor Affect, based on predominating separation mechanism
S/N Ratio	Somewhat Proportionally More, based mostly on reduced baseline noise.
Quantitation	Relatively Unaffected in the Flow Rate Range of 0.4 – 0.9 mL/min

From the results described in this white paper, the most obvious change in the chromatography by increasing the delivered flow rate is that the analysis time is proportionally decreased. As explained in Table 2, the elution time is based on the volume of mobile phase delivered that gets a material through the column and to the detector. If no other processes are affected, the relative degree of chromatographic performance will be the same at all flow rates within the range defined by kinetics of interaction.

If it can be concluded that the increased loss of quantitative consistency at 1.0 mL/min is an indication that this flow rate may be high enough to start affecting the kinetics of separation, then it may also indicate the operational highest flow that should be used with this methodology.

Keeping in mind that this was a very limited study and only one HPLC system and one HPLC column was used, the results point to the method flow rate as having a **consistent performance range of 0.6 – 0.9 mL/min**. As previously pointed out, using a flow rate less than 0.6 mL/min is likely to not provide any chromatographic benefit, but will lead to longer analysis times.

- Biggest Advantage of Higher Flow Rate: Shorter analysis time, leading to more analyses per system, per day.
- Biggest Disadvantage of Higher Flow Rate: When analysis is not being performed, mobile phase is being expended at a faster rate.

■ Future Studies

- Part 3 – What Effect Does Column Dimension Have on the Chromatography of Fermentation Samples?
- Part 4 – What Effect Does the Mobile Phase Sulfuric Acid Concentration Have on the Chromatography of Fermentation Sample?

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