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PEOPLE > PROCESS > PERFORMANCE

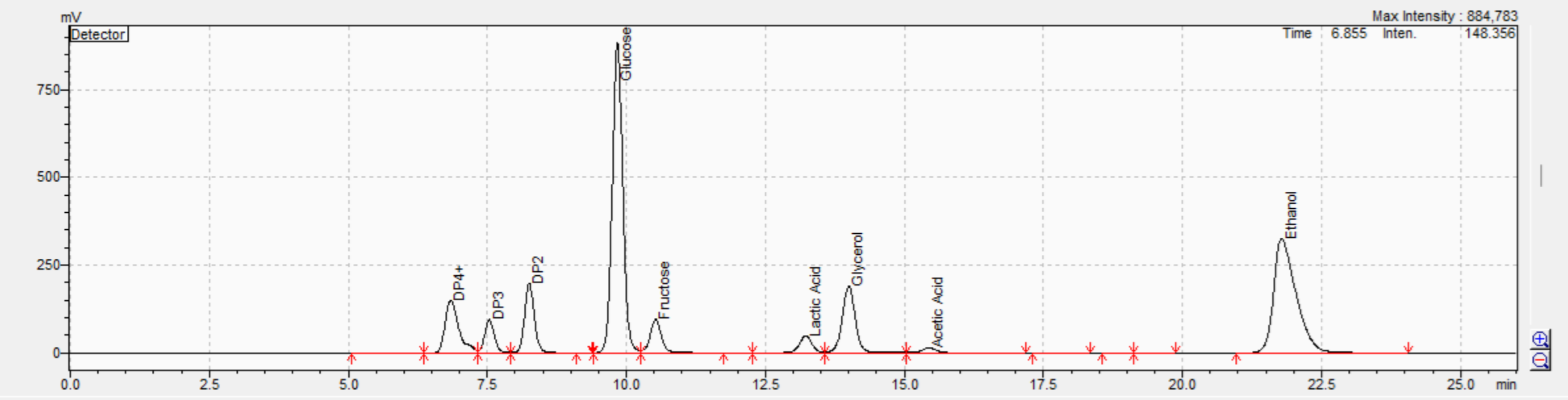
HPLC Calibration and Column Optimization

Andy Hodac

High Performance Liquid Chromatography

Liquid chromatography is a separation technique for nonvolatile liquid mixtures

High-performance liquid chromatography (HPLC) is the modern form of LC and is able to separate complex mixtures in a short amount of time



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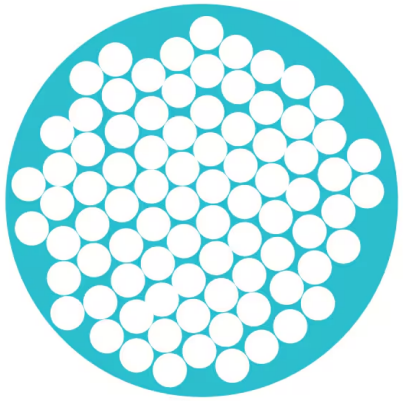
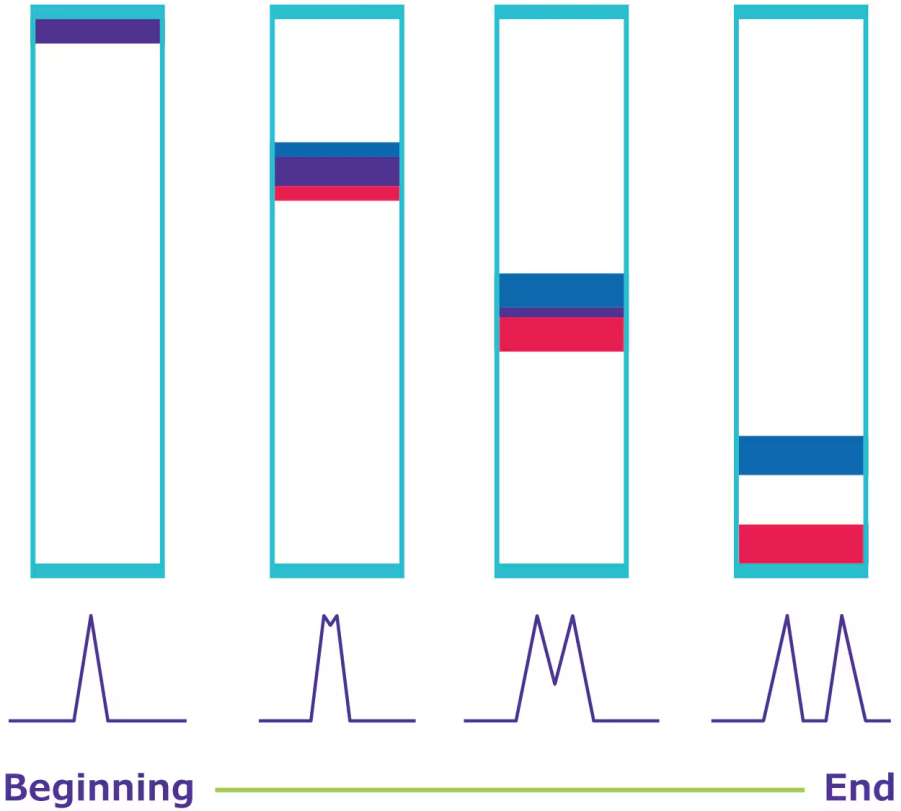
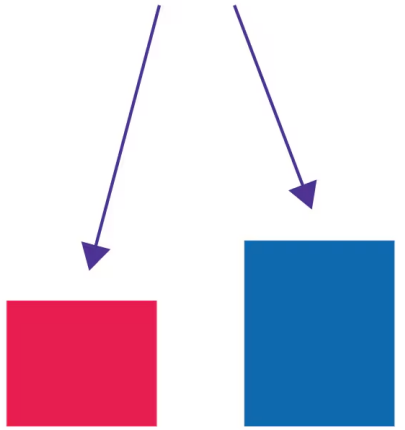
How does the HPLC Work?

Particles in the HPLC columns





Resolved Components



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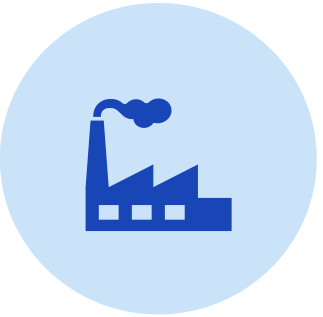
Why is HPLC Data So Important?

QUANTIFICATION OF RELEVANT COMPOUNDS

PROCESS EFFICIENCY

DETECTING BACTERIAL CONTAMINATION



ETHANOL YIELD

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HPLC Calibration

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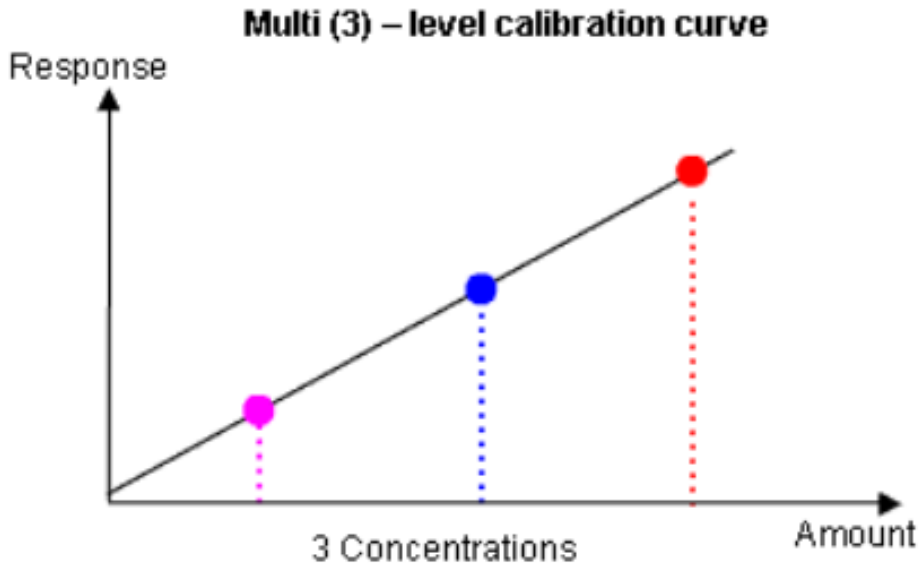
Calibration Basics

- Calibration at its basics is to determine the relationship between the analytical response and the analyte concentration
- There are different types of calibration, but for HPLC we generally use a direct, external-standard calibration
- Ideally, three or more standards are used in the calibration process[1]
  - Other governing bodies recommend even more



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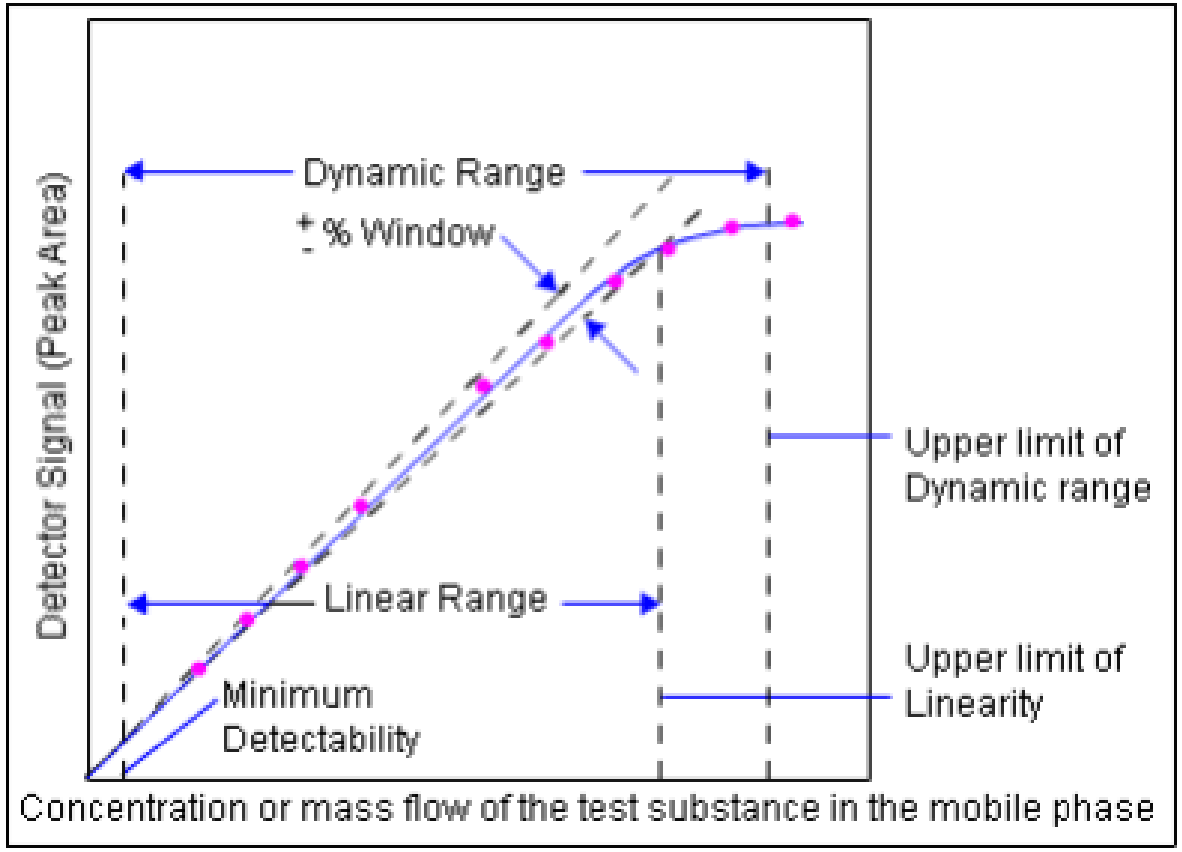
1. Skoog, D. A., West, D. M., Holler, F. J., & Crouch, S. R. (2021). Fundamentals of Analytical Chemistry (10th ed.).
2. ChromAcademy (Crawford Scientific). *Theory of HPLC - External Standard Quantitation*. (Accessible explanations on the principles of single-point and multi-point external calibration curves in chromatography)

Fundamentals of Calibration Curves

- Multi point calibration allows the establishment of a linear range
- Linear range represents the range of concentrations of a substance in the mobile phase at the detector over which the sensitivity of the detector is constant[5]
- It is crucial to establish relationship between the detector response and known concentrations of the calibration standard



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2. ChromAcademy (Crawford Scientific). *Theory of HPLC - External Standard Quantitation*. (Accessible explanations on the principles of single-point and multi-point external calibration curves in chromatography)
5. "Nomenclature and Presentation of Results in Chemical Analysis. Recommendations 1993, Section 9: Chromatography," in Pure and Applied Chemistry, 1993, 65(4), pp. 819–842

Leading Authorities on Calibration

- Governing bodies like IUPAC, ISO and the FDA along with regulated environments like GLP/GMP have set out to define calibration requirements[3]
  - IUPAC and FDA advises for six or more calibration standards[4]
  - ISO guidelines vary with some recommending five or more[3]
- Routine calibrations can be less intensive after initial validation of the calibration curve[1]
  - two- or one-point standard calibrations are implemented with some considerations

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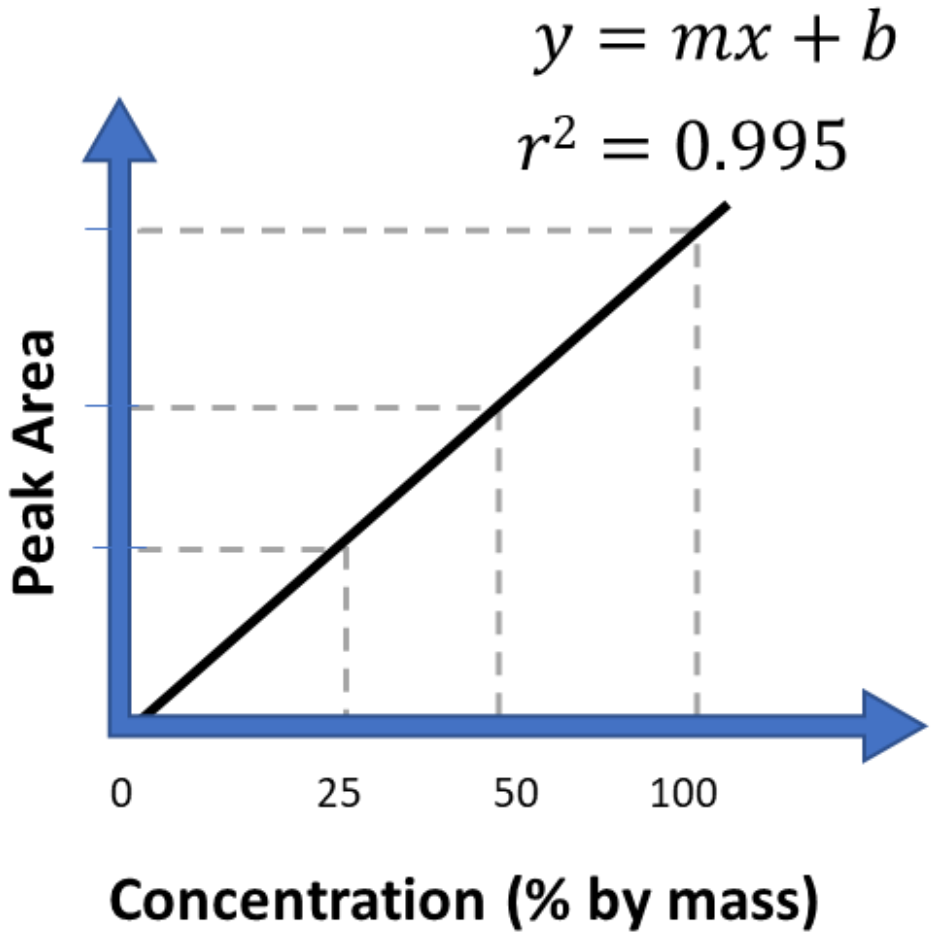
3. Cuadros-Rodríguez, L., Bagur-González, M. G., Sánchez-Viñas, M., González-Casado, A., & Gómez-Sáez, A. M. (2007). Principles of analytical calibration/quantification for the separation sciences. Journal of Chromatography A, 1158(1-2), 33-46.
4. Food and Drug Administration (FDA). (2018, May). Bioanalytical Method Validation Guidance for Industry. U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER).

HPLC: Calibration Standards

Run calibration standards that cover the expected range of all compounds.

Analyze the peak areas vs concentrations and ensure there is a linear regression with a minimum r^2=0.995

Multi-Point Calibration establishes your linearity



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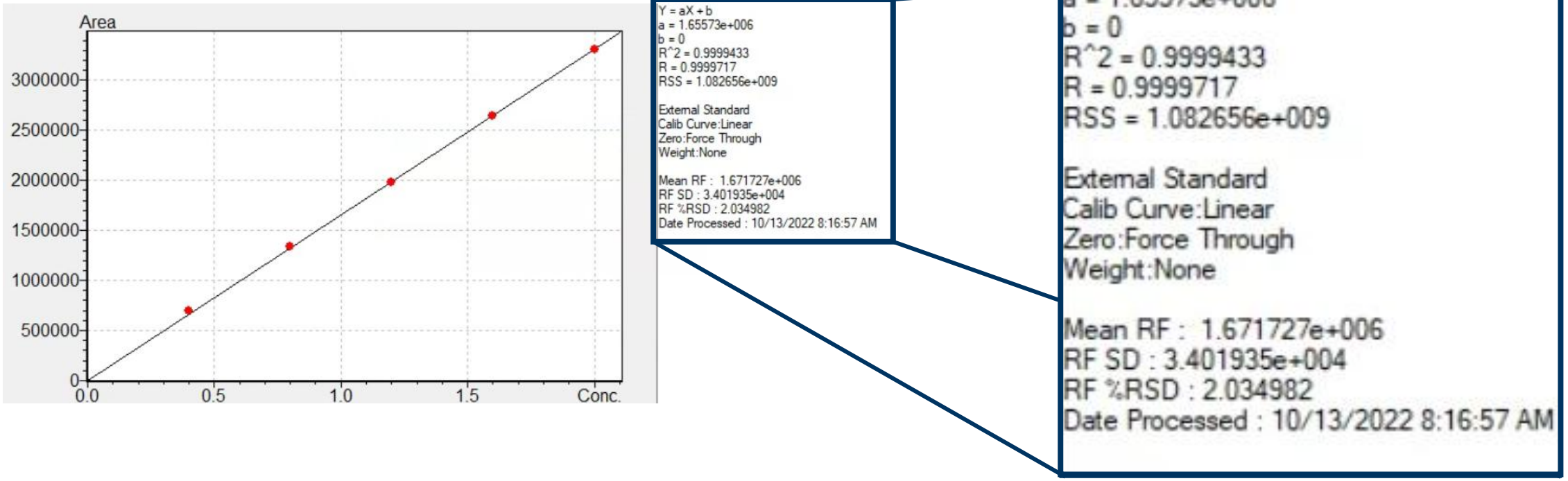
HPLC: Quantitative Analysis



Review calibration curves for linearity and R^2 values for all compounds

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HPLC: Standard Calibration Handling

- In the ethanol industry it is common for calibration standards to be purchased from an outside vendor
- It is important to verify the COA and expiration date of the calibration standards
- Store according to vendor recommendations
- Have a written SOP (standard operation procedure) on how to run a calibration curve based on your HPLC instrument
  - Contact the HPLC manufacture on calibration practices

Expanding the Linear Range

- Calibration Curves are only reliable and effective in their linear range.
- Current 3rd party calibration standards did not reflect values being quantified in some R&D work at the Technology Center
- Expanded calibration curve to 6 points, and a larger linear range

Compounds	3rd party standard range(%w/v)	CTE standard range (%w/v)
DP4+	0.40-2.00	0.10-10.00
DP3	0.20-1.00	0.05-5.00
DP2	0.40-2.00	0.10-10.00
Glucose	2.00-10.00	0.50-10.00
Fructose	0.20-1.00	0.05-1.00
Lactic Acid	0.20-1.00	0.10-1.00
Glycerol	0.60-3.00	0.10-3.00
Acetic Acid	0.10-0.50	0.05-0.50
Ethanol	2.00-18.00	0.50-18.00

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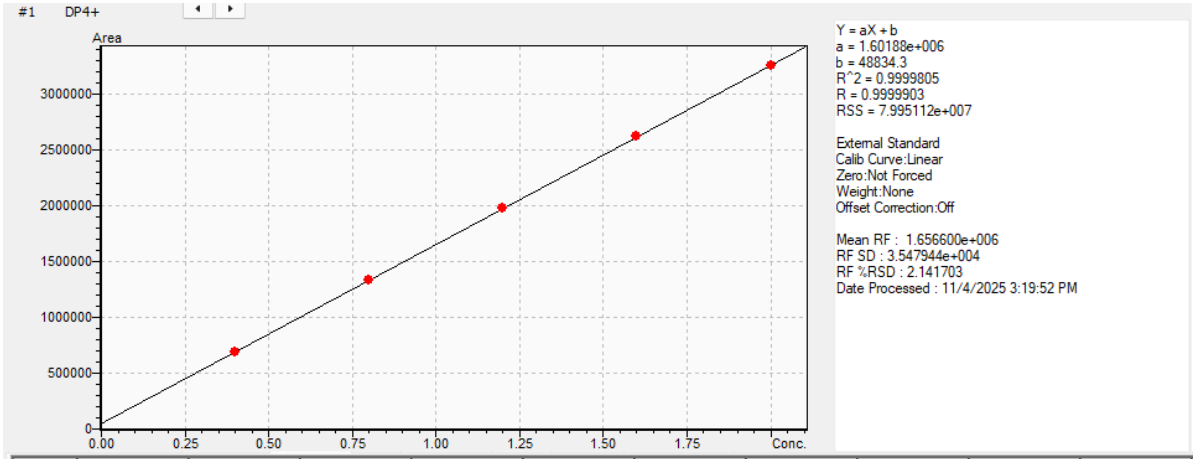
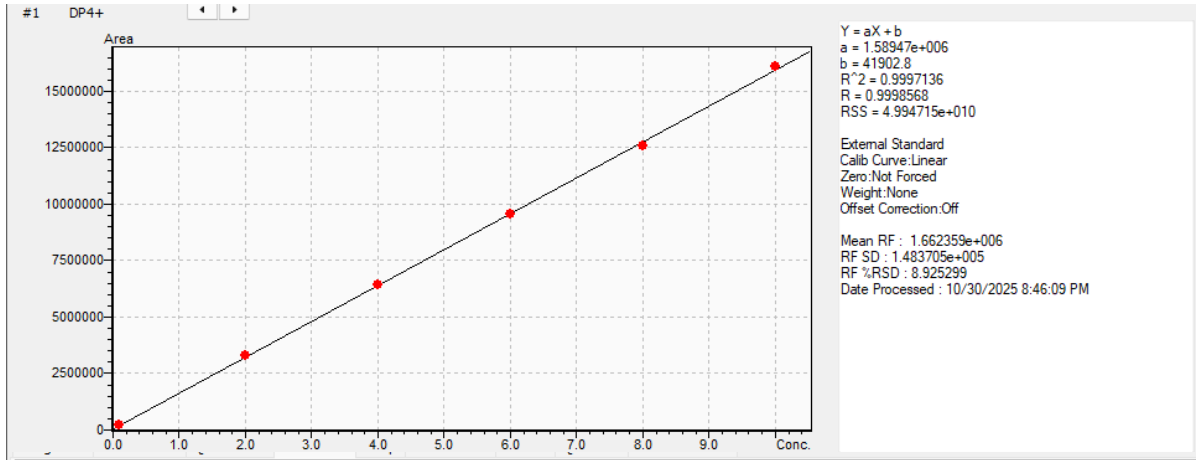
Cal Curve Extension of Linear Range

- Modified calibration curve from Bion to encompass a larger DP4+ range that we observe at the Technology Center



ID#	Name	Type	Channel	Ret. Time	Conc.(1)	Conc.(2)	Conc.(3)	Conc.(4)	Conc.(5)	Fit Type
1	DP4+	Target	Detector - Ch1	6.909	0.4	0.8	1.2	1.6	2	Default
2	DP3	Target	Detector - Ch1	7.663	0.2	0.4	0.6	0.8	1	Default
3	DP2	Target	Detector - Ch1	8.387	0.4	0.8	1.2	1.6	2	Default
4	Glucose	Target	Detector - Ch1	9.968	2	4	6	8	10	Default
5	Fructose	Target	Detector - Ch1	10.687	0.2	0.4	0.6	0.8	1	Default
6	Lactic Acid	Target	Detector - Ch1	13.386	0.2	0.4	0.6	0.8	1	Quadratic
7	Glycerol	Target	Detector - Ch1	14.127	0.6	1.2	1.8	2.4	3	Default
8	Acetic Acid	Target	Detector - Ch1	15.597	0.1	0.2	0.3	0.4	0.5	Quadratic
9	Ethanol	Target	Detector - Ch1	21.879	2	6	10	14	18	Default



ID#	Name	Type	Channel	Ret. Time	Conc.(1)	Conc.(2)	Conc.(3)	Conc.(4)	Conc.(5)	Conc.(6)
1	DP4+	Target	Detector - Ch1	6.909	0.1	2	4	6	8	10
2	DP3	Target	Detector - Ch1	7.663	0.05	1	2	3	4	5
3	DP2	Target	Detector - Ch1	8.387	0.1	2	4	6	8	10
4	Glucose	Target	Detector - Ch1	9.968	0.5	2	4	6	8	10
5	Fructose	Target	Detector - Ch1	10.687	0.05	0.2	0.4	0.6	0.8	1
6	Lactic Acid	Target	Detector - Ch1	13.386	0.1	0.2	0.4	0.6	0.8	1
7	Glycerol	Target	Detector - Ch1	14.127	0.1	0.6	1.2	1.8	2.4	3
8	Acetic Acid	Target	Detector - Ch1	15.597	0.05	0.1	0.2	0.3	0.4	0.5
9	Ethanol	Target	Detector - Ch1	21.879	0.5	2	6	10	14	18

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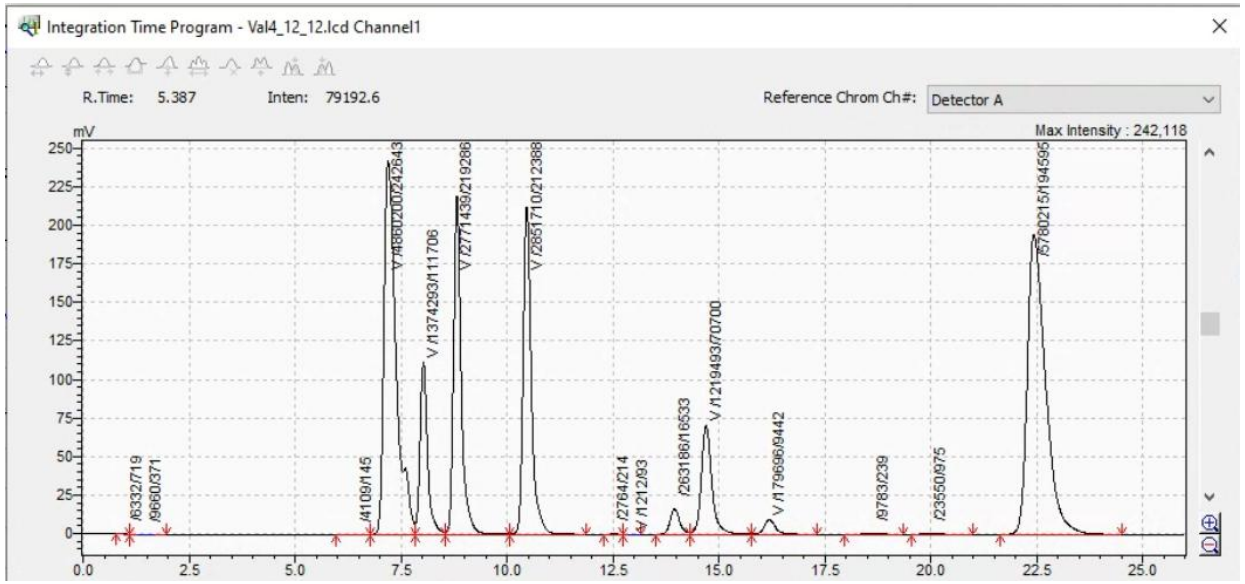
Data Analysis: Lab Solutions Automation

- Integration of peaks are automated to ensure consistency in data analysis
- This allows for a quicker turn around time and removes any manual bias
- Once automation is set within the method file, it will be applied to all samples



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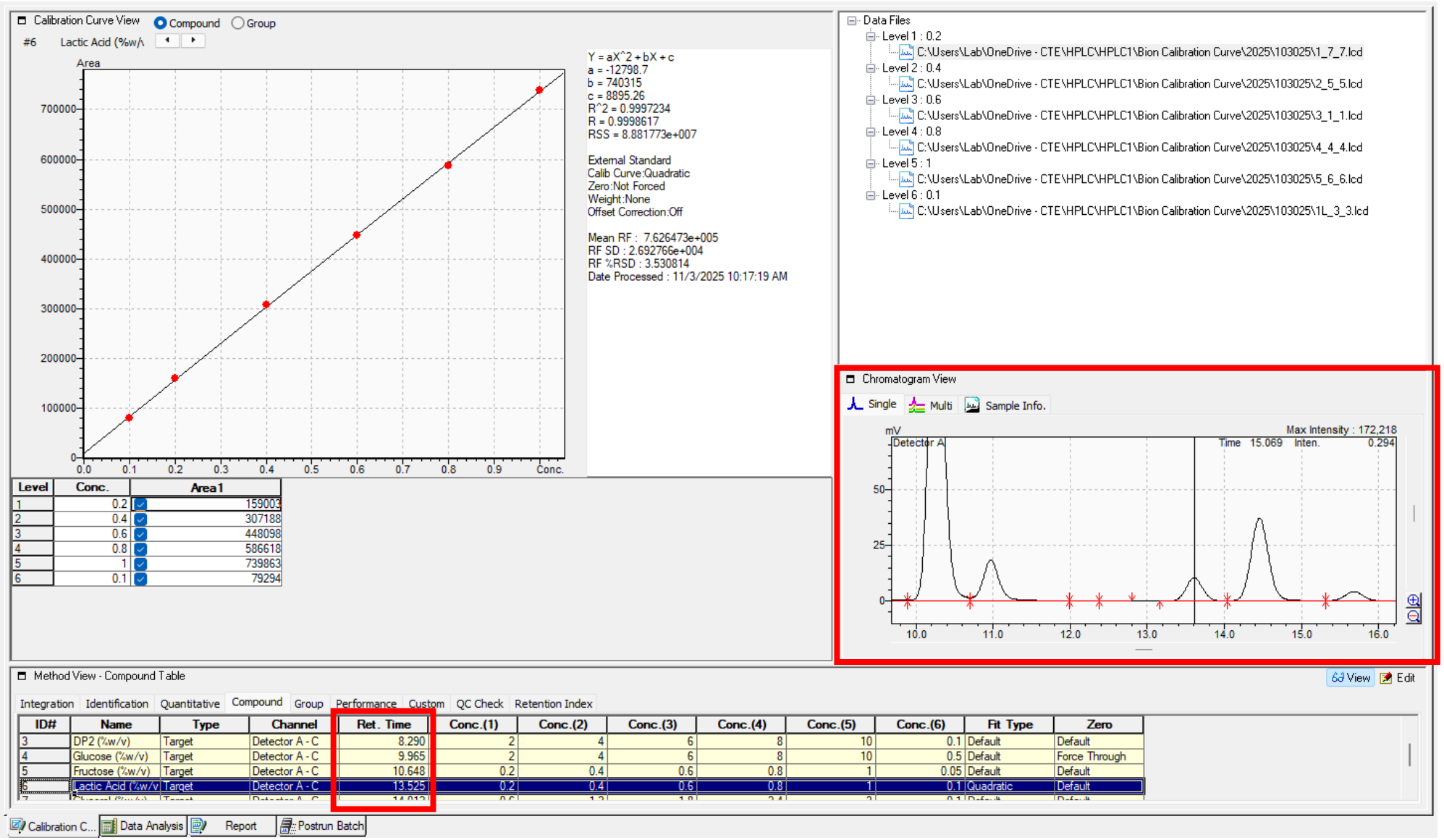
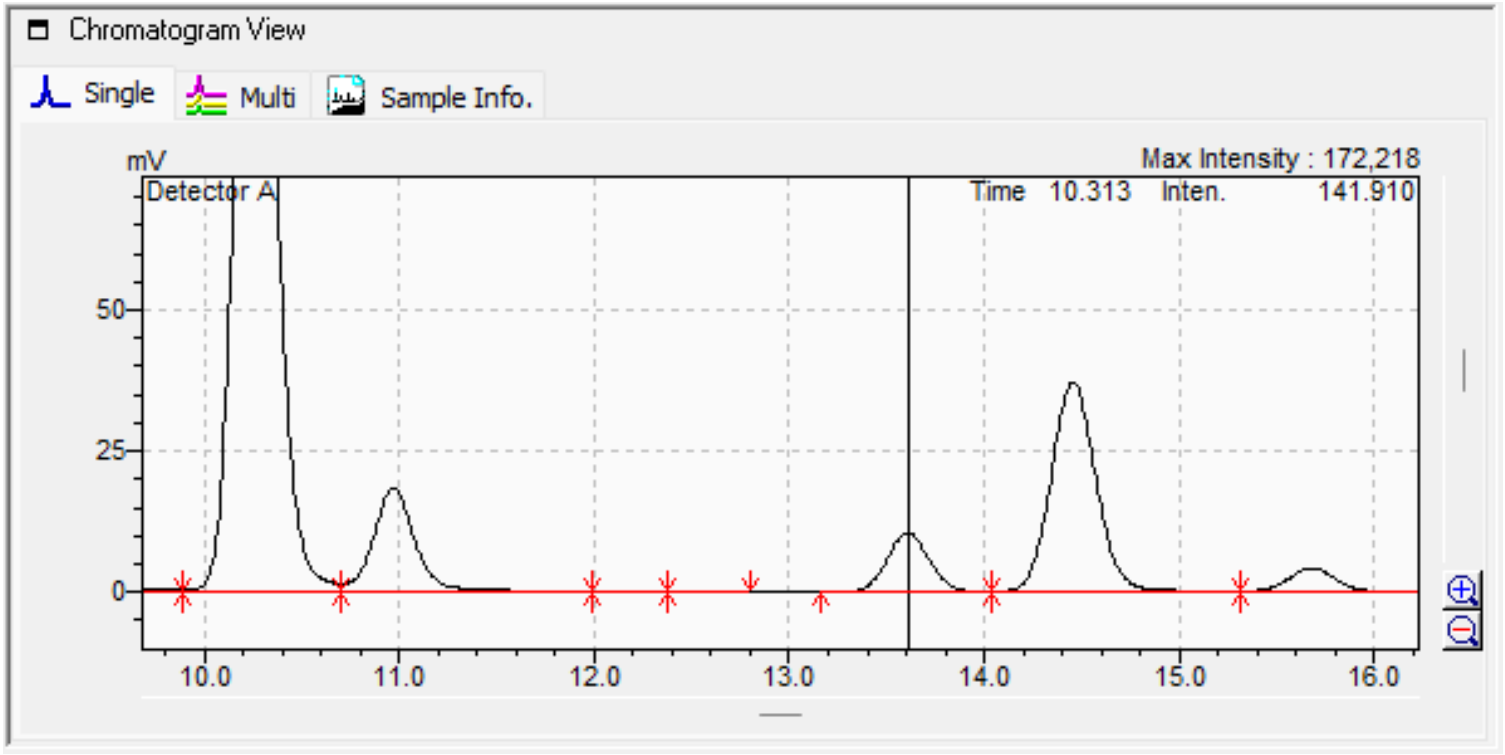
Defining Retention Times



Defining Retention Times



Method View - Compound Table

View Edit

Integration | Identification | Quantitative | Compound | Group | Performance | Custom | QC Check | Retention Index

ID#	Name	Type	Channel	Ret. Time	Conc.(1)	Conc.(2)	Conc.(3)	Conc.(4)	Conc.(5)	Conc.(6)	Fit Type	Zero
3	DP2 (%w/v)	Target	Detector A - C	8.290	2	4	6	8	10	0.1	Default	Default
4	Glucose (%w/v)	Target	Detector A - C	9.965	2	4	6	8	10	0.5	Default	Force Through
5	Fructose (%w/v)	Target	Detector A - C	10.648	0.2	0.4	0.6	0.8	1	0.05	Default	Default
6	Lactic Acid (%w/v)	Target	Detector A - C	13.525	0.2	0.4	0.6	0.8	1	0.1	Quadratic	Default

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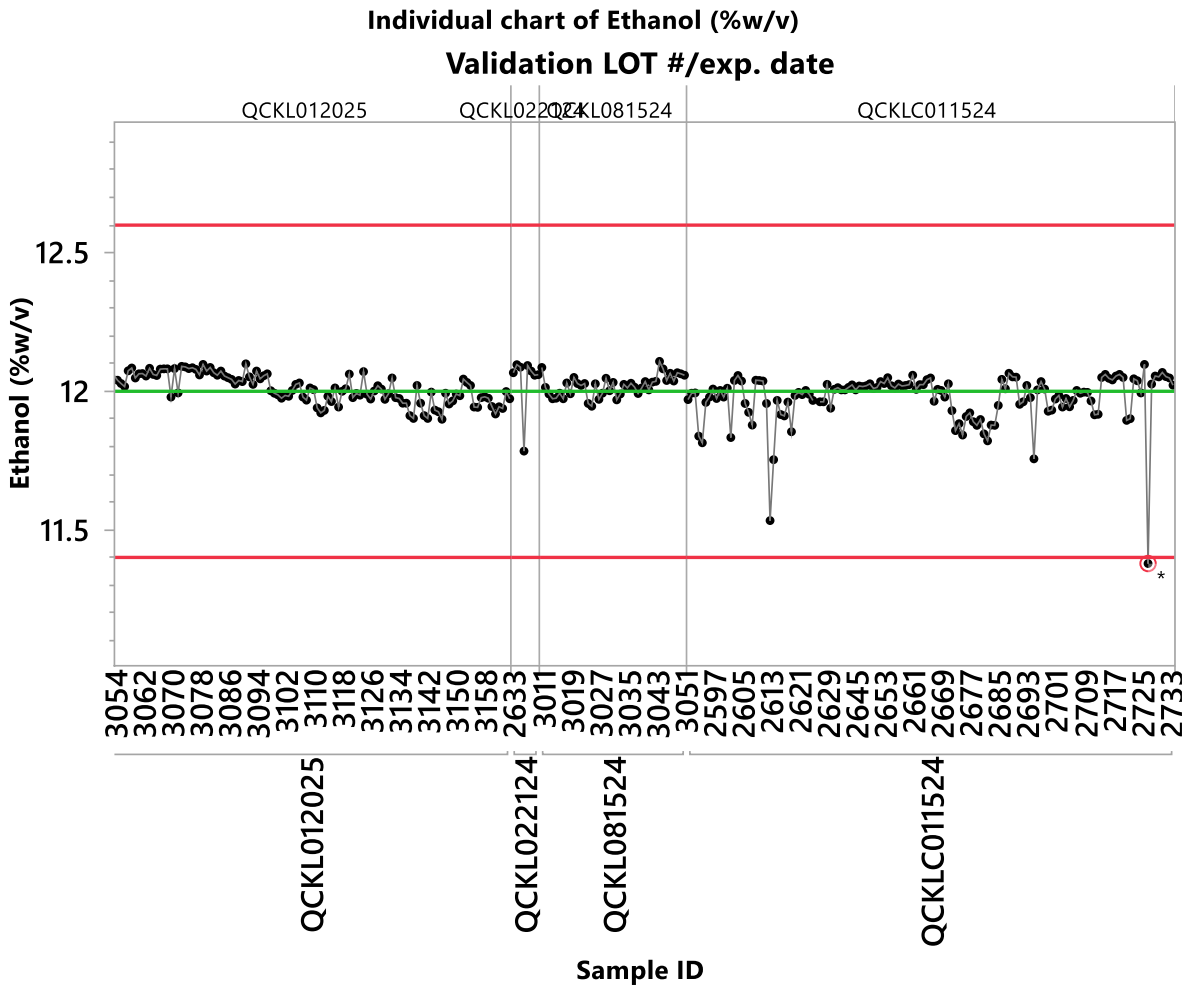
Data Analysis: Control Charts

- A validation or check standard increases the confidence in our HPLC data
- After a fixed number of samples are injected, a validation sample injection will follow within ever batch
- The concentration of the validations are plotted on a control chart and monitored over time
- An upper and lower control limit of 5% or 2σ was used



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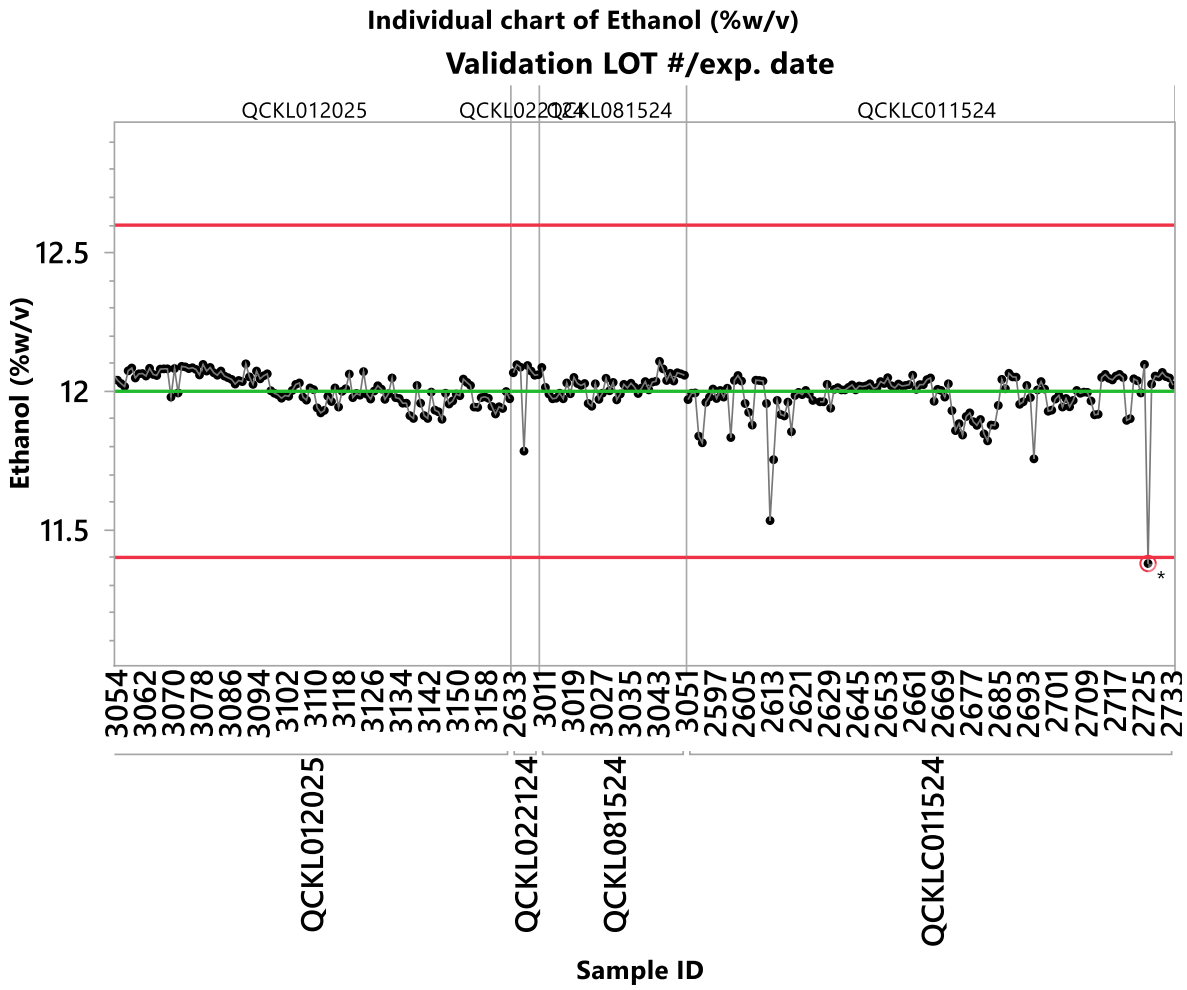
Identifying Variation

- **Common-cause variation** is the expected variation in a process
- **Special-cause variation** is unexpected variation in a process



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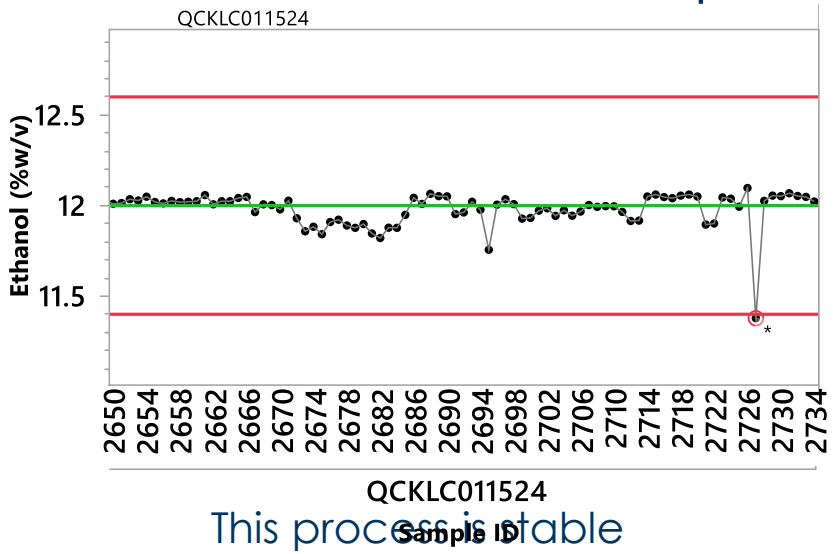
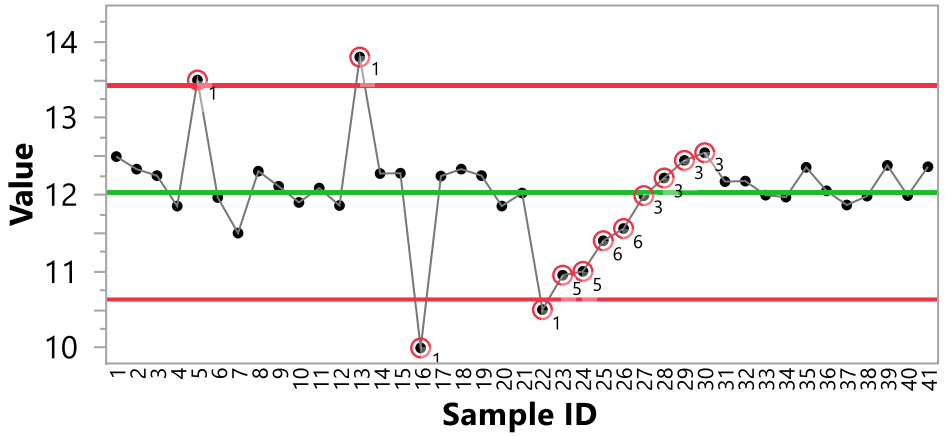
Identifying Variability Within Your Control Chart

- A process is stable if it does not contain any special-cause variation
- Control charts are visually informative tools that will help determine special-cause variation
- You will need an adequate set of historical data



This process is stable
Data is distributed randomly and does not violate any of the control chart tests



This process is not stable
Data is erratic and violates several control chart tests

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Column Optimization

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Column Care

- **Increasing lifetime**
  - Check column pH limitations
    - Typically safe between pH 2 – pH 8 for silica-based columns
  - Do not exceed temperature limits
    - < 80°C
  - Do not exceed pressure limits
    - Record pressure for each newly installed and equilibrated column
    - Gradual 15% increase of pressure over time indicates build-up on column. Rinse per manufacturer's instructions.
  - Use UHPLC/HPLC grade solvents
  - Do not dry out columns
- **Maintenance and Clean up**
  - Monitor system pressure
  - Column cleaning: flushing/back flushing
    - Flush with appropriate solvents at least 10-20 column volumes
  - Keep Records
- **Store columns in proper solvent**

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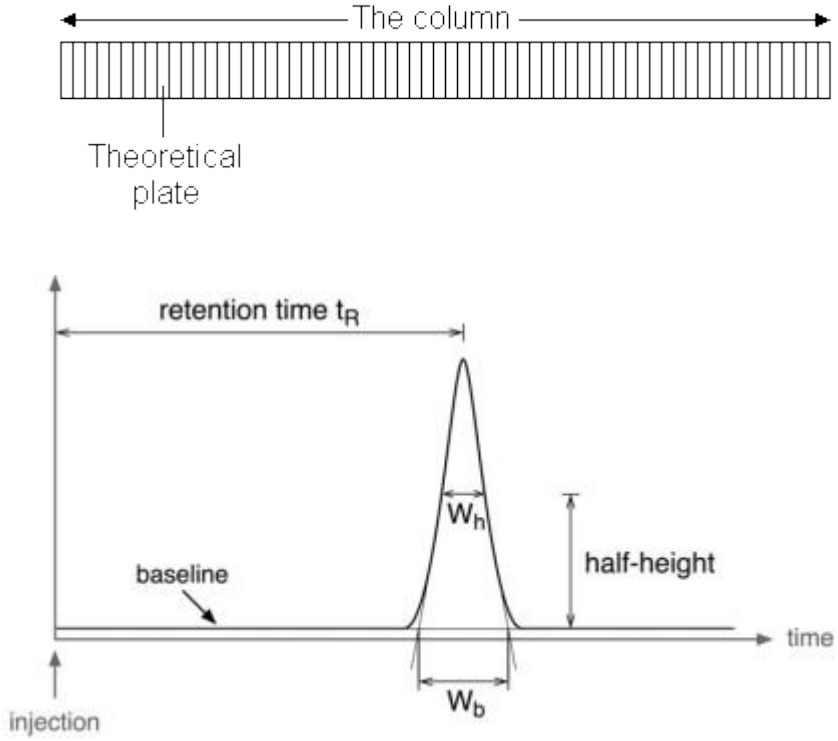
Column Efficiency – Theoretical Plate Model

- The theoretical plate model assumes a column contains a large number of separating layers called theoretical plates
- theoretical plate number (N) can be calculated using retention time and peak width ($W_{1/2}$ = peak width at half the peak height)



$$N = 5.54 \frac{(t_r)^2}{(w_{1/2})^2}$$

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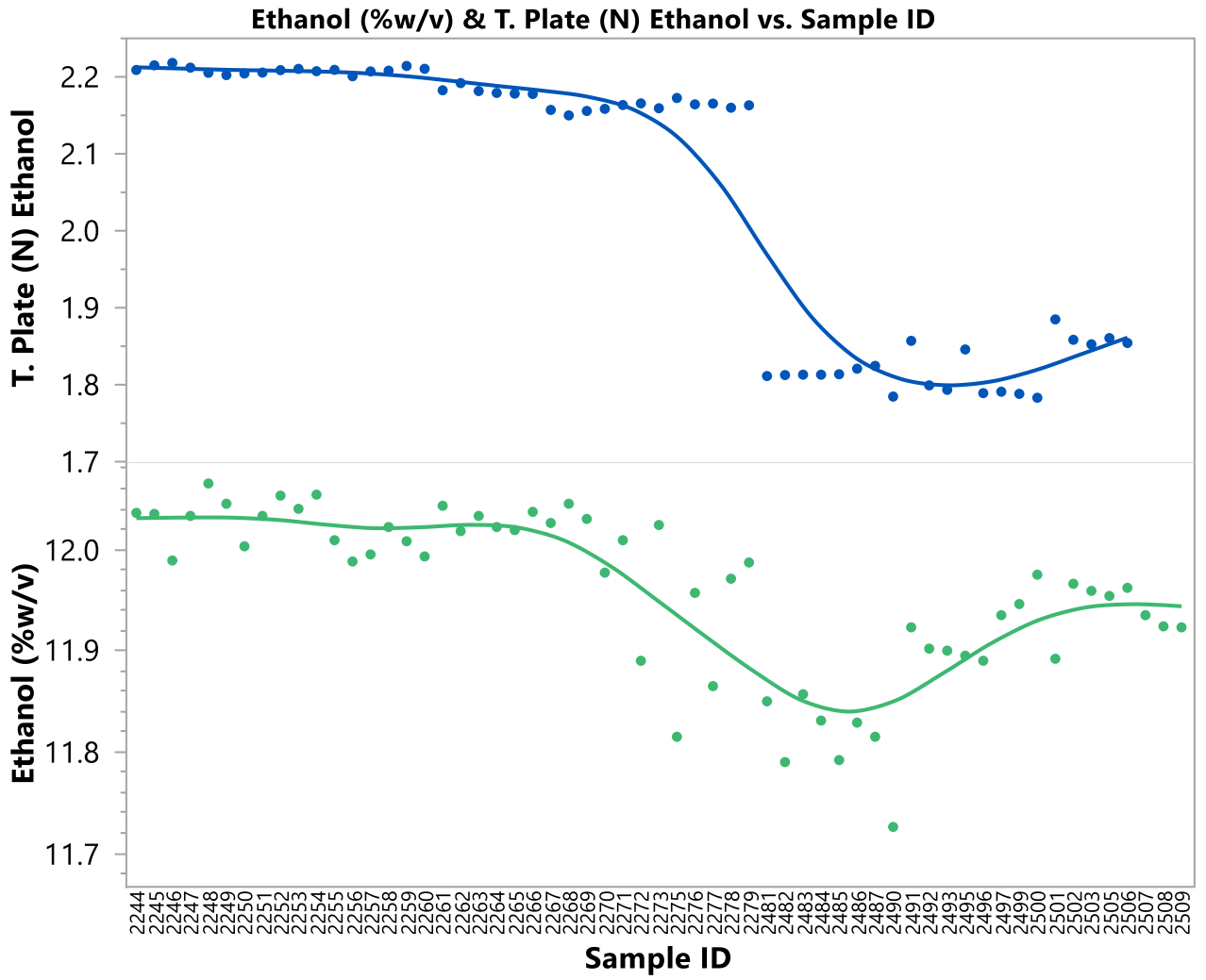
Theoretical Plate vs Concentration

- Determine the initial theoretical plate number and monitor inefficiency over time
- A less efficient column will contribute to peak broadening and lower resolution between peaks



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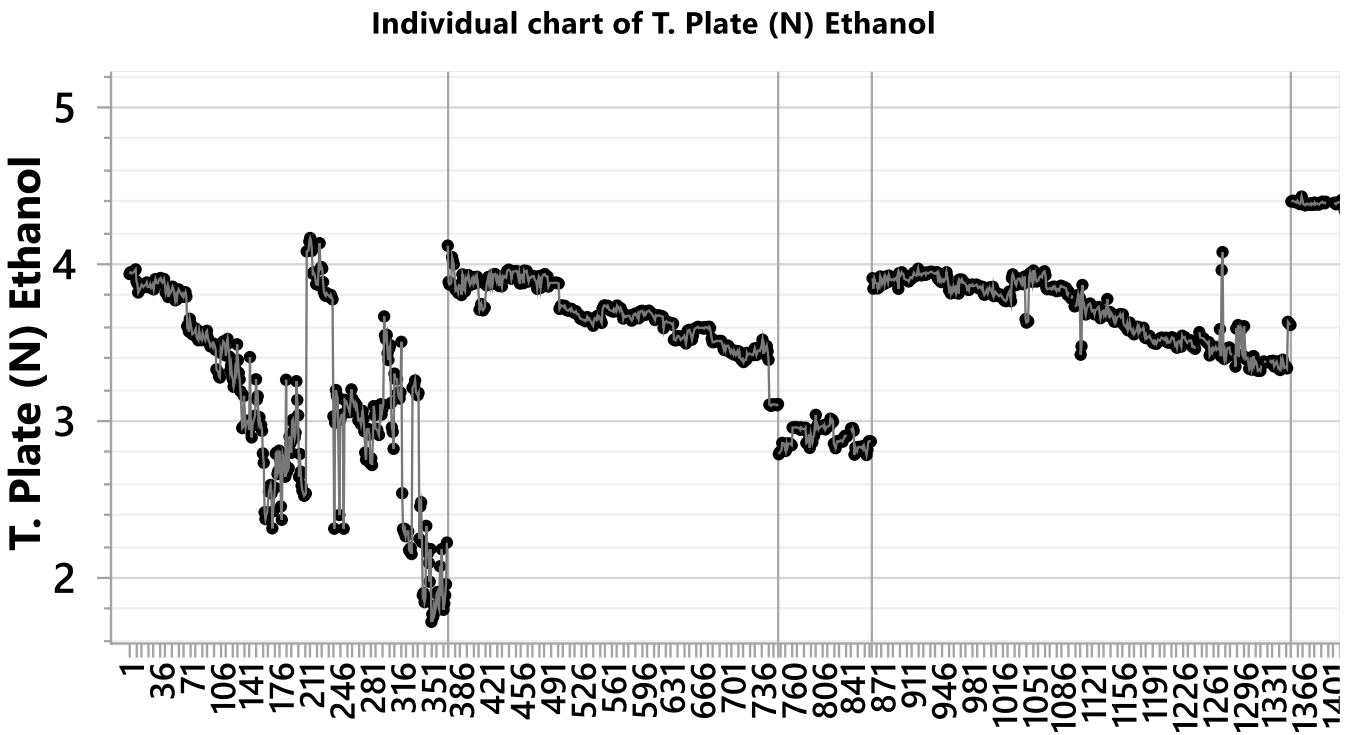
Theoretical Plate Data Tracking

- Historical data of column theoretical plates allows for confirmation of column degradation
- Determine the initial theoretical plate number and monitor inefficiency over time
- A less efficient column will contribute to peak broadening and lower resolution between peaks



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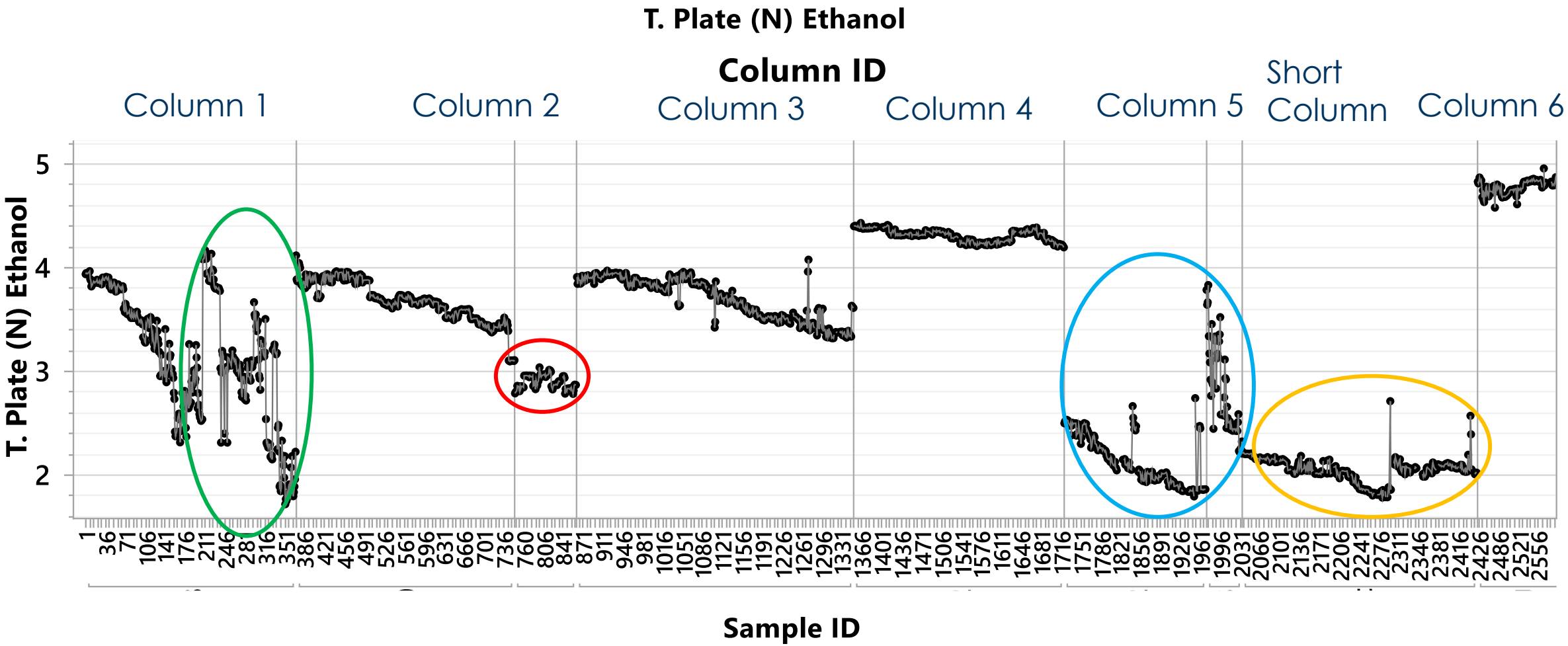
Identifying Problems with Theoretical Plates



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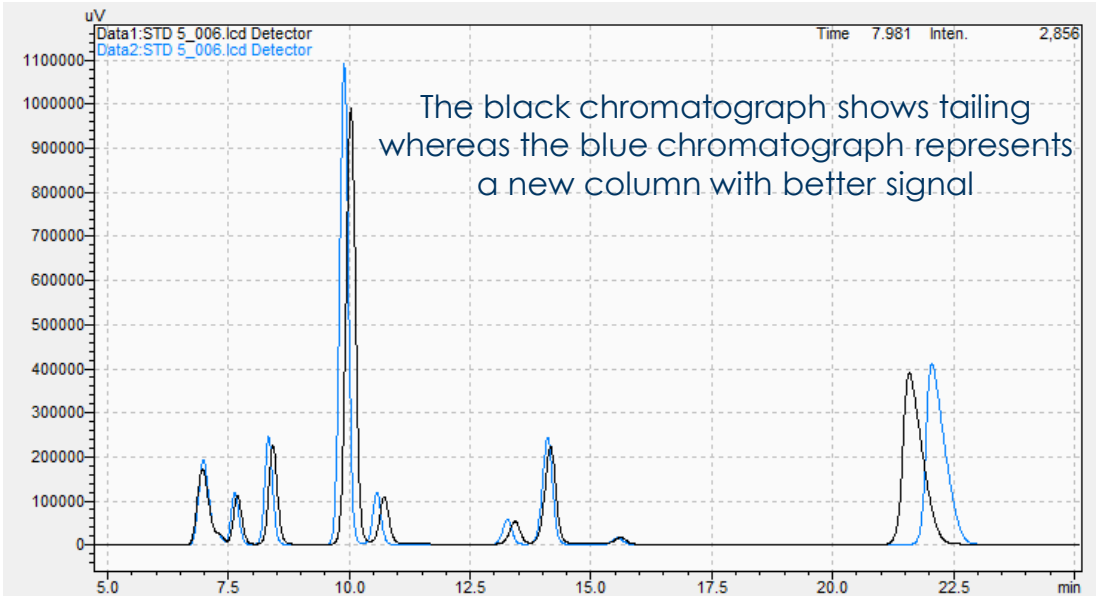
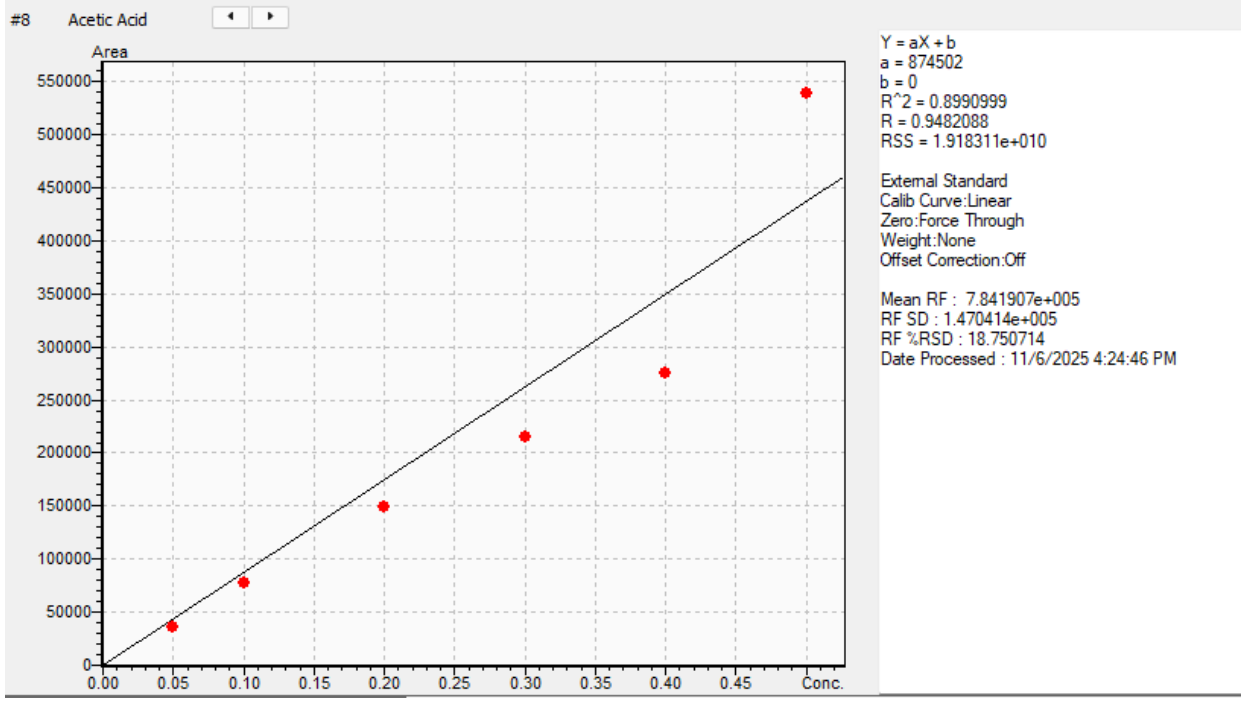
Chromatogram – Theoretical Plates

Case Study in Problem Identification

- Noticed peak broadening/tailing and saw a poor correlation for acetic acid calibration curve
- Column change corrected this





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Long vs. Short Column

Long Columns (300mm):

- Increase Analysis Time (~22-23 minutes)
- Improved sensitivity
- Improved resolution

Short Columns (150 mm):

- Faster Analysis Time (~11-12 minutes)
- Decreased sensitivity
- Decreased resolution

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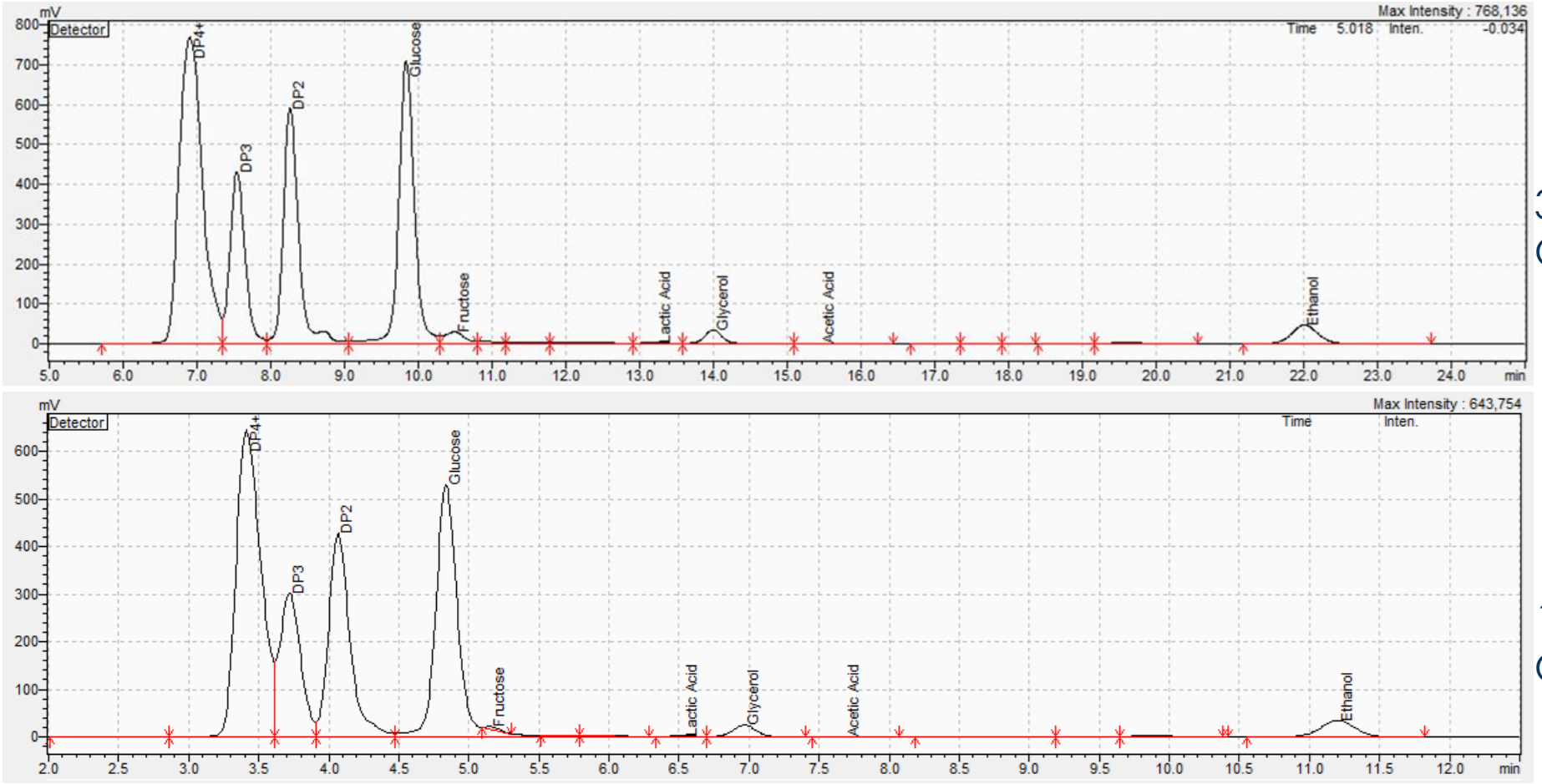
Factors Affecting Resolution

- **Retention Factor**
  - Also known as "capacity factor"
  - Describes how well the analyte is retained on the column
  - Dependent upon solvent strength, not column length or flow rate
- **Selectivity**
  - Ability to distinguish between different compounds
  - Depends on column chemistry and mobile phase
- **Efficiency**
  - Related to theoretical plates
  - Describes the distribution of the analyte's particles; how narrow is the peak
  - Depends on flow rate, column length, and particle size

Visual Comparisons- 10-Hour Samples



300mm Column

150 mm Column

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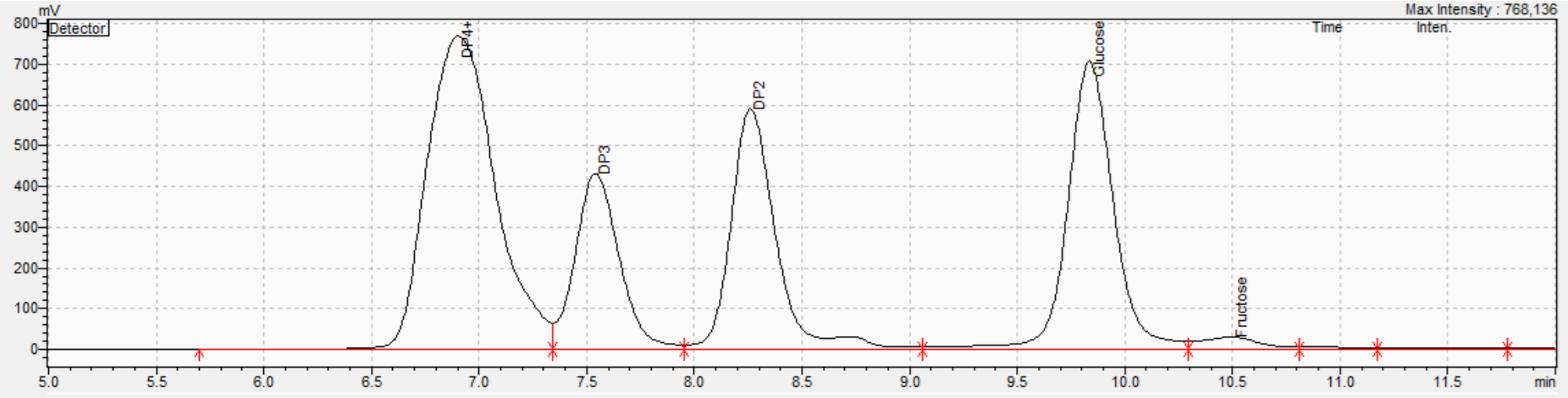
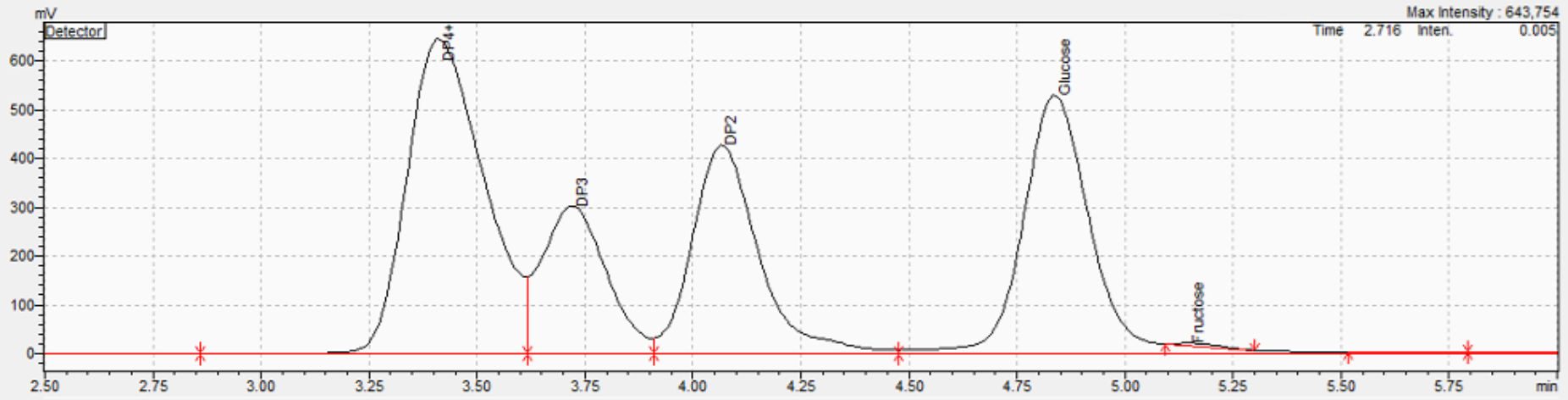
Visual Comparisons- 10-Hour Samples



300mm Column



150 mm Column

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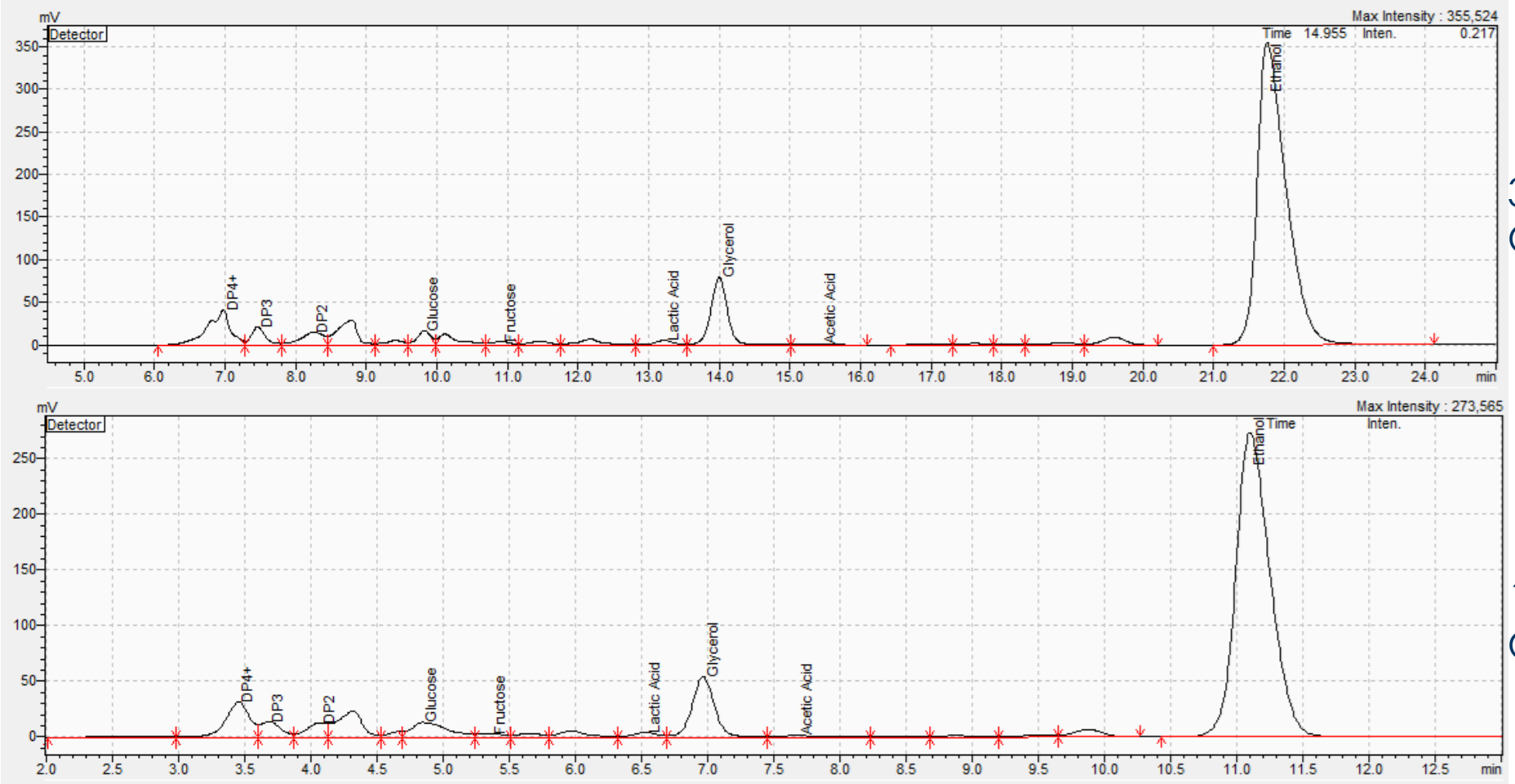
Visual Comparisons- Drop Samples



300mm Column

150 mm Column

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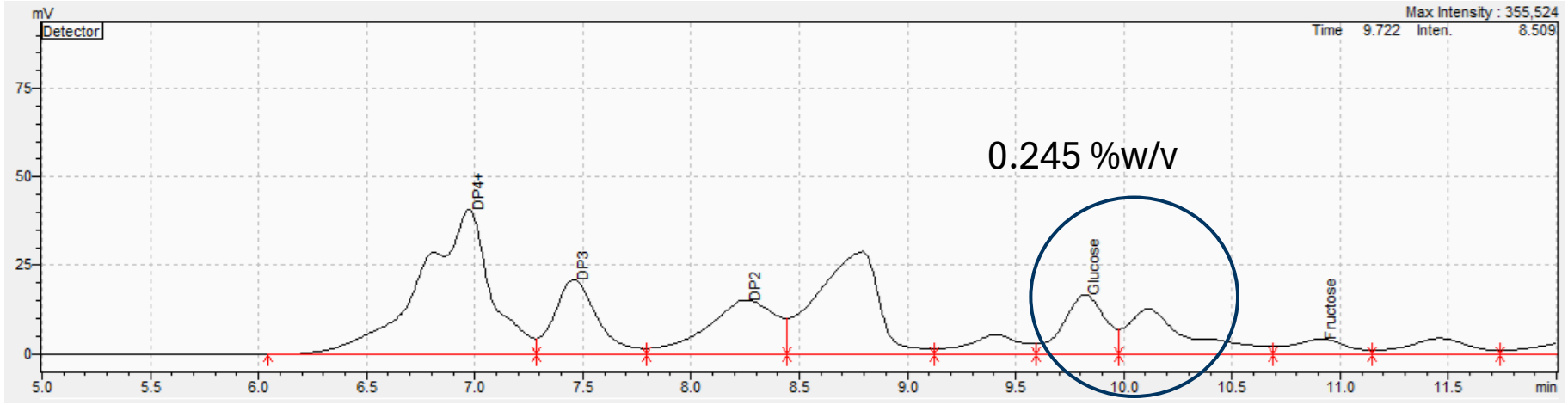
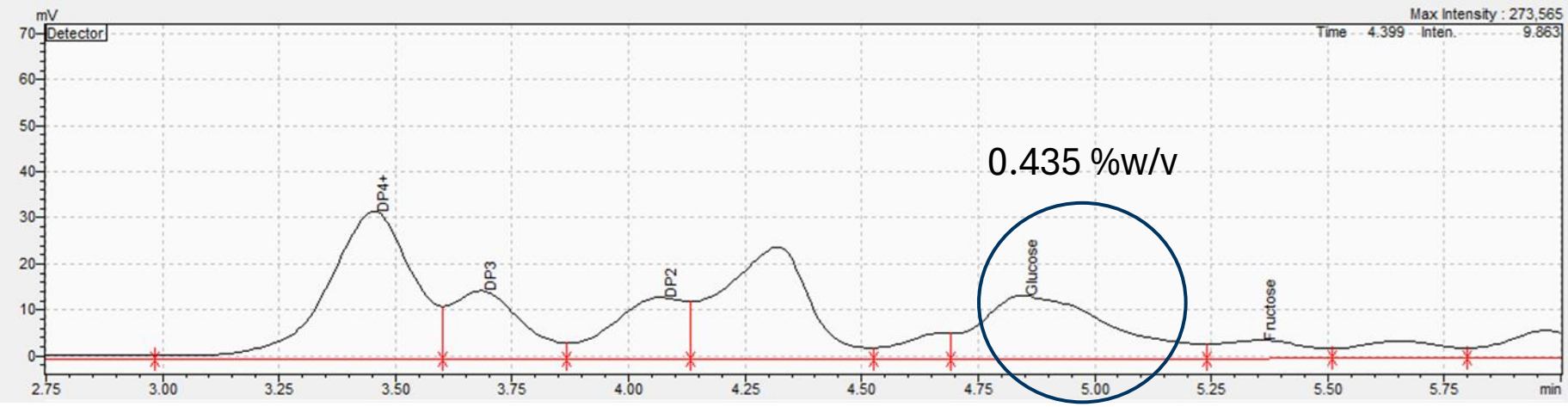
Visual Comparisons- Drop Samples



300mm Column



150 mm Column

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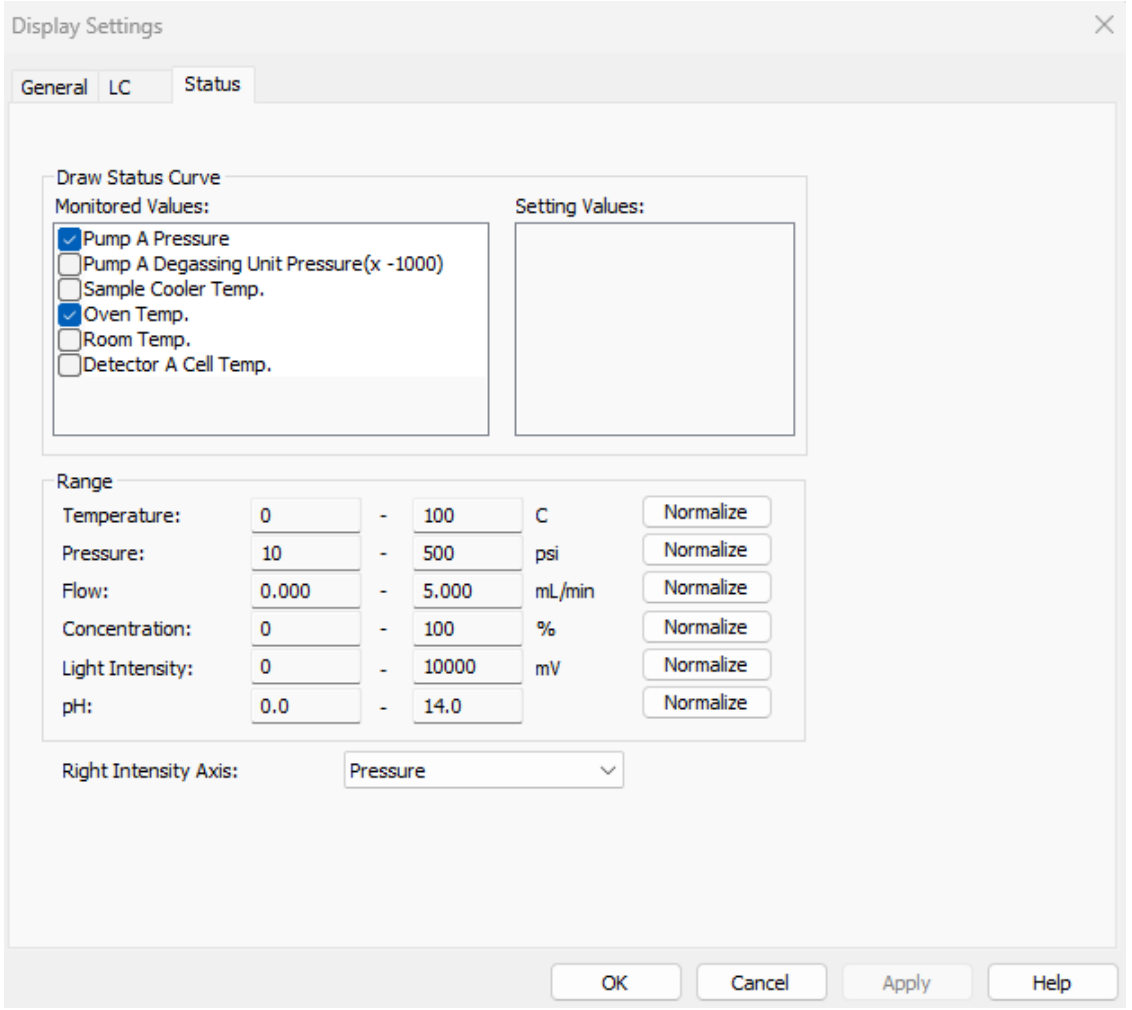
Optimization in General

Some tips, tricks and considerations

Adjusting the Display on the HPLC Screen

- LabSolutions software for Shimadzu Instruments
- Right click Real time chromatograph > Display Settings > Status > Check Pump A Pressure and or Oven Temp.
  - Allows real time visuals of pump pressure and oven temps
  - Pressure and Oven temperature are good culprits to track in case of HPLC issues



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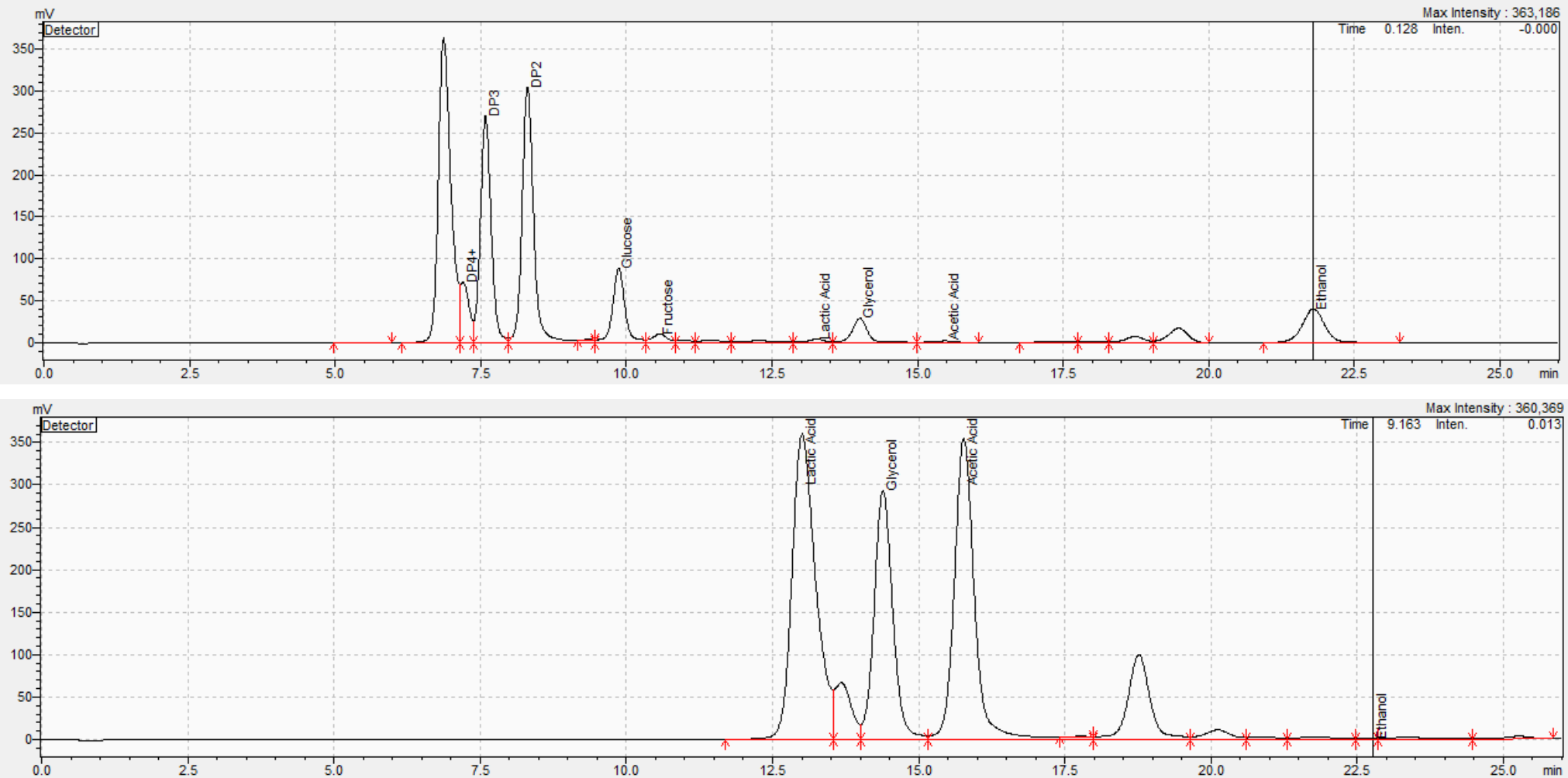
Realtime and Postrun Optimization



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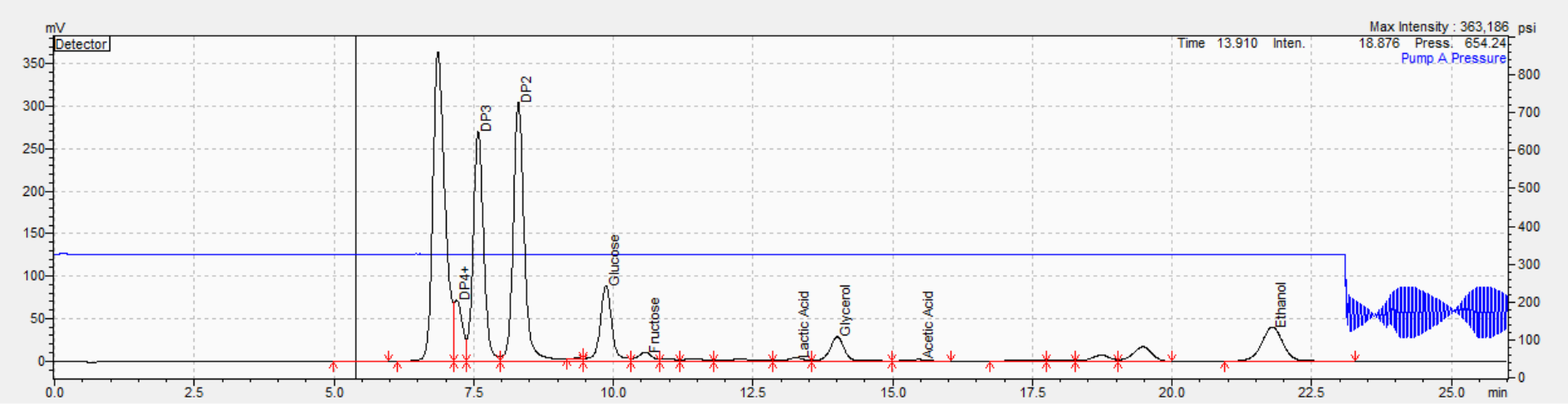
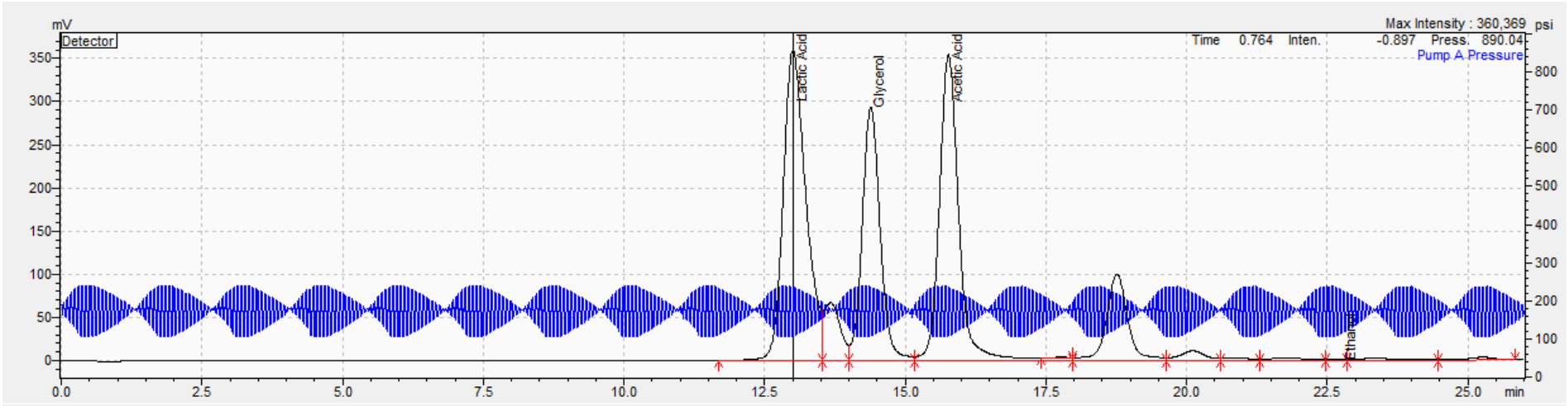
HPLC Chromatogram - Pressure





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- Pressure started to oscillate mid-sample run.
- All subsequent samples had pressure issues.

HPLC Chromatogram - Pressure

Likely Culprit: Check Valves

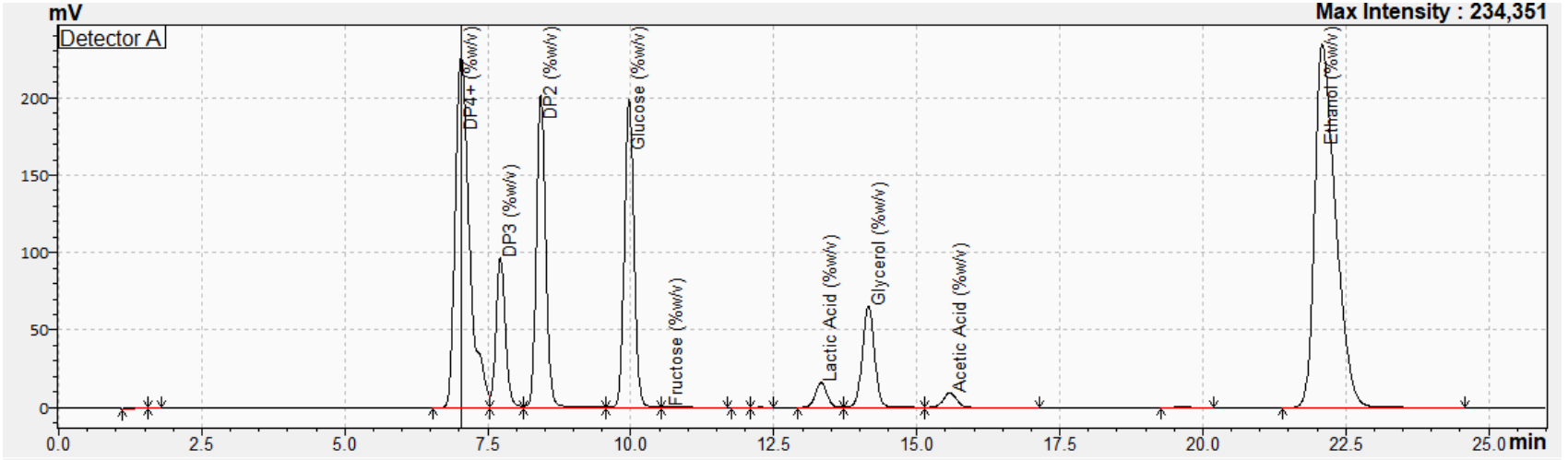
Oscillations can happen with pressure due to several factors-

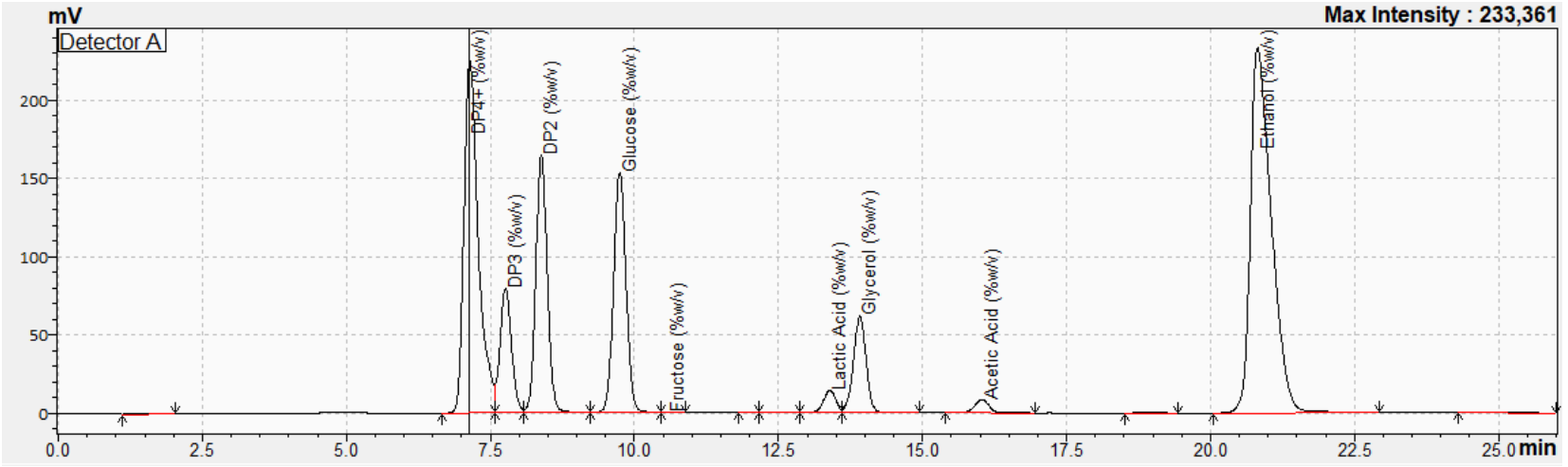
- Check Valve Malfunction
- Air Bubbles or Cavitation
- Blockage or Contamination
- Issues with Pumping System

Troubleshooting Steps:

- Check pump performance for irregular flow or pulsation.
- Flush or purge the system with fresh solvent to remove debris.
- Inspect and replace the check valve if worn or damaged.

Realtime and Postrun Optimization





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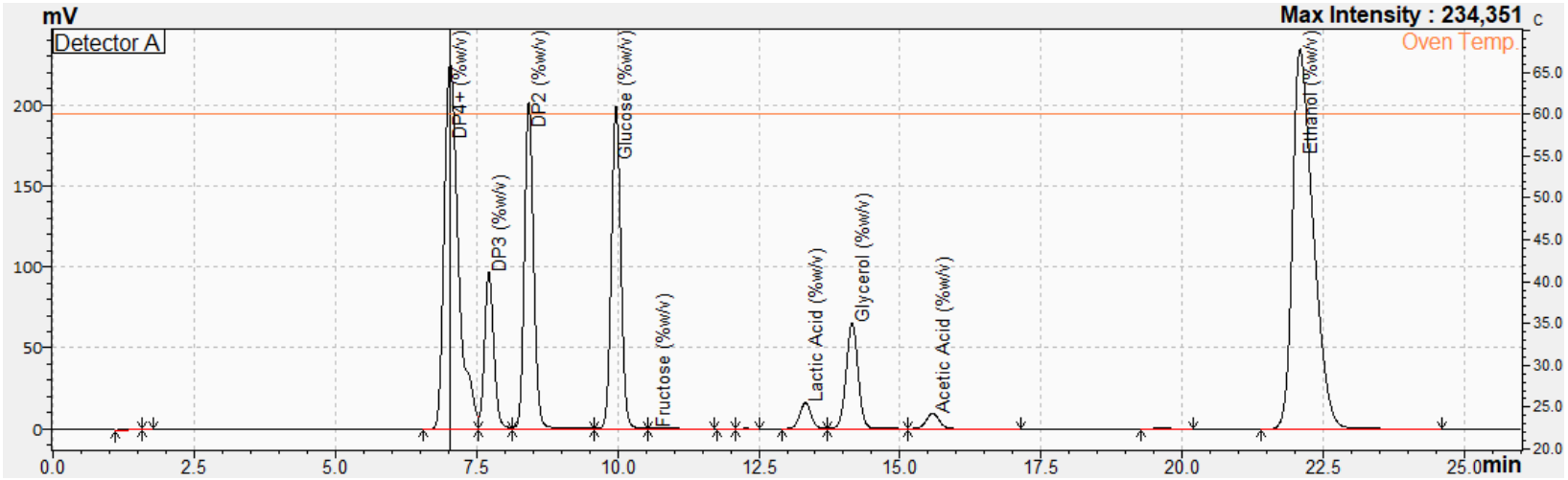
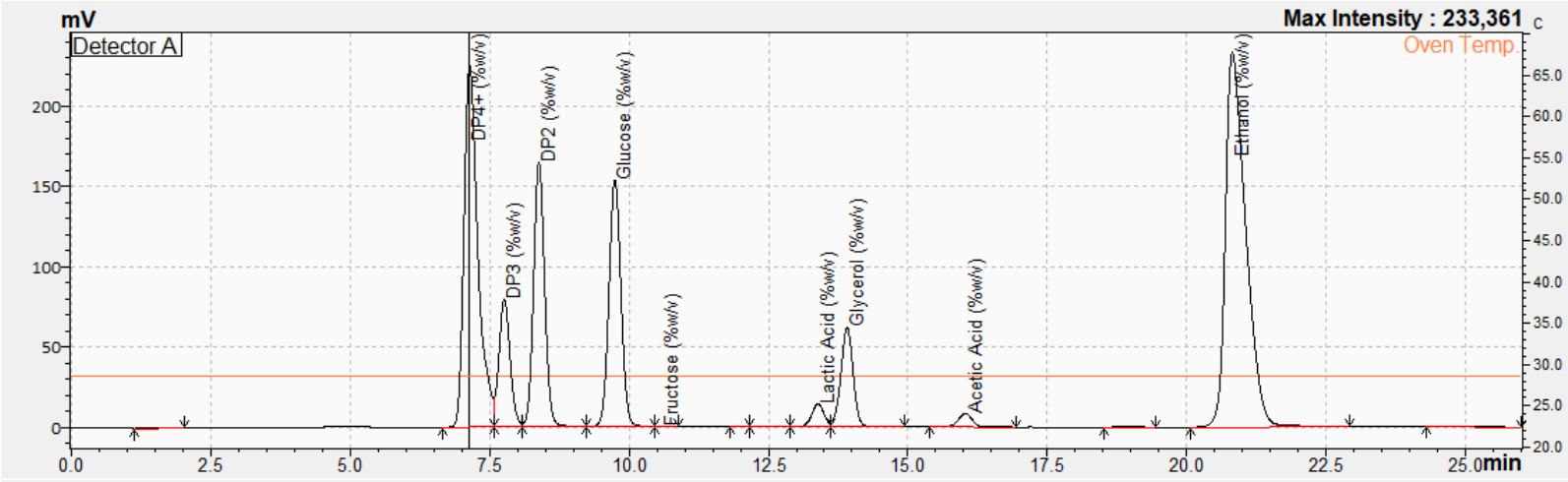
HPLC Chromatogram - Temperature





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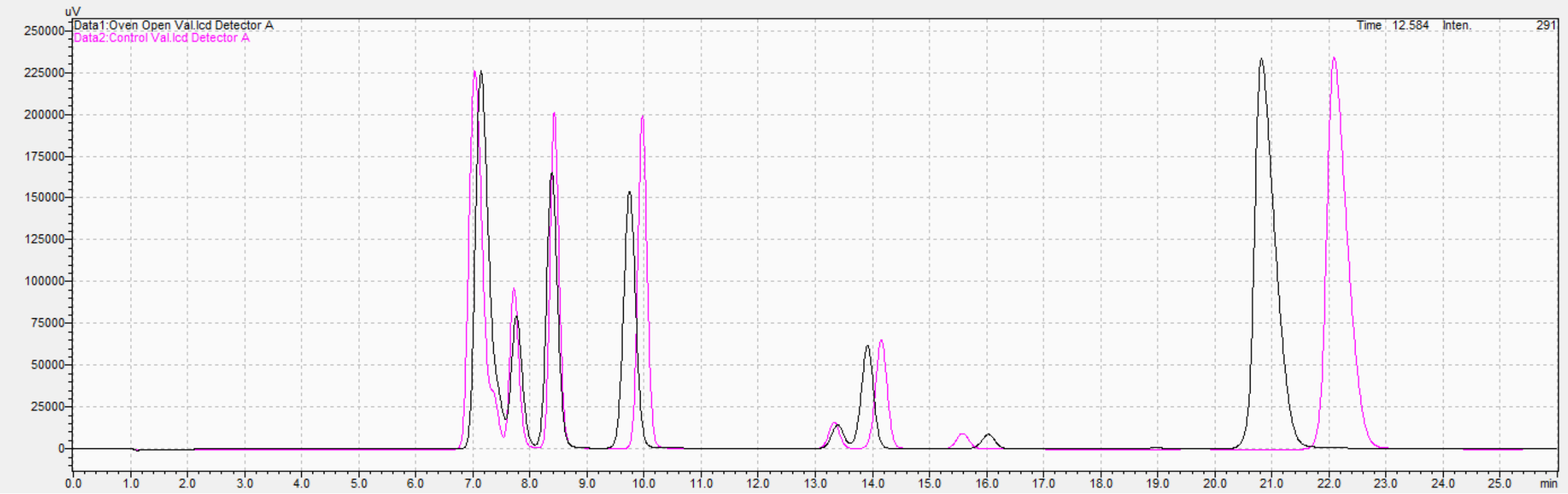
HPLC Chromatogram - Temperature



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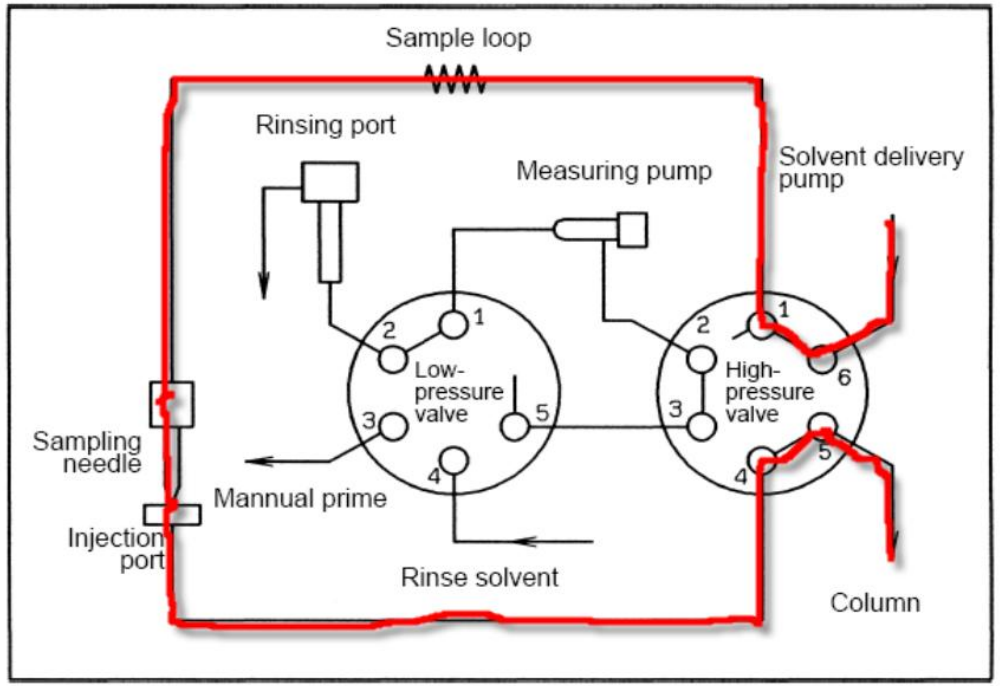
Troubleshooting HPLC

- Always work backwards from the column to identify issues
- Common culprits of clogs
  - High pressure valve
  - Check valves
  - Degassing unit
  - Quick connect to mobile phase



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Summary

Considerations :

- Recognizing Limitations:
  - Shorter columns may provide insufficient resolution for complex mixtures.
  - Proposed Solutions: Utilizing longer columns can help improve resolution and enhance the separation of compounds.
- Calibration Considerations:
  - Multi-Point Calibration:
    - Implementing 3- to 6-point calibration curves offers better representation across different analyte concentrations, improving precision and reducing error.
  - Monitoring Instrument Parameters:
    - Regular checks of key instrument settings and knowing when to recalibrate ensure accurate and reliable results.

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Citations

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Thank you!

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