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Evaluation of Animal Feeds:

Carbohydrate Analysis

on BEH Amide Prototypes

1/14/2010

Review

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- Sample preparation is critical for accurate quantitative analysis of carbohydrates in feed samples
- Enzymatic hydrolysis needs to be arrested for accurate determination of carbohydrates present
  - Use heat or 50% Acetonitrile extraction solvent
  - Un-arrested hydrolysis might provide useful information for total carbohydrates present
- Recommended sample preparation:
  - Extraction of fats may be necessary
  - Extract sugars with **50% ACN**
  - Homogenize for sample for 3 minutes
- Samples analyzed on BEH Amide prototypes
  - Gradient conditions necessary to elute larger polysaccharides

Review

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- First gradient method developed could NOT resolve the glucose/galactose peak pair as required by the Method Needs Statement
- Second gradient method developed utilizing linked columns
- Moderate resolution of these peaks has been obtained
  - Requires longer column (or linked columns)
  - Requires longer gradient method
  - Uses alternative mobile phase system

Carbohydrates: Modified Gradient Conditions

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Carbohydrates: Gradient Conditions	
Mobile phase	A2) 50:50 acetone/acetonitrile with 0.1% NH_4OH B2) 100% MilliQ Water with 0.1% NH_4OH
Gradient	10 minute hold at 80% A2 10 minute Gradient from 80% to 40% A2 5 minute hold at 40% A2 15 minute re-equilibration in 80% A2
Flow rate (mL/min)	0.10 mL/min
Columns	(2 x) 2.1 x 150mm BEH Amide Prototype (1.8µm)
Temperature	35°C
Injection	2µL
Detector	Acquity ELSD
Gain	200
Pressure	40 psi
Drift Tube	40°C
Nebulizer	Cooling
Standard Sample	Food Sugars in 50:50 Acetonitrile/Water – 1mg/ml each (Fructose, Glucose, Sucrose, Maltose and Lactose with p-toluamide)

Total method time: 40 minutes

Results from Previous Method

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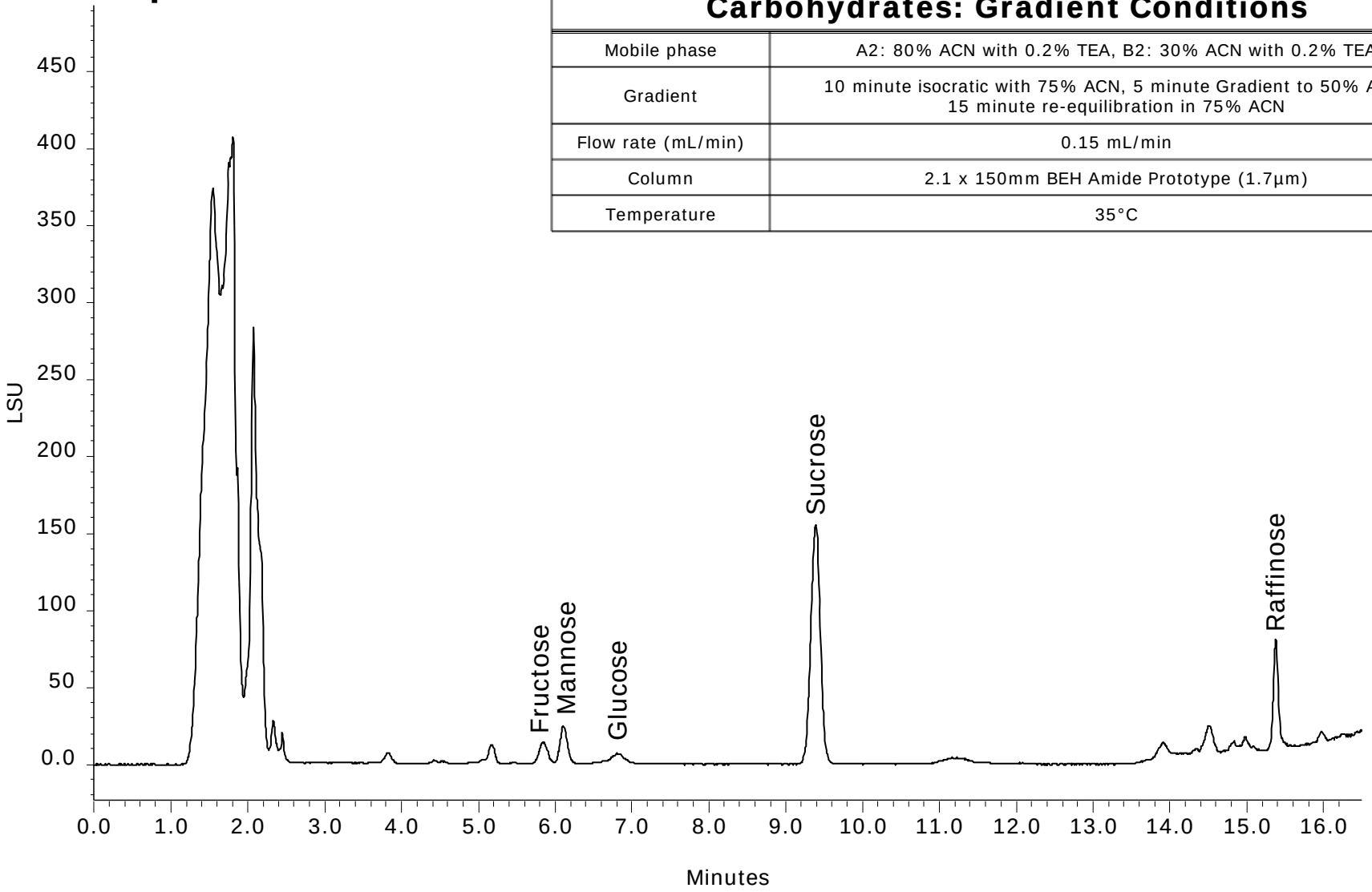
Feed Sample A

Carbohydrates: Gradient Conditions	
Mobile phase	A2: 80% ACN with 0.2% TEA, B2: 30% ACN with 0.2% TEA
Gradient	10 minute isocratic with 75% ACN, 5 minute Gradient to 50% ACN, 15 minute re-equilibration in 75% ACN
Flow rate (mL/min)	0.15 mL/min
Column	2.1 x 150mm BEH Amide Prototype (1.7µm)
Temperature	35°C



Results from Current Method

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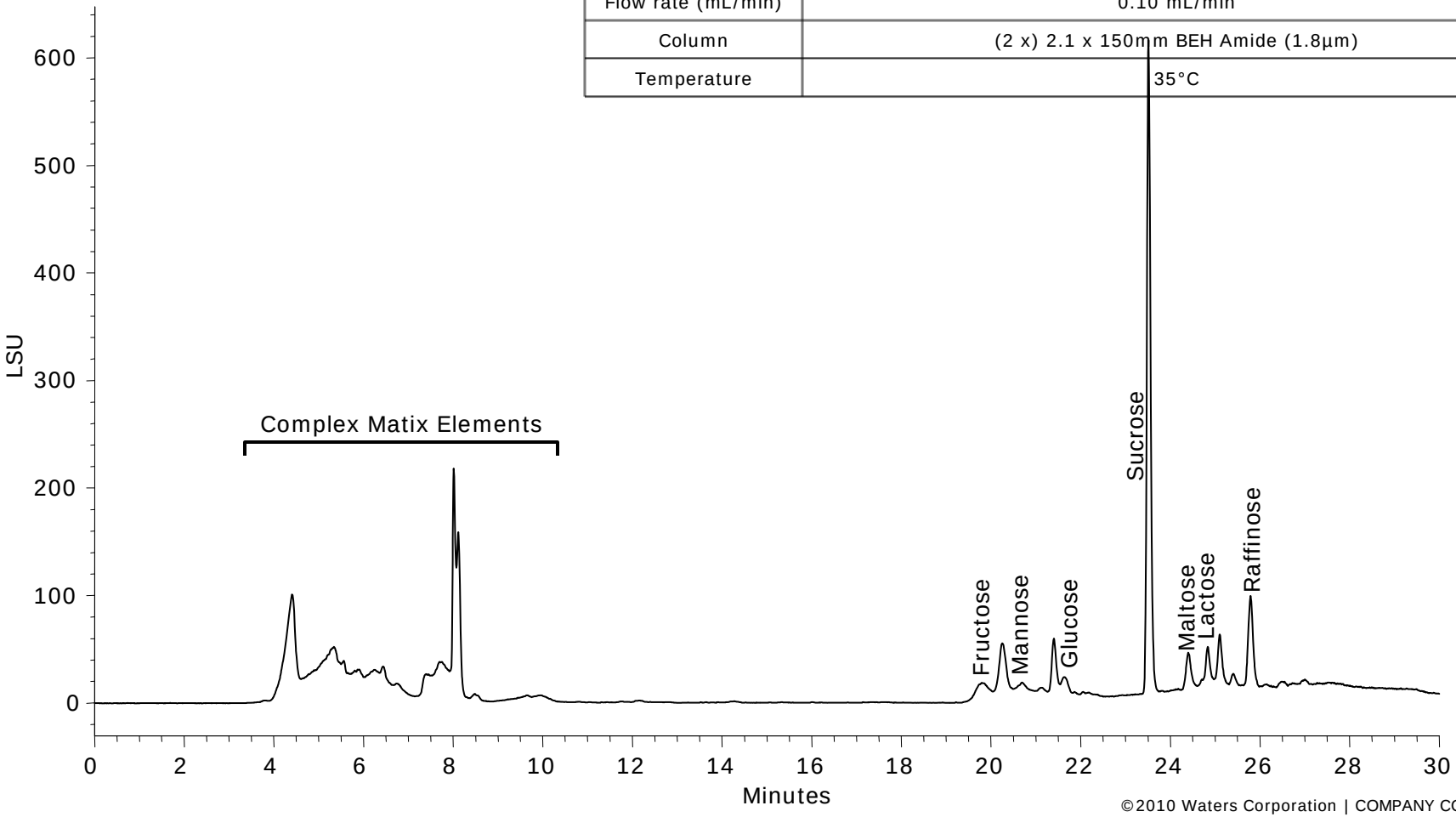
Feed Sample A

Carbohydrates: Gradient Conditions	
Mobile phase	A2: 50:50 Acetone/ACN with 0.1% NH_4OH, B2: 100% H_2O with 0.1% NH_4OH
Gradient	10 minute isocratic with 80% A2, 10 minute Gradient to 40% A2, 5 minute hold at 40% A2, 15 minute re-equilibration in 80% A2
Flow rate (mL/min)	0.10 mL/min
Column	(2 x) 2.1 x 150mm BEH Amide (1.8µm)
Temperature	35°C



Increased Peak Resolution

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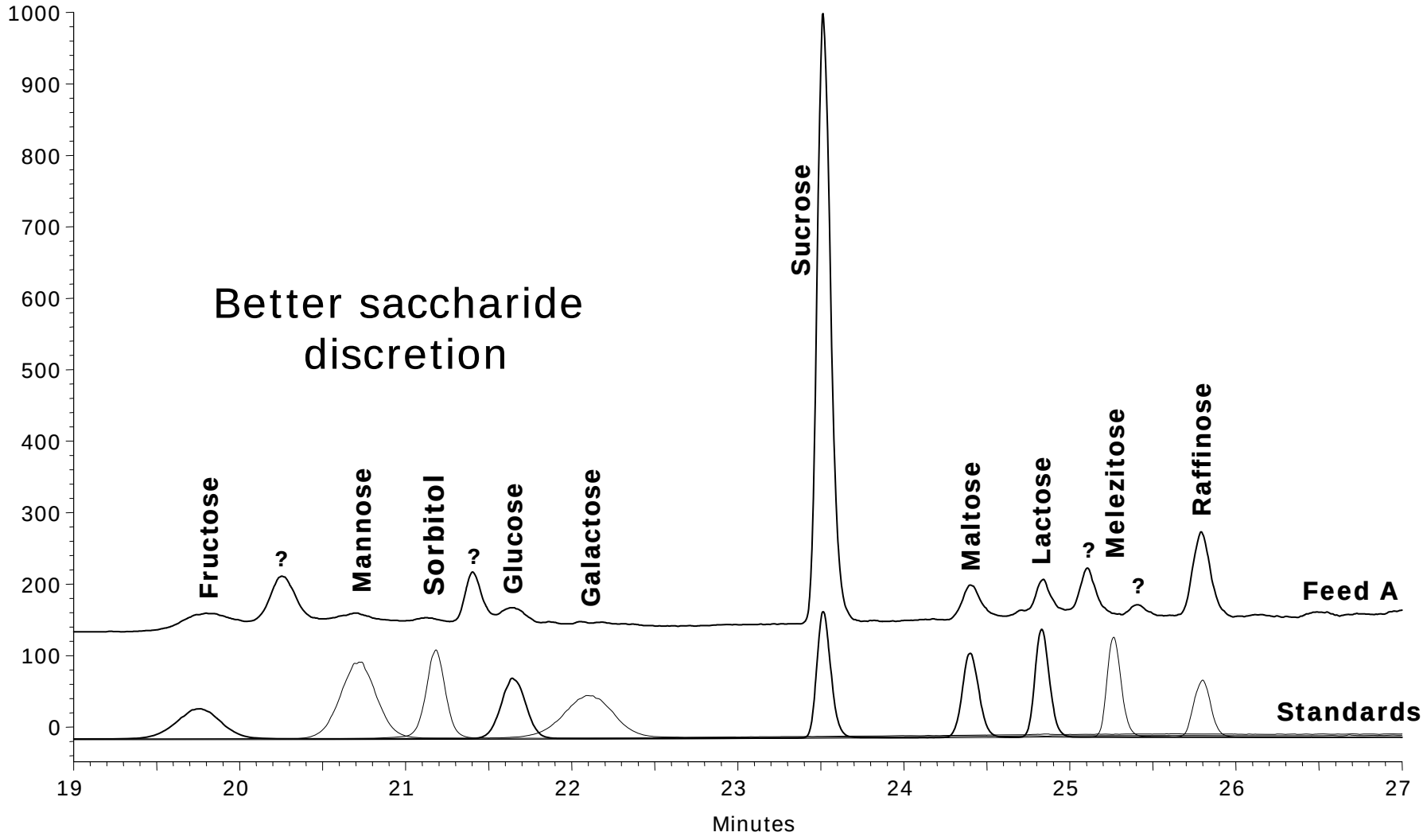

Spiking Experiments

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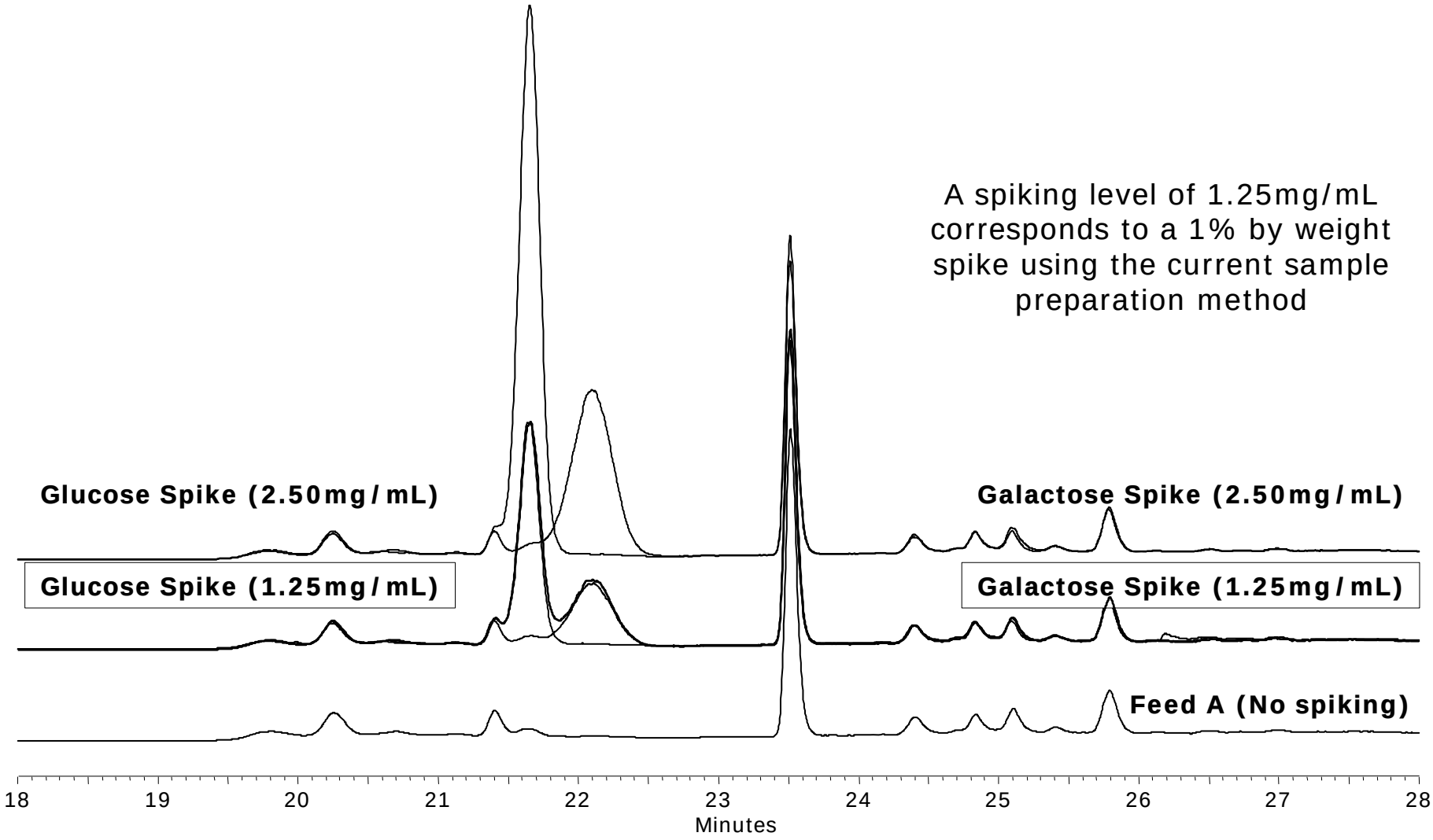
A spiking level of 1.25mg/mL corresponds to a 1% by weight spike using the current sample preparation method



Summary

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- Gradient method using 0.2% TEA modified mobile phase provides excellent injection to injection and column to column reproducibility.
- Glucose/Galactose resolution is not sufficient with the this method.
- Using two 4.6 x 150mm columns with 0.1% NH_4OH modified mobile phase provides significant improvement in the resolution of the glucose/galactose pair
  - Provides better overall discretion between saccharide identities
  - Requires longer time (~ 40 minutes) than previous method
- Spiking experiments show clear distinction between glucose and galactose peaks at 1% by weight spiking level
- Using pyrrolidine as a mobile phase modifier may change selectivity and resolution enough so that a single column may be used to meet required criteria on a single 4.6 x 150mm column.

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Food Sugars Analysis in Feed Samples
New Data

January 18, 2010

Chromatographic Analysis of Carbohydrates

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- Summary Highlights
  - Improved chromatographic conditions
  - Sample preparation
  - Analysis of seed Samples A-K
  - Retention time reproducibility
  - USP resolution of critical pairs

Method

ACQUITY UPLC System with ELS Detection

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- Waters ACQUITY UPLC® system
- Evaporative Light Scattering Detection (ELSD)
  - Not dependent on UV absorbance
  - No derivatization is necessary
- Gradient compatible
- More sensitive than Refractive Index (RI) detection
  - At least an order of magnitude more sensitive
- Non-linear response
  - Requires a calibration curve for quantification

Detector Conditions

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Waters ACQUITY ELS Detection:

Gain:	200 for sucrose and 1000 for the other 5 sugars
Pressure:	40 psi
Drift Tube:	40°C
Nebulizer:	Cooling
Data Rate:	10pps
Filter Time Constant:	Normal
Data Processing:	Savitsky-Golay Smoothing (Level 17)

Gradient Conditions Food Sugars (ELSD)

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Carbohydrates: Gradient Conditions	
Mobile phase	A) 80:20 ACN/H_2O with 0.1% Pyrrolidine, B) 30:70 ACN/H_2O with 0.1% Pyrrolidine
Column	2.1 x 150 mm BEH Amide (1.7 µm)
Sample	Food Sugar Mixture (Fructose, Glucose, Galactose, Sucrose, Maltose and Lactose, each at 1 mg/mL in 50:50 ACN/H_2O)
Temperature	25°C
Injection	1.0µL (5µL loop with partial needle overfill)
Detector	Acquity ELSD
Detector Settings	Data rate: 10 pps, Time Constant: Normal
Gain	200-300*
Pressure	40 psi
Drift Tube	40°C
Nebulizer	Cooling
Needle Wash	800µL Strong needle wash of 20:80 ACN/H_2O, 500µL Weak needle wash of 75:25 ACN/H_2O
Seal Wash	Seal wash in 50:50 ACN/H_2O
Data Processing	Savitsky-Golay Smoothing (Level 17)

•*ELSD normalized and calibrated to Acetaminophen Test Solution (Part No. 700002387)*

Gradient Conditions

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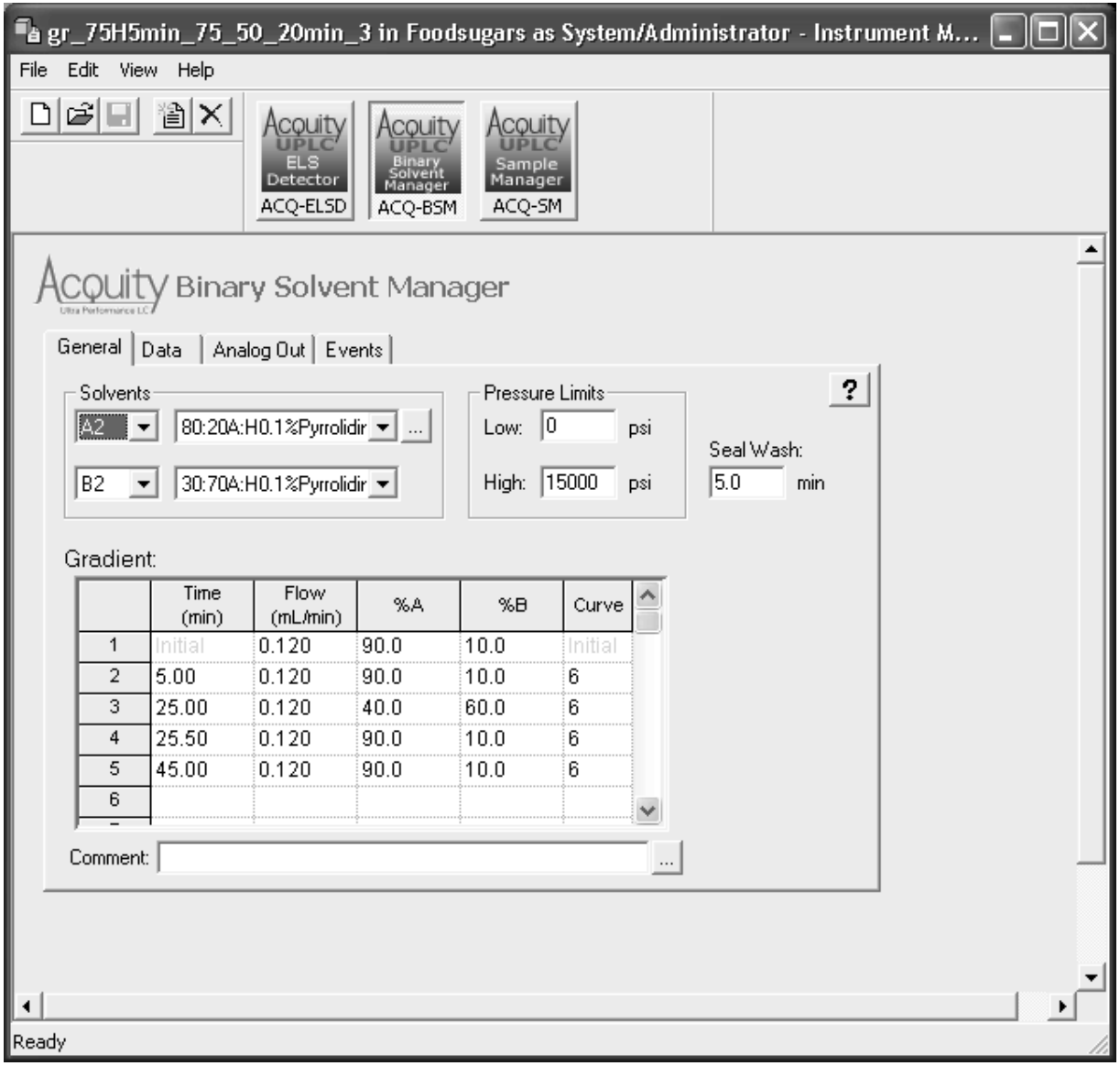

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.120	90.0	10.0	Initial
2	5.00	0.120	90.0	10.0	6
3	25.00	0.120	40.0	60.0	6
4	25.50	0.120	90.0	10.0	6
5	45.00	0.120	90.0	10.0	6
6					

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Sample Preparation

Sample Preparation

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- Very simple sample preparation
- Extraction of Solid Samples
  - Weigh out sample (~3g) into 50mL centrifuge tube
  - Add 25mL of 50:50 ACN/H_2O and homogenize for at 3 minutes (mechanically)
  - Centrifuge at 4000rpm for 30 minutes
  - Collect supernatant and filter using 0.45µm PVDF syringe filter
- Depending on sample or detection method, additional sample dilutions may be necessary

Chromatograms

FEED SAMPLES (A-D)
Full Scale

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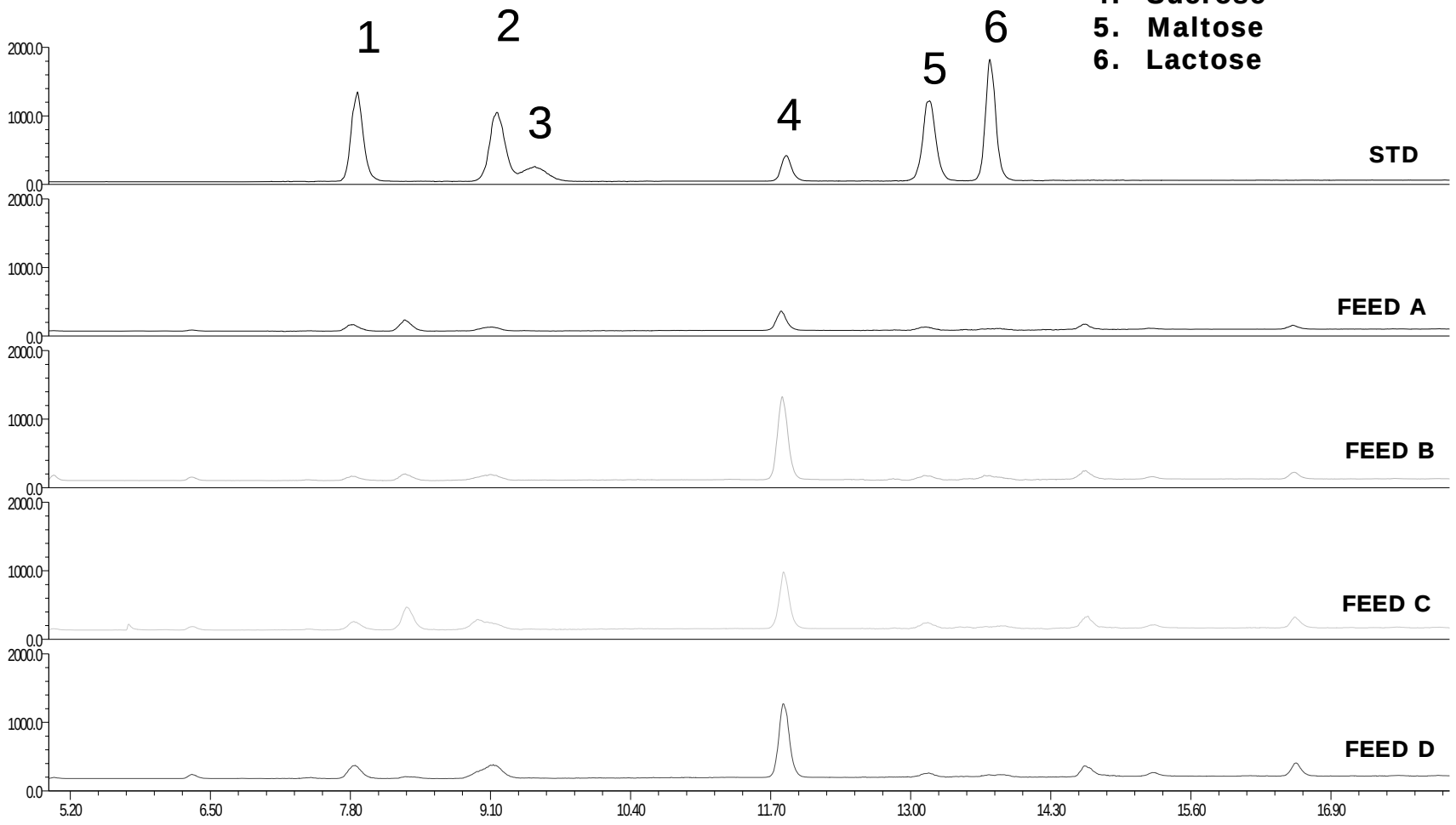
1. Fructose
2. Glucose
3. Galactose
4. Sucrose
5. Maltose
6. Lactose



FEED SAMPLES (A-D)
Expanded Scale

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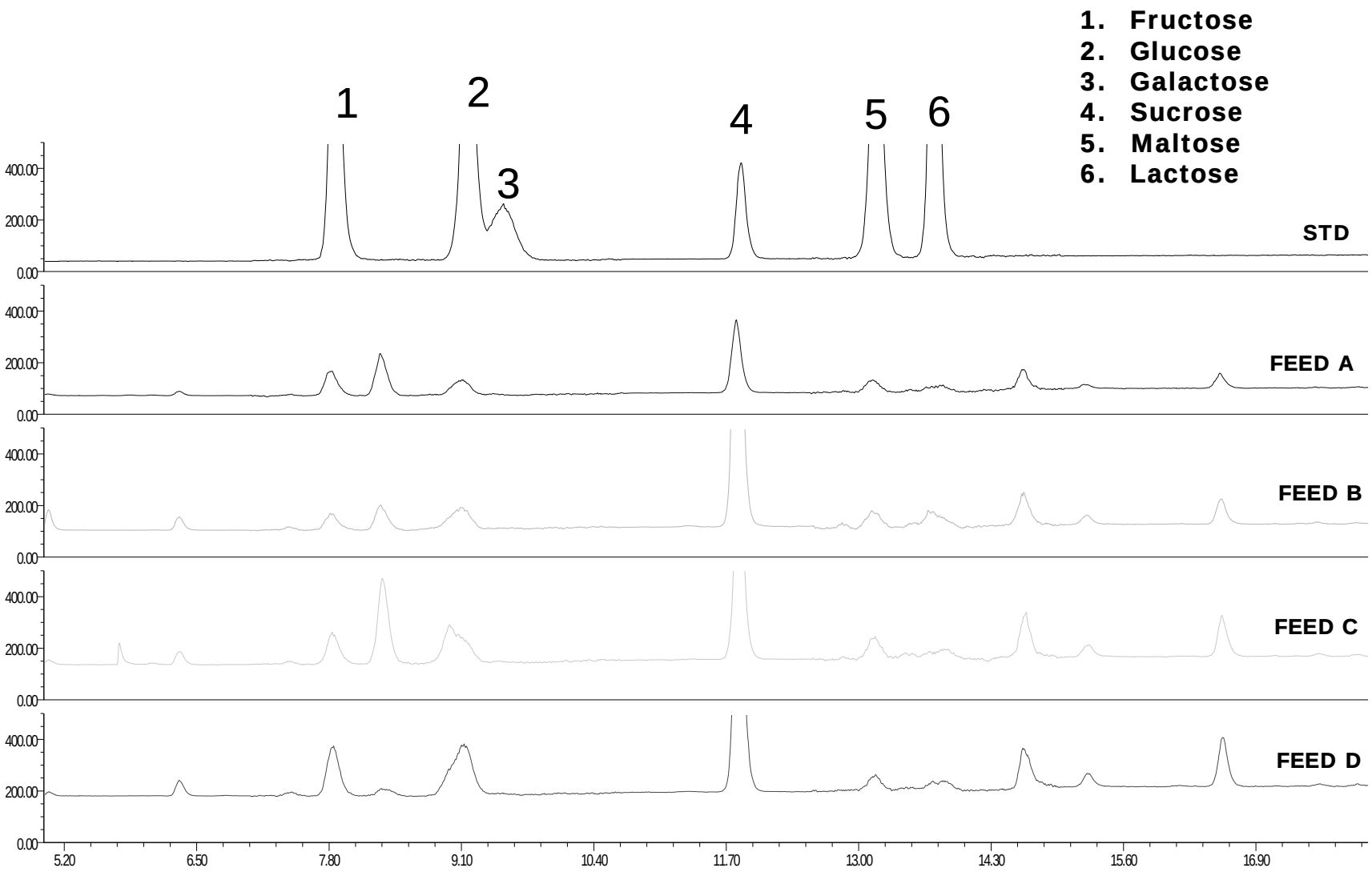

FEED SAMPLES (E-H)
Full Scale

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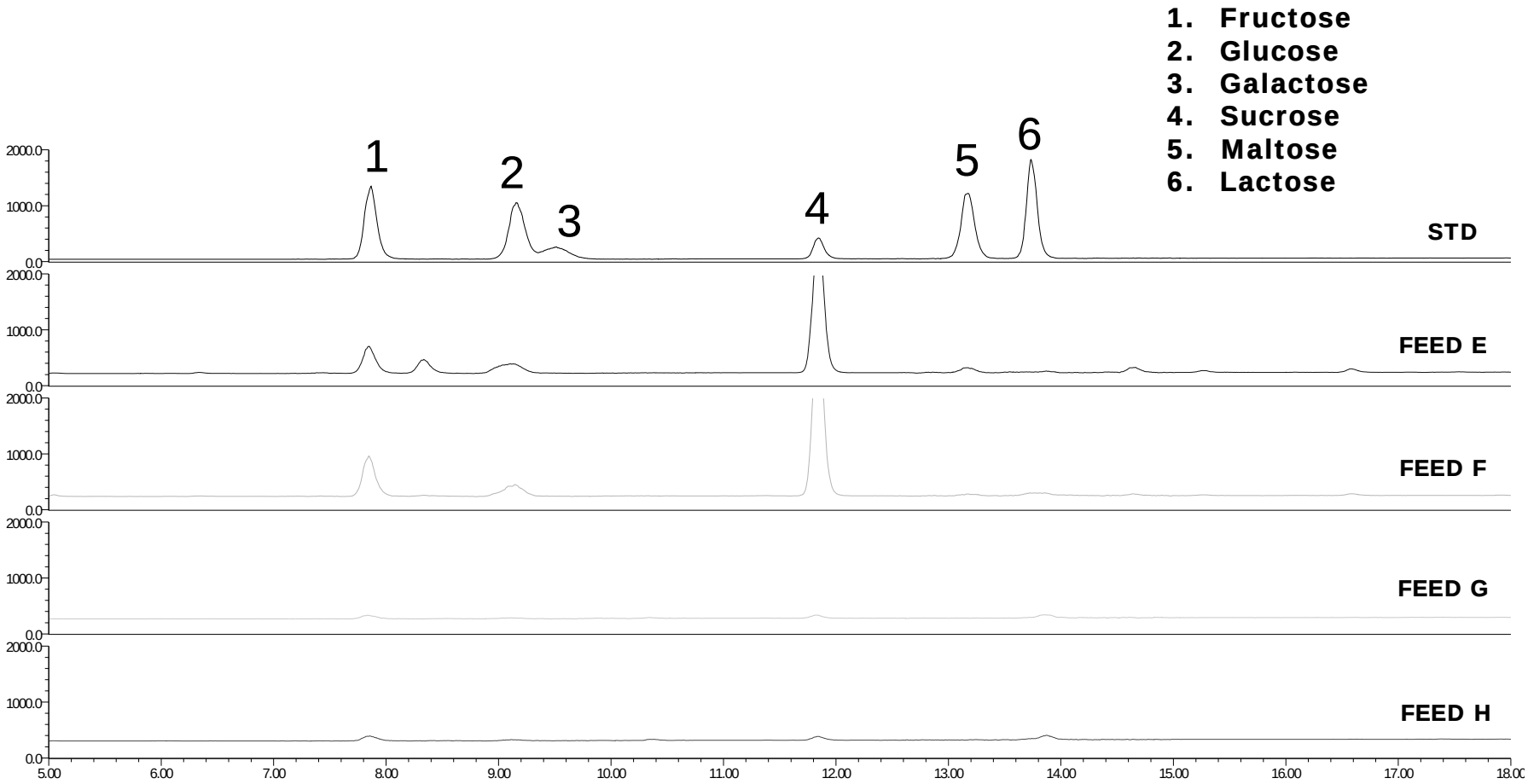

FEED SAMPLES (E-H)
Expanded Scale

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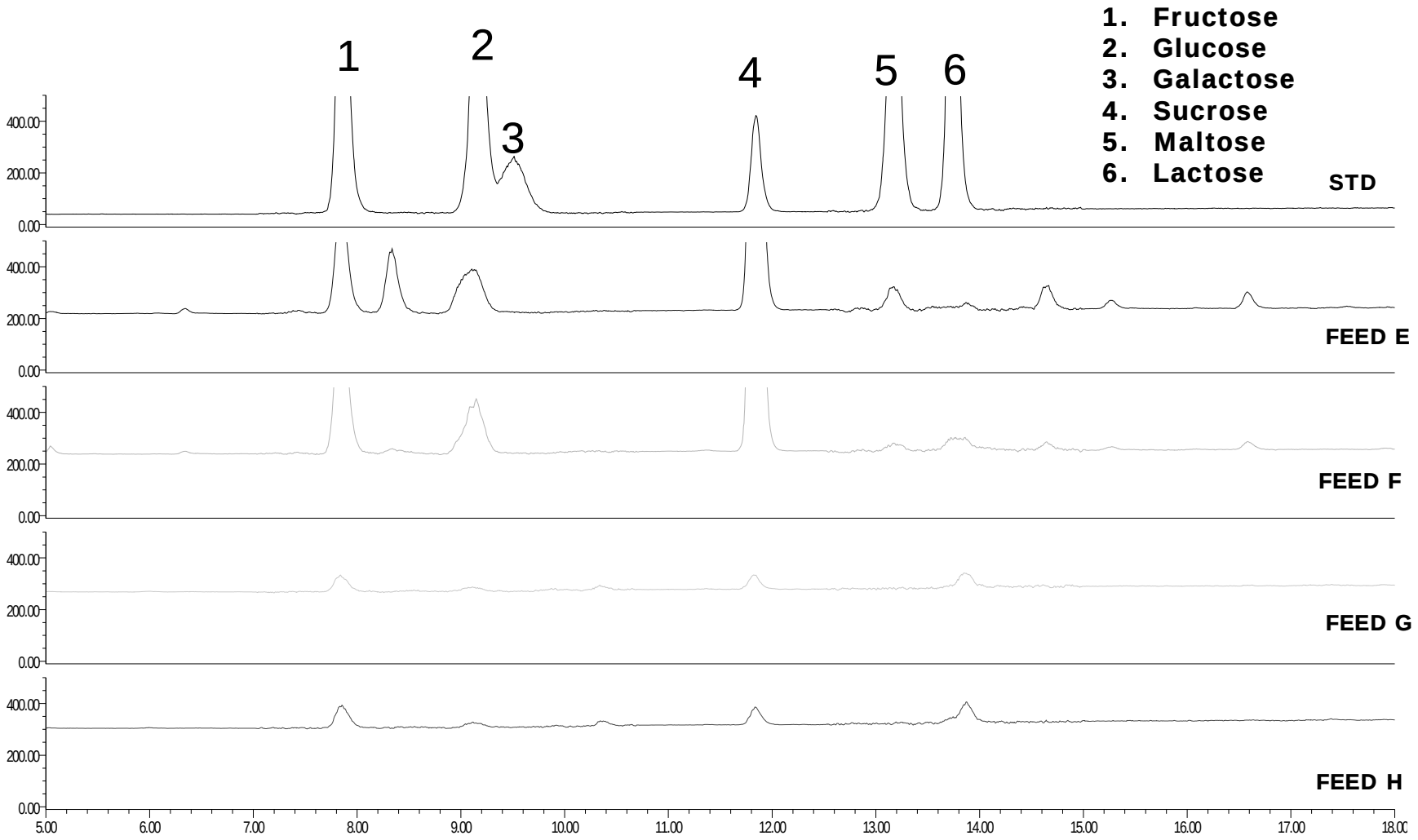

FEED SAMPLES (I-K)
Full Scale

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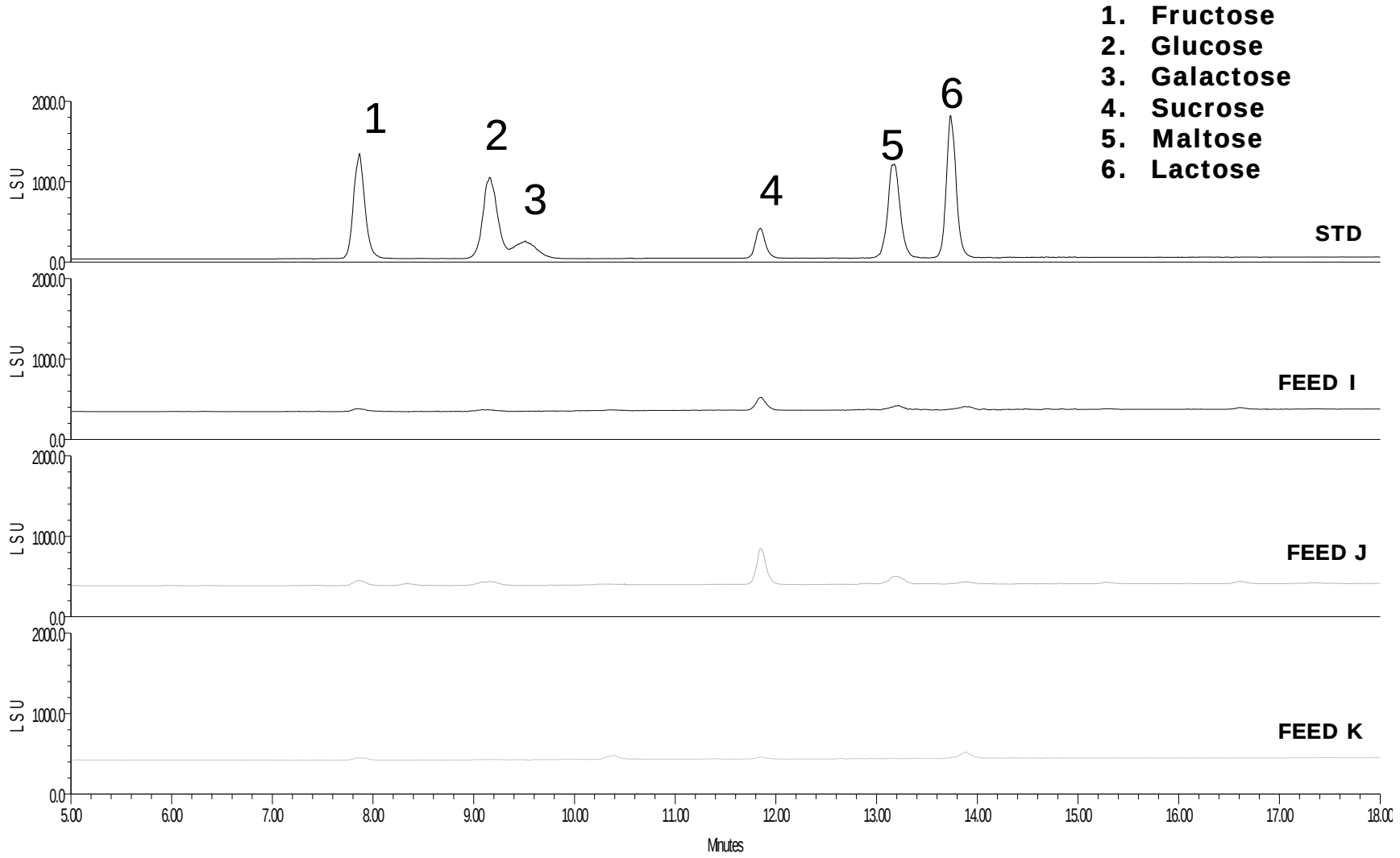

FEED SAMPLES (I-K) Expanded Scale

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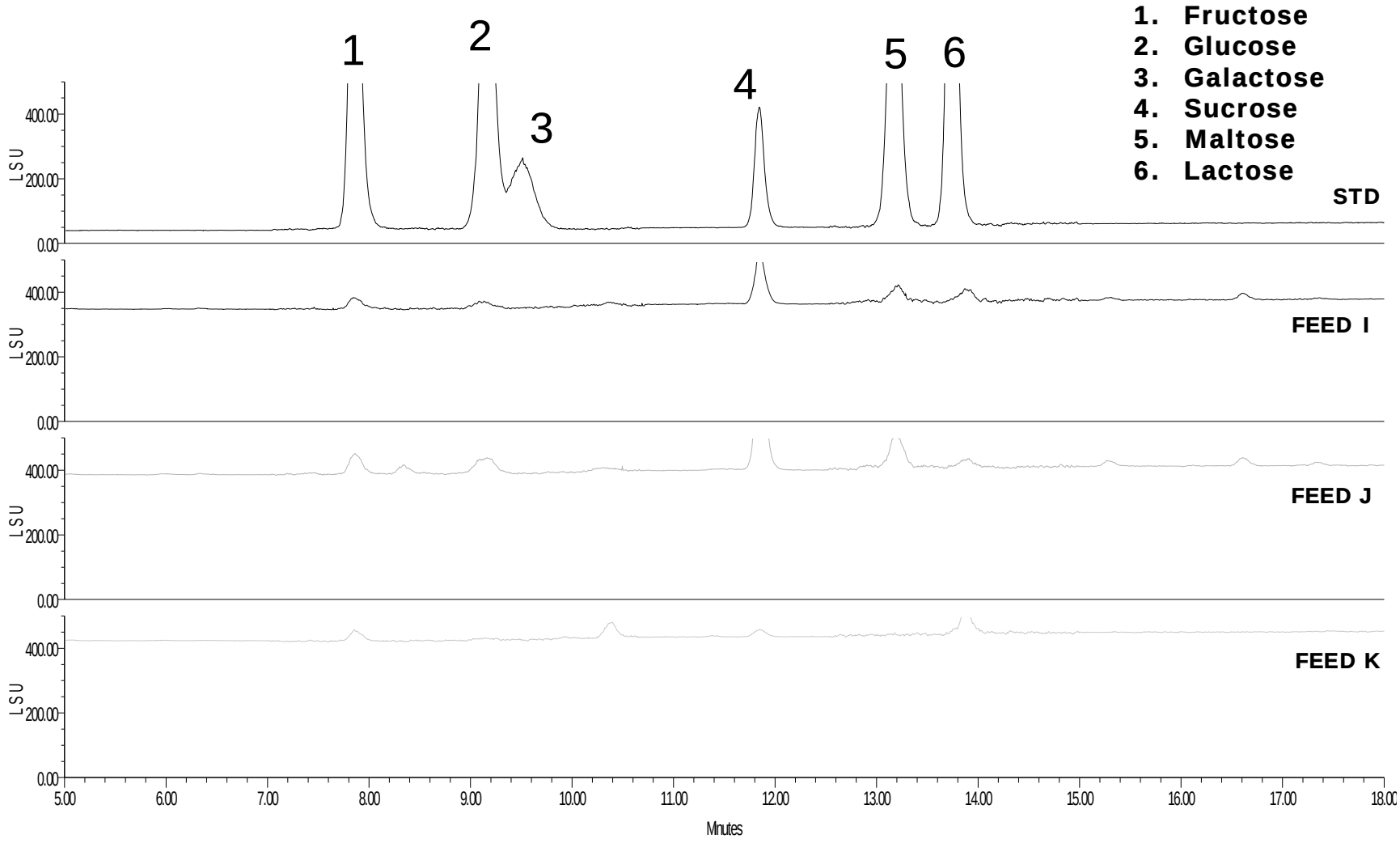

Retention Time Reproducibility

Retention Time Reproducibility (Sugars Spiked into Feed Sample K)

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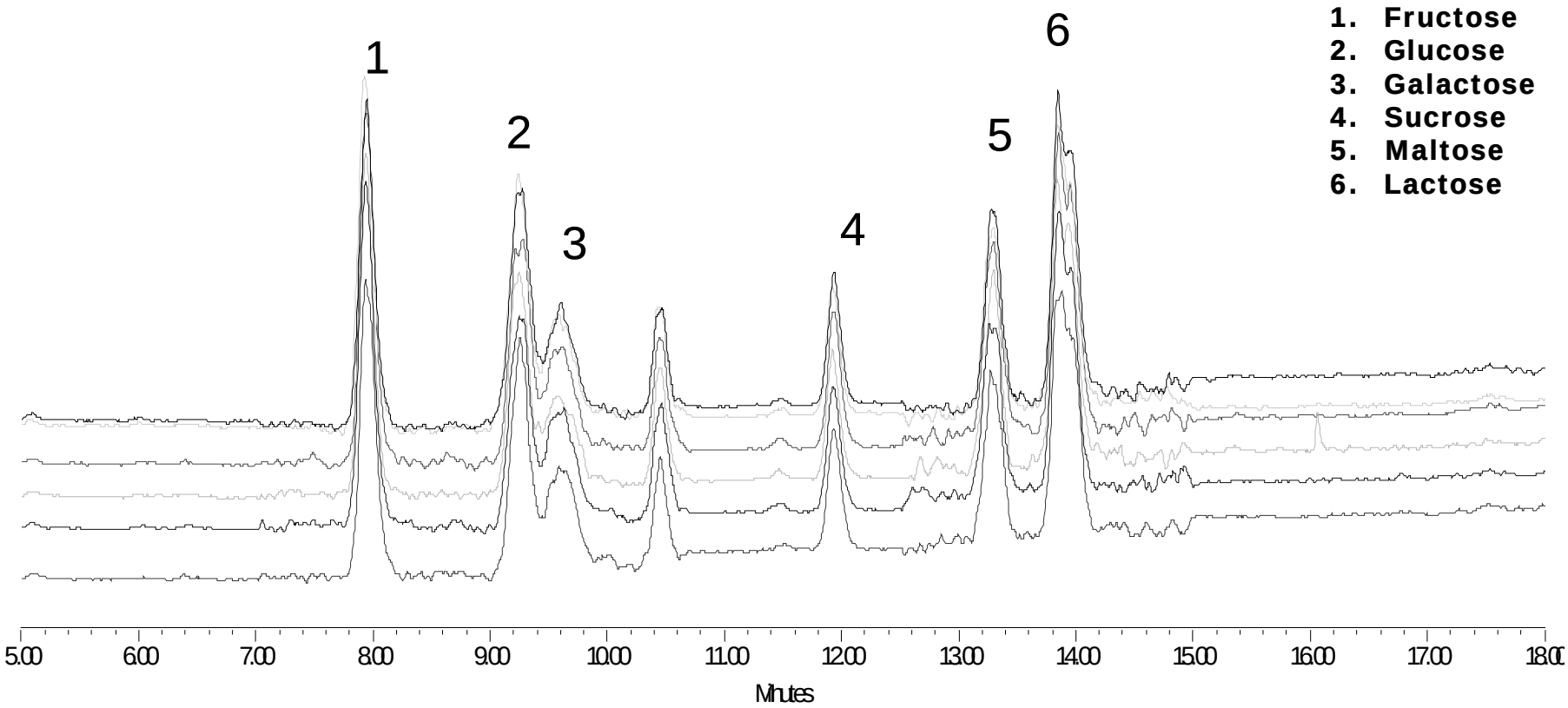

Each sugar was spiked at 0.1% (galactose spiked 0.15%)

Precision of Retention Times in Spiked Samples

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Name	Retention Time	%RSD
Fructose	7.95	0.12
Glucose	9.28	0.18
Galactose	9.60	0.25
Sucrose	11.93	0.05
Maltose	13.28	0.13
Lactose	13.85	0.10

- Average retention times in the spiked samples of feed K (six replicas)
- Each sugar was spiked at 0.1% except galactose (spiked at 0.15%)

Resolution between Glucose and Galactose

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Feed Sample K	USP Resolution of Glucose & Galactose Peaks
Spiked # 1	0.891
Spiked # 2	0.721
Spiked # 3	0.676
Spiked # 4	0.829
Spiked # 5	0.809
Spiked # 6	0.660

Acknowledgements

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