The Use of Hydrophilic Interaction Chromatography with Mass Spectrometry Detection for the Separation of Basic Pharmaceuticals and Food Toxins

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Outline of Talk

- Problems with polar compounds and RPLC
- What is HILIC?
- What types of samples can it be used for?
- What types of columns and mobile phases can be used?
- Advantages & Disadvantages of the Technique
- Comparison of RPC and HILIC
- Some applications using LC-MS/MS
- Conclusions

Polar Compounds and Reversed-Phase HPLC

- 92% of all users employ RPLC in their laboratory at one time or another*
- Separation based on hydrophobicity of analytes
- Polar compounds tend to elute early; the more polar, the earlier the elution
- For very polar compounds, in order to increase retention, the amount of organic modifier in mobile phase is reduced
-
  - When % organic becomes very low (say <5%), phase collapse (solvent dewetting) may occur
  - Quantitation is impaired for analytes with k'<1
- There are special RPC phases such as polar-embedded, hydrophilic endcapped, wide pore/lower density bonded phases, & short chain phases to increase retention at high aqueous but there may be a decrease in MS sensitivity due to poor mobile phase desolvation and ion suppression

*** R.E. Majors, LCGC Survey, 2007**

Another Way to Increase Retention for Polar, Ionizable Compounds is to Use Ion Pair RPC

- Adjust pH to ionize analyte and add to the mobile phase an alkyl counterion (e.g. sulfonate for cations or tetrabutyammonium for anions) to form ion pair which increased retention
  - Ion pair reagents modify columns
  - Equilibration time is slow, esp. for long alkyl chain IP reagents
  - MS systems don't like typical ion pair reagents typically used with UV detection

A Third Way to Increase Retention is to Derivatize the Analytes to Provide Increased Retentioin

- Requires an additional chemical synthesis step; a hassle
- Derivatization is not always quantitative
- Sometimes extraneous peaks are introduced

Could Use Normal Phase HPLC

- Must carefully control water content of mobile phase (especially with silica)
- Many chromatographers aren't familiar with the use of NP HPLC
- Some of the normal bonded phase columns aren't very stable
- Water-immiscible organic solvents are frequently used (e.g. hexane, dichloromethane)

...Or Use Hydrophilic Interaction Liquid Chromatography (HILIC)

- What is HILIC?
  - A liquid chromatographic technique that has been around for a couple of decades (Andy Alpert's early work or sugars on amino columns using H20/ACN)
  - Uses a polar stationary phase such as silica, amino, mixed mode, zwitterionic, etc.
  - Uses a water-miscible, non-polar mobile phase containing a small amount of water (at least 2.5% by weight)
  - May use volatile buffers for compatibility to MS detection

Characteristics of HILIC

- **Hydrophilic, polar and charged compounds are retained preferentially compared to hydrophobic, neutral compounds---opposite of RPLC.**
  - **e.g. toluene is unretained while uracil is retained**
- **Addition of water to mobile phase reduces retention**
- **Provides good peak shape for strongly polar solutes compared to normal phase**
- **Complements RPLC; retains hydrophilic compounds and often reversed elution order**
- **Sometimes called “aqueous normal phase (ANP)”**

Parameters That May Need Optimization

- Type of stationary phase
- Organic solvent concentration
- Type of buffer
- Buffer (salt) concentration
- pH
- Temperature

Advantages of HILIC

- Good peak shape for basic compounds where RPC may give tailing and/or low efficiency
- Higher flow rates and/or long columns can be used due to low viscosity mobile phases with high organic content; greater efficiency
- Enhanced detection sensitivity with MS
  - Efficient spraying and desolvation in electrospray MS
  - As much as 3X sensitivity
- Can directly inject extracts from C18 SPE cartridges or acetonitrile precipitated plasma supernatant
- Orthogonal to RPC (2D separations); elution order reversal

Disadvantages of HILIC

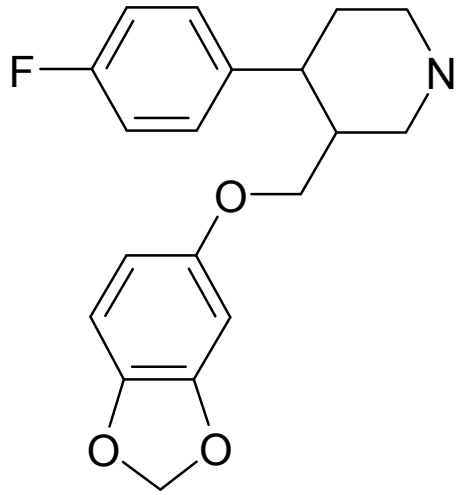
- Mechanism not entirely understood; mixed mechanisms; studies are being done here
- Column overloading can be a problem
  - Similar to silanol overloading in RPC
  - Overloaded HILIC peaks show fronting
- Equilibration times can be long for certain column types

Typical Columns Used in HILIC
(Silica- and Polymer-Based)

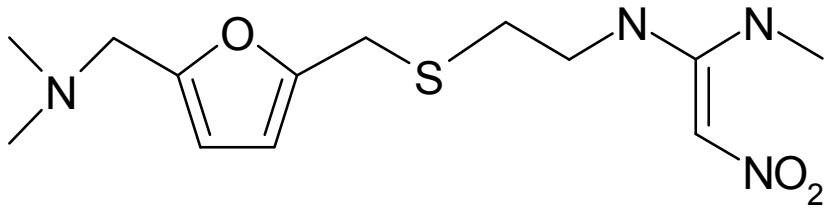
- Silica
- Amino
- Mixed-mode
  - Alkyl-diol - C18-amide
  - Alkyl-carboxyl - Phenyl-amino
  - Aromatic-cyano - Alkyl hydroxyl
  - C18-SCX - Polyhydroxyethylaspartamide
- Hydride and modified hydride
- Zwitterionic (e.g. tetraalkylamine-sulfonic)
- Proprietary phases

Structures of Drug Compounds Studied

a) Paroxetine
Antidepressant
MW 329.36



b) Ranitidine
Antiulcerative
MW 314.41



Chromatographic Conditions

RPLC

- Instrument: Agilent Series 1100LC
- Column: ZORBAX Eclipse XDB-C18, 2.1 mm × 150 mm, 5 µm)
- Mobile phase: A: 8-mM HCOONH4 in water
  B: 8-mM HCOONH4 in 95% acetonitrile (ACN)/5% water
- Gradient: 5% B to 90% B in 10 min
- Column temperature: 40 °C
- Sample volume: 5 µL
- Flow rate: 0.3 mL/min

HILIC

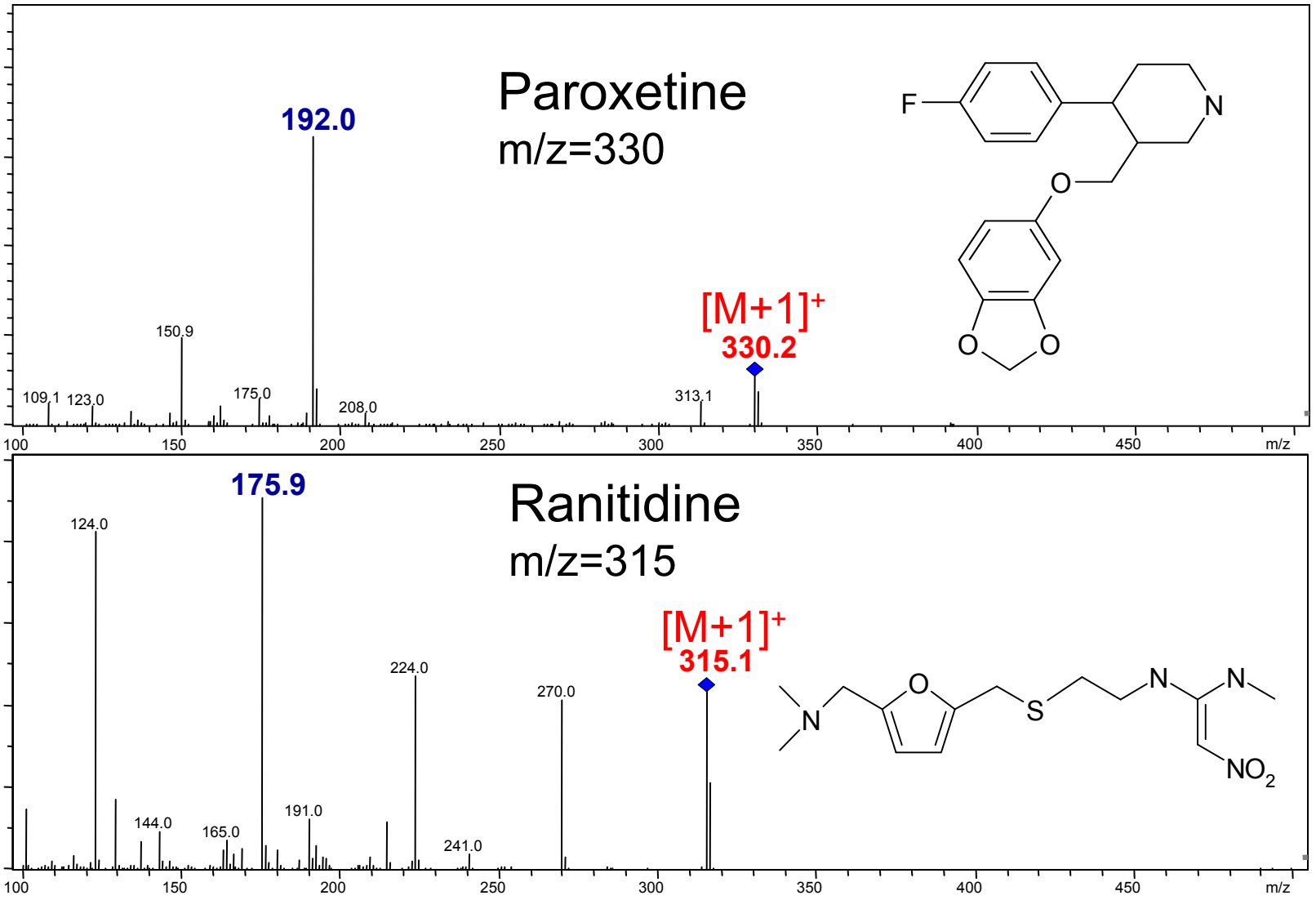
- Everything same as for RPLC except for column and gradient
- conditions:
- Column: ZORBAX RX-SIL, 2.1 mm × 150 mm, 5 µm
- Gradient: 100% B to 50% B in 10 min

Mass Spec Conditions

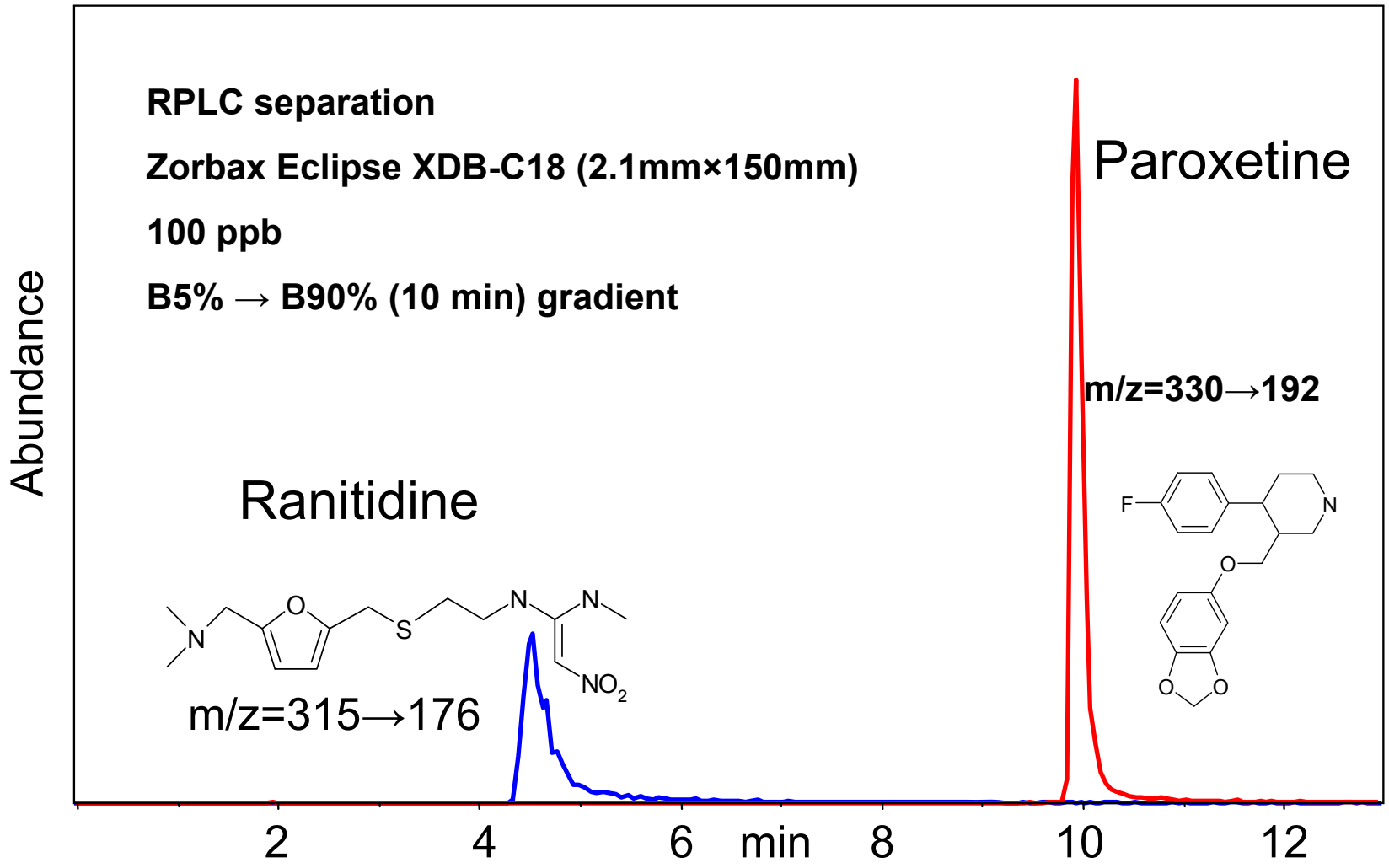
- Instrument: Series 1100 LC/MSD Trap
- Ionization: Positive ESI
- Scan range: 100–500 *m/z*
- SIM ions: *m/z* = 315, 330
- Drying gas: 10 L/min at 350 °C
- Nebulizer gas: 45 psi
- Fragmentor voltage: 0.25 V

MS/MS Spectra of Drug Standards

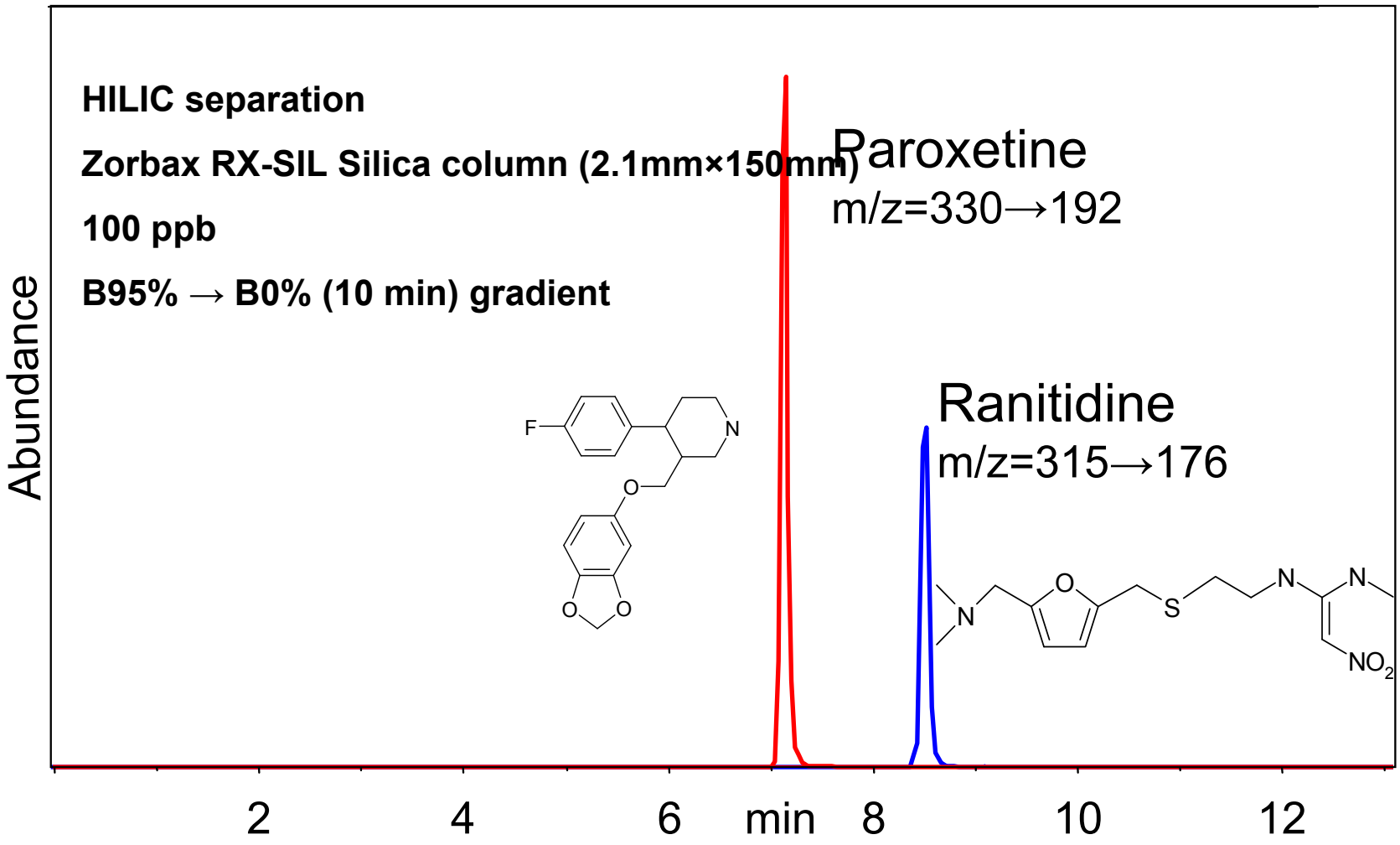
□□precursor ion selected for subsequent fragmentation



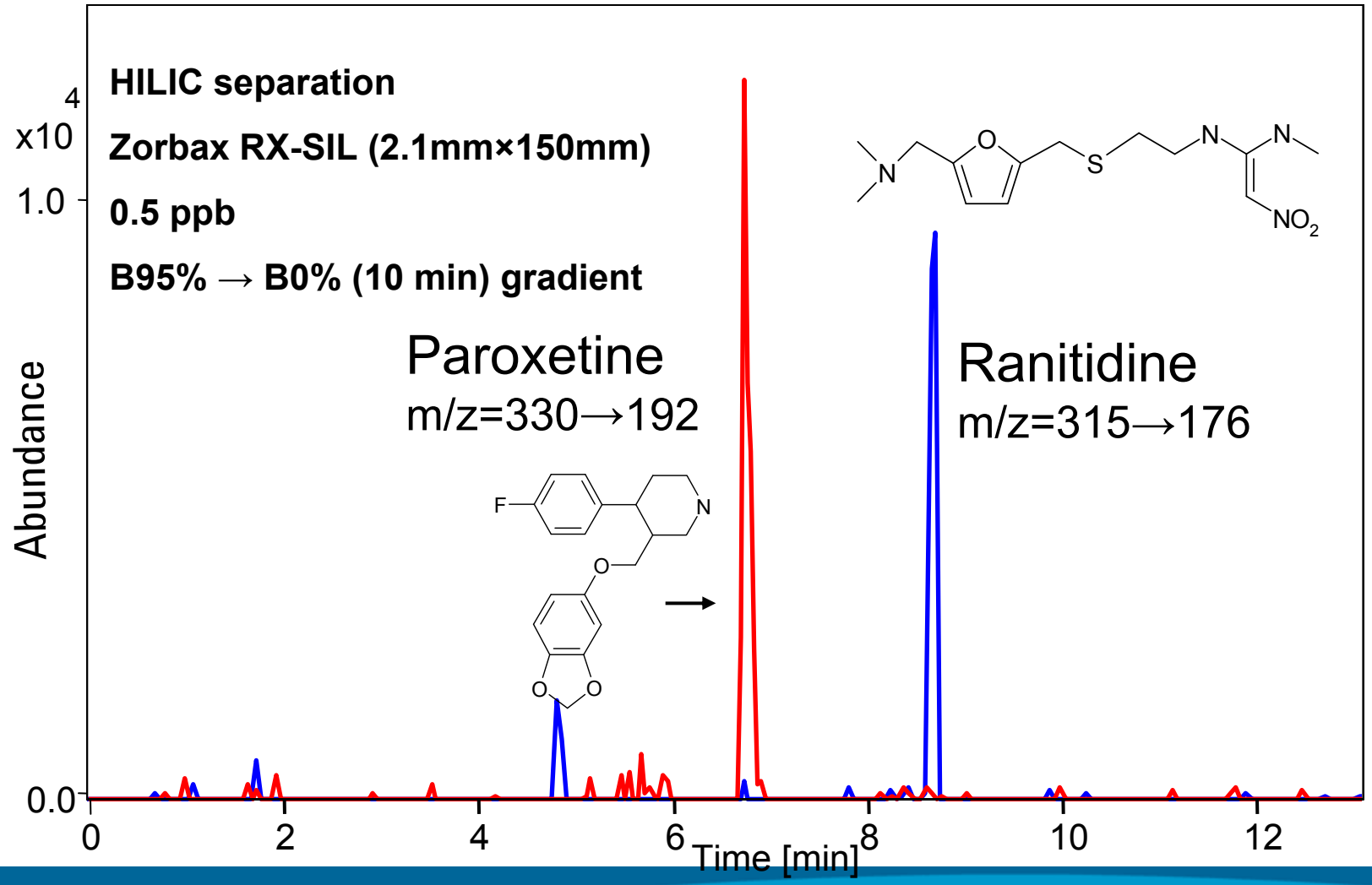
LC/MS/MS Separation of Paroxetine and Ranitidine on Zorbax Eclipse XDB-C18 Column (RPLC Mode)



LC/MS/MS Separation of Paroxetine and Ranitidine on Zorbax RX-SIL Column (HILIC Mode)- 100 ppb Level



LC/MS/MS Separation of Paroxetine and Ranitidine on Zorbax RX-SIL Column (HILIC Mode)- 0.5 ppb Level



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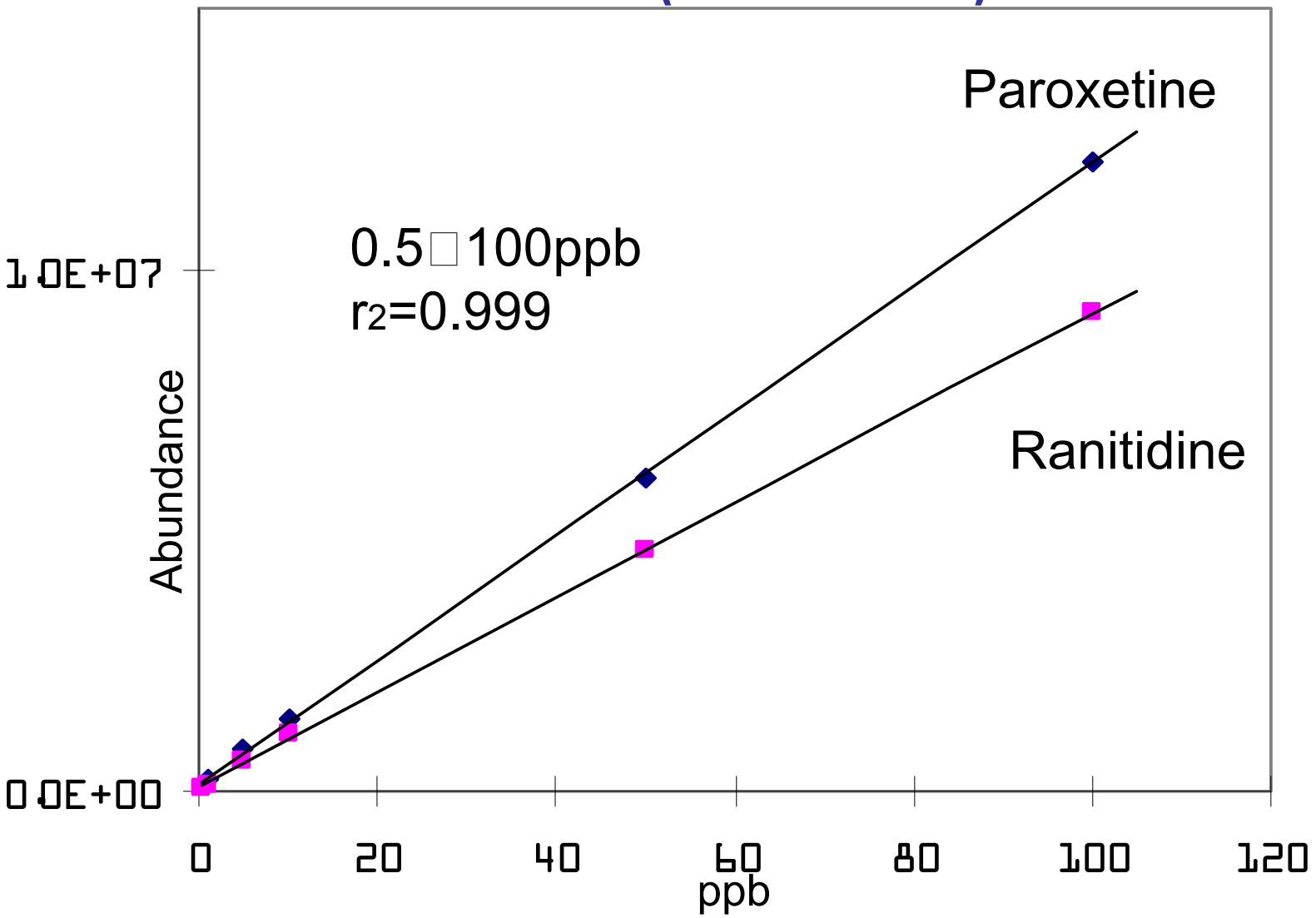
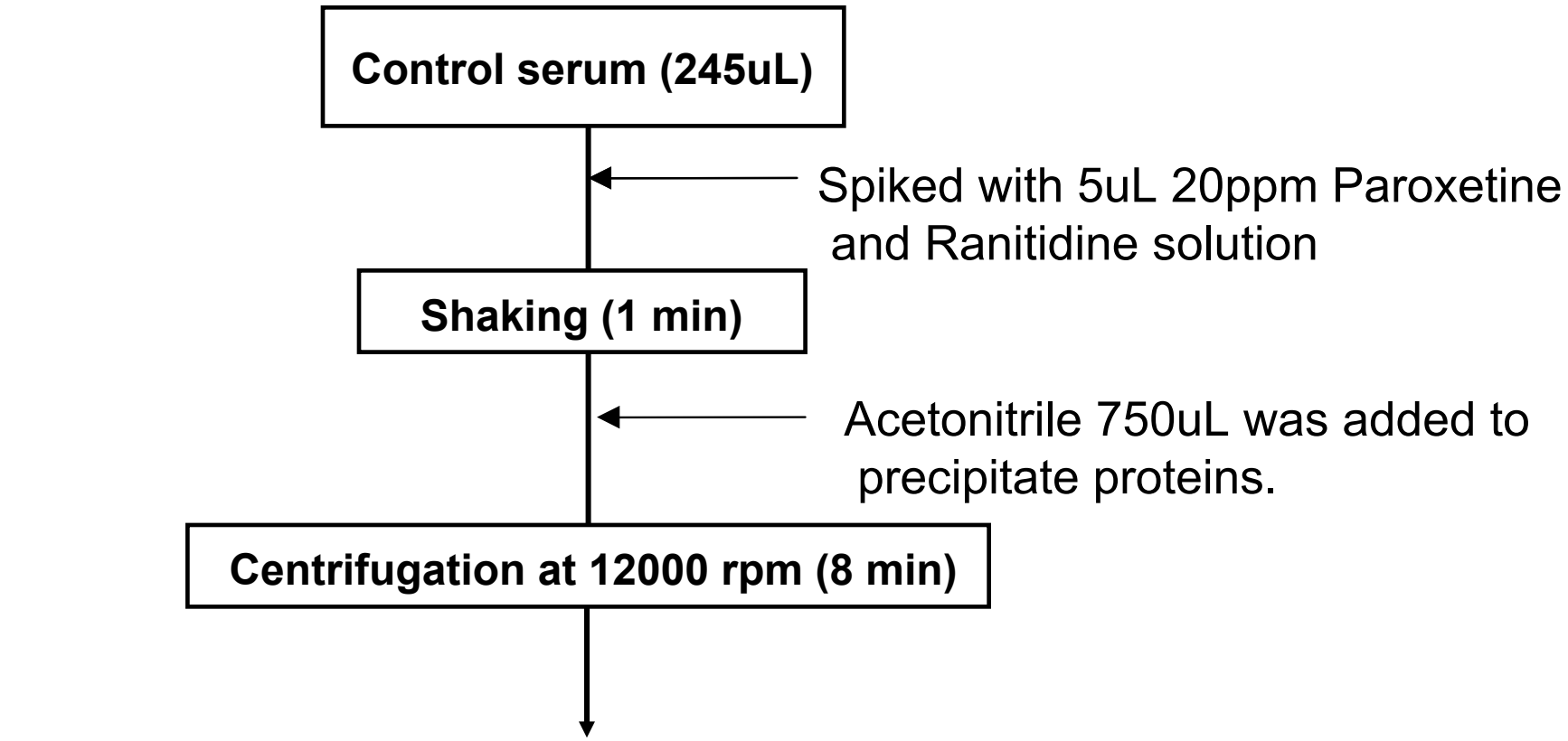
Linearity of Paroxetine and Ranitidine on Zorbax RX-SIL Column (HILIC Mode)

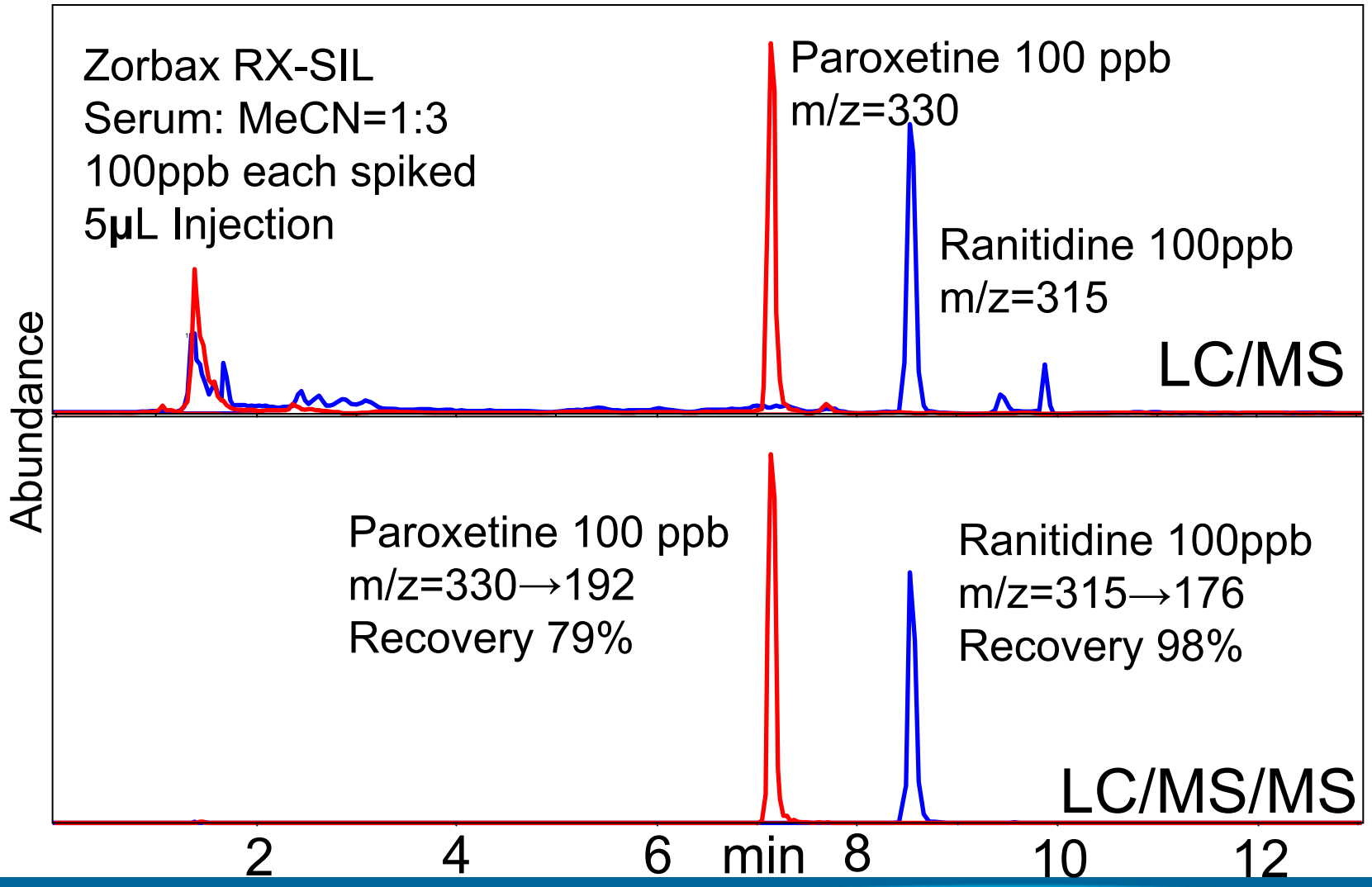


Sample Preparation Procedure of Spiked Serum

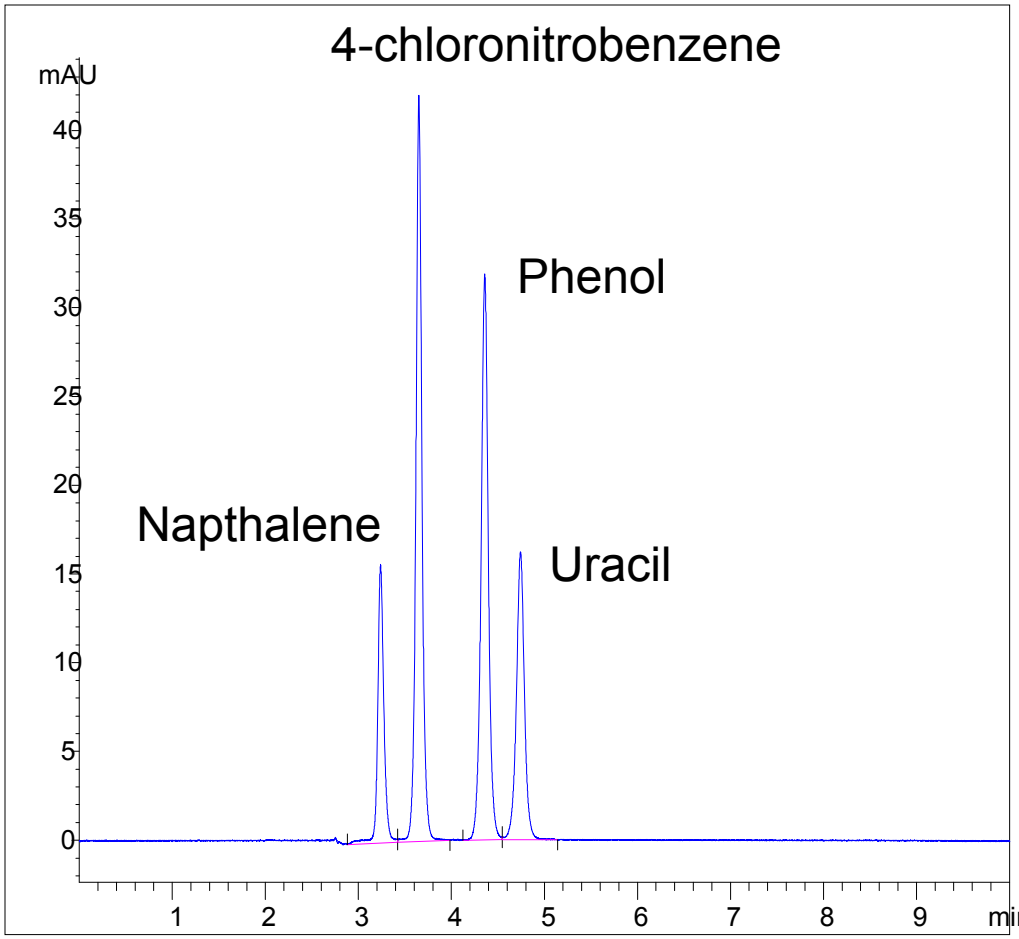


Solution phase (5uL) was directly injected into HPLC.

LC/MS and LC/MS/MS Analysis of Paroxetine and Ranitidine Spiked into Human Serum with Zorbax RX-SIL Column

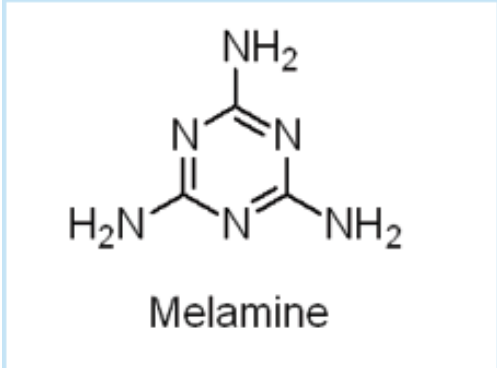


Typical Elution Order for Test Compounds on HILIC Column

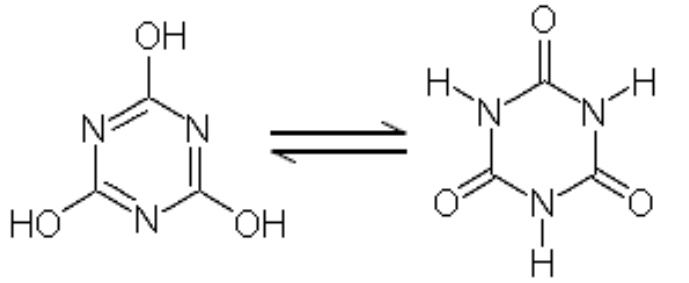


Column: Experimental Modified Hydride, 100 Å,4.6 x 150 mm; 4 um
Mobile phase:75% ACN /25% H2O
Flow Rate = 0.5 ml/ml
Temp = 25 °C
Injection Volume = 5 µl

Melamine and Cyanuric Acid Analysis in Pet Food



Melamine

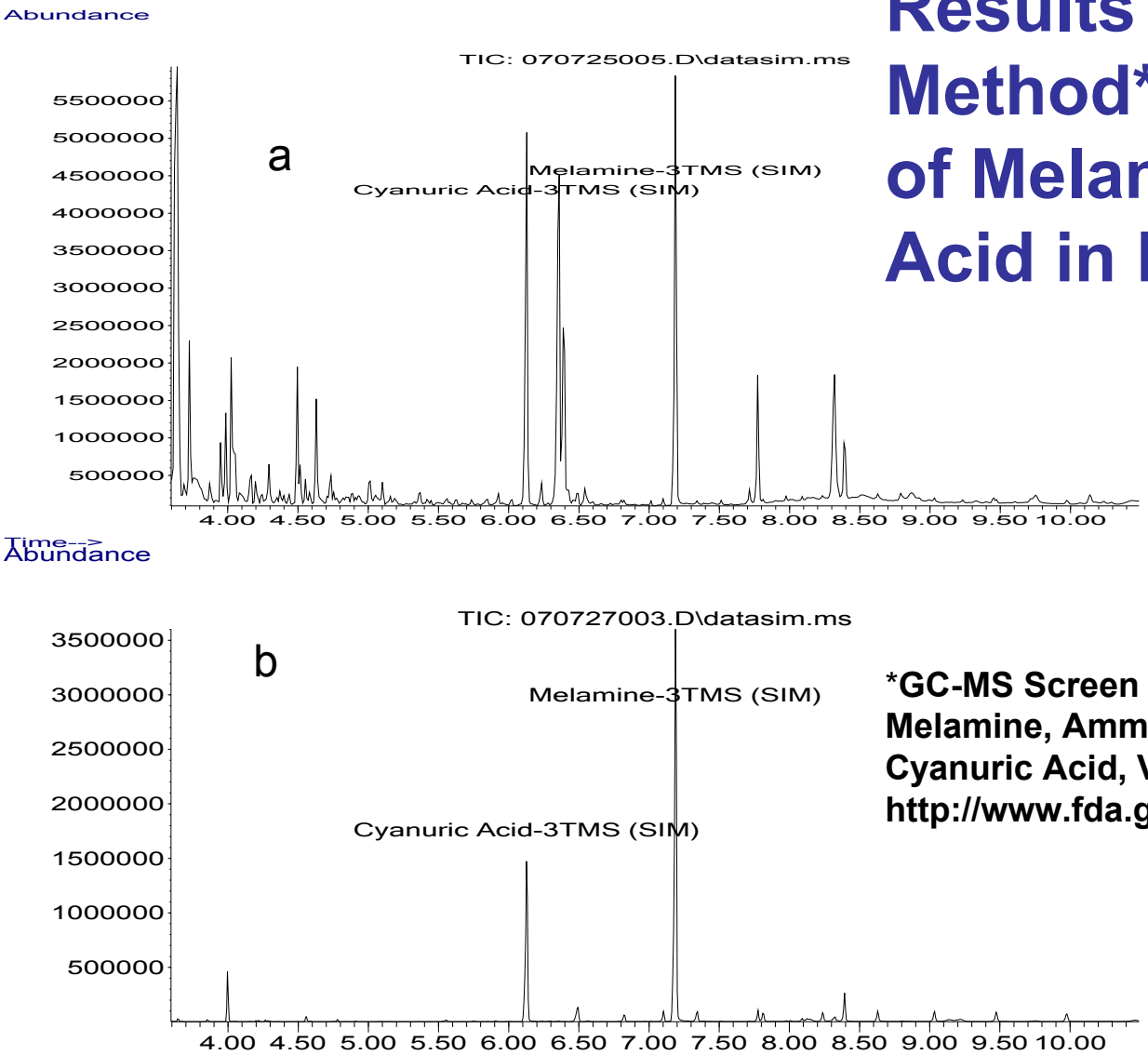


s-triazine-2,4,6-triol s-triazine-2,4,6-trione

Cyanuric Acid

- Combination of these two resulted in renal failure of cats/dogs
- FDA published GC/MS method using silylation; 10-ug/g detection limit
  - Confirmation using SIM/SCAN

Results from FDA GC/MS Method* for the Analysis of Melamine and Cyanuric Acid in Pet Food



***GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide, & Cyanuric Acid, Version 2, May 7, 2007 http://www.fda.gov/cvm/melaminepresence.htm**

The influence of the selection of monitoring ions in SIM mode shown with spiked dry cat food; a) the ions of highest abundance were used (cyanuric acid: m/z 73, 147, 171; melamine: m/z 327, 330, 342, 345) b) characteristic ions were used(cyanuric acid: m/z 188, 330, 345; melamine: m/z 197, 285, 327, 342)

HPLC Parameters

HPLC system	: Agilent 1200 RRLC
Column	: Agilent Zorbax-Rx Sil, 2.1×150 mm, 5-um
Injection Volume	: 10 uL
Temp	: 40□
Flow rate	: 0.2 mL/min
Mobile phase	: A - 5 mM Ammonium acetate in Water
	: B - 5 mM Ammonium acetate in ACN
Isocratic	: 95%B

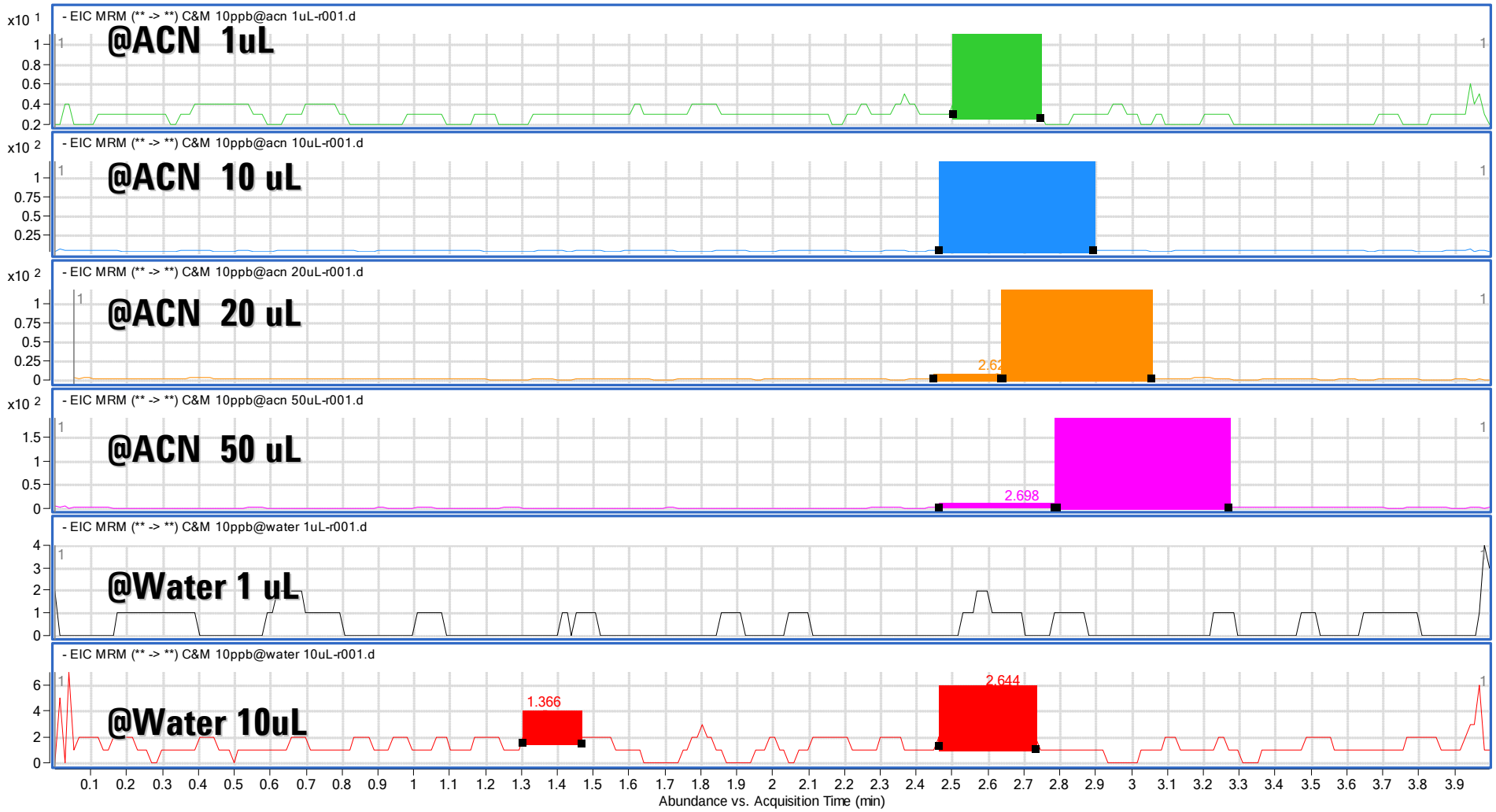
MS Parameters

MS system	: Agilent 6410 LC/MS/MS
Ion source	: ESI
Polarity	: Positive and Negative
Nebulizer gas	: Nitrogen
Ion spray voltage	: 4000V
Source temperature	: 350□
Resolution	: Q1 (unit) Q3 (unit)
Scan mode	: Multiple Reaction Monitoring (MRM)
Segment	: Segment 1= 0~4min negative for cyanuric acid
	Segment 2= 4~6min positive for melamine
Delta EMV	: 600V

MRM Conditions

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Engergy
2.6	Cyanuric Acid	128	85	200	100	5
		128	42	200	100	30
5.9	Melamine	127	85	200	100	20
		127	68	200	100	35

Effect of Injection Solvent & Volume on Cyanuric Acid Retention



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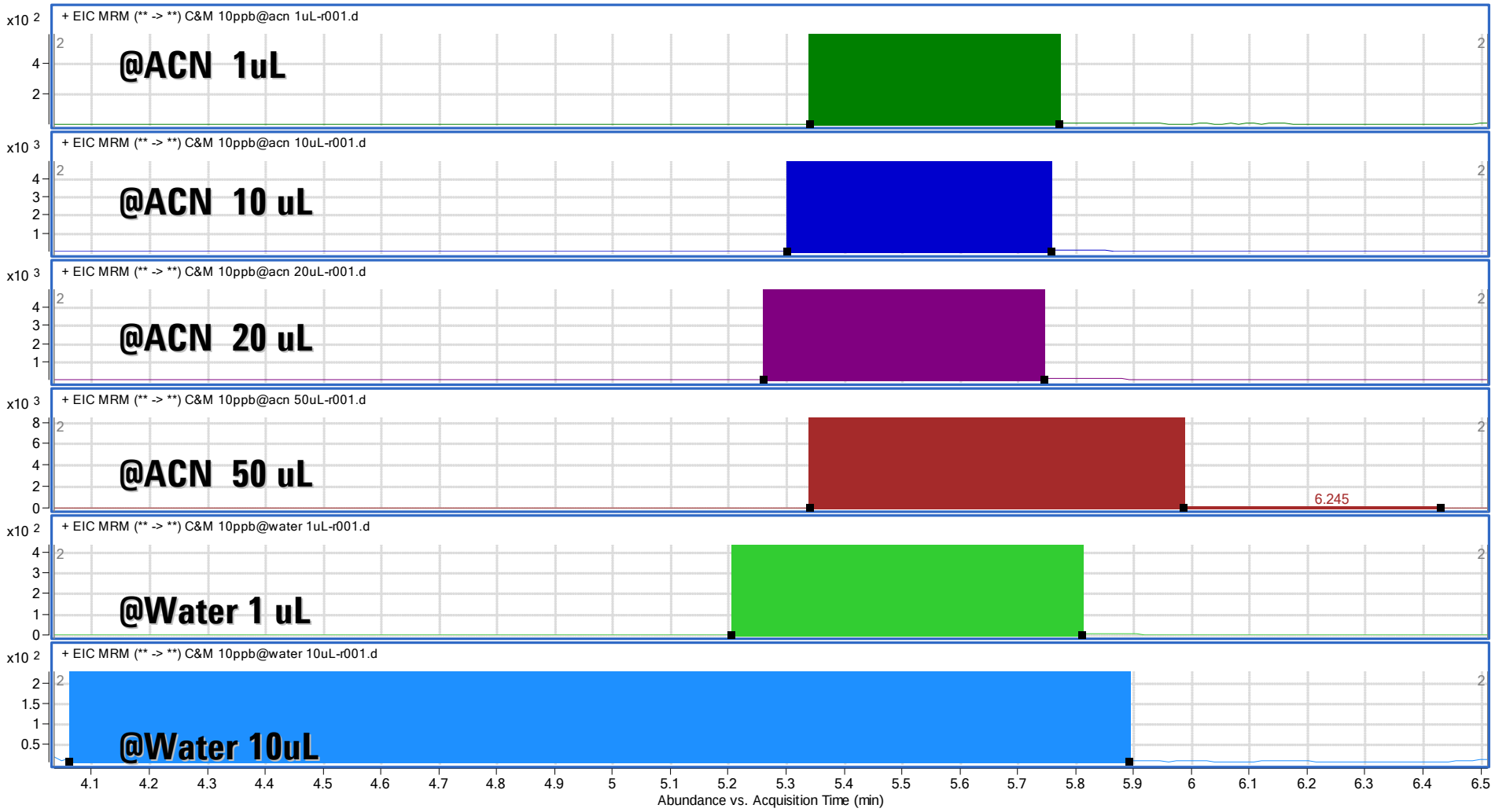
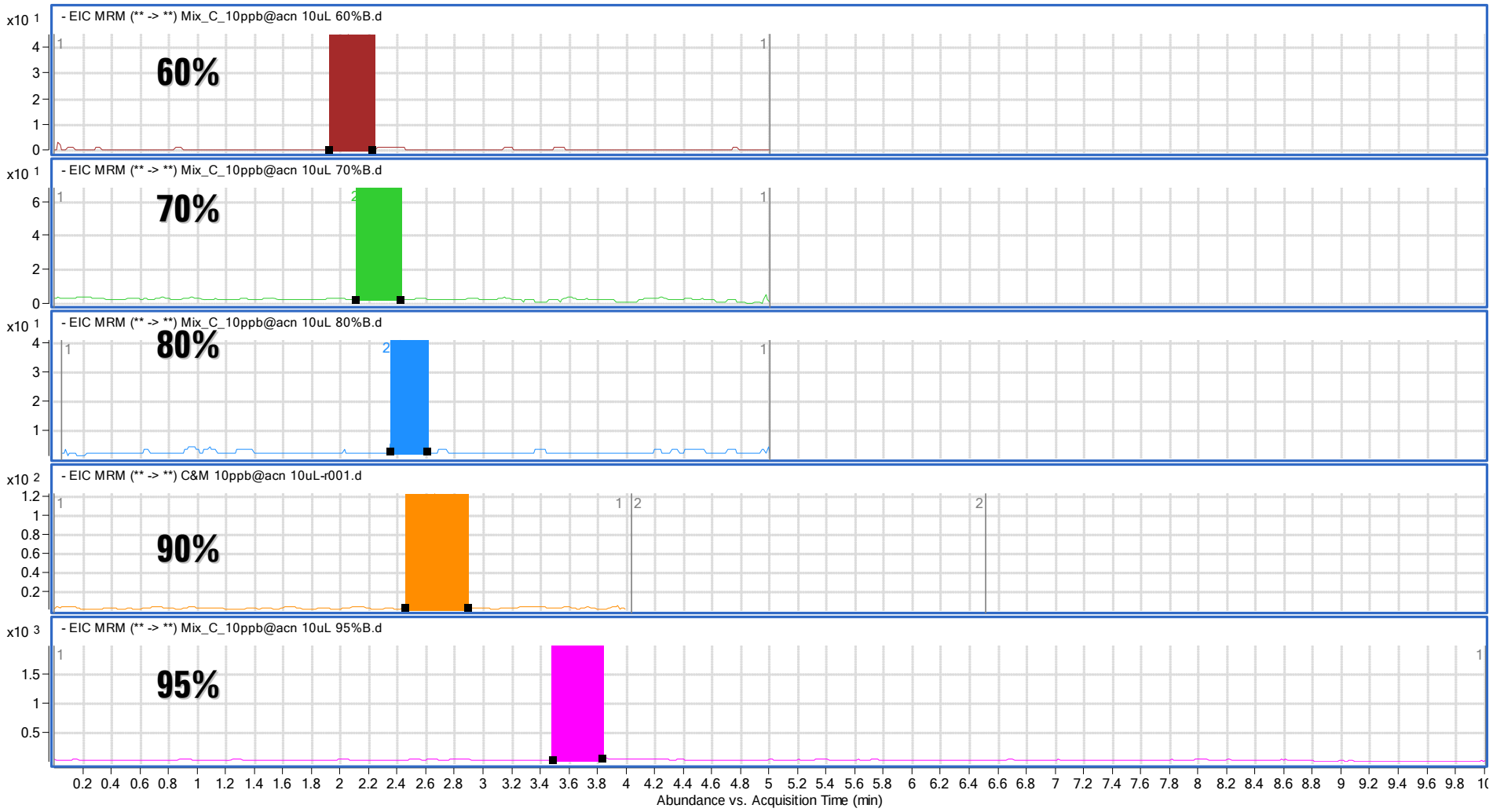
Effect of Injection Solvent & Volume on Melamine Retention



Effect of Increasing % ACN on Cyanuric Acid Retention



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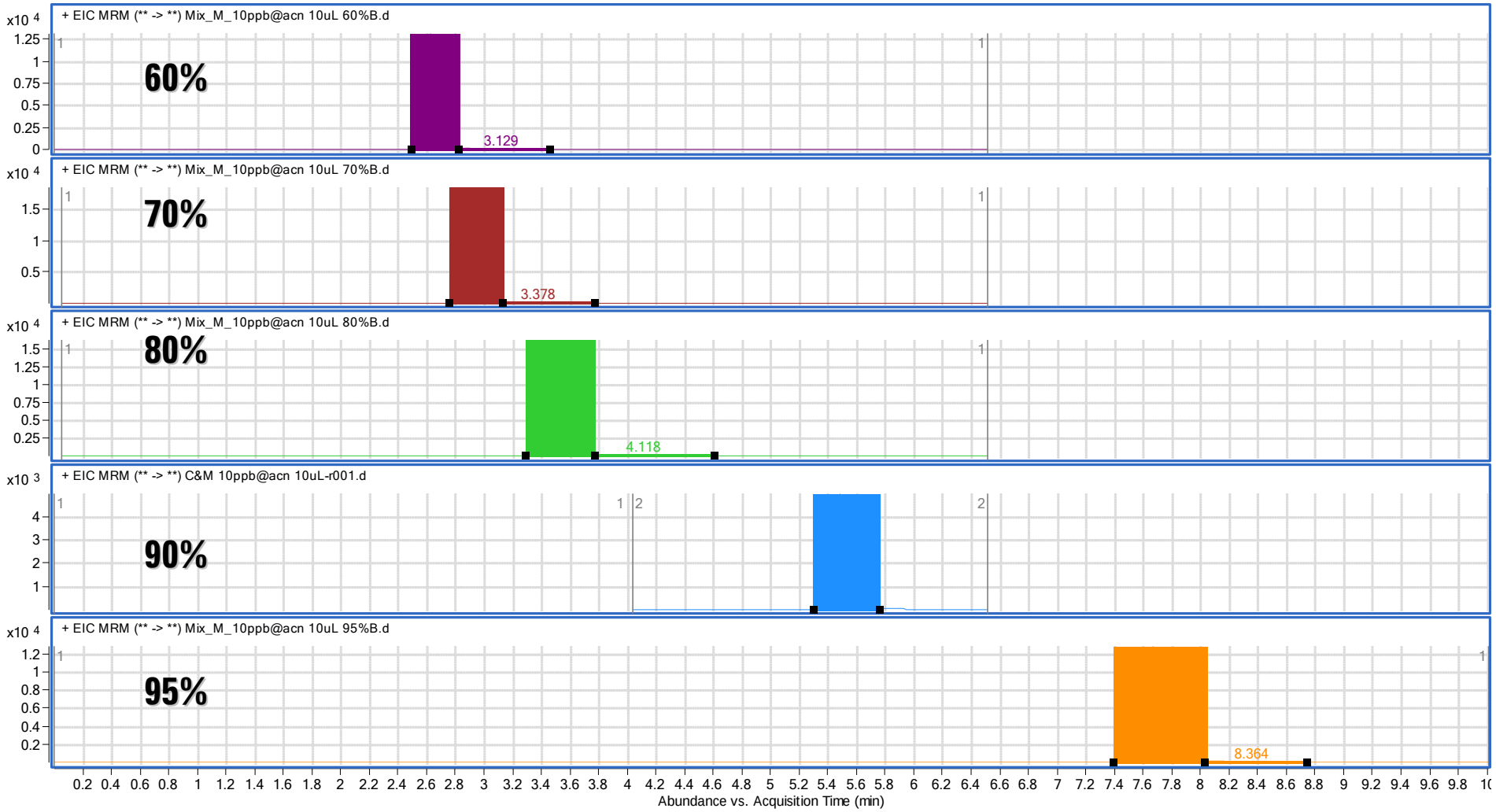
Effect of Increasing %ACN on Melamine Retention



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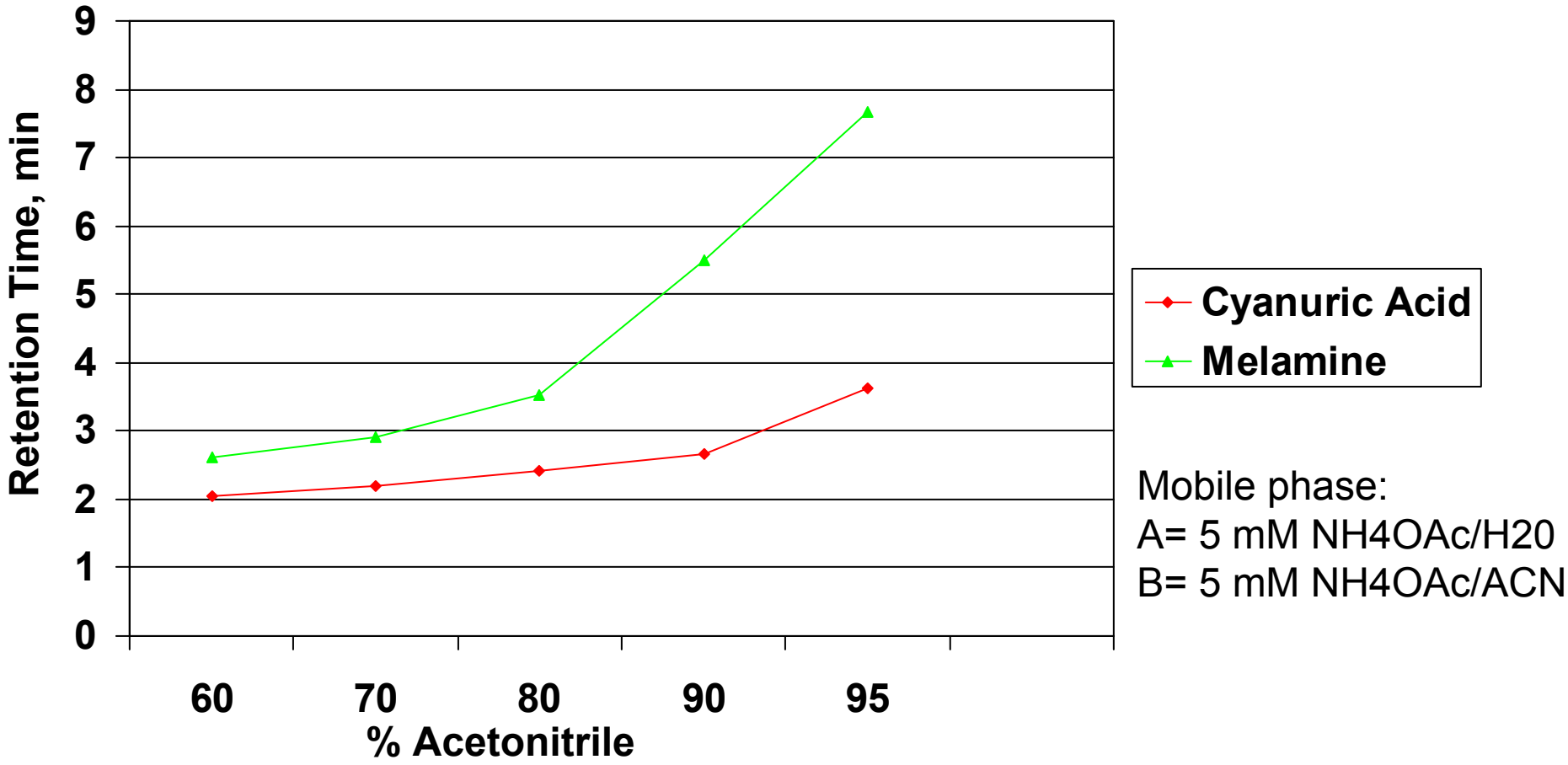
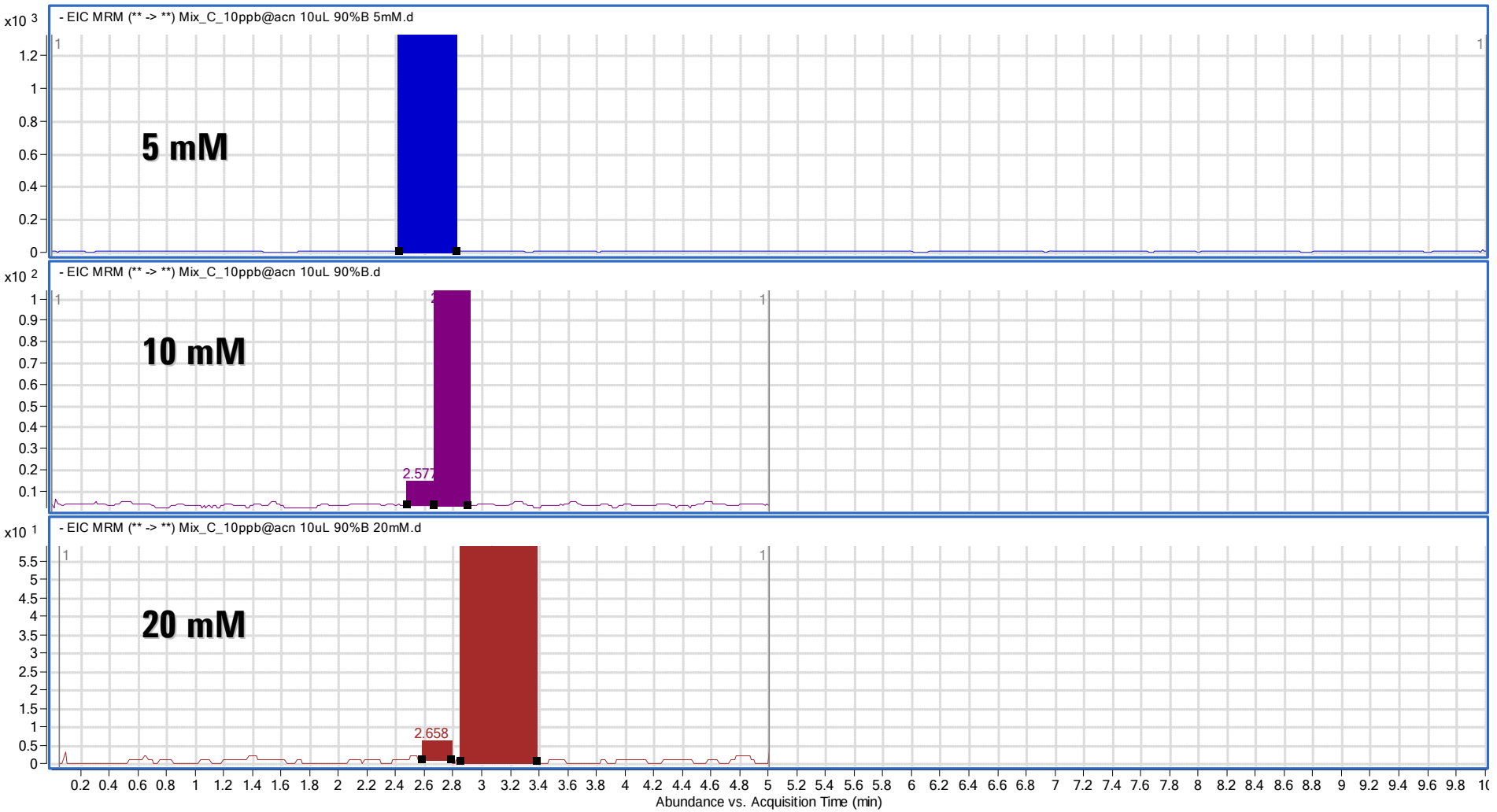
Retention Time Changes with Changes in Mobile Phase Composition



Mobile phase:
A= 5 mM NH4OAc/H20
B= 5 mM NH4OAc/ACN

Effect of CH3COONH4 Concentration on Cyanuric Acid Retention



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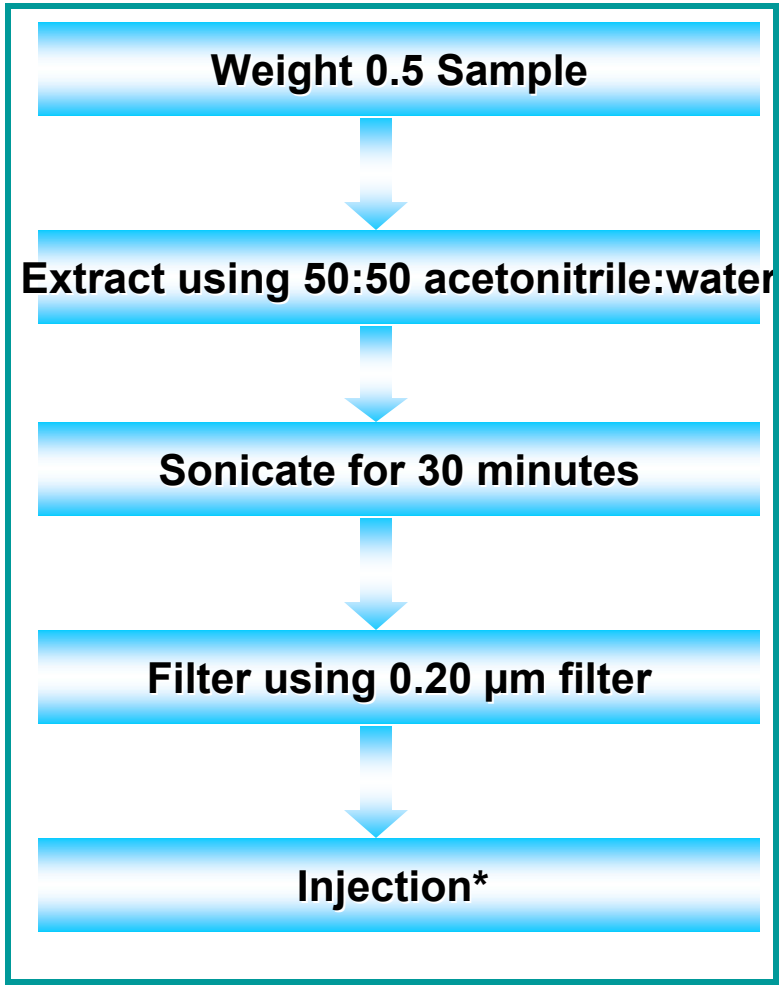
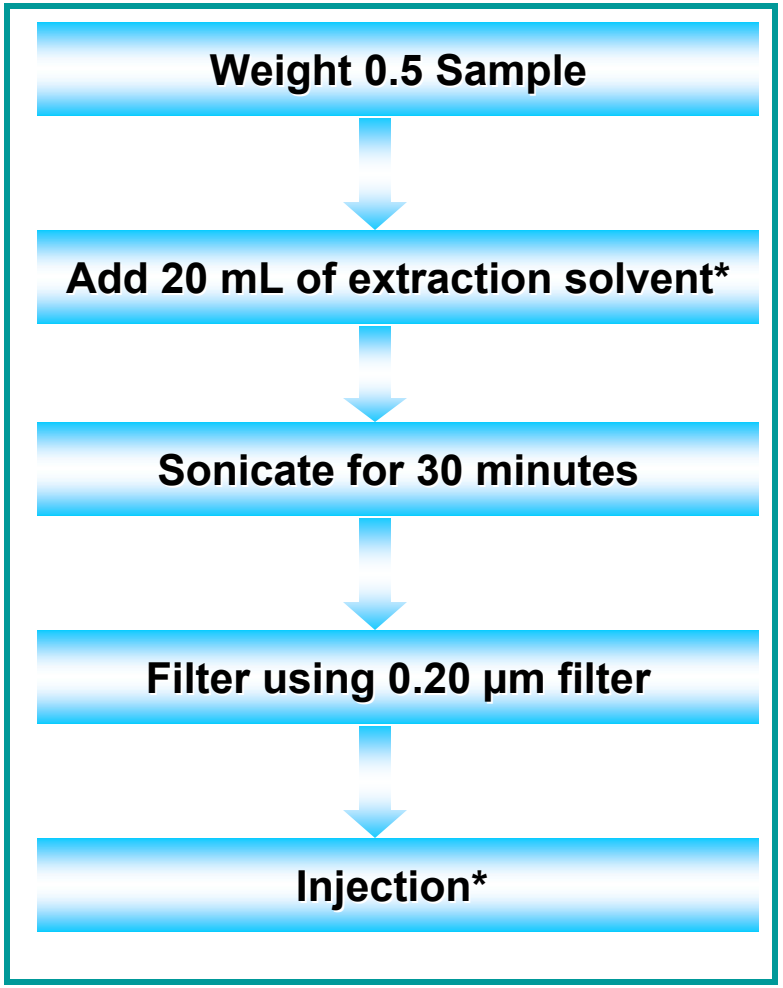
Sample Treatment



1

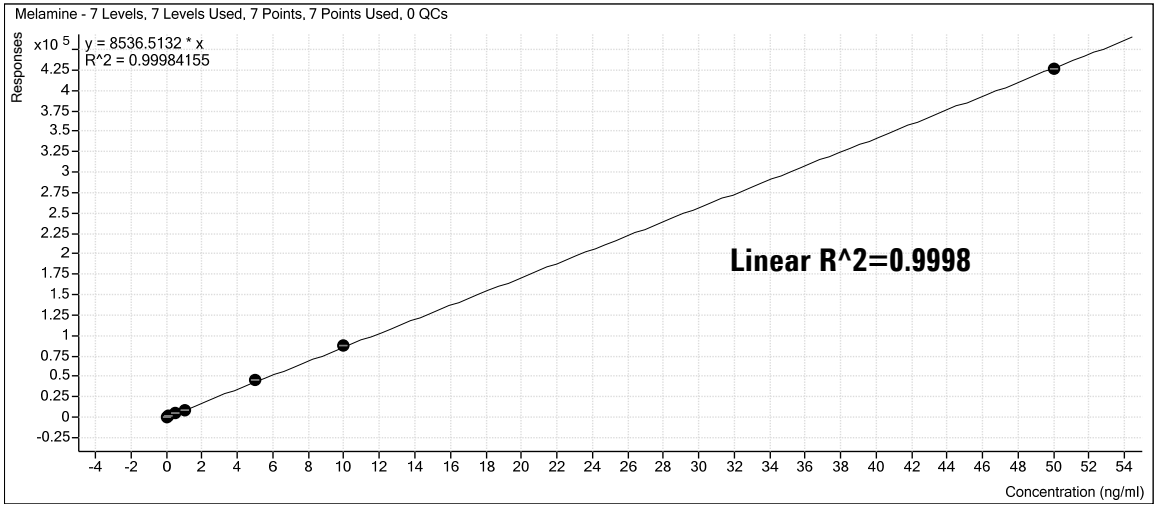


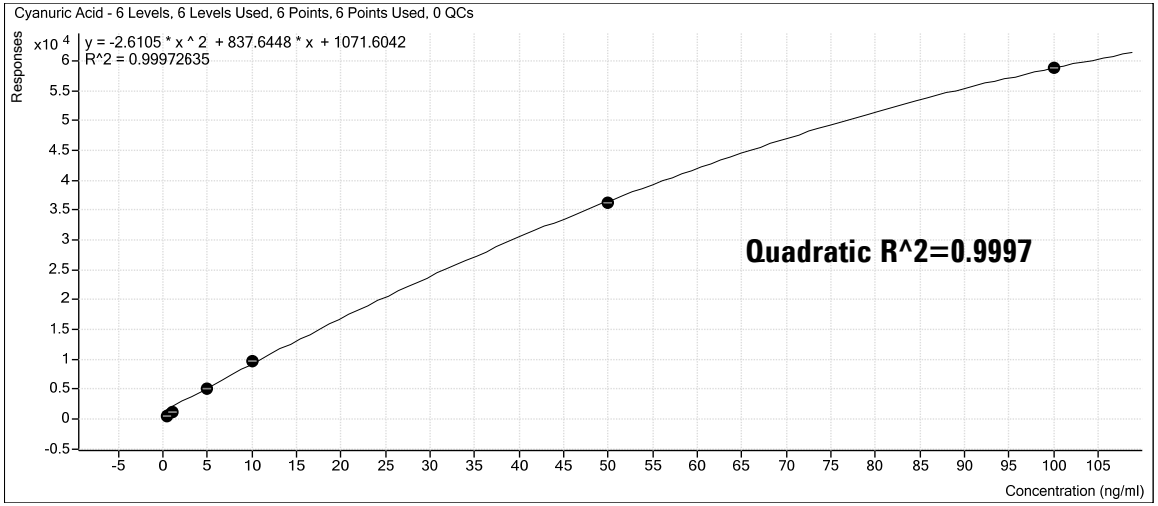
2

Matrices studied: wheat gluten, corn meal
And rice protein

*10:40:50 TEA : H_2O : Acetonitrile

Calibration Curves for Melamine (Top) and Cyanuric Acid (Bottom) By LC-MS/MS





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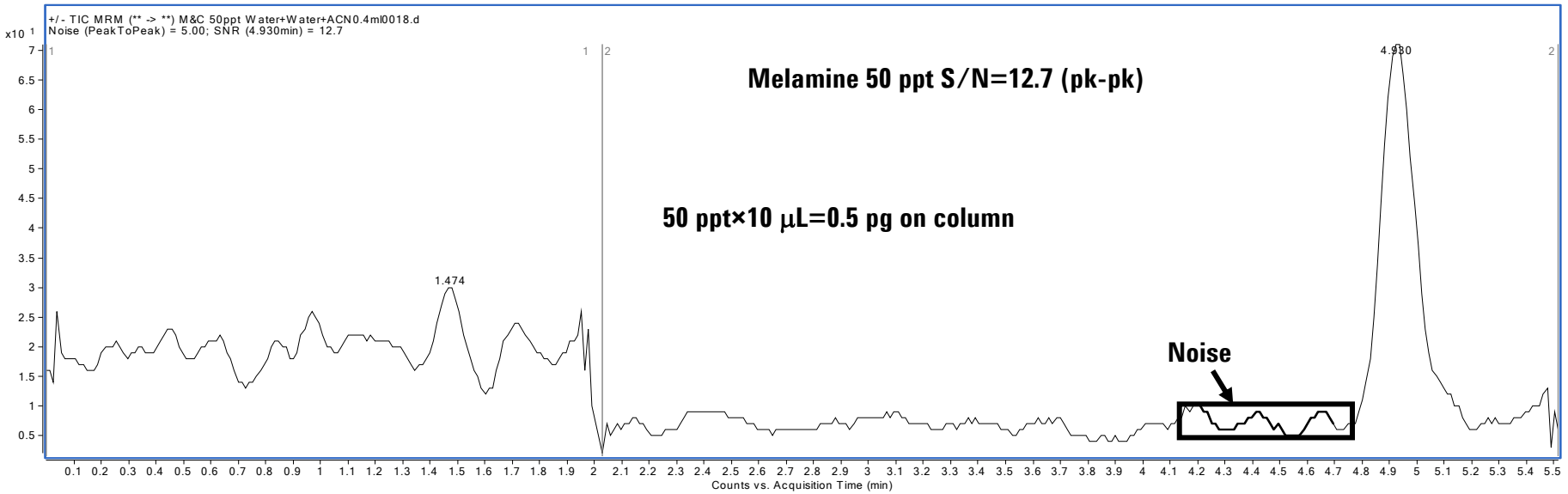
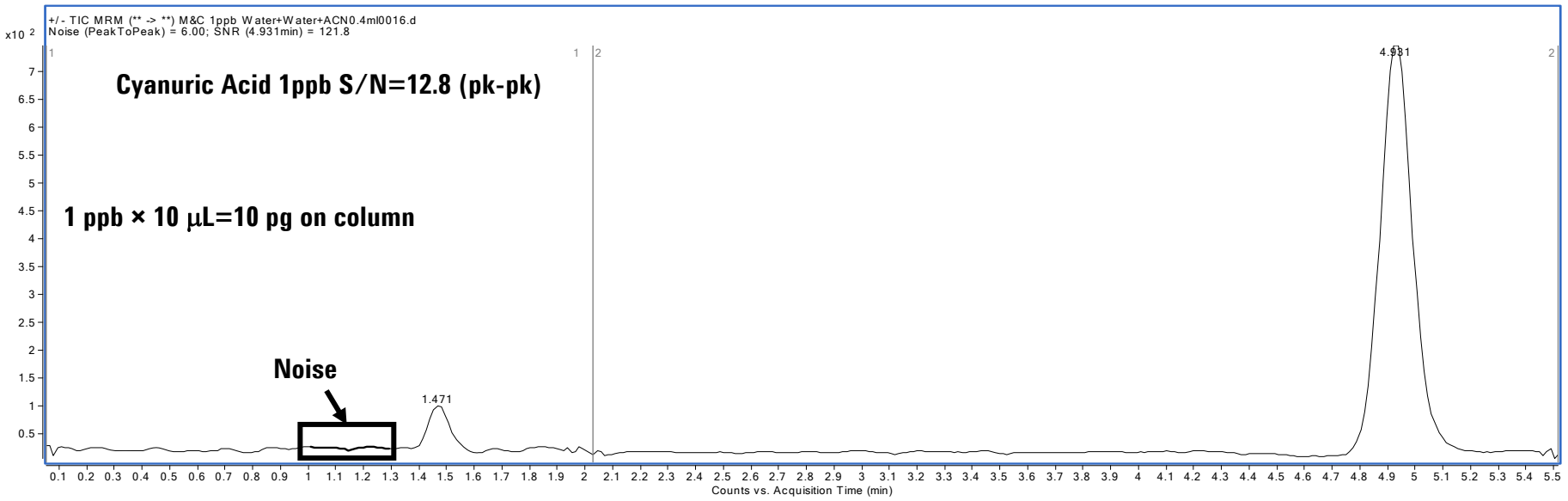
Detection Limits for Melamine and Cyanuric Acid





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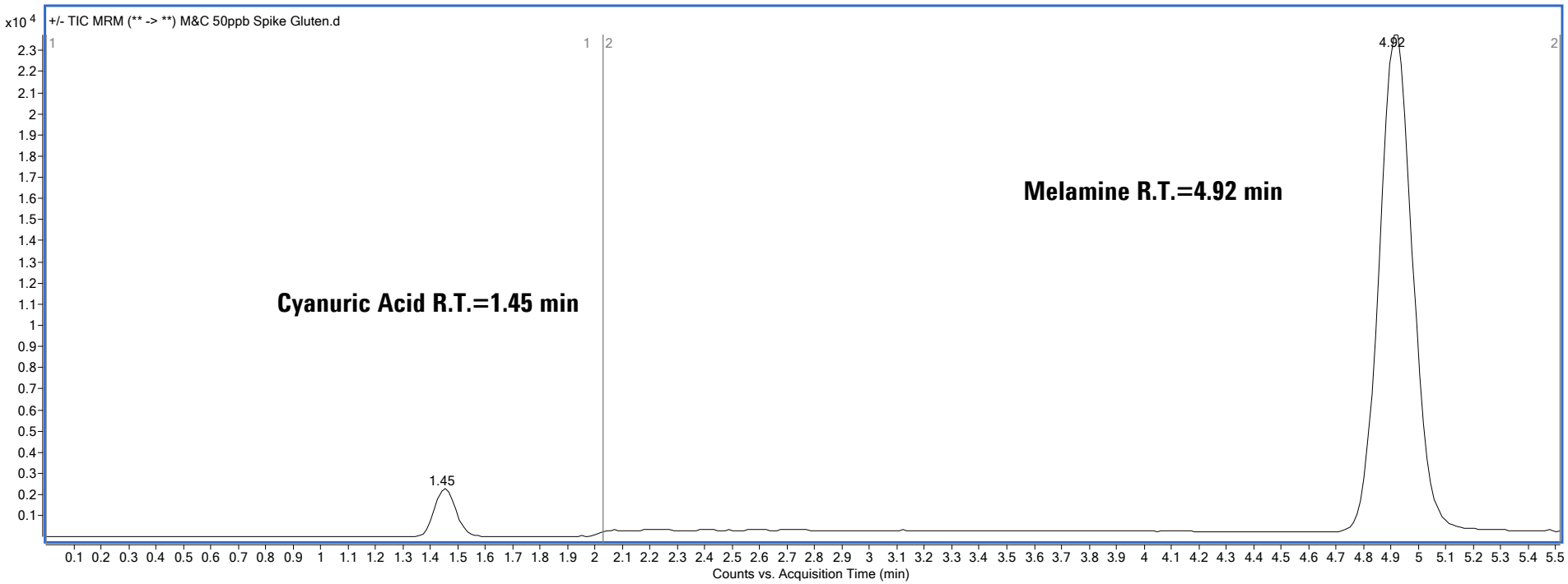
Total Reaction Monitoring Chromatogram for Spiked Wheat Gluten Sample

(50 ppb level)



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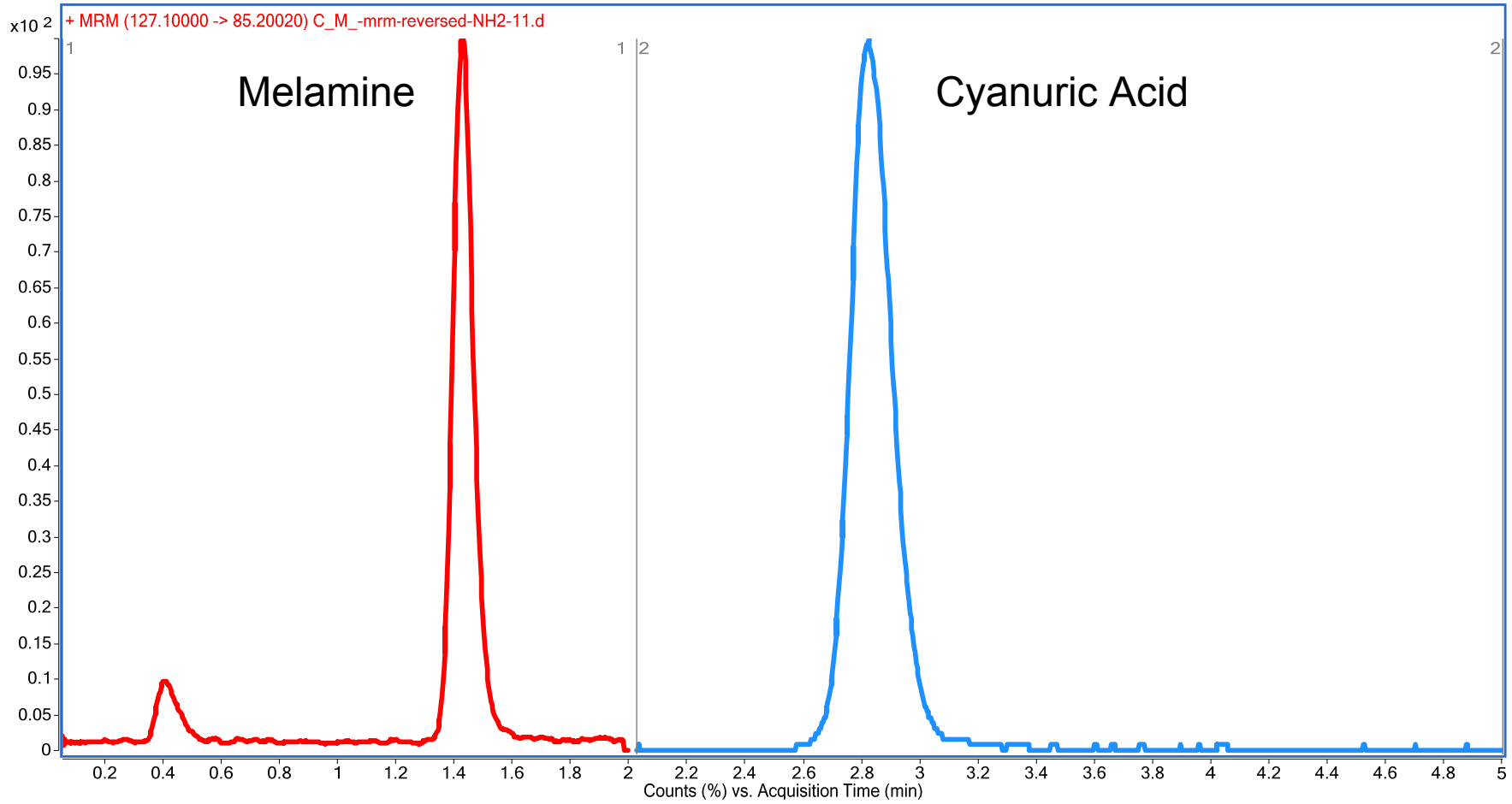
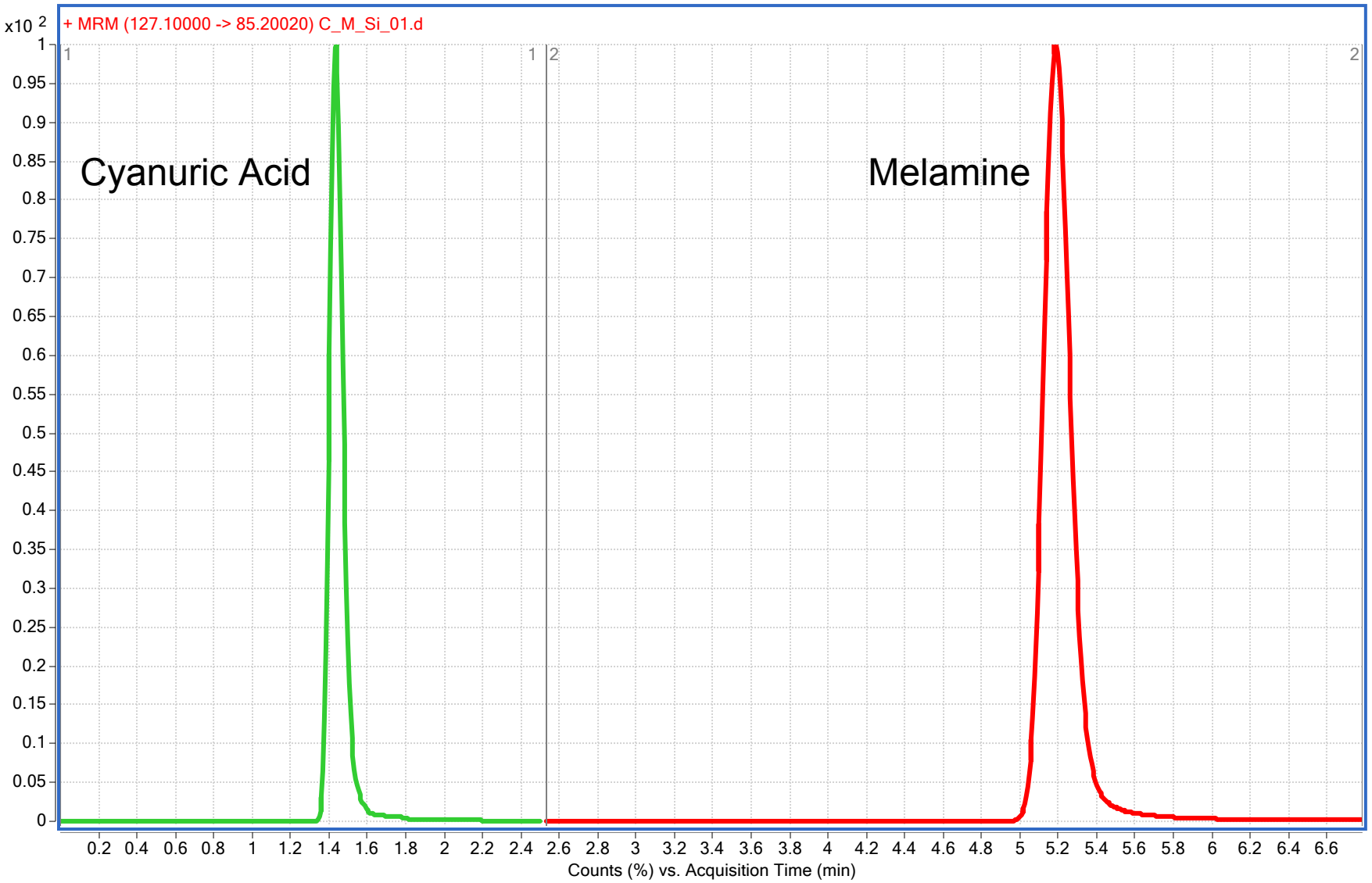
Comparison of Silica and Amino HILIC Columns

HPLC system	Agilent 1200 RRLC
Column 1	Agilent Zorbax-NH2, 2.1×50 mm, 5 um
Column 2	Agilent Zorbax-Rx Sil, 2.1×150 mm, 5 um
Injection Volumn	2 uL
Temp	25□
Flow rate	0.4 mL/min
Mobile phase	A - 5 mM Ammonium acetate in Water
	B - 5 mM Ammonium acetate in ACN

HILIC Separation Using Zorbax NH_2, Column 1



HILIC Separation Using Zorbax Rx-Sil Column 2



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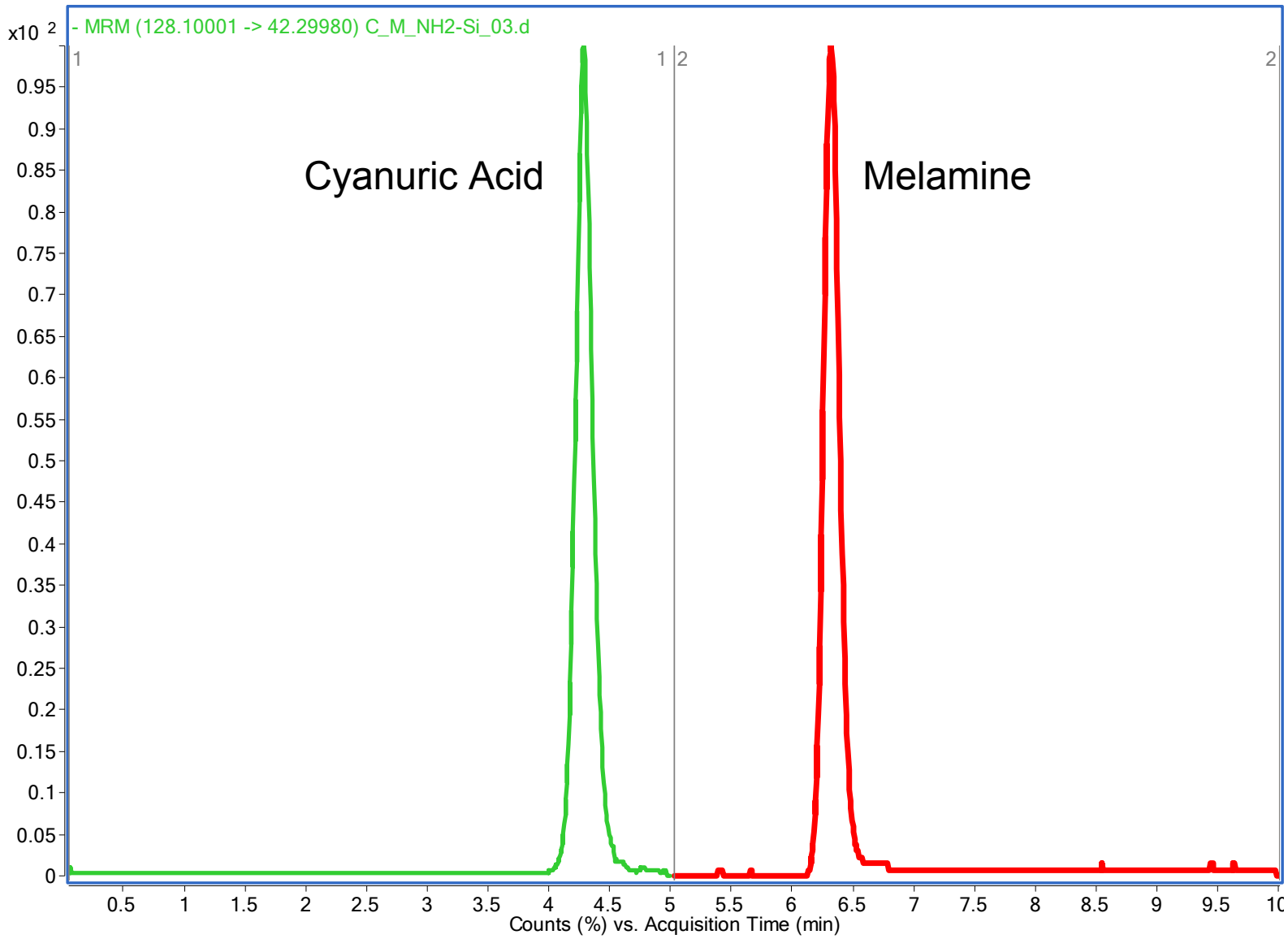
HILIC Separation with Both Columns in Series



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Conclusions

- HILIC has received renewed attention for the analysis of polar compounds that are unretained or poorly retained on RPC columns
- The technique is readily adaptable to MS and MS-MS detection
- 13 of the 23 new “specialty” columns introduced at Pittcon 2008 by 9 companies*
- HILIC used by 4% of all users**
- Publications are accelerating at exciting pace

* Pittcon 2008 Report, LCGC No. America, March, 2008
** R.E. Majors, LCGC No. Amer. Column Survey,2007

Acknowledgements

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