

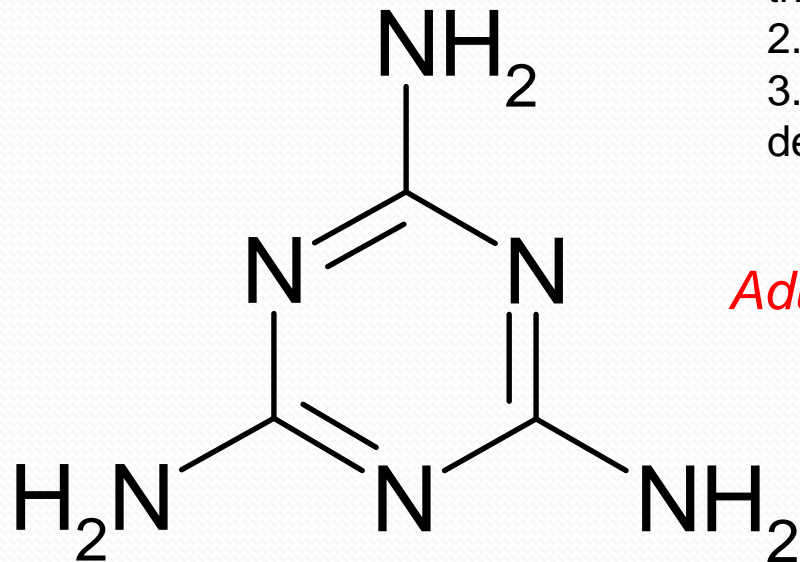
Chemical Testing of Melamine in Dairy Products



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Melamine



M.W.= 126

Uses:

1. Combined with formaldehyde to produce melamine resin which is a very durable thermosetting plastic.
2. Fertilizers
3. Use as non-protein nitrogen for cattle as described in a 1958 US patent.

Adulteration to Milk product to "fake" protein

Water solubility: 3240mg/L

Tolerable Daily Intake:
0.63 mg/kg per body weight per day

EC-directive 2007/72/EC, 6 August 2002
Melamine specific migration restriction
30mg/kg food.



Method References:

US FDA Method:

Updated FCC Developmental Melamine Quantitation (HPLC-UV) (for Wheat gluten and Moist Pet Food)

Extraction Solvent:
50% Acetonitrile



Ion Pair
chromatography

Column: Zorbax Rx C8 (retention is too high on C18 column)

Buffer: 10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0

Mobile phase: 85:15 buffer:acetonitrile

Flow rate: 1.0 ml/min.

Injection volume: 10 µl

Column thermostat: 40 °C (column thermostating is necessary for ion-pair separations)

Detection wavelength: 240 nm

Spectral collection: 200 – 400 nm (look for I_{\max} near 236 nm)

Retention time: 4.2 - 4.3 min.

Run time: 10 min.

<http://www.fda.gov/cvm/melamine04022007.htm>

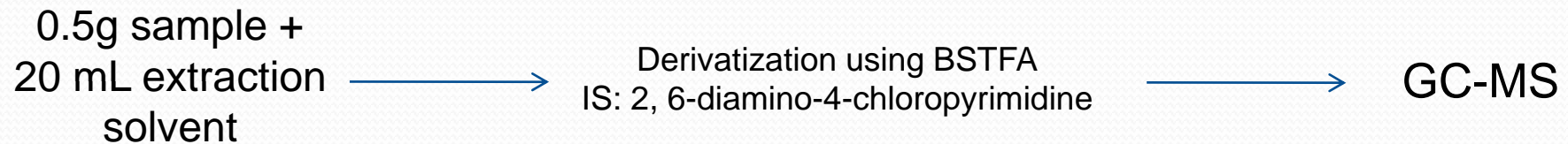


Method References:

US FDA Method

GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric acid

(for Wheat gluten and Moist Pet Food)



Extraction Solvent: 10/40/50: Diethylamine/water/Acetonitrile

Column: DB5-MS capillary

Reporting Limit: ~10 ppm in sample

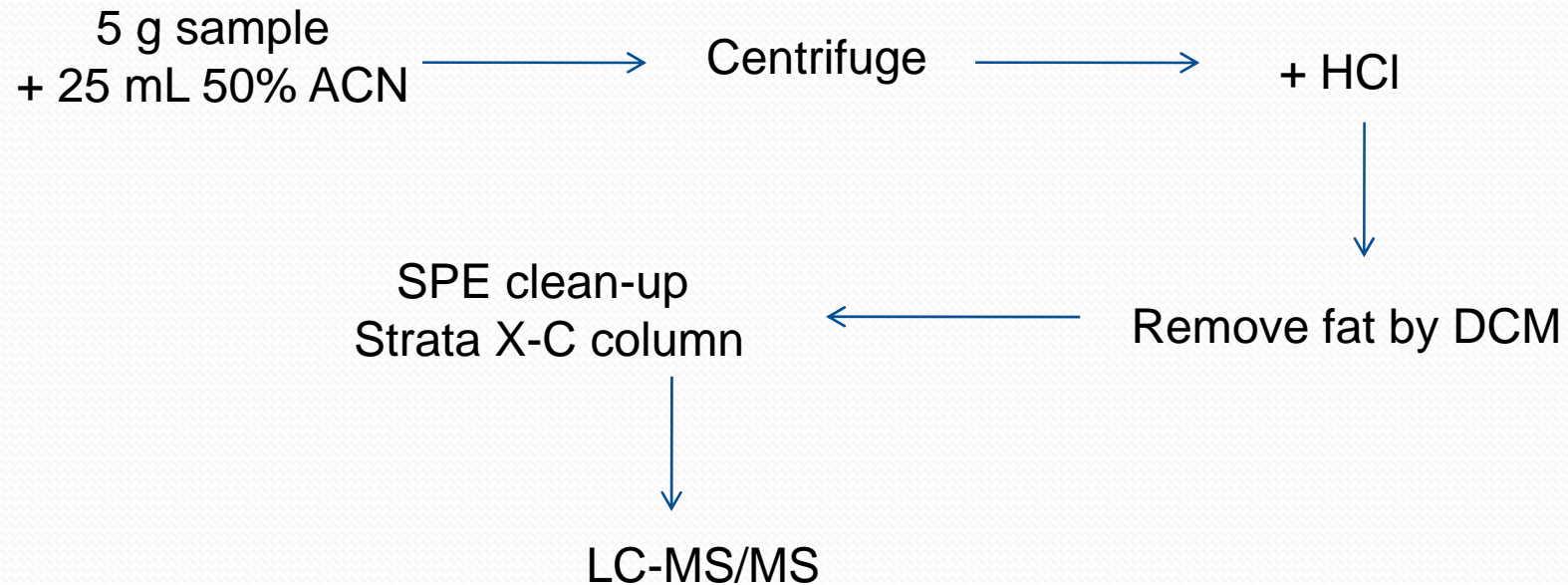
<http://www.fda.gov/cvm/melamine04022007.htm>



Method References:

US FERN Method:

LC/MS/MS Screen for the Presence of Melamine in swine and poultry tissues



Column: Phenomenex Synergi Polar RP(150 x 4.6mm 4mm)

Minimum proficiency level (MPL): ~50 ppb in sample

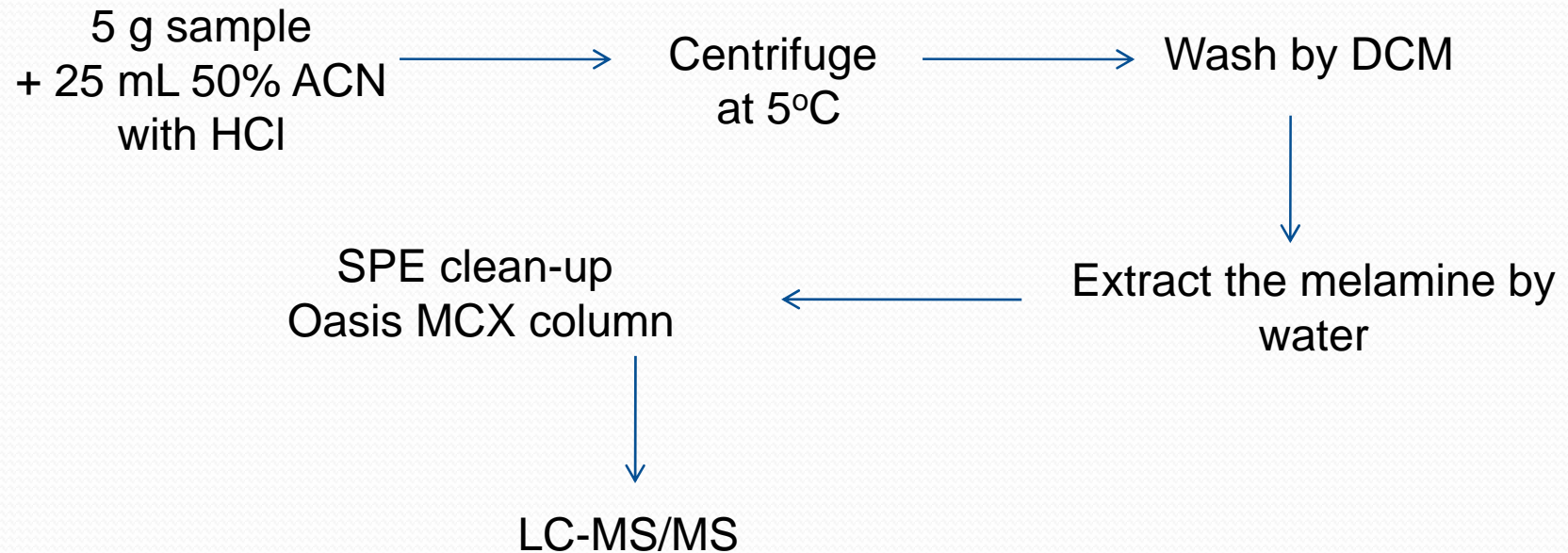
http://www.fsis.usda.gov/PDF/FERN_CHE_0003.pdf



Method References:

US FDA Method:

Determination of Melamine Residues in Catfish Tissue by Triple Quadrupole LC-MS-MS with HILIC Chromatography



Column: Atlantis HILIC Silica column (50 x 3.0 mm 3 μ m)

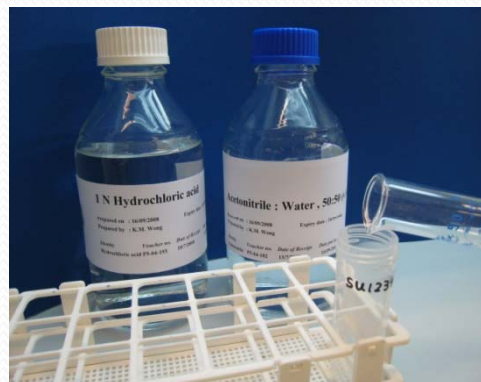
Average Recovery of about 76% over the concentration range 10 ppb to 500 ppb in sample

<http://www.cfsan.fda.gov/~frf/lib4396.html>



GL Testing Methods: (Fast method) Based on US FDA LC-MS-MS method

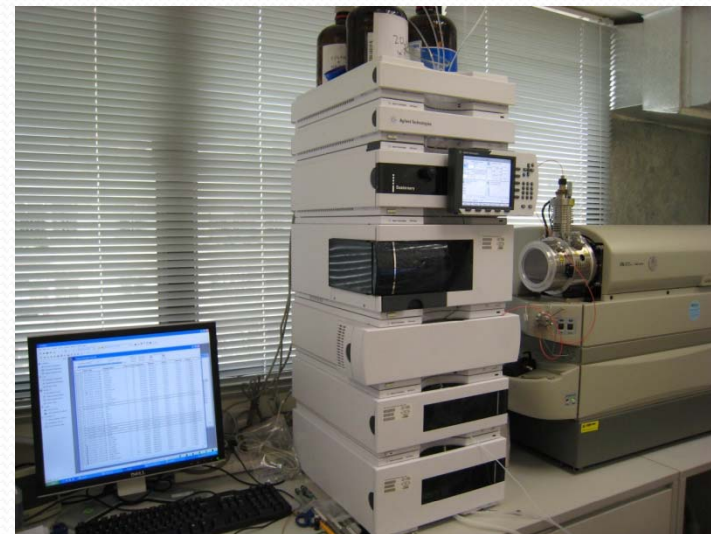
1. Weigh 0.5 g sample into a 50-mL polypropylene centrifuge tube
2. Add 24 mL a 50:50 (v:v) solution of acetonitrile:water and 1 mL of 1.0 N hydrochloric acid.
3. Cap the sample and shake vigorously for 30 seconds and then vortex mixed for 1 minute.
4. Centrifuge the sample at 3200 x g (4000 rpm) for 5 minutes at 4°C.
5. Transfer 5 mL aliquot of aqueous layer to a 15-mL polypropylene centrifuge tube.



7. Add 5 mL dichloromethane to the 15-mL polypropylene centrifuge tube and then shake the sample for two minutes.
8. Centrifuge the sample at 3200 x g (4000 rpm) for 5 minutes at 4°C.
9. Transfer carefully the upper aqueous layer (about 2.5 mL) to 10-mL volumetric flask.
10. Add 2.5 mL water to the dichloromethane layer and re-extract that sample by shaking for 1 minute.
11. Centrifuge the polypropylene tube at 3200 x g (4000 rpm) for 5 minutes at 4°C.



11. Repeat Clause 9 to 11 once.
12. Transfer the entire upper aqueous layer and combine with the previous aqueous extract into the 10-mL volumetric flask and make up to mark with water.
13. Transfer 2.5 mL of the sample to a 25-mL volumetric flask and make up to mark with acetonitrile for dilution factor x 1000.
Or transfer 1 mL of the sample to a 5-mL volumetric flask and make up to the mark with acetonitrile for dilution factor x 500.
11. Filter the sample with a 0.45 μm filter disc and transfer 1 mL of the filtrate to a LC vial. Add 10 μL of 2000 ppb internal standard (Melamine $^{13}\text{C}_3$, Amino- $^{15}\text{N}_3$). The solution is ready for LC-MS/MS analysis.



Testing Methods: HPLC condition for Melamine determination

Column : Waters Altantis HILIC column, 3 μm , 50 mm x 2.1 mm or other equivalent column

Mobile phase Sol A : 20 mM NH_4OAc in water

Sol B : Acetonitrile

Gradient program	:	<u>Time (min)</u>	<u>% Solvent B</u>
		0	95
		3.8	5
		2.2	5

Flow rate : 0.5 mL/min.

Injection volume : 10 μL



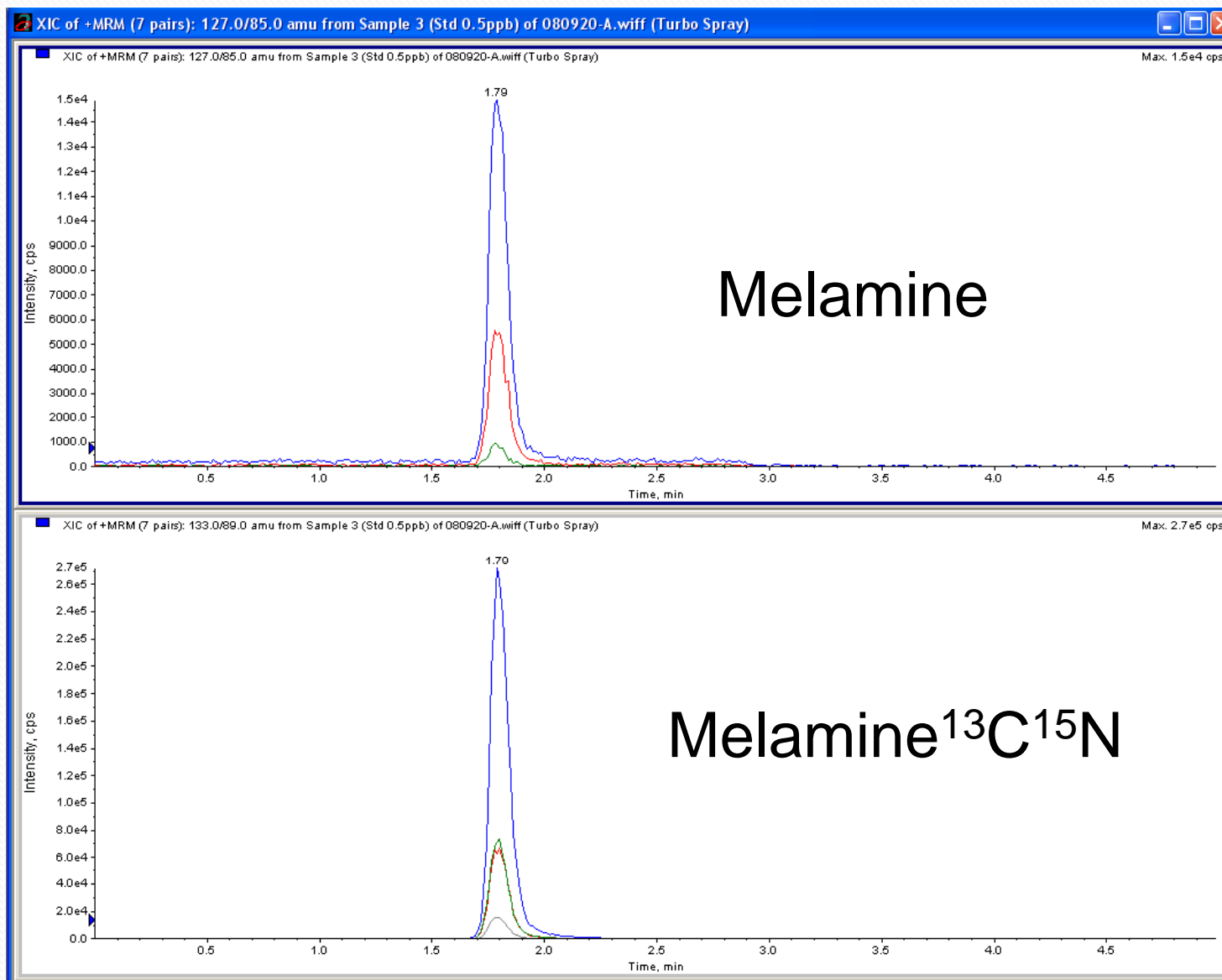
Testing Methods: LC-MS/MS conditions

System : Applied Biosystem 3200QTrap
Ionization : Turbo Spray
Flow rate : 0.5 mL/min.
Curtain Gas (CUR) : 35
Temperature (TEM) : 700
Gas 1 (GS1) : 50
Gas 2 (GS2) : 70
Collision Gas Supply (CAD) : High

Analyte	Purpose ¹	Parent Ion (Q1)	Daughter Ion (Q3)	Dwell Time	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)
Melamine	Q	127.0	85	100 ms	30	7	24
	C	127.0	68	100 ms	30	7	42
	C	127.0	60	100 ms	30	7	27
Melamine- ¹³ C ¹⁵ N	IS	133.0	89	100 ms	33	7	27

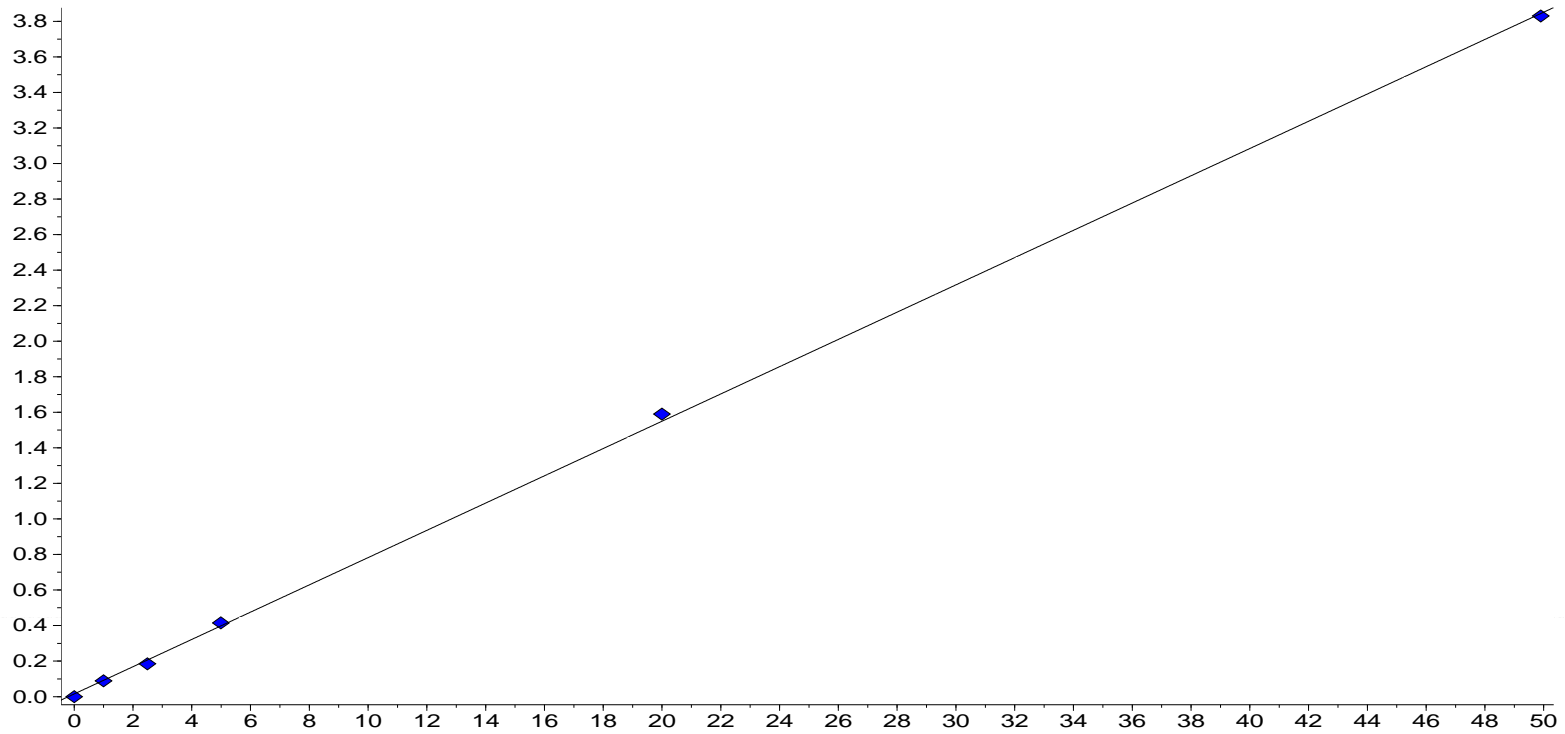


0.5 ppb Standard

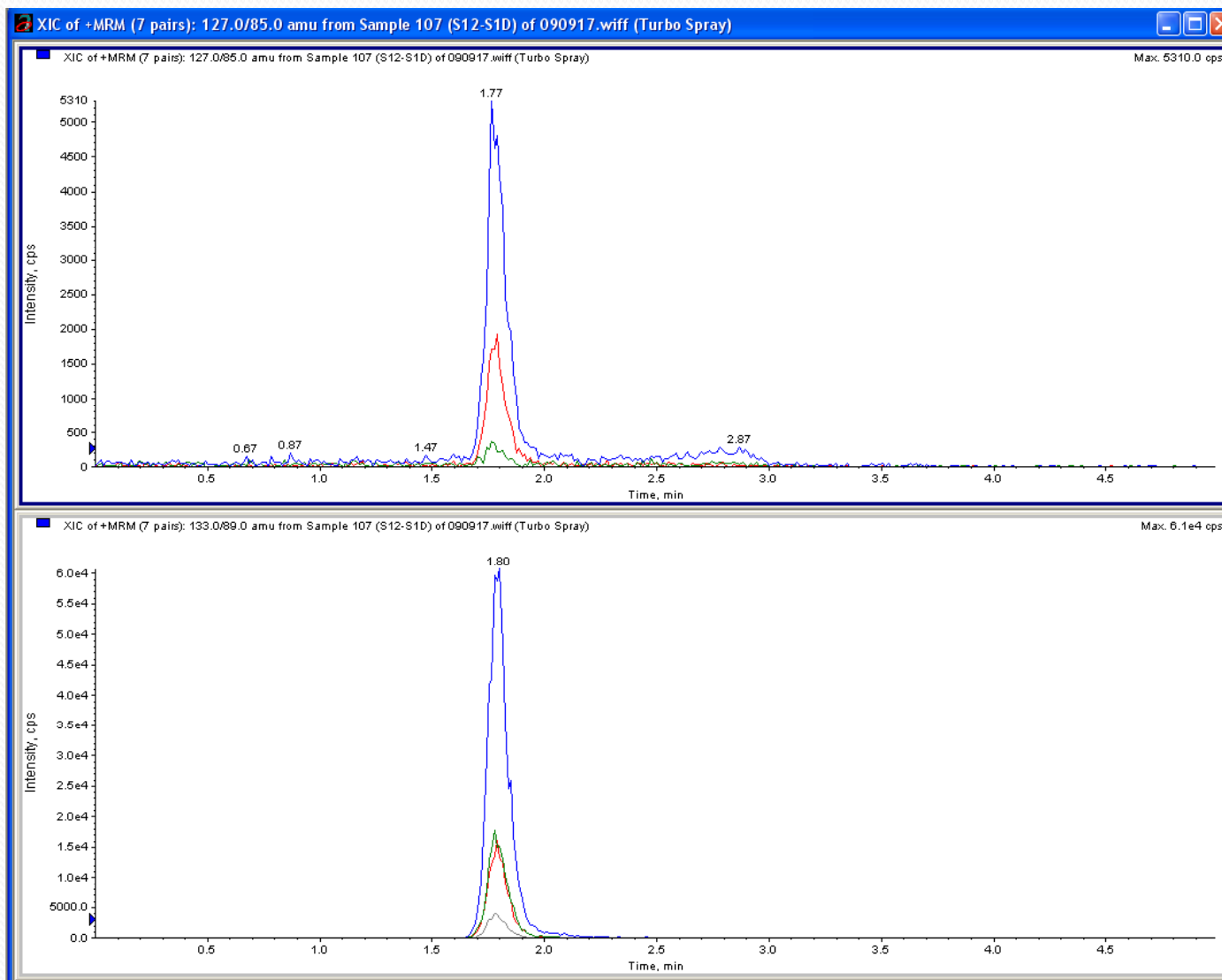


Linearity

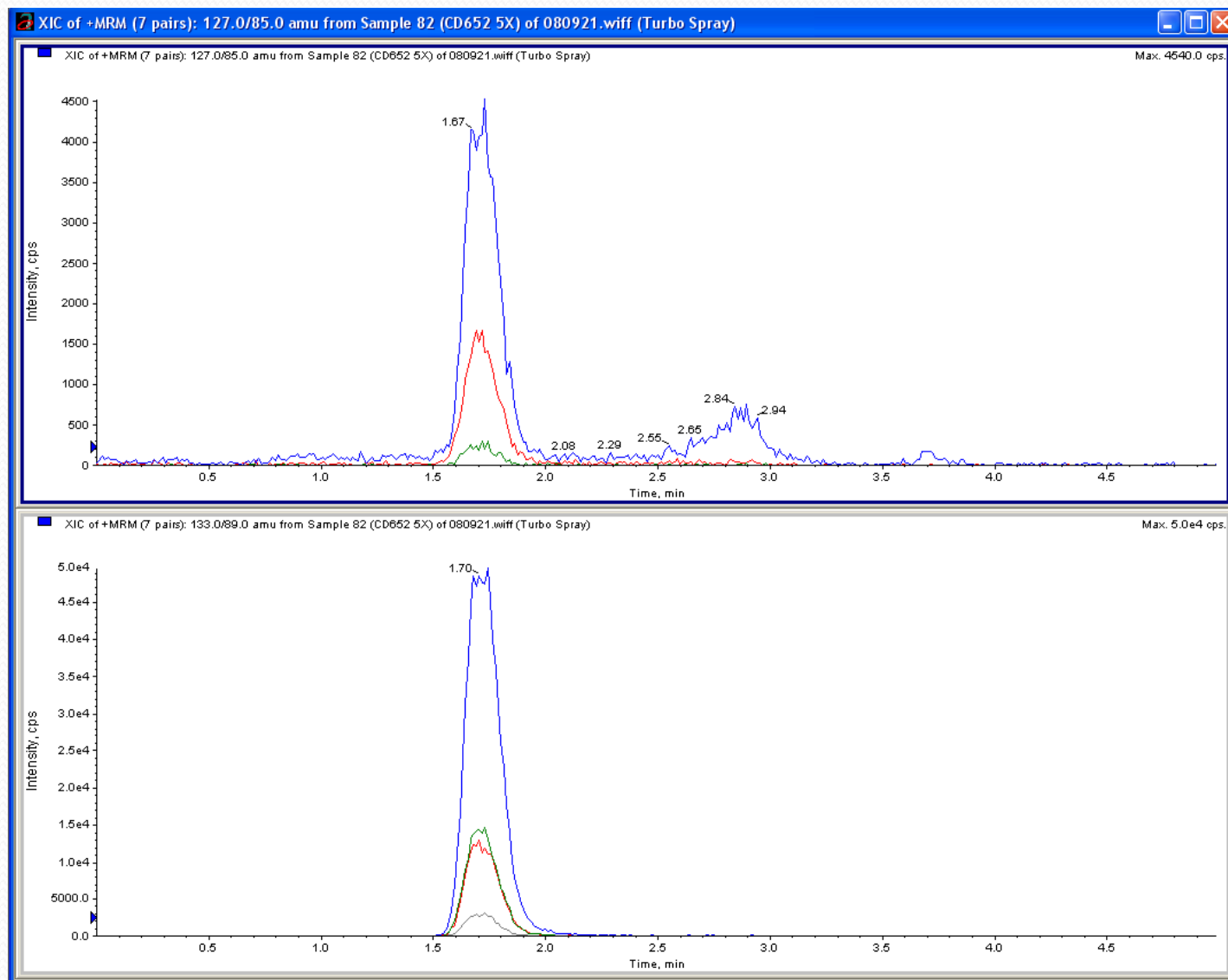
090917.rdb (127.0 / 85.0): "Linear" Regression ("No" weighting):
 $y = 0.0767 x + 0.0151$ ($r = 0.9999$)

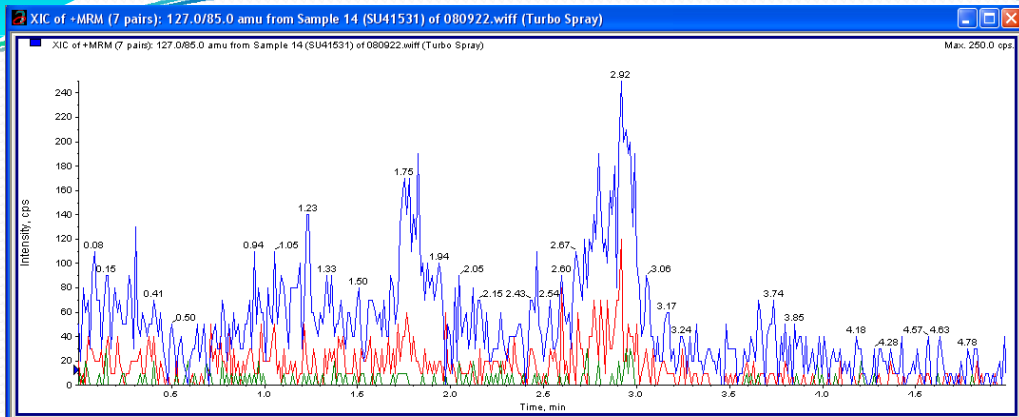


1 ppm spike sample – Dilution x 1000

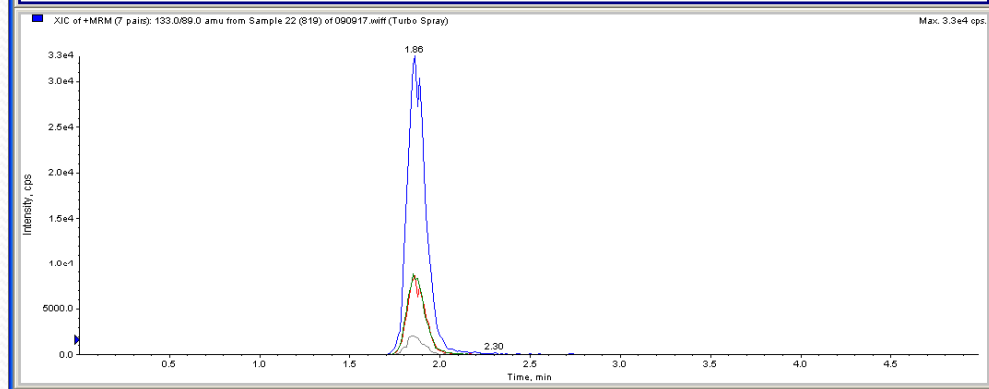
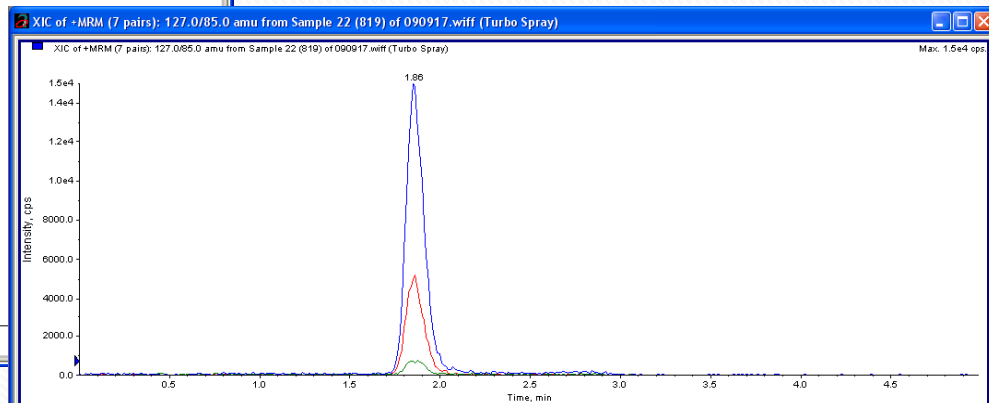
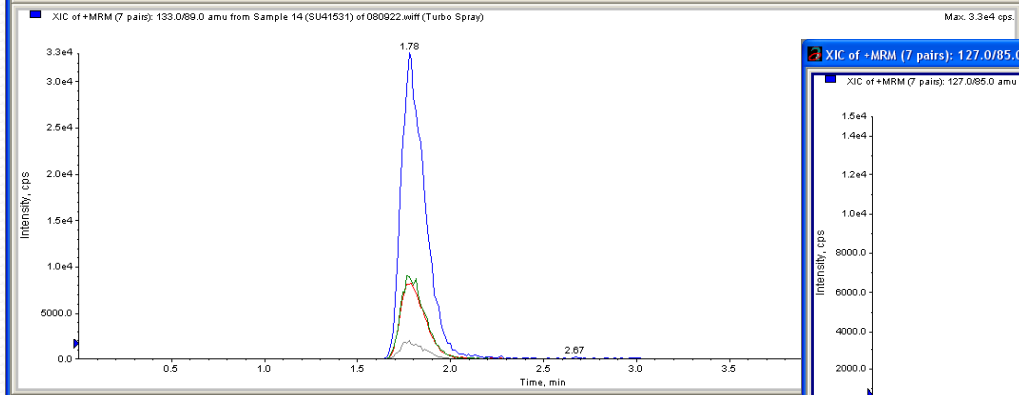


0.5 ppm Spiked Milk Powder Samples – Dilution x500





Blank milk sample



High calcium milk sample containing 5 ppm

Testing Methods: LC-MS/MS Conditions (II)

System : Thermo TSQ Quantum Discovery MS system
Ionization : ESI
Spray voltage : 3500V
Sheath Gas : 50 (arb unit)
Aux Gas : 20 (arb unit)
Capillary Temperature : 280°C
Collision gas pressure : 1.2 mtorr

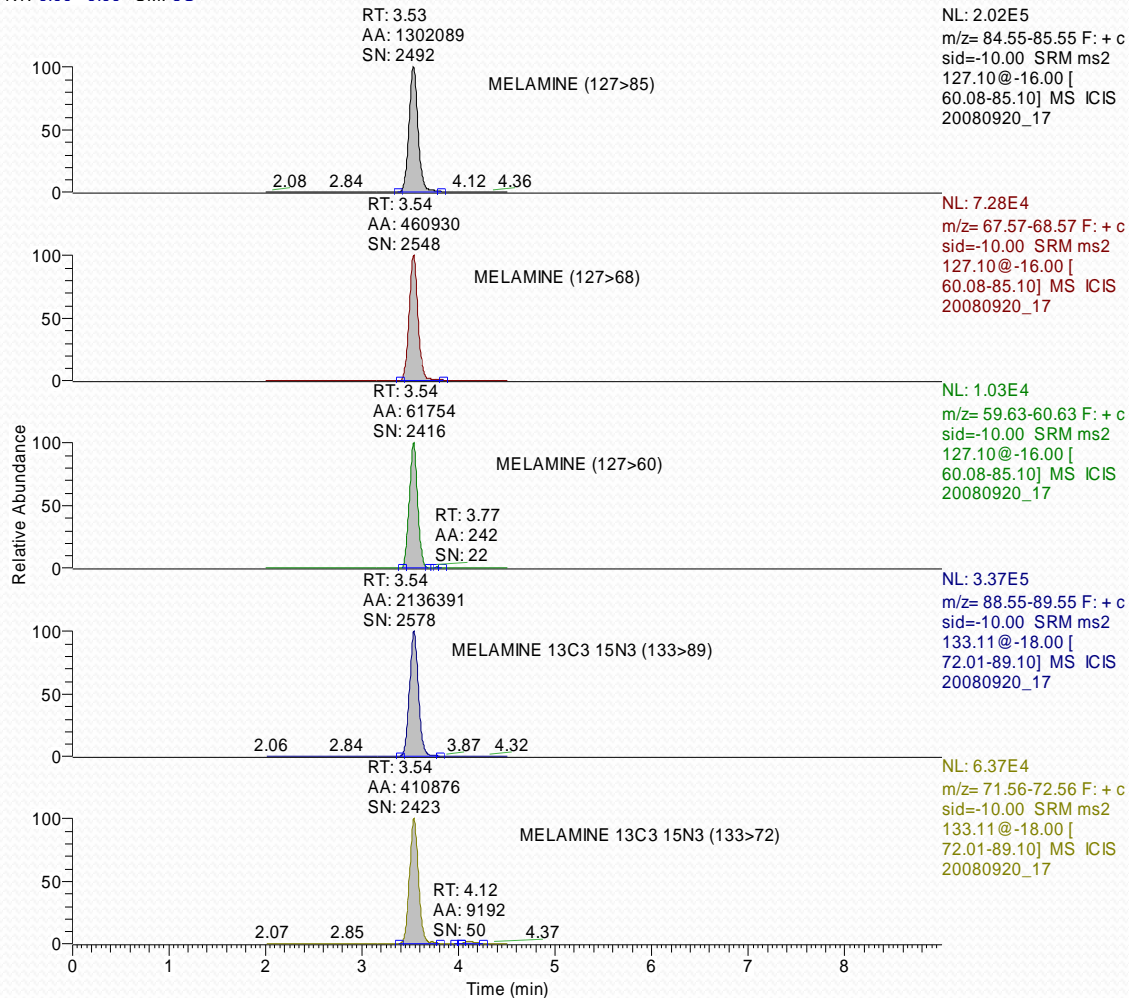
Analyte	Precursor ion	Product ion	Scan width	Scan time	Collision energy	Q1/Q3 PW	Tube lens offset
Melamine	127.1	85.0	0.1amu	30ms	16	0.7amu	100
		68.1	0.1amu	30ms	27	0.7amu	100
		60.1	0.1amu	30ms	18	0.7amu	100
Melamine , ¹³ C ¹⁵ N	133.1	89.1	0.1amu	30ms	18	0.7amu	102
		72.1	0.1amu	30ms	25	0.7amu	102



Testing Methods: chromatograms

Milk Powder 1 ppm spike

RT: 0.00 - 9.00 SM: 5G



Testing Methods: LC-MS/MS Conditions (III)

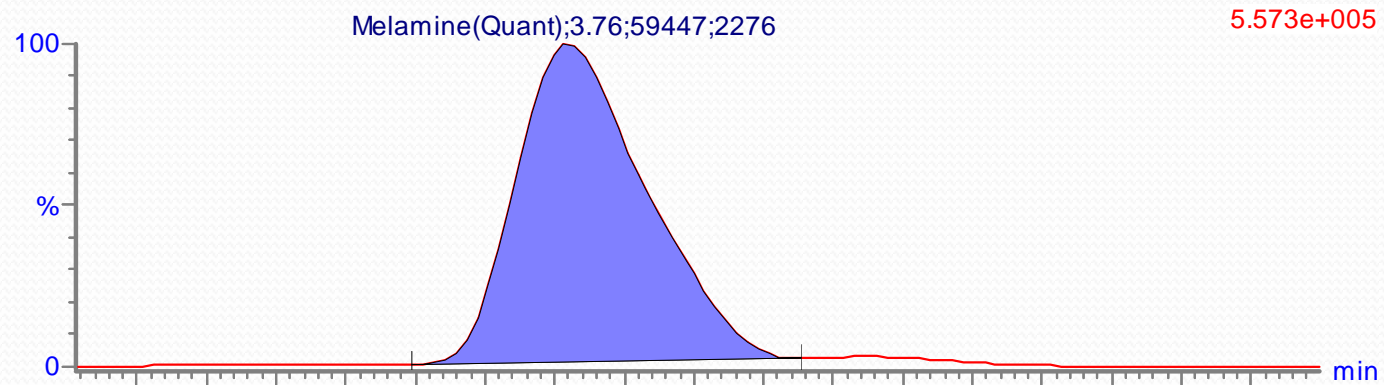
System : Waters Quattro Premier XE
Polarity : ES+
Capillary : 3.5 kV
Cone : 35 V
Extractor : 5.0V
RF lens : 0.1V
Source Temperature : 120°C
Desolvation Temp. : 430°C
Cone Gas Flow : 48 L/hr
Desolvation Gas Flow : 841 L/Hr

MRM	Dwell (s)	Cone Volt	Col. Energy	Delay (s)
127.00 > 60.25	0.080	35	18	NA
127.00-> 68.18	0.080	35	25	NA
127.00-> 85.12 (Q)	0.080	35	16	NA
133.02-> 72.00	0.080	35	17	NA
133.02-> 89.00	0.080	35	17	NA



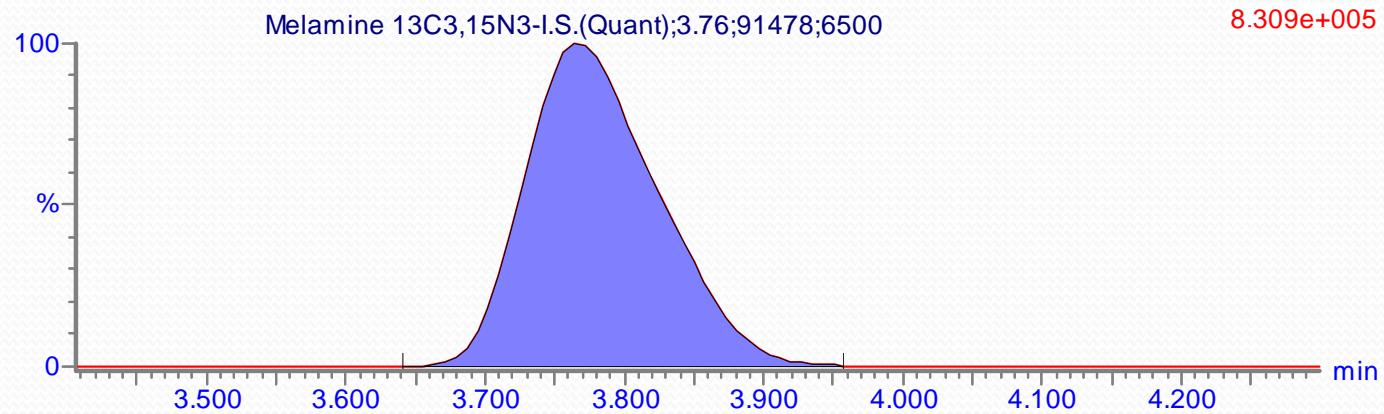
M20080919_49
SG51992R

MRM of 5 channels,ES+
127 > 85.12
5.573e+005



M20080919_49
SG51992R

MRM of 5 channels,ES+
133.02 > 89
8.309e+005



Recovery Rate

<u>Spiked level</u>	<u>Average Recovery</u>	<u>N</u>
10 ppm	97%	10
1 ppm	78%	3
0.5 ppm	101%	2



MCX Clean-up method

- Use 1g sample to a 50mL centrifuge tube.



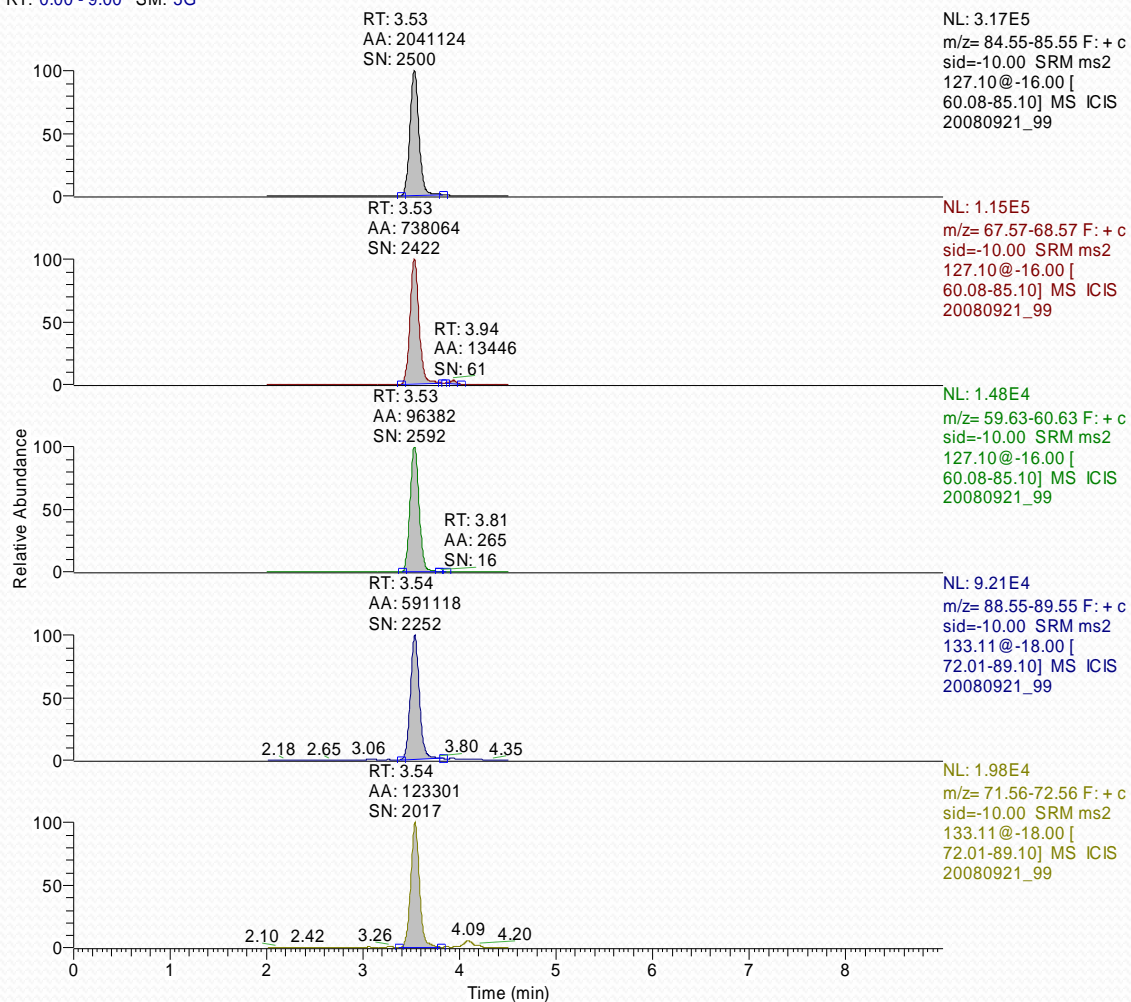
- Condition the Waters Oasis MCX (60mg, 3mL) column with 3mL of methanol and 3mL of water.
- Add the aqueous aliquot into the column at gravity flow.
- Wash the column with 3mL of 0.1N HCl and 2mL of methanol.
- Vacuum dry the column. Do not over dry.
- Elute the analyte with 3mL of 4% NH₃ in MeOH.
- Evaporate the eluent to just dryness at a gentle stream of nitrogen at about 40-50°C.
- Reconstitute the residue with 1mL of 95:5 v/v 0.1% Formic acid in acetonitrile: 20mM NH₄OAc buffer solution. Vortex mix to dissolve well.
- Filter when necessary and analyse the final extract by LC-MSMS.



Testing Methods: chromatograms with MCX clean-up

Milk beverage 60 ppb in sample

RT: 0.00 - 9.00 SM: 5G



Notes:

- *Final injection solution should be 95% Acetonitrile for giving better peak shape.*
- *Use 2.1mm id column will give higher peak height → better sensitivity*
- *You can use other isotope internal standards*

$^{13}\text{C}_3$ $^{15}\text{N}_3$ (amino labeled)-Melamine,
 $^{13}\text{C}_3$ -Melamine, $^{15}\text{N}_3$ (ring labeled)-Melamine, $^{15}\text{N}_6$ -Melamine
Avoid d_6 -Melamine in which the deuterium may be exchanged

Cost for 1.2 mL 100 $\mu\text{g}/\text{mL}$ $^{13}\text{C}_3$ $^{15}\text{N}_3$ (amino labeled)-Melamine ~ HK\$2700 - 3500
Cost for d_6 -Melamine ~ 1 mg (~US\$250.0 each) or 10 mg (~US\$1300.00 each).

Sources: Cambridge Isotope Laboratory, Sigma-Aldrich, www.amxpress.com

- *You can achieve lower detection limit by varying the dilution factor or with MCX SPE clean-up.*





Thank you

*Thank to every staff in Food Safety Sections
who have contributed their holidays and worked very
hard in this incident.*

