Total Solution for Melamine Analysis by Agilent SPE, LC, GC/MS and Triple Quadrupole LC/MS

Food Safety Limit

- EU has set safe dose level at 0.5 mg/Kg body weight per day
- US FDA has set same at 0.063 (10 X safety factor)
- None say it is safe, just what can be tolerated before impacting health
- WHO has most recently set limit at
  - 2.5 mg/Kg of food for adults
  - 1.0 mg/Kg of food for children

Agilent Solutions for Analyzing Melamine in Food

Broadest portfolio of solutions lets you select the protocol that meets your needs

- **GC/MS method**: rapid screening and confirmation
- **LC method**: routine quantitation
- **Triple Quadrupole LC/MS**: sensitive and selective for screening, confirmation, and quantitation
- **SampliQ SPE:** removes milk matrix interferences

Agilent innovative technologies for better lab productivity and faster results

- **Backflush** using innovative Capillary Flow Technology minimizes the requirement for sample preparation and shortens the GC cycle time up to five-fold
- **Fast data review** software for faster analysis time.
- High-sensitivity melamine analysis with **Triple Quadruopole LC/MS**
- **Broad column portfolio** to meet different analysis needs

Why Agilent for Melamine Analysis?

Agilent expertise in food safety:

- Experience with melamine analysis: supported US FDA and Chinese government to develop standard methods (China GB)
- Extensive collaborations with government and private food labs around the world
- Hundreds of publications by Agilent scientists

Reliable analytical systems for continuous lab operation:

- Proven reliability through a large installed base in food laboratories worldwide: private labs, government labs, and industry labs

Agilent support:

- Support to keep systems running at top performance and ensure uninterrupted lab operation
- Training courses to meet customers' needs
- A large selection of columns for LC- and GC-based applications and SPE sample preparation for melamine analysis

Agilent Offers Solutions to Meet Different Needs

- **SPE Sample Preparation:** SampliQ SCX is used to remove complex sample interferences.
- **GC/MS:** J&W DB5-ms capillary column, TMS derivatization for sample screening and confirmation.

  Optional backflush based on Agilent's Capillary Flow Technology can minimize the requirement of sample preparation and shorten the GC cycle time up to five-fold.
- **HPLC:** Reversed-phase Zorbax SB-C8 column in ion-pair mode for routine quantitation

  Optional rapid resolution LC method: RRHT column to significantly increase speed

  Alternative Ion-Exchange LC method: Zorbax 300SCX Ion-Exchange column to eliminate the need for ion-pair reagent. Easy to match with China GB LC-QQQ method for confirmation.
- **Triple Quadrupole LC/MS:** Zorbax Rx-Sil column is employed to run in hydrophilic interaction mode (HILIC) to better match with electrospray (ESI) LC/MS, provides extremely simple, sensitive, and selective testing for both melamine and cyanuric acid analysis.

  Alternative Ion-Exchange Zorbax 300SCX column can be used for the analysis of melamine in dairy products.

GC/MS Methods

GC/MS is used for sample screening and confirmation.

Improved method using Capillary Flow Technology to reduce run time and sample preparation

Standard GC/MS Method

- Screening, confirmation, and quantitation in various matrices for the presence of melamine and related compounds
- US FDA published method has detailed procedure http://www.cfsan.fda.gov/~frf/lib4423.html
- Sample needs to be derivatized before injection into GC/MS
- Analysis time can be long due to sample matrix

GC/MS Sample Preparation Procedure

Extraction

- Weigh ~ 0.5 g sample
- Add 20 mL of methanol
- Sonicate for 30 minutes
- Centrifuge for 6 minutes
- Filter using 0.45 µm filter

TMS-Derivatives

- Transfer 40 µL filtrate to a GC vial
- Evaporate to dryness (70 ˚C, nitrogen)
- Add 50 µL pyridine, 50 µL BSTFA
- Incubate at 70 ˚C for 30 min

GC/MS Experimental Conditions

GC Conditions

Inlet Temp EPC,	Split/Splitless @ 250 ºC
Injection Volume	1 µL, Split 3:1
Carrier Gas	Helium, Constant Flow Mode, 1.3 mL/min
Oven Program	75 ºC (1 min); 30 ºC /min to 300 ºC (1 min)
Post Run	300 ºC hold 5 min
Transfer Line	280 ºC

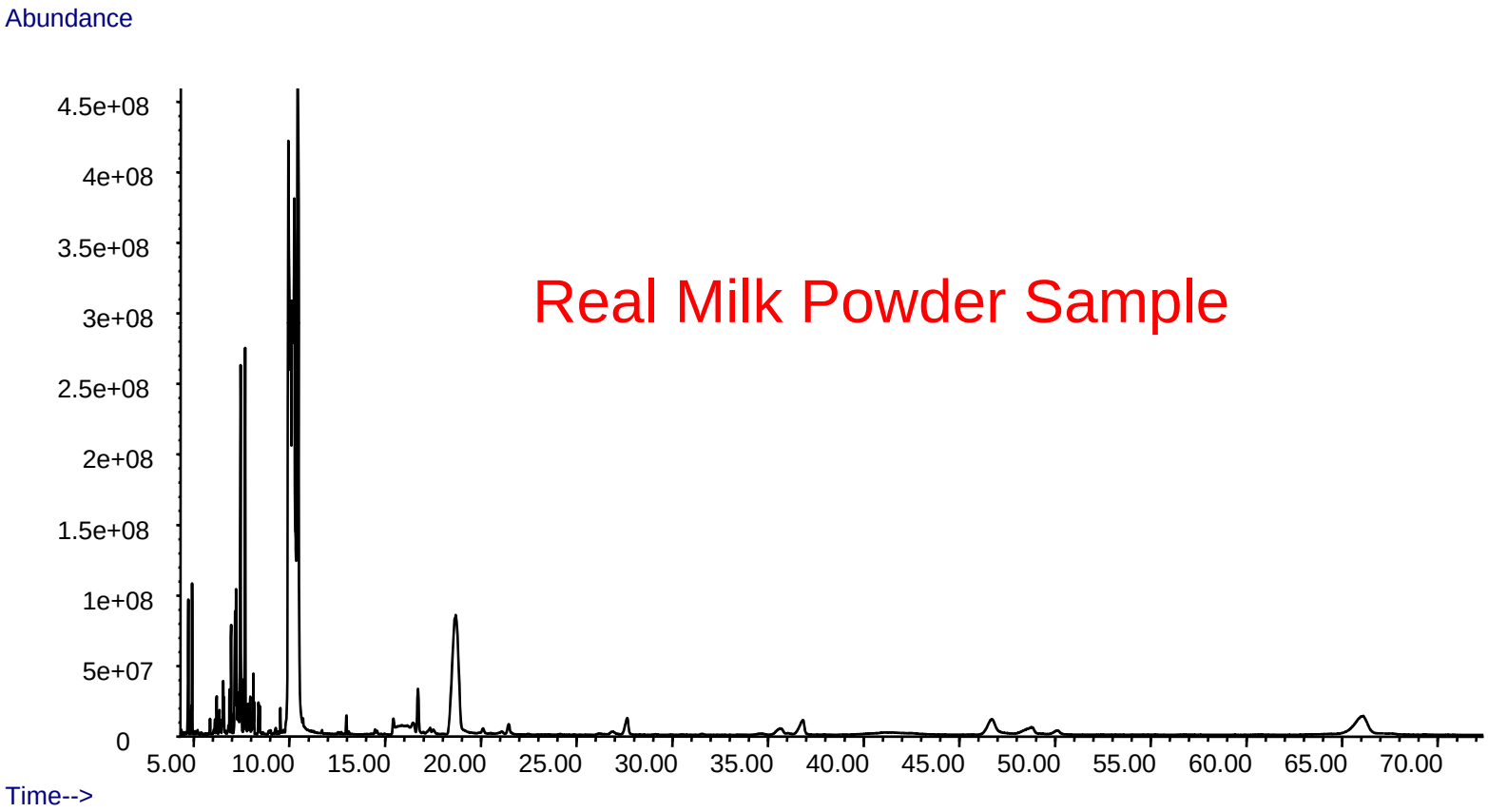
MS Conditions

MS	EI, SIM/Scan
Solvent Delay	4.2 min
MS Temp	230 ºC (Source); 150 ºC (Quad)
Scan Mode	mass range (40-450 amu)
SIM Mode Ion	melamine: 342, **327***, 171, 99; cyanuric acid: **345***, 330, 188)

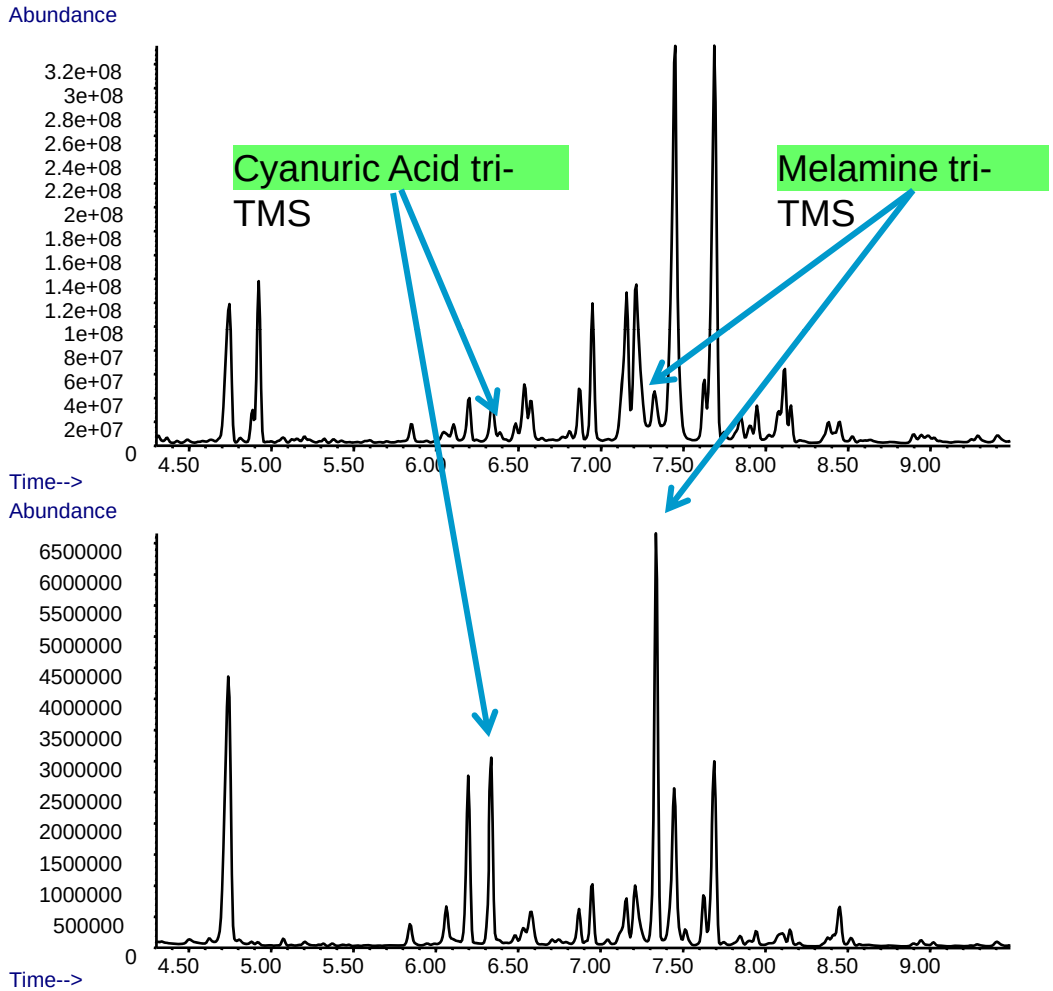
Ordering information:

		7890A/5975C with MSD ChemStation E.01.00 or later
Column:	HP-5ms 19091S-433	30 m x 0.25 mm x 0.25 µm
Or	DB-5ms 122-5532	30 m x 0.25 mm x 0.25 µm

Chromatogram of GC/MS Method



Data Analysis: SIM/Scan for Simultaneous Confirmation and Quantitation with Backflush



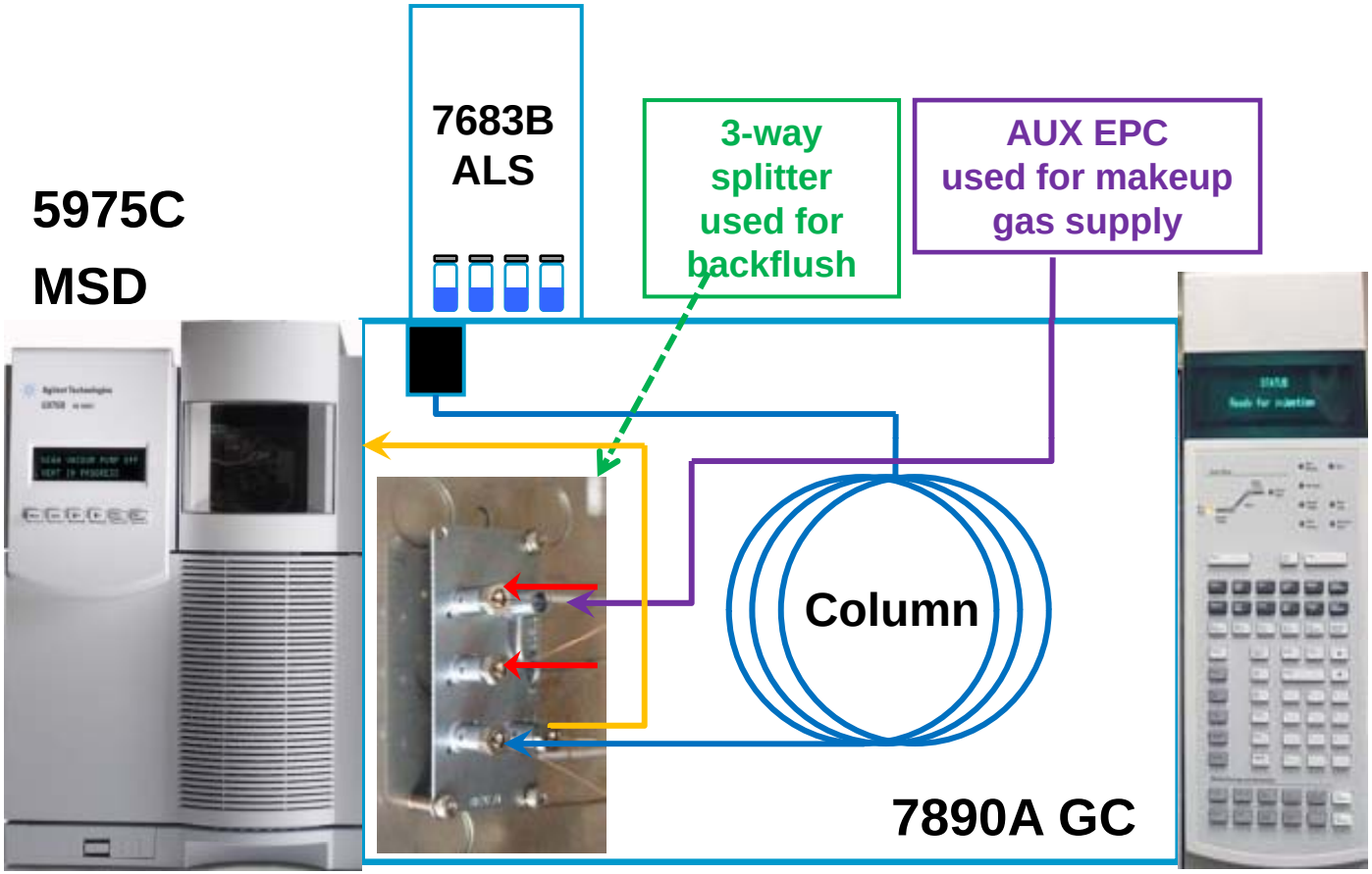
Real Milk Powder Sample

Improved GC/MS Method with Backflush*

- Optional backflush to improve lab productivity
- Compliant with FDA, China GB method but faster
- Agilent reliable and easy-to-use Capillary Flow Technology based backflush can minimize the requirement of sample preparation and shorten the GC cycle time up to five-fold
- Requires additional hardware and method setup

*See detail in application note on www.agilent.com/chem/melamine

Backflush GC/MS Configuration



Ordering information:

Same as FDA, China GB GC/MS configuration **PLUS**:

Capillary Flow Technology 3-way splitter: P/N G3183B + Aux EPC: G1530-63309

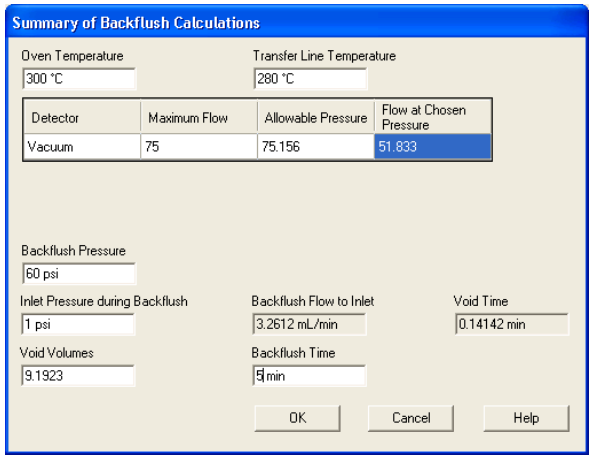
Experimental Conditions

GC/MS conditions:

Same as standard GC/MS method

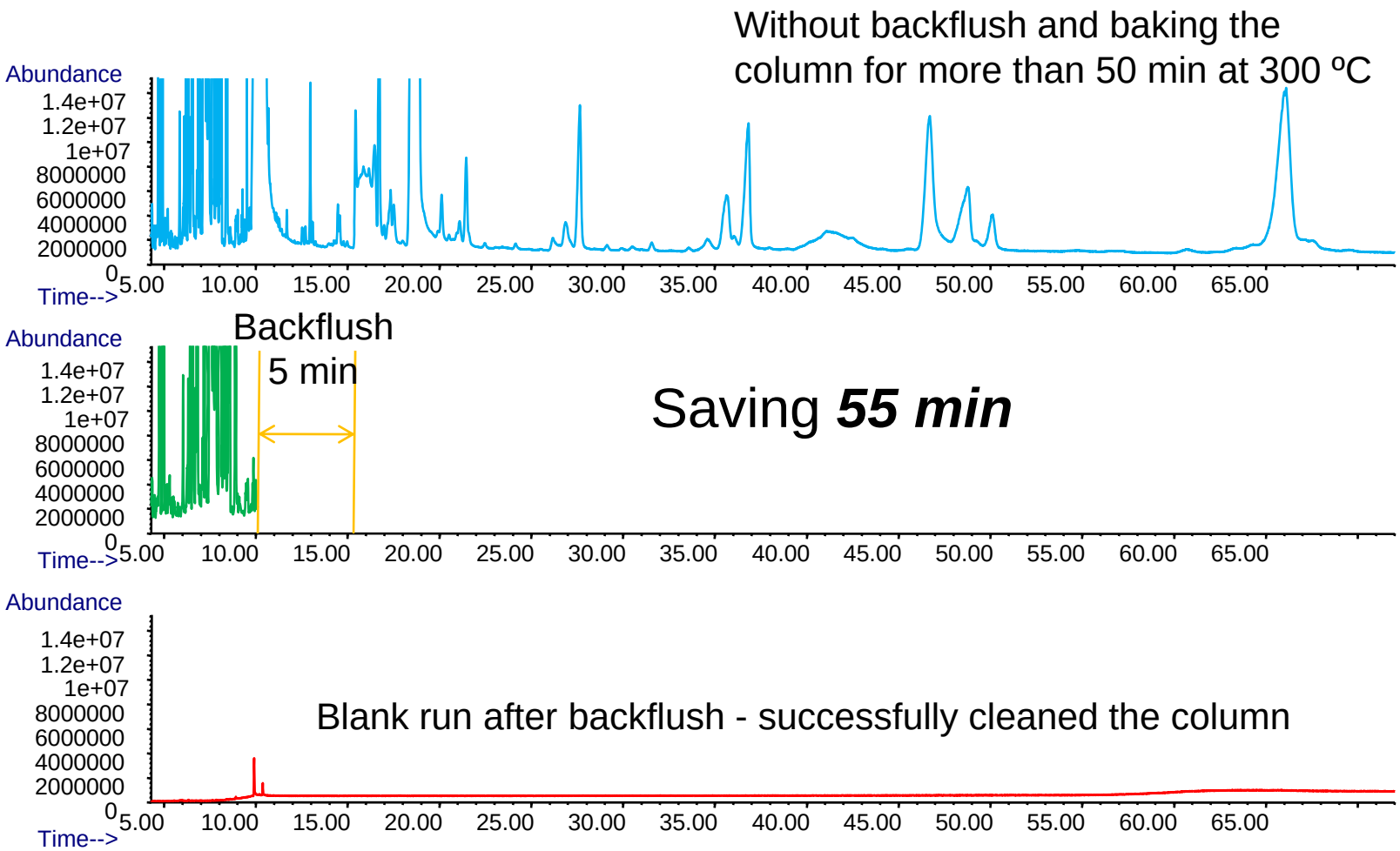
Backflush Conditions

Restrictor	0.71 m x 180 µm id
Outlet pressure	2 psi (60 psi for post-run)
Backflush duration	5 min



Easy tool to develop a backflush method using your MSD ChemStation

Using Backflush Can Reduce Run Time 5-fold



Summary for GC/MS Methods

- Rapid screening and confirmation for melamine and related compounds.
- SIM/Scan can be used for simultaneous confirmation and quantitation.
- Backflush helped reduce run time from 75 min to 15 min.
- No need for SPE clean-up, but requires derivatization.
- Agilent's improved method is fully compliant with FDA, China GB methods.

SPE Sample Preparation

Agilent's newly introduced SampliQ SCX SPE removes complex sample matrix interferences from milk and related products.

SPE Method for LC and LC/MS/MS

Step1:Extraction

Milk, milk powder, yogurt & ice-cream

↓

Weigh 2 g into 50-mL tube

↓

add 15 mL 5% TCA solution
+ 5 mL acetonitrile

↓

Sonicate for 10 min, vertically shake for 10 min, centrifuge at 4000 rpm for 10 min

↓

Filter the supernatant, bring to 25.0 mL with 5% TCA solution

↓

(5.0 mL of the above extract
+ 5.0 mL)[1] water for SPE clean-up

→

Step2: SPE Clean-up

SampliQ SCX cartridge Condition:
using 3 mL methanol + 3 mL water

↓

Load: the above extract[1]

↓

Wash:
using 3 mL water + 3 mL methanol at <1 mL/min
Dry the cartridge by applying vacuum

↓

Elute: using 6 mL 5% ammonium hydroxide in methanol

↓

Collect:
Evaporate to dryness under N_2 (50° C), reconstitute in 1.0 mL mobile phase and filter into a LC vial

Ordering information: SampliQ P/N: 5982-3236 (3 mL, 60mg)

HPLC Methods*

FDA, China GB standard reversed-phase method is used for routine quantitation

Optional Rapid Resolution LC (RRLC) method can significantly increase sample throughput

Agilent-developed Ion Exchange Chromatography (IEC) method is a better match with China GB LC-QQQ method for melamine analysis in dairy products

*See detail in application note on www.agilent.com/chem/melamine

Reversed-Phase LC (recommended by FDA, China GB)

- Routine quantitation method in various matrices for the presence of melamine
- US FDA reference method with detailed procedure

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

- No need for derivatization, but requires SPE cleanup

Reversed-phase LC Conditions

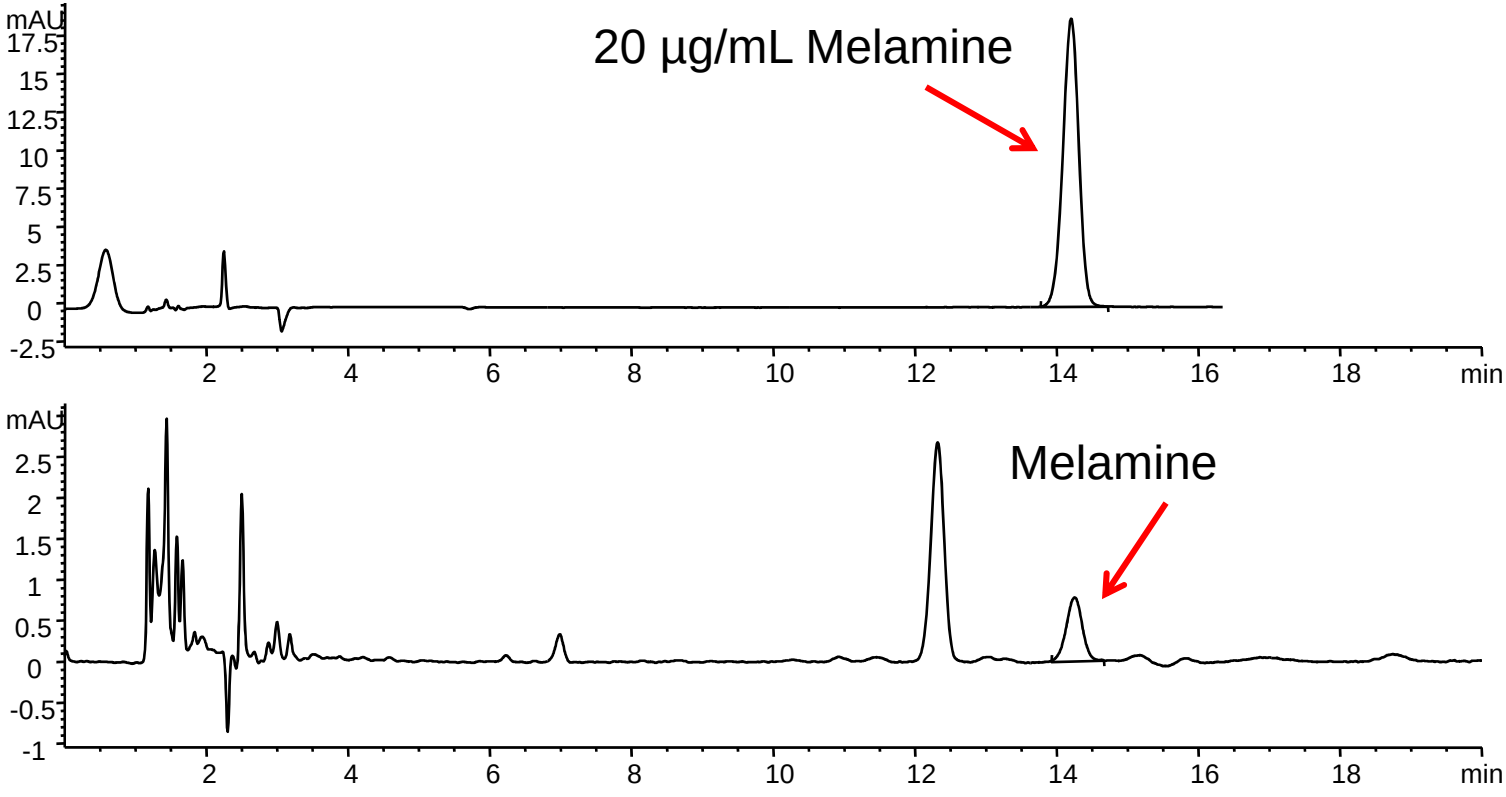
Column:	Zorbax SB-C8, 4.6 mm x 250 mm x 5 µm
Buffer:	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase:	92:8 buffer: acetonitrile
Flow rate:	1.5 mL/min
Injection volume:	20 µL
Column thermostat:	30˚C
Detection wavelength:	240 nm
Run time:	20 min

Ordering information:

LC system: Any Agilent LC (1200 HPLC, 1200SL RRLC or 1120 Compact LC)

Column: Zorbax SB-C8 4.6 mm x 250 mm x 5 µm (P/N: 880975-906)

Reversed-phase LC: yogurt Sample



Contaminated yogurt sample
after clean-up by SampliQ SCX SPE cartridge (PN: 5982-3267)

Optional Rapid Resolution LC (RRLC) Method

- Rapid routine quantitation in various matrices for the presence of melamine
- Total analysis time less than 6 min
- No need for derivatization, but requires SPE cleanup

RRLC Conditions

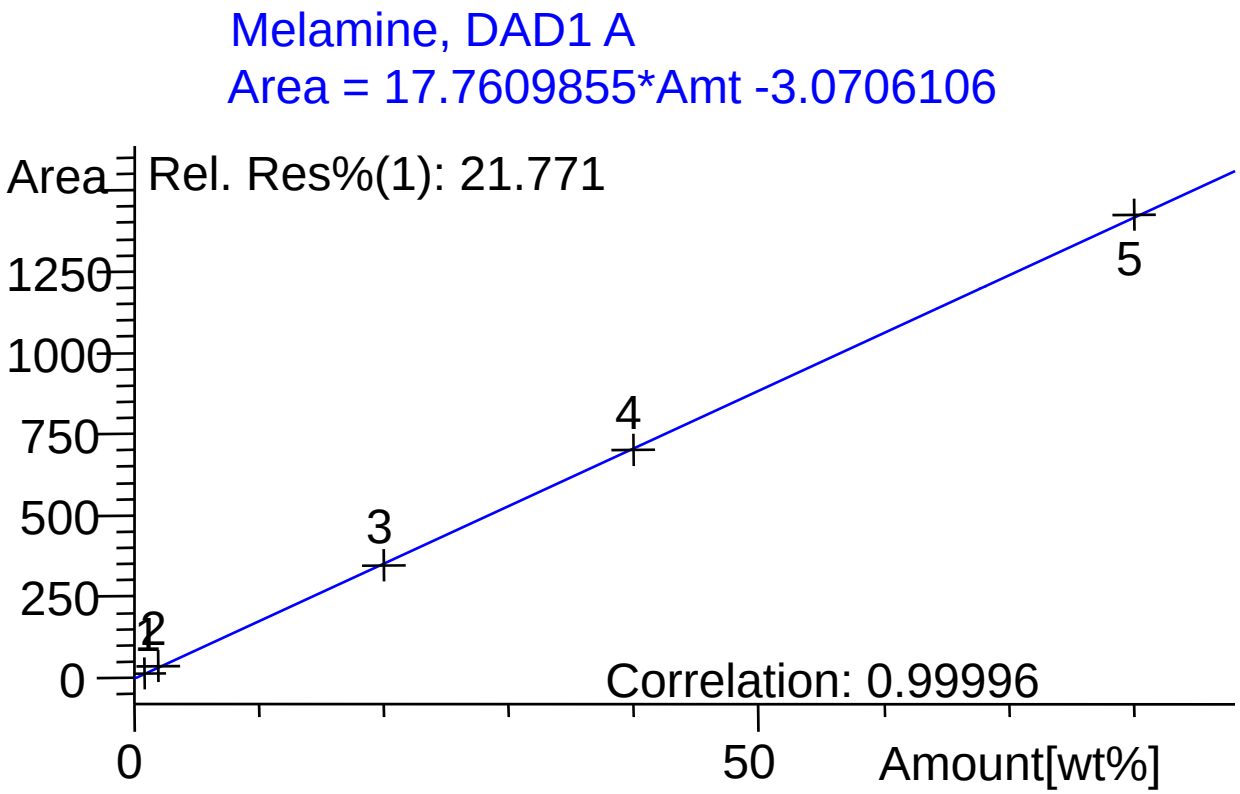
Column:	Zorbax SB-C8 RRHT, 4.6 mm x 50 mm x1.8 µm
Buffer:	10 mM citric acid, 10 mM sodium octane sulfonate, adjusted to pH 3.0
Mobile phase:	92:8 buffer: acetonitrile
Flow rate:	1.5 mL/min
Injection volume:	8 µL
Column thermostat:	30 ˚C
Detection wavelength:	240 nm
Run time:	6 min

Ordering information:

LC system: Agilent 1200 RRLC system

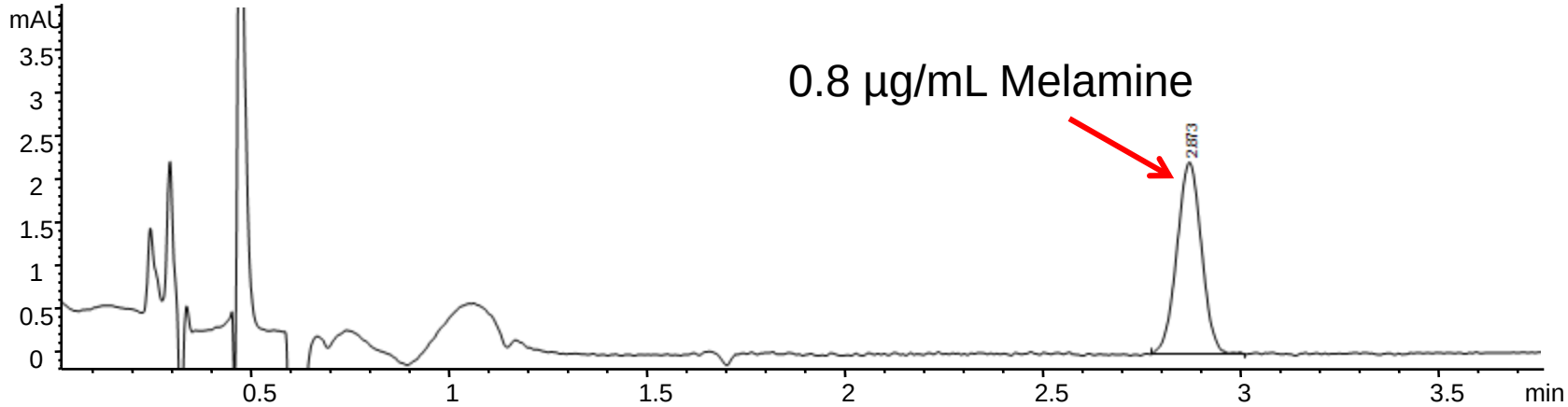
Column: Zorbax SB-C8 RRHT, 4.6 mm x 50 mm x 1.8 µm (P/N:827975-906)

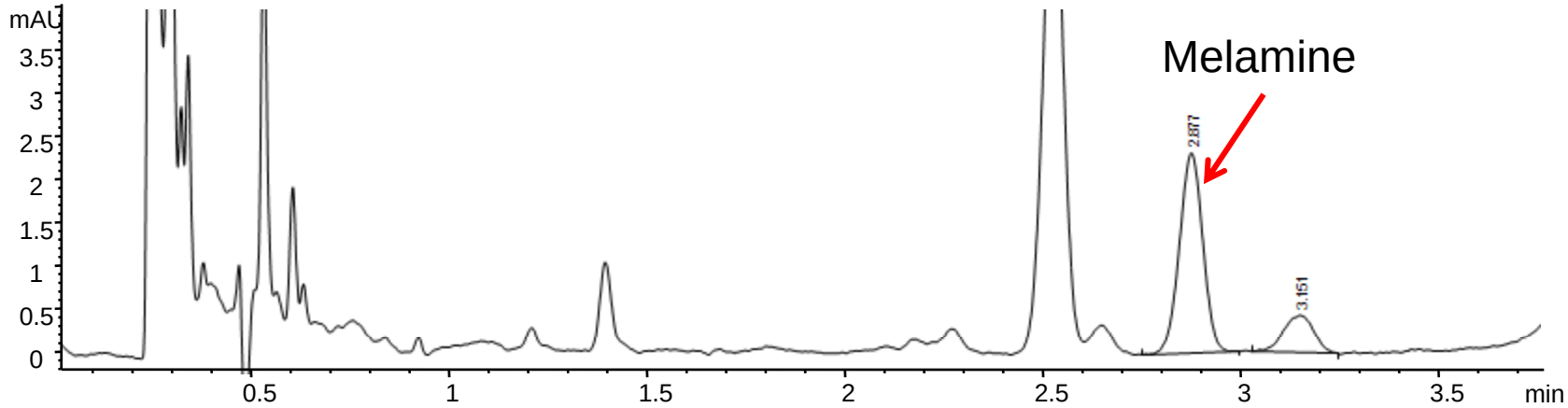
RRLC Method – Linear Dynamic Range



RRLC Result: Yogurt Sample

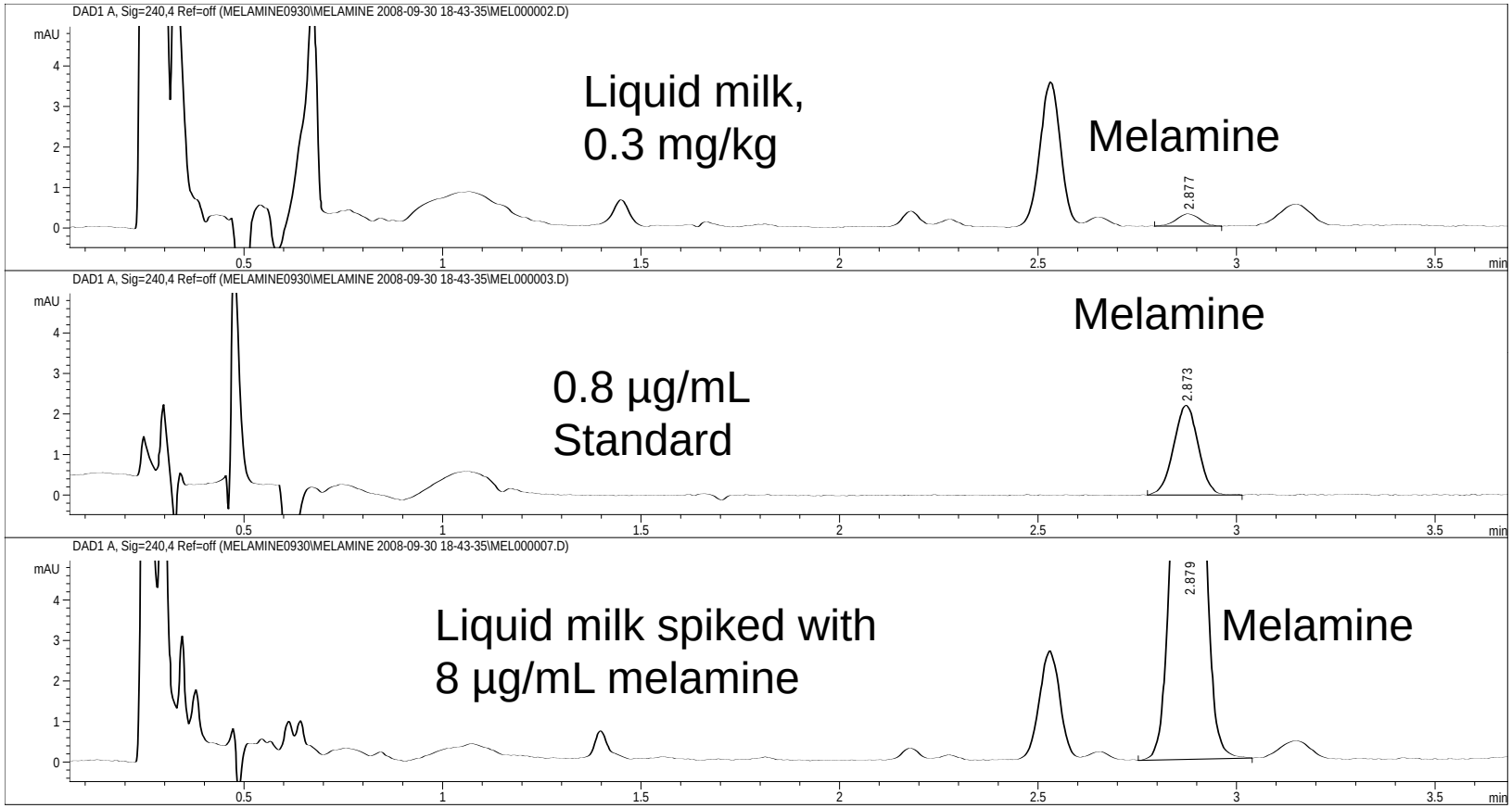
-same sample, more than 3 times faster





Contaminated yogurt sample
after clean-up by SampliQ SCX SPE cartridge (PN: 5982-3267)

RRLC Result : Liquid Milk



Alternative LC Method – Ion Exchange Chromatography (IEC)

- Fast , simple way for routine quantitation of melamine in milk and related products
- Compatible with China GB LC-QQQ method for confirmation
- Not an FDA or China GB standard LC method but a simple and faster method for the analysis of melamine in milk
- Less interference from milk matrix components

IEC Conditions

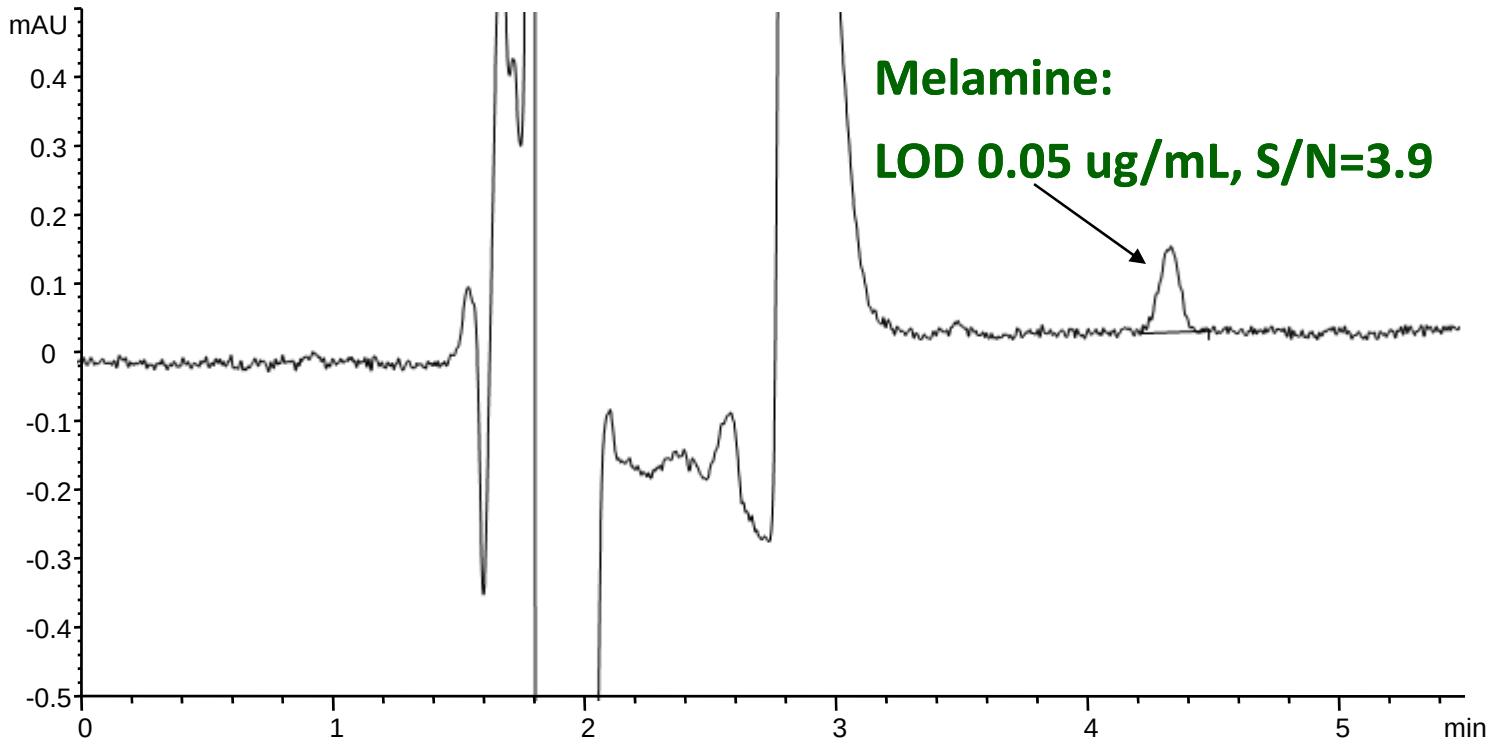
Column:	ZORBAX 300SCX 4.6 x 150 mm, 5 µm
Buffer:	50 mM ammonium formate solution, adjust pH to 3.0 with formic acid
Mobile phase:	15:85 buffer: acetonitrile
Flow rate:	1.0 mL/min.
Injection volume:	10 µL
Column thermostat:	30 ˚C
Detection wavelength:	240 nm
Run time:	5.5 min

Ordering information:

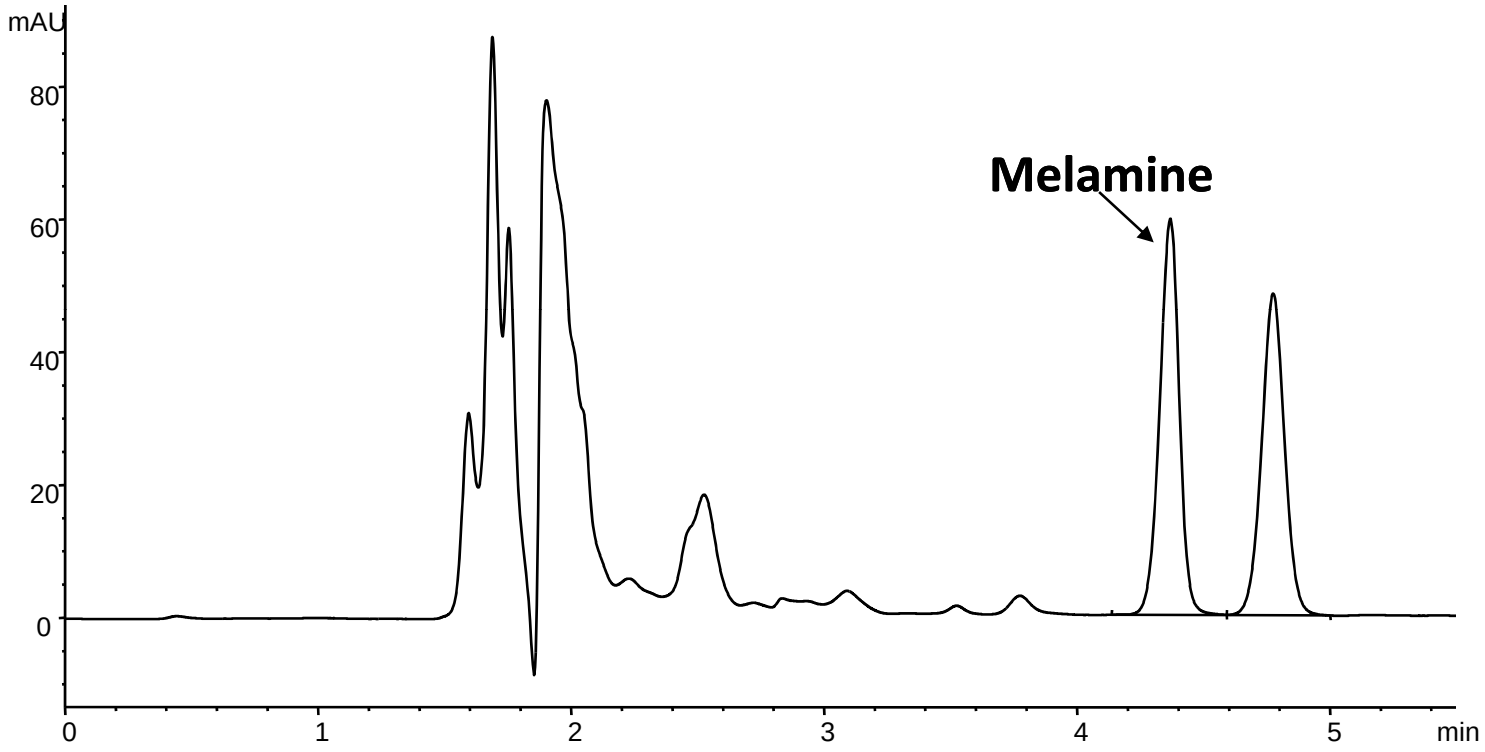
LC system: Any Agilent LC (1200 HPLC, 1200SL RRLC, or 1120 Compact LC)

Column: ZORBAX 300SCX 4.6 mm x 150 mm x 5 µm (P/N 883952-704)

IEC Method Result



IEC – Milk Powder Sample

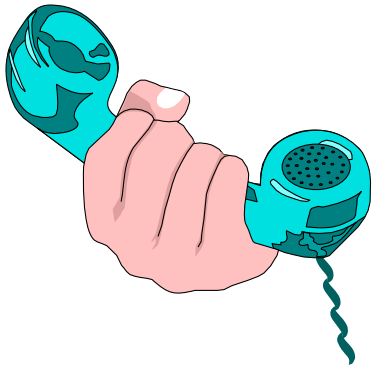


Note: no SPE clean-up was used for sample

HPLC Methods Summary

- LC methods are suitable for routine quantitation.
- SPE sample cleanup to eliminate matrix interferences.
- FDA, China GB Reversed-phase LC method is easy to set up with the most commonly used HPLC mode and column, but requires the use of an environmentally unfriendly ion-pairing reagent as buffer and longer retention time.
- Optional RRLC significantly improved the speed of analysis.
- Agilent-developed IEC method eliminates the need for the ion-pair reagent; it is simple, fast, more forgiven to matrix interferences and a better match with LC/MS/MS.

Break Number 1



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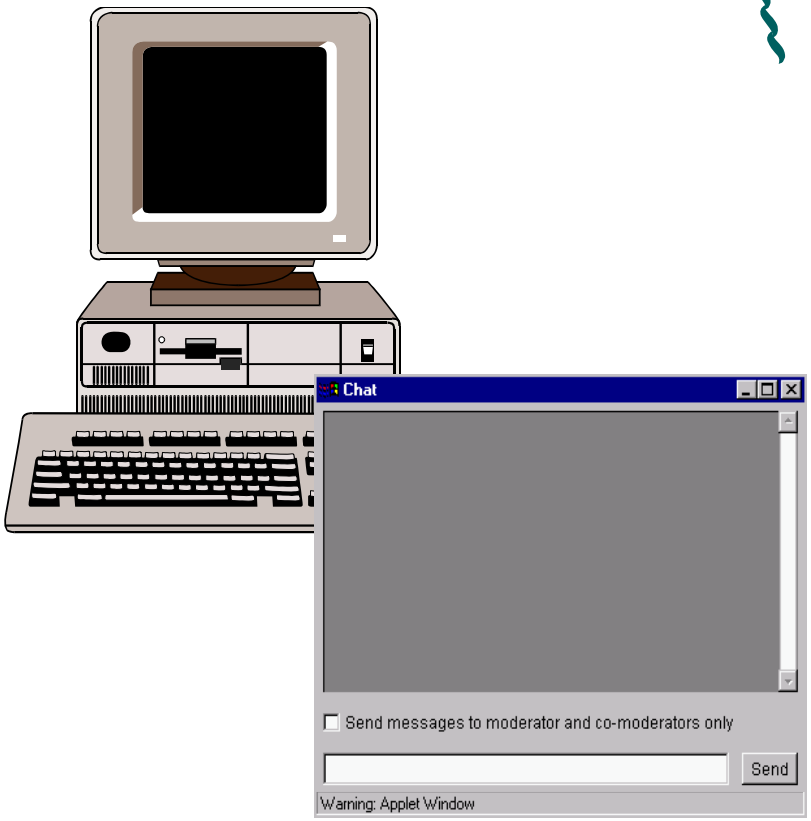


Triple Quadrupole LC/MS Methods*

Highly sensitive and selective method for screening, quantitation and confirmation

HILIC method is referenced by FDA research method for the analysis of melamine & cyanuric acid

Ion-exchange is referenced by China GB method for the analysis of melamine in dairy products

*See detail in application note on www.agilent.com/chem/melamine

Triple Quadrupole LC/MS with HILIC Chromatography

- Simple, sensitive, and selective for screening, confirmation, and quantitation
- No need for derivatization but requires SPE cleanup
- Simultaneously analyze melamine and cyanuric acid

HPLC Parameters for Triple Quadrupole LC/MS HILIC Method

HPLC system	**: Agilent 1200 RRLC**
Column	**: Agilent Zorbax-Rx Sil, 2.1 x 150 mm, 5 µm**
	P/N 883700-901
Injection Volume	**: 10 µL**
Temp	**: 40 ˚C**
Flow rate	**: 0.2 mL/min**
Mobile phase	**: A - 5 mM Ammonium acetate@Water**
	: B - 5 mM Ammonium acetate@ACN
Isocratic	**: 95% B**

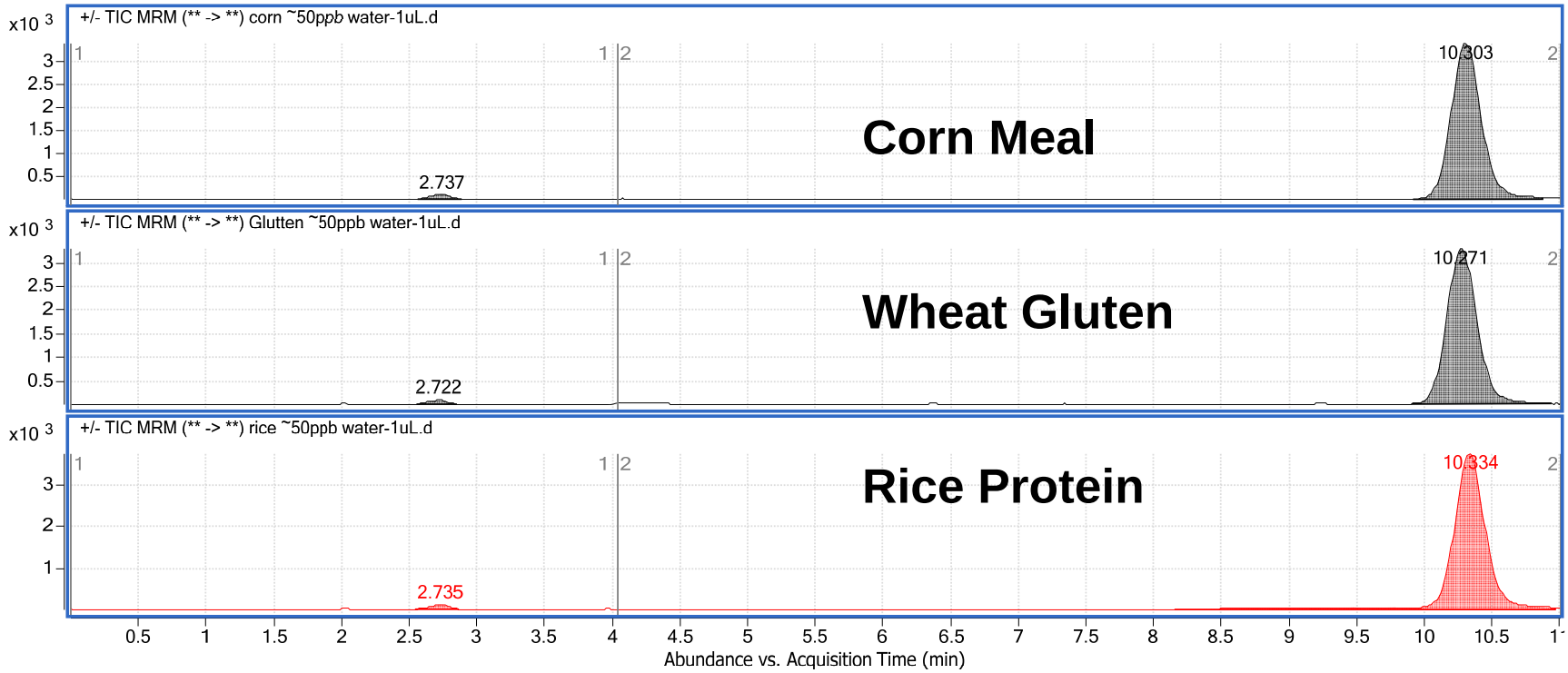
MS Parameters for Triple Quadrupole LC/MS HILIC Method

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

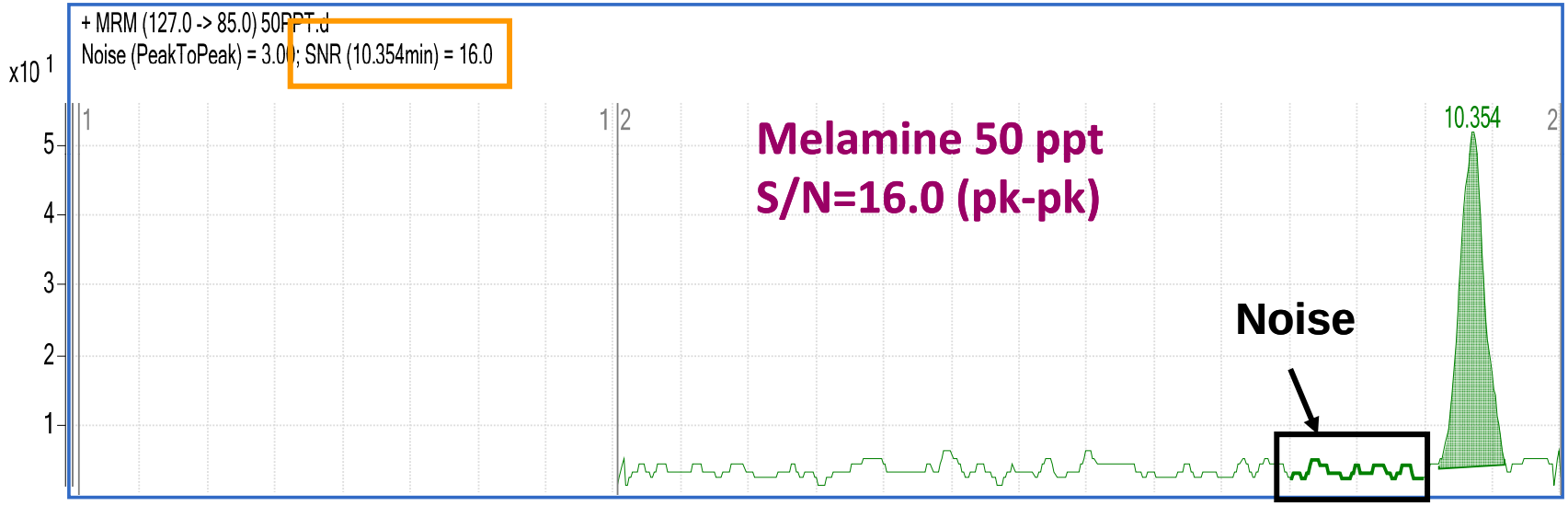
MRM Conditions

Time	Compound	Precursor	Product	Dwell (ms)	Fragmentor (V)	Collision Engergy
9.9	Melamine	127	85	200	100	20
		127	68	200	100	35

Triple Quadrupole LC/MS HILIC Method Result - Spiked 50 ppb



Triple Quadrupole LC/MS with HILIC Chromatography - High Selectivity and Sensitivity



Triple Quadrupole LC/MS – Ion-Exchange LC-MS

- Simple, sensitive, and selective for screening, confirmation, and quantitation of melamine in milk products
- No need for derivatization but requires SPE clean up

Triple Quadrupole LC/MS Ion-Exchange LC Conditions

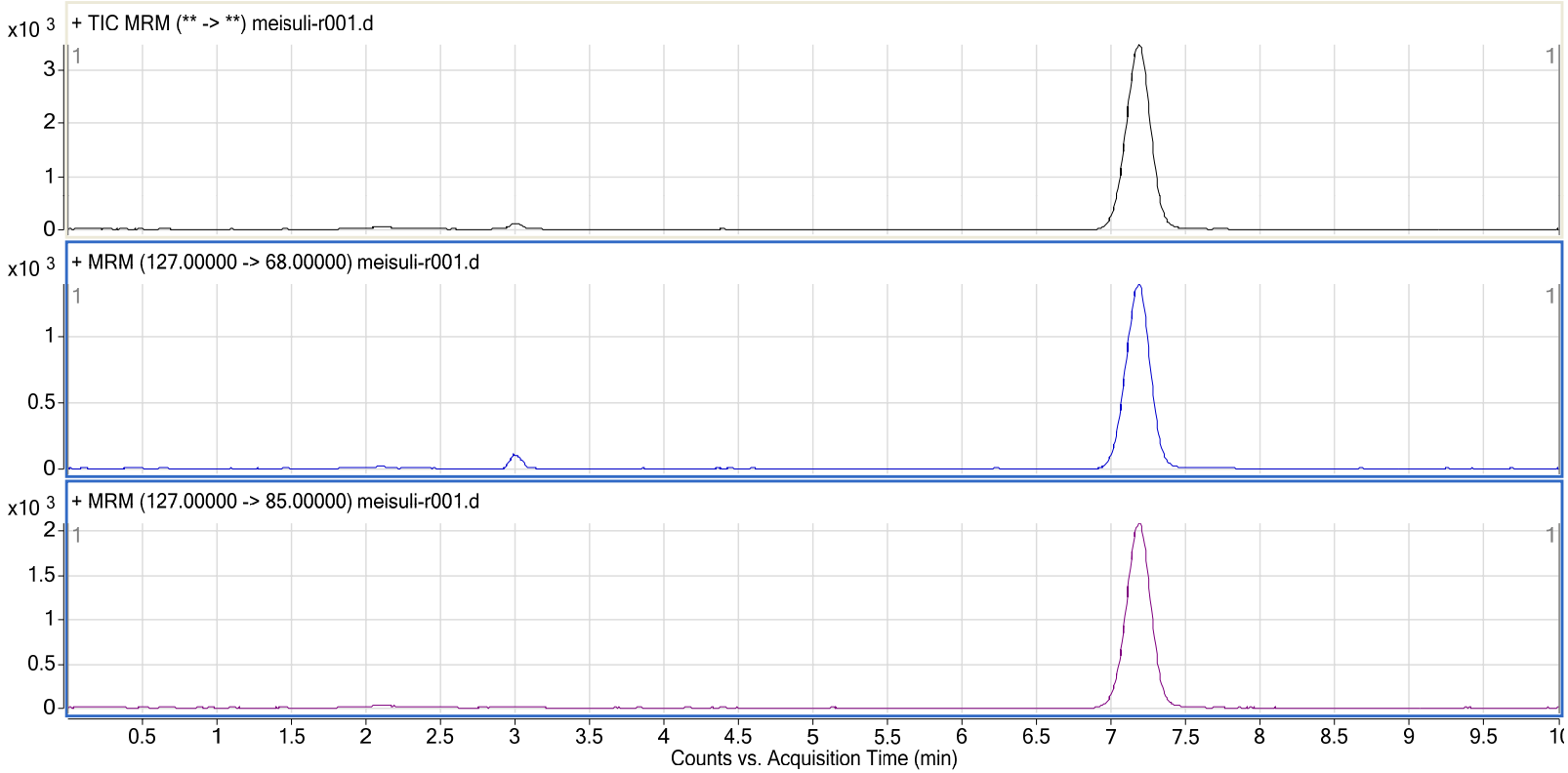
HPLC system	**1200 LC system with binary pump**
Column	**Agilent Zorbax 300SCX, 2.1×150 mm, 5 µm** **P/N: 883952-704**
Injection Volume	**10 µL**
Flow rate	**0.2 mL/min**
Temperature	**40 °C**
Mobile phase	**A---10 mM NH_4Ac/acetic acid pH adjusted to 3.0;** **B---ACN** **A:B=20:80**
Run time	**10 min**

Triple Quadrupole LC/MS Ion-Exchange MS Conditions

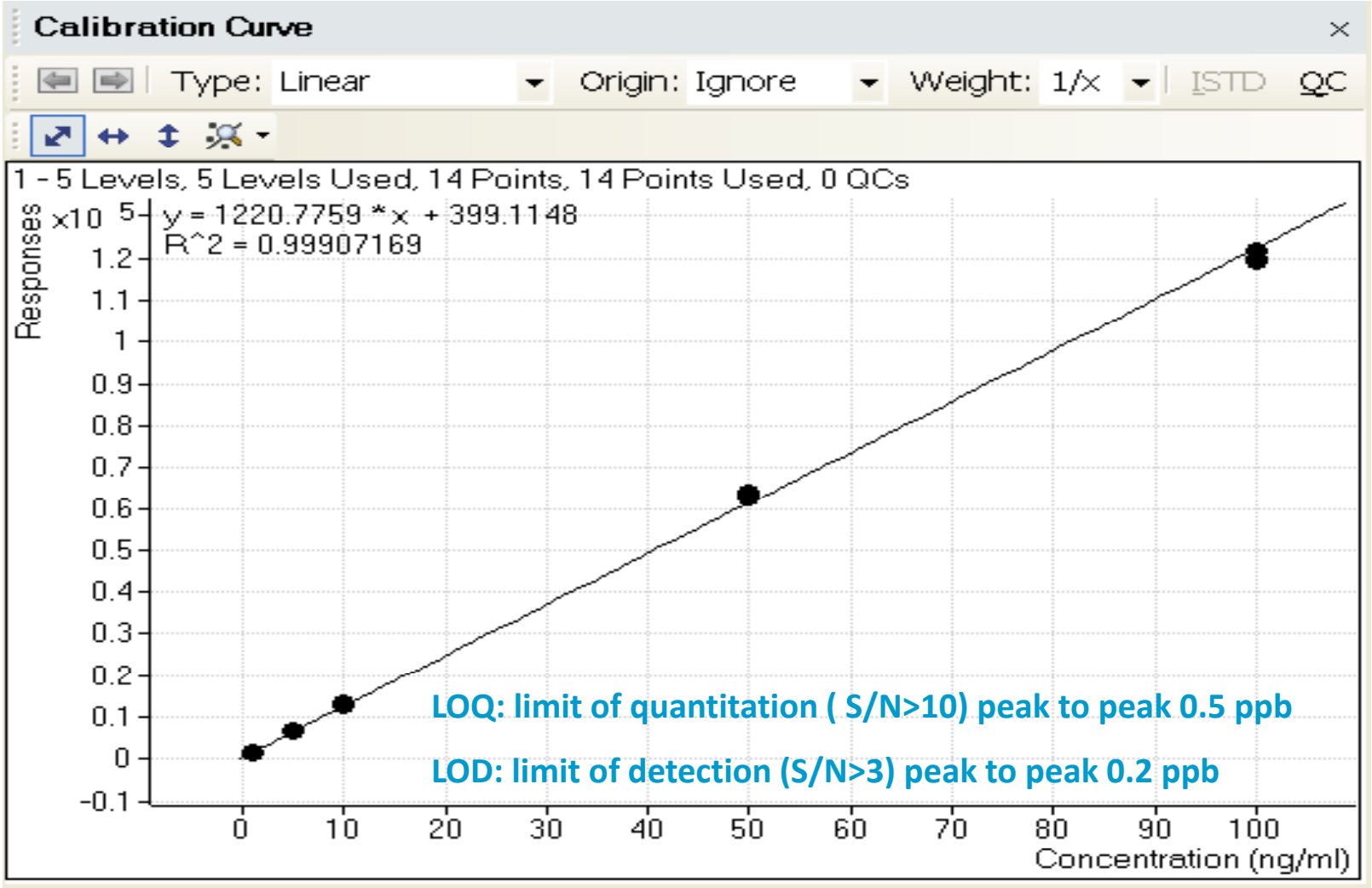
MS system	**Agilent 6410A LC/MS/MS**
Ion source	**ESI**
Polarity	**Positive**
Nebulizer gas	**Nitrogen**
Ion spray voltage	**4000V**
Dry gas temperature	**350 °C**
Dry gas flow rate	**9 L/min**
Nebulizer pressure	**40 psi**
Resolution	**Q1 (unit) Q3 (unit)**
Scan mode	**Multiple Reaction Monitoring (MRM)**

Triple Quadrupole LC/MS Ion-Exchange Result – Milk Powder

Melamine assay: 18.3 ppb



Triple Quadrupole LC/MS Ion-Exchange Result – Calibration Curve



Triple Quadrupole LC/MS Summary

- Simple, sensitive, and selective for screening, confirmation, and quantitation.
- No need for derivatization but requires SPE cleanup.
- Zorbax Rx-Sil normal-phase column is employed to run in hydrophilic interaction mode (HILIC) to match with electrospray (ESI) LC/MS and simultaneously analyze melamine and cyanuric acid.
- Ion-exchange Zorbax 300SCX column-based method is a simple and fast equivalent to China GB method.

Total Solution for Melamine Analysis

- **SPE Sample Preparation:** to remove complex sample interferences.
- **GC/MS:** for sample screening and confirmation.

  Optional backflush to shorten the GC cycle time up to five-fold.
- **HPLC:** for routine quantitation

  Optional RRLC method: to significantly increase speed

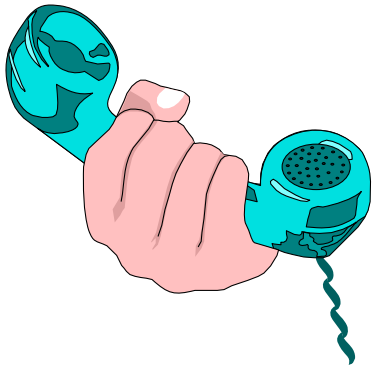
  Ion-Exchange LC method: Fast, simple, robust and compatible with LC-QQQ
- **Triple Quadrupole LC/MS:** HILIC to better match with electrospray (ESI) LC/MS for both melamine and cyanuric acid analysis.

  Ion-Exchange – MS for the analysis of melamine in dairy products.

For More Information:

Please visits www.agilent.com/chem/melamine.

Break Number 2



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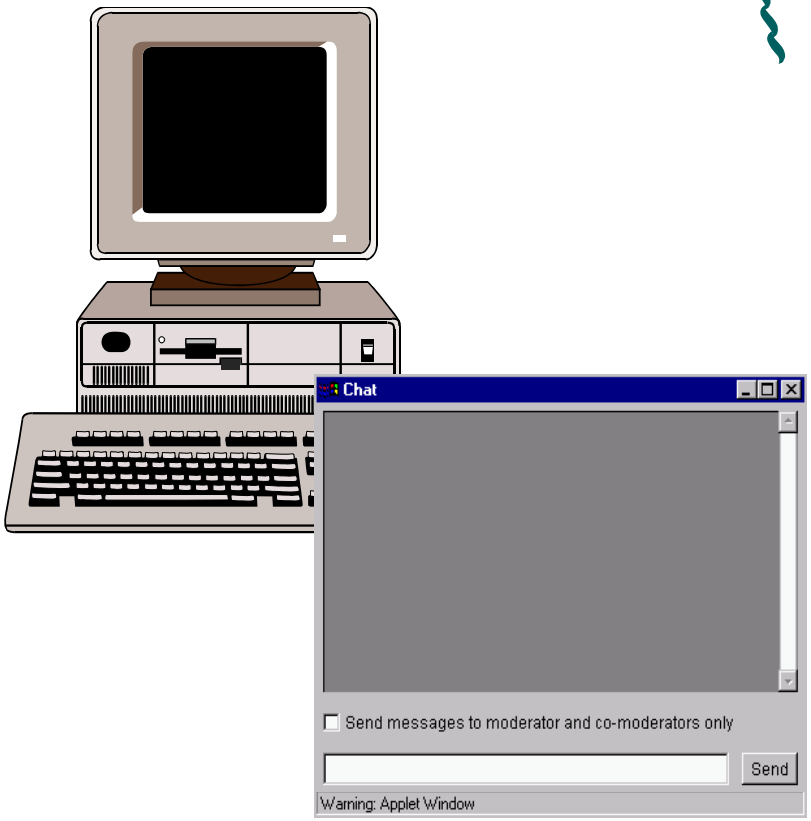

Wrap-up E-Seminar Questions

Thank you for attending Agilent e-Seminars. Our e-Seminar schedule is expanding every week. Please check our website frequently at:

www.agilent.com/chem/eseminars