Determination of Melamine in Powdered Milk by LC-MS/MS Using a Core Enhanced Technology Solid Core HPLC Column

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Key Words
Melamine, milk, HyperSep, Retain-CX, Accucore HILIC

Abstract
In recent years melamine has been found in adulterated milk and milk based products, especially in infant formula milk, causing thousands of children to become ill. Limits have now been set for the amount of melamine that can be present in foodstuffs; for infant formula milk this level is zero and for other food stuffs there is a 2.5 ppm maximum. An LC/MS method for melamine in infant formula milk has been developed. Using Thermo Scientific™ HyperSep™ Retain-CX solid phase extraction cartridges sample preparation is fast and efficient giving an average recovery of 121%. The Thermo Scientific Accucore™ HILIC column provided a fast run time of 2 minutes. The dynamic range was linear between 10 and 1000 ng/g with a correlation coefficient (r2) of 0.9913 and accuracies of +/- 20% for standards.

Introduction
Melamine is a compound commonly used as a fire retardant in plastics or as a fertilizer due to its nitrogen rich structure. As melamine is nitrogen rich it has been used to fraudulently increase the detected protein level in milk so that the milk can command a higher price. The ingestion of melamine can cause renal complications including kidney and bladder stones and even bladder cancer. In 2008, (13000) children were taken ill in China as a result of drinking infant formula milk containing melamine. Limits have now been set for the amount of melamine that can be present in foodstuffs, for infant formula milk this level is zero (although a level below 1 ng/g does not present a concern) and for other food stuffs is 2.5 ppm. It is therefore necessary to have a fast and sensitive method that can be conducted quickly and gives reproducible results.

The method described in this application note allows the testing of infant formula milk for the presence of melamine. Using HyperSep Retain-CX for the extraction of melamine followed by analysis using an Accucore HILIC column, a fast two minute run time was achieved.

Experimental Details

Consumables	Part Number
Fisher Scientific LCMS grade methanol	M/4062/17
Fisher Scientific LCMS grade water	W/0112/17
Fisher Scientific LCMS grade acetonitrile	51101
Fisher Scientific analytical reagent grade formic acid 100 %	F/1900/PB08
Fisher Scientific HPLC grade ammonia solution	A/3295/PB05
Fisher Scientific analytical reagent grade acetic acid	A/0415/07
Melamine, Sigma Aldrich	30130TR
Infant Formula Milk – Stage 2	

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Sample Handling Equipment	Part Number
Liquid handling hardware: Thermo Scientific FinnPipette (100-1000 µL)	642090
Thermo Scientific FinnPipette (10-100 µL)	4642070
Thermo Scientific FinnPipette (1-10 µL)	4642040

SPE Hardware	Part Number
Thermo Scientific Ultra Vap	CLS-229070
Thermo Scientific HyperSep Glass Block Manifold	60104-232

SPE Cartridge	Part Number
HyperSep Retain-CX, 60 mg/3 mL	60107-303

Vials and Closures	Part Number
Thermo Scientific Chromacol storage vial	22-SV-CP
Thermo Scientific premium vial	60180-600

Sample Preparation	
Compound:	Melamine
Matrix:	Milk powder
Weigh	1 g of milk powder. Dissolve in 4 mL deionised water. Mix well
	Spike in appropriate amount of melamine calibrator. Add 6 mL of 2.5% formic acid (aq). Mix well. Sonicate for 10 minutes and centrifuge for 20 minutes at 2000 rpm
SPE cartridge type:	HyperSep Retain-CX 60 mg/3 mL
Conditioning stage:	Load 3 mL of methanol onto the SPE cartridge under vacuum followed by 3 mL of water
Application stage:	Load all supernatant onto the SPE cartridge, trying not to disturb the top layer
Washing stage:	Load 3 mL of water followed by 3 mL of methanol onto the SPE cartridge
Elution stage:	Load 2 x 1 mL 5% ammonia in methanol onto the SPE cartridge
Additional stage:	Evaporate the collected sample to dryness under nitrogen. Reconstitute in 1 mL 90:10 acetonitrile: 50 mM ammonium acetate pH 5. Gently vortex mix and sonicate for 5 minutes.

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific Accela™ 600 pump, Thermo Scientific CTC autosampler	
Column:	Accucore HILIC 2.6 µm, 100 x 2.1 mm	17526-102130
Mobile phase A:	50 mM ammonium acetate adjusted to pH 5 with acetic acid	
Mobile phase B:	Acetonitrile	
Isocratic:	(10:90)	
Flow rate:	1 mL/min	
Run time:	2 minutes	
Column temperature:	40 °C	
Injection details:	Inject 10 µL	
Loop Size:	100 µL	
Injection wash solvent:	Acetonitrile: water (50:50)	
Preparation of mobile phase A:	Weigh 3.854 g of ammonium acetate and dilute into 1 L of water. Adjust to pH 5 with acetic acid	

MS/MS Conditions

Instrumentation:	Thermo Scientific TSQ Vantage
MS/MS run time:	2 minutes
Ionization conditions:	APCI Positive
Discharge current:	4 eV
Vaporizer temperature:	350 °C
Sheath gas pressure:	50 arbitrary units
Auxiliary gas pressure:	15 arbitrary units
Capillary temperature:	300 °C
Collision pressure:	1.2 mTorr

Ions Monitored

Precursor (m/z)	Product (m/z)	Collision Energy (eV)	S-lens (Arb)
127.1	68.213	32	32
127.1	85.171	17	44

Scan time:	0.1 s
Q1 peak width:	0.20 m/z
Q3 peak width:	0.70 m/z
Chrom filter:	5 arbitrary units

Solutions

Melamine stock:	Weigh 1 mg and dissolve in 1 mL of 50 mM ammonium acetate pH 5.

Data Processing

Software:	Thermo Scientific LCQuan 2.6
Integration algorithm:	ICIS

Parameter	Value
MRM	127.1 (68.208-68.218, 85.166-85.176)
Retention Time (minutes)	0.59
Retention Window (seconds)	15
Smoothing (arbitrary units)	1
Baseline Window (counts)	75
Area Noise Factor (arbitrary units)	28
Peak Noise Factor (arbitrary units)	10
Constrain Peak Width	No
Component Type	Target Compound
Calibration Curve Type	Linear
Response	Area
Origin	Ignore
Weighting	1/X2

Results

Chromatography

The Accucore HILIC column gave excellent peak shape. The chromatography of Melamine can be seen in figure 1 and the chromatography of a milk blank can be seen in figure 2.

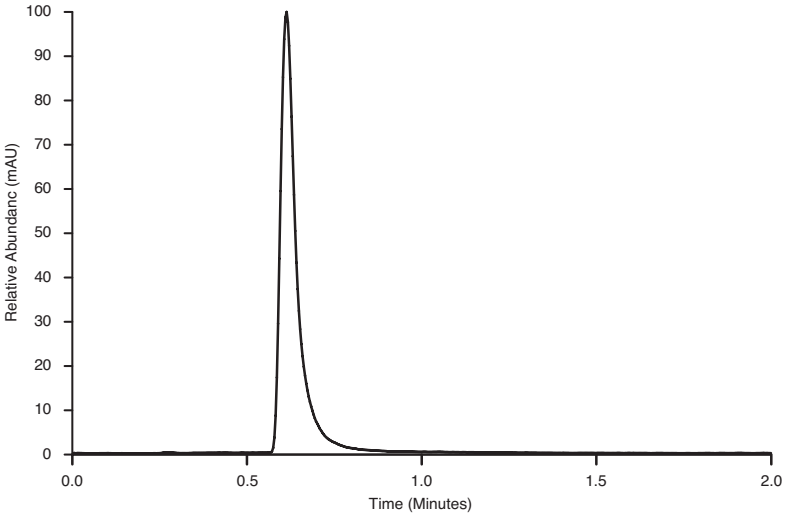

Figure 1: Selected Reaction Monitoring chromatogram of melamine at 500 ng/mL

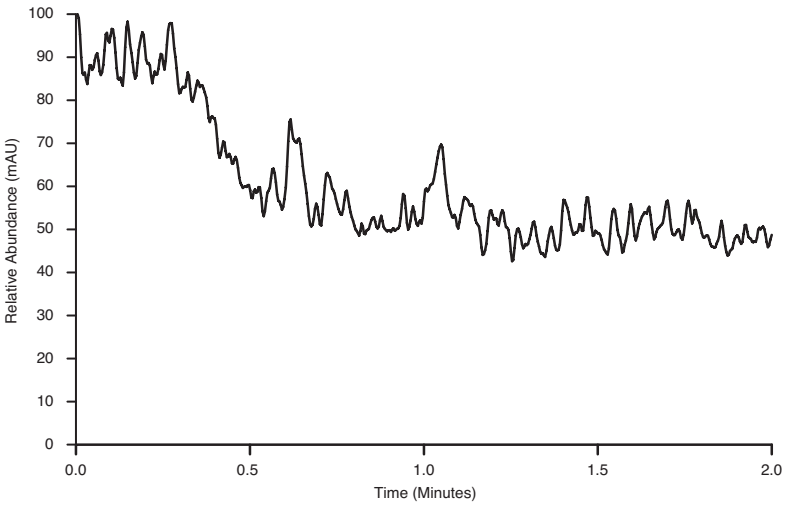

Figure 2: Selected Reaction Monitoring chromatogram of milk blank

Linearity

Standards of melamine extracted from spiked milk powder gave a linear calibration curve over the dynamic range of 10 to 1000 ng/mL with an r^2 of 0.9928 (figure 3 and table 1).

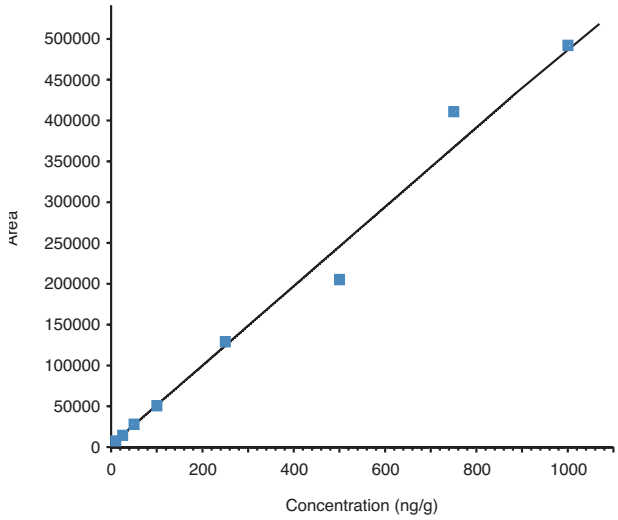

Figure 3: Linearity over the dynamic range of 10 ng/mL to 1000 ng/mL

Sample	Melamine Response (peak area)	Specified Concentration (ng/g)	Calculated Concentration (ng/g)	% Diff
Extracted S1	7770	10	10	1
Extracted S2	14549	25	24	-4
Extracted S3	28124	50	52	4
Extracted S4	50881	100	98	-1
Extracted S5	129277	250	260	4
Extracted S6	205400	500	420	-17
Extracted S7	411165	750	840	12
Extracted S8	492232	1000	1000	1

Table 1: Accuracy of extracted standards

Recovery

An average Recovery of 121% was observed.

Conclusion

Retain CX SPE cartridges and Accucore HILIC HPLC columns allow for a simple extraction and rapid quantification melamine from milk powder.
Accucore HILIC gives a fast runtime of 2 min with excellent peak shape for melamine.
An LLOQ of 10 ng/mL was achieved with an extraction recovery of above 100%.

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