Rapid Screening and Confirmation of Melamine and its Analogs in Baby Formula and Soy Products Using Triple Quadrupole GC/MS and Backflushing

Application Note

Food

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Abstract

A rapid method for the screening and confirmation of melamine, ammelide, ammeline and cyanuric acid in baby formula and soy meal was developed using the Agilent 7890A/7000A Series Triple Quadrupole GC/MS and backflushing with a Purged Ultimate Union. The extraction and derivatization procedures are the same as those used in the FDA GC/MS method. Excellent linearity ($R^2 > 0.99$) was obtained in the range of 0.16 to 2.5 ppm, with run times less than 15 minutes.

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Introduction

The adulteration of food with melamine has quickly become an international problem as it has been detected in baby formula produced in the US, chocolates distributed in Canada, biscuits sold in the Netherlands, condensed milk in Thailand and eggs in Hong Kong. In response, many countries have established allowable limits for melamine, with the FDA maximum residue limit (MRL) as 1 part per million (ppm) for infant baby formula and 2.5 ppm for other products. The FDA GC-MS screening method [1] is capable of detecting melamine and its analogs (ammeline, ammelide and cyanuric acid) at 2.5 ppm. However, the FDA import alert of February 2009 requires that a testing method with a sensitivity of 0.25 ppm for melamine and its analogs be used to assure compliance to the MRLs. Therefore this method cannot be used to screen for melamine and its analogs under the new regulations, and confirmation would require an additional orthogonal method.

This application note describes a modification of the FDA GC-MS method for use on the new Agilent 7000A Series Triple Quadrupole GC/MS. The new method, which does not require a change in sample extraction and derivatization procedures, employs a purged union GC column configuration and backflushing to provide run times under 15 minutes. Melamine and its analogs can all be detected at 0.25 ppm, with highly reproducible and accurate quantification. Most importantly, this method provides screening, quantification and confirmation of melamine and its analogs, all in one short run.

Experimental

Standards and Reagents

The standards and reagents used are listed in Table 1. Stock solutions of melamine, ammelide, ammeline and cyanuric acid, each at a concentration of 1,000 µg/mL, were separately prepared in a mixture of DEA/H20 (20/80) and stored at 4 °C. Internal standard (2,6-Diamino-4-chloropyrimidine, or DACP) was prepared at a concentration of 57.7 ng/mL in pyridine. The above solutions were used to prepare matrix-matched standards as described in the FDA method [1]. Matrix samples were generously provided by the FDA.

Table 1. Standards and Reagents

Standard	Melamine	Sigma-Aldrich	>99% purity
	Cyanuric acid	TCI-America	>98.0%
	Ammelide	TCI-America	>98.0%
	Ammeline	TCI-America	>95.0%
	Internal standard†	Sigma-Aldrich	98%
Solvent	Diethylamine (DEA)	Sigma-Aldrich	SigmaUltra grade
	Pyridine	Fisher Scientific	Certified A.C.S. reagent
	Acetonitrile	Fisher	HPLC grade
Silylating reagent	BSTFA with 1% TMCS* (SYLON BFT)	Sigma-Aldrich	Derivatization grade

† DACP (2,6-Diamino-4-chloropyrimidine)
* BSTFA: bis(trimethylsilyl)trifluoroacetamide, TMCS: Trimethylchlorosilane

Instruments

The experiment was performed on an Agilent 7890A gas chromatograph equipped with a split/splitless capillary inlet, an Agilent 7000A Series Triple Quadrupole GC/MS with Triple-Axis Detector, and an Agilent 7683B automatic liquid sampler (ALS). The split/splitless inlet was fitted with a long-lifetime septum (p/n 5183-4761) and a deactivated, splitless single taper injection liner (p/n 5181-3316). Injections were made using a 10-µL syringe (p/n 9301-0714). The instrument conditions are listed in Table 2.

Table 2. Gas Chromatograph and Mass Spectrometer Conditions

GC Run Conditions	
Column	Two 15 m × 0.25 mm × 0.25 µm HP-5ms columns (p/n 19091S-431)
Inlet temperature	280 °C
Inlet pressure	12.9 psi
Carrier gas	Helium, constant flow mode, 1.2 mL/min
Pulsed splitless	25 psi at 0.5 min
Oven program	100 °C (1 min hold), 10 °C/min to 210 °C
Column velocity	41 cm/s
Injection volume	1 µL
Transfer line temperature	290 °C
GC Post-Run Conditions	
Backflush device	Purged Ultimate Union (p/n G3186-60580) controlled by a Pressure Control Module (p/n G3476-60501)
Backflush conditions	–3.6 mL/min at 300 °C for 1.3 min
MS Conditions	
Tune	Autotune
Delta EMV	400 V
Acquisition parameters	EI; selected reaction monitoring
Solvent delay	6 minutes
MS temperatures	Source 230 °C; Quadrupoles 150 °C

Sample Preparation

A 0.5-g amount of a representative portion of the sample was weighed into a 50-mL polypropylene centrifuge tube. An extraction solvent of DEA/Water/Acetonitrile (10/40/50) was prepared, and 20 mL added to the weighed sample. Diethylamine dissociates the melamine-cyanuric acid complex, thus reducing the risk of false negative measurements. DEA also improves the solubility of ammelide and ammeline, which have extremely low solubility in traditional extraction solvents. The sample was capped, vortex mixed, and then sonicated for 30 minutes. After the sample was centrifuged at 5,000 g or higher for 10 minutes, the supernatant fluid was filtered through a 0.45-µm nylon filter.

Derivatization

A 160-µL amount of the filtrate was transferred to a glass GC vial. The extract was evaporated to dryness under a stream of nitrogen at approximately 70 °C, and 600 µL of ISTD and 200 µL of BSTFA with 1% TMCS were added. The sample was vortex mixed and incubated at 70 °C for 45 minutes before injecting.

Analysis Parameters

The parameters used in the analysis of melamine and its analogs, as well as the internal standard, are shown in Table 3.

Table 3. Analysis Parameters

		Triple Quadrupole GC/MS		
Compound	**RT**	**SRM**	**Dwell time (ms)**	**Collision energy (EV)**
Melamine	12.467	327 → 171	20	17
		342 → 285	150	20
		342 → 213	150	22
Ammelide	10.801	344 → 171	50	22
		344 → 214	50	15
		329 → 171	50	20
Ammeline	11.748	328 → 171	50	25
		343 → 214	50	20
		343 → 171	50	30
Cyanuric acid	9.613	345 → 215	50	8
		345 → 188	50	12
		330 → 215	50	4
DACP (ISTD)	11.185	273 → 237	150	12
2,6-Diamino-4-chloropyrimidine		273 → 99	150	20

Results and Discussion

Backflushing with a Purged Ultimate Union System

A backflushing configuration was employed to remove higher boiling substances from the column prior to each subsequent run by flushing late eluting peaks out of the inlet split flow vent instead of driving them though the entire column and into the MSD. Backflushing reduces chemical noise and the cycle time of the analysis, thus increasing throughput. System uptime is also increased, due to reduced maintenance of the columns and MS detector. The suite of Agilent Capillary Flow Technology modules comprises a proprietary solution that enables easy and rapid backflushing with small dead volumes for improved resolution, and ferrules and fittings that eliminate leaks. All Capillary Flow Technology modules require the use of an Auxiliary Electronic Pneumatic Control (EPC) module or a Pneumatic Control Module (PCM) to provide a precisely-controlled second source of gas that directs the column flow to the appropriate column or detector. In normal operation, the PCM pressure is at or slightly above the pressure of the carrier gas through the column. During backflush, the inlet pressure is dropped to 1 psi and the PCM pressure is increased, forcing the flow to reverse through the column and out the purged inlet.

A unique, alternative approach to backflushing is the use of a Capillary Flow Technology device in the middle of the analytical column [2, 3]. Instead of using a 30-m column, two 15-m columns are used and connected by an ultra-low dead volume Purged Ultimate Union (Figure 1). The PCM adds just enough makeup gas to match that from the first column. Therefore, there is very little flow addition and subsequent decrease in sensitivity due to sub-optimal carrier gas flows into the mass spectrometer. Backflushing in this configuration is accomplished by reducing the flow and pressure in the first column and increasing them in the second column.

Figure 2 shows an example of backflushing with the purged union configuration. The top chromatogram shows six standards, where the third peak is considered the last analyte of interest and the fourth peak is the first of the late-eluting interferences. The middle chromatogram shows (a) the same

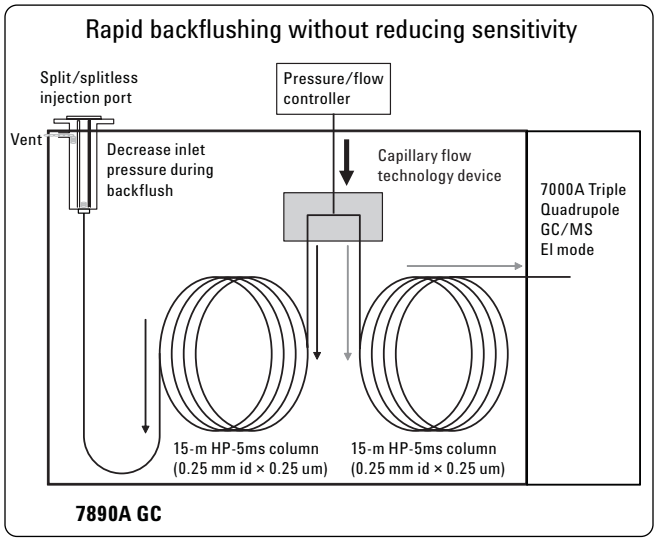

Figure 1. Schematic of the Purged Ultimate Union GC/MS configuration.

standard with backflushing beginning at 10.1 minutes, where flow is dropped in the first 15-m column and (b) where the flow in the second column is increased. The time between points a and b is the residence time of the last analyte compound in the second column. The last analyte is retained, but the late eluters never enter the MS detector. The bottom chromatogram demonstrates the lack of carryover in a subsequent blank run. Alternatively, backflushing can begin after the last peak of interest has eluted (point b). This eliminates the need to experimentally determine the residence time of the last target compound in the second column, while slightly increasing the cycle time.

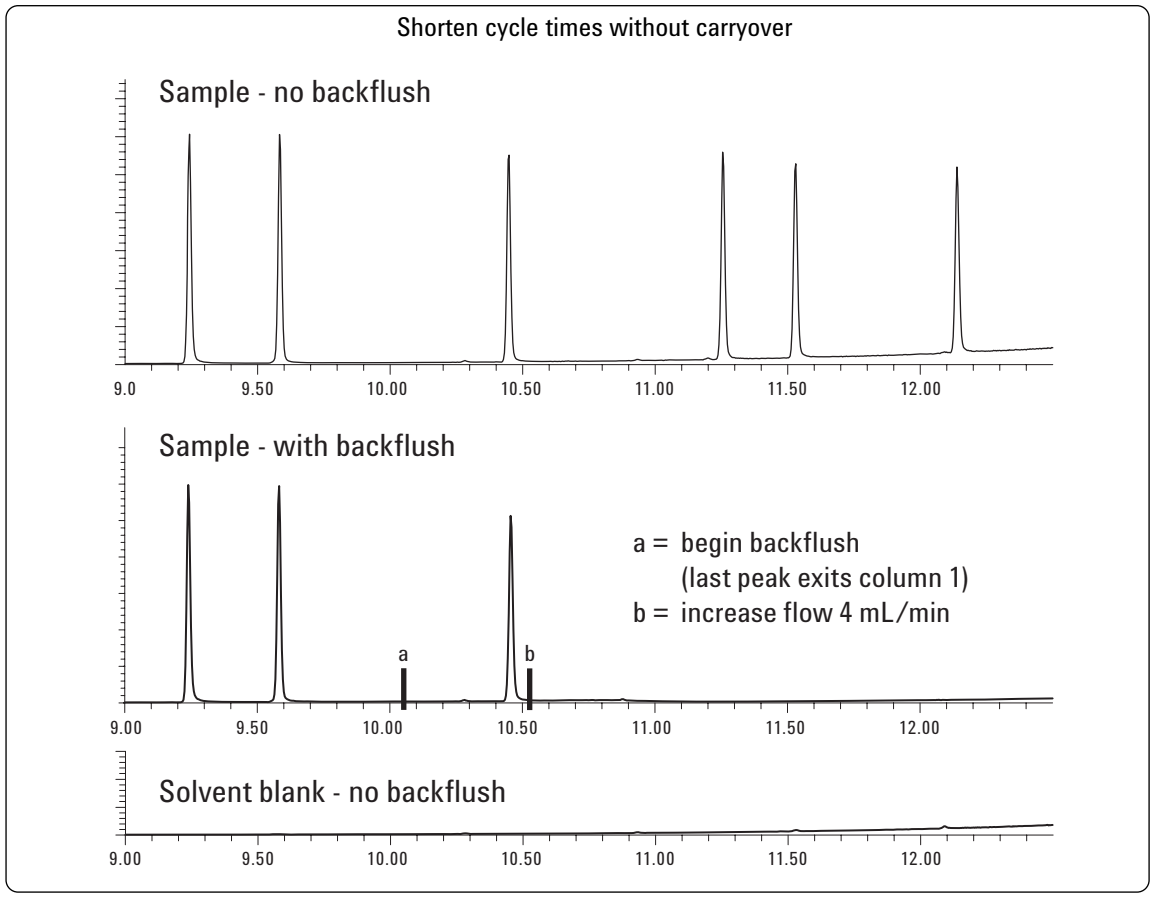

Figure 2. Backflushing with a purged ultimate union configuration. Top: no backflushing. Middle: Backflushing beginning at 10.1 minutes (a) until the third analyte elutes off the second column (b). Bottom: Subsequent blank injection showing no carryover.

Analysis of Melamine and its Analogs

The method developed on the Triple Quadrupole GC/MS system provides excellent separation and analysis of melamine, ammelide, ammeline and cyanuric acid in one run, and in less than 15 minutes (Figure 3). The significant improvement in the sensitivity and selectivity of the new Triple Quadrupole GC/MS method versus the GC/MS SIM method is vividly illustrated in Figure 4. While the new method provides a very clean analysis of the quantifying transition of melamine at 0.25 ppm, the GC/MS SIM method is less effective at reducing chemical noise at 2.5 ppm, using any of the SIM ions.

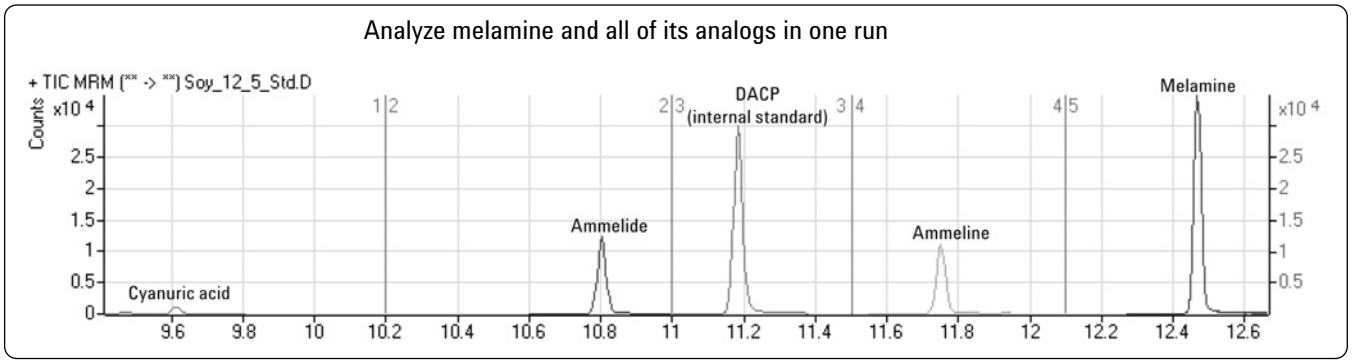

Figure 3. Reconstructed Total Ion Current Chromatogram (RTICC) resulting from SRM analysis, illustrating the resolution of melamine and its analogs.

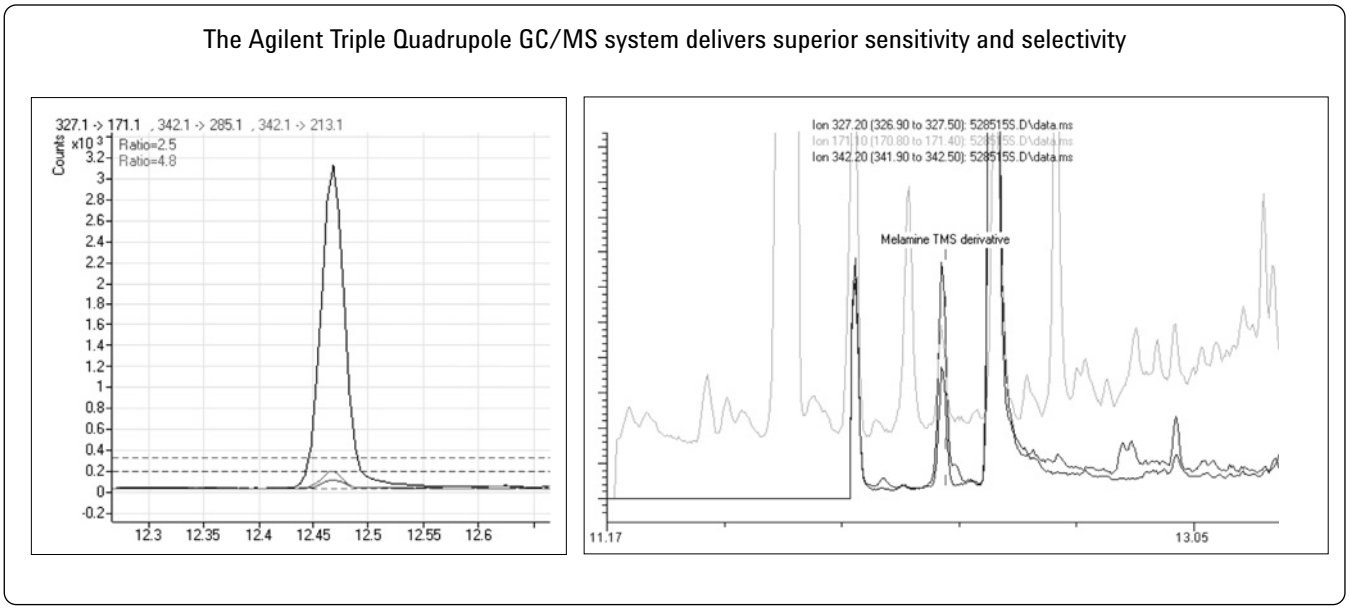

Figure 4. Comparison of detection of 0.25 ppm melamine in soy meal using the Triple Quadrupole GC/MS method (a), versus the GC/MS SIM method at 2.5 ppm (b). The quantifying transition used with the Triple Quadrupole GC/MS method was m/z 327.1→171.1, and the qualifying transitions were m/z 342.1→295.1 (2.5% of the peak area of the quantifying transition) and m/z 342.1→217.1 (4.8% peak area). The uncertainty bands are shown in (a) as well. The SIM ions used in the GC/MS method were m/z 342.2, 327.2, and 171.1 (b).

Sensitivity and Quantification

Each of the standards for melamine and its three analogs was added to matrix (both baby formula and soy meal) at concentrations of 0.78, 1.25, 3.9 and 12.5 ng/mL, corresponding to detection levels of 0.16 to 2.5 ppm. Calibration curves were constructed for each of the four compounds in each matrix.

Figures 5 and 6 illustrate the excellent linearity obtained for melamine and its three analogs, with R^2 values very close to 1.00. The accuracy of quantification was also very good for all four compounds in both matrices as illustrated in Tables 4 and 5.

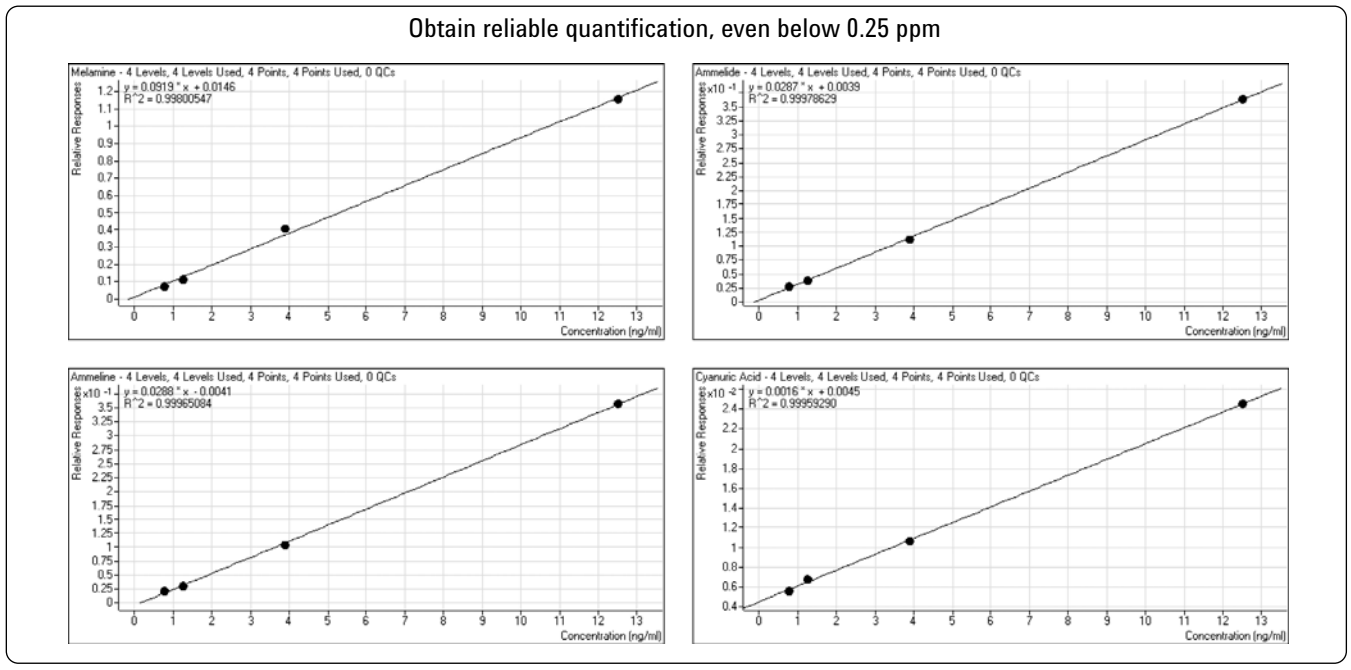

Figure 5. Calibration curves for quantification of melamine and its derivatives in baby formula based on a linear fit.

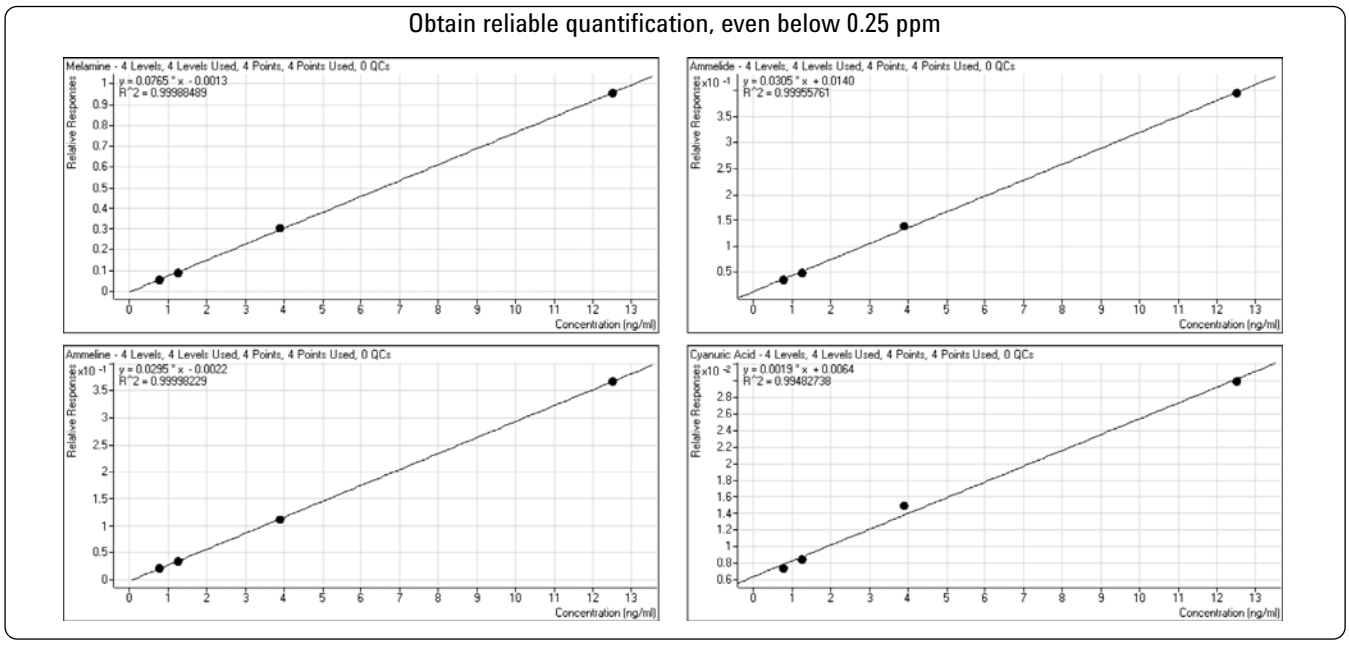

Figure 6. Calibration curves for quantification of melamine and its derivatives in soy meal based on a linear fit.

Table 4. Calibration Data for Quantification of Melamine and its Derivatives in Baby Formula Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.79	101.3
	1.25	1.23	99.1
	3.90	4.39	112.5
	12.5	12.50	100.0
Ammelide	0.78	0.86	110.3
	1.25	1.25	99.9
	3.90	3.79	97.2
	12.5	12.52	100.2
Ammeline	0.78	0.90	115.5
	1.25	1.22	97.2
	3.90	3.78	97.0
	12.5	12.52	100.3
Cyanuric acid	0.78	0.67	86.1
	1.25	1.40	111.9
	3.90	3.85	98.8
	12.5	12.51	100.1

Table 5. Calibration Data for Quantification of Melamine and its Derivatives in Soy Meal Based on Matrix-Matched Standards

	Standard Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy of Quantification (%)
Melamine	0.78	0.76	97.7
	1.25	1.20	96.3
	3.90	3.98	102.2
	12.5	12.48	99.8
Ammelide	0.78	0.72	92.9
	1.25	1.18	94.2
	3.90	4.07	104.4
	12.5	12.46	99.7
Ammeline	0.78	0.81	103.7
	1.25	1.22	97.9
	3.90	3.90	99.9
	12.5	12.50	100.0
Cyanuric acid	0.78	0.71	91.3
	1.25	1.22	94.5
	3.90	4.49	115.1
	12.5	12.01	96.1

Confirmation

The identification point system was developed by EU scientists to define an acceptable procedure for scientifically confirming the presence of regulated substances. The more identification points provided by the analytical method, the more certain is the confirmation of the compound. Three points are required for compounds with an MRL. When no MRL can be defined because of the toxicity of the compound, it is banned at all levels. These compounds require four identification points. While four ions need to be monitored by GC/MS to provide four identification points, only two SRM transitions need to be monitored when using triple quadrupole GC/MS/MS. Analysis of melamine and its analogs was performed using at least two SRM transitions for each compound on the triple quadrupole GC/MS system to provide screening and positive confirmation in the same run.

Figures 7 and 8 illustrate the quantifying and qualifying transition profiles for the GC separation of each of the four compounds in both baby formula and soy meal. In each case the qualifying transitions have been normalized to the quantifying transition in order to better illustrate the identical peak shape obtained from both. These transitions therefore provide a positive confirmation of each of the four compounds in each of the sample matrices.

Conclusions

The FDA GC/MS method for screening for melamine, ammelide, ammeline, and cyanuric acid has been modified for use on the Agilent Triple Quadrupole GC/MS system in order to provide screening, quantification and confirmation in one short run. This method does not require any changes in extraction or derivatization procedures, and cycle time is about 15 minutes. In addition, this method meets the new FDA requirement for sensitivity of 0.25 ppm, and it demonstrates excellent linearity of quantification up to 2.5 ppm. Accuracy of quantification is greater than 97% and two SRM transitions for each of the four compounds have been demonstrated in order to provide sufficient identification points for a positive confirmation.

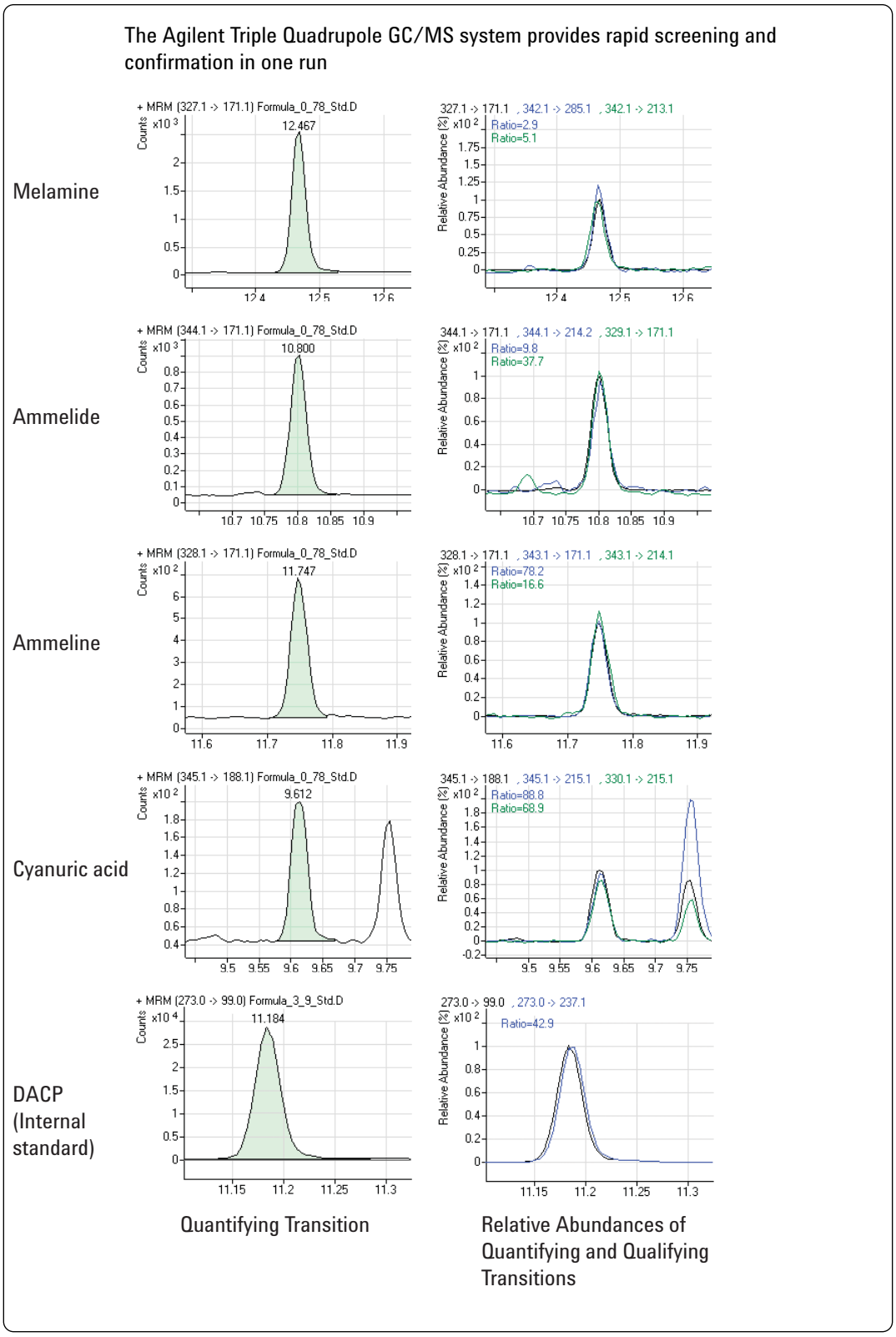

Figure 7. The Agilent Triple Quadrupole GC/MS system provides confirmation and screening in one run: quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in baby formula.

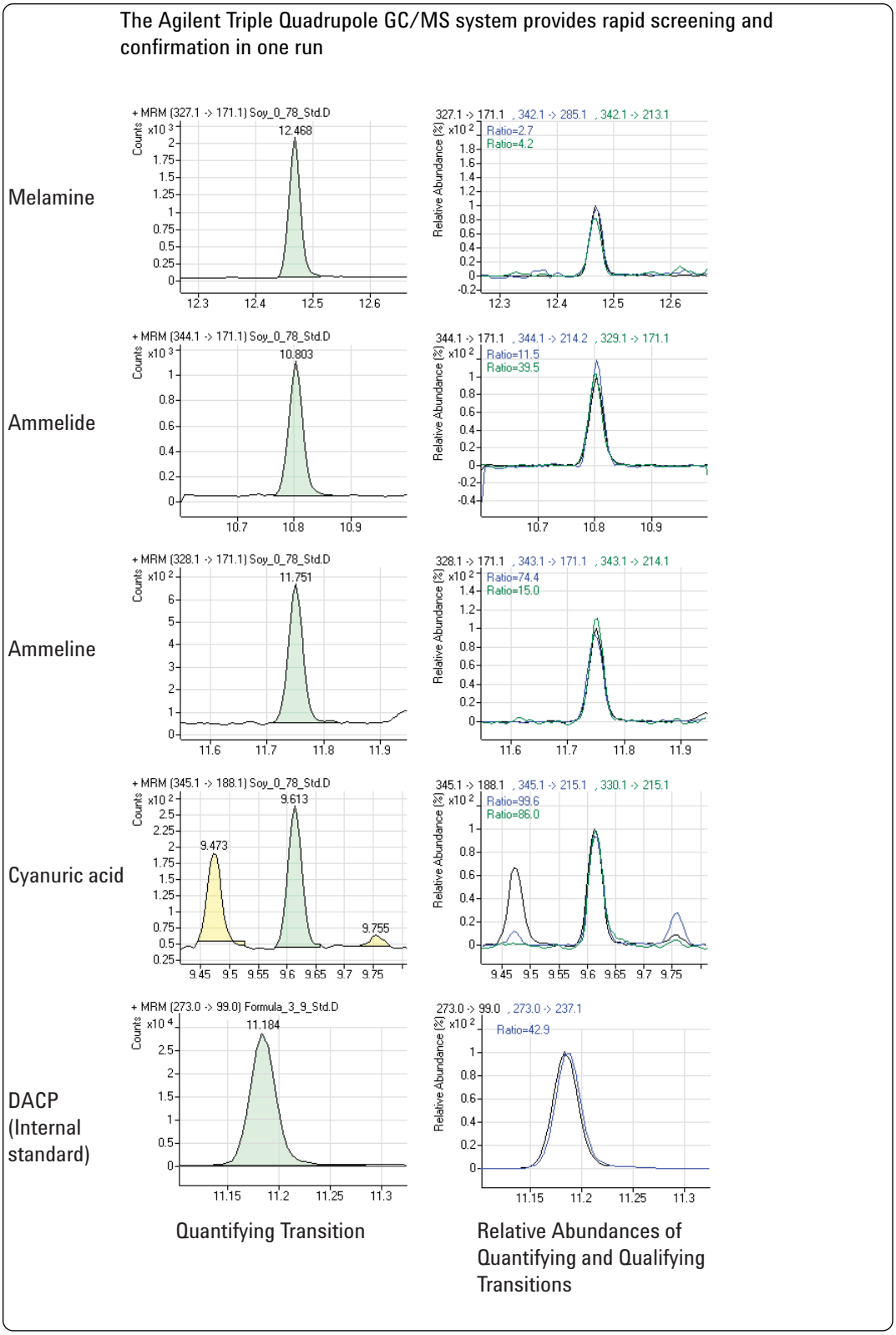

Figure 8. Quantifying and normalized qualifying transitions for melamine and its analogs at 0.78 ng/mL in soy meal.

Acknowledgement

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