Evaluation and single laboratory validation of an on-line turbulent flow extraction tandem mass spectrometry method for melamine in infant formula

John A.G. Roach [a],*, Joe M. DiBussolo [b], Alex Krynitsky [a], Gregory O. Noonan [a]

[a] USA Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Parkway, College Park, MD 20740, USA
[b] Thermo Fisher Scientific, 101 Constitution Boulevard, Franklin, MA 02038, USA

ARTICLE INFO

ABSTRACT

This report presents the single-laboratory validation of a method for the determination of melamine in dairy-based products using on-line turbulent flow extraction-tandem mass spectrometry. Liquid or powder test portions were dissolved in water, enriched with $^{13}C_3{}^{15}N_3$-Melamine internal standard, followed by protein precipitation and withdrawal of an aliquot for analysis. The turbulent flow method was validated by analyses of liquid and powdered proficiency test portions containing up to 10 mg/kg melamine. Accuracy of results ranged from 96 to 106% of the assigned values for the 6 proficiency test portions tested with relative standard deviations of 4–8%. Apparent recoveries based on addition of amino-$^{15}N_3$-Melamine to prepared test portions were between 98 and 114%. Based on the repeat analysis of a known blank sample the limit of detection and limit of quantification were determined to be 27 and 87 µg/kg, respectively. Additionally, this report demonstrates that turbulent flow chromatography is significantly faster than traditional LC–MS, with sample analysis times of less than 2 min.

Published by Elsevier B.V.

1. Introduction

A study of the 2004 and 2007 incidents of pet food-associated renal failure in cats and dogs led Brown to report compelling evidence that melamine and cyanuric acid were the causal agents [1]. Analyses for these chemicals in the ingredients used to manufacture pet food and trace back of these ingredients led to their discovery in other food products including animal feeds [2]. Cyanuric acid is permitted as a nitrogen supplement in animal feed [3]. However, its presence with melamine in these products was indicative of their intentional use as economic adulterants.

In response to the adulteration of pet food and feeds with melamine and cyanuric acid, various melamine assays were developed by U.S. Food and Drug Administration (FDA) Scientists. Heller and Nochetto developed a rapid method for the determination of melamine and cyanuric acid in animal feeds [4]. Smoker and Krynitsky collaborated to develop an in-house tandem mass spectrometry (MS) method, which used stable isotope dilution, for the determination of melamine and cyanuric acid residues in tissue [5]. This method was tested against incurred residues of these adulterants in fish and pork and performed well in a Fall 2009 collaborative study conducted by the Food Industry Analytical Chemists Committee (FIACC) of the Grocery Manufacturers Association (unpublished data) as well as the 2009 melamine proficiency test conducted by

the Joint Research Centre (JRC) of the European Commission Institute for Reference Materials and Measurements [6].

In 2008, an increased incidence of infant kidney disease in China led Chinese authorities to discover melamine adulteration of infant formula and milk by several Chinese producers [7,8]. Although there had been no indication that US food imports had been contaminated, part of the FDA response was a survey of American infant formulas. The Smoker and Krynitsky method was applied to the analysis of infant formulas for melamine. Every American infant formula product tested was found to be safe (unpublished data), but the manpower, time and resources required to prepare and analyze the extracts were considerable. Consequently, the 2009 report [9] of a 4-min, on-line turbulent flow isolation of melamine from dairy-based food extracts was a significant advance in the methodology for high-throughput melamine measurement. This report formed the basis for the collaborative effort described below, which had the goal of testing and validating turbulent-flow methodology for high throughput foods analysis applications.

In turbulent flow chromatography, high flow rates are used with large (ca 50 µm), chromatographically active, porous polymeric particles. The high flow rates produce a turbulent flow, instead of the laminar flow achieved in traditional chromatographic systems. The small (ca 75–125 Å) pores, turbulent flow and solvent selection allow the retention of small analytes, while the larger matrix compounds, such as proteins, are washed from the column. After elution of matrix compounds, the mobile phase is changed to elute the previously retained small analyte(s) [10]. Since Quinn

and Takarewski patented turbulent flow chromatography in 1997 [11], it has been used primarily for rapid determinations in pharmacokinetic studies and therapeutic drug monitoring [12–14]. For example, Srinubabu et al. described the development and validation of an automated method for loratadine and deloratadine in human plasma [15]. The extraction, separation and tandem MS quantification were completed within 8 min. On-line turbulent flow chromatography has been applied to environmental matrices such as the determination of pesticides in ground, waste, and drinking water [16,17]. Additionally, the application of turbulent flow chromatography to the analysis of food matrices, such as the determination of veterinary drugs in honey and milk, has been reported [18,19].

We report here the development of an on-line turbulent flow tandem MS method for the determination of melamine in dairy-based foods and its subsequent single laboratory validation. The resulting method is 15 times faster than the LC–MS/MS method of Smoker and Krynitsky and provides comparable results in analyses of proficiency test portions bracketing the Codex maximum level for infant formula [20].

2. Method

2.1. Reagents and materials

Melamine (99+%), acetic acid (99.7%), formic acid (98–100%), ammonium hydroxide (ACS reagent grade), ammonium acetate (99.999%) and HPLC grade acetone (99.9%) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). $^{13}C_3{}^{15}N_3$-Melamine ($^{13}C_3$, 99%; amino-$^{15}N_3$, 98%) 100 µg/mL in water was purchased from Cambridge Isotopes Laboratories (Andover, MA). Amino-$^{15}N_3$-Melamine was synthesized for FDA in June, 2007, by Goncalo Gamboa, at the FDA National Center for Toxicological Research (Jefferson, AR). Optima LC–MS grade acetone, acetonitrile, methanol, 2-propanol, and water were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Conical 50 mL screwcap polypropylene centrifuge tubes were purchased from VWR Scientific Products (Buffalo Grove, IL). Narrow-mouth 1 L Teflon wash bottles were purchased from Cole Parmer (Vernon Hills, IL) and fitted with narrow mouth solvent reservoir caps purchased from Waters (Milford, MA) for use as solvent reservoirs for basic solutions.

2.2. Sample preparation

Liquid concentrate and powder dairy products were prepared according to manufacturer's instructions prior to sampling. Liquid (2 mL) or solid test portions (1 g or less) of baking products were weighed into 50 mL centrifuge tubes. Liquid test portions were spiked with 200 ng $^{13}C_3{}^{15}N_3$-Melamine internal standard (200 µL), 1.8 mL 2% acetonitrile in water, and vortexed (0.5 min). For solid matrices, water (2 mL) was added to solid portions and vigorously vortexed for 0.5 min prior to and after addition of spiking solutions. To precipitate proteins a crash solution consisting of 30% aqueous solution of 50 mM ammonium acetate mixed with 70% acetonitrile (25 mL) was added to each test portion and vortexed for 1 min. The portions were then centrifuged at 9500 rpm for 10 min in a Beckman Coulter Allegra 21 centrifuge (Palo Alto, CA) to separate the precipitated solids from the supernatant. A 1 mL aliquot of clarified supernatant was withdrawn and transferred to an LC sample vial for analysis.

2.3. Instrumental analysis

Turbulent flow chromatography of the test portions was performed using a Transcend TLX-2 two-channel, two-dimensional LC
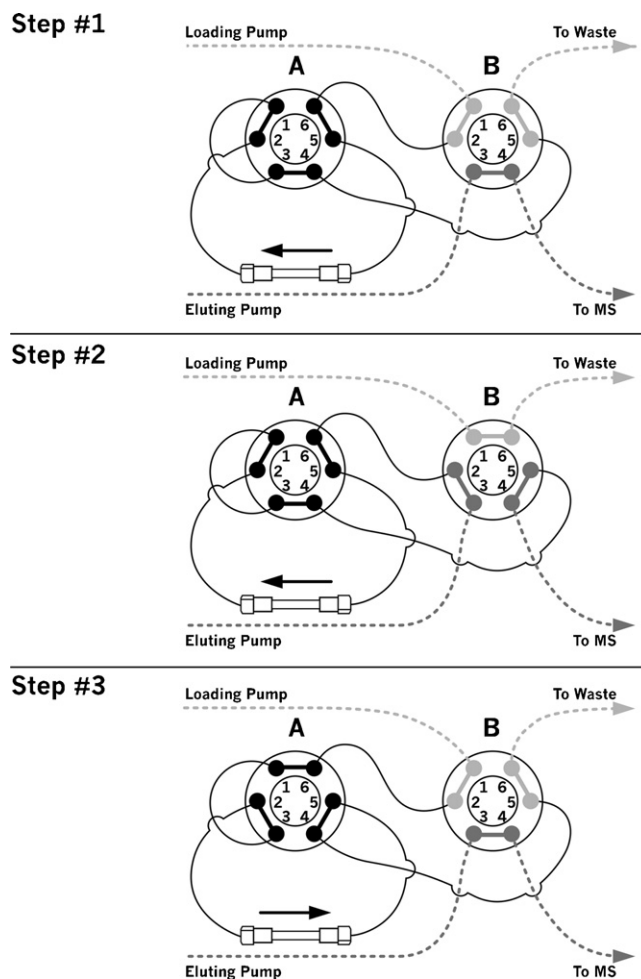

Fig. 1. Paths of loading and eluting pump flows during loading (step 1), elution (step 2), and backwash (step 3) of the TurboFlow™ column.

system configured for turbulent flow on-line solid phase extraction and fast liquid chromatography (Thermo Fisher Scientific, San Jose, CA). Each channel, designated loading or eluting, utilized a quaternary LC pump to combine and pump the appropriate solvents. For this analysis, 3 solvents were used from the loading channel and 2 were used in the eluting channel. Only loading pump flow passed through the 100 µL sample injection loop. The eluting pump flow entered the LC system downstream from the injection loop. A prototype Cyclone™ MCX-2 cation exchange TurboFlow™ column was used for on-line extraction.

The first step of on-line extraction sequence (Table 1) began with a 5 µL injection loaded onto the MCX-2 column with 100% loading solvent A (0.1% formic acid and 5% methanol) at a flow rate of 2 mL/min for 1 min. The column effluent was directed to waste during this step (Fig. 1a). In the second step, melamine was eluted from the column, into the mass spectrometer, with 100% eluting solvent A (0.1% NH₄OH and 5% methanol) at 1 mL/min for 1 min. Simultaneously, loading solvent B (100 mM $NH_4C_2H_3O_2$ and 5% methanol, pH 9), was directed to waste at a flow rate of 1 mL/min (Fig. 1b). In the third step, the MCX-2 column was backwashed with loading solvent B at 2 mL/min for 0.5 min (Fig. 1c). Eluting solvent flow was reduced from 1 mL/min to 0.5 mL/min and stepped from 100% eluting solvent A to eluting solvent A/B (methanol, 20/80). In the fourth step the column was backwashed with loading solvent C (2-propanol and acetone 50/50) for 1 min at 2 mL/min while eluting solvent (20/80) continued at 0.5 mL/min to the mass spectrometer. In the fifth step the MCX-2 column and valve system

Table 1

Turbulent flow LC method for in-line enrichment and elution of melamine. Loading and eluting pump flow compositions are shown for each method step in columns on either side of the action and flow direction columns.

Step	Duration (min)	Load flow mL/min	Load % A	Load % B	Load % C	Action	Flow direction	Elute flow mL/min	Elute % A	Elute % B
1	1.0	2.0	100			Load	Forward	1.0	20	80
2	1.0	1.0			100	Elute	Forward	1.0	100	
3	0.5	2.0			100	Load	Back	0.5	20	80
4	1.0	2.0		100		Load	Back	0.5	20	80
5	0.5	2.0	100			Load	Forward	0.5	20	80

was re-equilibrated with initial loading and eluting solvent compositions for 0.5 min. Eluting pump flow remained at 0.5 mL/min during step 5. Using these chromatographic conditions, melamine eluted in 1.35 min. MS data collection began at 0.75 min resulting in a detected elution time of 0.6 min. Aqueous (2% acetonitrile, 0.1% formic acid) and organic syringe wash (45:45:10 acetonitrile:2-propanol:acetone) were used after each injection.

The turbulent flow system was interfaced to an API 5000 tandem mass spectrometer from Applied Biosystems (Foster City, CA). The heated nebulizer (APCI) source operated in positive ion mode was held at 400 °C and a nebulizer current (NC) of 1. The collision gas (CAD) setting was 6, the curtain gas (CUR) setting was 10 and the nebulizer gas (GS1) setting was 30. The interface heater (ihe) was in the ON state. The declustering potential (DP) was 80 V, the entrance potential (EP) was 6 V and the collision cell exit potential (CXP) was 25 V. Tandem MS detection was in multiple reaction monitoring (MRM) mode. Transitions monitored for melamine, m/z 127.1–85 and 68, used collision energies of 30 and 40 V, respectively, and a dwell time of 100 ms. Corresponding labeled melamine transitions, m/z 133.1–89 and 71, used collision energies of 30 and 40 V, respectively and equivalent dwell times. The Turbolon spray (ESI) source was set at 650 °C with an ion spray (IS) potential of 4500 V.

As part of the method comparison and validation, test portions were also analyzed using LC–MS/MS conditions similar to those developed by Smoker and Krynitsky [7]. Separations were performed by liquid chromatography (Agilent 1100) using Sequant Zic-Hilic, 2.1 mm × 150 mm, 50 μ, 200 Å column from Merck (Southborough, MA). A flow rate of 300 μL/min of 100% mobile phase A (95% acetonitrile with 10 mM $NH_4C_2H_3O_2$) was maintained from injection until 11 min. At 11.1 min, flow switched to 100% mobile phase B (50% acetonitrile, 10 mM $NH_4C_2H_3O_2$) at 600 μL/min, and returned to 100% mobile phase A at 16.1 min. At 20.1 min, the 100% mobile phase A flow was reduced from 600 to 400 μL/min and held at that flow until 30 min. Syringe wash solution was 30% aqueous 50 mM ammonium acetate mixed with 70% acetonitrile. All separations were performed at 35 °C. Melamine eluted at 5 min. As with the turbulent flow system, melamine determination was performed using the API 5000 mass spectrometer and conditions listed above.

2.4. Stock solutions and calibration standards

Solid melamine standard was accurately weighed and dissolved in 2% acetonitrile in water. An aliquot was then diluted in 2% acetonitrile in water to prepare a 1.00 ng/μL stock spiking solution. $^{13}C_3\,^{15}N_3$-Melamine in water was diluted to 1.00 ng/μL with 2% acetonitrile in water to prepare internal standard stock spiking solution. Calibration standard solutions containing 10–8000 ng melamine were prepared by dilution of the stock spiking solution with 2% acetonitrile in water. $^{13}C_3\,^{15}N_3$-Melamine (200 ng) was added to each calibration standard, which was the same amount of internal standard added to each test portion. Once prepared, calibration standards were processed using the same dilution and protein precipitation procedure described above. The calibration standard solutions and extracts were stored in the dark at room temperature. Repeat analyses over the course of 5 months were

reproducible, indicating that the mixed standards solutions and extracts were stable for this length of time.

Calibration standards were analyzed daily, prior to and throughout the analysis of test portions. Calibration curves were prepared by plotting the non-weighted simple linear regression of the concentration ratio of melamine/labeled melamine versus the area ratio of melamine/labeled melamine. All of the peak integration and mass spectrometry data processing was performed with Analyst version 1.4.2 software. Melamine content of each test portion was calculated from the equation of the line of the standards curve(s) analyzed with the set. Microsoft Office 2003 Excel was used for all additional data processing. Confirmation of melamine identity in an extract was based on its co-elution with labeled melamine and agreement of its detected relative abundance of the product ions m/z 85 and 68 to within 10% of the relative abundance detected for melamine standard in the calibration standards.

2.5. Proficiency samples

Liquid infant formula test portions containing both melamine and cyanuric acid were the same test portions provided for participation in the Fall 2009 melamine/cyanuric acid proficiency study by the FFIACC of the Grocery Manufacturers Association. Test results including the mean result for each sample were provided to the participants in the study.

Powder test portions containing melamine were the same test portions provided for participation in the Joint Research Centre 2009 melamine proficiency test. Preparation of the test portions for the study was described in detail in the JRC report of the study results. In brief, melamine in solution was added to a suspension of skimmed milk powder, blended, and freeze-dried. This powder was then blended and re-blended with melamine-free milk powder to obtain milk powder contaminated with 10 mg/kg melamine. Several different baking mixes were purchased and combined prior to blending with contaminated milk powder to obtain a baking mix material contaminated at 3 mg/kg melamine. This baking mix composite contained >10% dried milk by weight. Assigned values and their uncertainties for melamine content of the milk powder and baking mix were obtained by analysis prior to release of the portions to the study participants [6].

3. Results and discussion

3.1. Choice of ionization source

The turbulent flow isolation of melamine from dairy-based food extracts protocol used APCI for MS detection [9]. Operation of the API 5000 in APCI mode and ESI mode differed in sensitivity by less than an order of magnitude in this laboratory, so APCI was retained as the ionization mode for this study.

3.2. Elution conditions

In order to reduce possible matrix effects and develop a more robust method a number of changes to the solvent system were evaluated. First, methanol was evaluated as a replacement for
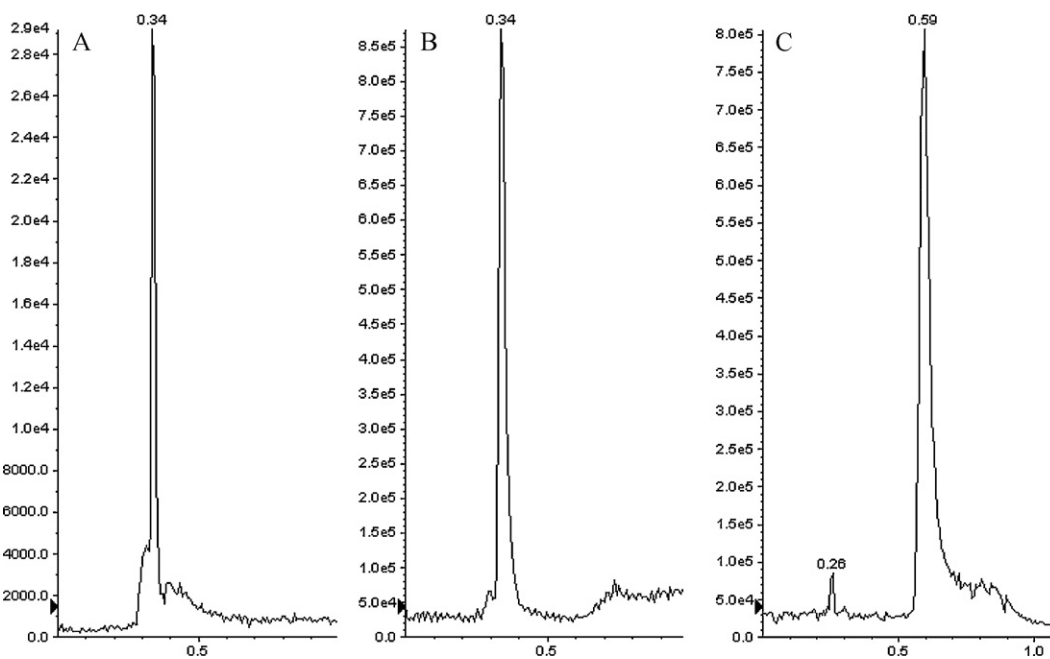
Fig. 2. TF-MS/MS MRM total ion data showing melamine retention time shift caused by varying NH$_4$OH content of eluting solvent A from (A) 1%, (B) 2%, and (C) 0.1%.

acetonitrile in all of the loading and wash solvents. These solvent changes showed no effect on melamine retention time. Secondly, given the ion exchange properties of the MCX-2 column, NH$_4$OH concentration was evaluated as a means to alter melamine retention time. The initial choice of elution solvent, 1% NH$_4$OH in 2% acetonitrile (solvent A) and acetonitrile (solvent B), was based on the solubility and chromatographic properties of melamine. This solvent system caused melamine to elute at the edge of the solvent front, co-eluting with co-extractives. Increasing NH$_4$OH content of eluting solvent A from 1%, to 2% did not shorten its retention time, suggesting that the analyte was already eluting with the leading edge of the basic mobile phase. Decreasing the NH$_4$OH concentration to 0.1% shifted the retention time of melamine, without degrading peak shape, from 0.3 to 0.6 min (Fig. 2). This change separated the melamine from co-extractives and improved the linearity and reproducibility of the solvent and matrix matched standard curves.

3.3. Interference/background

Initial TLX-2 analyses of reagent blanks, solvent blanks and test portion blanks showed a response consistent with melamine. The retention time of this response matched that of melamine and eluted with melamine when the concentration of ammonium hydroxide in eluting solvent A was changed. Additionally, the relative abundance of the product ions m/z 85:68 was comparable to the melamine standards. However, subsequent analysis of the same portions by LC–MS/MS did not show a response for melamine. Based on these results, two possible sources of the interference were evaluated, carryover from previously analyzed extracts and contamination within the TLX-2 system.

To address the issue of carryover, multiple vials of crash solvent, all with undetectable levels of melamine by LC–MS/MS, were run before and after the injection of a calibration standard. The melamine response was comparable for all of the blank injections, and there was no significant increase in response following the injection of melamine calibration standard (Fig. 3). These data indicate that the background is not due to carryover from previously analyzed positive samples. Attempts to identify the source of the

contamination within the TLX-2 system were unsuccessful. Solvent reservoirs were changed and filled with new solvent, seals were washed and a new column was placed in the system, however the background melamine signal remained unchanged in the short term. The signal did decline over the course of 3 months, but the background signal never completely disappeared.

Because the background signal was detectable in blanks and calibration standards and the standards and test portions were processed and analyzed in the same manner, the background should only affect the method limit of detection (LOD) and limit of quantification (LOQ), but not the accuracy of the method. The analysis of infant formula spiked at various levels (0–200 µg/kg) showed that calibration standard curves did not correct for the background melamine in the spiked portions. However, if matrix matched standards were used, the standard curve did correct for the background melamine response, resulting in accurate melamine quantification (Table 2). While the accuracy is not impacted at higher melamine concentrations (100 µg/kg), these data indicate that when using the TLX-2 system, there is a small, but detectable effect from the addition of matrix on the response of background melamine.

It should be noted that it was not necessary to utilize the same formula matrix to correct for the background response. Therefore, these results do not represent a back calculation from a standard addition curve, but melamine spiked into a known blank formula matrix for use as "matrix matched" calibration standards.

Table 2
Summary of calculated melamine content based on standards curves using mixed standards solutions prepared in solvent and matrix. Listed values summarize 8 independent data sets.

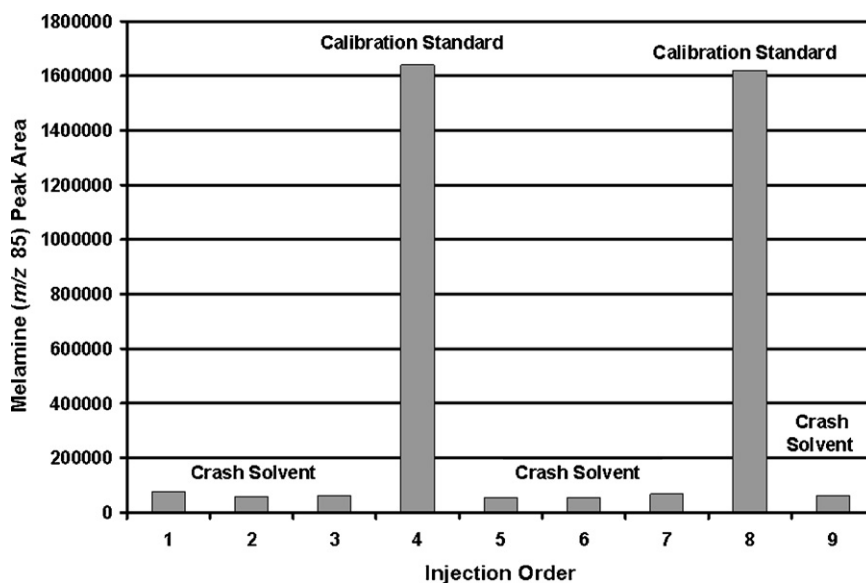| Actual melamine (µg/kg)	Calculated melamine (µg/kg)		Precision (% RSD)	
	Solvent	Matrix	Solvent stds	Matrix stds
0	9	0	45	*na*
10	18	9	21	28
20	30	21	15	16
50	58	50	10	8
100	104	98	6	4
200	204	201	9	1

Fig. 3. Melamine response (product ion m/z 85) from consecutive injections of blank crash solvent and 200 μg/kg calibration standard.

3.4. Robustness

The robustness of the method was evaluated in a series of experiments which altered the sample processing and analysis conditions. These changes included, decreasing the crash solution-to-sample volume ratio, decreasing the injection volume, and altering the solvents used in the TLX-2 sample loading and elution method and syringe wash.

Experiments in which methanol was replaced by acetonitrile as the organic solvent in both loading and eluting solutions, and column wash steps were varied showed that these changes did not affect method performance. The accuracy, overall % relative standard deviation, %RSD, and the %RSD for each experimental condition were comparable and acceptable. It was concluded from these results that the protocol was sufficiently rugged.

It was found that the ratio of crash solution to liquid milk test portion should approximate 9:1 in order to effectively precipitate milk proteins. The 9:1 ratio produced a clear supernatant above the precipitated solids, while ratios below 9:1 produced extracts with 3 phases and suspended solids, even after centrifugation. Analysis of these turbid supernatants increased co-extractive background signals and impacted protocol performance.

Melamine content of a 2 g portion of FIACC 213 was found to be at or above the highest point of the standard curve in these tests. Quantifications based on 5 and 10 μL injection volumes provided different TF-MS/MS results with 5 μL injections indicating melamine content beyond the standard curve. These results were investigated further in an examination of the linear dynamic range.

3.5. Linear dynamic range

The 10 point calibration curves (0–4000 ng) were linear ($r^2 = 0.999$) for 5 μL and 10 μL injections. When the calibration curve was extended to 8000 ng, curves based on 5 μL injections were linear, but curves derived from 10 μL turbulent flow injections were non-linear above 4000 ng. LC–MS/MS data did not mirror this trend. Calibration curves obtained by LC–MS/MS were linear for both 5 and 10 μL injections. This suggested that the linear range of the turbulent flow system was limited by MCX-2 column capacity, so subsequent injection volumes were limited to 5 μL.

3.6. Limit of detection and limit of quantification

FIACC sample 318 did not contain melamine, therefore its data were used to calculate the method limit of detection and limit of quantification. The blank value (1.6 μg/kg) was the average of these 47 results. Three times the standard deviation of the blank value (8.562 μg/kg) was added to the blank value to calculate the LOD. The sum of the blank value and ten times the standard deviation of the blank were used to determine the LOQ. The limit of detection of the protocol was 27 μg/kg. The limit of quantification was 87 μg/kg [21].

3.7. Recovery

Addition of labeled standard prior to extraction compensated for analyte losses prior to analysis as well as matrix effects during analysis post sample preparation addition of $^{15}N_3$-Melamine was used to estimate absolute recovery from FIACC proficiency test portions in order to assess the extraction efficiency of the sample preparation step. The portions were examined by TF-MS/MS and LC–MS/MS. The estimated absolute recoveries ranged from 98 to 114% (Table 3).

3.8. Quantification of mg/kg levels of melamine

Proficiency test portions were analyzed without prior knowledge of their melamine or cyanuric acid spike levels. Results of the analyses of all of the proficiency test portions by TF-MS/MS were within 102–110% of the study spiking levels (Table 4). The results were within 96–106% of the assigned study values. Some

Table 3
Summary of estimated absolute recoveries expressed in percent for JRC and FIACC test portions. LC–MS/MS and TF-MS/MS quantifications were based on addition of $^{15}N_3$-melamine standard to finished extract test portions. Values for FIACC 213 are single determinations. Values for all other samples are the average result for single determinations of 2 independently prepared test portions of each sample.

Data set	Absolute recovery (%)				
	JRC A	JRC B	FIACC 411	FIACC 111	FIACC 213
LC analysis	112	105	99	107	102
TF analysis	112	114	100	106	98

Table 4

TF-MS/MS and LC–MS/MS proficiency test results for 5 μL injections. Spiked and assigned melamine concentrations from the FIACC and JRC proficiency test portions.

Analytical method	Melamine concentration μg/kg (%RSD)					
	JRC A	JRC B	FIACC MC 09F6			
	Milk powder	Baking powder	411 Liquid	111 Liquid	213 Liquid	318 Liquid
TF-MS/MS	10,600 (6.4)	3150 (4.6)	276 (4.0)	1091 (6.1)	2667 (7.3)	<27 (na)
LC–MS/MS	10,500 (4.7)	3060 (5.3)	245 (na)	1040 (na)	2500 (na)	
Spike conc.	10,000	3000	250	1000	2500	0
Assigned conc.	10,000	3180	260	1040	2680	<LOD

Table 5

TF-MS/MS JRC proficiency sample results. Portions analyzed by TF-MS/MS were quantified by standard curve extrapolation, by dilution of standards and 1 g test portions, and by analysis of reduced portion sizes (0.1 g).

Sample	Melamine conc. μg/kg (%RSD)				
	Spike	Study assigned	Curve extrapolation ($n=4$)	Dilution ($n=2$)	Reduced aliquot (0.1 g) ($n=5$)
JRC A	10,000	10,000	9230 (1)	9830 (0)	10,100 (3)
JRC B	3000	3180	3140 (1)	3120 (0)	2850 (13)

proficiency portions contained up to 2500 μg/kg cyanuric acid. The agreement of our results with the assigned study values indicated that our method was not affected by the presence of cyanuric acid in these samples.

It should be noted that when JRC test portion A was found to contain melamine beyond the range of the calibration standards, smaller sample sizes (0.1 and 0.2 g) of JRC A and B were processed in order to bracket their melamine content with the standards curve. The 0.2 g portions were prepared by dissolving 1 g JRC powder in 10 mL water followed by aliquoting and spiking 2 mL sub-samples for analysis. The JRC milk powder samples contained melamine at a level that was well above the range of the standards curve as well as the present Codex limit of 1 mg/kg in infant formula. Test portion quantification by standards curve extrapolation, standards and sample dilution, and preparation of reduced portion sizes were compared for accuracy in the measurement of melamine in the JRC samples (Table 5). FIACC samples were analyzed concurrently in 1 and 2 g portion sizes. Extrapolation was the least accurate way to quantify the JRC milk powder. Dilution of the standards and test portions in combination with new calibration standards containing melamine at levels comparable to the melamine content of diluted portions of milk powder diminished the concentration of the internal standard to a level that was below the LOD of melamine. The internal standard was not subject to the matrix interference noted for melamine, but additional data would be required to determine the merit of quantifying with an internal standard at a level that was below the LOD of the analyte. On the basis of these comparisons, a reduced portion size was selected as the way to quantify samples such as the JRC milk powder that contained 10 mg/kg melamine.

3.9. Repeatability

We used the %RSD of results from a combined total of 20 analyses of 3 replicate 0.2 g sub samples of JRC A and B obtained on separate days to determine the precision for the JRC analyses. The mean value for 3 replicate portions of JRC milk powder was 10,600 μg/kg with %RSD 6.4. The mean value for the JRC baking powder analyses was 3150 μg/kg with %RSD 4.6. The results for the 5 μL injections of FIACC test portions acceptably and repeatedly quantified the FIACC study test portions prepared on different dates over a one year period.

3.10. Speed of analysis

In-line turbulent flow extraction tandem mass spectrometry provided a 4 min analysis time, including column re-equilibration, that was 7.5 times faster than the 30 min LC analysis. Four MCX-2 prototype columns were evaluated during this study. They showed uniform analytical behavior. This uniform behavior enabled multiplexing two analytical streams to the mass spectrometer, halving time between determinations to 2 min, which made the in-line turbulent flow – tandem mass spectrometry method 15 times faster than the LC method.

The 4 min analysis time permitted overnight multiple replicate analyses of test sets consisting of 7–10 process standards, 4–6 extracts, and crash solvent blanks. This regimen of analysis of more than 100 injections each night was used to check for drift in results that would signal system degradation [22]. Several days were required to obtain comparable data for a single one of these test sets by LC–MS/MS.

4. Conclusions

Sample preparation by protein precipitation and on-line turbulent flow analyte enrichment was simpler, faster, and cheaper than the interim method used by the FDA for its 2008 infant formula survey. The analytical results fell within the acceptable results range of the 2 proficiency studies for all studied spike levels. The very few product test portions in the 2008 infant formula survey that did merit closer examination could have been re-examined by LC–MS/MS. This approach would have completed the survey in a more timely and cost-effective manner.

The in-line turbulent flow extraction tandem mass spectrometry protocol demonstrated acceptable accuracy, reliability, ruggedness, and selectivity in this study. Its limit of detection and limit of quantification are appropriate for the melamine levels detected in the proficiency test portions. This single laboratory validation study demonstrates that this method is fit for purpose in the analysis of these dairy-based foods for melamine in the studied concentration range of 0.25–10 mg/Kg [23].

Melamine synthesized by Goncalo Gamboa, FDA Center for National Toxicological Research, Jefferson, Arkansas.

References

[1] C.A. Brown, K.-S. Jeong, R.H. Poppenga, B. Puschner, D.M. Miller, A.E. Ellis, K.-I. Kang, S. Sum, A.M. Cistola, S.A. Brown, J. Vet. Diagn. Invest. 19 (2007) 525.
[2] R. Reimschuessel, E. Evans, W.C. Andersen, S.B. Turnipseed, C.M. Karbiwnyk, T.D. Mayer, C. Nochetto, N.G. Rummel, C.M. Gieseker, J. Vet. Pharmacol. Ther. 33 (2009) 172.
[3] 21CFR573.220 Food additives permitted in feed and drinking water of animals.
[4] D.N. Heller, C.B. Nochetto, Rapid Commun. Mass Spectrom. 22 (2008) 3624.
[5] M. Smoker, A.J. Krynitsky, (2008) LIB No. 4422. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/DrugChemicalResiduesMethodology/ucm071673.htm (accessed 29.01.10).
[6] European Commission Joint Research Centre Institute for Reference Materials and Measurements, Melamine Proficiency Test 2009. http://irmm.jrc.ec.europa.eu/html/activities/melamine/EUR23809EN_melamine.pdf (accessed 1.02.10).
[7] H. Xin, R. Stone, Science 322 (2008) 1310.
[8] Y.C. Tyan, M.H. Yang, S.B. Jong, C.K. Wang, J. Shiea, Anal. Bioanal. Chem. 395 (2009) 729.
[9] J.M. DiBussolo, R. Rohm, poster W352, in: 57th Annual ASMS Conference on Mass Spectrometry and Allied Topics, St. Louis, MO, 2009.
[10] A. Liesener, U. Karst, J. Sep. Sci. 28 (2005) 1658.
[11] H.M. Quinn, J.J. Takarewski, Int. Pat. WO97/16724 (1997).
[12] S. Zhou, H. Zhou, M. Larson, D.L. Miller, D. Mao, X. Jiang, W. Naidong, Rapid Commun. Mass Spectrom. 19 (2005) 2144.
[13] U. Ceglarek, J. Lembcke, G.M. Fiedler, M. Werner, H. Witzigmann, J.P. Hauss, J. Thiery, Clin. Chim. Acta 346 (2004) 181.
[14] F.L. Sauvage, J.M. Gaulier, G. Lachatre, P. Marquet, Ther. Drug Monit. 28 (2006) 123.
[15] G. Srinubabu, R.S. Patel, V.P. Shedbalkar, A.A. Rao, M.N. Rao, V.V.R. Bandaru, J. Chromatogr. B 860 (2007) 202.
[16] A. Asperger, J. Efer, T. Koal, W. Engewald, J. Chromatogr. A 960 (2002) 109.
[17] T. Koal, A. Asperger, J. Efer, W. Engewald, Chromatographia 57 (2003) S93.
[18] P. Mottlier, Y. Hammel, E. Gremaud, P.A. Guy, J. Agric. Food Chem. 56 (2008) 35.
[19] A.A.M. Stolker, R.J.B. Peters, R. Zuiderent, J.M. Dibussolo, C.P.B. Martins, Anal. Bioanal. Chem. 397 (2010) 2841.
[20] CODEX ALINORM 10/33/41 Appendix IV Maximum Levels for Melamine in Food, Adopted 6 July 2010.
[21] AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, 2002.12.19.
[22] R. Bakhtiar, T.K. Majumdar, J. Pharmicol. Toxicol. Methods 55 (2007) 227.
[23] M. Thompson, S.L.R. Ellison, R. Wood, Pure Appl. Chem. 74 (2002) 835.