Simultaneous Determination of Melamine, Ammelide, Ammeline, and Cyanuric Acid in Milk and Milk Products by Gas Chromatography-tandem Mass Spectrometry

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Objective To develop an analytical method for simultaneously qualitative and quantitative determination of melamine and triazine-related by-products including ammelide, ammeline, and cyanuric acid in milk and milk products by gas chromatography-tandem mass spectrometry (GC-MS/MS). **Methods** Melamine and triazine-related by-products namely ammelide, ammeline and cyanuric acid in the samples were extracted in a solvent mixture of diethylamine, water, and acetonitrile (10:40:50, *V/V/V*). After centrifugation, an aliquot of the supernatant was evaporated to dryness under a gentle stream of nitrogen gas, and then melamine and triazine-related by-products were derivatized using BSTFA with 1% TMCS. The derivatives of melamine and its analogues were determined by gas chromatography/ tandem mass spectrometry using multiple reactional monitoring (MRM) with 2, 6-Diamino-4-chloropyrimidine (DACP) being used as an internal standard. **Results** The linear detectable ranges were from 0.004 mg/kg to 1.6 mg/kg for melamine, ammelide, ammeline, and cyanuric acid with a correlation coefficient no less than 0.999. The recovery rates of the four compounds in spiked blank milk powder at concentrations 0.5, 1, 2 mg/kg were between 61.4%-117.2%, and the relative standard deviation was no more than 11.5% (n=6). The detection limits of melamine, ammelide, ammeline and cyanuric acid in milk powder were 0.002 mg/kg with a ratio of signal to noise of 3. **Conclusion** This GC-MS/MS method for simultaneous determination of melamine, ammelide, ammeline, and cyanuric acid in milk and milk products is sensitive and specific.

Key words: Melamine; Ammelide; Ammeline; Cyanuric acid; GC-MS/MS; Milk products

INTRODUCTION

Melamine (CAS 108-78-1, $C_3H_6N_6$) is an industrial chemical commonly used in the manufacture of plastics, flame retardants and other materials. Melamine is also a metabolite of the insecticide cyromazine[1]. Cyanuric acid (CYA, CAS 108-80-5, $C_3H_3N_3O_3$) is also an important industrial product widely used as an ingredient in the production of scouring powders, household bleaches, industrial cleansers, and automatic dishwasher compounds[2]. CYA is a stabilizer in the water of swimming pool to prevent the destruction of chlorine caused by evaporation and sunlight[3]. Ammelide (CAS 645-93-2, C3H4N4O2), ammeline (CAS 645-92-1, C3H5N5O1) and CYA are the by-products from the manufacturing of melamine. Ammelide, ammeline and CYA are also the microbial metabolites of melamine[4-5]. The structures of the four compounds are shown in Fig. 1.

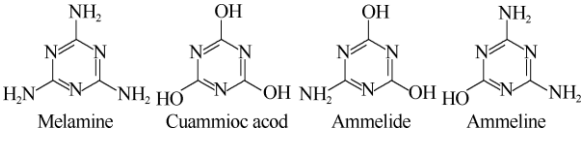

FIG. 1. Structures of melamine and related compounds.

Melamine and CYA are able to form an insoluble salt, may precipitate in kidneys, and cause renal functional failure[6-7]. In March of 2007, pet feed had caused an outbreak of renal disease and associated deaths of hundreds of cats and dogs in the United States. It was found that wheat gluten and other protein-based foods were contaminated with melamine and its analogues ammeline, ammelide, and CYA. Analytic methods for determination of melamine and related compounds were developed for samples such as wheat, rice gluten and other protein-based feed[8-27].

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The event of tainted milk powder which resulted in a large number of renal lithiasis in infants and young children in China in 2008 has attracted worldwide attention on melamine once again. In December of 2008, more then 294 000 cases of urinary tract stone were reported in China due to the consumption of melamne contaminated infant formula. As a temporary control measure, the Chinese government promulgated an interim control limit for melamine at 1 mg kg^{-1} for infant formula and 2.5 mg kg^{-1} for other milk products[28-30].

Melamine and CYA have been analyzed by gas chromatography/mass spectrometry (GC/MS)[9-12], high-performance liquid chromatography with UV detection[9,13], and LC/MS[9,14-17]. CYA has been analyzed by various methods including derivatization combining with GC/MS[11,18], high-performance liquid chromatography (HPLC) with UV detection[19-21], and LC/MS[16,22-23]. Of those methods, several have been developed for the Simultaneous analysis of melamine and its analogues[24-27], mainly using LC-MS/MS determination. Chinese government issued a national standard GB/T 22388-2008 *Determination of melamine in raw milk and dairy products*[9] on October 7, 2008, and another standard GB/T 22288-2008 *Determination of melamine, ammelide, ammeline and cyanuric acid in plant products-GC-MS method*[9], on August 12, 2008. In GB/T 22388-2008, melamine in milk and milk products are determined by HPLC-UV or DAD, HPLC-MS/MS and GC-MS or GC-MS/MS with quantification limits of 2 mg kg^{-1}, 0.01 mg kg^{-1}, and 0.05 mg kg^{-1}, respectively. In GB/T 22388-2008, melamine, ammelide, ammeline and CYA were determined by GC-MS only in original plant products with quantification limit of 2 mg kg^{-1} for the four compounds, respectively. However, those method officially issued in China was not suitable for simultaneously qualitative and quantitative determination of melamine and triazine-related by-products in milk and milk products.

The present study was to develop a sensitive and highly specific analytical method on the basis of the method of US FDA[10] for simultaneous determination of melamine, ammelide, ammeline and CYA in milk and milk products using gas chromatography-tandem mass spectrometry (GC-MS/MS). The method was successfully applied in the analysis of milk and milk products in the tainted infant formula event in 2008 in China.

MATERIALS AND METHODS

Materials

Reagents and chemicals Diethylamine and pyridine (GR), acetonitrile (HPLC grade), BSTFA with 1% TMCS (Supelco) were purchased from Supelco (Bellefonte, PA, USA). De-ionized water was prepared from a Milli-Q Plus system at 18.2 MΩ (MilliPore, Bedford, MA, USA).

Analytical standards Melamine, ammelide, ammeline and CYA were purchased from J&K CHEMICAL LTD with purity not less than 99%. The internal standard, 2, 6-diamino-4-chloropyrimidine (DACP), was purchased from Dr. Ehrenstorfer in Germany with a purity not less than 98%.

The stock solution of melamine or CYA (1000 mg L^{-1}) was prepared by dissolving 10.0 mg melamine (or CYA) into 10 mL of acetonitrile and water (1:1, *V/V*) and then stored at -20 ℃. Similarly, the stock solution for ammlide and ammeline was prepared by dissolving 10.00 mg of ammelide (or ammeline) in 10 mL solution of diethylamine and water (50:50, *V/V*) and stored at 4 ℃. A stock solution of DACP (50.0 mg L^{-1}) was prepared by dissolving DACP standard in pyridine, and stored at -20 ℃.

Matrix matched standards namely 0.5 g of the blank sample (or 2 mL if the sample was a liquid) was extracted according to the sample extraction procedure to obtain matrix blank solution. A multi-component standard solution was prepared by spiking the four standards into matrix blank solution separately to make the concentrations of the four compounds of 0.004, 0.01, 0.04, 0.1, 0.2, 0.4, 0.8, and 1.6 mg L^{-1}, respectively.

Samples Liquid milk, infant formula, milk powder, and other milk products were purchased from local supermarkets in Beijing in September 2008. And some of the infant formula samples were provided by our colleagues who conducted epidemiological studies in the affected areas.

One of the milk powder samples which was negative in melamine, ammelide, ammeline and CYA was extracted and used as blank matrix.

Methods

Sample extraction The extraction procedure was based on the method of US FDA (GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide and Cyanuric Acid) (Version 2.1)[10]. 20 mL solution of acetonitrile, water and diethylamine (5:4:1, *V/V/V*) was added into each 50 mL polypropylene centrifuge tube containing 0.5 g solid sample. In each tube, 50 μL DACP (internal standard, 5.0 mg L^{-1}) was added. The samples were capped, vigorously shaken for 30 s, ultrasonicated for 30 min, and then centrifuged at 8 000 g for 10 min at 4 ℃. An aliquot of the supernatant was filtered through 0.22 μm nylon syringe filter. 50 μL of the filtered solution was placed into a 2 mL vial and evaporated to dryness under a gentle flow of nitrogen gas for later derivatization.

Derivatization An aliquot of 150 μL pyridine and 150 μL acetonitrile was added to the sample extract, and vortexed for 30 s. Afterwards, 200 μL BSTFA with 1% TMS was added, vortexed for 1 min, and then incubated at 70 ℃ for 45 min.

Instrument Conditions

GC conditions Chromatographic separation was carried out on a Varian GC3800 system (Varian, USA) using a VF-5ms quartz capillary column (30 m×0.25 mm i.d. × 0.25 μm). High purity helium (>99.999%) was used as a carrier gas, a mode of constant flow was selected, and the flow rate was set at 1.0 mL/min. The column temperature was initially set at 75 ℃ for 1 min, then programmed to 300 ℃ at a rate of 15 ℃/min, and held at 300 ℃ for 10 min. The inlet temperature was set at 250 ℃, the injection volume was 1 μL.

MS conditions Mass spectrometry was carried out on a triple quadrupole spectrometer (1200L, Varian, USA) using the electrospray ionization mode (EI), and with MRM scan mode. The multiplier voltage was 1200 V. The transfer line was operated at 250 ℃. The temperature of manifold was set at 40 ℃. During tandem mass spectrometric analysis, an ultra high purity argon was used as the collision gas. Filament Delay was 9 min. Both Q1 and Q3 were operated with a peak width of 0.7 m/z units. The retention time, optimized collision energy and the precursor and product ions for each analyte were listed in Table 1.

TABLE 1

MS/MS Monitoring Parameters

Compounds	Retention Time (min)	Qualitative Transition	Quantitative Transition	Collision Energy (V)
Cyanuric Acid	9.29	345>73	345>73	15
		345>147		15
Ammelide	10.06	344>171	344>171	20
		344>329		10
DACP (Internal std.)	10.53	273>171	273>171	15
		273>237		5
Ammeline	10.73	328>171	328>171	20
		328>189		20
Melamine	11.34	342>327	342>171	12
		342>171		24

RESULTS

Validation of the Method

Calibration curves GC-MS/MS measurements were carried out with the matrix matched mixed standard solutions at a series of concentrations as described elsewhere above. The chromatograph and MS/MS spectrum of the four compounds with milk powder matrix were shown in Fig. 2. The ratios of the area of melamine, ammelide, ammeline and CYA to the area of internal standard DACP were found to be linearly response to its concentration in the range from 0.004 to 1.6 mg L^{-1} with a correlation coefficient of no less than 0.999, respectively (Table 2).

LOD and LOQ The limit of detection for melamine, ammelide, ammeline and CYA was 0.002 mg kg^{-1} with a ratio of signal to noise of 3. The limit of quantification for melamine, ammelide, ammeline and CYA was 0.005 mg kg^{-1} with a ratio of signal to noise of 10.

Recovery As there were no CRM for melamine, ammelide, ammeline and CYA, the recoveries of the four compounds were determined by experiments using fortified blank milk powder matrix as described above. The fortified levels were selected at 0.5, 1, and 2 mg kg^{-1} according to the interim control limit at 1 mg kg^{-1} for infant formula set by the Ministry of Health of China in September, 2008. The results were shown in Table 3.

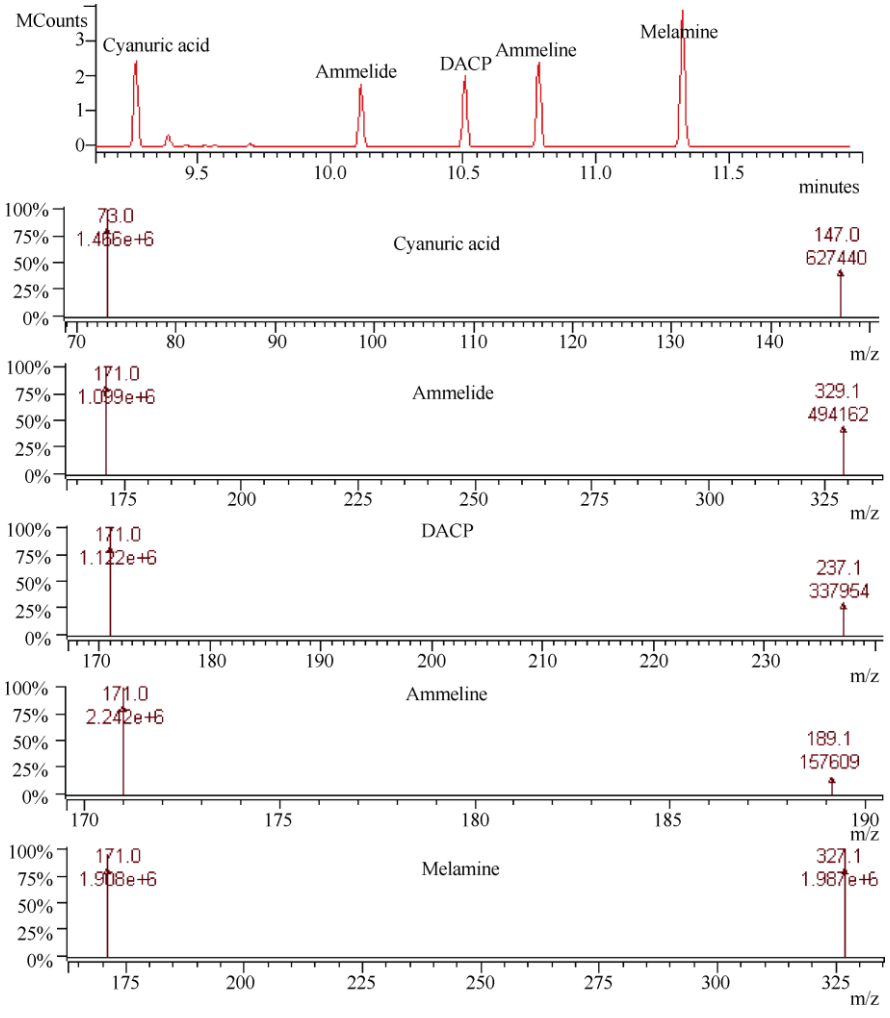

FIG. 2. Chromatograph and MS/MS spectrum of matrix (matched standards 0.1 mg L^{-1}).

TABLE 2

List of Linear Range, LOD, and LOQ

Compounds	Linear Range (mg L^{-1})	Calibration Curve	Correlation Coefficient (r)	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Melamine	0.004~1.6	Y=28.4521x-0.158	0.9998	0.002	0.005
Ammelide	0.004~1.6	Y=11.2892x-0.207	0.9993	0.002	0.05
Ammeline	0.004~1.6	Y=18.8117x+0.073	0.9994	0.002	0.05
Cyanuric Acid	0.004~1.6	Y=10.2103x-0.324	0.9994	0.002	0.005

TABLE 3

Recovery Rates of Melamine, Ammelide, Ammeline, and Cyanuric Acid in Fortified Blank Milk Powder Matrix

Compounds	Fortified Levels (mg kg^{-1})	Inter-day (n=6)		Intra-day (n=6)	
		Average Recovery (%)	RSD (%)	Average Recovery (%)	RSD (%)
Melamine	0.5	86.2	1.8	80.7	7.8
	1	87.9	1.9	83.5	8.2
	2	92.5	2.9	91.7	9.4
Ammelide	0.5	69.3	7.2	69.6	11.5
	1	75.9	4.5	75.0	5.5
	2	83.5	1.9	80.9	6.2
Ammeline	0.5	69.6	7.2	61.4	9.4
	1	83.0	1.8	80.7	4.2
	2	91.6	5.5	91.4	7.1
Cyanuric Acid	0.5	117.2	2.6	107.3	4.8
	1	99.0	1.4	104.0	4.2
	2	115.6	2.2	103.1	4.3

Analysis of Actual Samples

The method was successfully applied to the analysis of milk and milk products collected from local supermarket in September of 2008, including fresh milk, milk powder, toffee candy, milk tea powder, *etc*. All of the samples gave a clean extract, with no interferences for melamine, ammelide, ammeline and CYA, and produced an excellent baseline stability enabling easy quantification. Parts of the analytical results were shown in Table 4. Detailed results will be published separately. The chromatograms of the samples were shown in Fig. 3.

TABLE 4

Results of Milk Product Sample Analysis

Samples	Melamine (mg kg^{-1})	Ammelide (mg kg^{-1})	Ammeline (mg kg^{-1})	Cyanuric Acid (mg kg^{-1})
Fresh Milk	0.28	0.073	ND	1.64
Toffee	ND	ND	ND	0.86
Tea with Milk	11.07	ND	ND	ND

Note. ND - less than LOQ 0.005 mg kg^{-1}.

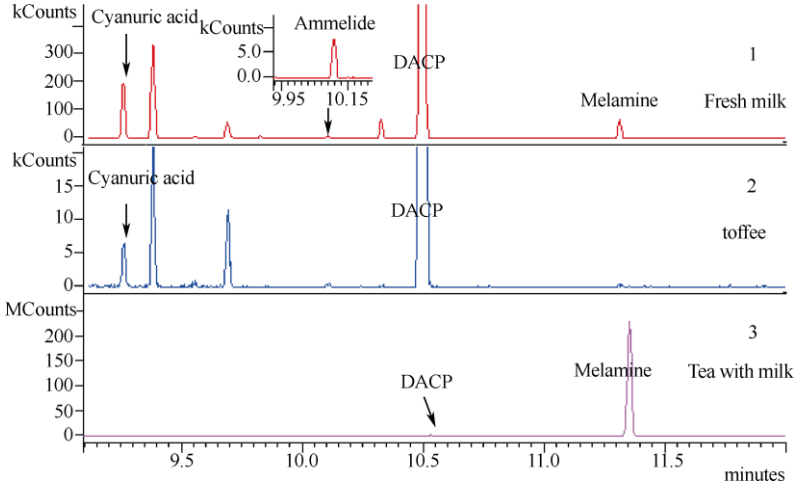

FIG. 3. The chromatographs of melamine analogs in milk product samples.

DISCUSSION

The present method was developed on the basis of the method of US FDA[10] *GC-MS screen for the presence of melamine, ammeline, ammelide, and cyanuric acid*, used for the analysis of pet foods. The method was modified and optimized. We found it was sensitive and specific for the analysis of four melamine analogs with minimum matrix interference.

Selection of Extraction Solvents

Melamine and its analogues are only slightly soluble in water, but they are easily soluble in organic solvents, such as methanol and acetonitrile. A mixture of acetonitrile, water, and diethylamine (5:4:1, *V/V/V*) was therefore selected as an extraction solvent mixture. Firstly, acetonitrile was used to precipitate protein in milk product samples to reduce the interference caused by the sample matrix. Secondly, diethylamine could maintain the extraction solvent at an alkaline pH to prevent melamine and cyanuric acid from forming an insoluble salt of melamine-cyanurate. Lastly, we found this solvent mixture could increase the extraction efficiency as the solubility of cyanuric acid, ammelide and ammeline from the matrix was maximized. We had tried to use a mxture of acetonitrile and water (1:1, *V/V*) as an extraction solvent; however, ammelide and ammeline could not be recovered well from milk product samples.

Optimization of Derivatization

As melamine, ammelide, ammeline and cyanuric acid have high boiling points and polarity, they can not be directly gasified in gas chromatography. So silylation reagent had to be used to derivatize them in order to increase their volatility.

Solvents used to re-dissolve the extracts before derivatization could affect the response of the four compounds in GC-MS/MS. For example, the response of cyanuric acid would be inhibited if it was re-dissolved in pyridine only while the response of cyanuric acid would be enhanced if it was re-dissolved in acetonitrile only. We found that a mixture of pyridine and acetonitrile (1:1, *V/V*) could enhance the response of the four compounds by 3-folds compared with acetonitrile or pyridine alone.

After derivatization, the derivatives were dried under a gentle stream of nitrogen gas followed by being re-dissolved in 200 μL toluene, and then subjected to GC-MS/MS analysis. However, there was no response observed for the four compounds. So the derivatives were injected into GC directly without re-dissolved in toluene.

Optimization of GC Conditions

According to the method of US FDA (version 2.1), the oven temperature for GC analysis was initially set at 75 ℃ for 1 min, and was programmed to 270 ℃ at a rate of 15 ℃/min and then held for 2 min. Under this condition, obvious interference which came from the last injection was seen after the two circles of injection of samples. To eliminate the interference and have a better separation, we changed the final temperature to 300 ℃ and the holding time from 2 min to 10 min.

Stability of the Extracts and Derivatives

Blank sample was spiked at levels of 0.05 mg kg^{-1} and 0.5 mg kg^{-1} and then extracted. An aliquot of the extracts was derivatized at varying time intervals of 0, 1, 2, and 3 days, and the derivatives were subjected to the GC-MS/MS analysis to check the stability of the extracts. The extracts were found stable within two days as a whole (Fig. 4). The lower the concentration was, the lesser stability was in the extraction solution. The stability of the derivatives in the matrix at concentrations of 0.005 μg L^{-1} and 1 μg L^{-1} was also tested. The derivatives were injected into GC-MS/MS at 0, 4, 8, 12, 24, 48, 72, 264, and 384 h after derivatization, showing that the derivatives were stable for at least 16 days (Fig. 5).

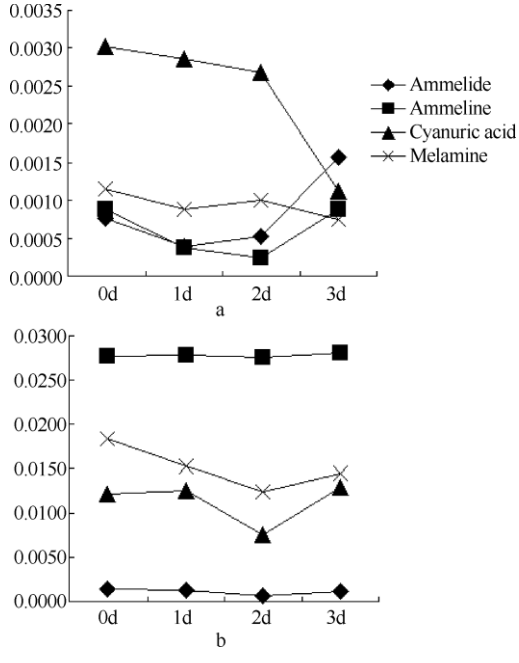

FIG. 4. The stability of melamine analogs in extraction solvent at different concentrations in the sample (a, 0.05 mg kg^{-1}; b, 0.5 mg kg^{-1}).

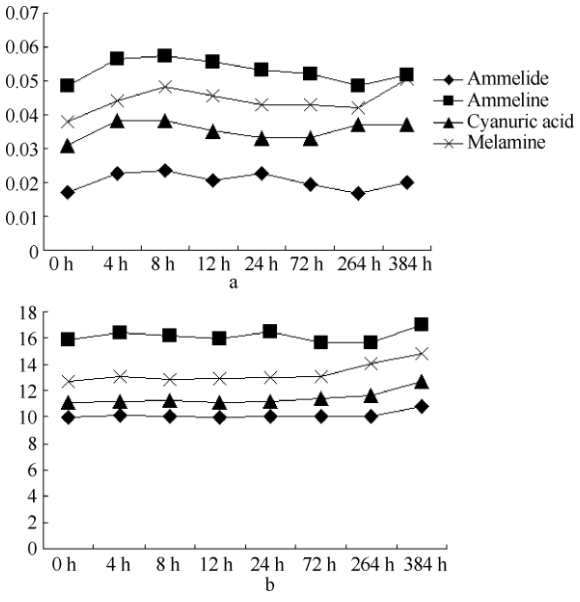

FIG. 5. The stability of melamine analogs (a, 0.005 μg L^{-1}; b, 1 μg L^{-1}).

Performance Test

During the crisis of melamine-tainted milk powder in China, we participated in the validation test of melamine organized by the General Administration of Quality Supervision, Inspection and Quarantine of P.R.C (AQSIQ) and the results were satisfactory.

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