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High Sensitivity Quantitation Method of Dicyandiamide and Melamine in Milk Powders by Liquid Chromatography Tandem Mass Spectrometry

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Zhi Wei Edwin Ting[1], Jing Cheng Ng[2*],
Jie Xing[1] & Zhaoqi Zhan[1]
[1] Customer Support Centre, Shimadzu (Asia Pacific) Pte Ltd, 79 Science Park Drive, #02-01/08, SINTECH IV, Singapore Science Park 1, Singapore 118264
[2] Department of Chemistry, Faculty of Science, National University of Singapore, 21 Lower Kent Ridge Road, Singapore 119077, *Student

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Introduction

Melamine was found to be used as a protein-rich adulterant first in pet-food in 2007, and then in infant formula in 2008 in China [1]. The outbreak of the melamine scandal that killed many dogs and cats as well as led to death of six infants and illness of many had caused panic in publics and great concerns in food safety worldwide. Melamine was added into raw milk because of its high nitrogen content (66%) and the limitation of the Kjeldahl method for determination of protein level indirectly by measuring the nitrogen content. In fact, in addition to melamine and its analogues (cyanuric acid etc), a number of other nitrogen-rich compounds was reported also to be potentially used as protein-rich adulterants, including amidinourea, biuret, cyromazine, dicyandiamide, triuret and urea [2]. Recently, low levels of dicyandiamide (DCD) residues were found in milk products from New Zealand [3]. Instead of addition directly as an adulterant, the trace DCD found in milk products was explained to be relating to the grass "contaminated by DCD". Dicyandiamide has been used to promote the growth of pastures for cows grazing. We report here an LC/MS/MS method for sensitive detection and quantification of both dicyandiamide (DCD) and melamine in infant milk powder samples.

Experimental

High purity dicyandiamide (DCD) and melamine were obtained from Sigma Aldrich. Amicon Ultra-4 (MWCO 5K) centrifuge filtration tube (15 mL) obtained from Millipore was used in sample pre-tretment. The milk powder sample was pre-treated according to a FDA method [1] with some modification as illustrated in Figure 1. The final clear sample solution was injected into LC/MS/MS for analysis. Stock solutions of DCD and melamine were prepared in pure water.

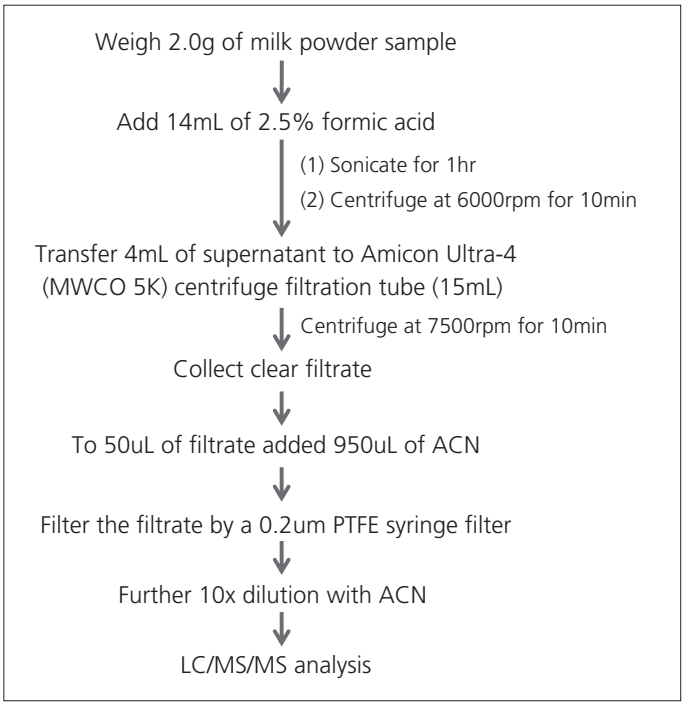

Fig 1: Sample pre-treatment workflow

Table 1: Analytical conditions of DCD and melamine in milk powders on LCMS-8040

LC conditions

Column	Alltima HP HILIC 3µ, 150 x 2.10mm
Flow Rate	0.2 mL/min
Mobile Phase	A: 0.1 % formic acid in H_2O/ACN (5:95 v/v) B: 20mM Ammonium Formate in H_2O/ACN (50:50 v/v)
Elution Mode	Gradient elution: 5% (0.01 to 3.0 min) → 95% (3.5 to 5.0 min) → 5% (5.5 to 9.0 min)
Oven Temperature	40ºC
Injection Volume	5 µL

MS conditions

Interface	ESI
MS mode	Positive
Block Temperature	400ºC
DL Temperature	300ºC
CID Gas	Ar (230kPa)
Nebulizing Gas Flow	N2, 2.0L/min
Drying Gas Flow	N2, 15.0L/min

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An LCMS-8040 triple quadrupole LC/MS/MS (Shimadzu Corporation, Japan) was used in this work. The system is consisted of a high pressure binary gradient Nexera UHPLC coupled with a LCMS-8040 MS system. An Alltima HP HILIC column was used for separation of DCD and melamine with a gradient program developed (Table 1). The details of the LC and MS conditions are shown in Table 1. A set of calibrants (0.5, 1.0, 2.5, 5 and 10 ppb) was prepared from the stock solutions using of ACN/water (90/10) as diluent.

Results and Discussion

MRM optimization

MRM optimization of DCD and melamine were performed using an automated MRM optimization program of the LabSolutions. The precursors were the protonated ions of DCD and melamine. Two optimized MRM transitions of each compound were selected and used for quantitation and confirmation. The MRM transitions and parameters are shown in Table 2.

Table 2: MRM transitions and optimized parameters

Name	RT (min)	Transition (m/z)	Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
DCD	2.55	85.1 > 68.1	-15	-21	-26
		85.1 > 43.0	-15	-17	-17
MEL	6.29	127.1 > 85.1	-26	-20	-17
		127.1 > 68.1	-26	-27	-26

Method Development

A LC/MS/MS method was developed for quantitation of DCD and melamine based on the MRM transitions in Table 2. Under the HILIC separation conditions (Table 1), DCD and melamine eluted at 2.55 min and 6.29 min as sharp peaks (see Figures 4 & 5). Figures 2 and 3 show the calibration curves of DCD and melamine standard in neat solutions and in milk matrix solutions (spiked). The linearity with correlation coefficient (R2) greater than 0.997 across the calibration range of 0.5~10.0 ng/mL was obtained for both compounds in both neat solution and matrix (spiked).

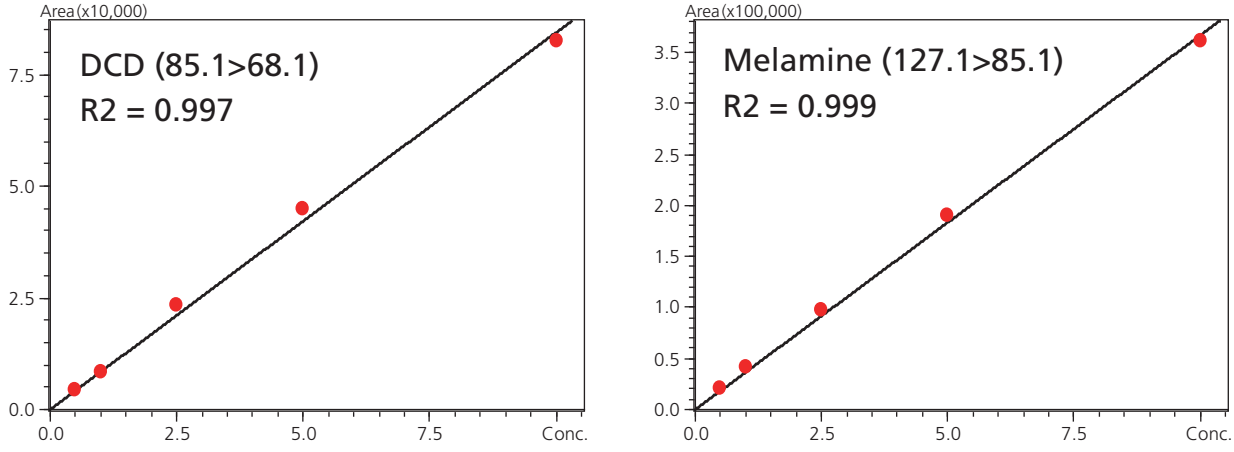

Figure 2: Calibration curves of DCD and melamine in neat solution

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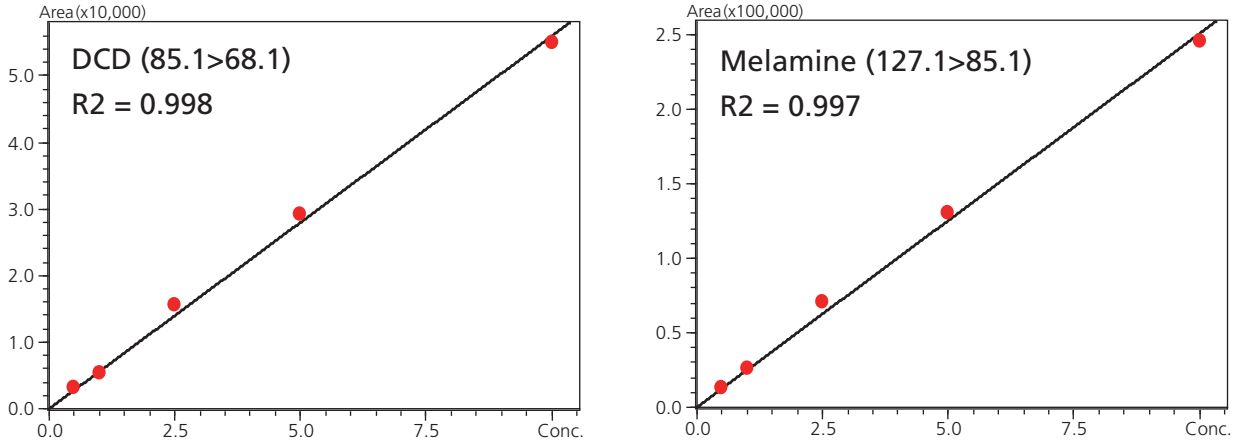

Figure 3: Calibration curves of DCD and melamine spiked in milk powder matrix

Performance Evaluation

The repeatability of the method was evaluated at the levels of 0.5 ng/mL and 1.0 ng/mL. Figures 4 & 5 show the MRM chromatograms of DCD and melamine of six consecutive injections of 0.5 ng/mL level with and without matrix. The peak area %RSD for the two analytes were lower than 9.2% (see Table 3).

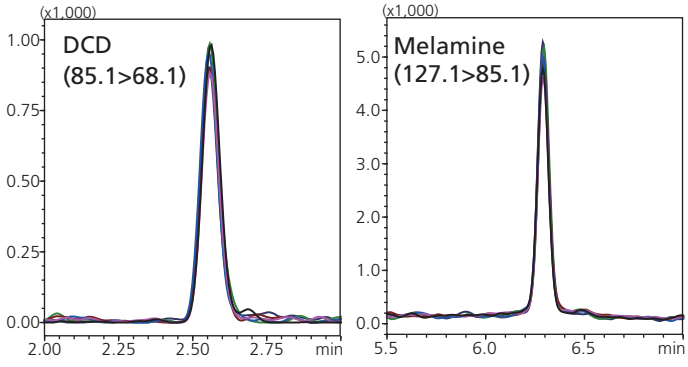

Figure 4: Overlapping of six MRM peaks of 0.5 ng/mL DCD and melamine in neat solution

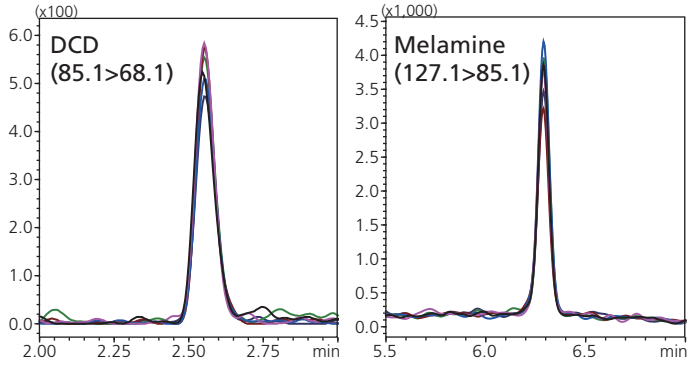

Figure 5: Overlapping of six MRM peaks of 0.5 ng/mL DCD and melamine in milk powder matrix

Table 3: Results of repeatability and sensitivity evaluation of DCD and melamine (n=6)

Sample	Compd.	Conc. (ng/mL)	%RSD	LOD (ng/mL)	LOQ (ng/mL)
In solvent	DCD	0.5	5.9	0.03	0.10
		1.0	5.3		
	MEL	0.5	5.5	0.03	0.09
		1.0	2.6		
In matrix	DCD	0.5	5.9	0.05	0.16
		1.0	8.2		
	MEL	0.5	9.2	0.05	0.15
		1.0	2.4		

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The LOD and LOQ were estimated from the results of 0.5 ng/mL in both neat and matrix solution. The LOD and LOQ results were summarized in Table 3. The method achieved LOQs (in matrix) of 0.16 and 0.15 ng/mL (ppb) for DCD and melamine, respectively. Tables 4 & 5 show the results of matrix effect and recovery of the method. The matrix effects for DCD and melamine in the whole concentration ranges were at 64%~70% and 62%~73%, respectively.

The recovery was determined by comparing the results of pre-spiked and post-spiked mixed samples of DCD and melamine in the milk powder matrix (2.5 ng/mL each compound). The chromatograms of these samples are shown in Figure 6. The recovery of DCD and melamine were determined to be 103% and 105% respectively.

Table 4: Matrix effect (%) of DCD and melamine in milk powder matrix

Conc. (ng/mL)	0.5	1	2.5	5	10
DCD	70.4	65.4	66.9	64.8	66.6
MEL	62.2	62.5	73.1	68.9	68.0

Table 5: Recovery of DCD and melamine determined with spiked sample of 2.5 ng/mL

Compound	Pre-spiked Area	Post-spiked Area	Recovery (%)
DCD	14,393	13,987	102.9
MEL	65,555	62,659	104.6

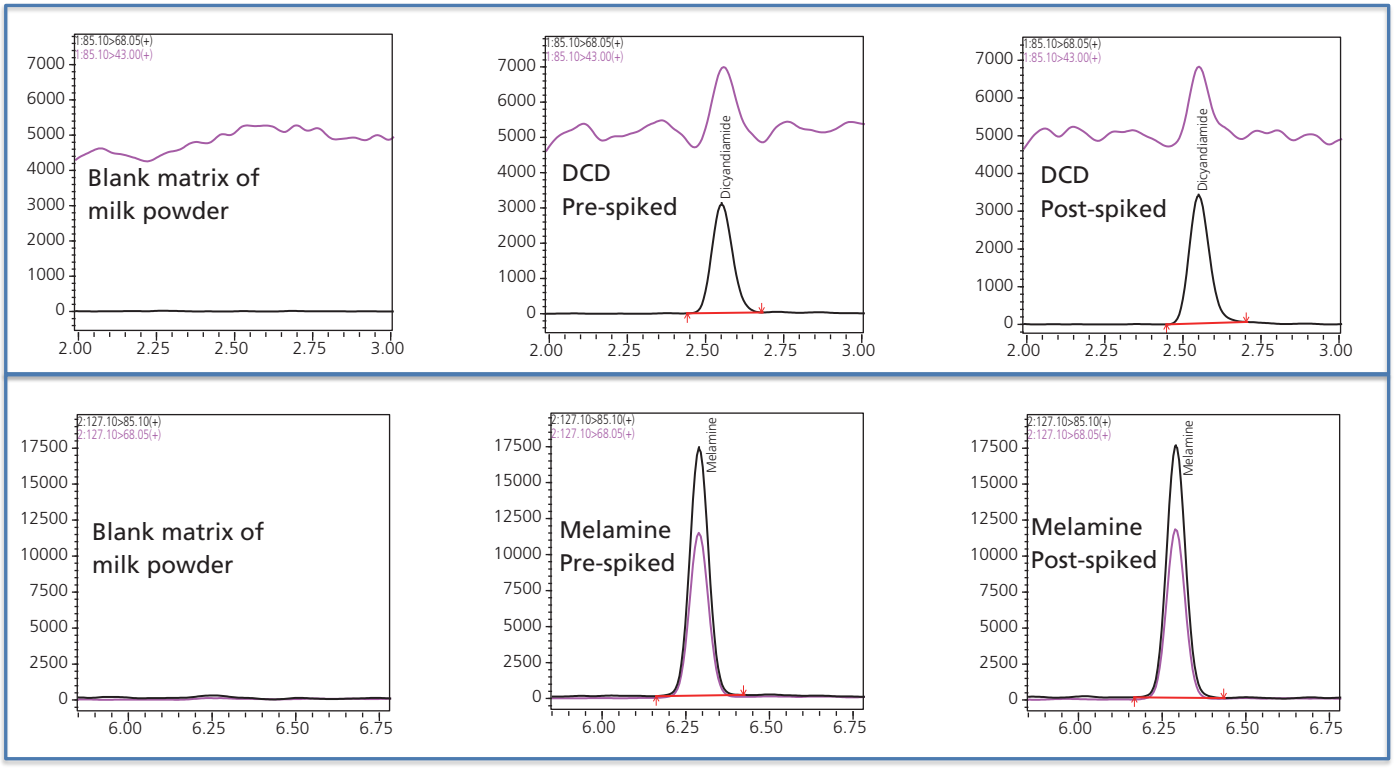

Figure 6: MRM peaks of DCD and melamine in pre- and post-spiked samples of 2.5 ng/mL (each). DCD and melamine were not detected in blank matrix of milk powder.

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Conclusions

A high sensitivity LC/MS/MS method was developed on LCMS-8040 for detection and quantitation of dicyandiamide (DCD) and melamine in milk powders. The method performance was evaluated using infant milk powders as the matrix. The method achieved LOQ of 0.16 ng/mL for both compounds in the matrix, allowing its application in simultaneous analysis of melamine, a protein adulterant in relatively high concentration, and dicyandiamide residue in trace level in milk powders samples.

References

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