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# Application News

# No. AD-0063

**LCMS-8040 UFMS**

# Analysis of Dicyandiamide and Melamine in Milk Powders by LC/MS/MS Method

## ❑ 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 [3-4]. Instead of addition directly, the DCD present in the milk products was explained to be due to that cows eating the grass "contaminated by DCD" may produce milk containing traces of DCD residues. Dicyandiamide is a toxic agrichemical compound and could be used to promote the growth of pastures where cows graze. We report here a LC/MS/MS method for sensitive detection and quantification of both dicyandiamide (DCD) and melamine in infant milk powder samples.

## ❑ Experimental

### Preparation of standards and Samples

Dicyandiamide (DCD) and melamine were obtained from Sigma Aldrich. Amicon Ultra-4 (MWCO 5K) centrifuge filtration tubes (15 mL) were obtained from Millipore. Stock solutions of DCD and melamine were prepared in pure water. 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. 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.

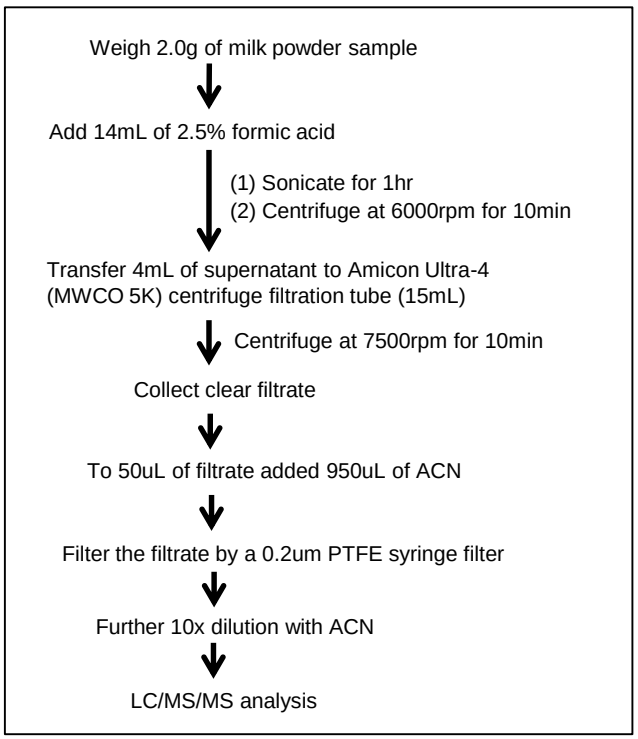


Figure 1: Flow chart of sample pre-treatment method

A 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 UHPLC coupled with a LCMS-8040 system. An Alltima HP HILIC column was used for separation of DCD and melamine with a gradient program developed in house. The details of the LC and MS conditions are shown in Table 1.

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

**LC conditions**

| | |
|---|---|
| Column | 2.1mm x 150 mm, Alltech |
| Flow Rate | 0.2 mL/min |
| Mobile Phase | A :0.1 % formic acid in H2O/ACN (5:95 v/v)<br>B :20mM Ammonium Formate in H2O/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 9.0min) |
| Oven Temperature | 40ºC |
| Injection Volume | 5 uL |

**MS conditions**

| | |
|---|---|
| Interface | ESI |
| MS mode | Positive |
| Block Temperature | 400ºC |
| DL Temperature | 300ºC |
| CID Gas | Ar (230kPa) |
| Nebulizing Gas Flow | $N_2$, 2.0L/min |
| Drying Gas Flow | $N_2$, 15.0L/min |
| MRM | DCD: 85.1 ➔ 68.05, 43.00<br>Melamine: 127.1 ➔ 85.10, 68.05 |

## ❑ 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 & Performance Evaluation

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 ($R^2$) 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).

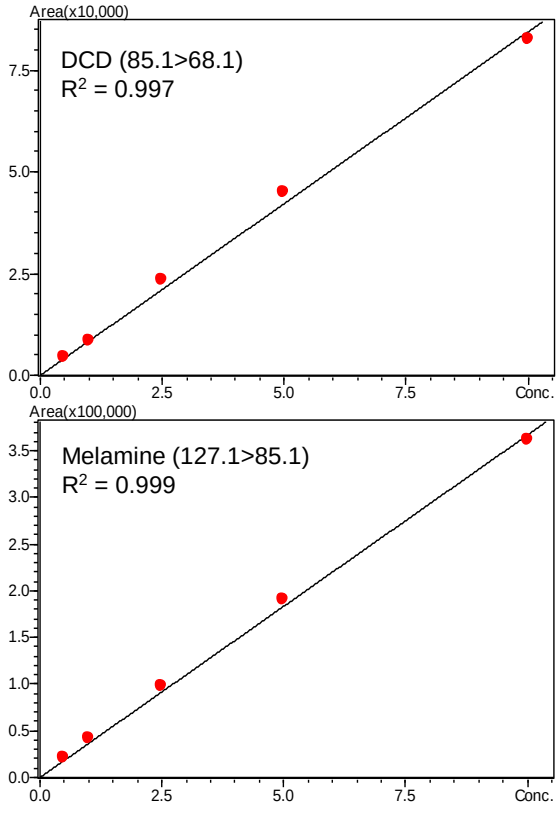


Figure 2: Calibration curves of DCD and melamine in neat solution

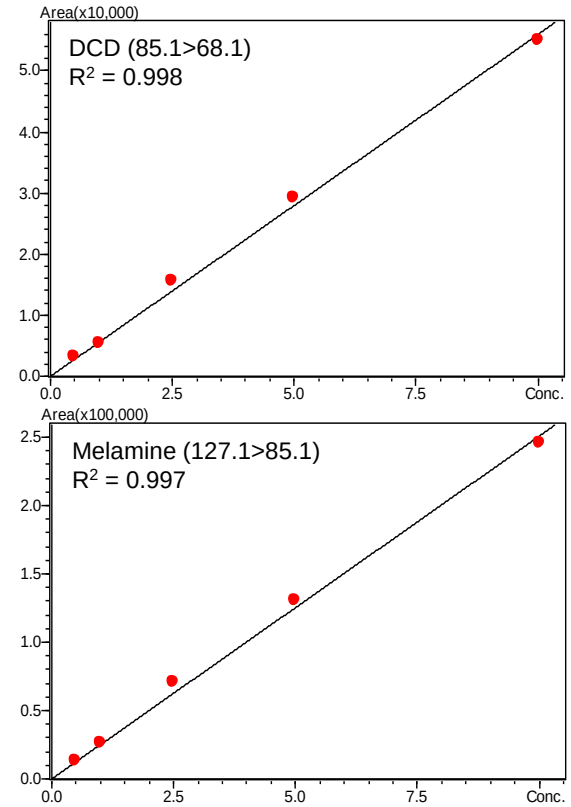


Figure 3: Calibration curves of DCD and melamine spiked in milk powder matrix

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).

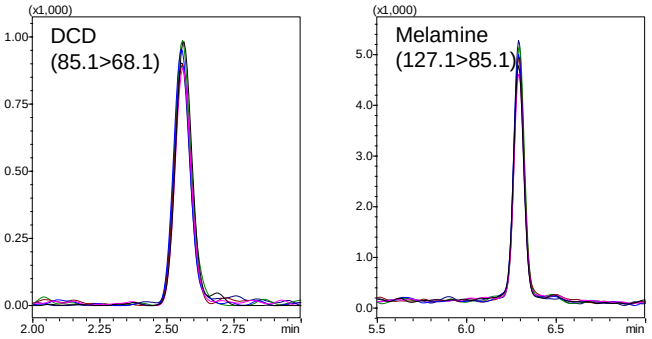


Figure 4: Overlapping of six MRM chromatograms of 0.5 ng/mL DCD and melamine in neat solution

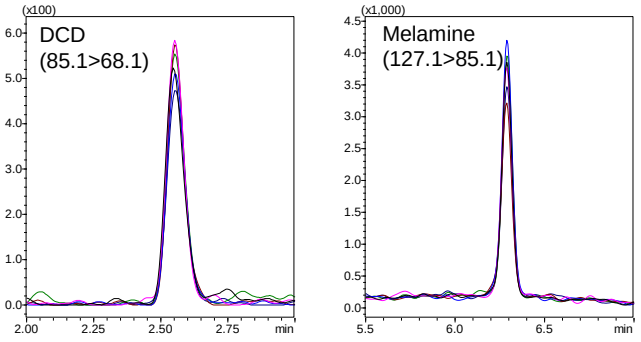


Figure 5: Overlapping of six MRM chromatograms of 0.5 ng/mL DCD and melamine in milk powder matrix

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 | | |

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 102% 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 |

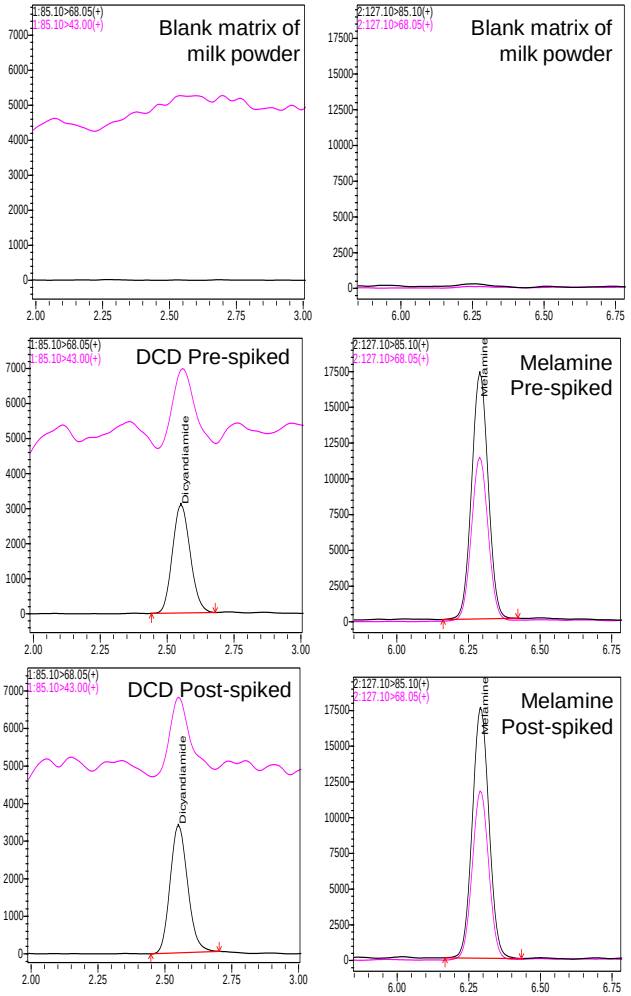


Figure 6: MRM peaks of DCD and melamine in pre- and post-spiked samples of 2.5 ng/mL (each). Noted that, DCD and melamine were not detected in the blank matrix of milk powder sample (top).

## ❑ 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 residues in trace concentration in milk powders samples.

## ❑ References

1.S. Turnipseed, C. Casey, C. Nochetto, D. N. Heller, FDA Food, LIB No. 4421, Volume 24, October 2008.
2.S. MachMahon, T. H. Begley, G. W. Diachenko, S. A. Stromgren, Journal of Chromatography A, 1220, 101-107 (2012).
3.http://www.naturalnews.com/041834_Fonterra_milk_powder_dicyandiamide.html
4.G. N. Lucas, Sri Lanka Journal of Child Health, 2013; 42(2): 63-64.

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