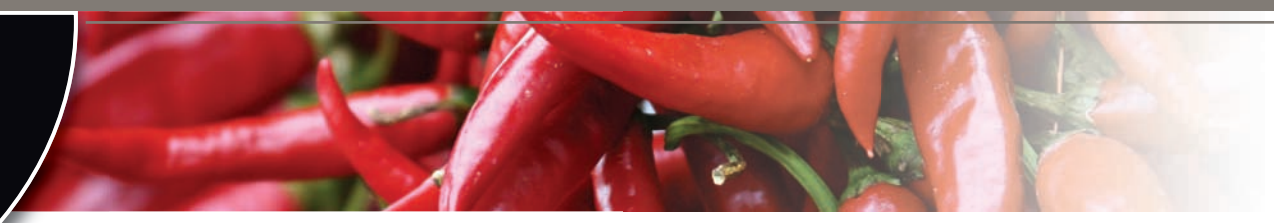

LC-MS/MS Analysis of Emerging Food Contaminants

Quantitation and Identification of Dicyandiamide in Milk and Other Protein-Rich Foods

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Recent issues with adulteration of food using nitrogen rich compounds to make the protein content of food appear higher than the actual value highlighted the need for both food manufacturers and regulatory agencies to utilise fast and accurate analytical techniques to proactively ensure product safety.

In 2007, melamine and cyanuric acid in wheat gluten added to pet food caused renal failure and sickened and killed large numbers of cats and dogs. In 2008, Chinese authorities discovered the adulteration of milk and infant formula with melamine by several Chinese producers. There were hundreds of thousands of victims and six confirmed deaths in China, as well as product recalls in many countries [1-4].

In response to the melamine contamination a large number of analytical methods were developed for the detection of melamine and its analogues, including several published by the United States Food and Drug Administration (FDA) that also targeted cyanuric acid [4-8].

However, the Kjeldahl method, the traditional standard technique for measuring protein content by indirectly measuring the nitrogen content in food, remains the most widespread methodology. As long as protein content in food is not determined directly, economic adulteration with nitrogen rich compounds will continue to be a serious concern.

Analytical methods to detect potential adulterants (non-protein nitrogen sources), including amidinourea, ammelide, ammeline, biuret, cyanuric acid, cyromazine, dicyandiamide, melamine, triuret, and urea *(Figure 1)* have been developed and validated to test milk products and bulk protein [4, 5].

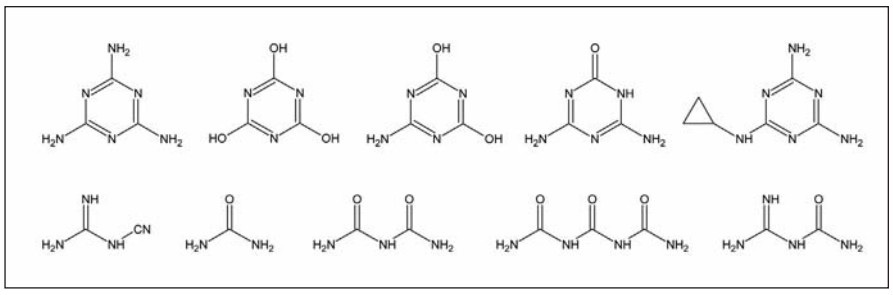

Figure 1. Potential adulterants (non-protein nitrogen sources), including melamine, cyanuric acid, ammelide, ammeline, cyromazine, dicyandiamide, urea, biuret, triuret, amidinourea, (top left to bottom right).

Recently, traces of dicyandiamide were found in milk produced in New Zealand. Milk producers and government agencies moved quickly to reassure there was no risk to health. Here we present a fast, easy, and sensitive LC-MS/MS method for the detection of dicyandiamide and other nitrogen rich compounds in milk and other protein-rich foods with limits of quantitation down to low µg/kg.

Experimental

Sample Preparation

Simple liquid extraction of food samples was performed using the following procedure [4]:

- Add 10mL of acetonitrile containing 2% formic acid to 1 g of a homogenised sample
- Mix thoroughly and sonicate for 10 minutes
- Centrifuge for 10 minutes
- Transfer an aliquot of 50µl of the extract into and autosampler vial and dilute with 950µL acetonitrile resulting in a total dilution factor of 200

Further dilution of the extract might be necessary if the sample is heavily contaminated.

LC

The target compounds were separated using a normal phase gradient on a Hydrophilic Interaction Chromatography (HILIC) column. LC separation was achieved using the Eksigent ekspert™ ultraLC 100 system with a Phenomenex LUNA HILIC 3u (100 x 2mm) column with a mobile phase of acetonitrile and water containing 0.1% formic acid and 10mM ammonium formate at a flow rate of 0.2mL/min *(Table 1)*. A sample volume of 10µL was injected.

Table 1. LC gradient used for the separation of dicyandiamide and other potential adulterants MS/MS

Time (min)	Mobile phase A (%): water with 0.1% formic acid and 10 mM ammonium formate	Mobile phase B: 95% actetonitrile with 0.1% formic acid and 10 mM ammonium formate
0.0	0	100
2.0	0	100
2.1	50	50
4.3	50	50
4.4	0	100
10.0	0	100

MS/MS

The AB Sciex QTRAP® 5500 was used with the Turbo V™ source and an Electrospray Ionisation (ESI) probe. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode using fast switching between negative and positive polarity. Two selective MRM transitions were monitored for each analyte using the ratio of quantifier and qualifier ion for identification *(Table 2)*. 13C3 15N3-melamine was used as an internal standard.

LC-MS/MS data was processed using the MultiQuant™ software version 2.1.

Table 2. MRM transitions used for the detection of dicyanamide and other potential adulterants

Time (min)	Mobile phase A (%): water with 0.1% formic acid and 10 mM ammonium formate	Mobile phase B: 95% actetonitrile with 0.1% formic acid and 10 mM ammonium formate
0.0	0	100
2.0	0	100
2.1	50	50
4.3	50	50
4.4	0	100
10.0	0	100

Results and Discussion

First, the limit of detection (LOD) and reproducibility were evaluated using injections of dicyandiamide standards and spiked matrix samples.

Figure 2 shows a chromatogram of dicyandiamide spiked into milk at 2µg/kg with a Signal-to-Noise (S/N) of 54 and 13 for the quantifier and qualifier ion, respectively.

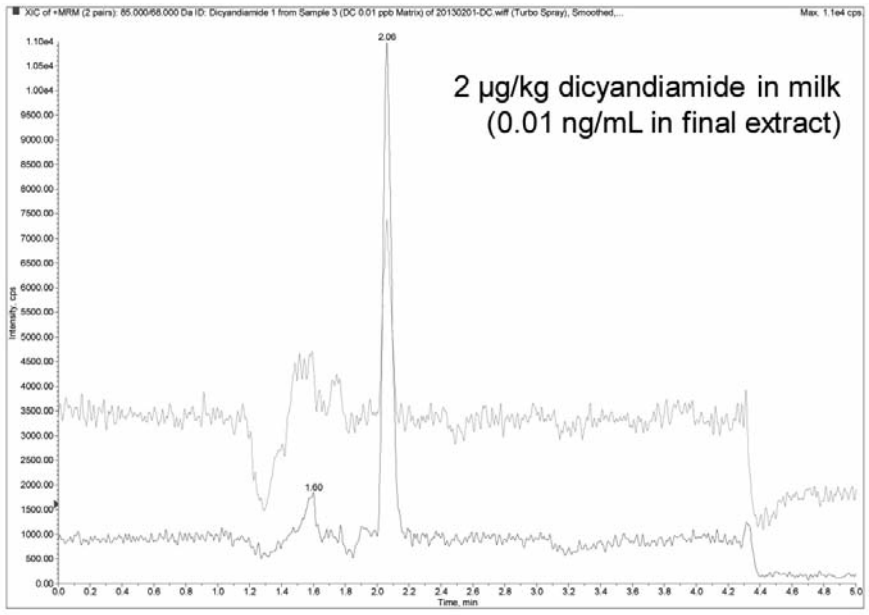

Figure 2. LC-MS/MS chromatogram of 2g/kg dicyanamide spiked into milk with a concentration of 0.01ng/mL in the final extract after 200x dilution

Figure 3 shows calibration lines for dicyandiamide spiked into milk, extracted using the described procedure with a total dilution factor of 200x. Extensive dilution is recommended to accurately quantify the target analyte in matrix samples to minimise possible ion suppression effects which cannot be compensated using an internal standard.

Coefficients of regression were determined to be greater than 0.997 for both transitions.

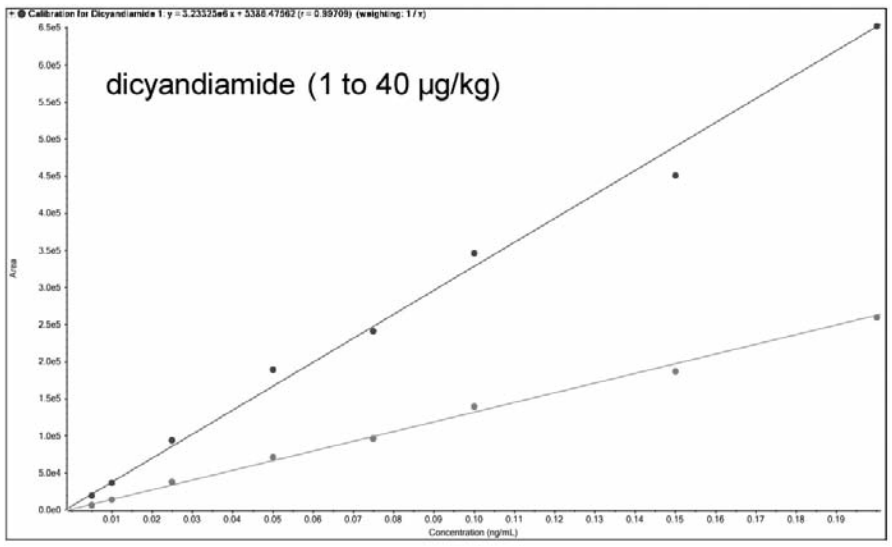

Figure 3. Calibration lines for dicyanamide spiked into milk and analysed after 200x dilution

The MRM ratios calculated across the dynamic range for identification were found well in between the expected 25% tolerance [9] of the standard ratio of 0.392. The MRM ratios were automatically calculated and reported using the 'Multicomponent' query in the MultiQuant™ software.

In a second step the method was extended to also detect other known potential adulterants. An example chromatogram is shown in *Figure 4*.

Dicyandiamide (retention time, RT=2.0 min), melamine (RT=4.6 min), ammeline (RT=4.7 min), ammelide (RT=4.8 min) were detected in positive polarity and cyanuric acid (RT=2.1 min) in negative polarity. The fast polarity switching of the QTRAP® 5500 system was used to detect dicyandiamide and cyanuric acid in a single run.

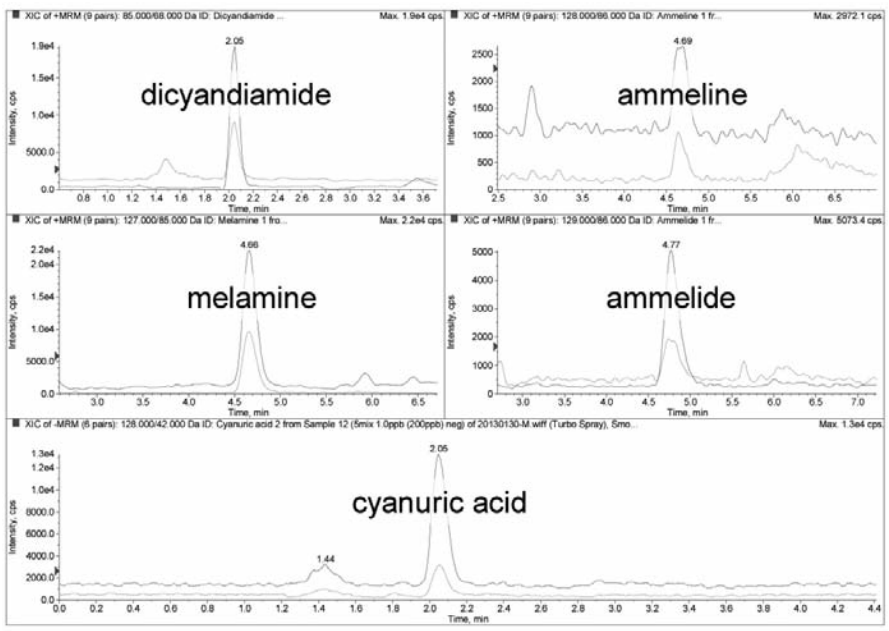

Figure 4. Quantitation of five potential adulterants (non-protein nitrogen sources) in a single run using fast polarity switching with the AB Sciex QTRAP® 5500 system

Figure 5 shows example calibration lines for melamine (positive polarity) and cyanuric acid (negative polarity). All calibration lines had r-values of greater than 0.998.

Note that the spiked matrix contained traces (< 10µg/kg) of cyanuric acid and the calibration line does not go through zero.

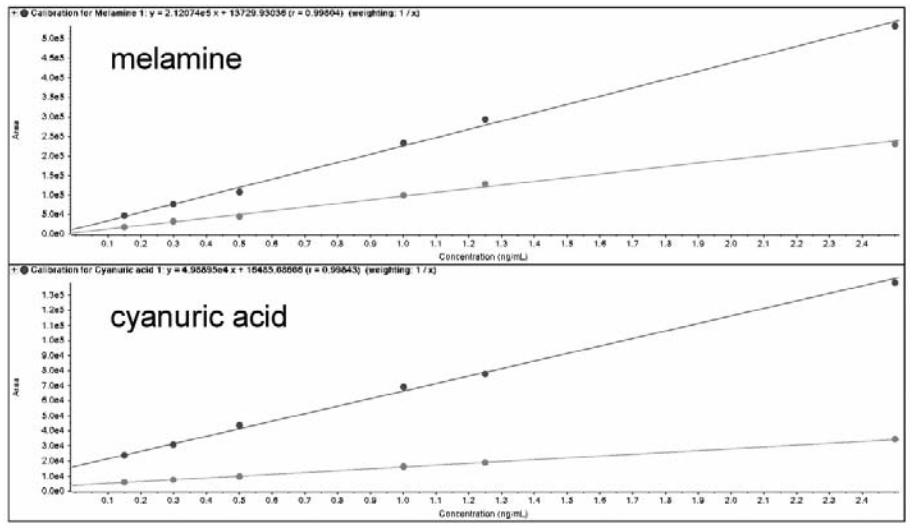

Figure 5. Calibration lines for melamine and cyanuric acid spiked into milk and analysed after 200x dilution

Milk samples were analysed using the developed method and tested positive for dicyandiamide. The 'Multicomponent' query was used to automatically calculate ratio of quantifier and qualifier ion for identification *(Figure 6)*.

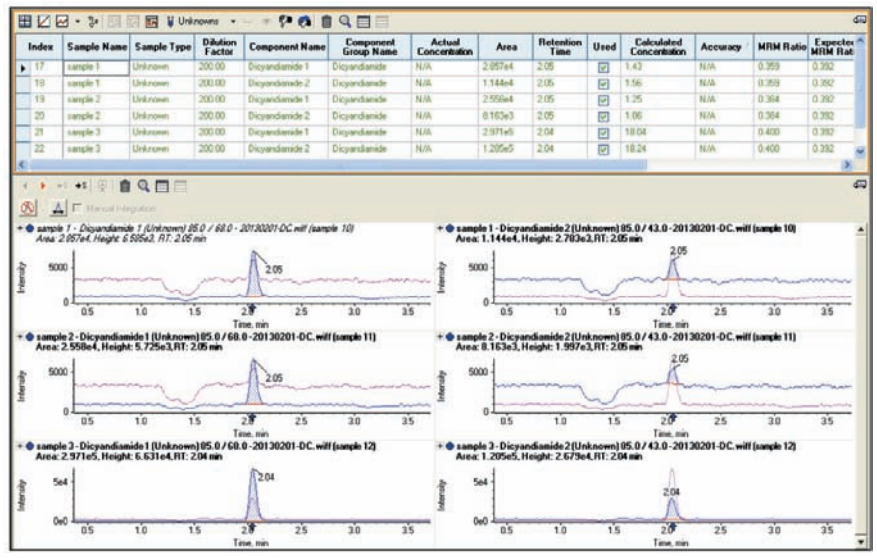

Figure 6. Milk samples tested positive for dicyandiamide, the 'Multicomponent' query was used to automatically calculate MRM ratios for compound identification

Summary

The method and data presented here showcase the fast, easy, and accurate solutions for the analysis of dicyandiamide and other nitrogen rich compounds in milk and other protein rich foods by LC-MS/MS. The AB Sciex QTRAP® 5500 systems provide excellent sensitivity and selectivity for this analysis, with minimal sample preparation allowing maximised throughput for the analysis of many samples in a short time period.

Dicyandiamide was quantified in milk samples. Automatic MRM ratio calculation in MultiQuant™ software was used for compound identification.

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