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# Highly sensitive detection of melamine based on reversed phase liquid chromatography mass spectrometry

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**In this work, we developed a highly sensitive method to detect melamine based on reversed phase liquid chromatography mass spectrometry. A mass spectrometry compatible ion pair, heptafluorobutyric acid(HFBA), was used to separate melamine by reversed phase liquid chromatography prior to electrospray mass spectrometry. The incorporation of isotope internal standard and multiple reaction monitoring improved the accuracy and linearity of quantification. Based on this strategy, the method limit of quantification was 0.1 ng/g. The limits of quantification were 8 ng/g for liquid milk and 15 ng/g for dry milk powder. This method provided a reproducible and stable approach to sensitive detection and quantification of melamine.**

reversed phase liquid chromatography, mass spectrometry, melamine, multiple reaction monitoring, ion pair

Recently, high amount of melamine has been found in the infant milk and other milk products. Due to the high amount of nitrogen (66.7%)[1] in the structure of melamine, it is used to boost the proteins levels in foods. However, in human and animal systems, an overdose of melamine will generate urolithiasis and have influences on health[2]. Therefore, it is an important task to detect melamine in the milk and other food products. Melamine was found toxic in the animal test during the 1980s and several detecting methods were developed such as gaschromatography[3], liquid chromatography[4–6], immunoassay[7], gas chromatography-mass spectrometry[8–9], and liquid chromatography-mass spectrometry[10,11]. Currently, there are three major methods for melamine detection.

(1) Reversed phase liquid chromatography (RPLC): The FDA of USA published a high performance liquid chromatography (HPLC-UV) method for the detection and quantification of melamine in food products[12]. Melamine is a very hydrophilic compound and is not retained on reversed phase columns, thus it cannot be separated from other impurities. In general, an ion pair reagent was added in the mobile phase to retain hydrophilic compounds and achieve the separation in reversed phase chromatography. For melamine detection, sodium octane sulfonate[12] was used as ion pair reagent. The limit of quantification (LOQ) of this method is 2 μg/g[12,13]. One of the setbacks of this method is the identification of the purity of peaks. Therefore, any co-eluted impurity can affect the accuracy of measurement and the sensitivity of UV detection only reaches μg/g level as well.

(2) Gas chromatography-mass spectrometry: This method separates the analytes by gas chromatography and then the targets are detected by mass spectrometry. Gas chromatography has a high separation power and can separate a complicated mixture. Mass spectrometry

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enables the identification of compounds with sensitivity down to the ppb level. When these two technologies were combined, it was a routine method to detect melamine at ng/g level[9]. However, melamine is not a volatile compound, so it requires derivatization before GC-MS analysis and therefore affects its reproducibility and detection limit. In the report of FDA in 2007, the minimum reporting level (MRL) of melamine by GC-MS was 10 μg/g[14].

(3) Liquid chromatography-mass spectrometry: This method is to separate mixture by high performance liquid chromatography, followed by mass spectrometric identification. It does not require derivatization of melamine and its detection limit is also in the ppb level. The most commonly used HPLC method is the reversed phase chromatography. However, melamine cannot be retained on reversed phase column and the current ion pair reagent is not compatible with the LC/MS analysis. Therefore, current LC-MS method uses hydrophilic interaction chromatography (HILIC) for HPLC separation[10]. The FDA of USA published an HILIC method for melamine detection in October, 2008. The LOQ for liquid sample can reach 25 ng/g (ppb) and for solid sample is 200 ng/g[15]. However, HILIC method is not stable and requires a long equilibration time, and its peak efficiency and separation power are lower than RPLC. The high organic mobile phase also reduces the ionization efficiency on electric ionization and therefore reduces the sensitivity.

Another method used the cation exchange and C18 mixed bed column for the separation[11,13]. The mix-bed column is difficult to be commercially available and reproduced. So far, the FDA has not adopted this method. Therefore, it is necessary to develop a method based on reversed phase liquid chromatography mass spectrometry for the highly sensitive detection of melamine. Here, we describe the method using heptafluorobutyric acid (HFBA) as a buffer additive for the reversed phase liquid chromatography mass spectrometry (RP-LC-MS) analysis of melamine. Melamine compound can be bound to HFBA and then retained on the reversed phase column. HFBA is easy to evaporate due to its low boiling point, so it does not interfere with the mass spectrometric detection. In addition, using the melamine isotope internal standard increases the accuracy of quantification[10]. In this report, the limit of quantification (LOQ) is 0.1 ng/g (ppb)for melamine standard. The LOQ is 8 ng/g for liquid milk and 15 ng/g for dry milk powder. This method utilizes routine C18 reversed phase liquid chromatography for the separation coupling with mass spectrometry. Therefore, it is robust and reproducible, with high sensitivity and accuracy to be a better option for the detection and quantification of melamine.

## 1 Experimental

### 1.1 Materials

Methanol was from Fisher (HPLC grade), and Heptafluorobutyric Acid (HFBA) was from Fluka (HPLC grade). Water was purified by Millipore. Trichloromethane (analytical grade) and melamine (purity ⩾ 99%) were from Sigma. Isotope melamine standard was from Cambridge Isotope laboratories, Inc. ($^{13}C_3$; $^{15}N_3$, 98%). Infant milk powder and liquid milk were obtained from supermarket.

### 1.2 LC/MS condition

For LC-MS analysis, an Agilent 1100 HPLC system was coupled with 6410 Triple Quad mass spectrometer (Agilent. Technologies, Palo Alto, CA, USA). Reversed phase C18 columns (150 mm×2.1 mm (i.d.), 5 μm, 120 Å) were from Column Technology Inc. (Fremont, CA, USA). Mobile phase: 0.1%HFBA (*v*/*v*)-methanol (70∶30), isocratic gradient. Flow rate: 0.20 mL/min. Temperature: Room temperature. Injection: 20 μL. Run time: 5.5 min. For mass spectrometry detection, electrospray ionization and positive ion mode were applied. Gas temp: 350℃; Gas flow: 8 L/min; Gas pressure (Nebulizer): 30 psi. Multiple reaction monitoring (MRM) scan parameters are listed in Table 1.

### 1.3 Preparation of melamine standard

Melamine stock solution: 5 μg/mL melamine solution was prepared in 0.1% HFBA (*v*/*v*) and stored at 4℃. Melamine isotope stock solution: 100 μg/mL [$^{13}C_3$;

**Table 1** Parameters for mass MRM scan

| Compound name | Precursor (*m*/*z*) | Product ion (*m*/*z*) | Dwell (ms) | Fragmentor (V) | Collision engergy (V) |
|---|---|---|---|---|---|
| Melamine | 127 | 85 | 300 | 100 | 20 |
| [$^{13}C_3$; $^{15}N_3$]-melamine | 133 | 89 | 300 | 100 | 20 |

$^{15}N_3$]-melamine was prepared in 0.1% HFBA solution (*v*/*v*) and then diluted to 5 μg/mL and stored at 4℃. Melamine isotope working solution was prepared by diluting melamine stock solution to 10 ng/mL by 0.1% HFBA. Melamine working solution was prepared by diluting melamine stock solution to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL by 0.1% HFBA.

### 1.4 Liquid and dry milk sample preparation

Dry milk powder: 25 mg of milk powder was accurately weighed in a 1.5 mL plastic tube, then 1 mL 1% HFBA solution, 0.25 mL methanol and 2.5 μL melamine isotope stock solution were added, followed by vortexing for 5 min, sonicating for 10 min and centrifuging for 5 min at 12000 r/min. 700 μL supernatant liquid was transferred into a 1.5 mL centrifuge tube, and 100 μL chloroform was added, followed by vortexing for 3 min and centrifuging at 12000 r/min for 5 min. Finally, 200 μL supernatant liquid was filtered through 0.22 μm membrane and ready for LC-MS analysis.

Liquid milk: 25 mg sample (~25 μL) was accurately weighed in a 1.5 mL tube, and 975 μL 1% HFBA solution, 0.25 mL methanol and 2.5 μL melamine isotope stock solution were added, followed by vortexing for 5 min, sonicating for 10 min and centrifuging for 5 min at 12000 r/min. 700 μL supernatant liquid was transferred into a 1.5 mL centrifuge tube, and 100 μL chloroform was added, followed by vortexing for 3 min and centrifuging at 12000 r/min for 5 min. Finally, 200 μL supernatant liquid was filtered through 0.22 μm membrane and ready for LC-MS analysis.

## 2 Results

### 2.1 Linearity

The MS/MS spectrum of melamine shows peaks at *m*/*z* 85 and 68 (Figure 1(a)). The peak at *m*/*z* 85 is $C_2N_4H_5^+$ ion and is the ion formed due to the loss of $NCNH_2$ from melamine. This is the highest peak in the spectra and is used for the quantification of melamine. The ion of *m*/*z* 68 is formed by the loss of $NH_3$ from melamine. The MS/MS spectrum for the melamine isotope is shown in

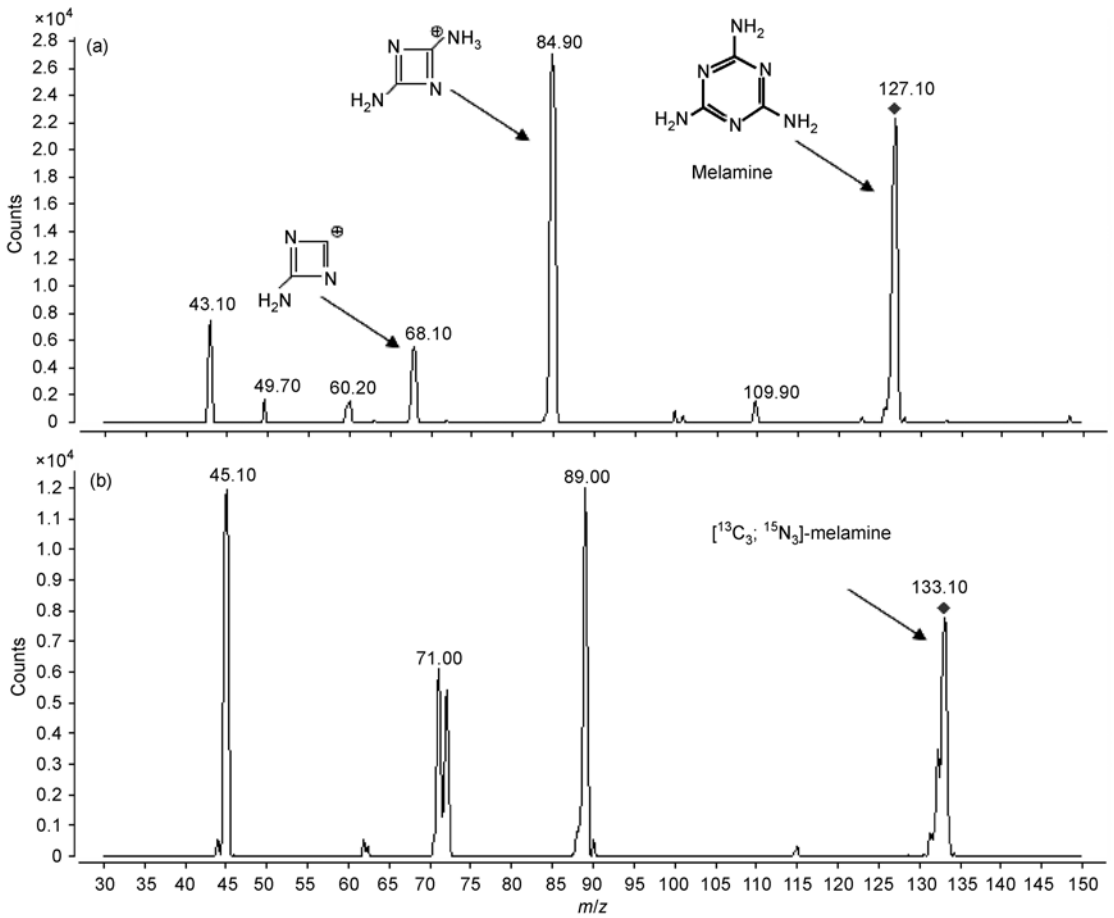


**Figure 1** MRM spectra of melamine standard (a) and melamine isotope (b).

Figure 1(b). Three ions (*m*/*z* 89, 70 and 71) are observed in the spectrum. The ion whose peak is at *m*/*z* 89 is chosen as the internal quantification ion. The linearity is obtained by plotting of melamine standard solutions at the concentrations of 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL using triple quadruple mass spectrometry in MRM mode ($R^2$ = 0.99946). The further linearity is plotted using the ratio between melamine standards and melamine isotope. Using isotope internal standard for quantification, the method has better linearity ($R^2$ = 0.99993) and accuracy.

### 2.2 Limit of quantification

Figure 2 shows the MRM chromatogram at the 0.2 ng/g level. The signal to noise ratio is 11.9. In terms of the FDA criteria ($S/N \geqslant 5$)[14], the limit of quantification (LOQ) of this method is at 0.1 ng/g (ppb). Figure 3 shows the MRM chromatogram for the liquid and dry milk powder with 50 ng/g melamine spiking. In the liquid milk at 50 ng/g level, the *S*/*N* ratio of melamine is 32.1 (Figure 3(a)). The LOQ for the liquid milk is 8 ng/g (8 ppb). For the dry milk powder at 50 ng/g level, the *S*/*N* is 16.9 (Figure 3(b)). The LOQ for the dry milk powder is 15 ng/g (15 ppb).

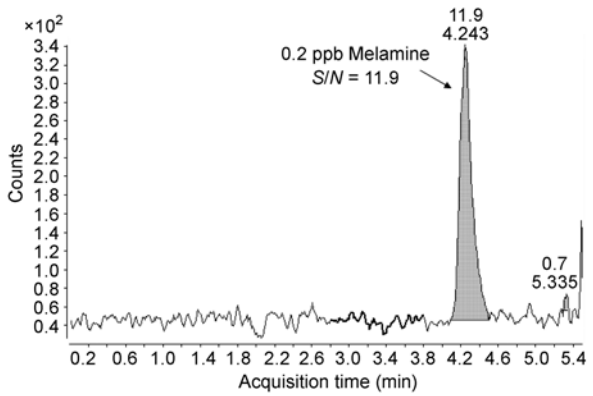


**Figure 2** MRM chromatogram of melamine at 0.2 ng/ mL.

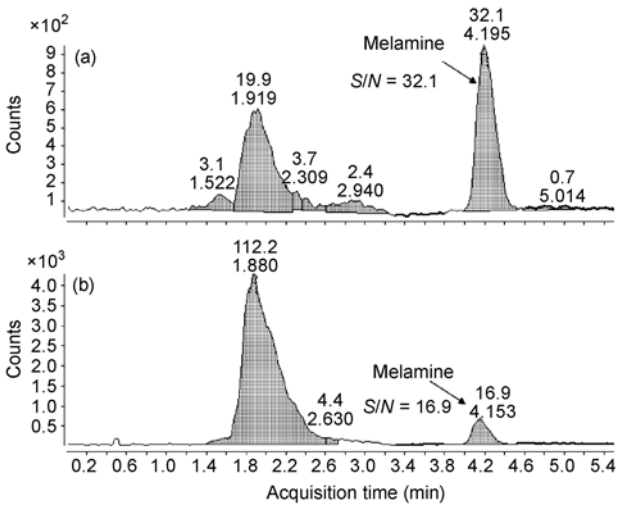


**Figure 3** 50 ng/g melamine MRM chromatogram in liquid milk (a) and dry milk powder (b).

### 2.3 Reproducibility

Figure 4 shows the MRM chromatogram of seven melamine standards at different levels (0.2, 0.5, 1, 2, 5, 10, 20 ng/mL). The average retention time is 4.201 min and the relative standard deviation is 0.40% (Figure 4(a)). For the melamine isotope, the average retention time is 4.200 min and the relative standard deviation is 0.41% (Figure 4(b)). All the data show a great reproducibility of the analysis of melamine by the new method. Both the melamine standard and the melamine isotope show the same retention time in LC-MS analysis.

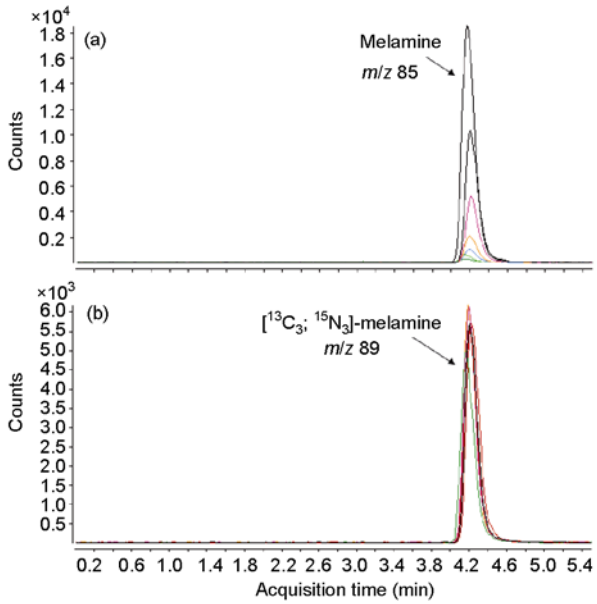


**Figure 4** Reproducibility test: MRM chromatograms of seven melamine standards in different concentrations (a) and seven melamine isotope standards (b).

### 2.4 Recovery

Figure 5 shows the MRM chromatograms of five 25 mg melamine samples in dry milk powder. The average recovery rate is 99.4% with relative standard deviation which is 1.87% (Table 2). This demonstrates that the method has good stability and excellent recovery rate.

### 2.5 Real sample applications

Following the steps in experimental section 1.4, melamine in three samples of infant milk powders and two samples of liquid milk were measured. According to MRM and isotope internal standard, the amounts of

**Table 2** Recovery rate with isotope internal standard[a)]

| Samples | Amount of milk (mg) | Amount of melamine in milk (ng) | Theoretical amount of melamine (ng/g) | Actual amount of melamine detected (ng/g) | Recovery rate (%) |
|---|---|---|---|---|---|
| 1 | 25 | 12.5 | 500 | 492.57 | 98.51 |
| 2 | 25 | 12.5 | 500 | 508.43 | 101.67 |
| 3 | 25 | 12.5 | 500 | 494.92 | 98.97 |
| 4 | 25 | 12.5 | 500 | 503.35 | 100.66 |
| 5 | 25 | 12.5 | 500 | 484.71 | 96.94 |

a) Average recovery rate: 99.4%, RSD: 1.87%.

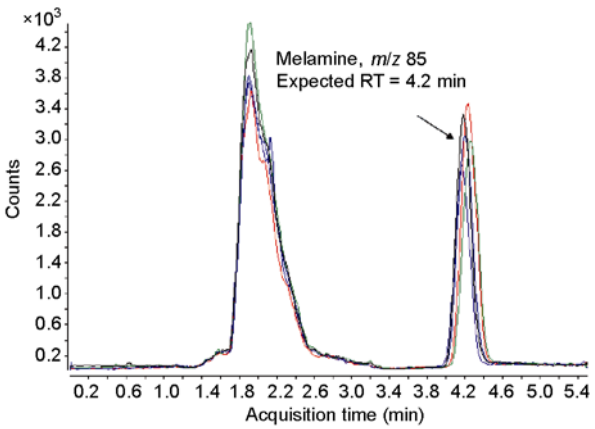


**Figure 5** Recovery rate: MRM chromatograms for five 50 ng/g melamine in dry milk powder.

melamine in these three milk powder samples are 611.6 ng/g (Figure 6(a), 545.5 ng/g (Figure 6(b)), and 227.5 ng/g (Figure 6(c)), respectively. One sample of liquid milk contains 11.2 ng/g melamine (data not shown) and the melamine amount of the other liquid milk is undetectable.

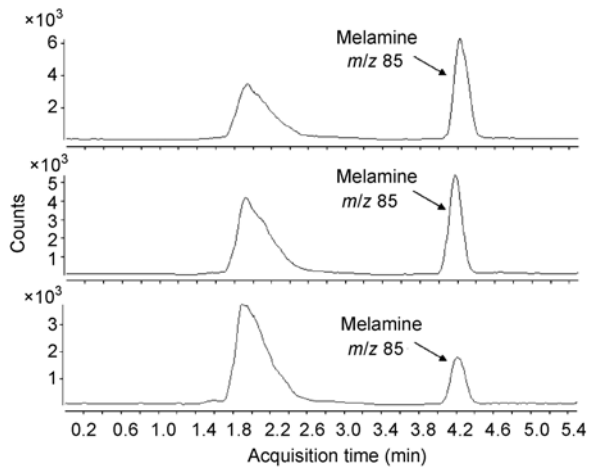


**Figure 6** MRM chromatograms of milk sample. Amounts of melamine detected in the milk samples are 611.6 ng/g (a), 545.5 ng/g (b) and 227.5 ng/g (c).

## 3 Discussion

### 3.1 Sample preparation

The melamine sample preparation using a cation exchange reversed phase and mixed bed solid phase extraction has been reported[15]. The process is tedious, time-consuming, with reproducibility and recovery rate. In this experiment, 1% HFBA-methanol (80∶20) was used for the extraction of melamine from food products. The solution not only had good solubility for the polar melamine molecule, but also could precipitate the protein mixture in the matrix. In this experiment, we compared the extraction effect of different types of acids (formic acid, trifluoroacetic acid, trichloroacetic acid and heptafluoro butyric acid) and the ratio between acid and organic solvent (20%, 30% and 50%). All acids at 1% level could precipitate proteins in the matrix while trifluoroacetic acid and trichloroacetic acid caused the shift of retention time and sometimes generated two peaks of melamine. The high organic contents reduced the solubility of melamine. Therefore, the current 1% HFBA-methanol (80∶20) is the best system for the sample extraction. After the extraction and centrifugation, the melamine sample was further washed by chloroform to remove esters from melamine sample. A double layer was formed when mixing chloroform with 1% HFBA-methanol (80∶20) solution. Most of esters dissolved in the bottom chloroform layer. The melamine remains in the top layer. After the extraction, the chloroform layer contains only about 0.58% of melamine which was not significant to the results.

### 3.2 Optimization of LC-MS condition

In this experiment, we used reversed phase liquid chromatography for the separation of sample mixture. We added HFBA as a mobile phase additive which could increase retention time of melamine on the C18 column and therefore increased the resolution and reproducibility of the method. HFBA is a volatile compound and is compatible with any mass spectrometry. The C18 column used for the separation has small pore size at 120 Å with high surface area and high coverage. It is suitable for the separation of small molecules.

In this report, 0.1% HFBA-methanol (70∶30) is used

as a mobile phase. We found acetonitrile reduced peak efficiency and mass signal. Different amounts of methanol were also tested in this work. Finally, a mixture of methanol with 0.1% HFBA (30∶70) generated the best separation and had short retention time.

### 3.3 Reproducibility, recovery and accuracy

Reproducibility, recovery and accuracy are the most important issues in analytical method development. As a good analytical method, its relative standard deviation of reproducibility and accuracy should be less than 20%. In this method, the recovery rate is from 96.94% to 101.67% with an average recovery rate of 99.4%. The relative standard deviation is less than 1.87%, which is far lower than that the regular method allows.

In this report, the use of triple quadruple mass spectrometer under MRM mode suits the analysis of low concentration samples in the complicate matrix. The high selectivity of MRM mode can eliminate the background noise and enhance detection sensitivity. The use of melamine isotope as the internal standard can reduce the variation in the experiments and instrument, and therefore increases the accuracy of detection and quantification.

## 4 Conclusion

Here, we describe an analytical method for the detection of melamine. The sample preparation step effectively removes proteins and esters, thus increasing the detection sensitivity. Using MRM mode in the triple Q mass spectrometry with isotope internal standard also further increases the accuracy and linearity in the quantification.

This analytical method for melamine detection is simple, stable (recovery rate: 99.4%, RSD: 1.87%), fast (sample preparation < 30 min, LC-MS detection < 6 min) and sensitive (limit of quantification for standard is 0.1 ng/g, liquid milk 8 ng/g, and dry milk powder 15 ng/g). It is suitable for the detection of melamine and should be widely used in the inspection of food products.


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