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Review:

Recent developments in the detection of melamine*

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Abstract: In recent years, there were two reported outbreaks of food borne illness associated with melamine. The presence of melamine and its related compounds in milk, feed, and other foods has resulted in the need for reliable methods for the detection and accurate quantification of this class of contaminants. The sample pretreatment for melamine in a complex matrix usually involves a liquid extraction by a polar solvent, followed by a further clean-up with solid phase extraction. Analyses of melamine and related compounds are commonly carried out by liquid or gas chromatographic methods conjugated with mass spectrometry. Other innovative screening methods, which use antibodies, molecularly imprinted polymers, capillary electrophoresis, and gold nanoparticles, are also used to develop assays and biosensors to melamine. However, many of these methods have been hindered by matrix effects, the solubility of melamine-cyanuric acid complex, and background contamination. This article reviews recent developments for detecting melamine and discusses future directions.

Key words: Melamine, Detection, Confirmation methods, Screening methods, Sample pretreatment
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1 Introduction

In recent years, there were two reported outbreaks of food borne illness associated with melamine-contaminated food. In March 2007, pet food ingredients contaminated with melamine and its analogues ammeline, ammelide, and cyanuric acid (Fig. 1) resulted in a major outbreak of renal disease and associated deaths in cats and dogs in the United States. As a result, several major companies have recalled more than 5300 pet food products (FDA, 2009b). In September 2008, Sanlu and 21 other diary companies in China were selling milk and infant formula that had been contaminated with melamine, which leads to kidney stones and renal failure. By November 2008, nearly 300000 people had become ill, and six infants died. The situation has since become an international health scare (Look Back '08:10 Big Events in CHINA (II), 2008). It was determined that melamine had been deliberately added to pet food and raw milk to give the false appearance of a high level of protein. Melamine alone is of low toxicity; however it is able to form an insoluble complex with cyanuric acid, which can lead to kidney toxicity.

The World Health Organization (WHO) has recommended the tolerable daily intake (TDI) for melamine to be 0.2 mg melamine per kg of body mass. Meanwhile, maximum residue levels (MRLs) for melamine have also been set worldwide. In China and the US, the MRL for infant formula has been set at 1.0 mg/kg and at 2.5 mg/kg for milk and other milk products, while in Europe, the Food Safety Authority has set the limit to 2.5 mg/kg for all products containing greater than 15% milk (WHO, 2009b).

Considering the presence of melamine in milk and animal feeds, and their possible contamination in other foods, there is a need for establishing sensitive and reliable methods that are capable of screening

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samples and confirming the presence and quantities of melamine and other metabolites. This article aims to review the recent detection methods for melamine and its related compounds in different matrices.

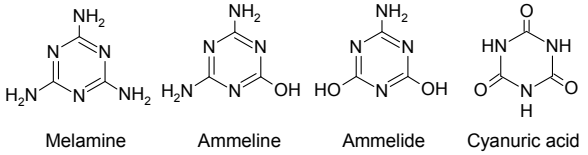

Fig. 1 Structures of melamine, ammeline, ammelide, and cyanuric acid

2 Sample pretreatment

The strategies of sample pretreatment depend on the different matrixes and the selective abilities of detection methods. Melamine (2,4,6-triamino-1,3,5-triazine; $C_3H_6N_6$; relative molecular weight (M_W): 126.12) is a small molecular compound with a polar compound. The sample pretreatment for melamine usually involves liquid extraction by a polar solvent, and some complex matrixes also require further clean-up with solid phase extraction (SPE) (WHO, 2009a). The solution for melamine extraction includes a mixture of acetonitrile/water/diethylamine (50:40:10, v/v) solution in feed (Squadrone *et al.*, 2010), kidney (Filigenzi *et al.*, 2008), and milk (Miao *et al.*, 2009), and trichloroacetic acid solution (some coupled with lead acetate) in milk or milk-based products (Ibáñez *et al.*, 2009; Sun H.W. *et al.*, 2010) and eggs (Xia *et al.*, 2009). Some have also reported using methanol (Venkatasami and Sowa, 2010) or hydrochloric acid (Wei *et al.*, 2010) to extract melamine from matrixes. The organic solutions or acids are used to extract melamine and precipitate protein in the samples to reduce the interference caused by the sample matrix. Diethylamine is used to maintain the extraction solvent at an alkaline pH to prevent melamine and cyanuric acid from forming an insoluble salt of melamine cyanurate (Miao *et al.*, 2009). Some tissue samples also require dichloromethane (Andersen *et al.*, 2008) or hexane (Karbiwnyk *et al.*, 2009) to act as a defatted reagent. Mixed-mode cation exchange solid-phase extraction cartridges are the most popular cleaning-up method for melamine samples. However, some highly selective methods (Yang S.P. *et al.*, 2009) or nondestructive methods (Mauer *et al.*, 2009) do not require any pretreatment before detection.

3 Confirmation methods

Some recent publications of instrumental methods for analysis of melamine and related compounds are summarized and compared in Table 1.

3.1 Liquid chromatographic (LC) methods

In most laboratories, melamine and its metabolites are routinely separated by LC and detected by selective techniques. Tandem mass spectrometry (MS/MS) provides the highest degree of selectivity, followed by single-stage MS, diode array detection (DAD), and lastly ultraviolet (UV) absorption.

An interim method for the determination of melamine and cyanuric acid residues in foods using LC-MS/MS was proposed by the Food and Drug Administration (FDA) at levels of 0.25 mg/kg in infant formula and other dairy-containing food products or ingredients (FDA, 2009a). In 2008, a national standard method for rapid determination of melamine in raw milk using LC-MS/MS has been issued in China with the limit of detection (LOD) of 50 μg/kg (AQSIQ and SAC, 2008c).

In the early studies, an analysis method was developed as a degradation product of the cyromazine (CYRO) and *S*-triazine herbicides. Since the 2007 pet food recall, LC method has been more widely applied to detect melamine in feed samples. Due to the polar features of melamine, it is difficult to obtain sufficient retention time by the traditional C_{18} column (Ehling *et al.*, 2007). Many methods utilize ion-exchange (Varelis and Jeskelis, 2008), ion pair chromatography (Ibáñez *et al.*, 2009), or hydrophilic interaction chromatography (HILIC) (Andersen *et al.*, 2008; Heller and Nochetto, 2008) to overcome these issues. Considering the potential for high concentrations of melamine and cyanuric acid to form crystals in tissues, it is necessary to detect melamine and its relate compounds in combined crystals (Filigenzi *et al.*, 2008). Dane and Cody (2010) also designed a method by using direct analysis in real time (DART) MS to eliminate the interference from 5-hydroxymethylfurfural with direct analysis of melamine in powdered milk.

Table 1 Liquid and gas chromatographic methods for detecting melamine and related compounds

Method	Matrix	Analyst	LOD (mg/kg)	Comments	Reference
LC-MS/MS	Human urine	MEL, CYA	0.00066	Cation-exchange solid-phase extraction	Panuwet *et al.*, 2012
LC-MS/MS	Rat plasma, liver, kidney, spleen, bladder, and brain	MEL	0.00560–0.0083l	Evaluated matrix effect of biological samples; background contamination was observed	Wu *et al.*, 2009
LC-MS/MS	Milk-based products and other food and beverage products	MEL	0.01–0.10	Ion-pair liquid chromatography; no SPE clean-up step	Ibáñez *et al.*, 2009
LC-MS/MS	Milk and dairy products	MEL, CYRO	10	Purified by Waters Oasis MCX column	Feng *et al.*, 2008
LC-MS/MS	Kidney tissue	MEL, AMD, AML, CYA	0.092–0.140	Validated by analysis of samples fortified with MEL-CYA powder; multi-residue analysis; polar RP column	Filigenzi *et al.*, 2008
LC-MS/MS	Catfish, trout, tilapia, salmon, and shrimp	MEL	0.0032	Hydrophilic interaction chromatography; MEL was determined in fish dosed with MEL and a combination of MEL and CYA	Andersen *et al.*, 2008
LC-MS/MS	Porcine muscle	MEL	0.0017	Separated by ether-linked phenyl column	Filigenzi *et al.*, 2007
LC-MS	Catfish, pork, chicken, and pet food	MEL, CYA	0.01	Ion exchange solid phase clean-up	Varelis and Jeskelis, 2008
HPLC-DAD	Milk and diary products	MEL	0.035–0.110	Separated by Nucleosil C_8 column	Filazi *et al.*, 2012
HPLC-DAD	Liquid milk	MEL	0.018	Purified by Cleanert PCX-SPE cartridges; separated by C_{18} column	Sun H.W. *et al.*, 2010
HPLC-DAD, LC-MS/MS	Plant origin protein powder	MEL	10 and 0.5	HPLC-DAD for screening; LC-MS/MS for confirmation	Ding *et al.*, 2008
HPLC-DAD	Rice protein concentrate feeds	MEL, AMD, AML, CYA	55–113	Separated by polar selectivity column; eight validation data	Muñiz-Valencia *et al.*, 2008
HPLC-UV	Infant formula	MEL	0.1	Acetonitrile-free; Kromasil C_{18} column	Venkatasami and Sowa, 2010
HPLC-UV	Diary products	MEl	0.003	HILIC; NH_2 column	Zheng *et al.*, 2012
HPLC-UV	Cereal flours	MEL, AMD, AML, CYA	5–90	Multi-residue analysis; the formation of MEL-CYA was prevented by alkaline conditions	Ehling *et al.*, 2007
GC-MS/MS	Milk and milk products	MEL, AMD, AML, CYA	0.002	Multi-residue analysis	Miao *et al*., 2009
GC-MS	Milk products	MEL	0.0003	Zirconia hollow fiber sorptive microextration; simple and solvent free pretreatment	Li *et al.*, 2009
GC-MS	Liquid milk and milk powder	MEL	0.01	Coupled column separation; no derivatization step	Xu *et al*., 2009
GC-MS	Eggs	MEL	0.01	SPE pretreatment	Xia *et al.*, 2009

LC: liquid chromatography; MS: mass spectrometry; HPLC: high performance liquid chromatography; DAD: diode array detection; UV: ultraviolet; GC: gas chromatography; MEL: melamine: CYA: cyanuric acid; CYRO: cyromazine; AML: ammeline; AMD: ammelide; LOD: limit of detection; SPE: solid phase extraction; MCX: mixed-mode cation exchange sorbent for bases; RP: reverse phase; PCX: polymer based cation exchange cartridge; HILIC: hydrophilic interaction chromatography

3.2 Gas chromatographic (GC) methods

GC is another common confirmation method used to analyze melamine and related compounds, since the instruments are relatively cheap to maintain and use. However, since melamine is a strong polar substance, it usually needs an extra derivatization step with three methyl silanized reagents in a sample pretreatment phase.

In 2007, the FDA developed a GC-MS screening and confirmation method for the presence of melamine, ammeline, ammelide, and cyanuric acid (Smoker and Krynitsky, 2008). In 2008, the Chinese

government issued two national standards to determine melamine in raw milk, diary products (AQSIQ and SAC, 2008b), and plant products (AQSIQ and SAC, 2008a) by GC-MS and GC-MS/MS methods.

Miao *et al.* (2009) described a method to simultaneously determine the presence of melamine, ammelide, ammeline, and cyanuric acid in milk and milk products by GC-MS/MS. Xu *et al.* (2009) determined melamine in milk products by GC-MS with a coupled capillary column system with a limit detection of 0.01 mg/kg. In addition, hollow fiber sorptive extraction (HFSE) followed by GC-MS was developed for the determination of melamine residues from milk products (Li *et al.*, 2009).

4 Screening methods and other techniques

In most cases, the type of method selected will depend on cost, labor, and the available facilities. Alternatives to costly chromatographic methods include cheaper screening methods, which have sufficient sensitivity and selectivity for melamine and have also been developed. Immunological assays, capillary electrophoresis (CE), molecularly imprinted polymer (MIP), and some visual detection are the most popular screening methods for melamine. These methods are compared in Table 2.

4.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a good screening method used for large amounts of samples because of its simplicity and low cost. Liu *et al.* (2010) and Lei *et al.* (2010) separately reported the synthesis of different haptens and production of polyclonal antibodies to melamine, the half maximal inhibitory concentration (IC_{50}) values of the two types of antibodies are 13 and 70.6 ng/ml, respectively. Wang *et al.* (2011) developed a fluorescence polarization immunoassay (FPIA) for the determination of melamine in milk with the LOD of 9.3 ng/ml. In addition, commercial ELISA kits based on melamine antibodies are also available (Garber, 2008; Kim *et al.*, 2008). The sensitivities of many ELISA methods are equivalent or even superior to LC methods. However, most of the antibodies show cross reactivity to the structure of related compounds of melamine; therefore, the positive samples need be rechecked by a confirmation method.

Table 2 Some screening methods and other techniques for analyses of melamine and related compounds

Method	Matrix	Analyst	LOD (mg/kg)	Comments	Reference
FPIA	Milk	MEL	0.0093	Fluorescence polarization immunoassay	Wang *et al.*, 2011
ELISA	Animal muscle tissues	MEL, CYA	0.0018 (MEL) 0.0045 (CYA)	Hapten synthesis	Liu *et al*., 2010
ELISA	MEL standard	MEL	0.0026	Hapten synthesis	Lei *et al*., 2010
ELISA	Dog food	MEL	0.009	Commercial kit	Garber, 2008
MIPES	Milk	MEL	8.82×10^{-5}	High sensitivity	Li *et al.*, 2011
MIPCD	Liquid milk and milk powder	MEL	0.02	The 96-well micro-plate was modified with sol-gel film	Yu *et al*., 2009
CE-MS	Milk powder	MEL, AML, AMD, CYA	0.0022–0.0194	Mass spectrometric detection	Huang *et al.*, 2012
CE-UV	Infant formula	MEL	0.0005 and 0.0092	Two on-line preconcentration steps	Tsai *et al.*, 2009
CZE-DAD	Egg, dairy products and pet feed	MEL, AML, AMD, CYA	0.045–0.342	Without solid phase extraction step	Xia *et al*., 2010
Ag NP	MEL	Raw milk	0.292	Label-free silver nanoparticles as a probe	Ping *et al.*, 2012
Au NP	Milk	MEL	0.006	Based on the 18-crown-6 ether functionalized Au NP	Kuang *et al.*, 2011
Au NP	Milk	MEL	0.04	Colorimetric visual detection, label-free Au NP	Wei *et al.*, 2010
Microfluidic device	Milk products	MEL	0.23	No sample pretreatment, UV detection	Zhai *et al*., 2010

FPIA: fluorescence polarization immunoassay; ELISA: Enzyme-linked immunosorbent assay; MIPES: molecularly imprinted polymer-electrochemical sensor; MIPCD: molecularly imprinted polymer-chemiluminescence detection; CE: capillary electrophoresis; MS: mass spectrometry; UV: ultraviolet; CZE: capillary zone electrophoresis; DAD: diode array detection; NP: nanoparticle; MEL: melamine; CYA: cyanuric acid; AML: ammeline; AMD: ammelide; LOD: limit of detection

4.2 Molecularly imprinted polymer (MIP)

In recent years, MIP has been used to select melamine in the pretreatment step due to its molecular recognition functions. Yang H.H. *et al.* (2009) prepared MIPs with high affinity to melamine and used them to selectively extract melamine in dairy products and feed samples. Yu *et al.* (2009) also prepared a 96-well micro-plate modified with molecularly imprinted melamine sol-gel film, by which the highly selective and high throughput chemiluminescence detection of melamine was achieved. The linear range of detection for melamine was between 0.1–50 mg/kg, with a lower detection limit of 0.02 mg/kg.

4.3 Capillary electrophoresis (CE)

Melamine can also be separated using CE, which is an efficient and cost-effective analytical method for small molecules with many advantages, including minimum sample and reagent consumption, and fast separation speed. The pretreatment steps for melamine in milk and other matrices are quite simple since they do not require the SPE step after extraction. However, if the method was coupled with the commonly used UV detection, the detection sensitivity and reproducibility would become a problem (Tsai *et al*., 2009).

4.4 Visual detection

Melamine could decrease the stability of citrate-stabilized gold nanoparticles (Au NPs), thus causing dramatic and visible color changes and indicating the presence of melamine. Guo *et al.* (2010) and Wei *et al.* (2010) have reported using the label free Au NPs to detect melamine in milk with a simple sample preparation step. The aggregation of Au NPs could be visually detected after several seconds; however, many compounds could cause the aggregation of Au NPs. To increase the specificity of the method, Wei *et al.* (2010) introduced the cyanuric acid to the detection system and used the dual signal readout to determine melamine. In addition to Au NPs, silver nanoparticles (Ag NPs) could also be used for visual detection of melamine (Ping *et al.*, 2012). Although, the sensitivity and specificity of visual detection are not as precise as chromatographic methods, the results can be directly observed by the naked eye, therefore offering a simple and promising method to be used for on-site screening melamine contamination in milk products or at home diagnosis.

4.5 Other alternative methods

Some other methods by electrochemical, spectrophotometric, and nondestructive detection have also been used to develop assays or biosensors to melamine. Cao *et al.* (2009) reported a novel electrochemical method, which used interaction between $d(T)_{20}$ and melamine. This method was applied successfully to the determination of melamine in milk products with a recovery of 95%. A spectrophotometric method based on the Mannich reaction has been reported for melamine detection. The method was tested to determine the level of melamine in a contaminated fish sample with a recovery value and LOD of 97% and 0.063 μg/ml, respectively (Rima *et al.*, 2009). Mauer *et al*. (2009) also described a nondestructive and rapid method to detect melamine in infant formula powder using near- and mid-infrared spectroscopy with an LOD of 1 mg/kg with little to no sample preparation. Raman spectrometry is another rapid and cost effective method for the rapid screening of melamine with minimum sample preparation (Lin *et al*., 2008; Cheng *et al*., 2010).

5 Conclusions

Nowadays, there are a lot of methods available to detect melamine and its related compounds (Sun F.X. *et al*., 2010; Tittlemier, 2010). They included some highly selective confirmation methods and rapid screening methods. Currently, LC and GC conjugated with mass detection are the most powerful confirmation methods to analyze melamine and related compounds. However, these methods can be expensive and time-consuming, and often require sample pretreatment to extract analytes and eliminate matrix effects. Rapid screening methods like ELISA are faster and less expensive than the traditional instrumental methods, but the positive samples from these screening methods must be confirmed by selective methods, for their lack of selective ability of mass-based detections. Newer technologies such as biosensor and nondestructive methods are also becoming available, with sufficient sensitivity and specificity to enable rapid on-site screening with or

without simple pretreatment.

Even so, there are some common difficulties with melamine analysis, such as the matrix effects (Wu *et al.*, 2009), solubility of melamine-cyanuric acid complex (Filigenzi *et al.*, 2008), and background contamination, and some existing detection methods still need to be validated. The future work should focus on resolving these difficulties and establishing mobile, simple, and reliable screening methods, which could be used outside the laboratories and operated by someone who is not a technician (WHO, 2009a). Establishing protein detection methods, which are not affected by melamine and its metabolites, is another approach to prevent the risk of melamine to human (Field and Field, 2010).

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