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Application of Tandem Mass Spectrometry for Analyzing Melamine

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1. Introduction

Melamine (MEL; 1,3,5-triazine-2,4,6-triamine, CAS No. 108-78-1) is a heterocyclic triazine compound containing 67% nitrogen by weight (Figure 1). It is a polar compound and slightly soluble in water and ethanol. MEL forms synthetic resins with formaldehyde which are used in the fabrication of laminates, glues, adhesives, and surface coating resins (WHO, 2008). The pesticide cyromazine (CYRO) can degrade to form MEL (Chou et al., 2003).

In terms of toxicity, MEL is not metabolized and is rapidly eliminated in the urine. Animal studies have shown that the oral LD₅₀ in rats is 3,161 mg/kg body weight. High doses of MEL have an effect on the urinary bladder, in particular causing inflammation, the formation of bladder stones and crystals in urine. With regards to carcinogenicity, the International Agency for Research on Cancer (IARC) has concluded there is inadequate evidence for carcinogenicity in humans (WHO, 2008).

Fig. 1. Structure of melamine.

There are no approved uses for the direct addition of MEL to food or feed. However, in 2007, MEL was found in pet food causing the death of a large number of dogs and cats due to kidney failure. In September of 2008, MEL tainted milk resulted in nephrolithiasis and renal failure in infants in China. More than 50,000 infants and young children were hospitalized for urinary problems, possible renal tube blockages and possible kidney stones related to the consumption of melamine contaminated infant formula and related dairy

products. Six infant deaths were confirmed in mainland China (WHO, 2008). The adverse renal effects of melamine-tainted formula have also found in children in Taiwan, where cross-strait dairy trade with China had bloomed in recent years (Wang et al., 2009). The dietary exposure based on the consumption of MEL tainted infant formula in China at the median levels of MEL reported in the most contaminated brand was estimated to be 40-120 times the tolerable daily intake (TDI) of 0.2 mg/kg-body weight, explaining the dramatic health outcomes in Chinese infants (WHO, 2009).

Currently, the amount of protein in foodstuffs is measured indirectly using the Kjeldahl method, which is based only on nitrogen content in samples regardless of whether or not it is incorporated into protein. For example, the factor is 6.38 for milk, of which the protein content is around 16%. However, since MEL is intentionally added to foodstuffs and pet food in order to increase the nitrogen (protein) content, other factors clearly need to be considered for the calibration of protein content.

Many countries have reported finding MEL in milk containing products. Samples testing positive for MEL include biscuits, cakes and confectionery (0.6–945.86 mg/kg); liquid milk and yoghurt products (0.5–648 mg/kg); frozen desserts (39–60.8 mg/kg); powdered milk and cereal products (0.38–1143 mg/kg); processed foodstuffs (0.6–41 mg/kg); food-processing ingredients (1.5–6694 mg/kg); and animal feed (116.2–410 mg/kg) (Hilts & Pelletier, 2009).

2. Analytical method

Many methods have been developed to analyze MEL since the contamination of pet food in 2007 and milk in 2008, including screening and selective quantitative methods (Table 1). Screening methods have the advantages of simplicity, cost-effectiveness, time saving, and labor-saving, where selective methods provide much more information on identification with reliable, reproducible results. Current methods focus on MEL, cyanuric acid (CYA), and related compounds, such as ammelide (AMD) and ammeline (AME) content in food, feed and biological tissues or body fluids.

2.1 Screening and qualitative methods

In screening methods, commercial enzyme-linked immunosorbent assay (ELISA) has been used to detect MEL in dog food (Garber, 2008), infant formula (Garber & Brewer, 2010), muscles, the liver, kidney, plasma, and urine (Wang et al., 2010). Choi and Lee (2010) developed a competitive chemiluminescent enzyme immunoassay (CLEIA) with detection of 1,1'-oxalydimidazole (ODI) derivatives to analyze MEL in milk. This method is capable of rapidly quantifying and screening MEL.

Other studies have introduced alternative qualitative methods, such as surface-enhanced Raman spectroscopy (SERS), matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), and direct analysis in real time (DART) coupled with time of flight mass (TOFMS). SERS has been used to screen MEL in a number of food matrices including gluten, chicken feed, and processed foods (Lin et al., 2008). Vail et al., (2007) used DART ion source coupled with TOFMS to detect MEL in pet food. Dane and Cody (2010) also employed DART to selectively ionize MEL directly from powdered milk. To eliminate the interference of 5-hydroxymethylfurfural (5-HMF) produced during heating of the milk, TOFMS with a higher resolving power has been used to distinguish MEL (m/z 127.0732) and 5-HMF (m/z 127.0395) which have very close molecular weights. Tang et al., (2009) employed MALDI-

MS for direct analysis of biochemical crystals containing melamine/cyanuric acid complexes in urine samples. MEL can be desorbed/ionized upon N₂ laser irradiation by simply mixing the commonly adopted MALDI matrixes with melamine cyanurate in urine residue. The high-throughput MEL analysis method using low-temperature plasma (LTP) coupled with an ion trap mass spectrometer of bench top type (Huang et al., 2009) or handheld mass spectrometer (Huang et al., 2010) have also been introduced recently. The mechanism involves thermally-assisted (from 20 °C to 170 °C) vaporization and plasma ionization of MEL. The characteristics are direct sampling, no solvents used, air serving as the plasma support gas, low power consumption, small size and rapid analysis.

Screening and quantitative methods provide an effective alternative for the analysis of samples suspected of containing MEL without extensive sample preparation or expensive instrumentation. The cost, selectivity and sensitivity are low, but the speed is fast. However, positive results should be confirmed using a confirmatory method in most cases.

2.2 Selective quantitative methods

For selective methods, high performance liquid chromatography (HPLC) coupled with ultraviolet absorption (UV) and diode array detection (DAD) have been used to analyze food and feed. MEL has UV absorbance at approximately 240 nm. In addition to retention time confirmation, the spectrometric profile of MEL recorded between 200 and 400 nm has been used to confirm the identity of a selected peak. However, the detection limit is around the parts per million (ppm) level (Kim et al., 2008; Muniz-Valencia et al., 2008; Chou et al., 2003; Lutter et al., 2011; Venkatasami & Sowa, 2010).

Gas chromatography (GC), and HPLC coupled with single stage mass spectrometry (MS) or tandem mass spectrometry (MS/MS) are frequently used for the analysis of MEL and related compounds. In March 2007, the Forensic Chemistry Center was asked to assist in the investigation of pet foods responsible for the adverse effects in cats and dog. GC-MS methods to detect the presence of MEL and related compounds were developed for rapid screening. The selected ion monitoring mode (SIM) was used for quantification and full scan mode for further identification (Litzau et al., 2008).

LC-MS/MS has many applications in MEL analysis in food, feed and urine (Andersen et al., 2008; Heller & Nochetto, 2008; Somker & Krynitsky, 2008; Turnipseed, 2008; Cheng et al., 2009a; Ibáñez et al., 2009; Zhang et al., 2010; Lutter et al., 2011) LC is suitable for the separation of polar MEL, and double stage mass (MS/MS) analysis provides high selectivity and sensitivity, with a reported detection limit range from 0.01 to 0.1 µg/g.

3. Sample preparation and tandem mass spectrometry analysis

Biological samples, especially food and feed samples containing proteins, fats, mineral salts, carbohydrates and others are complex. The food constituents may result in contamination of the separation column and interface between inlet and detector. Co-elutents with MEL can result in severe interference during analysis, and matrix effects can affect the MEL recovery from a sample during preparation. For these reasons, sample preparation is performed in most studies before instrumental analysis, although some high-throughput methods using directly handheld mass spectrometers have also been introduced. This section firstly introduces extraction and clean-up procedures in sample preparation. The solvent used for extraction steps from food, which is the most complicated matrix, is discussed, as well as the ion-exchange cartridge used for clean up. Moreover, the raising the sensitivity and

	Matrices	Analytes	LOD (µg/g)	S/C	Ref.
LC-DAD	Wet pet food, animal feed	MEL, CYA, AML, AMD	1.2-113	C	Kim et al., 2008; Muñoz-Valencia et al., 2008
LC-UV	Poultry, eggs, milk, milk-based infant formula,	MEL	0.06-0.36	C	Chou et al., 2003; Lutter et al., 2011; Venkatasami & Sowa Jr, 2010
LC-MS/MS	Catfish, trout, tilapia, salmon, shrimp, infant formula, ,milk-based products, beverage products, animal feed	MEL, CYA	0.01-0.1	C	Andersen et al., 2008; Heller & Nochetto, 2008; Smoker & Krynitsky, 2008; Turnipseed, 2008; Ibáñez et al., 2009; Lutter et al., 2011
GC-MS	milk, milk products, milk-based powdered infant formula	MEL, CYA, AML, AMD	0.002-0.009	C	Lutter et al., 2011; Miao et al, 2009.
GC-MS	Wheat gluten, rice protein, wet pet food, corn gluten, soybean meal, dry cat food, salmon favor	MEL, AML, AMD, CYA	10	S,C	Litzau et al., 2008
ELISA	Infant formula, wheat food products, dog food	MEL	1-2.5	S	Garber & Brewer, 2010; Garber, 2008
ODI	Milk	MEL	1.12 ppb	S	Choi & Lee, 2010
CLEIA					
DAPCI-MS	Milk products	MEL	1.6X10 ⁻¹¹ g/mm ²	S	Yang et al., 2009
SERS	Gluten, chicken feed, processed foods	MEL	0.05-0.1 %	S	Lin et al., 2008
DART-TOFMS	Powdered milk	MEL	-	S	Dane & Cody, 2010
UPLC-MS/MS	Urine	MEL	0.01	C	Cheng et al., 2009a
LC-MS/MS	Urine	MEL, CYA	0.01	C	Zhang et al., 2010
LTP-MS/MS	Urine	MEL	0.01	S	Huang et al., 2009
MALDI-MS	Urine	MEL cyanurate	1.25	S	Tang et al., 2009
ELISA	Tissue, body fluid samples (urine)	MEL	0.05	S	Wang et al., 2010

LOD: Limits of detection; MEL: Melamine; CYA: Cyanuric acid; CYRO: Cyromazine; AMD: Ammelide; AME: Ammeline.
S: Screening method; C: Confirmation method.
DAPCI: Desorption atmospheric pressure chemical ionization; SERS: Surface enhanced Raman spectroscopy; DART: Direct analysis in real time; LPT: Low-temperature plasma; ELISA: Enzyme-linked immunosorbent assay; ODI: 1,1'-oxalydimidazole; CLEIA: Chemiluminescent enzyme immunoassay;

Table 1. Comparison of the methods used to analyze melamine in food, feed and urine.

strengthening the resolution power by high pressure liquid chromatography is also addressed. Secondly, the selective analytical method of tandem mass spectrometry is the most important analytical tool to trace contaminants in a biological matrix. In this section, the principle of the tandem mass spectrometric technique, including ionization, mass

analysis, fragmentation, and quantification, are also illustrated, along with detailed information on the advantages and drawbacks of tandem mass spectrometry.

3.1 Sample preparation

To analyze MEL, two steps are performed in most studies, including extraction and clean up in LC techniques. Derivation of the extracted MEL is necessary in GC techniques to improve the sensitivity. Sample preparation has positive effects on the instrumental analysis, and especially limits of detection (LOD).

Because MEL is a slightly polar molecule, 50% acetonitrile (Cheng et al., 2009a; Fujita et al., 2009; Gosciny et al., 2011; Mondal et al., 2010; Smoker & Krynitsky, 2008), 1-5% trichloroacetic acid (Juan et al., 2009; Xia et al., 2009), and 2.5% formic acid (Turnipseed et al., 2008) are used for extraction. Acetonitrile is the most frequently used organic solvent in reversed-phase liquid chromatography. Acetonitrile has the characteristics of miscibility with water, low viscosity and results in protein denaturation which is useful in food or feed preparation to eliminate protein in the sample solution. Some studies have also reported using 30-50% methanol to reduce the amount of acetonitrile combined with sonication or two step centrifugation to extract the MEL in liquid formula, dry powder or pet foods (Tran et al., 2010; Vendatasami & Sowa Jr 2010). In addition, the non-polar solvents dichloromethane and hexane are used in lipid-lipid extraction to reduce lipid effects in high-fat bio-samples, such as animal-derived food or bakery goods. (Deng et al., 2010; Gosciny et al., 2011, Zhu et al., 2009). To enhance the protonation of MEL before it is applied to cation solid phase extraction (SPE), hydrochloric acid, formic acid, and acetic acid are added in most cases. Acid also can denature protein and reduce interference.

For urine samples, solvent extraction is generally omitted because the level of proteins in urine is relatively low. Nevertheless, a clean up procedure is still necessary for the isolation and concentration of MEL before analysis. Hydrochloric acid is added to make sure MEL is protonated before SPE clean up. To eliminate renal stones in urine, especially for nephrolithiasis cases, centrifugation is performed at around 3,000 g for 10 minutes at 5 °C (Cheng et al., 2009a).

Aside from the solvent extraction, cation exchange SPE such as Oasis® MCX SPE cartridge (Waters Corp., Malford, MA, USA), Bond Elut®-SCX (Agilent Technologies, Inc. Santa Clara, CA, USA) is used to clean up extracts (Fujita et al., 2009; Smoker & Krynitsky, 2008; Cheng et al., 2009a) from food, feed, or urine samples. The Oasis® MCX cartridge contains a strong sulfonic (HSO_3) group bonded onto a poly(divinylbenzene-coN-polyvinylpyrrolidone) copolymer to extract the basic compounds with cation-exchange groups. Bond Elut-SCX contains a benzene ring in the functional group. The non-polar characteristic becomes particularly important when conducting ion-exchange from aqueous systems, where selectivity towards compounds exhibiting cationic and non-polar character is seen.

To shorten the preparation time, several improvements have been developed. MALDI-MS has been applied to the direct analysis of melamine cyanurate and MEL in urine (Tang et al., 2009). A low temperature plasma ambient ionization source, coupled to a portable mass spectrometer (Mini 10.5), has been used to determine MEL contamination in whole milk and related materials, such as synthetic urine and fish meat (Huang et al., 2010). However, the urine matrix results in interfering ion peaks and suppresses the ion intensity of MEL, while a clean up process consisting of simply washing with water eliminates such interference and enhances the ion intensity (Tang et al., 2009).

3.2 Chromatographic and mass analysis (ionization, fragmentation and mass analysis)

Mass spectrometry is a sensitive analytical technique that has been used extensively in chemical, biological, and environmental applications. Since public concern about food safety is growing, mass spectrometry is nowadays regarded as the most important and novel technique to detect trace residues or contaminants in food and the environment, such as pesticides in vegetables, veterinary drugs in animal derived foods, adulteration (e.g. MEL in pet food and milk), and environmental or processed food contamination (e.g. dioxins in fatty foods, acrylamide in starchy foods, 3-MCPD in soy sauce etc.) (Cheng et al., 2004; 2009b).

The basic structure of a mass spectrometer consists of an ionization source, mass analyzer(s) and detector. Inlets separate the desired compounds to deliver the analyte to the ion source. The devices should be chromatographs, such as GC, LC, HPLC, UPLC or CE. A direct probe is also an alternatively method to provide the samples in certain circumstances.

HPLC is the most frequently used inlet device to separate MEL from other compounds. However, MEL is not easily retained in a reverse phase chromatographic column. Mostly, zwitterionic HILIC (hydrophilic interaction chromatography) columns are chosen because of the high polarity and hydrophilic properties of MEL (Cheng et al., 2009a; Heller & Nochetto, 2008; Goscinny et al., 2011; Smoker & Krynitsky, 2008; Turnipseed et al., 2008; Xia et al., 2010; Zhou et al., 2009). HILIC is a variation of normal-phase chromatography where the stationary phase is polar material, such as silica, cyano, amino, or diol. Retention times are prolonged for polar compounds and sensitivity is enhanced in mass spectrometry. Other methods use reversed-phase C18 or C8 columns (Lin et al., 2008; Venkatasami & Sowa Jr, 2010; Ibáñez et al., 2009; Lutter et al., 2011). Recently, UPLC has been introduced in this field. The particle size in the column in UPLC is 1.7 μm , which can be used under high pressure and increases the sensitivity to MEL (Cheng et al., 2009a).

GC is used in the separation step; however, derivatization is needed to improve the sensitivity for involatile compounds such as MEL (Zhu et al., 2009; Miao et al., 2009). However, to reduce solvent waste, simplify sample preparation and avoid the need for hazardous reagents, a simple and reliable injection-port derivatization method coupled with GC-furan-CI-MS/MS has been developed to determine MEL and cyanuric acid in powdered milk samples (Tzing & Ding, 2010). Recently, direct determination (no derivation) of MEL in dairy products by GC-MS has been developed with coupled column separation. Thirty meters of DB-5ms ((5%-phenyl)-methylpolysioxane, 0.25 mm i.d., 0.25 μm df) coupled with 1.5 m of Innowax (polyethylene glycol, 0.32 mm i.d., 0.25 μm df) by a quartz capillary column connector has been introduced as separation column (Xu et al., 2009).

During ionization, samples migrate from the inlet system into the ionization chamber. A variety of ionization techniques are used for mass spectrometry, such as atmospheric pressure chemical ionization (APCI), chemical ionization (CI), electron ionization (EI), electrospray ionization (ESI), and MALDI. The ionization methods used for the majority of MEL analyses are EI and ESI.

EI is suitable for gas-phase ionization, although its use is limited to compounds that are sufficiently volatile and thermally stable. Samples are usually heated to increase the vapor pressure for analysis. The EI source consists of a heated filament giving off electrons. EI is gained by bombardment with electrons at 70 electron volts of energy, with the energy being transferred to the sample molecule leading to the ejection of an electron to form a molecular ion with a positive charge. The excess energy absorbed by the molecular ion will

continuously lead to extensive fragmentation to produce characteristic ions. If the molecular ion is unstable, nearly all of the molecular ions will decompose into fragment ions; if not, an intense molecular ion will be recorded. The characteristic ions of MEL derivatives (derived with *N, O*-bis(trimethylsilyl)trifluoroacetamide)) are m/z 327, 171, 99, and the molecular ion m/z 329. The most abundant ion is m/z 327, and it is considered to be the quantifying ion (Zhu et al., 2009). The characteristic ions for MEL alone are found at m/z 126, 85, 68, and 43. The molecular ion m/z 126 is most abundant and used as the quantitative ion. Because the full scan spectrum of EI is quite specific, single-stage mass analysis is normally enough to elucidate results. However, the selectivity is less than that for MS/MS methods. In MS/MS experiments, a first analyzer is used to isolate a precursor ion, which then undergoes spontaneous (or by some activation) fragmentation to yield product ions and neutral fragments.

For compounds that are thermally labile or are not volatile, ions of these compound must be extracted from the condensed to the gas phase. ESI and APCI are two examples of atmospheric pressure ionization sources. Such sources ionize the sample at atmospheric pressure and then transfer the ions into the mass spectrometer (De Hoffmann & Stroobant, 2001).

The molecular weight of MEL is low and not easily analyzed by GC-MS directly. Moreover, matrix interference will mask the signal unless there is a clear extract. However, fragmentation of MEL is sufficient for it to be detected using LC-MS/MS by collision-induced dissociation (CID). MEL has been analyzed using ESI in positive ion (ESI+) mode. The protonated molecular ion of MEL in ESI+ mode is m/z 127. The fragments of m/z 127 are m/z 127>85 ($[M+H-CH_2N_2]^+$) and m/z 127>68 ($[M+H-CH_5N_3]^+$), where m/z 85 is the dominant ion, as shown in Figure 2 in product ion scan mode.

Fig. 2. Mass spectrum of melamine (Cheng et al., 2009a).

4. Validation of analytical methods

To establish an accurate and precise method, the process of validation is necessary before an analytical method is adapted in routine practice. Generally, there are four validation steps; qualitative analysis, quantitative analysis, recoveries test, and detection limit evaluation. After the method has been developed, inter-laboratory or international precision test results are also applied for verification.

4.1 Qualitative analysis

Retention time is the major index used to identify analytes in chromatography coupled with a UV detector. However, the selectivity is low because of interference of the same retention

time, and even a specific wavelength that are selected for some compounds. Mass spectrometry is more selective, where not only the retention time, but also the mass spectrum can provide information on the chemical structure for identification. The four main scan modes which are available using MS/MS include product ion scan, precursor ion scan, neutral loss scan, and selected reaction monitoring. For tandem spectrometry, the mass fragments of the analytes offer the most important information for qualitative evaluation. However, the criteria of the precursor ion and product ion should be carefully considered. For example, the multiple reaction monitoring (MRM) mode is used during LC-MS/MS for MEL, with the characteristic fragmentation transition of m/z 127>85 for quantitative analysis and m/z 127>68 for confirmatory analysis. The ion ratio of transition ions m/z 127>68 and 127>85 is calculated for confirmation. For mass spectrometry techniques, the confirmation criteria applied are according to Commission Decision 2002/657/EC. Retention time, two transition reactions, and the peak area ratio from the different transition reactions are evaluated to identify the target compounds. Table 2 shows the maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques.

Relative intensity (% of base peak)	EI-GC-MS (relative)	CI-GC-MS, GC-MS ^a LC-MS, LC-MS ^a (relative)
> 50 %	± 10 %	± 20 %
> 20 % to 50 %	± 15 %	± 25 %
> 10 % to 20 %	± 20 %	± 30 %
≤ 10 %	± 50 %	± 50 %

Table 2. Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques (2002/657/EC).

4.2 Quantitative analysis

Because of the analytes losing in sample preparation and the presence of matrix effects, external standard method, matrix-matched external standard method or internal standard methods are used to correct analytical differences during analysis. Matrix effects, especially are relatively significant in LC-MS/MS analysis where the ionization of analytes may be enhanced or suppressed. Matrix effects are expressed as the ratio of the mean peak area of analytes between the post-extraction fortification and solvent standards (Heller & Nochetto, 2008; Xia et al., 2010). Matrix effects are more variable among different feeds with the most pronounced suppression of CYA in wheat gluten; of MEL in pelletized hog feed and fish feed respectively (Heller & Nochetto, 2008). Xia et al., (2010) reported that the matrix effects are in general around 10% for MEL, AML, AME and CYA in several matrices including cat feed, dog feed, egg, milk, yoghurt, ice cream and milk powder. Nevertheless, matrix-matched external standard or internal standards can be used to improve the accuracy and precision of the data. The application of isotope-labeled internal standards is the most ideal choice for MS based methods because their physical and chemical properties are close to the analytes. Generally, the internal standards are added prior to sample preparation to account

for the loss of analytes during the sample preparation process, such as extraction, clean up, or concentration and for the ionization step in LC-MS/MS. Stable isotope-labeled internal standards are currently used for MEL analysis including $^{13}\text{C}_3^{15}\text{N}$ -MEL, $^{13}\text{C}_3$ -MEL and $^{15}\text{N}_3$ -MEL (Jaco et al., 2011; Deng et al., 2010; Ding et al., 2009; Smoker & Krynitsky, 2008; Cheng et al., 2009a; Goscinny et al., 2011). For example, the internal standard $^{13}\text{C}_3\text{N}_3(^{15}\text{NH}_2)_3$ gives a protonated molecular ion of m/z 133, where m/z 133>89 and m/z 133>71 are the predominant fragments by CID during MS/MS experiments. The m/z 89 product ion is monitored for routine analysis. However, the isotopic internal standards are not economic and only can be used in MS spectrometry. Some of matrix matched standards or internal standards are alternatively used, such as resorcline in HPLC-DAD methods (Ehling et al., 2007; Muñiz-Valencia et al., 2008).

4.3 Recoveries test

In the recoveries test, samples are fortified with standard (or target) compounds at different concentrations, and then undergo preparation, clean up procedures and finally instrumental analysis. The calculated recoveries show the performance of the developed method and whether it is feasible for routine use. As shown in Table 3, yellow fish and infant formula were fortified with 50 or 200 $\mu\text{g/kg}$ of MEL, followed by the application of internal standards, homogenization, extraction, clean up procedure and finally injection into the instrument, in this example UPLC-MS/MS (Cheng et al., 2009c). The results showed that the recovery was quite good in the range of 101 to 107%, and the coefficient of variation (CV) was also lower than 5%.

Sample blank (MEL, $\mu\text{g/kg}$)	MEL added ($\mu\text{g/kg}$)	MEL measured ($\mu\text{g/kg}$ wet weight)	Recovery (%)	CV (%)
Yellow fish (ND)	50	52,56,53,52	107 ± 4	4
	200	208, 200, 200, 201	101 ± 2	2
Infant formula (ND)	200	211, 209, 214, 201, 204, 199	103 ± 3	3

ND: None detected

Table 3. Recovery test of the UPLC-MS/MS method (Cheng et al., 2009c).

According to the Commission Decision 2002/657/EC, in cases of repeated analysis of a sample carried out under within-laboratory reproducible conditions, the intra-laboratory coefficient of variation of the mean should not exceed the values shown in Table 4. The CV results fell in the range of 15% showing excellent reproducibility in this study.

Mass fraction	CV (%)
$\geq 10 \text{ } \mu\text{g/kg}$ to $100 \text{ } \mu\text{g/kg}$	20
$> 100 \text{ } \mu\text{g/kg}$ to $1\,000 \text{ } \mu\text{g/kg}$	15
$\geq 1\,000 \text{ } \mu\text{g/kg}$	10

Table 4. CVs for quantitative methods by element mass fractions (2002/657/EC).

4.4 Detection limit

The detection limit is the smallest sample quantity that yields a signal that can be distinguished from the background noise (generally a signal equal to ten times the background noise) (Hoffmann & Stroobant, 2001). In method detection limit (MDL) evaluation, the spiked (signal-to-noise, S/N ratio) and statistic method are usually used. The limit of quantification (LOQ) suggested by the Food and Drug Administration (FDA) in food is the level at which a 10:1 peak to peak S/N ratio is observed for the analyte quantification ion transition, and a 3:1 peak-to-peak S/N ratio observed for the analyte secondary ion transition. In the statistic method, matrices fortified with MEL are used to evaluate the MDL by using Students' values at the 99% confidence level for 6 degrees of freedom ($MDL=t(6,0.99) \times SD$) as shown in Table 5 (Cheng et al., 2009a). In this example, the MDL was 0.006 µg/mL in the sample. The calculated MDL concentration of MEL can be further fortified into the matrix and analyzed again to guarantee that the statistical results are feasible.

Test number	MEL* measured (µg/mL)
1	0.051
2	0.056
3	0.050
4	0.050
5	0.051
6	0.053
7	0.053
Mean ± SD	0.052 ± 0.02
CV%	4
MDL (3 × SD)**	0.006

* MEL spiked (50 ng/mL) urine
** $MDL = t_{(n-1, 1-\alpha = 0.99)} \times SD$, t: Student's value, SD: Standard deviation

Table 5. The method detection limit of UPLC-MS/MS methods (n=7) (Cheng et al., 2009a).

4.5 Performance test

Precision evaluation is measured as repeatability (intra-day) and reproducibility (inter-day) of samples as demonstrated by injection (in triplicate) of the sample on the initial day and three consecutive days (Venkatasami & Sowa, 2010). Moreover, participation in proficiency tests internationally or nationally can show the performance of the method. For example, the Food Analysis Performance Assessment Scheme (FAPAS) conducted by the Central Science Laboratory in the UK ran several tests involving MEL in pet food in 2008, chocolate in 2009/10, and they aim to test milk powder in 2012 (FAPAS, 2011). The European Commission's Joint Research Centre, and Institute for Reference Materials and Measure under a request by the European Commission's Directorate-General for Health and Consumer Protection also conducted melamine proficiency testing in 2009 (Breidbach et al., 2009). Laboratories from 31 countries including Taiwan and 21 of the 27 member states of the European Union participated. The purpose was to assess the capabilities of control laboratories to measure MEL in food samples, including skimmed milk powder and starch-containing foods. The method we developed during the MEL food safety scare in 2008 using UPLC-MS/MS was also applied to analyze

the MEL content in these two samples (Cheng et al., 2009d). The method was found to have good precision and accuracy as shown in Table 6. The SD and CV were quite low, and the Z-score fell between +/- 2. The report compared the methods used in the tests and concluded that isotope dilution mass spectrometry with stable-isotope labeled MEL was clearly advantageous with regards to the accuracy of the results (Breidbach et al., 2009).

	Material A Milk powder	Material B Baking mix
Mean (mg/kg) ± uncertainty	9.2 ± 0.40	3.0 ± 0.13
SD	0.1	0.02
CV	1.3	0.7
Z-score	-0.7	-0.4
Theoretical value (mg/kg) ± uncertainty	10.6 ± 0.6	3.18 ± 0.17

Table 6. Results obtained in the EU Melamine proficiency test 2009 (Cheng et al., 2009d).

5. Other applications of tandem mass spectrometry

Tandem mass spectrometry is frequently used in industry and in academic research. It has applied to identify protein, peptides, oligonucleotides in biotechnology, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and endocrine disrupting chemicals in the environment and food. After 2008 MEL tainted milk event, the tandem mass spectrometry becomes more and more important in food safety. Tandem spectrometry coupled with gas or liquid chromatography has been applied extensively in the determination of residues or contaminants, and even unknown contaminants in food. Moreover, it can be performed to measure biologically active compounds and their metabolites in the body fluid for clinical applications. The development of tandem mass spectrometry newborn screening led to a large expansion of potentially detectable congenital metabolic diseases that affect blood levels of organic acids (Chace et al., 2003; Wang et al., 2003). We have checked cord blood cotinine by HPLC-MS/MS for objectively measuring the prenatal tobacco smoke exposure (Wang et al., 2008). We have also checked cord blood perfluorinated compounds by UPLC-MS/MS and correlated its concentration with atopic disorders (Wang et al., 2011). The future of the analytical chemistry and food safety is closely associated with the development of mass spectrometry.

6. Summary

The MEL tainted milk event in 2008 raised public concerns about food safety. Because food is increasingly transported worldwide, governments are now paying more and more attention to safeguarding food products, and many novel methods have been introduced for this purpose. A lot of researchers are working on developing screening methods and selective methods to detect contaminants. Both methods have applications in different respects. To achieve reliable results, most studies have used selective methods to analyze MEL. Because of the chemical properties of MEL, organic solvents such as acetonitrile or methanol are used to extract it from food, feed or biological matrices followed by cleaning

up of SPE in the preparation steps. Gas or liquid chromatography are responsible for the separation of analytes from interference before the analytes enter the detection areas. To answer the demand for high selectivity, mass spectrometry has become the most popular method used to identify MEL and related compounds. The ionization process can charge molecules by using EI, ESI, CI, APCI, or MALDI. Either single stage mass or tandem mass are used in spectrometry. The application of tandem mass can increase the selectivity and raise the S/N , resulting in low LOD and LOQ. HPLC coupled with tandem mass has become the major analytical method in the analysis of MEL and related compounds. Before applying the method as a routine practice, researchers should perform a series of validation evaluations, including qualification, quantification, recoveries, reproducibility, and detection tests. International or inter-laboratory precision tests will provide confidence in the ability of the analytical method. As the technique of tandem mass spectrometry progresses, we can apply it in various biological and clinical fields. It is a small step for the technique, but a giant leap for the science. We look ahead to these and other emerging applications as the benefits of this technology become incorporated into current and future human health.

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