Rapid detection of melamine in untreated milk and wheat gluten by ultrasound-assisted extractive electrospray ionization mass spectrometry (EESI-MS)†

Liang Zhu,[a] Gerardo Gamez,[a] Huanwen Chen,*[b] Konstantin Chingin[a] and Renato Zenobi*[a]

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A novel method based on ultrasound-assisted EESI-MS has been developed and applied to rapidly detect the presence of melamine in raw milk, wheat gluten and milk powder with no or minor sample pre-treatment; the high sample throughput and figures of merit make it specially useful for screening melamine levels well below the current safety limit in various food matrices.

Melamine is a chemical compound commonly used as a fire retardant, and a component of fertilizer and plastic resin. Melamine can be found at ppm levels in food and beverages due to migration from melamine-containing resins,[1] or as a metabolite product of cyromazine, an insecticide used on animals and crops,[2] but these levels do not pose any danger to humans. Recently, intentional adulteration by a high melamine level has been discovered in pet food, wheat gluten, infant formula and various milk products.[3] Ingestion of melamine at levels above the safety limit (2.5 ppm in the USA and the EU; 1 ppm for infant formula in China) can induce renal failure and even death in pets and humans, as in the scandal involving melamine tainted infant formula in China in September 2008, which caused the death of several children.[3] These adulterated food products can contain melamine in concentrations as high as ~3300 ppm,[3] posing extreme danger to consumers. It has been reported that the combination of melamine at very high doses with another triazine, cyanuric acid, leads to the formation of insoluble crystals in the kidneys, causing renal failure in animals and humans.[4]

An interim method to screen for the presence of melamine in pet food by gas chromatography/mass spectrometry (GC/MS) has been published by the US Food and Drug Administration (FDA).[5] Moreover, several other methods including gas chromatography,[6] liquid chromatography/mass spectrometry (LC/MS),[7] capillary electrophoresis,[8] matrix-assisted laser desorption ionization (MALDI) MS[9] and enzyme-linked immunosorbent assay (ELISA)[10] have been developed for the detection of melamine in various matrices. Due to the complicated matrix, extensive sample pre-treatment, including extraction, pre-concentration, and derivatization, *etc*., which can take tens of minutes or even hours is usually required.

To rapidly screen for the presence of melamine in large batches of food products (milk, wheat gluten, *etc*.), high throughput analytical techniques are required. Neutral analytes in the gas phase, in solution, in the form of aerosols, or desorbed from a surface can be rapidly and directly detected by extractive electrospray ionization (EESI) MS,[11–17] without any sample pre-treatment. Liquid–liquid EESI has been successfully applied for continuous analysis of trace amounts of compounds directly in complex matrices.[11] However, for simplicity of sample manipulation and thus, for high throughput measurements of milk samples, rapid analysis of a single sample aliquot using a novel ultrasound-assisted EESI method is applied in this study. An ultrasonic transducer was directly utilized to nebulize a tiny droplet (~3 μL) of a liquid sample deposited on it. The dispersed neutral droplets then undergo extractive processes by contact with a conventional ESI plume and are ionized for subsequent MS measurement. The features of this new approach are that it consumes only small amounts of sample, can analyze liquids directly, and is also able to detect trace amount compounds present inside the liquid droplet, even in the case of complicated biological matrices. Most importantly, its application to rapid detection of melamine matches the urgent need for the tainted milk product issue. Other ambient pressure ionization techniques such as desorption electrospray ionization (DESI),[18] desorption atmospheric pressure chemical ionization (DAPCI),[19] electrospray-assisted laser desorption ionization (ELDI),[20] and atmospheric pressure glow discharge (APGD)[21] could also be suitable for this application.

In the optimized EESI source, the electrospray tip was placed at a distance of ~18 mm (*b*) from the cone inlet of the mass spectrometer, at a 140° angle (β) from the axis of the sampling cone (shown in Fig. 1). The ultrasonic transducer, which was taken from a commercially available ultrasonic nebulizer (U6000AT+, CETAC Technologies), was located about 5 mm (distance *a*) below the electrospray capillary. A 3 μL aliquot for analysis was deposited by a pipette tip (Gibson, France) onto the transducer surface, approximately in the middle between the cone inlet orifice and the ESI capillary end. During a single measurement, the transducer was switched on for about 1 s to avoid possible influences from thermal effects. After each measurement the transducer surface was thoroughly cleaned with Kimtech wipes (Kimberly-Clark, UK) wetted with methanol to avoid sample carry-over. There is almost no memory effect for this experimental setup, except in the case of highly volatile liquids which can remain in the surrounding air for a while. Further details regarding the experimental setup are included in the supporting materials.†

A stock solution of milk (different fat contents) spiked with melamine was prepared by dissolving 3 mg of melamine into

[a] *Department of Chemistry and Applied Bioscience, ETH Zurich, Switzerland. E-mail: zenobi@org.chem.ethz.ch; Fax: +41-44-632-1292; Tel: +41-44-632 4376*
[b] *Applied Chemistry Department, East China Institute of Technology, Fuzhou, China. E-mail: chw8868@gmail.com; Fax: +86-794-8258-320*

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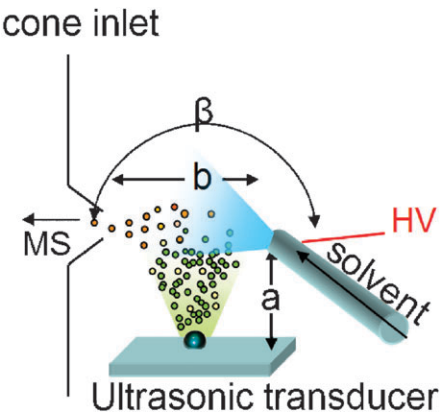

Fig. 1 Schematic view of setup: a tiny aliquot (~3 μL) of liquid sample was ultrasonically desorbed from the transducer surface.

6 mL raw milk (500 ppm). The stock solution was diluted 1 : 5 times, 1 : 50 times, and 1 : 500 times with pure milk to obtain samples spiked with 100, 10 and 1 ppm melamine, respectively.

A key point of the ultrasonic nebulization of the liquid aliquot is the high frequency (greater than MHz) oscillation able to break it up into μm sized droplets. This nebulization efficiency is much better than that of pneumatic nebulizers.[12] Compared to an application in which a commercially available ultrasonic nebulizer was utilized,[22] carry-over and thermal effects induced by long, continuous runs are eliminated in our setup, accompanied by much lower liquid sample consumption and the capability to carry out high throughput measurements.

To show the performance of this setup, a 3 μL aliquot of undiluted milk spiked with 100 ppm melamine (for details see supporting materials†) was analyzed and the corresponding mass spectrum, after background subtraction, is shown in Fig. 2a. Many different peaks appear. Protonated melamine was observed well above the noise level at m/z 127; its peak was distinctly separated from other signals originating from the milk. In addition, m/z 343 could be identified as lactose which is present as ~3% in cows' milk (supporting materials†). We did not attempt to identify other peaks, since the purpose of this study was the detection of melamine. The inset in Fig. 2a is the MS/MS spectrum of the protonated melamine, with m/z 85 representing the loss of a molecule of cyanamide, $N{\equiv}C{-}NH_2$, and the peak at 110 could be produced by loss of one NH_3 group from the molecular ion.[23] The ion at m/z 68 is $C_2N_3H_2^+$.[23] Fig. 2b is the mass spectrum of the same tainted milk sample, but with the instrumental settings optimized for the higher m/z range. We detect a broad ion distribution over a mass range of 900 to 1600, with the main peaks at m/z 1096 and m/z 1048. In the deconvoluted mass spectrum (Fig. 2b, inset), the peak at m/z 24 092 could be identified as β-casein, which is ~33% of the protein content in cows' milk. Minor peaks in Fig. 2b could be assigned to other molecules of the casein family. The charge state distribution of casein ions was from 15+ to 26+. The ability to detect high mass proteins opens up possibilities of this technique for detection of native biological samples in the liquid phase without drying.

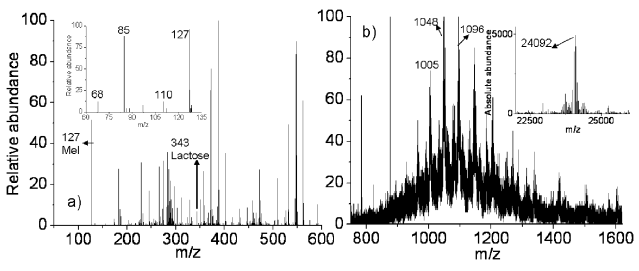

Fig. 2 (a) Mass spectrum of undiluted milk spiked with 100 ppm melamine. Protonated melamine was clearly observed at m/z 127, and its MS/MS spectrum is shown in the inset. The ion at m/z 343 could be identified as lactose. (b) Higher m/z range of the same milk, with a deconvoluted spectrum shown in the inset.

Pure milk spiked with melamine at different concentrations (500, 100, 10 and 1 ppm) was prepared by mixing different amounts of pure milk with a melamine stock solution. First, one 3 μL droplet of unspiked milk was deposited on the transducer surface by a pipette tip as a control. No distinguishable signal at m/z 127 was detected. Individual analyses from the four spiked milk samples were then performed in series. The melamine signal was monitored by following the m/z 127 signal in a single ion trace, as shown in Fig. 3a. The m/z 343 ion was also followed (lower trace). It took only *ca.* 2 min to run four measurements, including the time to deposit the sample and clean the transducer surface after each measurement. The inset in Fig. 3a shows the dependence of the melamine signal on the melamine concentration in the milk. The response exhibited a linear relationship, with a linearity coefficient of $R = 0.9998$, covering 3 orders of magnitude. A limit of detection (LOD) of 500 ppb (μg L^{-1}) was derived for a signal-to-noise (S/N) ratio of 3. For 1 ppm melamine in milk, the S/N is around 7. The LOD achieved here is well below the safety limit and sufficient for rapid screening of milk samples in a quick and simple manner. The sensitivity of melamine detection can be improved by diluting the milk with pure water instead of milk. It is also worth noting that the response for the same melamine concentration (10 ppm) in milk with different fat contents (0.1, 1.5 and 3.5%) was found to be similar (data not shown).

In the case of polluted wheat gluten, although very fine powders could in principle be nebulized directly from the transducer surface for subsequent mass measurement, the poor efficiency of powder desorption makes a simple melamine extraction step necessary. Following a simplified version of the FDA recipe,[5] methanol was chosen as the best solvent for

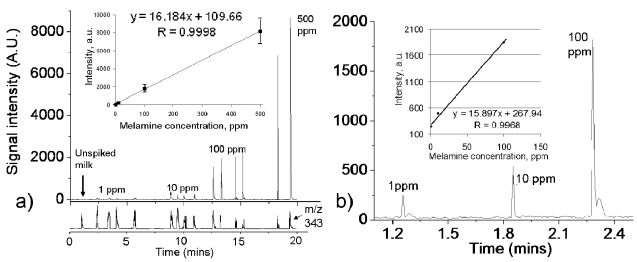

Fig. 3 (a) Single ion current (SIC) trace of m/z 127 and m/z 343 (bottom) from pure milk spiked with melamine at different concentrations (1, 10, 100 and 500 ppm). (b) SIC of m/z 127 during measurements of extracts of gluten containing melamine at 1, 10 and 100 ppm levels, respectively. Inset shows the dependence of the melamine signal on the melamine concentration in these extracts.

the extraction procedure and subsequent mass measurements (as shown in supporting materials†). There were some precipitates and floating particulates inside these extracts, but it was observed that these particulates did not affect the performance of the measurements. Thus, no filtration was performed. A melamine signal is clearly seen in the mass spectrum taken of a methanol extract from one portion of tainted wheat gluten (data not shown). Vanillin (m/z 153) can also be observed since the wheat gluten has a vanilla flavour. Taking everything into account, the time from opening the bag of wheat gluten to completing the corresponding analysis was less than 5 min. Methanol solutions (1, 10 and 100 ppm of melamine) were prepared following the procedure described in the supporting materials,† and then measured in sequence. Fig. 3b shows the SIC of m/z 127. The inset in Fig. 3b shows the dependence of the melamine signal on the melamine concentration in these extracts. The response exhibited a linear relationship, with a linearity coefficient of $R = 0.9968$, covering a dynamic range of 2 orders of magnitude. The data suggest a detection limit of around 200 ppb (μg kg^{-1}) with a signal-to-noise ratio of 3, while the S/N is around 15 for 1 ppm melamine methanol extracts. Also, measurement of tainted milk powder has been performed using the same procedure as that for wheat gluten. The experimental results show a LOD of 270 ppb (μg kg^{-1}) for melamine in milk powder (shown in supporting materials†). It is also worth noting that our extraction procedure is a simplified version of the FDA procedure, for the sake of time. Hence, the recovery might be lower than the value given by the FDA ($\sim$100%),[5] which may represent one possibility to improve the LOD for melamine detection in wheat gluten and milk powder.

Milk is an emulsion of fat globules and of casein micelles, all suspended in an aqueous phase which contains solubilized lactose, whey proteins, and some minerals. The casein micelles enclose water and other compounds; melamine, which is rich in amine groups, tends to form complexes with proteins. Due to intermolecular interactions, protein aggregation can occur at high concentrations. Such high molecular weight complexes, which may include bound melamine, may be difficult to desorb from a liquid surface by pure surface desorption techniques.[24] Drying of the liquid/keeping the liquid in a high temperature environment may be necessary for the detection of melamine (or other analytes) in pure milk in this case. Using ultrasound-assisted EESI, we found no particular difficulty in liberating and detecting such milk constituents. Thus, ultrasound-assisted EESI appears to be useful for the rapid detection of compounds even in heterogeneous, complex matrices such as emulsions.

The growing concern over tainted food products requires analytical methods for rapid screening of melamine and related compounds in various matrices. According to the FDA, a melamine concentration of less than 2.5 ppm in milk products does not raise concerns for human health. The amount of melamine in intentionally adulterated milk or related products has to reach a certain level to make the adulteration process profitable (*e.g.*, by adding 2.5 mg melamine into 1 kg milk, the "apparent protein content" is increased by only $\sim$0.001%). Thus, the sensitivity achieved herein is more than sufficient for detecting melamine adulteration in milk products. Detection of ppb or even ppt levels of melamine by advanced methods may be confusing for the public and for politicians, since melamine at completely safe levels may be found in many products, and "false positives" could result from such analyses. Ultrasound-assisted EESI has high specificity, excellent tolerance to complicated matrices, no requirement for sample pre-treatment, and high throughput capability, which make it suitable for rapid screening of melamine in large batches of tainted milk products: 120 samples could be processed in one hour utilizing this method, faster than ELISA. A portable miniaturized mass spectrometer[25] coupled with an ultrasound-assisted EESI source would be a good choice for field applications, such as high throughput quality control of milk products in manufacturing environments.

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