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## Accelerated Articles

# Detection of Melamine in Milk Products by Surface Desorption Atmospheric Pressure Chemical Ionization Mass Spectrometry

Shuiping Yang,<sup>†</sup> Jianhua Ding,<sup>†,‡</sup> Jian Zheng,<sup>§</sup> Bin Hu,<sup>†</sup> Jianqiang Li,<sup>†</sup> Huanwen Chen,<sup>\*,†,‡</sup> Zhiqian Zhou,<sup>||</sup> and Xiaolin Qiao<sup>\*,||</sup>

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Without any sample pretreatment, trace amounts of melamine in various milk products were rapidly detected noting the characteristic fragments (i.e.,  $m/z$  110, 85, and 60) in the MS/MS spectrum of protonated melamine molecules ( $m/z$  127) recorded by using surface desorption atmospheric pressure chemical ionization mass spectrometry. Signal responses of the most abundant ionic fragment ( $m/z$  85) of protonated melamine were well correlated with the amounts of melamine in milk products, showing a dynamic range about 5 orders of magnitude. The limit of detection (LOD) was found to be  $3.4 \times 10^{-15}$  g/mm<sup>2</sup> (S/N = 3) for the detection of pure melamine deposited on the paper surface, which was much lower than that for detection of melamine in powdered milk ( $1.6 \times 10^{-11}$  g/mm<sup>2</sup>, S/N = 3) or liquid milk ( $1.3 \times 10^{-12}$  g/mm<sup>2</sup>, S/N = 3). The significant difference in LOD was ascribed to the relatively strong molecular interactions between melamine and the matrix such as proteins in the milk products. As demonstrated using desorption electrospray ionization (DESI) for melamine detection, weakening the molecular interaction between analytes and proteins is proposed as a general strategy to improve the sensitivity of ambient mass spectrometry for direct detection of analytes bound in protein matrixes. The relative standard deviation (RSD) and the recovery of this method were found to be 5.2~11.9% and 87~113%, respectively, for the detection of melamine in milk products. A single sample analysis was completed within a few seconds,

providing a particularly convenient way to rapidly screen melamine presence in milk products.

Melamine (1,3,5-triazine-2,4,6-triamine, MW 126) is a basic organic compound, which has been widely used in polymer resins or as raw material in chemical industry. Because of its high nitrogen level (66% nitrogen by mass), melamine gives the analytical characteristics of protein molecules when the Kjeldahl method is used for protein analysis. Thus, melamine can be used to adulterate protein-rich diets by unethical manufacturers. For example, melamine was found in pet food and blamed for killing thousands of cats and dogs in the U.S. in 2007.<sup>1</sup> Recently, high levels of melamine were reported in pet food, wheat gluten, and various dairy products.<sup>2–4</sup> A safety limit of melamine ingestion has been officially set to be 1 ppm for infant formula.<sup>5,6</sup> However, melamine concentrations in the adulterated milk products were as high as ~3300 ppm,<sup>7</sup> posing extreme danger to consumers. In

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September 2008, milk products tainted by melamine induced renal failure and even death in pets and several infants in China. Therefore, fast, unambiguous detection of trace amounts of melamine in food products is of paramount importance.

Food is a typical sample of complex matrixes. Melamine in food can be detected by either mass spectrometry (MS),<sup>8–17</sup> chromatography,<sup>8–11,15,18–20</sup> or enzyme-linked immunosorbent assay (ELISA)<sup>21</sup> when the matrixes are cleaned up using separation techniques such as gas chromatography (GC),<sup>18,20,22</sup> liquid chromatography (LC),<sup>8–11,13,15,16</sup> and capillary electrophoresis (CE).<sup>23</sup> Mass spectrometry-based methods are preferable for melamine detection because of the high sensitivity and high specificity. However, because of the complicated matrixes, extensive multiple-step sample pretreatments (e.g., extractions, pre-concentration, derivatization, etc.) which take tens of minutes or even hours are normally required by conventional mass spectrometry-based methods. For example, the U.S. Food and Drug Administration (FDA) has published a method for screening melamine in pet food using gas chromatography/mass spectrometry (GC/MS),<sup>18</sup> which takes about 3 h for a single run due to the tedious sample pretreatment. Undoubtedly, these methods are not ideally suitable for rapid detection of melamine in a large number of milk products.

Because of the capability of high throughput analysis, ambient mass spectrometry is of increasing interest for analysis of samples with complex matrixes. Surface desorption atmospheric pressure chemical ionization mass spectrometry (DAPCI-MS) has been used for direct analysis of various samples<sup>24–28</sup> with neither

sample pretreatment nor chemical contamination. With the use of a low sheath gas flow or even without sheath gas, DAPCI can be used for direct analysis of powdered samples, sticky liquids, and biological tissues without a notable loss of sensitivity. Thus, DAPCI-MS is selected as a representative ambient ionization technique for fast screening of melamine in various milk products. Experimental data show that DAPCI-MS serves as a sensitive, rapid analytical tool for specific detection of melamine in both powdered milk and liquid milk, without any sample pretreatment.

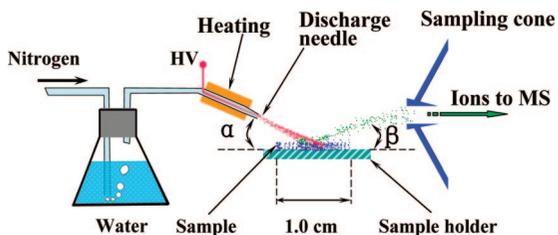
## MATERIALS AND METHODS

All the milk samples were bought in local super markets and were directly used without further treatment. Each measurement was performed with 20 mg of powdered milk or 20  $\mu$ L of undiluted milk suspension, which was deposited on a piece of clean filter paper to form a sample spot about 1 cm<sup>2</sup>. Melamine was added into water and then sonicated for 30 min until no crystal was visible to prepare the stock solution (3000 ppm). Nanograms of melamine were deposited on a filter paper surface and then dried in air before DAPCI-MS analysis. Chemicals such as melamine (A.R. grade), methanol (A.R. grade), acetic acid (A.R. grade) were bought from Chinese Chemical Reagent Co. Ltd. (Shanghai, China). All chemicals were directly used without any pretreatment other than dissolution and dilution with deionized water when it was necessary.

Commercial powdered/liquid milk products containing no melamine were used as blank powdered/liquid milk samples. Pure melamine fine powder was precisely added into blank powdered milk samples (1 + 9, w/w) to make a mixture containing melamine of high concentration. The mixture was then carefully ground and stirred to allow the even distribution of melamine in the whole mixture. The same procedure was followed when a portion of the mixture was diluted with blank powdered milk to make a mixture containing melamine of lower concentrations (e.g., 1%). This procedure was repeated several times to make a series of dilute melamine milk powder standards. A drop of water (20  $\mu$ L) was dripped onto a piece of filter paper to form a wet area (~1 cm<sup>2</sup>), which was saturated by pure water but no water oozing out. The wet side of the filter paper was pressed onto the dry powdered milk sample, resulting in an even distribution of a thin layer (about 1 mm in thickness) of the dry milk powders (15~20 mg) on the wet surface of the filter paper. The milk strip was immediately placed on a sample holder under the discharging needle of the DAPCI source for direct desorption/ionization, with a distance of 2–3 mm between the tip of the needle and the sample surface. Care was taken to ensure that the center of the sample area was right under the discharging needle. No powdered milk was blown off by the sheath gas of the DAPCI source. A certain amount of pure melamine was directly added into undiluted milk to form a heterogeneous liquid mixture, which was then sonicated for 30 min to prepare a standard liquid milk sample. The liquid milk sample was deposited on a piece of filter paper to form a sample spot around 1 cm<sup>2</sup>, which was then directly analyzed without drying the sample or any other treatment. Note that all the liquid milk standards were readily analyzed within 3 min after the sonication.

Experiments were carried out using a commercial linear ion trap mass spectrometer (LTQ-XL, Finnigan, San Jose, CA) installed

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**Figure 1.** Schematic illustration of the heated DAPCI source for melamine detection. The schematics are not proportionally scaled.

with a surface desorption chemical ionization source. The principle and experimental setup for the DAPCI source were previously described elsewhere.<sup>26–28</sup> Briefly, the DAPCI source (schematically shown in Figure 1) and the LTQ mass spectrometer were set to work in positive/negative ion detection mode. A gentle nitrogen sheath gas flow (0.15–0.2 MPa, 250–450 mL/min) was used to bring saturated water vapor as the chemical reagent for the production of primary ions in the DAPCI source. The gas flow tube was heated to 450 °C, and the ionization region of the DAPCI source was maintained at 150–175 °C, ensuring that the water droplets brought by the nitrogen gas flow were completely vaporized before ionization. This maximizes the ionization efficiency by facilitating the release of melamine from the sample and results in a stable signal level. The DAPCI source assembly was coaxially coupled to the LTQ mass spectrometer, allowing a 15 mm distance between the discharge tip and the instrument inlet. The milk samples, either as powders or as liquid suspension, were directly supplied to the DAPCI source using a paper surface placed at 2–3 mm from the discharge tip. The sample spot was located midway between the ion entrance of the LTQ instrument and the DAPCI discharge needle. The angle ( $\alpha$ ) formed between the discharge needle and the sample holder was 30–45°, and the angle ( $\beta$ ) formed by the ion entrance capillary with respect to the sample holder was 20–25°. The primary reagent ions, accelerated by the electric field, impacted the sample surface for desorption/ionization, so that protonated melamine ions ( $m/z$  127) were created at ambient pressure and then introduced through the ion guide system into the LTQ mass analyzer for mass analysis. The corona discharge voltage was  $\pm 4.5$  kV with a discharge current about 1–2 mA. The temperature of the heated capillary of the LTQ instrument was maintained at 150 °C. The default voltages for conversion dynode and detectors were used, but the voltages for the ion guide system (including voltages for the heated capillary, tube lenses, etc.) were optimized using the signal ( $m/z$  127) of authentic melamine (~5 ng) on a paper surface. Further optimization was not performed.

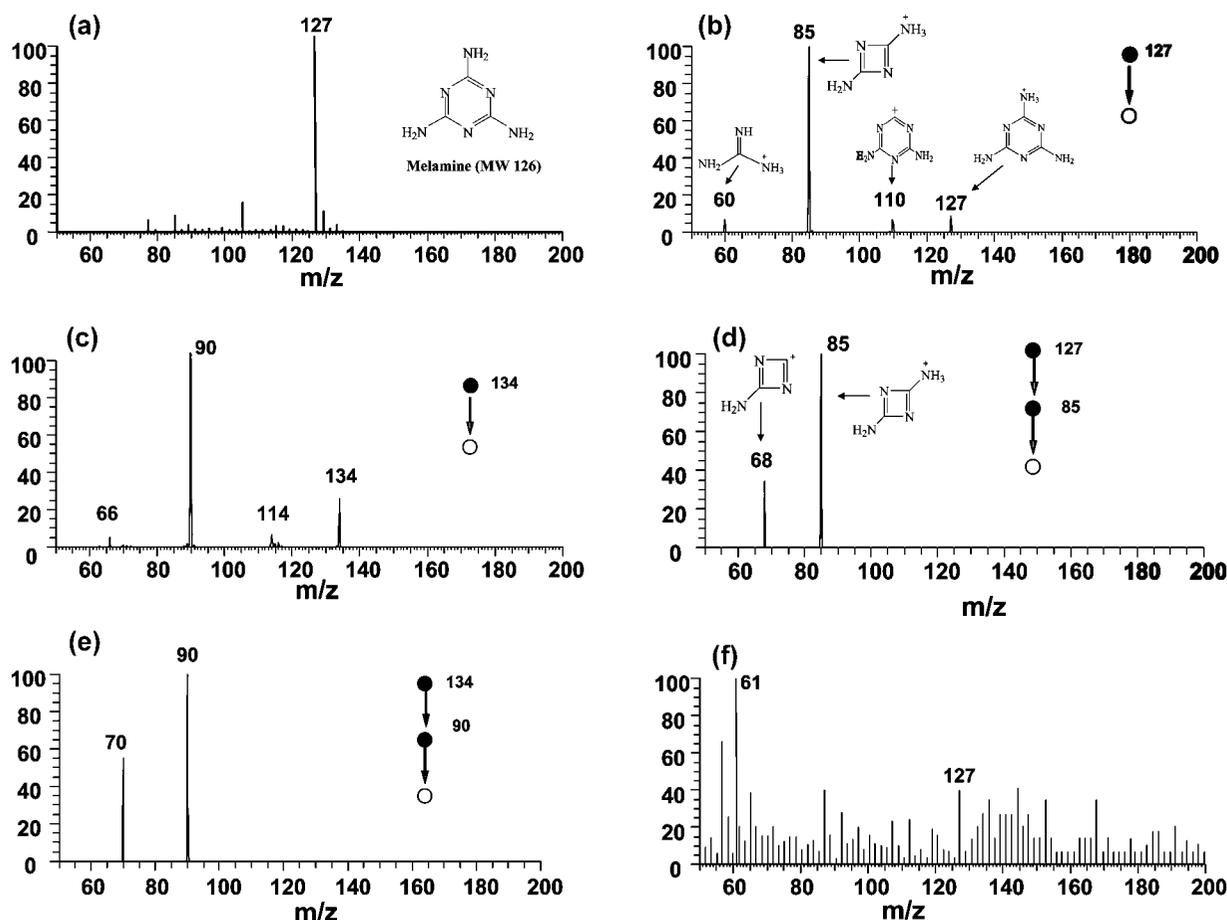
All the MS mass spectra were recorded with an average duration time of 0.1 min and were background subtracted. Collision-induced dissociation (CID) was performed with 20–35% collision energy (CE) to the precursor ions of interest, which were isolated with a mass window width of 1  $m/z$  unit. MS/MS spectra could be collected with a recording time more than 0.1 min if necessary. Compounds of interest were identified using MS and CID data matching of the unknown compounds against authentic standards.

## RESULTS AND DISCUSSION

**Direct Analysis of Powdered Milk.** *Ambient Ionization Techniques for Direct Analysis of Powdered Samples.* Many ambient

mass spectrometry techniques including desorption electrospray ionization (DESI),<sup>29–32</sup> direct analysis in real time (DART),<sup>17,25,33</sup> desorption atmospheric pressure chemical ionization (DAPCI),<sup>25–28,34</sup> atmospheric pressure glow discharge (APGD),<sup>35,36</sup> atmospheric solids analysis probe (ASAP),<sup>37,38</sup> and neutral desorption extractive electrospray ionization (ND-EESI)<sup>39–43</sup> are available for fast detection of analytes on various solid surfaces with minimal sample pretreatment. Nonfat powdered milk is a manufactured dairy product composed of very fine particles. Typically, the average amounts of major nutrients in the unreconstituted milk are (by weight) 36% protein, 52% carbohydrates (predominantly lactose), 1.3% calcium, and 1.8% potassium.<sup>44</sup> Milk powders contain all 20 standard amino acids and are high in soluble vitamins and minerals.<sup>44</sup> Therefore, powdered milk is a representative case of complicated biological samples, which challenge modern analytical tools for rapid analysis due to the matrixes and fine size of particles. For instance, milk powders cannot be directly supplied for fast analysis even using the well-established DESI technique, because the relatively strong sheath gas flow of an open-air DESI source<sup>30,45–50</sup> blows the powders away from the right position, resulting in serious contamination in the source region. Certainly, DESI and other techniques requiring high-pressure sheath gas can be used to analyze powdered samples after the samples are processed to form a solid surface (e.g., a tablet). However, the sample preparation steps decrease the analysis speed.

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**Figure 2.** Mass spectra of melamine recorded by DAPCI-MS: (a) mass spectrum of authentic melamine (1 ng) on filter paper surface; (b) MS/MS spectrum of protonated melamine ( $m/z$  127); (c) MS/MS spectrum of protonated deuterium melamine ( $m/z$  134); (d) MS/MS/MS spectrum of the ionic fragments ( $m/z$  85) produced from protonated melamine ( $m/z$  127); (e) MS/MS/MS spectrum of the ionic fragments ( $m/z$  90) produced from protonated deuterium melamine ( $m/z$  134); (f) mass spectrum of powdered milk on a filter paper surface, the signal detected at  $m/z$  127 yielded the same MS/MS spectrum as that of protonated authentic melamine (shown in Figure 2b).

Surface desorption atmospheric pressure chemical ionization mass spectrometry has been successfully applied to rapid detection of powdered samples such as amino acids and glucose using ambient moisture for primary ion production.<sup>26,28</sup> In autumn, the relative humidity of the local air is usually lower than 45%, which is not sufficient for primary ion production in DAPCI. To ensure sufficient reagent (i.e., water) in the nitrogen sheath gas, saturated water vapor was carried into the DAPCI source by the gentle nitrogen gas flow flushing through an acetic acid aqueous solution (250 mL, 1%, v.v).

**Characteristic Fragmentation of Protonated Melamine.** Under the experimental conditions, the DAPCI-MS spectrum of authentic melamine (1 ng) deposited on a filter paper surface was recorded, showing a predominant peak at  $m/z$  127 (shown in Figure 2a). Upon CID (25% CE, 30 ms), the precursor ions ( $m/z$  127) generated ions of  $m/z$  110, 85, and 60 as the major fragments (shown in Figure 2b), by the loss of  $\text{NH}_3$ ,  $\text{NH}_2\text{CN}$ , and  $\text{C}_2\text{HN}_3$ , respectively. In the MS/MS spectrum of the deuterium labeled melamine ions ( $m/z$  134), the precursor ions produced major fragments of  $m/z$  114, 90, and 66 (shown in the Figure 2c), which were ascribed to the loss of  $\text{ND}_3$ ,  $\text{ND}_2\text{CN}$ , and  $\text{C}_2\text{DN}_3$  from the parent ions, respectively. The fragmentation pattern was identical to that observed using the protonated melamine molecules ( $m/z$  127), validating the fragmentation pathways

of the precursor ions. These data suggest the fragment of  $m/z$  60 or 66 observed from the protonated melamine ( $m/z$  127) or the isotope labeled molecule ( $m/z$  134) contains 6 hydrogen or deuterium atoms. Its possible structure is proposed in Figure 2b. Interestingly, under the experimental conditions, the ionic fragment of  $m/z$  68, which was observed as a major fragment in MS/MS spectra recorded using triple quadrupole mass analyzers,<sup>9–11</sup> was almost undetectable. This difference was probably caused by the different CID conditions. For example,  $m/z$  68 showed up when the CID experiments were performed with either high collision energy (e.g., 35%) or high source/heated capillary temperature (e.g., 300 °C). In the MS<sup>3</sup> experiment, the parent ions of  $m/z$  85 ( $m/z$  90 for the deuterium labeled ions) yielded a predominant signal at  $m/z$  68 ( $m/z$  70), probably by the loss of  $\text{NH}_3$  ( $\text{ND}_3$ ). This confirmed that the fragment of  $m/z$  68 observed in the MS/MS spectrum was mainly derived from the fragment of  $m/z$  85. As  $m/z$  68 increased to high abundance, the intensity of  $m/z$  85 drastically decreased. This is also in agreement with previous studies.<sup>9–11</sup> However, it is beneficial to maximize the abundance of the fragment of  $m/z$  85 for sensitive quantification of melamine in our experiments. In the MS/MS spectrum of protonated melamine, the signal intensity ratio of  $m/z$  109 to  $m/z$  110 varied with CID conditions (i.e., collision energy, CID duration time). Preliminary data show that

the relative abundance of  $m/z$  109 is favored using high collision energy (e.g.,  $\geq 28\%$ ) and short collision time (e.g., 15 ms) and that  $m/z$  110 is more abundant at relatively low collision energy (e.g., 23%) and long collision time (e.g., 300–500 ms). This indicates that the weak signal ( $m/z$  109) might come from protonated melamine, possibly by successive loss of  $\text{NH}_3$  and a H radical. However, it was difficult to perform further CID experiments on the ions of  $m/z$  109, indicating that the ions of  $m/z$  109 were very fragile or of a very short lifetime. To validate the fragmentation pattern of protonated melamine ( $m/z$  127), a commercial ESI source was employed to repeat the MS/MS experiments using authentic melamine. The same fragmentation pattern of protonated melamine was experimentally obtained. Interestingly, similar behaviors of the fragment  $m/z$  109 were also observed by ESI-MS/MS. These data suggest that protonated melamine might fragment through different pathways, depending on the CID conditions. However, it is beyond the scope of this study to investigate this systematically.

Reference experiments were done using blank paper, wetted with water as described in the Materials and Methods. As a result, a small peak was found at  $m/z$  127 for most paper surfaces; however, no characteristic fragments such as  $m/z$  110, 85, 68, and 60 were found in the MS/MS spectrum of the precursor ions of  $m/z$  127. Thus, the melamine levels on the paper surfaces should be low enough to be neglected. Validation of the melamine content in the materials used for the experiments is crucial to eliminate false positive responses in MS/MS spectra, and any melamine contaminated material should not be used for the experiments.

**DAPCI-MS Spectra of Powdered Milk Samples.** Figure 2f shows a DAPCI-MS spectrum recorded from a powdered milk sample contaminated with melamine. Among many signals detected, protonated melamine shows up as an abundant peak (6920 cps, 45% relative abundance) at  $m/z$  127. The ions of  $m/z$  127 produced major ionic fragments of  $m/z$  110, 85, and 60 in the CID (23% CE, 300 ms) experiments by the loss of  $\text{NH}_3$ ,  $\text{NH}_2\text{CN}$ , and  $\text{C}_2\text{HN}_3$ , respectively. Clearly, the fragmentation pattern of the signal detected from the milk powder matches that observed using authentic melamine compound. This confirms that the melamine in milk powders is successfully detected by DAPCI-MS without any sample pretreatment.

Milk powders are complex biological samples which generate similar DAPCI-MS mass spectral fingerprints even when the samples are produced by different manufacturers. In the mass spectrum of blank powdered milk samples, a small peak ( $\sim 50$ – $1500$  cps) at  $m/z$  127 was also obtained (data not shown). The signal intensities ( $m/z$  127) in the most expensive powdered milk samples were significantly lower ( $\leq 50$  cps) than those detected in low-price products, suggesting the signal could be used as a molecular index of milk quality. In the CID mass spectrum, however, the ions ( $m/z$  127) generated fragments of  $m/z$  109, 84, and 71 probably by the loss of water,  $\text{CH}_3\text{CO}$ , and  $\text{CH}_2\text{COCH}_2$ , respectively. The fragment of  $m/z$  109 could lose CO to yield the major fragment of  $m/z$  81. Unlike the melamine experiment, no effect of CID conditions on the fragmentation was found. Thus, the MS/MS fragments supported that the signal ( $m/z$  127) detected from the blank powdered milk was not melamine. However, these CID data were identical to those obtained using

authentic hydroxymethylfurfural (HMF, MW 126) in terms of both fragments and relative intensity ratios (shown in Figure S1 in the Supporting Information). Thus, the signal ( $m/z$  127) found in most blank milk samples was most likely protonated HMF. Since HMF is commonly found in many powdered samples, the characteristic fragments (e.g., peaks at  $m/z$  110, 85, and 60) generated in the MS/MS experiment must be used to exclude false positive signals for detection of melamine. As found in this study, the relative intensity of the peak at  $m/z$  85 is significantly higher than the other two peaks, thus the signal of fragment of  $m/z$  85 is proposed for quantitative analysis of melamine in milk products using DAPCI-MS.

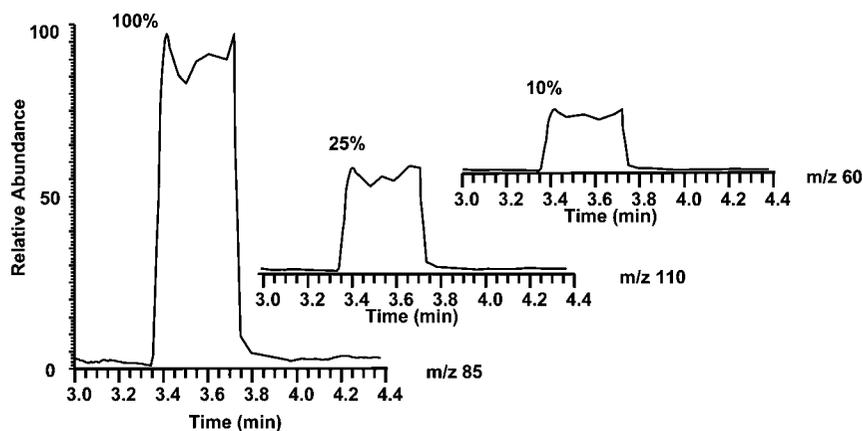
**Sensitivity and Dynamic Response Range.** In order to evaluate the sensitivity of the method, a powdered milk sample that did not produce any melamine signal (i.e.,  $m/z$  110, 85, and 60 in MS/MS) was spiked with melamine as described before. In the MS/MS experiments, the characteristic fragments (i.e.,  $m/z$  110, 85, and 60) of melamine were simultaneously detected starting from a concentration of 0.1 ppm (w:w) melamine in the spiked milk powder sample. It was found that the maximum yields of the most abundant fragment of  $m/z$  85 occurred under mild CID conditions (e.g., 23% CE and 300 ms duration) in our experiments. A working curve was drawn using a series of standard samples spiked with different amounts of melamine, and the calibration curve represented by the equation  $Y = 0.1402x$  (in mg/kg) + 1.734 ( $R^2 = 0.979$ ). The response range of this method was found to be about 5 orders of magnitude, 0.1–10 000 ppm. The LOD of this method was calculated to be  $8.0 \times 10^{-2}$  mg/kg ( $n = 30$ ,  $S/N = 3$ ) using the following equation based on measurements of a series of melamine-spiked samples.

$$\text{LOD} = \frac{c3\sigma}{S}$$

where  $c$  is the melamine concentration in the powdered milk sample,  $\sigma$  is the standard deviation of all the measurements ( $n = 30$ ), and  $S$  is the mean value of the 30 signals measured. According to the experimental conditions (20 mg sample on a spot of  $1 \text{ cm}^2$ ), the LOD was equal to  $1.6 \times 10^{-11} \text{ g/mm}^2$  ( $S/N = 3$ ).

**Response Time of DAPCI-MS.** As a sensitive method, DAPCI-MS shows no serious memory effects for fast analysis of typical solid surfaces.<sup>25–28,34</sup> After 1000 measurements of milk samples which were spiked with melamine at different levels (1–100 ppm), no memory effect was observed. Figure 3 presents the TIC (total ion current) traces of the characteristic ions generated in MS/MS experiments of the parent ions ( $m/z$  127). Notably, the ion current responded rapidly to the presence of milk samples. For example, the signal rose up to 90% in less than 1 s for the fragment of  $m/z$  85, and after the measurement ( $\sim 0.3$  min), the signal dropped down to the noise level in about 0.8 s when the sample was taken away. Therefore, the analysis speed is largely dependent on the measurement time, which can be shortened to less than 1 s using a turbo scan mode. For the current setup, the analysis speed is essentially limited by the sample delivery, which was done manually using a piece of filter paper as the sample holder. In this work, it took about 3 s for each sample loading.

After the measurement of 120 high-concentration samples (100 ppm) continuously, the memory effect starts to be detectable. For example, the signal decays in 10–20 s down to the noise level



**Figure 3.** TIC traces of the characteristic ions generated in DAPCI-MS/MS experiments of protonated melamine ( $m/z$  127).

**Table 1. Analytical Results of Powdered Milk Analysis**

sample code	MS/MS signal intensity <sup>a</sup> ( $m/z$ 85)	practical sample measurements			recovery experiments	
		values found <sup>b</sup> (mg/kg)	mean value (mg/kg)	RSD (% , $n = 6$ )	amount added (mg/kg)	recovery (%)
1	166, 165,	2818.4, 2691.5	3032.5	7.9	1000.0	106.2
	169, 168,	3235.9, 3069.0			2000.0	115.9
	168, 170	2754.2, 3311.3			3000.0	94.0
2	73.2, 73.4,	8.2, 8.4,	8.4	11.9	5.0	97.7
	74.9, 72.3,	9.7, 7.5,			10.0	103.3
	71.9, 74.6	7.2, 9.4			15.0	95.9
3	113, 112,	182.0, 170.3,	194.0	9.6	100.0	112.8
	115, 116,	205.6, 218.8,			200.0	102.5
	113, 115	182.0, 205.6			300.0	94.3
4	1.6		not detectable			
5	101, 103,	81.3, 93.3,	85.4	8.3	100.0	98.8
	103, 100,	93.3, 75.9,			200.0	92.6
	102, 101	87.5, 81.3			300.0	109.1

<sup>a</sup> Values were directly read from the software after measurements; <sup>b</sup> Values were calculated using the calibration curve shown in Figure 5.

when the sample with high melamine concentration is moved away. The decay time heavily relies on the concentration of the samples and the contamination of the source region by the samples. Low sheath gas pressure ( $\leq 0.2$  MPa) and short sample exposure time ( $\leq 1$  min) are advised to reduce the chances for the fine particles to contaminate the source, thus to minimize the memory effects. To be on the safe side, a high pressure nitrogen jet (e.g., 1.6 MPa) was suggested to be used to clean the source region for 2 min after analysis of 50~100 samples containing melamine of high concentrations ( $\geq 100$  ppm).

Within a given period, no notable depletion effect of DAPCI ionization was observed during the experiments; a steady state response was obtained when the exposure on a specific spot was extended to a few min. However, the duration time of a steady signal response was mainly dependent on the concentration of the melamine in the samples. The shortest duration time of the steady state was 38 s, which was observed for the samples of melamine concentrations lower than 1 ppm. The duration time could be more than 3 min if the melamine concentration was higher than 100 ppm. The sample spot was about 1 cm<sup>2</sup>, and for each spot the sampling area was about 10 mm<sup>2</sup>; the DAPCI sampling probe was relocated to a fresh sample spot to collect the next spectrum. As demonstrated previously,<sup>25-28,34</sup> non-destructive analysis is a general feature of DAPCI, because no high velocity gas beam is required for DAPCI experiments. However, the signal intensity decreases when a sample spot is sampled for

long time (shown in Figure S2 in the Supporting Information), which varies in 0.5~5 min, depending on the concentration of melamine in the sample. The half-life time of the signal decay correlates with the concentration of melamine in the samples. Probably, after being sampled for long time, the melamine is consumed to low levels, which sustain no constant signal intensities. However, reproducible results were obtained for multiple measurements performed before the signal decay.

**Practical Sample Analysis.** Five powdered milk products were rapidly analyzed using DAPCI-MS within a few minutes. As summarized in Table 1, the experimental results were satisfactory for fast screening of melamine in various powdered milk samples. Although the major ingredients of milk products are the same even in products manufactured using a different formula, the matrixes of a given milk product are not exactly identical to any other product. This probably explains the deviation in the RSD values obtained from different samples. Thus, the working curve obtained in this work might not be acceptable for accurate quantification of melamine in all types of milk powders. However, the data show that DAPCI-MS is still useful to obtain semiquantitative information about trace amounts of analyte such as melamine in milk powders.

**Direct Analysis of Liquid Milk Samples.** Liquid milk is widely supplied as a protein-rich diet. It has been demonstrated that trace amounts of analytes in undiluted liquid milk can be

**Table 2. Analytical Results of Liquid Milk Samples**

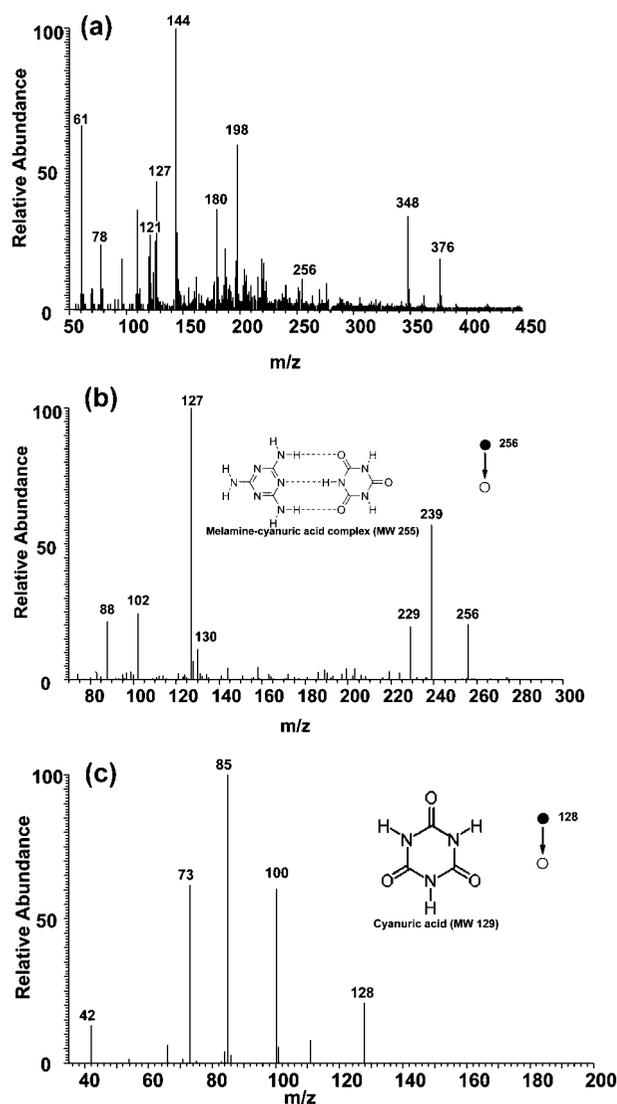
sample code	practical sample measurements			recovery experiments		
	MS/MS signal intensity <sup>a</sup> ( <i>m/z</i> 85)	values found <sup>b</sup> (mg/kg)	mean value (mg/kg)	RSD (% , <i>n</i> = 6)	amount added (mg/kg)	recovery (%)
raw milk	151, 147,	5.8, 5.1	5.6	6.1	5.0	102.4
	150, 151,	5.6, 5.8,			10.0	100.7
	152, 148	6.0, 5.3			15.0	103.8
homogenized milk	122, 125,	2.0, 2.2,	2.0	5.2	2.0	102.2
	121, 123,	1.9, 2.0,			5.0	98.9
	124, 122	2.1, 2.0			10.0	95.4
defatted milk	192, 191,	20.0, 19.5,	20.2	11.5	10.0	96.7
	199, 191,	24.0, 19.5			20.0	96.3
	186, 195	17.0, 21.4			30.0	99.0

<sup>a</sup> Values were directly read from the software after measurements. <sup>b</sup> Values were calculated using the calibration curve shown in Figure 5.

directly analyzed by EESI-MS.<sup>51</sup> Unquestionably, melamine in liquid milk can be sensitively detected by EESI-MS. However, it takes several minutes to flush the capillary after loading a sample, and this decreases the analysis throughput. Taking advantage of a very gentle reagent gas flow, liquids can be directly supplied for fast analysis by DAPCI-MS. To avoid liquid splashing on the sample substrate, a piece of filter paper was used to hold liquid milk (0.02 mL) for direct analysis. However, ultraeven surfaces such as glassware and stainless steel plane are avoided for supporting the milk suspension solution in the DAPCI experiment since the liquid droplet can be easily blown away, especially for the cases where a strong sheath gas or a large amount of milk (0.1 mL) is used. Because the matrixes are different between the liquid milk samples and powdered milk samples, the calibration curve for milk powder samples cannot be used for quantitative detection of melamine in liquid milk. Thus, a working curve for quantification of melamine in liquid milk samples was made using a series of authentic liquid milk samples spiked with different amounts of melamine. Accordingly, a working curve  $y = 0.196x$  (in mg/kg) + 2.029,  $R^2 = 0.986$  shown in logarithm scales was obtained using the experimental data, providing a dynamic response range of 6 orders of magnitude (i.e., from 6.6 ppb to 10 000 ppm).

With the use of the working curve specifically made for analysis of liquid milk products, melamine in liquid milk was rapidly semiquantified using DAPCI-MS. Table 2 summarizes the analytical results of three liquid milk samples. It was found that the RSD values for liquid milk analysis were relatively smaller than those obtained for powdered milk analysis, probably because the surface provided by the liquid samples was more homogeneous than that provided by the powdered milk samples.

**Direct Analysis of Cyanuric Acid in Milk Products.** It has been found that melamine itself is not very toxic at low concentration levels. However, the melamine–cyanuric acid complex, formed from a 1:1 mixture of melamine and cyanuric acid, is believed to be more toxic to health, because the complex is held together by an extensive two-dimensional network of hydrogen bonds between the two compounds and has been implicated as a causative agent for the toxicity of adulterated milk products.<sup>3</sup> DAPCI-MS is also useful for fast detection of cyanuric acid (MW 129) in milk products. Under positive ion detection mode, the protonated molecules of melamine, cyanuric acid, and the melamine–cyanuric acid complex (MW 255) were simultaneously



**Figure 4.** Detection of melamine and cyanuric acid in milk products by DAPCI-MS/MS: (a) mass spectrum of melamine and cyanuric acid coexisting in a liquid milk sample; (b) MS/MS spectrum of the protonated melamine–cyanuric acid complex (*m/z* 256); (c) MS/MS spectrum of deprotonated cyanuric acid (*m/z* 128) recorded in negative ion detection mode.

detected at *m/z* 127, 130, and 256 (shown in Figure 4a) from a 20  $\mu$ L liquid milk sample, which contained melamine (50 ppm) and

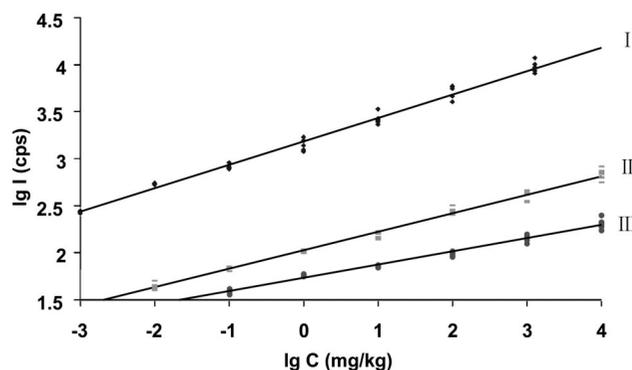
(51) Chen, H. W.; Venter, A.; Cooks, R. G. *Chem. Commun.* **2006**, 2042–2044.

cyanuric acid (10 ppm). Upon CID, the parent ions of  $m/z$  127 gave characteristic fragments of melamine, which was in good agreement with previous experiments (shown Figure 2). The signal intensity of the peak at  $m/z$  130 was fairly weak, but it yielded major fragments of  $m/z$  102 and 88 by the loss of CO and NCO, respectively. The signal level of the protonated melamine–cyanuric acid complex ( $m/z$  256) was much higher than that of the protonated cyanuric acid ( $m/z$  130), indicating that the most cyanuric acid interacted with melamine to form the complex. As shown in Figure 4b, the precursor ions of  $m/z$  256 generated major fragments of  $m/z$  239, 229, 127, and a small peak at 130, probably by the loss of  $\text{NH}_3$ , HCN, cyanuric acid, and melamine, respectively. The signal intensity of peak at  $m/z$  127 was considerably higher than that of peak at 130, suggesting that the melamine has higher proton affinity. The fragments of  $m/z$  239 and 229 indicated that the complex was strongly bound. The fragments of  $m/z$  102 and 88 were probably generated by the loss of CO and NCO from protonated cyanuric acid ( $m/z$  130). These data confirmed the successful detection of cyanuric acid in milk products.

Cyanuric acid is also detectable using DAPCI-MS under the negative ion detection mode. For example, in a powdered milk sample spiked with trace amounts of cyanuric acid (e.g., 1 ppm) and a relatively high amount of melamine (2 ppm), the most abundant signal was detected as  $m/z$  128, which was ascribed to the deprotonated cyanuric acid molecules. Upon CID, the parent ions of  $m/z$  128 produced major fragments at  $m/z$  100, 85, 73, and 42 (shown in Figure 4c), probably by the loss of CO, HNCN,  $\text{C}_2\text{HNO}$ , and  $\text{C}_2\text{H}_2\text{N}_2\text{O}_2$ , respectively. The identical fragmentation pattern was observed using authentic cyanuric acid (20 ng) deposited on a paper surface. Thus, the experimental data demonstrated that DAPCI-MS was able to detect trace cyanuric acid present in milk products.

Further experiments showed that cyanuric acid could be detected with similar sensitivity in either a positive ion detection mode or a negative ion detection mode when it was added into milk without the presence of melamine. However, once the melamine–cyanuric acid complex is formed, it is better to detect the protonated melamine–cyanuric acid complex in a positive ion detection mode. So far, our data showed that cyanuric acid levels around 10 ppb were detectable in MS/MS experiments with a good signal-to-noise ratio ( $S/N = 10$ ).

**Matrix Effects.** Milk is an emulsion that also contains a suspension of multiple components including proteins, fats, casein micelles, and lactose. The big molecules such as protein micelles enclose water and other compounds, presenting a complex matrix challenging the detection of trace amounts of melamine in milk products. Numerous applications performed by using DESI,<sup>29–32</sup> DART,<sup>17,25,33</sup> DAPCI,<sup>25–28,34</sup> EESI,<sup>39–43,52–55</sup> electrospray-assisted



**Figure 5.** Signal responses of the fragment of  $m/z$  85 detected by DAPCI-MS/MS: curve I,  $y = 0.2488x + 3.183$ ,  $R^2 = 0.984$ , recorded from dried pure melamine deposited on a paper surface; curve II,  $y = 0.196x + 2.029$ ,  $R^2 = 0.986$ , recorded from liquid milk spiked with melamine; curve III,  $y = 0.1402x + 1.734$ ,  $R^2 = 0.979$ , recorded from powdered milk spiked with melamine.

laser desorption ionization (ELDI),<sup>56–59</sup> or other techniques<sup>37,38,60–62</sup> have shown that matrixes usually form no complex with the analytes in complex samples, and thus the matrixes have no significant interference to rapid analyte detection using ambient ionization techniques. In the case of melamine detection using DAPCI-MS, the sensitivity of detection was changed when the same amount of melamine was present on the same paper surface but with or without powdered milk as matrixes. For example, the LOD for a standard melamine sample on a paper surface was  $3.4 \times 10^{-15}$  g/mm<sup>2</sup> ( $S/N = 3$ ) while the LOD was  $1.6 \times 10^{-11}$  g/mm<sup>2</sup> ( $S/N = 3$ ) for melamine in powdered milk samples. Note that the melamine was distributed homogeneously in the powdered milk samples because the resulted mixture was well ground when milk powder was added as a matrix. As shown in Figure 5, however, linear signal responses were observed in both cases (curves I and III) but with a much wider response range when pure melamine was directly deposited on a paper surface. The huge difference in sensitivity was also reflected by the slope of the two calibration curves. Experimental evidence proved that the difference was not caused by the competition of other chemical species for the reagent ions. A possible explanation was that melamine interacted with proteins in powdered milk to form strongly bounded complexes. Multiple binding sites can be expected in the complex formed between melamine and protein because melamine contains three amino groups, which easily interact with proteins by forming multiple hydrogen bonds. With dependence on the size and conformation of protein, several melamine molecules can be attached to a single protein. In such a complex, the melamine caged in protein is unlikely to be

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released unless the molecular complex is disrupted. DAPCI is also a nondestructive technique for surface analysis; the melamine cannot be freed from the melamine–protein complexes during a DAPCI process. This resulted in an overall lower sensitivity for detection of melamine in powdered milk.

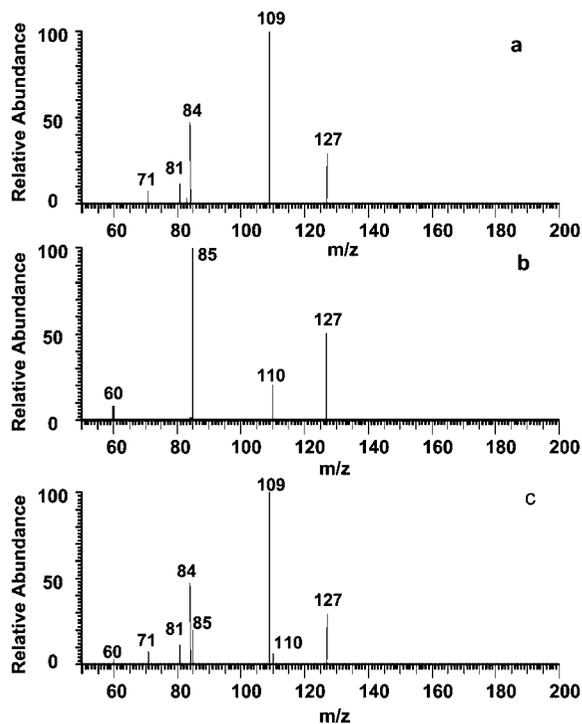
As shown in Figure 5, the slopes of the calibration curves obtained using different samples are different. Since the protons created by DAPCI were sufficient for ionization of the analytes (i.e., proper signal response in samples of high melamine levels), the slope variation could be attributed to the matrixes of the samples, where the total water content differed significantly. Water forms strong hydrogen bonds with amino groups in melamine and protein, thus water is expected to compete with melamine to bind with protein molecules in a solution phase. Accordingly, another response curve (curve II) of melamine in liquid milk was obtained. As shown in Figure 5, the slope of the curve II is located between curves I and III, showing that the sensitivity of DAPCI-MS for detection of melamine in liquid milk is lower/higher than that for matrix-free/powdered milk samples. For comparison, liquid milk samples (10 mg/kg) were dried at 60° in a vacuum (10 mTorr) to produce solid samples containing high melamine content (80 mg/kg) due to the loss of water. Surprisingly, the melamine signal detected in the dried sample was about 92 cps ( $m/z$  85), which was lower than that of the liquid samples (about 162 cps, see Table S1 in the Supporting Information for details). These data show that the molecular interactions of melamine between water and proteins affect the sensitivity of melamine detection; and the melamine–protein complexes could be partly disrupted by other ligand compounds such as water.

Molecules move much faster at high temperature than at low temperature. Therefore, heat can be used to disrupt hydrogen bonds and nonpolar hydrophobic interactions. This occurs because heat increases the kinetic energy and causes the molecules to vibrate so violently that the noncovalent bonds are disrupted. When the movement of molecules in a melamine–protein complex is increased, the liberation of melamine is also improved, and thus better sensitivity can be obtained. Further experiments were done to investigate the temperature (from 25 to 300 °C) effect on the signal intensity levels by changing the temperature of a heated substrate carrying a thin-layer of powdered milk. As a result, it was found that the sensitivity was enhanced significantly (almost 2 orders of magnitude) when the source was heated to 125~175 °C (shown in Figure S3 in the Supporting Information). The signal decreased when a higher source temperature (e.g., 200 °C) was used, probably due to the pyrolysis of melamine. Thus, the source temperature was maintained at 150 °C. This experiment also supports our hypothesis that melamine forms tightly bound complexes with proteins in milk, which break easily at relatively high temperature.

Experimental data show that even the inherent background noise was associated with all the samples, and the technique provided reproducibly satisfactory results of the characteristic fragments across the range of response. Typical RSD values of the fragment of  $m/z$  85 were found to be 2.0~12.6% and 1.5~11.9% at different concentrations (6 measurements for each sample) for powdered milk and liquid milk, respectively. Table S2 in the Supporting Information summarizes the details of the data. Note that every measurement is based on an average of five mass

spectra, and each mass spectrum was recorded using an averaged scan time of 0.1 min. As mentioned before, the product ions ratios depend on the parameters such as energy and duration of the CID experiments. Under fixed operational conditions, the product ion ratios of the fragments were constant, showing that the product ions were not affected by the matrixes. However, the ratios of other peaks (e.g.,  $m/z$  109–85 and  $m/z$  84–85) varied from different samples, because the peaks at  $m/z$  109 and 84 were not intrinsic fragments of protonated melamine. These data confirm that it is necessary to use the characteristic product ions of melamine to exclude false positive signals and to yield trustworthy results.

Other techniques such as DESI are widely employed to analyze surfaces, without a serious matrix effect. With the use of a traditional open-air DESI source, no signal of melamine was detected from liquid milk samples (10  $\mu$ L, 10 ppm) although a few peaks showed up in the mass spectrum (data not shown). In the DESI mass spectrum, a predominant peak (890 cps) showed up at  $m/z$  127, which was almost exactly the same mass to charge ratio as that of protonated melamine detected by our instrument. More experiments found that this peak was detectable with similar abundances ( $900 \pm 15\%$ , cps) by DESI-MS from all the blank liquid milk samples tested. According to the major fragments such as  $m/z$  109, 84, and 81 observed in the CID mass spectra (data not shown), the signal detected at  $m/z$  127 was tentatively assigned to protonated HMF. Normally, a high-pressure sheath gas (e.g., 1.6 MPa) is required for open-air DESI experiments on dry surfaces.<sup>29–32</sup> In this study, the signal intensity was heavily dependent on the sheath gas pressure (i.e., velocity of the gas jet) used to perform the desorption electrospray ionization. For example, it was found that the TIC trace of the signal ( $m/z$  127) rapidly reached the maximum (100%) from a low level (20%) when the gas pressure was increased from 0.6 to 0.8 MPa and decreased down to a low level (15%) when the gas pressure was further increased to 1.0 MPa. The signal level dropped slightly again if the sheath gas pressure was higher than 1.2 MPa. In the DESI MS/MS spectrum (shown in Figure 6a), the precursor ions ( $m/z$  127) detected from the blank liquid milk samples yielded major fragments of  $m/z$  109, 84, and 81. However, protonated melamine molecule gave major fragments of  $m/z$  110, 85, and 60 in DESI-MS/MS experiments (shown in Figure 6b). This was in good agreement with the fragmentation pattern recorded using DAPCI-MS (shown in Figure 2). Therefore, the DESI-MS signal detected at  $m/z$  127 was not melamine. This suggested that DESI was able to detect signals directly on sticky liquid surfaces such as milk on filter paper, but signals detected were most likely to be volatile and semivolatile compounds. Note that the DESI experiment should be executed on a coarse filter paper surface (Teflon surface gave no signal due to the quick sample spreading), on which the liquid milk forms a thin film rather than a droplet so that the liquid milk sample tolerates the sheath gas flow. The total amount of liquid milk dropped on the paper surface should be no more than 20  $\mu$ L to avoid the formation of small droplets upon the sheath gas flow; and the sticky droplets are likely to be sucked into the ion entrance, causing serious blocking. No powdered milk should be directly analyzed by the open-air DESI source because the fine particles started to contaminate the source region when the sheath gas pressure was higher than 0.2 MPa. The DESI experiment was



**Figure 6.** MS/MS spectra of signal detected at  $m/z$  127 by DESI-MS from various samples: (a) MS/MS spectrum of DESI signal detected from blank milk samples on a paper surface; (b) MS/MS spectrum of DESI signal detected from authentic melamine on a paper surface; (c) MS/MS spectrum of DESI signal detected from milk samples spiked with authentic melamine on a paper surface.

performed at room temperature, and no trial was made using a milk sample containing melamine more than 10 ppm. However, when the liquid milk was dried by heating at 120 °C, a weak melamine signal was detected, showing all the characteristic fragments (such as  $m/z$  110, 85, and 60) in the MS/MS spectra of precursor ions ( $m/z$  127) (shown in Figure 6c). Other ions detected at  $m/z$  109, 84, and 81 were attributed to an isobaric interference for melamine detected from the dried milk sample. Once a blank milk sample was used to generate the background for subtraction, these false signals of melamine were eliminated from the DESI-MS/MS spectrum, resulting in a MS/MS spectrum similar to that shown in Figure 6b.

Additional experiments found that the DESI signal intensity level was not vitally dependent on the temperature ( $\leq 100$  °C) of the sample surface, and the signal level was maintained almost constant once the liquid sample was dried thoroughly at 120 °C. However, if the same amount of liquid milk (e.g., 0.2 mL) on the same sample substrate was kept at 120 °C for a longer time (e.g., 30 min), the melamine signal level was about 2–3 times higher than that obtained from the sample heated for a short time (5 min). This is probably because the destruction of the melamine–protein complexes takes about 10 min at the temperature (120 °C). These findings indicate that melamine–protein complexes have the tendency to liberate melamine under a high temperature environment, probably because the proteins are denatured at high temperature. On the basis of the experimental results, it is difficult to evaluate the heating time required to liberate melamine from the protein matrixes in milk products.

**Analysis Speed.** Melamine detection is usually performed by using tandem techniques involving sample pretreatment such as

preparation and preconcentration. Melamine can be directly detected using ambient mass spectrometry-based methods, particularly DAPCI-MS and DESI-MS as demonstrated in this study. A typical DAPCI-MS mass spectrum was obtained within 1–3 s. A single sample analysis, plus MS/MS experiment (about 300 ms) for elimination of false positive alerts, was completed within 3 s using a selective ion monitoring mode. Therefore, the throughput of melamine detection is dramatically improved by using ambient mass spectrometry. So far, the sample analysis speed is limited by the sample loading, which takes usually about 3–30 s to deliver a solid/liquid milk sample manually. It is reasonable to enhance the throughput by using an automatic sample delivery system, which has been used for high-throughput DESI analysis of pharmaceutical preparations.<sup>30</sup>

## CONCLUSIONS

Milk adulteration presents a typical challenge in food regulation, which requires novel analytical methods for sensitive, specific, and high-throughput detection. Recently developed ambient mass spectrometry certainly becomes a good choice since these techniques tolerate complex matrixes and require minimal or no sample pretreatment. As a useful tool for food analysis, DAPCI-MS was chosen to rapidly detect melamine in milk products. Under the experimental conditions, powdered milk and liquid milk as well were rapidly analyzed by DAPCI-MS. A linear dynamic response of more than 5 orders of magnitude was found for authentic melamine standards, powdered milk, and liquid milk samples, respectively, using the characteristic fragments obtained in MS/MS experiment. However, the LOD for pure melamine on a paper surface ( $3.4 \times 10^{-15}$  g/mm<sup>2</sup>) differed from that for melamine in either powdered milk ( $1.6 \times 10^{-11}$  g/mm<sup>2</sup>) or liquid milk ( $1.3 \times 10^{-12}$  g/mm<sup>2</sup>) samples. Experimental data suggest that melamine forms strongly bound complex with proteins in milk. This is in accordance with the recent DAPCI-MS imaging study, showing an extremely strong propensity of melamine to preferably locate in the protein-rich region (i.e., the egg white) of the egg samples.<sup>63</sup> Extra experiments done with DESI and DAPCI under different working conditions confirm that melamine cannot be readily detached from the complex matrixes for post ionization in mild conditions, particularly in DESI or DAPCI process at room temperature. The preliminary results show that heating the sample surface and/or adding chemicals which weaken the molecular interaction between melamine and protein facilitate sensitive detection of melamine in milk products. These findings suggest a general strategy for the direct detection of trace amounts of analytes strongly bound in complex matrixes. Once this strategy is implemented, DESI is anticipated to be an alternative tool for rapid detection of melamine in liquid milk products.

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