Detection of Melamine in Gluten, Chicken Feed, and Processed Foods Using Surface Enhanced Raman Spectroscopy and HPLC

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ABSTRACT: Melamine, a nitrogen-rich chemical, was implicated in pet and human food recalls in 2007, which caused enormous economic losses to the food industry. In this study, melamine concentration in wheat gluten, chicken feed, and processed foods (that is, cake and noodle) was measured by surface enhanced Raman spectroscopy (SERS) in combination with SERS-active substrates. SERS was able to rapidly detect 0.1% melamine in wheat gluten, 0.05% in chicken feed, 0.05% in cakes, and 0.07% in noodle, respectively. A partial least squares (PLS) model was established for the quantification of melamine in foods by SERS: \( R = 0.90, \text{RMSEP} = 0.33 \). In addition, SERS results were verified by HPLC analysis based on a simplified FDA method. Compared with HPLC, the SERS method is much faster and simpler, requires minimum sample preparation, but still yields satisfactory qualitative and quantitative results. These results demonstrate that it is an applicable approach to use SERS to screen foods, eliminate presumptive negative samples of melamine contamination from the sample population, and then verify presumptive positive samples using HPLC protocols. Combining these 2 methods could provide a more rapid and cost-effective way for monitoring melamine contamination in increasingly large numbers of imported foods and feed products.

Keywords: cake, chicken feed, gluten, HPLC, melamine, noodle, SERS

Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is a nitrogen-rich chemical commonly used to produce melamine resin, a synthetic heat-tolerant polymer (Sugita and others 1990). Melamine is not a permissible substance in foods at any level and was implicated in the 2007 pet and human food recalls in North America (FDA 2007a). In these incidents, melamine and its related compounds (for example, cyanuric acid) were deliberately added to protein ingredients to elevate the measured protein content (Weise and Schmit 2007). The economic impact of these recalls on the pet food market and the food industry has been enormous.

Previous studies on melamine and its derivatives were mostly performed in environmental and chemical sciences (Ono and others 1998; Yokley and others 2000; Leidl and Schwarzinger 2005; Sancho and others 2005; El-Sayed and others 2006). High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) is the principal analytical method currently used by the Food and Drug Administration (FDA) for detection and quantification of melamine in foods. The limit of detection (LOD) of this method is as low as 10 parts per billion (ppb) (FDA 2007b). However, this standard method involves time-consuming and labor-intensive procedures, especially in terms of sample preparation and clean-up steps. In addition, it is not a cost-effective screening tool for large numbers of samples as typically encountered in imported food and feed ingredients. Therefore, it is of critical importance to develop simpler, quicker, cost-effective, and sensitive methods for melamine detection in food systems.

Recently, vibrational spectroscopic methods such as Raman spectroscopy have been increasingly used as analytical techniques for evaluating food safety and quality. Raman signals arise from the inelastically scattering of the incident light from a sample and the frequency or wavelength of the scattered light shifts in a manner of characteristic molecular vibrations (Kneipp and others 1999). The “fingerprint-like” Raman spectrum is able to provide overall and specific information on various chemical and biochemical components in a complex system without destroying the sample (Naumann 2000) and requires little or no sample preparation (Li-Chan 1996).

However, only one out of 1 million photons results from Raman scattering. Therefore, only bulk samples or concentrated solutions are applicable for traditional Raman spectroscopic methods (Li-Chan 1996). To solve this problem, much effort has been expended on developing a much more sensitive method—surface enhanced Raman spectroscopy (SERS) which could reach a LOD potentially to the ppb level or even a single molecule level (Kneipp and others 2002a). With the aid of metallic nanostructures, Raman scattering signals of probed molecules within highly localized optical fields of metallic structures can be enhanced by more than a million times due to the effects of electromagnetic field and chemical enhancement (Kneipp and others 2002b; Haynes and others 2005). SERS is one of the few phenomena that can truly be described as nanoscience because the SERS enhancement mechanism depends on the metal surface proximity with an optimum distance in nanometers between the nanostructure surface and the probed sample molecules (Naja and others 2007).
The objective of this study was to develop and evaluate a new approach for detection and quantification of melamine in food and feed products by the SERS methods combined with novel SERS-active substrates. The HPLC protocol was used to verify SERS results. Various food and feed models were selected for SERS and HPLC analysis, including wheat gluten, chicken feed, and processed foods (that is, cake and noodle).

Materials and Methods

Melamine was purchased from Fisher Scientific Inc. (Pittsburgh, Pa., U.S.A.). Wheat gluten imported from overseas was purchased from a local oriental grocery store. A commercial type chicken basal diet was prepared using corn (52.7%), soybean meal (28.7%), pork meal (4.5%), fish meal (3.5%), corn oil (6%), mineral (0.1%), and vitamin premixes (0.08%), and others (4.4%). The basal diet was formulated to include 3% sand. Melamine was substituted for sand in the basal diet to achieve the desired melamine concentrations so that each diet had the same amount of nutrients. Commercial premium cake mix (J. M. Smucker Co., Orrville, Ohio, U.S.A.), cooking oils, large farm eggs, and table salt were purchased from a local supermarket.

Sample preparation

Melamine was incorporated into wheat gluten at the levels of 2.0%, 1.0%, 0.5%, 0.1%, and 0% (w/w) and chicken feed at levels of 2.0%, 1.0%, 0.5%, 0.1%, and 0% (w/w). To determine how food processing conditions affect melamine recovery in foods made with contaminated food ingredients, 2 food models, home-baked cake and noodles, were selected. Melamine was added into 5 batches of cake mix (34.5 g) at the levels of 2.0%, 1.0%, 0.5%, 0.1%, and 0% (w/w), respectively. A mixture of tap water (100 mL), oil (26 mL) and 1 egg (egg white and egg yolk, 40 g) was prepared by hand whisking and then divided evenly into 5 portions. Each portion (32.76 g) was blended with the cake mix (34.5 g) containing different concentrations of melamine. Melamine content in each batter was estimated to be 1.020%, 0.508%, 0.256%, 0.051%, and 0% (w/w), respectively. These percentages were calculated as the weight of added melamine in each batter divided by the total weight of cake mix, liquid portion (water, oil, and egg), and the added melamine. Each cake batter was then baked in an oven at 170 °C for 15 min. After baking, cakes were air-dried for 24 h and ground with a mortar and pestle before extraction. The dry basis of melamine content in each batter was estimated to be 1.680%, 0.841%, 0.424%, 0.085%, and 0%, respectively. Noodles were made from wheat gluten spiked with different concentrations of melamine (2.0%, 1.0%, 0.5%, 0.1%, and 0% w/w), tap water, and salt (gluten:water:salt = 100:25:3.6, w/v/w). All ingredients were then put in a blender (Kitchen Aid, St. Joseph, Mich., U.S.A.) and mixed at a low speed for 1 min and at a high speed for 4 min to allow dough formation. Melamine contents in dough were estimated to be 1.30%, 0.65%, 0.31%, 0.07%, and 0% (w/w), respectively. These percentages were calculated as the weight of added melamine divided by the total weight of the dough. The dough was hand-kneaded for 1 min, shaped into a rectangular form and stored inside a closed plastic bag at room temperature for 1 h. The dough sheets were passed through the sheeting rolls (Kitchen Aid) 3 times at increasing roll settings of 1 to 3 to 5 to produce a 5-mm-thick dough sheet. Raw noodles were air-dried for 24 h and ground with a mortar and pestle before extraction. The dry basis of melamine content in each batter was estimated to be 2.513%, 1.088%, 0.547%, 0.110%, and 0%, respectively. Triplicate samples were prepared for each treatment.

Melamine extraction

Extraction of melamine in samples was based upon a standard FDA method for melamine detection with some modifications (FDA 2007b). Briefly, 50% (v/v) acetonitrile in water was used to extract melamine from all tested samples. For wheat gluten and chicken feed samples, melamine was extracted at a ratio of 0.1 g sample to 10 mL extraction solvent. The samples were then sonicated using an ultrasonic processor equipped with a 6.5-mm tappered microtip (Sonic & Materials, Inc. Newton, Conn., U.S.A.) for 2 min with 30 s working and 30 s interval at an amplitude of 36%. For cake and noodle samples, melamine was extracted at the ratio of 2.0 g sample with 15 mL extraction solvent, and sonicated for 3 min with 1 min working and 1 min interval at amplitude of 36%.

For SERS analysis, samples were set for 30 s to allow for the setting of large particles, then 0.3 μL of the upper layer of the extract was deposited onto a substrate. For HPLC analysis, extracts were further purified by centrifugation at 2266 × g for 20 min. The supernatant of each sample was then diluted with 0.1 N HCl and filtered through a 0.45 μm nylon syringe filter. Melamine standard stock solution (1.0 mg/mL) was prepared with an acetonitrile:water (60:40, v/v). A series of concentrations of standard melamine solutions were prepared by diluting the stock solution with 0.1 N HCl to obtain concentrations of 1, 5, 10, 25, 50, 75, 100, 200, 300, 400 μg/mL, respectively.

SERS measurement

A Renishaw RM1000 Raman spectrometer system (Gloucestershire, U.K.) equipped with a Leica DMLB microscope (Wetzlar, Germany) was used in this study. This system is equipped with a 785-nm near-infrared diode laser source. During the measurement, light from the high power (maximum at 300 mW) diode laser was directed and focused onto the sample at a microscope stage through a 50× objective. Raman scattering signals were detected by a 578 × 385 pixels CCD array detector. The size of each pixel was 22 × 22 μm. Spectral data were collected by WIRE 1.3 software (Gloucestershire, U.K.). In this study, spectra of samples were collected using a 50× objective with a detection range from 300 to 1800 cm⁻¹ in the extended mode. The measurement was conducted with a 10 s exposure time and approximately 20 mW laser power.

SERS-active substrates

Klarite™ SERS-active substrates (DiTechologies Ltd., Hampshire, U.K.) were used. These devices were fabricated on silicon wafers coated with gold. A 6 × 10 mm chip including a 4 × 4 mm patterned SERS-active area and an unpatterned gold reference area was adhered to a standard 25 × 75 mm microscope slide (Figure 1). Pyramidal subunits begin as approximately 1.8 μm openings and arranged in a square lattice configuration at a separation of approximately 0.4 μm. Sharp edges, or “hot spots,” of this gold metal surface can produce surface plasmon resonances induced by the incident excitation laser, generating an enormously enhanced electromagnetic field of signals that take place within the highly localized optical fields of metallic structures.

HPLC analysis

An Agilent 1100 series HPLC system with a 1200 series automatic injector was used. The system consisted of a quaternary pump, a degasser, a column oven, and a diode array detector. The mobile phase was an 85:15 (v/v) buffer containing 10 mM citric acid and 10 mM sodium octanesulfonate (pH 3.0): acetonitrile. Test conditions: Zorbax SB-C8 (4.6 × 75 mm, 3.5 μm particle, Agilent) column;
Detection of melamine in foods by SERS... column temperature of 40 °C; flow rate of 1.0 mL/min; DAD spectra, 190 to 400 nm, detected at 236 nm.

Statistical analysis

SERS spectral data were analyzed by Delight version 3.2.1 (D-Squared Development Inc., LaGrande, Oreg. U.S.A.). Data preprocessing algorithms including polynomial subtract, binning, smoothing, and Savistky-Golay 2nd derivative transformation were employed to subtract the baseline shift and eliminate high-frequency noises from the instrument. A multivariate statistical model, the partial least square (PLS) model, was constructed to predict melamine concentrations in tested samples. The PLS model was validated by leave-one-out cross-validation, which uses all but one sample to build a calibration model and repeats for each sample in the data set (Martens and Naes 1986). The number of PLS latent variables was optimized based on the lowest root mean square error of prediction (RMSEP) values to avoid overfitting of spectral data.

\[
\text{RMSEP} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (\hat{c}_i - c_i)^2}
\]

In this equation, \( n \) is the number of samples, \( \hat{c}_i \) is the predicted melamine concentration (percent), and \( c_i \) is the actual melamine concentration (percent). The correlation coefficient (\( R \)) and RMSEP were used to evaluate the model. The higher the \( R \) value or the lower the RMSEP value, the better predictability for the model.

For HPLC analysis, a melamine standard curve was obtained by establishing a plot correlating the concentrations of standards to the peak areas. Melamine in samples was confirmed by retention time and a specific absorbance spectrum with a \( \lambda_{\text{max}} \) at 236 nm. Melamine contents in samples were quantified by plotting peak areas into the standard curve. Recoveries of melamine in chicken feed and gluten samples were calculated by dividing quantified melamine content by spiked melamine content in the samples. Recoveries in homemade cakes and noodles were calculated on a dry matter basis. The LOD for melamine was calculated at a signal-to-noise ratio of 3. The limit of quantification (LOQ) for melamine was estimated as the lowest value of melamine content in the linear part of the standard curve.

Results and Discussion

In this study, chicken feed and wheat gluten were selected as model food systems for studying melamine contamination since both of them were implicated in the safety incidents of imported foods in 2007. Homemade cakes and noodles were selected as food models to evaluate how food processing procedures affect melamine recovery in processed foods containing contaminated food ingredients. The spiked melamine concentrations on a dry basis for gluten, chicken feed, cake, and noodle samples are shown in Table 1.

SERS measurement

A normal Raman spectrum of melamine in solid form is shown in Figure 2a. The most intense peak around 682 cm\(^{-1}\) is assigned to the ring breathing mode I and involves in-plane deformation of the triazine ring in melamine molecules. A barely visible peak around 989 cm\(^{-1}\) arises from the ring breathing mode I of the triazine ring (Koglin and others 1996). Normal Raman spectra of chicken feed containing 2.0% melamine and the control (melamine-free chicken feed) are shown in Figure 2b and 2c. Detecting melamine in chicken feed directly by normal Raman is challenging because melamine powders are not evenly distributed in the feed and the normal Raman signals are too weak. To solve this problem, an extraction protocol using a solution of acetonitrile in water (50%, v/v) was employed, followed by the SERS measurement to acquire tremendously enhanced Raman signals of trace amounts of melamine in the extract.

Differen concentrations of melamine in wheat gluten, chicken feed, cake, and noodle were extracted and analyzed by SERS. Average SERS spectra (\( n = 4 \)) of melamine extracted from gluten samples are shown in Figure 3. The melamine featured peak at around 682 cm\(^{-1}\) was present in the SERS spectra collected from the extracts of samples spiked with different concentrations of melamine in gluten, chicken feed, cake, and noodle.

Table 1—Recoveries of melamine concentrations (percent) in gluten, chicken feed, cake, and noodles using HPLC.

<table>
<thead>
<tr>
<th>Spiked (%)</th>
<th>Quantified (%)</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td><strong>Gluten</strong></td>
<td></td>
<td></td>
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<tr>
<td>2.00</td>
<td>1.849 ± 0.265</td>
<td>92.4</td>
</tr>
<tr>
<td>1.00</td>
<td>0.858 ± 0.007</td>
<td>85.8</td>
</tr>
<tr>
<td>0.50</td>
<td>0.556 ± 0.004</td>
<td>111.2</td>
</tr>
<tr>
<td>0.10</td>
<td>0.097 ± 0.018</td>
<td>97.0</td>
</tr>
<tr>
<td><strong>Chicken feed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>2.150 ± 0.050</td>
<td>107.5</td>
</tr>
<tr>
<td>1.00</td>
<td>0.933 ± 0.026</td>
<td>93.3</td>
</tr>
<tr>
<td>0.50</td>
<td>0.463 ± 0.024</td>
<td>92.6</td>
</tr>
<tr>
<td>0.10</td>
<td>0.099 ± 0.004</td>
<td>99.0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.047 ± 0.009</td>
<td>94.0</td>
</tr>
<tr>
<td><strong>Cake (dry basis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.680</td>
<td>1.550 ± 0.019</td>
<td>92.3</td>
</tr>
<tr>
<td>0.841</td>
<td>0.773 ± 0.007</td>
<td>91.9</td>
</tr>
<tr>
<td>0.424</td>
<td>0.373 ± 0.007</td>
<td>88.0</td>
</tr>
<tr>
<td>0.085</td>
<td>0.071 ± 0.002</td>
<td>83.5</td>
</tr>
<tr>
<td><strong>Noodle (dry basis)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.153</td>
<td>1.710 ± 0.050</td>
<td>79.4</td>
</tr>
<tr>
<td>1.088</td>
<td>0.867 ± 0.022</td>
<td>79.7</td>
</tr>
<tr>
<td>0.547</td>
<td>0.536 ± 0.035</td>
<td>98.0</td>
</tr>
<tr>
<td>0.110</td>
<td>0.090 ± 0.004</td>
<td>81.8</td>
</tr>
</tbody>
</table>

*aQuantified values are shown as mean ± standard deviation (\( n = 3 \)).

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Melamine, but absent in the control. Similar results were obtained from chicken feed, cake, and noodle samples. SERS was able to rapidly detect melamine concentrations as low as 0.1% in wheat gluten, 0.05% in chicken feed, 0.05% in cakes, and 0.07% in noodle, respectively. Lower concentrations of melamine could be detected by SERS if better performing nanosubstrates are used. In our previous study (He and others 2008), the LOD of SERS combined with Klarite substrate for detecting melamine in aqueous solutions was estimated to be approximately 53 ppb (0.033 μg/mL). These results demonstrate that SERS can effectively detect trace amounts of melamine in feed and food samples with the aid of a simple and rapid extraction procedure and Raman signal enhancement on SERS-active substrates.

Figure 4 shows the RMSEP values obtained from the PLS models with different latent variables. The spectral data were preprocessed with binning at 2 cm⁻¹, smoothing at 6 cm⁻¹, and a 2nd derivative transformation at 12 cm⁻¹ in the spectral region between 500 and 1700 cm⁻¹. The lowest RMSEP value was obtained when 5 latent variables were used, indicating that the optimal number of latent variables is five. Figure 5 shows the PLS prediction results (n = 74) by constructing the log values of predicted melamine concentrations against the log values of spiked melamine concentrations. The prediction result was obtained with \( R = 0.90 \) and RMSEP = 0.33, indicating that satisfactory quantitative results for melamine contamination in foods by SERS could be obtained.

**HPLC analysis**

Figure 6 shows the HPLC chromatogram of chicken feed containing 0.1% melamine and the DAD spectrum of melamine. The
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SERS compared with HPLC

Both SERS and HPLC methods could detect trace amounts of melamine in tested wheat gluten, chicken feed, and processed foods. The LOD of using HPLC to measure melamine standard solution was 1.0 μg/mL. The LOQ was the same as the LOD due to linear property of the standard curve. HPLC was able to detect the lowest concentrations of melamine spiked into the samples: 0.1% melamine in wheat gluten, 0.05% in chicken feed, 0.05% in cakes, and 0.07% in noodle, respectively. The recoveries of melamine were between 85.8% and 111.2% for gluten, 92.6% and 107.5% for chicken feed, 83.5% and 92.3% for homemade cakes (dry basis), and 81.8% and 98% for raw noodles (dry basis) (Table 1). By comparison, the standard FDA method typically produces recoveries of melamine in gluten and moist pet food in the range of 90% to 110%. In simple food systems such as chicken feed and gluten, melamine can be easily extracted with 50% acetonitrile in water followed by ultrasonication for 2 min, instead of using a FDA protocol, which takes more than 30 min. Thus, our results show that the simplified extraction procedure compares favorably with more time-consuming and tedious FDA standard protocol for quantitative estimation of melamine in foods and feed ingredients. This result also indicates that food processing does not have a significant effect on melamine recovery in foods since the differences in recoveries between raw and processed foods were fairly negligible (P value > 0.05).

![Figure 6](image)

Figure 6—HPLC chromatogram of chicken feed containing 0.1% melamine and the DAD spectrum (inset).

![Figure 7](image)

Figure 7—A proposed protocol for monitoring melamine contamination in imported foods and food ingredients by SERS and HPLC.

Conclusions

SERS is a novel and rapid analytical technique. It can be used to detect trace amounts of melamine in foods, food ingredients, and feed. SERS is much faster and simpler than HPLC, and still provides accurate results if partial quantification is needed. An applicable approach would be to use SERS to screen foods first, eliminate presumptive negative samples of melamine contamination from the sample population; then HPLC is used to verify and quantify presumptive positive samples. By doing so, melamine contamination in large numbers of food and feed products could be detected accurately, quickly, and efficiently.

Acknowledgment

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References


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