Selective melamine detection in multiple sample matrices with a portable Raman instrument using surface enhanced Raman spectroscopy-active gold nanoparticles

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HIGHLIGHTS

- Initial work for field deployable screening of melamine in multiple matrices.
- Without extensive sample preparation, colorimetric screening offered false results.
- Surface enhanced Raman spectroscopy was performed with a portable Raman system.
- Gold nanoparticles as SERS substrates offered selective detection of melamine.
- Limits of detection in seven food and pharmaceutical matrices were 100–200 mg L⁻¹.

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ABSTRACT

Melamine adulteration of food and pharmaceutical products is a major concern and there is a growing need to protect the public from exposure to contaminated or adulterated products. One approach to reduce this threat is to develop a portable method for on-site rapid testing. We describe a universal and selective method for the detection of melamine in a variety of solid matrices at the 100–200 μg L⁻¹ level by surface enhanced Raman spectroscopy (SERS) with gold nanoparticles. With minimal sample preparation and the use of a portable Raman spectrometer, this work will lead to field-based screening for melamine adulteration. Citrate coated gold nanoparticles (Au NPs) were investigated for both colorimetric and Raman-based responses. Several non-hazardous solvents were evaluated in order to develop a melamine extraction procedure safe for field applications. Au NP agglomerates formed by the addition of isopropanol (IPA) prior to sample introduction enhanced the Raman signal for melamine and eliminated matrix interference for substrate formation. The melamine Raman signal resulted in a 10⁷ enhancement through the use of Au NP agglomerates. To our knowledge, we have developed the first portable SERS method using Au NPs to selectively screen for the presence of melamine adulteration in a variety of food and pharmaceutical matrices, including milk powder, infant formula, lactose, povidone, whey protein, wheat bran and wheat gluten.

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1. Introduction

Melamine is a chemical most commonly used in the synthesis of a resin with fire retardant properties. However, in 2007, the economically motivated adulteration of various milk products, baby formula, wheat gluten, and pet foods arose as a major health concern for consumers. Available at low cost and with 66% nitrogen by mass, melamine can raise the nitrogen level of the Kjeldahl protein test and falsely indicate a high protein content in food and animal feed [1]. Ingestion of melamine levels above the World Health Organization (WHO) safety limits of 1 mg L\(^{-1}\) for infant formula and 2.5 mg L\(^{-1}\) for milk and food products can cause renal failure and even death in humans and pets when melamine and cyanuric acid combine to form insoluble crystals in the kidneys [2,3]. According to WHO, more than 50,000 children were hospitalized and six deaths occurred due to melamine adulteration of infant formula in China [3].

A variety of analytical methods are available for melamine detection and include gas chromatography (GC) [4,5], liquid chromatography/mass spectrometry (LC/MS) [6], matrix-assisted laser desorption ionization (MALDI) [7], capillary electrophoresis (CE) [8], and enzyme linked immunosorbent assays (ELISA) [4,9]. However, these methods require extensive sample preparation and data analysis as well as extended measurement times, and are therefore not well suited for the rapid evaluation of numerous food and pharmaceutical products. Thus, there is a need for a rapid, sensitive, portable and selective universal method that can routinely screen products for melamine adulteration without using special chemicals or expensive laboratory equipment.

Recently, gold (Au) and silver (Ag) nanoparticles (NPs) have been used for colorimetric detection of melamine due to their unique optical properties [10–14]. Au NPs are more commonly employed due to their ease of preparation, biocompatibility and stability. The optical properties of Au NPs strongly depend on the surrounding dielectric medium and physical characteristics such as interparticle distance, size and shape [15]. Colloidal Au NPs in solution are a wine red color and have interparticle distances greater than the average particle diameter. When aggregation of Au NPs occurs, a red to blue color change is induced, and the interparticle distance becomes smaller than the particle diameter [10]. To improve selectivity, Au NPs can be modified with reporter groups that favorably interact with the target molecule. Au NPs have been tailored with 1-(2-mercaptoethyl)-1,3,5-triazine-2,4,6-trione, p-nitroaniline, uracil-5-carboxylic acid, 2,4,6-trinitrosulfenylsulfonic acid and 4-mercaptopypyridine for the trace detection (mg L\(^{-1}\) to \(\mu\)g L\(^{-1}\)) of melamine [10,11,13,16–18]. Unfortunately, synthetic procedures for modified Au NPs require special chemicals that may be costly and are often multi-step and time consuming.

In addition to modified NPs, melamine has a very strong binding affinity to the surface of unmodified Au NPs. Colorimetric assays using unmodified Au NPs for the detection of melamine in infant formula and milk products have been shown to have detection limits in the mg L\(^{-1}\) to \(\mu\)g L\(^{-1}\) range. A major drawback to the use of colorimetric assays as routine screening methods for food and pharmaceutical products is that they lack selectivity. False positive results for melamine can occur when NPs interact with other molecules found in complex sample matrices associated with food and pharmaceutical products. As a result, low limits of detection are only achievable when extensive sample preparation techniques such as sample extraction, centrifugation, and pH adjustments are employed.

Raman spectroscopy is rapid, has high chemical and structural information content and requires minimal sample preparation. Its primary shortcoming is lack of sensitivity due to the low efficiency of inelastic scattering. Surface-enhanced Raman spectroscopy (SERS) exploits the dramatic increase in the Raman scattering efficiency of molecules adsorbed to rough metal surfaces. Carefully designed SERS substrates have been developed to achieve \(10^4\)–\(10^6\) enhancement factors [19–23]. Metal NPs are widely used as SERS substrates because they enhance Raman signal through electromagnetic, chemical, or electronic enhancement [24,25]. SERS analysis on roughened or patterned gold surfaces have been used for the trace detection of melamine after extensive extraction procedures from food products that include wheat gluten (33 \(\mu\)g L\(^{-1}\)), chicken feed (33 \(\mu\)g L\(^{-1}\)), and milk powder (200 \(\mu\)g L\(^{-1}\)) [21,22,26]. Unfortunately, these methods are not adaptable for routine field screening due to the use of expensive gold substrates that also require a Raman microscope system for sample analysis. In addition, to achieve such low limits of detection the methods required sample extraction procedures that used hazardous chemicals and employed complex multivariate statistical analysis techniques, such as partial least squares regression (PLS). Conversely, Wen et al. developed a portable Raman screening method that utilized SERS to detect trace levels (10 \(\mu\)g L\(^{-1}\)) of melamine in several commonly used materials for protein pharmaceutical formulation and purification that included sucrose, urea, and arginine [27]. Unfortunately, this same method failed to detect melamine at low concentrations in histidine and cell culture media due to the interference/adsorption of amino acid molecules onto the surface of gold nanoparticles in suspension. Thus, there are no rapid on-site analytical methods that require minimal field-safe sample preparation procedures and are capable of screening trace levels of melamine in complex sample matrices that may include amino acid interferents.

Several studies have shown that Au or Ag NPs solutions that undergo aggregation or agglomeration prior to sample introduction can act as SERS active substrates and significantly increase the observed Raman signal [28,29]. Au NPs used in colorimetric assays are easy and inexpensive to produce and have been shown to have high sensitivity for melamine. Altering the environment around Au NPs can cause the primary particles (PPs) to aggregate or agglomerate and result in an inexpensive and ideal SERS substrate that can be used for trace level detection. Although several SERS methods have been utilized for melamine screening, no rapid field deployable Raman SERS method has been developed that can survey both a variety of food and pharmaceutical matrices.

The purpose of this work is to develop a universal field deployable method for the trace level detection of melamine in both simple and complex food and pharmaceutical products. We will demonstrate that melamine of a known concentration spiked into matrix samples including milk powder, infant formula, lactose, povidone, whey protein, wheat bran and wheat gluten can be extracted from the matrices using a simple one-step extraction procedure that can be implemented into the field. The extracted melamine samples were mixed with Au NP agglomerates, analyzed using a portable Raman spectrometer, and the limit of detection for melamine in the various matrices has been determined to be under the WHO limits. Au NP agglomerates in solution have also been characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), phase analysis light scattering (PALS), and inductively coupled plasma mass spectrometry (ICP–MS).

2. Experimental

2.1. Materials

Sodium citrate, chloroauric acid (HAuCl\(_4\)), melamine, polyvinylpyrrolidone with an average molecular weight of 3500 g mol\(^{-1}\) (povidone), trace metal grade nitric and hydrochloric acids, and HPLC grade isopropanol (IPA) were purchased through
2.3. Preparation of gold nanoparticles

Prior to use, all glassware used in the synthesis and storage of Au NPs were soaked in aqua regia, thoroughly rinsed with water and dried. Gold NP synthesis was based on a previous method [17,30], but reactant concentrations were increased in this work in order to yield a greater number of NPs. Briefly, 100 mL of 2 mM HAuCl₄ was brought to a boil and 10 mL of 77.6 mM sodium citrate was rapidly added and allowed to reflux for 15 min. During this time, the initial golden colored solution turned a wine red color. The NP solution was cooled to room temperature with constant stirring and then stored at 4 °C. All Au NP solutions were warmed to room temperature prior to use.

2.3. Nanoparticle characterization

Transmission electron microscopy (TEM) was performed on three NP suspensions with a JEOL 100 CS Electron Microscope equipped with an IA L9c camera. First, the synthesized NPs, or primary particles (PPs) were taken directly from the original stock. A second sample was prepared by mixing the PPs with IPA (Au NP agglomerates) in a 1:2 ratio while the third sample was prepared by mixing the Au NP agglomerates with a 1 mg L⁻¹ melamine standard (PPs:IPA:1 mg L⁻¹ melamine) in a 1:2:2 ratio, respectively, to study the interaction of melamine with the NPs in an IPA solvated environment. Samples were prepared by adding 15 μL of each suspension on a holey carbon-coated 200 mesh copper grid. For each sample, a minimum of 6 images were taken at 80 kV and 100k× magnification. For particle size determination, a minimum of 30 primary particles were analyzed. Lower magnifications were used to visualize the total size of nanoparticle agglomerates.

NP suspensions were also evaluated using dynamic light scattering (DLS) and phase analysis light scattering (PALS) to determine the hydrodynamic size and zeta potential of the particles, respectively. Samples were run on a Malvern Instruments Nano-ZS (λ: 633 nm, 173° scattering angle) in disposable folded capillary cells. All measurements were performed at 25 °C and consisted of three consecutive measurements of at least 10 runs per measurement. Viscosity, refractive index, and dielectric constants were used as provided by the manufacturer (Malvern for water, Fisher Scientific for IPA). Additionally, an Agilent 8453 UV–Visible spectrophotometer operated at room temperature was used to measure the absorption frequency of the primary and agglomerated NPs.

To estimate the concentration of Au in the PPs, ICP-MS was performed using an Agilent 7700x instrument. NP samples were weighed into metal free polypyrrole centrifuge tubes and digested at an elevated temperature in aqua regia (1:3 HNO₃: HCl) for 2 h. The digestates were diluted to concentrations appropriate for ICP-MS analysis in 2% nitric acid. Two dilutions were prepared with target gold concentrations of 20 and 5 μg L⁻¹ (weight/weight). Gold standards from 0 to 20 μg L⁻¹ were prepared from a certified 10 mg L⁻¹ Au standard. Thallium internal standard (10 mg L⁻¹) was added to all standards and samples at a nominal concentration of 100 μg L⁻¹. A calibration curve over the 0 to 100 μg L⁻¹ range was found to be linear (R² = 0.999). Gold standards were used within 4 h of preparation due to their instability at low concentration.

2.4. Preparation of melamine standards and pure component samples

A standard stock solution of 1018 mg L⁻¹ melamine was prepared by dissolving 0.509g of melamine in 500 mL of water. Water, MeOH and IPA were used as diluents for standard preparations. 10 mg L⁻¹ melamine standards were prepared from the standard stock solution by dilution with either water or MeOH. A 100 mg L⁻¹ melamine standard was prepared by diluting the standard melamine/water stock solution with IPA. A subsequent series of concentrations that included 0.05, 0.1, 0.15, 0.2, 0.5, 1, 5, 10, 15, 20, and 25 mg L⁻¹ were prepared by dilution of the 100 mg L⁻¹ melamine/IPA standard.

2.5. Preparation of matrix samples

Known melamine concentrations were spiked into matrix samples including milk powder, lactose, povidone, infant formula, whey protein, wheat gluten, and wheat bran by suspending approximately 50 mg of the matrix powder into 5 mL of the neat extraction solvent. Once in solution, all samples were mixed by inversion for approximately 10 s. Sample solids were allowed to settle for 5 min and the supernatant was decanted into a clean 16 mm × 100 mm glass test tube.

2.6. Preparation of NP samples for colorimetric and Raman analysis

Samples for colorimetric analysis were prepared in plastic disposable cuvettes in a 1:2 ratio by adding 300 μL of NPs to 600 μL of the decanted test sample preparation. Colorimetric samples were mixed by inversion and allowed to interact for approximately 5–10 min before analyzing using UV–Vis spectrophotometry. For Raman analysis, samples were prepared in a 1:2:2 ratio of NPs/IPA/decanted test sample. Sample preparation began by adding 200 μL of NPs to an 8 mm × 40 mm glass vial. Next, 400 μL of IPA was added and thoroughly mixed using the pipette to draw and release the solution approximately 5 times. After mixing, 400 μL of the decanted sample was added. The vial was capped and the solution was mixed by inversion for 10 s. Finally, the solution was allowed to sit for 5–10 min before analyzing by Raman spectroscopy.

2.7. Raman spectroscopy

Raman spectra were recorded using a B&W Tek MiniRAM II portable Raman spectrometer. The unit was equipped with a fiber optic probe, a 785 nm diode laser excitation source with a maximum output power of approximately 275 mW at the sample and a thermoelectrically cooled 2048 pixels CCD detector. The spectrometer was operated on AC power and controlled by a Microsoft Excel-based user interface developed in-house. Spectra were measured with the fiber optic probe in a backscattering geometry. The average spectral resolution measured with a neon lamp was 8 cm⁻¹, and the spectra were truncated to a common spectral range of 300–1700 cm⁻¹. The exposure time was set to 2.5 s per scan, and each spectrum was the average of 10 scans for a total integration time of 25 s. A background scan with the same integration time was collected before each sample acquisition to remove dark noise.

2.8. Data analysis

Raman spectra were analyzed by first subtracting the measured background spectrum from the sample spectrum and then applying a wavelet signal-to-noise peak detection algorithm [31] developed
in house to identify the melamine peak at 712 cm⁻¹. The Mexican Hat function was used as the mother wavelet. Two wavelet transform filters were applied to the spectrum to separate signal (10 pixel wavelet width) and noise (2 pixel wavelet width). The width of the signal wavelet was optimized to match the resolution of the spectrometer and the minimum width wavelet was selected to characterize the noise. All maxima in the signal wavelet space found between 690 and 730 cm⁻¹ were identified, and the signal-to-noise ratio (SN⁻¹) was determined for each peak. The noise at each signal peak was characterized by averaging 21 points in noise wavelet space centered on the signal peak to avoid spurious noise spikes at the signal peak frequency resulting from photon counting statistics. Positive identification for melamine occurred when the SN⁻¹ was greater than or equal to 9 and the peak position was within 10 cm⁻¹ of 712 cm⁻¹. These characteristics were determined empirically, and three to five replicate measurements were taken for each sample.

### 3. Results

#### 3.1. Gold nanoparticles

Gold NPs are commonly used for colorimetric testing due to their ability to undergo a color change upon aggregation/agglomeration that results from a shift in the surface-plasmon absorption band. Agglomerates are clusters of individual nanoparticles that reversibly interact with each other without changing the size or shape of the particles whereas aggregation involves permanent changes in the structure or shape of the NPs [32]. As no permanent changes were seen during NP characterization, the clusters were termed agglomerates. The PPs used in this work resulted in a wine red suspension that had a surface plasmon resonance band at 522 nm. To help prevent agglomeration, the NPs were stabilized during the synthesis process by a negatively charged coating of citrate ions [17]. In the presence of melamine, immediate agglomeration of the PPs occurred and the wine red suspension turned a violet blue color that corresponds to a shift of the plasmon band from 522 nm to 793 nm. Agglomeration of the NPs with melamine can be attributed to a ligand exchange process that occurs between the negatively charged citrate ions and the amino groups from the melamine molecules. Electrostatic repulsion is decreased and agglomeration of the NPs occurs resulting in a color change and a positive identification for the presence of melamine [17]. At low concentrations of melamine, NP agglomeration resulted in a red to violet color change, whereas at high concentrations a blue color was observed.

The current sample preparation procedure developed for the colorimetric assay utilizes 10% trichloroacetic acid and chloroform in order to extract melamine from liquid milk and infant formula [17]. Due to the hazardous nature of these solutions, the current sample preparation procedure is not acceptable for field applications. Instead, melamine was extracted from several food and pharmaceutical products, including lactose, milk powder, infant formula, whey protein, wheat gluten, and wheat bran and providone using non-hazardous solvents. Because melamine has low solubility in most solvents, water, MeOH and IPA were investigated as possible field acceptable extraction solutions. Control and melamine spiked samples were analyzed using the NP colorimetric method. Ideally, a color change should not be observed when the control samples are mixed with the Au NPs, whereas, the wine red colored AuNP solution should change to a violet blue after the addition of melamine. Color changes were determined by comparing the control samples to the melamine spiked samples and the results for the colorimetric analysis using field acceptable solvents for the extraction procedure are listed in Table 1. False positive results for the NP colorimetric assay occurred when the control samples displayed a color change from red to violet/blue in the absence of melamine whereas false negatives resulted when a known melamine spiked sample did not undergo a color change and remained a wine red color. From the colorimetric assay results in Table 1, the use of water as an extraction solvent resulted in false positives for both wheat matrices and false negatives for milk powder, infant formula, and whey protein. Wheat samples extracted in

### Table 1

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Observed color</th>
<th>Colorimetric result</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>10 ppm melamine</td>
</tr>
<tr>
<td>Extraction solvent – H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ppm melamine standard</td>
<td>Red</td>
<td>Blue</td>
</tr>
<tr>
<td>Lactose</td>
<td>Red</td>
<td>Blue</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>Infant formula</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>Whey protein</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>Povidone</td>
<td>Red</td>
<td>Violet</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>Violet</td>
<td>Violet</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>Violet</td>
<td>Violet</td>
</tr>
<tr>
<td>Extraction solvent – MeOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ppm melamine standard</td>
<td>Red</td>
<td>Blue</td>
</tr>
<tr>
<td>Lactose</td>
<td>Red</td>
<td>Blue</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Red</td>
<td>Violet</td>
</tr>
<tr>
<td>Infant formula</td>
<td>Red</td>
<td>Violet</td>
</tr>
<tr>
<td>Whey protein</td>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>Povidone</td>
<td>Red</td>
<td>Blue</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>Violet</td>
<td>Violet</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>Violet</td>
<td>Blue</td>
</tr>
<tr>
<td>Extraction solvent – IPA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ppm melamine standard</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Lactose</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Milk powder</td>
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<td>Whey protein</td>
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<td>Povidone</td>
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<td>Wheat gluten</td>
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</tr>
<tr>
<td>Wheat bran</td>
<td>Violet</td>
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</tbody>
</table>
MeOH underwent a slight color change in the absence of melamine giving a false positive result, and no color change was observed for the melamine adulterated whey protein samples. False positive results for lactose, milk powder, wheat bran, and wheat gluten were observed for control samples extracted in IPA. Due to the high number of false positive results observed for the control samples, the neat extraction solvents themselves were tested using the NP colorimetric assay. Water and MeOH gave no apparent color change when mixed with the PPs whereas a distinct blue color change associated with NP agglomeration was observed for the IPA solvent. Consequently, the colorimetric assay failed to produce reliable results for adulterant detection when modifying the sample preparation procedure to use a field safe solvent for melamine extraction. The combination of a solvent and a particular matrix may produce uncertain results. This survey only includes one brand of each matrix and different brands may include additional components that could also interact with the NPs and induce false results.

3.2. Characterization of Au NPs

Primary NPs were characterized using DLS, TEM, PALS and ICP-MS. Both DLS and TEM indicate two size populations of particles. Intensity weighted values indicated particles ~1.2 nm and 25.6 nm in diameter. A representative TEM image of the Au PPs is shown in Fig. 1A. Most PPs had an average size of 10.4 nm ± 3.4 nm (N = 30), as seen in Fig. 1A. Smaller particles were also observed with an average particle size of 0.75 nm ± 0.16 nm (N = 7). The zeta potential of the PPs was determined to be ~44.43 mV, which is consistent with a citrate-capped surface. ICP-MS was used to investigate the concentration of Au in our primary NP suspension. The elemental Au concentration in the NP solution was found to be 344 ± 6 mg L⁻¹ (RSD = 2%). The expected concentration of Au in the NP solution depends on the water composition of the HAuCl₄·xH₂O starting material, and is 358 mg L⁻¹ for x = 3 and 342 mg L⁻¹ for x = 4. This result correlates nicely with the total amount of gold added to the system during the synthesis process.

DLS, TEM and PALS were also used to further investigate the effects of IPA on the PPs. Au NP agglomerates were prepared in a 1:2 ratio (PPs:IPA) for all characterization experiments. The DLS experiments resulted in larger particle sizes of over ~1200 nm (intensity weighted) than those observed for the PPs, thus supporting agglomeration associated with the observed color change. A representative TEM image for the Au NP agglomerates is shown in Fig. 1B. Loose NP agglomerates were observed from 1 to 3 μm in size. When a 1 mg L⁻¹ melamine standard prepared in IPA was added to the Au NP agglomerates in a 1:2:2 ratio (PPs/IPA/melamine), it bound to the NP agglomerates producing a tighter conformation of clusters with agglomerate sizes ranging from 350 nm to 1.5 μm in size as measured and shown in the TEM image of Fig. 1C. The zeta potential again changed from ~36.13 mV in IPA to ~17.67 mV with the addition of a 1 mg L⁻¹ melamine standard. Thus, the observed increase in zeta potential supports the interaction of melamine with the surface of the NPs within the Au NP agglomerates.

3.3. Raman spectroscopy

The Raman spectra of solid melamine, IPA, Au NP agglomerates, and Au NP agglomerates mixed with 1 mg L⁻¹ melamine standard are displayed in Fig. 2. The spectra are vertically offset for clarity. Melamine is only slightly soluble in IPA and as a result no Raman bands associated with melamine were observed in the spectrum of a saturated melamine solution prepared in IPA, therefore the solid melamine spectrum was used for peak identification. In the solid melamine spectrum peaks at 380, 582, 674, and 982 cm⁻¹ were observed. The most intense peak at 674 cm⁻¹ can be assigned to the ring breathing mode and involves in-plane deformation of the triazine ring in melamine molecules [22,33]. Raman bands for IPA were observed at 372, 430, 488, 816, 952, 1132, 1344, and 1454 cm⁻¹. Most of the peaks observed in the Raman spectrum of Au NPs in IPA can be attributed to the IPA solvent, with the exception of the Raman bands located at 1020 and 1386 cm⁻¹. These bands are also observed in the Au NP control solution and are therefore associated with the Au NPs. The most significant difference between the Raman spectra of the Au NP agglomerates and the 1 mg L⁻¹ melamine standard mixed with Au NP agglomerates is the Raman band at 712 cm⁻¹. The peak at 712 cm⁻¹ is due to melamine and corresponds to the ring breathing mode and the in-plane deformation vibrational modes [21,22,27]. This band was shifted from the normal mode at 674 cm⁻¹ in the conventional Raman spectrum.

![Fig. 1. TEM images of Au NP suspensions. (A) PPs imaged at 100k×, (B) Au NPs agglomerates at 29k×, (C) Au NP agglomerates with 1 mg L⁻¹ melamine (29k×).](image1)

![Fig. 2. Raman spectra of (A) solid melamine, (B) IPA, (C) Au NP agglomerates, and (D) Au NP agglomerates mixed with 1 mg L⁻¹ melamine standard. Spectra are vertically offset for clarity.](image2)
of the solid melamine sample in Fig. 1. The 38 cm\(^{-1}\) difference in the Raman shift suggests a weak interaction of the melamine molecules with the surface of Au NP agglomerates.

3.4. Enhancement factor and linearity

Signal enhancement is observed when analyzing NP–melamine solutions using Raman spectroscopy. Because of the random nature of agglomerate formation of the NPs upon addition of the IPA clustering agent, the number of NPs probed is not known and the typical enhancement factor calculation for SERS substrates cannot be used [34]. To determine the enhancement factor of the Au NP agglomerates for melamine, aqueous melamine solutions were used due to the poor solubility of melamine in IPA. Saturated (1875 mg L\(^{-1}\)) and low level melamine samples were used in the enhancement studies and analyzed using UV–Vis spectrophotometry and Raman spectroscopy. The 1875 mg L\(^{-1}\) aqueous melamine sample is a clear colorless solution that was analyzed, as is, and resulted in an absorbance value of 0.069 AU at 785 nm. The low level aqueous melamine sample was determined as the concentration of melamine that when interacted with Au NP agglomerates resulted in a similar Raman peak intensity for the in-plane deformation vibrational mode as the 1875 mg L\(^{-1}\) aqueous melamine solution. This low level sample had a melamine concentration of 0.025 mg L\(^{-1}\), was blue in color due to the Au NP agglomerates and had an absorbance value of 0.646 AU at 785 nm. Because the signal intensities for both samples were similar, the enhancement factor of the NP agglomerates for melamine can be approximated by calculating the ratio of the melamine concentrations in the two samples using the following equation:

\[
\text{Enhancement factor} = \frac{C_{\text{Ref}}}{C_{\text{Sig}}}
\]

where \(C_{\text{Ref}}\) is the concentration of the saturated melamine solution and \(C_{\text{Sig}}\) is the concentration of the melamine solution interacted with the Au NP agglomerates. Based on the most intense peak for melamine, there is approximately a 10\(^3\) signal enhancement in the NP spectra of melamine over the normal spectra was calculated.

Although the Au NP SERS method is intended for the screening and identification of melamine adulteration, it is important to determine whether a quantitative relationship between the NP-enhanced spectrum intensity and the melamine concentration exists. Since IPA is used as a clustering agent to form NP agglomerates prior to melamine binding, differences in agglomerate shapes and sizes can dramatically alter the enhancement factor by affecting the roughness of the Au NP agglomerates. To further investigate the enhancement property of NP/melamine interactions, quantitative analysis was performed by correlating the concentration of melamine prepared with Au NP agglomerates to the intensity of the melamine band at 712 cm\(^{-1}\) for several known melamine standard concentrations (0.05–25 mg L\(^{-1}\)). Fig. 3 shows the relationship of Raman peak intensity with varying concentrations of melamine. A linear concentration dependence (\(R^2 = 0.9983\)) was observed for samples prepared with melamine standards in the range of 0.05–0.5 mg L\(^{-1}\). The Raman signal observed for melamine reached a maximum at approximately 13,000 counts and the peak intensity remained constant for all melamine concentrations greater than 10 mg L\(^{-1}\). Due to the non-linearity of the NP/Raman method at high melamine concentrations, only a qualitative response as to whether or not melamine is detected in a sample can be answered.

3.5. Selectivity and limits of detection

To test the selectivity of the Au NP SERS method for melamine, a variety of different matrices were investigated that included food products and pharmaceutical excipients such as milk powder, whey protein, wheat gluten, wheat bran, lactose and povidone. Fig. 4 is an overlay of the control and 1 mg L\(^{-1}\) melamine spiked Raman spectra for the 7 different matrices in the spectral region of 550–1050 cm\(^{-1}\). The control samples exhibited similar Raman spectra as the Au NP spectrum in Fig. 2. No additional peaks from the matrix constituents were observed suggesting that effective isolation and extraction of melamine was achieved during the sample preparation process. Each spiked sample exhibited a Raman band at ~712 cm\(^{-1}\) due to the presence of melamine. The 1 mg L\(^{-1}\) melamine standard solution, illustrated in Fig. 2, resulted in a SN\(^{-1}\) ratio of approximately 40.8. If constituents in the matrices do not affect the recovery of melamine during the extraction process, the SN\(^{-1}\) ratios for the spiked samples should result in a similar SN\(^{-1}\) ratio as the 1 mg L\(^{-1}\) melamine standard (SN\(^{-1}\) = 40.8). A comparison of the SN\(^{-1}\) ratios for the melamine spiked samples are given in Table 2. A decrease in the SN\(^{-1}\) ratios was observed for melamine spiked samples prepared in infant formula, whey protein, wheat gluten and wheat bran. A 25–50% decrease is noted in the SN\(^{-1}\) ratios, which suggests that other molecules are being extracted from the complex food matrices and interacting with the binding sites of Au NP.
agglomerates. Since the current method is designed for field use and not quantitative purposes, a quick and simple extraction using IPA should be acceptable for melamine adulteration identification when directly followed by Au NP SERS analysis.

The recommended limit of melamine concentrations by the US Food and Drug Administration is 1 mg L⁻¹ in infant formula and 2.5 mg L⁻¹ in food and pharmaceutical products [35,36]. SERS detection utilizing Au NP agglomerates and a portable Raman system provides excellent sensitivity for the detection of trace amounts of melamine in IPA extracted samples down to the µg L⁻¹ level. The LOD for melamine in each of the seven sample matrices was determined as the concentration of the melamine standard that resulted in a peak at approximately 712 cm⁻¹ with a S/N⁻¹ ratio equal to 9. The LODs for each of the sample matrices are listed in Table 2. Lactose and milk powder resulted in the lowest LODs of ~100 µg L⁻¹, whereas the more complex matrices of infant formula and wheat gluten resulted in LODs of ~200 µg L⁻¹.

4. Discussion

SERS analysis of food and pharmaceutical products extracted in IPA resulted in a universal field deployable screening method for melamine adulteration. Au NP agglomerates, formed in an IPA solvated environment, have been shown to be an excellent and inexpensive substrate for SERS applications. The in-house synthesis of a single batch yields enough PPs for the SERS analysis of approximately 550 samples and has a very low cost of production. Colloidal Au NP solutions prepared for these studies were used over the course of approximately 10 weeks and remained stable. Agglomerates remained in solution for approximately 3 h after the addition of IPA to the PPs. A major advantage to using Au NP agglomerates as SERS substrates is that any portable Raman instrument capable of analyzing liquid samples can be used. Portable Raman devices are easy to use, convenient for on-site analysis and cost-effective when compared to chromatographic equipment.

For SERS enhancement to occur, Au NP agglomerates must be formed prior to sample introduction. The closely packed spheres, illustrated in Fig. 1B, form a roughened surface where periodic junctions or “hot spots” can aid in increasing the SERS signal of the adsorbed melamine molecules. The multi-dimensional agglomerate structures provide larger surface areas that can allow more target molecules to adsorb. Since the surface of each agglomerate is different, the location and orientation of melamine molecules can vary. Melamine molecules are unlikely to adsorb on the surface of the agglomerates in a flat manner, but more likely to adsorb on the edges of hot spots formed by the Au NPs [27]. The TEM image in Fig. 1C shows the variability in agglomerate structures after the surface interaction of melamine.

Since SERS is relative to the target molecule’s surface orientation, each agglomerate can result in a slightly different SERS signal. As a result, the recorded spectrum is the expected average of all agglomerates analyzed for a particular sample. The different orientation and placement of melamine molecules on agglomerates can cause changes in the SERS spectra. Shifts in frequency and variations in intensity can be observed in SERS spectra as compared to the normal Raman spectrum of melamine. In Fig. 2, the Raman spectrum of solid melamine exhibited an intense peak at 674 cm⁻¹ that is due to the ring breathing mode and the in-plane deformation vibrational modes [21,22,27]. When melamine was adsorbed to the surface of Au NP agglomerates, the same Raman band was shifted to 712 cm⁻¹. The 38 cm⁻¹ difference in Raman shift of the SERS spectrum can be attributed to the adsorption of melamine molecules to the surface of Au NP agglomerates. These results can further be supported by the zeta potential, where the zeta potential changed from −36.13 mV for Au NPs agglomerates to −17.67 mV for melamine/agglomerate surface interactions.

SERS with Au NP agglomerates provides excellent sensitivity to rapidly detect trace levels of melamine in IPA solvent down to 100–200 µg L⁻¹ depending on the sample matrix. As a universal screening method, a wide variety of food and pharmaceutical products can be tested with the application of a single procedure. The method does not require timely extraction procedures or multivariate statistical analysis techniques in order to achieve these limits. Many of the current SERS methods use costly solid substrates that typically can be used for one sample analysis and may require a Raman microscope, not rendering the technique as a screening method for easy field deployment. In addition, quantitative results are often obtained using complex chemometric models that includes partial least squares regression (PLS). Because PLS models are matrix dependent they may require laborious extraction procedures to remove all sample components except for melamine or they may not be suitable for a wide variety of samples in which new models would need to be developed for each sample product.

The benefit of using wavelet transform is that melamine detection is based on peak identification and does not require the development of chemometric models for its application. Care must be taken so that the specified peak for melamine (~712 cm⁻¹) is not in the spectral region where interferent peaks from the samples could be observed. The spectral window for melamine peak identification was set at 690–730 cm⁻¹. This region was selected to compensate for small shifts in the SERS signal that could arise from the sampling of different matrices, from batch to batch variability of the Au NPs, and differences in agglomerate formation. The typical S/N⁻¹ ratio for quantitative analysis at the LOD is 3, however, the S/N⁻¹ ratio for the universal screening method was determined experimentally due to variability of the SERS signal for melamine extracted from different sample matrices. The S/N⁻¹ ratio (S/N⁻¹ = 9) for the method was determined as the value where a sample at the LOD resulted in a positive response for the presence of melamine based on the 95% confidence interval. To promote the user-friendliness of a field deployable method, the data analysis procedure, as described, has been automated in the excel-based user interface and results in a pass/fail analysis for the screening of melamine adulteration.

5. Conclusions

Quality screening of food and pharmaceutical products is an increasing challenge due to the vast number of products being imported into the United States. In this paper we have explored the capabilities of using Au NP agglomerates as SERS substrates for trace level detection of melamine adulteration in a variety of food and pharmaceutical matrices. On-site analysis can be performed using portable Raman instruments and non-hazardous field safe solvents can be used to extract the melamine adulterant from various sample matrices. Agglomeration of the Au NPs prior to sample introduction improves the selectivity and sensitivity of the

Table 2
Signal to noise (S/N⁻¹) ratio and limit of detection (LOD) for melamine in each sample matrix.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 mg L⁻¹ melamine S/N⁻¹ ratio</th>
<th>LOD (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg.</td>
<td>Std. dev.</td>
</tr>
<tr>
<td>Standard</td>
<td>40.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Lactose</td>
<td>35.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Milk powder</td>
<td>37.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Infant formula</td>
<td>25.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Whey protein</td>
<td>31.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Povidone</td>
<td>35.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>20.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>22.7</td>
<td>5.5</td>
</tr>
</tbody>
</table>
screening method resulting in detection limits in the μgL⁻¹ range. To try and improve the selectivity and sensitivity of the screening method, further studies on the formation of Au NP agglomerates using various clustering agents in combination with non-hazardous extraction solvents with higher solubilities for melamine are being investigated.

Acknowledgements

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

References