IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 13, Issue 7 Ser. I (July. 2020), PP 01-07
www.iosrjournals.org

Detection of Melamine in Solid Pet Food Using Gold Nanoparticles and Method Validation with HPLC

E. Goodrow[1], A. Hummert[1], S. Adem[1*]
[1](*Department of Chemistry, Washburn University, Topeka, KS, USA)*
**Corresponding author at seid.adem@washburn.edu*

ABSTRACT: *We report a simple, easy and sensitive method for detection of melamine in a rather complex pet food sample using gold nanoparticles (AuNPs). The sample preparation was relatively short and effective in removing interference species from the sample. The aggregation of gold nanoparticles in the presence of melamine caused a color change from red to purple and a shift of the absorption band to longer wavelengths. The observable color change and the shift in wavelength was used for melamine detection in solid pet food sample. The limit of detection (LOD) based on visual color change and UV-Vis spectroscopy was found to be 0.25 ppm and 0. 14 ppm, respectively. This LOD is well below the threshold limit set by FDA of USA. The method was highly selective to melamine against other substances present in pet food. HPLC using resorcinol as internal standard was used to validate the performance of the developed method and the percent recovery of melamine ranges from 95.6% to 101.5% with CV less than 5%.*

Keywords: *Gold nanoparticles (AuNPs), pet food, melamine, HPLC, method validation, TEM, UV-Vis*

Date of Submission: 05-07-2020 Date of Acceptance: 21-07-2020

I. Introduction

Melamine is a nitrogen rich industrial chemical commonly used in the manufacture of plastics, flame retardants, eating utensils and other material important in everyday life. It is a metabolite of the insecticide cyromazine. Despite its wide applications, melamine is incriminated as a source of the 2007 outbreak of renal diseases and deaths of pet foods such as cats and dogs in the USA and subsequently for a major pet food recall in the USA and Canada. Melamine is also implicated in tainted milk powder that resulted in a large number of renal lithiasis in infants in China in 2008. In 2008, more than 250,000 urinary tract stone cases were reported in China alone caused by consumption of infant formula tainted with melamine [1]. What happened in 2007/2008 was a wake-up call to monitor different food samples containing wheat flour, wheat gluten, rice protein concentrate, corn flour, which are commonly used in animal feed and also in human food such as bread, pasta and baby food formula.

There are several analytical methods for the analysis of melamine and structurally related compounds such as cyanuric acid in foods and animal feeds. A series of methods including gold nanoparticle based sensors were reported following the 2007/2008 pet food and infant food incidents. Prior to this incident, the methods developed were mainly used to study the fate of the pesticide cyromazine, of which melamine is a degradation product. Methods were also developed for the analysis of melamine in beverages and liquid food simulants to evaluate the migration of melamine from melamine-formaldehyde resin used to make food contact materials such as eating utensils [2]. Current techniques to quantify melamine are all instrumental methods that combine either GC or HPLC with different detection schemes such as UV-Vis and MS. Both GC-MS [3,4] and LC-MS [5-7] require expensive, complex instrumentation and skilled personnel to run them. Furthermore, GC-MS requires sample derivatization and most of the reported LC-MS methods involve gradient elution and repeated column clean up. Most of the reported LC-MS methods involve gradient elution and repeated column clean up. Enzyme-linked immunosorbent assay (ELISA) based methods have also been reported [8,9]. These methods, however, can be labor intensive, time consuming, expensive and complex. Therefore, there is a need for simple, sensitive, portable and selective techniques for analysis of melamine.

The development of new materials in the nanometer scale (nanoparticles) is one of the most important developments in recent years. It is believed that nanoparticles would form the basis of many of the technological and biological innovations of this century with their unique physical, chemical, biological and optical properties. A large number of nanoparticles especially from noble metals such as gold have been synthesized. They can be manufactured in a variety of sizes and shapes including nanosphers, nanorods, nanocages. Gold nanoparticles are red wine in color unlike the bulk yellow colored gold that we all know. As the size or shape of the nanoparticles change, their observed color also changes. Moreover, they also change their color and optical properties when the medium surrounding them changes. Because of these unique properties, they got attention

in a number of fields such as medicine, engineering, material science, environmental science and chemistry for a number of applications. There are many reports on the application of gold nanoparticle based sensors for analysis of melamine in milk samples though there is not yet a well-established protocol on their application. To mention some, the applications of crown ether [10], riboflavin [11], cysteamine [12], ss DNA, [13], 3-mercapto-1-propanesulfonate [14] and Chtosan [15] modified, and unmodified bare gold nanaoparticles [16-19] for detection of melamine in milk and infant formula have been reported.

Herein, we report a method based on citrate stabilized gold nanoparticles for detection of melamine in a rather highly complex pet food sample. To our knowledge, this is the first detailed report on the development of sensor based on gold nanoparticles for detection of melamine in pet food sample and its validation using HPLC. Here, we also reported a simple and efficient sample preparation method for both spectroscopic and HPLC analysis of melamine in pet food. It is our belief that recommendations can be made by concerned regulatory organizations based on cost effectiveness, time and practicality of the proposed method for portable on-site screening of melamine adulteration to ensure food safety.

II. Experimental

2.1 Reagents and Materials

Melamine, sodium citrate, $HAuCl_4$ and activated black charcoal were purchased from Sigma-Aldrich. Acetonitrile and trichloroacetic acid (30% w/v) were purchased from Fischer Chemical. Resorcinol was purchased from Merck. Pet Pride cat food was purchased from a local grocery store. All glassware was cleaned with aqua regia (a mixture of HCl/HNO_3, in a 3:1 ratio, by volume), rinsed with Milli-Q water (18.2 MΩ cm) and then dried prior to use. All solvents and other chemicals are of analytical grade and used as received without further purification. Millipore Milli-Q water was used in all experiments in this report.

2.2 Apparatus

A Perkin-Elmer Lambda 465 UV-Vis spectrophotometer was used to collect absorption spectra. TEM images were taken at the Nebraska Nanoscale Facility (Lincoln, NE). Agilent 1260 series HPLC system with manual injector was used for method validation. The HPLC system consists of a quaternary pump, degasser, a column thermostat and a diode array detector. A C-18 column (4.6 x 250 mm, 3.5 µm particle, Agilent Pursuit XRS, column temperature of 25°C) was used. The mobile phase was a mixture of commonly used solvents (61% H_2O/38% methanol/1% glacial acetic acid) and separation was carried out at a flow rate of 1.0 mL/min. Detection was conducted with diode array detector at 236 nm using resorcinol as internal standard.

2.3 Preparation of Gold Nanoparticles

The Turkevich method was used to synthesize citrate-stabilized AuNPs [20]. On a hot/stir plate, an Erlenmeyer flask containing 250-mL of 0.01% gold chloride (w/v) solution was brought to boiling before rapidly adding 10 mL of 1% trisodium citrate (w/v). The solution was then heated and stirred for another 10 minutes, and was then allowed to return to room temperature while stirring. The resulting gold nanoparticle solution is used in all experiments in this report.

2.4 Preparation of Pet Food Solution

A pet food solution was made by mixing 0.30 g of Pet Pride cat food, 3.00 mL of Milli-Q water, and 25 mL of acetonitrile:water (50:50) mixture in a 50 mL centrifuge tube. The solution was then vortexed for about a minute, sonicated for 30 minutes, and vortexed again for another minute. Then, 0.25 g of activated black charcoal was added to the mixture to remove the colorant from the solution. The solution was vortexed and then centrifuged for additional 10 minutes at 3500 revolutions per minute (rpm). After filtering the supernatant through a 0.22 µm filter, it was treated with 30% trichloroacetic acid (w/v). The supernatant was then vortexed and brought to a neutral pH using 1.0 M NaOH. Finally, the mixture was centrifuged and the supernatant was filtered. For the analysis of melamine, varying volumes of 10 ppm melamine were taken and added to 1.0 mL of AuNP solution. Each mixture was brought to a total volume of 5.0 mL with the pet food solution. The solution was allowed to stand for a minute before the UV-Vis spectrum was taken. The LOD of melamine was determined from the plot of the absorbance ratio (A_{780}/A_{530}) vs. concentration of melamine or from naked eyes observation of the color change. For percent recovery studies by reversed phase HPLC to validate the method, varying concentrations of melamine was added to the pet food sample and underwent the same sample treatment as discussed above.

III. Results and Discussion

3.1 Characterization of AuNPs in the Presence and Absence of Melamine

Figure 1 shows the scheme for sample preparation used in the analysis of melamine in pet food samples. The initial food sample solution was colored which could interfere with the analysis of melamine. It was thus needed to treat the colored food sample solution with activated charcoal to remove the coloring material. The clear solution obtained was used for all the experiments in this report. The citrate stabilized gold nanoparticles prepared by established methods were characterized in pet food solution before and after addition of melamine.

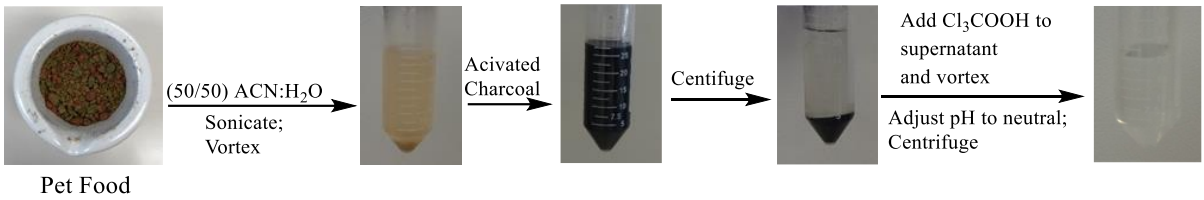

Figure 1: Schematic representation of the steps involved in pet food sample preparation.

Figure 2 shows photographs of the AuNP solution after 0.0 ppm, 0.10 ppm, 0.25 ppm, 0.75 ppm, and 1.50 ppm of melamine was added into the pet food solution. The red color of the dispersed gold nanoparticles solution is due to a plasmon peak around 530 nm with very high molar absorptivities (2.7×10^8 $M^{-1}cm^{-1}$), which is several times higher than that of highly absorbing organic dyes [21-23].

Figure 2: Photograph of color change of pet food samples spiked with different concentrations of melamine. (From left to right: 0 ppm, 0.10 ppm, 0.25 ppm, 0.75 ppm, and 1.50 ppm.

As can be seen, there was a distinct color change of the solution from red to purple and a bathochromic shift of the absorption band in the presence of melamine (Fig. 4A). The observed color change and the shift of the absorption band to longer wavelengths is the result of aggregation of AuNPs upon the addition of melamine. To corroborate this aggregation effect, TEM images of AuNPs in the presence and absence of melamine were obtained. Figure 3A depicts the AuNPs in the absence of melamine, whereas Fig. 3B and 3C display the TEM images of AuNPs in the presence of melamine. As the TEM images show, the AuNPs are well dispersed in the absence of melamine and are aggregated in the presence of melamine. The mechanism of aggregation of citrate stabilized gold nanoparticles in the presence of melamine was discussed in previous reports [23, 24].

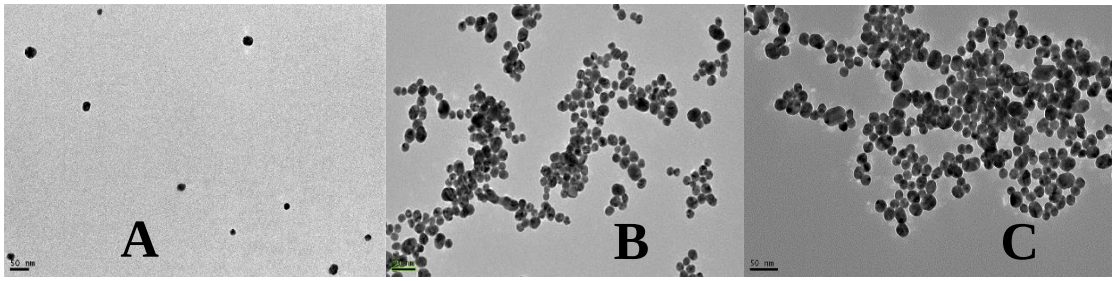

Figure 3: A) TEM image of AuNPs in pet food solution. B) TEM image of AuNPs in pet food solution with 1ppm melamine. C) TEM image of AuNPs in pet food solution with 5 ppm melamine.

3.2 Sensitivity of Melamine Detection

We used the synthesized gold nanoparticles for detection and quantification of melamine in pet food samples. Both visual color change observation along with photos with student cell phone and UV-Vis spectroscopy were used to quantify melamine and determine its LOD in pet food sample. As discussed above, the color of the gold nanoparticle solution changed from red to purple upon addition of melamine as the solution

transitions from dispersed state to an aggregated state. The lowest concentration of melamine that caused observable color change of the gold nanoparticles with naked eye was 0.25 ppm (Fig. 2), which we called the visual limit of detection. Figure 4A displays the absorption spectra of AuNPs in the presence of melamine ranging from concentrations of 0.0 ppm to 3.5 ppm.

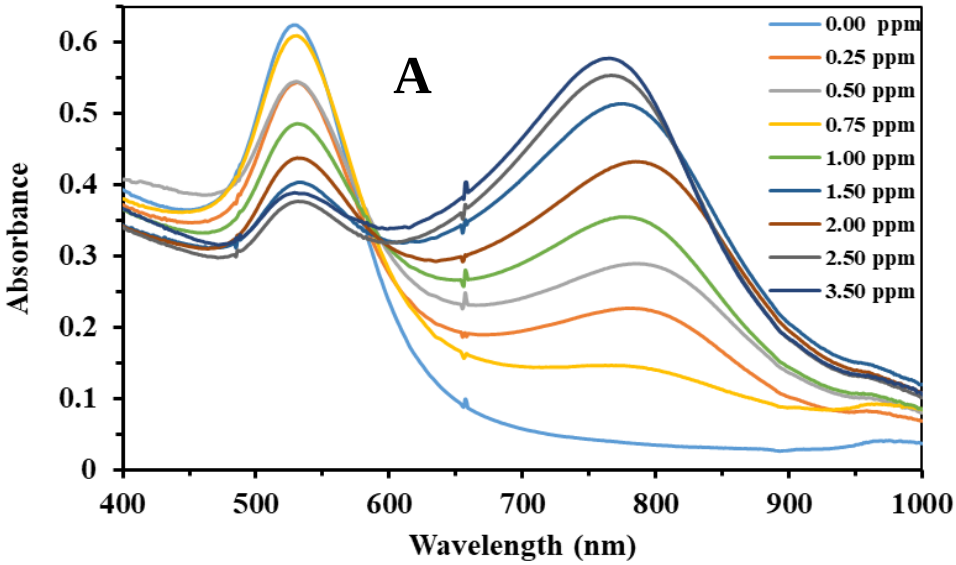

Figure 4A: UV-Vis spectra of AuNPs in pet food solutions containing different concentrations of melamine.

The peak centered about 530 nm is due to the surface plasmon resonance (SPR) of the AuNPs in their dispersed state [21]. In the presence of melamine, the AuNPs were aggregated causing a noticeable redshift of the SPR band to a wavelength around 780 nm. It can easily be noticed that the absorbance decreases at 530 nm while it increases around 780 nm as the concentration of melamine increases. Figure 3B shows the absorbance ratio, A_{780}/A_{530}, which was calculated based on the data shown in Fig. 4A increases with increasing concentration of melamine in the range of 0.0-3.0 ppm (Inset, Fig. 4B). The corresponding calibration equation was $y = 0.551x + 0.118$ with R^2 value = 0.993. The limit of detection (LOD = 3σ/slope) was calculated to be 0.14 ppm, which is much lower than that observed by visual color change with naked eyes. However, both the visual LOD and the one calculated from UV-Vis spectrophotometric data are well below the safety limit of 2.5 ppm by the FDA for non-infant products [16].

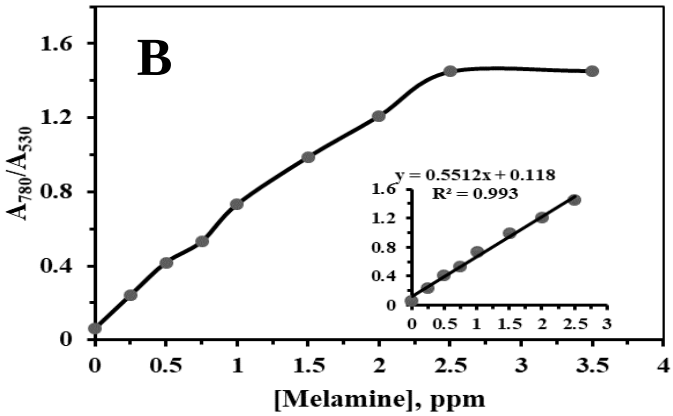

Figure 4B: Plot of absorbance ratio of A_{780}/A_{530} vs. melamine concentration. Inset in B displays the linearity of the curve of the absorption ratio of A_{780}/A_{530} vs. melamine concentration. Error bars represent standard deviations from three measurements.

3.3 Selectivity of Gold Nanoparticles to Melamine Detection

Food and feed samples have very complex matrices consisting of proteins, mineral salts, fats, carbohydrates and other compounds that may interfere with the analysis and cause false positive results. A previously reported milk sample treatment method [16, 19, 25] to remove interferences in milk was not effective

to be used for analysis of pet food samples, at least in our case. Thus, we developed a simple sample treatment procedure as discussed in section 2 and evaluated the selectivity of the developed method for melamine detection. The gold nanoparticle solution was mixed with a pet food solution that has been spiked with various compounds listed on the labels of the solid pet food and cyanuric acid, one of the structural analogues and hydrolysis product of melamine. Both visual color change observation and UV-Vis measurements were employed for this study, too. As can be seen in Fig. 5A and 5B, the only substance that caused color change from red to purple and a shift to longer wavelength of the absorption spectrum of the gold nanoparticle solution was melamine. It can also be seen from Fig. 5C that melamine showed the highest absorbance ratio, A_{780}/A_{530}, value of 1.34 and the others have a very low absorbance ratio value. Even increasing the concentration of the compounds 200-fold that of melamine didn't cause a color change or a shift in absorption spectrum indicating that they didn't cause aggregation of gold nanoparticles. These results clearly show that the compounds present in the food sample didn't interfere with melamine detection and the method is selective enough for routine analysis of melamine in rather very complex pet food samples.

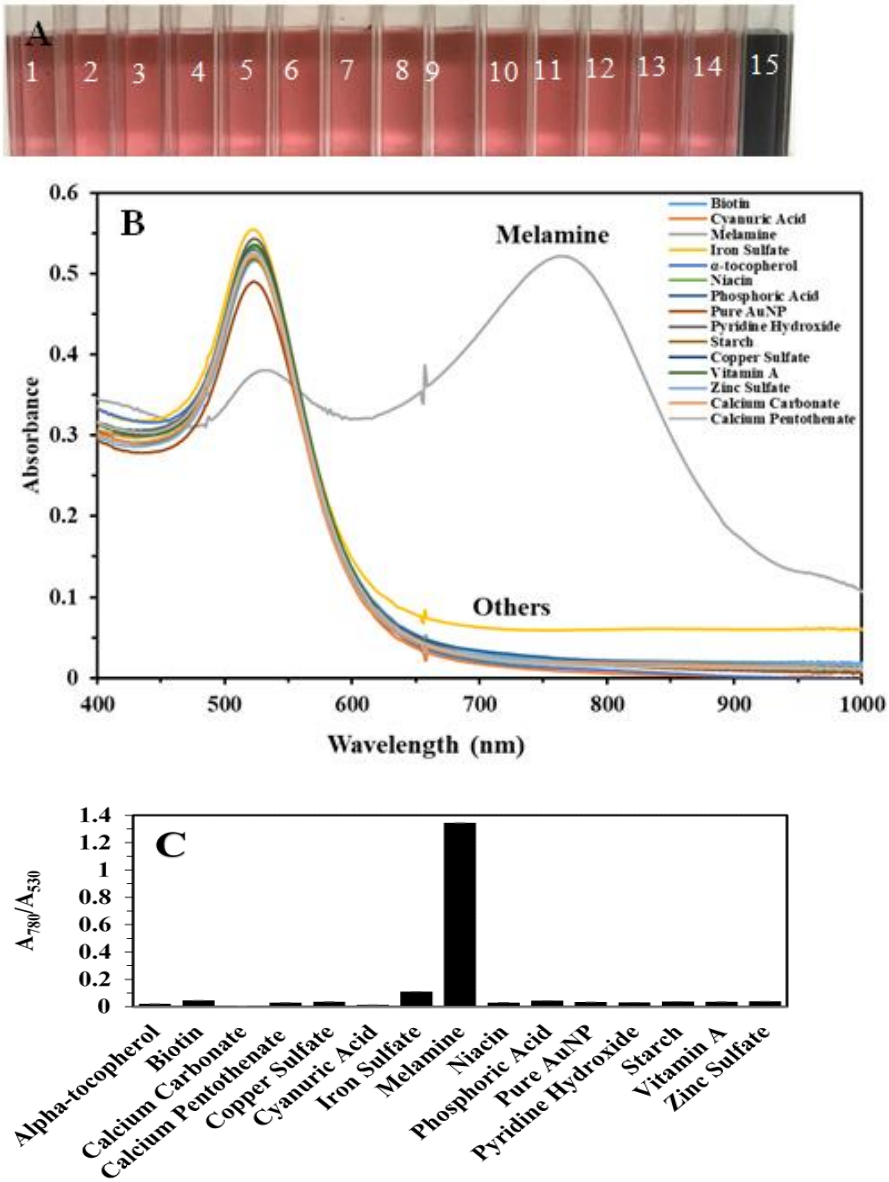

Figure 5: A) Photographs of color change of AuNPs in pet food samples in the presence of melamine and other interference substances B) UV-Vis spectra of AuNPs solution in pet food solution in the presence of melamine and other interferences substances and C) the corresponding absorbance ratio A_{780}/A_{530} for each substance. Error bars represent standard deviations from three measurements.

3.4 Method Validation

The colorimetric method developed based on gold nanoparticles for detection of melamine in pet food samples is fast that requires inexpensive instrumentation. This would make it an ideal tool for on-site screening of melamine adulteration in food samples. However, it has to be noted that food samples such as pet food have complex matrices that can greatly affect the results of analyses. As a result, time consuming and multistep sample preparation techniques need to be developed to remove or minimize matrix effects to get a good quality of data in terms of sensitivity, precision and accuracy. Thus, it is important to validate the method with established techniques for a better detection and quantification. For this purpose, Agilent 1260 series HPLC system with experimental conditions as mentioned in section 2 was employed. To evaluate accuracy of the method, a varying concentration of melamine was added into the pet food sample, passed through the same sample preparation step as mentioned in section 2 and then filtered before injected into the HPLC system. As shown in Fig. 6A, no interfering peaks were observed at the retention times of both melamine and resorcinol indicating the effectiveness of the sample preparation method in removing interfering species.

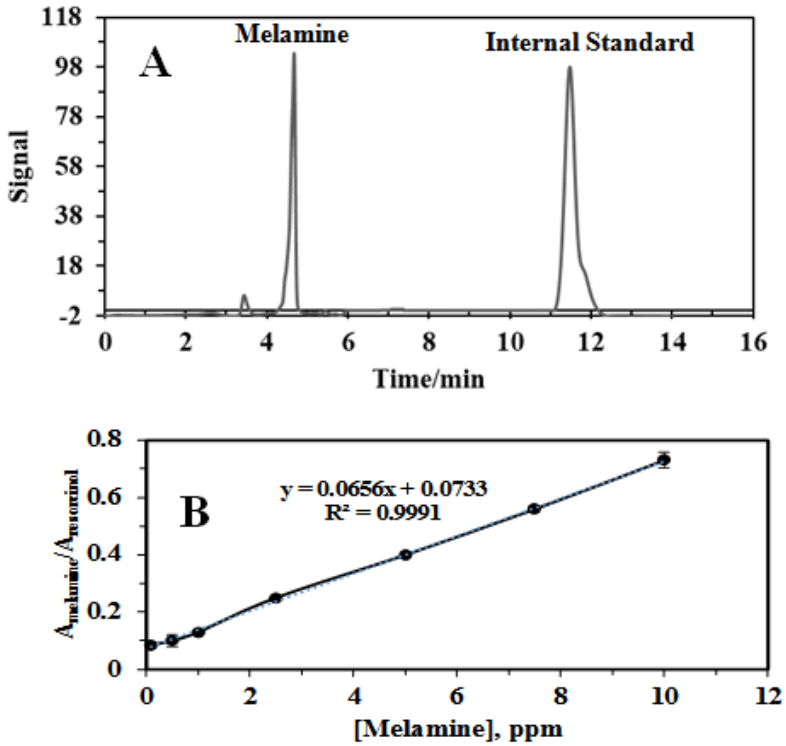

Figure 6: A) Chromatogram of melamine and resorcinol (internal standard) in pet food sample obtained using Reversed Phase Chromatography: Experimental conditions: C-18 column; mobile phase (61%H_2O, 38% methanol, 1% galcial acetic acid) at 1 mL/min flow rate, detection at 236 nm. B) A plot of the ratio of area of melamine to the area of resorcinol vs. concentraion of mealmine. Error bars represent standard deviations from six measurements.

A calibration curve (Fig. 6B) from the plot of the ratio of the mean peak area of melamine to that of the internal standard ($A_{melamine}/A_{resorcinol}$) as a function of concentration of melamine was used to calculate percent recovery using the equation:

$$\% \text{ recovery} = \frac{C_{\text{spiked sample}} - C_{\text{unspiked sample}}}{C_{\text{added}}} \text{ x } 100 \text{ (C = concentration)}$$

As can be seen in Table 1, the percent recoveries vary from 97 to 101% (with coefficient of variation CV less than 5%) indicating the accuracy and reproducibility of the developed method and its feasibility for routine detection of melamine in pet and other food samples. Furthermore, the LOD of detection using the chromatographic method was calculated to be 0.28 ppm which is very similar to that of the visual LOD obtained with the gold nanoparticle sensor.

Added Melamine, ppm	Measured Melamine, ppm (Average, n = 6)	Percent Recovery (Average, n = 6)	% CV (n = 6)
0.10	0.09	100	1.8
0.50	0.44	95.6	1.9
1.0	1.2	101.5	3.2
2.5	3.0	98.3	4.9
5.0	5.1	101.1	0.8
7.5	7.3	96.9	1.8

Table 1: Percent recovery of melamine from pet food solution using reversed phase HPLC.

IV. Conclusion

In this report, a simple, sensitive and reliable colorimetric probe based on gold nanoparticles for detection of melamine in pet food samples is demonstrated. It has the potential to be developed as a new technique for the detection of melamine in pet food and could possibly be used for portable on-site detection as well. This would be particularly important for those regions of the world which increasingly rely on imported food for both human consumption and animal feed but unable to afford expensive instrumentations which require skilled personnel to operate them to ensure food safety. In the proposed method, a visual limit of detection of 0.25 ppm and a further lower limit of detection of 0.14 ppm was obtained with the help of UV-Vis spectroscopy. We have also showed that other substances present in pet food don't interfere with the analysis of melamine. However, there are instances when compounds in the food sample cause color change and thus it is appropriate to use the proposed method for screening purposes and validate it with established techniques such as HPLC for accurate determination and quantification of melamine in complex pet food samples.

Acknowledgements

This work was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20 GM103418. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

References

[1]. M. Hong, F. Sai, W. N. Young, Z. Lei, Z. P. Ping, L. G. Jing, C. J. Hui and Z. F. Yun, *Biomedical and Environmental Sciences*, 2009, **22**, 87-94.
[2]. D. M. Mohamed and H. E. Roquala, *J. Environ. Anal. Toxicol.*, 2012, **2**, 1-4.
[3]. J. Li, H. Y. Qi and Y. P. Shi, *J. Chromatogr. A*, 2009, **1216**, 5467-5471.
[4]. X. Xu, Y. Ren, Y. Zhu, Z. Cai, J. Han, B. Huang and Y. Zhu, *Anal. Chim. Acta*, 2009, **650**, 39-43.
[5]. M. Ibanez, J. V. Sancho and F. Hernandez, *Anal. Chim. Acta*, 2009, **649**, 91-97.
[6]. A. Desmarchelier, M. G. Cuadra, T. Delatour and P. Mottier, *J. Agri. Food Chem.*, **2009**, 57, 7186-7193.
[7]. B. N. Tran, R. Okoniewski, R. Storm, R. Jansing and K. M. Aldous, *J. Agri. Food Chem.*, **2010**, 58, 101-107.
[8]. E. A. Garber, *Journal of Food Prot.*, 2008, **71**, 590-594.
[9]. J. Liu, Y. Zhong, J. Liu, H. Zhang, J. Xi and J. Wang, *Food Control*, 2010, **21**, 1482-1487.
[10]. H. Kuang, W. Chen, W. J. Yan, L. G. Xu, Y. Y. Zhu, L. Q. Liu, H. Q. Chu, C. F. Peng, L. B. Wang, N. A. Kotov and C. L. Xu, *Biosens. Bioelectron.*, 2011, **26**, 2032-2037.
[11]. B. Roy, A. Saha and A. K. Nandi, *Analyst*, 2011, **136**, 67-70.
[12]. X. Liang, H. Wei, Z. Cui, J. Deng, Z. Zhang, X. You and X. E. Zhnag, *Analyst*, 2011, **136**, 179-183.
[13]. H. Huang, L. Li, G. H. Zhou, Z. H. Liu, Q. Ma, Y. Q. Feng, G. P. Zeng, P. Tinnefeld and Z. K. He, *Talanta*, 2011, **85**, 1013-1019.
[14]. H. C. Su, H. Fan, S. Y. Ai, N. Wu, H. M. Fan, P. C. Bian and J. C. Liu, *Talanta*, 2011, **85**, 1338-1343.
[15]. H. Guan, J. Yu and D. Chi, *Food Control*, 2013, **32**, 35-41.
[16]. L. Guo, J. Zhong, J. Wu, F. Wu, G. Chen, X. Zhang and S. Lin, Talanta, 2010, **82**, 1654-1658.
[17]. Q. Zhou, N. Liu, Z. Qie, Y. Wang, B. Ning and Z. Gao, *J. Agric. Food. Chem.*, 2011, **59**, 12006-12011.
[18]. H. Chi, B. Liu, G. Guan, Z. Zhang and M. Y. Han, *Analyst*, 2010, **135**, 1070-1075.
[19]. W. Chen, H. H. Deng, L. Hong, Z. Q. Wu, S. Wang, A. L. Liu, X. H. Lin and X. H. Xia, *Analyst*, 2012, **137**, 5382-5386.
[20]. H. Wolfgangan, N. T. K. Thanh, J. Aveyard and D. G. Femig, *Anal. Chem.*, 2007, **79**, 4215-4221.
[21]. Y. L. Hung, T. M. Hsiung, Y. Y. Chen, Y. F. Huang and C. C. Huang, *J. Phys. Chem. C*, 2010, **114**, 16329-16334.
[22]. L. D. Liu, W. Qu, W. Chen, W. Zhang, Z. Wang and X. Jiang, *Anal. Chem.*, 2010, **82**, 9606-9610.
[23]. L. Li, B. Li, D. Cheng and L. Mao, *Food Chemistry*, 2010, **122**, 895-900.
[24]. K. Ai, Y. Liu and L. Lu, *J. Am. Chem. Soc.*, 2009, **131**, 9496-9497.
[25]. K. Wagers, T. Chui and S. Adem, *IOSR-JAC*, 2014, **7**, 15-20.

E. Goodrow,et. al. "Detection of Melamine in Solid Pet Food Using Gold Nanoparticles and Method Validation with HPLC." *IOSR Journal of Applied Chemistry (IOSR-JAC),* 13(7), (2020): pp 01-07.