Analytical Methods for the Evaluation of Melamine Contamination

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ABSTRACT: There is an urgent need for the analysis of melamine in the global pharmaceutical supply chain to detect economically motivated adulteration or unintentional contamination using a simple, nondestructive analytical technique that confirms the extent of adulteration in a shorter time period. In this work, different analytical techniques (thermal analysis, X-ray diffraction, Fourier transform infrared (FT-IR), FT-Raman, and near-infrared (NIR) spectroscopy) were evaluated for their ability to detect a range of melamine levels in gelatin. While FT-IR and FT-Raman provided qualitative assessment of melamine contamination or adulteration, powder X-ray diffraction and NIR were able to detect and quantify the presence of melamine at levels as low as 1.0% *w/w*. Multivariate analysis of the NIR data yielded the most accurate model when three principal components were used. Data were pretreated using standard normal variate transformation to remove multiplicative interferences of scatter and particle size. The model had a root-mean-square error of calibration of 2.4 ($R^2 = 0.99$) and root-mean square error of prediction of 2.5 ($R^2 = 0.96$). The value of the paired t test for actual and predicted samples (1%–50% *w/w*) was 0.448 ($p < 0.05$), further indicating the robustness of the model. Published 2013. This article is a U.S. Government work and is in the public domain in the USA 103:539–544, 2014

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INTRODUCTION

The global nature of pharmaceutical supply makes contamination of pharmaceutical ingredients of prime importance. Product adulteration with melamine is a serious public health concern as it is a known nephrotoxin.[1] Economically motivated melamine contamination of various products has been in the spotlight for the past several years owing to a series of highly publicized incidents. Besides the pet food recalls in the United States in 2007, China reported in 2008 adulteration of milk, infant formula, and other milk-derived products that affected over 300,000 infants worldwide.[2] Melamine is an industrial chemical used in the manufacturing of resins for surface laminates and adhesives in the production of wood-based panels. Melamine or its resins are also used in the manufacture of dinnerware, additives for textiles, and as flame-retardant in foam mattresses. Hence, analytical methodologies are needed to rapidly detect the presence of intentional adulteration or unintentional contamination from melamine.

Melamine or 2,4,6-triamino-*s*-triazine (Fig. 1) is a white crystalline solid with a molecular weight of 126.12 g/mol. Melamine has a pK_a of 5.35 at 25°C and its solubility has been reported to be 3.24 mg/mL at 20°C.[3,4] The Merck index reports the melting point of melamine as <250°C while Hawley's condensed chemical dictionary reports it as 354°C.[5,6] Mast et al. reported the elimination half-life of melamine as 2.7 h and the renal clearance as 2.5 mL/min. Following oral administration of 250 mg/kg melamine to rats, 50% of the parent compound was excreted in
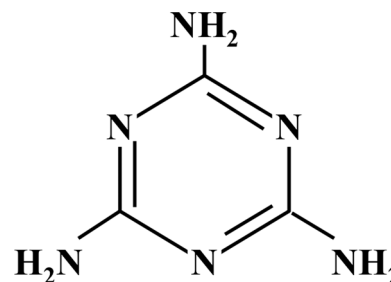

Figure 1. Chemical structure of melamine.

the urine in less than 6 h. LD_{50}, the lethal dose of a compound that would result in death in 50% of the tested animals, in rats has been reported as 3.2 g/kg body weight.[7]

In 2007, the Food and Drug Administration (FDA) published a guidance titled "Pharmaceutical Components at Risk for Melamine Contamination" which listed 23 components considered to be at risk for melamine contamination.[8] Gelatin is one of the pharmaceutical components on this list that may be vulnerable to melamine contamination as it is the main component in both hard and soft gelatin capsules. This inclusion is based upon the fact that gelatin has a high protein (nitrogen) content (98%–99% protein on dry weight basis), and the possibility exists that adulteration could be economically motivated as melamine is inexpensive and also has a very high nitrogen content of 66.6% by mass. Companies assaying for only nitrogen content would not be able to distinguish between melamine and the desired protein.

FDA has also developed and published a gas chromatography–mass spectrometry (GC–MS) method to screen for the presence of melamine and some related analogues in a variety of matrices at a minimum reporting level (MRL) of 10 μg/g or more, and guidance to extend the MRL

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to 2.5 μg/g are also provided.[9] Another approach to analyze melamine is based on surface-enhanced Raman spectroscopy with a limit of detection between 30 and 200 μg/mL depending on the sample.[10,11] A high performance liquid chromatography procedure with ultraviolet detection using an acetate buffer and acetonitrile mobile phase has been used to determine melamine in liquid milk and milk powder.[12] The analysis time is completed within 10 min and avoids the labor intensive derivatization that accompanies the GC–MS analysis. On the basis of this method using 10 injections of a 1 μg/mL standard, the minimum detectable level of melamine was 0.015 μg/mL. Currently, however, information is lacking on the analytical evaluation and assessment of melamine present in gelatin blends.

The purpose of this study is to evaluate various rapid and nondestructive analytical techniques, namely powder X-ray diffraction (PXRD), Fourier-transform infrared spectroscopy (FT-IR), Fourier-transform Raman spectroscopy (FT-Raman), and near-infrared spectroscopy (NIRS), for their utility in rapidly detecting and quantitating melamine using gelatin as a model matrix. Because melamine does not exhibit polymorphism, samples were also analyzed using differential scanning calorimetry (DSC) to clarify the apparent difference in the melamine melting point that has been reported in the literature.

MATERIALS AND METHODS

Materials

Gelatin (Type A, 300 Bloom), melamine, copper sulfate pentahydrate, borosilicate glass scintillation vials (15×45 mm^2), and methanol were purchased from local distributors and in-house distilled and de-ionized water was used for all studies.

USP Identification Tests

Identification tests A and B for gelatin NF were performed according to the USP 36/NF 31 procedure.[13]

Preparation of Melamine Blends

Blends of melamine and gelatin from 1% to 50% w/w were prepared by accurately weighing the individual components on an analytical balance and mixing by the method of geometric dilution into glass scintillation vials. Samples were secured in a plastic container and were blended at 100 rpm for five min using a shaker mixer. The concentration of all blends is expressed in terms of percentage w/w melamine unless stated otherwise.

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was performed to study gelatin's decomposition temperature. Samples (2–5 mg) were accurately weighed and heated from 25°C to 260°C using an underlying heating rate of 2°C/min. Nitrogen gas at a flow rate of 25 mL/min was used to maintain an inert atmosphere.

Differential Scanning Calorimetry

Accurately weighed samples (2–5 mg) were sealed in hermetic aluminum pans. The temperature range for gelatin and melamine-gelatin blends ranged from 25°C to 260°C, while for neat melamine, the temperature range was between 25°C and 400°C. An underlying heating rate of 2°C/min. was used. Nitro-

gen gas at a flow rate of 50 mL/min was used to maintain an inert atmosphere.

Powder X-Ray Diffraction (PXRD)

Powder X-ray diffraction (PXRD) patterns for pure melamine, gelatin, and their blends were collected using a Bruker D8 Advance with DaVinci design (Bruker AXS, Madison, Wisconsin) using Cu Kα radiation ($\lambda = 1.5405$ Å) at a voltage of 40 KV and current of 40 mA and equipped with the LYNXEYE scintillation detector. Corundum was used as an external standard to calibrate the PXRD instrument. Powder sample was placed in the sample holder, pressed, and scanned over 2θ range of 5°–60° with a step size of 0.01°C at 1 s/step. Samples were rotated at 15 rpm during measurements to get an average diffractogram of the sample.

Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectra (FT-IR) of pure melamine, gelatin, and their blends were collected using an attenuated total reflectance FTIR spectrometer (Tensor 27; Bruker Optics, Billerica, Massachusetts) by placing a small sample on the diamond crystal. Air in the sample was removed by pressing it with the attached arm. A total of 32 scans in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} were collected and averaged for each sample.

FT-Raman Spectroscopy

FT-Raman spectra were collected using a Raman spectrometer (Bruker Optics, Billerica, Massachusetts) equipped with a Nd-YAG laser source at 1064 nm and a liquid nitrogen-cooled germanium detector. The measurements were carried out at ambient temperature using a 310 mW laser source. The samples were packed in aluminum sample holders and measured over the Raman shift range of 100–3600 cm^{-1}. Each spectrum was the average of 32 scans with a resolution of 1 cm^{-1}. Polystyrene and naphthalene were used as reference material standards to monitor wave number accuracy.

Near Infrared Spectroscopy

Near infrared spectra (NIRS) for pure melamine, gelatin, and their blends were collected by placing the samples into 15×45 mm^2 borosilicate glass vials and scanning them in diffuse reflectance mode over the range of 1100–2500 nm. Duplicate scans, with a resolution of 2 nm, were taken of each sample and averaged into one spectrum.

Data Analysis and Interpretation

Spectral data were exported in American standard code for information interchange format for multivariate principal component analysis (PCA), which was performed before partial least squares (PLS) regression models were developed. PCA analysis only uses the spectral data and does not have the ability to predict the sample concentrations, whereas PLS analysis projects the spectral data on the sample concentration, thereby providing the ability to predict the sample concentration. PCA score plots were used to examine any relevant and interpretable structure in the data as well as for outlier detection. For spectroscopic data, calibration models were developed using PLS regressions with full cross-validation (10 samples/group). The optimum number of PLS factors was determined as indicated by the lowest number of factors that gave the closest to the