Potential of Raman Spectroscopy and Imaging Methods for Rapid and Routine Screening of the Presence of Melamine in Animal Feed and Foods

YONGLIANG LIU, KUANGLIN CHAO,* MOON S. KIM, DAVID TU- SCHEL, OKSANA OLKHOVYK, and RYAN J. PRIORE

Environmental Microbial and Food Safety Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, USDA, Building 303, BARC-East, 10300 Baltimore Avenue, Beltsville, Maryland 20705 (Y.L., K.C., M.S.K.); and ChemImage Corp., 7301 Penn Avenue, Pittsburgh, Pennsylvania 15208 (D.T., O.O., R.J.P.)

Index Headings: **Raman spectroscopy; Chemical imaging; Melamine; Contaminants; Food safety.**

INTRODUCTION

The impact of melamine-contaminated animal feed ingredients on food safety has become a major public concern ever since melamine was identified as the organic compound responsible for the deaths of a significant number of cats and dogs in 2007[1] by way of adulterated pet food. Melamine, a common industrial chemical often added to resins to improve flame resistance and proposed as an alternative form of fertilizer-N for plant growth,[2,3] was found to be intentionally added to animal feed in amounts ranging from 0.2% to 8% of total mass as a way to boost the products' apparent protein content. It was also used as a binder in the production of pelleted feed for animals.[4] In addition to melamine, a small amount of cyanuric acid, ammeline, and ammelide were also detected in pet feed and in the tissues and urine of dead pets that had consumed the contaminated food.[5] Even though it is possible that cyanuric acid, ammeline, and ammelide were added, their presence more likely resulted from the degraded derivatives of melamine.[6]

There is a great concern that melamine will again enter the food chain and be consumed by humans and animals. As part of the Food Protection Plan, US federal agencies such as the FDA and FSIS and other organizations have established GC-MS and LC-MS/MS procedures for the analysis of melamine in food/feed commodities.[7,8] Although they can detect melamine contaminants in trace amount, these time-consuming laboratory procedures require chemical solvents for the extraction steps and depend on expensive mass spectrometry.

Rapid, nondestructive, and routine methods for the specific detection of melamine in raw feed materials are increasingly important, not only for public health concerns but also for melamine screening to prevent protein fraud. Undoubtedly, the well-defined mass spectroscopic technique is preferred due to its low detection limit and capability for structural elucidation; however, since adulteration of raw materials by melamine usually occurs in higher concentrations in order to affect protein content, the high sensitivity of the mass spectroscopic technique may not be necessary. In addition, mass spectrometry might not be sufficiently rapid to screen for the presence of melamine in a large number of food/feed materials from very different sources, because the identification process includes sample-specific extraction procedures, which are labor-intensive and time-consuming.

Fast melamine screening requires minimal sample preparation (e.g., no extraction or centrifugation), routine analysis of a number of samples without reagents, minimal procedural steps, and easy operation and interpretation of results. The Raman technique, which has been used to obtain structural information on melamine,[9] is an alternative approach that can be applied to solid materials with no sample pretreatment. In addition, the use of the Fourier transform (FT) methodology and a 1064 nm excitation laser in the near-infrared (NIR) region provides precise wavenumber measurement and good-quality Raman spectra by reducing the interference from fluorescence and photodecomposition of colorants present in food and feed.[10,11]

Raman studies of melamine and melamine-modified resins have been reported in the literature, and the results have revealed the feasibility of the Raman technique for the structural characterization of melamine state in resins.[12,13] However, there have been few reports on Raman investigation and identification of melamine in complex food and feed systems.

The objectives of this study were (1) to identify the characteristic Raman bands in melamine-contaminated wheat flour, corn gluten, and soybean meal mixtures; and (2) to develop simple and universal ratio algorithms for qualitative and quantitative analysis of melamine in mixtures. The ultimate goal is to develop both Raman spectroscopy and Raman chemical imaging methods for rapid, accurate, nondestructive, specific, and routine screening of the presence of melamine in food and feed for public/animal safety and security.

MATERIALS AND METHODS

Chemicals, Feeds, and Glass Tubes. Chemical (melamine, >99+%) was purchased from Sigma-Aldrich Co. and used without further purification. Dry food/feed materials (wheat flour, corn gluten, and soybean meal) were obtained from a
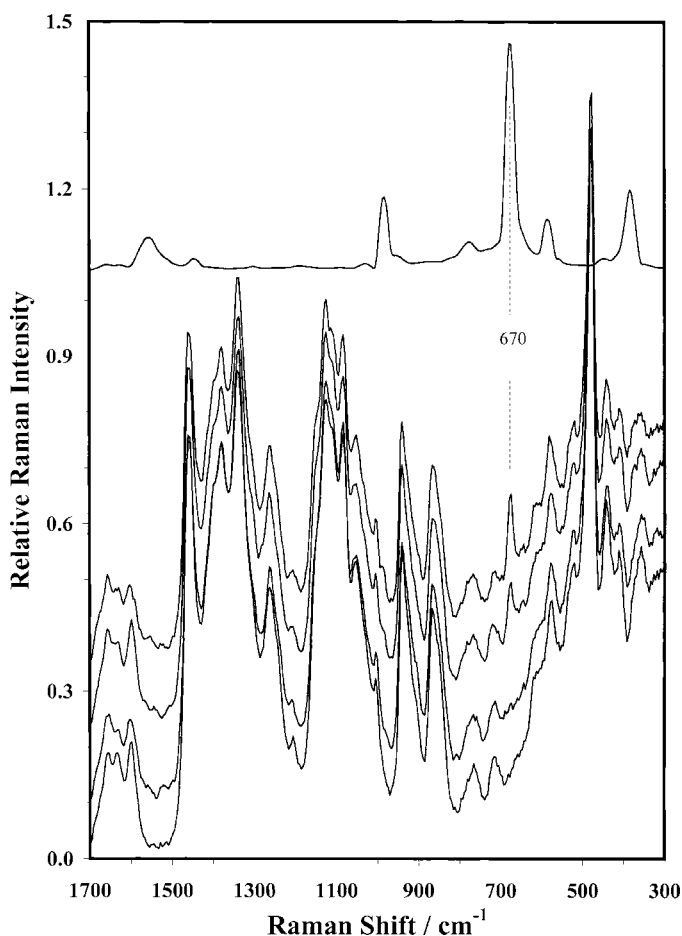
FIG. 1. Representative FT-Raman spectra of melamine-contaminated wheat flour at percentages (w/w) of 0 (pure wheat flour), 0.2, 0.5, 1.0, and 100% (pure melamine), from bottom to top. Raman intensity of melamine was scaled and all spectra were shifted vertically for direct comparison.

FIG. 2. Relationship between the intensity ratio and melamine concentration in wheat flour (●), corn gluten (○), and soybean meal (▲). Each data point was the average of ratios from three spectral measurements.

local store and USDA's dairy center in Beltsville, Maryland. Disposable glass tubes (6 mm outside diameter × 50 mm length) were obtained from Fisher Scientific (Suwanee, GA).

At least five mixtures were prepared for each food/feed material type by mixing melamine into the dry material to obtain contaminant concentrations (w/w) in the range of 0.2, 0.5, 1.0, 3.0, and 6.0%. A mortar and pestle were used to obtain homogeneous mixtures.

Fourier Transform Raman Spectra Collection. The FT-Raman spectra were acquired on a FT-Raman module for a Nicolet 670 FT-IR bench using an InGaAs detector and XT-KBr beamsplitter (Madison, WI). The samples in the glass tubes were illuminated using the Nd:YAG excitation laser operating at 1064 nm. Raman scattered light was accumulated using a 180° reflective mode with 0.5 W of laser power and 256 scans at 8 cm^{-1} resolution. Three measurements were taken for each mixture. All spectra were transformed into .spc files (Grams file format) and then smoothed with the Savitzky–Golay function of 2 polynomial and 11 points using Grams/32 software (Version 7.0, Galactic Industries Corp., Salem, NH). The data set was loaded into Microsoft Excel 2000 to execute a simple analysis algorithm using a two-band ratio.

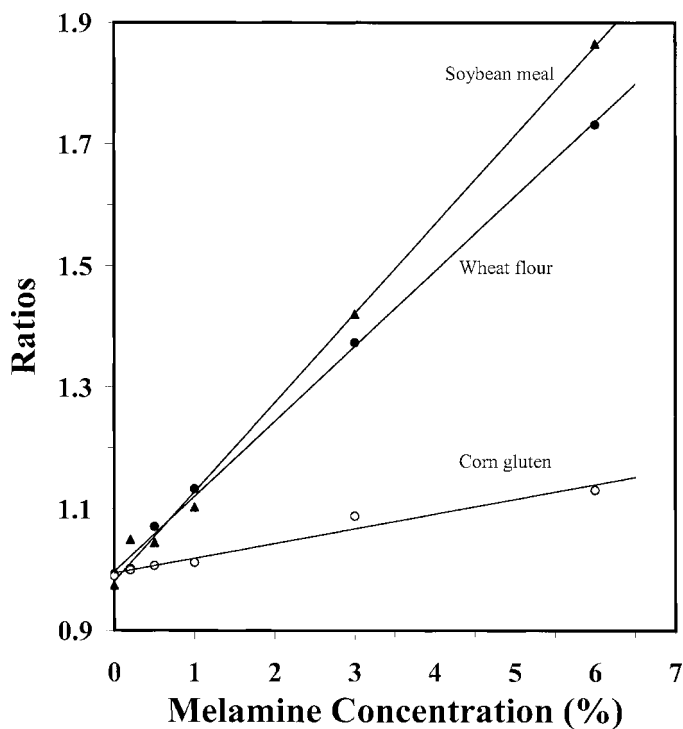**Raman Chemical Imaging.** Wide-field Raman chemical images were acquired on a Falcon I molecular chemical imaging system (ChemImage Corporation, Pittsburgh, PA) using a 785 nm laser as an excitation source. The laser power at the laser head was set to 460 mW and the laser beam was defocused through a 10× objective onto the sample. Backscattered Raman light was directed through an imaging spectrometer, a liquid crystal tunable filter with a 9 cm^{-1} spectral resolution, and collected by a charge-coupled device (CCD) camera. ChemExpert software (ChemImage Corporation, v.2.1.3) was used for acquisition and analysis of Raman chemical images.

RESULTS AND DISCUSSION

Fourier Transform Raman Spectroscopy. Figure 1 shows the representative melamine concentration-dependent FT-Raman spectra of the melamine and wheat flour mixtures, at melamine percentages (%, w/w) of 0, 0.2, 0.5, 1.0, and 100% in the 1700–300 cm^{-1} region. It indicates that melamine and wheat flour have unique Raman bands that increase or decrease in intensity in accordance with the relative amount of melamine. A dominant melamine Raman band at 670 cm^{-1} is of much interest because it is the strongest band and also is well separated from those ascribed to wheat flour. This intense melamine band arises from the breathing mode of the triazine ring.[9,12,13]

Careful comparison of spectra in Fig. 1 suggests that there exists a weak 670 cm^{-1} band in the spectrum of the mixture containing 0.2% melamine, and, as expected, the intensity increases with the melamine concentration. Therefore, the 670 cm^{-1} band could be used to identify the presence of a melamine component as low as a 0.2% in wheat flour.

In addition to determining whether melamine is present, it is of interest to assess its amount using a simple Raman measurement. Figure 2 depicts the melamine-concentration-dependent band ratio of I_{670}/I_{655}, by ratioing the Raman
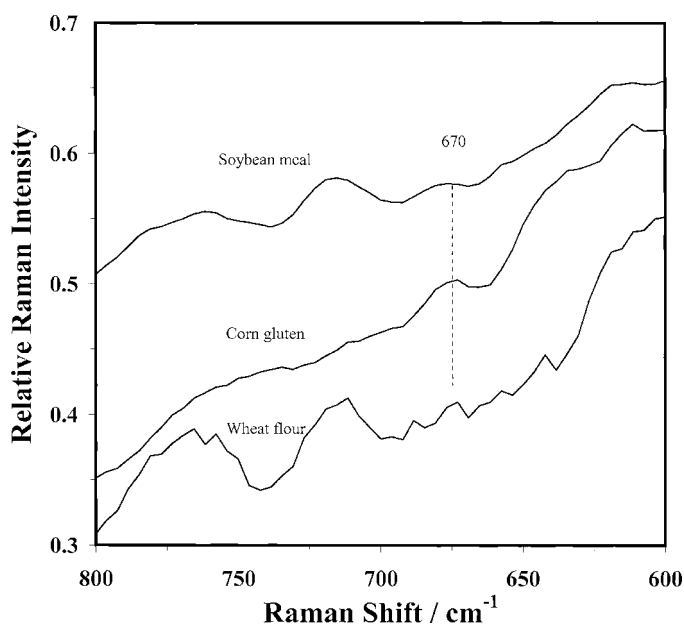
FIG. 3. Typical FT-Raman spectra of melamine-contaminated wheat flours, corn gluten, and soybean meal at the percentage of 0.2%. All spectra were shifted vertically for direct comparison.

intensity at 670 cm^{-1} against that at 655 cm^{-1} for the three mixture types. In general, the ratio yields a nearly linear relationship with a correlation coefficient (R^2) of 0.99. The use of the band at 655 cm^{-1} is somewhat subjective, as there are no apparent Raman peaks around this wavelength in either the pure compounds or the mixtures.

The FT-Raman spectra of melamine-contaminated corn gluten and soybean meal revealed the distinctive 670 cm^{-1} band, whose intensity increased as expected with increasing melamine concentration. For comparison, FT-Raman spectra of wheat flour, corn gluten, and soybean meal samples each containing 0.2% melamine are given in Fig. 3. Due to the large variations in chemical/ingredient constituents and color among wheat flour, corn gluten, and soybean meal, the differences observed in the relative intensity and position of the Raman bands are reasonable. Notably, the Raman bands near 670 cm^{-1} in the three mixtures are easily identifiable. Therefore, the dominant and well-isolated melamine band at 670 cm^{-1} could be used as a marker to detect the existence of melamine in the food/feed materials at levels as low as 0.2%.

Ratio values for melamine-contaminated corn gluten and soybean meal are also shown in Fig. 2. It can be seen that the ratio values are nearly linear with melamine concentration in the range of 0.2–6.0 %, with correlation coefficients (R^2) of 0.99, 0.96, and 0.99 for wheat flour, corn gluten, and soybean meal, respectively.

Comparison of the ratio values shown in Fig. 2 indicates variation between the three types of feed mixtures; such a distinction could reflect the chemical, physical, and structural
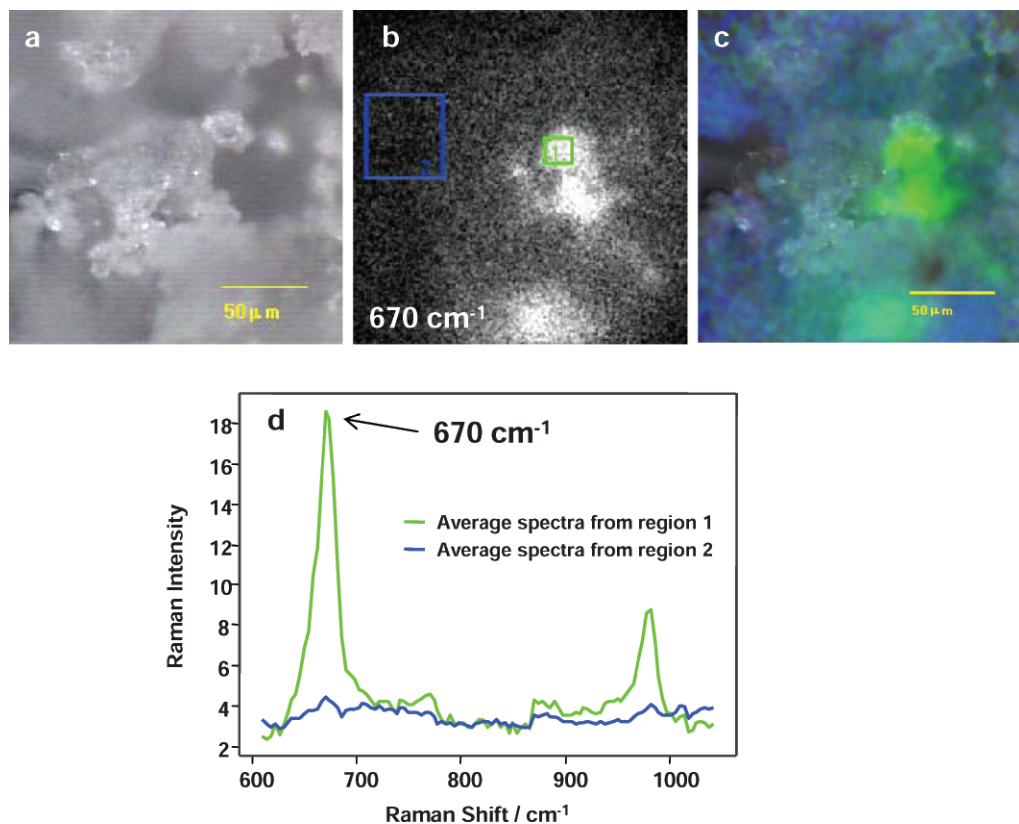

FIG. 4. (**a**) Bright field reflection image, (**b**) corresponding Raman chemical image at 670 cm^{-1}, (**c**) fusion of false-colored Raman chemical image with the corresponding bright field reflection image, and (**d**) image-spectrometer-derived spectra of selected regions imaged of a sample containing 6% wt. mixture of melamine in wheat flour. Raman hyperspectral image was obtained on a ChemImage Raman Imaging system equipped with 785 nm laser excitation. Laser power on the sample for the 785 nm excitation using a 10× objective was measured to be approximately 460 mW. Melamine in wheat flour mixture sample was prepared, characterized, and supplied to ChemImage by USDA-ARS.

differences of the three food/feed materials. However, careful examination of the ratio values reveals that all uncontaminated samples (no melamine added) had ratio values less than 1.00, and that the melamine-contaminated samples all had ratio values greater than 1.00. This was true for all three feed sample types—soybean meal, wheat flour, and corn gluten. Hence, it could be used as a universal value to identify melamine-contaminated materials from uncontaminated materials, even if the exact types of bulk materials are unknown.

Future Raman Detection Methods: Raman Chemical Imaging. Due to high specificity and sensitivity of spectroscopic methods for detecting melamine in mixtures, other innovative techniques that would allow nondestructive and highly reliable sensing of this contaminant is of great interest for further studies. One of the imaging techniques that holds this promise is global-illumination (or wide-field) spectroscopic imaging, which combines spatially and spectrally resolved information about molecular identities of mixtures. Raman chemical imaging[14] was used to assess the feasibility of imaging the melamine particles in wheat flour. Wide-field Raman chemical imaging offers an advantage of detecting a chemical of interest in the presence of complex backgrounds where spatial information is as important as spectral information. In addition, only a small portion of a spectrum featuring specific vibration modes in the form of their Raman bands can be used.

A 785 nm laser was used as the excitation source. The Raman scattering of melamine in wheat flour was collected through an electronically tuned liquid crystalline tunable filter over the spectral range from 610 cm^{-1} to 1040 cm^{-1} using 4 cm^{-1} steps with a 5 second integration per wavelength frame. This narrow spectral range was chosen to detect the specific melamine triazine ring breathing mode.[9,12,13] The resulting image presents a hyperspectral datacube possessing both spatial and spectral information.

Figure 4a shows a bright field reflection image of the wheat flour sample known to contain 6% melamine by weight. There is no noticeable heterogeneity in the bright field reflection image that can be attributed to the melamine contaminant. A Raman hyperspectral image was collected on the same field of view where each pixel of the Raman chemical image has an associated Raman spectrum. Figure 4b shows the 670 cm^{-1} band of this hyperspectral image. Spectra from neighboring pixels that show negative contrast at the 670 cm^{-1} band indicate chemical-specific spatial heterogeneity of the sample studied. Figure 4d shows two spectra derived from regions 1 and 2 (in green and blue boxes, respectively) in Fig. 4b. Figure 4c presents a bright field/Raman fusion image combining the image information shown in Figs. 4a and 4b, showing the melamine regions in green and the wheat flour regions in blue. Further investigation of this Raman chemical imaging technique is necessary in the development and validation of rapid methods

for routine large-volume high-throughput screening of biological and chemical contaminants in food and feed materials.

CONCLUSION

This study presented the usefulness of the FT-Raman technique for the screening of melamine contamination in food/feed materials. The characteristic melamine Raman band near 670 cm^{-1} was observed to be intense and well-separated from other bands attributed to food/feed components. This 670 cm^{-1} melamine band was used to develop simple ratio algorithms for melamine detection. The results revealed that the ratio algorithm could be used not only to perform the classification analysis between uncontaminated and melamine-contaminated classes at levels as low as 0.2% (w/w), but also to predict the melamine concentrations in contaminated products. Notably, the two-band algorithm approach is the most attractive and interesting since, in its simplest form, there is no calibration model that needs to be first built from a large sample set. In addition, the use of a ratio of wavelength intensities can reduce the influence measurement variation, and hence could be easily applied for fast, accurate, specific, and routine screening of melamine contamination in unknown products. A Raman chemical imaging technique was applied to identify and image melamine in a wheat flour matrix. Raman chemical imaging is demonstrated as a promising method to detect intentional tainting of agricultural commodities affecting the safety and security of foods and feeds.

1. FDA, available from http://www.fda.gov/bbs/topics/NEWS/2007/NEW01615.html (accessed July 7, 2008).
2. Toray Industries, Inc., "Flame Resistant Polyamides," Japan Tokkyo Koho JP 8222348, Chem. Abstracts **97,** 199037 (1982).
3. D. E. Freepons, "Fertilizer processes and compositions using s-triazines", US Patent 4,559,075 (1985).
4. FDA, available from http://www.fda.gov/bbs/topics/NEWS/2007/NEW01643.html (2007), (accessed July 7, 2008).
5. FDA, available from http://www.cfsan.fda.gov/~dms/melamra.html (accessed July 7, 2008).
6. D. R. Shelton, J. S. Karns, G. W. Mccarty, and D. R. Durham, Appl. Environ. Microbiol. **63,** 2832 (1997).
7. FDA, available from http://www.fda.gov/cvm/GCMSMelamine.htm (accessed July 7, 2008).
8. FSIS, USDA, available from http://www.fsis.usda.gov/PDF/FERN_CHE_0003.pdf (accessed July 7, 2008).
9. J. R. Schneider and B. Schrader, J. Mol. Struct. **29,** 1 (1975).
10. J. R. Ferraro and K. Nakamoto, *Introductory Raman Spectroscopy* (Academic Press, San Diego, CA, 1994).
11. Y. Ozaki, "Raman Spectroscopy", in *Spectral Methods in Food Analysis* (Marcel Dekker, New York, 1999), M. M. Mossoba, Ed., pp. 427–462.
12. C.-H. Tseng, C. K. Mann, and T. J. Vickers, Appl. Spectrosc. **48,** 535 (1994).
13. M. L. Scheepers, J. M. Gelan, R. A. Carleer, P. J. Adriaensens, and D. J. Vanderzande, Vib. Spectrosc. **6,** 55 (1993).
14. H. R. Morris, C. C. Hoyt, P. Miller, and P. J. Treado, Appl Spectrosc. **50,** 805 (1996).