

Rapid Analysis of Melamine Content in Powdered and Liquid Milk Using Fourier Transform Infrared Spectroscopy

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Abstract: Melamine is a chemical intermediate to manufacture amino resins and plastics, which cannot be used as food additive since it can cause kidney stones. A qualitative determination method of melamine residue in powdered and liquid milk was developed using Fourier transform infrared (FTIR) spectroscopic technique. The calibration sets consisted of 21 standard melamine solutions, in which 1% trichloroacetic acid aqueous solution and acetonitrile (3:1, v/v) were used as solvent. The model was validated using 10 standard melamine solutions which were unused to build up the calibration set. Infrared (IR) absorbance peaks specific to almost all chemical groups in melamine molecule were shown in the spectral range between 1,100 and 1,800/cm. Combined partial least squares (PLS)-2nd derivative calibration model coupled with mean centering (MC) mathematical enhancement showed the highest correlation coefficients ($R^2 > 0.99$). In brief, the FTIR technique can be used for quantitative analysis of melamine in milk samples.

Keywords: Fourier transform infrared spectroscopy, melamine, milk, food contamination

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is a chemical intermediate to manufacture amino resins and plastics (1). Prior to some research reports (2), melamine and its analogs had been regarded as non-toxic or minimally toxic. However, melamine has become a significant concern because of several food safety incidents. In the spring of 2007, many pets in the United States were harmed or killed after ingestion of pet food adulterated with melamine (3, 4). Food and Drug Administration (FDA) investigated the pet food incident and found that two ingredients imported from China and used in manufacturing pet foods were fraudulently labeled as wheat gluten and rice proteins but actually contained melamine and its analogs. Recently liquid and powdered milk products manufactured in China are also found to contain melamine. Mainly because protein content is traditionally determined by measuring nitrogen content in China, some unlawful businessmen add some cheap nitrogen-containing ingredients such as melamine to improve protein-content, which can save the suppliers money.

At present many analytical methods were used for analysis of melamine in high protein food. These methods mainly include gas chromatography-nitrogen phosphorus detection (GC-NPD) (5), gas chromatography-mass spectrometry (GC-MS) (6), liquid chromatography (LC)-ultraviolet (UV), diode array detection (DAD), and ion trap mass spectrometry (MS/MS) (6-8). Each method has disadvantages. For example, GC analysis, its sensitivity and reproducibility are poor. LC-MS and GC-MS can be proposed for the qualitative and quantitative analysis of melamine with

good sensitivity and reproducibility. However, the two methods have serious background interferences and their sample preparation process is complex and non-economical (7). Immunoassay method was used to determine melamine in milk by few researchers (9,10). Recently, near- and mid-infrared spectroscopic techniques were also applied to determine infant formula powder by Mauer *et al.* (11).

Fourier transform infrared (FTIR) spectroscopy with smart attenuated total reflectance kit (ARK) accessory is regarded as a relatively inexpensive technique capable of quickly and simply identifying materials without a need for direct contact with samples. In recent years, FTIR integrated with multivariate calibration analysis has been successfully used in many food analytical fields (12-20). Therefore, the objective of this study was to develop a feasible FTIR method for qualitative and quantitative analysis of melamine in powdered and liquid milk. The first step was intended to develop FTIR model integrated with spectral data compression. Standard calibration set of melamine solutions consisted of 21 different concentrations and its predictive ability was validated using 10 synthetic melamine solutions. The second step was aimed to apply the developed model for melamine analysis in commercial milk samples.

Materials and Methods

Preparation of standard solutions of melamine and powdered and liquid milk samples Calibration sets consisted of 21 melamine standard solutions including ranging from 2 to 200 mg/L. Validation sets consisted of 10 melamine standard solutions from 5 to 95 mg/L. A mixture of 1% trichloroacetic acid (TCA) aqueous solution and acetonitrile (3:1, v/v) was used as solvent.

Three different brands commercial powdered and liquid milk were purchased in Hawaii local retail stores. Each sample was spiked with melamine standards in known

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concentrations. The commercial milk samples did not contain melamine and its analogs, which were verified using LC-MS. Each adulterated milk (5 g) was extracted with 20 mL of 1% TCA aqueous solution and acetonitrile (3:1, v/v) and sonicated for 30 min. Extracted solutions were centrifuged at $2,800\times g$ for 10 min and then filtered (0.45- μm) for FTIR analysis (20).

FTIR-attenuated total reflectance (ATR) measurement

FTIR-ATR measurement was carried out according to the method of Khurana *et al.* (22). Briefly, a Nicolet 6700 spectrometer (Thermo Electron Corporation, Madison, WI, USA) was used to collect all the IR spectra reported in this study. The horizontal attenuated total reflectance (HATR) accessory was used integrated with a ZnSe crystal for sample containment with an aperture angle of 45° . The sample chamber always remained open during the analysis. Single beam spectra (4,000-400/cm) of melamine standard solutions and milk samples extracts in 1% TCA aqueous solution and acetonitrile (3:1, v/v) were obtained at a resolution of 8/cm and a total of 256 co-added scans after subtraction of 1% TCA aqueous solution and acetonitrile (3:1, v/v) background. An amount of 2 mL of sample was poured into the sample boat, enough to cover the length of the crystal. The ATR crystal was carefully cleaned with 100% ethanol to eliminate any residues on surface of HATR cell. Each sample had 4 replicates and the data were averaged by GRAMS software before preparing for multivariate analysis. The analyses were performed at room temperature (25°C). Temperature was not examined as a variable. ZnSe is a good HATR crystal material for analyzing solutions. Milk extracts were analyzed both immediately and 30 min after contact with the crystal. The 2 analysis spectra show no spectral differences. It was estimated that the FTIR spectral collection time for powdered or liquid milk samples was less than 4 min and total detection time including the sample preparation could be less than 10 min.

Quality control/quality assurance (QC/QA)

The pretreatments and analytical procedures for all milk samples adulterated with melamine were performed using QA and QC measures. Limits of detection (LODs) were derived from the blanks and quantified as within 3 standard deviations of the mean blank intensity based on IR absorbance peaks of main chemical groups in melamine molecule between 1,100 and 1,800/cm. Recovery tests of melamine for milk samples include 100 g of each of 5 milk samples spiked with the amount of 5 mg of melamine, extracted, and analyzed by using LC-MS technique.

Data analysis GRAMS spectroscopy software, (v. 8.0; Thermo Nicolet, Madison, WI, USA) was used for analysis of spectral data obtained for adulterated milk samples. The program requires no fewer than 30 samples to be used to generate a training set including 20 samples in the calibration set and 10 samples in the validation set. GRAMS software can offer partial least squares (PLS) and principal component regression (PCR) data compression methods. PLS and PCR methods were tested to develop the calibration model with the highest correlation coefficient (R^2) and the lowest standard error of calibration (SEC).

The 2 calibration models were further validated using the validation set. The model predictions were compared with the actual melamine concentrations spiked into the base foods, and the accuracy of prediction was assessed by the standard error of prediction (SEP) and the correlation coefficient.

Results and Discussion

Characterization of FTIR spectra of melamine In a representative IR spectrum of melamine solution (Fig. 1), 2 sharp bands in the wavenumbers above 3,400/cm can be assigned to the symmetric and asymmetric stretching vibrations of the terminal primary amine ($-\text{NH}_2$) groups (Fig. 1) (23,24). The other broad band in the wavenumbers above 3,000/cm is consistent with the modes involving O-H stretching vibrations, perhaps due to adsorbed water (Fig. 1). In particular, the IR spectrum of melamine is characterized by a series of bands in the region between 1,200 and 1,700/cm. These spectral bands are so distinctive for the molecules that contain aromatic C_3N_3 triazine-ring modes (24,25). The absorptions are consistent with conjugated C-N and C-C species (1,600-1,700/cm), aromatic rings (1,450-1,600/cm), aromatic C-N bonds (1,270-1,340/cm), and 1,3,5-substituted aromatic rings (810-950/cm) (24,26). In addition, the spectrum of melamine has 3 strong bands with similar intensities at 1,630, 1,550, and 1,445/cm (23-25). The band close to 1,650/cm is resulting from ring distortion modes and the one close to 1,550/cm originates from the NH_2 deformation. The selection of the spectral range between 1,100 and 1,800/cm was appropriate, which could cover the majority of spectral characteristics of melamine. Figure 2A and 2B show the original and the 2nd derivative spectra of 21 standard melamine solutions used for the calibration model. Figure 2C and 2D present the original and the 2nd derivative spectra of 3 different brand milk samples with melamine adulteration, which appear to be identical to those from the calibration set in the selected spectral region.

Extraction of melamine from milk samples Reproducible and quantitative extraction process of melamine from adulterated milk samples is the first, critical step in this study. Average recoveries of melamine from milk samples amounted to be between 80 ± 5 and $120\pm 5\%$. The results indicate that the melamine extraction method proposed in this study is appropriate. In addition, melamine standards with several low concentrations ranging from 0.25 to 2 mg/L were also tested for LOD estimation. Significant IR absorbance peaks specific to the melamine molecule were found in the range between 1,100 and 1,800/cm even at the melamine concentrations as low as 0.5 mg/L. Melamine and its analogues residual amounts in foods (not including infant formula) were temporarily limited 2.5 mg/L by the U.S. FDA. Very recently, a threshold of 1 mg/L for melamine in infant formula was set by the FDA (11). Our result shows the LOD value estimated in this approach satisfy the lower boundary of sensing limit required for detection of melamine in milk.

PLS calibration model Spectral profiling of the melamine during FTIR measurement, such as peaks, areas or valleys

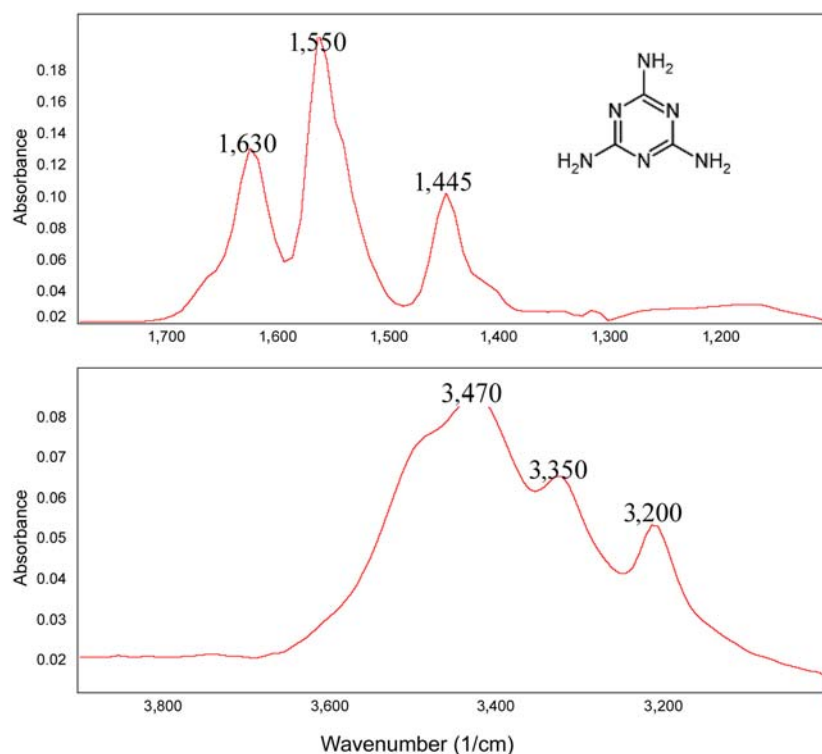


Fig. 1. Chemical structure and FTIR spectra of standard melamine solutions ranging from 1,100 to 1,800/cm and from 3,000 to 4,000/cm.

can be used to quantitatively describe the content of the melamine with aid of statistical software such as SAS or GRAMS. Table 1 shows validation of the calibration models based on original and the 2nd derivative spectra coupled with data compression methods such as PLS and PCR, and data enhancement methods such as mean centering (MC) and variance scaling (VS). The PLS calibration model coupled with the 2nd derivative spectra and MC data processing shows the lowest SEC value and highest R^2 value (Table 2). According to Sivakesava and Irudayaraj (27), the PLS regression model consider the reference values in constructing spectral components while

PCR does not use the reference values when selecting spectral data. This may clarify why PLS provided better prediction. Also by removing the mean from the data, MC data processing substantially enhances the differences between the samples in terms of both concentration and spectral response. This usually leads to calibration models that provide more accurate predictions.

Quantification of melamine in milk samples Predicted values of melamine in milk samples using the developed PLS-2nd derivative model are list in Table 3. The results show that the FTIR-PLS predictions of melamine are very close to the added melamine contents in 3 different varieties of milk products. The correlation coefficients (R^2) between the FTIR-PLS model predictions and the actual amount of melamine spiked into milk samples ranged from 0.97 to 0.99. Also the maximum percentage prediction error was found to be less than 15%, which would be acceptable, according to the previous studies (11).

In summary, the FTIR integrated with GRAMS spectroscopy software seemed to represent a reliable method for quantitative analysis of melamine in commercial milk products. The developed PLS-2nd derivative calibration model predicted the melamine concentrations more accurately than any other combinations of PLS, PCR, and spectral derivations. The selection of the spectral region to range between 1,100 and 1,800/cm could enhance the model prediction. The developed calibration model is expected to quantify the amount of melamine adulterated not only for milk but also for other foods in highly protein rich matrices. The proposed scheme of model prediction will enable its extension to other food products with different compositions because the FTIR spectral data of

Table 1. Validation sets (mg/L) of the calibration models based on original and the 2nd derivative data transformations

Actual	Origin ¹⁾			2 nd Derivative			
	PLS-MC	PLS-VS	PCR	Actual	PLS-MC	PLS-VS	PCR
5	4.2	2.9	4.2	5	3.4	18.8	3.4
15	14.3	13.2	14.2	15	14.7	28.1	14.7
25	25.6	24.7	25.5	25	25.7	36.5	25.7
35	32.4	37.4	32.1	35	35.6	53.9	35.6
45	46.1	44.0	46.0	45	45.0	56.5	45.0
55	55.4	55.0	55.3	55	56.3	72.2	56.3
65	67.4	65.8	67.4	65	66.4	78.7	66.4
75	77.7	75.5	77.7	75	77.2	88.4	77.2
85	86.9	85.7	86.8	85	87.4	95.6	87.4
95	98.9	97.3	98.9	95	98.0	105.4	98.0

¹⁾PLS, partial least squares; MC, mean centering; VS, variance scaling; PCR, principal component regression.

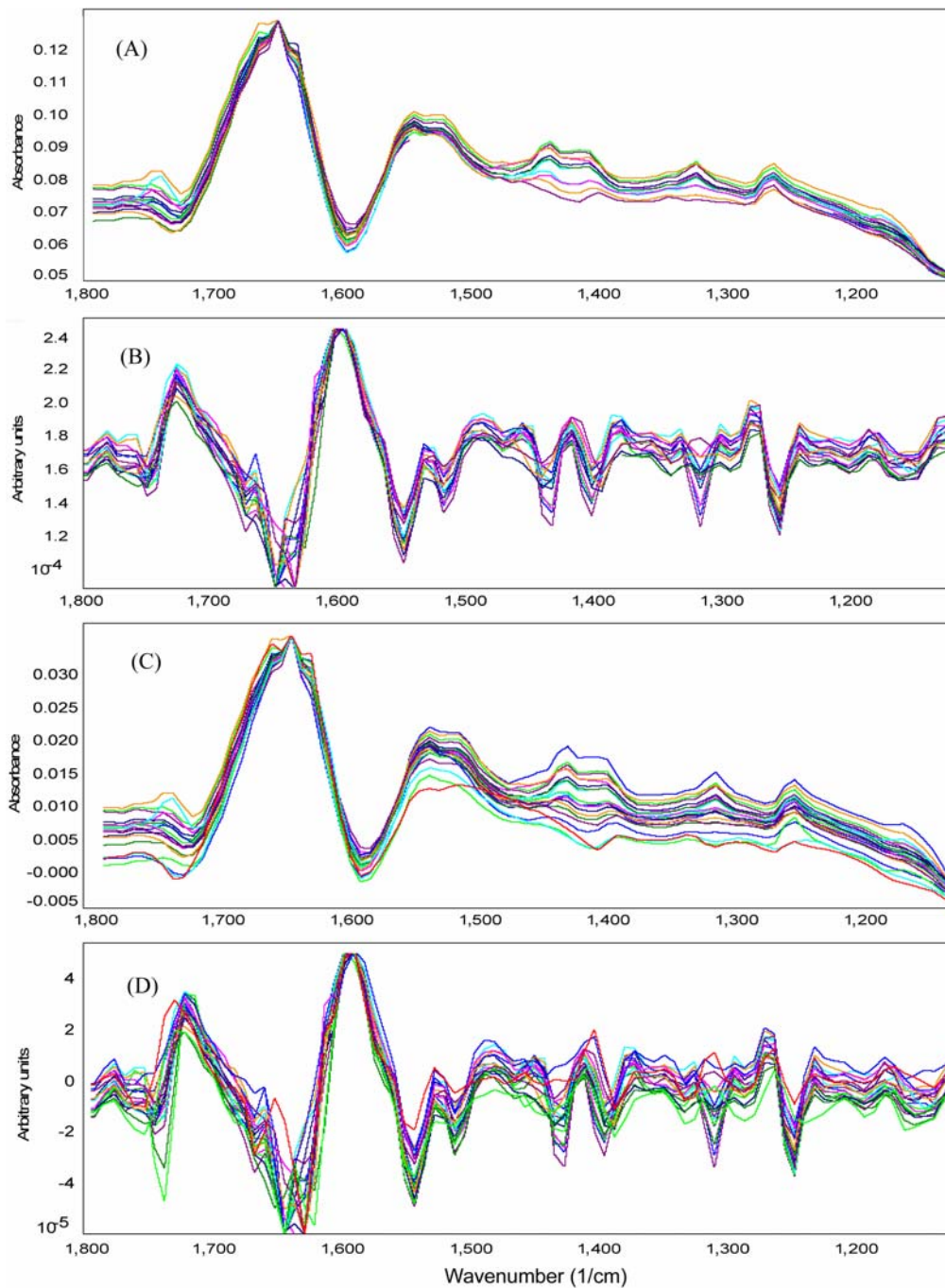


Fig. 2. Original and the 2nd derivative FTIR spectra of melamine. (A) original FTIR spectra of calibration sets, (B) 2nd derivative FTIR spectra of calibration sets, (C) original FTIR spectra of milk samples with melamine adulteration, and (D) 2nd derivative FTIR spectra of milk samples with melamine adulteration.

Table 2. Calibration and validation parameters for the models developed using original and the 2nd derivative spectral transformations and mean centering data processing method

Calibration method ¹⁾	Derivative transformation	Factor	Calibration		Validation	
			R ²	SEC	R ²	SEP
PLS-MC	Original	1	0.992	3.13	0.990	2.04
PCR		3	0.961	3.53	0.986	2.16
PLS-MC	2 nd	3	0.999	2.92	0.995	1.63
PCR		1	0.987	2.97	0.972	1.98

¹⁾PLS, partial least squares; MC, mean centering; VS, variance scaling; PCR, principal component regression.

Table 3. Predicted values (mg/L) of melamine in 3 commercial milk products spiked with known melamine concentrations using PLS-2nd derivative model with mean centering data processing method

Brand A		Brand B		Brand C	
Actual	Prediction	Actual	Prediction	Actual	Prediction
3.0	2.5	3.0	2.7	3.0	1.9
20.0	14.8	20.0	18.4	20.0	15.7
50.0	55.6	50.0	42.9	50.0	51.3
75.0	79.8	75.0	73.1	75.0	74.4
95.0	96.3	95.0	93.2	95.0	102.7
115.0	103.5	115.0	122.4	115.0	110.6
135.0	139.4	135.0	132.3	135.0	134.7
155.0	148.7	155.0	160.6	155.0	151.5
175.0	172.4	175.0	164.5	175.0	171.4
195.0	189.8	195.0	196.6	195.0	187.8
R ² =0.97		0.99		0.98	

adulterated foods negate the background of their base foods; thus, only the subtle peak changes specific to melamine contents can be taken into consideration during the quantitative analysis. The observation in this study shows little spectral interference between the melamine adulterant and existing dairy protein components, leading the model to be matrix-independent. The FTIR-PLS analysis is expected to complement traditional analysis of melamine in milk, dairy product, and furthermore, other protein base foods, which requires special skills, and long sample pretreatments.

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