Raman spectroscopy as an effective screening method for detecting adulteration of milk with small nitrogen-rich molecules and sucrose

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ABSTRACT

Adulteration of milk for commercial gain is acknowledged as a serious issue facing the dairy industry. Several analytical techniques can be used to detect adulteration but they often require time-consuming sample preparation, expensive laboratory equipment, and highly skilled personnel. Here we show that Raman spectroscopy provides a simple, selective, and sensitive method for screening milk, specifically for small nitrogen-rich compounds, such as melamine, urea, ammonium sulfate, dicyandiamide, and for sucrose. Univariate and multivariate statistical methods were used to determine limits of detection and quantification from Raman spectra of milk spiked with 50 to 1,000 mg/L of the N-rich compounds and 0.25 to 4% sucrose. Partial least squares (PLS) calibration provided limit of detection minimum thresholds <200 mg/L (0.02%) for the 4 N-rich compounds and <0.8% for sucrose, without the need for surface-enhanced Raman spectroscopy. The results show high reproducibility (7% residual standard deviation) and 100% efficiency for screening of milk for these adulterants.

Key words: milk adulteration, Raman spectroscopy, nitrogen-rich molecules, sucrose

INTRODUCTION

The adulteration of milk for economic gain has been an ongoing problem for more than 150 yr: dilution of milk with water was reported as early as 1857 (Gem, 1857) and, in 1890, *The New York Times* exposed the addition of compounds such as borax, soda, or salicylic acid to increase milk shelf life (New York Times, 1890). Unfortunately, adulteration of milk is still prevalent today with the fraudulent addition of a variety of

chemicals (Moore et al., 2012; Cattaneo and Holroyd, 2013; Santos et al., 2013). Recently, small nitrogen-rich compounds such as melamine (1,2,4-triazine) have been fraudulently added to milk to artificially boost its apparent protein content (Chan et al., 2008), which is routinely measured as total nitrogen content using the Kjeldahl or Dumas analytical test (Codex Alimentarius, 2010; Moore et al., 2010). As these test methods cannot distinguish between nitrogen in milk protein and other nitrogen compounds, more sophisticated laboratory test methods are necessary for sensitive detection of these compounds in milk. These include GC, HPLC, and hydrophilic interaction liquid chromatography (HILIC) methods, with MS detection (Desmarchelier et al., 2009; Abernethy and Higgs, 2013; Domingo et al., 2014). These techniques can measure melamine concentrations well below the World Health Organization's maximum residue levels of 2.5 mg/L in milk and 1.0 mg/L in infant formula (WHO, 2009). However, they require lengthy pretreatment of the milk samples, including centrifugation and addition of chemicals to remove the protein fraction, expensive laboratory equipment, and highly skilled operators.

Spectroscopic methods that do not require such sample preparation steps have been investigated as rapid and “greener” alternative methods for detecting these compounds in liquid or powdered milk; these include mid- and near-infrared spectroscopy (Mauer et al., 2009; Balabin and Smirnov, 2011; Cattaneo and Holroyd, 2013; Jawaid et al., 2013), Raman spectroscopy (Okazaki et al., 2009; Cheng et al., 2010), hyperspectral Raman imaging (Qin et al., 2010, 2011, 2012, 2013a,b; Kim et al., 2013), and Fourier transform-Raman spectroscopy (Srilakshmi et al., 2007; Cheng et al., 2010, 2012; Chao et al., 2013). Mid- and near-infrared spectroscopy combined with chemometric analysis have already been shown to be effective as a rapid, sensitive, robust, and low-cost alternative for analysis of melamine in milk and infant formula (Balabin and Smirnov, 2011; Abbas et al., 2013; Jawaid et al., 2013;

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Santos et al., 2013). However, different studies show considerable variation in the root mean square error of calibration and limit of detection (**LOD**) due to variations in instrumentation and method used for the measurements.

Compared with infrared spectra, Raman spectra generally contain fewer, sharper, and more-discrete bands that are significantly stronger than those from water. This affords Raman spectroscopy several advantages over infrared absorption: it enables greater sensitivity in measurement of low concentrations and the discrete and sharper spectral bands make Raman spectroscopy far more specific for the simultaneous analysis of mixtures of different adulterants in milk. Another advantage is the variety of robust mini and portable Raman systems that have become available commercially at increasingly competitive prices. For example, a portable mini Raman system with microscope and surface-enhanced Raman spectroscopy (**SERS**) has already been successfully used to detect melamine as a contaminant in eggs (Cheng and Dong, 2011). Combining partial least squares (**PLS**) and partial least squares discriminant analysis (**PLS-DA**) multivariate analysis methods with such mini Raman systems shows promise for the application of Raman spectroscopy as a robust technique for qualitative and quantitative screening of adulterants in milk. Further, the suitability of Raman microscopy with micro-fiber optics for analyzing aqueous fluids in microfluidics extends the usefulness of application to on-site analysis of milk, as has already been described for biological and medical screening applications (Johansson et al., 2007; Ashok et al., 2010; Chrimes et al., 2013).

The disadvantage of Raman spectroscopy for liquid milk is the relatively low signal-to-noise ratio (**SNR**) due to diffuse scattering by the milk matrix. Raman analysis has therefore been limited to powdered or dried milk. However, in these media, **LOD** are poor: those measured for melamine, dicyandiamide (**DCD**), ammonium sulfate (**AmS**), and urea in milk are between 1,000 and 1,300 mg/L (Cheng et al., 2010; Qin et al., 2013a,b). More sensitive detection of melamine in milk has been achieved using **SERS**, which utilizes the enhanced electromagnetic fields and chemical enhancement provided by gold or silver nanostructures; **LOD** as low as 2.4 µg/L have been reported (Zhao et al., 2013). However, most of the **SERS** measurements in the literature require sample preparation steps similar to those for **HPLC**, such as centrifugation and addition of chemicals to remove the protein fraction that may block analyte binding sites on **SERS** substrates. An exception is a **SERS** study using simple dilution of milk to which silver nanoparticles were added: a melamine **LOD** of 1 mg/L was obtained (Rajapandiyan et al.,

2015). Another disadvantage of **SERS** is low reproducibility (Guicheteau et al., 2011), which is also highly variable, due to the stochastic nature of the “hotspots” of intense electromagnetic radiation between the nanostructures (Moskovits, 2013). Variations can be as high as ±40% (Norrod et al., 1997).

The aim of this study was to find a robust, simple, and reliable method using Raman spectroscopy to screen for the adulteration of milk with N-rich compounds, without the need for **SERS**, dilution, or other preparation steps. Although baseline levels of these compounds in foods require more sensitive detection such as laboratory chromatographic techniques or **SERS**, adulteration levels used for commercial gain are hypothesized to be much higher, at levels between 90 and 4,000 mg/L (Abernethy and Higgs, 2013). The focus of the study was to improve both the reproducibility and sensitivity of Raman analysis for 4 N-rich compounds (melamine, **DCD**, **AmS**, and urea) and sucrose in milk without the use of **SERS**. Collection optics, instrument parameters, and sampling methods were optimized to improve reproducibility and sensitivity. Sucrose is often added to increase the solids content and sweeten milk, and is currently analyzed using **HPLC** with refractive index detection (Chávez-Servin et al., 2004) or using a modified Sewilanoff method requiring addition of harmful chemicals (Food Safety and Standard Authority of India, 2012). As far as we are aware, no quantitative study using Raman analysis of sucrose in milk has yet been reported.

MATERIALS AND METHODS

Sample Preparation

A commercial brand of homogenized full-cream milk was used for all experiments. Adulterated milk solutions were prepared by dissolving the appropriate amount of powdered adulterant in 100 mL of milk. The concentration of sucrose was varied between 0 and 40,000 mg/L to give a total of 7 samples, and 3 spectra were recorded for each sample, resulting in 21 spectra that were used for both univariate analysis and **PLS** analysis. For the 4 small N-rich molecules (melamine, **AmS**, **DCD**, and urea), the concentration of each was varied between 0 and 1,000 mg/L to give a total of 60 samples. Of these, 30 samples containing only one adulterant each and were used for univariate analysis, whereas the 30 samples that included additional mixtures were used for the **PLS** and **PLS-DA** models, 12 of which were pure milk samples from different dates over a period of 10 mo. Spectra were recorded from 2 different aliquots from each sample, resulting in 120 spectra in total. For the **PLS** and **PLS-DA** calibrations, these

were divided into a training set of 80 spectra and a separate validation set of 40 spectra, by selecting every third sample (row) in the data matrix for the validation set. An additional 4 spectra recorded of sucrose in milk (2,500 to 20,000 mg/L) were included in the validation set for the PLS-DA.

To assess the partitioning of the investigated N-rich small molecules into the cream fraction, 3,000 mg/L solutions of melamine (BDH Chemicals Ltd., Poole, UK), urea, and AmS (ECP Ltd., Auckland, New Zealand) and a 5,000 mg/L solution of DCD (Sigma-Aldrich Co., St Louis, MO) were made. Two milliliters of each solution was centrifuged at $8,050 \times g$ for 15 min, and spectra were recorded from the cream fraction.

Raman Spectroscopy

Drops of each sample (15 μ L) were placed individually into 4-mm chrome mirror-based sampling wells of a Thermo (Thermo Fisher Scientific, Waltham, MA) sample slide. These wells constrained the drops into 4-mm-diameter circles that dried over 1 h to an average thickness of 112 μ m. Raman spectra were recorded of the dried drops in backscattered configuration using a Renishaw System 1000 Raman microprobe (Renishaw plc, Wotton-under-Edge, UK) with a 25-mW 785-nm laser and 50 $\times$ objective, to give a spot size of 2 μ m at the center and 2.5 mW at the sample. The use of this excitation wavelength avoided fluorescence by milk proteins that would obscure the Raman bands. Reproducibility of the signal was optimized by keeping the sampling volume constant and recording spectra from the center of each dried drop. Reproducibility was parameterized as the relative standard deviation (%RSD) calculated from 3 spectra recorded from different drops of the same solution. The average thickness of the milk drops of 112 μ m was significantly greater than the depth of focus, Δf , of the focused laser spot, which was determined to be 8 μ m (Delhay et al., 1996):

$$\Delta f = \frac{\pi(d)^2}{2\lambda},$$

where d is the laser spot diameter and λ is the laser excitation wavelength.

For the backscattered geometry of the Raman microprobe, the intensity of the Raman spectra depends on several factors: laser power density, sample properties and geometry, spectrometer collection parameters, and integration time (McCreery, 2000). In our system, the only parameters that could be varied were the sample

volume, collection optics, and laser power, limited to a maximum of 25 mW. Under this constraint, a minimum integration time of 240 s was required for an adequate SNR of 3 for 200 mg/L of melamine. The optimum entrance slit width with acceptable resolving power for the Raman urea band at 1,008 cm^{-1} and milk protein phenylalanine 1,001 cm^{-1} band was 75 μ m. The spectral range obtained with 1,200 mm^{-1} grating in static mode and 785 nm excitation was 670 cm^{-1} . Due to overlap of some of the bands of melamine, DCD, urea, AmS, and sucrose with the milk bands, an averaged spectrum of 3 pure milk samples was subtracted from each adulterated milk spectrum to enable more accurate measurement of peak height for the univariate analysis. Peak heights were measured from these difference spectra at 683 cm^{-1} for melamine, 976 cm^{-1} for AmS, 1,008 cm^{-1} for urea, 836 cm^{-1} for sucrose, and 2,198 cm^{-1} for DCD. The latter band was used for univariate analysis of DCD as the band at 926 cm^{-1} overlapped with the milk bands (Figure 1). For PLS and PLS-DA, only the region 1,100 to 550 cm^{-1} was used.

PLS and PLS-DA

Partial least squares calibrations are generally more accurate and robust than univariate calibrations. These multivariate calibrations avoid the inaccuracies introduced in univariate analyses when the band due to the analyte of interest overlaps with bands of other analytes or milk, primarily because information from the entire spectrum is considered instead of only a single peak height. The PLS factors are calculated to maximize covariance between the spectral and analyte concentration matrices thereby excluding nonanalyte signal contributions such as instrument noise, consequently making the calibration more robust than univariate methods.

Spectral data were preprocessed with a 3-point baseline correction using the Omnic 7.1 spectroscopic software (Thermo Fisher Scientific) and smoothing using a Savitsky-Golay second-derivative algorithm (second-order polynomial over 9 points) in the Unscrambler X software (Camo Software AS, Oslo, Norway). After preprocessing, the data set was divided into a training set consisting of 80 spectra and a validation set of 40 spectra, by selecting every third sample (row) in the data matrix. Partial least squares 1 (PLS1) calibrations for melamine, AmS, DCD, and urea were calculated using the spectral region 600 to 1,092 cm^{-1} . Root mean square error of cross validation (RMSECV) was determined using an internal leave-one-out cross validation. These calibration models were then used to predict the concentrations of the separate validation

set. Each PLS1 calibration model was evaluated from the RMSECV for internal validation and the standard error of prediction (**SEP**) and ratio of prediction to standard deviation (**RPD**) for the separate validation prediction. The RPD is a nondimensional statistic used to evaluate PLS calibration models (Williams, 2008) and is calculated by dividing the standard deviation of the reference values used in the validation by the SEP. Values for RPD between 3.1 and 4.9 classify the model as suitable for screening, whereas values between 5.0 and 6.4 are suitable for quality control. Values between 6.5 and 8.0 classify a model as suitable for process control, and values of 8.1 or greater are considered suitable for any application. A separate PLS calibration model for sucrose was created from the training set of 21 spectra recorded from 3 separate drops of each of 7 different concentrations between 0 and 40,000 mg/L. For sucrose, the RMSECV for the internal leave-one-out cross validation was used instead of a separate validation set.

The PLS-DA calibration model was created using the same training set as for PLS calibrations; however, the validation set included an additional 4 sucrose spectra.

For the PLS-DA training set, a dummy concentration matrix was drawn up with values of -1 assigned to the pure milk spectra and $+1$ to the milk solutions containing one or more of melamine, AmS, DCD, and urea.

Limits of Detection and Quantification

The LOD and limit of quantification (**LOQ**) of the univariate analyses were measured according to International Conference on Harmonisation guidelines (ICH, 1996): $\text{LOD} = (3.3\sigma)/S$ and $\text{LOQ} = (10\sigma)/S$, where σ = standard deviation (**SD**) of the response and S = slope of the calibration curve of peak height vs. concentration; σ can be the SD of the blank (measured from the baseline noise for a blank over the range of the peak of interest) or the SD of the regression line, or the SD of the y -intercepts of the regression line. In this study, σ was the SD of the regression line as the LOD and LOQ values obtained compared best with visual estimation of the spectra and were most conservative.

For multivariate methods such as PLS calibration, the LOD can be roughly estimated as $3 \times \text{root mean SEP}$ (Cattaneo and Holroyd, 2013). A more rigorous

Figure 1. Spectra of the 4 N-rich adulterants: melamine (Mel), ammonium sulfate (AmS), dicyandiamide (DCD), and urea, and milk in the region 2,500 to 250 cm^{-1} . The strongest bands of each of the 4 N-rich compounds are labeled, as well as the 1,001 cm^{-1} phenylalanine band of milk. The spectra have been offset for clarity. Color version available online.

and IUPAC (*International Union of Pure and Applied Chemistry*)-consistent approach defines a set of LOD that are based on the set of samples in the training set and characteristic for the overall PLS calibration model (Allegrini and Olivieri, 2014). The LOD interval is obtained by finding the limits of the region in the hyperplane, π_0 , representing samples with zero analyte concentration, or sample blanks. This region is a function of the variance in the predicted analyte concentration for a blank sample, which in turn is a function of h_0 , the distance between π_0 and the center of a normalized calibration score space. Although these sample blanks contain none of the analyte of interest, they may well contain other analytes, and the LOD interval is determined by finding the smallest and largest extrapolated leverages to zero analyte concentration, h_{0min} and h_{0max} , for each calibration set. These values are then used to determine LOD_{min} and LOD_{max} , from (Allegrini and Olivieri, 2014):

$$LOD_{min} = 3.3 [\text{SEN}^{-2} \text{var}(x) + h_{0min} \text{SEN}^{-2} \text{var}(x) + h_{0min} \text{var}(y_{cal})]^{1/2}$$

$$LOD_{max} = 3.3 [\text{SEN}^{-2} \text{var}(x) + h_{0max} \text{SEN}^{-2} \text{var}(x) + h_{0max} \text{var}(y_{cal})]^{1/2}$$

where SEN is the inverse of the Euclidean norm of the regression coefficient, $\text{var}(y_{cal})$ is the variance in calibration concentrations, obtained here from the cross-validation variance, $\text{var}(x)$ is the variance in instrumental signals, which is the square of the signal noise, measured for a region of the baseline in the mean blank spectrum with no peaks, and h_{0min} and h_{0max} are the maximum and minimum extrapolated to zero leverages of each calibration training set.

Moreover, h_{0min} can be measured as follows:

$$h_{0min} = \frac{\bar{y}_{cal}^2}{\sum_{i=1}^l y_i^2},$$

where y_i is the mean centered concentration for the i th calibration sample; and h_{0max} is the maximum h_{0cal} value for the calibration set, obtained for each sample in the calibration set:

$$h_{0cal} = h_{cal} + h_{0min} 1 - \left(\frac{y_{cal}}{\bar{y}_{cal}} \right)^2.$$

Similarly, the LOQ interval (min and max) can be determined as follows:

$$LOQ_{min} = 10 [\text{SEN}^{-2} \text{var}(x) + h_{0min} \text{SEN}^{-2} \text{var}(x) + h_{0min} \text{var}(y_{cal})]^{1/2}$$

$$LOQ_{max} = 10 [\text{SEN}^{-2} \text{var}(x) + h_{0max} \text{SEN}^{-2} \text{var}(x) + h_{0max} \text{var}(y_{cal})]^{1/2}.$$

These values can be used to evaluate whether the predicted concentration of an unknown sample is below or above the detection limit. For samples with predicted concentrations falling within the range $LOD_{min} > y_i < LOD_{max}$, an LOD for the specific sample can be evaluated individually by approximating its real leverage, h , to the leverage h_0 , thus treating the sample as if it were a blank. The LOD obtained would then be used to check whether the analyte is present or absent depending on whether its predicted concentration is above or below the sample-specific LOD (Allegrini and Olivieri, 2014).

RESULTS AND DISCUSSION

Figure 1 shows the spectra of milk and pure samples of melamine, AmS, DCD, and urea. The strongest band for melamine appeared at 676 cm^{-1} and was due to in-plane deformation of the triazine ring, associated with C–N stretching of the C–NH₃ groups. The weaker band at 986 cm^{-1} is the ring-breathing mode due to C–N stretching of the triazine ring (Koglin et al., 1996). For DCD, 2 bands appeared at $2,198 \text{ cm}^{-1}$ and $2,165 \text{ cm}^{-1}$ due to C=N stretching, whereas another 2 bands occurred at 933 cm^{-1} and 668 cm^{-1} due to N–C–N stretching and NH₂ deformation, respectively (Lin et al., 2015). The strongest band in the spectrum of urea occurs at $1,008 \text{ cm}^{-1}$ and is due to the N–C–N symmetric stretch (Keuleers et al., 1999), whereas for AmS, the band at 976 cm^{-1} is due to the symmetric stretch mode of SO_4^{2-} , and the much weaker band at 620 cm^{-1} is due to the triply degenerate SO_4^{2-} deformation mode (Kettle et al., 1984).

The 982 cm^{-1} melamine and 976 cm^{-1} AmS bands are close in frequency, as are the melamine 683 cm^{-1} and DCD 666 cm^{-1} , and urea $1,008 \text{ cm}^{-1}$ and milk protein phenylalanine $1,001 \text{ cm}^{-1}$ bands. In mixtures of these compounds, the bands overlapped (Figure A1, Appendix), which introduced inaccuracies in peak height measurements from spectra of milk solutions adulterated with 2 or more of these compounds. This led to nonlinear calibration plots of peak height versus concentration, particularly for melamine and ammonium sulfate. Therefore, for reliable determination of LOD and LOQ from univariate measurement of peak height, spectra were recorded of milk samples spiked

individually with each compound to minimize component overlap.

Univariate Calibration Models

Figure 2 shows the spectra of milk spiked separately with different concentrations (50 to 1,000 mg/L) of melamine, AmS, DCD, and urea. Two different regions are shown for DCD, as strong bands in the $2,100\text{ cm}^{-1}$ region were isolated from any of the milk bands, which makes this region more suitable for univariate measurement of peak height. We note that some of the adulterant band frequencies and relative intensities shifted or changed when they were dissolved in milk; in particular, the melamine 676 cm^{-1} band shifted to 683 cm^{-1} , the DCD 930 cm^{-1} band shifted to 926 cm^{-1} , and the smaller $2,198\text{ cm}^{-1}$ DCD band increased to a greater intensity than that of the $2,165\text{ cm}^{-1}$ band.

Sucrose-specific peaks within milk were only observed for higher concentrations (2,500 to 40,000 mg/L). Because of the poorer Raman scattering cross-section of sucrose, its Raman bands were much weaker and broader than those of the 4 N-rich compounds; sucrose also exhibited a greater degree of overlap with the milk component bands (Figure 3a). An extended spectral range is shown for sucrose between 500 and $1,800\text{ cm}^{-1}$ to include all the strongest bands of sucrose, which can be more clearly seen in the difference spectra in Figure 3b and in the spectrum of sucrose granules in Figure 3c. The 836 cm^{-1} band was used for peak height measurements as this band showed the least overlap with the milk component bands.

Calibration plots of peak height versus concentration were constructed for each of the 5 components and are shown in Figure A2 in the Appendix. The plots showed good linearity over the whole concentration range; the R^2 values, %RSD, LOD, and LOQ determined from the slopes are given in Table 1.

The LOD obtained for each of the 4 N-rich compounds in milk are 4 to 5 times lower than those from Raman imaging experiments and Fourier transform Raman measurements of powdered milk samples (Cheng et al., 2010, 2012; Qin et al., 2010, 2012, 2013a,b; Chao et al., 2013). For sucrose, the LOD of 7,290 mg/L (0.7%) is an improvement on the results from a near-infrared study of sucrose in milk powder (Borin et al., 2006) for which the LOD can be approximated as $3 \times \text{RMSECV}$ and was 0.86 to 2.58%. Good linearity was obtained for each compound over the whole concentration range investigated, and the LOD were close to the lower limits expected for adulterated milk, especially in the case of DCD and melamine. The improvement in LOD was due to improving the SNR by optimizing the slit width, sample presentation, using a high numerical

aperture microscope objective, and a 4-min integration time. The reproducibility afforded by the consistent sampling volume of $15\text{ }\mu\text{L}$ resulted in a %RSD average of 7% for the 4 N-rich compounds and 3% for sucrose, lower than the reproducibilities previously reported for SERS (Norrod et al., 1997; Guicheteau et al., 2011). The $15\text{-}\mu\text{L}$ sample volume allowed reproducible filling of the wells and although we did not explore smaller volumes, they would not be expected to affect signal intensity unless the drop dried to a thickness close to or less than the depth of focus ($8\text{ }\mu\text{m}$). The criticality of the volumes and fragility of the sampling method could be explored in a future study by experimenting with volume sizes close to this to determine the effect on signal intensity. The only 3 limitations to a smaller drop size would be that (1) the drop needs to fill the well reproducibly and evenly, which may not happen spontaneously with smaller volumes, (2) the drop must dry evenly to a reproducibly flat surface, and (3) the thickness of the dried drop should not be less than the depth of focus of the laser.

Calibration plots were used to determine the fraction of melamine, DCD, AmS, and urea in the cream fraction after centrifugation of milk solutions containing 3,000 mg/L of one of either melamine, AmS, or urea, and 5,000 mg/L of DCD. Spectra were recorded of these solutions in the region from $1,150$ to 560 cm^{-1} before and after centrifugation (Figure A3, Appendix). For DCD, the spectra were recorded in the $2,300$ to $1,980\text{ cm}^{-1}$ region. The percentages of each compound remaining in the cream fraction after centrifugation were determined from peak heights in difference spectra, using the calibration plots and are given in Table 2.

The results show that a significant proportion of the compound remains in the cream fraction after centrifuging, particularly for melamine and DCD. The greater solubility of these 2 compounds in the cream fraction was expected as they are more nonpolar than urea and ammonium sulfate, with the latter being the most polar and thus least fat soluble, which explains its relatively low concentration in the fat fraction. The results show the need to consider the possibility of introducing inaccuracies in measurement of milk solutions using techniques that require centrifugation in the preparation steps.

PLS Calibrations

The 120 spectra used in the training and validation sets for the PLS analysis of the 4 N-rich compounds and for the PLS-DA, with an additional 4 sucrose validation spectra, are plotted in Figure 4. The spectra have been baseline-corrected and smoothed, and the strongest bands for each component have been labeled.

Figure 2. The spectra of milk solutions spiked with single components melamine (Mel), ammonium sulfate (AmS), dicyandiamide (DCD), and urea at concentrations between 0 and 1,000 mg/L. The bottom spectrum shows DCD-spiked milk in the region 2,300 to 1,900 cm^{-1} . The peak positions of the strongest adulterant bands are labeled in each spectrum. Color version available online.

Table 1. Limit of detection (LOD), limit of quantification (LOQ), coefficient of determination (R^2), and residual standard deviation (RSD) from calibration plots of peak height versus concentration

Item	Melamine	AmS ¹	DCD ² (2,198 cm ⁻¹)	Urea	Sucrose
LOD (mg/L)	180	230	130	280	7,290 (0.7%, wt/wt)
LOQ (mg/L)	560	710	410	840	22,090 (2.2%, wt/wt)
R^2	0.98	0.99	0.99	0.97	0.98
RSD (%)	5.0	7.9	10.6	7.9	3.4

¹Ammonium sulfate.²Dicyandiamide.

The figure clearly shows the extent of overlap of the strongest bands for the 5 different components with each other and with those of the milk. The PLS1 calibration regression coefficients for the optimum number of factors determined for each compound are plotted in Figure 5. As expected, the largest regression coefficients for each model were coincident with the spectral region of greatest intensity for the analyte of interest.

For melamine and AmS, 3 PLS factors were used for the calibration; these accounted for 97 and 93% of the variance in the data, respectively. In contrast, DCD and urea required more factors, with 5 PC proving optimum for DCD, explaining 97% of the data variance, and 6 PC for urea, explaining 92% of the data variance.

The PLS calibration model predicted concentrations versus reference concentrations for the validation set

Figure 3. (a) Spectra of milk spiked with 40,000 and 0 mg/L of sucrose, (b) difference spectra of 5 solutions ranging from 2,500 to 40,000 mg/L, and (c) spectrum of a sucrose crystal. Color version available online.

Figure 4. Overlaid spectra (124) used for partial least squares (PLS) analysis and partial least squares with discriminant analysis (PLS-DA) after baseline correction and smoothing using the Savitsky-Golay method with 9 points and a second-order polynomial. Color version available online.

are plotted in Figure 6, and the regression statistics are given in Table 3. The RPD for the sucrose PLS calibration model was excellent (>8.1), allowing it to be used for any application in the concentration range from 0 to 40,000 mg/L (or 0 to 4%). For melamine, the RPD was very good (6.5 to 8.0) and suitable for process control, whereas that for DCD was good (5.6 to 6.4), making it suitable for quality control. The fair RPD values for AmS and urea indicate that these PLS calibration models would be suitable for screening only.

The LOD intervals for each compound and the parameters used to determine them from the PLS calibrations are given in Table 4. The LOD values determined from the univariate calibrations are included in the table for comparison. The $\text{LOD}_{\min}$ and $\text{LOQ}_{\min}$ values

determined for the PLS calibration models were less than half those determined from the univariate measurements. The exceptions were for DCD and sucrose, where the univariate LOD value was closer to the $\text{LOD}_{\min}$ for the PLS calibration; this may be due to better SNR observed for these compounds. For DCD, the univariate LOD was determined using the strong and isolated peak at $2,198\text{ cm}^{-1}$ in a different spectral region than used for the PLS calibration; for sucrose, a much larger concentration range was used. The $\text{LOD}_{\min}$ sets the threshold level below which the analyte can be declared absent and should be more reliable than the univariate LOD values, considering the robustness of the PLS method in discounting instrument noise and inaccuracies from peak overlap. The 100 mg/L $\text{LOD}_{\min}$

Table 2. Concentration of each contaminant determined from spectra of the cream fraction, using calibration plots of peak height versus concentration

Adulterant	Concentration added (mg/L)	Amount remaining in cream fraction	
		Concentration (mg/L)	%
Melamine (683 cm^{-1})	3,000	783	26
AmS ¹ (976 cm^{-1})	3,000	251	8.4
DCD ² ($2,198\text{ cm}^{-1}$)	5,000	1,019	20
DCD (924 cm^{-1})	3,000	641	21
Urea ($1,008\text{ cm}^{-1}$)	3,000	424	14

¹Ammonium sulfate.

²Dicyandiamide.

obtained here for DCD is very close to the 100 mg/L value obtained in a recent study using SERS on diluted milk (Lin et al., 2015).

The LOD_{max} values were 2 to 3 times the LOD_{min} values and set the upper threshold level above which the analyte can be verified as present; LOD_{max} ranged from 310 to 450 mg/L for the 4 N-rich compounds and was 2.5% for sucrose.

PLS-DA for Screening of Adulterated Milk Samples

A PC1 versus PC2 plot of an exploratory PCA analysis of the entire data set of 120 spectra showed that the samples of the adulterated milk solutions were dispersed along the PC1 axis according to concentra-

tion of components. However, many of the samples below 500 mg/L were grouped together with more than one-third of the pure milk samples. Therefore, PLS-DA was needed to obtain better discrimination between the pure and adulterated milk samples. A PLS-DA calibration model was calculated using the training set of 80 samples, of which 24 were pure milk spectra and 56 were samples adulterated with one or mixtures of melamine, DCD, AmS, and urea. Ten PC were necessary and explained 80% of the data variance. The model was used to predict the separate validation set consisting of 11 pure milk samples and 33 adulterated samples, 4 of which were sucrose milk solutions at concentrations ranging from 2,500 to 20,000 mg/L (Figure 7). The predicted values for the pure milk samples were centered

Figure 5. Regression coefficients for melamine, ammonium sulfate, dicyandiamide (DCD), and urea, showing the bands corresponding to the vectors used by the models to calculate the concentrations. The number of principal components (PC) used in each partial least squares (PLS) calibration are also indicated.

Table 3. Root mean squared error of cross validation (RMSECV), R^2 , standard error of prediction (SEP) and ratio of SEP to SD (RPD) for partial least squares 1 calibrations of melamine, urea, dicyandiamide (DCD), and ammonium sulfate (AmS) between 0 and 1,000 mg/L and sucrose between 0 and 40,000 mg/L

Item	Melamine	AmS	DCD	Urea	Sucrose
RMSECV (mg/L; internal cross validation)	42	95	62	80	611
R^2 (internal cross validation)	0.97	0.93	0.97	0.92	0.99
R^2 (external validation set)	0.98	0.94	0.97	0.93	—
SEP (mg/L; external validation set)	39	78	56	67	1,001 ¹
RPD	7.0	4.4	5.9	4.0	13.2
Applicability	Process control	Screening only	Quality control	Screening only	Any application

¹For sucrose, the SEP was determined from a leave-one-out full internal cross validation.

Figure 6. Predicted versus reference plots for partial least squares (PLS) prediction of the separate validation set for melamine, ammonium sulfate (AmS), dicyandiamide (DCD), and urea. Color version available online.

Table 4. Limit of detection (LOD) and limit of quantification (LOQ) intervals (minimum and maximum) for melamine, ammonium sulfate (AmS), dicyandiamide (DCD), urea, and sucrose partial least squares calibrations

Item ¹	Melamine (mg/L)	AmS (mg/L)	DCD (mg/L)	Urea (mg/L)	Sucrose (mg/L)
LOD _{min}	100	150	140	120	7,205
LOD _{max}	260	350	600	490	25,245
LOD univariate	180	230	130	280	7,290
LOQ _{min}	310	450	440	350	21,834
LOQ _{max}	800	1,070	1,810	1,490	76,501
LOQ univariate	560	710	410	840	22,090

¹LOD and LOQ determined from the univariate analysis are included for comparison.

around -1 , and the adulterated samples around $+1$, except for the 2 high concentrations of sucrose. The threshold limit was set at 0, as it halfway between these 2, excluding those of sucrose, which was not included in the training set. High values were predicted for the 15,000 and 20,000 mg/L sucrose samples, as they were very different from the calibration set.

Figures of merit such as false positive rate, false negative rate, and efficiency were calculated to evaluate the effectiveness of the PLS-DA calibration model for screening milk. The false positive, false negative, and efficiency rates express the trueness in qualitative analysis, and the selectivity rate measures the selectivity of

the method (Botelho et al., 2015). The PLS-DA model correctly predicted 100% of the adulterated samples, with a selectivity rate of 100%, and false negative rate of 0%. The incorrect prediction of 2 false positives resulted in a false positive rate of 15.4% and an efficiency rate of 84.6%. All of the sucrose samples were correctly identified as adulterated, even though no sucrose solutions were included in the training set.

CONCLUSIONS

The results show that Raman spectroscopy with PLS and PLS-DA is feasible for fast, reliable, and sen-

Figure 7. Partial least squares with discriminant analysis (PLS-DA) prediction of the validation set consisting of 11 pure milk samples ($\square$), 29 milk samples adulterated with one or more of melamine, urea, dicyandiamide, and ammonium sulfate ($\blacksquare$) and 4 samples adulterated with sucrose ($\blacktriangle$). Samples with a value greater than zero are predicted to be adulterated and samples with a value less than zero are considered pure. Color version available online.

sitive screening of milk for adulteration of milk with the N-rich compounds melamine, ammonium sulfate, urea, and DCD, even at low levels of 50 to 1,000 mg/L (0.05–0.1%, wt/wt). Spectra were recorded from dried drops of milk with no need for sample preparation or addition of chemicals, making this a greener technique. Reliable and consistent sample presentation enabled an excellent reproducibility of 7% RSD. Limits of detection obtained from the PLS calibrations were determined as interval-specific for the sample concentration range, with an average LOD_{min} of 128 mg/L for the N-rich compounds and 7,250 mg/L (0.7%, wt/wt) for sucrose and LOD_{max} 2 to 3 times these values. Simultaneous analysis of the 4 small N-rich molecules in milk was possible at these low levels due to the high selectivity of Raman spectroscopy and the use of multivariate analysis. With the increasing availability of competitively priced portable mini-Raman systems, the technique shows promise for deployment on-site for rapid and reliable detection of adulterated milk and is easily incorporated into microfluidics.

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APPENDIX

Figure A1. The spectra of pure milk and milk spiked with equal mixtures of melamine, ammonium sulfate (AmS), dicyandiamide (DCD), and urea at concentrations of 0, 50, 100, 200, 300, 500, 750, and 1,000 mg/L. Color version available online.

Figure A2. Univariate calibration plots of peak height versus concentration (mg/L) for melamine, ammonium sulfate (AmS), dicyandiamide (DCD), urea, and sucrose. Color version available online.

Figure A3. Spectra of 3,000 mg/L solutions of melamine (Mel), ammonium sulfate (AmS), and urea and 5,000 mg/L solutions of dicyanamide (DCD) in milk before centrifuging, and the cream fractions from each after centrifuging at $8,050 \times g$ for 15 min. Overlaid with each spectrum is a spectrum of pure milk (light gray) for comparison. Color version available online.