J. Dairy Sci. 99:1–17
http://dx.doi.org/10.3168/jds.2015-10342

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

M. K. Nieuwoudt,*†‡[1] S. E. Holroyd,§ C. M. McGoverin,# M. C. Simpson,*†‡#‖ and D. E. Williams*‡
*MacDiarmid Institute for Advanced Materials and Nanotechnology, New Zealand
†The Photon Factory, and
‡School of Chemical Sciences, The University of Auckland, 23 Symonds St., Auckland, 1142 New Zealand
§Fonterra Research & Development Centre, Private Bag 11029, Palmerston North, New Zealand
#The Dodd Walls Centre for Photonic and Quantum Technologies, New Zealand
‖Department of Physics, The University of Auckland, 23 Symonds St., Auckland, 1142 New Zealand

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 quantitation 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

Received September 1, 2015.
Accepted December 10, 2015.
[1]Corresponding author: m.nieuwoudt@auckland.ac.nz

of melamine in milk and infant formula (Balabin and Smirnov, 2011; Abbas et al., 2013; Jawaid et al., 2013; 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-Servín 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 differ-