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Characterising variances of milk powder and instrumentation for the development of a non-targeted, Raman spectroscopy and chemometrics detection method for the evaluation of authenticity

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Abstract

There is a need to develop rapid tools to screen milk products for economically motivated adulteration. An understanding of the physiochemical variability within skim milk powder (SMP) and non-fat dry milk (NFDM) is the key to establishing the natural differences of these commodities prior to the development of non-targeted detection methods. This study explored the sources of variance in 71 commercial SMP and NFDM samples using Raman spectroscopy and principal component analysis (PCA) and characterised the largest number of commercial milk powders acquired from a broad number of international manufacturers. Spectral pre-processing using a gap-segment derivative transformation (gap size = 5, segment width = 9, fourth derivative) in combination with sample normalisation was necessary to reduce the fluorescence background of the milk powder samples. PC scores plots revealed no clear trends for various parameters, including day of analysis, powder type, supplier and processing temperatures, while the largest variance was due to irreproducibility in sample positioning. Significant chemical sources of variances were explained by using the spectral features in the PC loadings plots where four samples from the same manufacturer were determined to likely contain an additional component or lactose anomers, and one additional sample was identified as an outlier and likely containing an adulterant or differing quality components. The variance study discussed herein with this large, diverse set of milk powders holds promise for future use as a non-targeted screening method that could be applied to commercial milk powders.

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Keywords

Skim milk powder (SMP); non-fat dry milk (NFDM); authentication; Raman spectroscopy; chemometrics; PCA; variance

Introduction

Non-fat dry milk (NFDM) and skim milk powder (SMP) are important food ingredients in an array of food products, from infant formula to baked goods, with over 2 million metric tons of NFDM traded globally in the first half of 2015 (USDA 2015). The value of a milk powder is primarily determined by its protein content which is traditionally measured via non-specific nitrogen concentration evaluation including Kjeldahl or combustion (Dumas) (Thompson et al. 2002; Chan et al. 2008). Consequently, non-protein, nitrogen-containing compounds and foreign proteins, such as plant proteins, can be illegally added to increase the apparent protein content and thereby the price of the commodity (Moore et al. 2010). This economically motivated adulteration (EMA), in concert with an expanding global and complex market, increases the likelihood of fraudsters using new adulterants in order to evade existing targeted methods of detection. Recent events, including cyanuric acid and melamine contamination in pet foods in 2004 and 2007, which killed a large number of cats and dogs (Brown et al. 2007), as well as the melamine contamination of Chinese milk powder in 2008, which caused thousands of victims and four confirmed infant deaths in China (Xin & Stone 2008), highlight the risks of EMA. To evaluate the scope of EMA, the United States Pharmacopeial Convention (USP) established a database of food fraud and economic adulteration reports since 1980 (Moore et al. 2012a), which has documented 155 scholarly reports of dairy product EMA between 2012 and 2015 (USP 2015). As such, there is an urgent need to develop reliable, non-targeted complementary tools to screen milk powder for unknown adulterants and identify instances of EMA.

Raman spectroscopy and near-infrared spectroscopy (NIR) are two promising analytical techniques being investigated as screening tools to identify EMA in food products (Baeten et al. 2005; Kasemsumran et al. 2007; Cheng et al. 2010; Moore et al. 2012b; Azizian et al. 2015). Common benefits of these two methods, compared with other technologies such as LC-MS/MS (MacMahon et al. 2012), ELISA (Moatsou & Anifantakis 2003) and GC-MS/MS (Hong et al. 2009), include simplicity, limited need for sample pre-treatment, rapid measurements, rugged instrumentation, portable systems availability and high-throughput analysis. Indeed, Raman spectroscopy can be applied to a large number of sample types, including powders, and through transparent containers. These attributes make Raman spectroscopy especially well-suited to milk powder analysis. With regard to NIR, there have been a number of studies that analyse milk products for authenticity (Iñón et al. 2004; Souza et al. 2011; Botros et al. 2013; Harnly et al. 2014). Botros et al. (2013) analysed 41 SMP and NFDM samples collected using diffuse reflectance NIR, and the milk powder variance was characterised using PCA and varimax rotation. In general, the researchers observed no clear trends based on different milk powder categories including day of analysis, SMP versus NFDM, supplier, and condensing temperatures. However, chemical variance attributed to

milk powder moisture, lactose, sugars, lipids and protein was explained using spectral features in principal components. Additionally, Harnly et al. (2014) further studied the same set of milk powder NIR spectra using analysis of variance (ANOVA)- PCA which allowed for detailed insight into small variance contributors that are not generally observed when employing PCA alone. The ANOVA-PCA score plots showed clear separation of the milk powder samples with respect to milk class (SMP or NFDM), day of analysis, production site, processing temperature, and individual samples (Harnly et al. 2014). Raman spectroscopy could offer advantages over NIR, including minimal interference from sample moisture, ability to measure smaller volumes, and limited effects from packing density and particle size distribution, but these have yet to be fully characterised for milk powder from a diverse (e.g. multiple suppliers and geographical origins) set of samples. As such, the studies herein focus on evaluating a larger number of naturally variant commercial milk powders ($N = 71$) in order to determine Raman instrument variance and milk powder variance with the general goal of creating a non-targeted, Raman spectroscopic and chemometric detection method for evaluation of milk powder authenticity.

Milk can have varied composition as imparted by the species of a dairy animal, breed, age, diet, geographic origin, lactation cycle, and time of year. However, for further processed products such as SMP and NFDM, the basic chemical composition (e.g. total protein, lactose, fat) is somewhat limited by international standardisation measures for milk powder compositions (CODEX 2011). Nonetheless, these major components and their minor constituents can add variability to the milk powder and then to the observed Raman spectra (Moros et al. 2007; McGoverin et al. 2010). McGoverin et al. (2010) have shown that Raman spectroscopy can be used in a targeted evaluation of milk powder constituents; specifically they evaluated New Zealand milk powder samples, quantified protein and fat concentrations, and identified the additive calcium carbonate. Further, the manufacturing processes for SMP and NFDM involve several steps including concentrating the skim milk solids in an evaporator and then spraydrying the heated milk concentrate to produce a powder. Each of these steps may introduce physio-chemical variance to the milk powder and eventually to the measured Raman spectra. Particularly, the processing type (low, medium or high heat) could alter the composition, especially with respect to protein denaturation and particle size variation (Oldfield et al. 2005). Due to this natural diversity of milk powders and variability in processing conditions, conventional library comparison of a measured spectrum of an unknown test material against a library of spectra from unadulterated milk powders for non-targeted analysis may not be sufficient, and multivariate chemometric methods, such as PCA as an exploratory method and soft independent modelling of class analogy (SIMCA) as a classification method, have been employed (Almeida et al. 2011; Jablonski et al. 2014). Therefore, the first step to determining if a non-targeted detection method for investigating potential adulteration in milk powders is feasible is to fully evaluate a wide variety of commercial milk powder products and determine the effects of the physiochemical and production variations within this commodity by Raman spectroscopy and PCA.

In addition to the variability in milk powder, Raman instrument and measurement parameters (e.g. laser/ spectrograph stability, background/noise contributions, laser focus

variability) and the environment (e.g. slight changes in laboratory temperature) could potentially contribute to variance in the measured Raman spectra. Specifically, sample fluorescence can significantly affect the Raman spectrum, and proper wavelength selection and spectral pretreatment is essential to minimising variability. As such, this study additionally sought to optimise the operating conditions and then evaluate the instrumental and environmental components to reduce the variance and improve the detection method.

This manuscript focuses on characterising the commercial, expected authentic, milk powders and Raman instrumental variance for the development of a non-targeted Raman and chemometric detection method. Raman spectra of a diversified set of 71 commercial milk powders were acquired, and the optimal pre-processing tools were determined. These data were then used to evaluate both the physiochemical and instrumental variances to determine if variability could be minimised thus allowing for a dataset that includes the natural diversity of this sample set. Specifically, variance of Raman spectra collected in a single day from 71 different bovine SMP and NFDm was first evaluated to investigate processing conditions, sample geographic origin, supplier, and chemical sources of variance. Additionally, Raman spectra collected on three consecutive days with a subset ($N=39$) of the 71 products were investigated to determine the day-to-day variability and within-sample variability. These sources of variance were characterised using PCA, and the patterns in principal component scores along with the spectral features in loading plots were evaluated for identifying the potential sources of variance. In addition to characterising the variance, the Raman and PCA method developed herein shows promise for evaluating authenticity of samples, including the ability to identify potentially non-authentic materials, which may allow for future screening for adulteration of milk powders.

Materials and methods

Milk powder samples

A set of 71 commercial milk powders were provided by USP. The products consisted of 19 SMP, 51 NFDm, and 1 unknown type, commercial-scale produced materials from at least four countries, seven producers, and 11 production locations. Details on production scale and date, processing temperature, particle sizes, and weight percentages of moisture, fat, and protein were also provided, and the abbreviated sample information is summarised in Table 1. All 71 samples were used for the initial, single-day variance studies, and a representative subset ($N=39$) of the 71 samples was used for a three-day variance study.

Instrumentation

Raman spectra of the milk powder samples were acquired with a DXRxi Raman Imaging Microscope (Thermo Fisher Scientific, Madison, WI, USA) system run with the corresponding OMNICTMxi Raman Imaging Software. Alignment and calibration were automatically performed daily using the standardised, automated protocols to ensure proper spectrograph alignment, wavelength calibration, white light calibration, laser alignment, and laser calibration. Raman operating parameters (e.g. laser wavelength, acquisition time) were evaluated. The optimum settings were obtained by using a 780 nm laser at 24 mW with a

full range grating ($50\text{--}3300\text{ cm}^{-1}$ at a resolution of 5 cm^{-1} (FWHM)) to acquire spectra from 2 second exposures with 60 scans after an automatically acquired background spectrum was obtained. All measurements were performed by using the macro sample adapter that has a $4\times$ side-looking objective threaded into the microscope nosepiece.

Sample preparation and Raman spectral acquisition

Milk powder stock samples were stored in sealed glass jars, desiccated and under a nitrogen flow. Each stock sample was remixed by multiple inversions before sub-sampling. While Raman spectroscopy could be applied to the bulk container or to the powders themselves, the sample holder on the Raman instrument used for these analyses was most suitable to small glass vials. For each milk powder sample, a small portion of the stock powder was thus transferred into a 2 ml glass vial (Wheaton, Millville, NJ, USA; one vial per stock powder) until the vial was approximately half full. The vial was inverted multiple times and then placed in a sample holder that attaches to a base-plate that sits in the microscope chamber to allow interrogation with the side-looking objective. Replicates were obtained in random sample order, and the vials were inverted and repositioned at a new location prior to interrogation. The focus (X , Y and Z positioning) was set by evaluating an SMP sample to obtain the highest Raman signal intensity while ensuring that there is no background from the glass vial. To verify alignment per manufacturer protocols and evaluate instrument stability, a polystyrene microscope slide (Thermo) was interrogated at 0.5 s exposure with 10 scans. Successful focus and instrument function was achieved when the peak at approximately 1001.4 cm^{-1} had a signal intensity of approximately 2000 cts.

Prior to performing full sample analysis, sample heterogeneity was investigated. Six randomly selected samples were mixed well prior to the analysis, and six replicate measurements for each sample were collected at multiple locations by spinning the sample vial. This experiment was performed over three consecutive days with two samples collected each day ($2\text{ samples per day} \times 6\text{ spectra per sample} \times 3\text{ days} = 36\text{ spectra total}$). After use of a standard normal variate (SNV) followed by a gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9), a variance study was performed with these samples through PCA. No noticeable variations between replicate measurements of the same sample were observed in the PC scores plots with each sample replicates tightly clustered together (data not shown). As no significant variations due to sampling were observed, a singular Raman spectrum for each of the milk powder ($N = 71$) in the full sampling investigation was deemed appropriate.

A single Raman spectrum for each milk powder sample ($N = 71$) was acquired in a single day to explore the effects of class of milk powder (SMP versus NFD), supplier, processing temperature, and the SMP chemical composition as sources of variance. In a separate day-to-day variance study, each milk powder from a subset ($N = 39$) of samples was run a single time over each of three consecutive days, resulting in a total of 117 spectra. The milk powder samples were run in random order with a polystyrene standard measured intermittently (approx. every 10 samples). Two replicate spectra were identified as being outside the majority grouping on only one of the three days for each sample (S021 Run 1 and S031 Run 2) by an initial PCA (data not shown). This was found by applying the

Hotelling's T² statistic obtained from the first four principal component scores at a 95% confidence level. As such, these replicate spectra were not used for subsequent analysis. Polystyrene spectra acquired during the three days of data collection were used to evaluate the stability of the instrument and the repeatability of positioning samples.

Principal component analysis (PCA)

The use of chemometric tools that are based mainly on exploratory analysis has shown success in interpretation of analytical data (Ding & Xu 2000; Souza et al. 2011). In this study, PCA was used to characterise the variance of the milk powder samples and investigate repeatability of the instrument (MATLAB 8.3, The Mathworks Inc., Natick, MA; PLS_Toolbox_8.0.1, Eigenvector research Inc., Wenatchee, WA, USA). PCA is a dimension reduction methodology in chemometrics that computes a set of linearly orthogonal variables called principal components (PC) from the linear combinations of the original variables. The first PC contains the largest possible variance in the data set with each subsequent component in turn explaining the highest remaining variance. Generally, the higher-order PCs contain irrelevant or random variation, thus only the principal components that capture relevant variation are retained when evaluating the scree plot. The contribution of each original variable to each PC can be plotted graphically. These PC loading plots can be used as visualisation tools to identify the spectral regions and corresponding functional groups which contribute the most to the sample variance. For each sample, a score value can be computed for each PC based on the projection of the sample onto the PC, and these PC scores can be plotted against each other. In this work, a score on PC1 versus score on PC2 was used to create plots that showed inter-sample relationships for these milk powder samples. Both the spectral features in the loadings plots and the trends in the scores were explored to identify the possible sources of variances in the milk powder samples.

Results and discussion

Instrumental variance

In order to evaluate instrument variance, polystyrene spectra acquired over a three-day period were investigated. On each day, the automated alignment and calibration routine was performed to reduce variability due to instrument components, the macro accessory was installed, and the focus was established to be equivalent to previous settings. Acquired polystyrene spectral data were pretreated using gap-segment fourth derivative computation followed by sample normalisation (Hawkes et al. 1982; DeJong et al. 2015). These pre-processing tools, discussed in detail in the next section, were subsequently used to correct for unwanted background variations in milk powder spectral data. Pretreated spectral data were then mean-centred prior to PCA. Figure 1(a) shows the resulting scores for PC1 for each sample in their data acquisition sequence. The corresponding loadings plot for PC1 shown in Figure 1(b) contributed to about 74% of the total spectral variance, and the spectral features were similar to those seen in a polystyrene Raman spectrum (Figure 1(c)). Inspection of the trends in the first principal component scores revealed slight deviations across 3 days, even within a singular day (Figure 1(a)). Since no baseline artefacts were observed in the loadings plot for the polystyrene standard (Figure 1(b)), the slight variability

is attributed to minor differences in focus and interrogation position on the slide (e.g. not a uniform polystyrene standard) which lead to peak intensity variability. These factors are expected to be less prevalent in the milk powder samples due to depth of focus and improved uniformity of the powders. These data indicate that after careful alignment/calibration, as well as sample positioning, the instrument is stable within a day and from day-to-day and should have limited influence on the variance.

Milk powder variance determined by chemometrics

Data pre-processing—In Raman spectroscopy, fluorescence, minor laser fluctuations, and sample variability can result in unwanted signal differences in the measured spectra that need to be corrected prior to PCA analysis (Huang et al. 2010). Without pre-processing, uninformative signal variations such as fluorescence could be incorporated into the model and negatively impact the spectral feature characterisation. In the non-targeted model developed here, incorporation of fluorescence background variance would broaden the classification boundaries of authentic ingredients, thereby decreasing the method's sensitivity for detecting lower concentration adulterants. This is clearly seen in the unprocessed Raman spectra for the milk powder samples (Figure 2(a)), where native fluorescence, even when using the 780 nm, NIR laser, was observed as a sloping, inconsistent baseline. This spectral background difference became the major source of variance, and, when PCA was performed without any baseline correction, the sloping, inconsistent baseline accounted for 97.97% of the total variance (data not shown).

Additionally, the raw spectra showed high fluorescence and potential laser line bleed at lower wavenumbers as well as increased baseline noise and etaloning at higher wavenumbers. In order to overcome these issues, spectral pre-processing was applied only to the 154–3190 cm^{-1} wavenumber range, which consisted of 1574 out of 1686 total wavenumber data points. Higher order derivatives (e.g. fourth derivatives) have been successfully used in spectroscopic applications due to advantages such as: (1) having the fourth-derivative curves peak maxima located at nearly the same position as in the original spectrum; (2) obtaining greater resolution enhancement for overlapping peaks; (3) enhancing narrower features; and (4) revealing bands that are not apparent in the original spectrum (Butler & Hopkins 1970; Padrós et al. 1982). Further, the challenge of decreasing the signal-to-noise ratio in higher order derivatives can be reduced by applying smoothing and introducing a gap size for the derivative calculation (Rinnan et al. 2009; DeJong et al. 2015).

To correct for the sloping baseline from the MP background, a gap-segment fourth derivative (Hawkes et al. 1982; DeJong et al. 2015) was employed (gap size = 5, segment width = 9) that enhances the narrow Raman spectral features while concurrently eliminating the fluorescent, broadband signal. In this method, the derivative spectrum is obtained by computing a finite difference between intensity values averaged over segments separated by some gap. A number of gap sizes (i.e. 3, 5 and 7) and segment widths (i.e. 7, 9, 11, and 13) were visually evaluated to improve the spectra via higher peak resolution and lower noise baseline, spectral features. With these spectra, higher segment widths removed peaks and led to overlapping bands in the higher wavenumber range, while lower gap sizes resulted in

higher spectral noise (data not shown). The first two principal component scores plots for each combination of gap and segment sizes were also evaluated, but no clear differences in sampling patterns were observed with different gap and segment size combinations studied. Additionally, a Savitzky–Golay second-order derivative with a smoothing filter (second-order polynomial, window size = 35 points) was evaluated. The sample patterns in the first two principal component plots were the similar to those observed with gap-segment derivatives; however, poorer Raman spectral peak resolution was observed (data not shown). As such, the results for only the best gap-segment derivatives will be discussed further.

Due to the edge artefacts introduced during the spectral pretreatment, the end points of all pre-processed spectra (50 points from both higher and lower wavenumber regions) were removed prior to PCA so that the spectral range was from 251 to 3093 cm^{-1} . Finally, sample normalisation (area normalisation) was performed for each milk powder spectrum in order to correct for overall scaling effects. Prior to PCA, a mean centering was performed so that only the variation relative to the mean, which is not dependent on the absolute magnitude of the data, was modelled.

Upon pre-processing the spectra in Figure 2(a), the corresponding post gap-segment derivative and representative normalised spectra shown in Figure 2(b) were achieved. As can be seen, gap-segment derivative transformation followed by sample normalisation was effective in removing the irrelevant variability (i.e. baseline artefacts and overall scaling effects in the raw spectra). As previously reported (Susi & Ard 1974; Li-Chan 1996; Kirk et al. 2007; Moros et al. 2007; Almeida et al. 2011; Ji et al. 2014), milk powder Raman spectral bands were observed in the derivative spectra as follows: lactose contributions can be observed in the lower wavenumber region at 355 cm^{-1} ; the features at 1010 (phenylalanine ring breathing mode), 1260 (Amide III), 1665 (Amide C=O_{stretch}), and 3060 (N-H_{stretch}) cm^{-1} are indicative of milk powder proteins; the bands at 1080 (C-C_{stretching}, C-C_{deformation} and COH_{deformation}), 1130 (C-C_{stretching}, C-C_{deformation} and COH_{deformation}), 1260 cm^{-1} (CH₂twist), 1330 (C-O_{stretching} and COH_{deformation}), 1460 ((CH₂)scissoring), 2880 (C-H_{stretch}), and 2930 (C-H_{stretch}) cm^{-1} are attributed to milk carbohydrates. Due to the lower fat content in the milk powders (i.e. less than 1.5% (w/w), Raman bands at 1750 and 2853 cm^{-1} that are generally assigned to the CH₂ symmetric stretching and C=O stretching modes of lipids were not observed. Interestingly, and discussed further below, samples S081, S082, S086 and S087 had an additional band at about 476 cm^{-1} and more intense carbohydrate bands (Figure 2(b)).

Day-to-day variability—When the majority of background variations (i.e. fluorescence) were eliminated through the spectral pre-processing method developed herein and PCA performed on the 39 milk powder sample subset over 3 days, the scores plot in Figure 3(a) was obtained. Within PC scores, only slight day-to-day variations were observed and were not unexpected based on the polystyrene data that showed potential irreproducibility in focusing when using this non-fixed path length instrument. Nonetheless, this variability is inherent in the measurements and important to include in the model to create a robust non-targeted approach. While the majority of samples clustered well within the 95% confidence level, four samples (S081, S082, S086 and S087) were separated along

PC1 (i.e. x -axis in Figure 3(a)). When the PC1 loading plot was inspected, a peak at about 476 cm^{-1} was observed (Figure 3(b)) that is unique to these four samples. In addition, carbohydrate related spectral features could be observed; however, the spectral features related to milk powder proteins (i.e. 1665 cm^{-1}) were not found in PC1. Interestingly, these four anomalous samples were all from the same production site (B-2 as shown in Table 1). It is suspected that while these are commercial, authentic milk powders, they either contain an additional component (e.g. carbohydrate) (Kizil et al. 2002; Ji et al. 2014) or variances in the alpha to beta forms of lactose (Kirk et al. 2007) that yield a band attributed to the C-O-C bridge of the glycosidic bond. For example, Kizil et al. reported a Raman band at about 478 cm^{-1} attributed to the skeletal vibrational modes of the pyranose ring in starch (Kizil et al. 2002), and it is possible that this or a similar component is present in these four samples.

In order to investigate other chemometric methods and these outlying samples, a variance study was performed for the same sample set but with standard normal variate correction (SNV) followed by a gap segment derivatisation (gap size = 5, segment width = 9). As shown in Figure 3(c), samples 81, 82, 86 and 87 were still separated from the rest of the samples. Interestingly, these samples were now separated along PC2, and the first PC loading plot explained chemical variations due to lactose, protein and lipid constituents (data not shown). It was also noticed that the first two PCs explained a higher cumulative spectral variance of 85.82% over the 66.54% for the normalisation pre-processing used in Figure 3(a). It is theorised that these dissimilarities were due to the differences in correcting the signal variations due to physical phenomena such as sample heterogeneity and minor differences in sample focus. This highlights that pre-processing methods should be evaluated in building non-targeted models and that the most appropriate model for the research at hand should be selected based on its performance in predicting known adulterated/non-adulterated validation samples.

These results were not unexpected, as previous NIR studies by Botros et al. (2013) that used a subset of the same samples also evaluated in this research showed a similar separation for these four samples upon PCA analysis. Using these four samples to develop non-targeted classification methods could broaden the range and classification boundaries of authentic ingredients, thereby simultaneously decreasing the sensitivity and detection of lower concentration, adulterated samples. For this work, it was not necessary to use these 'high-leverage' samples (i.e. those with highly variant components) for evaluating the general variance and building the training set. Instead, these samples would need their own training model as a second set of authentic MPs; however, four samples were not sufficient to build such a model, and future work will investigate these further.

These four samples were removed from the 39 sample subset ($N = 35$), and a new PCA was performed. PC1 describes the milk powder chemical sources of variability more clearly (Figure 4(a)), where the peaks are at the same spectral location as those in the original pre-processed spectra (Figure 2(b)). These peaks revealed the features related to chemical sources of variance (PC1, 17.27%) of the milk powder samples, including lactose, proteins and carbohydrates (Figure 4(b)). Based on work by Almeida et al. (2011) and McGovern et al. (2010) the higher positive loadings at 355 cm^{-1} were attributed to lactose, while protein

variations were explained by the positive loadings at wavenumbers 1010, 1260 and 1665 cm^{-1} . Bands at 1303, 1778, 2853 cm^{-1} which would be attributed to the presence of fat were, as expected, not observed due to the lower fat content of the skim and non-fat milk powders. In the PC scores plot in Figure 4(b), samples are clearly clustered within the 95% confidence level although there is an additional spread along the y-axis (PC2, 14.47%) which appears to be due to day-to-day variability. When the spectra were pre-processed using SNV correction followed by the gap-derivative, similar sample patterns in the scores were observed, except the first two PCs explained a larger percentage variance (PC1, 77.28% and PC2, 3.92%) with PC2 also elucidating the day-to-day variability (data not shown). The minor variations observed within the three days of data collection, particularly between day one of data collection and the other two days, are likely due to the inhomogeneity in the powders as well as minor differences in repeatability of sampling position and laser focus, as seen in Figure 1(a) of the polystyrene samples. The clear clustering and minimal variations observed, however, indicate that Raman spectroscopy is a robust and stable method for the evaluation of milk powder variance.

Characterisation of commercial milk powder by PCA—To evaluate spectral differences and to explore the possibility of developing a non-targeted detection method for authentication of milk powder, the set of 67 samples, excluding the four samples identified above, was evaluated on the Raman spectrometer in a single day. During this study, the goal was to evaluate the effect of supplier, milk powder class (SMP versus NFDM), processing temperature (low, medium or high heat), and country of production on the milk powder variations. The effectiveness of the spectral pre-processing used on the 39-sample subset was confirmed by the absence of baseline artefacts in the first two PC loadings (data not shown), and, to better evaluate trends with regard to specific samples, scores plots were generated. Only 65 spectra were used, as samples S106 and S107 were omitted from further PCA data processing due to CCD detector saturation from fluorescence.

The two-dimensional PC scores plots for the first two principal components with labels adjusted based on the processing type (low, medium or high heat and set of unknown (UK) samples) for the two spectral pre-processing methods: (1) gap-segment derivatisation followed by normalisation and (2) SNV correction followed by gap-segment derivatisation are shown in Figure 5(a,b), respectively. Figure 5(c) shows the same PC scores plot of that in Figure 5(a) but relabelled based on the milk powder supplier (A–D, F, H and a set of unknown (UK) samples). With regard to the clustering of samples, there was no difference between SMP and NFDM forms of powder (data not shown). In Figure 5(a,b), it is clear there was no trending with regard to the processing type employed, and the samples clustered well with the exception of S136 along PC1 in Figure 5(a). The separation of this sample in Figure 5(b) is not as noticeable compared with Figure 5(a), likely due to more cumulative, spectral variance captured by the first two PCs in the SNV pre-processed data. This singular sample highlights the importance of studying different spectral pre-processing methods when developing the non-targeted models.

Similarly, as seen in Figure 5(c), no trending due to production country/supplier was observed with the exception of S136. This sample was not studied in the original 39 sample

subset and was a clear and surprising outlier along PC1. It is hypothesised that this sample may be adulterated with an adulterant which has spectral features similar to milk powder or that the quality of milk powder is not consistent with the other samples (i.e. lower or higher concentrations of components such as lactose). To investigate these hypotheses, further investigation is underway. This sample and the four samples which were flagged in the day-to-day variance study show the potential power of Raman and PCA analysis as a rapid screening and discriminating tool.

If sample S136 were ignored, comparison to a similar evaluation on a subset of 41 of these same samples using NIR diffuse reflectance spectroscopy can be made. In the study by Botros et al. (2013), NIR spectra were pre-processed and then evaluated with PCA and varimax factor rotation. Those NIR results also indicated the presence of significant chemical sources of variance in commercial samples and highlighted the same outliers found in the present study. However, as found with Raman in the present study, no clear trends with regard to milk type, supplier, or condensing temperatures were observed. While the complementary NIR approach also allows for a potential authentication model, Raman may offer advantages due to the ability to have less influence from sample moisture and ease in interpretation of loadings and bands identities. In a follow-up, more detailed NIR study performed by Harnly et al. (2014), ANOVA-PCA score plots showed clear separation for the same subset of 41 samples with respect to milk class (SMP or NFDM), day of analysis, production site, processing temperature, and individual samples. Botros et al. (2013) concluded that 'defining boundary conditions for classifying commercial milk powder (by NIR and chemometrics) is still challenged by the unknown chemical sources of variance that discriminate between authentic milk powders'. As in these authors' work, modelling these small variation contributors in detail was both outside the scope of the present study and would serve to weaken the non-targeted, rapid screening Raman method universality.

Conclusions

The research performed on these 71 commercial milk powder samples demonstrated that the main source of variance observed by Raman spectroscopic measurements was due primarily to background variations (i.e. fluorescence). This, however, can be corrected by utilising the spectral pre-processing applied herein which entailed employing a gap-segment derivative transformation (gap size = 5, segment width = 9, fourth derivative) in combination with sample normalisation. However, as discussed, investigation of different pre-processing methods and selection of the most appropriate model based on screening known adulterated/non-adulterated validation samples is required. Further, interpretation of the principal component loadings plots demonstrated that the chemical sources of variance within the milk powder matrix needed to be fully explored and accounted for in order to create a robust, non-targeted Raman detection method for milk powder authentication and potential use in determining adulteration. No clear patterns based on milk types, processing conditions, sample geographical locations, or suppliers were observed. Additionally, the largest day-to-day variability was likely due to the challenges in identical repositioning and focus of the samples, and efforts to create a more repeatable focusing protocol are underway. Further, studies to develop additional models to include diversity from potential additional

components or large variations in lactose forms which may have contributed to the separation of the four authentic samples from the majority of the MPs are being performed. Interestingly, this method has already shown success in identifying outliers as shown in the case of one sample (S136) potentially containing an adulterant. To our knowledge, this is the largest, most diverse set of milk powders used to characterise the associated product variance which could potentially improve adulteration detection by more fully classifying the natural variance in milk products and developing a calibration model for a multivariate detection method. In the future, this Raman and PCA characterisation will be tested against potential adulteration types at economically motivated adulteration levels as well as expanded to include additional milk powders to determine the true utility and universality of this non-targeted method.

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Figure 1.

(colour online) Principal component analysis (PCA) of a polystyrene standard acquired on three consecutive days of data collection ($N=17$): (a) scores on PC1 in the order of data acquisition, (b) a representative loadings plot and (c) a polystyrene spectrum.

Figure 2.

(colour online) (a) Raw Raman spectra (without spectral pre-processing) for a subset of 39 milk powder samples collected over three consecutive days of analysis in three replicate measurements ($N = 115$) showing similar spectra for all samples, but with inconsistently sloping, fluorescence baselines that necessitate pre-processing; and (b) pre-processed spectra after using a gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9) followed by sample normalisation. For clarity, one representative pre-processed spectra (S082; top, blue line) is visually offset from the other five, representative samples (S022, S051, S070, S106 and S149; bottom lines) to highlight the additional feature at 476 cm^{-1} .

Figure 3.

(colour online) (a) Scores plot of PC1 versus PC2 from PCA of Raman spectra of 39 different milk powder samples each acquired in three replicate measurements on three consecutive days (115 spectra). (b) Loadings plot of PC1. Spectra for (a) and (b) were pre-processed by the use of the gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9) and sample normalisation. (c) Scores plot of PC1 versus PC2 from PCA of same Raman spectra ($N=39$ samples, 115 spectra) when pre-processed by the use of SNV followed by a gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9).

Figure 4. (colour online) (a) Loadings plot of PC1 and (b) scores plot of PC2 versus PC1 with samples S081, S082, S086 and S087 excluded. Spectra were pretreated by the use of the gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9) and sample normalisation.

Figure 5.

(colour online) (a, b) Scores plots of PC1 versus PC2 from PCA of Raman spectra of 65 different milk powder samples acquired in a single day of analysis. Plots are labelled based on processing type (low heat (LH), medium heat (MH), high heat (HH), and unknown (UK)). Spectra were pre-processed by the use of (a) the gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9) and sample normalisation and (b) SNV followed by a gap-segment derivative transformation (fourth derivative, gap = 5, segment = 9). (c) Same plot as (a) but with the points labelled by the supplier code (A–D, F, H and UK).

Table 1

Sample information from 71 milk powders.

Sample	Class of milk powder ^a	Production country	Blinded production location code	Process type ^b	Moisture content (%)	Fat content (%)	Protein content (%)
S021	NFDM	USA	A-1	LH	3.6	0.65	35.67
S022	SMP	USA	A-1	–	3.8	0.4	33.71
S023	NFDM	USA	A-1	MH	3.3	0.67	35.4
S024	NFDM	USA	A-1	MH	3.1	0.66	35.56
S025	NFDM	USA	A-1	LH	3.6	0.64	35.46
S030	NFDM	USA	A-2	HH	3.87	1.05	–
S031	NFDM	USA	A-2	HH	3.49	0.69	–
S032	NFDM	USA	A-2	LH	3.92	0.99	–
S033	NFDM	USA	A-2	LH	3.71	0.95	–
S047	NFDM	USA	A-1	LH	3.68	0.59	35.5
S048	NFDM	USA	A-1	LH	3.68	0.69	35.21
S049	NFDM	USA	A-1	LH	3.59	0.67	35.66
S050	NFDM	USA	A-1	LH	3.62	0.73	35.32
S051	NFDM	USA	A-1	LH	–	–	–
S052	NFDM	USA	A-1	LH	3.39	0.73	35.59
S053	NFDM	USA	A-1	LH	–	–	–
S054	NFDM	USA	A-1	LH	–	–	–
S055	NFDM	USA	A-1	LH	3.63	0.83	35.69
S056	NFDM	USA	A-1	LH	3.69	0.73	35.37
S061	NFDM	USA	H-1	LH	3.4	0.54	–
S062	NFDM	USA	H-1	LH	3.434	0.634	–
S063	NFDM	USA	H-1	LH	3.346	0.59	–
S064	NFDM	USA	H-1	LH	3.443	0.723	–
S065	NFDM	USA	H-1	LH	3.518	0.615	–
S066	NFDM	USA	H-1	LH	3.188	0.51	–
S067	NFDM	USA	H-1	LH	3.255	0.605	–
S068	NFDM	USA	H-1	LH	3.46	0.573	–
S069	NFDM	USA	H-1	LH	3.302	0.594	–
S070	NFDM	USA	H-1	LH	3.294	0.62	–
S076	NFDM	USA	A-2	HH	3.5	0.61	–
S077	SMP	USA	A-1	LH	4	0.58	33.4
S078	SMP	USA	A-1	LH	3.6	0.67	34
S079	NFDM	USA	A-1	MH	3.7	0.73	36
S080	SMP	USA	B-1	LH	4	0.65	34.29
S081	NFDM	USA	B-2	HH	3.17	0.75	35.44
S082	NFDM	USA	B-2	LH	3.59	0.66	36.09
S083	SMP	USA	B-1	–	3.87	0.6	34.06
S084	SMP	USA	B-1	MH	3.66	0.6	34.06

Sample	Class of milk powder ^a	Production country	Blinded production location code	Process type ^b	Moisture content (%)	Fat content (%)	Protein content (%)
S086	NFDM	USA	B-2	HH	3.58	0.7	35.64
S087	NFDM	USA	B-2	LH	3.29	0.62	35.84
S088	SMP	USA	B-3	MH	3.65	0.74	34.48
S089	NFDM	USA	B-3	MH	3.6	0.78	35.73
S090	SMP	USA	B-3	MH	3.45	0.75	34.16
S091	NFDM	USA	B-3	MH	3.78	0.95	36.31
S092	NFDM	USA	A-1	LH	3.8	0.66	36.29
S093	NFDM	USA	A-1	LH	3.8	0.76	36.04
S094	NFDM	USA	A-1	MH	3.8	0.77	35.9
S095	SMP	New Zealand	D-1	MH	3.9	1	32.7
S096	SMP	USA	B-4	MH	3.96	0.67	34.12
S097	SMP	USA	B-4	LH	3.78	0.69	34.3
S098	SMP	USA	B-4	LH	3.92	0.75	34.4
S099	SMP	USA	B-4	MH	3.87	0.6	34.2
S106	SMP	Ireland	E-1	MH	3.82	0.95	37
S107	SMP	Ireland	E-1	MH	4.49	0.95	35.7
S108	NFDM	–	–	–	–	–	–
S109	NFDM	–	–	–	–	–	–
S110	NFDM	–	–	–	–	–	–
S111	NFDM	–	–	–	–	–	–
S112	NFDM	–	–	–	–	–	–
S116	SMP	Denmark	C-1	MH	4	0.5	–
S117	SMP	Denmark	C-1	MH	4	0.07	–
S133	SMP	New Zealand	D-1	MH	3.8	1	32.7
S134	SMP	New Zealand	D-1	MH	3.8	1	32.7
S136	–	–	–	–	–	–	–
S140	NFDM	–	–	HH	–	–	–
S141	NFDM	–	–	LH	–	–	–
S145	NFDM	USA	F-1	LH	1.8	0.01	–
S146	NFDM	USA	F-1	LH	0.93	0.03	–
S147	NFDM	USA	F-1	LH	1.82	0.02	–
S148	NFDM	USA	F-1	HH	1.55	0.02	–
S149	NFDM	USA	F-1	HH	2.37	0.02	–

Notes:

^aNFDM, non-fat dry milk; SMP, skim milk powder.

^bLH, low heat; MH, medium heat; HH, high heat.