Non-protein nitrogen determination: a screening tool for nitrogenous compound adulteration of milk powder

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ABSTRACT

Kjeldahl and Dumas crude protein measurement methodologies do not distinguish between nitrogen native to milk and nitrogen in low molecular mass, nitrogen-rich adulterants. Measuring the non-protein nitrogen (NPN) content is one possible means of closing this loophole. Four methods were considered, with three selected for further research and validation: protein precipitation using trichloroacetic acid, protein precipitation using tannic acid, and molecular mass cut-off filtration. Kjeldahl assay was used on the supernatant or filtrate for all three methods. An NPN reference concentration range was established using 15 milk powder samples. This was followed by spiking experiments using seven low-molecular-weight, nitrogen-rich adulterants. Tannic acid precipitation and molecular mass cut-off filtration, both simple techniques available for routine use in food analysis laboratories, proved to be suitable for detecting the adulteration of milk powders with a variety of nitrogenous contaminants. NPN concentrations of $\geq 0.34\%$ in milk powders are suspected to result from adulteration.

1. Introduction

The economically motivated adulteration of milk products with melamine in China in 2008 was a public health tragedy that resulted in more than 300,000 cases of illness among Chinese infants and young children, six reported deaths, significant impairment of international trade, and the temporary collapse of the Chinese dairy industry (Gossner et al., 2009; Ingelfinger, 2008; Moore, 2014). The incident revealed the vulnerability of the standard methods for the determination of the protein content of milk-based ingredients and infant formulas to verify product specifications. Methods based on the Dumas combustion principle and the Kjeldahl digestion principle measure the nitrogen content and calculate the protein content using suitable nitrogen-to-protein conversion factors. These methods lack the specificity to discriminate protein from other nitrogen-rich chemicals. Hence, the addition of melamine or other nitrogen-rich compounds to fraudulently inflate the apparent protein content of milk ingredients cannot be detected by these methods (Moore, DeVries, Lipp, Griffiths, & Abernethy, 2010). New analytical approaches are needed to more specifically characterize the inherent nitrogen composition of milk, milk ingredients, and milk products to detect economically motivated adulteration.

Milk ingredients contain nitrogen in the form of protein and non-protein nitrogen (NPN) components. Measurement of the quantity of the NPN fraction can be utilized to determine whether adulterants have been added. The addition of low-molecular-weight nitrogen-containing chemical(s) (e.g., melamine, urea, etc.), to increase the apparent protein concentration that is measured by these traditional nitrogen test methods, would increase the total NPN content and would result in a failure to meet product acceptance criteria. Likewise, milk powder dilution with a nitrogen-free adulterant (e.g., water or maltodextrin) would lower the NPN content and, if carried out at a significant level, would decrease the measured total NPN.

The NPN fraction of milk and other foods can be isolated using different techniques.

Trichloroacetic acid (TCA) was used by Rowland (1938) to separate the protein and NPN fraction of milk. This approach has been standardized in AOAC methods 991.21, 991.22, and 991.23 (AOAC, 2012a,b,c) to improve the accuracy of milk protein determination compared with crude total protein analysis by AOAC method 991.20 (AOAC, 2012d) or ISO 8968-1|IDF 20-1:2014 (ISO/IDF, 2014). A similar TCA procedure was recently used to calculate an NPN index to detect adulteration in raw fluid milk (Gao, Li, Zan, Yue, & Shi, 2015). Cationic zinc (Zn^{2+}) has been used to precipitate milk proteins followed by centrifugation, a second precipitation, and a filtration step (Scott, 1934). Tannic acids (TAs), which are used to precipitate proteins in beer production, have been used for milk protein isolation and for other applications (Courtney & Brown, 1930; Silanikove, Perevolotsky, & Provenza, 2001; Turley, Kelly, & Smyth, 1990). TA and Zn^{2+} procedures are of interest for milk screening, given their simplicity and use of nonhazardous reagents. Other milk protein recovery and precipitation techniques that include the use of organic solvents alone (e.g., acetone or ethanol; Beaudoin, St-Georges, & Martin, 2003; Hewedi, Mulvihill, & Fox, 1985), sulfosalicylic acid (Du & Bo, 2006; MacWilliam, 1891), phosphotungstic acid (Courtney & Brown, 1930), pH adjustments, salt treatments, or combinations thereof with or without heat (de Haast, Morressey, & Fox, 1987; McKenzie, 1967; Ottenhof, 1985; Palmer, 1934; Scott, 1952; Spreer & Mixa, 1998) have been reported. All of these additional protein recovery and precipitation approaches have low NPN recoveries or are considered to be too complicated for routine screening analysis. Methods for the precipitation of plant storage proteins from milk powders using borate buffers and turbidimetry (Cattaneo, Feroldi, Toppino, & Olieman, 1994; Scholl, Farris, & Mossoba, 2014) do not separate the NPN from the milk proteins.

In contrast to chemical procedures, molecular mass cut-off filtration is a physical approach that can be used to separate milk protein from the NPN fraction (Atkinson & Begg, 1988). A filtration approach using a 10 kDa or smaller membrane is attractive given its simplicity and the fact that it

requires no reagents other than water. The various methods discussed above have not been fully validated using known or potential adulterants found in milk powder.

The objectives of this study were to develop and validate NPN measurement methodologies and to establish reference NPN ranges that would be suitable for screening skim milk powder (SMP) and nonfat dry milk (NFDM) to detect economically motivated adulteration. Although SMP and NFDM are similar, SMP manufacturers are allowed to adjust the protein concentration to 34% by adding protein-rich or protein-reduced milk fractions (ADPI, 2016). Thus, it was deemed to be important to study both commodities. Four candidate approaches for isolating the NPN fraction of SMP and NFDM were investigated: precipitation with TCA, TA, or Zn^{2+} , and molecular mass cut-off filtration. Typical NPN ranges for authentic materials were established, upon completion of which studies were conducted using nitrogen-rich adulterants to assess the performance of each method.

2. Materials and methods

2.1. Chemicals

ACS reagent grade TCA was purchased from Alfa Aesar (Ward Hill, MA, USA). ACS reagent grade TA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, zinc sulfate, and sodium hydroxide solutions were all prepared from ACS reagent grade or equivalent chemicals. Melamine, urea, ammonium phosphate dibasic, dicyandiamide, and L-arginine were purchased from Sigma-Aldrich. Aminotriazole was purchased from Alfa Aesar. Isobutylidenediurea, in an agricultural grade of unknown purity, was procured from an agricultural supplier.

2.2. Milk powders

Fifteen commercial samples (six SMPs and nine NFDMs; Table 1) of various geographic origins, processing temperatures, and protein concentrations were obtained from manufacturers and purchasers of commercial SMP and NFDM commodities. Their protein contents were obtained either from the certificates of analysis or by the Kjeldahl method (AOAC, 2012d) using a nitrogen-to-protein conversion factor of 6.38.

2.3. *Wet-blended milk powders*

A series of wet-blended melamine-adulterated SMP samples was produced in batches. Aqueous slurries containing different amounts of melamine were produced by mixing > 99% reagent melamine (Sigma-Aldrich) with deionized water at ~ 45 °C. The slurries were quantitatively added to condensed skim milk (~ 40% total solids). The admixtures were heated to ~ 45 °C, stirred with a high-shear mixer for 10 min, and pumped into a custom pilot-scale spray-drier system under conditions that were optimized to produce commercial-like SMPs. The system included a 1.5 m (W) × 4.3 m (H) spray-drying chamber with one cyclone in parallel that was capable of evaporation rates of 136–181 kg H₂O h⁻¹. The spray-drying conditions included inlet air temperatures of 150–200 °C and outlet air temperatures of 65–80 °C. The wet-blended milk powders containing melamine were stored under ambient conditions before subsequent testing.

2.4. *Adulterants*

To evaluate method performance, seven nitrogenous materials were selected based on their history of use as adulterants (USP, 2016) or their potential for use as adulterants in milk, milk-based

ingredients, and milk-based food products (see Table 2). The materials were also selected based on commercial availability and the cost-per-unit of nitrogen relative to that of nitrogen in SMP or NFDM. The adulterants were tested for TN content (see Table 2) using a validated Kjeldahl method (AOAC, 2012d). The TN content data were used to calculate the recoveries of adulterant-spiked samples.

2.5. *TCA precipitation method*

Powdered milk samples and water, at a concentration of 10% (w/w), were blended using an Ultra Turrax T 18 with S18N-10 g dispersion tool (IKA, Wilmington, NC, USA) at 14,000 rpm for 20 s. Ten grams of the suspension was then combined with 10 g 24% (w/w) TCA (500 g of TCA in 1583 mL of water) in a screw-topped test tube, capped, shaken vigorously for 30 s, and then allowed to stand for 10 min. The contents were then remixed and immediately filtered through Whatman #1 filter paper. The TN content of a weighed aliquot (up to 5 g) of filtrate was then determined by Kjeldahl assay (AOAC, 2012d). The %NPN in the sample was calculated, correcting for the dilution, using the following equation:

$$\% \text{NPN in sample} = (\% \text{N in filtrate}) \times [(g \text{ Ss} + g \text{ TCAs})/g \text{ Ss}] \times \text{DF} \quad (\text{Eq. 1})$$

where Ss is the sample solution, TCAs is the TCA solution, and $\text{DF} = (g \text{ sample} + g \text{ water})/g \text{ sample}$.

2.6. *TA precipitation method*

Milk powder samples (200.0 mg, accurate to 0.1 mg) were weighed into 15 mL centrifuge tubes and 6.00 g of 1% (w/w) sodium chloride was added and mixed thoroughly with a vortex mixer. After

standing for at least 15 min, the suspension was mixed again briefly to ensure homogeneity. Then 3.00 g of 12% (w/w) TA solution (12 g of TA in 88 g of water; the solution may darken, but is stable indefinitely) was added, thoroughly mixed with a vortex mixer, and allowed to stand for at least 30 min. The precipitate was then separated from solution by centrifuging for 15 min at $1000 \times g$ and the supernatant (~ 6 mL) was carefully transferred to a container for nitrogen determination by Kjeldahl assay (AOAC, 2012d). The %NPN in the sample was calculated using the following equation:

$$\% \text{NPN} = (\% \text{N in supernatant}) \times (\text{g NaCl} + \text{g TA} + \text{g sample}) / \text{g sample} \quad (\text{Eq. 2})$$

where NaCl is the sodium chloride solution and TA is the tannic acid solution.

2.7. Zn^{2+} precipitation method

Reconstituted SMP (10%, w/w) (0.5 mL) was added to a 15 mL centrifuge tube containing 10 mL 0.45% zinc sulfate solution and the contents were mixed well. One tenth normal base of sodium hydroxide (2 mL) was added, and the contents were mixed well and allowed to stand for ≥ 1 min to ensure complete flocculation. The tube was centrifuged for 15 min at $1000 \times g$ and the supernatant was carefully removed. The procedure was repeated, and the supernatants were combined and subsequently analyzed using a validated Kjeldahl method (AOAC, 2012d).

2.8. Molecular mass cut-off filtration method

Milk powder samples were combined with water at a concentration of 10% (w/w) and blended using an Ultra-Turrax with S18N-10 g dispersion tool (IKA) at 14,000 rpm for 20 s. Amicon Ultra-15

Filter Unit with Ultracel-3K membrane or Ultra 4 Centrifugal Filter Unit with Ultracel-3K membrane (Millipore, Billerica, MA, USA) was filled with sample solution and centrifuged according to the manufacturer's instructions until ≥ 2.5 mL of filtrate had been collected. The TN content of ≥ 2.0 mL of the filtrate was determined by Kjeldahl assay (AOAC, 2012d) and the %NPN, corrected for dilution, was calculated using the following equation:

$$\% \text{NPN in sample} = (\% \text{N in filtrate}) \times \text{DF} \quad (\text{Eq. 3})$$

where $\text{DF} = (\text{g sample} + \text{g water})/\text{g sample}$.

2.9. *Statistical methods*

The NPN contents of commercial milk powders were measured in duplicate by the TCA, TA, and molecular mass cut-off procedures, whereas the NPN contents of spiked samples were analyzed in triplicate. Multi-factor analysis of variance was performed using Statgraphics software, version 16 (StatPoint Technologies, Warrenton, VA, USA).

3. **Results and discussion**

The key success factors for the application of an NPN technique to detect adulteration include: (1) accurate and precise measurement of the NPN; (2) satisfactory determination of the range of the NPN inherent in the SMP and NFDM; (3) satisfactory recovery of added adulterants through the measurement of the NPN fraction. Initial attempts using Dumas methodology for NPN determination were unsuccessful because of poor precision that resulted from the very small sample size used in the

combustion analyzer combined with the low nitrogen concentration in the filtrates and/or supernatants. Kjeldahl methodology was used for the entire study reported here. The Zn^{2+} protein precipitation procedure (Scott, 1934) failed to give reproducible NPN results (results not shown) and was eliminated from consideration.

The NPN concentrations obtained using the TCA, TA, and molecular mass cut-off methods were 0.25–0.33%, 0.26–0.36%, and 0.24–0.33%, respectively, and agreed very closely in their grand means and prediction intervals (Table 1). Overall relative standard deviations (including method and sample variability) were 5.3, 7.7, and 6.7% for the TCA, TA, and molecular mass cut-off methods, respectively. Ninety-five percent prediction interval ranges were 0.06–0.09, and would enable adulterant detection at concentrations lower than those expected to be used for economic adulteration. The methods will also detect adulterants when the NPN concentration falls below the normal range, which has a dilution effect on the NPN concentration. However, the concentration of detection will not nearly be as low as for NPN detection.

The data obtained for each method showed a small, but statistically significant, bias ($P = 0.0056$) between the molecular mass cut-off method (mean, 0.282; 95% confidence interval, 0.276–0.287) and the TCA (mean, 0.293; 95% confidence interval, 0.287–0.299) and TA (mean, 0.294; 95% confidence interval, 0.288–0.30) methods. The bias is not practically significant when method variability is combined with the natural variability of milk powders; the methods possess equal ability to detect NPN adulteration (Table 1). Further statistical evaluation showed method–sample interaction between the TCA method and the TA method (Table 3). A method-sample interaction indicates not all samples are consistent regarding method bias. For example, the TA method result was highest for some samples, whereas, the TCA method result was highest for other samples. The small P -value indicated that this was not likely to be due to random sampling variation. This interaction has no practical effect on method

performance as long as there are no adulterant–reagent interactions, such as that found for TCA and melamine, as discussed later.

Subsequent to establishing the NPN range of the milk powder samples, adulterant-spiked SMP or NFDM samples were prepared by using one lot of non-adulterated SMP or NFDM containing a level of NPN that was near the grand mean and the seven different potential adulterants listed in Table 2, each spiked individually at concentration levels designed to achieve similar percentage nitrogen to the naturally occurring NPN (0.3%, w/w, nitrogen spikes). The spike recoveries (Table 2) were 14.2–122.2% for TCA, 45.9–99.0% for TA, and 82.5–100.5% for molecular mass cut-off. The detection capability of each method was calculated (Table 2) using NPN data for unadulterated samples and spike recoveries for each adulterant.

The TCA method is not currently recommended as an SMP or NFDM screening method for NPN adulteration because of low melamine recovery (14.2%), although the result for other potential adulterants tested seemed promising. Low recovery of melamine from raw fluid milk was also evident in the work of Gao et al. (2015); samples spiked to match an apparent increase of 0.2% protein (672 ppm urea, 1198 ppm ammonium chloride, 1478 ppm ammonium sulfate) resulted in an NPN index increase to approximately 5 (unadulterated milk average index 1.2), whereas the addition of 2000 ppm melamine (0.85% apparent protein increase) increased the NPN index to only 2.6 rather than to > 10, as expected. In contrast, the TA and molecular mass cut-off methods are suitable melamine screening methods and have now been adopted as standard procedures in the Food Chemicals Codex of the USP (USP, 2015). SMP or NFDM would be classified as adulterated (at the 95% confidence level) when the NPN concentration is $\geq 0.34\%$.

To test method robustness, three milk powder samples spiked with melamine were prepared by “wet blending”, rather than by dry addition. Wet blending might be the preferred technique for adulterating milk because of the ease with which the adulterant can be evenly distributed and disguised

by mixing in the liquid state. The melamine contents of the wet-blended samples, as determined by a previously reported liquid chromatography–tandem mass spectrometry method (Turnipseed, Casey, Nochetto, & Heller, 2008) were 300, 1000, and 3800 mg kg⁻¹. The melamine recoveries obtained using each method are shown in Table 4.

The TCA, TA, and molecular mass cut-off methods were effective in detecting melamine at 1000 ppm, i.e., the method registered at $\geq 0.34\%$ NPN, but only the molecular mass cut-off and TA methods were effective at the 3800 ppm concentration level. Interestingly, all three methods agreed with respect to recovery at the 300 ppm level. The TCA method provided nearly complete recovery at 1000 ppm, but $\leq 10\%$ recovery at 3800 ppm, thus reinforcing the decision not to include the TCA method as a method for detecting NPN adulteration. The recoveries using the TA and molecular mass cut-off methods were similar to those obtained using dry spiked and blended melamine in SMP and NFDM. Further studies were carried out to determine the cause of the low melamine recoveries when using the TCA method. It was discovered that crystals were formed and interfered with the effective use of TCA for measuring NPN of milk powder spiked with melamine. The potential composition of the crystals may be a melaminium trichloroacetate salt or salts of unknown degrees of hydration. These studies are reported in the supplementary material for this manuscript.

4. Conclusions

Methodologies based on TA precipitation or molecular mass cut-off filtration, followed by Kjeldahl analysis for NPN determination, were effective in detecting nitrogen-containing adulterants in milk powder. Milk powders with NPN concentrations $\geq 0.34\%$ are suspected to be adulterated. The US Pharmacopeial Convention (USP) has adopted methods based on the TA and molecular mass cut-off principles as standard procedures in the Food Chemicals Codex of the USP.

Further validation work is currently underway under the aegis of the International Dairy Federation (IDF) and the International Organization for Standardization (ISO) to assess the potential of extending the scope of the TA method to raw milk. If successful, this opens the way to replacing the existing ISO/IDF joint standard (ISO/IDF, 2016), which uses TCA precipitation rather than TA precipitation. This would provide an internationally harmonized standard for use by the global dairy sector, enabling simultaneous measurement of the NPN content and screening for economically motivated adulteration of both source material (raw milk) and finished product (milk powders) for no extra cost compared with existing testing regimes used in the dairy sector.

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Table 1Commercial milk powder characteristics. ^a

Sample	Sample description	Geographic origin	Protein content (% weight)	Production date	Non-protein nitrogen content ^b		
					TCA	TA	MCO
1	HH NFDM	USA, location 2	35.64*	15 January 2011	0.30, 0.30	0.29, 0.29	0.27, 0.31
2	HH NFDM	USA, location 6	36.06	16 November 2009	0.32, 0.30	0.33, 0.29	0.27, 0.29
3	MH NFDM	USA, location 4	36.31*	26 December 2010	0.29, 0.29	0.32, 0.28	0.28, 0.30
4	MH NFDM	USA, location 5	35.9*	13 February 2011	0.29, 0.28	0.36, 0.32	0.27, 0.26
5	LH NFDM	USA, location 2	36.09*	27 February 2011	0.29, 0.29	0.27, 0.29	0.27, 0.26
6	LH NFDM	USA, location 6	34.25	19 June 2010	0.32, 0.30	0.33, 0.32	0.29, 0.27
7	LH NFDM	USA, location 7	35.18	21 February 2011	0.29, 0.30	0.26, 0.26	0.27, 0.31
8	LH NFDM	USA, location 8	35.14	12 May 2012	0.32, 0.33	0.28, 0.27	0.30, 0.33
9	NFDM	Unknown	35.81	Unknown	0.28, 0.29	0.27, 0.26	0.28, 0.33
10	MH SMP	Ireland	37*	17 August 2010	0.30, 0.30	0.29, 0.28	0.31, 0.29
11	MH SMP	New Zealand	32.7*	20 October 2010	0.26, 0.25	0.30, 0.27	0.25, 0.24
12	MH SMP	USA, location 1	34.12*	8 February 2011	0.28, 0.27	0.30, 0.32	0.27, 0.31
13	MH SMP	USA, location 3	34.06*	30 January 2011	0.30, 0.30	0.29, 0.29	0.29, 0.25
14	LH SMP	USA, location 1	34.4*	29 August 2010	0.28, 0.29	0.33, 0.32	0.28, 0.27
15	LH SMP	USA, location 5	33.4*	21 February 2011	0.30, 0.29	0.28, 0.27	0.27, 0.27
Grand mean					0.29	0.29	0.28
95% PI (prediction interval for typical samples)					0.26–0.32	0.25–0.34	0.24–0.32

^a Abbreviations are: LH, low-heat; MH, medium-heat; HH, high-heat; NFDM, nonfat dry milk; SMP, skim milk powder; TCA, trichloroacetic acid; TA, tannic acid; MCO, molecular mass cut-off. An asterisk against a protein content value indicates as reported on the certificate of analysis.

^b The non-protein nitrogen (NPN) contents determined by the three methods are expressed as percentage (w/w) NPN on an as-is basis.

Table 2

Theoretical and measured nitrogen contents, spiked recovery values for mixtures of milk powder and adulterants by different methods and the adulterant detection capabilities of the method. ^a

Adulterant	Nitrogen content (theoretical; measured) (% w/w)	Spike recovery values (%)			Detection capability (%)		
		TCA	TA	MCO	TCA	TA	MCO
Melamine	66.64; 66.16	14.2	66.0	98.3	0.38	0.07	0.06
Urea	46.65; 46.58	99.4	99.0	100.5	0.07	0.04	0.09
Ammonium phosphate dibasic	21.21; 21.05	103.1	95.4	91.0	0.16	0.05	0.21
Isobutylidenediurea	32.16; 32.35	100.7	45.9	82.5	0.11	0.09	0.15
Aminotriazole	66.64; 39.55 ^b	120.1	87.7	91.6	0.07	0.05	0.11
Dicyandiamide	66.64; 65.87	122.2	85.5	95.1	0.04	0.05	0.06
L-Arginine	32.16; 31.91	117.1	77.4	84.0	0.09	0.06	0.15

^a Abbreviations are: TCA, trichloroacetic acid; TA, tannic acid; MCO, molecular mass cut-off. The adulterant detection capability is the minimum level (% w/w basis) of adulterant that, when added to skim milk powder or nonfat dry milk, increases the nonprotein nitrogen content above the 95% confidence level determined for non-adulterated materials. The spiked samples were analyzed in triplicate.

^b The significant discrepancy between theoretical nitrogen and measured nitrogen is believed to be due to incomplete conversion of nitrogen to ammonia under Kjeldahl conditions. Low recovery was replicated in additional experiments.

Table 3Analysis of variance for non-protein nitrogen – type III sums of squares. ^a

Source	Sum of squares	Df	Mean square	F-ratio	<i>P</i> -value
Main effects					
A: sample	0.0107433	14	0.000767379	3.22	0.0014
B: method	0.00277253	2	0.00138627	5.83	0.0056
Interactions					
AB	0.0207190	28	0.000739964	3.11	0.0003
Residual	0.0107077	45	0.00023795		
Total (corrected)	0.0449426	89			

^a All F-ratios are based on the residual mean square error.

Table 4

Melamine recovery for milk powder containing melamine incorporated by wet blending prior to drying.

^a

Sample	Melamine (mg kg ⁻¹)	Recovery (%) using method based on		
		TCA	TA	MCO
A	300	50.0	56.7	50.5
B	1000	97.6	86.0	84.3
C	3800	7.9	84.9	95.7

^a Abbreviations are: TCA, trichloroacetic acid; TA, tannic acid; MCO, molecular mass cut-off. R^2 values for TA and molecular mass cut-off were 0.9701 and 0.9967, respectively.