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True protein value of milk and dairy products

The authors

Duncan A. Rouch,[1] Peter Roupas[2] and Hubert Roginski[1]

1. School of Agriculture and Food Systems, University of Melbourne, Victoria 3010, Australia.
2. Food Science Australia, 671 Sneydes Road, Werribee, Victoria 3030, Australia.

Corresponcence to: Duncan Rouch, School of Agriculture and Food Systems, University of Melbourne, Victoria 3010, Australia.
Phone: + 61 3 8344 5784; Fax: + 61 3 9347 5219;
e-mail: duncanar@unimelb.edu.au

Summary

A statistical analysis was performed on results produced by a range of methods used to calculate the value of the protein content of infant formulae that were quoted in a report by the European Commission (2003, Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae). This report recommended that the nitrogen conversion value be reduced from 6.38 to 6.25. It appears that the data presented in the report do not support this recommendation. Nevertheless, the report's data underscore the importance of true protein and Non-Protein Nitrogen analyses of milk products. For Australia to gain the most benefit from true protein analysis, a number of standards will need to be revised.

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Correction

This article was published in the *Australian Journal of Dairy Technology*, Vol 61, No. 1, pages 37-41, with the incorrect title.

Introduction

Owing to the growing market value of milk components in milk and dairy products, a number of countries now require the measurement of true protein content, rather than crude protein content. In this context, we have examined the issue of estimating the protein values of infant formulae and follow-on formulae (formulae used for children who have developed beyond the infant phase). This issue was examined in a report by the European Commission (EC) (2003) which recommended altering the nitrogen conversion factor used to estimate protein concentration from 6.38 to 6.25 for both infant formulae and follow-on formulae. That recommendation reduces calculated protein concentrations by 2% and has been opposed by the International Dairy Federation (IDF) (2003, 2004, 2005).

The nitrogen conversion factor is used to quantify protein concentration by the long-standing and widely used Kjeldahl method (Karman and van Boekel 1986), which is the basis of key national standards (Australian Standard 1988; 1991), as well as international standards (ISO-IDF 2001; Association of Official Analytical Chemists (AOAC) 2003).

For many years, the protein content of dairy and other foods has been measured as crude protein. For milk and milk products, the conversion factor of 6.38 has been used to give the crude protein values, following the work of Hammarsten (1883) on purified casein. In contrast, a general conversion factor of 6.25 has been used for determining crude protein values in food products that contain uncharacterised proteins (United Nations University 1980). Different conversion factors for different foods have been proposed for two reasons, namely (a) different foods contain proteins that contain different amounts of nitrogen, and (b) different foods contain different amounts of other compounds that contain nitrogen (United Nations University 1980). Given that milk protein values have usually been used for comparing similar milk samples, the use of crude protein values has been a reasonable compromise for accuracy of protein measurement and minimising the cost of analysis.

Nevertheless, for a number of reasons, there have been moves to use more accurate protein values, described as true protein. While the Kjeldahl method is time consuming, newer infra-red spectroscopy methods are now able to offer faster analyses (Sørensen *et al.* 2003) and are routinely used in the US, though still calibrated with standards analysed by the Kjeldahl method (Shen 1999). The combustion nitrogen analysis method (Dumas and Gay-Lussac 1831) has also been introduced for milk protein analysis (Jakob *et al.* 1995; Wiles *et al.* 1998).

The concepts of crude protein and true protein result from the fact that, apart from protein, foods contain other compounds that contain nitrogen. As the Kjeldahl method measures all nitrogen atoms in a food sample (known as Total Nitrogen, TN) the results of such an analysis includes both protein and other nitrogen-containing components. The nitrogen content of other components is known as Non-Protein Nitrogen (NPN), which in milk includes urea, amino acids, small peptides, creatine, orotic acid, creatinine, ammonia, uric acid, hippuric acid, nucleosides and nucleotides (Wolfschoon-Pombo and Klostermeyer 1981; Schlimme *et al.* 2002). The NPN content of a food sample can be reasonably closely estimated using an addition to the standard Kjeldahl method, in which proteins and large peptides are precipitated by trichloroacetic acid (Australian Standard 1988). This leaves the NPN components in solution, for which the nitrogen content can then be measured by the standard procedure. The NPN components of milk average 4.9% of Total Nitrogen, with a range of 2.8% to 10.6% (Cerbulis and Farrell 1975).

Two standard methods are used for calculating true protein values in milk, the direct and indirect methods, which are equivalent (ISO-IDF 2001). In the direct method, used in the US (Malinak 2000), the protein component of milk is extracted by acid precipitation and the nitrogen content measured: the protein nitrogen content is multiplied by the standard conversion factor 6.38 to calculate the

true protein value. In the indirect method, as used in Australia (Australian Standard 1988), the true protein value is calculated by subtracting the NPN number from the Total Nitrogen number, which is then multiplied by the standard nitrogen conversion factor. If a milk sample contains 5% NPN the concentration of protein is calculated as (1- 0.05) x TN x 6.38.

Miera (1998) introduced the term 'real protein content' to refer to the actual protein content of infant formulae. The real protein content, which is the sum of the mass of anhydrous amino acids present in the sample after hydrolysis (i.e. amino acids from proteins and peptides, as well as free amino acids), is meant to reflect more accurately the nutritional value of protein in infant formulae; it differs from the concept of 'true protein', as outlined above, in that the latter does not include amino acids.

The aim of this study was to analyse the data presented in the European Commission's report, and discuss the implications of true protein analysis for both dairy farmers and manufacturers.

Methods

Data source

Protein values of infant formulae resulting from nine methods of calculation were taken from Table 8 of the report by the European Commission (2003), which was based on data from Tables 3-1 and 3-9 of Miera (1998). These nine methods are:

1. Crude Protein-N x 6.38.
2. Crude Protein-N x 6.25.
3. True Protein-N x 6.38.
4. True Protein-N x 6.25.
5. Total α-Amino Acid-N x 6.38.
6. Total α-Amino Acid-N x 6.25.
7. (True Protein-N + 46% NPN) x 6.38.
8. (True Protein-N + 46% NPN) x 6.25.
9. EC (European Commission: TN x 0.9 x 6.25).

True Protein nitrogen was generally reported as Total Nitrogen – Non Protein Nitrogen. An exception to this was the European Commission method, which used an alternative measure of True Protein based on the definition used by the report of the Life Science Research Office (1998). The protein content in the sample of milk or a dairy product can be determined as the sum of anhydrous amino acids (Sum AA) present in the sample after hydrolysis (one water molecule is lost when a peptide bond is formed, hence the need to calculate the sum of *anhydrous* amino acids). Sum AA corresponds to Miera's (1998) concept of 'real protein content', mentioned above. Sum AA data from Table 8 in the European Commission's report were used to calculate a standard to which other results were compared. Sum AA data was reported for only nine infant formulae of the total of 16, so only data for these nine (product numbers 3, 4, 6, 7, 10, 12-15) were available for statistical analysis. Thus, there were nine points of data for each method. From Table 6 in the report we calculated data for a further two methods:

10. (True Protein-N + 34% NPN) x 6.38.
11. (True Protein-N + 34% NPN) x 6.25.

While a value of 46% NPN was used for methods 7 and 8, following Dewey *et al.* (1996), the actual mean value for NPN content in the nine selected infant formulae was 34%, as calculated from Table 6 of the report. Thus, methods 10 and 11 were included to represent the revised NPN value. In summary, the protein data represented five pairs of methods that used either 6.38 or 6.25 as nitrogen conversion factors, with the exception of the EC method that used only a factor of 6.25.

Statistical methods

Three approaches were used to compare data from the 11 protein calculation methods to the Sum AA standard, namely linear regression and difference, using Excel version 2002, and one-way Analysis of Variance with post-hoc Tukey Honestly Significant Difference (HSD) analysis by Statistical Package for the Social Sciences (SPSS) version 9.0. In the linear regression protocol, the data from each method were aligned pair-wise with the Sum AA standard data, followed by analysis of variance in the slope and intercept factors in each case. The difference protocol, described here, used the results from subtracting the values from the 11 methods from the Sum AA standard. While the original data were not considered to be normally distributed (due to legal minimum and maximum limits), subtraction removed the underlying trend values, leaving results with normal distributions, the differences, according to the principles of multiple sampling. The variances of the difference data were calculated directly, then the one-way ANOVA protocol was performed using the difference data, providing Tukey HSD results.

Results and discussion

Comparing the results of the 11 calculation methods to the Sum AA standard data by linear regression, all the methods showed high levels of correlation, with R values ranging from 0.95 to 0.99, and R^2 values of 0.90 to 0.98. The nature of the alignments was further examined by statistical analysis of the intercept and slope values (Figure 1A and B). The values are plotted as mean ± 1 standard deviation (SD). For a perfect alignment, the value for the intercept variable is 0.0, and for the slope variable 1.0, as marked. For the intercept results, mean values within 1 SD for the theoretical best value were shown by six of the methods, including four of the seven True Protein methods, namely:

5. Total α-Amino Acid-N x 6.38.
6. Total α-Amino Acid-N x 6.25.
7. (True Protein-N + 46% NPN) x 6.38.
8. (True Protein-N + 46% NPN) x 6.25.
10. (True Protein-N + 34% NPN) x 6.38.
11. (True Protein-N + 34% NPN) x 6.25.

Of these six methods, the last two gave mean values closest to 0.0, both around 0.004. In contrast, both Crude Protein methods gave the greatest deviation from 0.0. However, if the deviation range was extended to ±2 SD, the mean for each method may lie near the Sum AA standard value, with the exception of only one method giving a result clearly outside 1 SD, namely 9, EC (Figure 1B).

The differences between the results of the 11 methods to the Sum AA standard were examined (Figure 2). The differences were plotted as mean ± 1 SD, with the theoretical best value being 0.0, as marked. The differences showed a similar pattern to the linear regression intercept results, with the same six methods giving means within 1 SD for the theoretical best value, 0.0. Unlike the linear regression analysis, however, difference protocol separated the mean values of paired methods that differed only by 6.25 or

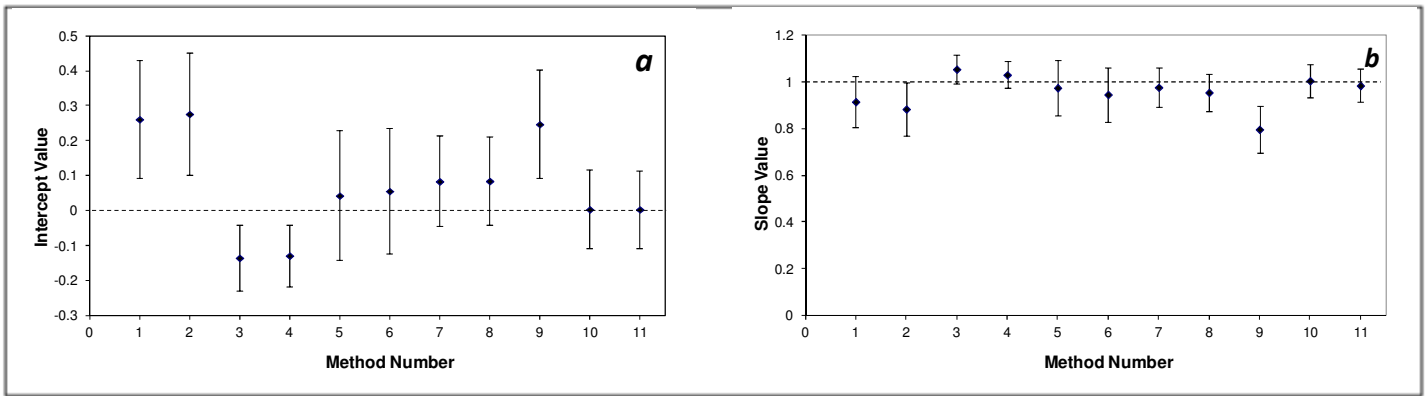

Figure 1: Regression analysis of 11 protein analysis methods. Protein value results of the infant formulae from the 11 test methods were compared to the values from the standard Sum AA method: (a) variation in intercept value, dotted line marks the predicted 0.0 protein value for a correlation coefficient value of 1.0; (b) variation in slope value, dotted line indicates the slope 1.0 value for a correlation coefficient value of 1.0. Diamonds (◆) mark mean values, and vertical bars mark ± 1 Standard Deviation. Numbers on the X axis correspond to the 11 protein analysis methods:
1, Crude Protein-N x 6.38; 2, Crude Protein-N x 6.25; 3, True Protein-N x 6.38; 4, True Protein-N x 6.25;
5, α-Amino Acid-N x 6.38; 6, α-Amino Acid-N x 6.25; 7, (True Protein-N + 46% NPN) x 6.38;
8, (True Protein-N + 46% NPN) x 6.25; 9, European Commission (TN x 0.9 x 6.25); 10, (True Protein-N + 34% NPN) x 6.38;
11, (True Protein-N + 34% NPN) x 6.25.

6.38 nitrogen conversion factors. The two methods with closest mean values to 0.0 difference were:

5. Total Amino Acid-N x 6.38.
10. (True Protein-N + 34% NPN) x 6.38.

As with intercept analysis, both Crude Protein methods gave the greatest deviation from the best theoretical value. Again, if the deviation range was extended to ±2 SD, the mean for each method may lie near the Sum AA standard value.

The difference means values were also analysed in one-way ANOVA with Tukey HSD. The results showed six overlapping subsets of the similar methods, with the two Crude Protein methods being most separated (data not shown). Overall, the groupings were comparable to the results from the linear regression for intercept and difference analysis.

Together, the results suggest that Crude Protein methods may not give acceptable data for infant formulae, due to over-estimation of the protein content. In contrast, a True Protein method may give accurate results. It also appears that the 6.25 nitrogen conversion factor may not be more effective than 6.38 in accurately calculating protein values for infant formulae. Moreover, the difference analysis suggests that the 6.38 factor may give more accurate data than 6.25. For example, the most accurate True Protein method was 10. (True Protein-N + 34% NPN) x 6.38.

Further work is required to draw firm conclusions, as the limited amount of data, only nine samples with 11 related measurements of them for the infant formulae, has led to wide confidence limits. For example, the overlapping of results of all methods at ± 2 SD, and the overlap of methods in the ANOVA test result are most likely due to the small sample size. If there were data for a reasonably higher number of samples, we could apply multiple regression methodology, which would allow calculation of the optimal equation for predicting true protein values.

The analysis of nitrogen conversion factors in the European Commission's report used, in part, a different method to calculate true protein content of infant formulae. The report used analysis of amino acid content to derive global nitrogen conversion factors to estimate true protein content in the presence of known average NPN content. For example, if milk samples contain an average 5% NPN, the global conversion factor to estimate the true protein content in different milk samples is 6.06, rather than 6.38. This method obviates the need to routinely measure the NPN value, as an average NPN value is assumed, but it is less accurate than the direct approach described in the introduction and used for methods 3, 4, 7, 8, 10 and 11.

The average global conversion factor for infant formulae to predict the 'real protein' value from TN was calculated as 5.90 (Miera 1998; European Commission 2003), which is considerably below that expected for a normal milk product. The difference is mainly due to the inclusion of whey and other components in infant formulae. Whey has more NPN compared to milk, at around 24% to 30% of TN, or around five to six times more than milk. The NPN values of various milk-based infant formulae quoted in the report ranged from 7.5% to 17.6% of TN (Miera 1998; European Commission 2003).

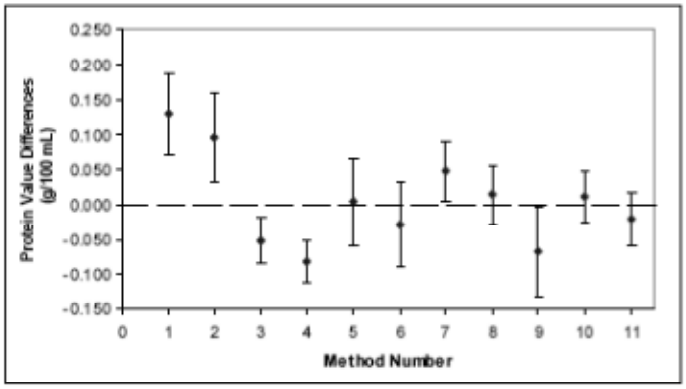

Figure 2: Difference analysis of 11 protein analysis methods. Protein value results of the infant formulae from the 11 test methods were compared to the values from the standard Sum AA method. Diamonds (◆) indicate mean values, and vertical bars mark ± 1 Standard Deviation. Numbers on the X axis correspond to the 11 protein analysis methods, as shown in Figure 1 legend. The dotted line marks the line of 0.0 difference compared to Sum AA values.

The accuracy of crude protein conversion factors for infant formulae can be adjusted by the known casein:whey protein ratios and demineralisation techniques (Miera 1998). Thus, for bovine milk-based formulae without added whey protein a factor of 5.93 is recommended, while for those with the addition of whey protein, a conversion factor of 5.70 is proposed (Miera 1998). With this approach, the error in protein estimation will be approximately ±3%. This difference follows from the different proportions of amino acid N in the total NPN in these two types of infant formulae.

With purified casein, a conversion factor of 6.37 is indicated (Harmmarsten 1883). Interpretation of this result is complicated by the use of the Dumas method by Hammarsten (Dumas and Gay-Lussac 1831) as the standard method for protein analysis in dairy food is that of Kjeldahl. Jacob *et al.* (1995) and Thompson *et al.* (2002) reported the Dumas method can produce significantly higher values than the Kjeldahl method for dairy foods, though in a cross-laboratory test Wiles *et al.* (1998) found no significant differences between data resulting from the two methods.

Since the proportion of nitrogen differs among amino acids, the amino acid composition of a protein can be used to derive a more accurate conversion factor. This has been investigated for milk proteins and concluded that a more accurate True Protein nitrogen conversion factor is 6.35 (Karman and van Boekel 1986; van Boekel and Ribadeau-Dumas 1987).

Since a number of components in the milk NPN group are available as human nutrients, such as amino acids and amino-sugars, they can be included with the protein content for more accurate nutrient value (Fomon *et al.* 1995; 1999). Miera (1998) and the Life Science Research Office report (1998), along with Fomon and colleagues, concentrate on human nutrition aspects without substantially addressing practical industrial food analysis.

The high levels and variability of NPN compounds in infant formulae render crude protein measurements far more inaccurate than for standard milk. Additionally, as an infant formula is used as the major part of the diet for an infant, it is prudent to utilise more accurate measurement. While analysis of total amino acid content is the most accurate method for evaluating 'real protein' values, the complexity and cost of this method renders it impractical for standard food analysis. In addition, while relevant information on product composition can allow adjustment of conversion factors to result in higher accuracy (Miera 1998), such information, however, is not always available. In these terms, true protein methods offer an advantage in the drive to obtain a higher accuracy in food protein analysis, as they obviate the need to rely on manufacturers' product information. Moreover, the costs of analysis are contained as only a single analysis of a sample is required, along with standards, as in the evaluation of crude protein. Also, reliability has been established in many laboratories through many years of use of true protein analysis methods.

It is curious that while the focus of the argument in the European Commission's Report concerns true protein analysis, a crude protein conversion factor is recommended, without supporting reasons. This is a clear inconsistency in the report which needs addressing. Our statistical analysis of the data in that report suggests that such a recommendation for a crude protein method is not valid. Currently, most countries in the European Union still use crude protein measurements for milk and milk products, while only France and Hungary require true protein data (VanRaden and Powell 2000).

The report also recommended that the NPN content of infant formulae should not be higher than 15% of Total Nitrogen content. This requires measurement of both NPN and true protein, which is also inconsistent with the recommendation for measuring crude protein.

What is the standard protein method in Australia for infant formula? The then Australia New Zealand Food Authority (ANZFA), now Food Standards Australia New Zealand (FSANZ), standard for infant formulae (Australia New Zealand Food Authority 2002a) requires only crude protein measurement, even though the inaccuracy of that method for infant formulae is well known. Nevertheless, another Federal Government agency, the Therapeutic Goods Administration (2002), requires true protein data for milk products. Moreover, the Australian Standards (1988; 1991) for milk analysis contain protocols for measuring true protein content.

While use of true protein data is prudent for infant formulae, there are other reasons for the accurate determination of protein and NPN contents in milk, both on the dairy farm and in manufacturing. DePeters and Ferguson (1992) proposed that determination of milk urea, a major component of the NPN group, in conjunction with true protein, could be used to improve management of the protein and energy status of lactating dairy cows. Subsequent investigations showed that milk urea data allowed improved balancing of diet to achieve greater efficiency of nitrogen utilisation and lower feed costs, while maintaining high levels of milk production (Godden *et al.* 2001).

Within Australia, true protein measurements have been required for domestic milk supply in both Western Australia (Olney *et al.* 1998) and Queensland (Queensland Government 1993).

Multiple Component Pricing (MCP) recognises the importance of protein, along with other milk solids. This pricing system began in Canada in 1992 and the US in 2000 (Agriculture and Agri-Food Canada 1996; Manchester and Blayney 2001). This occurred first due to the increasing trend towards low-fat dairy products. In the past, butterfat was usually the only component measured for milk quality and government financial support over many years. Second, Multiple Component Pricing leads to greater equity in milk pricing for both producers and processors. Producers are rewarded for the true value of milk solids, including protein, in the milk they supply, while manufacturers can purchase milk that has the optimal properties required for efficient production.

Conclusion

Data from the European Commission report on infant formulae and follow-on formulae, as statistically analysed here, support the importance of true protein and NPN analysis for milk products, despite its contrary recommendation. For Australia to gain the most benefit from true protein analysis, a number of FSANZ standards need to be improved, namely that of milk and milk products (Standards 2.5.1 to 2.5.7; Australia New Zealand Food Authority 2002b), as well as infant formula products (Standard 2.9.1; Australia New Zealand Food Authority 2002a). Furthermore, new Australian Standards are required to support the advanced infra-red spectrometry methods for milk analysis. Similar standards are also required at the international level, as IDF and ISO standards.

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