

Protein Analysis revisited*

Deficiencies of traditional nitrogen-based methods for detection of adulteration of food and feed have led to a search for alternative methods and an attempted redefinition of the term 'Protein'. Can traditional methods like Kjeldahl still be used? What about calibrations for indirect NIR and FTIR methods – and what are the alternatives?

The fraudulent addition of non-authentic material to food and feed has been a problem for over a century. Recent cases such as Melamine in Wheat Gluten, Melamine in Dairy, Leather Waste in Dairy and Urea in Wheat are new examples of a problem that dates back to before the Kjeldahl method was introduced in 1883. The modifications made, including fractionation schemes, have contributed to the lasting success of this method.

Even older (some say 1833, some 1848) is the Dumas combustion method, where the organic sample is burned at high temperature and the resulting gases are analysed. This method had its commercial breakthrough in the 1980's and 1990's.

So how good or bad are the traditional methods like Kjeldahl and Dumas? Do they have any chance of surviving, and what are the alternatives?

Existing analytical infrastructure

The Kjeldahl method is well known in the food and agri industry and is still the reference method for crude protein determinations, both by Dumas and NIR/NIT. Most NIRS calibrations should also be regarded as nitrogen-based methods as they are based on either Kjeldahl or Dumas. However, as a response to modern day fraud, such old nitrogen-based methods are naturally open to question.

Any answer to this must take into account that, perfect or not, these are the techniques out there working in the field. At an educated guess, there are more than 50,000 instruments for this type of protein analysis, at receiving stations for grain, dairy laboratories and such like, probably performing more than 50 million analyses per year.

The question then, is not how to replace them (because that will not be easy), but how to use them more effectively in our fight against fraud. Before jumping

to conclusions let's have a look at these techniques, their present status and their possibilities.

Developments in methods and standards

In the 1990's, Mercury was banned. Since most of the AOAC official methods were (and are still) based on Mercury as catalyst, this was considered the end of the Kjeldahl method. But then CuSO₄, TiO₂ and Selenium came in and in recent times newer standards have become available using copper sulfate and/or a mixture of CuSO₄/TiO₂ as catalyst.

Other milestones in protein analysis include pioneering work in crude protein analysis, starting in the 1970's with the introduction of block digestion and steam distillation and the UDY dye binding assay, continuing in the 1980's with infrared techniques (NIR/FTIR) and elemental analysis (Dumas combustion method).

Standard methods have gradually been updated to reflect instrumental and methodological improvements in crude protein analysis and due to the need for well defined reference methods for indirect spectroscopic methods.

In the first decade of this century a number of new Kjeldahl standards have been issued (references 1 & 2). These include standards for Non-Protein-Nitrogen using TCA precipitation (example: Reference 3). This is of interest as it shows how to differentiate between protein and non-protein by using a fractionation scheme.

Other standards are based on the Dumas combustion method. As this method uses the same factors as the Kjeldahl method for the calculation of the crude protein content, it principally results in higher protein values than Kjeldahl. The Kjeldahl method does not recover all organic nitrogen. Heterocyclic compounds are only partially recovered and inorganic

nitrogen fractions such as nitrate and nitrite are not determined by Kjeldahl. This makes Kjeldahl the recognised method for determining crude protein content. The European Commission confirmed the Kjeldahl method as the community method for official controls (Commission Regulation (EC) No 152/2009).

Amino acid analysis

The AAFCO (Association of American Feed Control Officials) PTS is one of the most comprehensive proficiency testing schemes with over 200 laboratories and more than 100 reported methods. On the basis of eighteen reported amino acid concentrations and their nitrogen content, protein values have been calculated using the factor 6.25 and compared with the reported crude protein contents using the Kjeldahl (AOAC 2001.11) and Dumas methods.

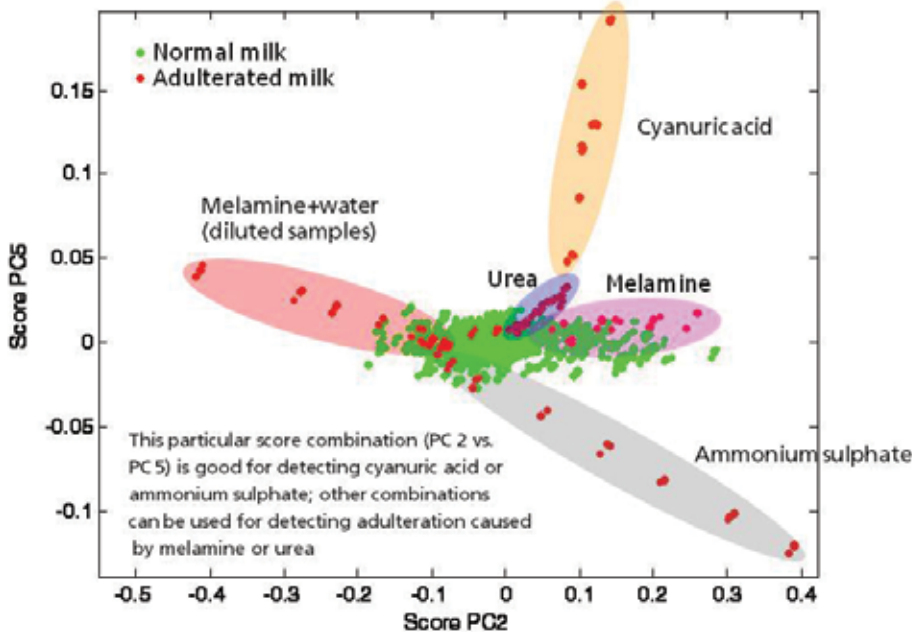
The protocols for the amino acid analysis (AAA) are in most cases AOAC 994.12 using post column Ninhydrin or pre column AQC derivatization. For tryptophan alkaline hydrolysis in combination with ion exchange chromatography (AOAC 988.15) is used and cysteine and methionine have been determined after oxidation and subsequent hydrolysis and ion-exchange chromatography (AOAC 999.13). There are therefore up to three different protocols for AAA. The differences compared to Kjeldahl and Dumas are not explained by non-protein nitrogen, but by incomplete recoveries in AAA.

Amino acid analysis maybe a tool for protein identification, but it is questionable whether AAA can replace Kjeldahl and other nitrogen-based methods in routine analyses. In addition to accuracy, sample throughput, precision and cost per analysis play a role.

Furthermore, nitrogen-based methods are still so widely used for crude protein analysis that they would be difficult to

Sample	Type	Kjeldahl	Dumas	Amino Acid
AAFCO 200921	Chicken	17,29 (0,15)	17,64 (0,33)	14,22 (0,17)
AAFCO 200922	Pig starter	23,94 (0,33)	24,51 (0,39)	19,73 (1,18)
AAFCO 200923	Chow	12,3 (0,52)	12,51 (0,65)	7,16 (0,19)

Table 1: Protein content (%) of feed samples using different methods (in parenthesis standard deviations)



replace. And, when discussing alternative solutions, it should be considered that most NIR calibrations for protein are based on Dumas or Kjeldahl results. The FOSS grain ANN calibration, for example, is based on data from 50,000 samples gathered over some 25 harvest years.

Dye binding

In recent years, dye binding capacity (DBC) assays have been re-launched as a solution for tracing intentional adulterations. DBC detects proteins and not added nitrogen sources. The dye binds not to the alpha amino nitrogen, but to the basic nitrogen (epsilon N) which is found in certain amino acids. The decolourisation of the dye due to its binding to these sites is measured.

As the sequence of amino acids is genetically determined, calibration graphs vs Kjeldahl for a certain species/variety can be generated. But this may be problematic for products of varying composition.

Infrared

FTIR and NIR have shown quite a potential for the detection of adulterants. Adul-

terants have different spectral signatures which makes it easy to distinguish even related compounds.

PCA calibrations can successfully detect 'abnormal' samples. However, it is critical that no adulteration is present in such data sets as this will 'poison' the calibration, i.e. make it impossible to detect adulterations (intoxicated calibrations).

FTIR and NIR are usually optimised for quality parameters such as protein and fat, but have potential as a fast and cost effective screening for adulterants. The application of FTIR and NIR will be most successful at the point of collection of the raw material or at the intake, partly because of higher concentrations, and partly because of more effective traceability.

Fraudulent additions of adulterants are most probably done at levels above the measurement uncertainties of the nitrogen based methods, i.e. > 0,2-0,4% CP or >0,05-0,1% melamine. The addition of 0,1% melamine will correspond to 0,4% higher crude protein values. Different fractionation schemes are being investi-

gated for the determination of crude protein in the presence of illegal adulterants.

The analysis of spectral integrity offers a good chance of detecting higher levels of a broad range of adulterants, including those of unknown nature. In addition, specific calibrations can be developed and used for known adulterants.

A combined strategy

Kjeldahl - including non-protein fractionation techniques - will still be the most important reference method. This is mainly due to the lack of alternatives. To respond to the ongoing threat of adulteration of food and feed, this reference method must be combined with rapid methods.

Kjeldahl based schemes and fast NIR and FTIR techniques are already in place in thousands of sites. These could be used for screening, preferably as close as possible to raw material sources or intake although they will have to be complemented with more advanced techniques for the confirmation of different adulterants.

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References:

- (1) Möller J., Kjeldahl - still going strong, In Focus, Vol 33, No 1, 2009, p 14-16.
- (2) Möller J., Traceability in modern Kjeldahl analysis, In Focus, Vol 29, No 1, 2005, p 4-5.
- (3) ISO 8968-4/ IDF 20-4/ AOAC 991.21
- (4) USP workshop, including the presentations given, can be found under www.usp.org/goto/proteins

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