KJELDAHL'S NITROGEN DETERMINATION METHOD

Anna Politis, graduate engineer of nutrition technology and of biotechnology, reports

For more than 120 years, Kjeldahl's nitrogen determination method has been an internationally accepted standard. In 1883, chemist Johan Kjeldahl developed this method for the quantitative determination of nitrogen. In the dairy industry, Kjeldahl's nitrogen determination method is used to determine protein content. The process is applied in accordance with ISO, DIN 8968-2/8968-3. To calculate the protein content, the nitrogen value obtained is multiplied by the product-specific factor for milk and dairy products, **6.38**. Nowadays, the entire determination can be automatised, even with multiple samples.

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1. Principle

The sample is solubilised with concentrated sulphuric acid and potassium sulphate in the presence of the catalyst copper sulphate. This causes the nitrogen bonded in the organic compounds to convert into the inorganic compound ammonium sulphate. Boiling the solution with sodium hydroxid releases ammonia from the ammonium sulphate. This is then led together with water vapour through a distillation apparatus. An ammonia-water solution results, which is introduced into a exactly defined amount of boric acid solution. By acidimetric titration, the amount of bonded boric acid and the nitrogen content is determined. Then protein content of the sample can be calculated with the help of the protein specific conversion factor.

2. Chemicals needed

2.1 Potassium sulphate (K_2SO_4) with a low nitrogen content.

2.2 Copper sulphate solution ($CuSO_4.5H_2O$): 5.0 g copper sulphate pentahydrate are dissolved and stirred into 100 ml of water.

2.3 Sulphuric acid: with 98 % by weight, nitrogen free, ρ20(H_2SO_4)~1,84 g/ml

2.4 Sodium hydroxid: with a low nitrogen content and a percent by weight of 30 g of sodium hydroxide per 100 g.

2.5 Indicator solution: 0.1 g of methyl red is dissolved into 95 % ethanol and diluted to 50 ml with ethanol. 0.5 g of bromcresol green are dissolved into 95 % ethanol. One part methyl red solution is mixed with five parts bromcresol green.

2.6 Boric acid solution (H_3BO_3): 40.0 g of boric acid are dissolved in one litre of hot water. The solution is cooled and the volume is adjusted to one litre. 3 ml of indicator solution (2.5) are added and the solution is stirred and stored in a borosilicate glass bottle (the solution is light yellow). The solution must be kept away from light and ammonia vapour during storage.

2.7 Hydrochloric acid: the concentration must be 0.1±0.0005 mol/L.

2.8 Ammonium sulphate [$(NH_4)_2SO_4$]. Before use, the ammonium sulphate must be dried for at least two hours at 102 ±2°C and cooled in a dehydrator to room temperature. The purity of the dried substance must equal 99.9 %.

2.9 Water: distilled or fully desalinized water or water of equivalent purity.

2.10 Sucrose: with a nitrogen content of less than 0.002 %.

2.11 Tryptophan or lysine hydrochloride with a purity of at least 99 %.

3. Equipment and appliances

3.1 Laboratory scale: suited for weighing increments of 0.1 mg.
3.2 Boiling stones: grain size 10; (boiling stones not for reuse).
3.3 Water bath, adjustable to (38±1)°C, suited for tempering milk and dairy products.
3.4 Digestion apparatus (art. no. 4200) consisting of a metal block, equipped with a heater and temperature regulator, and an emissions collector (supply voltage 230 V, temperature range up to 450°C).
3.5 Extraction apparatus with suction system (Behrosog 3, art. no. 4203): this neutralises dangerous vapours.
3.6 Distillation apparatus suitable for connecting to the 250 cm^3 digestion flask (art. no. 4210).
3.7 Titrator: an automatic titrator or a burette with a nominal volume of 50 ml and a scale division calibre of at least 0.1 ml in accordance with ISO 385, class A (art. no. 4220).
3.8 Digestion flask with a nominal volume of 250 cm^3
3.9 Pipette: suited for dispensing 1 ml of copper sulphate solution (2.2).
3.10 Erlenmeyer flask, nominal volume of 500 ml.

4. Preparation

The milk sample is heated to 38±1°C in a water bath, stirred gently, and then cooled to room temperature. (5± 0,1) g are weighed out to exactly 0.1 g into a digestion flask.

5. Procedure

1 **Digestion**
Total solubilization time: 1.75-2.5 hours. **Note: must be executed under a flue.**
Proteins + H_2SO_4 + K_2SO_4 + $CuSO_4$ → $(NH_4)_2SO_4$

12 g of potassium sulphate, 1 ml of copper sulphate solution (2.2), about (5± 0,1 g) of the heated and mixed milk sample and 20 ml of sulphuric acid are introduced into a digestion flask (the exact amount of milk must be determined to ± 0.1 mg and recorded because it will later be the basis for the nitrogen calculation. See "calculation"). The digestion flask is mixed carefully.

A suitable temperature program is selected on the digestion device, and then the digestion flask is set on the heating block. A glass emissions collector is carefully fixed onto every digestion flask. The entire apparatus is connected to a second apparatus (Behrosog 3, art. no. 4203) by a hose which can neutralise dangerous vapours. Selecting the following temperature program is recommended:

1) Preheat the heating block at 200°C for 10 min.
2) Heat the sample to 200°C for approx. 30 min.
3) Continue at 420°C for approx. 90 min for digestion (at a capacity of 10°C/min).

The digestion time is to be adjusted so that the maximum nitrogen contents are obtained. Too short or too long digestion times can lead to low values.

After digestion, the samples are removed from the heating block and cooled at room temperature for 25 minutes. Afterwards, they are put into the distillation apparatus.

- *The addition of potassium sulphate serves to increase the boiling temperature of the sulphuric acid and the addition of copper sulphate serves as an oxidation catalyst. They are also available as Kjeldahl tabs (art. no. 4230/4231). If the process is executed with tabs, then 5± 0.1 g of milk are mixed with 20 ml of sulphuric acid and 2 Kjeldahl tabs and left to sit for 5 min. Then the temperature program can be carried out.*
- *During the heating of the sample, foam is not allowed to climb higher then 4 - 5 cm below the flask opening.*
- *The sulphuric acid is added in such a way that any copper sulphate solution, potassium sulphate or milk which may have adhered to the flask neck is flushed down. If the flask is sealed airtight, it can also be stored for later digestion.*
- *To determine the specific digestion time, it is advisable to execute preliminary tests with samples high in protein and fat.*
- *Substantial crystallisation is a sign of too little sulphuric acid and can lead to low protein values. It is therefore advisable to reduce the loss of sulphuric acid by minimising the amount sucked.*
- *Before the hot digestion flasks can be taken from the digestion block, it must be ensured that no condensed fluid has collected in the extraction apparatus. If it has, the suction volume must be increased and the condensed liquid removed.*
- *The undiluted digestion should not be stored in the flask for a long period of time (overnight) for any reason. There is a risk that the sample will solidify and it is difficult to bring it into solution form again. After the sample is cooled down and dilluted with 70 ml of water, there is no problem to keep it overnight.*

2 **Distillation**
Total distillation time: 5-7 min

Ammonia release $(NH_4)_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + 2\ NH_4OH$
Ammonia absorption $NH_4OH + H_3BO_3 \rightarrow H_2O + NH_4H_2BO_3$

Do not touch any parts of the distillation apparatus either during a distillation or for a while after.
They could be hot.

The amount of water and sodium hydroxid, the reaction time, the distillation time, the heat output of the vapour generator and the suction time for the remains distilled out of sample are programmable. With the push of a button, the desired menu is found and by pushing the button again it is selected. Another push changes the value and a final push saves it.

The distillation apparatus must be degassed if it has not been used for a long time or if it is being used for the first time. To do this, "options", and then "direct input" are selected. Then, "H_2O in sample" is selected and the knob is held down until water runs into the digestion apparatus. Next, "NaOH" is chosen and the button is pushed down until soda lye runs into the digestion vessel. Finally, the menu "extractionPro" is selected by pushing "next" and the operating knob is pushed down until the chemicals are sucked from the digestion vessel. The aeration process is now finished.

A trial run without a sample must be executed daily before beginning the distillation process. To do this, a discharge hose is introduced into an empty digestion vessel, the main menu "options" and then "direct input" are selected. Then, "vapour" is chosen. By quickly pushing the operating button again, the vapour discharge is begun. Renewed pushing of the button ends the process. The process should not be ended until there is 1 cm of distillate in the Erlenmeyer flask. Finally, "extractionPro" is selected and the operating button is pushed down until the water is extracted from the digestion vessel.

After degasing and the test run, the distillation of the sample is carried out. A 500 ml Erlenmeyer flask for the boric acid solution is placed under the distillation apparatus outlet pipe. With the "start" menu option, the distillation process is begun. It is recommendable to run the following distillation program:

Bidest water: 70 ml (5 sec)
NaOH: 70 ml of a 30 % solutin (7 sec)
Distillation time: 5 min
Vapour capacity: 90 %
Sample suction: 30 sec.
Boric acid addition: 50 ml (4 sec)

The vapour distillation is begun and the ammonia, released through the addition of sodium hydroxid, is distilled with vapour. The distillate is absorbed in the boric acid solution (2.6). After the distillation program terminates, the digestion flask is removed from the apparatus, the distillation hose is flushed with distilled water and the Erlenmeyer flask containing the sample is also removed from the apparatus.

- *The water distiller must be set up on a stable laboratory bench with an even, horizontal support which is located near a cold water supply and a drain. The water pressure must be at least 0.5 bar.*
- *Before starting the operation, all hoses must be connected and the coolant engaged. The storage tank must be correctly positioned and the fluid level checked. The water vapour discharge hose must be introduced into the digestion flask. The water distiller is equipped with a safety gate.*
- *During the first distillation, the water vapour comes into contact with cold pipes and glass parts. This leads to increased build-up of condensation which can in turn lead to excessive sample dilution and liquid volume in the digestion vessel. A trial run is therefore essential. The discharge of water vapour with a temperature of approx. 106°C creates loud noises. This is no cause for alarm.*
- *The distillation is conducted until a distillation volume of 150 ml is obtained.*
- *About 2 minutes before the end of the distillation, the Erlenmeyer flask is lowered in such a way that the end of the discharge pipe is no longer submerged in the acid solution. The pipe must be flushed with water. This water is collected in the Erlenmeyer flask.*

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3 Titration 1 min

$NH_4H_2BO_3 + HCl \rightarrow NH_4Cl + H_3BO_3$

The boric acid absorption solution (which contains the indicator) is titrated with 0.1 M standard hydrochloric acid. The hydrochloric acid is added until the first trace of pink coloration arises. The volume of the hydrochloric acid consumed is read off on the burette at 0.05 ml. An illuminated disc can serve as a neutral background and enables the user to determine the colour change accurately at the end of the titration.

- *The mixture of methyl red and bromcresol green (see 2.5, 2.6) serves as the indicator. The indicator is responsible for the colour change and signals the end of the titration.*
- *The neutral background improves the accuracy and reproducibility of the results. This means that the titrations are always carried out under optical conditions that are as similar to each other as possible.*

Blind trial

The blind trial is carried out in the same way as above. The sample is replaced with 5 ml of water and 0.85 g of sucrose. The volume of hydrochloric acid consumed during titration is recorded.

- *The blind trial is important for the calculation of the nitrogen content of the sample.*

Calculation and analysis

The nitrogen content, given in g of nitrogen per 100 g of the product, is calculated using the following numerical equation:

$$Wn = \frac{1{,}4007\ (V - Vo)\ Cs}{Wt}$$

Wn: the nitrogen content of the sample

V: the volume of hydrochloric acid consumed during titration of the sample

Vo: the volume of the hydrochloric acid consumed during titration of the blind trial (see blind trial)

Cs: the exact molarity of the hydrochloric acid, given to four decimal places

Wt: the mass of the test sample in grams, given within 0.1 gram

To calculate the protein content of the sample, the Wn value must be multiplied by 6.38.

Model calculation
If the Kjeldahl determination yields a nitrogen content of 55 %, a protein content of 3.5% results (55% x 6.38).

- *Kjeldahl's digestion method is not specific to amino acids and proteins and includes all organically bonded nitrogen. Other non-protein compounds are also are digested and collected (NPN: non-protein nitrogen). However, the proportion of these compounds is very small and is disregarded in the calculation.*
- *If the non-protein containing nitrogen should also be established, then method must be executed in accordance with DIN EN ISO 8968-4. If only the protein nitrogen should be determined, then the milk proteins must first be separated. 5±0.1 ml of milk diluted with 5±0.1 ml of water is washed in stages with in total 60 ml of 15 % (w/v) trichloroacetic acid in accordance with DIN EN ISO 8968-5, the proteins are precipitated and finally filtered out into a hard paper filter. The filtrate contains the components of the non-protein nitrogen and the filtered-out precipitate contains the protein nitrogen. The filter with the precipitate is put into a digestion vessel and Kjeldahl's nitrogen determination method is carried out as described above. The protein content is calculated by multiplying by a factor of 6.38.*
- *The value 6.38 is specific to milk and dairy products and was established because milk proteins have a nitrogen content of 15.65 % (100:15.65 = 6.38).*

Alternatives, rapid methods

There is a faster way to execute Kjeldahl's method than the standard process. In accordance with ISO 8968-3, smaller milk samples are used (2 g, exactly weighed within 1 mg). 2 g of the sample, a catalyst tablet (consisting of 5 g K_2SO_4, 0.105 g $CuSO_45H_2O$ and 0.105 g TiO_2), 10 ml of 98% sulphuric acid, and a few drops of antifoam agent (30% silicon compound) are introduced into a 250 ml digestion flask. The contents are mixed carefully, 5 minutes are allowed to pass and then 5 ml of 30 % hydrogen peroxide are added at the side of the flask. The sample is left to sit for 10-15 minutes before digestion. The heating block is preheated to 400°C for 10 minutes and the sample is heated at 400°C for 60 minutes. Afterwards, the sample is cooled to room temperature and diluted with 50 ml of water. 55 ml of 30 % NaOH and 50 ml of 4 % boric acid are used to absorb the distillate. HCL 0.05 M is used for titration. 0.2 ml of a mixture of 0.03 % methyl red and 0.17 % bromcresol green in 95 % ethanol serves as the indicator.

- *Caution! The addition of hydrogen peroxide causes an intense reaction.*
- *The blind trial is executed with 2 ml of water and 0.25 g of sucrose. The effectiveness of the decomposition is tested with 0.08 g of tryptophan or 0.06 g of lysine hydrochloride.*

Monitoring the process

The accuracy of the process should be reviewed regularly. A loss of nitrogen should be tested for. As a sample, 0.12 g of ammonium sulphate [$(NH_4)_2SO_4$] and 0.85 g of sucrose are used here. Kjeldahl's method is carried out under the same conditions as with normal samples and the percentage of nitrogen content must be between 99.0 % and 100 %. The determination with ammonium sulphate is of use in detecting nitrogen losses during decomposition or distillation and differences in concentrations in the titration agent.

- *In order to monitor the effectiveness of the decomposition, 0.18 g of tryptophan or 0.16 g or lysine hydrochloride and 0.67 g of sucrose are used. 98 % of the nitrogen content must be recovered. If that is not the case, either the decomposition temperature or time is insufficient or the sample is charred.*

Determining the nitrogen content of dairy products

The reference standard process can also be adapted for other milk products. The only difference in the method is the sample amount. The sample must show a protein amount of 0.15-0.30 %. It is therefore advisable to use the following sample amounts:

Condensed milk:	3 g
Powdered skim milk:	1 g
Whey:	10 g
Cream cheese:	3 g

The method is carried out in the same way as the standard process.