

best@buchi No. 58 Nitrogen Determination with Kjeldahl





How to Achieve Low Detection and Quantification Limits for the Nitrogen Determination with Kjeldahl

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Abstract

Kjeldahl is one of the most commonly used techniques to determine the protein content in food and feed samples. The detection and quantification limits are important characteristics of analytical methods. The impact of the concentration of boric acid, the addition of potassium chloride, and the concentration of the titration solution on the detection and quantification limits were investigated. The best results were obtained by using 2 % boric acid with 3 g potassium chloride per liter. A titration solution of 0.005 M HCI worked best. With these parameters, detection limits for distillation of standard solutions as low as 0.008 mg nitrogen and quantification limits of 0.02 mg nitrogen can be achieved.

Introduction

Kjeldahl

For almost 130 years, the determination of nitrogen using the method developed by the Danish chemist Johan Kjeldahl (1849–1900) has been an internationally accepted standard. The method, which is named after its inventor, has since found widespread application in life science and chemistry and has extended its scope to the determination of nitrogen and proteins in dairy products, meat products, beer, cereals, and other food materials [1].

The Kjeldahl procedure involves three major steps:

In the digestion step, the organically bonded nitrogen is converted into ammonium ions by oxidation with concentrated sulfuric acid.

In the distillation step, the sample is alkalinized to convert the ammonium ions to ammonia. The latter is then distilled into a boric acid solution (via steam distillation).

In the final titration step, the ammonia is titrated and the nitrogen content can be calculated.

Theoretical background of pH measurements and boric acid titration

The pH value is the negative logarithm of the hydronium ion activity and is measured with an electro-chemical sensor. In practice this is a measurement of a potential difference between a reference electrode E_{ref} and the measuring electrode E. The measured voltage U is the potential difference of E and E_{ref} . The calculation of pH is performed according to the following equations (1 - 2), which are derived from the Nernst equation [2 - 4].

$$pH = pH_0 - \frac{E - E_{ref}}{slope} \tag{1}$$

The quotient in equation (2) represents the slope of the pH function and shows that the slope is a function of the temperature.

$$slope = \frac{f_{\rm ln-log} \bullet R \bullet T}{z \bullet F} \qquad (2)$$

- *pH* negative logarithm of the hydronium ion activity
- *pH*_o pH at zero point of pH sensor (the pH when the sensor signal is 0 mV)
- E potential at measuring electrode
- *E_{ref}* potential of the reference electrode (should be constant)
- f_{ln-log} conversion factor for the change of the natural (ln) to the common logarithm (2.303)
- R universal gas constant (8.3145 J/(K[•]mol))
- T absolute temperature [K]
- z number of electrons transferred (for pH: 1)
- F Faraday constant (9.6485[•]10⁴ C/mol)

Often the potential of the reference electrode E_{ref} , which should be constant, shows a small variability which can lead to measurement variations. The variability of the potential is largest when the solution is stirred. To demonstrate the stirring effect, a detailed view of the pH sensor is shown in Fig. 1.



Figure 1: Schematic representation of the pH electrode

- 1 measuring electrode (e.g., Ag/AgCl)
- 2 internal reference solution
- 3 pH sensitive glass membrane
- 4 sample solution
- (e.g., boric acid as receiving solution)
- 5 liquid junction
- (e.g., ceramic diaphragm)
- 6 reference electrolyte (e.g., 3 M KCl)
- 7 reference electrode (e.g., Ag/AgCl)
- U voltage measurement

The variability of the potential is produced at the liquid junction (zeta potential, different mobility of borate and hydronium ion, etc.). In diluted solutions, the variability is higher than in concentrated solutions. If the solution is not stirred, a cloud of potassium and chloride ions (black dots in Figures 2 and 3) is created at the exterior of the liquid junction and reduces the surface potential. If the solution is stirred, the cloud of potassium and chloride ions is removed from the surface so that the potential increases and the measured pH value decreases.





Figure 2: Liquid junction without stirring

1. The pH increase due to dilution of the receiving solution by distillate is less important in low concentrated boric acid. The variability of the amount of distilled water has less impact on the pH value and will therefore lead to less variability of the blank values. The pH change related to dilution is shown in Figure 4.



Figure 4: pH change when diluting 60 ml receiving solution at different concentrations of boric acid

2. The blank values in less concentrated boric acid are smaller for the same reason as above. This is particularly important because usually low concentrated titration solutions are used for the determination of low nitrogen amounts. For the determination of low nitrogen contents it is advantageous to have smaller blank values, because the difference in titration volumes between the blanks and the samples gets larger.

3. The pH change caused by the distilled nitrogen is more important the lower the concentration of the receiving solution is. Small amounts of nitrogen cause a considerable increase in pH, thus making the titration more accurate.

Detection limit and quantification limit

The so-called detection limit (limit of detection LOD) and quantification limit (limit of quantification LOQ) are important characteristics of analytical methods. They have to be determined for each method, analyte, and matrix.

The DIN 32 645 standard defines the two terms and describes the procedure used to calculate these values based on analytical results [5]. In this best@buchi, the definitions of the aforementioned standard are used (the terminology used in other standards may be slightly different).

Definitions

Detection limit:	The smallest content of the analyte that is significantly different from the blank value.
Quantification limit:	The smallest content of the analyte that can be determined quantitatively.

In general, the quantification limit is three times higher than the detection limit [5]. There are two ways to calculate these limits. The results achieved from these two methods are not equal but are equivalent:

with stirring



Figure 3: Liquid junction with stirring

The stirring effect can be minimized by adding potassium chloride to low concentrated (< 4%) boric acid to ensure that a sufficient amount of potassium chloride is always at the surface of the liquid junction.

The use of diluted boric acid is beneficial for the determination of low nitrogen amounts for the following three main reasons:

Direct method ("Blank method")

With the determination of a large number of blanks ($n \ge 10$), the detection and quantification limit can be calculated based on the standard deviations of the blank measurements and the slope of the calibration line. For Kjeldahl the slope would be the linearity between the nitrogen content and the consumption of the titration solution. The calibration line of the entire working range is used.

This method can only be used if a suitable blank is available. A blank should have identical properties to those of the actual sample, but without any analyte. This is rarely the case, as most analyses are done in complex matrices such as food or environmental samples, which cannot be imitated easily.

The calculations of the detection and quantification limits are performed according to equations (3 - 5).

$$x_{(LOD)} = \Phi_{n;\alpha} \bullet \frac{s_{\perp}}{b}$$
(3)

$$\Phi_{n;\alpha} = t_{f;\alpha} \bullet \sqrt{\frac{1}{m} + \frac{1}{n}}$$
(4)

(5)

$$x_{(LOQ)} = x_{(LOD)} \bullet k$$

 $x_{(LOD)}$ detection limit

 $X_{(LOQ)}$ quantification limit

- $\mathcal{O}_{n,\alpha}$ factor, depending on number of blank measurements (*n*), sample replicates (*m*), and significance level (α)
- ^SL standard deviation of blank measurements
- b slope of the calibration line; for Kjeldahl the relation between titration solution consumption and the nitrogen content (example: 14.28 ml of 0.005 M HCl corresponds to 1 mg nitrogen, b= 14.28).
- $t_{f;\alpha}$ quantile of the Student t-distribution, depending on degree of freedom f (f = n-1) and significance level α

nnumber of blank measurementsmnumber of sample replicates

factor used to calculate the $x_{(LOQ)}$ based on $x_{(LOD)}$; the factor is usually k=3 [5].

k

Indirect method ("Calibration line method")

A calibration line (in the range of the limit of quantification) is established (range 0 to 10 times x LOD). Based on the slope of this line, the detection and quantification limit can be calculated. In this case, the uncertainty of the blank is estimated by extrapolation of the calibration data. This method is more laborious and needs more statistical know-how than the direct method, but is often necessary due to the reasons mentioned above.

The calculations are explained in detail in the DIN 32 645 standard. Several statistical programs can be used to calculate the detection and quantification limit using the indirect method according to DIN 32 645.

In this study, the LOD and LOQ were always calculated according to the direct and the indirect method to be able to compare the findings. For Kjeldahl, the direct method is well suited, because the matrix is completely destroyed by the digestion with sulfuric acid.

Experimental

Equipment

AutoKjeldahl Unit K-370 with Kjeldahl Sampler K-371; Schott Titronic Universal, dosage instrument (Buchi P/N 043596); Analytical balance, reading precision +/- 0.1 mg; Statist24cp, Version 2.0., statistical program for method validation for analytical laboratories, ©2000-2005, Georg Schmitt, Michael Herbold, Arvecon GmbH, Walldorf, Germany.

Chemicals

Ammonium dihydrogen phosphate 99.99 % (Merck, 1.01440), dried; boric acid (Brenntag, 80948-155); potassium chloride (Merck, 104936); 0.05 M hydrochloric acid (Riedel de Haën, 35320), the titration solutions were prepared by diluting this standard solution.

Samples

Solutions of ammonium dihydrogen phosphate were diluted to obtain an absolute nitrogen amount per sample between 0.005 mg and 0.5 mg. Each sample was determined in triplicate. The solution was dosed into the Kjeldahl flasks using the Titronic Universal dosage instrument.

The determination was carried out with the AutoKjeldahl unit K-370 with Kjeldahl Sampler K-371 using the parameters given in Table 1.

Table 1: Parameters for the Kjeldahl sampler system K-370/K-371

Distillation		Titration	
Water	50 ml	Туре	Boric acid
NaOH	90 ml	Titration solution	HCI 0.005 M
Reaction time	5 s	Volume receiving sol.	60 ml
Distillation time	240 s	Titration mode	Standard
Steam power	100 %	End-point pH	4.65
		Algorithm	1

The experiment was set up in the following way: 1) optimization of the boric acid concentration 2) optimization of the KCI addition and finally 3) optimization of the titrant concentration.

Impact of boric acid concentration

Boric acid solutions of 4 %, 2 %, and 1 % and pure water (0 % boric acid) were compared. The pH of the boric acid was adjusted to 4.65. To compare the impact of the concentration of boric acid, 10 blanks and a sample series of 5 samples with different nitrogen contents were analyzed.

Impact of KCI addition

Different amounts of potassium chloride were added to 2 % boric acid. The final concentrations of KCI in the boric acid were 0.01 M, 0.02 M, 0.04 M, 0.06 M, and 0.1 M. A concentration of 0.01 M corresponds to the addition of 0.75 g / liter boric acid. The pH of the boric acid was adjusted to 4.65.

Impact of the titration solution

The following concentrations were used to investigate the impact of the titration solution on the detection and quantification limits: 0.0025 M HCI, 0.005 M HCI, 0.01 M HCI, and 0.05 M HCI. The analyses were carried out using 2 % boric acid with 0.04 M KCI.

Calculations

All calculations of the detection and quantification limits according to the direct method (blank method) were performed using the equations (3-5). The calculations according to the indirect method (calibration method) were carried out using the statistical program "Statist24cp." For both cases, a significance level of 99 % was used.

Results and Discussion

Impact of concentration of boric acid

In Table 2, the mean values of the blanks and their relative standard deviation (rsd) are given using different concentrations of boric acid. The results in Table 2 show that the higher the boric acid concentration, the higher the blank value. As shown in Figure 4, the pH increase due to the dilution of the boric acid by the distillate becomes more important the higher the concentration. Therefore, more titration solvent is needed to get back to the endpoint pH of 4.65. The relative standard deviations are higher the lower the boric acid concentration.

Table 2: Mean values of blank analyses with different boric acid concentrations (titration solution 0.005 M HCl, n=10)

	4 % boric acid	2 % boric acid	1 % boric acid	0 % boric acid
mean value [ml]	9.291	0.995	0.503	0.366
sd	0.216	0.033	0.026	0.037
rsd [%]	2.3	3.3	5.1	9.5

In Figure 5, the mean values of the recoveries of the sample series with different boric acid concentrations are shown.



Figure 5: Mean values (n=3, except for 0.2 mg in 4 %boric acid, n=1) of nitrogen recovery in samples with their standard deviation when using 0 % - 4 % boric acid as receiving solution In Table 3 and Figure 6, the detection and quantification limits calculated based on the data of the blank analyses and the sample series according to both methods proposed by DIN 32645 are shown.

Table 3: Detection limit and quantification limit calculated using the direct and indirect method.

Direct method (blank method) ¹

	4 % boric acid	2 % boric acid	1 % boric acid	0 % boric acid
Detection limit [mg N]	0.053	0.008	0.006	0.009
Quantification limit [mg N]	0.159	0.024	0.019	0.027

Indirect method (calibration method)

	4 % boric acid	2 % boric acid	1 % boric acid	0 % boric acid
Detection limit [mg N]	0.071	0.010	0.012	0.019
Quantification limit [mg N]	0.239	0.032	0.041	0.063

¹ A factor $\Phi_{n,\alpha}$ of 3.5 was used, which is valid for 4 blanks and a triplicate determination of the samples, which are typical conditions of Kjeldahl determination; for duplicate determination of the samples, the factor $\Phi_{n,\alpha}$ would be 3.9.



Detection limit and quantification limit

Figure 6: Calculated detection and quantification limits according to the direct and indirect method based on the data of the sample series using 0 % - 4 % boric acid as receiving solution

Taking into account the blank values presented in Table 2, the mean values of the recovery rates (Figure 5), and the calculated detection and quantification limits (Table 3 and Figure 6), it is evident that the best results are obtained by using 2 % boric acid. The blank value around 1 ml is in a good range, as well as its relative standard deviation of approx. 3 %. The recovery rates and their standard deviations are better than those obtained with the other concentrations of boric acid. The calculated detection and quantification limits are lowest, but comparable to the ones with 1 % boric acid. The subsequent analyses were therefore carried out using 2 % boric acid.

Impact of KCI addition

The measured pH shift caused by the stirring effect (using different concentrations of KCl in 2 % boric acid) is presented in Table 4.

	not stirred	stirred	ΔpH
No KCI	4.66	4.48	-0.18
0.01 M KCI	4.66	4.60	-0.06
0.02 M KCI	4.66	4.62	-0.04
0.03 M KCI	4.66	4.64	-0.02
0.04 M KCI	4.66	4.64	-0.02
0.06 M KCI	4.66	4.64	-0.02
0.1 M KCI	4.65	4.63	-0.02
No KCl + 150 ml H ₂ O	5.31	5.03	-0.28
0.04 M KCl + 150 ml H ₂ O	5.38	5.34	-0.04
0.06 M KCl + 150 ml H ₂ O	5.36	5.37	+0.01
0.1 M KCl + 150 ml H ₂ O	5.25	5.27	+0.02

Table. 4: pH shift caused by stirring using different KCI concentrations in 2 % boric acid, with and without dilution by distillate

As predicted by theory (see chapter "Theoretical background of pH measurements and boric acid titration"), the addition of potassium chloride (KCI) minimizes the stirring effect. In 2 % boric acid, a concentration of 0.03 M KCI is sufficient to decrease the pH shift to 0.02. If the boric acid is diluted with 150 ml water, which corresponds to the approx. amount of distillate after 4 min distillation time, 0.06 M KCI is needed to minimize the stirring effect.

The amount of added KCl also influences the titration speed and the blank values (see Tables 5 and 6).

Table 5: Titration time of a blank value, 2 % boric acid with different concentrations of KCI

	Titration time
No KCI	105 s
0.02 M	52 s
0.06 M	62 s
0.1 M	59 s

Table 6: Mean values of blanks (n=10) in 2 % boric acid with different concentrations of KCI.

	No KCI	0.01 M	0.02 M	0.04 M	0.06 M	0.1 M
mean value	0.881	1.174	1.272	1.268	1.489	1.518
s	0.074	0.060	0.017	0.025	0.039	0.052
rsd	8.438	5.076	1.298	1.967	2.643	3.437

The more KCl is added to the boric acid, the higher the blank values. This phenomenon is related solely to the stirring effect. If no KCl is added, the measured pH of the boric acid at the end of the distillation and start of titration is approx. 5.03 instead of 5.34 (0.04 M KCl, see Table 4). In this case, less titration solution is needed to reach the endpoint of 4.65. Although the blank values are higher with larger amounts of added KCl, the titration is faster due to a more stable pH measurement (see Table 5). For the determination of low nitrogen contents it is advantageous to have smaller blank values, because the difference in titration volumes between the blanks and the samples becomes more important. The ideal concentration of KCl in the boric acid is a compromise between the desirable (stable pH measurement) and the undesirable (increase of blank value).



Figure 7 shows the mean values of the recoveries of nitrogen in the sample series.

Figure 7: Mean values of samples (n=3) with their standard deviation when using 0 - 0.1 M KCl in 2 % boric acid as receiving solution

The best results are obtained using 0.02 – 0.06 M KCl in 2 % boric acid. Based on the data of the blank analyses and sample series, the detection limit and the quantification limit were calculated (see Table 7 and Figure 8).

Table 7: Detection limit and quantification limit calculated according to the direct and indirect method using boric acid with different KCI concentrations

Direct method (blank method)

	No KCI	0.01 M	0.02 M	0.04 M	0.06 M	0.1 M
Detection limit [mg N]	0.018	0.015	0.004	0.006	0.010	0.013
Quantification limit [mg N]	0.055	0.044	0.012	0.018	0.029	0.038

Indirect method (calibration method)

	No KCI	0.01 M	0.02 M	0.04 M	0.06 M	0.1 M
Detection limit [mg N]	0.014	0.003	0.004	0.005	0.005	0.007
Quantification limit [mg N]	0.046	0.010	0.014	0.016	0.018	0.025



Figure 8: Calculated detection and quantification limits according to the direct and indirect method using 0 M - 0.1 M KCl in 2 % boric acid as receiving solution

Taking into account the blank values presented in Table 6, the mean values of the recovery rates (Figure 7), the minimized pH shift (Table 4), the titration time (Table 5), and the calculated detection and quantification limits (Table 7 and Figure 8), it is clear that the best results are obtained by using 0.04 M KCl in 2 % boric acid. Although the detection and quantification limits are slightly lower with 0.02 M KCl and the blank values are comparable, the mean values of the recoveries as well as the pH shift are more promising with 0.04 M KCl. The subsequent analyses were therefore carried out with 2 % boric acid, with a concentration of 0.04 M KCl.

To determine the exact detection and quantification limits according to the indirect method, the highest value in the calibration line shall not exceed ten times the detection limit. If the value turns out to exceed this limit afterwards, a new calibration line needs to be established [5]. In the data shown in Table 7, the limit of detection is 0.005 mg and 0.006 mg, respectively. Therefore, the highest value in the calibration line should not exceed 0.06 mg. Consequently, new calibration lines needed to be established with lower nitrogen concentrations. The final calibration line is presented in Figure 9, the corresponding detection and quantification limits in Table 8.



Calibration line

Figure 9: Calibration line with concentrations between 0.0025 and 0.025 mg nitrogen. The mean values of triplicate determinations and their standard deviations are shown.

Table 8: Detection limit and quantification limit calculated according to the direct and indirect method using the data of the final calibration line.

	Direct method (blank method)	Indirect method (calibration method)
Detection limit [mg N]	0.008	0.004
Quantification limit [mg N]	0.023	0.012

Based on the calibration line data (calibration method), the detection and quantification limits are approx. half of the values obtained by the blank method. Considering the recoveries and the standard deviations of the measured concentrations of the calibration line, it is obvious that accurate quantifications cannot be performed for concentrations below 0.02 mg. Only at concentrations > 0.0225 mg, the recoveries are around 100 % with rsds lower than 5 %. In this case, the direct method gave more realistic detection and quantification limits.

Impact of the titration solution

In Table 9, the mean values of the blanks and their relative standard deviation (rsd) are given. Figure 10 shows the recovery rates of the sample series using different concentrations of titration solution.

Table 9: Mean values of blank analyses with different titration solutions (n=10)

	0.05 M HCI	0.01 M HCI	0.005 M HCI	0.0025 M HCI
mean value [ml]	0.134	0.687	1.396	2.744
sd	0.002	0.014	0.021	0.053
rsd [%]	1.8	2.1	1.5	1.9



Mean value of nitrogen recovery

Figure 10: Mean values of samples (n=3) with their standard deviation when using 0.0025 M – 0.05 M HCl as titration solution

The standard deviations are larger when using a higher concentrated titration solution, due the fact that very small differences in titration volume cause large differences in the calculated nitrogen content. The accuracy of the titration consumption using the integrated titrator with a 20 ml burette in the AutoKjeldahl Unit K-370 is limited to three digits (e.g., 0.001 ml). For concentrations higher than 0.01 M HCl, a higher accuracy would be necessary to obtain satisfying results. The disadvantage of highly diluted titration solutions (0.0025 M HCl) is that large volumes are titrated (higher costs per sample) and that the titration volumes of samples with low nitrogen content (0.005 mg N and 0.01 mg N) are within the statistical spread of the high blank values.

The most promising titration solutions were 0.01 M and 0.005 M HCl. With these solutions, more sample series needed to be analyzed to establish a calibration line to calculate the limit of detection and quantification (data not shown). The calculated detection and quantification limits are presented in Table 10.

Table 10: Detection limit and quantification limit calculated according to the direct and indirect method using different titration solutions

Direct method (blank method)

	0.01 M HCI	0.005 M HCI
Detection limit [mg N]	0.007	0.005
Quantification limit [mg N]	0.022	0.015

Indirect method (calibration method)

	0.01 M HCI	0.005 M HCI
Detection limit [mg N]	0.005	0.003
Quantification limit [mg N]	0.013	0.009

The detection and quantification limits are in the same order of magnitude as previous values (see Table 7 and Table 8). There is also no significant difference between the 0.01 M HCl and 0.005 M HCl titration solutions.

Conclusions

The detection limit and the quantification limit are as low as approx. 0.008 mg nitrogen and 0.02 mg nitrogen when 2 % boric acid with 3 g of KCI (0.04 M) is used as receiving solution. Titration solutions of 0.005 M HCl provide good results; however, the detection and quantification limits are not significantly influenced by the choice of the titration solution.

The above-mentioned parameters are suitable for low nitrogen concentrations. For nitrogen concentrations usually found in food samples, the standard application using 4 % boric acid, without addition of KCI. is recommended.

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