Application Note
No. 264/2016

Total nitrogen determination in pharmaceutical active peptides

KjelMaster System K-375 / K-376, SpeedDigester K-439, Scrubber K-415.

1. Introduction

Nitrogen and total protein determination in pharmaceutical products are described in the European pharmacopoeia (Ph.Eur.) methods 2.5.9. and 2.5.33. method 7 [1, 2]. In the pharmacopoeia of the United States (USP), nitrogen determination is described in method 461 [3]. Therein, different titration techniques are specified. The Ph.Eur. is using back titration in the presence of the indicator methyl red mixed solution and the USP is using potentiometric boric acid titration.
The aim of the following study was to determine the reproducibility and to compare the different titration techniques. Therefore, the nitrogen content of a biotechnologically prepared peptide sample for intravenous use was determined by sulfuric acid digestion with the SpeedDigester K-439, followed by steam distillation and titration applying the BUCHI KjelMaster System K-375 / K-376.

2. Samples

The nitrogen content of the following sample was analysed:

- Pharmaceutical active peptide, expected nitrogen content: 6.5 mg N /mL

The sample is a biotechnologically prepared peptide which is used for treatment of ischemic and hemorrhagic stroke, traumatic brain injuries and to treat different forms of dementia disorder.

3. Equipment

- KjelMaster System K-375 / K-376 with potentiometric and colorimetric sensor
- SpeedDigester K-439
- Micro Kjeldahl sample tubes (11057442)
- Insulation plate micro (11055204)
- Scrubber K-415 TripleScrub^{ECO}
- Analytical balance (accuracy ± 0.1 mg)

For back titration in addition:

- Dosing unit for back titration (11056836)
- Laboratory vessel (053203)
- Driving motor for dosing unit (11056835)

4. Chemicals and Materials

- Kjeldahl Tablets Titanium Micro BUCHI (11057981)
- Sulfuric acid conc. 98 %, Merck (1007482500)
- Sodium hydroxide 32 %, Brenntag (81980-452)
- Hydrochloric acid 0.01 M, volumetric solution, VWR Chemicals

For boric acid titration (potentiometric determination) in addition:

- Boric acid 2 %, Brenntag (80948-155) with 3 g/L KCl, pH adjusted to 4.65

For boric acid titration (colorimetric determination) in addition:

- Boric acid 2 %, Brenntag (80948-155), pH adjusted to 4.65
- Sher indicator (003512), 2.5 mL/L boric acid

For back titration (potentiometric determination) in addition:

- Sodium hydroxide 0.01 M, volumetric solution, VWR Chemicals

For safe handling please pay attention to all corresponding MSDS.

5. Experimental

5.1. Digestion of the samples

- Pipette 0.35 mL of the sample in the micro Kjeldahl sample tube.
- Add 1 Kjeldahl Tablet Titanium Micro and 5 mL of sulfuric acid (conc. 98 %) to each sample tube.
- Prepare blanks (chemicals without sample).
- Insert the rack containing the samples into the preheated digester.
- Digest the samples according to the parameters listed in Table 1.
- Let the samples cool down to ambient temperature before starting the distillation.

Table 1: Digestion parameters for Micro Kjeldahl digestion.

Step	Temperature [°C]	Time [min]
Preheat	480	–
1	500	5
2	480	45
Cooling	–	30

NOTE: If the liquid inside the sample tube is not clear and blue-green, digest for additional 10 min at 480°C.

5.2. Boric acid titration (potentiometric)

Boric acid titration can be performed by potentiometric or colorimetric determination. The difference between the two methods is the applied sensor to determine the endpoint of the titration. Potentiometric titration is based on the measurement of the electrical voltage using a pH electrode.

Boric acid titration is used for the direct determination of ammonia that is transferred to the receiving vessel by steam distillation. Ammonia and boric acid form a complex which is titrated with an acid e.g. hydrochloric or sulfuric acid. An excess of boric acid guarantees that ammonia is completely bound in a complex.

Before sample distillation, perform at least 3 blank determinations. All distillation parameters are listed in Table 2.

Table 2: Parameters for distillation and boric acid titration with potentiometric detection.

H_2O volume	25 mL	Sensor type	Potentiometric
NaOH (32 %) volume	30 mL	Titration mode	Standard
Reaction time	5 s	Measuring mode	Endpoint pH
Distillation mode	Fixed time	Endpoint pH	4.65*
Distillation time	180 s	Stirrer speed titration	7
Stirrer speed distillation	5	Titration start volume	0 mL
Steam output	100 %	Titration algorithm	Optimal
Titration type	Boric acid	Aspiration sample tube	Yes
Receiving solution vol.	50 mL (boric acid)	Aspiration receiving vessel	Yes
Titration solution	HCl 0.01 mol/L		

*NOTE: USP 461 describes the endpoint determination by potentiometric titration. However, no specific pH endpoint is mentioned. The point of inflection of the boric acid titration is found at pH 4.65. Hence, it is recommended to adjust the pH of the boric acid to 4.65 before distillation and use the same endpoint pH = 4.65 for the titration [4, 5].

5.3. Boric acid titration (colorimetric)

For the colorimetric titration an indicator is used to detect the pH-dependent color changes with a colorimetric sensor that is measuring the absorbance of the light. However, the color change is a result of a pH change of the receiving solution. Hence, the colorimetric detection allows a visible detection of the pH endpoint = 4.65 and is an indirect detection. For further information about colorimetric setpoint determination before measurements see technical note No.181/2015 [6].

Before sample distillation, perform at least 3 blank determinations. All distillation parameters are listed in Table 3.

Table 3: Parameters for distillation and boric acid titration with colorimetric detection.

H_2O volume	25 mL	Titration solution	HCl 0.01 mol/L
NaOH (32 %) volume	30 mL	Sensor type	Colorimetric
Reaction time	5 s	Titration mode	Standard
Distillation mode	Fixed time	Stirrer speed titration	10**
Distillation time	180 s	Titration start volume	0 mL
Stirrer speed distillation	3**	Titration algorithm	Optimal
Steam output	100 %	Aspiration sample tube	Yes
Titration type	Boric acid	Aspiration receiving vessel	Yes
Receiving solution vol.	60 mL (boric acid)*		

*NOTE: For colorimetric titration, a minimum of 60 mL receiving solution (boric acid) is recommended to avoid the accumulation of air bubbles which would falsify the measurement.

**NOTE: The stirrer speed during distillation is reduced to level 3 in order to avoid the immigration of air bubbles into the protection mesh. However, for titration, the speed level is increased (level 10) to improve indicator blending and increase reproducibility.

5.4. Back titration (potentiometric)

For back titration, an excess of standard acid solution, e.g. sulfuric acid or hydrochloric acid, reacts with the NH_3 from the distillate. The residual acid is back titrated with sodium hydroxide standard solution. Consumption of sodium hydroxide solution reversely correlates with amount of ammonia. Back titration can only be performed by potentiometric detection.

First, the nitrogen content of the 3 blanks is determined, then the nitrogen content of the samples. All distillation parameters are listed in Table 4.

NOTE: For back titration, a second burette is needed for accurate dosage of the receiving solution (hydrochloric acid).

Table 4: Parameters for distillation and back titration with potentiometric detection.

H_2O volume	25 mL	Titration solution	NaOH 0.01 mol/L
NaOH (32%) volume	30 mL	Sensor type	Potentiometric
Reaction time	5 s	Titration mode	Standard
Distillation mode	Fixed time	Measuring mode	Endpoint pH
Distillation time	180 s	Endpoint pH	5.6**
Stirrer speed distillation	3*	Stirrer speed titration	5*
Steam output	100 %	Titration start volume	0 mL
Titration type	Back titration	Titration algorithm	Optimal
Receiving solution	HCl 0.01 mol/L	Aspiration sample tube	Yes
Receiving solution volume	20 mL	Aspiration receiving vessel	Yes

*NOTE: The stirrer speed is decreased (level 3 during distillation and level 5 during titration) to avoid splashing of receiver solution.

**NOTE: The endpoint pH 5.6 is selected in accordance to Pharmacopoeia 2.5.9. Depending on the regulation, other pH endpoint e.g. pH 7 (neutral point), can be chosen.

6. Results and Discussion

The results of the three titration techniques, boric acid titration by potentiometric, colorimetric detection and back titration by potentiometric detection are shown in Table 5. Samples were measured three-fold (n=3).

Table 5: Results of the three determination methods.

	Nitrogen content [mg N/mL]
Boric acid titration (potentiometric)	**6.24 ± 0.01 (RSD 0.16)**
Boric acid titration (colorimetric)	**6.25 ± 0.01 (RSD 0.23)**
Back titration (potentiometric)	**6.23 ± 0.04 (RSD 0.58)**

A nitrogen content of 6.24 ± 0.01 mg N/mL, 6.25 ± 0.01 mg N/mL and 6.23 mg N/mL ± 0.04 was determined in the peptide sample applying boric acid titration (potentiometric), boric acid titration (colorimetric) and back titration (potentiometric), respectively. In general, all three titration techniques lead to comparable results. Advantages and disadvantages of each titration technique is listed in Table 6.

Table 6: Advantages and disadvantages of the three titration techniques.

	Boric acid titration (potentiometric)	Boric acid titration (colorimetric)	Back titration (potentiometric)
Advantages	· Direct detection. · No exact volume dosage for receiver solution required → quick and reliable method. · Time saving: Distillation and titration can be synchronized by means of the “Online-Titration mode”. · Fast calibration of pH sensor. · Lower detection limit than colorimetric detection. · IntelliDist possible	· No exact volume dosage for receiver solution required → quick and reliable method. · Time saving: Distillation and titration can be synchronized by means of the “Online-Titration mode”. · Longer lifetime of the sensor compared to pH electrode (approx. 4 years). · Endpoint visible by eye.	· Back titration can be used when boric acid should be avoided. · The acid immediately neutralizes the ammonia. The distillate receiver remains acidic, thus there are no losses even for large amounts of ammonia. · No pH adjustment of the receiving solution necessary.
Disadvantages	· The pH of the boric acid must be adjusted to 4.65. · 6- 12 months lifetime of pH electrode. · Daily calibration of the electrode.	· The pH of the boric acid must be adjusted to 4.65. · Setpoint determination takes longer than pH electrode calibration. · Protection mesh required to prevent from air bubbles. · Indicator required → additional task. The correct ratio needs to be considered.	· Indirect titration. · No colorimetric titration possible (potentiometric titration only). · The acid concentration and volume must be precisely measured and is a potential source of errors → second burette required. · 6- 12 months lifetime of pH electrode.

7. Conclusion

The nitrogen content of the peptide sample was successfully determined using the SpeedDigester K-439 with Micro Kjeldahl sample tubes, Scrubber K-415, and the KjelMaster System K-375 / K-376. All experiments were performed smoothly and reproducibly applying automated distillation and titration procedures in accordance to Ph.Eur. and USP.

Depending on the standard followed, boric acid or back titration are required for nitrogen determination. Boric acid titration (potentiometric and colorimetric) and back titration lead to similar results using the KjelMaster System K-375 / K-376.

A point-wise discussion of the Ph.Eur. 2.5.9. and USP 461 requirements and the compliance with the BUCHI instrumentation is detailed in the appendix in Tables 7 - 9.

8. References

[1] European Pharmacopoeia (01/2008:20509, 9th edition) 2.5.9.; Determination of nitrogen by sulfuric acid digestion.
[2] European Pharmacopoeia (01/2008:20533, 9th edition) 2.5.33. Method 7, Total protein.
[3] Pharmacopoeia of the United States 461, Nitrogen determination (2016).
[4] Kjeldahl Guide, BUCHI Labortechnik AG (2008).
[5] Kjeldahl Practice Guide, From sample preparation to result calculation, www.buchi.com
[6] Technical Note, Points to consider for colorimetric titration, www.buchi.com
[7] V. P. Singh; Metal Toxicity and Tolerance in Plants and Animals, (2005).
[8] Ugrinovits, M.; Kjeldahl nitrogen determination with various catalysts, Band 71, 124-139, (1980).
[9] Technical note, New Assortment of Kjeldahl Tablets 70/2011, www.buchi.com
[10] KjelOptimizer App: http://www.buchi.com/en/service-support/scientific-mobile-apps

Appendix
Compliance to Pharmacopoeia

European Pharmacopoeia

In the European Pharmacopoeia (Ph.Eur.), a method for nitrogen (2.5.9.) and total protein (2.5.33.(7)) determination using Kjeldahl apparatus are described. The latter method is based on nitrogen analysis and describes to follow method (2.5.9.) or to use a commercial instrumentation for Kjeldahl nitrogen assay. Hence, for nitrogen and total protein determination in pharmaceutical products, the method 2.5.9. (Determination of nitrogen by sulfuric acid digestion) is the standard to be followed.

This method 2.5.9. describes a semi-micro Kjeldahl method, i.e. the use of a reduced amount of concentrated sulfuric acid for sample digestion (5 mL).

A point-wise discussion of the Ph.Eur. 2.5.9. requirements and the compliance with the BUCHI instrumentation is detailed in Table 7.

Table 7: Compliance of the BUCHI instrumentation and the Ph.Eur. requirements.

Ph.Eur. 2.5.9.	BUCHI compliance
Place a quantity of the substance to be examined (mg) containing about 2 mg of nitrogen in a combustion flask…	For digestion the following BUCHI sample tubes are offered: Micro Kjeldahl: 100 mL sample tubes (11057442). Standard Kjeldahl: 300 mL sample tubes (037377). For low nitrogen containing or strong foaming samples: 500 mL sample tubes (043982). For further information see Kjeldahl Practice Guide (page 4) [5].
…add 4 g of a powdered mixture of 100 g of di-potassium sulfate, 5 g of copper sulfate and 2.5 g of selenium, and three glass beads.	4 g of the powder described in the Ph.Eur. contain: 3.71 g K_2SO_4, 0.186 g $CuSO_4$ and 0.093 g selenium. Typically selenium, mercury, titanium and copper are the catalysts of choice to increase the efficiency and speed of the Kjeldahl digestion process. However, mercury and selenium are to be avoided for toxicity and environmental reasons. It is recommended to use titanium and copper which are of low toxicity and equally effective [7 - 9]. The Kjeldahl Tablet Titanium (total weight 3.71 g) contains: 3.50 g K_2SO_4, 0.105 g $CuSO_4$ and 0.105 g TiO_2 instead of selenium. The Kjeldahl Tablet Missouri (total weight 5.0 g) contains: 4.98 g K_2SO_4 and 0.02 g $CuSO_4$ The Kjeldahl Tablet ECO (total weight 4.0 g) contains: 3.998 g K_2SO_4 and 0.002 g $CuSO_4$ Glass beads are not needed for solid and creamy samples. Digestion rods (043087) are recommended to avoid boiling delays for liquid samples (>10 mL) only.
Wash any adhering particles from the neck into the flask with 5 mL of sulfuric acid, allowing it to run down the sides of the flask, and mix the contents by rotation.	The constricted condensation zone allows a constant back flow / rinsing during digestion process. Therefore, no additional rinsing is required. The amount of sulfuric acid conc. used for digestion depends on the sample weight and matrix. The more sample analyzed, the more sulfuric acid is used. Furthermore, high-fat containing samples require more sulfuric acid. Hence, the amount of sulfuric acid for successful digestion should be adjusted depending on sample matrix and weight. A helpful tool for sulfuric acid volume calculation is the KjelOptimizer App [10]. Sample tubes Micro (11057442) are recommended if ≤5 mL sulfuric acid is used.
Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid.	All BUCHI digesters are designed to keep the user and surrounding free from of acid fumes formed during digestion. Due to improved sealing properties of the suction module, the evaporation of sulfuric acid will be reduced to a minimum and no fumes leak out. Both, SpeedDigester and the KjelDigester, are equipped with suction modules.

Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed.	The temperature can be set on all BUCHI digesters. On the SpeedDigester K-439 and KjelDigester K-449 a fully automated temperature / time profile can be programmed and up to 50 methods can be stored on the instrument. The automated lift function of the K-449 allows preheating and starting digestion unattended. When digestion is finished, the digested samples are lifted automatically out of the heating block to accelerate the cooling step.
Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of water,…	The water is added to the digested sample to dilute the sulfuric acid and avoid too violent reactions. The water can be dosed in defined volume automatically using BUCHI distillation units (except K-350 and K-355). The Ph.Eur. standard describes to add 5 mL water per used mL sulfuric acid (25 mL water / 5 mL sulfuric acid).
…cool again and place in a steam-distillation apparatus. Add 30 mL of strong sodium hydroxide solution and distil immediately by passing steam through the mixture.	Sodium hydroxide is added to increase the pH from acidic to pH >12 to liberate the ammonia from the digested sample. All BUCHI distillation units are able to dose defined volumes automatically to the digested and diluted sample. Further, the reaction time can be programmed. The Ph.Eur. standard describes to add 6 mL NaOH per used mL sulfuric acid (30 mL NaOH / 5 mL sulfuric acid).
Collect about 40 mL of distillate in 20.0 mL of 0.01 M hydrochloric acid and enough water to cover the tip of the condenser.	The steam generator produces a volume of approx. 35 mL steam per minute when set to 100 % steam power. Hence, 40 mL of distillate is achieved in about 70 seconds. However, distillation time is dependent on the steam generator as well as on the digested sample and the nitrogen content. To ensure proper distillation we recommend a distillation time of up to 150 seconds (distillation unit only) and 180 seconds (with KjelSampler K-376 or K-377). On the K-375, a second burette with driving motor (11056836 and 11056835) can be connected allowing automated dosage of the 20 mL volumetric receiving solution (0.01 mol/L HCl). Extra water is not necessary as the condenser tip is covered by the 20 mL of hydrochloric acid.
Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid.	This is not possible with modern distillation units. However, no influence on recovery rates could be observed when the receiver vessel is kept on the same position and is not lowered towards end of the distillation. The stirrer of the K-375 and K-360 provide a homogenous mixing of the receiving solution during distillation and titration by the integrated stirrer.
Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver.	The BUCHI distillation units are developed in the way to avoid nitrogen losses, therefore no water contamination from the outer surfaces of the condenser is possible.
Titrate the distillate with 0.01 M sodium hydroxide, using methyl red mixed solution as indicator.	Methyl red mixed solution, contain 0.1 g methyl red and 50 mg of methylene blue in 100 mL of alcohol and has a color change from red-violet (pH 5.2) to green (pH 5.6). By setting the pH endpoint to pH 5.6 potentiometric titration can be applied. In practice, no colored indicator is needed but has no influence on the measurement if added. For the automated colorimetric determination with the KjelMaster K-375, two indicators can be used: - Sher indicator - Bromocresol green / methyl red
Repeat the test using about 50 mg of glucose in place of the substance to be examined.	It is recommended to do a least two blank determinations before measuring the samples. Applying back titration, the sample volume is subtracted from the blank average volume automatically when using the KjelMaster K-375.

US Pharmacopoeia

The Pharmacopoeia of the United States (USP) describes two boric acid titration methods for nitrogen determination (USP 461).

USP 461 Method I

In method I, the standard distinguishes between samples containing nitrates / nitrites and samples without these components. Samples containing nitrates and nitrites require a special digestion procedure prior to distillation and titration. For all types of samples, the distillation and titration procedure is identical. In Table 8, compliance of the USP 461 method I with the BUCHI instruments is discussed.

Table 8: Compliance of USP 461 Method I with BUCHI instrumentation.

USP 461 Method I - nitrates and nitrites absent	BUCHI compliance
Place about 1 g of the substance, accurately weighed, in a 500 mL Kjeldahl flask of hard borosilicate glass.	For sample digestion the following sample tubes, made of borosilicate glass 3.3, are offered: Micro Kjeldahl: 100 mL sample tubes (11057442). Standard Kjeldahl: 300 mL sample tubes (037377). For low nitrogen containing or strong foaming samples: 500 mL sample tubes (043982).
The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask.	For solid or semisolid samples, nitrogen-free weighing boats are available for convenient sample transfer (11060522).
Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid.	The Kjeldahl Tablet Missouri (total weight 5.0 g) contains: 4.98 g K_2SO_4 and 0.02 g $CuSO_4$ The Kjeldahl Tablet Titanium (total weight 3.71 g) contains: 3.50 g K_2SO_4, 0.105 g $CuSO_4$ and 0.105 g TiO_2 The Kjeldahl Tablet ECO (total weight 4.0 g) contains: 3.998 g K_2SO_4 and 0.002 g $CuSO_4$ The amount of sulfuric acid conc. used for digestion depends on the sample weight and matrix. The more sample analyzed, the more sulfuric acid is used. Furthermore, high-fat containing samples require more sulfuric acid. Hence, the amount of sulfuric acid for successful digestion should be adjusted depending on sample matrix and weight. Using 20 mL of sulfuric acid, most samples will be digested properly. To save chemicals, a helpful tool for sulfuric acid volume calculation is the KjelOptimizer App [10].
Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in color or almost colorless for 30 minutes.	The sample tubes can't be inclined in modern digesters. The sample tubes stand in an upright position of 90°. However, the angle of sample tubes has no influence on digestion quality. The temperature can be set on all BUCHI digesters. On the SpeedDigester K-439 and KjelDigester K-449 a fully automated temperature / time profile can be programmed and up to 50 methods can be stored on the instrument. The automated lift function of the K-449 allows preheating and starting digestion unattended. When digestion is finished, the digested samples are automatically lifted out of the heating block to accelerate the cooling step.
Allow to cool, add 150 mL of water, mix the contents of the flask, and again cool.	The water is added to the digested sample to dilute the sulfuric acid and avoid too violent reactions. The water can be dosed in defined volume automatically in all BUCHI distillation units safely (except K-350 and K-355). The USP standard describes to add 7.5 mL water per used mL sulfuric acid (150 mL water / 20 mL sulfuric acid). To save chemicals, 5 mL water per used mL sulfuric acid (25 mL water / 5 mL sulfuric acid) for digestion can be used without affecting the results [4, 5].

Add cautiously 100 mL of sodium hydroxide solution (2 in 5), in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution.	Sodium hydroxide is added to increase the pH from acidic to pH >12 to liberate the ammonia of the digested sample. All BUCHI distillation units are able to dose defined volumes automatically in to the digested and diluted sample. The USP standard describes to add 5 mL NaOH per used mL sulfuric acid (100 mL NaOH / 20 mL sulfuric acid). To save chemicals, 6 mL NaOH per used mL sulfuric acid (30 mL water / 5 mL sulfuric acid) for digestion can be used without affecting the results [4, 5].
Immediately add a few pieces of granulated zinc,…	Granulated zinc can't be added automatically to the sample with BUCHI distillation units. Zinc is added only, when selenium containing Kjeldahl tablets were used for digestion. However, no influence on distillation quality or recovery rates could be observed [7 – 9].
…and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube from which dips beneath the surface of 100 mL of boric acid solution (1 in 25) contained in a conical flask or a wide-mouth bottle of about 500 mL capacity.	For nitrogen amounts of 0.02 – 6.75 mg N / sample BUCHI recommends using 2 % boric acid with 3 g/L KCl. For higher nitrogen amounts, 4 % boric acid is used. To safe chemicals, 50 mL of boric acid for potentiometric determination is sufficient. For colorimetric determination, 60 mL of boric acid are recommended. The capacity of the standard receiving vessel (043390) is about 420 mL boric acid.
Mix the contents of the Kjeldahl flask by gentle rotation, and distil until about four-fifths of the contents of the flask has distilled over.	Distillation time is dependent on the distillation unit as well as on the digested sample and the nitrogen content. To ensure proper distillation we recommend distillation time up to 150 seconds (distillation unit only) and 180 seconds (with KjelSampler K-376 or K-377). Additional mixing and rotation is not required as the inserted vapor steam is homogenizing the sample with the added chemicals.
Titrate with 0.5 N sulfuric acid (volumetric solution) determining the endpoint potentiometrically.	Using the KjelMaster K-375, the samples can be determined automatically with a potentiometric sensor.
Perform a blank determination, and make any necessary correction.	It is recommended to do a least two blank determinations before measuring the samples. The average is calculated and subtracted from the sample results automatically by the KjelMaster K-375.
Each mL of 0.5 N sulfuric acid is equivalent to 7.003 mg of nitrogen. When the nitrogen content of the substance is known to be low, the 0.5 N sulfuric acid may be replaced by 0.1 N sulfuric acid. Each mL of 0.1 N sulfuric acid is equivalent to 1.401 mg of nitrogen.	The optimum consumption range of volumetric titration solution using 20 mL burettes is between 3 – 17 mL. Hence, it is recommended to choose a concentration of volumetric solution, which fulfils the recommendation.

USP 461 Method II

The method II of the USP 461 describes an accelerated digestion using hydrogen peroxide (H_2O_2) as an oxidizing agent and boric acid titration. The required apparatus is described as an appropriate 300 mL Kjeldahl flask, from which the nitrogen is first liberated by acid digestion and then transferred quantitatively to the titration vessel by steam distillation. In Table 9, the USP 461 method II is described and its compliance with the BUCHI instruments discussed.

Table 9: Compliance of the USP 461 Method II with the BUCHI instrumentation.

USP 461 Method II	BUCHI compliance
Place an accurately weighed or measured quantity of the material, equivalent to 2 to 3 mg of nitrogen, in the digestion flask of the apparatus.	Depending on the expected nitrogen content, optimal amounts of sample are in the range of 0.01 – 5 g. The accuracy of the balance needs to be considered.
Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10:1), and wash down any adhering material from the neck of the flask with a fine jet of water.	The Kjeldahl Tablet Titanium Micro (total weight 1.59 g) contains: 1.5 g K_2SO_4, 0.045 g $CuSO_4$ and 0.045 g TiO_2 The Kjeldahl Tablet Copper Micro (total weight 1.65 g) contains: 1.5 g K_2SO_4 and 0.15 g $CuSO_4$ The in the standard described ratio of potassium sulfate and cupric sulfate (10:1) is identical with the Kjeldahl Tablet Copper Micro. However, the total weight is higher (1.5 g instead of 1 g). No rinsing step is needed, when Kjeldahl Tablets are used.
Add 7 mL of sulfuric acid, allowing it to rinse down the wall of the flask, then, while swirling the flask, add 1 mL of 30 % hydrogen peroxide cautiously down the side of the flask. (Do not add hydrogen peroxide during the digestion).	The amount of sulfuric acid conc. used for digestion depends on the sample weight and matrix. The higher the sample amount the more sulfuric acid is used. Furthermore, high-fat containing samples require more sulfuric acid. Hence, for successful digestion the amount of sulfuric acid should be adjusted depending on sample matrix and weight. See Kjeldahl Practice Guide [5]. For optimum digestion, a ratio of 1:2 of catalyst and sulfuric acid is recommended. Hence, if 7 mL sulfuric acid is used, the amount of catalyst should be increased to 3.5 g. A helpful tool for sulfuric acid volume and catalyst weight calculation is the KjelOptimizer App [10].
Heat the flask over a free flame or an electric heater until the solution has a clear blue color and the sides of the flask are free from carbonaceous material.	On the SpeedDigester K-439 and KjelDigester K-449 a fully automated temperature / time profile can be created and up to 50 methods can be stored on the instrument. The automated lift function of the K-449 allows preheating and starting digestion unattended. When digestion is finished, the digested samples are automatically lifted out of the heating block to automate and accelerate the cooling step.
Cautiously add to the digestion mixture 70 mL of water, cool the solution, and arrange for steam distillation.	The water is added to the digested sample to dilute the sulfuric acid and avoid too violent reactions. The water can be dosed in defined volume automatically in all BUCHI distillation units (except K-350 and K-355). Further, the reaction time can be programmed. The USP standard method II describes to add 10 mL water per used mL sulfuric acid (70 mL water / 7 mL sulfuric acid). To save chemicals, 5 mL water per used mL sulfuric acid (25 mL water / 5 mL sulfuric acid) for digestion can be used without affecting the results [4, 5].
Add through a funnel 30 mL of sodium hydroxide solution (2 in 5) in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, rinse the funnel with 10 mL of water, tightly close the apparatus, and begin the distillation with steam immediately.	Sodium hydroxide is added to increase the pH from acidic to pH >12 to liberate the ammonia of the digested sample. All BUCHI distillation units are able to dose defined volumes automatically in to the digested and diluted sample. The USP standard method II describes to add 4.3 mL NaOH per used mL sulfuric acid (30 mL NaOH / 7 mL sulfuric acid). To ensure proper dilution and alkalization, 6 mL NaOH per used mL sulfuric acid (30 mL NaOH / 5 mL sulfuric acid) for digestion is recommended [4, 5].

Receive the distillate in 15 mL of boric acid solution (1 in 25), to which has been added 3 drops of methyl red-methylene blue and sufficient water to cover the end of the condensing tube.	For nitrogen amounts of 0.02 – 6.75 mg N / sample BUCHI recommends using 2 % boric acid with 3 g/L KCl. For higher nitrogen amounts, 4 % boric acid is used. The capacity of the receiving vessel “small” (043333) is about 340 mL. Nevertheless, the volume of boric acid for potentiometric determination should be at least 40 mL. For colorimetric determination, 60 mL of boric acid is recommended. The indicator mentioned in method II (methyl red - methylene blue) is comparable with the indicator described in the Ph.Eur. 2.5.9. methyl red mixed solution, containing 0.1 g methyl red and 50 mg of methylene blue in 100 mL of alcohol. The indicator has a color change from red-violet (pH 5.2) to green (pH 5.6). With setting the pH endpoint of titration to pH 5.6 on the KjelMaster K-375, potentiometric measurement can be applied. In practice, no colored indicator is needed. For the automated colorimetric determination with the KjelMaster K-375, two indicators can be used: - Sher indicator - Bromocresol green / methyl red
Continue the distillation until the distillate measures 80 to 100 mL.	The steam generator produces a volume of approx. 35 mL steam per minute when set to 100 % steam power. Hence, 80 – 100 mL of distillate is obtained in approx. 140 - 180 seconds. However, the distillation time is dependent on the distillation unit as well as on the digested sample and the nitrogen content. To ensure proper distillation we recommend a distillation time up to 150 seconds (distillation unit only) and 180 seconds (with KjelSampler K-376 / K-377).
Remove the absorption flask, rinse the end of the condensing tube with a small quantity of water, and titrate the distillate with 0.01 N sulfuric acid.	Using the fully automated distillation and titration unit K-375 with KjelSampler K-376, the titration can be performed on the distillation unit without removing the receiving vessel. The stirrer of the K-375 and K-360 provide a homogenous mixing of the receiving solution during distillation and titration. Hence, no rinsing of the condensing tube is necessary.
Perform a blank determination, and make any necessary correction.	It is recommended to do a least two blank determinations before measuring the samples. The average is calculated and subtracted from the sample results automatically when using the KjelMaster K-375.
Each mL of 0.01 N sulfuric acid is equivalent to 140.1 µg of nitrogen. When a quantity of material containing more than 2 to 3 mg of nitrogen is taken, 0.02 N or 0.1 N sulfuric acid may be employed, provided that at least 15 mL is required for the titration. If the total dry weight of material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide.	The optimum consumption range of volumetric titration solution using 20 mL burettes is between 3 – 17 mL. Hence, it is recommended to choose a concentration of volumetric solution, which fulfils the recommendation and adapt the sample amount accordingly.