Kjeldahl Guide

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Guide

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Dr. Huldrych Egli

Foreword

In the past 200 years the techniques supporting chemical analysis have made tremendous progress from the invention of the Bunsen-burner and its use for flame tests to the atomic force microscope sent to Mars for the exploration of martian soil. At the time when Johan Kjeldahl published his method for the determination of nitrogen in 1883 the electric lamp was just patented and the technical age in its childhood. Seldom in human history has an invention remained basically unchanged for such a long time as Kjeldahl's method for nitrogen determination. As in 1883 a Kjeldahl nitrogen determination starts with sample preparation, proceeds to the mineralization followed by separation using distillation and subsequent volumetric determination of the amount of ammonia formed in the process. Kjeldahl's visionary idea of providing a simple method for nitrogen and protein determinations, which also can be carried out by non academic lab personnel, has been put into practice by Büchi's Kjeldahl systems since 1961.
The Büchi Kjeldahl Guide you have in your hands is addressed to laboratory personnel, laboratory supervisors, students and teachers. It is our intention to revive the basic knowledge needed to understand the chemical and physical background associated with nitrogen determinations according to Kjeldahl and provide clear instructions in a wide area of Kjeldahl applications. The first theoretical part of the Kjeldahl Guide contains basic knowledge and the second half consists of a selection of Büchi Application Notes describing successful nitrogen determinations.
With this Kjeldahl Guide Büchi would like to live up to its company slogan

«Quality in your hands»

and support you in your daily work by not only providing high quality instrumentation but also offering comprehensible theoretical background information and showcase applications.

Dr. Huldrych Egli

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1 Introduction

This introduction gives an overview of the history of the Kjeldahl Method and of its evolution to the current state of the art.

1.1 History of the Kjeldahl Method

For almost 130 years the determination of nitrogen by means of the method developed by the Danish chemist Johan Gustav Christoffer Thorsager Kjeldahl (1849–1900) has been an internationally accepted standard. The method was introduced in 1883 at a meeting of the Danish Chemical Society by Johan Kjeldahl as a means to determine nitrogen in barley and yeast [1]. The method named after its inventor has since found wide-spread 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.

Kjeldahl was a member of the innovative laboratory team at the Carlsberg brewery in Copenhagen, also famous in microbiology for isolating the famous beer yeast *Saccharomyces Carlsbergensis* which is still used today. As the head of the chemistry department at the Carlsberg brewery he was involved in a very modern problem: quality management and optimization of productivity. Kjeldahl intended to determine the protein content of grain in order to find out how the protein content influences quality and quantity of the brewed beer.

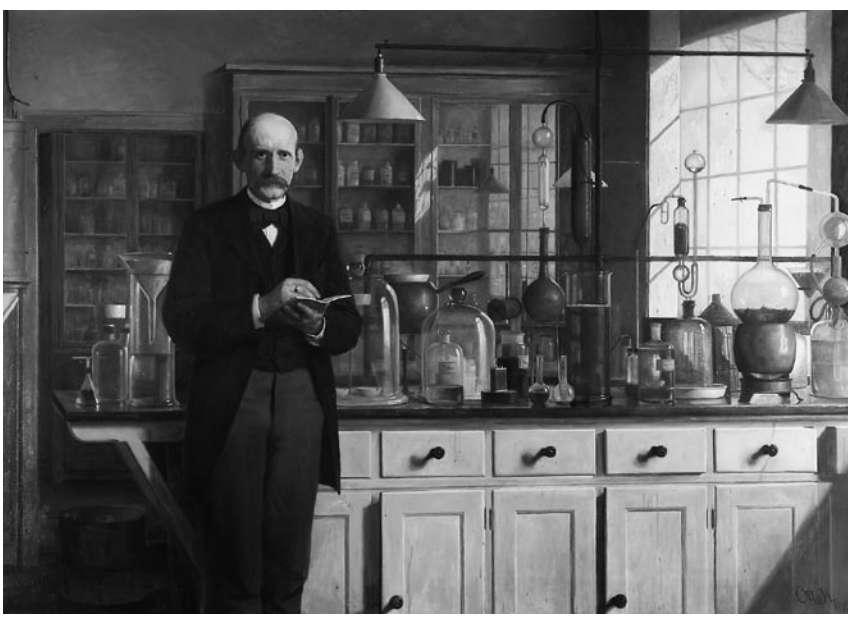


Figure 1: Johan Kjeldahl in his laboratory at Carlsberg Brewery in Copenhagen in the year 1880 (image by courtesy of Carlsberg Archives, Copenhagen).

Table 1: Citation from Kjeldahl's original publication of 1883.

Original citation	Translation
«Le principe de la nouvelle méthode consiste donc à chauffer pendant quelque temps la matière à analyser avec une forte proportion d'acide sulfurique concentré jusqu'à une température voisine du point d'ébullition de l'acide, et à oxyder la dissolution ainsi obtenue avec un excès d'hyperpermanganate de potasse sec en poudre. Dans ces conditions, l'azote des substances organiques, … , se transforme complètement en sulfate d'ammoniaque, qui, l'oxydation une fois terminée et après saturation avec la soude, peut être distillé et dosé par les méthodes ordinaires.»	*«The principle of the new method is to heat the test material for some time with a large quantity of concentrated sulfuric acid at a temperature close to the acid's boiling point and to oxidize the solution thus obtained with an excess of dry potassium per-manganate powder. Under these conditions the nitrogen of the organic substances, … , is completely transformed into ammonium sulfate which, once the oxidation is completed and after saturation with caustic soda can be distilled and determined by ordinary methods.»*

Although individual chemicals used in the Kjeldahl method have changed over the years it is possible to give a concise general definition:

> The Kjeldahl Method consists in a procedure of catalytically supported mineralization of organic material in a boiling mixture of sulfuric acid and sulfate salts at digestion temperatures between 340 and 370 °C. In the digestion process the organically bonded nitrogen is converted into ammonium sulfate. Alkalizing the solution liberates ammonia which is quantitatively steam-distilled and determined by titration.

1.2 Product Classes Amenable to the Kjeldahl Method

Proteins are of indispensable nutritional value for humans and animals and are contained in juices, dairy products, food and feed [2]. The Kjeldahl method allows the calculation of protein contents in food samples based on the determined nitrogen which is a general constituent of all proteins. Organically bonded nitrogen, amenable to the Kjeldahl method, is found in beer, yeast, barley, wine, wheat, corn, rice, vegetables, beans, soy and nuts, milk and related dairy products, eggs, meat and sausages, fish and seafood.

The scope of Kjeldahl nitrogen determinations today also includes applications in the fields of environmental analysis, research and development, pharmaceutical, chemical and cosmetics industries and is also used in governmental and regulatory laboratories.

Food	Protein [%]
Apple	0.3
Peach	0.8
Carrot	1.0
Raspberry	1.3
Potatoes	2.0
Elderberry	2.5
Spinach	2.7
Horse-radish	2.8
Rose hip	3.6
Milk	3.2
Pea sprouts	5.1
Corn	9.2
Flour	11.0
Oats	12.6
Chicken	19.9
Halibut	20.1
Red beans	21.2
Beef	22.0
Lentils (dry)	22.9
Cheese (eg. Cheddar)	24.7
Peanuts	24.7
Sunflower seed	26.5
Ostrich	35.3
Soybeans	37.6

Table 2: Protein contents of some foodstuffs.

For packed and processed food nutrition fact labels are in use almost everywhere in the world, examples are given in Figure 2. The protein content is one of the important parameters declared on nutrition fact labels.

Nutrition Facts
Serving Size 67 g

Amount Per Serving	
Calories 33	Calories from Fat 4
	% Daily Value*
Total Fat 0g	1%
Saturated Fat 0g	0%
Trans Fat	
Cholesterol 0mg	0%
Sodium 29mg	1%
Total Carbohydrate 7g	2%
Dietary Fiber 1g	5%
Sugars	
Protein 2g	

Vitamin A	20%	• Vitamin C	34%
Calcium	9%	• Iron	6%

*Percent Dialy Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

Nährwerte/valeurs nutritives/ valori nutritivi

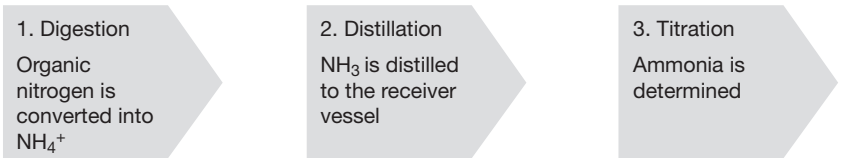
	100 g enthalten/ contiennent/ contengono:	1 Stängel (17 g) enthält/ bâton contient/ bastoncino contrine:
Energiewert/ valeur énergétique/ calore energetico	1870 KJ (447 kcal)	320 KJ (77 kcal)
Eiweiss/ protéines/ proteine	15 g	2.5 g
Kohlenhydrate/ glucides/carboidrati	56 g	10 g
davon/dont/di cui · Zucker/sucres/zuccheri	30 g	5 g
Fett/lipides/grassi	18 g	3 g
davon/dont/di cui · gesättigte Fettsäuren/ acides gras saturés/ acidi grassi saturi	6 g	1 g
· Cholesterin/ cholestérol colesterolo	0 mg	0 mg
Ballaststoffe/ fibres alimentaires/ fibres alimentari	6 g	1 g
Natrium/sodium sodio	0.15 g	0.02 g

Figure 2: Typical Nutrition Facts Labels as used in North America and Europe.

1.3 Procedures for Kjeldahl Nitrogen Determinations

The Kjeldahl procedure involves three major steps

Figure 3: The three major steps in Kjeldahl nitrogen determinations.



1.3.1 Digestion

In the digestion step the organically bonded nitrogen is converted into ammonium ions. Organic carbon and hydrogen form carbon dioxide and water, very much reminiscent to an incineration process. In this process the organic material carbonizes which can be visualized by the transformation of the sample into black foam. During the digestion the foam decomposes and finally a clear liquid indicates the completion of the chemical reaction. The generalized non-stoichiometric chemical equation (1) shows how a general nitrogen containing starting material (CHNO) is mineralized to dissolved ammonium ions.

$$(CHNO) + H_2SO_4 \rightarrow CO_2 + SO_2 + H_2O + NH_4^+ \quad (1)$$

In the original procedure published by Kjeldahl the mineralization was carried out in boiling sulfuric acid. The oxidation was supported by the addition of the strong oxidizing agent potassium permanganate. After its introduction by Kjeldahl, the digestion reaction was further improved and optimized. Examples were the addition of salts and the use of catalysts which allowed for shorter digestion time. The most common salt used historically was potassium sulfate and the catalysts were selenium and metal salts, particularly of mercury, copper or titanium.

Two types of heating units are used to heat up the sample together with the reagents to boiling temperatures of 340 to 370 °C. One type are IR-digesters and the other are block digesters (see «2.2.11 IR-Digestion versus Block-Digestion», p. 26).

Figure 4: IR-Digestion Unit K-424/435 (left) Block-Digestion Unit K-437 (right).

Figure 5:
Scrubber B-414 connected to a Digestion Unit K-438.

After the digestion has lead to a clear liquid, an additional digestion time of e.g. 30 minutes is usually added, in order to allow complete mineralization [3].

For the digestion working in a fume hood is highly recommended and the use of the Scrubber B-414 provides additional safety to laboratory personnel and environment as well as offering protection of the equipment against corrosion.

1.3.2 Distillation

After digestion the sample is allowed to cool to room temperature and the glass sample tube is transferred to a distillation unit.

Neutralization of sulfuric acid

Prior to the distillation the acidic sample is neutralized by means of concentrated sodium hydroxide solution (NaOH) as shown in equation (2).

$$H_2SO_4 + 2\ NaOH \rightarrow 2\ Na^+ + SO_4^{2-} + 2\ H_2O \qquad (2)$$

Distillation in glass sample tube

In the distillation step the ammonium ions are converted into ammonia which is transferred into the receiver vessel by means of steam distillation.

In a chemical equilibrium (see equation (3)) the solvated ammonium ions (NH_4^+) produce ammonia gas (NH_3) by reacting with hydroxyl ions (OH^-) of excess sodium hydroxide. By the steam distillation ammonia is separated from the glass sample tube and condensed together with water in the receiving vessel.

Figure 6:
Distillation units:
1. stand alone distillation (K-355)
2. with external titrator (K-360)
3. with built-in titrator (K-370)

$$NH_4^+ + OH^- \leftrightarrows NH_3(gas) + H_2O \quad (3)$$

Condensate collection in receiving vessel

A common procedure to collect the ammonia in the receiver involves the presence of boric acid $B(OH)_3$ dissolved in water which forms ions with ammonia according to equation (4). The ammonia is quantitatively captured by the boric acid solution forming solvated ammonium ions. See also «1.4 Blanks», p. 13.

$$B(OH)_3 + NH_3 + H_2O \leftrightarrows NH_4^+ + B(OH)_4^- \quad (4)$$

1.3.3 Titration

The concentration of the captured ammonium ions in the boric acid are determined by means of an acid base titration commonly using standard solutions of sulfuric or hydrochloric acid. Depending on the amount of ammonium ions present, concentrations in the range of 0.01 N to 0.5 N are used. Titrations may be carried out by means of a burette using an appropriate pH-indicator such as Sher mixed indicator [4] (Büchi 003512) to indicate the end point of pH = 4.65 (see «2.4.1 Boric Acid Titration», p. 31). For the preparation see «5.1 Two-Stage Mixing Indicator According to Sher for Boric Acid Titration» (p. 44). A second option is to attach a titration stand to the distillation unit and read the volume of consumed acid from the display of the titrator. The most sophisticated procedure is the use of a Kjeldahl distillation unit with a built-in titrator and have the calculation done by the software of the instrument. Whatever the choice of the determination method, the chemical reaction is described by equation (5) showing the reaction of the tetrahydroxyborate anion $B(OH)_4^-$ with a generalized strong acid HX (X = Cl^- etc.).

$$B(OH)_4^- + HX \rightarrow X^- + B(OH)_3 + H_2O \quad (5)$$

1.4 Blanks

Blanks, containing all reagents apart from the test material, are necessary if a Kjeldahl distillation is carried out. In the following outline this is discussed for the case of boric-acid titrations (see chapter «2.4 Titration», p. 30):
During distillation of a blank an increase of the pH-value in the receiving vessel is observed. This change in pH is due to dilution. The effect on the pH-value by diluting boric acid can be explained by equation (10) for the pH-value of a weak acid. In Table 3 the effect is demonstrated by means of experimental pH readings as a function of the volume of added water to 60 mL of 4%, 2% and 1% boric acid. In addition to the increase of pH due to dilution, also effects of traces of volatile bases, inevitably present in reagents and equipment, are taken into account by blank determinations.

Vol H_2O	pH exp		
	4%	2%	1%
0	4.65	4.64	4.65
10	4.85	4.74	4.72
20	5.02	4.84	4.80
30	5.14	4.91	4.83
40	5.25	4.96	4.85
50	5.35	5.00	4.88
60	5.42	5.05	4.92
70	5.48	5.08	4.93
80	5.54	5.11	4.95
90	5.59	5.13	4.97
100	5.64	5.15	4.99
110	5.67	5.17	5.01
120	5.71	5.18	5.03
130	5.75	5.19	5.04
140	5.78	5.20	5.05

Table 3: Measured pH-values as a function of the volume of added water to 60 mL of 4%, 2% and 1% boric acid.

In a distillation the extent of dilution depends on the distillation time and is identical for blank and sample determinations. The determination of the samples includes the pH increase due to dilution. This is taken into account in the calculations of the nitrogen contents in which the blank volumes are subtracted from the volumes found for the samples (see chapter «2.5.1 Calculation for Boric Acid Titration», p. 34)

As can be seen in Figure 7 the increase of the pH-value depends on the concentration of the boric acid. At lower concentrations the increase in pH due to the distillation is less than for higher concentrations. This also leads to lower blank consumptions if less boric acid is used.

A typical blank volume in a boric-acid titration, determined using a distillation time of 4 minutes, is in the range of 0.1–0.2 mL if 0.25 mol/L H_2SO_4 is used as a titrant.

For a better understanding of the chemistry involved in the boric-acid titration and the associated pH-increase effected by dilution during the distillation, a view at the chemical reaction and the chemical equilibria is given below:

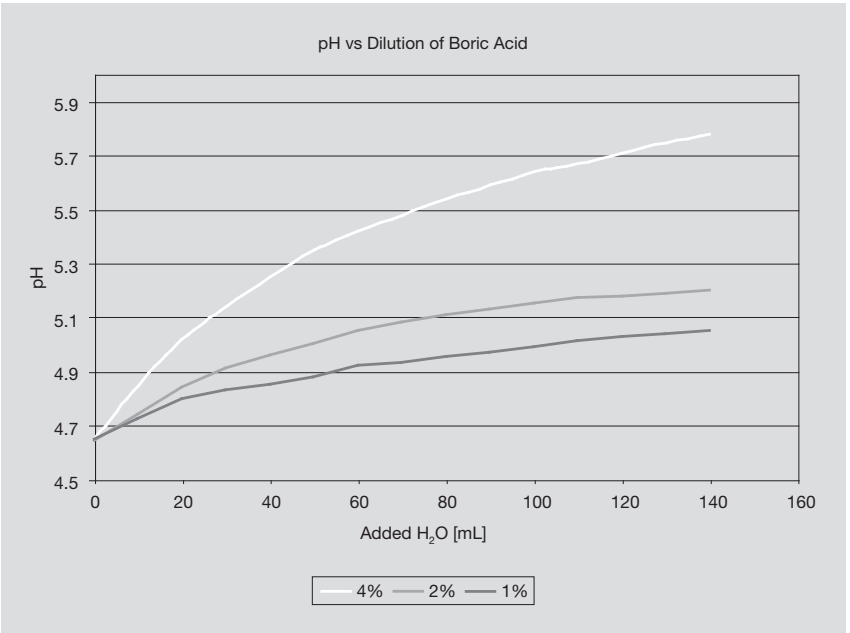


Figure 7: Representation of experimental pH-values as a function of the volume of water distillate added to 4%, 2% and 1% boric acid.

Boric acid acts as a Lewis acid, an electron pair acceptor, in the chemical equilibrium described by equation (6):

$$B(OH)_3 + 2\ H_2O \leftrightarrows B(OH)_4^- + H_3O^+ \qquad (6)$$

The chemical equilibrium is described by the law of mass action expressed by equation (7).

$$K = \frac{c(B(OH)_4^-) \cdot c(H_3O^+)}{c(B(OH)_3) \cdot c^2(H_2O)} \qquad (7)$$

The derivation of equation (10) is based on equations (8) and (9). The pK_a-value for boric acid can be found in the literature [5].

$$K_a = \frac{c(B(OH)_4^-) \cdot c(H_3O^+)}{c(B(OH)_3)} = 5.8 \cdot 10^{-10}\ \text{mol/L} \qquad (8)$$

$$pK_a = 9.27 \qquad (9)$$

$$pH = pK_a + \log \frac{c(B(OH)_4^-)}{c(B(OH)_3)} \qquad (10)$$

2 Determination of Nitrogen by the Kjeldahl Method

Nitrogen in the oxidized form of nitrates and nitrites and often nitrogen in aromatic heterocycles do not add quantitatively to the finally determined Kjeldahl reaction product ammonia. In Table 4 some of the most important refractory compounds for Kjeldahl nitrogen determination are summarized and in Table 8, p. 17, a classification of organic nitrogen containing compounds is given.

Compound	Recovery [%]
Nitrate, nitrite (organic and inorganic)	<1
Aromatic N-heterocycles: Pyridine, pyrimidine, thiazole, imidazole, pyrazole	1–<80
Azo compounds	30–85
Hydrazines	<50

Table 4: Refractory compounds which show poor contribution to Kjeldahl nitrogen.

In the context of studies on the oxidation of organic nitrogen containing substances, a general chemical equation was published by Jurecek et al. [6] [7] [8] in order to describe the oxidation. Kjeldahl digestions of nitrogen containing chemical compounds/substances can either lead to ammonia, nitrate or elemental nitrogen. A theoretical consideration on chemical reactions in the Kjeldahl digestion was published by Morita [9]. A short form of the chemical equations described in [8] and [9] is given in equation (11) and the indices a, b, c are explained to the right.

$$CHON_{a+b+c} + O \rightarrow a\ NH_3 + b\ HNO_3 + c/2\ N_2 \qquad (11)$$

a = groups producing NH_3
b = groups producing HNO_3
c = groups producing N_2

In Table 5 organic functional groups are listed and correlated to the respective nitrogen degradation products a, b, and c as described above in equation (11) [9] [10].

The chemical pathways of degradation processes include simultaneous reduction, dehydration, hydrolysis, substitution and other reactions. For ($-NH_2$, $=NH$, $\equiv N$, $[R_4N]_x$) the first step is protonation by sulfuric acid. The more alkaline an amine the easier it is protonated aiding the cleavage of the C-N bond. Primary amines are the easiest amines to be digested. In Table 6 the ease of reaction for a series of amines is expressed by relative recovery rates referenced to methylamine.

Table 5: Degradation products depending on functional groups.

Name	Functional Group	Degradation Product	Symbol
Amide	$-CONH_2$		
Amino	$-NH_2$		
Heterogenic Nitrogen	=N-		
Imino	=NH		
Isocyanide-	-NC	NH_3	a
Isooxocyanate	-NCO		
Isothiocyanate	-NCS		
Oxocyanate	-OCN		
Peptide	-CONH-		
Nitrile group	-CN		
Hydroxyamine-	-NHOH		
Isonitro	-NOOH		
Nitro-	$-NO_2$	HNO_3	b
Oxim-Group	=NOH		
Nitroso-	-NO		
Azo-	-N=N-		
Azino	=N-N=		
Diazonium-	$-N\equiv N^+$	N_2	c
Hydrazone	-N-NH-R		
Hydrazine-Group	$-NHNH_2$		

Table 6: Ease of degradation depending on type of amine.

1 100% is easiest degradation, results based on nitrogen recoveries of Kjeldahl reactions without catalyst.

Type of Amine	Ease of reaction[1] [%]
Primary Amines	90
Secondary Amines	80
Tertiary Amines	76
Quarternary Ammonium	54
Methylamine	100
Aniline	85
2-Nitroaniline	50

A comparison of recovery rates is given in Table 7 for a selection of typical amino acids. In amino acids neighboring carboxyl groups weaken the C-N bond and the conversion into ammonia is facilitated compared to primary amines. More difficult is the dissolution of a C-N bond if two amino groups are attached as in lysine shown in Figure 9 because a stable piperidine carboxylic acid is generated [11]. In this case only approx. 50% of the nitrogen is recovered. Amino acids containing aromatic heterocycles show a reduced nitrogen recovery, for tryptophan for instance a recovery of 67% is reported [10]. With modern optimized digestions e.g. by using selenium and mercury free Kjeldahl tablets, higher recoveries can be obtained for tryptophan but the problem of difficult digestions remains. In studies dealing with protein determinations an average nitrogen recovery of 98% can be reached [10], and protein contents are derived from experimentally found nitrogen contents by means of a protein factor (see chapter «2.5.3 Protein Content» p. 35)

Type of amino acid	Ease of reaction[1] [%]
Aspartic acid	100
Proline	98
Arginine	98
Tryptophan	67
Histidine	66
Lysine	50

Table 7:
Ease of degradation depending on type of amino acid.

1 100% is easiest degradation, results based on nitrogen recoveries of Kjeldahl reactions without catalyst.

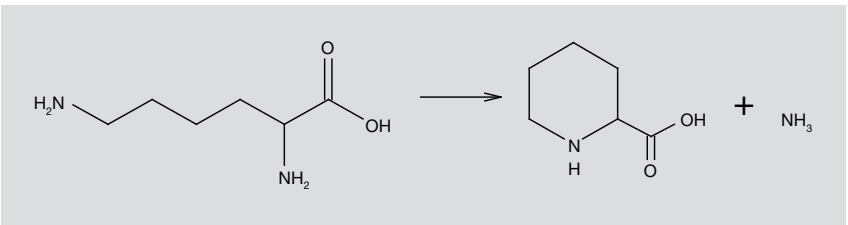


Figure 8:
Structure of the amino acid lysine which contains two amino-groups and formation of a stable piperidine carboxylic acid.

Depending on the ease of Kjeldahl degradations the duration of a particular digestion needs to be adjusted for the highest possible recovery rate (see «2.2.10 Optimization of the Digestion», p. 25). Ammonia salts do not need digestion.

In Table 8 the ease of degradation is given as a function of the nitrogen containing chemical groups [10]. As can be seen, especially nitrogen containing heterocycles exhibiting a strong stabilization by resonance of the aromatic system are not easy or even impossible to be digested. Organic nitrates and nitrites are not accessible to Kjeldahl because of the high oxidation state which does not allow the formation of ammonia.

Group	N recovery by Kjeldahl digestion
Azides (MeN_3)	Recovery approx. 20%[2]
Azo compounds (-N=N-)	only partly[3]
Carbamine group	very good
Heterocycles	The higher the resonance stability the worse
Hydrazine (NH_2-NH_2)	30-54%
Imides, oximes	up to 100%
Nitrates	1%
Nitrides	10%
Nitrites (Me-NO_2)	0%
Nitro (R-NO_2)	50%
Purines (uric acid, guanine, caffeine)	100%
Acid amides	100%

Table 8:
Nitrogen containing groups and typical recovery rates for nitrogen by means of Kjeldahl digestion.

2 Produces explosive HN_3
3 Produces nitrogen N_2

2.1 Sample Preparation

Two critical points involved in sample preparation are the amount of sample and its homogeneity which will be discussed below in chapters «2.1.1 Amount of Sample», p. 18, and «2.1.2 Mincing and Homogenization», p. 19. A further aspect is the expected titrant consumption which, for reasons of accuracy, should be in a range of 5 to 20 mL for titrant concentrations of 0.5 to 0.01 mol/L if a 20 mL burette is used. An immanent problem associated to Kjeldahl digestions is foam formation (see «1.3.1 Digestion», p. 10) and, especially if large sample volumes are present, the risk of foaming over into the suction module. In such cases the use of anti-foam agents can be of help. A common substance used as

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