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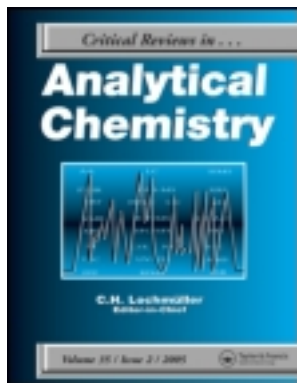
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An Overview of the Kjeldahl Method of Nitrogen Determination. Part I. Early History, Chemistry of the Procedure, and Titrimetric Finish

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In 1883 Kjeldahl devised a method for the determination of nitrogen, which has become a classical measurement in analytical chemistry and has been used extensively over the past 130 years. In the original method, sulfuric acid alone was used as a digestion medium. The use of a catalyst in Kjeldahl digestion accelerates oxidation and completes the digestion to allow the subsequent determination of nitrogen. Mercury (its use being in decline because of environmental concerns), selenium, and copper are the catalysts of choice, though for certain applications titanium has found some usage. Short digestion times in association with maximum nitrogen recovery may be achieved by using a methodology based on experimental design and response surfaces, with microwave digestion processes, and with the aid of the couple sulfuric acid-hydrogen peroxide without catalyst. The quantification of distilled ammonia is generally achieved by titration; the ammonia is absorbed in an excess of boric acid, followed by titration with standard acid in the presence of a suitable indicator. The Kjeldahl method can be done with limited resources; nitrogen determination with the Kjeldahl method does not require expensive devices nor specialized techniques and is precise and accurate. The Kjeldahl method is used for calibrating other protein assays; it is still the primary reference method for protein analysis today. The original method as presented by Kjeldahl has been continuously improved. Today's digestion systems offer safety both from a personal perspective and from an environmental point of view. The determination of nitrogen content is a frequently conducted analysis in industry and commerce, and numerous organizations have official methods. The use of instrumental finish in Kjeldahl applications will be the subject of the second part of this review.

Keywords Kjeldahl method, early history, chemistry, microwave digestion, titrimetric finish

INTRODUCTION

Nearly 130 years ago, the Danish chemist Johan Gustav Christoffer Thorsager Kjeldahl, Head of Chemistry Department of the Carlsberg Foundation Laboratory of the Danish Brewing Carlsberg Company, introduced a method known later under the eponym the Kjeldahl method that basically is still in use. It was first made public at a meeting of the Danish Chemical Society (Kemisk Forening) held on March 7, 1883 (Burns, 1984; Johannsen, 1900; McKenzie, 1994; Oesper, 1934; Ottensen,

1983; Veibel, 1949). Within the same year, the method was published in the German journal *Zeitschrift für Analytische Chemie* (Kjeldahl, 1883a), and written in French and Danish languages in communications from the Carlsberg Laboratory (Holter and Møller, 1976; Kjeldahl, 1883b, 1883c; Ottesen, 1983). Because of the respect that the founder of the laboratory, the Danish brewer J. C. Jacobsen, had for Pasteur and his work for the French wine industry (Burns, 1984), extensive French summaries of the Carlsberg papers were also published. As an extended summary of the Kjeldahl paper appeared in *Chemical News* in August (Kjeldahl, 1883d), the method was quickly taken up (Sella, 2008). The *Analyst* first gave details of the method in 1885 “for the benefit of those who may have missed the original paper” (Burns, 1984; Editor of The Analyst, 1885, p. 127), although the method had been briefly mentioned by Blyth (1884), who gave Kjeldahl's name incorrectly as Vjeldahl. A surprisingly short period went by between the

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publication of the Kjeldahl method and the appearance of publications effecting further improvements (Dyer, 1895; Hepburn, 1908; Kebler, 1891; Vickery, 1946a), both in Europe and the U.S., due to the tremendous impact that the Kjeldahl work had on others, especially in Germany (McKenzie, 1994). Most of the earlier contributions were discussed by Fresenius in the *Zeitschrift*, often to a length of several pages (Vickery, 1946a). Throughout the history of analytical chemistry, none of the methods has been as widely adopted, in so short a time, as the “Kjeldahl Method” for the estimation of nitrogen, as stated by Kebler (1891) at the beginning of an annotation in which he compiled references on the estimation of nitrogen by the Kjeldahl method (some 60) and by all other methods (about 200).

The Kjeldahl method was originally designed for the brewing industry as an aid in following protein changes in grain during germination and fermentation (Bradstreet, 1940; Kjeldahl, 1883b); the lower the amount of protein in the mash, the higher the volume of beer produced. It was Berzelius who suggested the use of the word “protein” in 1838 in a letter to Mulder because it was derived from the Greek word meaning “to be in the first place” (Zelitch, 1985). The Kjeldahl protein content is strictly dependent on total organic nitrogen content (Wong et al., n.d.); i.e., protein structure will not interfere with the accuracy of protein determination. The Kjeldahl method lack of analytical selectivity because do not distinguish protein-based nitrogen from nonprotein nitrogen. Adulteration incidents (e.g., adulteration of protein-based foods with melanine and related nonprotein compounds) exploiting this analytical vulnerability have been recently detected (Breidbach et al., 2010; Levinson and Gilbride, 2011; Moore et al., 2010; Tyan et al., 2009) and are new examples of a problem that dates back to before the Kjeldahl method was introduced (Möller, 2010a).

The presence of non-protein nitrogen (NPN) compounds in foods (aminoacids, ammonia, urea, trimethylamine oxide) overestimates their true protein content (Möller, 2010a; van Camp and Huyghebaert, 1996; Yuan et al., 2010) as derived from the current nitrogen determination methods. Separation of NPN from true protein nitrogen may be carried out by adding a protein precipitating agent such as trichloroacetic acid or perchloric acid (Rowland, 1938a, 1938b). The process conditions applied during protein precipitation, however, affect the composition and the amount of NPN, so it is mandatory to specify the type and concentration of precipitating agents used in each case. Alternative techniques such as dialysis and gel filtration are probably more accurate in removing the NPN fraction (van Camp and Huyghebaert, 1996), but they remain unacceptable for routine analysis. Reviews of NPN determination methods in cow milk, and on aspects concerning the composition of NPN fraction, are given by Wolfschoon-Pombo and Klostermeyer (1981, 1982). The Kjeldahl method measures what is termed total protein (American Jersey Cattle Association, n.d.). The alternative use of true protein (total nitrogen minus the NPN) has been under debate for some years (Grappin, 1992; Harding, 1992; Rouch

et al., 2007; Salo-Väänänen and Koivistoinen, 1996). A fundamental change in milk pricing in the U.S. was introduced January 1, 2000 with the implementation of producer payments in Federal Milk Marketing orders on the basis of the true protein content (American Jersey Cattle Association, n.d.; Stephenson et al., 2004; Zhao et al., 2010).

The protein content in a foodstuff is estimated by multiplying the nitrogen content by a nitrogen-to-protein conversion factor, usually set at 6.25 (*Comprehensive Review of Scientific Literature* . . . , 2006; Mariotti et al., 2008), which assumes the nitrogen content of proteins to be 16%. It is not clear who first reported such a factor for use (Moore et al., 2010). This general conversion factor is used for most foods because their non-protein content is negligible. However, pure proteins differ in terms of their nitrogen content because of differences in their amino acid composition, ranging from 13.4% to 19.3%; different multiplying factors are suitable for samples of different kinds. The factor 5.7 is applied for wheat and 6.38 for dairy products (O’Sullivan et al., 1999) and 6.394 ± 0.004 for cheddar cheese, as shown recently (Rouch et al., 2008). The proximate system where protein is measured as total nitrogen multiplied by a specific factor clearly dominates food composition studies (Greenfield and Southgate, 2003). As a matter of fact, most cited values for protein in food composition databases derive from total nitrogen or total organic nitrogen values.

A large variety of food proteins, either from animals (milk, meat, eggs, blood, fish) or plants (seeds, cereals), is nowadays available in the food industry. The determination of protein in foods and food products has important nutritional, functional, and technological significance (Van Camp and Huyghebaert, 1996). The protein content determines the market value (Krotz et al., 2008; Wiles et al., 1998) of major agricultural commodities (cereal grains, legumes, flour, oilseeds, milk, and livestock feeds). In addition, the quantitative analysis of protein content is necessary for quality control, and also a prerequisite for accurate food labeling (Owusu-Apenten, 2002). Protein analysis is required for a very wide range of animal and human nutrition products. Consumer interest in soy protein products has increased rapidly in Western cultures in recent years. This trend is due in part to the high-quality protein of soy foods and soy protein ingredients and in part to their associated health benefits (Jung et al., 2003); 25 g of soy protein per day may improve cardiovascular health (U. S. Food and Drug Administration, 1999). Consequently, precise determinations of protein content of soy products are very important. Total nitrogen concentration in soils is one of the most frequently measured nutrients in soil-testing laboratories (Sharifi et al., 2009). Determination of nitrogen content plays a key role in assigning values to insulin reference materials (Anglow et al., 1999). Primarily devised for the determination of protein nitrogen, the Kjeldahl method has been extended to include the determination of various other forms of nitrogen, e.g., in soils, plant materials, biological tissues, and wastewater matrices (Chemat et al., 1998).

Nitrogen is one of the most important elements for plant nutrition. Therefore, it is necessary to apply reliable methods for N determination in soils. Total nitrogen determination has been applied for geochemical, biochemical, and biogeochemical studies of this element in soils, sediments, and sedimentary rocks. Nitrogen determination is also very important for biosphere functioning and evolution forecasting studies (Pontes et al., 2009). Organic nitrogenous substances in surface waters arise mainly from animal and vegetable proteins and from synthetic organic compounds used in agricultural practices or used as dyes in various manufactures (Davi et al., 1993). Nitrogen is one of the specifications in the quality control of nuclear fuels (Verma et al., 2007). Ammonium is one of the most widely used industrial chemicals. It found used as fertilizer (either pure or as its derivatives) and for the synthesis of fibers, explosives, plastics, and animal foods (Meseguer-Lloret et al., 2005). It is also used as a food additive, in insecticides, in the manufacture of household detergents and cleaners, and as a major commercial refrigerant (Meseguer-Lloret et al., 2005). The ammonia concentration in water systems is regulated by legislation to guarantee the quality of the water supplied for human consumption; its maximum content is limited to 0.5 to 2 mg/L when expressed as total Kjeldahl nitrogen (TKN).

Table 1 gives the chemical methods most commonly used in protein determination. Some of the most significant methods (Dumas, Kjeldahl, and biuret assay) date from the late 1800s, although other analytical methodologies for total protein determination have been developed since then, relying on methods taken from biochemistry, biology, and proteomics. Most of these methods have been developed to address research needs and not

necessarily to determine the purity and/or adulteration of food products. Colorimetric methods of protein determination became available at the beginning of the past century and since then have been further developed for the analysis of protein in foods and food products (van Camp and Dierckx, 2004). In spite of the large number of techniques available for the quantification of total protein, only a few of them seem to be of interest for protein quantification in food products of different types (e.g., Kjeldahl, colorimetry, and infrared (IR) spectroscopy). The Kjeldahl, biuret, and dye-binding procedures (Ji, 2011; Li, 2006; Moser and Herman, 2011; Sherbon, 1974; Weaver et al., 1977) are classical methods that remain important in modern food protein analysis. The Lowry method is more frequently applied in biochemical research than in food analysis, but can be of importance for specific applications where high sensitivity is required. UV absorption (Rukke et al., 2010) is particularly suited when used in combination with a chromatographic separation technique, while IR spectrophotometry has become increasingly important during the past few years as a fast and nondestructive method for the quantification of total protein (Rayment et al., 2012; Santos, 2012) in food products. The best technique for precise determination of plant protein in plant materials is aminoacid analysis (Khanizadeh et al., 1995), given the small amount of protein present and the existence of many interfering substances.

For most methods of protein determination, proteins have to be solubilized in order to be determined. An exception to this rule is given by the Kjeldahl method and by near-infrared (NIR) reflection spectrophotometry, which allow the analysis of insoluble and eventually almost intact food samples. The use of NIR reflectance spectrophotometry for the quantification of

TABLE 1
Methods for Food Protein Analysis

Technique/eponym	Reference
Dumas method	Dumas, 1831; Tronimsdorff, 1832
Nessler reagent	Rammelsberg, 1843; Nessler, 1856a, 1956b
Biuret method	Wiedemann, 1849; Riegler, 1914; Gornall et al., 1949
Berthelot's method (alkali-phenol reagent)	Berthelot, 1859; Searle, 1984
Kjeldahl method	Kjeldahl, 1883b, 1883d
Folin-Ciocalteu	Folin and Ciocalteu, 1927
Dye binding	Fraenkel-Conrat and Cooper, 1944
Lowry method	Wu, 1922; Lowry et al., 1951; Peterson, 1979; Legler et al., 1985
Direct alkaline distillation	Developed 1960; Owusu-Apenten, 2002
NIR (near-infrared reflectance)	Developed 1960; Owusu-Apenten, 2002
Modified Berthelot reaction	1971; Searle, 1984
Modified Lowry method	Chandrarajan and Klein, 1975; Peterson, 1979
Bradford method (Coomassie blue dye-binding method)	Bradford, 1976; Compton and Jones, 1985
BCA (bicinchoninic acid method)	Smith et al., 1985
3-(4-Carboxy benzyl)quinoline-2-carboxaldehyde	You et al., 1997

Some key references: Chen (2000), Khanizadeh et al. (1995), Leca-Bouvier and Blum (2005), Möller (2010a), Moore et al. (2010), Noble and Bailey (2009), Owusu-Apenten (2002), Roberts and Jones (2008), Sapan et al. (1999), Searle (1984), Simonian and Smith (2006), Wallace and Fox (1998).

protein in cereals was first reported by Norris, who observed that cereal grains exhibit specific absorption bands in the NIR region (Ben-Gera and Norris, 1968; Williams, 1974; Williams and Norris, 1978) and suggested that NIR instruments could be used to measure grain protein, oil, and moisture. He invented a moisture meter based on the NIR principle (Norris, 1964).

However, despite the many methods proposed for protein determination in food samples, sample digestion by Kjeldahl's procedure is still the most frequently used one (Möller, 2009). Notwithstanding, some requests for environmentally friendly analytical processes (e.g., green methods) cannot be met by the conventional Kjeldahl method, since, for instance, sample preparation needs a metal or metalloid catalyst (Hg, Se, or Cu). This fact has contributed to the breakthrough of the Dumas combustion method and NIR methods at the turn of 1980s and 1990s. Modifications of the original Kjeldahl method have increased its versatility, so that analyses of many different sample types can now be accomplished (AOAC, 2011; Moore et al., 2010; Persson et al., 2008). Although there are several experimental approaches to evaluating nitrogen content in different kinds of samples, the Kjeldahl method still remains the reference method (Bravo et al., 2009; Lynch and Barbano, 1999), really being the "gold standard" for validating other quantifying methodologies in the biopharmaceutical and food industries (Möller, 2010a).

Although in the century following publication of the Kjeldahl method, almost every nitrogen-containing compound in biological fluids, such as blood and urine, can be determined separately and quantitatively, the need for estimation of total nitrogen is far from obsolete. For the study of nitrogen metabolism, total nitrogen determination is an essential tool (Smit, 1979). Kjeldahl nitrogen analysis is used for both calibration and validation of alternative methods for determining protein in various products. In either situation, however, success depends on the accuracy of the Kjeldahl-derived values. Methods that require calibration include mid-infrared spectroscopy, near-infrared spectroscopy, Fourier transform-infrared spectroscopy, and dye binding (Barbano and Lynch, 1992; Frank and Birth, 1982; Jarquín-Sánchez et al., 2011; Lefier et al., 1996; Möller, 2010b; Rayment et al., 2012; Rodríguez-Otero and Hermida, 1996; Santos, 2012; Schaalje and Mundell, 1991; Sherbon, 1974).

The Kjeldahl method is applied to nitrogen content measurement (AOAC, 2011; Möller, 2010b; Moore et al., 2010) in foods and many other samples: pharmaceutical, agricultural, biological sediments, and wastewater (Table 2). Since its introduction, the Kjeldahl method has been the subject of extensive research. The diversity of studies is attributable to the immense usefulness of the method, the need for its modification for applications to various types of organic and inorganic compounds, and the search for catalysts to provide such modifications and accelerate digestion (Ashraf and Shah, 1963; Hiller et al., 1948; McKenzie, 1994). Such a vast literature has been generated that it is not surprising that much irrelevant material exists, facts get rediscovered, and many potboilers

published (Belcher, 1976; Burns, 1984). Some review articles on this subject have been published from time to time (Table 3). An excellent book reviewing the Kjeldahl method for total organic nitrogen was written by Bradstreet (1965). Because it is impossible to review all the articles that have appeared since then dealing with the Kjeldahl procedure, only the most important ones, in the authors' opinion, shall be mentioned.

W. Johannsen (1857–1927), a pharmacist, wrote a Kjeldahl obituary, first published in German (Johannsen, 1900) and then translated into French (Johannsen, 1903) and English (Holter and Møller, 1976). Johannsen, one of the founders of the science of genetics, was in his beginnings an assistant in the chemistry department at the Carlsberg Laboratory under the chemist Johan Kjeldahl and is well known as the scientist who coined the term *gene* in 1909 (Dunn, 1971; Wilhelm Johannsen, n.d.). Oesper (1934), Veibel (1949), Szabadváry (1973, 1992), Ottesen (1983), Burns (1984), Pérez-Bustamante (2000), and Sella (2008) have given accounts of the life of Kjeldahl. He was elected to membership in the scientific academies of Denmark and Christiania and received an honorary doctorate from the University of Copenhagen (Burns, 1984; Oesper, 1934). Kjeldahl (like Nessler) has been turned into a verb, an honor not usually accorded to a chemist (Veibel, 1949). All chemists understand what it means when it is said that a substance was kjeldahled (Burns, 1984; Veibel, 1949), or that one kjeldahlizes a sample (Hansen, 1993). Kjeldahl was predecessor to S. P. L. Sørensen as Head of the Carlsberg Laboratory in Copenhagen, who introduced the notation of pH (Hansen, 1993; Rideal, 1940).

PRIOR TO KJELDAHL

The value of Kjeldahl's work becomes apparent when prior methods are considered. The state of the art in the area of nitrogen determination in the two decades preceding Kjeldahl's work exerted a notable influence on the development of his method (Stephen, 1984). The first method for the quantitative determination of the nitrogen content of an organic compound was the absolute method of Dumas, which dates from 1831 and provided the first scientific apparatus for the determination of nitrogen in varying samples. However, for some years prior to the publication of the Kjeldahl method, the Dumas method was rarely used due to the long time required for a determination and because of the difficulty in handling large amounts of materials with little nitrogen (Christensen and Fulmer, 1927). Moreover, the method was not suitable for use with the volumes of liquid used in biological work. As a matter of fact, Dumas's method was "a torment for everyone," as is evident from the contemporary literature, because it was so complicated (Szabadváry, 1973).

A weighed sample of substance is ignited with copper oxide in an atmosphere of carbon dioxide (generated by heating lead carbonate). The carbon is thus oxidized to carbon dioxide and the hydrogen to water, while the nitrogen is liberated as such and is determined volumetrically after collection over potassium

TABLE 2
Selected application of Kjeldahl methods

Samples	Reference
Agricultural products	Carson and Ozores-Hampton, 2012; Chemat et al., 1998; Watson and Galliher, 2001
Ammonium perchlorate in solid propellants	Ajaz, 1996
Animal feed	Etheridge et al., 1998; Figenschou et al., 2000; Guo et al., 2007; Kane, 1984; Marco et al., 2002; Thiex and Manson, 2002; Thiex et al., 2002
Aromatic amines	Pacheco et al., 2011
Atmospheric nitrogen depositon (wet, dry, and bulk deposition)	González-Benítez et al., 2009
Austenitic stainless steel	Ahmed et al., 2011
Barley, malt, beer	Buckee, 1994
Bean, soybean products	Cheng et al., 2008; Hartwig and Hurburgh, 1991; Jung et al., 2003; Korn et al., 2005; Schaalje and Mundel, 1991; Sessa, 2003;
Biological materials	Fleck and Munro, 1965; Krotz et al., 2008; Lang, 1958; Lange et al., 1979a, 1979b, 1979c, 1980a, 1980b, 1981, 1983a, 1983b; Quintela et al., 2009; Takatsu et al., 2008;
Bound protein phosphate	Dusek et al., 2003
Broiler litter	Kpombrekou, 2006
Cereals, oilseeds	Beljkaš et al., 2010; Bicsak, 1993; Daun and deClercq, 1994; Horino et al., 1992; Krotz et al., 2008
Cianobacteria	Ci, 2010
Coal	Markuszewski, 1986; Norton et al., 1987
Farmyard manure	Kemsley et al., 2001
Fish meal	Kubota et al., 2011; Martone et al., 1980; Miller et al., 2007; Oehlenschläger, 1997;
Foods and food products	Bellomonte et al., 1987; Chemat, 1998; Rossi et al., 2004; Simmone et al., 1997; Suard et al., 1993; Thompson et al., 2002;
Forages harvested from permanent meadows	Valdés et al., 2006
Forest streams and rainwater	Holcombe et al., 1986
Geological samples	Pontes et al., 2009, 2010
Grain proteins	Iverson, 1986
Leaf tissue	Ali and Lovat, 1995
Macaroni products	Hakoda et al., 2009, 2011
Manure samples	Pan et al., 2009
Meat, meat products	Benedict, 1987; Hakoda et al., 2010; McGill, 1980; Suhre et al., 1982; Thompson et al., 2004;
Milk, cheese, dairy products	Amancharla and Metzger, 2010; Ardo and Gripon, 1995; Botaro et al., 2011; Doultani et al., 2003; Inácio et al. 2011; Kamizake et al., 2003; Katsiari et al., 2000, 2001; Lynch and Barbano, 1999; Lynch et al., 1998, 2002; Macedo et al., 1997; O'Sullivan et al., 1999; Rohm et al., 1996; Wiles et al., 1998; Zhao et al., 2010
Nuclear fuels	Rizvi and Natrajan, 1986; Searle, 1984
Oil shale retort waters	Jones and Daughton, 1985
Pharmaceutical products	Chemat et al., 1998
Plant materials	Baethgen and Alley, 1989; Bremmer and Breitenbeck, 1983; Conklin-Brittain et al., 1999; Khanizadeh et al., 1995
Pulp mill effluents	Cook and Frum, 2004
Red wine	Smith et al., 2011
Seawater	Doval et al., 1997
Sewage sludge	Galvez-Sola et al., 2009

(Continued on next page)

TABLE 2
Selected application of Kjeldahl methods (*Continued*)

Samples	Reference
Soils	Baethgen and Alley, 1989; Bremmer, 1960; Bremmer and Tabatabai, 1972; Buondonno et al., 1995; Craft et al., 1991; Hood-Nowotny et al., 2010; Jarquín-Sánchez et al., 2011; Kpombrekou and Genus, 2012; Mason et al., 1999; Moraghan et al., 1983; Pereira et al., 2006; Skjemstad and Reeve, 1976; Sobiecka et al., 2007; Stark and Hart, 1996; Wickstrøm et al., 2004
Soy sauce	Nozawa et al., 2005, 2006, 2007
Tantalum alloys	Davis et al., 1971
Taurine	Quintela et al., 2009
Tripolyphosphate in meat	Jastrzebska, 2006
Vinegar	Hashimoto et al., 2009
Waters: waste and environmental water	Anderson and Möller, 1995; Bowman and Delfino, 1982; Campins-Falco et al., 2008; Chemat et al., 1998; Lo et al., 2005; Maher et al., 2002; Meseguer-Lloret et al., 2006; Morgan et al., 1957; Pehlivanoglu-Mantas and Sedlak, 2006; Ramon et al., 2005; Sharma and Gupta, 2004; Worsfold et al., 2008

hydroxide solution; the need for separate absorption of carbon dioxide is thus eliminated (Belcher and Godbert, 1945; Sherman, 1912). Any oxides of nitrogen that may be formed are reduced to nitrogen by means of a glowing copper spiral (after first activating the copper by heating in a stream of hydrogen). In the Dumas method it was very difficult to fill the combustion tube and then transfer the combusted gases over mercury into

TABLE 3
Reviews concerning Kjeldahl method

Keblor, 1891	Neill, 1962
Dyer, 1895	Ashraf and Shah, 1963
Hepburn, 1908	Bradstreet, 1965
Friedrich et al., 1933	Fleck and Munro, 1965
Oesper, 1934	Jacobs, 1965
Schuetz and Oppen, 1935	Fleck, 1970
Bradstreet, 1940	Fleck and Davidson, 1974
Drevon and Roussin, 1940	Lakin, 1978
Clark, 1941	Morries, 1983
Frankignoulle, 1942	Gaspar, 1984
Vickery, 1946a	Jones, 1987
Vickery, 1946b	Scarf, 1988
Seebold, 1947	Jones, 1991
Veibel, 1949	Kuzyk et al., 1995
Kirk, 1950	Horneck and Miller, 1998
Storvick, 1950	Conklin-Brittain, 1999
Middleton and Stuckey, 1951	Lynch and Barbano, 1999
Fontana, 1953	Owuso-Apenten, 2002
Hadorn et al., 1953	Amin and Flowers, 2004
Bradstreet, 1954	Möller, 2005
Eisses, 1960	Moore et al., 2010

a vessel to measure their volume. Many workers indicated the difficulties involved with this method later, for example, Dennstedt, who wrote, "The nitrogen determination was a punishment for everybody, until azotometers were introduced" (Szabadváry, 1992, p. 296). Remember Liebig's statement (1831, p. 10) concerning the nitrogen determination method: "Ich glaube nur, dass sie unter den schlechten die am wenigsten schlechte ist" [I just believe that it is among the poor choices, the least bad], which stresses the difficulties associated with nitrogen determination. The history of the convenient azotometer of Schiff (1868) traces its course from the inverted bell jars of Lavoisier and of Gay Lussac and Thénard, through the graduated cylinders of Liebig and Dumas (Schuette and Oppen, 1935). The Liebig publication of 1831 in which he introduces a new apparatus for the analysis of organic compounds (*kaliapparat*) is seen as a milestone in the development of chemistry (Usselman et al., 2005).

Historically, the original Dumas combustion method has proven to be time-consuming and laborious, with incomplete combustion giving nitrogen oxides and air contamination (Krotz et al., 2008). Numerous recent technical breakthroughs have significantly improved the method's accuracy, and the adoption of several automated features has made the method easy to use (Jung et al., 2003), as shown in the second part of this series.

The inaccuracies and difficulties encountered in the nitrogen combustion determination method turned interest towards the possibility of using a wet method of analysis. When certain organic nitrogen-containing substances were ignited, either alone or in the presence of other substances such as alkalis, the nitrogen was split off in the form of ammonia. Ammonia determination thus gives the nitrogen content.

Before the introduction of the Kjeldahl process, the soda lime method of Will and Varrentrap was commonly used,

almost displacing the Dumas method entirely. In a letter to Gay Lussac in 1831, Dumas mentioned that heating certain compounds with alkali evolves ammonia, suggesting that this gas be volumetrically determined, although no studies in this direction were undertaken (Szabadváry, 1992). A similar suggestion was made later, in October 1840, by Whöler to Liebig: the nitrogen might be determined accurately by heating an organic compound with slaked lime to form ammonia, which could be adsorbed in an acid of known strength. Liebig appears to have passed this information to his assistant, Henrich Will (Brock, 1997), who with the student Franz Varrentrapp perfected and published this technique (Varrentrapp and Will, 1841). The finely ground substance was heated to redness in a combustion tube with a large excess of barium hydroxide and the evolved ammonia was trapped in hydrochloric acid. The ammonium chloride formed was precipitated in excess platinum chloride as chloroplatinate and weighed. Liebig commented this on this method: "I do not doubt that it will replace very promptly and to the satisfaction of all analyses, the processes employed at the present time" (A.G.V., 1842, p. 25). John Lawrence Smith, Liebig's first American pupil (Van Klooster, 1956), arrived at Giessen in the summer of 1841. In November of that year, Smith sent to his country from Giessen a translation of Will and Varrentrapp's method, accompanying it with notes of his own, and once he returned to the U.S. he published a notice of M. Reizet's criticisms (Reizet, 1843) upon the limits of accuracy of this method (Smith, 1843). However, results obtained by the Will and Varrentrapp's method were usually somewhat low, mainly due to the air present in the tube (Prehn and Hornberger, 1880), and were obviated to a great extent by special manipulation.

The Will-Varrentrapp procedure was a refinement of an earlier method for organic nitrogen determination (Ihde, 1964) developed by the French industrial chemist Anselme Payen (1795–1801). The method was presented as an alternative to the laborious and experimentally difficult Dumas combustion process. However, the gravimetric finish was rather tedious, which limited the widespread adoption of the method in the more applied areas of analysis. For this reason, Eugene Péligot modified this process by using a known amount of standard acid and then titrating the excess of this acid (Péligot, 1847). The combustion tube was replaced later with a flask and lime water (soda lime) used instead of barium hydroxide. In order to insure the reduction of nitro-compounds or nitrates, this method was modified by the introduction of stannous sulfide, sodium formate and sodium thiosulfate, or sodium thiosulfate and a mixture of equal parts of sulfur and powdered sugar or charcoal (Sherman, 1912). Goldberg, Arnold, and Ruffle proposed these modifications, respectively, as indicated by Sherman (1912).

The method is too complicated for use in serial determinations, and it was stated later that was not quite as accurate as had previously been imagined, as part of the nitrogen is not converted to ammonia but is lost in the form of nitrogen. As long ago as 1860 Mulder called attention to the danger of loss by formation of volatile products that, escaping ammonifica-

tion within the tube, were either not caught by the acid or, if retained, escaped determination by either titration with alkali or precipitation by platinic chloride (Atwater, 1888–1889). A series of papers was published by Atwater concerning sources of error in the determination of nitrogen in protein compounds (especially those of animal origin) by soda lime and means for avoiding them (Atwater, 1888–1889; Atwater and Ball, 1888; Atwater and Woods, 1887–1888). Atwater studied under the chemist Samuel W. Johnson, a Leipzig graduate and America's leading authority on agricultural chemistry, and spent two years at Leipzig and Berlin (Rosenberg, 1970). In particular, there is a danger of nitrogen loss if there is not sufficient contact between the gaseous distillation products and the heated soda lime; the tube should be closely packed with soda lime to avoid open spaces. The method of Dumas and Will and Varrentrapp required that the compound be in a fine state of division (pulverization and intimate mixture of substance with soda lime); solutions had to be evaporated to dryness and the residue finely divided. Both of these methods were extensively modified, but both required the services of trained and expert chemists, and, although highly accurate, they were slow and extremely inconvenient (Vickery, 1946a).

Meulen (1924, 1925) devised a modification of the old soda lime process with some real improvement: a catalytic hydrogenation at 350°C with reduced nickel being carried out. He obtained very good results with this method in the analysis of several kinds of flour, coal, oil, cake, casein, gelatin, glue, and other materials, and stated that the analysis required only a few minutes. In general, higher results are obtained than by the Kjeldahl method. Colorimetry may also be applied as analytical finish for the microdetermination of nitrogen in the Will-Varrentrapp method (Remi and Pitiot, 1951).

At the University of Edinburgh, Wanklyn improved the Will-Varrentrapp method for estimation organic nitrogen as ammonia by adding potassium permanganate to increase the oxidative effect of soda lime (Brock, 1976). Organic nitrogenous compounds evolve ammonia when submitted to the action of potassium permanganate in strongly alkaline solutions (Wanklyn and Chapman, 1868; Wanklyn and Gamgee, 1868; Wanklyn et al., 1867). The ammonia is retained in the distillate and determined with Nessler's reagent. At that time, the Wanklyn method filled a great need, and within its limitations performed satisfactorily. The method, though severely criticized by Edward Frankland (Stephen, 1984), was claimed to give a measure of the albuminoid (protein) nitrogen and was useful for the rapid assessment of water quality in domestic water supplies (Wanklyn and Chapman, 1874). "The extreme minuteness of the quantities of substance subjected to quantitative determination is one of the most striking features of the method" (Wanklyn and Chapman, 1868, p. 501); it was convenient to operate on very small quantities of organic matter. Nevertheless, the yield of nitrogen generation was incomplete. The method, however, was scarcely used outside of England (Kjeldahl, 1883b). Wanklyn, a controversial figure (Editor of *The Analyst*, 1876), was a founder of

the Society of Public Analysis in 1874 (Chirnside and Hamence, 1974) and gained, according to Liebig, a European reputation as researcher, but was ignored and despised by British academic chemists (Brock, 1976).

The technique of effecting decomposition of an organic substance with a sulfuric acid and catalyst mixture appears to have been first employed by Berzelius (1837); i.e., the decomposition products from various animal and vegetable substances after digestion with a sulfuric acid-lead peroxide mixture were investigated, but focusing on the products of distillation from acid solution. Sulfuric acid digestion, followed by precipitation of the resulting ammonia as the chloroplatinate, was first applied in 1845 as a method for determination of urea (Heintz, 1845; Ragsky, 1845, 1846), which presages, in a certain sense, the later researches of Folin and colleagues (Schuette and Oppen, 1935), inasmuch as it was applied to the examination of urine and employed no catalyst. Later, Grete (1878) and Dreyfus (1883) gave their samples a preliminary treatment with concentrated sulfuric acid and subsequently treated the charred residue according to the Will-Varrentrapp method; the decomposition of hair, wool, leather, and similar substances could be expedited in this way.

However, Wanklyn's contribution is by no means unimportant, because it served to prepare the road for Kjeldahl's own contribution. If digestion with alkali and permanganate led only to partial success, it was not unreasonable to try an acid and permanganate treatment. In 1895 Dyer stated that "Kjeldahl's method for the determination of nitrogen has, in some one or another of its modifications, superseded the well-known soda lime process of Varrentrapp and Will in most agricultural laboratories, both in England and on the continent, and to a very large extent in America" (Dyer, 1895, p. 811).

Alkaline media have found a place again in the sample preparation field (Sharifi et al., 2009). The employment of an alkaline medium is a good alternative for some analytical tasks, as shown recently by Nobrega and colleagues (2006), and provides fast and simple preparation approaches.

THE KJELDAHL METHOD'S THREE STEPS

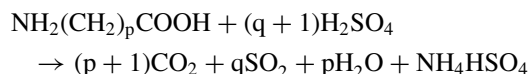
Although some analytical techniques allow the direct determination of species without sample treatment, it is usually necessary to dissolve the sample prior to analysis (Chemat et al., 1998; Lagha et al., 1999; Namiesnik and Szefer, 2008). The analytical adage "once the problem is dissolved, the problem is solved" clearly denotes this fact. Most analyses are preferentially performed on solution samples, a more homogeneous analysis sample representative of the bulk properties of a large solid sample being thus obtained, improving precision and accuracy (Sneddon et al., 2006). Digestion may be defined as the process in which a complex substance is decomposed into volatile gases and simple salts that are soluble in dilute acid solution (Brayton, 1992). Wet decomposition or acid digestion involves the use of mineral acid, alone or in combination with other acids and oxidizing agents, to effect the dissolution of a sample (Flores et al., 2007). Sample digestion still remains a

limiting factor in food control. It is time-consuming and widely based on empirical knowledge (Zorgati et al., 2000).

The quantitative conversion of organic nitrogen to ammonia was an obvious goal for chemists to achieve (Stephen, 1984). To study a subject, as claimed by H. C. Bolton, the first requirement is knowledge of the literature of that subject (Bolton, 2004). Kjeldahl (1883b) first used Wanklyn's method on plant tissues by working with greater amounts so that the ammonia evolved could be easily determined by ordinary methods, but found the yield of nitrogen to be incomplete. Kjeldahl obtained better results by boiling the sample with potassium permanganate and dilute sulfuric acid, making alkaline with a fixed alkali, distilling into standard acid, and titrating the excess of acid. Nevertheless, the production of ammonia was still incomplete. Finally, he heated the substance whose nitrogen content was to be determined with concentrated sulfuric acid and then completely oxidized it with permanganate. Kjeldahl said: "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'hypermanganate de potasse sec en poudre" [The principle of the new method, therefore, is to heat the substance in question for some time with a sufficient quantity of concentrated sulfuric acid, and then to oxidize the solution thus obtained with an excess of dry, powdered permanganate] (Egli, 2008; Kjeldahl, 1883b, p. 3).

The Kjeldahl method consists of three steps (Ashraf and Shah, 1963; Kjeldahl, 1883b, 1883d; Krotz et al., 2008; McKenzie, 1994; McKenzie and Wallace, 1954; Owusu-Apenten, 2002):

- (1) Wet acidic (conc. H_2SO_4) digestion (mineralization) of nitrogen-containing sample, in a long-necked digestion flask, causing its conversion into NH_4^+ ions. The Kjeldahl flask, which he constructed in 1888 to simplify the method, is still in use today (Kjeldahl, 1888a; Szabadváry, 1973). Traditionally, Kjeldahl flasks with a capacity of 500–800 mL and gas or electric heating have been used for the digestion. The reaction involved can be illustrated by reference to (Owusu-Apenten, 2002)



- The digest must contain residual H_2SO_4 to retain the NH_3 as NH_4^+ . Water is added manually or automatically to prevent the digest from solidifying, which also may cause bumping (APHA, 1998, 2012), and to avoid mixing concentrated alkali with concentrated acid during the distillation (Thiex et al., 2002). The initial digestion procedure, as the most important and complex step, is covered in several commercial sources (Egli, 2008; Labconco, 1998; Persson et al., 2008).
- (2) Transformation of NH_4^+ ions into NH_3 (neutralization with alkali) and distillation of the NH_3 . The flask is heated after

the addition of water and alkali to the digested sample in order to distill a volume of distillate (variable depending of the conditions of study) and collect NH_3 in the acidic distillation receiver. To carry out the distillation in a proper way, the digestion vessel should not be over one-third filled (Feinberg et al., 1993). Direct steam distillation drastically decreases the time needed for distillation (Thiex et al., 2002). Experimental conditions including rate of steam flow, vigor of distillation, and volume of solution must be correctly balanced to ensure efficient removal of ammonia without any carryover of alkaline spray (Doolan and Belcher, 1978).

- (3) Titration of the solution from the distillation receiver. The ammonia from the distillation is frequently collected in an excess of standard acid and determined by back titration with standard alkali solution. A more common practice is the use of boric acid for trapping ammonia. The titration in this case should be carried out as soon as possible after the distillation is complete, ensuring that the temperature of the distillate does not exceed 25°C . Under these conditions, losses of ammonia are avoided (ISO, 2009a). The reference methods for ammonium determination include other wet techniques such as colorimetry and ammonia-selective electrode (Sáez-Plaza et al., submitted).

Whereas the latter two steps indicated above are finished after only 15 minutes, digestion take at least 20–30 minutes up to several hours (Chemat et al., 1998), depending on the sample. Digestion is complete when the mixture turns char (light green color). A further (after boiling) period of heating is necessary to ensure quantitative recovery of nitrogen (Amin and Flowers, 2004; Belcher, 1976). Jones and colleagues (1991) reported that as much as 10% of the organic N may not yet be converted to NH_4^+ at clearing. Experience shows that the decomposition is most efficient when other means of preventing partial discharge of hot acid are used, such as the extension of the neck flask (Analytical Methods Committee, 1960). Factors in the digestion process have been controlled or modified by individual researchers (Jacobs, 1964; Middleton and Stuckey, 1951). Steam distillation, which may be carried out by methods ranging from macroscale (10–30 mg) to ultramicro scale ($4 \mu\text{g}$), is the most popular procedure (Fleck and Munro, 1965). Automation of the distillation and titration stages works well, but automation of the digestion has proved quite difficult. The ability to automate the digestion process provides increased safety as well as improved reproducibility. The micro methods can be automated at several levels (Chang, 1998; Egan et al., 1987). Procedures for the micro-determination of Kjeldahl nitrogen require specialized equipment and involved techniques. Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) were established by AOAC in methods 976.06, 976.05, and 960.52, respectively.

The distillation of ammonia by the Kjeldahl procedure is time-consuming because constant attention is needed. Conway (1957) developed a microdiffusion technique for determining

the nitrogen content of flour. This technique involves the release of ammonia from a Kjeldahl digested sample in a sealed container. The ammonia diffuses into a trapping solution and is titrated. Ammonia may thus be retrieved from complex mixtures and subsequently measured by several methods of varying specificity and sensitivity (Friedl, 1972). Numerous samples may be prepared routinely for simultaneous microdiffusion, and supervision is not required during the diffusion process (Lin and Randolph, 1978).

Large amounts of salts or solids may cause bumping during distillation. If this occurs, more dilution water should be added after digestion. The acid remaining in the test tube is not really critical from an environmental point of view (Persson et al., 2008) because it is converted to sodium sulfate by the alkali added during distillation. The major concern is the amount of acid that evaporated into the environment. When many sample digestions are in progress at the same time is advisable to trap the fumes, dilute them with water, and dispose of them down drains rather into the atmosphere (Analytical Methods Committee, 1960).

A series of factors such as sample origin, homogeneity, stability, laboratory skillfulness, sample handling procedures, and composition are of critical importance, as are the size of the test portion taken for analysis and concentration of the titrant used in the Kjeldahl analysis (Lynch and Barbano, 1999). Definite specifications by which the method is to be used may be outlined by industries or associations representing industries in order to assure comparative results between various laboratories. Some difficulties arise from the available sample size and a wide variability of nitrogen content. A relatively large analytical sample (1–2 g) was used in the original method, requiring large amounts of acid. Micro Kjeldahl methods are much more commonly used as they produce a reduced amount of acid fumes and also require less acid and catalyst mixture. Environmental considerations exert considerable pressure to ensure the safe disposal of mercury (when used as catalyst) and, especially, to minimize acid usage (Greenfield and Southgate, 2003).

Many laboratories are required to perform Kjeldahl analysis on a wide range of different sample types, and it is not desirable or practical for them to optimize each type of procedure. It is more advisable to have at one's disposal a single robust procedure that can be applied to all types of samples (Persson et al., 2008). However, little work has been published on the development of comprehensive systems that could be used for all classes of samples (Kramme et al., 1973). It is even doubtful if any digestion method is universally applicable (APHA, 1998, 2012; Kirk, 1950).

Kjeldahl originally directed that the substance be heated with sulfuric acid, with or without the addition of phosphoric anhydride, until a clear solution is obtained; then potassium permanganate is added in small portions to the hot solution until it remains permanently colored, the permanganate being added very cautiously (Kjeldahl, 1883b, 1883d; Sherman, 1912) because of the danger of a loss of nitrogen if the reaction

becomes too vigorous. Kjeldahl employed small special long-necked flasks (about 100 mL) for the treatment with acid. The addition of a little granulated zinc prevented bumping during distillation. The ammonia could be determined by direct titration of the excess of acid, or, as Kjeldahl himself preferred, inasmuch as the titration could be carried out by artificial light, by an iodometric method (Kjeldahl, 1883b, 1888b).

The necessity of carrying out blank determinations was emphasized by Kjeldahl, given the possible impurities in the available reagents, especially sulfuric acid being a sugar blank digested in this respect. The data that were presented in support of the method were remarkable (Vickery, 1946a), 23 aliphatic and aromatic compounds, as well as naturally occurring bodies. A comparison of a group of proteins and protein-containing materials analyzed by both the new Kjeldahl method and the Will and Warrenttrapp method, showed complete agreement. Low results were obtained only with alkaloids, such as morphine and quinine, and cyanogen and nitro compounds.

The Kjeldahl method has many reported advantages over the soda lime process, not being prone to errors arising from variations of temperature in combustion, and obviating the necessity for reducing the substance to a fine state of division (Dyer, 1895). The greatest advantage of the new method was the speed with which results could be obtained. Kjeldahl was able to carry out as many as 14 determinations a day, without assistance. This was a remarkable achievement compared to the Vill-Varrenttrapp method, which had a normal output of 4 determinations in a day.

The method rapidly gained acceptance because it overcame many of the limitations—notably, accuracy, simplicity, and speed—of nitrogen determination by combustion analysis (Dyer, 1895; Rhee, 2001; Szabadváry, 1992). Since its introduction, the Kjeldahl method has been the most widely accepted method for total protein determinations in feeds, foods, and food ingredients. It first appeared as an official Association of Official Agricultural Chemists (AOAC) method in 1887 for milk protein determination (Richardson, 1887). Note that AOAC was formed in 1884 to adopt uniform method of analysis for fertilizers, but in 1887 it also published a method of analysis for feeds and dairy products (AOAC, n.d.).

The Kjeldahl method has been the subject of many modifications and improvements; some of them are summarized in Table 4. The Kjeldahl procedure and most modifications for determining total nitrogen in soil and plants published prior to 1950 specified the use of macro Kjeldahl flasks (350–800 mL) for digestion and distillation (Amin and Flowers, 2004). The introduction of aluminum blocks for Kjeldahl digestion in the early 1970s is a most noteworthy development (Bremner and Breitenbeck, 1983; Douglas et al., 1980; Stumpe et al., 1985; Wang et al., 2004). The first block digestors were developed by Roger Mossberg at Tecator, now FOSS (Thiex et al., 2002). The block digestors made it possible to improve the speed and accuracy of the digestion procedure, as well as save space, chemicals, and energy (Persson et al., 2008). The digestion system has since

been improved by the addition of exhaust systems, heat shields, reagent handling systems, tube racks, etc. (Wang et al., 2004; Wu et al., 2008). By replacing classical Kjeldahl digestions systems with aluminum block digestors and efficient exhaust heat, most of the environmental issues related to the Kjeldahl method can be minimized.

A simple and efficient steam distillation apparatus is appropriate for micro Kjeldahl analyses (Markham, 1942). The distillation used to be tedious but has been much speeded up by the use of the semiautomated systems now available (Barlow et al., 1981; Watson and Galliher, 2001). Wide ranges of block digestion methodologies take less time than classical macro Kjeldahl digestions, and bench-top steam distillation units in use are faster than classical distillation (Labconco, 1993; Persson et al., 2008). For these reasons, block digestions followed by steam distillation are called rapid Kjeldahl. As a result of technical innovations, also available are fully automated protein analysis systems that are based on the classical Kjeldahl procedure (Rhee, 2001). For example, FOSS Tecator manufactures a wide range of Kjeldahl equipment in the Kjeltec series (FOSS, 2010). In the attempt to reduce the costs involved in large numbers of Kjeldahl determinations there have been three main approaches: continuous flow digestors, block digestors, and discrete digestion in flasks with conventional heating and automated stepwise movement (McKenzie, 1994).

THE PERMANGANATE DILEMMA: TO USE OR NOT TO USE

Chemical oxidants (hydrogen peroxide, perchloric acid, persulfates, chromic acid) can be added to the sulfuric acid to achieve acceleration and completion of the decomposition step (Bradstreet, 1965; Gaspar, 1984), which additionally minimizes foaming. However, there appears to be little advantage in the use of other acids or oxidants in conjunction with sulfuric acid for Kjeldahl digestion. Phosphoric acid has the disadvantage of etching the digestion flask (Hiller et al., 1948), while perchloric acid is an extremely hazardous strong oxidizing agent. Sulfuric/phosphoric acid mixtures have been used in semimicro Kjeldahl procedures (Honda 1962; Skjemstad and Reeve 1976). Tsap et al. (1969) and Batey et al. (1974) advocated the use of an $\text{H}_2\text{SO}_4\text{-HClO}_4$ mixture for Kjeldahl analysis of agrochemical and plant materials, although the procedure has not been widely adopted. Kruger (1894) introduced the use of potassium dichromate in the Kjeldahl method, though this method also seems not to have been widely adopted. Several other oxidizing agents (V_2O_5 , CrO_3 , MnO_2) have been used, but they did not appear to have any beneficial effect and were even detrimental when added in large amounts.

Kjeldahl's original method added potassium permanganate to complete oxidation of the sample (Kjeldahl, 1883b, 1883d). The use of permanganate, however, has been the subject of much controversy and cannot be recommended. It has a tendency to cause low results, probably by oxidizing the ammonia (Dyer,

TABLE 4
Some milestones in the Kjeldahl method

Year	Comment	Reference
1883	Original Kjeldahl paper	Kjeldahl, 1883b
1885	Application to such compounds as nitrobenzol, anilins, xylydins	Dafert, 1885
	Metal oxides as catalysts of the oxidation. Mercuric oxide was clearly superior to the others	Wilfarth, 1885a, 1885b
	Both copper and mercury as catalysts; phosphorous pentoxide	Arnold, 1885
1886	Sucrose added to the digestion as organic accelerator in some cases and benzoic acids in others	Von Asboth, 1886
	Phenol and zinc dust: modifies Kjeldahl's method for estimating nitrogen in nitrates	Jodlbauer, 1886
1887	First appeared as an official AOAC method for milk protein determination	Richardson, 1987
1888	Describes a bulb tube for distillation in which the vapors are passed through boiling water before reaching the condenser	Kjeldahl, 1888a
1889	Nesslerization of the distillate from a Kjeldahl determination was advocated as a means to estimate the nitrogen present	Drown and Martin, 1889
	The use of potassium sulfate is first proposed	Gunning, 1889
1892	The combination of the Gunning and Arnold methods shorten the time necessary for complete digestion	Arnold and Wedemeyer, 1892
1895	Kjeldahl-Gunning-Arnold-Jodlbauer process	Dyer, 1895
1913	The use of boric acid as an adsorbent is introduced	Winkler, 1913
1920	Hypohalite for the determination of ammonia to the Kjeldahl process applied for the first time	Willard and Cake, 1920
1930	Review of the early work on micro-determination of nitrogen by Kjeldahl method; gives detailed directions for the analytical procedure	Pregl, 1930
1931	Selenium is proposed as catalyst	Lauro, 1931
1933	Extension of the general application of the process by employing a preliminary reduction with hydriodic acid	Friedrich, 1933
1935	Study of the effects of metal oxide catalysts	Osborn and Wilkie, 1935
1960	Automating the Kjeldahl nitrogen procedure	Ferrari, 1960
1970	FOSS introduced Tecator™ block digestion to replace Bunsen burners and 800 mL Kjeldahl tubes for digestion; developed by Roger Mossberg	Möller, 2009; Persson et al., 2008; Thiex et al., 2002
1974	Direct steam distillation was introduced with the FOSS Tecator Kjeltac™ system	Möller, 2009; Persson et al., 2008
1980s	NIR/FT-IR methods for crude protein	Osborne and Fearn, 1983
1980	Semiautomated systems are subjected to collaborative study using fish meal	Bjarno, 1980
1982	The work of McGill validates the Kjel-Foss instrument for meat product analysis	McGill, 1980; Suhre et al., 1982
1990s	At the end of the 1990s FOSS developed a new standard method in cooperation with the AOAC using block digestion with copper sulfate as catalyst	Möller, 2009
2009	The latest generation of modern Kjeltec analyzers is introduced	Kjeldahl Analysis Goes Online, 2009

1895), unless the quantity used is very moderate. In the early days of Kjeldahl's method it represented an important advance, and, at least historically (Bradstreet, 1965), deserves mention. The addition of solid salt in the final oxidation instead of use of a saturated solution was recommended by Czczetka (1885). Care must be taken to avoid explosive violence. The time elapsing between cessation of boiling and the addition of permanganate influence the results. As Cochrane (1920, p. 1195) said: "There is a wide difference of opinion among analysts as to proper

interpretation of 'immediately' as used by Kjeldahl in this original description of the method."

Collaborative tests on the use of permanganate in the modified method for nitrates showed variable results (Phelps, 1920). It has been pointed out that if the permanganate is added before the digestion is completed, low results will be obtained (Beet, 1934). A semimicro method for nitrogen in coal and other carbonaceous materials has been described (Beet, 1954, 1955a). For a wide range of substances (cereals, feeding stuffs, coals,

alkaloids) potassium permanganate added gradually to the hot acid digest has several advantages over the usual catalyst employed in the Kjeldahl method on both the semimicro and microscale (Beet, 1955b). This permanganate digestion technique has proved to be satisfactory for routine analysis of coffee-leaf material (Robinson, 1956). As Belcher (1976, p. 156) has said: "It is of interest that the late A. E. Beet, who spent most of his life studying this process, after examining many catalysts eventually concluded that the best results were obtained by adding permanganate. Unfortunately, he had never read the original Kjeldahl paper and it was only after he had introduced the permanganate method that he discovered that this had been Kjeldahl's original recommendation."

Very low recovery of $\text{NH}_4^+\text{-N}$ (attributed to the formation of NCl_3) was observed in total N determination of $(\text{NH}_4)_2\text{SO}_4$ in KCl solutions by a semimicro Kjeldahl method using permanganate and reduced iron to recover NO_3^- and NO_2^- (Mulvaney et al., 1992).

ACID REQUERIMENTS AND SALT ADDITION: THE BLACK JACK GAME

Hot, concentrated sulfuric acid acts as a weak oxidizing agent. The carbon of organic matter should be oxidized without oxidizing the liberated ammonia to gaseous nitrogen. A fine balance between conditions for oxidation and reduction is needed (Fleck and Munro, 1965), and the oxidation-reduction range, within which nitrogen is reduced to ammonia, is a narrow one. This may explain the loss of nitrogen that sometimes occurs when oxidizing groups or halogens are present in a compound. Charring generally takes place in the hot acid, and the resulting carbon acts as a reducing agent. Oxidized forms of nitrogen are only partially reduced under these conditions, and are usually subjected to pretreatment with a suitable reducing agent. Another factor to take into account is the temperature, which at all times should be high enough to induce and ensure pyrolytic decomposition of the sample (Bradstreet, 1957, 1965; Lei, 2003; Morita, 1968).

The boiling rate and the total digestion time determine the loss of acid during digestion and the boiling period. Self (1912) and Carpiaux (1912) reported losses of nitrogen when the final digest was solid, and Self (1912) noted one earlier reference from the *Transactions of the Guinness Laboratory* of 1903 concerning the loss of nitrogen at higher temperatures (McKenzie, 1994). Potassium sulfate was used to elevate the boiling point of sulfuric acid and to increase the oxidizing power of the digestion mixture (Table 4) as early as 1889, having proved to be the most efficient salt to use because of its high solubility in sulfuric acid. Yoshikuni (1992a, 1992b, 1994) employed lithium sulfate and silver sulfate. A high boiling point reduces sample digestion time. The maximum amount of potassium sulfate that can be used without solidification of the digestion mass on cooling is 10 g per 25 mL of sulfuric acid. Sodium sulfate requires a higher ratio of acid to salt than an equivalent amount of potassium sulfate or potassium sulfate-sodium thiosulfate mixture

(Bradstreet, 1957). The acid and salt content of the Kjeldahl digestion reagent is intended to produce a digestion temperature of about 380°C . If the sample contains a very large quantity of salt or inorganic solids that dissolve during digestion, the temperature may rise above 400°C , at which point pyrolytic loss of nitrogen begins to occur (APHA, 1998, 2012; U. S. Environmental Protection Agency, 2001).

The total amount of acid necessary for the decomposition of the sample will vary depending upon the size of the sample, ease of decomposition, rate of heating, overall time of digestion, and use of salicylic acid or related compounds and length of the boiling period (Amin and Flowers, 2004; Egli, 2008; Labconco, 1998; Persson et al., 2008). The quantity of ammonia formed depends essentially upon the length of the period of digestion; if this is sufficiently long, nearly all nitrogenous bodies will undergo complete conversion of their nitrogen content into ammonia. Data from McKenzie and Wallace show that adding x (mg) of potassium sulfate per mL of sulfuric acid increases its boiling point according to the relation $Y(^{\circ}\text{C}) = 55.8x + 331.2$ (Owusu-Apenten, 2002). Digestion temperature increases from 332°C with no salts to 371°C when potassium sulfate is added at 1 g mL^{-1} sulfuric acid. Typical initial acid/salt ratios range from 1.4 to 2.0. For samples where acid consumption is higher, e.g., samples with high fat content, initial acid salt ratios may be in the range 2.5 to 2.8 (Persson et al., 2008). There are large differences in the boiling period after clearing, which is recommended for various semimicro Kjeldahl procedures (Amin and Flowers, 2004). Selected articles dealing with digestion, salt and temperature are summarized in Table 5.

THE TEMPERATURE/ DIGESTION PARTNER: NOT TOO LITTLE, NO TOO MUCH

High-temperature digestion ensures recovery of nitrogen compounds, which do not decompose at the boiling point of concentrated sulfuric acid, and markedly reduces the time required for complete digestion of samples (Amin and Flowers, 2004; Bradstreet, 1965). The temperature range $360\text{--}410^\circ\text{C}$ is optimal for this method (Fleck and Munro, 1965; McKenzie, 1994). It is possible by appropriate choice of temperature to obtain rapid digestion and full recovery of nitrogen even from refractory materials, e.g., tryptophan, lysine, or nicotinic acid (McKenzie, 1994), even mostly nitro and azocompounds without reductive pretreatment, as claimed by Morita (1968). Temperature must be measured in the actual digestion mixture (not in the digestion heating block). Adequate digestion heaters affording sufficient energy to give vigorous boiling conditions are necessary, especially at higher ratios of $\text{K}_2\text{SO}_4/\text{H}_2\text{SO}_4$. Because of potential problems of radiation and conduction error in thermocouples and problems concerning temperature standards, small glass-sheathed platinum resistance thermometers have been used (McKenzie, 1994). The digestion could be made very rapid by careful choice of catalyst, so that conversion to ammonium bisulfate was speeded up, but the temperatures

TABLE 5
Selected applications concerned with digestion, salt, and temperature

Sample	Comments	Reference
Water	Digestion of water for total Kjeldahl nitrogen analysis with a solution containing copper sulfate, potassium sulfate, and sulfuric acid to accomplish the conversion of organic nitrogen to its inorganic form (NH_4^+)	Roper et al., 2011
Maize, corn protein powder, corn lees	Digestion time is reduced from 1 h to 15 min by using concentrated sulfuric acid and hydrogen peroxide	Wang, 2010
Marine organisms	Results for total nitrogen analysis generated by the Hach method are compared with CHN elemental analysis Digest time of Kjeldahl determination was reduced from 2.5–3 h to 1 h. by using sulfuric acid and hydrogen peroxide. The results obtained are fast, simple, accurate, and exact	Barbarino and Lourenço, 2009 Zhang and Zhang, 2008
Animal foodstuffs	Adding hydrogen peroxide:sulfuric acid 3:2 (volume) can effectively speed up the digestion time in the protein determination of animal foodstuffs	Li et al., 2006
Hay, cabbage powder, Midelney soil	Evaluation of Kjeldahl digestion method using three salt and catalyst mixtures in standard Kjeldahl digestion method and salicylic acid modification	Amin and Flowers, 2004
25 foods with a wide range of protein and moisture content	The N contents by fast method of Hach and the traditional Kjeldahl method are compared allowing for the influence of the variation in structure and macronutrient in the food content	Rossi et al., 2004
Soil extracts	A high-temperature catalytic oxidation method is evaluated and compared with the Kjeldahl method for determining total dissolved nitrogen.	Alavoine and Nicolardot, 2001
Feedstuffs	The Hach et al. (1987) technique was tested on a number of samples that were also analyzed by a more conventional Kjeldahl method and by a Dumas method type analyzer	Figenschou et al., 2000
Soil	A two-step digestion with $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ (peroxy treatment) in the absence of heavy metal catalysts is proposed as alternative to the Cu/Se-catalyzed digestion process for the routine determination of soil Kjeldahl-nitrogen	Buondonno et al., 1995
	Review of evolution of the Kjeldahl method, toxicity problems, current status of automated methods, and microwave digestion	McKenzie, 1994
IH-1,2,4 Triazole, 2,4,6 trimethylbenzene-sulfonyl-triazoline, pyridazine	Quantitative decomposition of heterocyclic ring compounds containing N-N bonds in molten acidic lithium sulfate flux containing a catalyst such as silver sulfate	Yoshikuni, 1994
Nitrogen compounds including pyridines, pyrazolones, 1,2-diazines, and 1,2,3-triazines	A comprehensive Kjeldahl digestion method based on the criteria of oxidation number is formulated, resulting in a satisfactory nitrogen determination of almost all types of organic nitrogen compounds	Shirai and Kawashima, 1993
Pecan, corn, and turnip leaves	Influence of catalyst (containing $\text{K}_2\text{SO}_4/\text{Cu}/\text{Ti}$, $\text{K}_2\text{SO}_4/\text{Se}$, or Se alone), sample weight, and digestion blocks on Kjeldahl-nitrogen determination.	Simmons et al., 1993
Organic compounds and natural materials (residual fuel oil and coal)	Heterocyclic ring compounds were rapidly decomposed in the molten state with mixtures of various ratios of concentrated sulfuric and lithium sulfate flux-containing catalysts	Yoshikuni, 1992a, 1992b

(Continued on next page)

TABLE 5
Selected applications concerned with digestion, salt, and temperature (*Continued*)

Sample	Comments	Reference
Pure di- and tripeptides and aminoacids	H ₂ SO ₄ + H ₂ O ₂ without catalyst or salts	Kaltenborn and Hütter, 1992
Complex fermentation media	Samples are digested in tubes with sodium selenite (in sulfuric-phosphoric acid mixture) plus hydrogen peroxide for one hour at 380°C	Guebel et al., 1991
20 samples	Comparison among the Hach, Kjeltec, and Kjeldahl methods	Watkins et al., 1987
Coal	Modification of the Hach digestion method was effective in the rapid determination of total Kjeldahl N	Norton et al., 1987
Soybean, fish, and feather meals, bovine liver (NBS 1577), orchard leaves (NBS 1571), tryptophan	Rapid micro Kjeldahl digestion using sulfuric acid and hydrogen peroxide as the sole digestion reagents with complete recovery of nitrogen from the refractory compound nicotinic acid	Hach et al., 1987
Grain, cereal, and feed samples	Kjeldahl nitrogen determination by using peroxymonosulfuric acid (Caro's acid) as a strong oxidant for rapid sample decomposition without added salts or metal catalysts, and rapid colorimetric determination by improved Nessler method	Hach et al., 1985
Chickpeas, pigeon peas	Use of H ₂ O ₂ for the digestion and determination of total nitrogen, and comparison with mineralization by using mercury or selenium	Singh et al., 1984
Feedstuffs and biological products	A note on the use of H ₂ O ₂ for the determination of Kjeldahl organic nitrogen	Sricar and Chandru, 1983
	Procedure for the determination of nitrogen on a semimicro scale by Kjeldahl digestion with the use of H ₂ SO ₄ /H ₂ O ₂ without the use of a catalyst	Florence and Milner, 1979
Feedstuffs	Kjeldahl digestion time can be reduced to 10 min after clearing by using a phosphoric-sulfuric acid mixture	Skjemstad and Reeve, 1976
	Hydrogen peroxide without catalyst. Results are in good agreement with a standard method except for fishpaste, and a poor recovery nitrogen for tryptophan was reported	Tomomari et al., 1976
Plants	Hydrogen peroxide method and Kjeldahl digestion method. The treatment resulted in excessive foaming and loss of sample from digestion tubes	Hambelton and Noel, 1975
Many agricultural samples	Automatic analyzer for the measurement of total nitrogen after Dumas combustion method in vacuum. Reproducibility and accuracy compared with those of the Kjeldahl method	Fiedler et al., 1973
Selected forest soils	Estimates of total soil nitrogen by a standard Kjeldahl procedure and a modified procedure employing packets of premixed digestion salts were closely correlated	Blackmon, 1971
	Amount of sulfuric acid consumed versus ammonia produced. The temperature at the initial stage determines the ratio of oxidative action of hot concentrated sulfuric acid to reductive action of sulfur dioxide produced in the system	Morita, 1968
Biological materials, (containing a large excess of carbohydrate)	Effect of temperature on the digestion of a refractory test substance with concentrated sulfuric acid, in the presence and absence of efficient catalysts	Jacobs, 1964
Grasses	Total nitrogen content was determined by the Kjeldahl method and the more rapid H ₂ SO ₄ /H ₂ O ₂ method in its original form and with samples pretreated with salicylic acid and sodium thiosulfate	Ekpete and Cornfield, 1964

(Continued on next page)

TABLE 5
Selected applications concerned with digestion, salt, and temperature (*Continued*)

Sample	Comments	Reference
Nicotinamide, histidine, lysine . . .	Examination of the effects of concentration of potassium sulfate and of 21 single and mixed catalysts on the recovery of nitrogen by the Kjeldahl method	Baker, 1961
Organic matter	Recommended methods for the destruction of organic matter, both wet and dry, with a statement of the type or types of organic material to which each may be applied	Analytical Methods Committee, 1960
Amino acids and proteins	Study of temperature on the rate of Kjeldahl digestions in the absence of catalyst and oxidizing agent. Micro-apparatus for the distillation of ammonia from Kjeldahl digestions. Acidimetric methods for the determination of the ammonia. A review of limitations of existing procedures for the Kjeldahl determination of nitrogen organic compounds, with a general method of digestion in mind	McKenzie and Wallace, 1954 Middleton and Stuckey, 1951

chosen did not cause pyrolytic loss of nitrogen (APHA, 2012; McKenzie, 1994).

Great care must be taken when small volumes of digestion mixture are used, the situation becoming particularly complex for materials such as foods, plants, and fertilizers, where appreciable amounts of fats and carbohydrates are present. Consumption of sulfuric acid in the oxidation may occur to such an extent that there is considerable rise in temperature due to increase in the K_2SO_4/H_2SO_4 ratio. Preliminary oxidation with an auxiliary oxidant, e.g., hydrogen peroxide, may be required to solve this problem, in order to effect rapid oxidation of fats and carbohydrates and thus avoid an excessive consumption of sulfuric acid. Another alternative involves lowering the $K_2SO_4-H_2SO_4$ ratio, with acid being consumed in the preliminary stages thus increasing the $K_2SO_4-H_2SO_4$ ratio in the later stages, with resultant higher temperature.

The temperature may rise above 400°C, as shown above, when the sample contains a very large quantity of salts or inorganic solids that dissolve during digestion. In those cases, more H_2SO_4 acid is added to maintain the acid-salt balance, in order to avoid pyrolytic loss of nitrogen. In the absence of a universally recommended digestion time, its choice relies on the recommendations of some standard methods for Kjeldahl nitrogen (AFNOR, 1982; APHA, 1998; ISO, 1984).

ACCELERATING THE PROCESS/CHANGING THE CATALYST: "LE MIEUX EST L'ENNEMI DU BIEN"

The addition of a neutral substance, such as potassium sulfate, raises the boiling point of the digesting acid and the temperature of the reaction, promoting acceleration, as shown above. Because the initial digestion of the sample is slow, many different catalysts have been proposed to speed up the sample digestion process. A summary of selected articles is compiled in Table 6. In 1885, i.e., only two years after the original method

was published (Table 4), some articles appeared describing catalysts being added to increase the speed and efficiency of the digestion procedure.

Nearly all elements of the periodic table have been tried as catalysts for Kjeldahl digestion. The order of the effectiveness for metal oxide catalyst is $Hg > Se > Te > Mo > Fe > Cu > V > W > Ag$ (Ashraf and Shah, 1963; Bradstreet, 1965; Lakin, 1978; Owusu-Apenten, 2002). Results from many investigations have established mercury as being the most effective catalyst, and it is universally used in routine laboratory work. However, it is very toxic and expensive to use, and consequently much research has been undertaken to find an efficient substitute of low toxicity and cost (Florence and Millner, 1979). Moreover, distillation of ammonia by the sample addition of alkali is incomplete when mercury is used as catalyst, due to the fact that mercury forms a complex (precipitate) with ammonia; sodium sulfide, sodium thiosulfate, or zinc dust must be added then to the digest to decompose the mercury complex. Mercury and salts of mercury are apparently the only commonly used catalysts that require treatment before distillation.

Selenium, first suggested by Lauro (1931), has considerable merit as a catalyst, although it has been the subject of some controversy, generating many reports about erratic results, incomplete recovery, or loss of nitrogen (Ashraf and Shah, 1963; Davis and Wise, 1933; Seebold, 1947; Tingvall, 1978). In addition, selenium is also hazardous to health. A working mixed selenium-mercury catalyst can be formulated with potassium sulfate (32 g), mercuric sulfate (5 g), and selenium powder (1 g). To shorten the time needed for digestion in a Kjeldahl flask, the selenium reagent mixture (Na_2SO_4 , Hg_2SO_4 , $CuSO_4$, Se) is usually applied (Baker, 1961; McKenzie, 1994). Sulfuric acid-Se digestion mixtures have been employed for multi-elemental analysis in a simple plant digest (Sahrawat et al., 2002). The optimum amount of selenium in a power catalyst

TABLE 6
Selected applications involving the use of catalysts

Sample	Comments	Reference
Water	Digestion of water for total Kjeldahl nitrogen analysis with a solution containing copper sulfate, potassium sulfate, and sulfuric acid to accomplish the conversion of organic nitrogen to its inorganic form (NH_4^+)	Roper et al., 2011
Protein	The optimum quantities of catalyst found by a 3×3 orthogonal design were 2.5 g $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.1 g K_2SO_4 , and 4.0 mL sulfuric acid	Wang, 2005
	The use of Se powder as a catalyst for protein determination is advocated and results showed that the method was fast and exact	Lu et al., 2003b
	The optimum quantities of a catalyst for quick determination of protein determined by a single factor analysis were 1.5 g Se powder, 1 g K_2SO_4 , and 10 mL H_2SO_4	Lu et al., 2003a
Plants	Sulfuric acid-Se digestion mixtures for preparing plant materials for manual determination of N, P, K, Ca, and Mg in the same digest	Sahrawat et al., 2002
Soil extracts	A high-temperature catalytic oxidation method evaluated and compared with the Kjeldahl method for determining total dissolved N (to assess soil microbial biomass nitrogen)	Alavoine and Nicolardot, 2001
Plant tissue	Digestion medium of concentrated H_2SO_4 and a mixed catalyst of Na_2SO_4 for macronutrient analysis	Hu and Barker, 1999
Waste and wastewater	1 mL of saturated solution of titanium dioxide was added to the digestant mixture, instead of 2 g of mercuric oxide	Anderson and Möller, 1995
Meat and meat products	Copper catalyst for use in AOAC Method 928.08, Nitrogen in Meat, Kjeldahl Method, was subjected to a collaborative study in which it was compared to the standard Hg catalyst. Nine laboratories performed blind duplicate determinations of the protein content of four samples from each of six products	Price et al., 1994
IH-1,2,4-triazole, 2,4,6-trimethylbenzenesulfonfyltriazolide, and pyridazine	Heterocyclic ring compounds containing nitrogen-nitrogen bonds were completely decomposed in the molten state with mixtures of various ratios of concentrated sulfuric acid and lithium sulfate flux containing a catalyst such as silver sulfate (with quantitative recovery of nitrogen)	Yoshikuni, 1994
Pecan, corn, and turnip leaves	Influence of catalyst (containing $\text{K}_2\text{SO}_4/\text{Cu}/\text{Ti}$, $\text{K}_2\text{SO}_4/\text{Se}$, or Se alone), sample weight, and digestion blocks on Kjeldahl-nitrogen (N) determination	Simmons et al., 1993
Pyrazine, pyrimidine, purine, quinoxaline, quinaxoline, and phthalaxine	Heterocyclic ring compounds are rapidly decomposed in the molten state with mixtures of various ratios of concentrated sulfuric and lithium sulfate flux containing catalysts such as cupric sulfate, elemental selenium powder, selenium dioxide, mercury(II) oxide, and mercuric sulfate, with quantitative recovery	Yoshikuni, 1992a
Organic reagents, residual fuel oil, and coal	Organic compounds and natural materials rapidly decomposed in the melting state with various ratios of a sulfuric acid-lithium sulfate (v/w) mixture containing catalysts, oxidizing agents, or reducing agents such as elemental selenium powder, cupric sulfate, ceric sulfate, and stannous sulfate, with quantitative recovery	Yoshikuni, 1992b
Pyridine derivatives	Three hours of digestion at approximately 320°C using a Pd(II)-Cu(II) catalyst were found to be sufficient for quantitative liberation of ammonia from pyridines	Oishi and Komatsuzaki, 1991

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TABLE 6
Selected applications involving the use of catalysts (*Continued*)

Sample	Comments	Reference
Ecological materials	Copper sulfate as a catalyst in Kjeldahl digestion can be effective when a salt/acid ratio of 1 g/mL is used. Copper sulfate in combination with zirconium sulfate is no more effective than copper sulfate alone	Jones and Bradshaw, 1989
Milk	Copper sulfate was substituted for mercury as the catalysts in the International Dairy Federation (IDF) Standard 20A: 1986 method for the determination of nitrogen content Procedure for the post-reactive selenium elimination from Kjeldahl digests for nitrogen determination by using a reductant with a further step of degradation of its excess to avoid the disturbance of the ammonium ion analysis	Grappin and Horwitz, 1988 Engler et al., 1986
Animal feed	A different catalyst combination, CuSO ₄ -TiO ₂ , makes 40 min digestion feasible	Kane, 1986
Sewage effluents, trade effluents, sewage sludges	Method that uses a block digester and a low-toxicity copper-titanium catalyst with results comparable with those obtained by the widely accepted mercury catalyst method	Croll et al., 1985
Wide variety of cheeses	Copper sulfate successfully replaces mercury compounds as catalysts in the Kjeldahl digestion	Florence et al., 1985
Fertilizers, feeds, foods and beverages, animal feeds	A collaborative study compares the official HgO-catalyzed method and an alternative using CuSO ₄ . Fifty-four samples (blind duplicates) and results returned by 22 laboratories	Kane, 1984
Coal and coke	K ₂ SO ₄ (anhydrous), V ₂ O ₅ , and Se finely ground and mixed in 93:5:2 weight ratio Short digestion method for Kjeldahl nitrogen determination, using a trace of selenium as catalyst is described	Doolan and Belcher, 1978 Marshall and Walker, 1978
Foods, protein concentrates, rat diets, and samples of animal or human origin.	Samples digested in a sulfuric-phosphoric acid mixture in glass tubes in a thermostated aluminum block using selenium as catalyst. The ammonia liberated was determined colorimetrically using an alkaline phenol reagent	Hambraeus et al., 1976
Barley, malt, and beer	TiO ₂ in mixture with cupric sulfate and potassium sulfate in the ratio 0.3:0.3:10	Klopper, 1976
Lysine-HCl, trypto-phan, NBS standards, urea, meals, mixed feed grains, forage	Pollution from the Kjeldahl method for crude protein was reduced by substituting a low level of copper (0.04 g CuSO ₄) for the mercury (0.7 g HgO) specified in the AOAC official method 2.049	Rexroad and Cathey, 1976
Feed, food, and fertilizer samples	A new mixed catalyst (0.60 g cupric sulfate and 0.20 g zirconium dioxide in combination or 0.70 g zirconium dioxide alone) is recommended to replace the 0.70 g or more of mercuric oxide in all official macro Kjeldahl methods	Glowa, 1974
Cereal grains	Use of titanium dioxide as a catalyst in the Kjeldahl determination of total nitrogen, in the place of the efficient but highly toxic mercuric oxide	Williams, 1973
Nicotine acid, benzyli-sothiouronium chloride, and ammonium sulfate	Effects of concentration of potassium sulfate and of 21 single and mixed catalysts on the recovery of nitrogen by the Kjeldahl method	Baker, 1961
Cereals, feeding stuff, coals, alkaloids	Potassium permanganate added gradually to the hot acid digest has several marked advantages over the usual catalysts employed in the Kjeldahl method	Beet, 1955a

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TABLE 6
Selected applications involving the use of catalysts (*Continued*)

Sample	Comments	Reference
	Effect of temperature on the rate of Kjeldahl digestions in the absence of catalyst and oxidizing agent and the use of hydrogen peroxide as an oxidant in Kjeldahl digestions.	McKenzie and Wallace, 1954
Total protein prepared from plasma	Use of a mercury-containing digest in: (1) macroanalyses, (2) microanalyses with distillation and titration of the ammonia, and (3) microanalyses with gasometric determination of the ammonia by hypobromite reaction	Hiller et al., 1948
	A review of the literature on the use of selenium as catalyst in the Kjeldahl method	Seebold, 1948
Flour, wheat, cottonseed meal, cracklings	Use of selenium as catalyst in determination of nitrogen by the Kjeldahl method.	Lauro, 1931

has been determined by single-factor analysis (Lu et al., 2003a, 2003b).

Copper was mentioned as an alternate catalyst for the determination of nitrogen in early editions of the *Official and Tentative Methods of Analysis* (AOAC, 1935), and the use of copper sulfate for the determination of nitrogen content in milk was first proposed almost 75 years ago by Rowland (1938a). Replacement of mercury by copper as catalyst has also been proposed (Florence et al., 1985; Jones and Bradshaw, 1989; Rexroad and Cathey, 1974, 1976). Copper sulfate has been substituted for mercury as the catalyst in the International Dairy Federation (IDF) Standard 20A:1986 method for the determination of nitrogen content in milk (Grappin and Horwitz, 1988). The Kjeldahl method for nitrogen determination in meat and meat products using copper catalyst was adopted as a first action by AOAC International (Price et al., 1994). Mixed $\text{Na}_2\text{SO}_4\text{-CuSO}_4$ catalyst has been employed in plant tissue digestion for macronutrient analysis (Hu and Barker, 1999). Although the copper catalyst exhibited a negative bias when compared to the mercury catalyst, its magnitude is less than the reproducibility standard deviation of the methods. The optimum amounts of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, K_2SO_4 , and H_2SO_4 in a digestion measurement method for protein in samples have been evaluated by means of an orthogonal design (Wang, 2005). The use of a Pd(II)-Cu(II) catalyst allows degrading refractory compounds such as pyridine to ammonia (Oishi and Komatsuzaki, 1991).

Zirconium dioxide was found to possess a satisfactory catalytic potential for the Kjeldahl determination of total nitrogen. The white microcrystalline material is readily soluble in sulfuric acid, nontoxic, and reasonably priced, with a 20:1 cost ratio in favor of zirconium dioxide over mercuric oxide (Glowa, 1974). Repeatability, interlaboratory variation, and variation versus manual Kjeldahl were equal or better with an antimony-based catalyst (0.8 g potassium antimonate hemihydrate, 0.16 g zinc sulfate heptahydrate, 0.02 g cupric sulfate pentahydrate, and

49 g potassium sulfate) than with mercury catalysts (Bjarno, 1980).

The titanium dioxide is incorporated into a catalyst mixture with cupric sulfate and potassium sulfate (Croll et al., 1985; Kane, 1986; Klopper, 1976; Williams, 1973) and well as with copper and selenium (Simmons et al., 1993). The choice of titanium as a catalyst alternative to mercury was shown to be excellent (Anderson and Möller, 1995). Titanium occurs naturally in soils (0.3 to 6%), and titanium dioxide is a brilliant white pigment used in paint, plastics, paper coatings, detergents, and foods. It can be used as either a color stabilizer or a food additive. Titanium dioxide is used quite safely in large quantities in dairy products and bread flour where whiteness is desired; it is exceptionally nontoxic. The U.S. Code of Federal Regulations allows titanium dioxide as a food additive up to 1% by weight.

Catalysts can be purchased as prepared material under the brand names "Kjel-tab," "Kel tab," and "Kelmate" distributed by various chemical suppliers.

REFRACTORY COMPOUNDS AND NITRATE NITROGEN RECOVERY: TAKING EXTREME CONDITIONS

The ease of digestion varies with the nature of the sample, i.e., in some proteins it is more difficult to convert the organic nitrogen to ammonia. Histidine- and tryptophan-rich proteins generally require long or harsh digestion conditions (Pomeranz and Meloun, 2002). The simple procedure of digestion with concentrated sulfuric acid in the presence of a catalyst is applicable to amines, amino acids, amides, and their simple derivatives (Bradstreet, 1965). However, the nitrogen that occurs in organic compounds with N-N and N-O linkages (e.g., azo-, nitro-, and nitroso compounds, hydrazines, hydrazones, oximes, pyrazolones, isooxazoles, and dia- and triazines) is not completely recovered. Aromatic compounds are all difficult to digest; also, removing heterocyclic nitrogen is even more difficult and entails breaking every molecular bond in the compound (Schöniger, 1960; Suard et al., 1997). It would appear very

desirable that when the Kjeldahl method is used for the analysis of cyclic nitrogen derivatives, a suitable control analysis with pure substances should be carried out. Thus, the Kjeldahl procedure will not recover 100% of the N in most samples; nitrate and nitrite are not recovered unless a predigestion procedure is conducted, which reduces NO_3^- to NH_4^+ (Horneck and Miller, 1998); common areas of usage are fertilizers, soil, and water.

The reduction of refractory compounds can be effected in two ways, either by pretreatment before the digestion or during the digestion itself (Amin and Flowers, 2004). Commonly used reducing agents are chromium, zinc, iron sulfate, hydriodic acid, salicylic acid, and sucrose (Dalal et al., 1984; Goyal and Hafez, 1990; Pace et al., 1982; Pruden et al., 1985; Wyland et al., 1994). Satisfactory results can often be obtained by adding pure glucose to the digestion mixture. Sucrose or glucose is also commonly included in digestions of blank samples in order to get an acid consumption similar to the samples. Practically, it is preferable to do it during digestion, but many compounds cannot be reduced by this procedure and must be reduced by other means.

Three modifications of the Kjeldahl method have been used to recover nitrate and nitrite nitrogen in total nitrogen analysis (Amin and Flowers, 2004): (i) the salicylic acid modification introduced by Cope, where the sample is preheated with salicylic acid and sodium thiosulfate; (ii) the alkaline reduction modification of Davison and Parsons in which Devarda's alloy and alkali are used to reduce nitrate and nitrite to ammonia, which is then collected in sulfuric acid and added to the digest; and (iii) the Olsen permanganate-reduced iron modification in which potassium permanganate is used to oxidize nitrite to nitrate-nitrogen, which is then reduced to ammonium by reduced iron.

Nitrogen determination with the Kjeldahl method does not require expensive devices or specialized techniques, being in addition precise and accurate. However, it has been almost ignored as a means of organic elemental analysis. One reason for this may be the difficult degradation of pyridine derivatives to ammonia (Oishi and Komatsuzaki, 1991).

CLEANER DIGESTION: HYDROGEN PEROXIDE

Kleemann (1921), Heuss (1923), and Berman (1924) reported the use of hydrogen peroxide during sample digestion. Hydrogen peroxide has proven to be a rapid and powerful oxidizer of organic matter (Analytical Methods Committee, 1967) and has been used successfully in Kjeldahl digestion to decompose organic samples. Kleemann used addition of hydrogen peroxide to the sample followed by digestion with sulfuric acid and potassium sulfate to obtain quick conversion of nitrogen to ammonia (Kleemann, 1921). Koch and McMeekin (1924) carbonized the sample in sulfuric acid and then added hydrogen peroxide dropwise with heating to obtain rapid oxidation with complete retention of the nitrogen as ammonia. Bradstreet (1940) reported on the use of hydrogen peroxide without a catalyst in the macro-digestion of samples taken from a variety of products. A careful study of peroxide use proved

the importance of pre-carbonization and multiple additions for full nitrogen recovery (Miller and Miller, 1948). Sulfuric acid-hydrogen peroxide methods in their original form as well as in modified forms by pretreatment of samples with salicylic acid and sodium thiosulfate have been applied to ensure complete recovery of nitrate-N (Ektepe and Cornfield, 1964). Hydrogen peroxide assists in clearing the digests (Parkinson and Allen, 1975; Thomas et al., 1967), thereby decreasing digestion times. However, Nelson and Sommers (1973) obtained poor total N recoveries (84%) from plant materials by using sulfuric acid-hydrogen peroxide digestion procedures, without potassium sulfate or catalysts. Hydrogen peroxide treatment resulted in excessive foaming (Hambelton and Noel, 1975) and loss of sample from digestion tubes. Florence and Milner (1979) reported the need to heat the digestive mixture after clarifying for full nitrogen recovery. This procedure is preferable to those employing highly toxic catalysts (Florence and Milner, 1979; Singh et al., 1984).

Hach et al. (1985, 1987) and Hach Company (1997) outlined the peroxy reagent digestion method. The Hach technique utilizes 5 mL of concentrated sulfuric acid to carbonize the sample and then uses continuous-flow addition of 10–15 mL of hydrogen peroxide delivered into the digest via a capillary funnel. This reagent contains peroxymonosulfuric acid (H_2SO_5 , Caro's acid), which is a more powerful oxidant than hydrogen peroxide, requiring careful handling and storage in a vented container (Hach, 1987). It does not seem easy or safe to handle 50% hydrogen peroxide at high temperatures. However, the use of hydrogen peroxide as an oxidizing reagent was reported to greatly shorten the digestion period. Various modifications of the Hach digestion method were shown to be effective in rapid determination by the Kjeldahl method in a variety of samples (Barbarino and Lourenço, 2009; Buondonno et al., 1995; Figenchou et al., 2000; Norton et al., 1987; Rossi et al., 2004; Walkins et al., 1987). Hydrogen peroxide is used in the Digestahl^R apparatus (Brayton, 1992; Hach Company, 1982), in which the digestate is analyzed for ammonia-N by direct nesslerization. This makes possible a 10-minute Kjeldahl process possible (Hach Company, 1982; Pomeranz and Meloan, 2002). Digestion was integrated into a total analytical system based on spectrophotometric analysis (Brayton, 1992). Treatment of plant material with a mixture of H_2O_2 - H_2SO_4 in the absence of metal catalyst has been proposed as an alternative digestion procedure for determination of nitrate-nitrogen in plants (Carter and Gregorich, 2006; McGill and Figuerido, 1993). Many other elements can also be determined in peroxy digests prepared without mercury or other metal catalyst, like phosphorus, potassium, calcium, iron, and copper (Brayton, 1992; Carter and Gregorich, 2006). Hydrogen peroxide and concentrated sulfuric acid mixed solution has recently been used to digest samples for protein analysis (Li et al., 2006; Wang, 2010; Zhang and Zhang, 2008), the digestive time being substantially reduced.

Nontoxic Kjeldahl digestion with the aid of hydrogen peroxide has also been reported a number of times (Table 5).

Complete acid decomposition must always be achieved before adding hydrogen peroxide in order to lessen the risks of possible explosive reactions (Analytical Methods Committee, 1967), which are always possible when hydrogen peroxide is added to undecomposed organic matrices. A numerical rating scale for classifying nitrogen compounds, the digestion index, based on their resistance to decomposition (Brayton, 1992), has been devised. It is an arbitrary scale in which zero is assigned to compounds not needing digestion (ammonium salts) and ten to nicotinic acid, depending on the minimum peroxide reaction time required to obtain full nitrogen recovery relative to nicotinic acid.

OUT OF THE BEAKER AND INTO THE OVEN: MICROWAVE DIGESTION

In the more recent attempts at developing sample preparation procedures for the subsequent determination of Kjeldahl nitrogen, a significant amount of effort has been directed toward the use of microwave irradiation (Hilz et al., 2012). Analytical chemists first began using microwave heating as a means of applying energy in 1975 to digest biological samples (Abu-Samra et al., 1975). Since then, the microwave oven has been introduced to the chemical laboratory as a new way of conducting chemical reactions (Barclay, 2004; de Castro and Priego-Capote, 2011; Eskilsson and Björklund, 2000; Kingston and Jassie, 1988; Rawa-Adkonis et al., 2003; Zlotorzynski, 1995). The significance of microwave for analytical chemistry grows continuously and rapidly (Kubrakova, 2002; Navas et al., 2012a, 2012b). This method of electromagnetic heating is a “cold” in situ process based on the direct absorption of energy by the sample; heat absorption is instantaneous, and, as a consequence, more energy is collected by the solution, decreasing digestion time. Thereby, thermal phenomena like conduction, convection, or radiation play only a secondary role in temperature control and equalization. Microwave ovens have successfully found a way out of the kitchen, and MEC was recently proposed as a new acronym, standing for microwave-enhanced chemistry (Nobrega et al., 2002). Dipole rotation and ionic conductance are the two primary mechanisms for the absorption of microwave energy by a solution (Richter et al., 2001).

Most experiments are carried out using cavity- or focused-microwave systems, usually referred to as closed- or open-vessel systems, respectively. Note that the so-called open vessels in focused systems are not completely open. In addition, it is also possible to operate closed vessels in a focused microwave way. For these reasons that terminology is not totally correct (Nobrega et al., 2002). However, open-focused systems allow microwave energy to be more efficiently transferred to the sample and are safer and easier to automate (Mermet, 1997). Kjeldahl nitrogen has been determined in food (Chemat et al., 1998; Collins et al., 1996; Domini et al., 2009; Feinberg, 1999; Feinberg et al., 1993; Theiller, 1993), industrial wastewater (Ramon et al., 2005), and geological (He et al., 1990) and

reference samples (Theiller, 1993; Cerdà et al., 1997) by using focused microwaves in open systems. There are also on-line analyzers for this purpose, which, however, use pressurized pumps (Cerdà et al., 1997). The advantages of working at atmospheric pressure, including the ability to add reagents sequentially and the safety improvement in avoiding high pressures, makes it an attractive alternative (Collins et al., 1996).

The closed-vessel microwave digestion method (Lo et al., 2005; Matusiewicz and Sturgeon, 1989; Sneddon et al., 2006) has the advantages of minimizing contamination and analyte loss, as well as maintaining the digestion conditions at specific temperatures and pressures. Moreover, the closed-vessel method is more suitable for studying the chemical reactions of microwave heating under different operative conditions. However, though this approach expedites analyses substantially, it has some disadvantages such as the hazards involved in working at high pressures and temperatures with organic matrices, the requirement to use small amounts of sample, the inability to add reagents between steps of the process, and difficult optimization. Ultrasonication has been used in analytical chemistry to accelerate many steps of an analytical procedure, such as the extraction of organic and inorganic compounds (Luque-García and de Castro, 2003). Simultaneous direct irradiation with microwaves and ultrasound has also been recently applied in Kjeldahl determination (Chemat et al., 2004; Domini et al., 2009; Lagha et al., 1999).

The development of microwave digestion has given an important new dimension to Kjeldahl determinations (McKenzie, 1994). The operating conditions for the digestion itself are different from those of classical methods (Feinberg et al., 1994). Thus, a fully automated microwave digester can improve both accuracy and precision of determinations, but consists of more than just an automated sampler or a computer-driven instrument control. The analysis in situ of temperature and pressure measurements allows an understanding of the processes that occur during microwave digestions (Kingston and Jassie, 1988). In addition, the use of the couple sulfuric acid-hydrogen peroxide in digestion (Table 7) reduces the Kjeldahl digestion time and the chemical reagents needed.

Short digestion times in association with maximum nitrogen recovery were sought using methodology based on experimental design and response surfaces, i.e., fractional, complete factorial designs, and Doehlert designs (Feinberg et al., 1993; Korn et al., 2005; Mohd et al., 1992; Segade et al., 2003); a rotatable orthogonal Box-Wilson composite plane design (Ramon et al., 2005); a three-component Scheffé simplex-centroid design (Zorgati et al., 2000); Box-Wilson protocol (Ramon et al., 2005); and a Plackett-Burman saturated design (Domini et al., 2009; Feinberg et al., 1993; Lavilla et al., 1998). Expert database systems have been devised to assist microwave sample separation (Feinberg et al., 1994; Settle et al., 1989, 1992). Safe laboratory procedures should always be used when performing

TABLE 7
Microwave-assisted digestion applications

Sample	Comments	Reference
Animal fodder	The crude protein retained in the digestion residues increased by 19.2% as microwave treatment increased from 0 to 30 min. Barbano and Clark (1990) method was applied	Brodie et al., 2012
Amino acids and certified reference materials (peach leaves and soil)	Simultaneous direct irradiation with microwaves and ultrasound was used to determine total Kjeldahl nitrogen; two-step mineralization with sulfuric acid and oxidation with H ₂ O ₂ was involved	Domini et al., 2009
Soil and plant material	Conventional Kjeldahl wet digestion followed by steam distillation and titration of ammonium (oxidized forms of nitrogen such as nitrate and nitrite that might be present in the samples not detected)	Yardim, 2009
Milk	Microwave digestion conditions were optimized; relative standard deviation and recovery were 1.82%, and 98.8–101.7%, respectively	Shen et al., 2009
Beans	Focused-microwave Kjeldahl digestion procedure without metal catalyst for nitrogen determination. Temperature at which the decomposition plateau occurs, mass of potassium sulfate, and volume of either sulfuric acid or hydrogen peroxide were optimized	Korn et al., 2005
Sewage and wastewater	Closed-vessel microwave digestion process for the determination of total Kjeldahl nitrogen. Temperature at which the decomposition plateau occurs, mass of potassium sulfate, and volume of either sulfuric acid or hydrogen peroxide were optimized	Lo et al., 2005
Industrial wastewater	Demonstrated the use of a focused microwave method involving chemical digestion in two steps, mineralization and oxidation with H ₂ O ₂ , for the determination of Kjeldahl nitrogen	Ramon et al., 2005
Powdered milk, cow's milk, rice, corn, flour, beef, corned beef	Simultaneous microwave and ultrasound irradiation as a new technique for digestion of solid and liquid samples (food products), compared with the classical total Kjeldahl nitrogen analysis	Chemat et al., 2004
Synthetic foods	Scheffé mixture experimental designs were used to detect the influence and variables interactions using focused microwave	Zorgati et al., 2000
Soil	Open-vessel single-mode microwave technology applied and the extracted nitrogen determined colorimetrically in a flow injection system making use of the Berthelot reaction	Mason et al., 1999
Pharmaceutical, agricultural, and food products	Kinetics of microwave and classical Kjeldahl digestion for nitrogen determination. Effects of reaction conditions (microwave power, concentration, reaction time) on the microwave Kjeldahl digestion	Chemat et al., 1998
Amino acids	Open-vessel microwave digestion follows the stepwise degradation of amino acids in the presence of H ₂ SO ₄ and H ₂ O ₂ . The first step consists in charring the matrix, the second in oxidizing	Suard et al., 1997
Soybeans, miso, wheat flour, rice, loin ham, shiba-shrimp, and powdered cream	Microwave-assisted Kjeldahl digestion of food materials were successfully accomplished within 35 min at atmospheric pressure using an open-focused system	Suzuki, 1997
Tryptophan, bovine liver, oyster tissue, citrus leaves	Atmospheric pressure microwave digestion method for analysis of total phosphorus and Kjeldahl nitrogen in complex matrices (EPA Method 351.1 for Kjeldahl nitrogen determination)	Collins et al., 1996

(Continued on next page)

TABLE 7
Microwave-assisted digestion applications (*Continued*)

Sample	Comments	Reference
Fish, chicken, beef, liver, rice, butter, paté, soy flour	A vessel microwave digestion system carrying out two periods of decomposition of the organic matrix with sulfuric acid without catalyst, and oxidation with hydrogen peroxide	Feinberg et al., 1993
Six samples : flour, milk, meat . . .	Use of H ₂ SO ₄ and H ₂ O ₂ without catalyst for the rapid mineralization of samples in an interlaboratory study	Suard et al., 1993
Natural water	On-line microwave digestion of natural water samples with detection of urea as ammonia by colorimetric detection by flow injection	Schmitt et al., 1993
	Implementation of standard methods relies upon analyst expertise (conversion into usable procedures). Application of software techniques to standard methods for microwave sample dissolution	Settle et al., 1992
Meat products	The mineralization is carried out in 20 min. Results agree with the classic method of mineralization	Bermond and Ducauze, 1991
Soil	Microwave digestion procedure proposed for total N analysis by using HF-HCl, H ₃ BO ₃ , and H ₂ O ₂ , giving much lower values than conventional Kjeldahl digestion	He et al., 1990
Milk	Microwave digestion using within-laboratory study and the Kjel-FAST (CEM Corp.) in four stages. Total digestion plus dilution and cool-down time was about 8 min	Barbano and Clark, 1990
Wheat flour matrix	Expert system assists the analyst in the identification of four analytical descriptors necessary to develop procedures for sample preparation, with a PC-AT microcomputer and commercially available software	Settle et al., 1989
Essential amino acids, vegetables, milk products, seafood, canned meat	Efficiency of the microwave method of sample treatment in regard to the speed of the dissolution process and the precision and accuracy of the nitrogen determination	Alvarado et al., 1988

the microwave sample preparation methods described (Collins et al., 1996), and care will be needed in the choice of materials for digestion vessels and in adequate monitoring of the safety of the equipment with respect to microwave emission and potential explosion. Commercial microwave digestion systems (both of closed- and open-vessel types) are being marketed by a number of companies. Optimization, validation, and automation of this atmospheric pressure single-mode microwave method have led to its incorporation in the French AFNOR (1992) method, as an alternative sample preparation procedure (Collins et al., 1996; McKenzie, 1994).

MECHANISM: THE FORGOTTEN TOPIC

Comparatively few articles has been published on the mechanism of the Kjeldahl reaction. The actual decomposition reaction is mainly oxidation accompanied by reduction, dehydration, hydrolysis, substitution, and other complicated reactions, and cannot be simply expressed by a single equation (Morita, 1968). The decomposition reaction is extremely complicated and only very few studies concerning kinetics have been made (Bredig and Brown, 1903; Schwab and Caramanos, 1955; Schwab

and Neuwirth, 1957; Schwab and Schwab-Agadillis, 1951, 1953).

Microwave digestion studies carried out with sulfuric acid and hydrogen peroxide show that two steps are involved in the digestion process (Alvarado et al., 1988; Feinberg, 1999; Feinberg et al., 1993; Ramón et al., 2005): (a) mineralization, by which organic matter in the sample is decomposed in the presence of sulfuric acid, and the nitrogen in a difficult-to-degrade form is released; and (b) oxidation with hydrogen peroxide, which cleaves the bonds resisting acid attack (Colina and Gardiner, 1999; Collins et al., 1996; Nobrega et al., 2002; Ramon et al., 2005). Some amino acids contain two different kinds of nitrogen atoms, with their transformation into ammonium sulfate in the presence of sulfuric acid, easy and difficult, respectively (Kell and Steinhart, 1990). For several amino acids, only a stoichiometric fraction of nitrogen (called the easy fraction) is digested then when sulfuric acid is used alone. The other, difficult, fraction is totally dissolved when hydrogen peroxide is added (Suard et al., 1993, 1997). The digestion kinetic curves differ enormously between classical Kjeldahl and microwave methods. The conventional Kjeldahl method gives a kinetic curve

related to second-order reaction, whereas the microwave digestion seems to be a first-order reaction (Chemat et al., 1998), as it is derived from kinetic study of the arginine digestion.

AMMONIA DETERMINATION: TITRIMETRIC FINISH

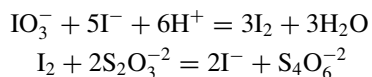
Transformation of NH_4^+ into NH_3 , caused by addition of NaOH (pH growth) into a Pregl-Parnas-Wagner (Parnas and Wagner, 1921) (semimicro distillation) apparatus (Andraos, n.d.; ASTM, 2011; Hakoda et al., 2009; Hoskins, 1944; Pregl, 1899, 1930; Scandrett, 1953; Sheers and Cole, 1953; Steyermark and McGee, 1960; Steyermark et al., 1951) results from the equation

$$\frac{[\text{NH}_3]}{[\text{NH}_4^+]} = 10^{\text{pH}-\text{p}K_1}$$

where $\text{p}K_1 = -\log K_1$; K_1 refers to reaction $\text{NH}_4^+ = \text{H}^+ + \text{NH}_3$, $\text{p}K_1 = 9.35$ at 20°C .

After collection in an acid solution, ammonia may be titrated with strong base or strong acid, depending on whether strong acid (HCl or H_2SO_4) or boric acid (H_3BO_3) is applied in the distillation receiver; H_3BO_3 is commonly used to trap ammonia (Beljkaš et al., 2010; Meeker, and Wagner, 1933; Stover and Sandin, 1931; Takatsu et al., 2008; Thiex et al., 2002), although some reports claimed that collection of ammonia in H_2SO_4 and subsequent titration of an excess acid with standard alkali gives better results (Fleck and Munro, 1965). Optionally, a titration with sulfamic acid (as primary standard) solution was also suggested (Milner and Zahner, 1960).

After distillation, the excess of standard acid may be determined iodometrically, according to consecutive reactions:



The high precision of the titration with starch as an indicator enabled Kjeldahl to work with small samples of material or to aliquot the distillate before titration. Nevertheless, as carbon dioxide is usually present in the distillate, it is advisable to boil the solution prior to the addition of the standard iodide-iodate solution (Wilson and Mattingley, 1926). For this reason, this method is seldom used. However, it has been stated that the iodometric method applied to the microdetermination of nitrogen is to be preferred to the acidimetric method because the risk of a displacement of the end-point by carbon dioxide absorption is much lower (Bradstreet, 1965; MacInnes and Granick, 1958; Michaelis and Maeda, 1923).

Titration of ammonia absorbed in H_3BO_3 solution was proposed first by Winkler (1913, p. 231), who noted, "Boric acid is indeed such a weak acid, that its solution does not cause a noticeable color change of certain indicators. Ammonia is, however, completely fixed by it, provided that a suitable excess of acid is present." One of the advantages of such a choice is that only one standardized solution (e.g., HCl or H_2SO_4) is needed (as titrant) for this purpose (Ma and Zuazaga, 1942), and the

results of titrations are obtained directly. When a strong acid (HCl or H_2SO_4) is applied in the receiver (i.e., in the titrand), two standard solutions are needed: (1) titrant (NaOH solution) and (2) the strong acid in the receiver; in (2), the results are obtained in an indirect manner. Other adsorbents have been used, e.g., Doyle and Omoto (1950) used, with good results, a solution of KHSO_4 instead of boric acid to trap ammonia. Blom and Schwarz (1949) used $\text{NiSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, and Urban (1971) suggested a solution of p-hydroxybenzoic acid.

There were some proposals involving the choice of a proper indicator applied in these (visual) titrations. For example, mixtures of methyl red and tetrabromophenol blue (Stover and Sandin, 1931), or methyl red and methylene blue (2:1) (Sobel et al., 1937) or methyl red alone (Meeker and Wagner, 1933; Wagner, 1940) were suggested (Ma and Zuazaga, 1942); the latter changes its color at about pH 5.2 (Sher, 1955). Alcohol (ethanol or methanol) solution containing a mixture of methyl red and methylene blue was known later as the Tashiro indicator. After addition of HCl, the Tashiro indicator changes its color from green to violet. Knowing the number of milliliters of the standardized acid used in the titration, the amount of ammonia neutralized with boric acid can be calculated; alternatively, a pH titration with end-point (V_e) at pH = 5.0 is also done. A mixed indicator (bromocresol green and methyl red) has been recently used by Beljkaš and colleagues (2010) to locate the end-point.

Three drawbacks of the usual Kjeldahl receiving solution (standard sulfuric or hydrochloric acid) are that it must be quantitatively standardized and quantitatively measured into the receiving flask, and that additional titrations and calculations are required if a blank is run. Also, when using this solution, it is necessary to have on hand standard sodium hydroxide solution (which can be difficult to keep) for forward titration to the methyl red end-point. Two drawbacks of the boric acid solutions that are frequently used are that boric acid is volatile, introducing an error due to acid evaporation, and that additional titrations and calculations are required if a blank is run.

A new standard method was developed by FOSS at the end of 1990s, in cooperation with the AOAC (Möller, 2009), using block digestion with copper sulfate catalysts, a minimum amount of sulfuric acid, a digestion time of 1 hour, steam distillation into boric acid receiver solution, and simultaneous titration with photometric end-point. The distillation plus titration time was reduced to some 3–4 minutes. It has been recommended that 1% H_3BO_3 as an optimal alternative to 4% boric acid trapping solution be allowed for automatic titrators that titrate through the distillation (Thiex et al., 2002). This standard was later adopted, first in 2005, by ISO and the European Committee for Standardization (CEN) as EN ISO 5983–2 (ISO, 2009b), establishing a global standard for protein determination. An analogous procedure with copper sulfate/titanium oxide catalyst was adopted in EN ISO 20483 (ISO, 2006) for the determination of the nitrogen content and calculation of crude protein in cereals and pulses. The Kjeldahl method has been widely used for protein estimation in cereals (Vivek et al., 2008) because: (i) it allows

quantifying nitrogen from either soluble or insoluble samples, (ii) the nitrogen from cereal samples is mainly derived from protein, and (iii) the amino acid composition of endosperm protein is constant enough to have a relatively fixed nitrogen:protein ratio within a given cereal.

Selected applications of titrimetric methods applied in Kjeldahl analysis are shown in Table 8. The distillation titration method is a standard procedure used by most laboratories to measure ammonium (NH_4^+)-nitrogen (N) in the total Kjeldahl nitrogen digests of various kinds of agricultural and environmental samples, as shown recently by Saha et al. (2012).

Conductivity measurements have also been the subject of study for the analytical finish of the Kjeldahl method. Hendricks and Thomas (1942) described a vacuum distillation procedure in which the ammonia was collected in boric or sulfuric acid and determined by the change in conductivity of the acid. The conductivity of boric acid is very small and practically independent of its concentration, whereas ammonium borate has the conductivity of a well-ionized salt. Shaw and Staddon (1958) used, in a same way, a diffusion cell to transfer ammonia from the sample to sulfuric acid for its subsequent determination by conductivity. Sarudi and Siska (1970) carried out the analysis of ammonium salts from the protein Kjeldahl method by measuring the specific conductance of the solution once the ammonia was separated from samples by distillation and collected in boric acid solution.

Friedl (1972) determined ammonia from the rate of change in conductivity of a small volume of sulfuric acid as it absorbed ammonia from a sample in a diffusion cell. Carlson (1978) collected Kjeldahl digests into a solution, the gaseous ammonia evaporated by the addition of sodium hydroxide was re-collected through a porous silicon membrane tube into deionized water inside the porous membrane tube, and the conductivity was then measured. The performance of the diffusion conductivity method in measuring NH_4^+ -N in the total Kjeldahl nitrogen digest of 29 different samples (surface water, lagoons, manure, poultry litter, and environmental wastes) was recently compared with that of the colorimetric method with the distillation titration method (Saha et al., 2012).

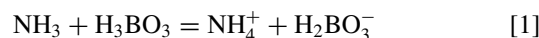
BASE OR ACID AS TITRANT: THAT IS THE QUESTION

Ammonia can be distilled into an excess of standard H_2SO_4 or HCl, and the excess determined by back titration with a standard base. Since the solution at the equivalence point contains $(\text{NH}_4)_2\text{SO}_4$, or NH_4Cl , which hydrolyzes, methyl red (transition range 4.4–6.2) is used as an indicator (Blaedel and Meloche, 1957). A mistake appears in Bradstreet (1965, p. 152): “in the case of back titration of a distillate, the equivalence point will occur at pH 7, since this is the point of neutralization of a strong acid by a strong base. Any indicator, therefore, changing color at or close to pH 7 is suitable.” The millimoles of NH_3 in the sample are equal to the total millimoles of HCl added minus millimoles of base used in back titration. This method has two

disadvantages: (i) the amount of NH_3 is obtained as a difference and (ii) two standard solutions are required.

Preferably, the distilled ammonia can be absorbed into a solution of boric acid (Winkler, 1913) or other weak acid (Laitinen and Harris, 1975). Boric acid is sufficiently acidic to react with ammonia and prevent loss by volatilization, but it is too weak an acid to interfere with the titration of ammonium borate with diluted hydrochloric acid. When boric acid is used instead of standard acid as the recipient of the distillate, the use of a weak standard alkaline solution is avoided. The alkaline solution is vulnerable to the absorption of atmospheric carbon dioxide with subsequent changes in molarity. Neither the amount nor the concentration (about 4%) of boric acid in the receiving bottle has to be precise.

Then, in the Winkler modification, the NH_3 is caught in an unmeasured excess of boric acid:



The borate formed is determined by titration with standard HCl, one mole of HCl being required for each mole of NH_3 :



The solution at the equivalence point contains H_3BO_3 and NH_4Cl , a mixture of two weak acids, so that an indicator transiting in the acid region (pH 5–6) is satisfactory (Blaedel and Meloche, 1957).

The equilibrium constant of Equation (1) is given by

$$K_{eq} = \frac{K_{1,\text{H}_3\text{BO}_3}}{K_{1,\text{NH}_4^+}} = \frac{10^{-9.24}}{10^{-9.35}} = 1.288 \quad [3]$$

Although this equilibrium constant is low, the fraction of ammonia converted into ammonium ion increases with increasing the difference of concentrations between boric acid and ammonia. However, hydrochloric acid titrates the borate ion from the above reaction (Equation (2)) as well as the ammonia, which is not converted into ammonium ion in Equation (1), i.e., $\text{H}_2\text{BO}_3^- + \text{H}^+ = \text{H}_3\text{BO}_3$; $\text{NH}_3 + \text{H}^+ = \text{NH}_4^+$ (the sum of both being equivalent to the ammonia distilled).

Equations for drawing the rigorous and complete titration curves corresponding to back and direct ammonia titrations have been recently derived (Michałowski et al., 2013), together with their corresponding derivative curves. Details are too lengthy to include here. The pH_{eq} increases in the back titration of ammonia (trapped in either hydrochloric or sulfuric acid) with sodium hydroxide, as the ammonia concentration decreases, reaching a value of 7, corresponding to the titration of a strong acid with a strong base, when the ammonia concentration is zero. The pH_{eq} in the titration with hydrochloric acid of ammonia trapped in boric acid remains practically unchanged and close to 5.2.

Two standardized solutions (trapping and titrant) are required in the titration of ammonia trapped in HCl solution with NaOH (back titration), whereas only one standard solution (titrant)

TABLE 8
Selected titration applications

Sample	Comments	Reference
Surface water, lagoons, manure, poultry litters, and environmental wastes	The performance of the diffusion conductivity method and colorimetric method is compared with that of the distillation titration method. Agricultural and environmental samples of various kinds are analyzed	Saha et al., 2012
Milk and milk products	Nitrogen content and crude protein calculation by using traditional and block digestion methods with CuSO ₄ as catalyst and titrimetric finish with HCl as titrant (steam distillation of ammonia into excess boric acid)	ISO/TC 34/SC 5, 2011
Cereals and oilseeds	Suitability of Dumas combustion method to completely substitute the Kjeldahl method in routine laboratory determination of nitrogen, evaluating the validation parameters, which are not outlined in the official method	Beljkas et al., 2010
Corn distillers dried grains and corn distilled dried grains with solubles	AOAC 990.03 Protein (Crude) in Animal-Feed Combustion and AOAC 2001.11 Protein (Crude) in Animal Feed and Pet Food Copper Catalyst and Steam Distillation into Boric acid can be used interchangeably to provide accurate and precise protein results.	Thiex, 2009
Animal feeding stuffs	Semimicro rapid routine using block digestion, copper catalysts, and distillation into boric acid. In the case of manual or semiautomatic steam distillation, distillation of the ammonia into an excess of boric acid solution is followed by titration with hydrochloric acid solution to a colorimetric end-point	ISO, 2009b
Creatinine and urea	Development of two CRMs. The certified values were assigned based on the results of two different methods: acidimetric titration and nitrogen determination by the Kjeldahl method	Takatsu et al., 2008
Cereal and pulses	Digestion with sulfuric acid in the presence of a catalyst trapping, then the ammonia distilled into boric acid and using sulfuric acid in the titration finish	ISO, 2006
Milk, egg, yogurt, soybean milk, almond	The method of Kjeldahl is more accurate than UV spectrophotometry, isoelectric focusing, biuret reaction, or Commassie Brilliant Blue reaction	Li, 2006
Foodstuffs	Both the block digestion and Kjeltex Auto Analyzer and the traditional Kjeldahl methods were used to determine the protein content of foodstuffs with satisfactory results	Wang et al., 2004
Milk, animal feed, forage, grain, and oilseed	Test portions digested, cooled, and diluted, the ammonia distilled, trapped in a weak boric acid solution, and titrated with hydrochloric acid (colorimetric end-point detection) (collaborative study)	Thiex et al., 2002
Blood	Flow-injection analysis for the rapid and direct determination of ammonium in Kjeldahl digests, on-line by a bulk acoustic wave (BAW)-impedance sensor. Results in good agreement with those obtained by the conventional distillation/titration method	Su et al., 1997
Cheese	Boric acid is used as the recipient of the distillate in an interlaboratory study carried out to establish precision parameters for a standard method for nitrogen in cheese employing copper sulfate as Kjeldahl catalyst	Florence and Harris, 1987
Iron(II) ammonium sulfate, thiourea, ammonium sulfate, sulfamic acid . . .	Inexpensive trap-de-mister assembly utilizing solid crystalline boric acid as ammonia absorbent. Ammonium ion determined by acidimetric titration, conductance measurement, or spectrophotometric measurement after the addition of Nessler's reagent	Siemer, 1986

(Continued on next page)

TABLE 8
Selected titration applications (*Continued*)

Sample	Comments	Reference
Animal feeds	Block digestion followed by steam distillation; collaborative study	Noel, 1979
Soils and plant extracts	The form of nitrogen under analysis (nitrate, nitrite) is converted to NH_4^+ , separated by steam distillation, collected in boric acid, and determined by titration with standard acid	Bremmer and Keeney, 1965
Samples with known amounts of TRIS	Solutions of tris(hydroxymethyl)aminomethane (TRIS) were shown to be a convenient primary standard for Kjeldahl analysis	Rodkey, 1964
Milk protein	High correlation results between Orange G dye and Kjeldahl methods of protein determinations	Castillo et al., 1962
Petroleum	Method employing Kjeldahl distillation and an acidimetric finish by sulfamic acid as titrant	Milner and Zahner, 1960
Pure aromatic nitrocompounds	A procedure is described (acidic reduction with Zn in organic solvents) for the determination of nitrogen in aromatic nitro compounds by a modified micro Kjeldahl method. Bromocresol green as indicator	Ma et al., 1957
Ammonia	Bromocresol green, new cocine, and p-nitrophenol are used as a mixed indicator in Kjeldahl nitrogen titration	Sher, 1955
	Boric acid for trapping ammonia in micro Kjeldahl determination is used, describing suitable titration procedure with methylene blue-methyl red indicator, together with a modified type of micro-distillation apparatus	Yuen and Pollard, 1953
Proteins	Mercury-containing digest with three types of Kjeldahl analyses: macroanalyses, microanalyses with distillation and titration of the ammonia, and microanalyses with gasometric determination of the ammonia by hypobromite reaction	Hiller et al., 1948
Several substances, amphibian embryos	Use of potassium biiodate in the micro Kjeldahl determination of nitrogen (titration of ammonia). Since biiodate simultaneously serves as a iodometric and an acidimetric reagent, the whole method requires only one standard solution	Ballentine and Gregg, 1947
Benzamide, phthalimide acetanilide, urea, urotropin . . .	Sample digested with sulfuric acid in the presence of selenium, copper sulfate, and potassium sulfate. The ammonia is distilled into boric acid solution and titrated directly with hydrochloric acid, using a mixed indicator methyl red and bromocresol green	Ma and Zuazaga, 1942
Organic compounds	Accuracy of the color matching end-point with methyl red indicator in the titration of ammonia in boric acid solution, and its applicability to the macro, semimicro, and micro Kjeldahl procedures.	Wagner, 1940
Urine	Mixed indicator containing methyl red and tetrabromophenol blue with good results in boric acid solution	Stover and Sandin, 1931
Serum	Mixture of methyl red and methylene blue as indicator in the titration of bases in boric acid solution	Sobel et al., 1937
Urea, glycine, uric acid . . .	Titration procedure extended to both macro- and microtitrations, with methyl red as indicator, giving a definite and unmistakable end-point without use of special light	Meeker and Wagner, 1933
Vaccines	Accuracy of the "formol titration" method, and especially its application to the estimation of nitrogen in ammonium sulfate	Shaw, 1924
Crops and soil	Distillates received in boric acid and titrated with standard sulfuric acid, using bromophenol blue as an indicator	Scales and Harrison, 1920
	Use of boric acid in the absorption of ammonia, which has the advantage that only one solution is necessary	Winkler, 1913

is required in the titration with HCl solution of the ammonia trapped in H_3BO_3 . However, the two options considered from a theoretical viewpoint provide good results (Michałowski et al., in press) of analyses if the pH value at the end-point is close to 5.2. Sulfuric acid may also be used instead of hydrochloric acid for trapping ammonia in the first case or for direct titration when ammonia is trapped into boric acid. When a strong acid is employed for trapping ammonia it is necessary to know beforehand the approximate amount of ammonia to be absorbed, so that the proper quantity of acid could be taken in order to obtain satisfactory results (Hendricks and Thomas, 1942). When boric acid is used for trapping ammonia, large excess is present and room temperature is not exceeded in order to avoid instability of ammonium borate solutions.

KJELDAHL WITHOUT DISTILLATION WITH TITRIMETRIC FINISH

In the Kjeldahl process, some means could be devised of estimating the ammonia formed, which would render distillation unnecessary, saving considerable time and attention (Shaw, 1924). Some approaches have been applied for this purpose. L. Legler in Germany used the condensation reaction of ammonia with formaldehyde to form hexamethylenetetramine for the alkalimetric assays of formalin solutions in 1883 (Laitinen and Ewing, 1977). The early history of this reaction is shown with detail in the review of Schuette and Oppen (1935). The displacement reaction, known as "formol titration" (Kolthoff, 1921; Marcali and Rieman, 1946, 1948; Shaw, 1924; Sørensen, 1907), occurs with the liberation of acid equivalent to the nitrogen present as ammonium salt, once the free sulfuric acid of the Kjeldahl digest is neutralized:

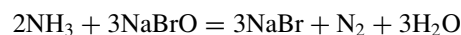


The digest is sufficiently diluted and carefully neutralized, formaldehyde is added, and the acid formed titrated with standard alkali. Further information relating to formol titration is found in Taylor (1957). The theoretical bases of the analytical reaction between ammonium and formaldehyde, including its kinetics and the optimum conditions for the reaction, have been recently considered (Tur'yam, 2010). Principles of realizing an equivalent amount of acid in reaction of ammonium salts with formaldehyde have recently been applied to the determination of crude protein in maize, corn powder, and corn lees (Wang, 2010). The formaldehyde method has also been applied to the rapid determination of ammonia nitrogen in high-concentration ammoniated wastewater in the ammonia industry and urban sewage (Huang et al., 2003).

A simple and rapid method for determining the ammonia content in the Kjeldahl digest by differential pulse polarography on the basis of the formol reaction has been devised. The hexamethylenetetramine produced by the ammonia contained in the digest and formaldehyde reaction in acetate buffer was determined electrochemically at -0.824 V (He et al., 2005), -0.9 V (Me and He, 2000), or -1.04 V (Fosdick and Pike,

1982). The method has been applied to nitrogen in foods, human milk, and foodstuffs, and the results obtained agree with the usual Kjeldahl method.

Ammonia may also be oxidized to nitrogen in alkaline solution with an excess of hypobromite solution (Adams and Spaulding, 1955; Osborn and Wilkie, 1935):



Potassium iodide is then added and the iodine formed titrated with sodium thiosulfate. This is a reaction with a dual role (Schuette and Oppen, 1935), because the nitrogen evolved may also be determined gasometrically.

Willard and Cake (1920) appear to be the first to apply the above reaction to the Kjeldahl process. As hypobromite solutions are not very stable, arsenite-hypochlorite titration in mildly alkaline solution with tartrazine as visual indicator has been proposed (Cohen and Connors, 1965). The reaction of ammonia with hypochlorite is not affected by large amounts of sodium sulfate formed in the neutralization of the digest. A hypobromite method (Bosch and Querol, 1947) employing brominating mixture and potassium tetraborate has been proposed, which has been modified and used for the determination of organic nitrogen on both the micro and the semimicro scales (Ashraf et al., 1968). Accurate results are also obtained when potassium bromate is replaced with potassium permanganate or dichromate (Sidiqui et al., 1970). The difficulties associated with the conservation of hypochlorite and hypobromite solutions (Kolthoff et al., 1957) do not arise when bromate, permanganate, or dichromate is used in the presence of potassium bromide.

Coulometric titration methods offer several advantages over standard volumetric procedures; they don't require standard solutions, and the only volumetric measurement needed is the sample measurement (Christian et al., 1965; Kaltenborn and Hütter, 1992; Lengerken et al., 1982). The elimination of the preparation and storage of standard solutions avoids the inherent volumetric error and danger of atmospheric and other contamination. Analysis of nitrogen immediately after the decomposition is possible, and the expensive distillation step of the original Kjeldahl procedure can be avoided. The hypobromite reaction has found an application in coulometric titrations of ammonia nitrogen and in amperometric titrations. The manufacturer Mitsubishi uses the diluted digest for a direct coulometric titration (Pansu et al., 2001).

Selected applications of titrimetric methods in Kjeldahl without distillation, both including visual indicator and coulometric methods, are summarized in Table 9.

CONCLUDING REMARKS

The early history of nitrogen determination methods ranging from the Dumas, Will and Varrentrapp, and Wanklyn methods to the Kjeldahl method has been outlined. Protein analysis has legal, nutritional, health, safety, and economic implications for the food industry (Owusu-Apenten, 2002), for example, wheat quality is significant for its purchasing, storage, processing, and

TABLE 9
Selected applications without ammonia distillation

Sample	Comments	Reference
Coulometric Titrations		
Pure di- and tripeptides, aminoacids	The total procedure for analysis of nitrogen represents in combination of digestion and electrochemical analysis a physical method, which is absolutely adjustable and does not need any chemical calibration. The titration was done in a cell consisting of two chambers, which were filled with carbonate buffer and the reactive element	Kaltenborn and Hütter, 1992
Animal feed	A comparison of the hypobromite coulometric procedure analysis and the original Kjeldahl procedure	Lengerken et al., 1982
Nitrogen in lunar igneous rocks	Coulometric titration with OBr^- and bioamperometric end-point detection. The technique is superior to the conventional Kjeldahl technique	Müller et al., 1976
Nitrogen in organic compounds	A rapid coulometric method based on the hypobromite reaction allows performing 20–30 nitrogen determinations per hour	Boström et al., 1974
Serum (non-protein nitrogen)	The digest from an aliquot of the protein-free filtrate is titrated with electrogenerated hypobromite after adjusting the pH to 8.6	Christian and Jung, 1966
Human serum samples	A coulometric procedure for the titration of ammonia formed from protein nitrogen following micro Kjeldahl digestion. A method is described for the direct titration of the ammonia in the digest. Results are compared with standard macro and micro Kjeldahl procedures.	Christian et al., 1965
Spiked solutions similar to Kjeldahl digests	Titration with electrolytically generated bromine of microgram quantities of ammonia in solution with a pH of 8.5. Neither copper sulfate nor mercuric nitrate affects the accuracy of ammonia determinations	Arcand and Swift, 1956
Amperometric Titrations		
Soils, fertilizers	Titration with NaBrO in the presence of $\text{Na}_2\text{B}_4\text{O}_7$ and NaBr buffer solution with end-point determined amperometrically	Tsap et al., 1968
Organic compounds	Current voltage curves of sodium hypobromite solution at a rotating platinum electrode as indicator were determined. This is used in the amperometric titration of ammonia and of nitrogen after Kjeldahl digestion	Kolthoff et al., 1953
Visual Indicator Titrations		
Maize, corn protein powder, corn leas	Formol titration is applied after $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ digestion	Wang, 2010
Wheat, rice, graham, peas, milk, blood, tea leaves, meat	Nitrogen converted into ammonium sulfate by Kjeldahl digestion. Potassium bromate and bromide were added in excess, and the ammonium sulfate reacts quantitatively with the hypobromite produced; the unutilized potassium bromate was determined iodometrically	Siddiqui et al., 1970
Serum	Nitrogen is determined by oxidation of the resultant ammonium sulfate with hypobromite produced in situ by the addition of an excess of potassium bromate	Ashraf et al., 1968
Nitrogen-containing organic compound	Ammonia is oxidized in a basic medium with hypobromite (generated by adding an excess of bromide to the hypochlorite), then an excess of standard arsenious oxide is added and the titration completed with hypochlorite with tartrazine as indicator	Cohen and Connors, 1965
Blood	Elimination of interference from the mercury catalyst. Complexation of mercury, followed by precipitation of ammonium tetraphenylborate in the resulting solution	Crane and Smith, 1964

(Continued on next page)

TABLE 9
Selected applications without ammonia distillation (*Continued*)

Sample	Comments	Reference
Nitrogenous compounds (leucine, glutamic acid, tyrosine . . .)	Method for the determination of nitrogen that does not involve any temperature and pH control. Mercury has been found to be the best catalyst as it does not necessitate any filtration	Hashmi et al., 1962
Nitro, nitroso, and azo compounds	Compounds reduced with glucose or zinc in methanol and hydrochloric acid. The ammonium sulfate produced after the usual digestion determined by hypochlorite-arsenite titration	Ashraf et al., 1961
Some pure organic compounds	Titration of ammonium salts with hypochlorite (Kolthoff and Stenger method) with mercury as catalyst (other catalysts interfered in the titration) and without prereduction; both in the micro and semimicro scale	Belcher and Bhatti, 1956
Pure organic compounds	Modification of the nondistillation method of Marcali and Rieman so that selenium may be used as the catalyst by the elimination of the interference of selenium	Adams and Spaulding, 1955
Pure organic compounds and dried blood	A procedure is described that eliminates the interference of phosphate and that due to the precipitation of sulfates of calcium and barium and of hydroxides of iron and aluminum	Marcali and Rieman, 1948
11 organic compounds	The ammonium ion is directly titrated with standard sodium hydroxide in the presence of formaldehyde with phenolphthalein as indicator, so that the reaction to form hexamethylenetetramine goes rapidly, with previous neutralization and addition of sodium bromide	Marcali and Rieman, 1946
	Oxidation of ammonia by an excess of hypobromite; addition of iodate plus iodide and titration of iodine with sodium thiosulfate	Haanappel, 1938
	Oxidation of ammonia by hypobromite and formation of iodine by the addition of iodide and iodate, which is titrated with sodium thiosulfate	Teorell, 1928
Nitrogenous substances	Neutral hemamethylene tetramine if formed by the reaction of ammonium salts with formaldehyde with the liberation of acid equivalent to the nitrogen present (formol reaction)	Shaw, 1924
Alanine, acetanilid, wheat flour, sawdust, egg albumen . . .	Sulfuric acid is added to the sample and heated until the organic matter is completely charred. It is cooled, and solid potassium persulfate free from ammonium salts is added to the dark solution through a thistle tube. Ammonia is treated with hypobromite	Willard and Cake, 1920

consumption. In spite of the fact that Kjeldahl's nitrogen determination method was developed nearly 130 years ago, it is still widely used, as shown in the introductory part of the article. Although the technique and apparatus have been modified over the years, the basic principles introduced by Johan Kjeldahl still remain unchanged (Belcher, 1976; Egli, 2008; McKenzie, 1994). In Kjeldahl's original method of nitrogen analysis, sulfuric acid alone was used as a digestion medium; the digestion in boiling H_2SO_4 converts organic nitrogen into NH_4^+ .

The Kjeldahl method has been the subject of more studies than many other methods in analytical chemistry (Chen et al., 1988) due to the usefulness of the method; the need for improvement, e.g., the search for catalyst to speed up the digestion process; and applications to nearly all forms of organic nitrogen

and to inorganic matrices as well. These facts have led to improvements in safety, speed, and applicability of the method; the analytical procedure is also simplified in this way (Persson et al., 2008; Thiex et al., 2001, 2002). Kjeldahl's process, as originally devised, had the disadvantage of being very slow. The most important and fundamental aspect of the Kjeldahl method is the digestion of nitrogen compounds with hot concentrated sulfuric acid (Morita, 1968). An alternative to save time is the use of devices that allow the digestion of many samples simultaneously, as well as the use of mechanically automated techniques, e.g., Kjeltec (Barbarino and Lourenço, 2009; FOSS, 2009; Watkins et al. 1987). Other elements may also be determined in the digest prepared without catalyst (Özer and Güçer, 2011). It should be noted that the critical temperature for decomposition is as high

as 373°C. The Kjeldahl method does not recover fraction species like nitrite and nitrate and not all organic nitrogen is recovered, i.e., all those compounds in the molecules in which a nitrogen atom is linked to an oxygen atom or to a second nitrogen atom. The ease of digestion varies with the nature of the sample. Thus, aromatic compounds are all difficult to digest; also, removing heterocyclic nitrogen is even more difficult and entails breaking every molecular bond in the compound (Amin and Flowers, 2004; Schöniger, 1960; Suard et al., 1997).

Many systematic studies on the efficiency of various catalysts have been made, mercury, selenium, and copper being the catalysts of choice. For certain applications titanium has also found some usage. Mercury is the most efficient catalyst. The use of mercury has several disadvantages, including its high cost, disposal problems arising from its toxicity, and the requirement that it should be completely precipitated as a sulfide. Alternative catalysts were investigated because of environmental concern. The use of copper as catalyst is becoming more common, as it is recognized to be more environmentally friendly. Today, selenium and copper are used as catalysts in more than 90% of the Kjeldahl digestions being performed all over the world (Persson et al., 2008).

The determination of ammonia by means of microdiffusion has proved to be a useful and time-honored method. Not only do the physical and chemical properties of ammonia lend themselves well to its separation by diffusion, but also ammonia itself can be quantitatively isolated in a designated solution.

Kjeldahl analysis gives accurate protein results, no matter what the physical state of the sample (Owusu-Apenten, 2002). This technique has approved status and is the reference method adopted by many national and international organizations (Möller, 2010a; Moore et al., 2010). The method is the basis for routine nitrogen assays and is used in many cases for the calibration of modern instruments used for rapid protein measurement, e.g., the Milkoscan for milk protein, and mid- and near-infrared instruments (Hughes and Gray, n.d.). Nitrogen determination with the Kjeldahl method does not require expensive devices or specialized techniques and is precise and accurate. Proper sampling and sample handling are crucial to successful analysis and must be addressed individually by type of material (Lynch and Barbano, 1999). The traditional macro (manual) Kjeldahl method is seldom performed in modern commercial laboratories because the numbers of samples per analysis are too large. On the other hand, laboratory health and safety regulations are becoming severe, and the use of hazardous and potentially toxic chemicals in Kjeldahl analysis is creating some concern (Owusu-Apenten, 2002). Several steps, from sampling in the field to the final analysis report, may have influence on the quality: sampling procedure, physical pretreatment (drying, sieving, milling, or grinding), digestion procedures, chemical analysis, and calculations (Carter and Gregorich, 2006; McDonald, 1977; Wickstrøm et al., 2004). The heightened focus on quality and the accreditation of laboratory systems have contributed to increased knowledge about the uncertainty in the

digestion procedures and the chemical analysis (Asuero et al., 1988; CITAC, 2002; Gonzalez et al., 2005; Herrador et al., 2005).

In spite of all the recent advances in instrumentation, the development of commercial sample digestion systems has been relatively slow, requiring further impetus (Flores et al., 2007). Kjeldahl microwave digestion is considered as a worthy alternative to overcome the drawbacks related to the use of a catalyst, since H₂O₂ has been used for complete decomposition of organic compounds. Some other advantages of Kjeldahl microwave digestion are decrease of the digestion time and improved precision. Chemometrics offers a sound theoretical basis for the optimization of chemical systems and processes (Feinberg, 1996; Wernimont and Spendley, 1985) and has been employed to optimize microwave analytical methods, especially with regards to the saving of resources (time, reagents, experimental work). Chemometrics can assess the statistical significance of the independent variable effects being investigated as well as evaluate their interaction effects by employing multivariate optimization designs by which the levels of all the variables are changed simultaneously (Domini et al., 2009; Korn et al., 2005; Ramon et al., 2005; Wang, 2005).

To avoid influence on the variation of the result obtained during the Kjeldahl analysis because of the determination of the ammonium content in the digestion solution, the approved distillation/titration as the determination method is taken up as the reference method (Thiex and Manson, 2002). The ammonia distilled may be trapped in standardized acid, and then back titration with standardized sodium hydroxide is carried out. It may be also received in boric acid solution; there is no back titration as the ammonia in the boric acid is titrated directly, and the need to standardize one reagent is eliminated. In order to improve both laboratory efficiency and the quality of the data, most laboratories today implement quality assurance/quality control procedures (Holcombe et al., 1986; Möller, 2005; Thiex et al., 2002). To support this improvement, however, it is important that officially approved methods are updated so that available technology can be used in the laboratory (Möller, 2009; Thiex et al., 2002).

The advances in microwave digestion and methods for determination of the ammonium bisulfate generated will ensure the future of the Kjeldahl method (McKenzie, 1994). It will be complemented by use of automated combustion methods for some purposes, given the improvement in dry combustion nitrogen analyzer technology (Krotz and Giuzzi, 2011). More sophisticated equipment than the Kjeldahl method is required in this later case, and thus may be beyond the capacity of many laboratories, especially those that carry out only intermittent analyses. Several alternatives to the classical approach for total nitrogen have been suggested, including the alkaline persulfate digestion technique, which oxidizes all the nitrogen in the sample to nitrate as the sole product, using potassium persulfate in a strongly alkaline environment (Kowalenko and Babuin, 2003; Maher et al., 2002) under high pressure and temperature.

However, the Kjeldahl method is still the primary reference method for protein analysis today (Möller, 2010a; Moore et al., 2010).

Murphy (1956) wrote in *Analytical Chemistry*: “Jet- and atomic-age analysts using instruments costing in the range of \$10,000 to \$100,000 actually are beginning to believe and say that anyone who uses chemical wet methods of analysis is either stupid or cannot afford to be up do date,” and at the end of the same editorial: “In our enthusiasm for modernity, let us not forget that the chemical procedures have a very important role to play in their own right, and when they are combined with the physical approach the sensitivity, specificity, and speed are greater than when each method is left into its own peculiar limitations.” The applications of spectrophotometric, electrochemical, FIA, chromatographic, and chemiluminescent methods in Kjeldahl applications will be the subject of the second part of this review, which also will cover aspects related to automation, sample preparation, working scale, and quality control.

This article forms part of an investigation into the applications of solution equilibria to titrimetric potentiometric determinations undertaken by Michałowski more than 30 years ago (Michałowski, 1981), and whose most recent references are Michałowski (2011), Asuero and Michałowski (2010), Michałowski and Asuero (2012), Michałowski et al. (2010a, 2010b, 2011, 2012a, 2012b, in press).

Commercial companies are mentioned in this review solely for the purpose of providing specific information. The use of trade, product, or firm names in this review is for descriptive purpose only, and does not imply endorsement by the University of Seville or by the Technical University of Cracow.

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