A Powerful Kjeldahl Nitrogen Method Using Peroxymonosulfuric Acid

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A method for the determination of Kjeldahl nitrogen uses peroxymonosulfuric acid (Caro's acid) as a strong oxidant for rapid sample decomposition without added salts or metal catalysts. Peroxymonosulfuric acid is formed in a hydrogen peroxide–sulfuric acid mixture and flows at 2 mL/min into a sample carbonized in concentrated sulfuric acid. The peroxy method obtains full recovery of nitrogen from nicotinic acid in 17.5 min. Decomposition occurs 5–10 times faster in the peroxy method than with conventional Kjeldahl methods. The digestion is followed by rapid colorimetric determination by an improved Nesslerization. A Digestion Index rates the difficulty of digestion of compounds and enables the digestion time and reagent to be minimized. Results with this method are accurate, fast, and comparable to standard Kjeldahl methods. The digest is not contaminated by salts or metal catalysts and can be further analyzed for other elements.

INTRODUCTION

In the conventional Kjeldahl method a sample is heated in concentrated sulfuric acid with salt and metal catalysts added to speed the decomposition of organic material. Organic nitrogen present is converted to ammonia, distilled into an acid solution, and titrated. The determination of nitrogen content is a frequently conducted analysis in industry and commerce, and numerous organizations have official methods. The crude protein content of foods and feeds are measured by the Kjeldahl method (AOAC, 1984). Total nitrogen is reported for fertilizer and plant material, and Kjeldahl nitrogen is determined in water and wastewater (APHA, 1985a). The classical Kjeldahl method involves a troublesome digestion requiring a large amount of equipment, reagents, and time. Conventional procedures use a 2-h digestion to ensure complete decomposition of organic material. Research in our laboratories has studied several oxidants to increase the speed, ease, and utility of the digestion.

A major objection to the Kjeldahl method is the time required for the digestion and distillation. Much work has been done to increase the rate of decomposition. Kjeldahl's original method added potassium permanganate to complete oxidation of the sample (Kjeldahl, 1883). Shortly after Kjeldahl published his method, two major improvements were proposed that used metal catalysts (Wilfarth, 1885) and potassium sulfate (Gunning, 1889). Since that time many oxidants and catalysts have been studied (Bradstreet, 1965).

Hydrogen peroxide has proven to be a rapid and powerful oxidizer of organic matter (Analytical Methods Committee, 1967). It has been used successfully in the 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 get quick conversion of nitrogen to ammonia (Kleemann, 1921). Koch and McMeekin 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 (Koch and McMeekin, 1924). A careful study of peroxide use proved the importance of precarbonization and multiple additions for full nitrogen recovery (Miller and Miller, 1948). More recent work used dropwise addition of hydrogen peroxide for the initial clearing, followed by a 30-min digestion to obtain good recoveries (Florence and Milner, 1979).

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The method of addition and digestion temperature are important when using hydrogen peroxide. The effective use of hydrogen peroxide requires that (1) there is a high residual concentration of peroxide (2) at high temperature (3) for sufficient time to decompose the organic material. Peroxide methods in the literature generally use the peroxide in an ineffective manner. The "peroxy" method in this study carbonizes the sample in concentrated sulfuric acid and then uses continuous-flow addition of a hydrogen peroxide–sulfuric acid reagent. This reagent contains peroxymonosulfuric acid (H_2SO_5, Caro's acid), which is a more powerful oxidant than hydrogen peroxide.

In conventional Kjeldahl methods it may be necessary to continue heating after the digest clears (the afterboil period) to obtain full nitrogen recovery. The digestion time required depends on the nature of the sample, temperature, salt concentration, and catalyst. Using the peroxy digestion method, nitrogen recovery curves are reported for a variety of samples. The Digestion Index—a rating indicating the difficulty of digestion—has been determined for each sample. With the Digestion Index, Kjeldahl determinations by the peroxy method can be customized for a sample, minimizing the time and reagents and eliminating the afterboil period.

After digestion the ammonia is determined by colorimetric analysis using Nessler's reagent. Several improvements have been made to the Nessler's method, one of which is greater reagent stability by removing any mercurous ion impurity in the reagent. Polyvinyl alcohol stabilizes the colloidal product of Nessler's reagent and ammonia, resulting in Nesslerized solutions with greater clarity and stability. A diluter/dispenser and flow-through spectrophotometer cell enable fast, accurate spectrophotometric measurement of the ammonia.

EXPERIMENTAL SECTION

Equipment. Kjeldahl digestions were done in 100-mL volumetric flasks on a 25–250-W disk element heater with a solid-state controller (Hach Model 21400 Digesdahl). A glass digestion manifold, see Figure 1, was used, which has an outer manifold with a side-arm vent to a water aspirator for fume removal and a capillary tube/funnel in an inner manifold to control the reagent addition (Hach 22142 manifold). A peristaltic pump with timer/controller also was used to add the digestion reagent. Digest aliquots were withdrawn, diluted with a polyvinyl alcohol solution, and Nesslerized by a Hach Model 42800 diluter/dispenser. For the colorimetric assay, absorbance measurements were made with a single-beam spectrophotometer at 460 nm with a 2.5-cm path length flow-through cell.

Chemicals. *Digestion Reagents.* Concentrated sulfuric acid was used for the carbonization step. Hydrogen per-

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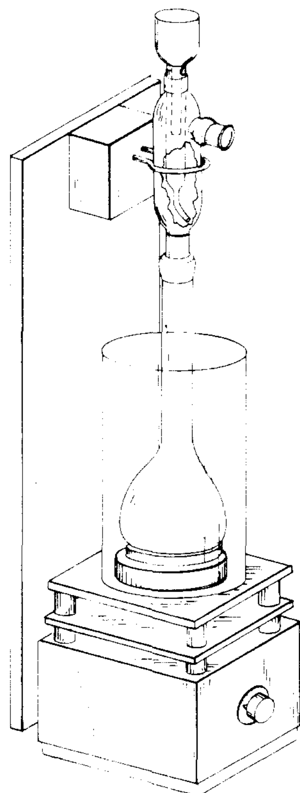


Figure 1. Capillary-flow digestion manifold, digestion flask, and heater.

oxide (50%) and concentrated sulfuric acid were premixed 4:1 to make the peroxy digestion reagent. The peroxy reagent was prepared by addition of 400 mL of 50% hydrogen peroxide to a 500-mL graduated cylinder and then slow addition of 100 mL concentrated sulfuric acid. After cooling, the reagent can be stored in an empty vented peroxide bottle. *Caution*! This reagent is a strong oxidizer and should be handled carefully. Eye protection should be worn while handling the peroxy reagent. Colorimetric assay used Nessler's reagent, Hach Co., catalog no. 21194 (treated during manufacture to remove mercurous ion). Polyvinyl alcohol, average mol wt 10 000, was used as a 0.1 g/L solution.

Samples. Reagent grade organic compounds such as amines, amino acids, nitrogen heterocycles, and porphyrins were used as received. The amino acids were obtained from Crescent Chemical Co. and Calbiochem-Behring, purity 99%. Nicotinic acid was recrystallized before use. Standard reference materials were obtained from the National Bureau of Standards (NBS) and the Association of American Feed Control Officials (AAFCO). All grain, cereal, and feed samples were ground in a chopper mill to a fine powder before digestion. Coarse samples such as hay were first ground in a food blender and then transferred to the mill.

PROCEDURE

Digestion. A 0.25–0.50-g sample was weighed and transferred to a 100-mL volumetric flask (the digestion flask), either with a funnel and brush or by folding the sample in the weighing paper and adding the folded packet to the flask. Two milliliters of concentrated sulfuric acid were added to the flask, and the flask was placed on the heater. The digestion manifold was placed on top of the digestion flask and connected to a water aspirator. Samples were allowed 2–5 min of heating on a 150-W heater to char. If any organic material was present, the sample became black as it carbonized. If the digest became dry, it was discarded and the digestion repeated using 4 mL of sulfuric acid. An appropriate volume (2–30 mL) of peroxy reagent was then added to the capillary funnel on the digestion manifold and flowed into the sample at 2 mL/min. When all the reagent had been added, heating was continued for 1–2 min to remove excess peroxide. The flask was then removed from the heat, cooled, and carefully diluted to the volumetric mark with deionized water. No change in the volume of Pyrex volumetric flasks has been observed after repeated heating and cooling cycles.

Nitrogen-Recovery Curves. Digestion and analysis were repeated for each sample with different volumes of peroxy reagent (varying digestion times). Typically, a sample would be digested for 2.5, 5, 7.5, 10, 12.5, and 15 min, corresponding to 5, 10, 15, 20, 25, and 30 mL of peroxy reagent. The nitrogen recovery vs. digestion (peroxy reagent flow) time was graphed to determine the 99% recovery time.

Colorimetric Assay. Typical procedure with a diluter/dispenser follows: A 0.400-mL aliquot of the digest was withdrawn and mixed with 24.60 mL of 0.1 g/L polyvinyl alcohol solution and 1.00 mL of Nessler's reagent. [The manual procedure for Nesslerization: pipet 0.4 mL of digest into a 25-mL mixing graduated cylinder, dilute to the 25-mL mark with 0.1 g/L polyvinyl alcohol, and mix; pipet 1.0 mL of Nessler's reagent into the cylinder, stopper, and mix; use a repeatable mixing action.] The solution was poured into a flow-through spectrophotometer cell and the absorbance recorded. On the basis of a linear calibration curve, the absorbance was converted into concentration (percent nitrogen or percent protein). The nitrogen content of the reagents can be determined and a reagent blank correction applied. Blank corrections were not used in this work due to the low reagent blank of less than 0.01% N.

Colorimetric Calibration. A series of standard ammonium chloride solutions with nitrogen contents equivalent to 0–100% protein were prepared (% protein = % nitrogen × 6.25). A 0.15276 g/L NH_4Cl solution has 40.0 mg/L of N and as analyzed in this procedure (0.25-g sample digested and diluted to 100 mL) contains 10% protein. Standards were analyzed by Nesslerization, and a best-fit linear calibration line determined. A series of standard solutions (Hach catalog no. 22204), containing 0–100% protein, 0–6% phosphorus, 0–12% calcium, and 0–3% magnesium were used for calibrations when the digest would be analyzed for phosphorus, calcium, and magnesium.

RESULTS AND DISCUSSION

Peroxymonosulfuric Acid Digestion. The peroxy method is a semimicro scale procedure, using 0.25–0.50-g samples in 2–4 mL of sulfuric acid. The procedure uses a carbonization time period, a peroxy digestion time, and a residual heating time. Heterocyclic nitrogen compounds

$$\text{sample} \xrightarrow[\text{carbonization time}]{} \xrightarrow[\text{peroxy digestion}]{} \xrightarrow[\text{residual heating}]{} \text{digest}$$

with a pyridine nucleus are difficult to break down and generally require long digestion times. The required digestion times for the refractory compound nicotinic acid (3-pyridinecarboxylic acid) have been used to compare many modifications of the Kjeldahl method. By the peroxy method, rapid and complete breakdown of nicotinic acid, and recovery of the nitrogen as ammonia, is demonstrated by the nitrogen-recovery curve in Figure 2. Complete recovery is obtained with a 5-min carbonization and 12.5 min of peroxy digestion time. Nitrogen recovery from nicotinic acid by the standard AOAC Kjeldahl method (AOAC, 1984) requires about 2 h. Recovery curves

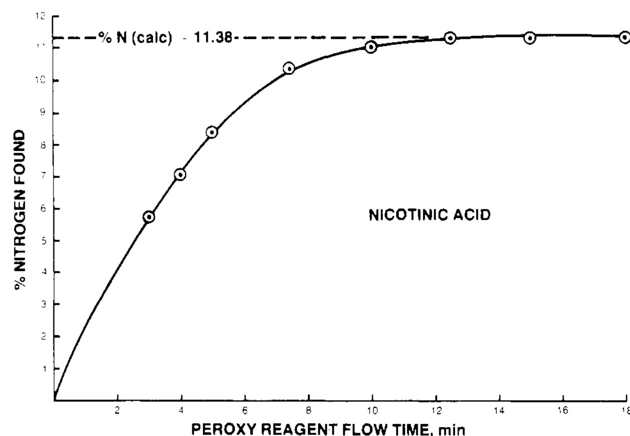


Figure 2. Nitrogen-recovery curve for nicotinic acid with continuous-flow peroxy digestion.

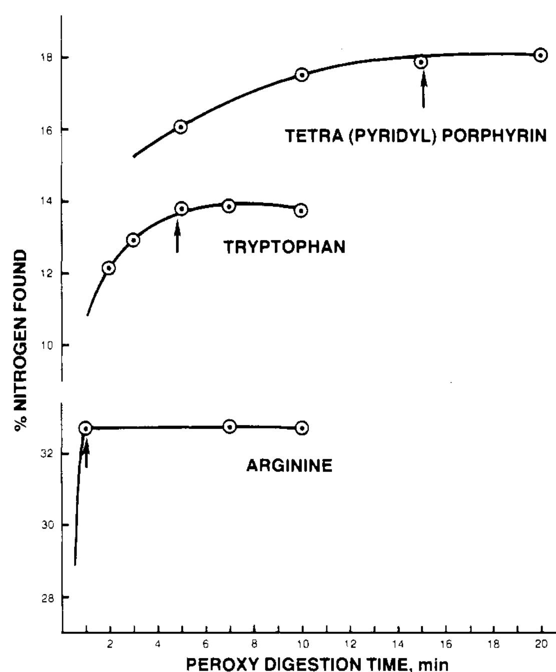


Figure 3. Nitrogen-recovery curves for easy, moderate, and difficult to digest samples. Arrows indicate 99% nitrogen recovery.

for an easy, a moderate, and a difficult to digest compound are shown in Figure 3. About half of the amino acids analyzed were classified as "easy", with peroxy digestion times of 3 minutes or less for full recovery. Conventional Kjeldahl digestion of tryptophan, another resistant compound, requires 55 min with an acid to salt ratio of 1 (McKenzie and Wallace, 1954). Peroxy digestion takes 5 min of carbonization and then 4.7 min of peroxy flow time to give full nitrogen recovery.

The digestion of refractory pyridine compounds using conventional procedures has been reported to require from 1 to 4 h, depending on the digestion conditions. Peroxy digestion of pyridinium *p*-toluenesulfonate is complete with 10 min of reagent flow time. It is evident the peroxy reagent gives rapid decomposition of organic matter. On the basis of an average of literature digestion times for these refractory compounds, the peroxy digestion appears to be 5–10 times faster than conventional Kjeldahl procedures.

The percent nitrogen calculated, percent nitrogen found, and Digestion Index for various samples are listed in Table I. The percent protein found and Digestion Index (DI) for food and feed samples are in Table II. A group of 20 food and feed samples including meat, grain, fish, and soy

Table I. Nitrogen Recovery with Continuous-Flow Peroxy Digestion

compound	% N calcd	% N found	% recovery	DI, min
arginine	32.16	32.71	101.7	1.0
aspartic acid	10.52	10.48	99.6	1.0
alanine	15.72	15.42	98.1	2.0
phenylalanine	8.48	8.56	100.9	2.5
serine	13.33	13.46	101.0	2.5
nitroaniline	20.28	20.28	100.0	2.7
valine	11.96	11.87	99.3	3.0
acetanilide	10.36	10.41	100.4	4.4
proline	12.17	11.95	98.2	4.6
tryptophan	13.72	13.68	99.7	4.7
methionine	9.39	9.41	100.2	6.3
histidine	27.08	27.75	102.4	6.5
pyridinium *p*-toluenesulfonate	5.58	5.61	100.5	10.0
leucine	10.67	10.85	101.7	10.5
tetraphenylporphyrin	9.11	9.10	99.9	11.9
nicotinic acid	11.38	11.43	100.4	12.5
lysine hydrochloride	15.34	15.27	99.6	15.0
tetrapyridylporphyrin	18.11	18.05	99.7	15.1

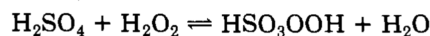
reference materials	% N calcd	% N found
NBS Orchard leaves #1571	2.76 ± 0.05	2.77
NBS Bovine liver #1577	10.6 ± 0.6	10.40
AAFCO Milk replacer #7925	3.28 ± 0.04	3.28
AAFCO Cattle supplement #7926	8.84 ± 0.08	9.26
AAFCO Swine feed #7928	2.52 ± 0.04	2.53

Table II. Nitrogen Recovery from Food and Feed Samples Using Peroxy Digestion

samples	% protein (found)	DI, min
barley	12.8	3.5
beef supplement	43.2	3.5
cattle feed	44.1	9.6
clover, sweet	19.3	4.2
corn silage	10.4	4.5
corn, sweet leaves	19.1	2.5
feather meal	85.3	5.5
fish meal	65.5	10.0
leather	52.1	5.0
meat meal	52.0	4.9
milk, instant dried	33.6	6.8
rice, enriched	8.1	8.5
sludge, aerobic	26.6	2.5
sludge, anaerobic	23.4	7.0
soybean meal	46.4	3.3
wheat mids	18.5	2.0
wheat, whole flour	16.7	2.0

were analyzed by two independent consulting laboratories, one using the peroxy method and the other the AOAC Kjeldahl method. The results compared by regression analysis (see Figure 4) have a coefficient of determination (r^2) of 0.9996 and a slope of 0.995 (±0.010 at 95% confidence limits). The sample means are 42.99% protein by AOAC Kjeldahl and 43.01% protein by peroxy determination, showing good agreement between the peroxy and AOAC methods.

Peroxy Reagent. An important aspect of the peroxy chemistry is the mixture of hydrogen peroxide and sulfuric acid to form peroxymonosulfuric acid (H_2SO_5). The peracid is known to be a stronger oxidant than hydrogen peroxide, and the peroxy reagent digests organic material faster than hydrogen peroxide alone. This is apparent with refractory compounds (see Figure 5, using pure hydrogen peroxide as reagent). Hydrogen peroxide and concentrated sulfuric acid react at room temperature to form peroxymonosulfuric acid.



$$H_2SO_4 + H_2O_2 \rightleftharpoons HSO_3OOH + H_2O$$

The equilibrium constant has been reported as 0.315

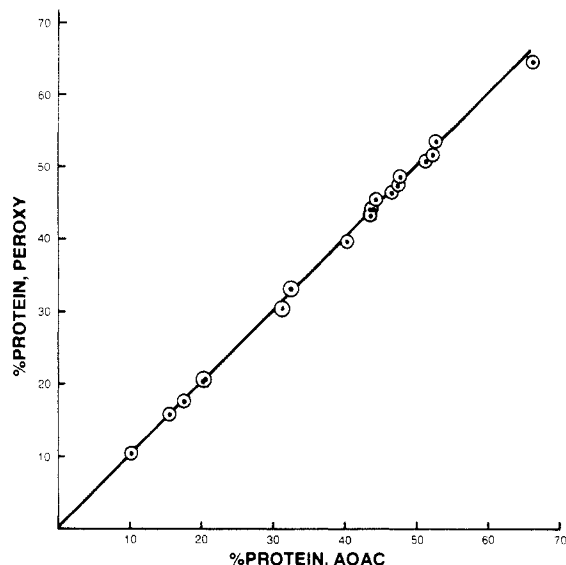


Figure 4. Comparative data for peroxy and AOAC Kjeldahl methods: $r^2 = 0.9996$, $a = 0.25 \pm 0.51$, $b = 0.995 \pm 0.010$.

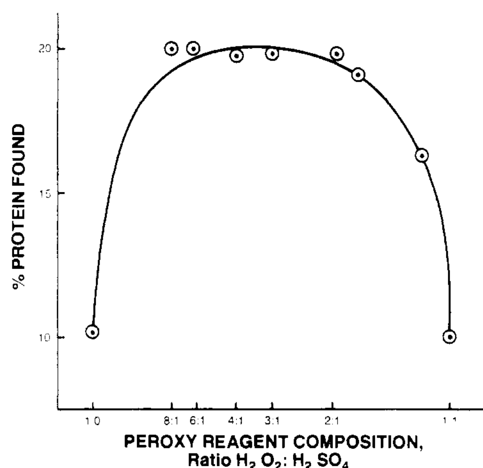


Figure 5. Peroxy reagent composition effect on nitrogen recovery from a nicotinic acid–succinic acid sample.

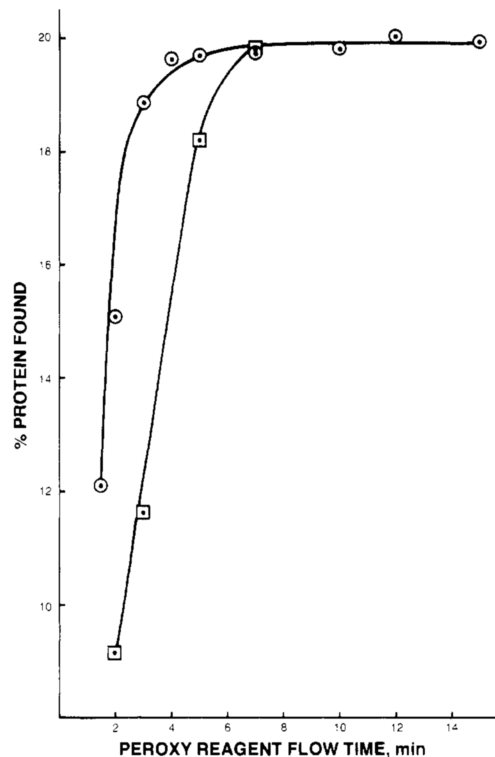


Figure 6. Nitrogen recovery from a nicotinic acid–succinate sample with ⊙ 50% peroxide and ▣ 30% peroxide reagent.

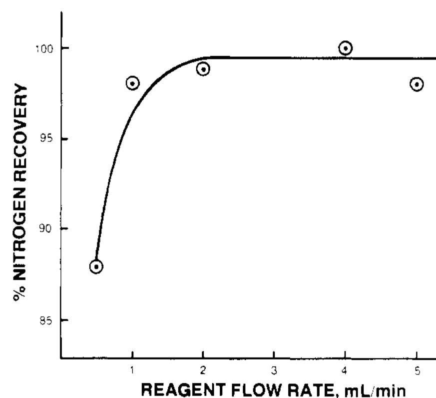


Figure 7. Peroxy reagent flow rate effect on nitrogen recovery from tryptophan.

(Ahrle, 1909) and 0.1 (Monger and Redlich, 1956). The H_2O_2 and H_2SO_5 concentrations in the 4:1 reagent were determined by a modification of the potassium permanganate–thiosulfate method (Greenspan and MacKellar, 1948). The H_2O_2 was titrated with permanganate to a pink end point and potassium iodide added. The H_2SO_5 oxidized iodide to iodine, and the iodine was titrated with phenylarsine oxide to a starch end point. The concentration of H_2SO_5 reached an equilibrium value of 0.68 M after 50 h. The equilibrium constant was found to be 0.32, in good agreement with Ahrle. Freshly mixed peroxy reagent, with an H_2SO_5 concentration of only 0.11 M, was used to digest nicotinic acid. The nitrogen recovery—and reagent effectiveness—was identical with that of peroxy reagent mixed for several days. The equilibrium constant for H_2SO_5 increases with temperature, and it is likely the rate of formation also increases. Apparently, peroxymonosulfuric acid is rapidly formed in the hot digest, which would explain the equal reactivity of the digestion reagent with different peroxymonosulfuric acid concentrations.

The composition of the peroxy reagent was varied from 1:1 50% hydrogen peroxide–concentrated sulfuric acid to pure 50% hydrogen peroxide. Nitrogen recovery from a 20% equivalent protein nicotinic acid–succinic acid sample is shown in Figure 5. The 4:1 peroxide–sulfuric acid reagent was an effective oxidant for all samples digested. Although good recovery can be obtained with a lower peroxide–sulfuric acid ratio, samples high in minerals and oxidizable species will require more peroxy reagent. In similar fashion, there is a loss of oxidizing strength when 30% hydrogen peroxide is used in place of the 50%. The resistant amino acid leucine requires 10.5 min of digestion with 50% reagent and 14 min with 30% reagent (see Figure 6). The 50% peroxide reagent gives faster digestions with difficult samples, requiring less reagent. The peroxy reagent flow rate has a slight effect on recovery from tryptophan, shown in Figure 7. While flow rate is not critical, the minimum effective flow rate will vary with the sample size and composition. Digestions for this study used 4:1 50% hydrogen peroxide–concentrated sulfuric acid at a 2 mL/min flow rate.

Continuous-Flow Addition. The physical method of adding the peroxymonosulfuric acid is important to using the oxidizing power of the reagent. An early method developed in our laboratory used the peroxy reagent added in two increments of 3 mL (Hach, 1981). Low nitrogen recovery with refractory samples using this "incremental" addition method is illustrated by data in Table III. The

Table III. Low Nitrogen Recovery with Incremental Peroxy Addition

sample	% N[a] calcd	% N[a] found
nicotinic acid	11.38	6.21
tryptophan	13.72	12.48
soybean meal	7.42	7.33

Nicotinic Acid [% N (calcd) = 11.38][b]

no. addns	% N (found)	no. addns	% N (found)
1	0.72	5	10.66
2	2.08	6	11.04
3	5.97	7	11.36
4	8.51		

[a] Two 3-mL additions of 2:1 50% H_2O_2–concentrated H_2SO_4. [b] 3-mL additions of 3:1 reagent.

recovery increased with the number of peroxide additions. Procedures in the literature vary in their use of hydrogen peroxide, with low recoveries reported by some workers using "incremental" addition (McKenzie and Wallace, 1954). Peroxide addition to a cold sample followed by heating decomposes the peroxide before the digest reaches temperature. Dropwise addition to a hot digest rapidly volatizes the peroxide. The method of reagent addition and the temperature of the digest control the residual peroxy concentration. The continuous-flow addition used in this method provides the high peroxy concentration in the hot digest needed for rapid digestion.

Digestion Temperature. Digestions usually were done with a heater temperature about 590 °C with 150-W heater input. The solution temperature during digestion was measured by a thermocouple in a sealed glass tube immersed in the digest (Figure 8). The digest temperature for a 0.25-g sample in 2 mL of sulfuric acid rises to 322 °C during the carbonization step. The slow, controlled reagent flow into the digest allows the temperature to remain high. In the conventional Kjeldahl digestion the severity of the decomposition reaction is governed by the temperature. Since some compounds do not completely decompose in boiling sulfuric acid, standard procedures raise the temperature as much as possible by salt addition. The temperature, controlled by the acid to salt ratio, is usually 370–410 °C. Low recovery may occur below 370 °C, and there is risk of nitrogen loss at temperatures above 410 °C. An advantage of the peroxy method is that complete decomposition is obtained at lower digestion temperatures.

Carbonization in Sulfuric Acid. The carbonization process provides a reducing environment that helps convert organic nitrogen to ammonia. Experiments have shown that the carbonization step is important for digestion of organic samples (Miller and Miller, 1948; McKenzie and Wallace, 1954). For many samples, 2 min of heating with a 150-W heater raises the digest temperature enough to char the sample and affect full recovery. A longer 5-min carbonization step is necessary for refractory compounds. Most samples contain enough carbon to char, but additional organic material such as weighing paper, succinic acid, or salicylic acid can be added with the sample to ensure carbonization. The reducing environment present is evident by the reduction and nitrogen recovery of the nitro group in nitroaniline, with no special pretreatment [20.28% N (calcd), 20.28% N (found), DI = 2.7 min]. A series of nitroaniline digestions with carbonization times of 0–10 min indicated that under the digestion conditions 2 min of charring was sufficient for complete nitrogen conversion.

Conventional procedures require a lengthy reducing pretreatment for nitro compounds. The regular peroxy

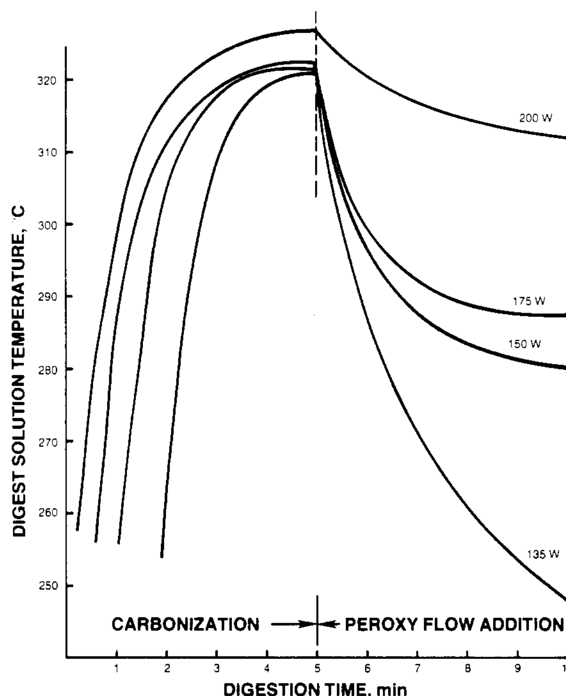


Figure 8. Digest solution temperature during capillary-flow peroxy addition with 135-, 150-, 175-, and 200-W heaters.

method recovered 85% of the nitro group nitrogen from *m*-dinitrobenzene. Addition of 0.25 g of salicylic acid to the dinitrobenzene and a longer 15-min carbonization time were tried. This procedure gave 97.6% recovery, and no further work was done to optimize the procedure. Amino nitrogen in glycine is apparently easier to reduce to ammonium ion than nitro nitrogen, as carbonization times from 0 to 5 min showed no difference in nitrogen recovery. Conversion occurred even with immediate peroxy reagent addition to the sample in sulfuric acid.

Digestion Index. The digestion time/reagent volume required for complete (greater than 99%) recovery for each compound can be determined from the recovery curve. The Digestion Index (DI) is the peroxy reagent flow time required, not including the carbonization time. The Digestion Index with the peroxy method ranges from 1 to 15 min, covering the easiest to the most difficult compounds (see Table I). In our work, compounds in which full nitrogen recovery was not obtained by 15 min did not give complete recovery by either peroxy or conventional Kjeldahl methods. Examples of such compounds include triazoles [1,2,4-triazole: 60.84% N (calcd), 29.80% N (found)] and pyrazolones [antipyrine: 14.88% N (calcd), 8.64% N (found)]. The conditions and time necessary for complete nitrogen recovery are simply expressed by the DI. The DI is especially useful to analysts performing multiple Kjeldahl determinations on the same or similar samples. Once the DI has been determined for a sample, the required digestion conditions are known. This minimizes the time and reagent requirements and helps ensure complete nitrogen recovery.

In the digestion process compounds may be degraded into intermediates more difficult to digest than the original compound. Standard practice is to continue the digestion for a period of time after clearing (the afterboil period). With the peroxy method and a Digestion Index, digestions are complete when peroxy reagent addition is finished. The afterboil period was not found to be necessary. The peroxy procedure generally followed allowed the digest to remain on the heater for 1–2 min after reagent flow stopped, to minimize the residual peroxy concentration. This

Table IV. Colorimetric Nitrogen Analysis[a]

Calibration Data (λ = 460 nm, 2.5-cm Cell)

% protein	abs,[b,e] A	% protein	abs,[b,e] A
5	0.03833	40	0.42933
10	0.08890	50	0.53740
20	0.20060	60	0.65103
30	0.31537	70	0.75953

Precision of Data

measurement		% RSD
diluter/dispens aliquot vol	0.4000 ± 0.008 mL	0.20
Nesslerized soln abs[c]	0.9677 ± 0.0030 A	0.31
protein determination[d]	15.94 ± 0.084%	0.53

[a] Nesslerization using polyvinyl alcohol and a diluter/dispenser. [b] Mean of three readings. [c] Triplicate nesslerization and measurement on a single digest. [d] Five digestions and analyses of a sample. [e] r^2 = 0.99993.

may not be necessary depending on the sensitivity of the subsequent analytical method to the peroxy species. Colorimetric analysis of calcium and magnesium was sensitive to residual peroxy species, presumably due to oxidation of the indicator. Longer residual heating does not cause nitrogen loss. The residual heating period was varied from 0 to 15 min in the analysis of leucine, with no effect on the nitrogen determined by Nesslerization.

Colorimetric Analysis. Digests were analyzed by Nesslerization. Nessler's reagent reacts with ammonia to form a colored colloidal species. The addition of polyvinyl alcohol—a colloid stabilizer—gives Nesslerized solutions with better clarity and stability. Digest aliquots are diluted with 0.1 g/L polyvinyl alcohol solution prior to addition of the Nessler's reagent. The intensity of the apparent color varies with the intensity of mixing the Nessler's reagent; therefore, the precision of the analysis increases with a mechanical diluter/dispenser. Typical nitrogen calibration data are given in Table IV. Calibration data with a coefficient of determination (r^2) greater than 0.9999 could be obtained with the diluter/dispenser. Data with an r^2 greater than 0.999 were obtained when manually pipetting, diluting, and dispensing the digest and reagent. In routine work the colorimetric analysis required less than 1 min and was performed in triplicate. The average precision for triplicate measurements on a single digest was 0.31% relative standard deviation. The precision for five repeat digestions and analyses on a feed sample was 0.53%. An interlaboratory study with organic nitrogen samples showed Nesslerization is more accurate and precise than titrimetric analysis (APHA, 1985b). Calibration curves with Nessler's reagent tend to vary slightly from time to time; for maximum accuracy standard solutions should be analyzed daily to maintain an accurate calibration.

The peroxy method does not add any mercury to the digest, which interferes with the Nessler's reagent. All determinations in this study used direct Nesslerization of the diluted digest without distillation. There is substantial time saving by not requiring distillation of the ammonia before determination. The short digestion time enables protein analyses to be completed in 15–20 min with most samples. The anticipated sample thruput for one analyst with a six-station block digester is 15 samples/h.

Colorimetric methods have been developed for phosphorus, calcium, magnesium, potassium, iron, copper, and manganese in the digest (Hach, 1984). These procedures have been modified from regular techniques for use with strongly acidic digests with residual peroxy species.

Analytical Standards. The Kjeldahl procedure is a difficult method, due in part to the troublesome digestion, the fact that not all forms of organic nitrogen are determined, and the inhomogeneity of real samples. The method can have a large degree of variability, as illustrated by the range in protein values from 45.06% to 47.55% reported by five major testing laboratories on a well-homogenized soybean meal sample. The Kjeldahl procedure actually consists of two separate processes: the digestion and the analysis. In this study two types of calibration were used. The colorimetric analysis was calibrated by generating a working curve from standard solutions of known nitrogen content. The performance of the digestion step was verified by digesting primary standard compounds of known nitrogen content and carrying them through the entire procedure. These standard compounds have high molecular weight, high purity, and varying difficulty of digestion. The compounds were carried through both digestion and analysis, and the performance of the entire analytical system was checked.

Table V. Primary Standard Compounds for Digestion

compound	% N	% protein	DI, min
ammonium chloride	26.18	163.62	0
ammonium *p*-toluenesulfonate	7.40	46.25	0
tryptophan *p*-toluenesulfonate	5.19	32.44	4.7
glycine *p*-toluenesulfonate	5.65	35.31	5.0
nicotinic acid	11.38	71.12	12.5
nicotinic acid *p*-toluenesulfonate	4.74	29.62	12.5

Primary standard digestion and analysis is an absolute method of assessing the accuracy of the analytical system which does not rely on comparative testing of samples by laboratories. If 100% recovery is obtained with a difficult standard compound, accurate results for other samples are likely. The use of pure compounds of known nitrogen content and known digestibility can be a valuable aid in verifying the accuracy of the technique. As standard compounds, the ammonium, glycine, and nicotinic acid salts of *p*-toluenesulfonic acid have been prepared and purified by recrystallization (Table V). These compounds provide three levels of digestion difficulty. Ammonium *p*-toluenesulfonate can be used as an ammonia standard with or without digestion, glycine *p*-toluenesulfonate is moderately difficult to digest, and nicotinic acid *p*-toluenesulfonate is a difficult sample.

The method of standard additions, where known amounts of nitrogen are added to a sample and analyzed to determine whether full recovery of the addition is obtained, should also be a viable technique for validating the analytical accuracy. However, in our work the standard additions technique was not used. The dual approach of colorimetric calibration and digestion accuracy verification works well for the Kjeldahl method, which consists of the two processes of digestion and ammonia analysis. Standard nitrogen solutions calibrate the colorimetric analysis, and primary standard compounds are digested and analyzed to verify the accuracy of the entire procedure.

CONCLUSION

Hydrogen peroxide mixed with sulfuric acid (peroxy reagent) forms peroxymonosulfuric acid and provides a powerful and rapid means of digesting samples for determining their nitrogen content. Sample carbonization in sulfuric acid and continuous-flow addition of peroxy reagent have been found the best procedure for accurate results using hydrogen peroxide. Digestion times for refractory compounds are reduced from 2 h to 15 min or less.

Another advantage of this method is that the digests are not contaminated by salts or metal catalysts. The digest can be analyzed for other species in addition to nitrogen such as phosphorus, calcium, magnesium, and metals. The need for distillation is eliminated. An improved Nesslerization procedure gives precise ammonia analyses

quickly. This method requires less equipment, reagents, and time than conventional Kjeldahl procedures.

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Registry No. N_2, 7727-37-9; H_2SO_5, 7722-86-3; H_2SO_4, 7664-93-9; H_2O_2, 7722-84-1; arginine, 74-79-3; aspartic acid, 56-84-8; alanine, 56-41-7; phenylalanine, 63-91-2; serine, 56-45-1; nitroaniline, 29757-24-2; valine, 72-18-4; acetanilide, 103-84-4; proline, 147-85-3; tryptophan, 73-22-3; methionine, 63-68-3; histidine, 71-00-1; pyridinium p-toluenesulfonate, 24057-28-1; leucine, 61-90-5; tetraphenylporphyrin, 917-23-7; nicotinic acid, 59-67-6; lysine hydrochloride, 657-27-2; tetrapyridylporphyrin, 16834-13-2; polyvinyl alcohol, 9002-89-5; ammonia, 7664-41-7.

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In Vitro Assessment of Zinc Binding to Protein Foods as a Potential Index of Zinc Bioavailability. Comparison of in Vitro and in Vivo Data

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An in vitro equilibrium dialysis test for estimating the strength of zinc binding to protein foods was developed for predicting zinc bioavailability. Soy flour, soy concentrate, casein, and dried egg white were labeled with $^{65}ZnCl_2$ before dialysis. The conditions included 24-h dialysis at pH 7.4 against 0.05 M tris(hydroxymethyl)aminomethane buffer (Tris), Tris plus 0.01 M L-histidine hydrochloride (Tris–His), and Tris plus 0.01 M Na_2EDTA (Tris–EDTA). Dialyzate and retentate ^{65}Zn were measured. The protein foods retained ^{65}Zn in the following decreasing order according to treatment: Tris > Tris–His > Tris–EDTA. The bioavailability of residual ^{65}Zn in casein, egg white, soy concentrate, and soy flour after each buffer treatment was determined by giving single doses of the protein foods to young Japanese quail. For these protein foods, the best agreement between in vitro and in vivo data was with Tris–His-dialyzable ^{65}Zn values and the whole-body ^{65}Zn retentions from the labeled casein and egg white (no treatment). The data suggest that this in vitro test could be useful for preliminary assessment of zinc bioavailability of protein foods.

The extent of a nutritional concern with low bioavailability of zinc from plant seed foods is presently not well-defined but would be expected to increase with expanded use of food products made from soybeans and other seeds to extend and/or replace traditional protein foods. O'Dell and Savage (1960) showed that phytic acid in isolated soybean protein contributed to low bioavailability of zinc. Evidence was presented by Oberleas et al. (1966a,b) that insoluble and nonabsorbable zinc–phytate complexes were formed in the gastrointestinal tract of rats, which rendered the zinc unavailable for absorption. Increased calcium accentuated the decreased zinc bioavailability by forming calcium–zinc–phytate complexes that were less soluble than zinc–phytate or calcium–phytate alone. Reinhold and co-workers (1976), in an in vitro study, found that zinc bound to the fiber of wholemeal breads remained unavailable.

There have been few in vitro studies dealing with the strength of zinc binding to protein foods. Kratzer et al. (1961) shook an isolated soybean protein homogenate with $^{65}ZnCl_2$ solution and then centrifuged it; the radioactivity of the supernatant was assumed to be the amount of ^{65}Zn not bound by the protein. This method was rapid, convenient, and reproducible for estimating the amount of zinc bound by insoluble, isolated soybean protein (Allred et al., 1964). Lease (1967) developed an in vitro digestion technique, which simulated the initial digestive processes of the chick. With this procedure, ^{65}Zn in soybean meal was bound as a water-soluble, dialyzable complex and was shown to be readily absorbed under the conditions present in the intestinal tract of chicks. However, when casein was used with this technique, little zinc dialyzed from the

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