High-Throughput Analysis of Total Nitrogen Content that Replaces the Classic Kjeldahl Method

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A high-throughput method for determination of total nitrogen content has been developed. The method involves decomposition of samples, followed by trapping and quantitative colorimetric determination of the resulting ammonia. The present method is rapid, facile, and economical. Thus, it can replace the classic Kjeldahl method through its higher efficiency for determining multiple samples. Compared to the classic method, the present method is economical and environmentally friendly. Based on the present method, a novel reactor was constructed to realize routine high-throughput analyses of multiple samples such as those found for pharmaceutical materials, foods, and/or excrements.

Keywords: *High-throughput total nitrogen determination; colorimetric analysis; Kjeldahl method; reactor for multiple samples*

INTRODUCTION

Determination of total nitrogen amounts is still very important in food and nutrition science, in addition to the quality control of pharmaceutical products especially proteins. For this purpose, the Kjeldahl method has been widely used since 1883 (*1*); that is, samples containing nitrogen compounds are decomposed by heating with concentrated sulfuric acid. The resulting ammonium sulfate, diluted with water, is heated with concentrated sodium hydroxide to release ammonia, which is titrated to give quantitative data. The whole procedure is carried out in glassware specially designed for this purpose. Several improvements have been realized for smaller amounts of samples by the use of more sophisticated glassware, which is both fragile and costly. Additionally, the decomposition procedure is carried out at high temperature and generates toxic gas. Thus, the whole apparatus must be placed in a draft chamber that occupies a relatively large space. Because of the relatively tedious procedure, it is not practical to analyze a large number of samples. As a replacement of this Kjeldahl method, elemental analyses can be performed, but such instrumentation is even more costly. The present paper describes a novel method for determining the total nitrogen content in a high-throughput manner, which can replace the classic Kjeldahl method. For this purpose, we have designed a novel reactor that allows for the routine analyses of multiple samples.

EXPERIMENTAL SECTION

Materials. The chemicals used were analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan) or Kokusan Chemical Works (Tokyo, Japan). They were used without further purification. Water was purified through MilliQ (Millipore, Bedford, MA). Potassium sulfate and copper sulfate (9:1 w/w) were mixed well to accelerate decomposition. For decomposition, sulfuric acid was mixed with 30% hydrogen peroxide (1:2 v/v). For coloration, the following two reagent systems were prepared: Reagent 1 consists of phenol (1 g), which is toxic in contact with skin so that gloves should be used in handling, and sodium pentacyanonitrosyl ferrate dihydrate (5 mg), which were dissolved in water to 100 mL. Reagent 2 was prepared as a 10% solution of sodium hypochloride (1.0 mL) and sodium hydroxide (1.5 g) diluted with water to 100 mL. The protein diet standard sample used was P/N 1548a, Typical Diet (lot 792404) and was obtained from the U.S. Department of Commerce, National Institute of Standards and Technology (Gaithersburg, MD). All-bran and soybean powder were purchased from local outlets. Excrements used for the present analysis were generous gifts from H. Shinohara, Tohoku University, and M. Fuchigami, Tokyo University of Agriculture.

Instruments. Samples were placed in a glass decomposition vial, which is marked at 10-mL internal volume, with a screw cap and septum (18 mm i.d. × 160 mm). For high-throughput decomposition analysis, the vials containing the samples were placed in a reactor designed for this purpose that has a shaking component (up to 1000 rpm), a heating unit (aluminum block, temperature up to 210 °C) for the bottom of the vials, and a cooling unit for the upper part of the vials that was connected to a circulation unit for chilled liquids. The reactor constructed for the present study has 25 positions for vials to perform 25 samples simultaneously. The schematic drawing of the present system is illustrated in Figure 1. The reaction conditions can be adjusted by a microprocessor connected to the system. Colorimetric analyses were performed with a model MPR-A4i (Tosoh Co. Ltd, Tokyo, Japan).

General Procedure. The sample (20 mg) and accelerator (200 mg) were placed in the above glass decomposition vial, and a mixture of sulfuric acid and hydrogen peroxide (1 mL) was added. After the vial was sealed with a cap and the mixture was shaken for a short time using a Voltex, the tube was placed in the above reactor, in which the heating unit was held at 200 °C and the cooling unit was adjusted to 0 °C. After 15 min shaking decomposition was terminated. When the sample was carbonized, decomposition was incomplete. In such cases, additional hydrogen peroxide (100 μL) was added, and

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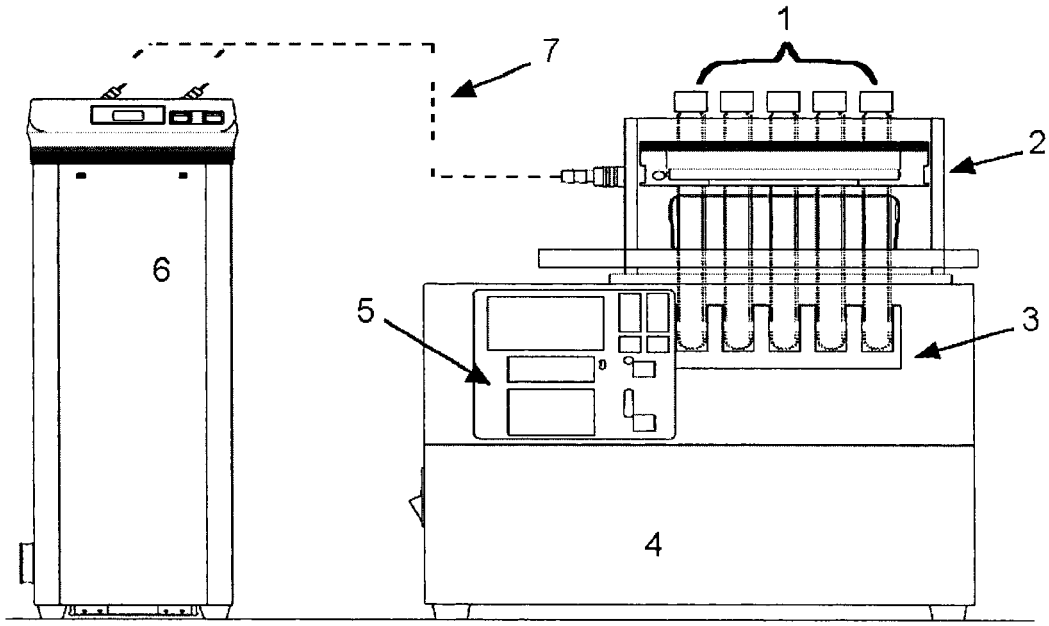

Figure 1. Schematic drawing of the present high-throughput sample decomposer: 1, sample vials; 2, cooling unit; 3, heating unit; 4, shaking unit; 5, control panel; 6, cooler; 7, tube for circulation of cold media.

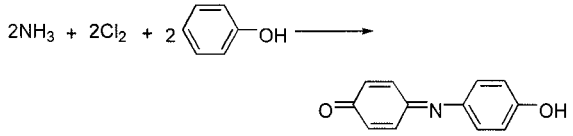

Figure 2. Colorizing reactions in the present procedure.

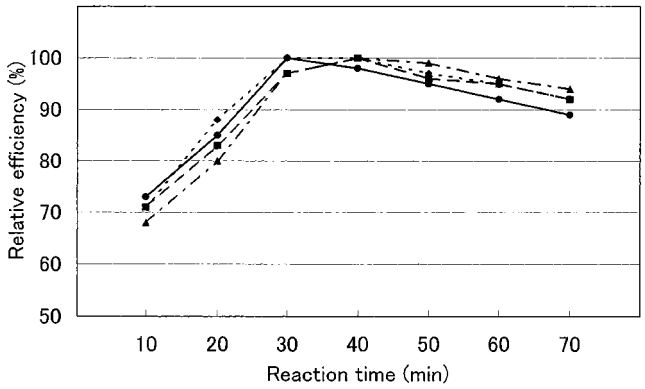

Figure 3. Optimization of reaction temperature for colorization. The maximum absorbance obtained in the experiments is calculated as 100%. 30 °C (◆), 40 °C (■), 50 °C (▲), 60 °C (●).

a further 15-min decomposition was used. The mixture was cooled to room temperature and neutralized with 1 M sodium carbonate (generally 1.7 mL was required). The remaining excess hydrogen peroxide was removed with manganese dioxide (100 mg) and then diluted to 10 mL with water. The resulting solution was further diluted to 5-, 25-, and 50-fold samples, and 100 μL of each was pipetted into a 96-well titer plate (Falcon #3075, Becton Dickinson, Lincoln Park, NJ), in which each well was filled with 50 μL of *n*-heptane before sample loading. The above reagent 1 (50 μL) and reagent 2 (50 μL) were added with gentle mixing at 50 °C for 40 min. Colorimetric analysis was then carried out at 640 nm using the plate reader described above.

RESULTS AND DISCUSSION

For the decomposition of the samples, a mixture of sulfuric acid and 30% hydrogen peroxide was used that

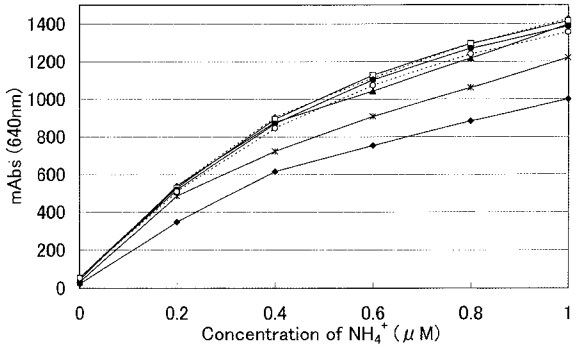

Figure 4. Optimization of reaction time for colorization at 50 °C: 10 min (◆), 20 min (∗), 30 min (▲), 40 min (◇), 50 min (□), 60 min (●), 70 min (○).

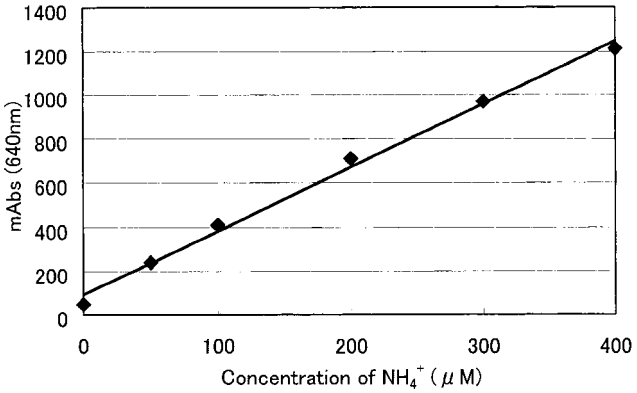

Figure 5. Calibration profile. Different concentrations of ammonium chloride solution were analyzed.

was stable for 2 days under dark and cool conditions. The ratio of the mixture should be between 1:1 and 1:4 v/v. The multiple-sample decomposing reactor was constructed for the present purpose. We preferred the Voltex shaking system rather than magnetic stirring because of its efficiency and easy use. The present apparatus permits the decomposition of resistant samples such as those containing fibrous material within 30 min. When samples are resistant to decomposition, the

Table 1. Nitrogen Content of Typical Diet[a]

analysis no.	1	2	3	4	5	6	avg	CV	standard deviation
nitrogen (%)	3.04	2.98	3.04	3.04	2.93	3.04	3.02	1.72	0.052

[a] Lot 792404, nitrogen content 3.03%, from the U.S. Department of Commerce, National Institute of Standards and Technology (Gaithersburg, MD).

Table 2. Nitrogen Content of Two Food Samples in Four Different Runs

sample	value indicated on package (%)	1 (%)	2 (%)	3 (%)	4 (%)	avg (%)	CV	standard deviation
roasted and ground soybeans	35.5	35.1	35.5	34.9	35.8	35.3	1.13	0.40
All-bran	13.0	13.2	14.3	13.8	13.3	13.7	3.92	0.51

sample is seen to become carbonized. In such cases, only sulfuric acid (0.5 mL) was used for decomposition initially, but after 15 min of shaking with bottom heating and top cooling, hydrogen peroxide (0.6 mL) was added, and complete decomposition was achieved by heating at 200 °C for a further 10 min. When necessary this procedure was repeated twice. The remaining hydrogen peroxide should be removed by addition of manganese dioxide as the excess H_2O_2 can affect the colorimetric analysis. Neutralization should be carried out slowly to trap all of the ammonia generated, as rapid neutralization often gives lower values through loss of ammonia gas. Beginning with the colorimetric analysis described in ref 2, we improved the procedure, especially through the use of microtiter plates. The coloration reagent 1 can be stored for ca. one month under cool and dark conditions, although it is recommended that reagent 2 be freshly prepared as the effective chlorine concentration should be 0.05−0.1% to give optimum sensitivity. The colorizing reaction is illustrated in Figure 2. *n*-Heptane was used to avoid diffusion of ammonia gas from the titer plate wells. This organic solvent can be replaced by any solvent that has no absorption at 640 nm, is not water miscible and does not affect the plastic titer plate. We used *n*-heptane, which is of low cost, nontoxic, and easy to obtain in high purity. With regard to the stability of the dye, the titer plate was treated at low temperature. Although determination using the plate reader proceeded very rapidly, we optimized the coloring reaction caused by ammonia ions at different temperatures and reaction times. As shown in Figure 3, the maximum efficiency (concentration of ammonia ion was 0.40 mM) can be seen at 30−50 °C for a 30−40 min reaction time. The optimization was further carried out at 50 °C with different times and concentrations of ammonia ion, as summarized in Figure 4. No significant difference is observed between 40 and 50 °C. Thus, the optimal conditions for the coloring reaction were 40−50 °C with a reaction time of 30−40 min. Under these conditions, the calibration profile is as shown in Figure 5. Here, different concentrations of standard ammonia solutions (50 μL) prepared by dilution of 2 mM ammonium chloride solution were analyzed after decomposition by the same procedure as used for the protein samples. They showed a correlation coefficient of 0.9953, thus confirming the high reliability of the present analytical method. Additionally, the standard reference material (P/N 1548a, Typical Diet) with 3.03% nitrogen content was analyzed. The analysis was carried out six times. As indicated in Table 1, the results were very close to this value and showed only small deviations. Thus, the present method is reliable and can replace conventional methods.

Table 3. Determination of Nitrogen (%) in Excrements of Individual Hamsters (Numbered)

	1	2	3	4	5	6	7	8
triplicate 1	3.30	3.45	3.17	3.46	2.99	2.98	3.05	3.33
triplicate 2	3.25	3.47	3.24	3.37	2.95	2.94	2.95	3.43
triplicate 3	3.28	3.45	3.22	3.43	2.98	2.97	2.99	3.39
average	3.28	3.46	3.21	3.42	2.97	2.96	3.00	3.38

As applications of the present method, two different food samples (20 mg), roasted and ground soybeans (Kawamitu Trading Co. Ltd) used by a Japanese delicatessen and All-bran (Kellogg's Japan Co. Ltd.), were analyzed. The analysis was carried out four times, and the results are shown in Table 2. The results obtained were reproducible and corresponded to those indicated on the package. For All-bran, which contains a lot of fibrous material, grinding was found to be the key to obtaining reproducible results.

Nitrogen content often reflects the efficiency of protein uptake through digestion. Hence, such analyses are particularly important in feeding. The excrements (20 mg) of eight individual hamsters were collected, dried, milled, and analyzed by the present method. Table 3 shows the results for triplicate analyses for each animal. The classic Kjeldahl method requires 1−2 g of excrements, whereas the present method requires much less (1/50−1/100 times) and is considerably quicker in both drying and nitrogen determination. As indicated in this table, individual differences reflect the digestive functions of each animal rather than differences for the same animal. Thus, the method is reliable.

In conclusion, we have developed a rapid, economical, sensitive, and reproducible quantitative analytical system for nitrogen content determination. Additionally, use of the apparatus described above does not require fume hoods. Thus, the method is relevant and can replace the classic Kjeldahl method. As it has high-throughput capabilities, this is a powerful tool for the analysis of digestion efficiency of feed in the field of animal husbandry in addition to quality control of protein samples. Using a 25-position reactor, the method allows more than 250 samples to be run daily. The present method and apparatus, designated the Nitro-Ace System, will be commercialized.

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