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Protein Nitrogen Determination by Kjeldahl Digestion and Ion Chromatography

Hsiaoling Wang, Nagarani Pampati, William M. McCormick, Lokesh Bhattacharyya*

Division of Biological Standard and Quality Control, Office of Compliance and Biologics Quality, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland 20993

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ABSTRACT

We report development and validation of a simple, rapid, and accurate method for the quantitation of protein nitrogen, which combines Kjeldahl digestion and ion chromatography with suppressed conductivity detection and requires nanomolar amount of nitrogen in samples (≥10 μg protein). The mechanism of suppressed conductivity detection does not permit analysis of samples containing copper (present in Kjeldahl digestion solution) and aluminum (present in many vaccines as adjuvants) due to precipitation of their hydroxides within the suppressor. We overcame this problem by including 10 μM oxalic acid in Kjeldahl digests and in the eluent (30 mM methanesulfonic acid). The chromatography is performed using an IonPac CS-16 cation exchange column by isocratic elution. The method reduces the digestion time to less than 1 h and eliminates the distillation and titration steps of the Kjeldahl method, thereby reducing the analysis time significantly and improving precision and accuracy. To determine protein nitrogen in samples containing non-protein nitrogen, proteins are precipitated by a mixture of deoxycholate and trichloroacetic acid and the precipitates are analyzed after dissolving in KOH. The method is particularly useful for biological samples that are limited and can also be applied to food, environmental, and other materials.

Introduction

The determination of total protein in pharmaceutical, biological, food, environmental, and other materials is performed widely by the Kjeldahl method.[1-5] The method indirectly quantifies the total protein content from nitrogen measurement and involves 3 major steps: digestion, distillation, and titration. The original method has been subject to many modifications and improvements. In its most recent form, the samples are digested in sulfuric acid/potassium sulfate in the presence of a catalyst to quantitatively convert nitrogen in proteins to ammonium sulfate. The solution is then distillated in the presence of excess of sodium hydroxide and the liberated ammonia is absorbed in an acid, which is titrated to quantitate ammonia. The method is time consuming and requires large sample quantities. For example, an experienced chemist would spend about 10 h for determination of nitrogen using the manual micro-Kjeldahl method, which typically requires about 0.1-0.7 mg nitrogen (0.6-4.0 mg protein) per sample.[5] Thus, the technique is often unsuitable for biological and environmental samples where only small sample quantities are available.

Ion chromatography (IC) has become a well-established technique for the quantitation of ammonium ion. The use of IC to determine total nitrogen as ammonium ion after Kjeldahl digestion can significantly improve the speed, accuracy, and precision of analysis by eliminating distillation and titration steps. In addition, because only nanomolar amounts of ammonium are required for analysis,[6,7] the amount of sample required for the digestion can be significantly reduced, which is of critical importance for biological and environmental samples. The IC method for the determination of ammonium ion typically involves separation by cation-exchange chromatography using a dilute solution of a strong acid followed by suppressed conductivity detection using a Cation Electrolytically Regenerated Suppressor (CERS) [formerly called Cation Self-regenerating Suppressor].[8] The effluent from the column passes through CERS before entering into the conductivity detector. In CERS, all anions, including the anion(s) present in the eluent, are replaced by OH^-, which forms H_2O with H^+ present in the eluent thereby essentially eliminating any background conductivity due to eluent and increasing the signal intensity (because ion conductance of OH^- is greater than all other anions).[9] Although this improves sensitivity and accuracy considerably, the technique poses a

* *Correspondence to:* Lokesh Bhattacharyya (Telephone: +1-240-402-9399; Fax: +1-301-595-1489).
E-mail address: lokesh.bhattacharyya@fda.hhs.gov (L. Bhattacharyya).

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significant problem to analyze Kjeldahl digests, which typically use copper sulfate as the catalyst. In the presence of hydroxide ion, Cu^{2+} forms sparingly soluble copper hydroxide that would precipitate to choke the eluent flow and clog CERS.

Another problem of using IC to analyze Kjeldahl digests is that the digests typically contain large molar excess of copper,[2-5] which reacts with ammonium ion to form a blue cupramine complex in neutral and acidic solutions.[10] Because copper sulfate is used in large excess, there will be no detectable free ammonium left in the solution when eluted with a dilute acid during IC. In addition, copper being a bivalent transition metal ion is expected to bind strongly to the cation exchange stationary phase, which could result in either irreversible damage to the column or require frequent column clean up.

Analyses of Kjeldahl digest using IC have been reported.[11-13] The authors attempted to circumvent the above mentioned problems by using catalysts other than copper. Jackson et al.[11] reported digestion with sulfuric acid-acetic acid mixture in the presence of either hydrogen peroxide or Hg^{2+} as catalysts, for determination of total nitrogen in food and environmental samples. Kjeldahl digests were analyzed by IC using indirect conductivity determination and obtained 5%-14% difference in results from the Kjeldahl method in samples containing 136-307 μg/mL ammonium ion. Although indirect conductivity determination does not require CERS, it requires large amount of samples for accurate quantitation due to high background conductance.[9] The reported assay range is too high for the analysis of most of the biological and environmental samples.

Furthermore, the use of mercury or hydrogen peroxide as catalyst requires either significant safety precaution due to the toxicity of mercury or complex and expensive equipment because of potential explosion due to the presence of H_2O_2.[12] de Medina et al.[12] reported determination of total nitrogen in water samples by oxidizing organic nitrogen to nitrate by potassium peroxodisulfate under high temperature and pressure using a Parr-type bomb and determining nitrate by IC with suppressed conductivity detection and obtained results that are comparable with the standard Kjeldahl method for river water containing 0.5-5.3 μg/mL of total nitrogen. Although the concentration range is suitable for analyzing biological samples, operation and maintenance of a Parr-type bomb is expensive and complex, and requires taking significant safety precautions.

Pontes et al.[13] reported analyses of soil and sandstone samples for the determination of total nitrogen, in which the traditional distillation step in the Kjeldahl method is replaced by distillation using a purge-and-trap ultrasonic system and ammonia in the distillate is quantitated using IC. The method used a calibration curve in the range of 0.03-0.80 μg/mL of ammonium. Although the method requires small amount of sample, it does not provide any advantage over the traditional micro-Kjeldahl analysis.

We report a simple and rapid method for the determination of nitrogen or protein concentration in biological samples, including vaccines, by a two-step procedure, Kjeldahl digestion followed by IC analysis of the digest after dilution. The method provides accurate and precise results in the range of 0.01-1.0 μg/mL of ammonium and requires very small amount of protein. The method eliminates the need for distillation and titration steps, thereby reducing the run time considerably and improving accuracy. We overcame the problem of having copper in the digest by simply adding oxalic acid to the Kjeldahl digest and the eluent. In the presence of oxalic acid, copper forms a stable copper bioxalate complex, $[Cu(OX)_2]^{2-}$.[14,15] Copper bioxalate being an anion does not bind to the column and is replaced by OH^- by CERS. In addition, complexation with oxalate prevents formation of cupramine complex and concomitant loss of ammonia. Furthermore, aluminum present in many vaccines as adjuvants also forms anionic complex with oxalate[15] and is removed by CERS, which permits application of the method to vaccines.

Many biological products contain non-protein nitrogen in excipients.[16] Because proteins constitute the active components, it is important to determine protein nitrogen in such product. In the food industry, it has also become critical to monitor protein nitrogen instead of total nitrogen after a few infamous food adulteration cases.[17] In this communication, we also report a simple procedure for the selective determination of protein nitrogen in samples that also contain non-protein nitrogen by precipitating protein with deoxycholate (DOC) and trichloroacetic acid (TCA) before Kjeldahl digestion and analyzing the precipitates after dissolving in KOH.

Materials and Methods

Proteins

Bovine serum albumin (BSA), human serum albumin (HSA), and ovalbumin (OVA) were of the highest purity available from Sigma-Aldrich (St. Louis, MO). Two "mock" lots of Anthrax vaccine were prepared in our laboratory, each by mixing contents of multiple vials from different lots of the vaccine from the approved manufacturer thereby removing identity traces of the lots. Five mock lots of influenza (Flu) vaccine antigens were prepared similarly by mixing monovalent bulks of the vaccines from approved manufacturers without noting the strain identifications, identity of the manufacturers, or protein concentrations. Each of these monovalent bulks contains influenza proteins from a strain but no live or attenuated viruses. The excipients present in these preparations are essentially the same as those present in actual vaccines. Thus, these samples can be considered as representatives of corresponding vaccines for our method.

Reagents and Standards

Ammonia standard (100 ppm, NIST traceable) was from Ricca Chemical Company (Arlington, TX). Methanesulfonic acid (MSA), sodium DOC, 0.1 M oxalic acid, and concentrated sulfuric acid were from Sigma Aldrich. Potassium sulfate, potassium hydroxide, TCA, 50% sodium hydroxide solution, copper (II) sulfate pentahydrate, and 0.01 N HCl were obtained from Fisher Scientific (Waltham, MA). The reagents needed for Lowry method are from Thermo Scientific (Waltham, MA). Deionized water (18.2 MΩ·cm) was used to prepare all prepared solutions.

Kjeldahl digestion solution is prepared as described previously[2] by adding 10 mL of a saturated solution of copper (II) sulfate to 500 mL of concentrated sulfuric acid, incubating for 7 days at room temperature so that excess copper sulfate is precipitated out, and collecting the clear solution from the top.

Equipment

The chromatography was performed using an ICS-3000 Ion Chromatography System, IonPac CS16 cation-exchange analytical column (5 × 250 mm), IonPac CG16 guard column (5 × 50 mm), CERS-500 (4 mm), and Chromeleon software for the system operation and data analysis, all from Thermo Scientific (Dionex, Sunnyvale, CA). The ion suppressor, CERS-500, is inserted in-line between the column outlet and the detector. The Micro-Kjeldahl Digestor and RapidStill I distillation unit were from Labconco (Kansas City, MO). An Agilent (Santa Clara, CA) 8453 UV-Visible Spectrophotometer was used to measure absorbance at 280 nm.