Improved Method for Manual, Colorimetric Determination of Total Kjeldahl Nitrogen Using Salicylate

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A manual colorimetric method for analyzing ammonia or total Kjeldahl nitrogen is described that eliminates irreproducibility due to variability in time between additions of reagents. Variability is negligible when trisodium phosphate is substituted in the buffer in place of the usual disodium phosphate and sodium hydroxide. The resulting procedure is very simple and reliable and uses salicylate, which is the most environmentally acceptable of the alternative colorimetric reagents. Only two reagents are used, the first containing trisodium phosphate, salicylate, and sodium nitroprusside and the second containing hypochlorite. The accuracy of the method was verified in two ways: first, by analyzing the digestate of three NBS standards and comparing the results to the NBS certified value; second, by analyzing the digestate of in-house samples and comparing the results using this procedure to the results using two other colorimetric procedures.

Manual colorimetric methods of measuring total Kjeldahl nitrogen (TKN) or ammonia are often criticized because they lack reproducibility or demand inordinate attention to detail such as precise timing at certain steps in the procedure and the requirement of vigorous stirring.

In this paper, we present a manual method to measure TKN that is very simple, is as sensitive as other published methods, and reduces irreproducibility. This method uses salicylate, which is considered the most environmentally acceptable of the alternative reagents. Its applicability in measuring TKN was evaluated for two different methods of digestion.

Historically, nesslerization has been the usual colorimetric method of measuring TKN. Nesslerization requires precise timing and generates waste containing mercuric compounds that are detrimental to the environment and costly to dispose of properly. Two reagents currently accepted as alternatives are salicylate (Baethgen and Alley, 1989; Fukumoto, 1982; Nkonge and Ballance, 1982; Xing-Chu and Ying-Quan, 1987) and phenol (Scheiner, 1976; Harwood and Kuhn, 1970; Hinds and Lowe, 1980). We chose salicylate because it is reputed to have higher sensitivity (Rowland, 1983; Crooke and Simpson, 1971), greater stability (Sutcliffe and Jones, 1968), and lower toxicity (Nkonge and Ballance, 1982). However, a problem with current salicylate and phenol methods is irreproducibility that is, at least partially, explained by variation in the timing of certain steps in the procedure (Bolleter et al., 1961; Fawcett and Scott, 1960; Smith, 1980; Weatherburn, 1967; Horn and Squire, 1967; Searcy et al., 1965).

In this paper, we present a new procedure using trisodium phosphate (TSP) as the buffer instead of a combination of disodium phosphate and sodium hydroxide. This procedure has several advantages: (1) The necessity of promptly adding the hypochlorite is very significantly reduced. (2) The acid content of the samples can vary widely without changing the amount of reagent being added or closely matching the acid content of the standards to the acid content of the samples. (3) The necessity of thorough mixing such as that obtained with a vortex mixer is eliminated. (4) The number of reagents being added is only two, which keeps the procedure simple and reduces the chance of error.

The method described here was used to analyze nitrogen of both plants and animals after digestion by either of two methods: acid−salt digestion with a block digestor (Smith, 1980) and acid−hydrogen peroxide digestion as described by Hach (Watkins et al., 1987; Hach, 1987). The validity of the procedure was verified by analyzing NBS standards (tomato leaves, pine needles, bovine liver) and by comparing the results of the analysis of our normal samples to the results obtained using two other procedures.

EXPERIMENTAL PROCEDURES

Reagents. *Salicylate.* Using either no heat or very little heat, dissolve 32 g of sodium salicylate (anhydrous), 40 g of TSP (trisodium phosphate, sodium phosphate tribasic dodecahydrate, or $Na_3PO4\cdot12H_2O$), and 0.5 g of sodium nitrosylpentacyanoferrate(III) (sodium nitroprusside) in 1 L of water.

Hypochlorite. Add 50 mL of commercially available bleach such as Clorox containing 5−5.25% sodium hypochlorite to water and dilute to 1 L. This reagent has remained stable as long as 2 months when protected from light and kept at room temperature.

Nitrogen Standard. Prepare from stock solution of ammonium chloride (1000 mg of N/L) and dilute with water. Compare to commercially available nitrogen standards.

Procedure. The colorimetric procedure described in this paper can be used for determining dissolved ammonia for which no digestion is necessary or organic samples that require digestion. If digestion is required, use any method you may prefer. When digesting animal matter, we used a 40-tube block digestor available from Tecator (Smith, 1980), and when digesting plant material, we used the digestion apparatus available from Hach Co., Loveland, CO. (Watkins et al., 1987). The procedure for the block digestor is take 200-mg to 1-g samples; add 10 mL of concentrated sulfuric acid, 3.5 g of potassium sulfate, and 35 mg of selenium [Potassium sulfate and selenium were added in tablet form. The tablets are available from Fisher Scientific Co., Pittsburgh, PA, Catalog No. 13-159B; Kjeltabs ST or from Perstorp Analytical, Silver Springs, MD, Catalog No. PA 1527-0003 (Kjeltabs-Selenium)]; heat at 425 °C for 1 h or until clear; wait 10 or 15 min and carefully add water (Caution. Solution will get hot and, if water is added too quickly, will immediately boil.); mixing as

the water is added, to a final volume of 100 mL. Usually, a small amount of undissolved material remains. Allow the mixture to cool and settle and then decant enough solution to make any dilution that is needed. When digesting plant matter, we used acid−hydrogen peroxide digestion and followed the well-described procedure (Watkins et al., 1987; Hach, 1987) except that we used 5 mL of 50% hydrogen peroxide instead of the recommended 10 mL. Solutions were always clear and thoroughly digested. Our choice of digestion procedure was for convenience and we know of no reason why either digestion technique will not work with all types of organic matter.

For the colorimetric procedure, samples of dissolved ammonia that were not digested will usually not require any dilution. Samples of digested organic matter often require dilution to bring the concentration within the working range of this procedure. The dilutions made for samples we analyzed required 0.2−1 mL of digestate plus 10 mL of water. Place 0.2 mL of the ammonia-containing solution (diluted if necessary) in a test tube, disposable cup, or other suitable container. Add 4 mL of salicylate reagent followed by 1 mL of hypochlorite reagent. Wait a minimum of 12 min and measure the absorbance at 685 nm. A calibration curve is linear to 20 ppm. We recommend using disposable cups and lightly swirling the mixture after the addition of each reagent. If test tubes are used, the solution should be vortexed after the addition of each reagent. The solution is stable for at least 18 h. In our laboratory, no effort was made to control the temperature. However, two or more standards were analyzed with every batch of samples so that compensation could be made for any variation that might occur.

Comparison with Other Procedures. We compared the results using the procedure proposed in this paper to results using two other procedures, Nkonge and Ballance (1982) and Baethgen and Alley (1989). The working buffer and the salicylate−nitroprusside reagent of Nkonge and Ballance were prepared as described. The hypochlorite solution was prepared by taking 11 mL of a commercially available bleach containing 5−5.25% sodium hypochlorite and diluting to 100 mL with water. We took 0.2 mL of digestate, which had been diluted 11 times with water, and added to it 1 mL of working buffer and 0.4 mL of salicylate−nitroprusside reagent, vortexed the mixture, added 0.6 mL of hypochlorite, and vortexed it again. After the solution sat for 30 min at room temperature, we added 10 mL of water, vortexed the solution, and measured the absorbance at 660 nm. We found that it was very critical that the time between addition of salicylate−nitroprusside and the addition of hypochlorite be constant for all samples and standards. This was achieved by adding the hypochlorite reagent immediately after the salicylate reagent.

The procedure of Baethgen and Alley (1989) was used without modification.

RESULTS AND DISCUSSION

Importance of Timing. In our opinion, the most critical problem with current procedures for manual colorimetric analysis of nitrogen is the need for precise timing to eliminate variable results. Most researchers who have addressed this problem advocate a certain order of addition of reagents. The usual order is as follows: sample, buffer, salicylate (or phenol if phenol is being used), nitroprusside, and hypochlorite. Some of the reagents may be combined, and the order of addition may vary from one method to another. But, the single most critical factor is the time interval between the addition of sodium nitroprusside and hypochlorite while the solution is alkaline. This holds true even if these components are mixed with other reagents. Because of its importance, we have named this interval the critical time interval. Usually, the longer the critical time interval, the lower the absorbance of the final colored solution. This decrease in absorbance has been attributed to the alkaline decomposition of nitroprusside (Harwood and Kuhn, 1970).
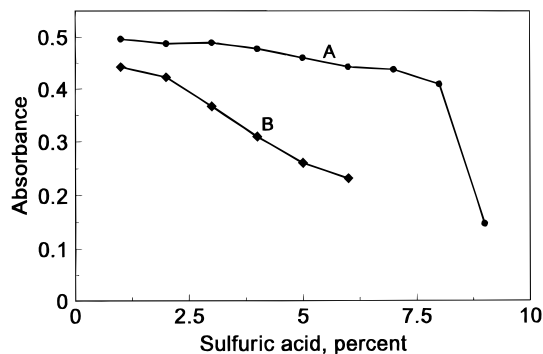

Figure 1. Absorbance of 9.0 mg/L nitrogen as a function of the percent sulfuric acid in the sample. Concentrations and quantities used are the same as described in the procedure except that, for (A), the salicylate reagent does not contain TSP but does contain 0.1 M sodium hydroxide and 0.1 M disodium phosphate. For (B), the salicylate reagent does not contain TSP, but the hypochlorite reagent contains 0.4 M sodium hydroxide and 0.4 M disodium phosphate in addition to the bleach.

A recommendation to eliminate the need for precise timing is to mix the buffer with the hypochlorite and add it last instead of first (Smith, 1980; Weatherburn, 1967). We tried this and can testify that it does eliminate alkaline decomposition. When the buffer was included with the salicylate and added first, the absorbance was 6.4% less for a solution with critical time 20 min compared to a solution with critical time 0 min. When the buffer was included with the hypochlorite reagent and added last, there was no difference in absorbance for a solution with critical time 20 min compared to critical time 0 min. (Concentrations are the same as those given in Figure 1.)

Although reversing the order solved the problem of decomposition, it was found to be unacceptable for three reasons. First, the absorbance of the color solution is more sensitive to the acid content when the buffer is added with hypochlorite (Figure 1). Second, if the buffer is mixed with hypochlorite, the maximum concentration of acid allowable in the sample is 2.8% compared to 11% or more, if the buffer is mixed with the salicylate. This is because acid in the sample must be neutralized with sodium hydroxide and disodium phosphate. Using the maximum amount of buffer that is soluble in the hypochlorite solution, the amount of acid that can be neutralized is 2.8%. If the buffer is dissolved in the salicylate, the maximum concentration of acid in the sample that can be neutralized is more than 11%. No attempt was made to determine a maximum acid concentration since 11% is the maximum one can expect using the usual procedure for Kjeldahl digestions. The final reason for not mixing sodium hydroxide and disodium phosphate with hypochlorite is that if the acid content of a sample is greater than 3%, a precipitate forms as the salicylate reagent is added to the sample. Thus, unlike others, we do not recommend changing the order of addition. Instead, we recommend changing the buffer from a combination of sodium hydroxide and disodium phosphate to TSP. Using TSP, decomposition, as determined by measuring absorbance, is 0.06%/min, which is a negligible level.

We hypothesize that sodium hydroxide is the cause of the decomposition of nitroprusside. Support for this hypothesis is provided by adding sodium hydroxide to the buffer (0.1 M TSP), increasing the rate of decomposition from 0.06 to 0.26%/min. Additional support for the hypothesis is the procedure of Nkonge and Ballance

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Table 1. Comparison of Relative Standard Deviation for Samples Mixed by Different Techniques[a]

mixing technique	rel std dev (%)
test tubes with no mixing	1.1 ± 0.4
test tubes with mixing	0.8 ± 0.3
cups with slight swirling	0.19 ± 0.07

[a] Results are the average of five samples, each sample replicated three times.

(1982). They used a buffer containing 1.35 M sodium hydroxide and 0.1 M disodium phosphate. We measured the rate of decomposition using their procedure and found it to be 0.57%/min, which is considerably higher than our procedure using TSP. Fawcett and Scott (1960) used the same buffer, but used phenol instead of salicylate, and reported that the decomposition rate was 1.5%/min. Baethgen and Alley (1989) used a buffer containing sodium hydroxide, disodium phosphate, and sodium potassium tartrate and, in addition, a slightly different procedure. Using their method with a critical time of 15 min, we observed no decomposition, which contradicts the hypothesis that sodium hydroxide is the cause of decomposition.

Mixing Requirement. The second critical problem with manual procedures for nitrogen is that thorough mixing of the solution is very important after the addition of each reagent; if the mixing is not adequate, a significant decrease in precision results. The procedure described in this paper does not require thorough mixing or vortexing after the addition of each reagent. A comparison was made using acid−salt digestate of gypsy moth pupae diluted 5% in addition to the dilution required by the digestion. The average relative standard deviation for five samples, each sample replicated three times, was determined for three different mixing techniques (Table 1). From this, it was concluded that sufficient accuracy could be obtained using disposable cups and vortexing was not necessary.

Extending the Concentration Range. The usual recommendation for extending the range of nitrogen concentrations is to dilute the sample before doing the final analytical test using smaller or larger samples as necessary to achieve the dilution desired. Using the procedure described in this paper, it is possible to extend the upper range of nitrogen concentrations that can be measured by diluting the hypochlorite reagent with water and adding greater volumes of the reagent. For example, if 5 mL of hypochlorite solution diluted one-fifth is used instead of 1 mL of undiluted hypochlorite, the range of nitrogen can be extended from 20 ppm to about 65 ppm. The calibration curve is linear to 65 ppm using this modification, and the time of color development was not increased, being about 12 min. A disadvantage is that the diluted hypochlorite is no longer stable and must be freshly prepared each day. The effect of the diluted hypochlorite is illustrated with the example of a 15 ppm standard analyzed 1 day after preparation of the diluted hypochlorite. This sample had an absorbance 6% less than the same standard analyzed immediately after preparation of the hypochlorite solution.

Verification of Accuracy. Results of analyses of digestate solutions were compared to results using two other procedures: Nkonge and Ballance (1982) with modifications as described in the procedure above and Baethgen and Alley (1989). Paired *t*-tests were made between the test method and each of the two alternatives, resulting in *p*-values pf 0.54 and 0.40, respectively.

Table 2. Nitrogen in Frass or Pupae Using the Procedure Described in This Paper Compared to Nitrogen Found Using the Procedure of Nkonge and Ballance or Baethgen and Alley[a]

			mg of N		
sample ID	type of sample	mg sampled[b]	this procedure	Nkonge & Ballance	Baethge & Alley
63	frass	236.07	19.2	19.3	19.3
63	pupa	629.59	17.8	17.8	17.6
61	frass	278.72	19.9	20.3	20.2
61	pupa	512.47	16.3	16.2	16.4
35	frass	660.12	52.3	52.5	51.4
35	pupa	2041.51	59.8	60.0	59.9
53	frass	1300.18	55.1	54.6	56.6
53	pupa	2713.03	91.7	90.4	92.5

[a] Each pupa sampled is one complete pupa. [b] Milligrams of sample digested for frass (insect excrement) is the dry weight, dried at room temperature, and represents total frass excreted during the life of the larva; for pupae, it is the live weight. Results are the average of three replications. Relative standard deviation for each procedure is as follows: this procedure 0.26; Nkonge and Ballance, 1.17; Baethgen and Alley, 1.03.

Table 3. Percent Nitrogen Found in NBS Standards[a]

reference material	this procedure	NBS value
tomato leaves (SRM 1573)	5.03	5.0
bovine liver (SRM 1577b)	10.4	10.6
pine needles (SRM 1575)	1.18	1.2

[a] Results are the average of three replications.

There are no grounds for rejecting the hypothesis that the methods are measuring the same quantity. Table 2 shows the value obtained for each sample by each analytical procedure.

Additional verification of the procedure was done by analyzing the NBS standards, tomato leaves, pine needles, and bovine liver. Samples (0.2 mg) of these were digested using the Digesdahl digestion apparatus from Hach Chemical Co. (Hach, 1987) following their direction, except that 5 mL of 50% hydrogen peroxide were used instead of 10 mL. We found that, using 5 mL, the solvent was clear and results were the same as when 10 mL was used. The digested solution was analyzed for nitrogen as described in this procedure. The results (Table 3) show good agreement with NBS values.

Reagent Composition. The initial concentrations of the reagents were taken from a previous work using an automated technique (Gentry and Willis, 1988), and tests were done to determine the optimum concentration of the compounds for this manual procedure. The concentration of salicylate was tested using a 16 ppm N standard, varying the concentration of sodium salicylate, and keeping the concentration of all other compounds as described in the procedure. A plot of absorbance vs molarity of sodium salicylate shows the optimum concentration of salicylate is between 0.15 and 0.4 M (Figure 2). We recommend a concentration of 0.2 M.

The concentration of TSP in the salicylate reagent depends on the concentration of acid in the sample being analyzed (Figure 3). As shown, the maximum concentration of sulfuric acid is about 2.5, 5.0, and 10.0% for TSP concentrations of 0.05, 0.1, and 0.2 M. Typical digestion procedures such as the two used in this paper result in digestate solutions having an acid concentration of about 10%. Biological samples having a low nitrogen content would have digestate not requiring further dilution and therefore require 0.2 M TSP in the salicylate reagent. Most biological material, such as that used in this paper, after digestion, have digestates
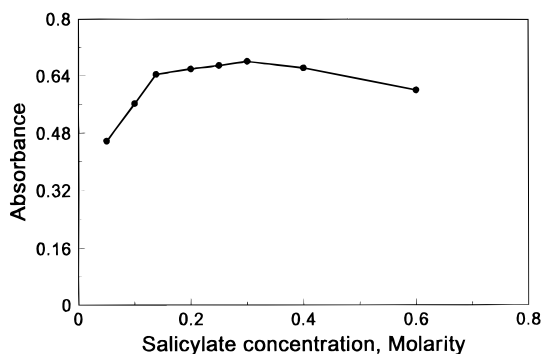
Figure 2. Absorbance as a function of salicylate concentration in the salicylate reagent. Nitrogen concentration is 15.8 ppm.
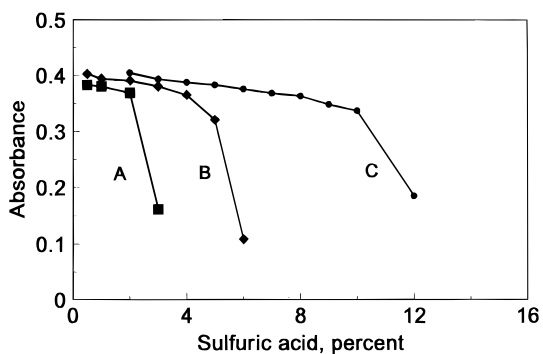

Figure 3. Absorbance of 9 mg/L nitrogen as a function of the percent sulfuric acid in the sample. Concentration of TSP is as follows: A, 0.05 M; B, 0.1 M; C, 0.2 M. In all cases, the TSP along with sodium nitroprusside and sodium salicylate were dissolved in the salicylate regent. Other reagents and the procedure are the same as described under Experimental Procedures. Each data point is the average of three replicate samples. The average standard deviation for all data points is 0.0019.

requiring 50% or more dilution. In these cases, 0.1 M TSP is the recommended concentration.

Prevention of Precipitation. For some samples, if the concentration of certain divalent or trivalent ions is sufficiently high, precipitation will occur. Citrate has been recommended as the best complexing reagent for elimination of precipitation (Gentry and Willis, 1988; Willis et al., 1993; Kempers and Kok, 1989). No precipitate formed for the digestate samples of animal and plant samples analyzed in this laboratory, and the measured concentration of nitrogen in the samples was identical whether or not citrate was used. Citrate was necessary when potassium chloride extracts of soil samples (Gentry and Willis, 1988; Willis et al., 1993) were analyzed. If precipitate does form, it is recommended that 40 g of sodium citrate dihydrate/L be added to the salicylate reagent. If sodium citrate is added, no problem is encountered with alkaline decomposition. Adding 40 g of sodium citrate/L to the salicylate reagent increases the rate of decomposition from 0.06 to 0.11%. This is still considerably less than the rate using other published procedures as described earlier.

CONCLUSION

We believe the most critical problem today with manual colorimetric procedures for ammonia or TKN is the lack of reproducibility. Use of TSP instead of disodium phosphate and sodium hydroxide solved the problem. Previously, it was thought that timing was the critical factor, but this change of buffers eliminates the need for precise timing in addition of reagents. It also is apparent that many scientists have ignored the problem and we feel that anyone publishing a paper describing a manual colorimetric method should address this problem.

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