An Improved Zinc Reduction Method for Direct Determination of Nitrate in Presence of Nitrite

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Experimental evidence, based on zinc reduction method, has revealed that currently used metal reduction technique for determination of nitrate in presence of nitrite provides an unreliable estimate for nitrate; **a**) slopes over linear range for nitrite and nitrate are strikingly different such that the slope for nitrite is about 17 times steeper than that for nitrate, **b**) the reduction process significantly reduces nitrite ($p < 0.01$) and **c**) absorbance values for nitrite and nitrate in combination are significantly lower than the sum of absorbance values obtained by separately measuring two analytes ($p < 0.01$). Modified technique using urea treatment followed by metal-reduction provides more accurate and direct method for nitrate determination in presence of nitrite. Samples containing nitrate and nitrite when added urea (0.1 mL of 2 % solution) and concentrated HCl (0.2 mL) per mL sample on incubation in boiling water bath for 10 min caused complete removal of nitrite without affecting recovery of nitrate compared to untreated standard nitrate ($p > 0.1$). Standard urea treatment removed at least 500 μg NO_2-N from the sample. Urea did not affect absorbance of NO_3-N; the value for 10 μg NO_3-N in presence of urea, 0.149 ± 0.002, did not differ from the value, 0.146 ± 0.002, obtained in absence of urea ($p > 0.1$). Nitrate recovery at 5, 8 and 10 μg NO_3-N in presence of varying concentrations of nitrite, 0.5 or 50 μg NO_2-N, with modified protocol has been overall 98 ± 1 per cent of basal nitrate levels.

Key Words: Nitrate, Determination, Zinc reduction, Urea treatment, Nitrite.

INTRODUCTION

The technique commonly used for nitrate determination is based on metal reduction of nitrate to nitrite followed by coupling of diazotized aromatic amine with a coupling agent. The reduction employs zinc[1,2], cadmium columns[3] or granules[4] or vanadium(III)[5]. These methods and several others for nitrate determination are considered semi-quantitative and not

exact[6]. The samples containing nitrate and nitrite are first analyzed for nitrite and then subjected to reduction process to convert nitrate into nitrite; thereafter the sample is assayed for total nitrite. The nitrate is determined indirectly as a difference between total nitrite following reduction and that before reduction with either metal reductants[3,5,7]. The current technique is based on two assumptions: (**a**) regression lines for two analytes are parallel assuming 100 % reduction of nitrate to nitrite and (**b**) metal reduction is selective for nitrate and does not affect nitrite. The technique would work well for nitrate determination in absence of nitrite, but is theoretically erroneous in presence of nitrite. Metal reduction is not specific for nitrate, because nitrite is also reduced to ammonia by metal reduction. Therefore, assaying nitrate in terms of nitrite in presence of nitrite would yield unreliable values. The rationale for the present investigation was accordingly mooted based on the theoretical considerations: (**a**) metal reduction is unlikely to be specific for nitrate, (**b**) interference caused by nitrite can be eliminated by selective removal of nitrite from the sample containing both nitrate and nitrite and (**c**) remaining nitrate can be then estimated directly by metal reduction. The investigations were accordingly designed to: (**a**) compare regression coefficients for nitrate and nitrite over their linear ranges; (**b**) find effect of reduction process on nitrite estimation, (**c**) compare absorbance values of mixtures of nitrate-nitrite subjected to metal reduction as compared to absorbance values obtained separately for the two analytes and (**d**) estimate nitrate in presence of nitrite by first rendering samples free of nitrite by a standardized urea treatment and then subjecting samples to metal reduction.

EXPERIMENTAL

The experiments were carried out at an ambient temperature of 20.9 ± 0.3ºC. The chemicals used were of standard purity. Double-distilled water was used for preparation of reagents, dilutions and for washing of glassware.

Stock nitrate and nitrite solutions: The stock solutions for nitrate and nitrite were made, respectively, 500 and 100 ppm (as nitrogen) in water using potassium nitrate and sodium nitrite and contained 0.02 % (v/v) chloroform as preservative. Working solutions were made by dilution in water as 100 ppm NO_3-N and 10 ppm NO_2-N from respective stock solutions.

Diazotization powder (DP): It contained barium sulfate 10 g, citric acid 7.5 g and sulfanilamide 0.5 g. The mixture was finely ground to a homogeneous powder and well stoppered in an amber-coloured glass bottle.

Reductant diazotization powder (RDP): Each 500 mg of diazotization powder was added 30 mg mixture of zinc dust and manganese sulfate

(1:5 ratio) thoroughly mixed to a homogeneous mass and well-stoppered in a blackened amber-coloured glass bottle.

Coupling reagent: The reagent was prepared by dissolving 200 mg of N-(1-naphthyl)ethylenediamine dihydrochloride ((NEDA) in 20 mL of 1% HCl. It was stable for at least 1 week when kept at refrigerating temperature when not in use.

Acetic acid solution: Glacial acetic acid was diluted in water to provide 20 % (v/v) solution.

Urea solution: It was prepared by dissolving 200 mg urea in 10 mL of water (2 %). The solution was stable for at least 1 month when stored at refrigerating temperature when not in use.

Nitrite determination: The technique employed for nitrite determination has been zinc reduction protocol (1) with some modifications. Each mL of nitrite sample was added one scoopful (454 ± 10 mg) of diazotization powder followed by 9 mL of acetic acid solution. The mixture was shaken well for about 1 min and then centrifuged at 6000 rpm for 5 min. The supernatant was filtered vide Whatman Filter No.1. Each 5 mL filtrate was added 0.2 mL of coupling reagent. The well-mixed sample was allowed to stand for about 20 min at room temperature and read spectrophotometrically at 540 nm.

Nitrate determination: The technique for nitrate determination has been identical to that for nitrite except reductant diazotization powder was used instead of diazotization powder to ensure reduction of nitrate to nitrite for diazotization and subsequent coupling.

Urea treatment for nitrite containing nitrate samples: To render nitrite containing nitrate samples free of nitrite, the following procedure yielded consistent and reproducible results. Each mL sample containing nitrate and nitrite was added 0.1 mL urea solution; mixed up and added 0.2 mL concentrated HCl. The well-mixed reaction mixture was incubated in boiling water bath for 10 min. This procedure was found to destroy at least 500 μg of NO_2-N per mL. Urea-treated samples were employed for nitrate determination. Standard nitrate solution and water blank were subjected to identical treatments.

Experiment 1: Absorbance values for nitrate and nitrite were determined to estimate regression coefficients for the two analytes using NO_2-N as 0.25, 0.5.1, 2 and 3 μg per mL and NO_3-N as 2.5, 5, 10, 20 and 40 μg per mL. The number of samples per each concentration was six.

Experiment 2: Standard samples in two sets, containing 0.5, 1, 2 and 3 μg of NO_2-N per mL, were treated with diazotization powder. One set was added zinc-manganese mixture (1:5 ratio) at two different concentrations, 30 mg and 60 mg, providing, respectively zinc 5 mg and 10 mg per sample. Zinc treated samples were compared with untreated samples, second set, to find effect of reduction process on nitrite estimation.

Experiment 3: Absorbance values for nitrate and nitrite were determined at various concentrations of NO_2-N (0.5 μg and 0.8 μg) and NO_3-N (2.5, 5 and 10 μg) separately and in combination using diazotization powder for nitrite determination and reductant diazotization powder for nitrate determination. The sum of individual absorbance values for nitrate and nitrite measured separately were compared with the values when the two analytes were present together.

Experiment 4: Samples of nitrite containing 100, 200, 300, 400, 500 and 600 μg NO_2-N per mL were subjected to urea treatment protocol as outlined already. The resultant samples were analyzed for residual nitrite if any.

Experiment 5: 10 μg NO_3-N was treated with 0, 2 and 10 mg urea under test conditions and nitrate recovery affected with urea treatment evaluated compared to untreated standard (0 mg urea) to optimize urea concentration.

Experiment 6: Samples containing 5, 8 and 10 μg NO_3-N per mL without or with 0.5 or 50 μg NO_2-N were subjected to urea treatment. Control sample, 1 mL water, was subjected to identical urea treatment. Per cent recovery of nitrate was estimated in samples containing nitrate and nitrite together compared to those containing nitrate alone.

RESULTS AND DISCUSSION

The protocol adopted for the investigation employed zinc-manganese sulfate reduction process[1]. The technique is simple, inexpensive, obviates use of toxic and hazardous cadmium metal and is more amenable to experimental control than use of cadmium- copper sulfate columns or granules. The original technique was modified to the extent that **a**) sulfanilic acid was substituted with a more stable aromatic amine *i.e.*, sulfanilamide, **b**) 1-naphthylamine, a listed carcinogen[3], was substituted with more widely used coupling agent NEDA and **c**) the coupling agent was added separately to avoid adsorption of azo-dye by the filter paper.

Results of Experiment 1 are presented in Table-1. NO_2-N was linear over 0.25 to 3 μg per mL while NO_3-N was linear over 2.5 to 40 μg per mL. As evident, the slopes for two analytes are quite different with regression estimate, b ± S.E., in terms of absorbance unit per μg NO_2-N as 0.258 ± 0.005 being about 17 times steeper than that for NO_3-N, 0.015 ± 0.001. The comparison of absorbance values at lowest detection limits for the analytes indicated that lowest detection limit for NO_2-N was 16-17 times more than that for NO_3-N. 2.5 μg NO_3-N was comparable to estimated 0.13 μg NO_2-N yielding mean absorbance value 0.034 and 0.25 μg NO_2-N was comparable to estimated 3.93 μg NO_3-N yielding mean absorbance value 0.059.

TABLE-1
COMPARISON OF ABSORBANCE VALUES AND REGRESSION
COEFFICIENTS FOR NITRITE AND NITRATE OVER
LINEAR RANGES

Analyte	NO_2-N		NO_3-N	
	μg	Absorbance*	μg	Absorbance*
Linear range	0.25	0.059 ± 0.002	2.5	0.034 ± 0.005
	0.50	0.116 ± 0.002	5.0	0.072 ± 0.003
	1.00	0.264 ± 0.005	10.0	0.156 ± 0.005
	2.00	0.463 ± 0.002	20.0	0.330 ± 0.004
	3.00	0.784 ± 0.011	40.0	0.604 ± 0.021
r	–	0.995	–	0.993
$b \pm SE$	–	0.258 ± 0.005	–	0.015 ± 0.001

*The absorbance values are mean $\pm$ SE of six observations each.

Experiment 2 (Table-2) revealed that nitrite too was reduced by metal reduction process. Reduction in nitrite estimate was both function of nitrite as well as that of metal reductant concentrations. At 5 mg zinc concentration, 0.5. 1.0, 2.0 and 3.0 μg NO_2-N basal concentrations were, respectively, recovered as 0.23 ± 0.01, 0.65 ± 0.01, 1.00 ± 0.06 and 1.55 ± 0.08 μg NO_2-N with *ca.* 35 to 50 per cent decrease in recovery from basal concentrations with mean regression coefficient of 0.500 μg NO_2-N decrease per μg NO_2-N taken (r = 0.99). At double zinc concentration (10 mg), 3 μg NO_2-N was reduced about 68 % showing a recovery of only 0.95 ± 0.06 μg NO_2-N. The decrease in nitrite estimate with metal reduction was highly significant (p < 0.01). This is expected since metal-reduction is non-selective. The nitrate gets reduced to nitrite while nitrite gets reduced to ammonia. Nitrite formed from reduction of nitrate is also likely to be reduced by metal reduction if contact time with the reducing metal is prolonged.

TABLE-2
EFFECT OF ZINC REDUCTION ON NITRITE ESTIMATE

Zinc added* (mg)	NO_2-N added (μg)	NO_2-N recovered (μg)**	Decrease in NO_2-N (mean, μg)
5	0.5	0.23 ± 0.01	0.27
	1.0	0.65 ± 0.01	0.35
	2.0	1.00 ± 0.06	1.00
	3.0	1.55 ± 0.08	1.45
10	3.0	0.95 ± 0.06	2.05

*Zinc added as dust with 5 times manganese sulfate in diazotization powder.
**The values are mean $\pm$ SE of six estimates each.

Experiment 3 (Table-3) revealed that sum of the absorbance values for nitrite and nitrate estimated separately were significantly higher than when the two analytes were put together and subjected to metal reduction ($p < 0.01$). There was 10 to 50 % decrease in estimated values when nitrite and nitrate were taken in different concentrations together as compared to measurements made separately at the two concentrations for the two analytes. The results from these three experiments provide consistent evidence that

TABLE-3
EFFECT OF ZINC REDUCTION ON ABSORBANCE VALUES FOR NITRITE AND NITRATE DETERMINED SEPARATELY AND IN COMBINATION

NO_2-N (µg)		NO_3-N (µg)	Absorbance*	
0.5		–	0.123 ± 0.002	–
–		2.5	0.030 ± 0.001	0.153 ± 0.003^a
0.5	+	2.5	0.137 ± 0.002^b	
0.5		–	0.120 ± 0.006	
–		5.0	0.072 ± 0.003	0.192 ± 0.007^a
0.5	+	5.0	0.098 ± 0.003^b	
0.8		–	0.199 ± 0.003	
–		10.0	0.163 ± 0.003	0.363 ± 0.004^a
0.8	+	10.0	0.260 ± 0.004^b	

*Absorbance values are mean $\pm$ SE of six observations; sum of individual values superscripted (a) are significantly higher than those superscripted (b) obtained for nitrite and nitrate taken together ($p < 0.01$).

nitrate can not be estimated reliably without error in terms of nitrite by metal reduction process: **a**) regression slopes for two analytes are strikingly different and so are their detection ranges; **b**) metal-reduction *per se* causes significant loss of nitrite hence estimated value from a mixture of nitrate and nitrite is not accurately measuring nitrate and **c**) sum of individual absorbance values for nitrate and nitrite measured separately are significantly higher than the values obtained when two analytes are present together and subjected to metal reduction. The results are in conformity to the theoretical considerations that metal reduction is non-selective. Nitrite and nitrate are equally vulnerable to metal reduction. In fact, metal reduction, employing Devarda's alloy (Al + Cu + Zn) or (Zn + $FeSO_4$) has been used for determination of nitrate/nitrite wherein the analytes are completely reduced to ammonia by metal reduction[8]. In separate experiments the author has observed that metal reduction converted major portion of nitrate or nitrite to ammonia (exceeding 60 %) and only a smaller portion of nitrate (< 30 %) was reduced to nitrite (unpublished data). The results are consistent with the observation that under optimal conditions, only about

20 % of nitrate is converted to nitrite by metal reduction[2]. Therefore, estimation of nitrate by metal reduction in terms of nitrite in presence of nitrite is highly erroneous.

The results provided rationale for standardizing an alternative protocol to enable nitrate determination by metal reduction in presence of nitrite. A pre-requisite for this accomplishment was to standardize a procedure that would eliminate nitrite from the sample containing nitrate and nitrite. The use of four chemicals including sodium azide, sulfamate, urea[9] and ethyl alcohol[10], with known potentials to remove nitrous acid, were screened to remove nitrite. Of these ethyl alcohol and urea proved promising. For the present investigations, urea treatment was standardized using 0.1 mL 2 % urea and 0.2 mL HCl per mL sample with incubation in boiling water bath for 10 min. Urea treatment protocol was standardized on the basis of following rationale: **a**) nitrite is converted to nitrous acid under dilute acid conditions, **b**) urea reacts instantly with nitrous acid to form nitrogen and carbon dioxide and **c**) controlled heat hastens the reaction, significantly removes urea through acid hydrolysis and facilitates removal of gases from the reaction mixture Experiment 4 revealed that the urea treatment (2 mg) was sufficient to destroy at least 500 μg NO_2-N from 1 mL sample. There is no detectable nitrite was observed in samples containing 100-500 μg nitrite nitrogen per mL. Experiment 5 revealed that the urea at standard level (2 mg) did not affect absorbance value of nitrate. The absorbance value of 10 μg NO_3-N in treated samples, 0.149 ± 0.002, was not significantly different from the value, 0.146 ± 0.002, obtained with untreated nitrate samples ($p > 0.1$). However, absorbance value at concentration of 10 mg urea, 0.123 ± 0.006, was significantly lower ($p < 0.01$) and recovery of nitrate was reduced to 85 ± 4 %. It is recommended not to exceed urea amount beyond 2 mg. Large amounts of urea would leave residual urea in the sample that would destroy some nitrite generated from nitrate by metal reduction.

TABLE-4
NITRATE DETERMINATION IN PRESENCE OF NITRITE
FOLLOWING UREA TREATMENT

Experiment no.	Analyte (μg)		Recovery	
	NO_3-N	NO_2-N	NO_3-N (μg)	Added (%)
1	5			
	5	+ 0.5	4.97 ± 0.07	97 ± 1
2	8			
	8	+ 0.5	7.85 ± 0.06	98 ± 1
3	10			
	10	+ 50.0	9.86 ± 0.33	99 ± 3

*The values are mean ± SE of six observations each.

Estimation of nitrate in presence of nitrite at various concentrations (Table-4) revealed that standardized urea pretreatment recovered nitrate efficiently; 5, 8 and 10 μg NO_3-N was recovered in presence of 0.5 or 50 μg NO_2-N as 4.97 ± 0.07, 7.85 ± 0.06 and 9.86 ± 0.33 indicating estimated recovery of 97 ± 1, 98 ± 1 and 99 ± 3 per cent of untreated controls, respectively.

Conclusion

The present investigation has revealed that the estimation of nitrate in presence of nitrite by metal reduction process is erroneous. A modified technique has been standardized to enable error-free estimation of nitrate by metal-reduction process in presence of nitrite. An aliquot of sample containing nitrate and nitrite be first analyzed for nitrite. Second aliquot be subjected to urea treatment to eliminate nitrite. Urea treated sample can be subjected to metal reduction for direct determination of nitrate. This would provide a reliable estimation for nitrate that is otherwise impossible with currently employed metal reduction techniques.

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