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Extraction of NO_x and Determination of Nitrate by Acid Reduction in Water, Soil, Excreta, Feed, Vegetables and Plant Materials

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ABSTRACT: Different methods are available for extracting NO_x from different samples. A judicious combination of lead acetate, sodium hydroxide and magnesium chloride has been devised to enable extraction of NO_x from different samples ensuring removal of potential interfering agents. The method provides over 95 per cent mean recovery with nearly 3 per cent accuracy and precision. Nitrite is determined by Griess reaction, and removed from samples by urea treatment to obviate any interference by nitrite in nitrate determination. Nitrate is determined by acid reduction method with minimum detection limit 0.5 ppm as N. The methods have been applied to selected environmental samples including food materials and excretory products. The average nitrate levels (as ppm N) found in water (0.8), soil (9), human urine (43), sheep excreta (2654), chicken feed (29), radish (270), spinach (222), carrot (194), potato (41), cabbage (11), tomato (2), Bermuda grass (175) and morning-glory leaves (576) have been within safe and documented limits. The average levels of nitrite, as ppm N, have generally ranged from 0.04 to 2.1 with highest content, 13, in sheep fecal matter. The protocol is intended for general use in environmental analysis, toxicological investigations and risk assessments. @JASEM

Water, soil, plant materials, feed and excreta are monitored for levels of NO_x for their toxicological implications and risk assessments (Schuddeboom, 1993; WHO, 2007). Nitrate is a more stable anion than its toxic metabolite nitrite and is dominant in environmental and biological samples. Methods based on the principle of Griess reaction are widely employed for their determinations as these are simple, specific and sensitive enough for routine use. However, for determination of nitrate, these methods require prior reduction of nitrate to nitrite which is accomplished by cadmium (APHA, 1998; AOAC, 2000; Cortas and Wakid, 1990; Green et al*.,* 1982; Schneider and Yeary, 1973), vanadium (Miranda et al., 2001), zinc (Nelson et al., 1954; Diven et al., 1962; Mir, 2007), hydrazine (Oms et al., 1995), or specific nitrate reductases (Guevara *et al.,* 1998). Of late, an inexpensive, rapid acid reduction method has been optimized for determination of nitrate (Mir, 2008). Standard methods commonly used for determination of nitrate have been rated as "semi quantitative" (Sawyer and McCarty, 1978). A major technical limitation in the methods is that the nitrate is measured indirectly with measurements made twice, before and after reduction, and the nitrate determined as a difference of NO_x and NO_2 (Sun et al*.,* 2003). This approach has serious limitations while determining nitrate in presence of nitrite (Mir, 2007). Differential abilities of nitrite and nitrate to engage in diazotization of sulfanilamide under different experimental conditions have provided a rationale for their sequential analysis in test samples (Mir, 2008). Urea pretreatment enables direct determination of nitrate in presence of nitrite (Mir, 2007, 2008). The maximum safe and / or regulatory limits for nitrate in drinking water, vegetables, forage/foodstuffs (Bagley et al., 1997; Schuddeboom, 1993; Stallings, 2006; WHO, 2007) or normal levels in human urine (Cortas and Wakid, 1990; Green et al., 1982; Guevara et al., 1998; Tsikas et al, 1994), chicken feeds (Okafor and Okorie, 2006) and soils (Chiroma et al., 2007; Jaiswal, 2003) or for maintaining aquariums (Sharpe, 2008) are well within the detection range, 0.5-10 ppm N, of acid reduction method for nitrate (Mir, 2008).

Perusal of literature reveals use of different extractants for extracting NO_x from different samples. These include water (Bhargava and Raghupathi, 1993), 0.1M HCl (Krishna and Ranijhan, 1980; Nelson et al., 1954), $CdCl_2$ + $BaCl_2$ (AOAC, 2000), Somogyi reagent (Cortas and Wakid, 1990), $CuSO_4$ + Ca $(OH)_2$ + $MgCO_3$ (Gupta, 2004), methanol/diethyl ether (Guevara et al., 1998), calcium chloride + charcoal (Chiroma et al., 2007) or Carez reagent (ISO, 1984). A need to develop a uniform protocol for extracting NO_x from different samples was mooted on the considerations of ensuring stability of the anions particularly of nitrite, removal of potential interfering agents, minimize losses and obtain colorless transparent extracts. A combination of lead acetate and activated charcoal (Oser, 1976; Pomeranz and Meloan, 1996) coupled to alkaline extraction achieved by sodium hydroxide and magnesium chloride (Mir, 2004) was found to provide an effective alternative extraction method for application to majority of the test samples.

Consequently, the investigations were designed to: (1) investigate the efficiency of the extraction method and to apply it to different test samples; (2) employ acid reduction method coupled to urea treatment for determination of nitrate, and (3) compare data generated for nitrate by acid reduction method in selected samples with those obtained by comparable metal reduction method.

MATERIALS AND METHODS

The experiments were carried out at an ambient temperature of 20.6 ± 0.7 ^{0}C. The chemicals used

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were of analytical grade purity. Appropriate working solutions of nitrate and nitrite were made by dilution in water, respectively, from stock solution of 500 ppm as nitrate N (potassium nitrate 0.361 % in water) and of 700 ppm as nitrite N (sodium nitrite 0.345 % in water) with 0.2 % chloroform as preservative in stock solutions. The strength of HCl used was found to be approximately 11.7 M. The basal diazotization powder contained finely pulverized homogeneous mixture of barium sulfate 10 g, citric acid 7.5 g and sulfanilamide 0.5 g. For reduction purpose, finely pulverized homogenous mixture of zinc dust and manganese sulfate (1:5 by weight) was added @ 30 mg per 500 mg of the diazotization powder (RDP). The other reagents included 1 %(w/v) sulfanilamide in 1 % (v/v) HCl, 1 %(w/v) N-1-(naphthyl)-ethylenediamine dihydrochloride in 1% (v/v) HCl (NEDA), 2 % (w/v) urea in water, 20 % (v/v) glacial acetic acid in water, 40% (w/v) lead acetate trihydrate in water, 20 % (w/v) sodium hydroxide in water (ca.5M NaOH) and 50 %(w/v) magnesium chloride hexahydrate in water. The activated charcoal was purified by sequential treatments with 0.1M NaOH, 0.2 M HCl, and deionized water till effluent failed to show any detectable nitrate and nitrite. The purified charcoal was drained off its liquid, dried, cooled to room temperature, and stored till use.

The samples included freshly collected local vegetables viz., potatoes, cabbage, radish, carrots, spinach and tomatoes, aerial parts of *Cynodon dactylon* (Bermuda grass, locally called Dramoon), leaves of *Ipomoea purpurea* (morning-glory, locally called Ashqpaechan), Formulated chicken feed (labeled composition approximately as maize 62%, ground nut cake 13%, soya meal 13 %, fish meal 10 % and mineral mixture 2 % with crude protein 21.78 %), top soil samples from apparently fertile premises of the Faculty, tap water, fecal pellets from sheep, dominantly Corriedale germplasm maintained at the Faculty Farm, and freshly collected urine samples from apparently healthy young male volunteers of the Faculty. Properly cleansed vegetables were made to pulpy homogenate in electric blender. All solid samples were air-dried and made to fine powder. Powdered soil samples were sieved to remove visible debris. Tap water was directly processed, or following its 5- or 10-folds concentration by evaporation. Vegetable homogenates 5g each, tomato pulp homogenate 10 g, chicken feed powder 1.25 g, Bermuda grass powder 0.5 g, morning-glory leaves powder 0.25 g, fecal powder 0.1g and composite soil powder 12.5 g were extracted with 15 ml or 20 ml water with intermittent shaking for about 1 hour at room temperature. Urine samples were diluted with three volumes of water. Urine samples and extracts were added lead acetate to provide 10 % salt concentration, shaken well, centrifuged at 6000 rpm for 10 minutes, and filtered vide Whatman filter paper No.1. Each eight milliliter of the extract was sequentially added 1 mL 5 M NaOH, 1 mL 50% magnesium chloride solution, and purified charcoal @ 10-30 mg mL^{-1} solution depending upon degree of pigmentation. Water did not require lead acetate and charcoal treatments. Each nine milliliter water sample was added 0.5 mL each of sodium hydroxide and magnesium chloride solutions. All the samples were well shaken, allowed to stand 2-3 minutes following each addition, centrifuged and filtered vide Whatman filter paper No.1. The sample extracts were assayed for NO_x.

The efficiency of the outlined extraction processes was studied by estimating per cent recovery of the anions from aliquots of water containing nitrate and nitrite, respectively 50 and 2 µg as N. The values obtained from the samples subjected to the extraction processes were compared with the values obtained from unprocessed aliquots of the same cocktail. The reagent blanks for each process were run simultaneously. The purified charcoal was used at maximum concentration limit, 30 mg mL^{-1}. Appropriate aliquot of assay extract (0.5 to 2 mL), depending upon nitrite concentration as checked by trial tests, was made 2 mL with distilled water, added 0.1 mL sulfanilamide solution and then 0.3 mL HCl, allowed to stand 2-3 minutes, and added 0.2 mL coupling agent. The samples were monitored at 540 nm (UV-Visible Spectrophotometer SL-150, Elico (India) Ltd, Hyderabad) after 20 minutes standing against reagent blank. Standard nitrite 0.1 and 0.2 ppm as N in 2 mL volume were simultaneously processed. Extracts containing excessive nitrate were appropriately diluted with water so that nitrate content remained within the linear detection range of acid reduction method (0.5-10 ppm as N). Urea treatment protocol optimized for determination of nitrate by metal reduction (Mir, 2007) or by acid reduction (Mir, 2008) has been followed in this work. Each milliliter of the sample extract was added 0.1 mL of urea solution and 0.2 mL HCl, and incubated in boiling water bath for a period of 10 minutes to get rid off any nitrite. The urea treated samples were assayed for nitrate.

Nitrate was determined by optimized acid reduction technique employing one milliliter sample extract (Mir, 2008). Nitrite free samples were added each 0.1 mL sulfanilamide solution and 2 mL HCl. The mixture was incubated in boiling water bath for exactly 10 minutes, cooled to room temperature, and added 0.2 mL of coupling agent, and color monitored at 540 nm after 20 minute standing. Water blank and standard nitrate solutions, 1 and 3 ppm as N, were subjected to identical treatments. The extracts from selected samples including soil, carrot, morning-glory, and feed were simultaneously assayed for nitrate by modified (Mir, 2007) metal reduction technique (Nelson et al., 1954).The modifications included: a) substituting sulfanilic acid with a more stable aromatic amine, sulfanilamide, b) substituting 1-naphthylamine with a widely employed safer coupling agent NEDA, c) separately adding coupling agent to avoid adsorption of the azo-dye by the filter paper, and d) using optimized mass of zinc in the reduction powder to curtail furthering of reduction process by the metal (Mir, 2007). Urea-

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treated sample extracts were each added 9 mL of acetic acid solution, and a scoopful, ca. 500 mg, of RDP, shaken well for about 1 minute, centrifuged at 6000 rpm for 5-10 minutes and filtered over Whatman No.1 filter paper. Each 5 mL filtrate was added 0.2 mL NEDA, and color monitored at 540 nm after 20 minute standing. Water blank and nitrate standards, 1 and 3 ppm as N, were subjected to identical treatments. The concentrations of nitrate and nitrite in the samples were calculated in terms of simultaneously processed standards:

$$X = (A.B)/(C.D.E)$$

Where

X = Concentration of the analyte in the original sample, ppm N.

A = Absorbance of assay extract.

B = Concentration standard as µg N.

C = Absorbance of standard sample.

D = mL sample extract used for the assay.

E = Concentration of sample extract, g or mL original sample per mL sample extract

RESULTS AND DISCUSSION

Lead acetate was chosen for its ability to precipitate proteins, organic acids, and chromogenic phenolics (Pomeranz and Meloan, 1996) that are most likely to be present in plant extracts. Lead acetate has been employed as protein precipitant for clarification of blood (Hoffman, 1925), potato extracts (Kinter, 1972) and for determination of nitrate in rumen liquor (Lewis, 1951). Poor decolorizing ability of lead acetate (Pomeranz and Meloan, 1996) has been compensated by using activated charcoal wherever necessitated. Lead acetate provides additional protection by removing sodium azide, phosphates and, as hydroxide, ascorbate (Oser, 1976). These substances interfere with the Griess technique (Cortas and Wakid, 1990; Miranda *et al.,* 2001). Sodium hydroxide serves to remove lead as hydroxide, as well as to remove other interfering metals including Bi^{3+}, Ag^{+}, Hg^{2+}, Fe^{3+}, Au^{3+} (APHA, 1998), Cu^{2+}, Zn^{2+} (Cortas and Wakid, 1990), Al^{3+}, Fe^{2+} (author, unpublished) likely to be present in water and soil samples. Magnesium chloride removes residual hydroxide, and prevents any solubilization of lead hydroxide in fixed alkali hydroxide. Combined use of sodium hydroxide and magnesium chloride has been demonstrated to remove various metallic ions including Zn^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+} from samples (Mir, 2004). The protocols ensured high alkalinity (Protocol A, pH 9.9 ± 0.1; protocol B, pH 10.9 ± 0.1, n = 4 each) and high electrolyte concentration in the extracts, maximally ca. 0.42 M CH_3COONa and ca. 0.21 M $MgCl_2$. These conditions have been found to prevent any adsorption of nitrate and nitrite to activated charcoal (unpublished data), and to maximize recovery. Insoluble hydroxide matrices provided by lead hydroxide and magnesium hydroxide contribute to clarity of the extracts. The proportions of sodium hydroxide and magnesium chloride can be varied depending upon the metal contents in the test samples. The recovery with protocol A, relevant to majority of the sample, has been 98.8 ± 1.1 % for nitrite nitrogen and 95.4 ± 1.7 % for nitrate nitrogen with per cent accuracy 3.3 ± 1.0 (n = 10). With protocol B, relevant to apparently clean water samples, the recovery has been 99.6 ± 1.1 for nitrite nitrogen and 99.8 ± 1.6 % for nitrate nitrogen with per cent accuracy 2.2 ± 0.4 (n=10) (Table 1). Overall per cent accuracy has been 2.8 ± 0.5 (n=20), and precision as estimated from values of coefficient of variation as 3.0 ± 0.5 for four sets of experiments. Per cent recovery with different extraction procedures have ranged from 85 to 112 (Green et al., 1982; Guevara et al., 1998; Lewis, 1951; Nelson et al., 1954). Reagent blanks did not show any background coloration indicating the reagents were free of any detectable nitrite/nitrate.

Table 1: Recovery of nitrate and nitrite with the extraction processes

Extraction			Extraction efficiency		
Protocol	Relevance	Analyte	Added	Recovered (µg)	Recovery (%)
A[a]	Vegetables, plants, feed, urine, feces, soil	NO_3–N	50	47.7 ± 0.9	95.4 ± 1.7
		NO_2-N	2	2.00 ± 0.02	98.8 ± 1.1
B[b]	Water	NO_3–N	50	50.0 ± 0.7	99.8 ± 1.6
		NO_2-N	2	2.00 ± 0.02	99.6 ± 1.1

[a] Lead acetate, sodium hydroxide, magnesium chloride and purified activated charcoal.
[b] Sodium hydroxide and magnesium chloride. The values are mean ± se of five observations each.

Urea treatment has not affected nitrate determination in presence of nitrite whether nitrate was determined by metal reduction (Mir, 2007) or by acid reduction (Mir, 2008). With employed procedures, the detection ranges for nitrite and nitrate have been, respectively, as 0.05 -1 and 0.5 -10 µg as N (Mir, 2008). The detection range for nitrate by metal reduction has been 2.5 – 40 µg N (Mir, 2007). For present investigation incubation of test samples in boiling water bath for maximal limit of ten minutes, range 3 – 10 (Mir, 2008), was found advantageous as it obviated any interference from lower levels of nitrite, < 0. 5 ppm N, and caused 97.4 ± 0.6 per cent loss in diazotization of nitrite at higher levels, 0.75 to 3.0 ppm N (n=15), compared to values observed at room temperature. This implies that urea treatment may not be required at low nitrite levels while employing maximal limit of incubation period for acid reduction.

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Table 2: Nitrate and nitrite contents in selected test samples

Sample category	Samples	Concentrations, ppm	
		NO_3–N	NO_2–N
Environmental	Water	0.8 ± 0.03	0.1 ± 0.01
	Soil	9 ± 0.1	0.7 ± 0.01
		9 ± 0.5[a]	NA
Vegetables and plants	Radish	270 ± 3	0.3 ± 0.02
	Spinach	222 ± 12	0.7 ± 0.04
	Carrot	194 ± 12	0.5 ± 0.02
		185 ± 11[a]	NA
	Potato	41 ± 1	0.3 ± 0.01
	Cabbage	11.3 ± 0.1	< 0.05
	Tomato	2.2 ± 0.1	ND
	Bermuda grass	175 ± 6	1 ± 0.2
	Morning-glory	576 ± 13	2 ± 0.1
	(leaves)	477 ± 61[a]	NA
Formulated feed	Chicken feed	29 ± 1	0.1 ± 0.02
		28 ± 2[a]	**NA**
Excreta	Human urine	43 ± 10	ND
	Sheep fecal pellets	2654 ± 94	13 ± 1

[a] The data values generated by metal reduction technique (P >0.1); NA not analyzed; ND not detectable.
The values are mean ± se of six observations each.

The choice of different samples has been made for testing applicability of envisaged methods for extraction and determination of nitrate, and for their relevance in environmental and toxicological investigations, and risk assessments. The significance of nitrate load in samples particularly in vegetables (Schuddeboom, 1997), forage (Stallings, 2006), drinking water (Blake and Hess, 2006; Okafor and Okorie, 2006, WHO, 2007), and sheep fecal material ((Ecochem, 2008) are well documented. Soil NO_x is important for plant nutrition and health, and contribute significantly to nitrate content in plants and in ground water. Toxicological implications and risks associated with high nitrite/nitrate intake in human and animals have been documented (Clarke and Clarke, 1975; Grizzle et al., 1997; IPCS, 1978, 1999; Okafor and Okorie, 2006; Schuddeboom, 1997; WHO, 2007). In general, nitrate induces toxicity through its toxic metabolite nitrite. Ruminants are more susceptible to nitrate poisoning (due to microbial reduction of nitrate to nitrite in rumen) than to nitrite poisoning (due to their higher capacity to reduce nitrite to ammonia) while fish and monogastrics including human, swine and chicken are more vulnerable to nitrite than to nitrate poisoning owing to lack of these capabilities.

Nitrate and nitrite contents in the tap water have been within permissible safe limits with mean levels, respectively, 0.84 and. 0.13 ppm N. The guideline values for nitrate and nitrite for safe drinking water are, respectively, 10 ppm and 1 ppm as N in human (WHO, 2007) and chicken (Blake and Hess, 2006), 10 ppm N of either in ruminants (Bagley et al., 1997, Stallings, 2006) and 5 ppm nitrate N in fish aquaria (Sharpe, 2008). Nitrate nitrogen in arable soils is reportedly in the range of ca. 0.7 – 13.6 ppm (Jaiswal, 2003; Chiroma et al., 2007) while the levels of nitrite are normally negligible (Jackson et al., 1986). The mean concentrations of 9.1 ppm as nitrate N and 0.7 ppm as nitrite N in the present study would suggest good nitrogen quality of the test soil. However, nitrite levels exceeding about 0.3 ppm as N in top soil is considered toxic for growing plants (Singh et al., 2007).

Vegetables contribute dominantly, 60 to 90 % or above, to dietary nitrate intake in humans followed by water (Schuddeboom, 1993; Chiroma et al., 2007; Lukassowitz, 2005). The present study has revealed mean concentrations of nitrate and nitrite (as ppm N) highest, ca.185 to 270 and 0.3 to 0.7, in radish, spinach and carrot, intermediate, ca. 40 and 0.3 , in potatoes, and lower, ca. 11 and < 0.1, in cabbage. (Nitrate content has been lowest, ca. 2 ppm N, in tomatoes with no detectable nitrite. The observed nitrate concentrations are within the normal documented ranges in fresh vegetables: radish 40.7 to 1211.4, spinach 4.5 to 1220.4, carrots 13.6 to 248.6, potatoes 10.4 to 67.8, cabbage 2.3 to 822.6 and tomatoes 1.4 to 36.2 (Anjana et al., 2007; Chiroma et al., 2007; Chung et al., 2003; Schuddeboom, 1993). Nitrite nitrogen in the vegetables has been either undetectable (Schuster and Lee, 1987) or in the range of 0.06 to 1.2 ppm N (Rao and Puttanna, 2000; Chung et al., 2003; Lyons et al., 2006). The regulatory levels of nitrate in vegetables vary with respect to type of vegetable and from country to country with range from about 55 to 1100 ppm N while nitrite levels are usually < 3 ppm N (Schuddeboom, 1993). The mean nitrate content in the grass, 175 ppm as N, is almost equal to that in carrots with nearly twice the concentration of nitrite (Table 2). The grass contributes significantly to dietary nitrate requirements of grazing livestock in the region. The observed levels do not exceed the safe limit of about 1500 ppm as nitrate N for livestock grazing (Bagley et al., 1997). Monitoring of nitrogen content in the grass is also important for its efficient management as turf grass (Bergareche and Simon, 1989) and for assessment of any inherent risks to water quality and the environment following its nitrogenous fertilization (Lee et al., 2003). *Ipomoea* species are listed amongst plants that can accumulate nitrate to dangerous concentrations endangering livestock safety (Clarke and Clarke, 1975). Present investigation confirms this assertion;

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mean NO_x content in leaves, ca. 576 ppm nitrate N and ca. 2 ppm nitrite N, exceed the levels observed in all test plant materials. The levels are, however, within safe limits, 600 to 1500 ppm nitrate N, permissible for livestock grazing (Bagley et al., 1997; Stallings, 2006). Higher concentrations of nitrate, ca. 880 ppm N, have been found in a related species, *Ipomoea aquatica* (Schmidt et al., 1971).

The concentrations of nitrate nitrogen in the chicken feed by either of the techniques have been around 29 ppm with no significant difference between mean values ($P>0.1$) while mean nitrite content has been < 0.1 ppm N. The levels are within safe guideline limits (Bagley et al., 1997; Stallings, 2006). Average concentrations of nitrate and nitrite, ppm N, in chicken feeds have ranged, respectively, from about 50 to 200 and from not-detectable levels to about 64 (Okafor and Okorie, 2006). Nitrate and nitrite to chicken is contributed by its ingredients principally by fish meal, corn, ground nut cake and soya meal. Human and ruminant excreta contribute significantly to environmental nitrogen pollution. The levels of NO_x in urine and fecal samples are indicators of status of dietary NO_x (Schuddeboom, 1993; IPCS, 1999), and would also aid in diagnosis in the event of nitrate/nitrite poisonings. The mean concentration of nitrate in human urine, ca. 43 ppm N with no detectable nitrite would indicate optimal dietary intake status in concerned volunteers. Normal documented levels in urine are 3.5-75.5 ppm as nitrate N (Green et al., 1982; Cortas and Wakid, 1990; Guevara et al., 1998; Tsikas et al, 1994) and < 0.13 ppm as nitrite nitrogen (Tsikas et al, 1994). Sheep fecal pellets were found to contain highest content of NO_x among all tested samples with mean nitrate nitrogen about 2654 ppm and mean nitrite nitrogen about 13 ppm. This suggests dominance of nitrate rich herbage in the available grazing areas for the sheep since high nitrogen excretion in sheep is directly linked to high nitrogen intake through forage (Orr et al., 1995). Nitrogen content in sheep fecal matter is as high as in chicken manure, ca. 9000 ppm, and nearly double than the levels found in cattle or horse manure (Ecochem, 2008). Sheep manure has contributed significantly to the nitrate content of plants (Pavlou et al., 2006) and to that of groundwater (Rosen et al., 2004).

As evident the mean values for nitrate obtained with acid reduction technique on selected samples, viz soil, carrot, morning-glory, and feed, did not vary significantly from those obtained with metal reduction technique ($P>0.1$). However, overall coefficient of variation with metal reduction technique, 21 ± 4 %, has been about 4 times of the value, 5 ± 1 %, obtained with acid reduction. Besides, acid reduction technique is 5 to 6 times more sensitive (Mir, 2008) than metal reduction technique (Mir, 2007) with respect to its minimum detection limit and the value of regression coefficient. The coefficients of variation in determinations of NO_x based on Griess reaction have ranged from 1.3 – 9.0 % (Cortas and Wakid, 1990; Guevara et al., 1998). Nitrate content is known to vary by factors between 2 and 60 between vegetables, and by factors between 5 and 70 within any given type of vegetable (Lukassowitz, 2005) or even over a 100 fold (Schuddebom, 1993).

The study has demonstrated use of alkaline extraction method independently or in combination with lead acetate and activated charcoal for extraction of NO_x from a variety of samples, and of acid reduction method for determination of nitrate in these samples. Urea treatment serves to remove any interference from the presence of nitrite while nitrate is being subjected to chemical reduction. Lead acetate besides removing proteins has potential to remove gums, organic acids, and chromogenic phenolics that are most likely to be present in plant extracts. It also removes interfering agents like sodium azide, phosphates and as hydroxide ascorbic acid. Hydroxide matrices of lead and magnesium contribute to clarity of extracts. Alkalinity is favorable particularly for the extraction of nitrite, in precipitating interfering metals as hydroxides, and in presence of high ionic load has prevented adsorption of nitrite and nitrate to activated charcoal (unpublished data). The values for nitrite and nitrate in water, soil, radish, carrots, spinach, cabbage, potatoes, tomatoes, Bermuda grass, morning-glory leaves, chicken feed, human urine and sheep fecal pellets from the local temperate region of Kashmir have been within the normal ranges documented in the literature from other areas of the world. These observations validate the applicability of the proposed protocol for environmental investigations of toxicological concern or for assessment of risks.

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