Colorimetric Determination of Nitrate in Soil and Plant Extracts with Brucine

A. S. Baker

Brucine alkaloid was used to determine nitrates colorimetrically in soil and plant extracts. Reproducible results were obtained by maintaining a low reaction temperature using prediluted H_2SO_4. The accuracy of nitrate determinations by the proposed procedure on soil extracts made with 0.2% $CaSO_4$ was equal to the accuracy obtained by the phenoldisulfonic acid procedure and was less cumbersome. Fructose and sucrose, present in plant extracts, cause a negative error in the proposed procedure. For maximum accuracy, a simple ion exchange procedure is recommended to obtain a plant extract free of sugars and some other interfering substances. Lower apparent nitrate levels are obtained when plant tissue is extracted with 0.25*N* NaCl and Darco G 60 owing to the presence of interfering sugars in the extract.

Interest in toxic nitrate levels in grass forages has stimulated studies requiring the analysis of a very large number of plant tissue samples. The phenoldisulfonic acid procedure (Johnson and Ulrich, 1950) involving precipitation of chloride, H_2O_2 oxidation, and evaporation proved too cumbersome for the number of analyses required. Brucine colorimetric procedures appeared promising from the standpoint of simplicity and sensitivity. Although brucine procedures have been published for waters and soil extracts (Fisher *et al.*, 1958; Jenkins and Medsker, 1964; Robinson *et al.*, 1959) and water extracts of meats (Landmann *et al.*, 1960), no satisfactory brucine procedure has been worked out for plant tissue. A study was initiated to test the applicability of brucine colorimetric procedures for nitrate analyses of plant tissue and soil extracts.

EXPERIMENTAL

Reagents. Brucine Alkaloid, 2% w./v. Solution in 0.05*N* HCl. Dissolution is speeded with heat. Use care in transferring, so as not to inhale reagent dust which is highly toxic.

Primary Nitrate Standard for Soils, 20 μg. of Nitrate-N per Ml. Dissolve 144.4 mg. of KNO_3 in sufficient 0.2% $CaSO_4 \cdot 2H_2O$ w./v. to make 1 liter of solution.

Working Standards for Soils. Dilute 5, 10, 15, 20, and 25 ml. of the primary nitrate standard for soils to 100 ml. with 0.2% $CaSO_4 \cdot 2H_2O$.

Extracting Solution for Soils, 0.2 μg. of Nitrate-N per Ml. Dilute 10 ml. of the primary nitrate standard for soils to 1 liter with 0.2% $CaSO_4 \cdot 2H_2O$.

Nitrate-Free 1*N* NaCl. Transfer 117 grams of NaCl to a liter beaker and dissolve in 300 ml. of distilled water and 5 ml. of concentrated HCl. Take to complete dryness at 100° C. Dissolve the NaCl in sufficient distilled water to make 2 liters of solution.

Western Washington Research and Extension Center, Puyallup, Wash.

Primary Nitrate Standard for Plant Material, 20 μg. of Nitrate-N per Ml. Dissolve 144.4 mg. of KNO_3 in sufficient distilled water to make 1 liter of solution.

Dilute Nitrate Standard for Plant Material, 1 μg. of Nitrate-N per Ml. Dilute 50 ml. of the primary nitrate standard for plant material to 1 liter with distilled water.

Working Standards for Plant Material (Methods A and B). Transfer 5, 10, 15, 20, and 25 ml. of the primary nitrate standards to a series of 100-ml. volumetric flasks. Add 40 ml. of 1*N* NaCl to each and bring to volume with distilled water.

Extracting Solution for Plant Material (Method A), 0.2 μg. of Nitrate-N per Ml. and 0.4*N* NaCl. Dilute 10 ml. of the primary nitrate standard for plant material and 400 ml. of 1*N* NaCl to 1 liter with distilled water.

Darco G 60. Washed with 0.4*N* nitrate-free NaCl and then with distilled water until the $AgNO_3$ test for chloride is negligible. Dry at 110° C.

Cation Exchange Resin. Dowex 50W-X8, hydrogen saturated, 20- to 50-dry-mesh size, dried at 105° C.

Anion Exchange Resin. Dowex 1-X8, chloride saturated, 100- to 200-dry-mesh size, maintained in moist state.

Apparatus. Exchange columns are constructed with filter tubes (Corning No. 9480, smallest size) and rubber tubing with an inside diameter of 3 to 4 mm. (Figure 1). To prevent occlusion of air, water-saturated glass wool and the anion exchange resin are inserted under water. Insertion of the exchange resin is facilitated with the aid of an ordinary thin-walled, translucent, plastic soda fountain straw and a glass rod that fits snugly into the straw to act as a piston (Figure 1). The straw is calibrated to deliver 0.2 cc. of moist anion exchange resin. Any resin adhering to the sides of the expanded portion of the funnel should be wiped out with absorbent paper. Five milliliters of water should drain in about 15 minutes. If the flow rate is excessive, it can be slowed by packing additional glass wool at the top of the column and/or by additional compaction of the column with a glass rod. Remove air trapped in the stem upon addition of solution. When not in use, the column is protected from evaporation and dust by placing Parafilm over the funnel and inserting a short glass rod into the open end of the rubber tubing.

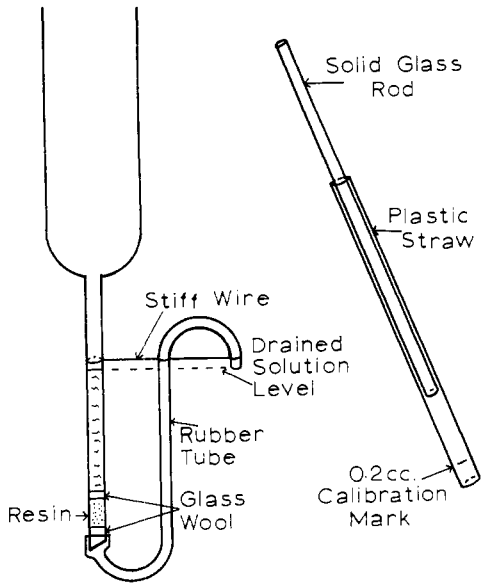

Figure 1. Filter tube–exchange column and plastic straw for transferring the anion exchange resin to the column

Extraction of Soils. Transfer 2.5 grams of air-dried soil to a shaking bottle. Add 25 ml. of the extracting solution for soil. Stopper and place in a reciprocal shaker for 15 minutes. The long axis of the container should be oriented parallel to the direction of movement of the shaker. Filter through S & S 602 filter paper. If the soil contains more than 48 p.p.m. of nitrate-N, use a smaller amount of soil or dilute the extract with soil-extracting solution.

Extraction of Plant Material, Method A. Transfer 50 mg. of dried plant tissue, ground to pass at least a 20-mesh screen, and 50 ± 5 mg. of Darco to a shaking bottle. Add 25 ml. of the extracting solution for plant material. Shake for 15 minutes in the same manner as for soils. Filter through S & S 602 filter paper. If the plant tissue contains more than 0.24% nitrate-N, use less plant tissue or dilute the extract with the extracting solution for plant material.

Extraction of Plant Material, Method B. Transfer 125 mg. of dried and ground plant tissue and about 50 mg. of cation exchange resin to a shaking bottle. Add 25 ml. of distilled water. Shake for 15 minutes in the same manner as for soils and filter through S & S 597 filter paper. Transfer an aliquot of the filtrate containing less than 120 μg. of nitrate-N (10 ml. if the tissue contains less than 0.24% nitrate-N and generally 5 ml. if higher) and 5 ml. of the dilute nitrate standard for plant material to the exchange column. When drained, wash the column with 5 ml. of distilled water, then 10 ml. of 0.01*N* HCl followed by 10 ml. of water. Discard the leachate. Place a 25-ml. volumetric flask in position to receive the leachate. Elute the nitrate with two successive 5-ml. aliquots of 1*N* NaCl. Wash the excess NaCl from the column with 9 and then 5 ml. of distilled water. Bring the solution to volume with distilled water. Wash the column with about 20 ml. more of distilled water, and seal it until it is to be used again.

Color Development. Transfer 20 ml. of H_2SO_4 (1 to 1, v./v., cooled to room temperature) to a 50-ml. Erlenmeyer flask. Add 1 ml. of the brucine reagent and swirl to mix. Add 2 ml. of the appropriate extract or working standard, swirl, and immediately add 5 ml. of concentrated H_2SO_4. Swirl and place the Erlenmeyer flask in a dark cabinet. Read transmittance at 410 mμ at least 2 hours after the flask was placed in the dark. If transmittance is not to be read within 5 hours after placement in the dark, seal the flask with Parafilm after it has reached room temperature. Use the color developed with the appropriate extracting solution as a blank and adjust the instrument so that color developed with the extracting solution reads 100% transmittance. The extracting solution used for extracting method A may be used as a blank when extraction method B is used. When calculating the concentration of nitrate in the soil or plant material, note that the extracts contained an extra 0.2 μg. of nitrate-N/ml.

The highly toxic brucine reagent is transferred with a repeating syringe-type pipet. The H_2SO_4 solutions are conveniently dispensed from Machlett-type automatic pipets with Teflon stopcocks.

RESULTS AND DISCUSSION

Brucine Reagent. Although Fisher *et al.* (1958) suggest the use of brucine hydrochloride, no source of this reagent could be located. Brucine sulfate proved very unsatisfactory because it lacked sensitivity, especially with lower standards. Brucine alkaloid produces satisfactory sensitivity but shows a discontinuity in the standard curve at concentrations below 0.2 μg. of nitrate-N per ml. (Figure 2). For this reason, all unknowns and standards are read above this concentration. The brucine reagent may be stored for at least 6 months at 5° C. It takes on a reddish cast on storage, but this does not impair its sensitivity to nitrate.

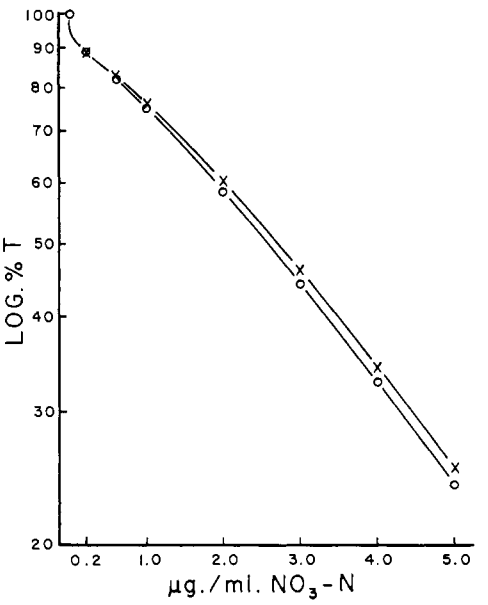

Figure 2. Curves obtained with brucine using 0.4*N* NaCl standard solutions

○ = 4 hours after color development
× = 24 hours after color development

Color Stability. The initial reddish color formed with brucine and nitrate in H_2SO_4 is very unstable and changes to yellow with an absorption peak at 410 mμ. This transition is complete in 2 hours. Fading of the yellow (Figure 2) is relatively slow so that samples may be run as much as 72 hours after color development with no significant changes in apparent nitrate level from those run 2 hours after color development. Standards must be run with each set of samples to compensate for fading. If flasks are left unsealed for more than 5 hours after color development, anomalous changes in the standard curve occur with low standards decreasing and high standards increasing in transmittance. The latter is probably related to absorption of atmospheric moisture by the strong H_2SO_4 solution.

Sources of Error. Initially, an attempt was made to follow the procedure of Fisher *et al.* (1958), but results were too variable. Possibly, HNO_3 is volatilized at the high temperatures attained during the dilution of concentrated H_2SO_4 involved in their procedure. This might explain the decrease in sensitivity with increase in temperature observed by Fisher *et al.* (1958). By substitution of 1 to 1 H_2SO_4 in the recommended procedure, much of the heat of dilution is dissipated beforehand. When 5 ml. of concentrated H_2SO_4 are added in the final step to develop the color, the temperature attained is well below the 86° C. boiling temperature of HNO_3.

Most of the literature states that chloride does not interfere in the brucine-nitrate reaction. However, Jenkins and Medsker (1964), working with marine water samples which are much higher in chlorides than would ordinarily be found in soil or plant extracts, found the contrary. The present author found that both the rate of color development and fading were increased slightly at chloride levels of the order used in the proposed precedures for plant extracts. Varying the NaCl concentration between 0.36 to 0.44*N* did not cause any change in transmittance of the color developed with standard nitrate solutions. This variation in chloride is well above the limits of variation due to the soluble chlorides in plants and nonsaline soils or the pipetting and recovery error in method B.

Nitrites will produce a positive error with the proposed procedure. Although procedures for the reduction of nitrite have been proposed and appear simple (Black, 1965a; Jenkins and Medsker, 1964; Landmann *et al.*, 1960; Robinson, 1959), this has not been considered owing to the purposes for which these tests have been used. Healthy plant tissue and air-dried soils do not contain significant quantities of nitrite.

There is an unknown agent extracted from green plant tissue with water or salt solutions that produces a deep yellow color in sulfuric acid. This agent is completely removed by Darco in an amount equal in weight to the weight of the dried plant tissue.

Very significant amounts of nitrate are sorbed by Darco when water is used as the extracting solution. Complete recovery of 125 μg. of nitrate-N per 25 ml. of solution is obtained from 50 mg. of Darco when the solution is at least 0.25*N* with respect to NaCl. Complete recovery is not possible from a similar system in which saturated $CaSO_4$ solution is substituted for 0.25*N* NaCl. The Darco added in method A must be 50 ± 5 mg. Large deviations from this may cause errors owing to sorbed nitrate (larger amounts) or background color (smaller amounts).

To determine whether there was sufficient colored matter in the $CaSO_4$ extracts of soils to interfere with the brucine determination, an extract was obtained from a Chehalis silty clay loam. This soil contained 7.8% organic matter. The procedure for color development was followed substituting 0.05*N* HCl for the brucine reagent. Two milliliters of the Chehalis soil extract were added to one Erlenmeyer flask and 2 ml. of pure extracting solution to another. Transmittance at 410 mμ was the same for both. Nevertheless, if a soil sample contains a considerable amount of fresh green plant tissue, a significant positive error might occur. In this case, soil samples should be extracted with 50 mg. of Darco and at least 0.25*N* NaCl. Standard solutions of comparable NaCl concentrations should then be used.

Sucrose and fructose caused a negative error in this analysis. Glucose produced no measurable error while sucrose and fructose caused the same amount of error when present in equal molar amounts. Because sucrose is rapidly inverted in strong H_2SO_4 solution, fructose probably is the cause of the interfering action of sucrose. The cause of this type of error may be the reducing action of fructose, since it is much stronger in this respect than glucose. Also, brucine reagent in strong H_2SO_4 develops a yellow color on addition of a strong oxidizing agent—e.g., H_2O_2 or metal peroxide—even in the absence of nitrate. Apparently, the color reaction is influenced by the redox potential of the system. Despite the fact that the brucine-nitrate color reaction has been known for many years, there is nothing in the literature to illuminate the exact nature of this reaction.

Method B was devised to obtain sugar-free extracts of plant tissue. The H-saturated cation exchange resin is used primarily to make the extract more acidic without adding a soluble anion. This reduces the ionization of sugars thus reducing adsorption on the anion exchange resin. The cation exchange resin also sorbs some of the coloring matter from the extract. Very little sugar is adsorbed on a chloride-saturated strong anion exchange resin and that which is can easily be eluted with 10 ml. of 0.01*N* HCl. When 250 μg. of nitrate-N (twice the maximum amount ordinarily added) were adsorbed on 0.2 cc. of the anion exchange resin, no nitrate was eluted until 25 ml. of 0.01*N* HCl were passed through the column. Almost all of the nitrate was eluted with the first 5-ml. aliquot of 1*N* NaCl, and complete recovery was obtained with the second 5-ml. aliquot of this reagent. The 10 ml. of NaCl are added to the column in 2 aliquots because it has a higher mass than the water left above the resin column. The water phase rises into the NaCl solution when the first aliquot is added. None of the plant coloring agents sorbed by the anion exchange resin is removed by 1*N* NaCl. It is not possible to remove all of the coloring agents from the exchange column with sensible amounts of strong acid, strong base, acetone, or 95% ethanol. Using one of the more highly colored extracts, complete recovery of nitrate was obtained from a column seven times without recharging the column with clean resin. By this time, the resin column became so highly colored it appeared advisable to recharge it.

Reagent grade NaCl contains small amounts of nitrate.

It is not necessary to purify the NaCl for method A when standards and extracting solution are made from the same NaCl source. It is advisable to purify the NaCl used in method B. This is easily accomplished by volatilization of HNO_3 in the presence of HCl.

Nitrates in Soils. Soil samples from a nitrogen fertilizer trial on a Sultan silt loam (4.6% organic matter) were extracted with 50 ml. of the proposed extracting solution and also with 50 ml. of the extracting solution containing an extra 1.5 μg. of nitrate-N per ml. (75 μg. per 50 ml.). Nitrate determinations were made on these extracts by the proposed brucine procedure and a phenoldisulfonic acid procedure (Black, 1965b). Table I indicates that nitrate levels determined by both procedures did not differ markedly or consistently from each other. Recovery by both procedures was quite good, and there was no real difference in this respect between the two procedures.

Nitrates in Plant Tissue. The control sample (Table II) is a mixture of forages used as a reference for various plant analyses in this laboratory. The remainder of the samples listed in Table II are from nitrogen fertilizer trials with orchard grass. The first three listed are from plots that received little or no nitrogen fertilizer and thus contain a large admixture of weeds and volunteer white clover. The remaining samples were almost pure orchard grass with no clover and only a small number of weed species. Because of the difference in sugar levels, the apparent nitrate levels were consistently lower for method A than for method B. Recovery of added nitrate is improved somewhat by the use of method B, but was still poor for the first four samples listed in Table II. As indicated in Table III, there is nothing inherent in plant tissue which is

Table I. Soil Nitrate Levels and Recovery of Nitrate by the Proposed Brucine and the Phenoldisulfonic Acid Procedure

Sample No.	Soil Nitrate-N Level, P.P.M.		Recovered,[a] μg.		Recovery, %	
	Brucine	PDSA[b]	Brucine	PDSA	Brucine	PDSA
1	29.2	30.5	75	73	100.0	97.3
2	14.5	14.2	73	76	97.3	101.3
3	11.4	11.0	75	74	100.0	98.7
4	48.4	50.8	74	72	98.7	96.0
5	41.7	42.0	76	73	101.3	97.3
6	33.2	31.4	73	76	97.3	101.3
7	1.1	0.5	70	70	93.3	93.3
8	1.5	1.0	78	76	104.0	101.3
9	2.2	2.0	77	74	102.7	98.7
10	14.9	14.0	77	78	102.7	104.0
	Average % recovery				99.7	98.9
	Average deviation from 100% recovery				±2.4	±2.7

[a] Soils extracted with 50 ml. of extracting solution containing an extra 1.5 μg./ml. of nitrate-N. 50 × 1.5 = 75 μg. of nitrate-N added.
[b] PDSA refers to phenoldisulfonic acid procedure (Black, 1965b).

Table II. Nitrate and Recovery Tests on Oven-Dry Plant Tissue Samples

Sample Designation	Apparent Nitrate-N Level, %			% H_2O-Soluble NH_3-N[a]	Nitrate-N Recovery Tests					
	Brucine		PDSA		Method A			Method B		
	Method A	Method B	Method[b]		Added, μg.[c]	Recovered, μg.	Recovery, %	Added, μg.[d]	Recovered μg.	Recovery, %
Sp 5 P_3T_3	0.006	0.006	0.009	1.18	45	38	84.4	25	22	88.0
								50	42	84.0
Sk 5 P_3T_6	0.015	0.017	0.019	0.82	45	36	80.0	50	45	90.0
Sk 5 P_2T_5	0.024	0.028	0.033	0.96	45	35	77.8	50	44	88.0
Control	0.044	0.046	0.055	0.67	45	38	84.4	50	47	94.0
			Average % recovery				81.7			88.8
			Average deviation from 100% recovery				−18.4			−11.2
Sp 5 P_6T_9	0.148	0.157	0.197	2.66	45	43	95.5	25	24	96.0
								50	48	96.0
Sk 5 P_9T_{10}	0.157	0.178	0.229	2.86	45	45	100.0	50	51	102.0
Sp 4 P_6T_6	0.180	0.196	0.231	1.50	45	43	95.5	25	25	100.0
								50	48	96.0
Sp 5 P_6T_9	0.240	0.250	0.300	2.96	45	44	97.8	50	49	98.0
Sk 5 P_9T_{10}	0.256	0.272	0.329	2.91	45	43	95.5	25	24	96.0
								50	48	96.0
Sk 5 $P_{13}T_9$	0.325	0.344	0.388	2.84	45	44	97.8	50	51	102.0
			Average % recovery				97.0			98.0
			Average deviation from 100% recovery				−3.0			±2.9

[a] Crude protein-N by Kjeldahl procedure, excluding nitrate-N.
[b] PDSA refers to the phenoldisulfonic acid procedure (Johnson and Ulrich, 1950).
[c] Added by extracting with 25 ml. of extracting solution containing an extra 1.8 μg. nitrate-N/ml. or 45 μg. nitrate-N/25 ml.
[d] Added in solution to the exchange column.

low in nitrate to cause low recovery. The poor recovery obtained with impure samples (first four listed, Table II) is possibly due to some reducing compound which is strongly to moderately ionized—e.g., oxalic acid—present in the weed species. From the practical standpoint, this is unimportant with samples which are very low in nitrates, but it may lead to serious errors with high nitrate samples which contain high weed populations. This latter seldom occurs on seeded meadows and pastures since grasses generally predominate in forages heavily fertilized with nitrogen. For nitrate studies on plant species other than those listed here, preliminary recovery tests are recommended to ascertain the presence of interfering compounds. When maximum accuracy is required, brucine method B should be used. When maximum accuracy is not required, brucine method A can be used with much saving in time and labor. For orchard grass samples, nitrate levels very close to values obtained using method B can be obtained by multiplying the results obtained using method A by 1.07.

Nitrate concentrations determined by the phenoldisulfonic acid procedure (Johnson and Ulrich, 1950) were consistently higher than those determined by the proposed brucine methods (Table II). There is good reason to suspect that the apparent nitrate level of plant tissue determined by procedure of Johnson and Ulrich (1950) is often erroneously high owing to oxidation by H_2O_2 of the various forms of reduced N in plant extracts. As noted above, there was no significant difference in the determination of nitrate levels of soils when the brucine procedure was compared with a phenoldisulfonic acid procedure (Black, 1965b) that did not involve H_2O_2 oxidation of organic residues. Johnson and Ulrich (1950) provided evidence that the addition of nitrate-free reduced N compounds resulted in positive errors. The amounts of NH_3-N which they added in the form of amino acids and protein were less than the amounts found in the extracts of high nitrate samples shown in Table II (last six). The difference in the apparent nitrate levels between the two procedures was greater when the samples contained high amounts of soluble NH_3-N compounds (Table II). The present author has found other evidence that alkaline oxidation of amino acids with H_2O_2 results in the conversion of a portion of the reduced N to nitrate.

Table III. Nitrate Levels and Recovery of Nitrates Added to Samples of Pure Grasses and Alfalfa Using Method B

Species	Nitrate-N Level, %	Recovery of Nitrate-N		
		Added, μg.[a]	Recovered, μg.	Recovery, %
Orchard grass	0.002	75	74	98.7
Timothy	0.000	50	50	100.0
Alta fescue	0.041	50	49	98.0
Beni grass	0.008	50	48	96.0
Alfalfa	0.023	50	49	98.0
		Average % recovery		98.1
		Average deviation from 100% recovery		−1.9

[a] Added in solution to the exchange column.

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