Determination of Nitrate Nitrogen in Bovine Blood, Milk, Urine, and Rumen Liquor

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A cattle feeding experiment required that nitrate be determined in bovine milk, blood, urine, and rumen liquor. Attempts to apply the phenoldisulfonic acid or brucine procedures to these fluids gave erratic results, despite protein precipitation, chloride removal, and heavy metal precipitation. Application of a xylenol extraction procedure to the clarified solutions gave reproducible results and satisfactory recoveries of added nitrates.

When a recent cattle feeding study (*1*) required the determination of nitrate in blood, milk, urine, and rumen liquor, no suitable method of analysis was found available. These biological fluids are sufficiently complex to require removal of many interfering substances prior to the determination of their nitrate content regardless of the method used for analysis. Protein may be removed by tungstic acid precipitation, chlorides with silver, and some of the remaining organic matter by a copper precipitation as suggested by Harper (*3*). Despite the cleanup thus accomplished, attempts to determine nitrate in the clarified solutions using either the phenoldisulfonic acid procedure (*5*) or the DeVarda distillation (*5*) were unsuccessful. Both methods gave extremely high results apparently as a result of the decomposition of substances not removed in the cleanup. Recoveries of nitrates added to the solutions were very erratic when the above procedures were used. Because of these difficulties, a modified xylenol extraction procedure was applied to the clarified body fluids. Use of this method gave good recoveries of added nitrates with acceptable precision.

Sample Preparation

Reagents. Disodium EDTA. Reagent-grade disodium salt of (ethylenedinitrilo)tetraacetic acid.

Sodium Dichromate. One-half grain tablets.

Sodium Tungstate Solution, 10%. Dissolve 100 grams of $Na_2WO_4 \cdot 2H_2O$ in 1 liter of distilled water.

Sulfuric Acid, 0.66*N*. Dilute 19 ml. of concentrated reagent-grade H_2SO_4 to 1 liter with distilled water.

Copper-Silver Reagent. Dissolve 20 grams of $CuSO_4 \cdot 5H_2O$ and 1 gram of Ag_2SO_4 in 100 ml. of distilled water.

Procedure. Blood. Collect the blood in a tube containing 2 mg. of disodium EDTA per ml. of blood to act as an anticoagulant. Refrigerate or freeze until analyzed. Transfer 5 ml. of blood to a 100-ml. centrifuge tube or a 125-ml. Erlenmeyer flask; add 35 ml. of distilled water, and mix. Add 5 ml. of 10% sodium tungstate solution, mix; add 5 ml. of 0.66*N* sulfuric acid, and immediately mix again. Let stand 20 minutes, and centrifuge until a clear supernatant liquid is obtained, or filter through a nitrate-free filter paper. Pipet 25 ml. of the clear solution into a 100-ml. centrifuge tube; add 1 ml. of the copper-silver reagent, mix, and let stand for about 20 minutes. Add about 0.3 gram each of calcium hydroxide and magnesium carbonate, mix well, let stand 10 minutes, and centrifuge. Take a 20-ml. aliquot and proceed as directed in the nitrate determination described below.

Milk. Add a 0.5-grain tablet of sodium dichromate per pint of milk as a preservative and keep sample refrigerated until analyzed, or analyze fresh samples immediately.

Dilute 10 ml. of milk with 20 ml. of distilled water and precipitate proteins by adding 10 ml. each of sodium tungstate solution and 0.66*N* sulfuric acid, mixing well after each addition. After 20 minutes centrifuge or filter, transfer a 35-ml. aliquot to a 100-ml. centrifuge tube, add 2 ml. of copper-silver reagent, mix, let stand 20 minutes, and then precipitate copper and excess silver by mixing in about 0.5 gram each of calcium hydroxide and magnesium carbonate. If the analysis is not to be continued immediately, filter or centrifuge and decant before letting stand. Use a 10-ml. aliquot of the clear supernatant liquid for the determination of nitrate.

Urine and Rumen Liquor. Transfer 2 ml. of urine or filtered rumen liquor to a 50-ml. centrifuge tube, add 2 ml. of the copper-silver reagent, dilute to 20 ml. with distilled water, mix, and let stand 20 minutes. Add about 0.5 gram each of calcium hydroxide and magnesium carbonate, mix several times during 10 minutes, and centrifuge to obtain a clear supernatant liquid. Take a 10-ml. aliquot for the determination of nitrate.

Determination of Nitrate

Reagents. Xylenol Solution. Dissolve 2 grams of 3,4-xylenol (3,4-dimethyl phenol) in 100 ml. of reagent-grade acetone.

Sulfuric Acid, 3 + 1. Cautiously add 300 ml. of concentrated reagent-grade H_2SO_4 to 100 ml. of distilled water. Mix and allow to cool before using.

Sodium Hydroxide, 0.4% Solution. Dissolve 4 grams of reagent-grade NaOH in 1 liter of distilled water.

Nitrate Standard Solution. Weigh 0.0408 gram of KNO_3 into a 1-liter volumetric flask, dilute to volume with distilled water, and mix. Add 1 ml. of chloroform as a preservative. This solution contains 25 μg. of NO_3 per ml.

Procedure. Transfer an aliquot of clarified solution containing 5 to 200 μg. of NO_3 to a 125-ml. separatory funnel and dilute to 20 ml. with distilled water. Add 1 ml. of the xylenol reagent, mix, and then add 60 ml. of 3 + 1 H_2SO_4, mix again, and let stand 20 minutes. Dilute with 20 ml. of distilled water, mix, and let cool. Add 25 ml. of CCl_4, shake vigorously for about 30 seconds, and then allow the layers to separate. Drain off the CCl_4 layer into a beaker, discard the aqueous layer, rinse the separatory funnel with distilled water, and allow it to drain dry. Pipet 10 to 50 ml. (depending on the amount of nitrate

Table I. Recovery of Nitrates Added to Solutions

Added, μg.	Total NO_3 Found, μg.	NO_3 Recovered, μg.	Recovery, %
	BLOOD		
0	46	...	...
50	90	44	88
100	144	98	98
250	283	237	95
500	514	468	94
	BLOOD		
0	159	...	...
50	206	47	94
100	257	98	98
250	386	227	91
500	642	483	97
	MILK		
0	4	...	...
50	56	52	104
100	104	100	100
	URINE		
0	12	...	...
50	61	49	98
100	116	104	104
	RUMEN LIQUOR		
0	2	...	...
50	48	46	92
100	100	98	98

expected) of 0.4% NaOH into the funnel, add the CCl_4 extract, shake vigorously for about 30 seconds, and allow the layers to separate. Discard the CCl_4 layer and filter the yellow sodium hydroxide extract through a slow paper (S & S No.605 or equivalent). Measure the transmittance of the filtered solution at 420 mμ *vs.* distilled water and compare the results to calibration curves established by carrying zero to 200 μg. of NO_3 through the procedure. A series of curves covering a large range of NO_3 concentration may be established by varying the final volume of added NaOH from 10 to 50 ml.

Table II. Comparative Analyses of Three Blood Samples

Sample No.	μg. per Ml. NO_3 Found: Microbiological method	Xylenol method
273	77	70
274	24	25
284	9	6

The above method is a modification of a procedure developed by Morris for the determination of water-soluble nitrate in plant tissue. Several solvents may be substituted for carbon tetrachloride for the extraction of the nitrated xylenol, including chloroform, toluene, benzene, Skelly Sol-B, xylene, and petroleum ether. Carbon tetrachloride was chosen because it showed less tendency to form emulsions than the other solvents and because it gave a lower blank value.

With the exception of the original aliquot and the final NaOH, the solutions added may be measured with a graduated cylinder. The aqueous solution after the final extraction with NaOH must be alkaline. If no yellow color appears at this point, the solution should be tested with pH paper before drawing off the CCl_4. If the solution is not alkaline, solid NaOH should be added and the solution shaken again.

Originally, two 15-ml. CCl_4 extractions were used; however, later tests showed a single 25-ml. extraction was equally effective. The yellow color of the sodium salt of nitro-xylenol is quite stable; no change occurs in the transmittance of the yellow solution over several days.

As recommended by Holler and Huck (*4*), 3,4-xylenol was used rather than the less reliable *m*-xylenol. Tests showed that the final sulfuric acid concentration in the nitration step could be varied between 54 and 80%. Above 80% there appeared to be some loss of nitrate. For best results, the temperature of nitration should be kept from 40° to 50° C. Lower temperatures slowed the reaction considerably. Above 60° C. some loss of nitrate occurred. A reaction time of 20 minutes was found sufficient when the temperature remained between 40° and 50° C. The concentration of NaOH used to extract the nitrated xylenol is immaterial so long as an excess is present to neutralize any acid carried over in the CCl_4 extract.

Recoveries of nitrates added to the solutions tested are listed in Table I. In addition, three samples of blood were analyzed by this method while subsamples were frozen and sent to the University of Missouri for analysis by the microbiological method of Garner and coworkers (*2*). Comparison of results obtained by the two procedures is shown in Table II.

The precision of the method was tested by repeated analyses of several samples. The average coefficient of variation was found to be 7.8%.

Literature Cited

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FERTILIZER PROCESS CONTROL

Analytical Procedures for Nitrogen Fertilizers Containing Phosphates and Sulfates

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NEW PROCESSES for producing granular nitrogen fertilizers have recently been developed at TVA. These fertilizers include ammonium nitrate, ammonium phosphate nitrate, and ammonium nitrate sulfate. Although analytical methods were available for determining the composition of these fertilizers, improvements in these methods were needed to reduce the time and effort required for maintaining adequate chemical control.

In the production of granular nitrogen fertilizers, an acid—either phosphoric or sulfuric—is added to control the degree of granulation and to give a product of the desired chemical composition. In addition, the moisture content of the product must be kept at 0.5% or less.

Conventional methods of determining moisture, phosphate, and sulfate in nitro-