# Simple Colorimetric Method for the Determination of Nitrates in Forage Crops

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A simple, rapid colorimetric method for the determination of nitrates in fresh forage crops has been devised by modifying and incorporating previously published procedures. The proposed method has an accuracy to ±4%, can be carried out within 2 hours, and meets the requirements of many biological investigations. Its accuracy and rapidity make it highly suitable for following the accumulation and decline of nitrates in forage crops growing under high levels of nitrogen fertilization.

Investigators in the fields of agriculture (*3*), coatings and plastics (*11*), fertilizer manufacture (*2*), and public health (*8*) have pointed out their need for a simple, rapid method for the quantitative determination of nitrates. Classical methods either lacked specificity (*3*), or the desired accuracy was difficult to obtain (*2*). Investigators in the field of forage crop production and characterization are using at least seven different methods for the determination of nitrates in plant materials (*1*, *4–7*, *12*, *13*). The implication is that no completely satisfactory quantitative method is yet available.

Swann and Adams (*11*) recently published a very simple quantitative method for the determination of nitrates in coating materials and nitrocellulose. They based their method on the absorbance, at 525 mμ, of the red compound formed when nitrates are treated with a solution of ferrous sulfate in concentrated sulfuric acid. Except for nitrites and thiosulfates, the common inorganic ions do not interfere. The possibility of broad applications of this method was pointed out. With a few modifications, the method of Swann and Adams can be used for the determination of nitrates in forage crops and other plant tissues. The nitrate concentration in highly colored tissue extracts can be determined after the extracts have been rendered colorless with hydrogen peroxide. The common, water-soluble components of plant tissues do not interfere.

### Analytical Procedure

Macerate a sample of plant tissue in a high-speed blender for 5 minutes, using 10 ml. of water per gram of tissue. Filter and place 5.0 ml. of the clear filtrate in a 25-ml. glass-stoppered Erlenmeyer flask. Add three drops of 0.5*N* sodium hydroxide and 1 ml. of 30% hydrogen peroxide, and evaporate almost to dryness on a hot plate. Do not overheat. Add 5 ml. of water and evaporate to dryness. Add rapidly 20.0 ml. of reagent prepared from 750 ml. of concentrated sulfuric acid and 250 ml. of water containing 5.0 grams of ferrous sulfate heptahydrate (*11*). Shake vigorously for 30 seconds, and allow the color to develop for 1 hour. If a very faint pink color develops, read transmittance at 355 mμ. If a medium to dark pink color develops, read transmittance at 525 mμ. Determine the quantity of nitrate in the aliquot from the appropriate standard graph. The 355-mμ graph is prepared from transmittance data obtained on solutions containing 25, 50, 100, 150, 200, and 250 γ of nitrate, and the 525-mμ graph from solutions containing 0.3, 0.5, 1.0, 1.5, 2.0, and 2.5 mg. of nitrate.

### Discussion

The compound which is formed when nitrates are treated with concentrated sulfuric acid containing ferrous sulfate exhibits characteristic light absorption. This property has been used as a qualitative test for nitrates for almost a century and is the basis of the widely used "brown ring" test for nitrates. The absorption spectrum of this compound exhibits a very sharp peak at 355 mμ and a broad absorption band between 450 and 550 mμ. The system is very sensitive to changes in nitrate concentration, stable for several hours, and adheres to the usual absorption laws. Single measurements at 355 mμ make it possible to determine quantities as small as 50 γ with an accuracy to ±4%. When aliquots contain more than 0.3 mg. of nitrate, transmittance data is taken at some point along the broad weaker band. Twenty replicate determinations on a standard solution of potassium nitrate containing 1.000 gram per liter ranged from 0.98 mg. per ml. to 1.03 mg. per ml. and averaged 1.01 mg. per ml.

The possibility that plant extracts contained substances that would interfere at 355 and 525 mμ was investigated. The absorption spectra exhibited by the treated residues of several different grasses were compared with the spectrum obtained with pure potassium nitrate. The identical nature of these spectra demonstrated that the color developed from treated plant extracts was due to the presence of nitrates, and that the plant extracts had been freed of interfering substances. The interference due to plant nitrites was negligible. Concentrations of chlorides, several times that usually found in forage grasses, did not

**Table I. Accuracy and Precision of Colorimetric Method for the Determination of Nitrates in Plant Tissues**

| Sample Analyzed | $NO_3^-$ Added, Mg. | $NO_3^-$ Found, Mg.[a] | | |
|---|---|---|---|---|
| Pure $KNO_3$ | 0.050 | 0.050 | 0.052 | 0.052 |
| Pure $KNO_3$ | 2.500 | 2.41 | 2.56 | 2.58 |
| 10% $KNO_3$; 90% NaCl | 0.100 | 0.096 | 0.096 | 0.098 |
| 10% $KNO_3$; 90% $NH_4Cl$ | 2.500 | 2.55 | 2.60 | 2.68[b] |
| Leaf extract a | none | 0.647 | 0.665 | 0.670 |
| Leaf extract a + NaCl | none | 0.638 | 0.653 | 0.660 |
| Leaf extract a + $KNO_3$ | 0.100 | 0.741 | 0.748 | 0.773 |
| Leaf extract a + $KNO_3$ | 2.000 | 2.54 | 2.71 | 2.76 |
| Leaf extract b | none | 1.37 | 1.50 | 1.50 |
| Leaf extract b + NaCl | none | 1.48 | 1.55 | 1.61 |
| Leaf extract b + $KNO_3$ | 0.100 | 1.67 | 1.69 | 1.71[c] |
| Leaf extract b + $KNO_3$ | 1.000 | 2.39 | 2.51 | 2.55 |

[a] Recoveries on 34 of 36 single determinations range from 96 to 104%.
[b] Recovery, 107%.
[c] Recovery, 109%.

interfere when the color was developed in the closed system as recommended (Table I). The only compound which interfered seriously with this method was β-nitropropionic acid, a nitro-aliphatic acid found in a few species of legumes (*10*). The method as given above is not applicable for the determination of nitrates in these particular species of legumes. Occasionally a single treatment with hydrogen peroxide does not completely decolorize an aliquot of leaf extract. This faint residual color does not lead to significant errors when milligram quantities of nitrates are present in the aliquots. However, with microgram quantities, this residual interference must be eliminated by treating the residue a second time with hydrogen peroxide.

The proposed method is not as accurate as some of the more exacting, time-consuming procedures which were developed for industries that must impose a post decimal point stringency on analytical results. Its accuracy of ±4% does, however, meet the requirements of many biological investigations. The rapidity and accuracy of the method make it highly suitable for nutritional studies involving the feeding of fresh forage crops produced under high levels of nitrogen fertilization. Its use permits one to follow very closely the build-up of nitrates in forage crops following the application of nitrogen fertilizers. Correlations between the results of animal feeding tests and the nitrate concentration of the ration being fed can be readily observed. In several cases, the results of animal feeding tests involving toxic quantities of nitrates have been accurately predicted from the results of chemical analyses. It is now possible to make valid recommendations concerning the feeding quality of forage crops insofar as nitrates are concerned (*9*). These recommendations can be made usually within 2 hours after a representative sample is received at the laboratory.

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**COUMARIN ASSAY**

# Determination of Coumarin in the Presence of Sterols


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**Colorimetric methods for the determination of coumarin, which involve a color reaction with diazotized *p*-nitroaniline, give inaccurate results when applied to sweet clover and other sterol-containing plant materials. Sterols as well as several other classes of compounds, give the same color reaction as coumarin with this agent. A procedure is described for the quantitative removal of "free" and "combined" coumarin from ground sweet clover seeds and the subsequent spectrophotometric determination of coumarin in the extract.**


Coumarin and its derivatives occur abundantly in nature, both in the free state and as glycosides. The principal natural sources are legumes, citrus fruits, orchids, and grasses. Coumarin has a sweet clover odor which is often referred to as the odor of new-mown hay. The tonka bean, a seed of a tropical South American tree, *Dipteryx odorata*, is the richest natural source of coumarin. Natural coumarin has been largely displaced by the cheaper synthetic product. Coumarin, the first natural perfume to be synthesized from coal tar raw materials, is used widely as a perfume in soaps and cosmetics. Until recently it was used as an ingredient of artificial vanilla extracts, but this is no longer permitted in the United States.

The increasing importance of Hubam clover as a southern forage and seed crop has prompted investigations to evaluate it as a potential source of valuable industrial raw materials. Modifications of two methods for the determination of coumarin in Hubam clover seeds were tried. Coumarin was determined colorimetrically by a modification of the method of Roberts and Link (*2*), wherein color is developed by a reaction with diazotized *p*-nitroaniline. This reagent gives the same color reaction with sterols as with coumarin. To overcome this difficulty, a method was developed for the spectrophotometric estimation of coumarin using a Beckman DU ultraviolet spectrophotometer to measure the absorption maxima of coumarin at 275 mμ. The procedure is a modification of that used by Englis and Hanahan (*1*) for the estimation of coumarin in vanilla extracts.

### Colorimetric Method

The apparatus and reagents used were similar to those described by Roberts and Link (*2*) except that the Klett-Summerson photoelectric colorimeter, equipped with filter No. 54, and 12 colorimeter tubes calibrated for 5 and 10 ml. were used. A working curve was prepared from data obtained on a series of standardized solutions containing known quantities of coumarin.

A standard aqueous solution of chemically pure coumarin was prepared and measured into a series of colorimeter tubes so that the quantity of coumarin increased through the series in 25-γ increments—the first tube contained none and the last contained 225 γ. Exactly 1 ml. of 1% sodium carbonate solution was added to each tube and then enough