

## An automated method for determining nitrate nitrogen in cotton plant parts

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### Summary

Samples of cotton parts are extracted with boiling water and analysed colorimetrically by auto-analyser using a modification of the Greiss-Ilosvay reaction. Incorporation of a dialyser enables the method to be extended to a range of plant material including highly coloured tissue such as extracts of beetroot tuber. The method overcomes interferences due to potassium, sulphur, chloride and eliminates problems associated with precipitation of hydroxides.

The procedure is sensitive, precise, and suitable for rapid analysis of large numbers of samples.

### INTRODUCTION

Knowledge gained from plant tissue analysis of selected parts of the cotton plant, particularly levels of nitrate nitrogen in leaf petioles, can be vital to evaluating the nutritional status of cotton plants. Nitrate nitrogen is readily taken up by plants and in general is transported unchanged in the stem and petiole to the leaf.

Several workers (Tabor *et al.* 1984, Burhan and Babikir 1968) have related nitrate nitrogen in petioles at varying stages of plant growth to final crop yield and fertiliser requirements. This has resulted in the use of fast field tests for nitrate nitrogen e.g., the snappy sap test, which are especially useful for predicting deficiencies up to two weeks before visible symptoms appear.

Similarly, studies on cotton are currently under way in the Emerald Irrigation Area to assess the interaction between nitrogen and irrigation. To service these projects a rapid means of accurately determining nitrate nitrogen in cotton parts was required.

In the past, steam distillation was used to determine nitrate nitrogen (AOAC 1970*b*). Although this procedure is accepted as an accurate means of determining nitrate nitrogen, it is time consuming and does not suit a high sample through-put application.

Most methods for determining nitrate nitrogen concentration are based on the reduction of nitrate to nitrite using the Griess-Ilosvay reaction (Bremner 1965; Fox 1979). There are several sources of interference with the colorimetric finish namely the colour of plant extracts and precipitation of ions due to hydroxides in the sample line (Best 1976; Rowland *et al.* 1984).

Our aim was to develop an automated method for nitrate nitrogen in cotton plant and this paper describes a method and reports on its performance characteristics. Emphasis is placed on the removal of likely interferences for the colorimetric finish.

### MATERIALS AND METHODS

#### Apparatus

1. A Technicon auto-analyser II system was used with a manifold construction as depicted in Figure 1. Analysis times were; sample time 35 s, wash time 40 s. The nitrate

reduction coil was held at 45°C in a constant temperature bath. The dialyzer was a 6 inch type using a type C membrane.

2. 100 mL semi-micro Kjeldahl flasks (graduated to 135.0 mL).
3. Whatman No.41 ashless filter papers, 9.0 cm.
4. Auto-analyser plastic sampler tubes.

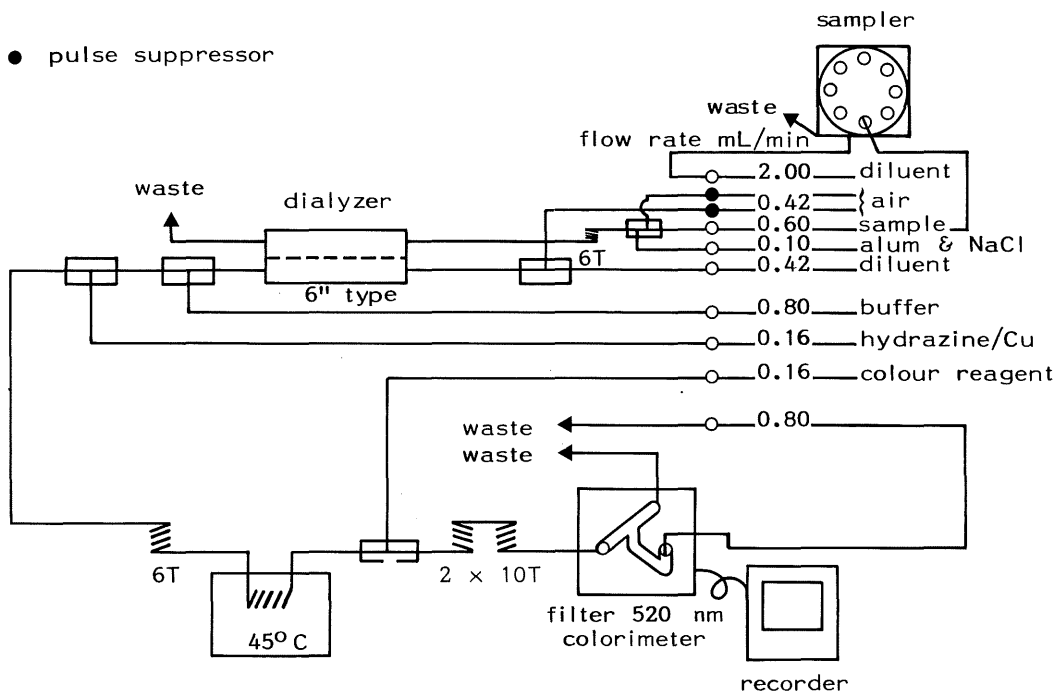


Figure 1. Flow diagram for nitrate nitrogen in plant extracts on the auto-analyser.

### Reagents

Unless otherwise specified, all reagents were analytical grade and the term 'water' implies de-ionised or distilled water.

**Buffer solution.** Dissolve 22.5 g sodium tetraborate decahydrate and 2.5 g sodium hydroxide in 1 L of water.

**Mixed alum and sodium chloride solution.** Dissolve 9.88 g  $\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  and 9.89 g sodium chloride in 1 L of water.

**Wetting agent (BRIJ 30% w/v).** Take 30 g of Brij 35 wetting agent and place in a sealable 250 mL glass container. Add 20 mL L.R. iso-propyl alcohol, mix and dilute to 100 mL with water.

**Wash solution and diluent.** Add 2 mL of wetting agent (Brij 30% w/v) to 1 L of water.

**Stock copper solution.** Dissolve 1.2 g cupric sulphate in 100 mL of water.

**Reducing solution.** Dissolve 1.3 g L.R. hydrazine sulphate in 900 mL of water. Add 1.3 mL of stock copper solution and dilute to 1 L with water.

**Colour reagent.** Dilute 20.0 mL phosphoric acid (sp.gr.=1.75) to approximately 100 mL with water. Add 2 g L.R. sulphanilamide and dissolve. Add 0.100 g N-1-napthylethylene diamine dihydrochloride and dilute to 200 mL with water. Store in glassware under refrigeration.

### Standard solutions

**Bulk nitrate nitrogen solution.** Prepare an aqueous solution containing 200  $\mu\text{g/mL}$  of nitrate nitrogen using potassium nitrate, dried at 105°C.

**Calibration standards.** Prepare solutions containing 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, and 10.0  $\mu\text{g/mL}$  of nitrate nitrogen by diluting appropriate amounts of the bulk standard with water.

### Procedure

Transfer 0.500 g sample of (<1 mm), previously dried at 105°C overnight, to a semi-micro Kjeldahl flask. Add 50 mL water and gently boil for one hour. Cool and dilute to volume (135 mL) with water. Mix well before filtering through Whatman No. 41 filter paper into an auto-analyser sample tube.

For extracts that are high in nitrate nitrogen, dilute (1+4) with water. Filtered solutions can be stored frozen.

### Interferences

Interference effects were assessed for calcium, magnesium, potassium, sulphur, phosphorus and chloride. All levels of interferences tested were higher than expected levels in cotton plant extracts.

In addition, enhancement of nitrate nitrogen response due to coloured soluble organic matter was assessed for a range of plant types. In this way a test of the applicability of the proposed method to other plant material was made.

### Calibration

Instrument response was tested using nine calibration standards. Each standard was prepared in duplicate. The linearity of calibration was assessed from a linear regression of response versus concentration of nitrate nitrogen in solution ( $\mu\text{g/mL}$ ). A lack-of-fit *F*-test was used to test the suitability of the linear model. The detection limit was estimated from twice the standard deviation of ten successive readings of a near blank nitrate nitrogen solution.

### Recovery

Recovery estimates were determined using three cotton plant samples (low, medium and high) which covered the expected range of plant nitrate nitrogen concentration. Additions of 100, 200, 300 and 400  $\mu\text{g/g}$  (low); 1000, 2000, 3000 and 4000  $\mu\text{g/g}$  (medium); 4000, 8000 and 12 000  $\mu\text{g/g}$  (high) nitrate nitrogen as potassium nitrate were made to the plant material before extraction. Each addition was replicated three times. The slopes of the regressions of total amount of nitrate nitrogen found versus amount of nitrate nitrogen added gave estimates of recovery for the method.

### Repeatability and reproducibility

Three samples of whole cotton were analysed three times on each of three days. A two-way analysis of variance (Youden and Steiner 1975) of the data was used to obtain precision estimates.

### Effect of sample fineness and means of extraction

Six samples of cotton plant were analysed in triplicate. Sub-samples of each were ground by a Christie and Norris mill to 1 mm, and by a shatter-box to a fine powder.

These were then extracted by boiling, as described in this method, and by shaking 0.4 g of material with 110 mL of water using a 'wrist action' shaker for a period of thirty minutes.

A three way analysis of variance was used to determine any significant variance due to sample preparation or extraction method.

### Comparison with reference method

Nine samples (leaf, petioles, stem and six separate whole plants) were analysed in duplicate by the proposed method and by a reference method using a barium chloride-cadmium chloride extracting solution (AOAC 1970*a*) followed by steam distillation finish (AOAC 1970*b*).

## RESULTS AND DISCUSSION

### Interferences

Several workers have adopted an automated colorimetric method of analysis for nitrate nitrogen based on the Greiss-Ilosvay reaction (Bremner 1965; Fox 1979); nitrate is reduced to nitrite using a copper sulphate-hydrazine mixture. The nitrite ions react with sulphanilamide and N-1-naphthylethylene diamine dihydrochloride in acid conditions. Most automated methods do not use dialysis. We have found that significant interferences from calcium, magnesium, chloride, potassium and sulphur can be experienced when water extracts are used.

Reagents described in this paper were selected to overcome interference effects due to calcium, magnesium, chloride, potassium and sulphur. The alum-sodium chloride solution (Reagent 2), which was mixed with the sample solution prior to dialysis, effectively reduced interferences due to potassium, sulphur and chloride. Chloride, potassium and sulphur ions then passed through the dialyser membrane at a constant rate. The proportion of sodium chloride and alum used in Reagent 2 was determined by adding varying amounts of sodium chloride to a predetermined fixed concentration of alum (0.988 % w/v) and observing the effect on the peak height of a sample whose concentration of nitrate nitrogen was 6.0 µg/mL. Results are shown in Figure 2.

A concentration of 6000 µg/mL chloride was selected for Reagent 2 since peak height remained relatively constant above this level.

The amount of buffer solution (Reagent 1) added was adjusted so that the pH of the reduction stream was within the pH range of 9.5 to 9.7 (Best 1976). This overcame the disruption of reagent stream flow caused by precipitation of calcium and magnesium hydroxides. These reagent changes were essential considering the relatively high concentration of each of these elements in cotton plant extracts.

A final interference test (Table 1) showed that there were no significant interferences for the concentrations tested. In that test, six solutions, each containing 4 µg/mL nitrate nitrogen and one of the potential interferents, were made up and analysed by the proposed method.

The use of a dialyser was shown to be essential for removal of colour due to soluble organic matter from extracts of a range of plant materials. Absorbances at 520 nm of extracts of cotton, cassava, sorghum, green peas, green beans, cabbages, Siratro leaves and beetroot tuber were 0.155, 0.023, 0.021, 0.158, 0.122, 0.394, 0.058 and 0.985 respectively before dialysis. After dialysis each extract gave an absorbance not different from water.

### Calibration

Instrument response in the range 0 to 10 µg/mL of nitrate nitrogen was linear and sensitivity was more than adequate for application to cotton plant parts. Detection limit was estimated as 0.016 µg/mL nitrate nitrogen.

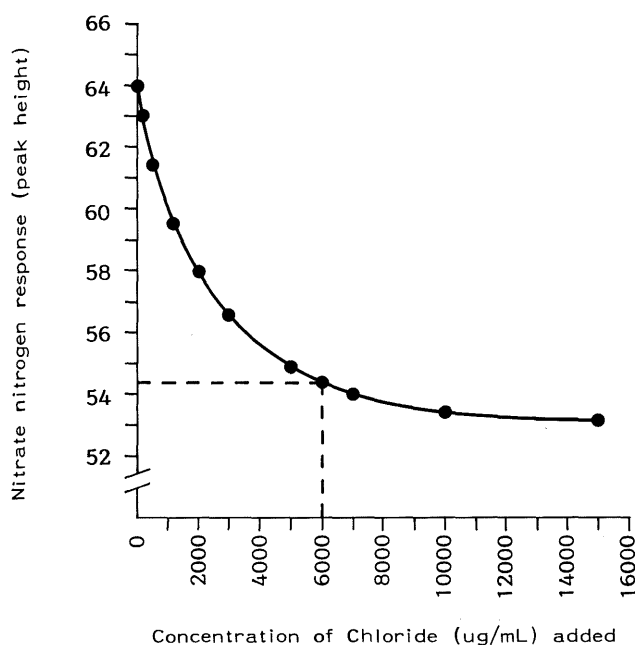


Figure 2. Effect of chloride (NaCl) concentrations on the reduction of  $\text{NO}_3\text{-N}$ .

Table 1. Effect of possible interferents on the determination of nitrate nitrogen in cotton plant using the proposed method

Typical level of interferent in cotton plant extract ( $\mu\text{g/mL}$ )	Concentration ( $\mu\text{g/mL}$ )	Auto-analyser result ( $\mu\text{g/mL NO}_3\text{-N}$ )
35 Ca	$4\text{NO}_3\text{-N}+180\text{ Ca}$	4.08
3 Mg	$4\text{NO}_3\text{-N}+ 15\text{ Mg}$	4.04
20 K	$4\text{NO}_3\text{-N}+100\text{ K}$	4.02
4 S	$4\text{NO}_3\text{-N}+ 20\text{ S}$	4.07
1 P	$4\text{NO}_3\text{-N}+ 5\text{ P}$	4.05
24 Cl	$4\text{NO}_3\text{-N}+120\text{ Cl}$	3.98

### Recovery

Recovery of added nitrate nitrogen was not significantly different from 100% for petiole, stem and leaf samples (Table 2). *F*-tests, for lack of fit of the linear model to each set of regression data, were not significant ( $P=0.05$ ).

### Repeatability and reproducibility

Analysis of variance of the precision test data (Table 3) showed that most of the variation was due to replicate error; repeatability standard deviation  $=\pm 3.04\%$  and reproducibility standard deviation  $=\pm 3.01\%$ . The relative confidence interval ( $P=0.05$ ) for a single estimate of nitrate nitrogen content, calculated from the reproducibility standard deviation, was  $\pm 6.1\%$ . Such good precision is more than acceptable for a routine method.

**Table 2. Evaluation of regression of nitrate nitrogen added to plant material**

Plant material	Regression*			F-value
	intercept (b <sub>0</sub> )	slope (b <sub>1</sub> )	Recovery, %†	
Petiole (high)	4163.03±125.15	0.985±0.017	98.5±1.7 d.f.‡=7	1.18 n.s.§
Stem (medium)	1359.20±32.50	1.001±0.013	100.1±1.3 d.f.=10	1.37 n.s.
Leaf (low)	25.94±5.97	0.982±0.026	98.2±2.6 d.f.=10	2.59 n.s.

\* The regression  $y=b_0 + b_1x$ , where  $y$ =nitrate nitrogen found ( $\mu\text{g/g}$ ) and  $x$ =nitrate nitrogen added ( $\mu\text{g/g}$ ).

†Recovery±95% confidence interval.

‡d.f.=degrees of freedom.

§n.s.=not significant.

**Table 3. Precision estimates for the determination of nitrate nitrogen in plant**

Day	Nitrogen nitrogen concentrations ( $\mu\text{g/g}$ )		
	Sample 1	Sample 2	Sample 3
1	94,81,75	977,929,945	4900,4900,5049
2	75,70,70	953,1080,985	4752,4900,4989
3	70,65,62	959,1012,977	5049,4900,4989

(b) Analysis of variance				
Source	d.f.	M.S.	F-ratio	
Days	2	1243.461	0.27	
Sample	2	60187224.15	-	
Day×sample interaction	4	4543.536	1.23	
Replicates	18	3684.29633		
Repeatability relative s.d.		=3.04%		
Reproducibility relative s.d.		=3.01%		

### Comparison of methods

Once an interference free analytical finish had been established a decision had to be made regarding the means of extraction of nitrate nitrogen from cotton plant material. The two extraction procedures examined were: extraction with boiling water, and shaking with water at room temperature. Both procedures completely recovered nitrate nitrogen, added as potassium nitrate, for low, medium and high ranges of nitrate nitrogen.

A comparison of nitrate nitrogen results using two extraction procedures and two grinding procedures, together with a three-way analysis of variance of the results is shown in Table 4.

Table 4. Comparison of results for two extracting procedures and two grinding procedures

Cotton material	Nitrate nitrogen concentrations ( $\mu\text{g/g}$ )			
	Boiling/C & N	Boiling	Shaking/C & N	Shaking/
	Mill as proposed	Shatter-box	Mill	Shatter-box
Petiole	496-4698 (4456)†	4404-4779 (4573)	4062-4619 (4304)	4314-4482(4388)
Leaf	338-378 (356)	270-297 (284)	338-381 (352)	279-16 (292)
Stem	2349-2430 (2385)	2160-2255 (2210)	2087-2284 (2162)	1974-2143(2069)
Plant 1	716-729 (725)	678-756 (726)	663-702 (681)	705-747 (726)
Plant 2	70-75 (72)	67-81 (72)	56-62 (59)	71-99 (85)
Plant 3	953-1080 (1006)	931-985 (963)	874-936 (908)	901-959 (935)

## (b) Analysis of variance

Source	d.f.	M.S.	F-ratio
Fineness	1	2556.2	1.943 n.s.†
Extraction	1	93384.1	70.971**
Sample	5	32198403.9	24470.6**
Fineness×sample interaction	5	19440.3	14.775**
Fineness×extraction interaction	1	5117.2	3.889 n.s.
Extraction×sample interaction	5	20881.3	15.87**
Fineness×extraction×sample interaction	5	1315.8	
Replicates	48	8541.44375	

\*\* significant at the 1% level.

†range (mean).

‡n.s.=not significant.

There was no significant difference between grinding procedures, so the grinding procedure using the C & N mill was adopted because it was a more convenient method. There was a significant difference between the shaking and the boiling procedures. Samples extracted using a wrist action shaker generally gave lower estimates of nitrate nitrogen than the boiling procedure. To further test the accuracy of the boiling procedure, a range of samples was analysed by this method and by the AOAC (1970a) extraction method followed by steam distillation.

The results and linear regression data are shown in Table 5.

There was excellent agreement between the proposed method and the reference method. Results were changing consistently between methods over the entire concentration range and there was no absolute bias between procedures.

As a result of the tests described in this paper we have adopted the proposed method for routine use in this laboratory.

**Table 5. Comparison of nitrate nitrogen results by proposed and reference methods**

Material	Proposed method*	Reference method*
Petiole	4296,4698 (4497)†	4426,4502 (4464)
Leaf	338,378 (358)	352,347 (350)
Stem	2349,2376 (2363)	2465,2243 (2354)
Plant 1	716,729 (723)	723,704(714)
Plant 2	70,75 (73)	71,68 (70)
Plant 3	1080,953 (1017)	990,1014 (1002)
Plant 4	19,27 (23)	50,30 (40)
Plant 5	1377, 1364 (1371)	1264,1398 (1331)
Plant 6	4900,4752 (4826)	4708,4919 (4814)

**Regression data‡**

Slope (b <sub>1</sub> )§	Intercept (b <sub>0</sub> )§	Coeff. of determination
1.0044	5.05	0.9981
(0.9809 to 1.0278)	(-51.43 to 61.54)	

\* duplicate determinations.

†mean.

‡The regression  $y=b_0+b_1x$  where  $x$ = result by reference method and  $y$ =result by proposed method.

§Estimate (95% confidence range).

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