

# AN IMPROVED METHOD FOR THE DETERMINATION OF NITROGEN IN CANE LEAVES\*

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## Abstract

A reliable routine method for the determination of total nitrogen in cane leaves using a discrete sample, automatic analyser system is described. Samples are prepared by means of a manual Kjeldahl digestion. This is followed by colorimetric estimation of the digestate ammonium content using a nitroprusside catalysed indophenol reaction. The colour complex is relatively stable and unaffected by normal fluctuations in digestate acidity, residual catalyst or mineral content of the samples. A total instrument time of 70 minutes per 200 samples is required for complex preparation and measurement. The test procedures used to evaluate the performance of this method in routine use are described in detail.

## Introduction

The estimation of total nitrogen in sugarcane third leaf samples forms an important part of the information provided by the Fertilizer Advisory Service (FAS) of the S.A. Sugar Association Experiment Station. Early assessment of how effective cane nitrogen fertilization has been is vital if remedial action is to be taken. During the 1975-76 season almost 9 000 leaf samples were analysed for total nitrogen content by the FAS. In the peak period as many as 2 500 samples per month may be received, and a rapid turn-round is required if results are to be utilised to assess the need for further nitrogen applications to the current crop.

Until recently the total nitrogen content of leaf Kjeldahl digestates was estimated by a distillation-titration procedure using microdistillation units (Bremner<sup>2</sup>). The method, although reliable, was very time-consuming, distillation and titration of each sample taking about five minutes. Maximum laboratory output was between 125-150 samples per day and this led to delays in sample analysis at peak periods. The recent introduction of semi-automated analytical equipment into the laboratory (Burrows and Meyer<sup>5</sup>) provided the opportunity of investigating the possibility of overcoming this bottleneck by the use of a colorimetric method for leaf nitrogen determinations.

There has been considerable interest recently in the use of the indophenol blue reaction for the estimation of ammonium although its application to the Kjeldahl procedure has presented several problems. The high, and often variable, residual acidity of the digestate (Novozamsky *et al*<sup>10</sup>) has necessitated special precautions as the formation of the colour complex is pH dependent. Heavy metals, usually derived from the catalyst, and the mineral content of the samples have interfered with complex formation (Davidson *et al*<sup>7</sup>) which results in anomalous values. In addition, the phenol-hypochlorite reagents used could be hazardous to laboratory personnel, and require careful preparation. The substitution of sodium salicylate for phenol, the use of sodium dichloroisocyanurate as a chlorine source and the catalytic effect of sodium nitroprusside (Reardon *et al*<sup>11</sup>) resulted in a more sensitive colour complex (Crooke and Simpson<sup>6</sup>) in which the reagents used were relatively safe and easy to prepare.

This paper describes a method, using these reagents, for which the colour complex is reasonably stable and unaffected by the normal fluctuations in digestate acidity, residual catalyst, or the mineral content of cane leaf samples.

## Material and methods

Reagent grade chemicals and twice deionised water are used throughout.

### Sample preparation

Digest 500 mg dried leaf powder in a 100 ml Kjeldahl flask with 4.5 ml selenised sulphuric acid (5 g selenium dissolved in 1 litre 98% sulphuric acid) for a minimum of 1½ hours, or until the digest clears. Cool the contents of the flask, transfer them to a volumetric flask and dilute to 50 ml with water.

### Reagents

#### Reagent A

Dissolve 170 g sodium salicylate and 2.5 g sodium lauryl sulphate and dilute to 5 litres with water. Activate this stock reagent by dissolving 50 mg sodium nitroprusside per 500 ml, immediately before use.

#### Reagent B

Dissolve 60 g sodium hydroxide and dilute to 5 litres with water. Activate this stock reagent by dissolving 500 mg sodium dichloroisocyanurate per 500 ml, immediately before use.

### Calibration standards

Dissolve dried ammonium sulphate in 0.05 M sulphuric acid to give equivalent nitrogen contents in the range 0-3% N. The acid is present only to stabilise the standards and inhibit algal growth.

### Analytical procedure

Transfer 3 ml of digestate or standard into LKB preparation tubes. Use the LKB 2071 sample processor to dilute these samples with water. Instrument settings and conditions are shown in programme 1 (Fig. 1). Use preparation tubes to collect the diluted samples. Change to programme 2 (Fig. 1) and prepare the colour complexes using LKB cuvettes to collect the final solutions.

Allow to stand at room temperature for one hour then read the complex absorbance values using the LKB 7400 calculating absorptiometer with a 665 nm interference filter. Use a 2% N standard to factorise the instrument.

### Synthetic standards for test purposes

To isolate possible interference effects during the development work, synthetic standards in the range 0-3% N were prepared, in four different matrices, from ammonium sulphate.

*Set A.* Full range of nitrogen equivalents in water only.

*Set B.* 0 and 2% N equivalents in 0.25, 0.50 and 0.75 M sulphuric acid.

*Set C.* Full range in a digest blank matrix prepared by substituting cellulose for leaf powder in the digestion procedure.

\* Post graduate material for submission to the University of Natal Applied Chemistry Department.

PROGRAMME 1 (dilution)															
PUMP D			PUMP C			PUMP B			PUMP A			RACKS			
R	1	2	R	1	2	R	1	2	R	1	2	R	R	R	S
												+	+	+	
						●	●		●	●				●	●
						D/0,2-3,0 ml			S/60-900 µl			PUMP TYPE			
						2000			360			VOLUME µl			
						Water			Sample			REAGENT			

PROGRAMME 2 (complex preparation)																	
PUMP D			PUMP C			PUMP B			PUMP A			RACKS					
R	1	2	R	1	2	R	1	2	R	1	2	R	R	R	S		
												+	+	+			
			●	●		●	●		●	●				●	●		
						D/0,2-3,0 ml			D/0,2-3,0 ml			S/10-150 µl			PUMP TYPE		
						1500			1500			50			VOLUME µl		
						Reagent B			Reagent A			Diluted sample			REAGENT		

FIGURE 1 Sample processor programmes for total nitrogen determination.

Set D. Full range in a leaf ash digest matrix. 0,5 g portions of dried leaf powder were ashed at 500° C to remove all nitrogen. The residue from this treatment was then digested, following the procedure for leaf samples, except that the digestion time at full heat was extended to drive off some of the extra residual sulphuric acid.

An analysis of the blank solutions in sets C and D is shown in Table 1.

TABLE 1 Matrix analysis of synthetic standards

	N%	P%	K%	Ca%	Mg%	Residual sulphuric acid (M)
Set C	0,07	0,00	0,02	0,01	0,02	0,67
Set D	0,08	0,16	1,29	0,18	0,17	0,56

Spiked Digests

Attempts to spike leaf digests by adding ammonium sulphate solution to leaf samples in the Kjeldahl flasks, then digesting in the normal manner, proved unsuccessful. Severe frothing occurred and sample material was lost from the flask mouth. Tests showed that the easiest way to overcome this problem was to partially digest the samples for about 30 minutes to remove the easily oxidised organic matter. After cooling, ammonium sulphate was added and the flasks were then reheated until digestion was complete.

Development of the method

The method described by Searcy *et al*<sup>12</sup> for the determination of serum urea nitrogen was used, with minor modification, as the starting point of this investigation. The colour complex absorbance peak was identified as 665 nm from a plot of absorbance vs wavelength, produced using a Beckman model 24S spectrophotometer and chart recorder. Subsequent changes in methodology had no effect on the wavelength of this peak.

Fig. 2 shows the nature of the absorbance spectra for colour complexes and reagent blank, with water as a reference, under the conditions which were finally adopted. Subsequent measurements were taken using the LKB 7400 calculating absorptiometer with a 665 nm filter.

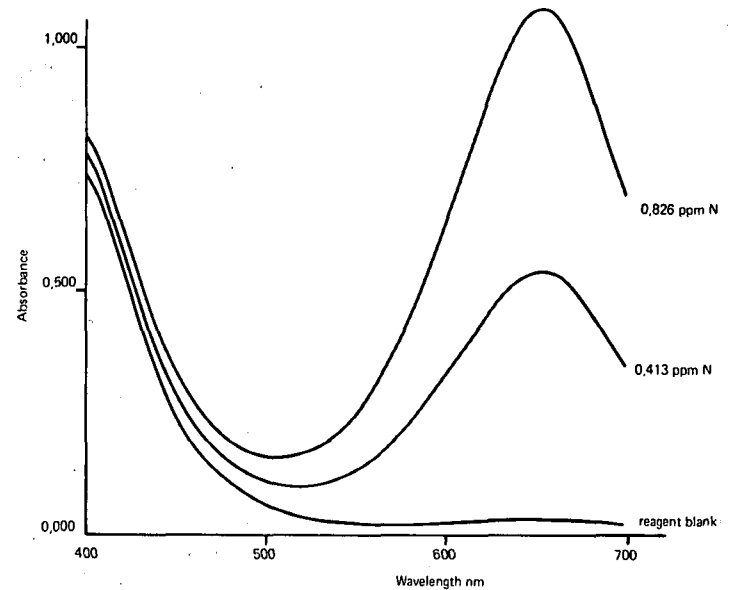


FIGURE 2 Complex absorption spectra.

In previous work, occasional anomalous high values were obtained when air bubbles adhered to the cuvette wall in the light path. Heating to 37°C for 10 minutes, as recommended by Searcy *et al*<sup>12</sup>, considerably aggravated this problem. A surfactant was used and successfully overcame the problem of cuvette bubbles in a method for soil phosphorus (Burrows<sup>4</sup>). In this case also, the addition of sodium lauryl sulphate to reagent A so reduced the surface tension of the final solution that cuvette bubbles were completely eliminated. Using reactant concentrations recommended by Searcy *et al*<sup>12</sup>, full colour was developed after standing for 30 minutes at ambient (22-25° C) and, since suitable heating facilities for batch use were not available, the heating step was discontinued.

Bietz<sup>1</sup> investigated the effect of reaction pH on the absorbance of the colour complex and recommended a pH of 12,48 for maximum colour development. Final solutions prepared using the modified Searcy's method had a pH of 12,25, and absorbance values could be increased by raising the sodium hydroxide content of reagent B. A plateau was reached at a concentration of 0,3 M sodium hydroxide and further increases had no effect on complex absorbance values.

Weatherburn<sup>13</sup>, in a comprehensive evaluation of reaction conditions for the phenol-hypochlorite reaction, reported an interaction between temperature, sodium nitroprusside concentration and the time taken to reach maximum absorbance. A similar effect was noted for the salicylate reaction, and Fig. 3 shows the effect of nitroprusside concentration on the time taken to reach maximum absorbance at ambient, and subsequent complex stability. Nitroprusside concentrations of 5 and 10 mg per 100 ml reagent A gave the highest absorbance values. An increase in the nitroprusside concentration to 20 mg, or greater, depressed absorbance values. It was necessary to strike a balance between the increased reaction speed at higher nitroprusside concentrations and the depressive effect of high concentrations on maximum complex absorbance. A standing time of about one hour presented no problems in the routine handling of this determination, so a nitroprusside concentration of 10 mg/100 ml reagent A was chosen. At this

concentration complexes were stable for at least a further two hours after formation.

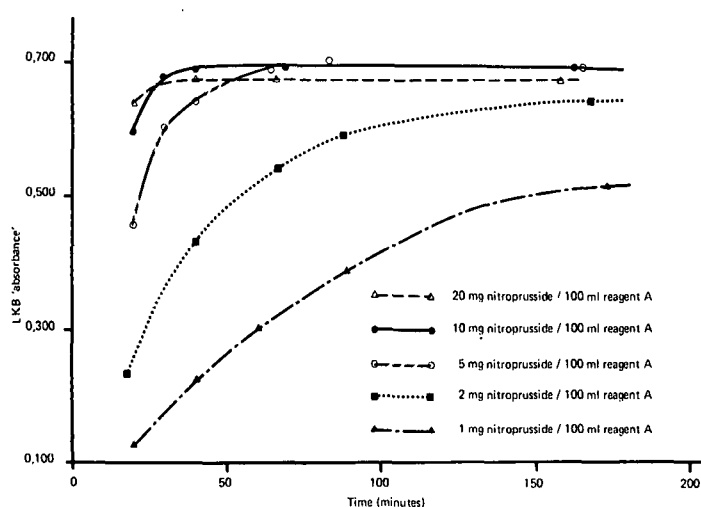


FIGURE 3 Effect of different nitroprusside concentrations on complex absorbance at 665 nm.

Both reagents had relatively poor storage properties. Reagent A was light-sensitive and slowly turned brown when kept in a clear glass bottle on the laboratory bench. Reagent B is reported to require refrigeration for satisfactory storage (Searcy *et al*<sup>12</sup>). Storage problems were simplified by keeping bulk stock solutions of the stable components and activating a small quantity of reagent by addition of sodium nitroprusside or sodium dichloroisocyanurate immediately before use.

Similar absorbance values were obtained for synthetic standards irrespective of matrix. A calibration curve for the reaction conditions adopted is given in Fig. 4. Quantitative nitrogen recoveries were also obtained from spiked digests (Table 2). These observations indicated that matrix interference effects were unlikely to be encountered during routine testing.

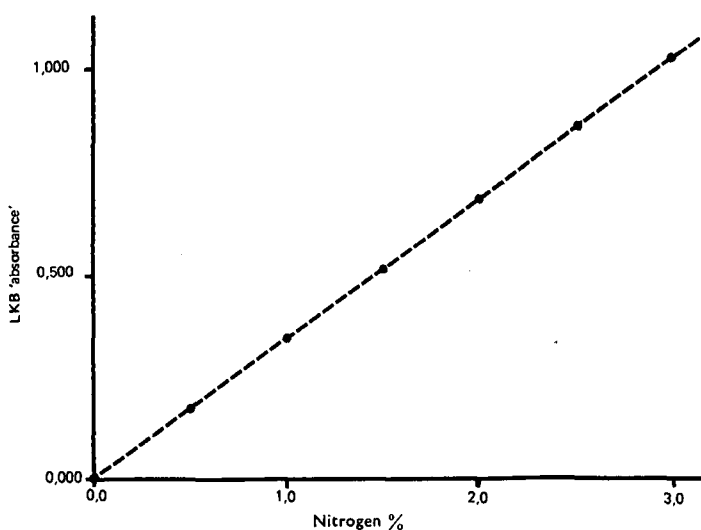


FIGURE 4 Nitrogen calibration curve.

### Routine testing

#### Precision evaluation

Fifty-six routine sample digests, with values ranging from 1,2-3,0% N were analysed twice in different batches, using the colorimetric and steam distillation methods. For statistical analysis, samples were divided into two groups with values above and below 2% N. Neither group showed a significant

TABLE 2  
Spiked digest recoveries using the colorimetric method

Sample	Untreated N%	0,5% N added			1,0% N added		
		N%			N%		
		Ex-pected	Meas.	Diff.	Ex-pected	Meas.	Diff.
E1	2,19	2,69	2,74	+0,05	3,19	3,22	+0,03
E4	1,97	2,47	2,48	+0,01	2,97	2,96	-0,01
E5	1,78	2,28	2,28	0,00	2,78	2,80	+0,02
E7	1,48	1,98	2,02	+0,04	2,48	2,45	-0,03
E10	1,35	1,85	1,86	+0,01	2,36	2,36	0,00
E11	1,69	2,19	2,21	+0,03	2,69	2,75	+0,06

difference between mean values obtained by the two methods (Table 3). Indications were that the colorimetric method was more precise. However, significantly different group mean values were obtained for the two batches measured by the colorimetric method. This difference was not apparent in the distillation results.

TABLE 3  
Precision comparison of distillation with the colorimetric method

	Less than 2% N		Greater than 2% N	
	Steam distil.	Colorim. method	Steam distil.	Colorim. method
Number of samples	24	24	32	32
Overall mean value	1,595	1,610	2,479	2,495
Between-batch diff.	mean value	0,003	0,026	0,041
	SE	±0,039	±0,021	±0,065
CV%	2,4	1,3	2,6	1,4

Broughton *et al*<sup>3</sup> consider that automatic analytical instruments show greater precision when samples are reanalysed within a batch rather than in a subsequent batch. Precision values shown in Table 3 contain mostly the within-batch variations. To obtain a more realistic assessment of the precision of the colorimetric method in routine use, three pooled digestate samples were reanalysed in duplicate on ten different days. The results (Table 4) confirm that greater precision is obtained when samples are reanalysed in the same batch. There was approximately a twofold difference between the within and between-batch precision values. However, the relative standard deviation values for the between-batch variability in the colorimetric method were of the same order as those calculated from the duplicate distillation results.

#### Accuracy

Steam distillation is a standard reference method (Horwitz<sup>8</sup>) for the determination of ammonium in Kjeldahl digestates.

TABLE 4  
Precision estimates for the colorimetric method using bulked digestates

	K1 N%	K2 N%	K3 N%
Mean value . . . . .	1,027	1,516	2,170
SE within batch . . . . .	±0,0177	±0,0149	±0,0235
SE between batch . . . . .	±0,0327	±0,0353	±0,0462
CV% within batch . . . . .	1,7%	1,0%	1,1%
CV% between batch . . . . .	3,2%	2,3%	2,1%

In addition, quantitative nitrogen recoveries were obtained from the spiked digests and synthetic standards when analysed by the existing distillation procedure. Since there was also good agreement between distillation and colorimetric results, the accuracy of the colorimetric method is inferred.

#### Total carryover

In sequential analysis, carryover can occur when different samples follow a common path. Using the method described by Broughton *et al*<sup>3</sup>, total carryover in the colorimetric method was found to be less than 0,5% which is consistent with the manufacturers specifications (LKB application notes<sup>9</sup>).

#### Performance in routine use

For approximately a month after the introduction of the new method into the laboratory procedure, 10% of all routine sample digestates (272 samples) were reanalysed by steam distillation. Only 18 of these samples gave values which differed by more than 0,10% N and the differences disappeared when the samples were reanalysed. Discrepancies in the distillation results accounted for 14 of these cases. Three of the four discrepancies in the colorimetric values were caused by high initial values. Cuvette ammonium contamination is suspected in these cases in view of the sensitivity of the method.

#### Advantages of the new method

A major limiting factor in leaf sample output of the FAS laboratory was removed with the introduction of the colorimetric method for leaf nitrogen. During the first month of its operation laboratory output increased by almost 40% (990 samples) over the maximum number of samples previously analysed in this period. Labour requirement for this measurement has been significantly reduced. A batch of 200 samples, previously requiring 11 man hours labour, is now handled with the intermittent use of one laboratory assistant in 90 minutes. A total of only 70 minutes of instrument time is required for complex preparation and measurement.

There has been no loss in precision due to automation and the new method performs reliably in routine use. The colour complex is unaffected by normal fluctuations in digestate acidity, residual catalyst or sample mineral content and is

formed from safe, easily prepared reagents. The equipment is robust, easily operated and gives a direct paper-tape printout of results in N%.

#### Acknowledgements

Thanks are due to Mr M. Murdoch for his help with the statistical analysis and to Dr R. A. Wood and Professor D. E. A. Williams-Wynn for their careful reading and constructive criticism of the manuscript.

#### REFERENCES

1. Bietz, J. A. (1974). Micro-Kjeldahl analysis by an improved automated ammonium determination following manual digestion. *Anal Chem* **46** (11): 1617-1618.
2. Bremner, J. M. (1965) in *Methods of Soil Analysis Part 2*, ed C. A. Black. Amer Soc of Agron Inc. p 1196.
3. Broughton, P. M. G., Buttolph, M. A., Gowenlock, A. H., Neill, D. W., and Skentelbery, R. G. (1969). Recommended scheme for the evaluation of instruments for automatic analysis in the clinical biochemistry laboratory. *J Clin Path* **22**: 278-284.
4. Burrows, J. R. (1976). Research project progress report No 6. Unpub. report.
5. Burrows, J. R. and Meyer, J. H. (1976). Semi-automation with reference to the determination of aluminium and phosphorus in soil and cane leaves. *SASTA Proc* **50**, 114-117.
6. Crooke, W. M. and Simpson, W. E. (1971). Determination of ammonium in Kjeldahl digests of crops by an automated procedure. *J Sci Fd Agric* **22**, 9-10.
7. Davidson, J., Mathieson, J. and Boyne, A. W. (1970). The use of automation in determining nitrogen by the Kjeldahl method with final calculations by computer. *Analyst* (London). **95**, 181-193.
8. Horwitz, W. (ed) (1975). *Methods of Analysis of the Association of Official Analytical Chemists*. 12th ed Section 2.048, p 15.
9. LKB Ultrolab System Application Notes (1973). An 94. File 1.
10. Novozamsky, I., von Eck, R., von Schouwenburg, J. Ch., and Walinga, I. (1974). Total nitrogen determination in plant material by means of the indophenol-blue method. *Neth J agric Sci* **22**, 3-5.
11. Reardon, J., Foreman, J. A. and Searcy, R. L. (1966). New reactants for the colorimetric determination of ammonia. *Clin Chim Acta* **14**, 403-405.
12. Searcy, R. L., Reardon, J. E., and Foreman, J. A. (1967). A new photometric method for serum urea nitrogen determination. *Am J med Technol* **31** (1), 15-20.
13. Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* **39** (8), 971-974.