

The Use of Hydrogen Peroxide for the Digestion and Determination of Total Nitrogen in Chickpea (*Cicer arietinum* L.) and Pigeonpea (*Cajanus cajan* L.)

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The use of hydrogen peroxide as an alternative to catalysts in the determination of nitrogen in plant materials has been investigated. Nitrogen determination in leaf, stem and seed samples of chickpea and pigeonpea was carried out by three digestion procedures, using hydrogen peroxide (H_2O_2 digestion) without a catalyst, and with mercury or selenium as catalysts (catalyst digestions). The nitrogen values obtained by the three digestion procedures did not differ significantly from each other when examined by microKjeldahl (MKJ) using mercury as a catalyst, and by a Technicon auto analyser (TAA) using selenium as catalyst. The gradual addition of H_2O_2 , as recommended previously, was found to be unnecessary. In view of the cost and health hazards implicated with the use of mercury and selenium for digestion, the procedure based on H_2O_2 digestion is preferable for large scale N determinations in plant tissue and seed samples of these pulse crops. The results suggest that the H_2O_2 digestion technique can be conveniently adapted for total N analysis in plant tissues and grain samples by either TAA or MKJ method.

1. Introduction

With the increased demand for N analyses in samples of plant origin, many attempts have been made to develop rapid and reliable techniques for determining N in the various parts. Although several rapid methods of N determination have been established, the use of Kjeldahl digests for the determination of total N remains a standard practice in many laboratories. Findings from a review on the use of a catalyst in Kjeldahl digestions established mercury as the most effective¹ and commonly used catalyst in routine laboratory work. The speed and dependability of N analysis of plant material, including grain samples, have been considerably enhanced with the availability of the Technicon auto-analyser, where selenium is used as a catalyst for digestion.² The procedure using the Technicon auto-analyser (TAA) has been found suitable for large-scale screening of grain from chickpea and pigeonpea.^{3,4}

Several studies have been undertaken to find suitable replacements for the poisonous catalysts, mercury and selenium in the analysis of feedstuffs and cereal grains.^{5,6} These workers have suggested titanium dioxide in combination with copper sulphate as an alternative catalyst for the determination of total N by Kjeldahl procedure. A comparison of mercury and selenium catalysts for the determination of Kjeldahl-nitrogen in leaf and stem tissues of pigeonpea has been reported.⁷ Other workers have found that, with foodstuffs, potassium-copper sulphate used as a catalyst gave better results than mercury.⁸

The digestion of plant tissue with sulphuric acid and H_2O_2 followed by direct nesslerisation for total N determination was thoroughly studied by Yuen.⁹ The determination of total N in dried plant

material by digestion with a sulphuric acid- H_2O_2 mixture was found to be rapid, and simpler, than the normal Kjeldahl procedure, in which sulphuric acid with addition of potassium sulphate and a catalyst is used.¹⁰ The sulphuric acid- H_2O_2 digestion method nearly always gave results that were identical with those obtained by the Kjeldahl method.¹¹ Florence and Milner found that N in biological materials can be routinely determined in Kjeldahl digestion using H_2O_2 .¹² The present authors have evaluated the use of H_2O_2 for the digestion and determination of a wide range of N values in leaf, stem and seed samples of chickpea and pigeonpea. Results from the H_2O_2 digestion technique and the mercury and selenium catalyst digestion methods, used as standards, were compared.

2. Materials and Methods

2.1. Plant Material

Leaf, stem and seed samples of chickpea and pigeonpea were obtained from crops grown under normal agronomic practices during the 1980–81 season at ICRISAT Centre, Patancheru, near Hyderabad. The samples were oven-dried at 65°C . Seed samples were ground to pass a 60-mesh screen using a Udy cyclone mill, the leaf and stem samples were ground to a fine powder (40–60 mesh) using a Pica mill.

2.2 Apparatus and reagents

The apparatus used included a 75 ml capacity digestion tube, an electrically heated block digester (BD-40 Tecator, Sweden) capable of holding 40 tubes (2–3 boiling chips were used per tube), and a technicon auto analyser I (TAA) (Technicon, New York).

The reagents used were 30% (by vol) AR grade hydrogen peroxide solution (Sarabhai, India); sulphuric and phosphoric acids (AR grade, BDH, England); mercury catalyst: Kjeltab MQ tablet, each containing 1.5 g K_2SO_4 and 0.075 g HgO ; selenium catalyst: Kjeltab auto-tablet, each containing 1.5 g K_2SO_4 and 7.5 mg Se. Both catalysts were obtained from Thomson and Capper Ltd, Cheshire.

2.3. Experimental procedures

2.3.1. Mercury catalyst digestion

A 40–50 mg sample was placed in the Kjeldahl digestion flask. One Kjeltab MQ and 3 ml sulphuric acid mixture 95:5 (by vol) conc. sulphuric acid: 85% phosphoric acid were added for digestion.

2.3.2. Selenium catalyst digestion

A 70–80 mg sample was placed in a digestion tube. One Kjeltab auto-tablet was added, followed by 3 ml of the sulphuric-phosphoric acid mixture as above. Digestion was performed at 370°C ; after 1 h the material became clear, indicating complete digestion.

2.3.3. H_2O_2 digestion

A 70–80 mg sample was placed in a digestion tube with 2–3 boiling chips, 1.5 g potassium sulphate and 3 ml sulphuric-phosphoric acid mixture added. The H_2O_2 was then added: 2 ml for leaf and seed samples, and 3 ml for the stem sample. Digestion was performed at 370°C for 1 h. Although the contents reached the clearing stage after about 40 min of digestion, heating was continued for 1 h in order to compare the results with catalyst digestion. After cooling, distilled water was added to the digest to 75 ml, and aliquots were taken for the determination of nitrogen. All determinations were carried out in triplicate.

2.3.4. Total N determination

Total N in all the H_2O_2 digests were determined by both Technicon auto-analyser (TAA)³ and microKjeldahl (MKJ).¹³ The catalyst digests prepared by using selenium were analysed by TAA,

Table 1. Ranges and means of percentage N of leaf, stem and seed samples of chickpea and pigeonpea estimated by H_2O_2 , mercury and selenium catalyst digestion procedures

Tissue	Nitrogen content (%) ^a		
	Catalyst digestion		
	H_2O_2 digestion ^b	Mercury ^c	Selenium ^b
Chickpea: Leaf ($n=40$)	2.59–4.43 (3.40)	2.66–4.56 (3.38)	2.58–4.50 (3.37)
Stem ($n=35$)	0.63–1.70 (1.18)	0.68–1.78 (1.26)	0.63–1.79 (1.21)
Seed ($n=60$)	3.20–4.59 (3.72)	3.26–4.57 (3.74)	3.28–4.52 (3.73)
Pigeonpea: Leaf ($n=40$)	3.06–3.26 (3.15)	2.84–3.28 (3.14)	2.90–3.28 (3.17)
Stem ($n=35$)	0.78–1.16 (0.89)	0.79–1.20 (0.94)	0.80–1.17 (0.91)
Seed ($n=60$)	3.08–4.22 (3.54)	3.16–4.24 (3.53)	3.12–4.20 (3.54)

^aFigures within parentheses indicate the mean values.^bAnalysed by TAA.^cAnalysed by MKJ.

and those prepared by using mercury were analysed by MKJ as above. The latter could not be analysed by TAA in view of the significant interference in the colorimetric estimation caused by the precipitation of mercury ions.²

The effect of gradually adding H_2O_2 during digestion was also studied. Initially, 1 ml of H_2O_2 was added, and the remainder drop by drop using a Pasteur pipette. The additions were trickled down the side of the digestion tube to control effervescence during digestion. The efficiency of digestion was also studied by adding 1, 2, 3, or 4 ml H_2O_2 to the flasks before digestion. Nitrogen recoveries were measured by adding ammonium sulphate and sodium nitrate (equivalent to 0.5–0.6 mg N) to the sample before the digestion.

Total N was also determined after adding salicylic acid, as a reducing agent, to include nitrate-N in the tissue. H_2SO_4 (3 ml) containing 5% (by vol) salicylic acid was added to a weighed sample by using a procedure described earlier.¹⁴ Salicylic acid was tested using H_2O_2 digestion and catalyst digestion.

3. Results and discussion

3.1. Comparison of H_2O_2 digestion and catalyst digestion procedures

Accuracy and precision are important considerations for any method used for routine analysis. To assess the accuracy of the H_2O_2 digestion procedure, samples of leaf, stem and seed of chickpea and pigeonpea were analysed by all three methods for N content: the results are presented in Table 1. A comparison of the two digestion procedures did not reveal large differences in the mean N values of leaf and grain samples of both chickpea and pigeonpea. However, the mean N values for stem samples were higher for both catalyst digests than with the H_2O_2 digest, even after adding slightly larger amounts of H_2O_2 . It may be that the stem tissue being fibrous and woody in nature was not digested properly without the catalyst. However, addition of larger volumes of sulphuric acid (5 ml) and H_2O_2 (5 ml) did not improve the N recovered in H_2O_2 digests of stem tissues (results not shown).

Further, the functional relationships between H_2O_2 digestion and catalyst digestions were worked out according to the procedure described earlier.¹⁵ It was observed that, with two exceptions, intercept and slope between the methods did not differ significantly ($P \leq 0.05$) from zero and unity, respectively, when comparisons between Hg catalyst and H_2O_2 digestion, and between Se catalyst

Table 2. Coefficients of functional linear relationships between H₂O₂ digestion and catalyst digestion for chickpea and pigeonpea seed^a

Tissue	Chickpea		Pigeonpea	
	Intercept	Slope	Intercept	Slope
H ₂ O ₂ digestion vs Hg catalyst				
Leaf	-0.06 (-0.24, 0.11)	1.01 (0.96, 1.06)	0.43 (-0.02, 0.86)	0.86 (0.72, 1.02)
Stem	-0.02 (-0.11, 0.07)	1.01 (0.94, 1.09)	0.04 (-0.11, 0.09)	0.95 (0.93, 1.01)
Seed	-0.16 (-0.40, 0.08)	1.03 (0.98, 1.10)	-0.06 (-0.12, -0.002)	1.01 (0.97, 1.03)
H ₂ O ₂ digestion vs Se catalyst				
Leaf	-0.13 (-0.28, 0.02)	1.02 (0.98, 1.07)	0.03 (-0.28, 0.34)	0.99 (0.89, 1.09)
Stem	-0.02 (-0.05, 0.09)	-0.96 (-0.90, 1.03)	0.02 (-0.05, 0.09)	0.98 (0.91, 1.06)
Seed	0.21 (-0.40, -0.01)	1.04 (0.99, 1.10)	0.05 (-0.09, 0.008)	1.01 (0.99, 1.02)

^aBoth H₂O₂ digests and selenium digests were analysed by TAA; mercury digests were analysed by MKJ.
^bValues within parentheses are the 95% confidence limits.

and H₂O₂ digestion were made (Table 2). The exceptions were with chickpea seed (H₂O₂ digestion versus Se catalyst) and pigeonpea seed (H₂O₂ digestion versus Hg catalyst). However, in these two instances the methods compared do not show highly significant differences at the $P \leq 0.01$ level in respect of intercept and slope from zero and unity, respectively. This indicated that the digestion procedures examined did not produce results which differed significantly from each other. Correlation coefficients between H₂O₂ digestion and catalyst digestion procedures for different plant tissues are given in Table 3. Highly significant correlations were observed for both chickpea and pigeonpea. However, correlations were lower for stem tissue than with leaf and seed samples. It appears that the H₂O₂ digestion procedure for the determination of N in stem samples is less accurate than for leaf and seed samples. But the correlations are reasonable enough to suggest that this procedure may be used where a large number of stem samples are to be analysed routinely. There were highly significant correlations between TAA and MKJ methods for leaf, stem and grain samples of both chickpea and pigeonpea. This confirms earlier findings,^{3,4} and further suggests that a rapid procedure of TAA could be used for N analysis of leaf and stem samples, although additional studies are required to test its suitability for a large-scale N determination in these tissues.

Table 3. Correlation coefficients between N values obtained by H₂O₂-digestion and catalyst-digestion procedures

Tissue	Correlation coefficient ^d		
	a	b	c
Chickpea: Leaf (n=40)	0.98	0.97	0.99
Stem (n=35)	0.84	0.85	0.97
Seed (n=60)	0.99	0.96	0.98
Pigeonpea: Leaf (n=40)	0.93	0.94	0.97
Stem (n=35)	0.83	0.82	0.95
Seed (n=60)	0.98	0.97	0.96

^aH₂O₂ digestion TAA (x) vs Se digestion (TAA) (y).
^bH₂O₂ digestion TAA (x) vs Hg digestion (MKJ) (y).
^cSe digestion TAA (x) vs Hg digestion (MKJ) (y).
^dSignificant at 1% level.

Table 4. Standard error of estimation of nitrogen in chickpea samples by different procedures^a

		H ₂ O ₂ -digestion		Catalyst-digestion	
Tissue		TAA	MKJ	TAA ^b	MKJ ^c
Leaf	Range	2.98–3.10	3.07–3.14	3.01–3.12	3.02–3.14
	Mean	3.04	3.10	3.05	3.08
	s.e. \pm	0.084	0.078	0.091	0.082
Stem	Range	1.07–1.12	1.08–1.14	1.12–1.18	1.16–1.21
	Mean	1.10	1.12	1.15	1.18
	s.e. \pm	0.038	0.036	0.039	0.035
Seed	Range	3.48–3.59	3.52–3.61	3.47–3.58	3.48–3.55
	Mean	3.54	3.59	3.53	3.52
	s.e. \pm	0.074	0.065	0.073	0.066

^aAnalysis of 10 independent determinations on a bulk sample.^bSe catalyst digests analysed by TAA.^cHg catalyst digests analysed by MKJ.

The standard error of determination for the H₂O₂ digestion procedure was calculated and compared with that of the standard catalyst digestion procedures. In order to confirm these results, both the TAA and MKJ methods were used for N determination. It was found that the error involved in both the digestion procedures did not differ greatly from each other when tested by either of the methods of N determination using chickpea samples (Table 4). A slightly higher error was obtained when both the digests were analysed by the TAA method indicating that the MKJ method is more accurate than TAA procedure.³ A similar difference was observed when the plant tissue and grain samples of pigeonpea were examined by different procedures.

3.2. Effect of different concentrations of H₂O₂ on N determination

Preliminary work was carried out to find the optimum volume of H₂O₂ needed for digestion. Table 5 shows the effect of adding different amounts of H₂O₂ on the determination of N. No measurable differences in N values were observed when more than 2 ml H₂O₂ was added to leaf or seed samples of chickpea and pigeonpea. However, with stem tissue increasing the volume of H₂O₂ up to 3 ml gave higher results. Such a response might perhaps be expected because of the fibrous nature of the stem tissue requiring more H₂O₂ for carbonisation. The use of 2 ml H₂O₂ for leaf and grain samples and 3 ml for stem tissues was found satisfactory: H₂O₂ was added at the beginning of digestion.

Table 5. Effect of different amounts of H₂O₂ on digestion and determination of N in leaf, stem and seed samples^a

Tissue	Nitrogen content (%)			
	Amount of H ₂ O ₂ (ml)			
	1	2	3	
Chickpea: Leaf	3.04 \pm 0.07	3.10 \pm 0.07	3.11 \pm 0.06	3.10 \pm 0.07
Stem	1.18 \pm 0.03	1.28 \pm 0.04	1.34 \pm 0.04	1.35 \pm 0.03
Seed	3.46 \pm 0.08	3.52 \pm 0.07	3.52 \pm 0.08	3.51 \pm 0.06
Pigeonpea: Leaf	3.12 \pm 0.06	3.18 \pm 0.05	3.20 \pm 0.06	3.21 \pm 0.07
Stem	0.78 \pm 0.02	0.82 \pm 0.03	0.84 \pm 0.03	0.84 \pm 0.04
Seed	3.02 \pm 0.05	3.03 \pm 0.07	3.03 \pm 0.05	3.04 \pm 0.07

^aAnalysed by TAA. Mean of three determinations.

Table 6. Effect of the method of addition of H₂O₂ on N values^a

Tissue	Nitrogen content (%)	
	H ₂ O ₂ added	
	Initially	Gradually
Chickpea ^b : Leaf	3.04±0.08	3.10±0.09
Stem	1.10±0.04	1.07±0.04
Seed	3.54±0.07	3.62±0.06
Pigeonpea ^c : Leaf	1.21±0.03	1.15±0.04
Stem	4.07±0.09	3.98±0.10
Seed	3.28±0.07	3.30±0.08

^aH₂O₂ digests analysed by TAA.^bMean of 10 independent determinations.^cMean of six independent determinations.

3.3. Effect of the method of addition of H₂O₂ on N determination

In order to study the effect of the method of adding H₂O₂, N was determined on the digests prepared in two ways: (1) the total amount of H₂O₂ was added at the beginning of digestion; (2) H₂O₂ was added gradually as described earlier. The results obtained on different tissues based on such a comparison are shown in Table 6. No significant differences in N values were observed when either the whole amount of H₂O₂ was added initially or added gradually as digestion proceeded. Florence and Milner, using a variety of animal and plant products, suggested a gradual drop by drop addition of H₂O₂ during the process of digestion, but this procedure¹² was cumbersome and would limit the number of analyses on a routine basis.

3.4. Recovery of added NH₄⁺-N and NO₃-N

Recovery of added NO₃-N was low with either H₂O₂- or catalyst-digestion procedures (Table 7). Small differences in N recovery values were observed when stem, leaf and seed samples of these crops were compared. Nitrogen recovery values of NH₄⁺-N obtained by either digestion procedure was good and did not differ significantly from each other. Because NO₃-N present in the tissues will not be detected by the procedures described here, an attempt was made to determine the NO₃-N by using salicylic acid as a reducing agent in the digestion mixture, but no noticeable differences were found (Table 8). While NO₃-N is usually absent in seed samples, NO₃-N ranging from 20 to

Table 7. Recovery (%) of added NO₃-N and NH₄⁺-N using H₂O₂-N and catalyst-digestion procedures^a

Tissue	Catalyst digestion					
	H ₂ O ₂ digestion ^a		Selenium ^a		Mercury ^b	
	NO ₃ -N	NH ₄ ⁺ -N	NO ₃ -N	NH ₄ ⁺ -N	NO ₃ -N	NH ₄ ⁺ -N
Chickpea: Leaf	40.8±1.2	98.5±2.1	61.6±1.4	99.3±1.9	59.4±1.8	98.7±2.1
Stem	40.4±1.5	100.4±1.9	56.2±1.5	98.4±2.0	57.5±1.9	99.0±1.6
Seed	39.3±1.5	97.8±1.8	64.3±1.8	97.4±1.7	63.6±2.0	96.5±2.0
Pigeonpea: Leaf	64.0±1.8	96.5±2.0	63.5±1.9	99.0±1.8	64.8±1.3	96.4±2.5
Stem	40.5±1.4	98.9±1.9	53.8±1.7	98.2±2.2	55.6±1.9	97.5±1.8
Seed	36.4±1.7	98.7±2.1	50.8±2.9	99.8±1.9	52.9±1.4	98.3±2.0

^aH₂O₂ digests and Se catalyst digests analysed by TAA.^bHg catalyst digests analysed by MKJ.^cMean of three determinations.

Table 8. Comparison of H₂O₂, catalyst, and catalyst+salicylic acid digestion procedures for total N determination.*

Tissue	Nitrogen content (%)				
	H ₂ O ₂ digestion ^a	Catalyst digestion		Catalyst+salicylic acid	
		Selenium ^a	Mercury ^b	Selenium ^a	Mercury ^b
Chickpea: Leaf	2.98±0.04	3.06±0.05	3.10±0.04	3.08±0.04	3.04±0.06
Stem	0.68±0.03	0.70±0.04	0.69±0.03	0.70±0.05	0.68±0.04
Seed	4.03±0.08	4.08±0.07	4.16±0.09	4.04±0.10	4.09±0.08
Pigeonpea: Leaf	4.15±0.12	4.20±0.09	4.28±0.10	4.17±0.12	4.14±0.09
Stem	1.25±0.05	1.28±0.04	1.25±0.04	1.24±0.03	1.22±0.05
Seed	3.26±0.08	3.30±0.07	3.29±0.08	3.28±0.06	3.27±0.06

*H₂O₂ digests and Se catalyst digests analysed by TAA.

^bHg catalyst digests analysed by MKJ.

^cMean of three determinations.

200 µg g⁻¹ has been found in leaf and stem samples of these crops (unpublished results). These levels are not large enough to cause significant errors against a background organic N content of 2–4%

4. Conclusion

The results presented here using diverse plant samples indicate that the H₂O₂-digestion method can easily be adapted for total N analyses using either the auto-analyser or distillation techniques.

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