韓草誌(J. Korean Grassl. Sci.) 16(4) : 253 ~ 259, 1996

Ammonia Microdiffusion and Colorimetric Method for Determining Nitrogen in Plant Tissues

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암모니아 확산 및 발색에 의한 식물조직의 질소분석 방법

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적 요

암모니아 확산 및 암모니아 발색후 자외선 분광광도계에 의한 질소분석방법을 설정하기 위한 실험을 실시하였다. 미세확산용기(Conway microdiffusion cell)를 이용해 켈달소화 후 무기질화된 질소를 NaOH에 의한 알칼리화 및 HCl에 의한 산화반응을 통하여 NH_4^+-N으로 유도하였다. 암모니움 베이스의 표준용액을 이용하여 확산시간에 따른 질소함량 및 회수율을 측정한 바 15시간 이상 반응으로 99% 이상의 질소회수율을 보여주었으며, 반복간 높은 재현성을 나타내었다. 회수된 NH_4Cl의 발색반응을 자외선 분광광도계에서 검토하여 발색반응제의 조성을 조정하였다. 0.5ml의 시료, 4.0ml의 증류수 및 0.5ml의 암모니아 발색제를 혼합하여 발색시켰을 때 410nm에서 최대흡광도를 주었고, 발색후 5~45분간의 흡광도는 매우 안정하였다. 표준용액의 NH_4^+-N의 함량과 흡광도 간에 고도의 정의 상관관계가 인정되었다. 미세확산－암모니아 발색반응에 의해 분석한 식물체 시료의 단백질태 및 총 질소함량은 켈달－증류방법에 의해 얻어진 분석값과 잘 일치하였으며 시료반복간에는 높은 재현성을 보여주었다.

I. INTRODUCTION

In principle, the Kjeldahl digestion and distillation are done in the same way as the ordinary organic nitrogen (amino acid-N, protein-N and total-N) determination of plant or soil samples. By the classical Kjeldahl digestion, sample is attacked and mineralized by the action of concentrated sulfuric acid and oxidizing catalyzer. The nitrogen in sample is transformed into ammonium sulphate by a mineralization procedure. In some methods, hydrochloride is used instead of sulfuric acid, resulting in the formation of ammonium chloride. Ammonium chloride, however, dissociates already in temperature below 100℃, making the volatilization of ammonia from the sample and sublimation of NH_3 from the surrounding air to the sample possible (Hauck, 1982). This can change the enrichment of sample, especially when ammonia is present in the air used for evaporation. Sulfuric acid is preferred, because $(NH_4)_2SO_4$ is stable in temperatures up to 235℃. After the Kjeldahl digestion the nitrogen becomes in the form of ammonium-N.

Many works applied the ammonia distillation method to convert ammonium-N in the digests to ammonia for the nitrogen determination. The digests is made alkaline with NaOH, and NH_4^+-N is steam

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distilled to an acid. Usually boric acid is used for collection of ammonia. This technique has some disadvantages of being fairly tedious because of taking more time and labor. The application of large volume of sample digests and high concentration of sodium hydroxide cause an inconvenience. Also vagueness of titration point (color changing or reading point) results in an inaccuracy of N quantification. Alternatively, there is a limitation to titrate for a the samples containing too small amount of nitrogen. For example, one drop (about 0.1~0.2 mL) corresponds to 140~280 μg N when 0.1 N of HCl is used as a titration solution. So it is logically impossible to determine smaller amount of nitrogen than this level. For the determination of ammonium or nitrate fractions separated by distillation, similar disadvantages above-mentioned are actually being followed. Various methods have been proposed for the improved direct determination by a colorimetetric measurement (Cataldo *et al.*, 1975; Sen and Donaldson, 1978; Lyons *et al.*, 1991).

A more simple, stable and rapid technique is required for the conversion of detectable ammonium salt and for the subsequent determination of ammonia. In this work most attention was paid to test the suitability for the ammonia diffusion of digests using a Conway microdiffusion cell and to establish a colorimetric method for the nitrogen determination.

Ⅱ. MATERIALS AND METHODS

Apparatus and reagents

1) Conway microdiffusion cell

Two types of Conway cell having different size were tested for determining the efficiency of ammonia diffusion. The external compartments is destined to received sample digests and sodium hydroxide. Sample digests are alkalized and produce ammonia gas. The central compartment of 85 mm cell can contain 2 mL of HCl N/10, while that of 110 mm cell contain until 5 mL. In this compartment ammonia, which is produced by microdiffusion in external compartment, is trapped and is converted to ammonium chloride.

2) Color reagent

Nessler's reagent (BDH, England) or ammonia color reagent (Sigma Diagnostics) are available. After incubation for coloration, the absorbance is proportional the amount of ammonium-N.

Preparation of sample and authentic standard solution

Plant tissues of alfalfa (*Medicago sativa* L.) that had been oven-dried were ground to fine powder. The ground samples were prepared for the determination of total nitrogen and protein-N. For total nitrogen determination, about 250 mg of sample were digested to reduce nitrate by 5% salicylic acid-H_2SO_4, and then mineralized by Kjeldahl procedure. The protein-N fraction was obtained from insoluble residues of 80% ethanol after centrifugation at 10,000 rpm. The vacuum-dried residues were mineralized by Kjeldahl procedure. The digests were diluted to 100 fold with distilled water, and then used for ammonia diffusion in a Conway dish.

For determining the diffusion efficiency, authentic chemical solutions were made from $(NH_4)_2SO_4$ following a dissolution with concentrated H_2SO_4. Two authentic solutions containing 500 μg and 1,000 μg N/mL were used for microdiffusion. The diffusion efficiency in a Conway cell was kinetically checked by measuring the percentage of nitrogen recovery.

Preparation of standard curve for N quantification

The mother solution containing 1 mg N-NH_4/mL was prepared with $(NH_4)_2SO_4$. The working standard solutions were made to 1, 5, 10, 20, 40, 50, 70, 90 and 100 μg N/mL with a careful dilution of mother solution into separate volumetric flask. The standards solutions were prepared fresh daily.

Nitrogen determination

1) Distillation of ammonia

The contents of mineralization tubes were mixed thoroughly and adjusted to 100 mL with distilled water. An aliquot of 10 mL of digest was pipetted into a distillation flask. The flask was connected to the spray trap and 10 mL of 10 N NaOH was added down the steam inlet tube. The steam was passed through until 25 mL of distillate had collected in a Erlenmeyer flask 3 mL of 1% boric acid-indicator solution. The amount of N was titrated against 0.1 N HCl.

2) Colorimetric determination by using micro-diffusion-nesslerization method

From previous experiments for checking the spectrum and the kinetics of absorbance, coloration method was slightly modified. An aliquot of 0.5 mL of digest and 4 mL of distilled water was pipetted into a test tube, and then 0.5 mL of ammonia color reagent were subsequently added. After mixing thoroughly and 10 minutes of coloration at room temperature, absorbance at 410 nm was read in a UV-spectrophotometer. N amount was calculated from determined absorbance by using the correlation equation of standard curve.

Ⅲ. RESULTS AND DISCUSSION

Standardization of coloration and standard curve making

The coloration and stability of absorbance were scanned by the function of spectrum and kinetics in a U. V spectrophotometer (Shimadzu 2200). The method of coloration was firstly adapted from that used by Konttinen (1967). Table 1 shows some analytical results observed from two different coloration methods. An aliquot of 0.3 mL of standard solution (50 μg N/mL), 0.7 mL of distilled water and 2.5 mL of 10N NaOH were well mixed. 1.5 mL of ammonia color reagent was finally added. After the color development at room temperature, absorption curve was checked. It showed maximal absorption at 425 nm. The kinetic measurement of absorbance showed a sufficient stability although a little decrease was observed for 45 minutes after coloration. Absorbance at 425 nm was linear from 1 to 50 μg N/mL, but aliquots containing up to 75 μg N were slightly lost the sensitivity and linearity between absorbance and N content. In aliquots containing up to 150 μg N, a turbidity also appeared. General inconveniences, (e. g., Imprecision caused by turbidity, difficulty of 10N NaOH employment, high consumption of ammonium color reagent) were presented in this method. Particular attention was therefore paid on replacement NaOH with distilled water and on the setting up of appropriate wavelength for absorbance reading.

Table 1. Comparison of two different coloration methods. Reaction was scanned with standard solutions containing 1 to 200 μg N/mL prepared from ammonium sulphate

Reagent composition	Maximal absorption	Observation
0.3 mL of standard solution +0.7 mL of distilled water +2.5 mL of 10N NaOH +1.5 mL of color reagent	425 nm (stable until 15 min.)	turbidity (non linear from 75 μg/mL)
0.5 mL of standard solution +4.0 mL of distilled water +0.5 mL of color reagent	410 nm (stable until 45 min.)	clear color development (non linear from 150 μg/mL)

The effect of reagent composition on color development was previously checked with modifying the ratio of NaOH from to 0% (Table 2). When absorbance was read at 425 nm, it slightly increased with decreasing the percentage of NaOH 10N and was relatively unstable. Alternatively, when absorbance was read at 410 nm most of absorbances reached to the maximum level at 425 nm. Also the absorbances at 410 nm were much less affected by the ratio of NaOH and color reagent, showing non-significant difference among reagent compositions. This clearly showed that a sufficient coloration could be obtained without an addition of NaOH. The results suggested that NaOH could be replaced with distilled water with controlling the wavelength for absorbance reading. Based on these results, reagent composition for coloration was set up as follows; 0.5 mL of standard solutions, 4 mL of distilled water and 0.5 mL of ammonia color reagent. The color development is rapid and is stable for at least 48 hours in light or dark. Maximal absorption occurred at 410 nm. The kinetic measurement of absorbance showed a high stability with a range from 1.0520 at 5 min to 1.0390 at 45 min after color development. Absorbance at 410 nm was linear with ammonium-N content from 1 to 100 μg N/ml.

Table 2. Effect of reagent composition on absorbance for Nessler reaction. The ratio of each treatment represents the percentage of standard solution: distilled water: 10N NaOH: ammonia color reagent. The standard solution containing 50 μg N/mL was used for coloration

Reagent composition	Absorbance at 425 nm	Absorbance at 410 nm
6:14:50:30	0.701	0.990
6:20:44:30	0.770	1.011
6:30:34:30	0.900	1.070
6:64:0:30	1.001	1.003
6:74:0:20	1.018	1.021
6:84:0:10	1.050	1.053

The working standard curve obtained from 1, 5, 10, 20, 40, 50, 70, 90 and 100 μg N/mL is shown Fig. 1. It shows the highly significant positive correlation between N content and absorbance at 410 nm. In addition, the N quantification using standard curve for authentic solutions or digests of plant sample also confirmed the precision and suitability of this method for N determination.

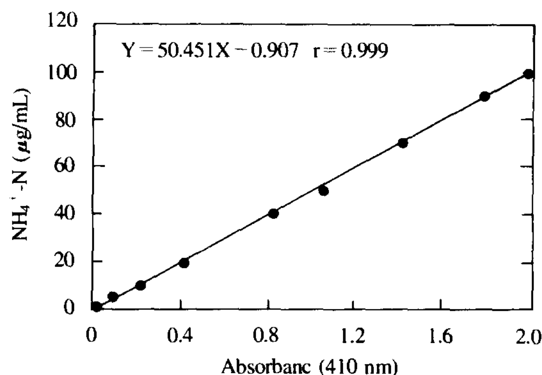

Fig. 1. Standard curve showing the linear response between absorbance and NH_4^+-N. Absorbance of each standard solution was corrected with that of distilled water.

Microdiffusion of ammonia in a Conway dish

For investigating the efficiency of ammonia diffusion in a Conway dish, appropriate deposing quantities of sample digests and NaOH in external compartment and HCl in central compartment were firstly examined. For 110 mm cell, 2 mL of HCl N/10 (corresponding 0.2 meq H^+ allowing to fix 3.6 mg NH_4 or 2.8 mg N) in central compartment. 2 mL of sample digest or authentic solution (previously diluted to 15 meq H^+) and 2 mL of NaOH 10N (corresponding 20 meq OH^-) were separately placed in external compartment. After a closure with keeping no leakage of gas, sample digest and NaOH 10N allowed to contact. The solution in central compartment, in form of ammonium chloride, was collected at interval until 18 hours after

microdiffusion. The collected solutions were diluted to 10 times and determined NH_4^+-N. Absorbance, N content and percentage recovery according to the microdiffusion time are shown in Table 3. The results showed a great reproductivity among sample repetitions. Under microdiffusion condition above mentioned, it is necessary up to 15 hours of microdiffusion to obtain above 99% of recovery. Microdiffusion with 85 mm cell (1 mL of HCl N/10 in central, 2 mL of authentic solution and 2 mL of NaOH 10N) also showed a high precision and reproductivity (data not shown).

Table 3. Quantification and percentage recovery of nitrogen in relation to microdiffusion time in a Conway dish. An aliquot of 2 mL of standard solution containing 500 μg N/mL was microdiffused with 2 mL of 10N NaOH. The HCl solution in the central compartment was collected and diluted to 10 mL. Nitrogen was quantified by using standard curve. Each value is the mean ± S.E for n=3

Diffusion time (hr)	Absorbance	N content (μg N/2mL)	Recovery (%)
2	1.3431±0.024	668.5±12.1	66.8±1.2
4	1.4718±0.180	733.5± 9.2	73.3±0.9
8	1.8027±0.017	900.4± 8.8	90.0±0.9
12	1.9287±0.008	963.9± 4.3	96.4±0.4
15	1.9860±0.009	992.9± 5.0	99.3±0.4
18	1.9967±0.047	998.3± 2.4	99.8±0.2

Nitrogen determination of plant samples

To verify precision and suitability of microdiffusion-nesslerization method for nitrogen determination, protein-N and total-N of dried alfalfa leaves were determined and the data obtained were compared with those of Kjeldahl distillation method (Table 4). The proposed microdiffusion-nesslerization method and Kjeldahl distillation method gave similar values in protein-N and total-N. However, values obtained with microdiffusion method were slightly higher than those with distillation method. Comparing a variability of sample repetition, microdiffusion method is more reliable and stable than distillation method. The results also suggests that a small loss of ammonia seems be occurred in the latter method. The possibility of ammonia loss during distillation were pointed out by several works. The surfaces of the apparatus and flasks used in distillation contain small amounts of negative charges, where a small fraction of the ammonia in the samples is absorbed (Buresh *et al.*, 1982; Pruden *et al.*, 1985). This may give erroneous results of N determination of subsequent samples. Reducing erroneous effect is especially important for distillation of ^{15}N enriched samples. The apparatus is suitable for the double distillation procedure suggested by Saffigna and Waring (1977). Also a distillation using ethanol steaming between samples gives equally reliable results, but this procedure is more laborious. In contrast, an alkalification of digests with NaOH and acidification of NH_4^+-N with HCl, which are main chemical reaction in the distillation method, could be much reliably obtained in a microdiffusion cell. Also a colorimetric measurement with ammonia color reagent gives a wide range of N determination. Thus, it is proposed that microdiffusion-nesslerization method can suitably replace a Kjeldahl distillation method. The proposed methods can be also easily used for sample preparation or fractionation of ^{15}N isotope, which is usually contained below microgram level.

Table 4. Comparison of two methods for nitrogen determination. Dried sample of alfalfa leaves used for protein-N and total-N determination

Fraction		Microdiffusion-nesslerization		Kjeldahl distillation	
		μg N	%	μg N	%
Protein-N	1	8,365	3.50	7,360	3.20
	2	9,025	3.61	8,164	3.14
	3	8,730	3.52	8,477	3.46
	4	8,700	3.48	7,995	3.29
	5	8,747	3.43	8,109	3.31
Total-N	1	11,952	4.80	10,632	4.43
	2	11,650	4.66	11,076	4.26
	3	11,334	4.57	11,246	4.59
	4	11,775	4.71	11,202	4.61
	5	11,343	4.63	10,902	4.45

Ⅳ. SUMMARY

Ammonia microdiffusion method and colorimetric measurement are described for the nitrogen determination. The diffusion of ammonia could be successfully induced by using a microdiffusion cell. It is a simple and rapid technique, which is suitable for transforming the nitrogen in digests into NH_4Cl for the colorimetric N determination with ammonia color reagent. Above 99% of N recovery were obtained with microdiffusion up to 15 hours. The coloration method of collected NH_4Cl for the colorimetric N determination was also estabilshed with a scanning in U.V. spectrophotometer. Under the proposed coloration method (0.5 mL of sample digest, 4.0 mL of H_2O and 0.5 mL of ammonia color reagent), a maximal absorbance was observed at 410 nm. The kinetic measurement of absorbance showed a high stability from 5 to 45 minutes after color development. Absorbance was directly proportional to the amount of NH_4^+-N present. The microdiffusion-ammonia coloration method was successfully applied to the nitrogen determination in the forms of protein-N or total-N in plant tissue. Comparing with Kjeldahl distillation method, the values obtained with described method were slightly higher and more reliable.

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