Direct Analysis of ^{15}N Abundance of Kjeldahl Digested Solution by Emission Spectrometry

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Summary

Direct analysis of ^{15}N abundance of Kjeldahl digested solution by emission spectrometry was established. Plant sample is digested by Kjeldahl digestion method using H_2SO_4 and H_2O_2, and an aliquot of digested solution including ammonium nitrogen (about 2μgN) is directly taken into a Pyrex glass tube with the outer diameter 4mm. After water in the Pyrex tubes is evaporated, the glass tubes are evacuated by the vacuum system for discharge tube preparation and sealed with oxidation reagent (CuO) and water absorbent (heated CaO at 950℃). The sealed tubes are heated at 560 ℃ for 30 min in a muffle furnace to oxidize ammonium into N_2 gas. The ^{15}N abundance can be measured by emission spectrometry (JASCO N-150 analyzer). This procedure is the simplest and accurate method for the determination of ^{15}N abundance of total N in the samples.

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Key words: ^{15}N, Emission spectrometry, Kjeldahl digestion

^{15}N is a stable isotope and its natural abundance is 0.366 % (99.634 % ^{14}N), although there are small variations due to isotope discrimination by some physical, chemical and biological processes. Recently precise mass spectrometric analysis is used for measuring the natural abundance ($\delta^{15}N$) of biological materials for estimating the origin of N such as N_2 fixation, organic materials or chemical fertilizers.

Nitrogen is one of the most important plant macronutrient, and the studies on the fate of N fertilizer applied are very important. Also, the absorption, transport and metabolism of nitrogen in plant are of a major interest in both fields of plant nutrition and physiology.

Dr. Kumazawa introduced emission spectrometric determination of ^{15}N by revising the precise analysis of a trace amount of samples and he applied this method for plant nutrition researches [1-4]. In this method sample is taken into a Pyrex glass tube with oxidizer (CuO) and water and CO_2 absorber (CaO) and evacuated, sealed followed by heating for converting sample N to N_2 in the tube. Spectroscopic measurement of the ^{15}N abundance is based on the measurement of the intensity of the emission spectra of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$ molecules by scanning of the wavelength from 299 nm to 297 nm. ^{15}N labeled compounds are available after condensation of ^{15}N by the exchange reaction between nitric acid and the oxides of nitrogen [5].

The advantages of emission spectrometry for ^{15}N measurement compared with mass spectrometry are as follows:

1) The amount of N required for the anaysis is only 1-2μgN. This is equivalent to about 0.1mg of dry matter, or one spot of amino acid separated by two-dimesional thin layer chromatography [6,10].

2) Low running cost and easy maintenance of the equipment compared with mass spectrometry.

The disadvantages of this method are as follows:

1) Less precision in low ^{15}N abundance samples. For quantitative analysis, the data lower than 0.1 atom % excess is not reliable [6]. Emission spectrometry cannot be used for determination of natural abundance of ^{15}N ($\delta^{15}N$).

2) The preparation of discharge tubes needs some practice and time consuming.

For the ^{15}N analysis of the total N in plant materials, a Pyrex glass tube with 8 mm diameter was originally used for making discharge tube by Dumas method[1]. Several mg of dry plant powder is taken into a 8 mm outer diameter Pyrex tube and air inside is evacuated and sealed with 0.5g of CuO and 1 g of CaO, and heated at about 650℃ for 2-6 hr for complete combustion of plant powder. This method needs skillful glass works and it is time consuming.

The alternative is Rittenberg method, in which ammonium sulfate solution by Kjeldahl digestion is converted to N_2 by the addition of NaOBr solution[1]. Relatively a larger amount of N (eg. 50-100μgN) is necessary for this analysis, and this method is also time consuming.

The condensation of ammonium in HCl solution was used for ^{15}N analysis, followed by diffusion from Kjeldahl digested solution by the addition of 10 M NaOH. The condensed solution was taken into a small capillary tube and dried under an infrared lamp[1].

We tried to use the Kjeldahl digested solution directly taking into a Pyrex glass tube with 4 mm outer diameter for discharge tube preparation. Although an aliquot of sulfuric acid remains in the bottom of the tube after evacuation, no

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interference occurred for emission and measurement due to the small amount of the existed sulfuric acid. So far, it is the easiest way to determine ^{15}N abundance of the total N in plant materials and other sources, such as animals or environmental materials.

MATERIALS AND METHODS

Sample preparation and grinding

Plant samples are thoroughly washed with tap water, then rinsed with de-ionized water and blotted by a paper towel. Plant samples or separated parts are packed in an envelope and dried in ventilation oven at 60-80 ℃ for several days until the dry weight becomes constant.

Dry plant materials are ground into a fine powder. Special care should be paid not to cross contaminate （not to mix） the samples during grinding. The grinder should be cleaned up completely for each sample by washing or wiping the container. The amount of N in a tube for ^{15}N analysis is very low and a trace amount of N contamination will give a significant error. So care should be taken not to contaminate N between samples or from equipments, water, reagents, air, dust, hands or other environmetal materials.

Kjeldahl digestion

Fig. 1 shows the out line of Kjeldahl Digestion using H_2SO_4 and H_2O_2 for plant samples containing low nitrate （Fig. 1. left: NO_3-N is less than 5% of total N） and high nitrate （Fig.1. right）[7]. The concentration of NO_3-N in plant powder can be analyzed by Cataldo's method followed by hot water extraction[8,9]. 50mg of fine dry powder with low nitrate, such as in seeds, was put into a test tube（approximately 20 mm in outer diameter and 20 cm in tall）. Then 1 mL of H_2SO_4 （Precise analytical grade with least ammonium contamination） is mixed, and kept over night with a parafilm lid on it to protect ammonia or other N contaminant from the outside air. Test tubes are set in an aluminum block heater and plant materials are digested at 180℃ for 10 min. Then the test tubes are taken out from the block heater and they are kept at room temperature for 5 min for cooling. Then 0.3 mL of 30 % H_2O_2 is added to the test tubes. Vigorous agitation should be avoided due to insufficient cooling. The test tubes are heated at 230 ℃ for 30 min. Then 0.3 mL of 30 % H_2O_2 is added again. The heating and adding H_2O_2 are repeated for several times until the solution becomes clear and transparent. Then the test tubes are heated at 230 ℃ for 60 min in order to degrade H_2O_2 completely. When H_2O_2 remains in the digested solution, it will interfere the Indophenol colorimetry for ammonium determination. The digested solution is filled up in a 25 mL volumetric flask.

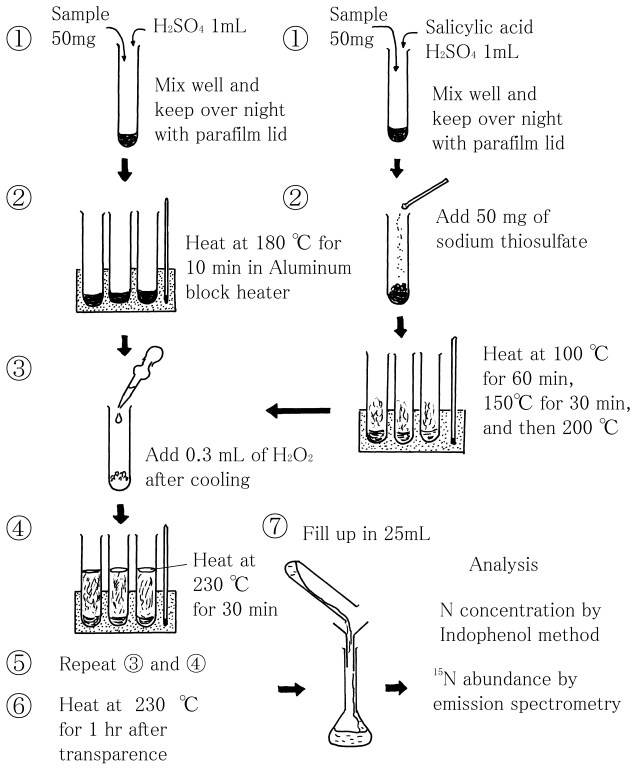

Fig.1. Kjeldahl digestion of plant samples with a low concentration of nitrate （left） and a high concentration of nitrate （right）.

In the case of plant materials which contain high levels of nitrate （NO_3^--N over 5% of total N） such as roots and stems, nitrate in the plant materials should be reduced to ammonium with reducing reagents （salicylic acid and sodium thiosulfate） under mild temperature conditions, unless nitrate will volatile from the acidic solution. 1 mL of salicylic-sulfuric acid （10 g of salicylic acid was dissolved in 300 mL of H_2SO_4） is used instead of H_2SO_4. Just before heating, about 50 mg of sodium thiosulfate is added. The temperature should be increased slowly, for example, 100 ℃ for 60 min, 150℃ for 30 min and up to 200 ℃ for 30 min, nitrate was then completely recovered[7]. Then the tubes are taken out to cool for 5 min, and 0.3 mL of H_2O_2 is added as same as mentioned before.

Determination of N concentration by indophenol method

Reagent

EDTA solution: Dissolve 25 g of EDTA（ethylenediamine-*NNN'N'*-tetraacetic acid）•2Na in about 800 mL of water. Adjust pH at 10 using 10 M NaOH solution. Add 20 mL of 0.25 % methyl red in 60 % ehtanol solution as a color indicator. Fill up to 1 L with water.

P-buffer（1 M of Potassium phosphate buffer）: Dissolve 136.09 g of KH_2PO_4 and 2.75 g of benzoic acid in 1 L of water.

Nitropursside reagent: Prepare liquid phenol. Dissolve 500 g of phenol in water bath and add 47mL of water.（Stable for a half year under room temperature）. Dissolve 10.25 mL of liquid phenol and 100 mg of sodium nitropursside in 1 L of water.（Stable for 2 weeks in a refrigerator）

Hypochlorite solution: Dissolve 10 mL of sodium hypochlorite solution（available Cl 5%<）, 10 g of NaOH, 7.06 g of $Na_2HPO_4 \cdot 7H_2O$ and 31.8 g of $Na_3PO_4 \cdot 12H_2O$ in 1 L of water.

1M NaOH: Dissolve 40 g of NaOH in 1 L of water.

Ammonium standard solution （100μgN mL^{-1}） : Dissolve 471.1

mg of ammonium sulfate in 1 L of 0.25 M sulfuric acid (Stock solution). Dilute 2.5 mL of stock solution into 50 mL. Put 0, 0.5, 1.0, 1.5, 2.0 mL of the diluted solution (5 μgN mL^{-1}) in 25 mL volumetric flask for calibration standard.

Procedure

Fig. 2 shows the procedure for determination of the concentration of ammonium in Kjeldahl digested solution as mentioned above. 0.1-1 mL of digested solution is put into a 25 mL volumetric flask. The volume depends on the ammonium concentration estimated. 0.5 mL of EDTA solution and 0.5 mL of P-buffer are added to the flask in this sequence. The solution is neutralized with 1 M NaOH by the indication of methyl red color reagent changed from red to yellow, then 2.5 mL of nitroprusside reagent is added immediately and stirred well. Then 2.5 mL of hypochlorite solution is added, the flask is filled up to 25 mL with water. The volumetric flasks are kept at room temperature or at 30℃ incubator for 3 hr or longer. The optical absorption at 625 nm is measured. The ammonium concentration can be calculated from the calibration line with ammonium standard solution.

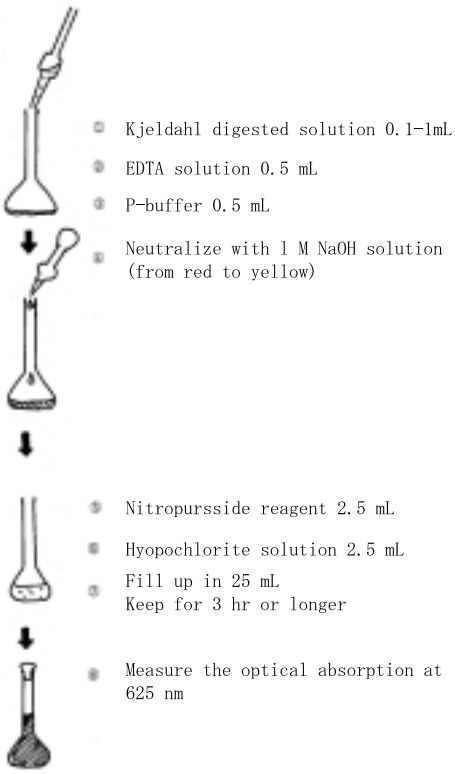

Fig.2. Indophenol method for determination of ammonium concentration in the Kjeldahl digested solution.

Determination of ^{15}N abundance of Kjeldahl digested solution by emission spectrometry

Preparation

Pyrex glass tubes: Pyrex glass tubes which have an outer diameter of 4mm and an inner diameter of 2mm are cut to 30 cm long pieces and submerged in a detergent solution overnight. The tubes are thoroughly washed with tap water, rinsed with deionized water, and dried in an oven. The center of the tube was melted and cut off by an oxygen burner to make two pieces of 15 cm long tubes one end of which is closed. Tubes are wrapped with aluminum foil, and heated at 560℃ for 3 hr in a muffle furnace to clean them up. Tubes in the aluminum foil can be stored in a plastic bag keeping them away from adsorption of contaminated nitrogen such as ammonia from air or dirt.

CaO reagent: Grind the calcium oxide blocks into a fine powder with mortar and pestle. Make a tablet (2 cm diameter and 1 mm thick) of CaO by pressing under the pressure at 4 ton cm^{-2}. Cut the tablet to 1 mm width sticks by a cutter. Heat the cut sticks in a crucible at 950℃ for 3hr in a muffle furnace to remove water and CO_2 and make them free from nitrogen contamination. CaO reagent should be kept at 950 ℃ furnace during preparation of the discharge tube.

CuO reagent: Wire type CuO reagent (about 3mm in long and 0.5mm in diameter) in a crucible and heated at 560℃ for 3 hr to make it free from nitrogen contamination. Then it can be stored in a glass bottle with airtight cap.

Procedure for making discharge tube

After N concentration was determined by indophenol method as mentioned before, an aliquot (usually 10-100 μL depending on the N concentration) of Kjeldahl digested solution, which contains about 2 μgN, is put into a clean Pyrex glass tube with 15 cm long. The volume of the sample solution should not exceed 100 μL. Duplicated analysis is necessary for each solution, because ^{15}N abundance is often decreased by trace amount of N contamination. Therefore, when the difference of ^{15}N abundance (calculated ^{15}N atom %) between duplicated tubes is over 10% of the average ^{15}N abundance, the data should be omitted and reanalyzed. Sample letter or number should be recorded by white marker pen on the lower part of the tubes, and the white letter remains after heating even at 560℃. A range of pressure at 2-6 Torr (270-800 Pa, about 1 to 3 μgN in a 4mm tube) is appropriate for discharge. When N is lower than 0.5 μg, a discharge emission is very weak and easy to fade out. On the other hand, when the N is overloaded in the tube, no discharge occur at all. The use of the mixed gases of He and Xe is beneficial for extending the range of N amount in the discharge tube by preventing N_2 gas adsorption on the glass wall[1)], however, it is time consuming to make sure the purity of the mixed gases at each preparation time. Therefore, we do not use the mixed gases. Instead if the N content is very low, we make a short discharge tube at 4 cm in long. If N concentration in the solution appeared to be low, the volume of fill up after digestion must be made to 10 mL instead of 25 mL (Fig. 1).

The Pyrex tubes with sample solution are bound with rubber band put into a 1 L round bottom flask with short neck and evaporated as shown in Fig.3. After evaporation about several hrs, most of the water will be transpired, but small drops of H_2SO_4 remains in the bottom of the tubes. These tubes are

stored in a round bottle flask with tight lid, or some airtight container to prevent ammonium absorption in H_2SO_4 from air.

After addition of several wires of CuO in the tubes, the hot CaO sticks preheated at 950 ℃ for 3hr are putted in the middle of the tubes. In total about 1cm long of CaO sticks are enough for each tube. The edge of the tube is heated with oxygen burner to smooth the collar of the edge not to damage the polyvinylacetate or silicon attachment tubes. Then CaO sticks are dropped down to the bottom, and the Pyrex tubes are attached to the attachment tubes of vacuum system with small amount of vacuum grease for making discharge tubes (Fig. 4). We don't use liquid N_2 trap, because neither water nor CO_2 was trapped in the liquid nitrogen trap (Fig. 4 ⑦) during 4 mm tube preparation. We make tubes under continuous vacuum conditions without closing the system. After evacuation of the air inside the tubes, the part of the outside tubes up to about 2cm-10 cm from the end are heated gently by oxygen gas burner from down up direction to remove the adsorbed water in the glass wall of the tubes. About 2 cm long from the bottom of the Pyrex tube should not be heated, because sample N will be converted to N_2 and lost. After the pressure inside the discharge tubes reaches below 10^{-4} Torr (mgHg), the tubes are cut off and sealed by oxygen gas burner, for about 4 cm (short tube for low N) to 8 cm (long tube for high N) from the bottom (Fig. 5).

The tubes are wrapped in aluminum foil and heated at 560 ℃ for 30 min in a muffle furnace. In this step NH_4^+ in the tube is

Fig.3. Evaporation of water in Kjeldahl digested solution in Pylex glass tubes by round bottom flask with short neck.

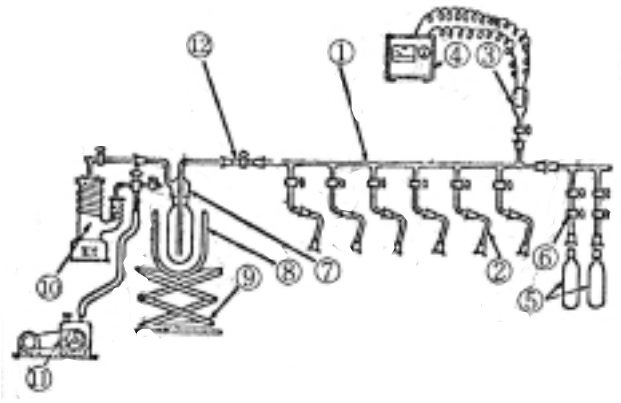

Fig.4. Vacuum system for preparation of discharge tubes devised by Dr. Kumazawa.
① glass tube, ② attachment for tubes ③ vacuum gauge ④ vacuum meter ⑤ bottles for He and Ar ⑥ cocks ⑦ liquid nitrogen trap ⑧ liquid nitrogen container, ⑨ lift ⑩ oil diffusion pump ⑪ vacuum pump ⑫ cock

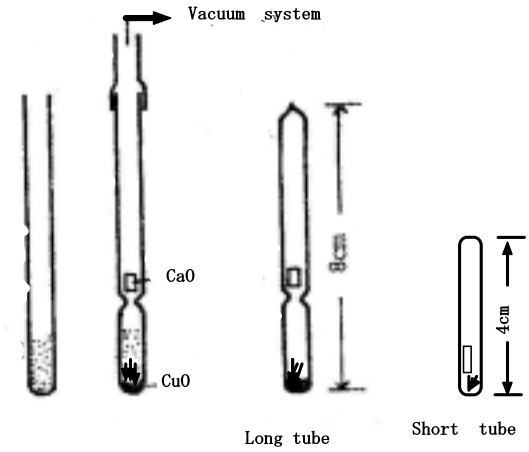

Fig.5. Discharge tube preparation for long and short tubes about 4 cm in length.

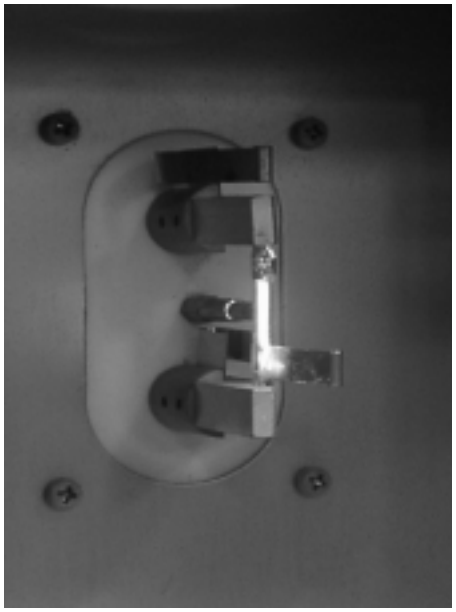

Fig.6. Photograph of emission by JASCO N-150 analyzer.
The position of the tube holder attachment was changed from the original position.

oxidized to N_2 and H_2O by CaO wire. After leaving overnight to ensure the absorption of water and impurities into CaO reagent, ^{15}N measurement can be done.

For obtaining good emission, we use aluminum foil cap at the top of the discharge tube while measuring as shown in Fig.6. At least four times stable scans peaks were collected for each tube and the height of the $^{28}N_2$ (I_{28}) and $^{29}N_2$ (I_{29}) peaks are measured as shown in Fig.7. The emitted light should not touch the CaO reagent, because it will make base line high due to gas emission.

^{15}N abundance can be calculated as following equation:

^{15}N (atom %) =100 / [2R (I_{28}/I_{29}) +1]

where, I_{28} and I_{29} are the peak height of $^{28}N_2$ and $^{29}N_2$ as shown in Fig.7.

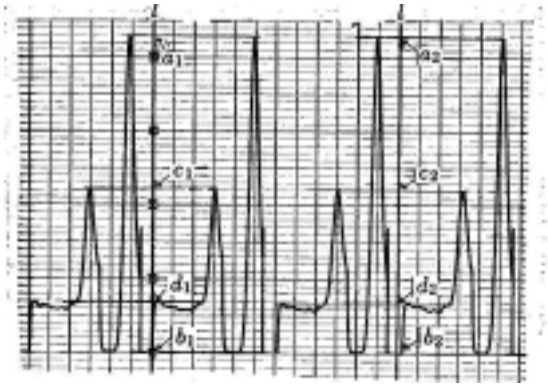

Fig.7. Measurement of the chart of ^{15}N emission spectrometry. Four stable scans are recorded and the peak height of a pair of peaks are measured.
I_{28}=a-b, and I_{29}=c-d where I_{28} and I_{29} indicate peak intensity of $^{28}N_2$ and $^{29}N_2$, respectively. The average value of ^{15}N atom % calculated from two pair of peaks is calibrated to obtain theoretical ^{15}N atom % and ^{15}N atom % excess.

R is the magnification factor of I_{29}. One of the number of 32, 16, 8, 4, 2 or 1 can be selected for the magnification factor R in N-150 analyzer.

The ^{15}N abundance obtained by this calculation is shifted from true value, therefore, a calibration curve should be made by using ^{15}N standard. The calibration curve is almost linear in the range of ^{15}N atom % of 1.0 - 15.0, but depart from linearity at higher atom % [1]. The ^{15}N abundance calculated in the above equation should be calibrated for fitting this curve to obtain the correct (theoretical) atom %. Atom % excess is calculated from theoretical atom % minus natural abundance of ^{15}N (0.366 atom %).

We use a series of linear calibration lines for correction as follows. The atom % calculated was separated into five equation due to the calculated atom % (C). In these equation, natural abundance of ^{15}N (0.366 atom %) is deduced to get theoretical ^{15}N abundance (T) in atom % excess.

① If C (atom %) <2.9,
T (atom % excess) = 0.9921 × C - 0.490

② If 2.9<C (atom %) <3.5,
T (atom % excess) = 0.9714 × C - 0.430

③ If 3.5<C (atom %) <18,
T (atom % excess) = 1.050 × C - 0.705

④ If 18<C (atom %) <28,
T (atom % excess) = 1.175 × C - 2.916

⑤ If 28<C (atom %) <35,
T (atom % excess) = 1.440 × C - 10.492

⑥ If 35<C (atom %),
T (atom % excess) can be obtained from a calibration curve.

RESULTS AND DISCUSSION

Chart of ^{15}N measurement by this method

Fig. 8 shows the examples of the chart of ^{15}N measurement by discharge tubes prepared from Kjeldahl digested solution of soybean roots (Fig. 8. left) and shoots (Fig.8. right) treated with ^{15}N labeled urea cultivated in the Nagaoka field in 2002. The discharge was stable and the back ground did not increase or disturb as shown in Fig. 8, although some sulfuric acid

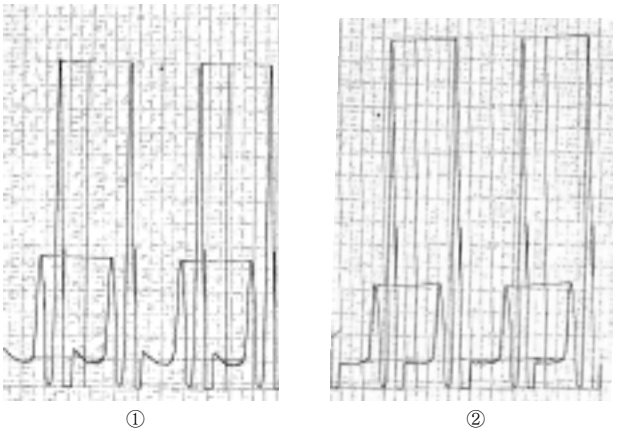

Fig.8. Examples of the charts of ^{15}N emission spectrometry of discharge tubes prepared from Kjeldahl digested solution.
Soybean roots (① left) and shoots (② right) treated with ^{15}N labeled urea in the field. Magnification factor R=16.

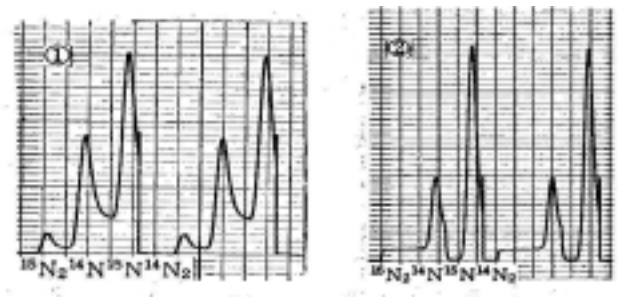

Fig.9. The charts of ^{15}N emission spectrometory of discharge tubes with high and low ^{15}N abundance.
27.3 atom % excess (① left, R=1) and 0.08 atom % excess (② right, R=32).

remained in the tube.

The appearance of peaks $^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$ are different depending on the ^{15}N abundance. When ^{15}N concentration is high at 27.3 atom % excess, $^{15}N_2$ ($^{15}N^{15}N$:298.9 nm) peak can be seen with $^{15}N^{14}N$ (298.3 nm) and $^{14}N_2$ ($^{14}N^{14}N$:297.7 nm) peaks (Fig. 9 left). When ^{15}N abundance is low (0.08 atom % excess), $^{15}N_2$ peak is not detectable and $^{15}N^{14}N$ peak need to be multiplied (Magnification factor: R= 16) as shown in Fig.9 right.

Usually peak height of $^{15}N^{14}N$ and $^{14}N_2$ ($^{14}N^{14}N$) are measured as shown in Fig. 7. The background of $^{14}N_2$ is baseline of the chart, however, the background of $^{15}N^{14}N$ is the front of the peak as shown by "d" in Fig. 7. The impurity gases such as CO (297.6 nm), H_2O (298.0 nm, 298.7 nm), O_2 and CO_2 (297.7 nm) increase and disturb the back ground level of $^{15}N^{14}N$. The height of background of $^{15}N^{14}N$ (d-b) should be lower than the peak height (c-d).

Calculation of N derived from labeled N and fertilizer efficiency

The percentage of N derived from labeled N can be calculated by the equation as follows:

100 × Atom % excess of the sample / Atom % excess of the labeled source.

For example, 10 atom % of ammonium sulfate (5 gN for one plant) is applied as a fertilizer. Then the ^{15}N abundance and total N of the harvested plant were 3 atom % and 4 gN, respectively.

The percentage of N derived from labeled N is 100 × 2.634 atom % excess / 9.634 atom % excess = 27.3 %.

The amount of N from labeled source can be calculated by total N × %N from labeled N / 100. In this example the amount of N from labeled source is 4 × 27.3 / 100 =1.092 g. Therefore the fertilizer efficiency can be calculated by the equation that 100 × amount of N from labeled source / amount of the applied N.

In this example: 100 × 1.092 / 5 = 22 (%)

Application to plant nutrition studies

We have used the direct analysis of Kjeldahl digested solution for ^{15}N emission spectrometry for studies on soybean, narcissus and tulip experiments [11-13].

Sato et al. (1999) [11] reported the nitrate absorption and transport in non-nodulated and nodulated soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. The nodulated and non-nodulated soybean isolines were hydroponically cultivated, and radioisotope labeled $^{13}NO_3^-$ or stableisotope labeled $^{15}NO_3^-$ was added to the culture solution. The accumulation pattern of absorbed ^{13}N was observed by positron emitting tracer imaging system (PETIS) as well as bioimaging analyzer (BAS). The ^{15}N abundance of the 80 % ethanol soluble and insoluble fractions of plant part was digested by Kjeldahl digestion and digested solution was prepared for emission spectrometry in the method described in this paper. In the case of 80 % ethanol extract, 1 mL of extract was taken to the test tube and dried by heating in the aluminum block, then sulfuric acid or salicylic-sulfuric acid was added as shown in Fig. 1. About 10-100 μL of 80 % ethanol extract was taken into a Pyrex tube for the preparation of discharge tube instead of Kjeldahi digested solution. The the residue of the 80% ethanol extract was dried in the oven and ground to a fine powder again, then 50 mg of powder was put in the test tube and digested with sulfuric acid.

RUAMRUNGSRI et al. (2000) [12] reported the ammonium and nitrate assimilation in daffodil (*Narcissus*) roots using ^{15}N labeled ammonium and nitrate. Ammonium was more rapidly absorbed in the roots than nitrate in 2 days after ^{15}N feeding. However, at 4-7 days after feeding, the amounts of N absorbed from ammonium and nitrate were almost equal.

KOMIYAMA et al. (2003) [13] reported the site of nitrogen accumulation in tulip roots during winter. Tulip plants absorb and accumulate N in the roots during winter. ^{15}N labeled nitrate and ammonium were supplied in a vertical split-root system (upper part of roots and lower part of roots). From the results obtained, tulip roots could absorb ammonium and nitrate either from the upper roots or from the lower roots and accumulate N in the absorption sites.

From the experiences of the above studies, we are convinced that the direct analysis of Kehldahl digested solution is the simplest and accurate method in which the chance of N contamination is least compared with other methods.

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発光分光法によるケルダール分解液の^{15}N濃度分析

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要　約

ケルダール分解液を直接パイレックスガラス管に導入して放電管を作成し、発光分光法により^{15}N濃度を測定することができた。植物試料を硫酸-過酸化水素を用いてケルダール分解し、一定量に定容後、インドフェノール法でアンモニウム濃度を測定した。測定後、窒素として約2μg程度を含む分解液を直接片側を閉じた外径４mmのパイレックスガラス管に入れ、水を減圧除去した後、酸化剤（線状酸化銅）と水吸収剤（950℃で加熱した酸化カルシウム）を入れて、真空封印した後、560℃で30分間加熱して、アンモニアを窒素ガス変えた。^{15}N濃度は、発光分光法（JASCO N-150アナライザー）で分析した。この方法は、従来の方法と比較して試料全窒素の^{15}N 濃度を測定するには最も簡単で正確である。

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