ADAPTATION OF THE MICRO-KJELDAHL METHOD FOR THE DETERMINATION OF NITROGEN IN PLANT TISSUES

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Introduction

The micro-Kjeldahl method for nitrogen determination has been widely used in organic chemistry laboratories in recent years. The value of this method, developed through the brilliant researches of Pilch, Bang, Parnas and Wagner, Pregl, and coworkers, has justified its application to industrial and clinical problems. Plant physiologists have not generally adopted quantitative micro-methods for nitrogen analysis. The purpose of the present study was to test the applicability of the micro-Kjeldahl method for the determination of nitrogen in plant materials.

Review of literature

Numerous modifications of micro or semi-micro methods have been proposed for the determination of nitrogen in small amounts of material. This paper is not concerned with clinical methods developed for specialized purposes.

Prebl (9) has reviewed the early work on micro-determination of nitrogen by the Kjeldahl method and gives detailed directions for the analytical procedure.

Mothes (7) employed the Prebl micro-Kjeldahl method in a study of nitrogen metabolism in plants. He states that the errors of the method were less than 1 per cent. but gives very few details of how the analyses were performed.

Hartley (5) has described a dilution method for micro-Kjeldahl determinations when no microchemical balance is available. In this method a certain number of drops of partially digested acid solution are taken for analysis so the results can not be strictly accurate.

Allen (1) modified the Parnas and Wagner (8) distillation apparatus used by Prebl, permitting the analysis of larger samples.

Cavett (2) developed a semi-micro-Kjeldahl method to analyze small quantities of nitrogen in large volumes of liquid. He claims for the method and apparatus an accuracy of 0.006 mg. of nitrogen when determining from 0.5 mg. to 14 mg. of nitrogen.

Morris (6) has reported successful use of a slight modification of the Cavett method in plant metabolism studies.

Emmert (4) has reported a rapid semi-micro method for determining...
nitrogen in plants. This method requires that the sample be oxidized with sodium chlorate and $H_2SO_4$. The nitrogen is estimated by the phenoldisulphonic acid colorimetric method.

Doneen (3) has described a micro-method for organic and nitrate nitrogen through the use of salicylic acid and perchloric acid. The nitrogen is estimated colorimetrically after Nesslerization.

**Materials and methods**

**PREPARATION OF SAMPLES.**—Fresh plant tissues used in this study were first passed through a Nixtamal mill. The method of extracting the non-protein nitrogen has been described in a previous publication (12). Portions of the macerated tissue were dried at 70° C. The dry tissue was finely ground in a hand mill and stored in small aluminum boxes which were placed in the case housing the microchemical balance. The amount of moisture in this tissue at the time the nitrogen analyses were made was determined by drying 1-gm. portions at 80° C. and a pressure of 3 to 4 cm.

**MACRO-METHODS.**—Total nitrogen in the dry tissue and in the non-protein extracts was determined by the Gunning modification of the Kjeldahl method not modified for nitrates. Basic nitrogen, precipitated by phosphotungstic acid, was determined as described in a previous publication (12). Amide nitrogen was determined by hydrolysis of aliquots of the non-protein extract with 10 per cent. $H_2SO_4$ followed by aeration of the alkaline solution for 16 hours by the Sessions and Shive method (11).

**MICRO-METHODS.**—Total nitrogen in the dry tissue was determined by the micro-Kjeldahl method described by Pregl (9). All weighings were made on a Kuhlmann microchemical balance. About 25 mg. of dry plant tissue were weighed in a Coors porcelain boat. Boat and contents were transferred to a microdigestion tube and digested with 1 ml. of low nitrogen concentrated $H_2SO_4$ and a few mg. of 3:1 $CuSO_4-K_2SO_4$ mixture. When the solution was partially clear, two or three drops of Merck's reagent Superoxol (30 per cent. hydrogen peroxide) were added and the digestion completed. The acid digest was diluted with 1 ml. of ammonia-free water and washed into the distillation apparatus.²

After adding 7 ml. of 30 per cent. NaOH containing 5 per cent. $Na_2S_2O_3$ the mixture was distilled with steam for 5 minutes. The ammonia was collected in 10 ml. of 0.01 N $H_2SO_4$. Distillates were boiled and titrated with 0.01 N NaOH to a methyl red endpoint. The indicator, introduced by means of a glass thread, was prepared by adding an excess of methyl red to 0.1 N NaOH. Acid and base were measured in 10-ml. microburettes. The apparatus was steamed for 30 minutes before each series of determinations. Blank determinations amounted to 0.05 to 0.06 ml. of 0.01 N NaOH.

² Manufactured by the firm of Paul Haack, Vienna.
The nitrogen content of 2-ml. aliquots of the non-protein extracts was determined by the micro-Kjeldahl method. The non-protein extracts were first concentrated in vacuo to a volume such that 2 ml. contained 0.3 to 0.5 mg. or more of nitrogen.

Basic nitrogen precipitated by phosphotungstic acid was determined as the difference between non-protein and non-basic nitrogen. The filtrate from the basic nitrogen was neutralized with NaOH, acidified with acetic acid, and concentrated in vacuo to the original volume of the aliquot precipitated with phosphotungstic acid. Aliquots of 2 ml. of this basic-free solution were analyzed for nitrogen content by the micro-Kjeldahl method.

Aliquots of 5 ml. of the non-protein extracts were hydrolyzed in micro-digestion tubes with 0.3 ml. of concentrated H₂SO₄ under reflux condensers for 2.5 hours. The solutions were washed into the distillation apparatus and distilled with 2.4 ml. of 30 per cent. NaOH for 5 minutes.

**Experimental results**

**Analysis of amino acids.**—The accuracy of the apparatus and method was first tested by determining the nitrogen content of several commercial preparations of amino acids. The results are shown in table I.

**Table I**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Wt. of Sample</th>
<th>Percentage of Nitrogen</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.940</td>
<td>7.64</td>
<td>7.73</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.846</td>
<td>7.70</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>6.488</td>
<td>10.52</td>
<td>10.68</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.647</td>
<td>10.50</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>6.987</td>
<td>11.56</td>
<td>11.66</td>
</tr>
<tr>
<td>Cystine</td>
<td>7.960</td>
<td>11.58</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>4.475</td>
<td>18.60</td>
<td>18.65</td>
</tr>
</tbody>
</table>

**Comparison of macro- and micro-methods for the determination of nitrogen in plants.**—Typical results, showing the precision of the micro-Kjeldahl method with reference to the macro-methods, are shown in tables II and III. Each value reported is the mean of duplicate determinations. Both macro- and micro-determinations were made from single samples which were collected at intervals during the year.
The micro-Kjeldahl method and apparatus used in this study are reliable and accurate within commonly accepted limits of error as shown by the data in table I.

Plant tissues are usually lower in nitrogen than many substances analyzed in organic chemistry laboratories. Many plant tissues contain less than 3.00 per cent. of total nitrogen in their dry tissue. Accordingly, 0.1 mg. of tissue would contain less than 0.003 mg. of nitrogen. The writer has found it possible to titrate duplicate ammonia distillates to within 0.02 ml. of 0.01 N NaOH. This amount of reagent is equivalent to 0.0028 mg. of nitrogen or about the amount contained in 0.1 mg. of tissue. Under these conditions it is necessary to weigh the sample accurately only to the first decimal of a milligram. This is best accomplished by weighing the

### TABLE II

**Comparison of macro- and micro-methods for the determination of total and non-protein nitrogen in plant tissues**

**Total nitrogen expressed as percentages of the dry weight and non-protein nitrogen as percentages of the fresh weight**

<table>
<thead>
<tr>
<th>Plant Tissues</th>
<th>Total nitrogen</th>
<th>Non-protein nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macro-method</td>
<td>Micro-method</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Clover roots</td>
<td>1.962</td>
<td>1.972</td>
</tr>
<tr>
<td></td>
<td>2.070</td>
<td>2.078</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover shoots</td>
<td>3.209</td>
<td>3.193</td>
</tr>
<tr>
<td></td>
<td>2.972</td>
<td>2.961</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snap beans</td>
<td>3.560</td>
<td>3.554</td>
</tr>
<tr>
<td>Snap beans and pods</td>
<td>2.266</td>
<td>2.276</td>
</tr>
<tr>
<td>Potato tubers</td>
<td>1.448</td>
<td>1.450</td>
</tr>
<tr>
<td></td>
<td>1.388</td>
<td>1.363</td>
</tr>
<tr>
<td></td>
<td>1.375</td>
<td>1.371</td>
</tr>
<tr>
<td>Peas</td>
<td>3.888</td>
<td>3.874</td>
</tr>
<tr>
<td></td>
<td>3.897</td>
<td>3.908</td>
</tr>
<tr>
<td></td>
<td>3.875</td>
<td>3.874</td>
</tr>
</tbody>
</table>
sample to the second decimal of a milligram. It is, of course, very important that the sample be very finely ground and thoroughly mixed. Since dry tissue is somewhat hygroscopic, it should be allowed to come into moisture equilibrium with the air where the weighings are made. The moisture content of the tissue is determined and the appropriate correction made.

The data in table II indicate that the micro-Kjeldahl method is as accurate as the corresponding macro-method for the determination of total nitrogen or non-protein nitrogen in plant materials. In addition, the micro-method is more economical of material, reagents, and time. The determination may be performed in any laboratory where water and gas are available since no hood is necessary.

The data in table III show that it is possible to employ the micro-Kjeldahl method for estimation of basic nitrogen of plant extracts without digesting the phosphotungstates and filter paper.

### TABLE III

**Comparison of Macro- and Micro-Methods for the Determination of Basic and Amide Nitrogen in Plant Extracts**

Results are expressed as percentages of the fresh weight

<table>
<thead>
<tr>
<th>Plant Tissue</th>
<th>Basic Nitrogen</th>
<th>Amide Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macro-METHOD</td>
<td>Micro-METHOD</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Clover roots</td>
<td>0.061</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>0.022</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover shoots</td>
<td>0.020</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>0.010</td>
<td>0.011</td>
</tr>
<tr>
<td>Potato tubers</td>
<td>0.026</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It is also possible to utilize the micro-Kjeldahl equipment for the estimation of amide and ammonia nitrogen of plant extracts (table III). This method cannot be used to determine the free ammonia since a portion of the amide nitrogen is hydrolyzed during the distillation. However, after the amide hydrolysis with H₂SO₄, total amide and ammonia nitrogen were determined just as accurately as in the lengthy aeration method. Recently
Pucher et al. (10) have shown that the conventional methods of determining amide and ammonia in plant extracts may be somewhat in error. Several precautions are necessary in order to distinguish between free ammonia and amide nitrogen. Where ammonia values are low, it is sometimes satisfactory to determine the combined total. In such cases the present method proved valuable though it is recognized that it might be inaccurate with some tissues.

The data in table IV reveal that the amide nitrogen may be accurately determined in 5-ml. aliquots of an asparagine solution as dilute as 0.01 M.

**TABLE IV**

| NaOH 30% ml. | Amide Nitrogen in 0.01 M Solution of Asparagine (mg.) | Amide Nitrogen in Plant Extracts
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potato Tubers</td>
<td>Clover Shoots</td>
</tr>
<tr>
<td>1.8</td>
<td>0.68</td>
<td>0.51</td>
</tr>
<tr>
<td>2.0</td>
<td>0.68</td>
<td>0.53</td>
</tr>
<tr>
<td>2.2</td>
<td>0.69</td>
<td>0.54</td>
</tr>
<tr>
<td>2.4</td>
<td>0.69</td>
<td>0.54</td>
</tr>
<tr>
<td>2.6</td>
<td>0.69</td>
<td>0.54</td>
</tr>
<tr>
<td>2.8</td>
<td>0.69</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Calculated 0.70</td>
<td></td>
</tr>
</tbody>
</table>

The data also show that 2.4 ml. of 30 per cent. NaOH are sufficient to neutralize the acid used for hydrolysis and to furnish sufficient alkalinity for removal of the ammonia in a distillation period of 5 minutes. Lesser amounts of alkali yielded slightly erratic results while larger amounts resulted in no greater yield of ammonia.

**Summary and conclusions**

The Pregl micro-Kjeldahl method has been applied to the determination of total organic nitrogen in plants. Modifications necessary for making micro-determinations of nitrogen in plant materials are discussed. Micro-methods are proposed for the determination of non-protein, basic, and amide nitrogen in plant extracts.

It is concluded that micro-methods are rapid, economical, and as accurate as macro-methods for the determination of total organic nitrogen in plant tissues and non-protein, basic, and amide nitrogen in plant extracts.

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STUART: MICRO-KJELDAHL METHOD

LITERATURE CITED