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Non-Protein Nitrogen (NPN) Test Protocol for Raw Materials of Feed

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ABSTRACT

Nutrients are the important factor for the formulation of feed. Nutritionist always tries to make a combination of nutrients in the feed during formulation. All nutrients such as protein, fat, calcium salt etc. are the equally important for the body growth of bird and animal (Feed acceptor). Among these nutrients, protein plays a vital role in the energy and body growth of bird and animal. Normally nutritionist makes the formula of feed from the analysis record of feed raw materials. Protein rich raw material contains nitrogen and the protein is measured on the basis of nitrogen content in the raw materials. But sometimes raw material contains non-protein nitrogen (NPN) which tends to make an error during protein measurement. In the feed raw materials urea, urea formaldehyde, melamine etc. are present as NPN. Actual protein cannot be measured by the conventional method (Micro Kjeldahl Method) due to the presence of NPN. This tends to have an error in the final formulation of feed and thus, nutrient values are deteriorated from the standard or claimed amount. NPN test finally helps the nutritionist to make an authentic formula for feed.

1. INTRODUCTION

Non-protein nitrogen (or NPN) is a term used in animal nutrition to refer collectively to components such as urea, biuret, and ammonia, which are not proteins but can be converted into proteins by microbes in the ruminant stomach. NPN can also be used to artificially raise crude protein values, which are measured based on nitrogen content as protein.

Proteins are complex organic compounds of high molecular weight. As with carbohydrates and fats, proteins contain carbon, hydrogen and oxygen, but in addition, they all contain nitrogen and generally sulphur. Proteins (also known as polypeptides) are made of amino acids arranged in a linear chain and folded into a globular form. Amino acids are produced when proteins are hydrolyzed by enzymes, acids or alkalis. Although over 200 amino acids have been isolated from biological materials, only 20 of these are commonly found as components of proteins. Animals cannot synthesize the amino group, and in order to build up body proteins, they must have a dietary source of amino acids. Within all known amino acids, there are 10 classed as "Essential Amino acids", as the animal can't produce them. Non-essential amino acids are also needed for animals but are synthesized from other amino acids. Proteins are present in animals cells and tissues and are continuously needed to replace dying body cells and to supply materials to build body tissue (ligaments, hair, hooves, skin, organs, and muscle are partially formed by protein). Thus, proteins have an important role as basic structural unit and are also needed for metabolism, hormone, antibody and DNA production. When proteins are fed in excess, they are converted to energy and fat.

Crude protein contains both true protein and other nitrogenous products (non-protein nitrogen), but only the true protein portion is able to be digested by animals. To determine the protein, content of a feedstuff, it is usual to ascertain first the percentage of nitrogen by chemical analysis [1]. This estimate is then multiplied by 6.25 as the average protein content of a feed is 16% nitrogen (6.25 x 16 = 100). The resulting value is called crude protein, as distinguished from true protein, because some of the nitrogen analyzed is not derived from protein [2]. In most grasses and other green feeds, only a part of the nitrogen comes from protein; the balance consists of inorganic nitrogen salts, amino nitrogen, amides and other forms [3, 4]. The adulteration and contamination of several feed ingredients with inexpensive melamine, urea and other

compounds, such as cyanuric acid, ammeline and ammelide are common practice. These adulterants can be used to inflate the apparent protein content of products so that inexpensive ingredients can pass for more expensive, concentrated proteins [5, 6, 7]. Melamine by itself has not been thought to be very toxic to animals or humans except possibly in very high concentrations, but the combination of melamine and cyanuric acid has been implicated in kidney failure [8, 9].

All proteins contain nitrogen, but not all nitrogen is contained in proteins. For example, urea and anhydrous ammonia are two compounds which contain significant amounts of nitrogen, but neither is a protein [10, 11, 12]. Instead, they are called nonprotein nitrogen (NPN) compounds. The common non-protein nitrogenous compounds are as follows:

- Ammonia

  NH_3 is a gas which usually dissolves in water. It is the cheapest source of nitrogen that can be used in feeding, but being toxic and difficult to handle it is mostly used to increase the nitrogen content of low-protein feeds by ammoniation on an industrial scale.
- Urea or carbamide

$CO(NH_2)_2$, the cheapest solid nitrogen source, is a white crystalline water-soluble powder that is used as a fertilizer. Urea contains 46% nitrogen; thus, each kilogram of urea is equivalent to 2.88 kg of crude protein (6.25 x 0.46), which in most rations equals a digestible crude protein content of 200%.

- Biuret

  NH_2-CO-NH-CO-NH_2, produced from urea by heating, contains 41% nitrogen (256% CP
- Diammonium phosphate

$(NH_4)_2HPO_4$, a white crystalline water-soluble powder, contains 21.4% nitrogen (134% CP) and 23.7% phosphorus.

- Ammonium polyphosphate

This is a common supplier of phosphorus and nonprotein nitrogen in liquid supplements. It contains 11% nitrogen (equivalent to 68.8% CP) and 16.1% phosphorus.

2. MATERIALS AND METHODS

Non-protein nitrogen test is not so sophisticated at all. Some common instrument and equipment are used to measure the NPN.

2.1 Instrument and equipment

- Stereomicroscope
- Laboratory balance
- Vortex mixer
- Sieve ~ 45 mesh
- Test tube
- Petri dish
- Dropper and rubber
- Spot plates
- General laboratory equipment and glassware

2.2 Chemicals

- Urease, Merck # 1.08489.00005 (or equivalent to)
- Bromothymol blue AR grade
- Sodium hydroxide (NaOH) AR grade
- Hydrochloric acid (HCl) 37% AR grade
- Diphenylamine AR grade
- Chromotropic acid ($C_{10}H_6O_8S_2Na_2$) AR grade
- Sulfuric acid (H_2SO_4) Conc.98% AR grade
- Potassium iodide (KI) AR grade
- Mercuric chloride ($HgCl_2$) AR grade
- Petroleum ether AR grade
- Urea (NH_2CONH_2) AR grade
- Potassium nitrate (KNO_3) AR grade
- Ammonium chloride (NH_4Cl) AR grade (or equivalent to)
- Melamine ($C_3H_6N_6$) AR grade
- ESB

- Urea formaldehyde
- Distilled water

2.3 Reagent preparation

Different types of reagents are prepared to perform the NPN test as follows:

2.3.1 0.4 % Urease enzyme

0.4 g of Urease is dissolved in 100 ml of distilled water and are mixed well. The prepared reagent is kept in a glass amber bottle in refrigerator.

2.3.2 0.1 % Bromothymol blue

0.1 gm of Bromothymol blue is dissolved in 1.6 ml of 0.1 N NaOH (NaOH 0.4 g in 100 ml distilled water), mixed well and the volume is adjusted to 100 ml with distilled water. pH is also adjusted from 2.3 – 2.4 with 0.1 N HCl (0.88 ml of HCl and 99.02 ml of distilled water). The prepared reagent is kept in a glass amber bottle in refrigerator.

2.3.3 0.5 % Diphenylamine

0.5 gm. of Diphenylamine is dissolved in 20 ml of distilled water and mixed well with 80 ml of H_2SO_4 and is kept in a glass bottle at room temperature.

2.3.4 0.5 % Chromotropic acid

0.5 gm of Chromotropic acid is dissolved in 100 ml of sulfuric acid and mixed well. The prepared reagent is kept in a glass bottle in refrigerator.

2.3.5 Nessler reagent

3.50 gm of KI and 1.25 gm. of $HgCl_2$ in 80 ml of distilled water are mixed well and stirred. 12 gm of NaOH is added and the volume is adjusted to 100 ml with distilled water. The solution is filtered through filter paper and is kept the filtrate in a glass amber bottle at room temperature.

2.3.6 50% HCl

50 ml of HCl in 50 ml of distilled water is mixed well. The reagent is kept in a glass amber bottle in refrigerator.

2.3.7 10% $AgNO_3$

10 gm of $AgNO_3$ is dissolved in 100 ml of distilled water and mixed well. The reagent is kept in a glass amber bottle in refrigerator.

2.4 Reference preparation

Different concentrations of NPN reference samples are prepared by mixing the NPN chemical at several levels as illustrated in the table-1 with NPN free raw material and the final weight is adjusted to 100 gm.

Table-1: Weight of chemicals according to the NPN

Type of NPN	Chemical	Weight of Chemical (g)			
		0.1% NPN	0.25% NPN	0.5% NPN	1.0% NPN
Urea	Urea	0.1g	0.25g	0.5g	1.0g
NO_3^-	KNO_3	0.16g	0.4g	0.8g	1.6g
NH_4+	NH4Cl	0.3g	0.74g	1.49g	2.96g
UF	UF	0.1g	0.25g	0.5g	1.0g
ESB	ESB	0.1g	0.25g	0.5g	1.0g
Melamine	Melamine	0.1g	0.25g	0.5g	1.0g

Note: To confirm the results of samples, NPN reference samples are needed to be tested together with samples.

2.5 Test protocol

This test method covers the determination of Urea, Ammonium salt, Nitrate-Nitrite, Urea-formaldehyde, ESB and Melamine. In the case of samples that contains fatty matter must be

defatted before test, there are 2 ways of defatting step. One is normal defatting and another one is fast defatting.

2.5.1 Normal de-fatting method

Small amount (Around 5 gm) of sample is taken on a filter paper and is wrapped.

The wrapped sample is then soaked with Petroleum ether.

Soaking should be continued for 2-3 hrs.

The wrapped sample is then taken out from petroleum ether and is left in a fume hood to remove the solvent.

De-fatted sample

2.5.2 Urea / Ammonium salt / Nitrate-Nitrite salt

2 gm of sample is taken into a test tube and 10 ml of distilled water is added.

↓

Mixed by vortex mixer 10-15 sec.

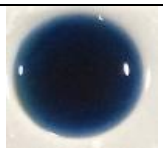
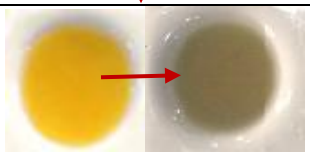
↓

Let it stand for sedimentation (~10 min).

↓

NPN test is performed by the supernatant.

↓

Urea test	Ammonium salt test	Nitrate test
Addition of 6-8 drops of the supernatant into a spot plate. ↓	Addition of 6-8 drops of the supernatant into a spot plate. ↓	Addition of 6-8 drops of the supernatant into a spot plate. ↓
Addition of 2-3 drops of 0.1% Bromothymol blue and 4-5 drops of 0.4% Urease. ↓	Addition of 2-3 drops of Nessler reagent. ↓	Addition of 2-3 drops of 0.5% Diphenylamine. ↓
Positive result: blue color. ↓	Positive result: deep orange color and then change to gray. ↓	Positive result: dark blue color in the middle. ↓
		
Detection limit 0.25%	Detection limit 0.1%	Detection limit 0.05%

2.5.3 Urea formaldehyde

There are two methods for the determination of urea formaldehyde.

Scope: for both animal by-product and plant samples.

De-fatting operation of sample of animal by-product.(Not necessary for plant sample).

↓

Sample is screened with normal sieve (45 mesh) to separate into fine portion and coarse portion.

↓

Addition of 6-8 drops of 0.5 % Chromotropic acid into spot plate.

↓

Fine portion of screened sample is added into 0.5 % Chromotropic acid in the above spot plate.

↓

Positive result: The present of violet color spread on the surface of reagent within 5–10 min.

Detection limit 0.15%

2.5.4 ESB Test

Scope: for both animal by-product and plant samples.

Sample is screened with normal sieve (45 mesh) to separate into fine portion and coarse portion.

↓

Fine portion of sample is taken into a spot plate.

↓

Addition of 0.5 % Diphenylamine to cover the sample.

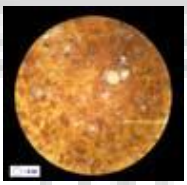
↓

The result is observed under microscope at several magnifications.

↓

Positive result: Floating white to gray or blue particle.

↓



Detection limit 0.1 %

3. RESULTS AND DISCUSSION

Naturally occurring raw materials contain very low level of NPN. That is not so harmful to the nutrient source. The value of NPN should be taken into consideration during formulation of feed to maintain the proper combination of nutrient values. Otherwise, the claimed values will be differentiated from the standard. Moreover, it is very serious when supplier intentionally mixes the NPN to their valuable raw materials. Normal NPN values of some raw materials are shown in the table-2.

Table-2: NPN values of raw materials

SL No.	Raw material Name	NPN values/ %	Origin
1	Corn	0.00	Bangladesh
2	Soybean meal	0.00	Bangladesh
3	Mustard meal	0.00	Bangladesh
4	Rice bran fine	0.10	Bangladesh
5	Rice bran coarse	0.00	Bangladesh
6	Rice bran solvent	0.00	Bangladesh
7	Paddy	0.00	Bangladesh
8	Broken rice	0.00	Bangladesh
9	Meat and bone meal	0.10	Bangladesh
10	Canola meal	0.00	Bangladesh
11	Fish meal	0.10	Bangladesh
12	Wheat grain	0.00	Bangladesh
13	Wheat bran	0.10	Bangladesh
14	Coconut meal	0.00	Bangladesh
15	Full fat soya	0.00	Bangladesh

Normally raw materials do not contain NPN, but raw materials are stored for a long time produce ammonia for the bacterial metabolism. This test is perfect to find out the intentional contamination and help to make quick decision.

4. CONCLUSION

Total protein or true protein content of a raw material is the sum of crude protein and the protein comes from the non-protein nitrogenous compound. Sometimes supplier intentionally mix NPN compound to the valuable raw material to enhance the total protein of raw material for getting extra benefit. Feed mills receive huge amount of raw materials every day for producing different types of feed. These NPN test protocol will help to sort out the contaminated raw materials with NPN within a very short time and thus helps the quality controller to reject that under grade raw material.

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