

CHAPTER-2

Proximate analysis of feeds

Objectives

1. To know the general precautions to be observed in the laboratory.
2. To define the proximate principles.
3. To collect and process the feed samples.
4. To analyse proximate principles (moisture, total ash, crude protein, ether extract, crude fibre and nitrogen free extract) in feedstuffs.

Introduction

Hanneberg and Stohmann working at the Weende Experimental Station, Germany devised proximate principles analysis system in 1865 for routine description of animal feedstuffs. The components are: moisture, total ash, crude protein, ether extract, crude fibre and nitrogen free extract.

A. General precautions for safety in the laboratory

1. Care and cleanliness must be practised by the Laboratory personnel at all times.
2. Staff should not indulge in smoking in a laboratory.
3. Electric switches and connections should be repeatedly inspected and kept free from corrosion so that no danger shall arise from short circuiting or due to the exposure of naked wire resulting from breaking of the insulation of corrosion.
4. When a laboratory is to be left unattended for any length of time, all water tap should be turned off.
5. Proper disposal of waste from a laboratory is a problem of considerable importance. There should be an ample supply of bins of suitable sizes for disposal of solid materials such as broken glass, porcelain, filter papers, etc. The bins should be cleaned often, at least daily.
6. If corrosive/poisonous liquids are being thrown down the sink, they should be accompanied by a generous supply of water to ensure that by the time they reach the main drain they will be too dilute to be dangerous.

7. When working in a laboratory, it is better to wear some sort of protective coat, apron or overall.
8. All bottles and containers should be carefully and distinctly labeled so that no confusion or possible error can arise when several containers are being filled with different chemicals.
9. Many accidents occur in transporting bulky containers from room to room or from one part of the laboratory to another. Bottles should never be carried by the neck. Trays or polythene buckets or suitable trolley should be used.
10. Adequate and conveniently accessible storage of both apparatus and chemicals is essential to the laboratory safety. Bottles containing liquids should be kept on the lower shelves whilst solids should be kept on the upper ones.
11. The floor space should be kept free from spillage, broken glass, straw, paper etc. as their presence may lead to accidents.
12. When dealing with volatile inflammable liquids, no flames should be allowed in vicinity and care should be taken to avoid inhalation of vapours.
13. Cylinders of gases under pressure are best kept upright in a suitable stand well away from any source of heat.
14. When pouring liquids from one container into another, keep both vessels well away from the body so that any spillage shall not fall upon the person. When corrosive chemicals are involved, carry out the operation over a sink so that any spillage can be easily flushed away.
15. It is often an advantage to use a glass rod to direct the liquid stream when pouring from one bottle to another.
16. When diluting concentrated sulphuric acid, always add the acid to the water kept in ice and not vice versa. Add the acid slowly, preferably down a glass rod with stirring to reduce the violence of the reaction.
17. When NaOH is dissolved in water, a lot of heat is evolved. Use cold water and add NaOH to it little by little. Do not handle NaOH with unprotected hands.
18. When pushing a glass tube or rod through a hole in rubber cork, lubricate well with water.
19. Poisonous and offensive reagents should never be pipetted by mouth, use propipettes or bulbs.
20. It is not advisable to use glass stoppers in bottles containing KOH and NaOH. The stoppers are prone to sticking and their removal may involve breaking the neck of the bottle. Use rubber stoppers or store them in polythene containers.

21. Fire buckets containing sand and water should always be available. Fire extinguishers should be kept at readily accessible points. They should be sent for filling immediately after use.
22. In case of emergency, medical attention should be quickly obtained. Meanwhile, first aid should be given to the person. A first aid cabinet should be readily accessible and be placed in the charge of a staff member capable of dealing with an emergency.
23. Many accidents are caused by failure to seek advice or information. Never attempt to use an equipment that you have not fully understood.
24. If acid falls on clothes, neutralize the same with few drops of dilute ammonia solution or some other weak alkali solution.
25. If you happen to such acid into your mouth during pipetting, wash your mouth quickly with water and then rinse with a weak solution of washing soda.
26. Laboratory floor, working tables and water sinks should be kept neat and lean and it should be well ventilated and provided with an exhaust fan to remove unwanted gases, fumes and smoke.
27. Store chemicals and glassware in alphabetical order in well protected cupboards/almirahs.
28. Systematic breakage record should be maintained.
29. Always use acid and alkali gloves while handling strong acids and alkalies.
30. While digesting the samples, use fume protecting face mask to avoid inhalation of highly irritating sulphur dioxide fumes.
31. Distilled water bottles should be kept tightly corked to avoid absorption of atmospheric gases.
32. While opening liquor ammonia bottles, especially during summer season, cool it for some time in a freezer to avoid sudden spurt of ammonia gas accumulated in the bottle.
33. After use of electronic balance, platform of the balance should be cleaned with a camel hair brush, if any spillage of chemical, sample etc. was found.
34. Proper record of usage of special equipment should be made in log book meant for it showing date, time and condition of the equipment.
35. Never blow the solution left at the tip of the pipette and delivery of the reagent drawn into pipette should be uniform giving appropriate time varying from 10 to 30 sec. for quantities of 2 to 50 ml.

36. Acid and alkali spillage on working tables, floor and clothes should be thoroughly washed with water after suitably neutralizing with either weak alkali in case of acid and weak acid in case of alkali.
37. Always use double glass distilled/HPLC grade water while analyzing minerals.
38. Always use self prepared reagents and indicators.
39. Consider lower meniscus for clear and colourless solutions and upper meniscus for coloured solutions while recording observations with the help of measuring glassware.
40. During cooling samples in a desiccator, the lid should be displaced to leave a small space which can be put after cooling.
41. Do not put on fans during decarbonization of the sample for ashing. Put on the exhaust fan during decarbonization and while handling fuming acids and other chemicals.
42. Always keep decarbonized samples in a closed container like desiccator while carrying to muffle furnace, otherwise being light the material in silica basin may be displaced due to external air movements.

Cleaning of glasswares and precautions in their use

Cleaning of Glassware

1. Ordinary glassware should be thoroughly cleaned with washing soda or any detergent followed by washing with ordinary tap water and rinsing with distilled water care should be taken to remove previous markings on the glassware, if any, while cleaning.
2. Cleaning of flasks, used while estimating ether extract, should be done by slight boiling with dilute alkali (NaOH) followed by same procedure adopted for cleaning of ordinary glassware. Care, however, should be taken not to use any brush for cleaning inside the flask to avoid scratch formations.
3. Graduated glassware may be cleaned by initially keeping in chromic acid solution (Dissolve about 60 g potassium dichromate in 300 ml of water by thorough stirring and boiling to which 460 ml of concentrated sulphuric acid is added slowly after cooling) kept in jar for reasonable time followed by washing and cleaning as per ordinary glassware. Discard chromic acid solution when it develops green colour.

Drying of Glassware

1. Ordinary glassware can be dried by keeping in hot air oven at low temperature.
2. Graduated glassware (pipettes, burettes, measuring cylinders, volumetric flasks etc.)

should never be dried in hot air oven as high temperature would change the volume for which they are graduated.

Precautions in the use of burettes and micro burettes

1. The burette must be clean. When a liquid is delivered from a burette, no adhering drops should be left on the walls. If drops are formed, the burette must be thoroughly cleaned before it is used.
2. Before use, the burette should be rinsed thrice with a small volume of the solution with which it is to be filled.
3. Fill a micro burette by attaching a rubber tube to the top. Suck up the solution from a beaker held beneath the tip. Remove the rubber tube and wipe off the burette tip with a clean filter paper before adjusting the meniscus to the zero mark.
4. After filling any burette, it is essential to remove all air bubbles from the delivery tip before measurement are made.
5. Be sure that the burette delivery tip or stop cock does not leak.
6. During titrations, do not empty the burette too rapidly. Accurate measurements depend largely upon uniform drainage.
7. Make careful readings. Have your eyes level with the bottom of the meniscus. Estimate the volume to the nearest 0.01 ml on an ordinary burette and to the nearest 0.001 or 0.002 ml on a micro burette.

Precautions in the use of pipettes

1. The pipette must be clean. No drops should adhere to the walls after the pipette has been drained.
2. Before use, the pipette should be either dry or rinsed three times with small volume of the solution to be measured.
3. While filling a pipette, be sure that its tip is well below the surface of the liquid. This is extremely important when strong acids, bases, or other corrosive or poisonous solutions are handled.
4. Draw the liquid a little way above the mark, then carefully remove all drops adhering to the outside of the pipette stem by wiping with a clean piece of filter paper.
5. Adjust the meniscus of the liquid to the mark a carefully while the pipette is held vertically.
6. Hold the pipette vertically while draining it. When the liquid has ceased to run, touch

the tip of the pipette to the wall of the receiving vessel. A small drop of liquid will remain on the tip of the ordinary transfer pipette. Do not try to remove this drop. However, when using an *Ostwald* pipette, this last drop is removed by blowing through the pipette.

7. Use care to prevent contamination of the pipette tip. Support in such a manner that tip does not rest on the bench top.
8. After using, pipettes should be kept in chromic acid solution (potassium dichromate 60 g distilled water, 300 ml sulphuric acid, 460 ml) at least for overnight and then should be washed thoroughly.

Principles of preparing standard solutions

The analytical procedures of feeds in the field of avian nutrition are done both quantitatively and qualitatively. In quantitative analysis, volumetric methods are mostly used and these are solely dependent upon standard solutions. The accuracy of analytical work and hence results depends to a greater extent on the accuracy of the standard solution used.

Expression of Concentration of Standard Solutions

A standard solution is one whose strength or concentration is known with sufficient accuracy to use in volumetric analysis. The concentration of solution can be expressed in various ways such as

Weight Per unit Volume

This is the simplest way of expressing concentration of solution in which a weighed quantity is dissolved and diluted to a definite volume giving a solution containing a known weight per unit volume. In this method, number of grams or milli grams) of solute per litre (or milli litre) of solvent are used.

Per cent Composition

By this method concentration is expressed in terms of grams of solute per 100 grams of solution. A 10 per cent solution of a given salt is made by dissolving 10 grams of the salt in 90 grams of water.

Volume Ratio

Occasionally, the concentration of a mineral acid or of ammonium hydroxide is given in terms of the volume ratio of the common concentrated reagent and water. Thus H_2SO_4 (1: 3) signifies a solution made by mixing one volume of the commonly used conc H_2SO_4 (sp. Gr. 1.84) three volumes of water.

Molar Solution

A molar solution contains one mole or a gram molecular weight of the solute in one litre of solution.

Molal Solution

A molal solution contains one mole or a gram molecular weight of the solute in 1 litre of the solvent.

Normal solution

This is the most widely used method of expressing the concentration of a standard solution. A normal solution contains one gram equivalent weight per litre of solution.

In case of acids, a 1 N solution contains 1.008 gram of replaceable hydrogen per litre of solution. A 1N solution of a base is one that contains 17.008 grams of hydroxyl ions per litre. A 1N solution of the precipitating agent contains a weight of precipitating agent equivalent to 1.008 gram hydrogen.

Equivalent Weight

An equivalent weight of a substance is that weight equivalent in reacting power to an atom of hydrogen.

$$\text{Equivalent weight of an acid} = \frac{\text{Molecular weight of acid}}{\text{basicity}}$$

Basicity of an acid is equal to the number of replaceable hydrogen atoms present in the mole of the acid.

Hence,

$$\text{Eq. wt. of HCl} = \frac{36.46}{1} = 36.46$$

$$\text{Eq. wt. of H}_2\text{SO}_4 = \frac{98.08}{2} = 49.04$$

$$\text{Eq. wt. of an alkali} = \frac{\text{Molecular weight of acid}}{\text{Acidity}}$$

Acidity of an alkali is equal to the number of replaceable hydroxyl groups present in one mole of the alkali.

Hence,

$$\text{Eq. wt. of NaOH} = \frac{40.00}{1} = 40.00$$

$$\text{Eq. wt. of Na}_2\text{CO}_3 = \frac{105.00}{2} = 53.00$$

Gram equivalent weight of an oxidizing substance is that weight of the substance in grams which is equivalent to 8 g of available oxygen.

$$\text{Eq. wt. of KMnO}_4 \text{ in acidic medium} = \frac{158}{5} = 31.6$$

$$\text{Eq. wt. of KMnO}_4 \text{ in alkaline medium} = \frac{158}{3} = 52.7$$

Titration

The process of adding one solution of known strength to another unknown one so that the reaction is just completed. In titration, we find the strength of unknown solution by measuring its volume with the help of burette, pipette and measuring flask. So it is also termed as volumetric analysis.

Titre

A titre is defined as the weight of solute contained in a milli litre of solution or the weight of any substance which will react with or be equivalent to 1 ml of the solution. It is seen that the litre of a normal solution is its milli equivalent weight. The relationship between titre and normality is shown by the expression.

$$\text{Normality} = \frac{\text{Titre} \times 1000}{\text{g. eq. wt. of substance in which the titre is expressed}}$$

For example, an acid solution with an "NaOH titer of 0.0040 g" means that 1 ml of the acid solution neutralized 0.0040 g of NaOH.

End Point

The point at which the colour change occurs and at which the titration is just completed is called the end point (the equivalence or stoichiometric point). The end point can be found by some change in colour or a coloured precipitate developed during the reaction either by one of the reagents added or by the addition of an auxiliary reagent known as the indicator.

Indicator

The analyst must have some way of knowing when an equivalent amount of reagent has been added. This is accomplished in several ways one of which is the addition of certain substances to a solution being titrated which will be indicated by their behaviors when the end point has reached. Such substances are called indicators. Usually the indication they give is that the right amount of reagent has been added which is shown by a distinct change in colour. Indicators may be internal, external or self indicators.

Internal Indicators

These are chemical substances which are added to the volumetric flask in which titration is carried out e.g. **phenolphthalein**, methyl orange, starch etc.

They may be further divided according to the types of reactions in which they are used (1) acid-base indicators (2) precipitation indicators (3) redox indicators (4) adsorption indicators.

- a. **Acid-base Indicators:** The indicators used in acidimetry and alkalimetry are either weak organic acids (indicator acids) or weak organic bases (indicator bases), the dissociated form of which has a colour different from that of the undissociated form. A basic indicator must possess a coloured cation while an acid indicator must possess a coloured anion. Eg.: methyl orange, methyl red, phenolphthalein etc.
- b. **Precipitation indicators:** They are employed in the fractional precipitation of two insoluble salts by the same reagent, e.g. in the case of silver nitrate-sodium chloride titration, potassium chromate is used as a precipitation indicator
- c. **Redox indicators:** An oxidation-reduction (redox) indicator should mark the sudden change in the oxidation potential in the neighbourhood of the equivalence point in an oxidation-reduction titration. e.g. orthophenanthroline ferrous ion.
- d. **Adsorption indicators:** Are those substances which adsorb certain ions at the equivalence point and a typical orientation is developed at the surface which gives a characteristic colour change indicating the end point e.g. fluorescein, eosin, rhodamine etc.

Some Common Indicators

Methyl Orange

It is the sodium salt of dimethyl-l-aminobenzene sulphonic acid. For the preparation of the indicator solution, 0.1 g of methyl orange is dissolved in 100 ml solution to be titrated. The colour change is from pink (acidic) to yellow (basic). Its pH ranges from 3.1 to 4.4. It is especially useful in titrating sodium carbonate with strong acids.

Methyl Red

It is dimethyl aminozobenzene -o- carboxylic acid. An indicator solution is prepared by dissolving 0.1 g of methyl red in 60 ml of alcohol after which 40 ml distilled water is added. Its colour change is from pink (acidic) to yellow (basic).

Phenolphthalein

The indicator solution of phenolphthalein is made by dissolving 0.2 g of phenolphthalein in 100 ml of 95% ethyl alcohol. The colour change is from colourless (acidic) to pink (basic). Its pH ranges from 8.2 to 10.0. It is especially useful in titrating weak acids with strong bases.

Bromocresol Green

It is tetrabromo-n-cresol sulpho-naphthalein. A suitable indicator solution is prepared by dissolving 0.1 g of bromocresol green in 100 ml of 95% ethyl alcohol. The colour change is from yellow (acidic) to blue (basic). Its pH ranges from 3.8 to 5.4. It is suitable for the titration of ammonia which has been absorbed in boric acid as in the Kjeldahl determination of nitrogen.

Primary Standard Solution

Primary standard are used for the standardization of solutions of unknown strengths.

Properties of Primary Standard Substances

1. Substances should be obtained in high purity.
2. They should be stable at temperature required for removal of moisture.
3. They should not oxidize on standing.
4. They should neither gain or lose weight on exposure.
5. A high equivalent weight is desirable in order to reduce the percentage errors in weighing.

Examples, Borax, potassium dichromate, potassium hydrogen phthalate, potassium hydrogen iodate, sodium carbonate, sodium oxalate.

B. Definition of proximate principles in feeds

Water

The moisture is determined as the loss in weight which results from drying a known weight of feed to constant weight at 100°C. Therefore, dry matter is estimated as a part of substance that does not evaporate at 100°C. The major difference in nutritional value on as fed basis is traceable to moisture content and dry matter. Calculation of relative cost of nutritional value involves consideration of moisture content. Determination of moisture is essential in bulk purchase of feed ingredients, when the grain crop is new, the moisture in feeds is very important. Feeds containing more than 14% moisture should be stored in bulk for the danger of development of moulds/fungi as well as spontaneous combustion.

Crude Protein

The crude protein content is calculated from the nitrogen content of the feed and knowing the protein content of a feed provides an idea about the class of feed it belongs to. In this method, the feed is digested with sulphuric acid which converts all nitrogen to ammonia. This ammonia is liberated by the addition of sodium hydroxide to the digest, distilled off and collected in standard acid. The quantity so collected is determined by titration. Determination of crude protein involves multiplication of estimated nitrogen content by a factor of 6.25 based on the assumption that all the proteins contains 16% nitrogen and all nitrogen is present in form of proteins. However, both the assumptions are not true. The term crude protein does not distinguish the nitrogen contribution from true protein and non-protein nitrogenous substances like urea, uric acid, free amino acids, ammonium salts etc. Non-protein nitrogen can be calculated by subtracting true protein content from crude protein.

Ether Extract

The ether extract of crude fat is determined by subjecting the feed to a continuous extraction with petroleum ether (boiling point = 40° - 60°C or 60° - 80°C) for a definite period. The residue after the evaporation of solvent is called ether extract. Ether extract is a source of energy and provides essential fatty acids. In addition to fat, ether extract contains waxes, organic acids, alcohols, pigments etc.

Crude Fibre

The carbohydrate of the feeds is contained in two portions, the crude fibre and nitrogen free extract. Crude fibre is determined by subjecting the residual feed from ether extraction to successive treatments with boiling acid and alkali of fixed concentration, the organic

residue is crude fibre. Crude fibre contributes to the bulkiness of feed. The crude fibre fraction contains cellulose, lignin and hemicellulose in variable proportions. Originally the crude fibre was intended to represent the indigestible portion of feed, however, a large part of it may in fact be digested by ruminants. Crude fiber estimation simulates digestion in monogastric animals than in ruminants.

Nitrogen Free Extract

Nitrogen free extract is obtained when a sum of moisture, ether extract, crude fibre, crude protein and ash (per cent) is subtracted from 100. The value of nitrogen free extract is affected by analytical errors of these five parameters as well as lack of precision in crude fibre determination in separating functional categories of carbohydrates. The NFE content is inversely related to protein in concentrate feeds.

Ash

The ash content is determined by ignition of a known weight of feed at 550° - 600°C. The residue obtained is ash and represents inorganic constituents of feeds. The ash, however, does not discriminate between the proportion of mineral matter and sand/silica due either to contamination or adulteration. The ash may contain materials of organic origin such as sulphur and phosphorus of proteins. Likewise some loss of sodium, chloride, selenium and iodine takes place on ignition. Indirectly, ash helps in calculating total carbohydrates, NFE and organic matter by difference.

C. Collection and processing of various samples for analysis

Feed Ingredients/Compounded Feeds

1. With an appropriate sampling or with a simple scoop the sample is withdrawn from the top, bottom and sides of selected bags. While selecting the number of bags following sequence may be taken into consideration.

Lot size (bags)	Selected number (bags)
Up to 50	1
51 to 100	3
101 to 300	4
301 to 500	5
501 to above	7

- The collected material (say 1500g) is equally distributed in 2-3 lots, packed separately in clean and dry polythene bags and sealed air tight. These polythene bags may further be packed in paper bags for suitable labeling and further safety.
- A small portion (say 100g) of sample may be ground using pestle and mortar or in an electric grinder to pass through 1 mm IS sieve or ASTM sieve 18 or BS sieve 16 or Tyler sieve 16. The ground sample is stored in a well stoppered glass/polythene bottle for chemical analysis.
- The following are the approximate quantities of sample of feed taken for proximate analysis.

Parameter	Dry rough ages (g)	Grains/grains by products (g)	Oil seed cakes (g)	Animal by products (g)
Moisture	10-15	10-15	5-10	5-10
Crude protein	2-3	1.5-2	0.5-1	1-2
Crude fat	3-5	2-3	1.5-2	2-3
Crude fibre	3-5	2-3	1.5-2	2-3
Total ash	5-10	5-10	5-10	5-10

D. Determination of proximate principles

Determination of dry matter

Principle

If a sample of feed is heated and dried in a hot air oven at the temperature of boiling water ($100^{\circ}\text{C} \pm 5^{\circ}\text{C}$), the water present in feed sample escapes as vapour. The loss in weight is considered as moisture and the residue is termed as dry matter (DM).

Requirement

- Aluminium moisture cup
- Analytical balance
- Desiccator
- Hot air oven
- Metal tongs
- Spatula

Procedure

1. Dry aluminium moisture cup (dish) in oven at 100°C for overnight, place in desiccator, cool and weigh.
2. Weigh in duplicate 2-10 g of sample into aluminium cup and shake the cup until the contents are evenly distributed.
3. **Place the cup with lid removed in the oven and dry the contents for 12-18 hours at 100° ± 5°C.**
4. Place cover on cup and transfer to desiccator to cool. Weigh as quickly as possible. Repeat the process of heating, cooling and weighing until the difference between two successive weighing is less than one milli gram.
5. Report loss in weight as moisture and it can be calculated using the following formula.

Calculation

$$\text{Moisture per cent} = \frac{100 (W_1 - W_2)}{W_1 - W}$$

Where, W = Wt. of moisture cup

W_1 = Wt. of moisture cup + Sample

W_2 = Wt. of moisture cup + sample after drying

DM% = 100 – Moisture.

Determination of crude protein

Principle

Crude protein constitutes a mixture of true protein and non protein nitrogenous substances and is determined by estimating nitrogen and then multiplying the nitrogen content of feed by the factor 6.25. The factor 6.25 is derived on the basis that all proteins contain 16% nitrogen.

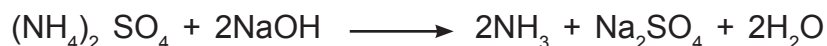
The total nitrogen content in the feedstuff is determined by Kjeldahl method which generally consists of three steps i.e. digestion, distillation and titration. The sample is digested with concentrated sulphuric acid which hydrolyses the protein, decomposes to ammonia and finally converts all nitrogen into ammonium sulphate. The acid digest is cooled, diluted

with distilled water and distilled with strong alkali (sodium hydroxide, 40%) which liberates ammonia to be absorbed quantitatively in a known volume of standard sulphuric acid again forming ammonium sulphate. The unreacted acid is then determined by back titration with standard sodium hydroxide. Alternatively, the ammonia released during distillation can also be taken up into a boric acid solution and such ammonia is titrated with standard sulphuric acid.

Boric acid procedure saves considerable time as the boric acid is not to be standardized and there is no problem of absorbing all of the ammonia distilled from acid digest of high nitrogen samples. While estimating the nitrogen content, following reactions take place.



Ammonium sulphate derived from feed nitrogen is converted into ammonia by adding 40% alkali.



Ammonia is received in boric acid solution by steam distillation. The ammonia boric acid complex (ammonium borate) is titrated with standard H_2SO_4 .



Equipment/glasswares

1. Analytical balance
2. Beaker (100 ml or 250 ml)
3. Burette
4. Conical flask (100 ml)
5. Digestion bench/automatic digestion unit
6. Glass beads
7. Glass rods
8. Kjeldahl flask (500 ml)/digestion tube
9. Micro Kjeldahl Distillation Assembly/automatic distillation assembly
10. Measuring Cylinder

11. Metal tongs
12. Spatula
13. Wash bottle

Reagents

1. Digestion mixture: (9.5 parts potassium sulphate/sodium sulphate + 0.5 parts copper sulphate). Potassium sulphate/sodium sulphate raises the boiling point of the contents for efficient oxidation of material. Copper sulphate is added as a catalyst to speed up the reaction.
2. Boric acid/mixed/Toshiro's indicator: 10 ml of 0.1 percent methyl red indicator + 5 ml of 0.1–0.5% bromocresol green indicator + 1000 ml of 2–4% boric acid solution.

For 0.1% methyl red, dissolve 100mg of methyl red in 100 ml of ethanol (95%). For 0.1–0.5% bromocresol green, dissolve 100 mg–500 mg of bromocresol green in 100 ml of ethanol (95%). For 2–4% boric acid, dissolve 20–40 g of boric acid in one litre of hot distilled water, cool and allow to mature for 2–3 days before decanting the clear liquid.
3. NaOH solution (40%): Dissolve 400 g of NaOH pellets in one litre of distilled water, cool and then use.
4. Concentrated H_2SO_4 (AR)
5. Standard N/10 or N/100 H_2SO_4 solution.

Procedure

This method of nitrogen and crude protein determination is applicable for feed, faeces and meat samples. Micro Kjeldahl nitrogen analysis comprises three different stages viz., digestion, distillation and titration.

Digestion

1. Weight 1–2 g of sample on a piece of butter paper depending upon the protein content and transfer it into a clean and dried Kjeldahl flask/digestion tube.
2. Add 5–10 g of digestion mixture to the sample taken in flask/tube.
3. Add 20–30 ml of conc. H_2SO_4 along the neck of the flask so as to wash down any particles of the sample/digestion mixture sticking to the sides (increase the amount of H_2SO_4 by 10 ml for each gram of sample).
4. Drop a few glass beads into the flask to prevent spurting while heating.

5. Place the flask/tube in an inclined position on a digestion heater and heat it below the boiling point of the acid until frothing ceases. If the froth of a sample starts up the neck of flask, remove it from the heater to allow froth to subside the return to heater. Increase heat until the acid boils vigorously and digests for a time after mixture is clear or until oxidation is complete (about 2 to 4 hours). If any material sticks to the neck of the flask cool, wash down with a small amount of water and redigest.
6. When the end point of digestion is reached, remove the Kjeldahl flask/tube and allow it to cool.

A blank digestion should always be conducted simultaneously using all reagents in the same quantities except sample to be tested.

Distillation

Distillation process can be accomplished using micro distillation method.

Micro-Kjeldahl method

1. Dissolve the digested material in a small quantity (say 50 to 100 ml) of distilled water, transfer into a volumetric flask (100 ml or 250 ml) and then make the volume upto the mark by adding distilled water.
2. Pour 5-10 ml of aliquot in receiving part of the micro-Kjeldahl distillation apparatus, add 10-20 ml of 40% NaOH solution and then add 5 ml of distilled water to the same receiver for rinsing.
3. Take 10-20 ml of boric acid indicator in a conical flask/beaker and dip the tip of collecting tube into it. Start the cold water circulation in the condenser.
4. Heat the alkaline liquid by passing steam into it through the boiling distilled water in a flask.
5. Faint reddish colour of boric acid starts changing to green colour since the released ammonia is absorbed by boric acid indicator. Released ammonia is allowed to distil till the original volume of boric acid indicator (10-20 ml) becomes three times more (30-60 ml).
6. Remove the conical flask/beaker after rinsing the tip with a little distilled water and preserve the same for titration.

Titration

Titrate the distillate using N/10, or N/100 standard sulphuric acid solution. The end point in all the cases is light pink.

Calculation

Two atoms of nitrogen (in the form of ammonia) neutralize one molecule of sulphuric acid and form ammonium sulphate. Therefore, each ml of 1N sulphuric acid neutralized by ammonia is equivalent to 0.014 g or 14 mg nitrogen. Amount of nitrogen and crude protein per ml of sulphuric acid of different strengths may be noted from the following details.

H₂SO₄		Nitrogen		Crude Protein	
Volume (ml)	Normality (N)	g	mg	g	mg
1	1.000 N or 1 N	0.0140	14.00	0.08750	87.50
1	0.100 N or 1 N/10	0.0014	1.40	0.08755	8.75
1	0.010 N or 1 N/100	0.00014	0.14	0.00087	0.87

$$\text{Nitrogen (per cent)} = \frac{140 \times V \times N}{m (100-M)} \quad \text{on moisture free basis}$$

Where, V = volume in ml of the standard H₂SO₄ used

N = normality of the standard used H₂SO₄ used

m = sample weight in g

M = moisture percentage

For conversion on nitrogen into crude protein

Where, CP (%) = N (%) x 6.25

CP = crude protein (%) content of feedstuff

N = total nitrogen (%) present in feedstuff

Determination of ether extract

Principle

When a sample of feed is extracted continuously with fat solvent like petroleum ether, the vapour from the ether boiling in the flask passes into the condenser where it condenses and drops back on the sample which dissolves ether soluble materials such as fat, sterols, lecithins, resins and volatile oils in the extraction flask. The

extract is termed as crude fat or ether extract (EE) because it also contains other fat like substances.

Equipment/glasswares

1. Analytical balance
2. Beaker
3. Cotton wool
4. Desiccator
5. Extraction thimble
6. Hot air oven
7. Oil flask (150-250 ml)
8. Soxhlet extraction assembly: This consists of 3 parts (a) condenser at the top (b) Soxhlet or extractor in the middle (c) receiver flask at the bottom. These three parts are assembled by means of their ground glass joints.
9. Heating mantle, provision for water supply to the condenser and electricity to sheating mantle is also to be made.

Reagent

Petroleum ether (boiling point = 40-60°C or 60-80°C)

Procedure

1. Weigh 2-5g of ground feed material on a butter paper, transfer it into an extraction thimble and plug the mouth of thimble with a piece of cotton wool. If the crude fat is to be determined directly on dry matter basis, place the thimble in a beaker and dry in oven for 6 hours at $100 \pm 1^\circ\text{C}$.
2. Weigh the empty oil flask and fill it with the petroleum ether (say 50-100 ml). Place the oil flask over the heating mantle. The amount of solvent taken is about 1.5 times the capacity of the Soxhlet extractor.
3. Introduce the extraction thimble with sample into Soxhlet extractor (middle piece) over a pad of cotton wool so that the top of thimble should be well above the siphon bend of extractor. Place the extractor alongwith the oil flask and connect it with the condenser.
4. Start the cold water circulation in condenser. Switch on the heating mantle or water bath. As soon as the ether begins to boil adjust the heat to 40-60°C or 60-80°C as per the boiling point of petroleum ether used.

5. Extract the sample for 4 hours (5 to 6 drops per second condensation) to 16 hours (2 to 3 drops per second condensation). In most of the cases 8 hours extraction is sufficient.
6. After the period of extraction, remove the thimble from extractor and recover through the extractor all the ether from the oil flask leaving only few drops of ether in it.
7. Evaporate the residual ether of oil flask on water bath and place the oil flask in hot air oven at 80-100°C for two hours, cool it in desiccator and weigh it.
8. Continue cooling and weighing of the oil flask until the difference between two successive weighings is less than one milligram. Never leave more amount of petroleum ether in the oil flask and don't keep the flask in hot air oven otherwise fire may take place. If necessary, reserve the extracted sample in the thimble for the determination of crude-fibre content.

$$\text{Ether extract, per cent on as such basis} = \frac{100 (W_1 - W_2)}{m} \quad \text{or} \quad \frac{100 \times \text{wt of ether extract}}{\text{wt. of sample}}$$

Where, W_1 = wt. in g of oil flask + extracted material

W_2 = wt. in g of empty oil flask

m = wt. in g of sample

If the thimble with sample has not been dried in hot air oven at 80-100°C for 10-12 hours prior to extraction, the crude fat (ether extract) value can be converted into dry matter basis.

$$\text{Ether extract on DM basis} = \frac{\text{Crude fat} \times \text{dry matter per cent}}{100}$$

Determination of crude fibre

Principle

When a sample of moisture and fat free feedstuff is boiled with dilute acid followed by dilute alkali, the soluble carbohydrates and proteins go into the solution and are extracted. The undissolved residue left behind represents cellulose, hemicellulose, lignin and mineral matter. The cellulose, hemicellulose and lignin put together are called crude fibre (CF). When the residue after drying and weighing is ignited, the fibre being organic in nature is burnt whereas the ash or mineral matter is left over. The fibre content is

obtained by deducting the weight of ash from the weight of the dried residue. In simple way it can also be explained that the crude fibre is determined gravimetrically as part of the substance which is left when protein, soluble carbohydrate, fat, salts and water have been removed.

In poultry feeding, the analysis of crude fibre content is very essential because the amount of crude fibre predicts the digestibility of the feedstuff. Higher the fibre content lesser will be the digestibility.

Equipment / glasswares

1. Analytical balance
2. Hot air oven
3. Hot plat heating mantle
4. Muffle furnace
5. Beaker (1000ml), tall form spoutless
6. Buchner funnel
7. Conical flask
8. Desiccator
9. Horn spatula
10. Measuring cylinder
11. Muslin cloth
12. Silica crucibles, large size
13. Suction flask with gasket
14. Suction pump
15. Wash bottle

Reagents

1. Acetone
2. Hydrochloric acid (10%)
3. Sodium hydroxide (10%)

Procedure

1. Weigh accurately about 2 g of dried feed material and extract the crude fat (ether extract) for about 8 hours with petroleum ether using Soxhlet extraction apparatus.
2. Transfer the extracted feed material into tall form spoutless beaker (1000 ml), mark the beaker for 200 ml with a marker.
3. Add 50-100 ml of boiling distilled water into the beaker, add 25 ml of H_2SO_4 (10 per cent) to the beaker and make the volume upto the mark (200 ml) with the help of distilled water. Boil the content of beaker over a hot plate for 30 minutes. In order to keep the volume constant in the tall form beaker or to check the evaporation, keep one flask filled with cold water over the beaker. Bulb condenser fitted over the beaker can also be used in lieu of round bottom flask.
4. Remove the beaker from the hot plate, filter the content through muslin cloth spread over the Buchner funnel fitted with suction pump and suction flask. Wash the residue with hot water for several times till the filtrate becomes acid free.
5. Transfer the residue (acid-free) by horn spatula from Buchner funnel to the beaker. Add 50-100 ml of boiling, distilled water, 25 ml of sodium hydroxide solution (10 per cent) and then make the final volume upto the mark with distilled water. Boil the content in beaker over the hot plate for 30 minutes. Use the round bottom flask with cold water or bulb condenser with running tap waer to maintain the constant volume in the beaker.
6. Remove the beaker form the hot plate and filter the content again through the same muslin cloth on filter flask connected to a filter pump. Wash the residue with hot water for several times till free from alkali. Wash the residue with acetone.
7. Transfer the residue to a clean dry silica crucible. Dry the crucible and contents at $100^\circ\text{C} \pm 1^\circ\text{C}$ in hot air oven for complete dryness. Cool the crucible in a desiccator and weigh the crucible containing dried residue.
8. incinerate the contents of the crucible at $600^\circ\text{C} \pm 20^\circ\text{C}$ for two hours in a muffle furnace until all the carbonaceous matter is burnt.
9. Remove the crucible from furnace, cool in a desiccator and weigh the crucible containing ash. The loss in weight on ignition is due to the amount of crude fibre present therein.

Calculation

$$\text{Crude fibre (per cent) on as such basis} = \frac{100 (W_1 - W_2)}{W}$$

Where, W = wt. in g of sample

W_1 = wt. in g of crucible + residue before ashing

W_2 = wt. in g of crucible + ash

$$\text{Crude fiber (per cent) on DM basis} = \frac{\text{Crude fat} \times 100}{\text{DM}}$$

When residue after fat determination is used.

$$\text{Crude fiber (per cent) on moisture free basis} = \frac{(W_1 - W_2) (100 - \text{EE})}{W}$$

Where, W = wt. in g of fat free sample

W_1 = wt. in g of crucible + residue before ashing

W_2 = wt. in g of crucible + ash

EE = Ether extract on moisture free basis

Determination of total Ash

Principle

Total ash (TA) is the inorganic residue which remains after a feed stuff is ignited to carbon free at 550°C in a muffle furnace. In other words, ash is that part of the material which does not disappear at 550°C.

Requirement

1. Analytical balance
2. Asbestos sheet

3. Desiccator
4. Hot air oven
5. Hot plate/gas flame burner
6. Metal tongs
7. Muffle furnace
8. Silica basin/crucibles

Procedure

1. Place silica basin/crucible in hot air oven for drying at $100^{\circ} \pm 1^{\circ}\text{C}$ for six hours, cool in desiccator and weigh it.
2. Weigh in duplicate 2-10 g of feed sample depending upon the type of feed into a pre-weighed silica basin/crucible.
3. To carbonize the contents, burn and char the organic matter by placing the basin with sample on a low gas flame or hot plate until all smoke and black portions escape out. Do not stir or disturb the material while charring as this will result in a loss of the substance.
4. Keep the crucible in a muffle furnace and raise the temperature upto $550\text{-}600^{\circ}\text{C}$ slowly by setting the regulator and complete the ignition of sample for 2-4 hours at the constant temperature.
5. Remove the silica basin/crucible holding with metal tong from muffle furnace and cool in desiccator and weigh.
6. Since ash is highly hygroscopic, note the lowest weight and report the total ash as per the calculation show below.

Calculation

$$\text{Total ash (per cent) on as such basis} = \frac{100 (W_2 - W)}{W_1 - W} \quad \text{or} \quad \frac{100 \times \text{wt. of ash}}{\text{Sample wt.}}$$

Where, W = wt. in g of crucible

W_1 = wt. in g of crucible + residue before ignition

W_2 = wt. in g of crucible + residue after ashing

$$\text{Total ash (per cent) on moisture free basis} = \frac{\text{Total ash} \times 100}{\text{DM}}$$

Determination of nitrogen free extract

Principle

Carbohydrates are divided into two groups i.e. crude fibre (CF) and nitrogen free extract (NFE). The NFE comprises the sugars, starch and a large part of the material classed as hemicellulose.

The NFE is not determined by analytical procedures. This is calculated as the figure obtained when the sum of water, ash, protein, fat and crude fibre of a feed is subtracted from 100 since the figure is determined by difference, it includes the cumulative errors of the other determinations and thus it is not an exact value.

Requirement

Following analyzed volumes of a feedstuff are very much essential to derive the nitrogen free extract of the same feedstuff.

Calculation

The nitrogen free extract can be derived from the following formula:

$$\text{NFE (\%)} = 100 - (\text{CP\%}) + \text{EE (\%)} + \text{CF (\%)} + \text{TA (\%)}$$

If NFE is to be expressed on as fed basis the following formula would be applicable:

$$\text{NFE\%} = \text{DM\%} - \text{CP\%} - \text{EE\%} - \text{CF\%} - \text{TA\%}$$

Sample questions

1. What are precautions to be taken in a laboratory?
2. Define various proximate constituents.
3. What is the sampling procedure for feeds?
4. What are the methods of estimation of proximate principles?
5. What is the significance for estimation of moisture in feedstuff?